THE MODULATION OF THE ACTIVITY OF INTRACELLULAR pH REGULATING MECHANISMS BY SECOND MESSENGER SYSTEMS IN RAT HIPPOCAMPAL CA1 NEURONS

by

Christopher L. Brett

B.Sc. (Physiology), The University of British Columbia, 1996

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Department of Physiology)

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

April, 1999

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of _PHYSIOLOGY_

The University of British Columbia
Vancouver, Canada

Date _April 30th, 1999_

ABSTRACT

The effects of modulating the activities of the cAMP/cAMP-activated protein kinase (PKA) and calmodulin (CaM)/Ca^{2+}-CaM activated protein kinase II (CaMK-II) second messenger systems on steady-state (SS) intracellular pH (pH_i) and on rates of pH_i recovery following imposed acid and/or alkaline loads were investigated in acutely dissociated adult rat hippocampal CA1 neurons loaded with the fluorescent hydrogen ion indicator 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein.

Alkali extrusion under HCO_3^-/CO_2-buffered conditions was dependent on the activity of a 4,4'-diisothiocyanatostilbene-2,2'-disulphonate (DIDS)-sensitive, Na^+-independent HCO_3^-/Cl^- exchanger (NIAE). Acting in concert with an acid-extruding Na^+-dependent HCO_3^-/Cl^- exchanger (NDAE) and a Na^+/H^+-exchanger (NHE), the NIAE functioned to maintain SS pH_i near the external pH value.

Under HCO_3^--free conditions, stimulation of cAMP/PKA increased both SS pH_i and rates of pH_i recovery from imposed acid loads. The increases in SS pH_i were enhanced by protein phosphatase inhibition, unaffected by changes in intracellular free calcium ($[Ca^{2+}]_i$), and dependent on the presence of external Na^+. These results indicate that stimulating cAMP/PKA activates the NHE in rat hippocampal neurons. Conversely, inhibition of cAMP/PKA failed to affect NHE activity. However, in the presence of HCO_3^-/CO_2, both stimulation and inhibition of cAMP/PKA evoked changes in SS pH_i and rates of pH_i recovery from internal acid (and alkaline) loads. Further study indicated that modulation of the activity of the cAMP/PKA pathway changes the activities of the NIAE and NDAE in a manner depending on resting pH_i.

Inhibitors of CaM evoked rises in SS pH_i that were dependent on the initial resting pH_i and external Na^+, and increased rates of pH_i recovery from acid loads imposed in either the presence or absence of HCO_3^-, indicating that CaM inhibitors activate the NHE. CaM inhibitor-evoked increases in SS pH_i were minimally affected by changes in $[Ca^{2+}]_i$, but were not mediated

by changes in the activities of the cAMP/PKA second messenger pathway, calcineurin or CaMK-II.

The results indicate that the activity of the NHE can be modulated by more than one intracellular second messenger system. Furthermore, the activities of the three pH_i regulating mechanisms can be altered concomitantly by a single second messenger system. Ultimately, the described second messenger control of the activities of pH_i regulating mechanisms present in rat hippocampal CA1 neurons may contribute to some of the effects of neurotransmitters on neuronal excitability and function.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

I would like to thank only one individual for the experimental work and scientific insight presented herein, a true scientist and an excellent mentor, Dr. John Church.

I would also like give my sincere gratitude to all who supported me for the past two and a half years, including: every member of my family, especially my parents Ed and Viive Brett; my best friend, Lu-Anne DaCosta; my lab companions, Claire Sheldon, Adam Sidky, Cynthia Fan, Jimmy Chin, Abdoullah Diarra, Keith Baxter, Tony Kelly; faculty and graduate students of the Department of Physiology, Gordon Rintoul, Simon Hinke, Frank Lynn, Craig Kamimura, Dr. Ken Baimbridge, Dr. Tony Pearson; and finally the members of my examination and research committees, Drs. Vladimir Palatý, Ed Moore, Alison Buchan, Ray Pederson, Peter Vaughan, Chris McIntosh.

Financial support was provided by a University Graduate Fellowship to myself from the University of British Columbia and an operating grant to Dr. John Church from the Medical Research Council of Canada.

INTRODUCTION

1. Neuronal function and extracellular pH (pH$_o$)

The precise regulation of acid-base balance within a narrow range is essential for proper function and survival of the brain. Pathological alterations of this homeostatic mechanism can lead to dramatic effects. For example, acidification of cerebrospinal fluid inhibits convulsions (Meyer *et al.*, 1961) and respiratory alkalosis can lead to seizure activity (Marcus & Watson, 1968). However, recent studies indicate that significant changes in pH can also occur during normal cerebral function; for example, a transient alkalinization followed by a sustained acidification of the extracellular space is associated with normal synaptic transmission (Kaila & Voipio, 1987; Chesler, 1990; Paalasmaa *et al.*, 1994; Smith *et al.*, 1994; Voipio *et al.*, 1995; Paalasmaa & Kaila, 1996). Additional evidence indicates that changes in pH$_o$ act to modulate various ion channels, including the *N*-methyl-D-aspartate (NMDA) and γ-aminobutyric acid$_A$ (GABA$_A$) receptor-operated channels (Kraig *et al.*, 1983; Siesjö, 1988; Tang *et al.*, 1990; Traynelis & Cull-Candy, 1990; Vyklický *et al.*, 1990; Chesler & Kaila, 1992; Traynelis *et al.*, 1995; Kashiwagi *et al.*, 1996; Vignes *et al.*, 1996) and voltage-activated calcium, sodium and potassium channels (Tombaugh & Somjen, 1996). These effects have been shown to be important functionally, because studies performed in the hippocampus indicate that extracellular alkaline shifts induced by activation of glutamate or GABA$_A$ receptors (Chen & Chesler, 1992a & 1992b; Kaila *et al.*, 1992; Kaila *et al.*, 1993; Kaila, 1994; Pasternack *et al.*, 1996) can modulate the functional properties of the NMDA receptor-operated channel within a physiologically relevant time frame (Traynelis & Cull-Candy, 1991; Gottfried & Chesler, 1994 & 1996). These findings suggest that induced changes in pH$_o$ can act to regulate the mechanisms that initially caused them, such that increases and decreases in pH$_o$ enhance and reduce neuronal excitability (see Balestrino & Somjen, 1988), respectively. For example, raising pH$_o$ can elicit epileptiform activity (which is sensitive to NMDA receptor antagonists; Church & McLennan, 1989), whereas lowering pH$_o$ completely and reversibly suppresses both low Mg$^{2+}_o$-induced,

NMDA receptor-mediated epileptiform bursting in cortex-hippocampal slices (Velísek *et al.*, 1994) and the efficacy of normal, low frequency neuronal transmission, thereby preventing the induction of long term potentiation (LTP) in the CA1 region of the hippocampal slice (Velísek, 1998).

Furthermore, more pronounced shifts in pH_o can be associated with neurological pathologies which, depending on their magnitude, may provide a mechanism of neuroprotection or mediate subsequent neuronal damage. For example, ischemia (*i.e.* oxygen-glucose deprivation) induced increases in the intracellular free calcium concentration ($[Ca^{2+}]_i$) (mediated by the influx of Ca^{2+} ions) can be inhibited by reducing pH_o (to ~6.8) and enhanced by increasing pH_o (to ~7.8) in rat hippocampal slices (Ebine *et al.*, 1994; also see Nemoto & Frinak, 1981; Siesjö, 1985). Thus, mild external acidosis may be neuroprotective by reducing Ca^{2+}-influx via NMDA receptor-operated channels and/or voltage-gated Ca^{2+} channels or by attenuating Na^+ entry through voltage-gated Na^+ channels (Giffard *et al.*, 1990; Andreeva *et al.*, 1992; Kaku *et al.*, 1993; Simon *et al.*, 1993; Kristián *et al.*, 1994; Tombaugh, 1994; Chidekel *et al.*, 1997; also see Yu & Salter, 1998); similar mechanisms may mediate the antiepileptogenic effects of reduced pH_o (Caspers & Speckmann, 1972; Somjen, 1984; Velísek *et al.*, 1994). However, further reduction of pH_o to levels which may occur during complete cerebral ischemia (< pH 5.3) causes local areas of tissue necrosis reminiscent of those observed after ischemic brain infarctions (Kraig *et al.*, 1987; also see Goldman *et al.*, 1989; Nedergaard *et al.*, 1991; Morimoto *et al.*, 1994).

Extracellular pH is therefore an important mediator of neuronal function and dysfunction. However, it has been suggested that intracellular pH (pH_i) may account for some of the effects caused by changes in pH_o described above (see Herman *et al.*, 1990; Nedergaard *et al.*, 1991). This possibility is supported by findings from studies performed in hippocampal neurons which indicate that changes in pH_o give rise to shifts in pH_i (Church & Baimbridge, 1991; Ou-yang *et al.*, 1993; Church *et al.*, 1998). Therefore, some of the noted effects of pH_o on both the normal

activity of neurons and on pathophysiological events may be mediated by alterations in pH_i (also see Lee *et al.*, 1996).

2. Neuronal function and pH_i

2.1 Changes in pH_i evoked by neuronal activity

Concomitant and opposite to the changes in pH_o described above, neuronal depolarization and/or the application of glutamate or GABA produces a decrease in pH_i, which is either sustained (Chesler, 1990; Irwin *et al.*, 1994; Yamamoto *et al.*, 1998; Pasternack *et al.*, 1993) or transient, and which may be followed by an increase in pH_i to (Wang *et al.*, 1994) or above (Hartley & Dubinsky, 1993; Ou-yang *et al.*, 1995) initial steady-state (SS) pH_i levels. The pH_i response to glutamate is largely NMDA receptor-mediated and dependent on a rise in $[Ca^{2+}]_i$ (Hartley & Dubinsky, 1993; Irwin *et al.*, 1994; Wang *et al.*, 1994; Ou-yang *et al.*, 1995; Yamamoto *et al.*, 1998; also see Dixon, 1993), although Amos *et al.* (1998) have reported that metabotropic glutamate receptors may also contribute to glutamate-induced pH_i responses in cultured neurons by causing the release of Ca^{2+} from intracellular stores.

A number of mechanisms can be proposed to account for the internal acidifications induced by neuronal activity and/or the application of excitatory and inhibitory amino acids. First, increased neuronal activity may lead to the increased production of metabolic end-products such as carbon dioxide and lactic acid which, in turn, can elicit a fall in pH_i (Siesjö, 1985; Irwin *et al.*, 1994; Wang *et al.*, 1994). Second, in the case of $GABA_A$ receptor activation, an efflux of bicarbonate ions occurs via $GABA_A$ receptor-operated channels and, possibly, other ligand- and voltage-gated channels (*e.g.* Chen & Chesler, 1992a; Kaila *et al.*, 1994; also see Bonnet *et al.*, 1998). Third, the rise in $[Ca^{2+}]_i$ evoked by the activation of glutamate receptors may lead to the release of protons from shared internal binding sites (Chesler, 1990) and/or the activation of Ca^{2+}/H^+ exchange (Schwiening *et al.*, 1993; Paalasmaa & Kaila, 1996; Trapp *et al.*, 1996). Reversed Ca^{2+}/H^+ exchange has also been suggested to mediate the internal alkalinizations which are sometimes observed following the application of glutamate (reviewed by Takahashi &

Copenhagen, 1996; also see Paalasmaa & Kaila, 1996; Trapp *et al.*, 1996). Furthermore, each of the aforementioned mechanisms may contribute to the concomitant changes in pH_o observed during neuronal excitation, which were discussed above.

As noted above, activation of either $GABA_A$ or NMDA receptors leads to a fall in pH_i attributable, respectively, to the efflux of HCO_3^- ions or to a rise in $[Ca^{2+}]_i$. Recent evidence also indicates that other neurotransmitters and neuromodulators may evoke changes in neuronal pH_i, but in these cases the effects arise via specific alterations in the activities of neuronal pH_i regulating mechanisms. Thus, noradrenaline and β-adrenoceptor agonists activate the acid-extruding Na^+/H^+ exchanger in rat hippocampal neurons and thereby raise SS pH_i (Smith *et al.*, 1998). The modulation of the activities of pH_i regulating mechanisms by receptor-linked intracellular signaling cascades has also been suggested to participate in glutamate-induced pH_i responses (Amos & Richards, 1994).

In summary, pH_i is a physiological variable that can be modulated by multiple voltage- and ligand-activated ion channels and by neurotransmitters and neuromodulators which act to alter the activities of membrane-bound ion transport mechanisms that function to regulate SS pH_i. The subsequent changes in pH_i may then ultimately modulate the neuronal events that initially caused them, thereby establishing a feedback mechanism.

2.2 Modulation of neuronal function by changes in pH_i

Ultimately, the activities of all enzymes and transport proteins, which contain charged ionizable groups (*e.g.* histidine) and which are exposed to the cytosol, are sensitive to changes in pH_i (Siesjö, 1985; Boron, 1989; Takahashi & Copenhagen, 1996). In fact, significant changes in neuronal activity can be attributed to relatively small perturbations in pH_i (*i.e.* within the physiological range; Chesler, 1990). For example, the activities of the Na^+/Ca^{2+} exchanger (Koch & Barish, 1994) and the plasmalemmal Ca^{2+} pump (Carafoli, 1987) are known to be highly dependent on pH_i in neurons. Changes in pH_i have also been shown to influence ionic currents in vertebrate neuronal preparations. For example, intracellular acidification inhibits rat

brain Na^+ channels incorporated into bilayers (Daumas & Andersen, 1993), and an increase and a decrease in high-$[K^+]_o$-evoked $[Ca^{2+}]_i$ transients occurs in rat hippocampal neurons during intracellular alkalosis and acidosis, respectively (Mironov, 1995; Church *et al.*, 1998). Similarly, cytoplasmic alkalinization increases high voltage-activated Ca^{2+} currents in chick dorsal root ganglion neurons (Mironov & Lux, 1991), whereas a decrease in pH_i leads to a reduction in the high voltage-activated, nifedipine- and Cd^{2+}-sensitive Ca^{2+} current in horizontal cells of fish retina (see Takahashi & Copenhagen, 1996). More recently, in rat hippocampal CA1 neurons, it was demonstrated that the activities of high voltage-activated Ca^{2+} channels (specifically, L- and N-type Ca^{2+} channels) were bidirectionally modulated by shifts in pH_i, whereas low voltage-activated (T-type) Ca^{2+} channels were relatively insensitive to changes in pH_i (Tombaugh & Somjen, 1997). Also in rat hippocampal CA1 pyramidal neurons, potentials mediated by Ca^{2+}-activated K^+ conductances appear sensitive to changes in pH_i (Church, 1992 & 1998; Church *et al.*, 1998). Voltage-gated K^+ channels and the inward rectifier K^+ current were also found to be modulated by changes in pH_i in rat dorsal vagal motoneurons (Cowan & Martin, 1996) and catfish retinal horizontal cells (see Takahashi & Copenhagen, 1996), respectively.

Given the pH_i-dependent nature of the activities of the aforementioned ionic conductances and transport mechanisms, changes in pH_i will ultimately affect neuronal excitability although, in light of the wide range of processes affected by pH_i, the net consequence of a change in pH_i on neuronal function is not easy to predict. Nevertheless, it is apparent that changes in pH_i (at a constant pH_o) do affect synaptic transmission; Lee *et al.* (1996), for example, reported that exposure to the weak base trimethylamine (which evokes an increase in pH_i) increased the slope of the field excitatory postsynaptic potential (fEPSP) in hippocampal slices by 50-70% whereas exposure to the weak acid propionate (which evokes a decrease in pH_i) decreased the slope of the fEPSP by 40-60%.

Changes in pH_i may also lead to pathophysiological alterations in neuronal function. For example, gap-junctional conductances, which are enhanced by increases pH_i, have been suggested to participate in the genesis of epileptiform activity under a variety of experimental

conditions (reviewed by Spray & Bennett, 1985; Dudek *et al.*, 1986; also see Church & Baimbridge, 1991; Dixon *et al.*, 1996; Pappas *et al.*, 1996; Bevans & Harris, 1999). Similarly, it has been suggested that the inability of Na^+/H^+ exchanger-deficient mutant mice to regulate pH_i may be causally associated with a reduced threshold for the initiation and/or propagation of epileptic discharges (Cox *et al.*, 1997). Changes in pH_i are also known to accompany ischemic and/or anoxic insults in neurons, and both the reductions in pH_i which occur during anoxia and the activation of Na^+/H^+ exchange which occurs immediately upon the return to normoxia contribute to anoxia/ischemia-evoked neuronal death (Siesjö, 1985; Nedergaard *et al.*, 1991; Tombaugh, 1994; Vornov *et al.*, 1996; Chidekel *et al.*, 1997; Diarra *et al.*, in press; also see Shen *et al.*, 1995).

In summary, changes in pH_i occur under both physiological and pathophysiological conditions in the mammalian central nervous system, and these changes in pH_i in turn can act to modulate the events which initially caused them. Importantly, protons are not passively distributed across neuronal membranes and a variety of mechanisms serve to regulate SS pH_i and to restore pH_i to normal values after internal acid or alkali loads. Given the established role of pH_i in modulating neuronal function, the mechanisms which act to regulate this variable, which include the Na^+/H^+ exchanger, the Na^+-dependent HCO_3^-/Cl^- exchanger and the Na^+-independent HCO_3^-/Cl^- exchanger, assume considerable physiological importance.

3. pH_i regulation in rat hippocampal CA1 neurons

The recovery from changes in pH_i to resting levels does not occur simply by the passive diffusion of protons across the cellular membrane (Roos & Boron, 1981; Aickin, 1984). Many studies have been performed in invertebrate neurons and vertebrate non-neuronal cell types to identify the mechanisms responsible for the recovery of pH_i to resting levels after internal acid and alkaline loads (*e.g.* Paradiso *et al.*, 1986; Olsnes *et al.*, 1987a; Boyarsky *et al.*, 1988a & 1988b; Frelin *et al.*, 1988; Reinertsen *et al.*, 1988; Ganz *et al.*, 1989; Tønnessen *et al.*, 1990a; Ou-yang *et al.*, 1993; Aniksztejn *et al.*, 1997). These, and many other, studies indicate that pH_i

regulatory mechanisms differ considerably across phyla and amongst tissues (see Lee *et al.*, 1991; Wakabayashi *et al.*, 1997a). Therefore, the present discussion will be limited to a description of only those mechanisms which have been shown to participate in pH_i regulation in rat hippocampal neurons.

Recent studies have identified two electroneutral mechanisms which contribute to acid extrusion from rat hippocampal neurons; an amiloride-*insensitive* Na^+/H^+ exchanger (NHE) and a Na^+-dependent HCO_3^-/Cl^- (*i.e.* anion) exchanger (NDAE) (Raley-Susman *et al.*, 1991; Schwiening & Boron, 1994, Baxter & Church, 1996; Bevensee *et al.*, 1996; Smith *et al.*, 1998). In addition, indirect evidence suggests the presence of an alkali extruding (*i.e.* acid-loading) Na^+-independent HCO_3^-/Cl^- exchanger (NIAE) (Raley-Susman *et al.*, 1993; Baxter & Church, 1996). Both HCO_3^--dependent pH_i regulating mechanisms are inhibited by stilbene derivatives such as 4,4'-diisothiocyanatostilbene-2,2'-disulphonate (DIDS; Schwiening & Boron, 1994; Baxter & Church, 1996). All three mechanisms also exist in a variety of non-neuronal cell types, where their activities have been shown to be highly regulated by pH_o, pH_i and a variety of intracellular second messenger cascades.

3.1 NHE

Since the first characterization of a NHE by Murer *et al.* in 1976, 6 mammalian and the β-trout isoforms of the NHE have been cloned (reviewed by Orlowski & Grinstein, 1997; Wakabayashi *et al.*, 1997a; also see Weinman *et al.*, 1987; Wang *et al.*, 1993; Borgese *et al.*, 1994; Klanke *et al.*, 1995; Bookstein *et al.*, 1996; Numata *et al.*, 1998;). The NHE extrudes one hydrogen ion in exchange for the influx of a single sodium ion; exchange is driven by the transmembrane electrochemical gradients for Na^+ and H^+, without an input of metabolic energy such as ATP hydrolysis (Roos & Boron, 1981; Aronson, 1985). The first NHE to be isolated, NHE-1, has been the focus of most structure-function relationship studies because it is believed to be expressed ubiquitously in most species and types of tissues (Yun *et al.*, 1995; Bookstein *et al.*, 1996). NHE-1 has been shown to form oligomers (Otsu *et al.*, 1989; Fafournoux *et al.*,

1994) and is thought to consist of twelve transmembrane segments (Bookstein *et al.*, 1996; also see Shrode *et al.*, 1998).

The kinetics of NHE-1 are complex, and its activity can be regulated by both pH_o and pH_i (Aronson, 1985; Vaughan-Jones & Wu, 1990). With regard to regulation by pH_i, the exchanger is quiescent at resting pH_i levels but as pH_i becomes more acidic, the exchanger becomes more active (reviewed by Grinstein & Rothstein, 1986; Wakabayashi *et al.*, 1997a). The site at which the kinetics of NHE-1 can be modulated with respect to pH_i has been identified as an internal H^+ binding site(s) on the cytosolic surface of the transmembrane portion of the NHE-1 molecule. This H^+ "modifier" site, which is distinct from the H^+ transport site on the exchanger, permits the allosteric activation of NHE-1 by internal H^+ (Wakabayashi *et al.*, 1994; Yun *et al.*, 1995). Further studies have identified two additional cytosolic sites on NHE-1 which appear to bind the calcium-calmodulin complex (Ca^{2+}-CaM; Bertrand *et al.*, 1994; Wakabayashi *et al.*, 1994 & 1995; Ikeda *et al.*, 1997); interestingly, from the perspective of the present study, Ca^{2+}-CaM also binds to NHE-2, NHE-3 and NHE-4 (Wakabayashi *et al.*, 1997b). Additional binding sites have also been suggested for NHE-3 (an NHE isoform predominantly expressed in the gut and kidney; see Orlowski & Grinstein, 1997) which interact with regulatory binding protein(s) (*e.g.* NHE regulatory factor, NHE-RF, and/or NHE-3 kinase A regulatory protein, E3KARP; Weinman *et al.*, 1993b & 1995; Cabado *et al.*, 1996; Robertson *et al.*, 1997; Yun *et al.*, 1997 & 1998; Hall *et al.*, 1998; Lamprecht *et al.*, 1998). Furthermore, phosphorylation consensus sequences for a variety of protein kinases (*e.g.* cAMP-dependent protein kinase; PKA) have also been identified on the trout β-NHE, NHE-1 and NHE-3 (Fliegel *et al.*, 1992; Fliegel & Frochlich, 1993; Borgese *et al.*, 1994; Bianchini *et al.*, 1997; Zhao *et al.*, 1999).

All NHE isoforms cloned to date are sensitive, to a greater or lesser extent, to the diuretic compound amiloride, its 5-*N*-substituted derivatives such as 5-(*N*-ethyl, *N*-isopropyl)-amiloride and 5-(*N*-methyl, *N*-guanidinocarbonylmethyl)-amiloride, and benzoyl guanidinium compounds (*e.g.* HOE694), which all compete with sodium for the external Na^+ transport site on the exchanger (Clark & Limbird, 1991; Orlowski & Kandasamy, 1996; Counillon *et al.*, 1997;

Orlowski & Grinstein, 1997). In distinct contrast, the NHE isoform present in the hippocampal CA1 neurons to be employed in this study is insensitive to amiloride, amiloride analogs and benzoyl guanidinium compounds (Raley-Susman *et al.*, 1991; Schwiening & Boron, 1994; Baxter & Church, 1996; Smith *et al.*, 1998). It has been suggested that NHE-4 and NHE-5 may be involved in pH$_i$ regulation in the hippocampus. Thus, the former has been localized to the rat hippocampus and mRNA for the latter has been found within mammalian brain, with low levels in the hippocampus (Klanke *et al.*, 1995; Bookstein *et al.*, 1996; Attaphitaya *et al.*, 1999; Baird *et al.*, 1999). However, NHE-4 appears to be activated not by changes in pH$_i$ but by changes in osmolarity, suggesting that it acts predominantly as a cell volume, rather than pH$_i$, regulating mechanism. Furthermore, although both NHE-4 and NHE-5 are *relatively* insensitive to inhibition by amiloride and amiloride analogs (compared to NHE-1), they are both inhibited by micromolar concentrations of amiloride, in contrast to the hippocampal NHE which is completely (up to 1 mM) amiloride-insensitive. Although the NHE isoform present in rat hippocampal neurons has not, to date, been identified, and despite the fact that its pharmacological sensitivities differ from other NHE isoforms, it shares a number of properties with other NHEs, including the ability to transport Li$^+$ in place of Na$^+$ (Raley-Susman *et al.*, 1991).

3.2 NDAE and NIAE

Since the first descriptions of the NDAE (Boron & DeWeer, 1976) and the NIAE (Cabantchik *et al.*, 1978), these HCO$_3^-$-dependent transport mechanisms have been found in a wide variety of cell types (*e.g.* Roos & Boron, 1981; L'Allemain *et al.*, 1985; Olsnes, 1987a; Cassel *et al.*, 1988), although only the NIAE has been cloned. Thus, three isoforms of the NIAE have been identified (AE-1 to 3), and it has been suggested that the AE-3 isoform may be present in rat hippocampal neurons (Kopito *et al.*, 1989; Raley-Susman *et al.*, 1993). The NIAE acts as an acid loader by expelling one bicarbonate ion in exchange for the influx of a single chloride ion in a manner independent of external Na$^+$ (the energy requirement for the exchange process is

supplied by the electrochemical gradients for HCO_3^- and Cl^- alone; see Boyarsky et $al.$, 1988b; Aronson, 1989; Boron & Knakal, 1989). Under extreme conditions, including low extracellular $[Cl^-]_o$ and/or low pH_i (pH_i <6.5), the NIAE may function in reverse mode ($i.e.$ as an acid extruder). Indeed, only reverse-mode NIAE has ever been demonstrated in rat hippocampal neurons (Raley-Susman et $al.$, 1993; Baxter & Church, 1996); no studies have examined forward-mode ($i.e.$ alkali extruding) NIAE activity in this cell type. In contrast to the NIAE, the NDAE acts as an acid extruder by expelling one chloride ion in exchange for the influx of a single bicarbonate ion; the energy requirement for this process is supplied by the electrochemical Na^+ gradient via the influx of sodium ions through coupled or direct mechanism(s) (see Boron, 1985; Aronson, 1989; Boron & Knakal, 1989). The NDAE present in rat hippocampal neurons appears to share the kinetic and pharmacological properties of NDAEs expressed in other cell types (see Schwiening & Boron, 1994).

In peripheral cell types, the activities of both HCO_3^--dependent exchangers have been shown to function in concert in order to accurately maintain SS pH_i at normal values ($e.g.$ Boyarsky et $al.$, 1988b; Cooper & Hunter, 1997). In Vero cells, for example, the NDAE is near quiescent at normal resting pH_i levels and its activity increases with an $acidic$ shift in pH_i; in contrast, although the NIAE is also near quiescence at resting pH_i levels, its activity increases with an $alkaline$ shift in pH_i (Tønnessen et $al.$, 1987 & 1990a; Reinertsen et $al.$, 1988). In a manner analogous to the control of the activity of the NHE, the activities of the NDAE and the NIAE can also be modulated by changes in pH_o (Cohen et $al.$, 1990b; Tønnessen et $al.$, 1990a; Lin & Miller, 1994; Zhang et $al.$, 1996). The pH_i dependence of the activity of the NIAE (AE-2) has been characterized in detail by Olsnes et $al.$ (1987b) and Zhang et $al.$ (1996), who reported not only that this mechanism is most active at high absolute values of pH_i but also that the pH_i-dependence of its activity is mediated by a "pH_i-sensor" analogous to the H^+ "modifier" site on various NHE isoforms (also see Boyarsky et $al.$, 1988b; Mugharbil et $al.$, 1990; Sekler et $al.$, 1995). The structural basis for the pH_i dependence of the NDAE has not been investigated. In addition, and in contrast to many NHE isoforms (see above), no information is available

concerning the structural features of NIAEs or NDAEs which might account for the known ability of intracellular second messengers to modulate their activities.

4. pH_i: a potential signal transduction mechanism

4.1 Modulation of the activities of pH_i regulating mechanisms in non-neuronal cell types

In non-neuronal cell types, the activities of the NHE, NIAE and NDAE have been shown to be highly controlled by a variety of intracellular second messenger systems. The latter include: (1) the cAMP/PKA pathway (Boron et al., 1978; Reuss & Petersen, 1985; Reuss & Stoddard, 1987; Vigne et al., 1988; Green et al., 1990; Helmle-Kölb et al., 1990; Casavola et al., 1992; Green & Kleeman, 1992; Weinman et al., 1993a; Benedetti et al., 1994; Désilets et al., 1994; Emmons & Stokes, 1994; Alvaro et al., 1995 & 1997; Azarani et al., 1996; Alpini et al., 1997; Kurashima et al., 1997; Strazzabosco et al., 1997; Nyberg et al., 1998); (2) the calmodulin(CaM) /Ca^{2+}-CaM-activated protein kinase II (CaMK-II) pathway (Little et al., 1988; Weinman et al., 1988; Emmer et al., 1989; Owen et al., 1989; Weissberg et al., 1989; Green & Kleeman, 1992; Chakraborty et al., 1994; Tanaka et al., 1994; Kawai et al., 1995; Yamada et al., 1996; Cooper & Hunter, 1997; Koren et al., 1997; Le Prigent et al., 1997; Shrode et al., 1997; Siczkowski et al., 1997; Robertson et al., 1997); (3) intracellular Ca^{2+} (Piwnica-Worms et al., 1985; Ober & Pardee, 1987; Green et al., 1990; Kikeri et al., 1990; Muallem & Loessberg, 1990); (4) the phospholipase C/protein kinase C (PKC) pathway (Gaillard & Dupont, 1990; Tønnessen et al., 1990b; Ludt et al., 1991; Casavola et al., 1992; Horie et al., 1992; Rajotte et al., 1992; Cooper & Hunter, 1997; Camilión de Hurtado et al., 1998); (5) receptor protein tyrosine kinases and the mitogen-activated protein kinase (MAPK) pathway (Matsuda et al., 1995; Hooley et al., 1996; Bianchini et al., 1997); (6) protein phosphatases (Grinstein & Rothstein, 1986; Bianchini et al., 1997); and (7) pathways involving GTP-binding proteins (Barber & Ganz, 1992; Davis et al., 1992; Felder et al., 1993; Dhanasekaran et al., 1994; Voyno-Yasenetskaya et al., 1994; Lin & Barber, 1996).

However, the effects of these intracellular messengers on pH_i regulating mechanisms vary and are highly dependent not only on the particular isoform of the exchange protein being studied but also on the cell type or cell line in which the isoform is expressed. For example, increased levels of $[cAMP]_i$ in rabbit kidney brush-border membranes (Weinman *et al.*, 1987), rat brain synaptosomes (Sánchez-Armass *et al.*, 1994) and epithelial cells (Casavola *et al.*, 1992) cause the activity of the NHE to decrease, remain the same and increase, respectively. Similarly, increasing $[cAMP]_i$ in aortic smooth muscle cells (Vigne *et al.*, 1988), rat cardiomyocytes (Désilets *et al.*, 1994) and Vero cells (Tønnessen *et al.*, 1990b) respectively decreases, increases and fails to affect the activity of the NIAE. Stimulation of the CaM/CaMK-II pathway in isolated rabbit proximal tubules (Yamada *et al.*, 1996) and rat astrocytes (Shrode *et al.*, 1995) causes the activity of the NHE to decrease and increase, respectively. Furthermore, the effects of the various signal transduction pathways on the activities of pH_i regulating mechanisms are not mutually exclusive. In cardiac Purkinje fibers, for example, increasing $[cAMP]_i$ evokes a decrease in the activity of the NHE, not by a direct effect involving PKA but via a pathway which involves changes in $[Ca^{2+}]_i$ and stimulation of the CaM/CaMK-II pathway (Wu & Vaughan-Jones, 1994).

The complexity of the regulation of the activities of pH_i regulating mechanisms by second messengers is further underscored by the fact that the regulation of NHE activity by second messengers is often different from the regulation of the activities of HCO_3^-/Cl^- exchangers in the same cell type (*e.g.* Green & Kleeman, 1992). In addition, concomitant regulation of the NHE, NIAE and NDAE by a given intracellular second messenger system varies between cell types. In cultured human intrahepatic bile duct cells, for example, elevation of $[cAMP]_i$ increases the activities of the NDAE and the NIAE (Strazzabosco *et al.*, 1997); in contrast, increasing $[cAMP]_i$ inhibits both the NHE and the NIAE in *Necturus* gallbladder epithelial cells (Reuss, 1987; Reuss & Stoddard, 1987).

In summary, the activities of the NHE, NIAE and NDAE in non-neuronal cell types are highly regulated by intracellular signaling systems. This second messenger regulation, however,

is: (1) highly variable between cell types and exchanger isoforms; (2) may or may not be mutually exclusive; and (3) may have different effects on different exchangers in the same cell type. In distinct contrast to non-neuronal cell types, the regulation of the activities of pH_i regulating mechanisms in central neurons by intracellular second messenger systems has not been rigorously investigated. This is surprising, given the importance of changes in pH_i for neuronal function under both physiological and pathophysiological conditions and the dependence of pH_i on the activities of pH_i regulating mechanisms. Furthermore, given the diversity of second messengers which control the activities of pH_i regulating mechanisms in non-neuronal cell types, it is impossible to predict which second messenger systems might control the activities of the pH_i regulating mechanisms present in a given type of neuron.

4.2 Cellular functions affected by second messenger-evoked changes in the activities of pH_i regulating mechanisms

It has been suggested previously (*e.g.* Frelin *et al.*, 1988) that pH_i is an important second messenger in various cell types. This possibility stems from the facts that: (1) the activities of pH_i regulating mechanisms are highly controlled by surface receptor-linked intracellular signaling cascades; and (2) subsequent exchanger-mediated changes in pH_i can modulate a host of cellular functions. For example, stimulation of Ca^{2+}-linked second messengers such as PKC and CaM and/or tyrosine kinases by growth factors and hormones leads to changes in pH_i regulation which, in turn, regulate growth/ proliferation (Moolenaar *et al.*, 1983; Moolenaar, 1986; Frelin *et al.*, 1988; Grinstein *et al.*, 1989; Wakabayashi *et al.*, 1992; Pappas *et al.*, 1994), DNA synthesis (Pouysségur *et al.*, 1985), cellular metabolism (Roos & Boron, 1981; Busa & Nuccitelli, 1984; Bazaes & Kemp, 1990) and/or cytoskeletal reorganization (Grinstein *et al.*, 1993; Murthy *et al.*, 1998; also see Regula *et al*, 1981). In addition, pH_i regulating mechanisms participate not only in the control of $[H^+]$ and $[HCO_3^-]$ but also in the regulation of $[Na^+]$ and $[Cl^-]$, features of their activities which are involved in the regulation of cell volume (Grinstein *et al.*, 1992; Demaurex & Grinstein, 1994; Shrode *et al.*, 1997) and which may be particularly

important in neurons. Finally, second messenger-evoked changes in the activities of pH_i regulating mechanisms have been causally linked to apoptosis (Rajotte *et al.*, 1992; Li & Eastman, 1995; Chen *et al.*, 1997) and anoxia/ischemia-evoked cellular injury (Ikeda *et al.*, 1988; Yasutake & Avkiran, 1995).

Changes in the activities of pH_i regulating mechanisms by second messengers can also act to modulate G-protein coupled receptor desensitization/resensitization (Kruëger *et al.*, 1997) and the activities of proteins associated with signal transduction cascades, including CaM, inositol triphosphate (IP_3), adenylyl cyclases, phosphodiesterases and calcineurin (Hjemdahl & Fredholm, 1977; Tkachuk & Men'shikov, 1981; Wilson & Gillette, 1985; Huang & Cheung, 1994; Speake & Elliot, 1998). These facts suggest the possibility that changes in pH_i consequent upon second messenger-evoked changes in the activities of pH_i regulating mechanisms may participate in a feedback mechanism which might act to alter the activities of the signal transduction cascades which initially caused them.

Despite the large number of studies which have been conducted in non-neuronal cell types, the possibility that changes in the activities of neuronal pH_i regulating mechanisms might represent a novel and physiologically-relevant means of regulating function in mammalian central neurons has not been explored. The present investigation of the second messenger control of the activities of pH_i regulating mechanisms in rat hippocampal neurons will lay the foundation from which to explore this possibility.

5. Overview

In mammalian central neurons, normal activity and pathological states (such as epilepsy and ischemia) are known to lead to changes in pH_i. In turn, these shifts in pH_i can modulate the events which initially caused them, and may ultimately participate in determining neuronal excitability and survival. In mammalian hippocampal neurons, the acid-extruding mechanisms that participate in pH_i regulation have been characterized; however, alkali extrusion has not been demonstrated. In non-neuronal cell types, second messenger systems alter pH_i by modulating the

activities of acid and alkali extrusion mechanisms; in turn, the changes in pH_i mediate widespread changes in cellular function. However, the possibility that intracellular second messenger systems might affect pH_i regulation in mammalian central neurons has not, to date, been rigorously explored.

In the first part of the present study, alkali extrusion by the NIAE was demonstrated in isolated adult rat hippocampal CA1 neurons. Next, the effects of modulating the activity of the cAMP/PKA second messenger system on SS pH_i and on the activities of the alkali-extruding NIAE and the acid-extruding NHE and NDAE were investigated. Finally, the effects of modulating the activity of the CaM/CaMK-II second messenger system on SS pH_i and the activity of the acid-extruding NHE were characterized, and the potential role of 'cross-talk' between the cAMP/PKA and CaM/CaMK-II was examined. The dual-excitation ratio method was used to measure pH_i employing the fluorescent hydrogen ion indicator 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein.

METHODS AND MATERIALS

1. Cell Preparation

1.1 Acutely dissociated adult rat hippocampal CA1 neurons

Experiments in this study utilized acutely dissociated adult rat hippocampal CA1 neurons. Already well-established in our laboratory (see Smith *et al.*, 1998), the neurons were isolated using a modified version of the procedure described by Köhr & Mody (1991). Male Wistar rats (approximately 40 days old; 180 - 200g) were obtained from the Animal Care Center (University of British Columbia, Vancouver, BC) and housed under conditions of controlled lighting (lights on 0600 - 1800 h) and temperature (20 - 22°C). Water and food (Lab Diet, PMI Feeds Inc., St. Louis, MO) were available *ad libitum*. On the day of an experiment, an animal was anesthetized with 3% halothane in air and decapitated. The brain was rapidly removed, placed in ice-cold (4 - 8°C) HCO_3^-/CO_2-buffered saline (Table 3, solution 11) and one of the hippocampi was removed from the surrounding brain tissue, as outlined by Tyler (1980). Nine to twelve transverse hippocampal slices, 450 μm thick, were obtained with a McIlwain tissue chopper and collected in ice-cold HCO_3^-/CO_2-buffered saline. The slices then were placed in an incubation chamber containing HCO_3^-/CO_2-buffered saline (32°C), and were allowed to recover for at least one hour. For a given experiment, three hippocampal slices were removed from the incubation chamber and enzymatically digested for 30 minutes in 2.0 ml of HCO_3^-/CO_2-buffered saline (32°C) containing 1.5 mg ml^{-1} of pronase (protease type XIV bacterial from *Streptomyces griseus*; Sigma-Aldrich Canada Ltd., Oakville, ON). The CA1 regions of each slice were then microdissected with a dissecting chisel and triturated with fire-polished Pasteur pipettes of diminishing tip diameters (~ 0.8, 0.6, 0.4, and 0.2 mm) in 0.5 ml of HEPES-buffered saline (Table 1, solution 1), pH 7.35 at room temperature (RT; 20-22°C). The tissue suspension was then deposited onto a 18 mm glass coverslip coated with poly-D-lysine (50 μg ml^{-1}; Sigma-Aldrich Canada Ltd.). The neurons were allowed to adhere to the substrate for 30 minutes at RT while exposed to a 100% O_2 atmosphere, during which time they were loaded with either

BCECF or Fura-2 (see Section 2). Although each trituration produced ~ 10 - 30 viable hippocampal CA1 neurons free from cellular debris, pH_i or $[Ca^{2+}]_i$ recordings could be made from only one to four neurons simultaneously in any given experiment. Based on morphological criteria established by Schwiening & Boron (1994) and Bevensee et al. (1996), a given freshly isolated hippocampal CA1 pyramidal neuron was chosen for study (see Fig. 1) if it had: (1) a smooth, non-granular appearance; (2) a soma which was triangular in shape; (3) a single major process (presumably an apical dendrite) projecting from one pole of the soma which was at least three times the length of the diameter of the cell body; and (4) the presence of two or more smaller processes (presumably basal dendrites) at the opposite pole.

1.2 Cultured postnatal rat hippocampal neurons

Calibration experiments (see Section 6.1) were performed using cultured neurons which were prepared and maintained by Ms. S. Atmadja (Department of Physiology, U.B.C.). As described in Sidky & Baimbridge (1997), primary cultures of postnatal hippocampal neurons were prepared from 4 to 5 day old Wistar rat pups. Cultures were maintained in serum-free, N2-supplemented Dulbecco's modified Eagle's medium (Life Technologies, Burlington, ON) at 36°C in 5% CO_2:balance air and were treated with 5-fluorodeoxyuridine to arrest glial cell proliferation. All other experiments employed acutely dissociated adult rat hippocampal CA1 neurons. Calibration experiments were performed in both types of neuronal preparation, and no significant differences between the results were observed (data not shown).

2. Neuron loading with BCECF and Fura-2

The microspectrofluorimetric technique was used to measure pH_i or $[Ca^{2+}]_i$ with the intracellular fluorescent hydrogen or calcium ion indicators, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF; Rink et al., 1982) or Fura-2 (Grynkiewicz et al., 1985; Kao, 1994), respectively. The membrane permeant acetoxymethyl esters of either fluorescent probe (BCECF-AM or Fura-2-AM; Molecular Probes Inc., Eugene, OR) were prepared initially as 1.0

mM or 1.2 mM stock solutions, respectively, in anhydrous dimethylsulphoxide (DMSO) and stored at -60°C. The acetoxymethyl ester form of the dyes is hydrophobic and uncharged; thus, it can pass easily through the plasma membrane and, upon entry into the neurons, is hydrolyzed by intracellular esterases to produce the polyanionic, hydrophilic free acid form of the dyes which is trapped within the neurons.

For the experimental recording of pH_i, 1.0 µl of 1.0 mM BCECF-AM stock solution was pipetted gently onto the plated neurons (which were contained in 0.5 ml of HEPES-buffered saline; Table 1, solution 1). The neurons were then allowed to load with 2.0 µM BCECF-AM for 15 minutes at RT in a 100% O_2 atmosphere. For the experimental recording of $[Ca^{2+}]_i$, 3.0 µl of 1.2 mM Fura-2-AM stock solution was pipetted onto the plated neurons and the neurons were allowed to load with 7.2 µM Fura-2-AM for 30 minutes at 37°C in a 100% O_2 atmosphere.

After the loading procedure was completed, the prepared chamber (containing neurons loaded with BCECF or Fura-2) was placed onto the stage of the microscope (see Section 3). The neurons were then superfused continuously with the initial experimental solution at a rate of 2.4 ml min^{-1} for 15 min at the appropriate temperature prior to the start of each experiment. The temperature-controlled perfusion chamber (shown in Fig. 2A) allowed for the relatively uninterrupted and rapid exchange of experimental perfusion solutions. When perfusing with HCO_3^-/CO_2-buffered media, the atmosphere in the perfusion chamber contained 5% (or 10%) CO_2 in balance air.

3. Experimental setup and the ratiometric method

The dual-excitation ratio method for estimating pH_i or $[Ca^{2+}]_i$ with BCECF or Fura-2 is based upon the relationship between the ratio of emitted fluorescence intensities at alternating wavelengths of excitation at 488 and 452 nm, or 334 and 380 nm, respectively. For BCECF, fluorescence emission during excitation at 488 nm is pH sensitive whereas emission during excitation at 452 nm is relatively pH insensitive. In contrast, for Fura-2, fluorescence emissions during excitation at both 334 and 380 nm are sensitive to changes in $[Ca^{2+}]_i$. In either case, the

emitted fluorescence intensities at both excitation wavelengths are from the same cell volume; thus, the ratio of fluorescence intensities emitted at two different excitation wavelengths will, in principle, not be susceptible to artifacts caused by variation in optical path length, local probe concentrations, illumination intensity and photobleaching (Bright *et al.*, 1987; Bright *et al.*, 1989; Silver *et al.*, 1992).

Throughout experiments using BCECF, the viability of the neurons was assessed by monitoring the stability of the fluorescence emission during excitation at 452 nm (I_{452}, pH_i - insensitive; see Bevensee *et al.*, 1995). According to Bevensee *et al.* (1995), experimental recordings from neurons showing rapid declines $\geq$ 2% min^{-1} in I_{452} indicated degradation of neuronal viability. Hence, in the present study, any experimental recordings failing to meet this criterion were discarded prior to further analysis.

The experimental apparatus, shown in Fig. 2B, consisted of an Attofluor Digital Fluorescence Ratio Imaging System (Atto Instruments Inc., Rockville, MD) used in conjunction with a Zeiss Axiovert 10 microscope (Carl Zeiss Canada Ltd., Don Mills, ON). Ultra violet light from a 100 W mercury arc lamp was alternately passed though one of two 10 nm interference filters centered at 488 and 452 nm, or 334 and 380 nm, depending on the dye used (see Section 2). Each wavelength of filtered light was then reflected by a dichroic beam-splitting mirror (FT > 495 nm for BCECF, 395 nm for Fura-2) into the objective lens (Zeiss LD Achroplan n.a. 0.60, 40x) for excitation of the fluorescent indicator loaded in the neurons. Fluorescence emitted by the neurons was passed back through the objective, the dichroic mirror, and a long band-pass barrier filter (λ > 520 nm for BCECF, 510 nm for Fura-2) to an intensified charge-coupled device camera which measured the intensity of light emitted. The camera gain was set by maximizing the image intensity while minimizing the possibility of camera saturation, and was held constant throughout an experiment. The camera output was digitized to a 512 x 480 pixel frame size with 8 bit resolution and emission intensity measurements were obtained from multiple neuronal somata simultaneously, with each soma delineated as a region of interest (ROI). A mean fluorescence intensity value for each ROI was calculated from fluorescence

intensity values (0 - 255) derived from each pixel within a given ROI (~ 20 x 20 pixels in size). Paired mean fluorescence intensity values (*e.g.* I_{488} and I_{452} for BCECF, and I_{334} and I_{380} for Fura-2) for each ROI were obtained every 3 - 10 s during the course of an experiment. Resulting experimental recordings of fluorescence intensity values were then further analyzed (providing the inclusion criterion was met; see above) to ultimately estimate pH_i or $[Ca^{2+}]_i$ (see Section 6).

In order to prevent UV-induced damage to the neurons and photobleaching of the dye: (1) neutral density filters were placed in the UV light path to reduce the intensity of incident light; (2) neuronal exposure to UV light was limited to image acquisition periods by a computer activated high speed shutter; and (3) the intensity of the 100 W mercury arc lamp was reduced by 50% using a variable intensity lamp control unit (Attoarc, Carl Zeiss Canada Ltd.).

4. Solutions and chemicals

The HEPES-buffered media employed in experiments and in the preparation of acutely dissociated neurons are listed in Tables 1 and 2. All HEPES-buffered solutions were prepared at RT and titrated to appropriate pH values using a Corning 240 pH meter (calibrated daily). Most HEPES-buffered solutions to be utilized at RT were titrated to pH 7.34 - 7.36, and most experimental HEPES-buffered solutions to be utilized at 37°C were titrated to pH 7.47 - 7.49 (at RT) in order for the media to be pH 7.34 - 7.36 at 37°C. For the latter solutions, the pH value to which the HEPES-buffered media were titrated (at RT) was determined by the equation:

$$pH_{37°C} = 0.1809 + (0.9573 \times pH_{RT}),$$ *(Equation 1)*

where $pH_{37°C}$ denotes the pH of the HEPES-buffered medium at 37°C and pH_{RT} represents the titrated pH of HEPES-buffered medium at RT (Baxter, 1995). All HEPES-buffered solutions were titrated with 10 M NaOH except for the high-K^+ calibration medium (Table 1, solution 5), the Na^+-free solutions substituted with $NMDG^+$ (Tables 1 and 2; solutions 2, 7 and 8) or Li^+ (Table 2, solution 10), and the Na^+-free, Cl^--free solution (Table 2, solution 9), which were titrated with 10 M KOH (or 10 M HCl), 10 M HCl, 2.0 M LiOH, and 0.5 M gluconic acid, respectively.

The HCO_3^-/CO_2-buffered solutions employed in experiments and in the preparation of acutely dissociated neurons are listed in Tables 3 and 4. To alter the pH of HCO_3^-/CO_2-buffered media, the concentration of $NaHCO_3$ was adjusted (at a constant P_{CO_2}) according to the equations:

$$pH_{37°C} = 6.017 + (1.036 \times \log [HCO_3^-]), \text{ and} \qquad (Equation\ 2)$$

$$pH_{RT} = 5.833 + (1.039 \times \log [HCO_3^-]), \qquad (Equation\ 3)$$

where $pH_{37°C}$ or pH_{RT} represents the pH of a HCO_3^-/CO_2-buffered solution at 37°C or RT, respectively, following equilibration with 5% CO_2 in balance air (Baxter, 1995). Solutions equilibrated with 10% CO_2 contained twice the $[HCO_3^-]$ as calculated from Equation 2. Changes in $[NaHCO_3]$ were balanced by equimolar changes in $[NaCl]$.

The stock solutions of the pharmacological agents employed are listed in Table 5. These were prepared in advance except for 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), which was prepared on the day of the experiment. Stock solutions were further diluted to the appropriate test concentration using experimental media on the day of the experiment. The final concentration of DMSO in the test solutions did not exceed 0.4%. Appropriate control experiments have been performed with DMSO and the results indicate that this agent evokes no significant changes in either SS pH_i or on the rate of pH_i recovery from imposed intracellular acid load loads (see Smith, 1996).

5. Experimental paradigms

Most experiments performed in this study involved examining the effects of a pharmacological agent and/or the addition or removal of a specific ion (e.g. Cl^- or Ca^{2+}) on SS pH_i. It has been shown in mesangial cells that the addition of arginine vasopressin elicits similar changes in the activities of opposing (i.e. acid loading and acid extruding) pH_i regulating mechanisms such that SS pH_i was affected minimally despite the fact that the activities of pH_i regulating mechanisms were altered (see Boyarsky et al., 1988a; Ganz et al., 1989). Therefore, the effects of a test treatment on pH_i recovery from acute internal acid loads or (if appropriate)

acute internal alkaline loads were also examined to directly assess changes in the activities of pH_i regulating mechanisms, regardless of whether a change in SS pH_i was observed or not. These SS pH_i and pH_i recovery experiments were performed under various conditions in order to examine the effect of a test treatment on the activity of either the NHE, NIAE or the NDAE in isolation.

5.1 Na^+/H^+ exchange

To examine the effects of a given treatment on Na^+/H^+ exchange independent of HCO_3^- /Cl^- exchange, experiments were performed under nominally HCO_3^-/CO_2-free, HEPES-buffered conditions. When a change in SS pH_i was observed, the potential involvement of the NHE was initially assessed by removing $[Na^+]_o$ (iso-osmotically replaced with N-methyl-D-glucamine, $NMDG^+$; Table 1, solution 2) to inhibit Na^+/H^+-exchange, as a pharmacological blocker of NHE in rat hippocampal neurons has not been identified (Raley-Susman et al., 1991; Schwiening & Boron, 1994; Baxter & Church, 1996; Bevensee et al., 1996). The effect of a test treatment on SS pH_i under 0 $[Na^+]_o/NMDG^+$-substituted conditions was then compared to observations made during perfusion with 0 $[Na^+]/Li^+$-substituted medium (Table 2, solution 10; in contrast to $NMDG^+$, Li^+ can act as a substrate for the NHE; Aronson et al., 1983; Wakabayashi et al., 1997a). Also, acid load recovery experiments were performed to quantitatively assess possible treatment-induced changes in the activity of the NHE.

Acid load recovery. Intracellular acidification was induced (without changing pH_o) by the NH_4^+ prepulse technique (Boron & De Weer, 1976; Boron, 1977; Boron, 1989) which involves acute exposure and subsequent removal of a medium containing the weak base, NH_4^+ (Table 1, solution 4; Table 3, solution 14; Table 4, solution 19). As illustrated in Fig. 3A: (1) Upon exposure to NH_4^+, the initial SS pH_i alkalinizes due to entry of NH_3 which forms NH_4^+ within the neuron; (2) In the continued presence of NH_4^+, a gradual acidification occurs because NH_4^+ follows its electrochemical gradient into the neuron causing the formation of NH_3/H^+ and/or acid-loading mechanisms are activated; (3) Upon the removal of external NH_4^+, pH_i rapidly acidifies to a pH_i value which undershoots the initial SS value because NH_3 rapidly exits the

neuron leaving H^+ behind; (4) After the maximum point of acidification is reached, pH_i recovers to initial SS levels due to the activities of acid-extruding pH_i regulating mechanisms (*e.g.* Na^+/H^+-exchange and/or Na^+-dependent HCO_3^-/Cl^--exchange, depending on experimental conditions; Roos & Boron, 1981; Boyarsky *et al.*, 1988a & 1988b; Boron, 1989; Schwiening & Boron, 1994; Baxter & Church, 1996; Smith *et al.*, 1998). This final recovery phase was employed to calculate rates of pH_i recovery, which provide estimates of the activities of acid-extruding pH_i regulating mechanisms (see Section 6.3).

5.2 HCO_3^-/Cl^--exchange

All experiments employed to examine the activities of HCO_3^--dependent mechanisms were performed under HCO_3^-/CO_2-buffered conditions. Initially, the effect of a test treatment on SS pH_i was examined. Any treatment-induced changes in SS pH_i were then compared to results observed in the presence of DIDS (an inhibitor of anion exchangers; Boyarsky *et al.*, 1988b; Schwiening & Boron, 1994), the absence of $HCO_3^-_o$ (see Section 5.1), and/or the absence of Na^+_o (choline-substituted; Table 3, solution 12), in order to determine if the activities of the NIAE and/or NDAE contribute to the SS pH_i response.

5.2.1 Na^+-independent HCO_3^-/Cl^--exchange.
The activity of the NIAE, an alkali extrusion mechanism, was assessed in two different ways: Firstly, the acute (~3 min) removal of extracellular Cl^- (Table 3, solutions 13 and 15) was used to reverse the activity of the NIAE, thus causing the NIAE to act as an acid extruder (see Boyarsky *et al.*, 1988b; Kramhøft *et al.*, 1994). Paired 0 Cl^-_o experiments were performed in the presence and absence of a test treatment and the changes in SS pH_i observed in the absence of Cl^-_o under each condition were compared to assess the effect of the test treatment on the activity of the NIAE. Secondly, alkaline load experiments (under both Na^+_o-containing and -free conditions) were performed to quantitatively assess the effects of a test treatment on the activity of the NIAE (Ganz *et al.*, 1989). The results from both experimental series were compared to parallel results obtained in the presence of DIDS.

Alkaline load recovery. Intracellular alkalization was induced (without changing pH_o) by the acute exposure and subsequent removal of a medium containing high concentrations of HCO_3^- and CO_2 (Table 4, solutions 16 and 17; see Ganz *et al.*,1989; Mellergård *et al.*, 1993). As illustrated in Fig. 3B: (1) Upon exposure to high $[HCO_3^-/CO_2]_o$, the initial SS pH_i acidifies slightly due rapid CO_2 entry into the neuron; (2) In the continued presence of an elevated $[HCO_3^-/CO_2]_o$, a sustained alkalinization is observed because CO_2 continues to enter the neuron (thus increasing $[HCO_3^-]_i$) and HCO_3^- influx occurs via membrane transport mechanisms; (3) Upon the removal of the high $[HCO_3^-/CO_2]$ medium, pH_i rapidly alkalinizes to a pH_i value which overshoots the SS value observed in the presence of an elevated $[HCO_3^-/CO_2]_o$ because CO_2 rapidly exits the neuron leaving behind elevated $[HCO_3^-]_i$; (4) After the maximum point of alkalinization is reached, pH_i recovers to initial SS levels due to the activities of alkali-extruding pH_i regulating mechanisms (*e.g.* the NIAE; Ganz *et al.*,1989; Raley-Susman *et al.*, 1993). This final recovery phase was further analyzed to assess the activity of the NIAE (see Section 6.5).

5.2.2 Na^+-dependent HCO_3^-/Cl^--exchange. Assessment of NDAE activity was accomplished by using two procedures: (1) Acid load recovery experiments (see Section 5.1) were performed in the presence of a test treatment at 37°C and RT (see Table 4, solutions 18 and 19). Room temperature is a condition under which the activity of the NHE is reduced and the NDAE is the dominant contributor to the recovery of pH_i from imposed internal acid loads (Baxter & Church, 1996). Results obtained under these conditions were compared to those observed in the presence of DIDS or the absence of HCO_3^-; any differences between the rates of pH_i recovery were assumed to be caused by changes in the activity of the NDAE (Raley-Susman *et al.*, 1991; Schwiening & Boron, 1994; Baxter & Church, 1996; Smith *et al.*, 1998). (2) At RT, $[Cl^-]_i$-depletion experiments were performed by repetitively exposing neurons (3 to 4 times), initially perfused with a Cl^--free, HEPES-buffered solution (Table 2, solution 6; used at RT), to HCO_3^-/CO_2-buffered medium also devoid of external Cl^- (Table 4, solution 20). As described by Schwiening & Boron (1994) and Bevensee *et al.* (1997), repeated stimulation of Na^+-dependent HCO_3^-/Cl^- exchange with addition of HCO_3^-/CO_2 leads to a decrease in $[Cl^-]_i$ after each

successive application until [Cl⁻]$_i$ is low enough to subsequently inhibit Na⁺-dependent anion exchange (which has an absolute requirement for Cl⁻$_i$). Parallel experiments were performed in the presence of a test treatment and any changes in the pH$_i$ response to Cl⁻$_i$-depletion were interpreted as being caused by changes in the activity of the NDAE (see Boyarsky *et al.*, 1988b; Mellergård *et al.*, 1993; Schwiening & Boron, 1994; Bevensee *et al.*, 1997). As the activities of both the NHE and the NDAE are dependent on Na⁺$_o$, and as a specific inhibitor for the NDAE is not available, the Na⁺$_o$-dependence of any effect due to the activity of the NDAE was not tested.

6. Data analysis

6.1 Calculation of pH$_i$

Initially, background-corrected ratio values (*BI$_{488}$/BI$_{452}$*) were calculated for each ROI by subtracting the respective background fluorescence intensity values (measured in a region devoid of cellular elements) from the recorded fluorescence intensity values at each excitation wavelength using Visual Basic macros running in Microsoft Excel v.5.0 (Microsoft Corp., Redmond, WA.). A normalizing ratio corresponding to pH$_i$ 7.00 was then calculated for each ROI by averaging the background-corrected ratios of emitted intensities recorded during a calibration period. This one point calibration was performed at the end of each experiment by exposing the neuron(s) to a high-K⁺, HEPES-buffered calibration solution (pH 7.00; Table 1, solution 5) containing 10 μM nigericin (Thomas *et al.*, 1979) for 5-10 minutes (*e.g.* Fig. 3A) at the appropriate experimental temperature. Nigericin, a K⁺/H⁺ ionophore, balances the ionic concentrations of cytoplasmic and extracellular K⁺ and, in doing so, equilibrates pH$_o$ to pH$_i$ (Chaillet and Boron, 1985).

Normalized ratio values were then calculated by dividing the experimentally-derived background-corrected ratio value (*BI$_{488}$/BI$_{452}$*) by the normalizing ratio values corresponding to pH 7.00. These resulting normalized ratio values were then converted to corresponding pH$_i$ values using equation 4, which is based on the Henderson-Hasselbalch equation for the dissociation of a weak acid:

$$pH_i = \log [(R_n - R_{n(min)} / (R_{n(max)} - R_n)] + pK_a, \qquad (\textit{Equation 4})$$

where R_n represents the normalized ratio value being converted to pH_i, $R_{n(min)}$ and $R_{n(max)}$ represent the minimum and maximum obtainable values for the normalized ratio, and pK_a represents the -log of the dissociation constant of BCECF (see Baxter, 1995). *In situ* calibration experiments were performed to derive values for $R_{n(max)}$, $R_{n(min)}$ and pK_a. Such experiments involved exposing neurons to a series of high-K^+, HEPES-buffered solutions (each containing 10 μM nigericin) titrated to ~pH 5.5 to 8.5 in ~0.5 pH unit increments (Fig. 4A). Normalized ratio values were calculated (see above) and a pH titration curve was produced (Fig. 4B). The lower and upper asymptotes of the resulting curve represent the $R_{n(min)}$ and $R_{n(max)}$, respectively, and the pH_i value corresponding to the point of inflection of the curve represents the pK_a of BCECF. The calibration parameters were not affected by temperature and were reassessed whenever the optical setup of the microscope was altered (*e.g.* replacement of the mercury arc lamp). Fourteen full calibration experiments were performed during this study and the average values for $R_{n(min)}$, $R_{n(max)}$ and pK_a (mean ± S.E.M.) were 0.43 ± 0.02, 1.89 ± 0.04 and 7.19 ± 0.02, respectively.

6.2 Estimation of [Ca^{2+}]$_i$

6.2.1 Presentation of Fura-2 data. When using Fura-2, background-corrected ratio values (BI_{334}/BI_{380}) were calculated by subtracting the respective background intensity values from recorded fluorescence intensity values at each excitation wavelength using Visual Basic macros running in Microsoft Excel. For most experiments, transformation of the BI_{334}/BI_{380} ratio values was not attempted, due to the facts that the affinity of Fura-2 for Ca^{2+} is dependent upon pH and that pH_i was often changing continuously during a given experiment (see Martinez-Zaguilán *et al.*, 1991; Church *et al.*, 1998). Therefore relative changes in [Ca^{2+}]$_i$ are presented as changes in BI_{334}/BI_{380} ratio values.

6.2.2 Estimation of [Ca^{2+}]$_i$. Under some experimental conditions (*e.g.* treatment with W-7, an inhibitor of calmodulin) where changes in both pH_i and [Ca^{2+}]$_i$ were observed, the inter-relationship between pH_i and [Ca^{2+}]$_i$ was further examined, and, thus, estimation of [Ca^{2+}]$_i$ was

required. As the dissociation constant (K_d) of Fura-2 for Ca^{2+} is sensitive to pH (see above), mean observed SS pH_i levels were used to estimate the K_d of Fura-2 for Ca^{2+} and, thus, allowed calculation of associated mean $[Ca^{2+}]_i$ values obtained from parallel experiments. With the application of pH-dependent corrections for the K_d of Fura-2 for Ca^{2+} (see Church *et al.*, 1998), BI_{334}/BI_{380} intensity ratio values representing $[Ca^{2+}]_i$ were converted to $[Ca^{2+}]_i$ values (at estimated pH_i values), using the equation:

$$[Ca^{2+}]_i = \beta \times K_d (R - R_{min}) / (R_{max} - R), \qquad (Equation\ 5)$$

where R represents the ratio value (BI_{334}/BI_{380}) being converted to $[Ca^{2+}]_i$, R_{min} and R_{max} represent the minimum and maximum obtainable values for the ratio, and β is a proportionality coefficient representing the ratio of $BI_{380(min)}$ to $BI_{380(max)}$ (Grynkiewicz *et al.*, 1985). Under all experimental conditions, the values for R_{min}, R_{max}, and β were estimated to be 0.15, 8, and 20, respectively (Martinez-Zaguilán *et al.*, 1991; Church *et al.*, 1998). However, depending on the experimental condition (and its effect on pH_i), the value for K_d was adjusted appropriately, *e.g.* at $pH_i = 7.35$, $K_d = 132.2$ (Abdel-Hamid, 1994).

6.3 The recovery of pH_i from imposed intracellular acid loads

Initially, the recovery portion of a given acid load experiment was fitted (using the least squares method; SigmaPlot v.3.0, Jandel Scientific, San Rafael, CA; see Fig. 5A) to a single exponential function of time having the form:

$$pH_i = a + b(1 - e^{-ct}), \qquad (Equation\ 6)$$

where *a* represents the pH_i at the point of maximum acidification ($pH_{i,t=0}$), *b* is the pH_i range of the recovery ($pH_{i,max} - pH_{i,t=0}$) and *c* is the time constant. The first derivative of Equation 6 yields the rate of change in pH_i at any given time:

$$\frac{dpH_i}{dt} = bc\ e^{-ct}, \qquad (Equation\ 7)$$

from which the instantaneous rates of pH_i recovery (dpH_i/dt) were calculated at 0.05 pH unit intervals from $pH_{i,t=0}$ to ~75% of $pH_{i,max}$ (*i.e.* 75% of pH_i recovery; Baxter, 1995).

All internal acid loads were performed in paired experiments; the first intracellular acid load was performed under control conditions and the second was performed in the presence of a test treatment. In this way, control rates of pH_i recovery could be compared to the rates of pH_i recovery in the presence of a test treatment within a single given neuron. Rates of pH_i recovery under control and test conditions were compared at the same absolute pH_i values as it has been shown that the activities of pH_i regulating mechanisms are dependent on pH_i (see Introduction). For each experiment in which paired internal acid loads were imposed, percentage difference values were calculated between the control and treatment rates of pH_i recovery at each absolute value of pH_i (in 0.05 pH unit increments). The average of the resultant percentage difference values was then calculated and used to describe the overall effect of a given treatment on rates of pH_i recovery.

It has previously been reported that, under HEPES-buffered conditions at 37°C, acutely dissociated adult rat hippocampal CA1 neurons can be classified into those which have low resting pH_i values (and which recover slowly from imposed internal acid loads) and those which have high resting pH_i values (and which recover more quickly from imposed internal acid loads; see Bevensee *et al.*, 1996). A similar classification was used in the present study because resting pH_i values for 517 neurons measured under HCO_3^-/CO_2-free, HEPES-buffered conditions were best fitted with the sum of two Gaussian distributions with means ($\pm$ S.E.M.) at $pH_i = 6.90 \pm 0.02$ and $pH_i = 7.37 \pm 0.01$ (see Results, Section 1, Fig. 7B). In addition, 'low' and 'high' resting pH_i neurons exhibit markedly different rates of pH_i recovery from imposed acid loads (*e.g.* Fig. 5B). Thus, rates of pH_i recovery were analyzed separately in neurons with initial resting pH_i values of ≤ 7.3 or > 7.35 under HEPES-buffered conditions at 37°C. However, under all other experimental conditions, rates of pH_i recovery were pooled in a single group because: (1) Although a similar bimodal distribution of resting pH_i values was observed in neurons perfused with HEPES-buffered medium at RT (mean $\pm$ S.E.M. values centered at $pH_i = 6.90 \pm 0.02$ and 7.23 ± 0.03; $n = 33$; Results, Section 1, Fig. 7D), rates of recovery of pH_i from acid loads imposed on all neurons were similar (*e.g.* Results, Section 2). (2) Neurons perfused with HCO_3^-

/CO_2-buffered media at either 37°C or at RT had resting pH_i values that were best represented by single Gaussian distributions (means ± S.E.M.s at pH_i = 7.20 ± 0.01, n = 370 and pH_i = 7.14 ± 0.06, n = 17, respectively; see Results, Section 1, Fig. 7A and C, respectively) and all neurons exposed to acute acid loads showed similar control rates of pH_i recovery at a given temperature (*e.g.* Results, Section 2).

In order to assess the intrinsic differences between rates of pH_i recoveries following two consecutive internal acid loads, rates of pH_i recovery were calculated for 20 control paired acid load recovery experiments where both acid loads were performed in the absence of any form of treatment under HEPES-buffered conditions at 37°C (see Fig. 3A). The neurons were classified into 'high' and 'low' resting pH_i groups and, for each group, the average rates of pH_i recovery at corresponding absolute pH_i values were then calculated separately for each acid load recovery (see Fig. 5B). Percentage difference values were calculated between the averaged first and second control rates of pH_i recovery at each absolute value of pH_i and the means (± S.D.) of the resultant percentage differences were -1 ± 29 % (n = 3) and 22 ± 16 % (n = 17) for the 'high' and 'low' resting pH_i groups, respectively. These mean percentage difference values were then utilized to establish criteria which determined whether a neuron did or did not respond to a given treatment (see Smith, 1996; Smith *et al.*, 1998). For neurons with high resting pH_i values, the mean percentage difference between the control and treatment rates of pH_i recovery had to be > 57% or > -59% (mean ± 2 S.D.) in order for a neuron to be classified as a 'responder'. For neurons with low resting pH_i values, the mean percentage difference between the control and treatment rates of pH_i recovery had to be > 54% or > -10% (mean ± 2 S.D.) in order for a neuron to be classified as a 'responder'. If the test treatment *failed* to cause a 'response' in all neurons examined, then data from all of these neurons underwent further analysis. However, if the test treatment *succeeded* in causing a 'response' in some neurons examined, then only data from 'responding' neurons underwent further analysis.

After sorting, where appropriate, neurons into low or high resting pH_i groups and after determining which neurons would undergo further analysis (see above), average rates of pH_i

recovery were calculated at each absolute value of pH_i (in 0.05 pH unit increments) for both the control and treatment acid load recoveries. At this point, 3 additional procedures were performed: (1) Percentage difference values were calculated between the averaged control and treatment rates of pH_i recovery at each absolute value of pH_i; the mean of the resultant percentage difference values was employed to describe the overall effect of a test treatment on rates of pH_i recovery; (2) The average rates of pH_i recovery in the absence and presence of a test treatment were plotted against corresponding absolute values of pH_i, and linear regression plots were fit to each data set using SigmaPlot 3.0 (*e.g.* Fig. 5B). The resulting linear plots were employed to estimate treatment-induced changes in both exchange activity (represented by a change in slope) and the pH_i set-point of the exchange mechanism (represented by a change in the x-intercept; see Paris & Pouysségur, 1984; Aronson, 1985; Grinstein *et al.*, 1986; Grinstein *et al.*, 1989; Levine *et al.*, 1993; Copper & Hunter, 1994; Wakabayashi *et al.*, 1997a); and (3) The statistical difference between control and treatment rates of pH_i recovery was assessed at each absolute pH_i value using paired two-tailed Student's *t*-tests. In order for a test treatment to have had a significant effect on the rate of pH_i recovery at a given pH_i value, the calculated *P* value had to be < 0.05.

6.4 Estimation of Fluxes

In experiments in which internal acid loads were imposed under HCO_3^-/CO_2-free, HEPES-buffered conditions at 37°C, rates of pH_i recovery were converted to net acid-equivalent (or H^+) fluxes (see Roos & Boron, 1981; Boyarsky *et al.*, 1988a; Boron, 1989) using the equation:

$$J_{total} = dpH_i /dt \times \beta_i, \qquad\qquad (Equation\ 8)$$

where J_{total} represents the net flux of H^+, dpH_i/dt is the rate of pH_i recovery from an acid load at a given pH_i value and β_i represents the intrinsic intracellular buffering power at that pH_i value. Bevensee *et al.* (1996) reported previously that in acutely dissociated rat hippocampal CA1 neurons under HEPES-buffered conditions at 37°C, β_i varies with pH_i according to the equation:

$$\beta_i = 94.45 - (pH_i \times 11.28),\qquad\qquad\qquad (Equation\ 9)$$

Fluxes were calculated only for experiments performed under HEPES-buffered conditions at 37°C (see Fig. 5C), as estimates of the pH_i-dependence of β_i are not available for acutely dissociated rat hippocampal CA1 neurons at RT or under HCO_3^-/CO_2-buffered conditions.

6.5 The recovery of pH$_i$ from imposed intracellular alkaline loads

Using a similar procedure as that used for acid load recovery data (see Section 6.3), the recovery portion of a given alkaline load experiment was fitted (using the least squares method; see Fig. 6A) to a single exponential decay function of time having the form:

$$pH_i = a + b(10^{-ct}),\qquad\qquad\qquad (Equation\ 10)$$

where a represents the pH_i at the point of maximum alkalization ($pH_{i,t=0}$), b is the pH_i range of the recovery ($pH_{i,t=0}$ - $pH_{i,min}$) and c is the time (or decay) constant. A variety of exponential decay functions were fitted to the data and the function shown as equation 10 provided the most accurate fit to the experimental data ($n = 26$, $r^2 = 0.98 \pm 0.02$ (mean $\pm$ S.E.M.); see Fig. 6B for residuals). The first derivative of Equation 10 yields equation 7 from which the instantaneous rates of pH_i recovery (dpH_i/dt) were calculated at 0.05 pH unit intervals from $pH_{i,t=0}$ to ~75% of $pH_{i,min}$ (i.e. 75% of pH_i recovery). Analysis was then identical to the procedures used to analyze acid load recovery experiments (see Section 6.3; also see Tønnessen et al., 1990a; Ludt et al., 1993; Zhang et al., 1996). Only paired alkaline load experiments were performed and only HCO_3^-/CO_2-buffered media (at 37°C) were used. Thirteen control paired alkaline load recovery experiments (see Fig. 3B) were used to derive the criterion for the identification of neurons 'responding' to a given treatment. Neurons (under Na^+_o-containing or Na^+_o-free conditions) were classified as 'responders' if the mean percentage difference between the control and treatment rates of pH_i recovery was > 21% or > -19% (= mean intrinsic percentage difference between control rates of pH_i recovery $\pm$ 2 S.D.; $n = 13$). Although the recovery of pH_i from alkaline loads is driven by acid loading mechanisms, whereas acid load recovery is driven by acid extruding mechanisms, rates of pH_i recovery from an alkaline loads were reported as positive values (rather

than negative; see Fig. 6C). This form of presentation was chosen because it better represents the activity of the NIAE (*i.e.* an increase in the rate of pH_i recovery = an increased NIAE activity).

6.6 Statistics

All statistical comparisons were made using two-tailed Student's *t* tests (paired or unpaired, as appropriate). If the resulting *P* value was < 0.05, then the 2 groups under investigation were considered significantly different. Errors are expressed as S.E.M., except where noted, and all reported linear regression lines have r^2 values > 0.75. All reported *n* values represent the number of neurons from which data were obtained. On average, each rat sacrificed yielded three to four neurons from which data were obtained; of these, ~50% were not formally analyzed in light of the exclusion criteria outlined above.

7. Data presentation

Each pH_i or *BI_{334} /BI_{380}* vs. time trace illustrated represents data obtained from a single neuron. In order to minimize variations in emission intensity ratio values (and, subsequently, pH_i) caused by brief fluctuations in the intensity of the UV light output from the 100 W mercury arc burner, moving averages (period = 5) were applied to all pH_i or *BI_{334} /BI_{380}* vs. time traces shown (see Boyarsky *et al.*, 1988a; Baxter and Church, 1996; Smith *et al.*, 1998).

Table 1. Composition of HEPES-buffered experimental solutions
(all concentrations are in mM):

	Solution				
	1 Standard	*2* Na$^+$-free (NMDG$^+$)	*3* Ca^{2+}-free*	*4* NH$_4$Cl	*5* High K$^+$
NaCl	136.5	-	136.5	96.5	-
KCl	3.0	3.0	3.0	3.0	-
CaCl$_2$	2.0	2.0	-	2.0	2.0
NaH$_2$PO$_4$	1.5	-	1.5	1.5	1.5
MgSO$_4$	1.5	1.5	3.5	1.5	1.5
Na Glu	-	-	-	-	10.0
K Glu	-	-	-	-	129.5
$^1/_2$Ca Glu	-	-	-	-	-
D-glucose	17.5	17.5	17.5	17.5	17.5
NMDG$^+$	-	138.0	-	-	-
NH$_4$Cl	-	-	-	40.0	-
HEPES	10.0	10.0	10.0	10.0	10.0
LiCl	-	-	-	-	-
Titrated with:	10 M NaOH	10 M HCl	10 M NaOH	10 M NaOH	10 M KOH or 10 M HCl

* Ca^{2+}-free medium also contained 200 μM ethylene glycol-bis(β-aminoethyl ether) *N, N, N', N'*-tetraacetic acid (EGTA).

All solutions were titrated at RT to the appropriate experimental pH (see Section 4). Solutions 2 and 3 were exclusively used at 37°C.

Abbreviations: Na Glu, sodium gluconate; K Glu, potassium gluconate; $^1/_2$Ca Glu, hemi-calcium gluconate; NMDG$^+$, *N*-methyl-D-glucamine; HEPES, *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid].

Table 2. Composition of HEPES-buffered experimental solutions
(all concentrations are in mM):

	Solution				
	6 Cl⁻-free	7 Na^+, K^+-free	8 Na^+, Mg^{2+}-free	9 Na^+, Cl⁻-free	10 Na^+-free (Li^+)
NaCl	-	-	-	-	-
KCl	-	-	3.0	-	3.0
$CaCl_2$	-	2.0	2.0	-	2.0
NaH_2PO_4	1.5	-	-	-	-
$MgSO_4$	1.5	1.5	-	1.5	1.5
Na Glu	136.5	-	-	-	-
K Glu	3.0	-	-	3.0	-
$^1/_2$Ca Glu	4.0	-	-	4.0	-
D-glucose	17.5	17.5	17.5	17.5	17.5
$NMDG^+$	-	141.0	139.5	138.0	-
NH_4Cl	-	-	-	-	-
HEPES	10.0	10.0	10.0	10.0	10.0
LiCl	-	-	-	-	138.0
Titrated with:	10 M NaOH	10 M HCl	10 M HCl	0.5 M Glu acid	2 M LiOH

All solutions were titrated at RT to the appropriate experimental pH (see Section 4). Solution 6 was exclusively used at RT; solutions 7 - 10 were exclusively used at 37°C.

Abbreviations: Na Glu, sodium gluconate; K Glu, potassium gluconate; $^1/_2$Ca Glu, hemi-calcium gluconate; $NMDG^+$, N-methyl-D-glucamine; Glu acid, gluconic acid; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid].

Table 3. Composition of HCO_3^-/CO_2-buffered experimental solutions
(all concentrations are in mM):

	Solution				
	11 Standard	*12* Na$^+$-free	*13* Cl$^-$-free	*14* NH$_4$Cl	*15* Na$^+$, Cl$^-$-free
NaCl	126.5	-	-	86.5	-
NaHCO$_3$	20.0	-	20.0	20.0	-
KCl	3.0	3.0	-	3.0	-
CaCl$_2$	2.0	2.0	-	2.0	-
NaH$_2$PO$_4$	1.5	-	1.5	1.5	-
MgSO$_4$	1.5	1.5	1.5	1.5	1.5
D-glucose	17.5	17.5	17.5	17.5	17.5
Na Glu	-	-	126.5	-	-
K Glu	-	-	3.0	-	3.0
$^1/_2$Ca Glu	-	-	4.0	-	4.0
NH$_4$Cl	-	-	-	40.0	-
Chol HCO$_3$	-	20.0	-	-	20.0
Chol Cl	-	126.5	-	-	-
Chol base	-	-	-	-	126.5
Glu acid	-	-	-	-	126.5

All HCO_3^--containing solutions were equilibrated with 5% CO_2 in balance air. The final pH of the experimental media was 7.35 at 37°C.

Abbreviations: Na Glu, sodium gluconate; K Glu, potassium gluconate; $^1/_2$Ca Glu, hemi-calcium gluconate; Chol HCO$_3$, choline bicarbonate; Chol Cl, choline chloride; Chol base, choline free-base; Glu acid, gluconic acid.

Table 4. Composition of HCO_3^-/CO_2-buffered experimental solutions
(all concentrations are in mM):

	Solution				
	16 ↑ HCO_3^-*	*17* ↑ HCO_3^-, Na^+-free*	*18* Standard @ RT	*19* NH_4Cl @ RT	*20* Cl^--free @ RT
NaCl	106.5	-	117.5	77.5	-
$NaHCO_3$	40.0	-	29.0	29.0	29.0
KCl	3.0	3.0	3.0	3.0	-
$CaCl_2$	2.0	2.0	2.0	2.0	-
NaH_2PO_4	1.5	-	1.5	1.5	1.5
$MgSO_4$	1.5	1.5	1.5	1.5	1.5
D-glucose	17.5	17.5	17.5	17.5	17.5
Na Glu	-	-	-	-	117.5
K Glu	-	-	-	-	3.0
$^1/_2$Ca Glu	-	-	-	-	4.0
NH_4Cl	-	-	-	40.0	-
Chol HCO_3	-	40.0	-	-	-
Chol Cl	-	106.5	-	-	-
Chol base	-	-	-	-	-
Glu acid	-	-	-	-	-

All HCO_3^--containing solutions were equilibrated with 5% CO_2 in balance air, with exception of ↑ HCO_3^- media (*) which were equilibrated with 10% CO_2 in balance air. The final pH of solutions 16 and 17 was 7.35 at 37°C; the final pH of solutions 18 - 20 was 7.35 at RT.

Abbreviations: Na Glu, sodium gluconate; K Glu, potassium gluconate; $^1/_2$Ca Glu, hemi-calcium gluconate; Chol HCO_3, choline bicarbonate; Chol Cl, choline chloride; Chol base, choline free-base; Glu acid, gluconic acid; @ RT, at room temperature.

Table 5. Compounds employed in this study:

Agent	[Stock] mM	Solvent	Storage Temp. (°C)	Supplier
Nigericin	10	ethanol	-60	Sigma-Aldrich Canada Ltd. (Oakville, ON)
DIDS	100	DMSO	-	Sigma-Aldrich Canada Ltd. (Oakville, ON)
Forskolin	50	DMSO	-60	Research Biochemicals International (Natick, MA)
IBMX	50	DMSO	-60	Research Biochemicals International (Natick, MA)
DDF	50	DMSO	-60	Research Biochemicals International (Natick, MA)
DDA	50	DMSO	-60	Biomol Research Laboratories Inc. (Plymouth Meeting, PA)
Sp-cAMPS	25	water*	-20	Biolog Life Science Institute (La Jolla, CA)
Rp-cAMPS	50	water*	-20	Biolog Life Science Institute (La Jolla, CA)
Okadaic Acid	1	water*	-60	Research Biochemicals International (Natick, MA)

* Ultrapure distilled water (Milli-Q UF Plus, Millipore Ltd., Mississauga, ON).

Abbreviations: DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; IBMX, 3'-isobutyl-1-methylxanthine; DDF, 1',9'-dideoxyforskolin; DDA, 2',5'-dideoxyadenosine; Sp-cAMPS, Sp-adenosine-3',5'-cyclic phosphorothioate; Rp-cAMPS, Rp-adenosine-3',5'-cyclic phosphorothioate.

Table 5 (continued). Compounds employed in this study:

Agent	[Stock] (mM)	Solvent	Storage Temp. (°C)	Supplier
W-7	50	ethanol:water* (1:1, v/v)	-60	Biomol Research Laboratories Inc. (Plymouth Meeting, PA)
TFP	50	water*	-60	Biomol Research Laboratories Inc. (Plymouth Meeting, PA)
FK-506	5	DMSO	-60	Calbiochem-Novabiochem Corp. (La Jolla, CA)
KN-62	10	DMSO	-60	Biomol Research Laboratories Inc. (Plymouth Meeting, PA)
KN-93	10	DMSO	-60	Research Biochemicals International (Natick, MA)
KN-92	10	DMSO	4	Research Biochemicals International (Natick, MA)
4Br-A23187	1	DMSO	-60	Molecular Probes Inc. (Eugene, OR)

* Ultrapure distilled water (Milli-Q UF Plus, Millipore Ltd., Mississauga, ON).

Abbreviations: W-7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; TFP, trifluoperazine ; FK-506, tacrolimus; KN-62, 4-[2-[(5-isoquinolinyl-sulfonyl)methylamino]-3-oxo-3-(4-phenyl-1-piperazinyl)-propyl]phenyl ester; KN-93, N-[2-[[[3-(4'-chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4'-methoxy-benzenesulfonamide phosphate; KN-92, 2-[N-(4'-methoxybenzenesulfonyl)]amino-N-(4'-chlorophenyl)-2-propenyl-N-methylbenzylamine phosphate; 4Br-A23187, 1-(4,5-dimethoxy-2-nitrophenyl)ethyl ester.

Figure 1. Examples of acutely dissociated adult rat hippocampal CA1 neurons.

Neurons were isolated according to procedures outlined in Section 1.1 and were chosen for study based on morphological criteria established by Schwiening & Boron (1994) and Bevensee *et al.* (1996). Note the smooth appearance, triangular-shaped soma, single major process projecting from one pole (= three times the length of the diameter of the soma) and multiple smaller projections projecting from the opposite pole.

Figure 2. The experimental apparatus.

A The temperature-controlled perfusion chamber. *B* The experimental apparatus consisting of an Attofluor Digital Fluorescence Ratio Imaging System used with a Zeiss Axiovert 10 microscope.

A
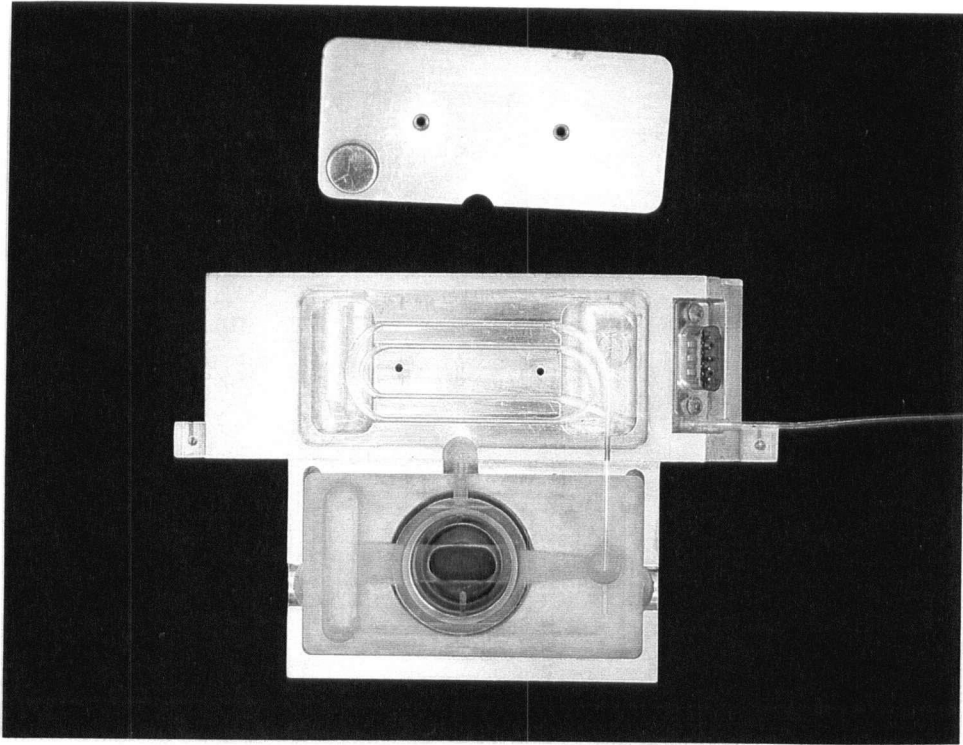
B
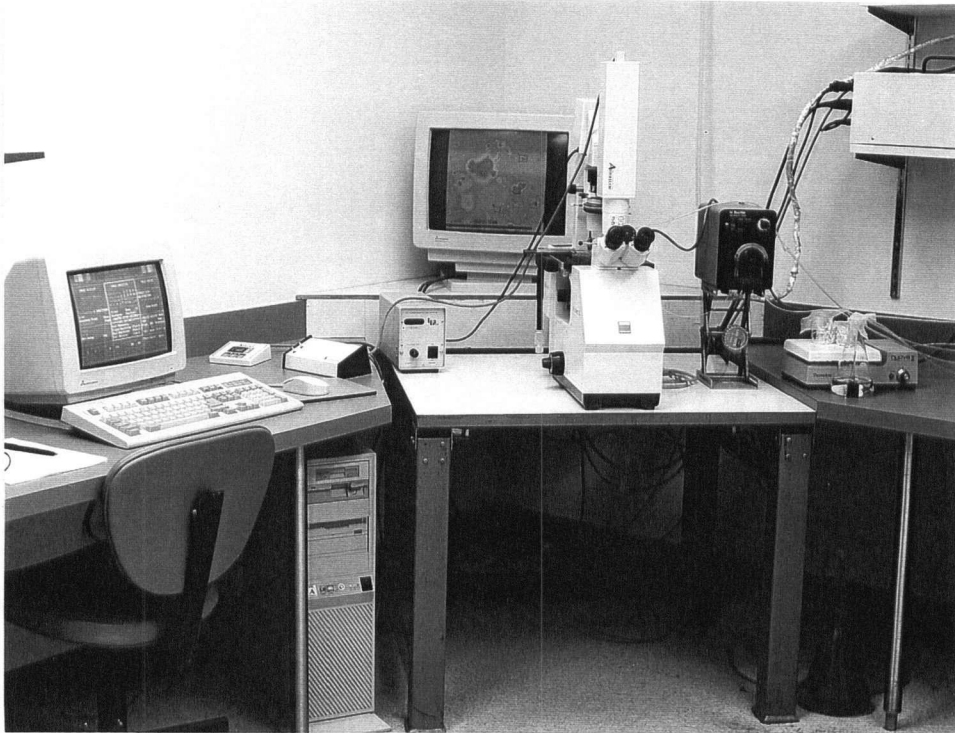
Figure 3. Examples of control internal acid and alkaline loads recovery experiments.

A When the neuron was exposed to NH_4^+ under HEPES-buffered conditions (pH_o = 7.35), pH_i initially increased and, in the continued presence of NH_4^+, pH_i fell slowly. Removal of NH_4^+ caused pH_i to fall rapidly below the initial SS level and after reaching a point of maximum acidification, it recovered to the initial resting value. This process was then repeated. Finally, at the end of the experiment, a single point calibration was performed during which the neuron was exposed to a high K^+, HEPES-buffered solution (containing 10 µM nigericin) in order to equate pH_o to pH_i (= 7.00). This procedure was performed in all subsequent experiments which employed BCECF, but is only illustrated here. The formal analyses of the pH_i recoveries illustrated here are presented in Fig. 5. **B** Upon exposure to a high $[HCO_3^-]$ medium (equilibrated with 10% CO_2 balance air) under HCO_3^-/CO_2-buffered conditions, pH_i initially fell and then quickly increased to a level ~0.20 pH units above the resting value. Removal of the high $[HCO_3^-]$ medium caused pH_i to increase further and, after reaching a peak alkalinization, it recovered to the initial SS level. The alkaline load procedure was repeated and no significant differences were observed between the first and second pH_i recovery portions of this experiment. External pH was 7.35 throughout. The formal analyses of the pH_i recoveries illustrated here are presented in Fig. 6.

A
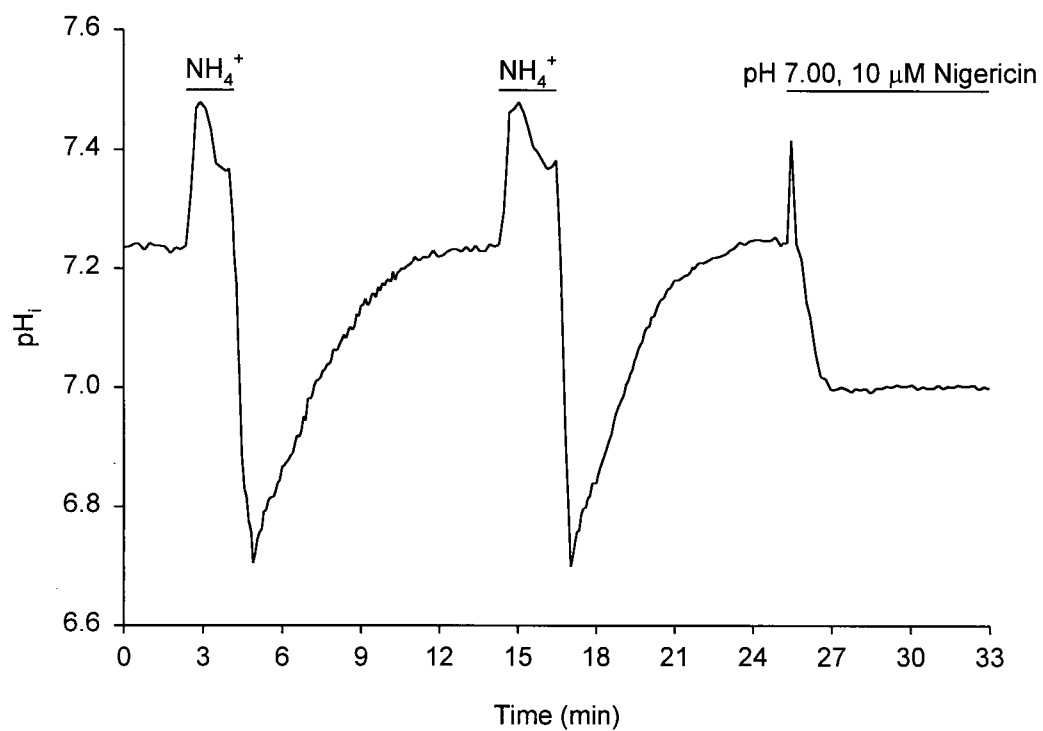
B
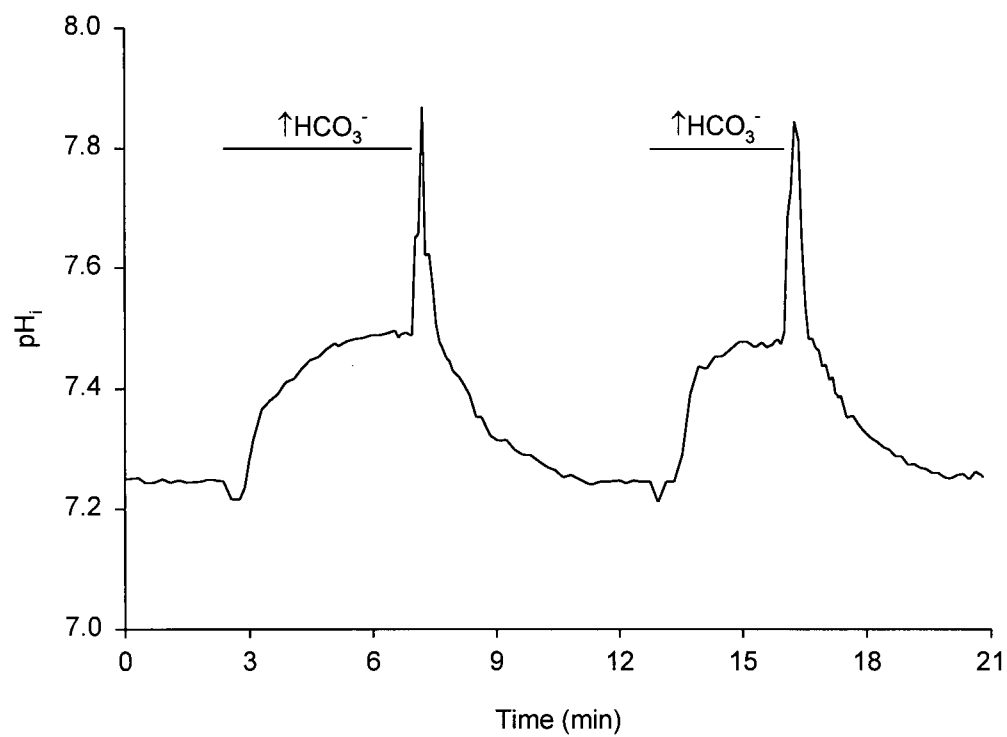
Figure 4. Sample full calibration experiment and calibration curve for BCECF.

A 26 cultured postnatal rat hippocampal neurons (on a single coverslip) were exposed to high K^+, HEPES-buffered media titrated to pH values within the range of 5.54 to 8.48 in ~0.5 pH unit intervals. All solutions contained 10 μM nigericin to equilibrate pH_o to pH_i. This trace represents the average background-subtracted ratio values (BI_{488}/BI_{452}) obtained over time (from all neurons). **B** Data from 3 experiments of the type shown in *A* were compiled and the average normalized ratios were plotted against the pH_i values at which they were obtained. This resulting calibration curve was used to convert BI_{488}/BI_{452} ratio values to pH_i values.

A
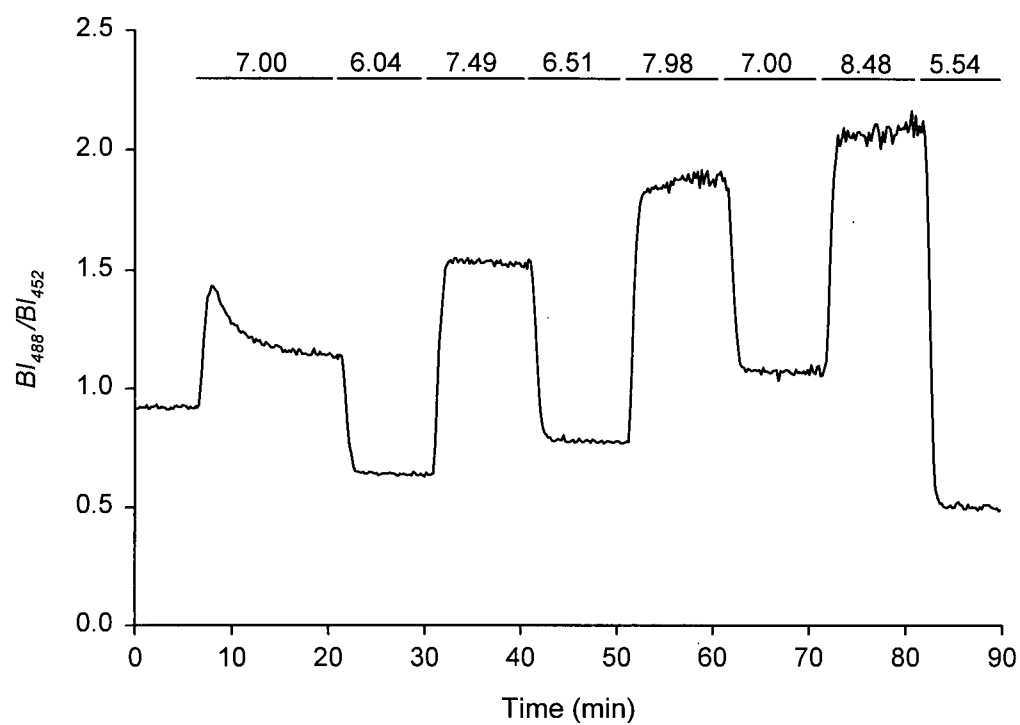
B
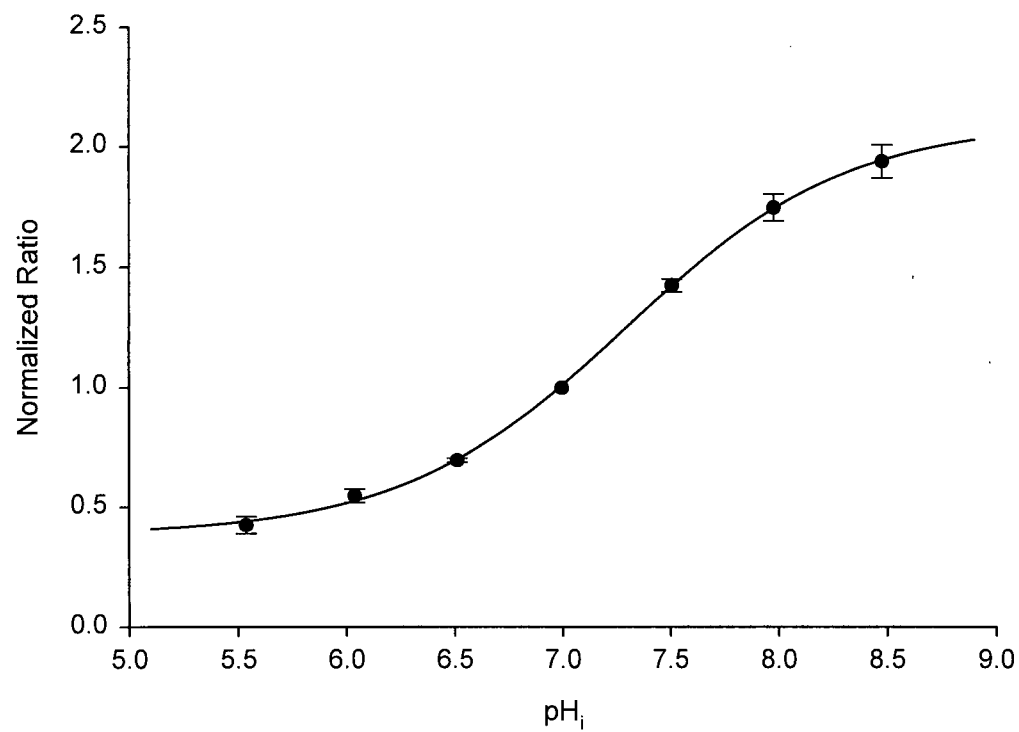
Figure 5. Analysis of control internal acid load recovery experiments performed under HEPES-buffered conditions at 37°C.

A The first acid load recovery portion of the experiment shown in Fig. 3A (O) was fit to a single exponential function of time (solid line) for further analysis. **B** Rates of pH_i recovery were calculated at each absolute value of pH_i (in 0.05 pH unit increments) from the curve fitted data representing each recovery portion of the pH_i trace from 20 experiments of the type shown in Fig. 3A. The mean rates of pH_i recovery from the first (filled symbols) and second (open symbols) acid loads were plotted against absolute values of pH_i and least square regression lines were fitted to the data points. Data obtained from neurons with low and high resting pH_i values are shown as circles and squares, respectively. No significant differences in rates of pH_i recovery between the first and second control acid loads were observed. **C** Net acid effluxes were calculated from rates of pH_i recovery shown in *B*.

A
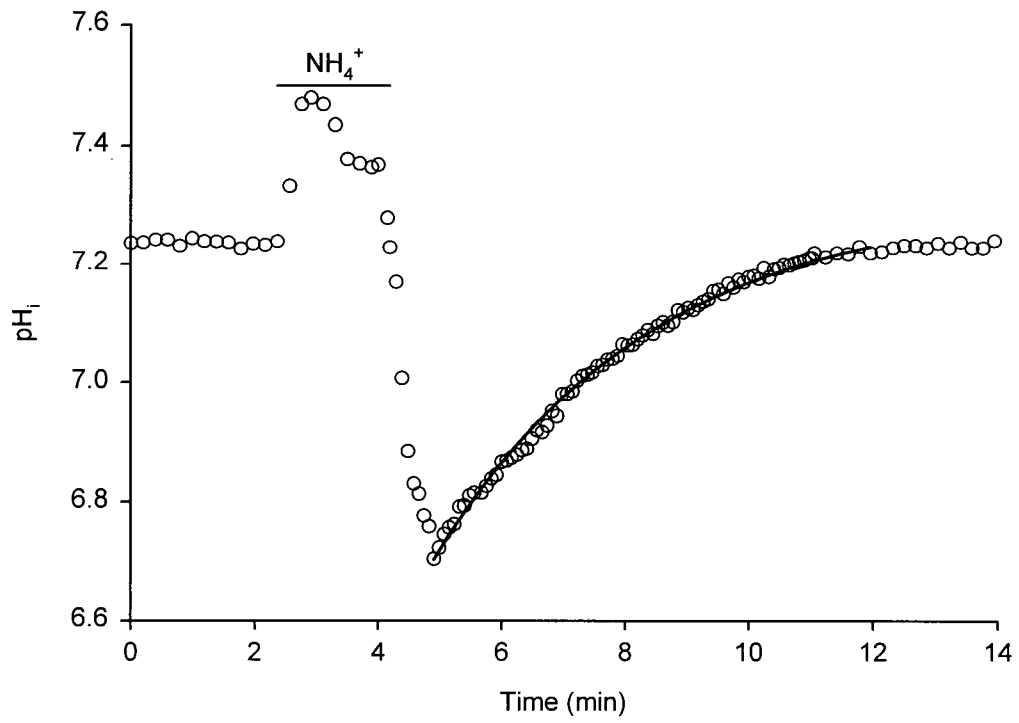
B
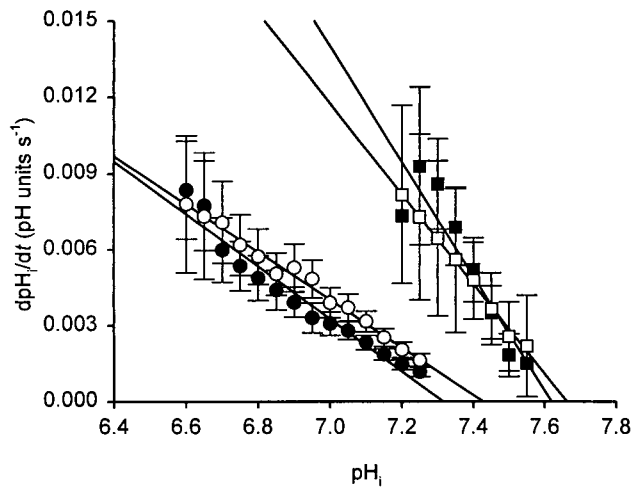
C
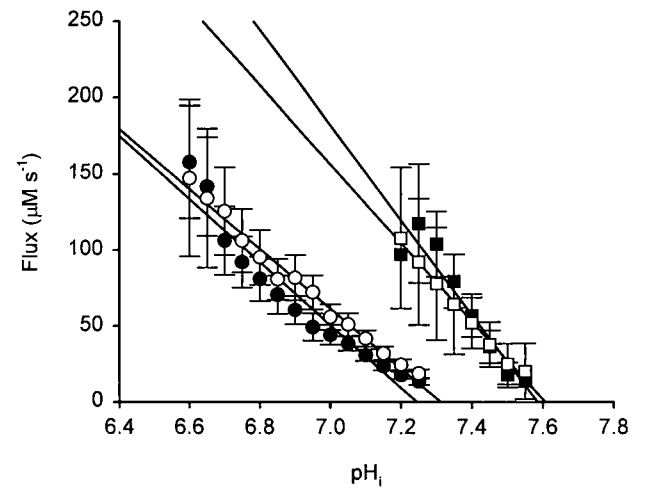
Figure 6. Analysis of control internal alkaline load recovery experiments performed under HCO_3^-/CO_2-buffered conditions.

A The pH_i recovery from the first alkaline load of the experiment shown in Fig. 3B (O) was fit to a single exponential decay function of time (solid line) for further analysis. **B** Mean residual values (●) were plotted against time from 26 single exponential decay functions of time fitted to pH_i recovery data obtained from 13 control alkaline load experiments performed under HCO_3^- /CO_2-buffered conditions. **C** Plotted are the mean rates of pH_i recovery from control alkaline loads against absolute pH_i values taken from 13 experiments of the type shown in Fig. 3B. The data points were fitted to least squares regression lines. The overall rate of pH_i recovery from the second alkaline load (O) was 1 ± 10% (mean ± S.D.) faster than the overall rate of pH_i recovery from the first alkaline load (●).

A
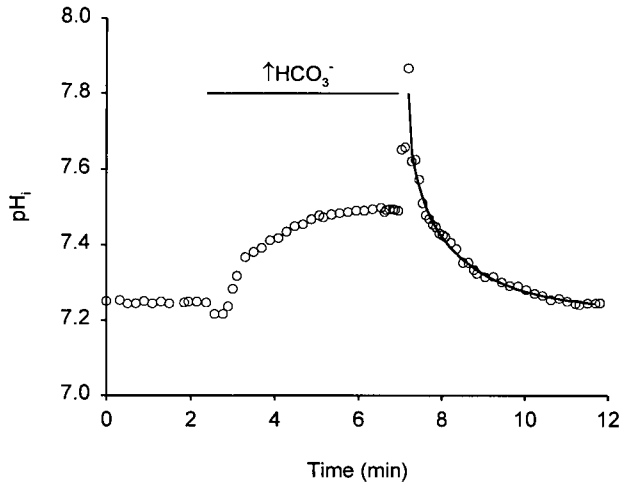
B
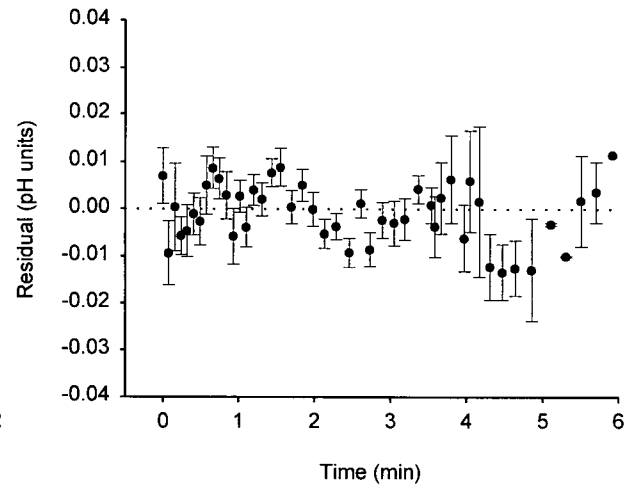
C
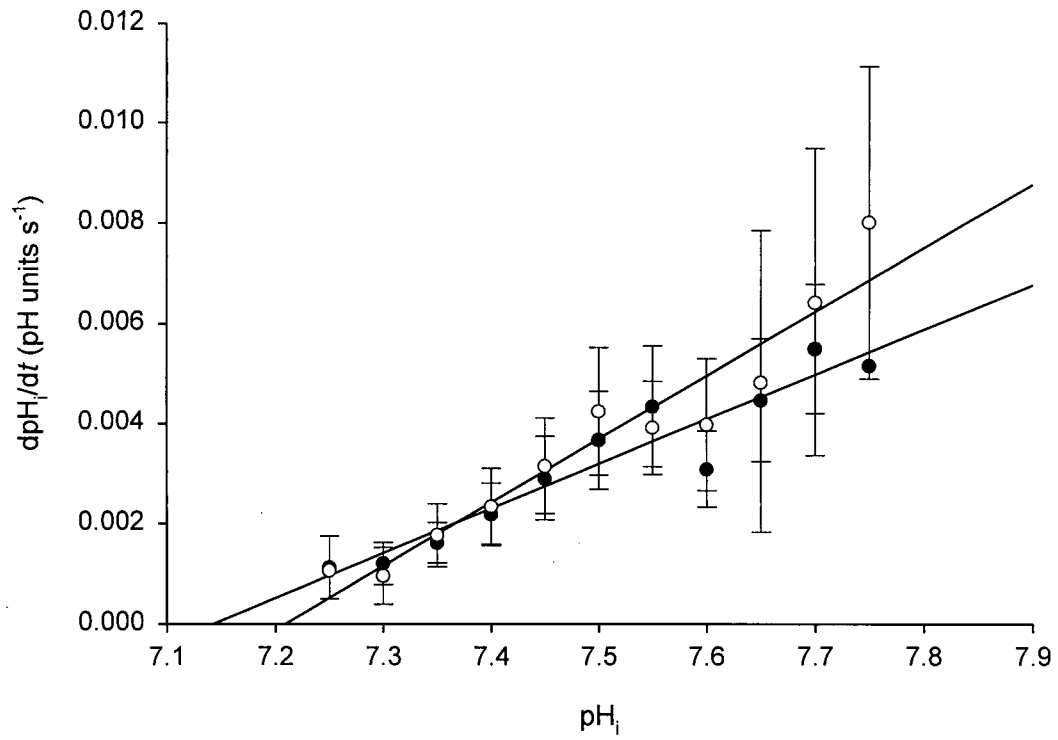
RESULTS

Although it is clear that Na^+/H^+ exchange and Na^+-dependent HCO_3^-/Cl^- exchange are involved in acid extrusion from rat hippocampal CA1 neurons (Raley-Susman *et al.*, 1991; Schwiening & Boron, 1994; Baxter & Church, 1996; Bevensee *et al.*, 1996; Smith *et al.*, 1998) and that a Na^+-independent Cl^-/HCO_3^- exchange mechanism exists in this cell type (Kopito *et al.*, 1989; Raley-Susman *et al.*, 1993; Baxter & Church, 1996), the specific contributions of the HCO_3^--dependent mechanisms to the maintenance of SS pH_i have not been established. Thus, the first part of the present study examined the role of both the NDAE and NIAE in the maintenance SS pH_i. The second part of the study examined the effects of modulating the activity of the cAMP/PKA second messenger system on the activities of: (1) the NHE; and (2) the NIAE and NDAE. Although it has already been established that noradrenaline, acting via G-protein coupled β-adrenoceptors, modulates the activity of the NHE in adult rat hippocampal CA1 neurons (Smith, 1996), this study did not examine the effects of modulating the cAMP/PKA pathway on the activity of the NHE (in isolation), nor did this study specifically examine the effects of the cAMP/PKA pathway on the activities of HCO_3^--dependent pH_i regulating mechanisms. In addition, the CaM/CaMK-II second messenger system is known to alter the activities of various NHE isoforms in peripheral cell types (Owen & Villereal, 1982; Little *et al.*, 1988; Weinman *et al.*, 1988; Bertrand *et al.*, 1994; Le Prigent *et al.*, 1997; Wakabayashi *et al.*, 1997a) but the effects of this intracellular pathway on SS pH_i and the activities of pH_i regulating mechanisms have not been examined in mammalian central neurons. As the components of this intracellular pathway are present (Scholz *et al.*, 1988) and play a functional role in hippocampal CA1 neurons (*e.g.* Shirke & Malinow, 1997; see Discussion), the final part of the study examined the role of the CaM/CaMK-II intracellular pathway in modulating the activity of the NHE in adult rat hippocampal CA1 neurons.

1. Contribution of HCO_3^--dependent pH_i regulating mechanisms to SS pH_i

The resting values of pH_i from neurons perfused with HCO_3^-/CO_2-buffered media at 37°C exhibited a mean at $pH_i = 7.20 \pm 0.01$ ($n = 370$) and were best fit to a single Gaussian distribution (Fig. 7A). In contrast, resting pH_i values in neurons perfused with nominally HCO_3^--free, HEPES-buffered solutions were best fit by the sum of two Gaussian distributions with mean pH_i values at 6.90 ± 0.02 and 7.37 ± 0.01 ($n = 517$, Fig. 7B). Similar results were obtained at RT (Figs. 7C and 7D). At 37°C, the ranges of resting pH_i values obtained under HCO_3^-/CO_2- and HEPES-buffered conditions were 6.60 - 7.84 and 6.20 - 7.89, respectively. The shift from a unimodal distribution to a bimodal distribution of resting pH_i values with significantly lower and higher mean values ($P < 0.00001$ for the differences to the mean pH_i value observed in the presence of HCO_3^- in each case) upon the removal of HCO_3^-/CO_2 from the perfusion medium has been reported previously (see Bevensee et $al.$, 1996; Smith et $al.$, 1998) and suggests that external HCO_3^- plays a role in the maintenance of SS pH_i in acutely dissociated adult rat hippocampal CA1 neurons.

A role for HCO_3^--dependent mechanisms in the maintenance of SS pH_i was also suggested by the finding of Smith et $al.$ (1998) that shifts in SS pH_i occur with the addition of HCO_3^-/CO_2 to neurons initially perfused with HEPES-buffered medium at $pH_o = 7.35$ and 37°C (also see Schwiening & Boron, 1994). In the study by Smith et $al.$ (1998), addition of HCO_3^-/CO_2 elicited a rise in SS pH_i in neurons with resting pH_i values (in HEPES-buffered medium) $< \sim7.3$ ($n = 13$, Fig. 8A) and a fall in SS pH_i in neurons with resting pH_i values $> \sim7.3$ ($n = 7$, Fig. 8B). The changes in SS pH_i elicited by the addition of HCO_3^-/CO_2 were plotted against resting pH_i values obtained under HEPES-buffered conditions and a regression line that has a negative slope and an x-intercept at $pH_i = \sim7.3$ was fitted to the data points (Fig. 8C). It was suggested that changes in the activities of HCO_3^--dependent pH_i regulating mechanisms or β_i may be responsible for these HCO_3^--induced changes in SS pH_i (see Smith et $al.$, 1998). To further investigate these possibilities, in the present study the effect of 200 μM DIDS on SS pH_i was

examined in neurons perfused with HCO_3^-/CO_2-buffered medium (pH_o = 7.35 at 37°C). Interestingly, the changes in SS pH_i evoked by DIDS were opposite to those reported by Smith *et al.* (1998; see above) with the addition of HCO_3^-/CO_2 to the perfusion medium. Thus, the addition of DIDS elicited a fall in SS pH_i in neurons with resting pH_i values < ~ 7.3 (n = 12/26, Fig. 9A) and a rise in SS pH_i in neurons with resting pH_i values > ~7.3 (n = 10/26, Fig. 9B). The SS pH_i of four neurons with resting pH_i values at ~7.25 was not affected by DIDS. The DIDS-evoked changes in SS pH_i were plotted against resting pH_i values observed prior to the addition of the drug and a least squares regression line was fitted to the data points (Fig. 9C). This line had a similar x-intercept (pH_i = ~7.3) as the line representing data obtained upon the addition of $HCO_3^-_o$ reported by Smith *et al.* (1998; see Fig. 9C) but an opposite slope. Taken together, the results of Smith *et al.* (1998) and the present study suggest that the activities of HCO_3^--dependent pH_i regulating mechanisms maintain the SS pH_i at a value near pH_o (= 7.35) in adult rat hippocampal CA1 neurons.

Two known pH_i regulating mechanisms that are HCO_3^--dependent and DIDS sensitive have been identified in adult rat hippocampal CA1 neurons (see Introduction): the NIAE (an acid loader) and the NDAE (an acid extruder). In peripheral cell types, the latter is most active at low pH_i values and, when active, causes an internal alkalinization, whereas the former is most active at high pH_i values and, when active, causes internal acidification (see Introduction). As both internal acidifications and alkalinizations were observed with the addition of $HCO_3^-_o$ (or DIDS), depending on the resting pH_i prior to the test maneuver, it was of interest to assess the possible contribution of the NIAE and NDAE to the maintenance of SS pH_i. Initially, experiments were performed in the absence of Na^+_o, under which condition neurons were perfused with HEPES-buffered medium and then exposed to $HCO_3^-_o$. Under these conditions, the activity of the NDAE (and the NHE) is blocked and any changes in SS pH_i evoked by addition of HCO_3^-/CO_2 reflect activation of the NIAE and/or changes in β_i. Addition of HCO_3^-/CO_2 in the absence of Na^+_o elicited a rise in SS pH_i in neurons with resting pH_i values (in HEPES-buffered medium) < ~7.5 (n = 13/17, Fig. 10A) and a fall in SS pH_i in neurons with resting pH_i values > ~7.5 (n = 2/17,

Fig. 10B). The SS pH_i of two neurons with resting pH_i values at 7.38 and 7.48 was not affected by the addition of HCO_3^-/CO_2 to the perfusion medium. These results suggest the possibility that the NIAE contributes to the maintenance of SS pH_i in rat hippocampal CA1 neurons. Changes in SS pH_i evoked by the addition of HCO_3^-/CO_2 under external Na^+-free conditions were plotted against resting pH_i values and the data were fitted to a regression line (Fig. 10C). Interestingly, the x-intercept of this regression line (pH_i = ~7.5) was more alkaline than that reported in the presence of Na^+_o (pH_i = ~7.3; see Fig. 10C). In addition, five neurons with resting pH_i values > ~7.3 showed increases in SS pH_i with the addition of HCO_3^-/CO_2 in the absence of Na^+_o, compared to none in the presence of Na^+_o (compare Fig. 10C with Fig. 8C). Thus, there was a difference in the effect on SS pH_i of adding external HCO_3^- under external Na^+-containing and external Na^+-free conditions. These results suggest the possibility that the NDAE also contributes to the maintenance of SS pH_i. Nevertheless, as noted above, it remains possible that changes in β_i consequent upon the addition of HCO_3^- may also contribute to the SS pH_i changes observed under both Na^+_o-containing and Na^+_o-free conditions.

Therefore, in the next series of experiments, neurons initially perfused with HEPES-buffered medium were exposed to HCO_3^-/CO_2 in the continued absence of Na^+_o and presence of 200 μM DIDS to inhibit the NIAE (pH_o = 7.35 at 37°C). Under these conditions, in which all known pH_i regulating mechanisms are blocked, the addition of HCO_3^-/CO_2 evoked a 0.21 ± 0.03 pH unit increase in SS pH_i in neurons with resting pH_i values in HEPES-buffered medium < ~7.5 (n = 9, Fig. 11A). No neurons with resting pH_i values > ~7.5 were observed. The SS pH_i response to HCO_3^-/CO_2 in the presence of DIDS and absence of Na^+_o likely reflects a change in β_i consequent upon the addition of HCO_3^-/CO_2, as the activities of all known pH_i regulating mechanisms in these neurons are inoperative under these experimental conditions. Changes in SS pH_i evoked by the addition of HCO_3^-/CO_2 under Na^+_o-free, DIDS-containing conditions were plotted against resting pH_i values and the data points were fitted to a regression line (Fig. 11B). Importantly, the equation representing this line (ΔpH_i = 5.93 - 0.78(pH_i)) had a greater slope than the linear equation representing the data obtained in the absence of DIDS (ΔpH_i = 2.79 -

0.37(pH_i); see Fig. 11B). Thus, at a given value of pH_i, the addition of HCO_3^-/CO_2 evoked a significantly smaller internal alkalinization in the absence than in the presence of DIDS under Na_o^+ -free conditions, suggesting that a DIDS-sensitive, HCO_3^--dependent, acid-loading mechanism, most likely the NIAE, also contributes to the maintenance of SS pH_i.

In summary, the activities of the NIAE and NDAE, and the HCO_3^-/CO_2 open buffering system, act to maintain pH_i near pH_o (= 7.35) in adult rat hippocampal CA1 neurons under normal HCO_3^-/CO_2-buffered conditions at 37°C.

Figure 7. Distributions of resting pH_i values for all neurons under HCO_3^-/CO_2- and HEPES-buffered conditions at 37°C and RT.

A Distribution of resting pH_i values from 370 neurons perfused with HCO_3^-/CO_2-buffered medium (pH_o 7.35) at 37°C (bin width = 0.05 pH units). The data were fitted to a single Gaussian distribution with a mean at pH_i = 7.18 and a range of pH_i = 6.60 to 7.84. **B** Distribution of resting pH_i values from 517 neurons perfused with HEPES-buffered medium (pH_o 7.35) at 37°C (bin width = 0.05 pH units). The data were fitted to the sum of two Gaussian distributions with means at pH_i = 6.90 and 7.35 and a range of pH_i = 6.20 to 7.89. **C** Distribution of resting pH_i values from 15 neurons perfused with HCO_3^-/CO_2-buffered medium (pH_o 7.35) at RT (bin width = 0.05 pH units). The data were fitted to a single Gaussian distribution with a mean at pH_i = 6.99 and a range of pH_i = 6.70 to 7.49. **D** Distribution of resting pH_i values from 33 neurons perfused with HEPES-buffered medium (pH_o 7.35) at RT (bin width = 0.05 pH units). The data were fitted to the sum of two Gaussian distributions with means at pH_i = 6.89 and 7.22 and a range of pH_i = 6.75 to 7.49.
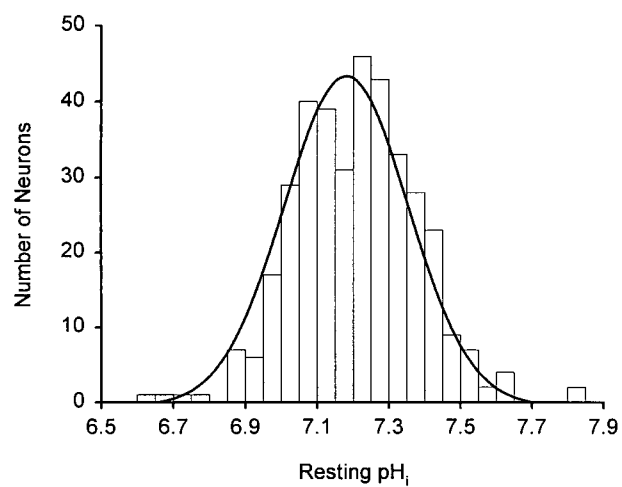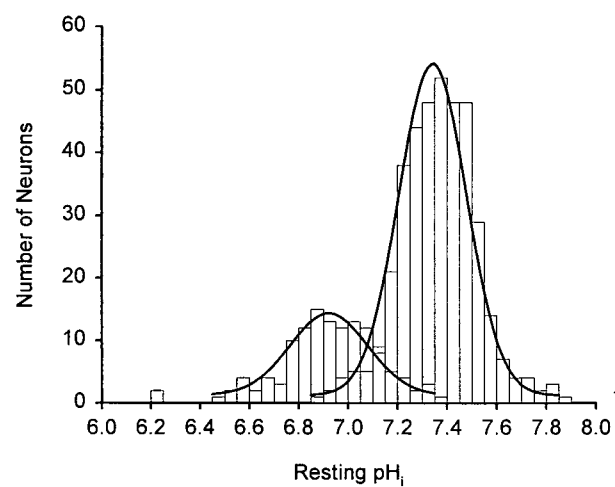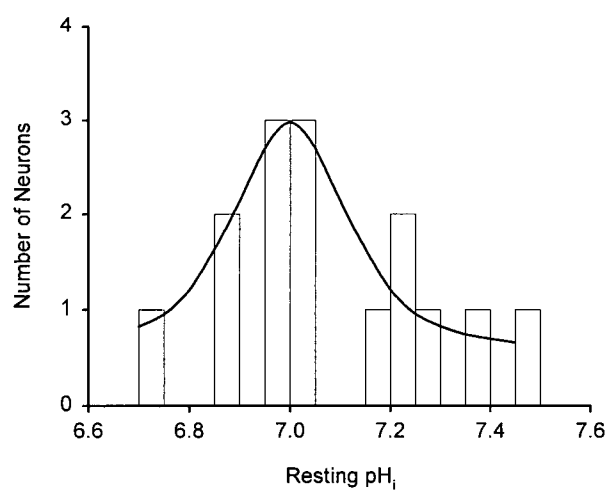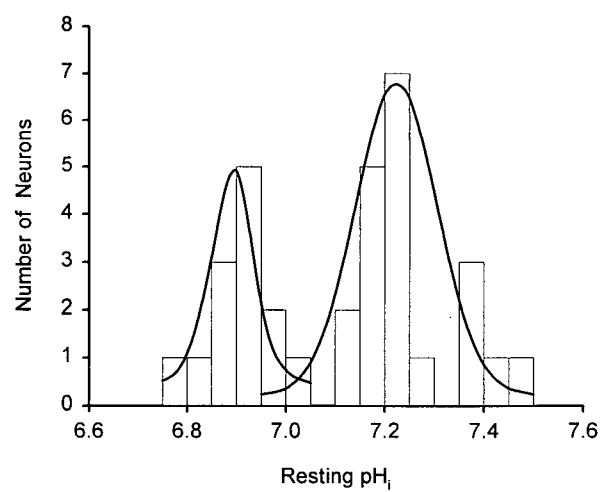
Figure 8. Examples of the effect of the addition of HCO_3^-/CO_2 on SS pH_i under HEPES-buffered conditions.

These results were previously reported by Smith *et al.* (1998). **A** A neuron perfused with HEPES-buffered medium (pH 7.35) had a resting pH_i at ~6.85. Upon perfusion with HCO_3^-/CO_2-buffered medium (also pH 7.35), pH_i initially fell to ~6.75 and then alkalinized to a new SS level at ~7.15. **B** A different neuron was initially perfused with HEPES-buffered medium and had a resting pH_i at ~7.60. Perfusion with HCO_3^-/CO_2-buffered medium at a constant pH_o (= 7.35) caused pH_i to fall and then stabilize at a new SS level of pH_i ~7.38. **C** Plotted against resting pH_i values observed under HEPES-buffered conditions are the changes in SS pH_i elicited by the addition of HCO_3^-/CO_2 at a constant pH_o (7.35; $n = 20$). A least squares regression line was fitted to the data points and had a negative slope and an x-intercept at pH_i = ~7.3.

A
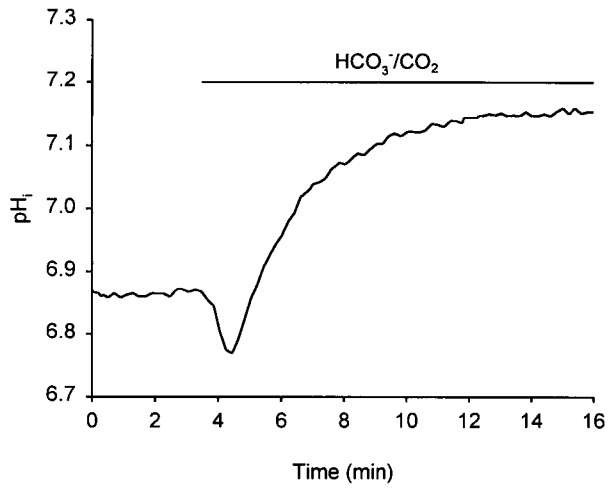
B
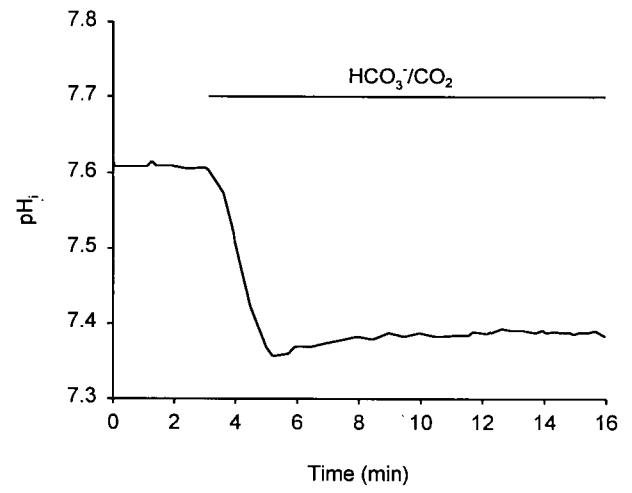
C
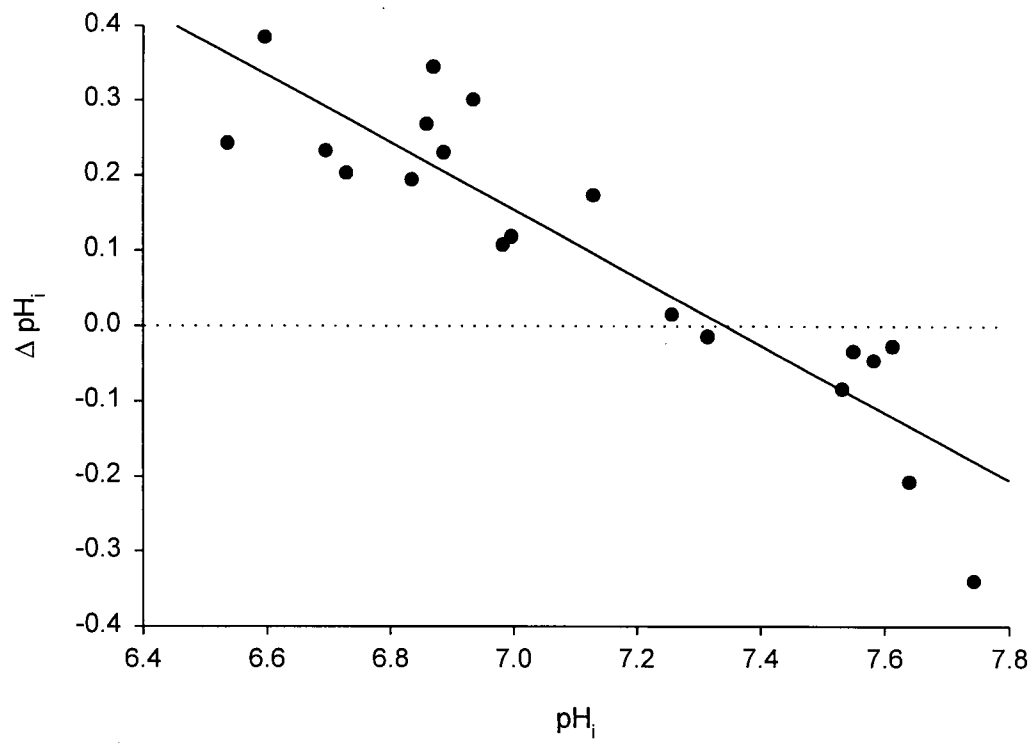
Figure 9. Effect of DIDS on SS pH_i under HCO_3^-/CO_2-buffered conditions.

A Under HCO_3^-/CO_2-buffered conditions (pH_o = 7.35), a neuron that had a resting pH_i of ~7.00 was exposed to 200 μM DIDS causing pH_i to decrease to a new SS level at ~6.75. **B** A different neuron perfused with HCO_3^-/CO_2-buffered medium (pH_o = 7.35) had a resting pH_i at ~ 7.52. Upon exposure to 200 μM DIDS, pH_i increased to a new SS level near 7.60. **C** Changes in SS pH_i elicited by 200 μM DIDS under HCO_3^-/CO_2-buffered conditions were plotted against resting pH_i values (n = 26). The solid line represents a least squares regression line which was fitted to the data points. Also shown is the regression line representing the SS pH_i response to HCO_3^-/CO_2 application in the absence of DIDS (dashed line; see Fig. 8C). The regression lines had similar, but opposite, slopes and a common x-intercept (pH_i = ~7.3).
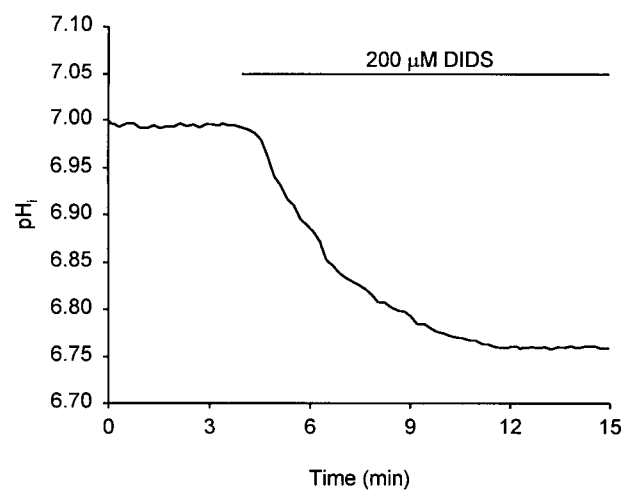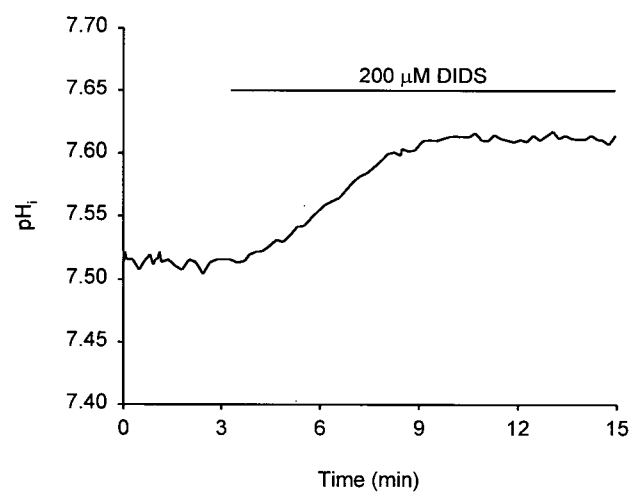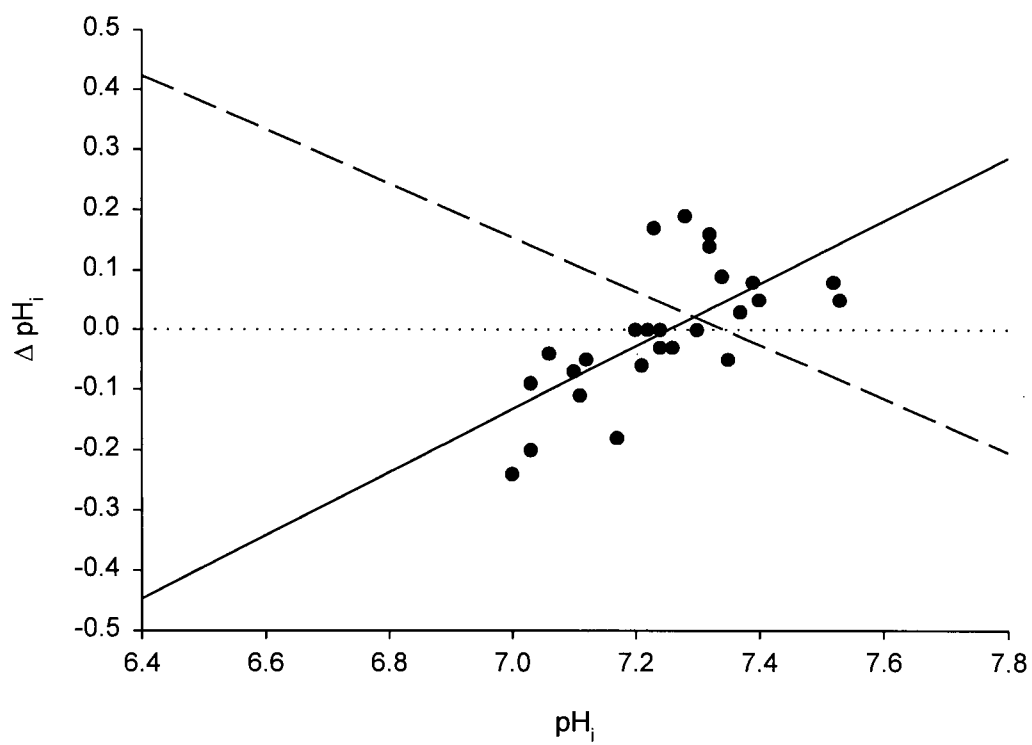
Figure 10. Effect of the addition of HCO_3^-/CO_2 on SS pH_i under Na^+_o-free, HEPES-buffered conditions.

A A neuron perfused with Na^+_o-free, HEPES-buffered medium had a resting pH_i at ~6.90. Perfusion with HCO_3^-/CO_2-buffered medium, in the continued absence of Na^+_o, caused pH_i to transiently fall ~ 0.10 pH units and then alkalinize to a new SS level at ~7.15. **B** A different neuron that had a resting pH_i at ~7.55 under Na^+_o-free, HEPES-buffered conditions was exposed to a HCO_3^-/CO_2-buffered medium. In the continued absence of Na^+, HCO_3^-/CO_2 caused pH_i to increase to a new SS level at ~7.48. In both *A* and *B*, pH_o was maintained at 7.35 throughout each experiment. Compare with Fig. 8A and B; similar experiments performed in the presence of external Na^+. **C** Plotted against resting pH_i values observed under HEPES-buffered conditions are the changes in SS pH_i elicited by the addition of HCO_3^-/CO_2 in the absence of Na^+_o ($n = 17$). The data points were fitted to a regression line (solid line) which had a negative slope and an x-intercept at $pH_i = ~ 7.50$. This regression line is compared to the regression line representing the SS pH_i response to HCO_3^-/CO_2 observed in the presence of Na^+_o (dashed line; see Fig. 8C). The regression line observed under Na^+_o-free conditions had a more alkaline x-intercept.

A
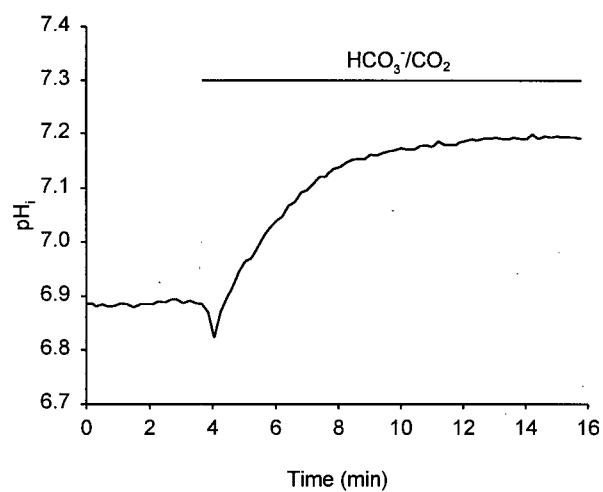
B
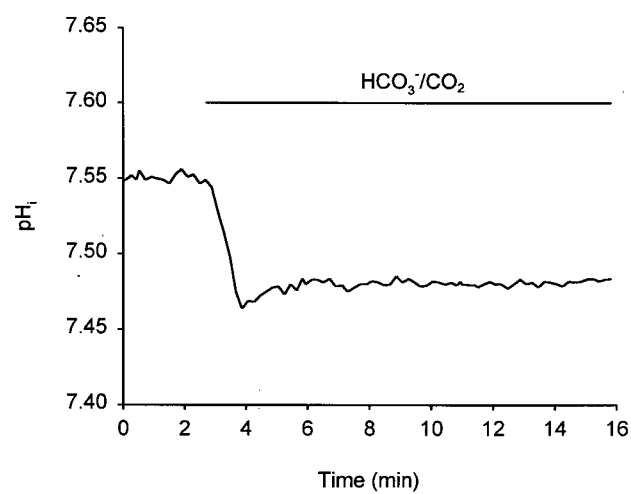
C
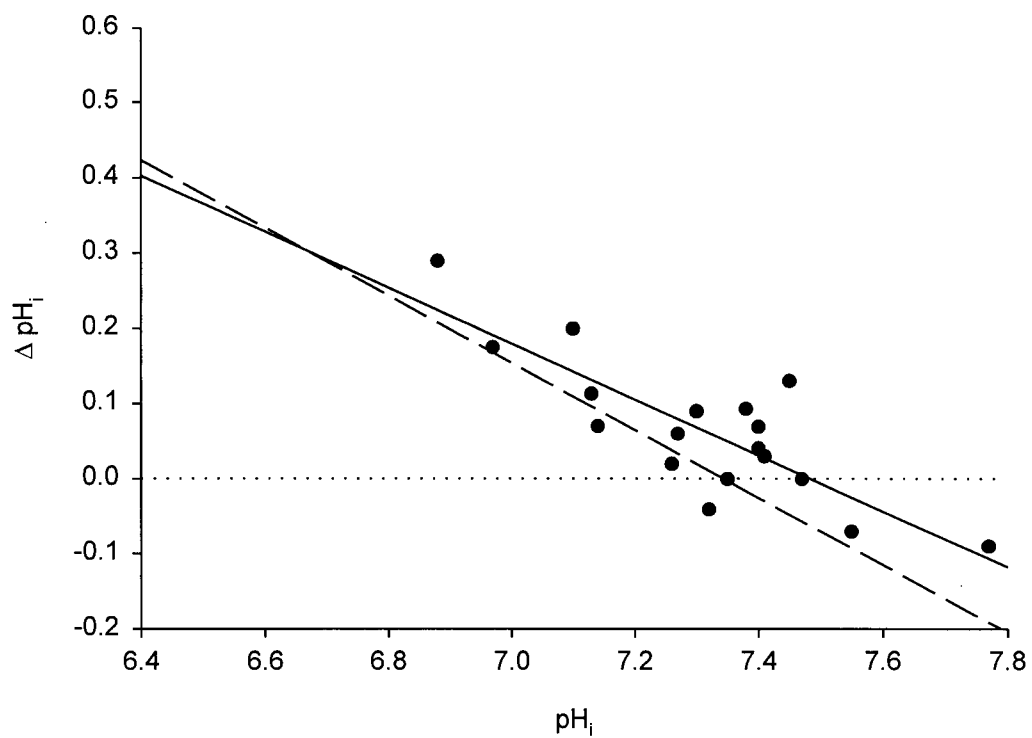
Figure 11. Effect of DIDS on the SS pH_i response to the addition of HCO_3^-/CO_2 under Na^+_o-free, HEPES-buffered conditions.

A A neuron perfused with Na^+_o-free, HEPES-buffered medium and *pretreated with 200 µM DIDS* had a resting pH_i at ~7.19. Perfusion with HCO_3^-/CO_2-buffered medium, in the continued presence of DIDS and absence of Na^+_o, caused pH_i to transiently fall ~ 0.07 pH units and then to alkalinize to a new SS level at ~7.46. pH_o remained constant (at 7.35) throughout the experiment. **B** Plotted against resting pH_i values observed under 0 Na^+_o, HEPES-buffered conditions are the changes in SS pH_i elicited by the addition of HCO_3^-/CO_2 in the presence of DIDS ($n = 9$). The data points were fitted with a regression line (solid line) which had a negative slope and an x-intercept at pH_i = ~7.55. This regression line is compared to the regression line representing the SS pH_i response to HCO_3^-/CO_2 observed in the absence of DIDS (and absence of Na^+_o; dashed line; see Fig. 10C); the regression line observed in the presence of DIDS had a greater slope.

A
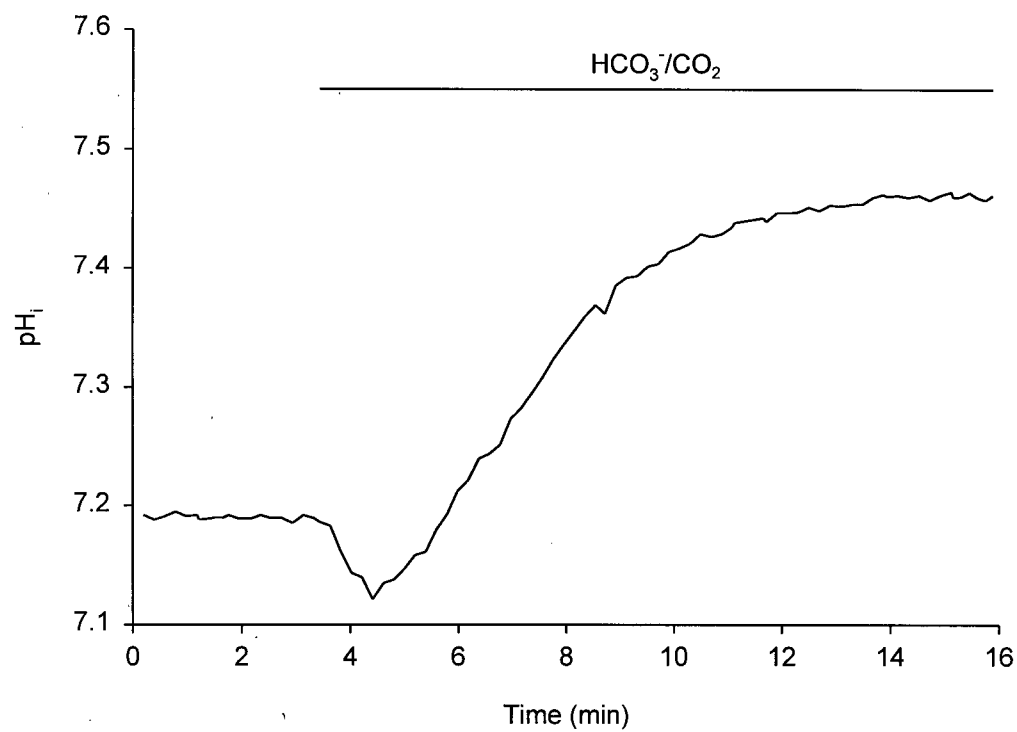
B
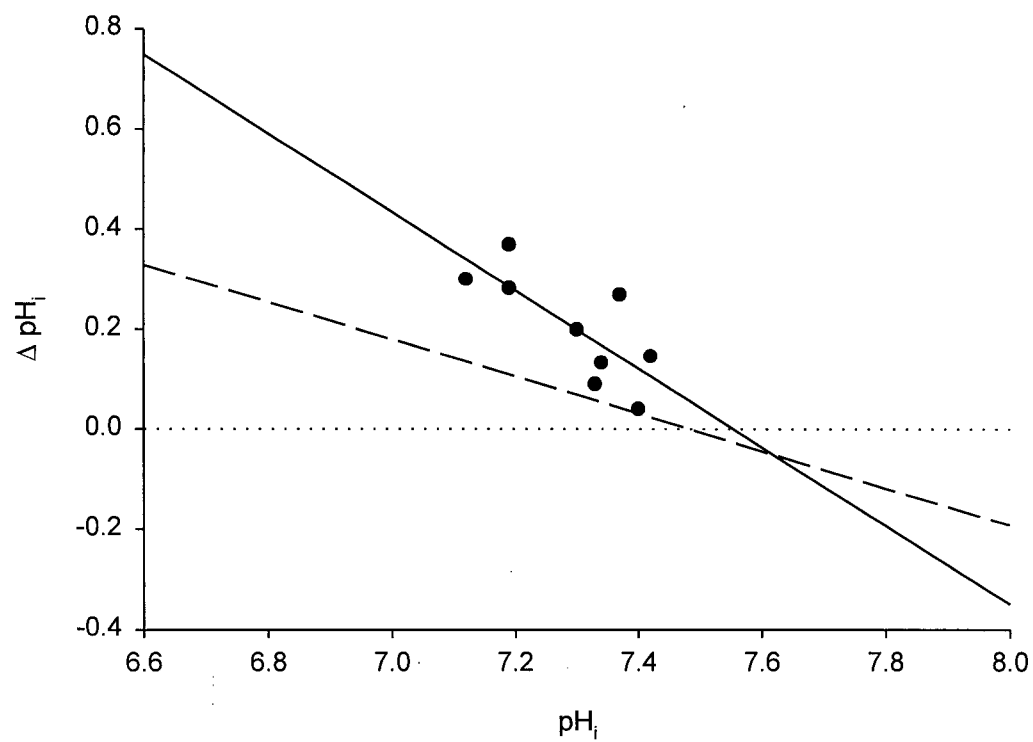
2. Modulation of the activities of pH$_i$ regulating mechanisms by the cAMP/PKA second messenger system

2.1 Na$^+$/H$^+$ exchange

In order to isolate the activity of the NHE from HCO$_3^-$-dependent pH$_i$ regulating mechanisms, all experiments in this section were performed using nominally HCO$_3^-$-free, HEPES-buffered solutions (see Method and Materials, Section 5.1).

2.1.1 The role of cAMP$_i$.

Increasing [cAMP]$_i$ levels. Increases in [cAMP]$_i$ were evoked using two approaches: (1) the activities of endogenous adenylyl cyclases were increased with forskolin (see Daley *et al.*, 1982); and (2) the activities of phosphodiesterases were decreased with IBMX (Smellie *et al.*, 1979; Wu *et al.*, 1982). The addition of 25 µM forskolin evoked a 0.27 ± 0.03 pH unit increase in SS pH$_i$ (n = 24/28, Fig. 12A). The remaining 4 neurons showed no change in SS pH$_i$ with the addition of forskolin. Of the 28 neurons examined, six were subjected to the subsequent removal of forskolin from the perfusion medium; none of these six neurons showed recovery of SS pH$_i$ to resting levels (*e.g.* Fig. 12A), suggesting that the effects of forskolin on SS pH$_i$ may be relatively prolonged. When neurons were exposed to 200 µM IBMX, an increase in SS pH$_i$ of 0.30 ± 0.03 pH units was observed (n = 19, Fig. 12B). Therefore, increasing [cAMP]$_i$ with either forskolin or IBMX caused an increase in SS pH$_i$ in the absence of HCO$_3^-$, suggesting that the activity of a HCO$_3^-$-independent pH$_i$ regulating mechanism, most likely the NHE, may contribute to this SS pH$_i$ response.

To examine the effect of increasing [cAMP]$_i$ on the activity of the NHE, acid load recovery experiments were performed with forskolin and IBMX. The overall rates of pH$_i$ recovery from internal acid loads imposed in the presence of 25 µM forskolin were 138 ± 23% (n = 6/10) and 110 ± 16% (n = 6/7) faster than control rates of pH$_i$ recovery in neurons with low and high resting pH$_i$ values, respectively (Fig. 13A; the remaining 5 neurons did not respond to

forskolin treatment). As shown in Fig. 13B, the rates of pH_i recovery (from both populations of neurons) were converted to net acid flux values and plotted against absolute values of pH_i in the absence and presence of 25 μM forskolin; regression lines then were fitted to the data points. The addition of forskolin significantly increased net acid efflux ($P < 0.05$ at each absolute value of pH_i for both populations of neurons) and caused the pH_i dependence of net acid efflux to shift ~0.2 and ~0.05 pH units in the alkaline direction in neurons with low and high resting pH_i values, respectively. Similarly, the presence of 200 μM IBMX evoked a 116 ± 9% ($n = 8/11$) and 180 ± 17% ($n = 2/3$) increase in the overall rates of pH_i recovery from imposed acid loads in neurons with low and high resting pH_i values, respectively (Fig. 13C). The remaining 4 neurons did not respond to treatment with IBMX. Calculated net acid flux values were plotted against absolute values of pH_i in the absence and presence of 200 μM IBMX (Fig. 13D) and regression lines then were fitted to the data points. The addition of IBMX: (1) significantly increased net acid efflux ($P < 0.05$ at each absolute value of pH_i for both populations of neurons); and (2) caused the pH_i dependence of net acid efflux to shift 0.05 - 0.1 pH units in the alkaline direction in both populations of neurons. These results suggest that increasing $[cAMP]_i$ with forskolin and IBMX elicits an increase in the activity of a HCO_3^--independent acid-extruding mechanism, most likely the NHE, which in turn accounts for the subsequent increase in SS pH_i.

Control SS pH_i and acid load recovery experiments were performed for forskolin with its inactive analogue, 1′,9′-dideoxyforskolin. The addition of 25 μM 1′,9′-dideoxyforskolin did not change SS pH_i ($n = 6$, Fig. 14A) and failed to significantly affect net acid efflux following imposed internal acid loads ($n = 4$, Fig. 14B and C).

Inhibition of adenylyl cyclase activity. 2′,5′-dideoxyadenosine (DDA) was used to non-selectively decrease the activities of endogenous adenylyl cyclases (Chijiwa *et al.*, 1990; Johnson & Shoshani, 1990 & 1994). In neurons with low resting pH_i values (*i.e.* < ~7.4), the addition of 100 μM DDA evoked little or no change in SS pH_i ($n = 20$; see Fig. 53A) in the absence of HCO_3^-. Although DDA, when applied alone, failed to affect SS pH_i, it has previously been reported that this adenylyl cyclase inhibitor is able to inhibit markedly the ability of

noradrenaline to increase both SS pH_i and rates of pH_i recovery from acid loads in adult rat hippocampal CA1 neurons (Smith *et al.*, 1998).

Taken together, the results detailed above indicate that cAMP is involved in the control of the activity of the NHE in rat hippocampal neurons, and that the increase in SS pH_i observed upon the application of forskolin or IBMX reflects an increase in the activity of the NHE.

2.1.2 The role of PKA

Stimulating PKA activity. Increases in PKA activity were elicited with Sp-cAMPS, a specific activator of PKA (Van Haastert *et al.*, 1984; Audesirk *et al.*, 1997). As illustrated in Fig. 15A, increasing concentrations of 5, 10, 25 or 40 µM Sp-cAMPS evoked 0.10 ± 0.01 ($n = 6/8$), 0.15 ± 0.01 ($n = 11/13$), 0.22 ± 0.03 ($n = 7/8$; all neurons had resting pH_i values below 7.4) and 0.20 ± 0.02 ($n = 2/2$) pH unit increases in SS pH_i, respectively. The remaining neurons did not show changes in SS pH_i with Sp-cAMPS treatment. As these experiments were performed in the absence of HCO_3^-, a change in the activity of the NHE was most likely responsible for the increase in SS pH_i evoked by stimulation of PKA activity. Also, because a maximal SS pH_i response was obtained with the addition of 25 µM Sp-cAMPS, this concentration was employed in most subsequent experiments.

To assess the effect of stimulation of PKA activity on the activity of the NHE, paired acid load recovery experiments were performed with Sp-cAMPS. When compared to control rates of pH_i recovery, the overall rates pH_i of recovery from acid loads imposed in the presence of 25 µM Sp-cAMPS were $168 \pm 16\%$ ($n = 5/7$) and $82 \pm 15\%$ ($n = 2/3$) faster in neurons with low and high resting pH_i values, respectively (Fig. 15B). The 3 remaining neurons did not respond to treatment with Sp-cAMPS. The rates of pH_i recovery were converted to net flux values and plotted against absolute values of pH_i in the absence and presence of Sp-cAMPS; regression lines were then fitted to the data points (Fig. 15C). The addition of Sp-cAMPS significantly increased net acid efflux ($P < 0.05$ at each absolute value of pH_i) and caused the pH_i dependence of net acid efflux to shift ~0.2 and ~0.05 pH units in the alkaline direction in neurons with low and high

resting pH_i values, respectively. These results suggest that stimulation of PKA activity elicits an increase in the activity of a HCO_3^--independent acid-extruding mechanism, most likely the NHE, which causes a subsequent increase in SS pH_i.

To confirm the involvement of the NHE in the SS pH_i response to Sp-cAMPS, experiments were performed under Na_o^+-free, $NMDG^+$-substituted conditions to block the activity of the NHE. Under these conditions, the addition of 25 μM Sp-cAMPS failed to change SS pH_i ($n = 4$, Fig. 16A), suggesting that the SS pH_i response to stimulated PKA activity was dependent on Na_o^+. Similar experiments were then performed under Na_o^+-free, Li^+-substituted conditions. Addition of 25 μM Sp-cAMPS evoked a 0.18 ± 0.03 ($n = 5$, Fig. 16B) pH unit increase in SS pH_i, an internal alkalinization which was similar to that observed in the presence of Na_o^+ ($P = 0.30$). As the SS pH_i response to Sp-cAMPS was re-established with the replacement of $NMDG^+$ by Li^+ in the continued absence of Na_o^+, these results confirm that Na^+/ H^+ exchange is involved in the SS pH_i response to Sp-cAMPS.

Inhibition of PKA activity. The activity of PKA was inhibited with Rp-cAMPS, a cAMP analogue which specifically and irreversibly inhibits PKA (see Botelho *et al.*, 1988). Addition of 50 μM Rp-cAMPS evoked little or no change in SS pH_i in the absence of HCO_3^- ($n = 22$; Fig. 17). This result was similar to the effect of DDA (an inhibitor of adenylyl cyclase activity) on SS pH_i (see Section 2.1.1). Although Rp-cAMPS was unable to alter SS pH_i when applied alone, it has previously been reported that Rp-cAMPS is able to inhibit markedly the ability of noradrenaline to increase both SS pH_i and rates of pH_i recovery from intracellular acid loads (Smith *et al.*, 1998).

Taken together, the results obtained with Sp- and Rp-cAMPS indicate that PKA is involved in the control of the activity of the NHE in adult rat hippocampal CA1 neurons.

2.1.3 The relationship between cAMP and PKA

The rise in SS pH_i evoked by 25 μM Sp-cAMPS was similar in magnitude to that evoked by 25 μM forskolin ($P = 0.40$) and 200 μM IBMX ($P = 0.12$). In addition, the extent to which

NHE activity (as assessed in acid load recovery experiments) was stimulated by 25 μM Sp-cAMPS was similar to that observed with 25 μM forskolin ($P = 0.95$) and 200 μM IBMX ($P = 0.26$). These results suggest the possibility that the responses evoked by increasing $[cAMP]_i$ were mediated by stimulation of PKA activity. To examine this possibility, experiments were performed in which neurons were pretreated with 50 μM Rp-cAMPS and then exposed to 25 μM forskolin. Under these conditions, the addition of forskolin either failed to change SS pH_i ($n = 6$) or evoked a 0.06 ± 0.04 pH unit increase in SS pH_i ($n = 4$, Fig. 17), a rise which was significantly smaller than the forskolin-evoked alkalinization observed in the absence of Rp-cAMPS ($P < 0.005$). Thus, inhibition of PKA activity attenuated the SS pH_i response to increasing $[cAMP]_i$, confirming that the effects of cAMP on SS pH_i are mediated, in large part, by PKA.

Nevertheless, in some cell types (e.g. cardiac Purkinje fibres; Wu & Vaughan-Jones, 1994), changes in $[cAMP]_i$ can modulate NHE activity, not via PKA but via a pathway which involves changes in $[Ca^{2+}]_i$. In order to examine whether altering the activity of the cAMP/PKA pathway affects $[Ca^{2+}]_i$, which in turn may affect NHE activity, experiments were performed to monitor $[Ca^{2+}]_i$ in the presence of either Sp-cAMPS or Rp-cAMPS. In 3 neurons with a mean resting BI_{334}/BI_{380} value at 1.20 ± 0.15, the addition of 25 μM Sp-cAMPS failed to significantly alter the BI_{334}/BI_{380} ratio values (Fig. 18A). Similarly, in 3 neurons with a mean resting BI_{334}/BI_{380} value at 1.16 ± 0.26, the addition of 50 μM Rp-cAMPS did not change BI_{334}/BI_{380} ratio values (Fig. 18B). These results suggest that altering the activity of PKA affects SS pH_i and the activity of the NHE independent of changes in $[Ca^{2+}]_i$. Further evidence of this possibility was obtained from experiments performed to monitor pH_i in the absence of external Ca^{2+}. Under these conditions, the addition of 25 μM forskolin and 25 μM Sp-cAMPS evoked a 0.25 ± 0.10 ($n = 3/4$, Fig. 18C) and 0.23 ± 0.03 ($n = 3/4$, Fig. 18D) pH unit increases in SS pH_i, respectively. The remaining 2 neurons did not respond to forskolin or Sp-cAMPS treatment. These results were not significantly different ($P = 0.47$ and $P = 0.68$, respectively) to the observed changes in SS pH_i evoked by either agent in the presence of external Ca^{2+}, further suggesting that the SS pH_i

response to stimulation of the cAMP/PKA pathway was independent of changes in intracellular $[Ca^{2+}]$.

Finally, experiments were performed with okadaic acid (OA; Cohen *et al.*, 1990a), a relatively non-specific inhibitor of serine/threonine protein phosphatase 1 and 2A activity, to assess whether a phosphorylation event may be involved in the observed SS pH_i response to stimulation of PKA activity. As shown in Fig. 19, the addition of 10 μM OA caused a 0.18 ± 0.05 (*n* = 4) pH unit increase in SS pH_i. The subsequent addition of 10 μM Sp-cAMPS (a sub-maximal [Sp-cAMPS]; see above) in the continued presence of OA evoked a 0.36 ± 0.10 (*n* = 4) pH unit increase in SS pH_i, which significantly greater (*P* < 0.0001) than the alkalinization evoked by 10 μM Sp-cAMPS in the absence of OA. Thus, OA enhanced the SS pH_i response to Sp-cAMPS, suggesting that a phosphorylation event is involved in the SS pH_i response to stimulation of PKA activity.

2.1.4 Summary of the effects of altering the activity of the cAMP/PKA pathway on the activity of the NHE

Increasing $[cAMP]_i$ or PKA activity evokes an increase in SS pH_i which reflects an increase in the activity of the NHE. The effect of cAMP on SS pH_i (and the activity of the NHE) was mediated by PKA in a manner that was independent of changes in intracellular $[Ca^{2+}]$, but which may involve a serine/threonine protein phosphorylation event. Thus, the cAMP/PKA second messenger system participates in the control of the activity of the NHE in adult rat hippocampal CA1 neurons.

2.2 Na^+-independent and Na^+-dependent HCO_3^-/Cl^- exchange

The results presented above (Section 2.1) indicate that stimulating the cAMP/PKA second messenger system increases the activity of the NHE. In peripheral cell types, the activities of HCO_3^--dependent pH_i regulating mechanisms can also be modulated by the cAMP/PKA pathway (*e.g.* Vigne *et al.*, 1988; Harada *et al.*, 1991; also see Introduction). As it

has been established that the activities of HCO_3^--dependent mechanisms contribute to the regulation of SS pH_i in adult rat hippocampal CA1 neurons (Section 1), in the next series of experiments I examined the effects of modulating the activity of the cAMP/PKA pathway on the activities of both the NDAE and NIAE. All experiments were performed using HCO_3^-/CO_2-buffered media, unless otherwise noted.

2.2.1 The role of $cAMP_i$

Increasing [cAMP]$_i$ levels. Addition of 25 μM forskolin or 200 μM IBMX under HCO_3^--containing conditions elicited a 0.21 ± 0.01 ($n = 11/13$, Fig. 20A; the remaining two neurons did not respond to forskolin treatment) and 0.37 ± 0.07 ($n = 7$, Fig. 20B) pH unit increases in SS pH_i, respectively. These changes in SS pH_i were similar to those observed under HCO_3^--free conditions ($P > 0.25$, in each case). Of the thirteen neurons that were exposed to forskolin, three were subjected to the subsequent removal of forskolin from the perfusion medium; none of these neurons showed recovery of SS pH_i to resting levels (*e.g.* Fig. 20A). Furthermore, the addition of 25 μM forskolin in the presence of 200 μM DIDS evoked a 0.18 ± 0.03 pH unit increase in SS pH_i ($n = 6/7$, Fig. 20C; the remaining neuron did not respond), a rise which was not significantly different to the forskolin-induced alkalinization observed in the absence of DIDS ($P = 0.53$). Taken together, the similar results obtained under HEPES-buffered and HCO_3^-/CO_2-buffered (in the presence and absence of DIDS) conditions suggest that the SS pH_i response to *increasing* [cAMP]$_i$ is mediated, in large part, by an increase in NHE activity.

To confirm this suggestion, acid load recovery experiments were performed with 25 μM forskolin and 200 μM IBMX under HCO_3^-/CO_2-buffered conditions. The overall rates of pH_i recovery from acid loads were 160 ± 20% ($n = 13/17$, Fig. 21A) and 164 ± 10% ($n = 10/14$, Fig. 21B) faster in the presence than in the absence of forskolin and IBMX, respectively. The remaining eight neurons did not respond to treatment with forskolin or IBMX. The rates of pH_i recovery were plotted against absolute values of pH_i in the absence and presence of forskolin (Fig. 21C) or IBMX (Fig. 21D) and regression lines were fitted to the data points. The addition

of forskolin and IBMX significantly increased rates of pH_i recovery ($P < 0.05$ at each absolute value of pH_i) and caused the pH_i dependence of pH_i recovery to shift ~0.2 and ~0.1 pH units in the alkaline direction, respectively. The overall increases in rates of pH_i recovery from imposed acid loads elicited by each drug under HCO_3^--buffered conditions were not significantly different ($P > 0.20$) to the overall increases in rates of pH_i recovery observed under HCO_3^--free conditions. Furthermore, 200 μM DIDS failed to affect the increase in the rate of pH_i recovery from imposed acid loads evoked by 25 μM forskolin under HCO_3^-/CO_2-buffered conditions. Thus, the overall rate of pH_i recovery from acid loads was increased by 147 ± 4% ($n = 7/8$, Fig. 22A; the remaining neuron did not respond to treatment) in the presence of 25 μM forskolin and 200 μM DIDS. The rates of pH_i recovery under both control conditions and in the presence of forskolin and DIDS were plotted against absolute values of pH_i and regression lines were fitted to the data points (Fig. 22B). The addition of forskolin and DIDS: (1) significantly increased rates of pH_i recovery ($P < 0.05$ at each absolute value of pH_i); and (2) caused the pH_i dependence of pH_i recovery to shift ~0.3 pH units in the alkaline direction. The effects of forskolin on rates of pH_i recovery elicited in the presence of DIDS under HCO_3^--containing conditions were not significantly different to those evoked by forskolin in the absence of DIDS ($P > 0.39$) and were similar to the effects of forskolin observed under HEPES-buffered conditions ($P > 0.15$; when compared to neurons with low resting pH_i values). These results further suggest that increasing $[cAMP]_i$ acts to increase the rate of pH_i recovery from acid loads imposed under HCO_3^--containing conditions by increasing the activity of the NHE.

Control SS pH_i and acid load recovery experiments for forskolin were performed with its inactive analogue, 1',9'-dideoxyforskolin. The addition of 25 μM 1',9'-dideoxyforskolin under HCO_3^-/CO_2-buffered conditions did not change SS pH_i ($n = 6$, Fig. 23A) or rates of pH_i recovery from intracellular acid loads ($n = 5$, Fig. 23B and C).

Inhibition of adenylyl cyclase activity. The results presented above suggest that the changes in SS pH_i and rates of pH_i recovery from acid loads imposed under HCO_3^-/CO_2-buffered conditions evoked by forskolin and IBMX (agents which act to *increase* $[cAMP]_i$) can be

accounted for by changes in the activity of the NHE rather than the activities of HCO_3^--dependent pH_i regulating mechanisms. In addition, under HEPES-buffered conditions, application of the adenylyl cyclase inhibitor, DDA, failed to alter SS pH_i (see Section 2.1.1). Therefore, it was of interest that, when applied under HCO_3^-/CO_2-buffered conditions, DDA elicited a 0.23 ± 0.08 ($n = 8/9$; the remaining neuron did not respond to DDA treatment) pH unit rise in SS pH_i in neurons with resting pH_i values $\leq \sim 7.4$ (Fig. 24A). No neurons with resting pH_i values > 7.4 were observed. When the changes in SS pH_i evoked by DDA in the presence of HCO_3^- were plotted against values of resting pH_i (Fig. 24B) and a regression line was fitted to the data, it was found that the SS pH_i response to DDA was dependent on the resting pH_i of a neuron. These results indicate that DDA was increasing SS pH_i in HCO_3^--dependent manner suggesting the possibility that inhibiting adenylyl cyclase activity may affect the activities of HCO_3^--dependent pH_i regulating mechanisms in neurons with resting pH_i values $\leq \sim 7.4$.

In summary, under HCO_3^-/CO_2-buffered conditions, *increasing* $[cAMP]_i$ (with forskolin or IBMX) evokes an increase in SS pH_i which most likely reflects an increase in the activity of the NHE. In contrast, *inhibition* of adenylyl cyclase activity (with DDA) causes SS pH_i to increase in neurons with resting pH_i values $\leq \sim 7.4$, an effect which may reflect changes in the activities of HCO_3^--dependent pH_i regulating mechanisms (such as the NIAE and NDAE).

2.2.2 The role of PKA

Inhibition of PKA activity. As detailed above, inhibition of adenylyl cyclase activity caused SS pH_i to change only when HCO_3^- was present in the perfusion medium. As it has also been shown that the effects of changing $[cAMP]_i$ on SS pH_i under HEPES-buffered conditions are mediated by changes in PKA activity (see Section 2.1.3), experiments were performed with Rp-cAMPS under HCO_3^-/CO_2-buffered conditions to investigate whether inhibition of PKA activity would also change SS pH_i in a HCO_3^-dependent manner. In contrast to the failure of Rp-cAMPS to change SS pH_i under nominally HCO_3^--free, HEPES-buffered conditions (see Section 2.1.2), the addition of 50 µM Rp-cAMPS under HCO_3^-/CO_2-buffered conditions elicited changes

in SS pH_i which varied depending on the resting pH_i of the neuron. Thus, Rp-cAMPS elicited a rise in SS pH_i in neurons with resting pH_i values $< {\sim}7.4$ ($n = 12$; Fig. 25A; compare with the results obtained with DDA, Fig. 24A) and a fall in SS pH_i in neurons with resting pH_i values $>$ ${\sim}7.4$ ($n = 3$; Fig. 25B). In an additional neuron with a resting pH_i value at ${\sim}7.4$, Rp-cAMPS failed to evoke a change in SS pH_i. When the Rp-cAMPS-elicited changes in SS pH_i were plotted against values of resting pH_i measured prior to the application of the compound and a regression line was fitted to the data points (Fig. 25C), the regression line had a similar profile and x-intercept as the lines representing both the effect of DDA on SS pH_i in the presence of HCO_3^- (Fig. 24B) and data obtained upon the addition of HCO_3^- to HEPES-buffered medium (a maneuver which leads to the activation of HCO_3^--dependent pH_i regulating mechanisms; see Fig. 8C). Furthermore, the changes in SS pH_i evoked by Rp-cAMPS in the presence of HCO_3^- were abolished by 200 μM DIDS ($n = 11$, Fig. 26). Thus, Rp-cAMPS alters SS pH_i in a HCO_3^--dependent, DIDS-sensitive manner which is dependent on the resting pH_i value of a neuron and which mimics the effect of HCO_3^-/CO_2 addition on SS pH_i. Taken together, these results suggest that inhibiting PKA activity may change SS pH_i by changing the activities of HCO_3^--dependent pH_i regulating mechanisms.

Stimulation of PKA activity. As shown in Section 2.1.2, under HEPES-buffered conditions, the addition of 25 μM Sp-cAMPS evoked a 0.22 ± 0.03 pH unit ($n = 7/8$; see Fig. 27A) increase in SS pH_i in neurons with resting pH_i values $< {\sim}7.4$; this change in SS pH_i was attributed to stimulation of the activity of the NHE. Because the effects of *inhibiting* the activity of PKA on SS pH_i are HCO_3^--dependent (see above), it was of interest to determine if stimulation of PKA activity also had any HCO_3^--dependent effects on SS pH_i. Thus, under HCO_3^-/CO_2-buffered conditions, addition of 25 μM Sp-cAMPS elicited a 0.14 ± 0.02 ($n = 6/8$, Fig. 27A; the remaining two neurons did not respond to Sp-cAMPS treatment) pH unit increase in SS pH_i in neurons with resting pH_i values $< {\sim}7.4$. This alkalinization was significantly smaller than that observed under HEPES-buffered conditions ($P < 0.05$), when considering that the mean resting pH_i values of the neurons examined under both HCO_3^--containing and HCO_3^--free conditions

were not significantly different (P = 0.76; pH_i = 7.25 ± 0.09 and 7.22 ± 0.05 under HEPES- and HCO_3^-/CO_2-buffered conditions, respectively). Thus, the presence of HCO_3^- caused a reduction in the magnitude of the rise in SS pH_i evoked by Sp-cAMPS, compared to the rise observed under HEPES-buffered conditions, suggesting the possibility that, under HCO_3^-/CO_2-buffered conditions, the Sp-cAMPS-evoked increase in the activity of the NHE may be opposed by changes in the activities of HCO_3^--dependent pH_i regulating mechanisms. Notably, the effects of Sp-cAMPS on SS pH_i in neurons with resting pH_i values < ~7.4 under HCO_3^-/CO_2-buffered conditions are opposite to those of Rp-cAMPS (see above).

Inhibiting protein phosphatase activity. I have shown previously that a protein phosphorylation event may be involved in the regulation of NHE activity (see Section 2.1.3). Given the fact that modulation of PKA activity affects SS pH_i in a HCO_3^--dependent manner, I performed preliminary experiments to assess whether inhibition of protein phosphatase activity (with OA) might also affect the activities of HCO_3^--dependent pH_i regulating mechanisms. Under HEPES-buffered conditions, the addition of 10 µM OA elicited a 0.23 ± 0.03 (n = 9, see Fig. 27B) pH unit increase in SS pH_i in neurons with resting pH_i values < ~7.4. However, under HCO_3^-/CO_2-buffered conditions, the addition of 10 µM OA elicited a 0.15 ± 0.03 (n = 6; Fig. 27B) pH unit increase in SS pH_i, a rise in pH_i which was significantly smaller than the alkalinization observed under HEPES-buffered conditions (P < 0.05) when considering that the mean resting pH_i values of the neurons examined under both HEPES- and HCO_3^-/CO_2-buffered conditions were not significantly different (P = 0.67; pH_i = 7.26 ± 0.11 and 7.32 ± 0.02 under HEPES- and HCO_3^-/CO_2-buffered conditions, respectively). Thus, similar to the results obtained with Sp-cAMPS (see above), the presence of HCO_3^- caused a reduction in the magnitude of the internal alkalinization evoked by OA, compared to the rise in pH_i observed under HEPES-buffered conditions, suggesting that the OA-evoked increase in the activity of the NHE (responsible for the increase in SS pH_i) is opposed by a HCO_3^--dependent acidification which most likely reflects changes in the activities of HCO_3^--dependent pH_i regulating mechanisms.

This result suggests the possibility that protein phosphorylation may also modulate the activities of HCO_3^--dependent pH_i regulating mechanisms.

2.2.3 Summary of the HCO_3^--dependent SS pH_i changes evoked by changing the activity of the cAMP/PKA pathway

The studies detailed above suggest the possibility that the cAMP/PKA pathway may participate in the control of the activities of HCO_3^--dependent pH_i regulating mechanisms (*i.e.* the NDAE and NIAE) in adult rat hippocampal CA1 neurons. In this regard, four different possibilities may account for the data presented in Sections 2.2.1 and 2.2.2: (1) In neurons with *low* resting SS pH_i values (< ~7.4), Rp-cAMPS may inhibit the activity of the NIAE and/or stimulate the activity of the NDAE, resulting in the observed *increase* in SS pH_i (see Fig. 25A); (2) In neurons with *high* resting SS pH_i values (> ~7.4), Rp-cAMPS may stimulate the activity of the NIAE and/or inhibit the activity of the NDAE, resulting in the observed *decrease* in SS pH_i (see Fig. 25B); (3) In neurons with *low* resting SS pH_i values (< ~7.4), Sp-cAMPS may increase the activity of the NIAE and/or decrease the activity of the NDAE, resulting in the observed reduction in the magnitude of the rise in SS pH_i evoked by Sp-cAMPS under HCO_3^-/CO_2-, compared to HEPES-, buffered conditions (see Section 2.2.2 and Fig. 27A); and (4) Although no neurons with *high* resting SS pH_i values (> ~7.4) were observed when the effects of Sp-cAMPS were examined on SS pH_i under HCO_3^-/CO_2-buffered conditions, in light of the previous three possibilities presented it is possible that, in neurons with high resting pH_i values (> ~7.4), Sp-cAMPS may decrease the activity of the NIAE and/or increase the activity of the NDAE, resulting in an *increase* in SS pH_i which would be greater than the increase in pH_i evoked in high pH_i neurons by Sp-cAMPS under HEPES-buffered conditions

To test the validity of each of these four possibilities, experiments were performed to assess the effects of modulating the activity of the cAMP/PKA pathway on the activities of both the acid-loading NIAE and the acid-extruding NDAE. As described in Methods and Materials (Section 5.2), Na^+-independent anion exchange was examined using alkali load recovery, acute

Cl^-_o-removal and Na^+_o-free experiments, whereas Na^+-dependent anion exchange was assessed using acid load recovery and Cl^-_i-depletion experiments. Rp-cAMPS and Sp-cAMPS were used to inhibit and stimulate the activity of the cAMP/PKA pathway, respectively.

2.2.4 Effect of changing PKA activity on the activity of HCO_3^--dependent pH_i regulating mechanisms.

2.2.4.1 The NIAE

Alkaline load recovery. Initially, to establish whether Na^+-independent HCO_3^-/Cl^- exchange contributes to pH_i recovery from alkaline loads in adult rat hippocampal CA1 neurons: (1) Paired alkaline load recovery experiments were performed in the presence and absence of DIDS; addition of 200 μM DIDS almost completely abolished recovery of pH_i from imposed internal alkaline loads ($n = 6$, Fig. 28); and (2) Alkaline load experiments were performed in the absence of Na^+_o (a condition which isolates the activity of the NIAE, as the activities of the NDAE and NHE are blocked). Under these conditions, pH_i continued to recover from imposed alkaline loads ($n = 5$; see Fig. 29C). Thus, taken together, these data suggest that the activity of a DIDS-sensitive, Na^+-independent acid loading mechanism, most likely the NIAE, is, at least in part, responsible for the pH_i recovery from imposed internal alkaline loads in adult rat hippocampal CA1 neurons.

Next, in order to assess the effect of inhibiting PKA activity on the activity of the NIAE, paired alkaline load experiments were performed in the presence and absence of Rp-cAMPS. In neurons with resting pH_i values $< \sim 7.4$, the overall rate of pH_i recovery from alkaline loads was $78 \pm 1\%$ ($n = 14$, Fig. 29A) slower in the presence than in the absence of 50 μM Rp-cAMPS. No neurons with resting pH_i values $> \sim 7.4$ were encountered in these experiments. When the rates of pH_i recovery from alkaline loads imposed in the presence and absence of Rp-cAMPS were plotted against absolute values of pH_i and regression lines were fitted to the data points (Fig. 29B), significantly reduced rates of pH_i recovery were observed in the presence than in the absence of Rp-cAMPS ($P < 0.05$ at all absolute values of pH_i). In addition, Rp-cAMPS caused

the pH_i dependence of pH_i recovery from alkaline loads to shift ~ 0.1 pH units in the alkaline direction. Similar results, also in neurons with resting pH_i values $< \sim 7.4$, were obtained in parallel experiments performed in the absence of Na^+_o ($n = 5$, Fig. 29C and D), thereby confirming the Na^+_o-independence of the effects of Rp-cAMPS on pH_i recovery from alkaline loads. Taken together, these data suggest that inhibition of PKA decreases the activity of the NIAE in neurons with resting pH_i values < 7.4.

In order to assess the effect of stimulating PKA activity on the activity of the NIAE, paired alkaline load experiments were performed in the absence and presence of Sp-cAMPS. In neurons with resting pH_i values $< \sim 7.4$, the overall rates of pH_i recovery following alkaline loads were $14 \pm 17\%$ ($n = 9$, Fig. 30A) slower in the presence than in the absence of 25 μM Sp-cAMPS. No neurons with resting pH_i values $> \sim 7.4$ were encountered. However, when the rates of pH_i recovery from alkaline loads imposed in the presence and absence of Sp-cAMPS were plotted against absolute values of pH_i and regression lines were fitted to the data points (Fig. 30B), it was found that the percentage change value representing the difference between overall rates of pH_i recovery in the absence and presence of Sp-cAMPS (14 ± 17 %) did not accurately reflect the actual changes in the rates of pH_i recovery evoked by Sp-cAMPS. Thus, the line representing data obtained in the presence of Sp-cAMPS had a greater slope than the line representing control data and rates of pH_i recovery from alkaline loads imposed in the presence of Sp-cAMPS were significantly *faster* than control rates of pH_i recovery at absolute values of $pH_i \geq 7.40$. The latter finding appears to reflect the ~ 0.3 pH unit alkaline shift in the pH_i-dependence of pH_i recovery from alkaline loads evoked by Sp-cAMPS (see Fig. 30B), a shift that can be accounted for by the (net) increase in SS pH_i also evoked by Sp-cAMPS in neurons with resting pH_i values $< \sim 7.4$ (see Section 2.2.2). When all of the aforementioned factors are taken into account, the results suggest that stimulation of PKA activity increases rates of pH_i recovery from imposed alkaline loads, suggesting, in turn, that stimulation of PKA activity increases the activity of the NIAE in neurons with resting pH_i values $< \sim 7.4$.

When considering the SS pH_i changes evoked by alterations in PKA activity under HCO_3^-/CO_2-buffered conditions (see Section 2.2.3), these results from alkaline load recovery experiments confirm that: (1) The increase in SS pH_i evoked by Rp-cAMPS in neurons with resting pH_i values $< \sim 7.4$ reflects, at least in part, a reduction in the activity of the NIAE; and (2) the HCO_3^--dependent decrease in SS pH_i evoked by Sp-cAMPS in neurons with resting pH_i values < 7.4 reflects, at least in part, an increase in the activity of the NIAE.

Removal of external Cl^-. To further examine the effect of PKA on the activity of the NIAE, in the next series of experiments, neurons were acutely exposed to solutions devoid of $[Cl^-]$ (a condition which leads to the reversal of the NIAE; see Ganz *et al.*, 1989; Kikeri *et al.*, 1990; Ludt *et al.*, 1991; Jiang *et al.*, 1994; Lin & Miller, 1994). Acute (3 - 5 min) exposure to Cl^--free medium in the absence of any treatment elicited a sustained 0.13 ± 0.01 ($n = 31$; see Fig. 31A) pH unit rise in SS pH_i and, upon reintroduction of Cl^-_o, pH_i recovered to the resting level. This 0 Cl^-_o-evoked rise in SS pH_i was blocked by 200 μM DIDS ($n = 8$; see Fig. 31C). When neurons were exposed to acute Cl^-_o removal in the presence of 50 μM Rp-cAMPS or 25 μM Sp-cAMPS, 0.07 ± 0.01 ($n = 4$, Fig. 31A) and 0.42 ± 0.07 ($n = 5$, Fig. 31B) pH unit increases in SS pH_i were observed, respectively. The 0 Cl^-_o-evoked internal 0 Cl^-_o-evoked alkalinizations observed in the presence of Rp-cAMPS and Sp-cAMPS were $30 \pm 5\%$ smaller and $304 \pm 91\%$ larger than the control alkalinizations observed in the same neurons, respectively ($P < 0.05$ in each case). In addition, the large 0 Cl^--induced rise in SS pH_i observed in the presence of Sp-cAMPS was abolished by 200 μM DIDS ($n = 4$, Fig. 31C). Taken together, these results suggest that stimulating PKA activity increases the activity of the NIAE and that inhibiting PKA activity decreases the activity of the NIAE (see Fig. 31D). As all neurons examined in this experimental series had resting pH_i values $< \sim 7.4$, these results are entirely consistent with the results obtained from alkaline load recovery experiments and, thus, further suggest the involvement of changes in NIAE activity in the HCO_3^--dependent SS pH_i responses evoked by modulating PKA activity (see above).

SS pH_i in the absence of Na^+_o. The final series of experiments used to examine the effect of altering PKA activity on the activity of the NIAE involved monitoring SS pH_i under Na^+_o-free conditions (under which conditions both the NHE and NDAE are blocked). Addition of 25 μM Sp-cAMPS under these conditions elicited a 0.12 ± 0.03 pH unit rise in SS pH_i in neurons with resting pH_i values > ~7.4 (n = 4, Fig. 32). As this rise in SS pH_i evoked by Sp-cAMPS can not be accounted for by stimulation of NHE activity (or changes in the activity of the NDAE), this result suggests (in accordance with the forth possibility presented in Section 2.2.3) that a decrease in the activity of the NIAE is, at least in part, responsible for the increase in SS pH_i evoked by Sp-cAMPS in neurons with resting pH_i values > ~7.4. Although no neurons with resting pH_i values < ~7.4 were observed under Na^+_o-free conditions, it was previously shown that Sp-cAMPS stimulates the activity of the NIAE in these low pH_i neurons as indicated by results obtained from alkaline load recovery and acute Cl^-_o removal experiments (see above).

Similar experiments were performed in the presence of 50 μM Rp-cAMPS and in the absence of Na^+_o. As observed in the presence of Na^+_o (see Fig. 25), Rp-cAMPS elicited a rise in SS pH_i in neurons with resting pH_i values < ~7.45 (n = 9, Fig. 33A) and a fall in SS pH_i in neurons with resting pH_i values > 7.45 (n = 3, Fig. 33B). Changes in SS pH_i elicited by Rp-cAMPS in the absence of Na^+_o were plotted against resting pH_i values and a regression line was fitted to the data points (Fig. 33C). This line had a similar profile and x-intercept as the line representing data obtained in the presence Rp-cAMPS and Na^+_o (see Fig. 33C). In addition, this Na^+-independent SS pH_i response to Rp-cAMPS was abolished in the presence of 200 μM DIDS (n = 4, Fig. 34; in neurons with both low and high resting pH_i values). Thus, these results confirm suggestions made previously (see Section 2.2.3) that, in neurons with resting pH_i values < 7.4, inhibition of PKA activity evokes a rise in SS pH_i by inhibiting the activity of the NIAE. Furthermore, in neurons with resting pH_i values > 7.4, the fall in SS pH_i evoked by inhibition of PKA activity most likely reflects an increase in the activity of the NIAE.

Summary. The results from the three experimental series detailed above confirm the involvement of the activity of the NIAE in all of the SS pH_i responses evoked by modulating the

activity of PKA and discussed in Section 2.2.3: In neurons with resting pH_i values > 7.4, stimulation of PKA with Sp-cAMPS evokes a rise in SS pH_i, at least in part by decreasing the activity of the NIAE, whereas inhibition of PKA with Rp-cAMPS evokes a fall in SS pH_i, at least in part by increasing the activity of the NIAE. Conversely, in neurons with resting pH_i values < 7.4, stimulation of PKA with Sp-cAMPS evokes a HCO_3^--dependent acidification, at least in part by increasing the activity of the NIAE, whereas inhibition of PKA with Rp-cAMPS evokes a rise in SS pH_i, at least in part by decreasing the activity of the NIAE.

2.2.4.2 The NDAE

Acid Load Recovery. In order to assess the effect of modulating PKA activity on the activity of the NDAE, a series of acid load recovery experiments involving the exposure of neurons to Rp-cAMPS and Sp-cAMPS was performed. It must be noted that under normal experimental conditions (*i.e.* 37°C), the effects of modulating the activity of PKA on rates of pH_i recovery from imposed acid loads will reflect not only potential changes in the activity of the NDAE but also (as detailed in Section 2.1) changes in the activity of the NHE. In acid load recovery experiments conducted on neurons with resting pH_i values < 7.4 under HCO_3^-/CO_2-buffered conditions, the presence of 25 μM Sp-cAMPS elicited a 70 ± 3% (n = 8/14, Fig. 35; the remaining 6 neurons did not respond to Sp-cAMPS treatment) increase in the overall rate of pH_i recovery when compared to overall control rate of pH_i recovery. This mean percentage increase value for rates of pH_i recovery observed in the presence Sp-cAMPS was smaller in the presence than in the absence of HCO_3^- (a 168 ± 16 % increase in neurons with resting pH_i < 7.4 under HEPES-buffered conditions, P = 0.08; see Section 2.1.2). Thus, the presence of HCO_3^- reduced the effect of Sp-cAMPS to increase rates of pH_i recovery from acid loads compared to the increase observed under HEPES-buffered conditions. In concert with the reduced SS pH_i increase evoked by Sp-cAMPS in neurons with resting pH_i values < ~7.4 under HCO_3^-/CO_2-buffered, compared to HEPES-buffered conditions (see Section 2.2.2), the results suggest that the activation of PKA inhibits the activity of the NDAE. This effect of PKA activation might act to counteract the concomitant increase in the activity of the NHE also evoked by Sp-cAMPS.

In order to further assess the effect of stimulating PKA activity on the activity of the NDAE, additional acid load recovery experiments were performed at RT. Under these conditions of reduced temperature, NHE activity is markedly reduced and the relative contribution of Na^+-dependent HCO_3^-/Cl^- exchange to acid extrusion following internal acid loads in greatly increased (Baxter & Church, 1996). At RT, and in neurons with resting pH_i values $< \sim7.4$, addition of 25 μM Sp-cAMPS caused a 50 ± 3 % ($n = 5$, Fig. 36A) *decrease* in the overall rate of pH_i recovery when compared to control rates of pH_i recovery. When these rates of pH_i recovery from acid loads imposed in the absence and presence of Sp-cAMPS were plotted against absolute values of pH_i and regression lines were fitted to the data points (Fig. 36B), it was found that the rates of pH_i recovery were significantly reduced in the presence compared to the absence of Sp-cAMPS ($P < 0.05$ at absolute pH_i values of 7.15 and 7.20). Similar paired acid load recovery experiments were performed in the absence of HCO_3^- at RT and, under these conditions, 25 μM Sp-cAMPS failed to evoke a significant change in the rates of pH_i recovery in neurons with resting pH_i values $< \sim7.4$ (an overall 2 ± 10 % decrease; $n = 9$; Fig. 36C and D), indicating that the Sp-cAMPS-induced decreases in rates of pH_i recovery from acid loads at RT were HCO_3^--dependent. These results therefore also suggest that, in neurons with resting pH_i values < 7.4, stimulation of PKA activity decreases the activity of the NDAE.

It should also be noted that although the control rates of pH_i recovery under both HCO_3^--free and HCO_3^--containing conditions were nearly the same (compare Fig. 36B and D). However, because intracellular buffering power is greater in the presence than in the absence of HCO_3^- (see Baxter & Church, 1996), there is a greater rate of acid extrusion in the presence than in the absence of HCO_3^- which reflects the additional contribution of the NDAE under HCO_3^--containing conditions (also see Boyarsky *et al.*, 1988b). In addition, the overall control rate of pH_i recovery under HEPES-buffered conditions at RT (see Fig. 36D) was $\sim$33% slower than that observed at 37°C (see Methods and Materials, Fig. 5B; *e.g.* in neurons with low resting pH_i values ($< \sim7.4$), the rates of pH_i recovery at an absolute pH_i value of 7.10 were $\sim$0.0008 and $\sim$0.0024 pH units s^{-1} at RT and 37°C, respectively). Hence, the low rate of net acid extrusion

observed at RT and the inability of Sp-cAMPS to evoke increases in rates of pH_i recovery from acid loads performed under HEPES-buffered conditions at RT confirm the findings of Baxter & Church (1996) in cultured foetal rat hippocampal neurons that the activity of the NHE is greatly reduced at RT and that the NDAE is the major contributor to pH_i recovery from acid loads at RT.

Next, the effect of inhibiting PKA activity on the activity of the NDAE was assessed by performing paired acid load recovery experiments in the absence and presence Rp-cAMPS at RT and 37°C. In contrast to Sp-cAMPS, the addition of 50 μM Rp-cAMPS caused 170 ± 33% ($n = 11$, Fig. 37A) and 239 ± 49% ($n = 4/5$, Fig. 37B; the remaining neuron did not respond to Rp-cAMPS treatment) increases in the overall rates of pH_i recovery from acid loads imposed at RT and 37°C, respectively, when compared to control rates of pH_i recovery. All neurons examined had resting pH_i values < ~7.4. The mean rates of pH_i recovery observed in the absence and presence of Rp-cAMPS were plotted against absolute values of pH_i and regression lines were fitted to the data points. The addition of Rp-cAMPS significantly increased rates of pH_i recovery ($P < 0.05$ for all absolute values of pH_i) at both RT (Fig. 37C) and 37°C (Fig. 37D). In addition, Rp-cAMPS caused the pH_i dependence of pH_i recovery to shift by ~0.20 pH units in the alkaline direction at both RT and 37°C. Similar experiments were performed in the presence of 200 μM DIDS at 37°C ($n = 4$, Fig. 38A and B) and in the absence of HCO_3^- at RT ($n = 10$; Fig. 38C and D) and, in neurons with resting pH_i values < 7.4, the addition of Rp-cAMPS failed to significantly change rates of pH_i recovery under either condition. Thus, Rp-cAMPS increased rates of pH_i recovery in a HCO_3^--dependent and DIDS-sensitive manner, suggesting that, in neurons with resting pH_i values < ~7.4, inhibition of PKA with Rp-cAMPS increases the activity of the NDAE.

The NDAE is dependent upon internal Cl^-. In the second series of experiments designed to examine the effects of altering PKA activity on the activity of the NDAE, neurons were depleted of internal Cl^- using a protocol described by Schwiening & Boron (1994; also see Bevensee *et al*, 1997). In these experiments, neurons were initially perfused with a Cl^--free, HEPES-buffered medium and then exposed repeatedly to a Cl^--free, HCO_3^-/CO_2-buffered

medium (see Fig. 39A). In this protocol, repeated activation of the NDAE (evoked by the addition of HCO_3^-/CO_2) depletes $[Cl^-]_i$ from within the neuron.

Under Cl^-_o-free, control conditions ($n = 7$, Fig. 39A), an initial (~ 7 min) exposure to HCO_3^-/CO_2 elicited an initial acidification (due to CO_2 entry) followed by a sustained 0.09 ± 0.02 pH unit increase in SS pH_i, likely reflecting activation of the NDAE (see above), which returned to the initial SS value upon the removal of external HCO_3^-. Subsequent second, third and fourth exposures to HCO_3^-/CO_2 caused 0.07 ± 0.02, 0.06 ± 0.02 and 0.05 ± 0.02 pH unit increases in SS pH_i, respectively, in neurons with resting pH_i values $< \sim 7.4$. Using the same protocol as that employed for analysis of pH_i recovery from acid loads (see Methods and Materials, Section 6.3), instantaneous rates of change in SS pH_i (indicating NDAE activity) during each exposure to HCO_3^-/CO_2 were calculated at an absolute value of $pH_i = 7.10$ and were then normalized to the mean rate of change in SS pH_i observed during the first addition of HCO_3^-/CO_2; during the first, second, third and fourth exposures, the normalized dpH_i/dt values were 100 ± 15, 77 ± 13, 44 ± 2 and 55 ± 4 %, respectively (see Fig. 40C). The progressive decline in the normalized rates of alkalinization evoked by repeated HCO_3^-/CO_2 applications suggests that the activity of the NDAE is progressively declining due to depletion of $[Cl^-]_i$ (see Schwiening & Boron, 1994). This result was in concert with the results from similar experiments performed in the presence of DIDS (to inhibit the activity of the NDAE). In the presence of 200 μM DIDS, 0.26 ± 0.05, 0.24 ± 0.06 and 0.25 ± 0.05 pH unit increases in SS pH_i were observed during the first, second and third exposures to HCO_3^-/CO_2^-, respectively ($n = 3$; Fig. 39B) in neurons with resting pH_i values $< \sim 7.4$. The instantaneous rates of change in SS pH_i were calculated at an absolute value of $pH_i = 7.10$ (same as control) during each exposure to HCO_3^-/CO_2 in the presence of DIDS and were then normalized using the mean rate of alkalinization observed during the first application of HCO_3^-/CO_2; during the first, second and third exposures to HCO_3^-/CO_2, the normalized dpH_i/dt values were 100 ± 18, 92 ± 15 and 98 ± 18 %, respectively (see Fig. 40C). As the normalized rates of alkalinization observed during each addition of HCO_3^-/CO_2 were similar (and not reduced with each subsequent HCO_3^-/CO_2 application, as observed under control conditions), the

results suggest that Cl_i^- was not depleted because the activity of the NDAE was not stimulated by the addition of HCO_3^-/CO_2 in the presence of DIDS.

Next, similar experiments were performed in the continued presence of 50 μM Rp-cAMPS and 0.21 ± 0.05, 0.11 ± 0.05 and 0.11 ± 0.01 pH unit increases in SS pH_i were observed during the first, second and third exposures to HCO_3^-/CO_2, respectively ($n = 3$, Fig. 40A), in neurons that had resting pH_i values $< \sim 7.4$. The normalized rates of change in SS pH_i were calculated at an absolute value of $pH_i = 7.10$ during each exposure to HCO_3^-/CO_2 in the presence of Rp-cAMPS; during the first, second and third exposures to HCO_3^-/CO_2, the normalized dpH_i/dt values were 100 ± 16, 59 ± 9 and 53 ± 8 %, respectively (see Fig. 40C). Thus, in the presence of Rp-cAMPS, the normalized rate of alkalinization was significantly decreased during the second exposure to HCO_3^-/CO_2^- whereas, under control conditions, the rate of alkalinization became significantly decreased only upon the third exposure to HCO_3^-/CO_2 (see Fig. 40C). These results indicate that internal Cl^- was depleted faster in the presence than in the absence of Rp-cAMPS, suggesting that the inhibition of PKA increases the activity of the NDAE in neurons with resting pH_i values $< \sim 7.4$.

In contrast to the results obtained in the presence of Rp-cAMPS, when similar experiments were performed in the continued presence of 25 μM Sp-cAMPS, 0.12 ± 0.06, 0.16 ± 0.06, 0.20 ± 0.03 and 0.27 ± 0.02 pH unit increases in SS pH_i were observed during the first, second, third and fourth applications of HCO_3^-/CO_2 respectively ($n = 3$, Fig. 40B), in neurons with resting pH_i values $< \sim 7.4$. Normalized rates of change in SS pH_i were calculated at an absolute value of $pH_i = 7.10$ during each exposure to HCO_3^-/CO_2 in the presence of Sp-cAMPS; during the first, second, third and fourth exposures, the normalized dpH_i/dt values were 100 ± 17, 51 ± 7, 106 ± 12 and 120 ± 15 %, respectively. As shown in Fig. 40C, the normalized rates of alkalinization observed in the presence of Sp-cAMPS during the third and fourth applications of HCO_3^-/CO_2 were similar to the rate of alkalinization observed during the first application of HCO_3^-/CO_2, a pattern of response which was also observed in the absence of Sp-cAMPS but in the presence of DIDS. This result contrasts with the results obtained in the presence of Rp-

cAMPS and suggests that internal Cl⁻ is not sequentially reduced with each subsequent addition of HCO_3^-/CO_2 because the stimulation of PKA activity decreases the activity of the NDAE in neurons with resting pH_i values $< \sim 7.4$.

Summary. The results described above confirm the involvement of the NDAE in the SS pH_i response evoked by modulating the activity of PKA in neurons with resting pH_i values $< \sim 7.4$ under HCO_3^-/CO_2-buffered conditions (see Section 2.2.3). Thus, in neurons with resting pH_i values $< \sim 7.4$, stimulation of PKA with Sp-cAMPS evokes a HCO_3^--dependent acidification, at least in part, by decreasing the activity of the NDAE. Conversely, inhibition of PKA with Rp-cAMPS evokes a rise in SS pH_i, at least in part, by increasing the activity of the NDAE. Unfortunately, neurons with resting pH_i values $> \sim 7.4$ were not encountered in these series of experiments. Thus, it remains unclear whether PKA-evoked changes in SS pH_i in neurons with resting pH_i values $> \sim 7.4$ might reflect alterations in the activity of the NDAE in addition to the observed changes in the activity of the NIAE (see Section 2.2.4.1).

2.3 Summary of the effects of modulating the activity of the cAMP/PKA pathway on the activities of the NHE, NIAE and NDAE

Initially, experiments performed in the absence of HCO_3^- (described in Section 2.1) indicated that either increasing $[cAMP]_i$ or stimulating PKA activity causes an increase in SS pH_i which reflects stimulation of the activity of the NHE. Conversely, inhibition of the activities of adenylyl cyclases or PKA fails to change SS pH_i under HCO_3^--free conditions Further examination of the effects of activating the cAMP/PKA pathway on SS pH_i under HEPES-buffered conditions showed that: (1) The effect of increasing $[cAMP]_i$ on the activity of the NHE is mediated by PKA; (2) Stimulation of NHE activity by activation of the cAMP/PKA pathway is not mediated by changes in intracellular $[Ca^{2+}]$; and (3) A serine/threonine protein phosphorylation event may be involved in the stimulation of the activity of the NHE by PKA.

When experiments were performed in the presence of HCO_3^-/CO_2 (described in Section 2.2), increasing $[cAMP]_i$ evoked no HCO_3^--dependent effects on SS pH_i. In contrast, inhibition

of adenylyl cyclase activities caused SS pH_i to change in HCO_3^--dependent and pH_i-dependent manners, suggesting that modulation of activity of the cAMP/PKA pathway may change the activities of HCO_3^--dependent pH_i regulating mechanisms. Further experimental examination of this HCO_3^--dependent effect showed that: (1) In neurons with resting pH_i values < ~7.4, stimulation of PKA activity evokes a HCO_3^--dependent acidification by increasing the activity of the NIAE and inhibiting the activity of the NDAE whereas inhibition of PKA activity evokes a rise in SS pH_i by decreasing the activity of the NIAE and stimulating the activity of the NDAE; (2) In neurons with resting pH_i values > ~7.4, stimulation of PKA activity evokes a rise in SS pH_i, at least in part, by decreasing the activity of the NIAE whereas inhibition of PKA activity evokes a fall in SS pH_i, at least in part, by increasing the activity of the NIAE. However, in neurons with resting pH_i values > ~7.4, it remains unclear whether PKA-evoked changes SS pH_i might also reflect changes in the activity of the NDAE.

Thus, when considering the activities of all three pH_i regulating mechanisms present in adult rat hippocampal CA1 neurons, stimulation of the cAMP/PKA pathway has the overall effect of increasing SS pH_i above the prevailing pH_o value (see Fig. 27A). This reflects not only modulation of the activities of the NIAE and NDAE, but also activation of the NHE. In contrast, inhibition of the cAMP/PKA pathway has the overall effect of clamping SS pH_i at the prevailing pH_o value (see Fig. 25).

Figure 12. Effects of forskolin and IBMX on SS pH$_i$ under HEPES-buffered conditions.

A With a resting pH$_i$ at ~7.22 under HEPES-buffered conditions, a neuron exposed to 25 μM forskolin showed a ~0.30 pH unit alkalinization. Upon subsequent removal of forskolin, SS pH$_i$ remained elevated for the remainder of the experiment. **B** A neuron with a resting pH$_i$ at ~7.00 was exposed to 200 μM IBMX under HEPES-buffered conditions. IBMX caused pH$_i$ to increase to a new SS level at ~7.35 after an ~9 minute delay. Data in *A* and *B* were obtained from different neurons.

A
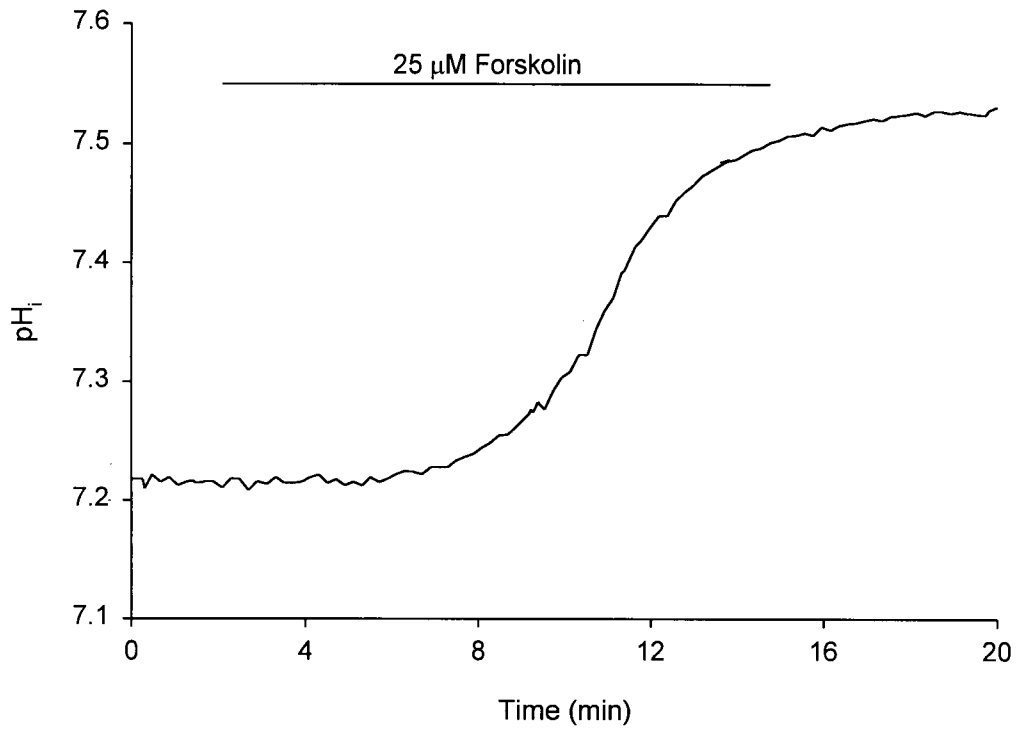
B
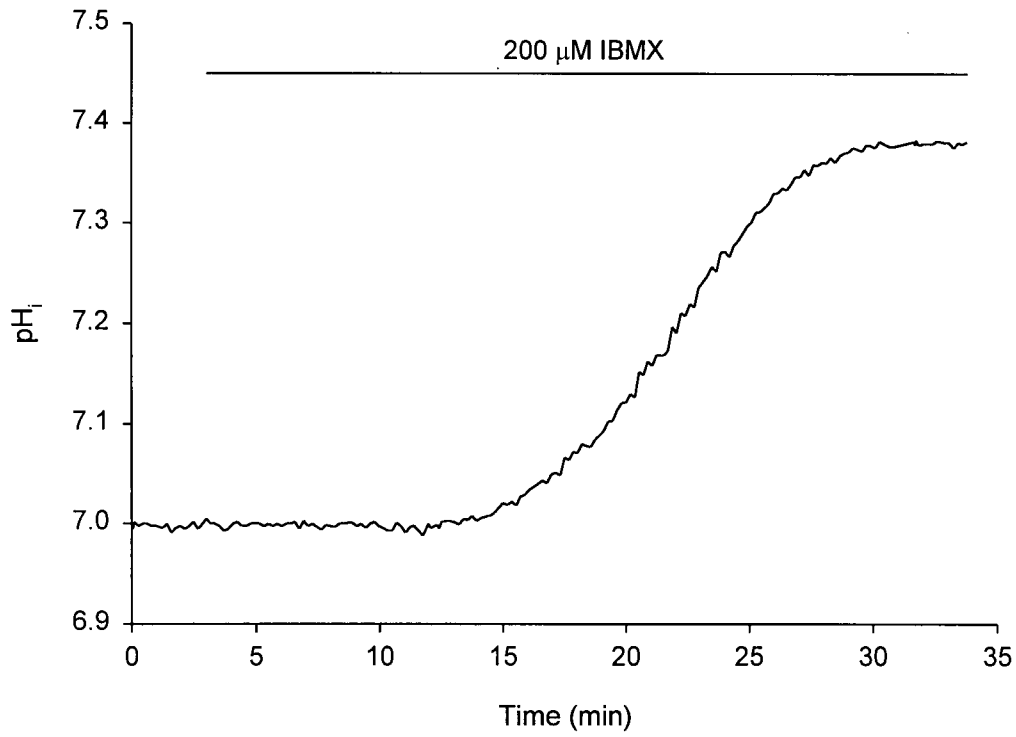
Figure 13. Effects of forskolin and IBMX on pH$_i$ recovery from imposed internal acid loads under HEPES-buffered conditions.

A A neuron with a resting pH$_i$ at ~7.10 was initially exposed to an internal acid load under HEPES-buffered conditions. After pH$_i$ recovered to the initial SS level, a second acid load was performed in the presence of 25 μM forskolin. pH$_i$ recovered faster in the presence than in the absence of forskolin. **B** Plotted are the mean calculated net acid fluxes against absolute values of pH$_i$ from 12 experiments of the type shown in **A**. The circles and squares represent the data obtained from neurons with low ($n = 6$) or high ($n = 6$) resting pH$_i$ values, respectively. In both groups of neurons, the net acid flux values observed in the presence of forskolin (open symbols) were significantly faster than those observed in its absence (filled symbols), at all absolute values of pH$_i$. When least squares linear regressions were fitted to the data points, it was found that the slopes of the lines were greater and the x-intercepts were more alkaline in the presence than in the absence of forskolin. **C** A neuron with a resting pH$_i$ of ~7.50 was initially exposed to an acid load under HEPES-buffered conditions. After pH$_i$ recovered to the initial SS level, a second acid load was performed in the presence of 200 μM IBMX. pH$_i$ recovered faster in the presence than in the absence of IBMX. **D** Plotted are the mean calculated net acid fluxes against absolute values of pH$_i$ from experiments of the type shown in **C**. The circles or squares represent the data obtained from neurons with low ($n = 8$) or high ($n = 2$) resting pH$_i$ values, respectively. In both groups of neurons, the net acid flux values observed in the presence of IBMX (open symbols) were significantly faster than those observed in its absence (filled symbols). When regression lines were fitted to the data points, it was found that the slopes of the lines were greater and the x-intercepts were more alkaline in the presence than in the absence of IBMX.
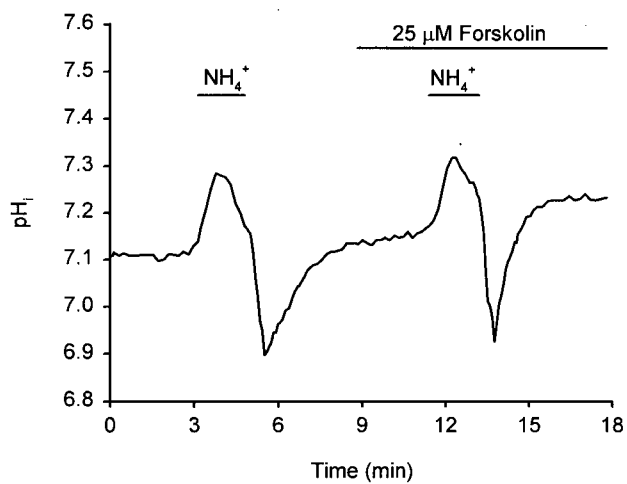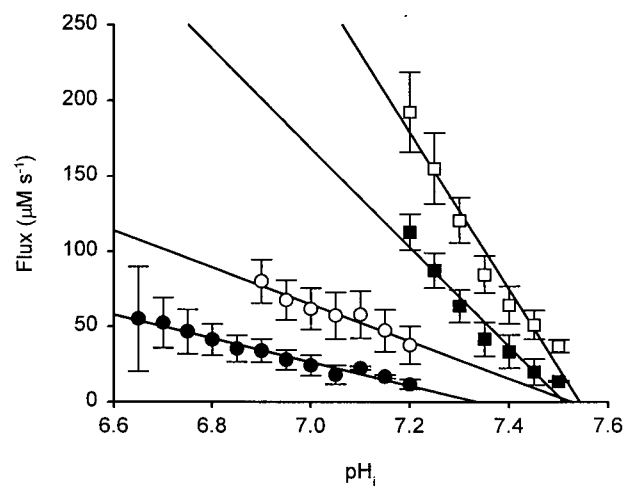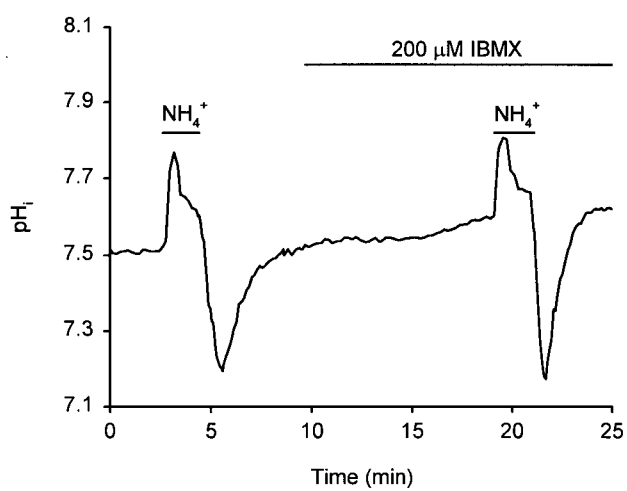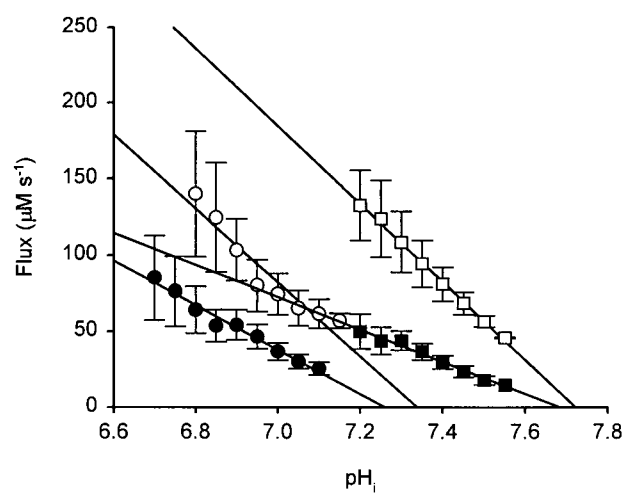
Figure 14. Effects of 1′, 9′-dideoxyforskolin on SS pH$_i$ and pH$_i$ recovery from internal acid loads under HEPES-buffered conditions.

A Addition of 25 µM 1′,9′-dideoxyforskolin had no effect on SS pH$_i$ in a neuron that had a resting pH$_i$ at ~7.13 under HEPES-buffered conditions. **B** In a different neuron with a resting pH$_i$ value at ~7.46 under HEPES-buffered conditions, pH$_i$ was allowed to recover after an initial acid load. A second acid load was performed in the presence of 25 µM 1′, 9′-dideoxyforskolin after which pH$_i$ recovered to initial SS levels at a similar rate to that observed under control conditions. **C** Mean calculated net acid fluxes were plotted against absolute values of pH$_i$ from 4 experiments of the type shown in **B**. Circles and squares represent the data obtained from neurons with low (n = 2) and high (n = 2) resting pH$_i$ values, respectively. The net acid flux values observed in the presence of 1′, 9′-dideoxyforskolin (open symbols) were not significantly different from control net acid flux values (closed symbols; P > 0.05 at each absolute value of pH$_i$). Regression lines that were fitted to the data points from neurons with low (circles) or high (squares) resting pH$_i$ values had x-intercepts at pH$_i$ ~7.1 and ~7.5, respectively.

A

25 µM 1',9'-Dideoxyforskolin

B

25 µM 1',9'-Dideoxyforskolin

NH$_4^+$ NH$_4^+$

C
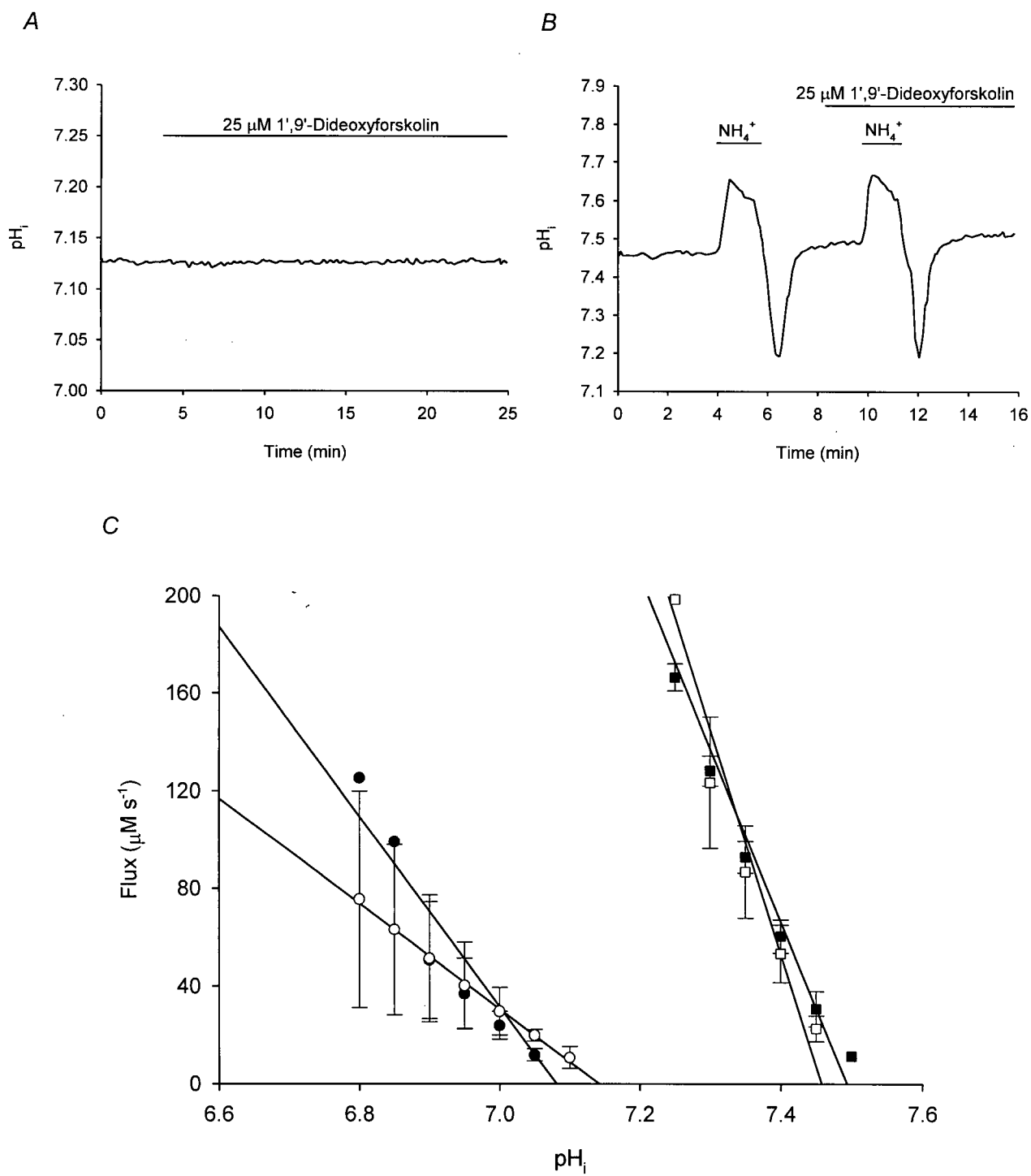
Figure 15. Effects of Sp-cAMPS on SS pH_i and pH_i recovery from internal acid loads under HEPES-buffered conditions.

A Under HEPES-buffered conditions, 4 different neurons (all with resting pH_i values at ~7.25) were exposed to increasing concentrations of Sp-cAMPS. Addition of 5 µM Sp-cAMPS (●) elicited a ~0.1 pH unit rise in SS pH_i and addition of 10 µM Sp-cAMPS (O) elicited a ~0.15 pH units rise. Addition of 25 µM Sp-cAMPS (▼) caused SS pH_i to reach ~ 7.45 within ~ 11 min. 40 µM Sp-cAMPS (▽) elicited a similar change in SS pH_i as 25 µM Sp-cAMPS but the new SS level was reached in only ~ 5 min. **B** A sample paired acid load recovery experiment performed under HEPES-buffered conditions in which the first acid load recovery was performed under control conditions and the second was performed in the presence of 25 µM Sp-cAMPS. pH_i recovery from the second acid load was faster than recovery from the first. **C** From 7 experiments of the type shown in *B*, mean calculated net acid fluxes were plotted against absolute values of pH_i and least squares regression lines were fitted to each set of data. The net acid flux values were significantly faster at each absolute value of pH_i in the presence (open symbols) than in the absence (closed symbols) of 25 µM Sp-cAMPS. The lines representing data from neurons with both low (circles) and high (squares) resting pH_i values had greater slopes and more alkaline x-intercepts in the presence than in the absence of Sp-cAMPS.

A
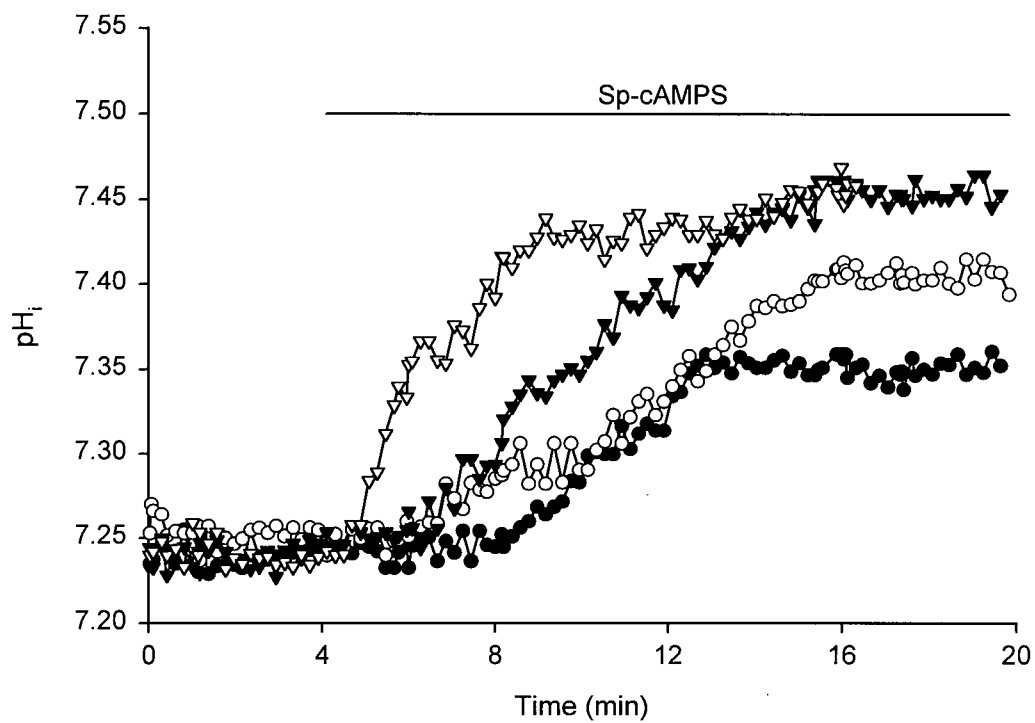
B
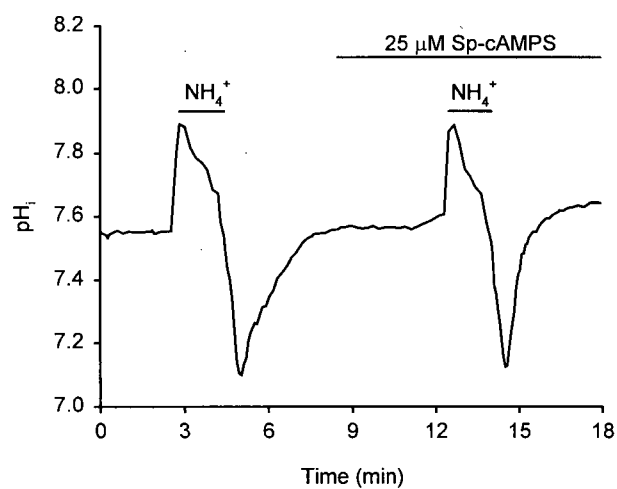
C
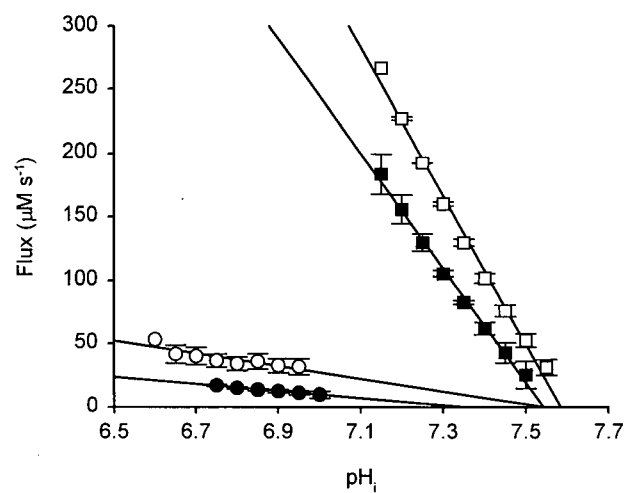
Figure 16. Effect of Sp-cAMPS on SS pH_i under Na^+_o-free, HEPES-buffered conditions.

A In a neuron with a resting pH_i at ~7.25, removal of Na^+_o (replaced with $NMDG^+$) caused SS pH_i to fall. After pH_i recovered to the initial SS level, the neuron was exposed to 25 μM Sp-cAMPS which had no effect on SS pH_i. **B** A different neuron with a resting pH_i at ~7.30 was exposed to a Na^+-free medium containing Li^+, which caused SS pH_i to initially fall. After pH_i recovered to the initial SS level, 25 μM Sp-cAMPS was added to the perfusion medium causing pH_i to increase to a new elevated SS level at ~7.48. Both experiments were performed under HEPES-buffered conditions, $pH_o = 7.35$ throughout.

A
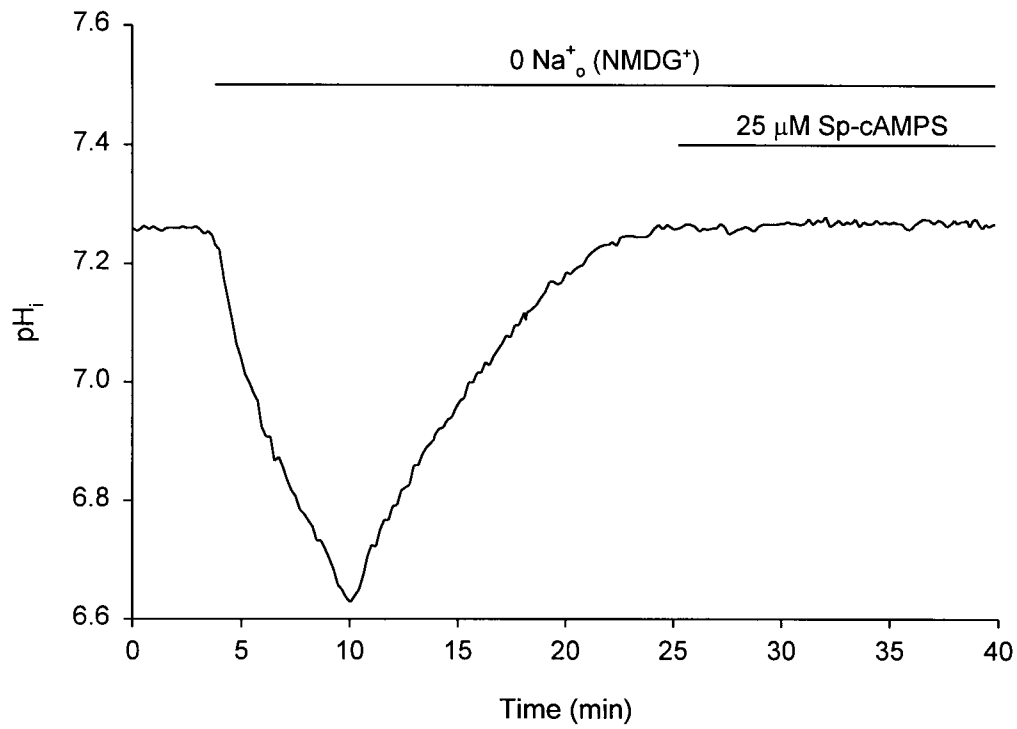
B
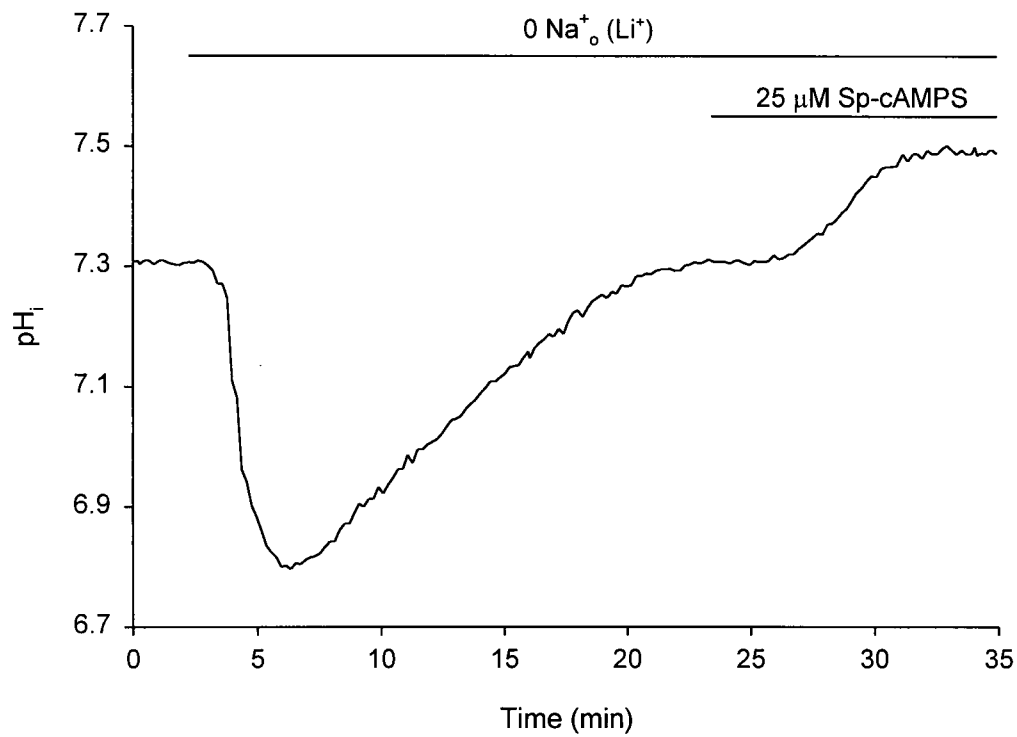
Figure 17. Effect of Rp-cAMPS on forskolin-induced changes in SS pH$_i$ under HEPES-buffered conditions.

Under HEPES-buffered conditions, a neuron with a resting pH$_i$ at ~7.20 was exposed to 50 μM Rp-cAMPS which did not alter SS pH$_i$. In the continued presence of Rp-cAMPS, the subsequent addition of 25 μM forskolin caused SS pH$_i$ to increase ~0.08 pH units.
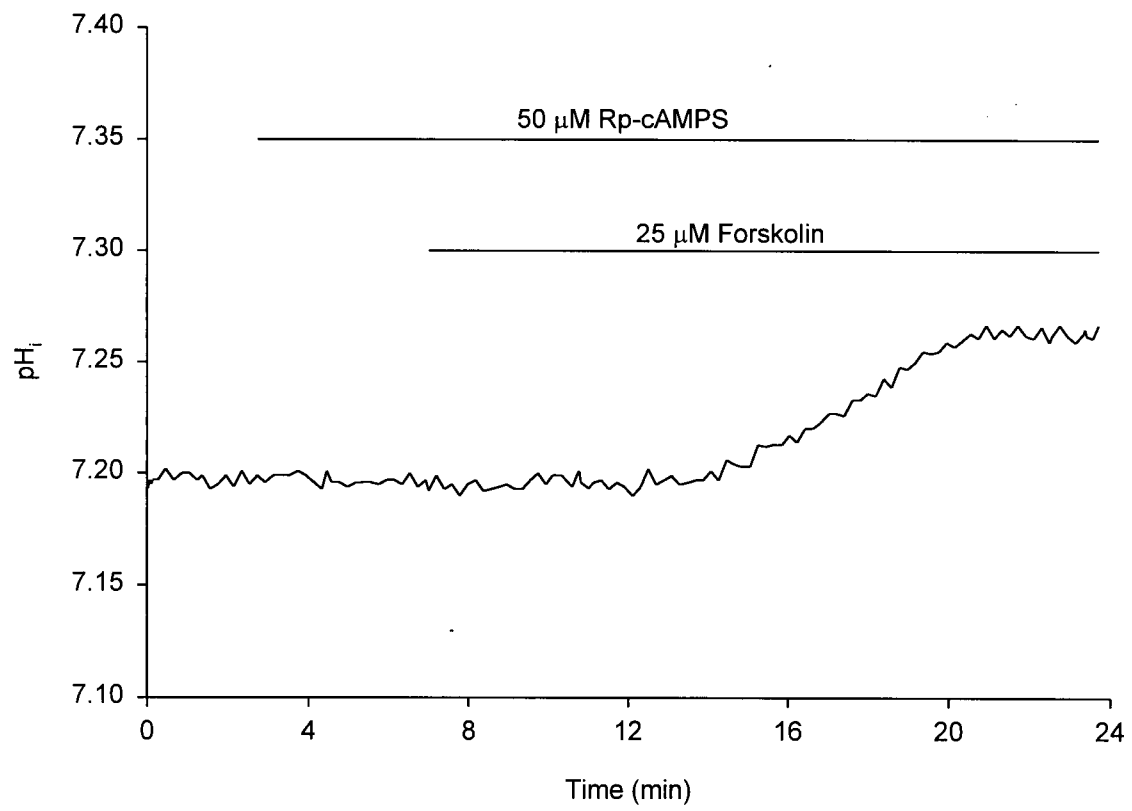
Figure 18. Effects of Sp-cAMPS and Rp-cAMPS on $[Ca^{2+}]_i$ and the effect of Ca^{2+}_o-removal on forskolin- and Sp-cAMPS-evoked changes in SS pH_i under HEPES-buffered conditions.

A The addition of 25 μM Sp-cAMPS elicited no change in the background-subtracted intensity ratios representing $[Ca^{2+}]_i$ in this neuron loaded with Fura-2 under HEPES-buffered conditions. *B* A neuron loaded with Fura-2 showed no change in BI_{334}/BI_{380} values when 50 μM Rp-cAMPS was applied under HEPES-buffered conditions. *C* Perfusion with a HEPES-buffered medium devoid of Ca^{2+} caused SS pH_i to increase in a neuron loaded with BCECF. Subsequent addition of 25 μM forskolin caused SS pH_i to increase a further ~0.25 pH units. *D* In a neuron loaded with BCECF, resting pH_i was at ~6.78. Removal of external Ca^{2+} caused an increase in SS pH_i. In the continued absence of Ca^{2+}_o, addition of 25 μM Sp-cAMPS caused a further ~ 0.20 pH unit increase in SS pH_i. Each trace shown was obtained from a different neuron.

A
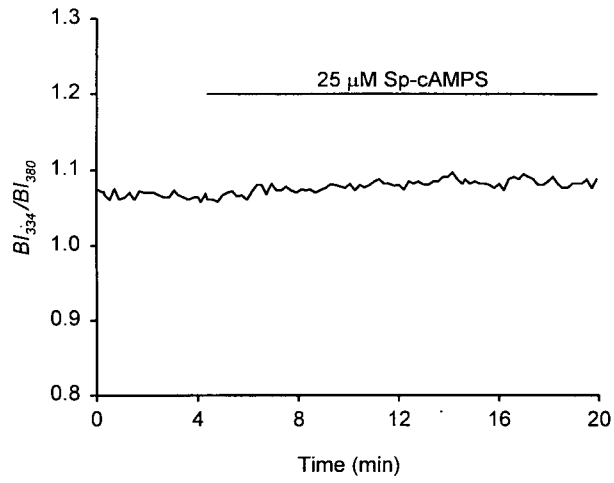
B
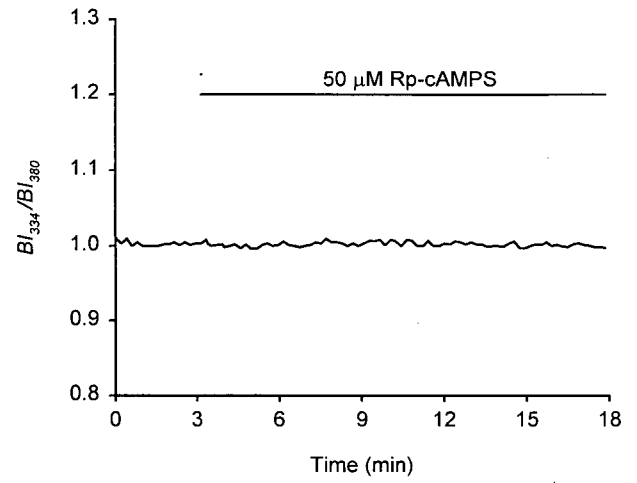
C
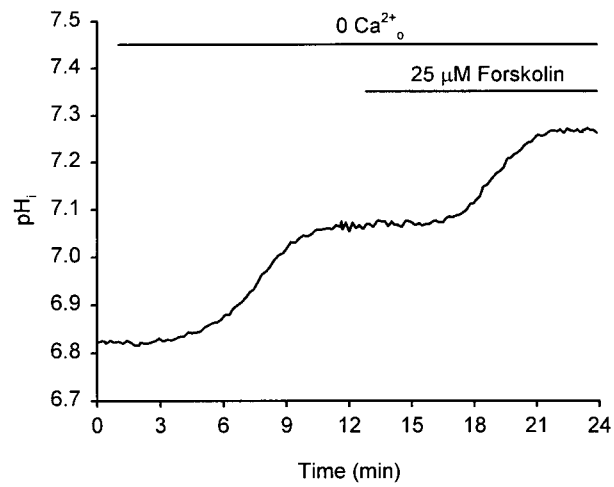
D
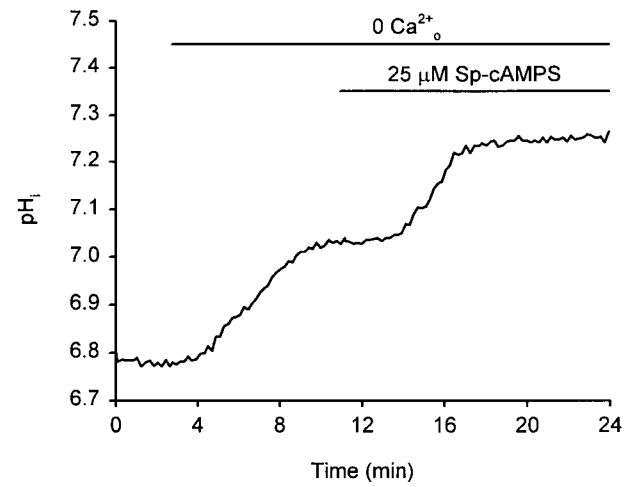
Figure 19. Effect of okadaic acid on Sp-cAMPS-evoked changes in SS pH_i under HEPES-buffered conditions.

A neuron with a resting pH_i at ~6.90 was perfused with a HEPES-buffered medium containing 10 μM okadaic acid which elicited an increase in SS pH_i. Subsequently, 10 μM Sp-cAMPS was added to the perfusion medium causing a further ~0.35 pH unit increase in SS pH_i.
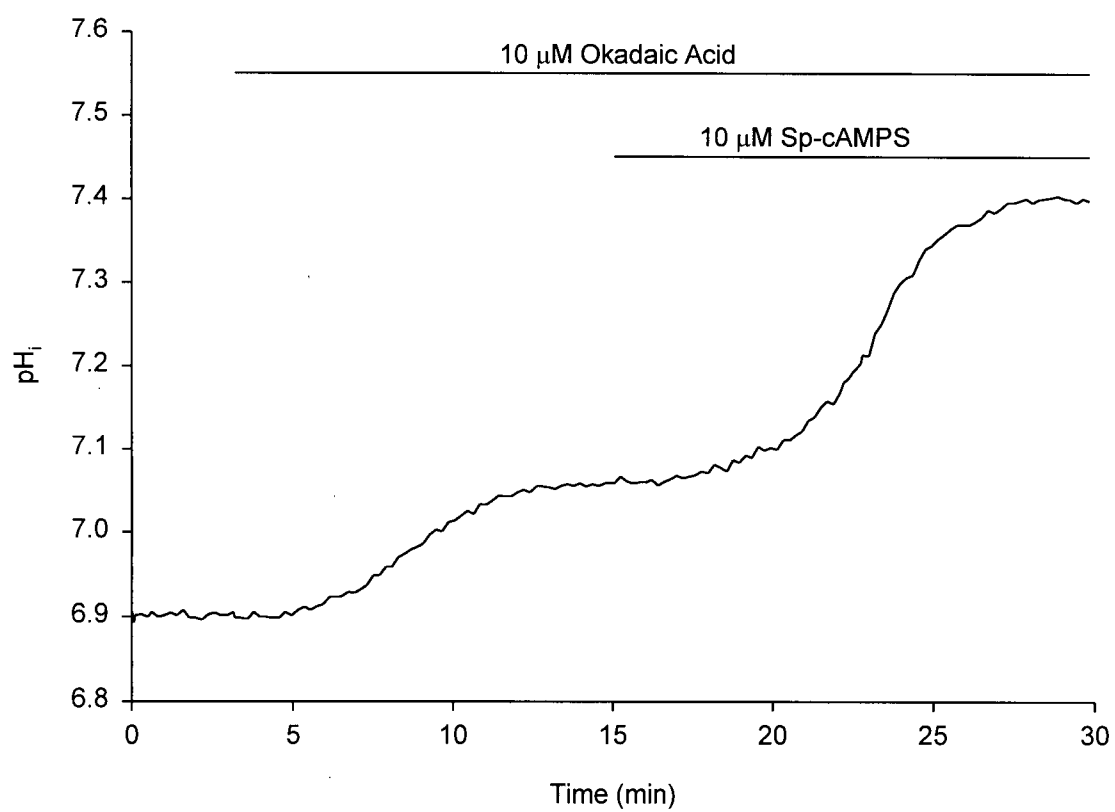
Figure 20. Effects of forskolin (in the presence and absence of DIDS) and IBMX on SS pH_i under HCO_3^-/CO_2-buffered conditions.

A A neuron that had a resting pH_i at ~7.27 was exposed to 25 μM forskolin which caused SS pH_i to increase under HCO_3^-/CO_2-buffered conditions. Upon subsequent removal of forskolin, SS pH_i remained elevated for the remainder of the experiment (~20 min). *B* Under HCO_3^-/CO_2-buffered conditions, a different neuron with a resting pH_i at ~7.05 was exposed to 200 μM IBMX which caused a ~0.35 pH unit increase in SS pH_i. *C* The SS pH_i of a different neuron, initially at ~7.07, decreased upon exposure to 200 μM DIDS under HCO_3^-/CO_2-buffered conditions. The subsequent addition of 25 μM forskolin caused a ~ 0.25 pH unit rise in SS pH_i.
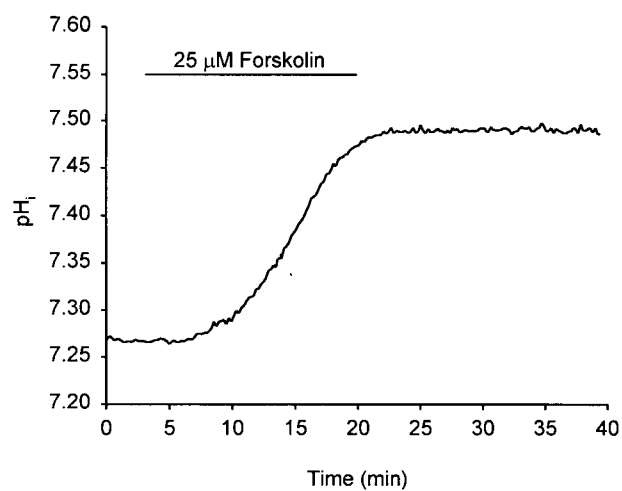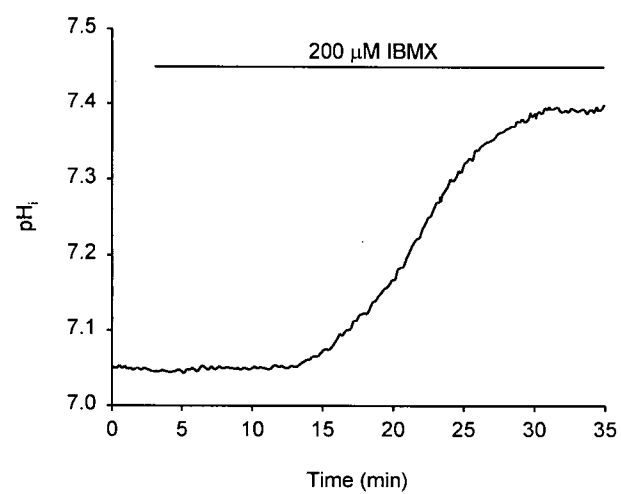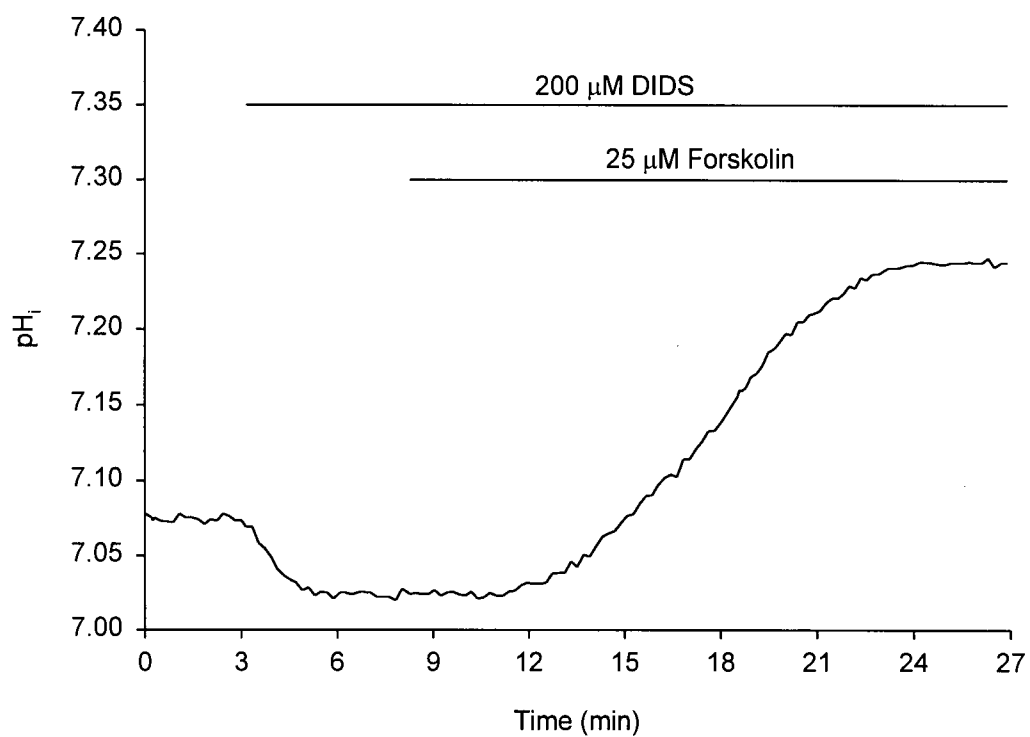
Figure 21. Effects of forskolin and IBMX on pH_i recovery from internal acid loads imposed under HCO_3^-/CO_2-buffered conditions.

A A neuron with a resting pH_i at ~7.17 was administered a control acid load under HCO_3^-/CO_2-buffered conditions. Upon pH_i recovery to the resting level, a second acid load was performed in the presence of 25 μM forskolin, after which pH_i recovered to a new SS level at pH_i = ~7.32. **B** After pH_i was allowed to recover from an acid load performed under control HCO_3^-/CO_2-buffered conditions, the different neuron was exposed to 200 μM IBMX and a second acid load was performed. pH_i recovery from the second acid load was faster when compared to the pH_i recovery from the first. **C** Plotted are mean rates of pH_i recovery against absolute values of pH_i from acid loads performed in the absence (●) and presence (○) of 25 μM forskolin (n = 13). The rates of pH_i recovery observed were significantly faster in the presence than in the absence of forskolin at all absolute values of pH_i. The data points were fitted by regression lines and the line representing the data obtained in the presence of forskolin had a greater slope and more alkaline x-intercept than the line representing the control data. **D** Plotted are mean rates of pH_i recovery against absolute values of pH_i from acid load experiments performed in the absence (●) and presence (○) of 200 μM IBMX (n = 10). The rates of pH_i recovery observed were significantly faster in the presence than in the absence of IBMX at all absolute values of pH_i. Regression lines were fitted to the data points and the line representing the data obtained in the presence of IBMX had a larger slope and more alkaline x-intercept than the line representing the control data.
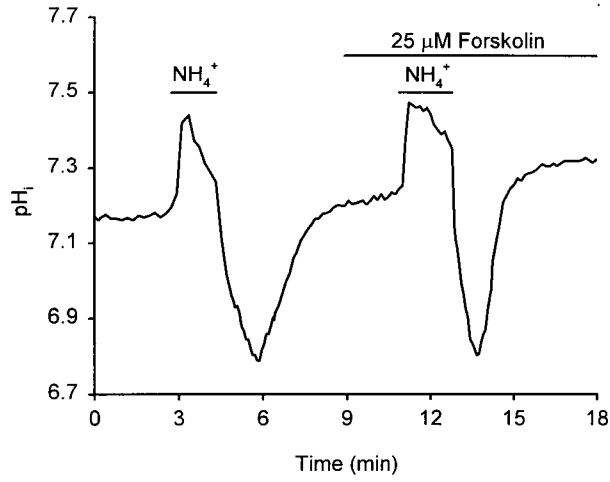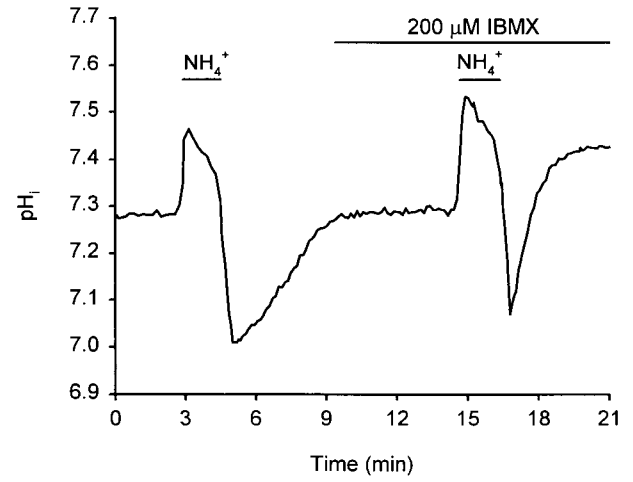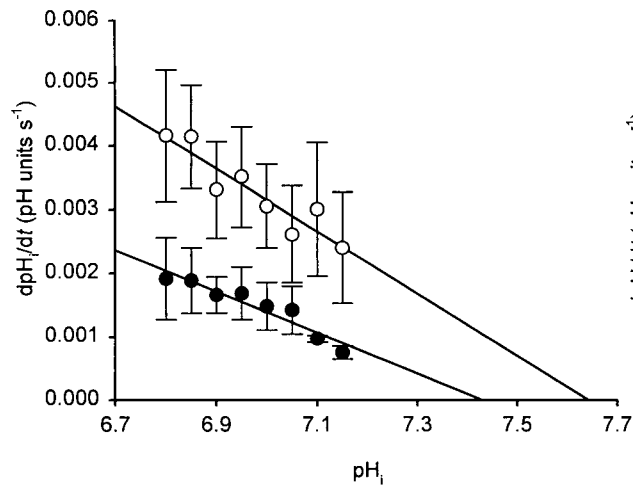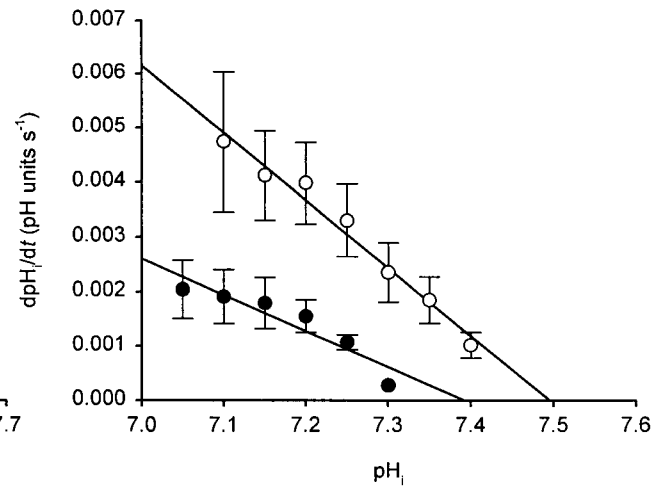
Figure 22. Effect of DIDS on the changes in rates of pH_i recovery from imposed internal acid loads evoked by forskolin under HCO_3^-/CO_2-buffered conditions.

A pH_i was allowed to fully recover after an initial acid load under control HCO_3^-/CO_2-buffered conditions. The neuron then was exposed to 200 μM DIDS and 25 μM forskolin which initially caused SS pH_i to fall. A subsequent acid load was then performed in the presence of these 2 agents, after which pH_i recovered to a new SS level at ~7.33. The rates of pH_i recovery were significantly faster in the presence than in the absence of forskolin and DIDS. **B** The mean rates of pH_i recovery obtained in the absence (●) and presence (O) of 200 μM DIDS and 25 μM forskolin were plotted against absolute values of pH_i ($n = 7$). Rates of pH_i recovery were significantly faster in the presence than in the absence of the 2 agents at all absolute values of pH_i. Linear regressions were fitted to the data points and the line representing the data obtained in the presence of forskolin and DIDS had a more alkaline x-intercept than the line representing the control data. Compare with Fig. 21C (the effect of 25 μM forskolin on rates of pH_i recovery from internal acid loads imposed under HCO_3^-/CO_2-buffered in the absence of DIDS).

A
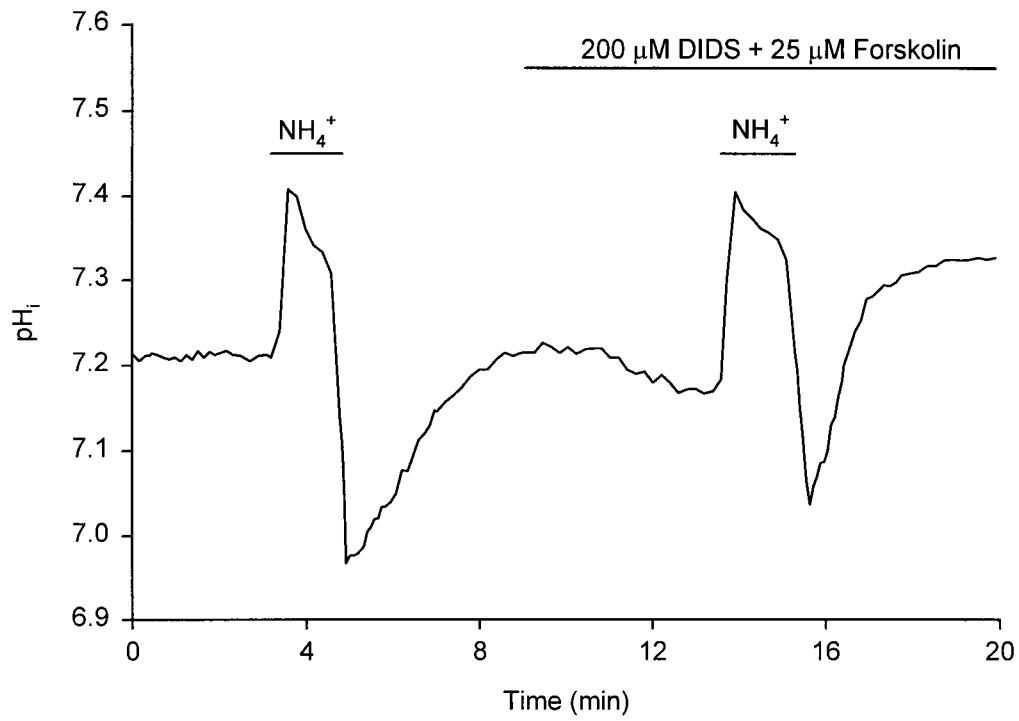
B
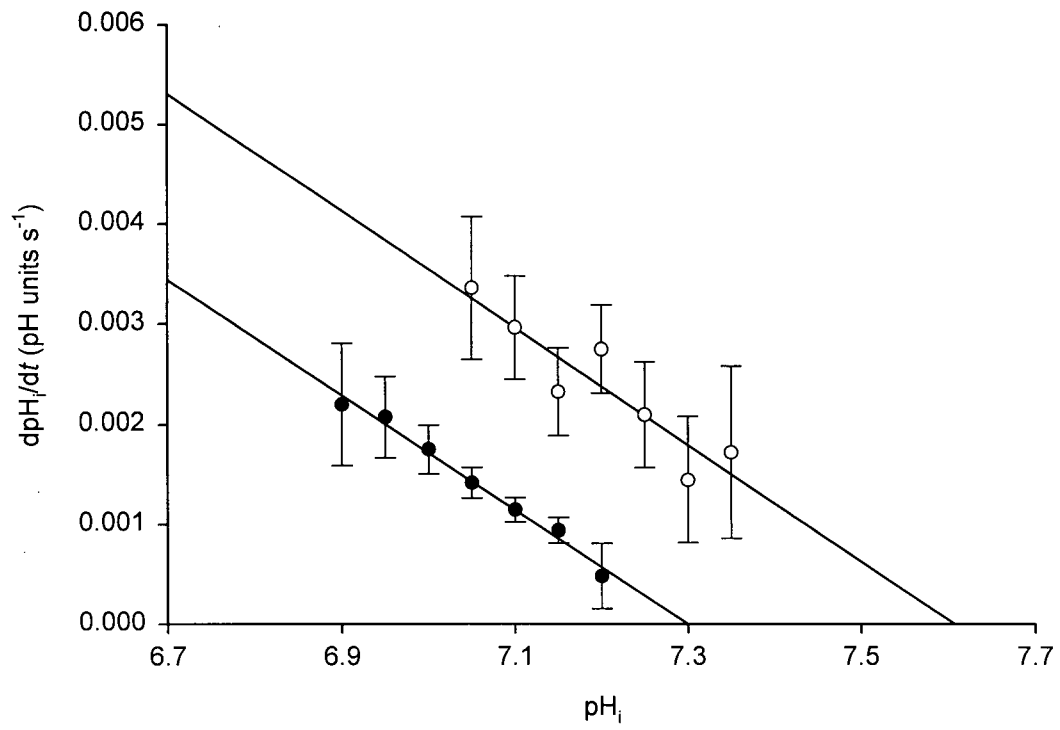
Figure 23. Effects of 1′, 9′-dideoxyforskolin on SS pH$_i$ and pH$_i$ recovery from internal acid loads under HCO$_3^-$/CO$_2$-buffered conditions.

A Addition of 25 µM 1′, 9′-dideoxyforskolin had no effect on SS pH$_i$ in a neuron that had a resting pH$_i$ at ~7.03 under HCO$_3^-$/CO$_2$-buffered conditions. **B** In a different neuron with a resting pH$_i$ at ~7.15 under HCO$_3^-$/CO$_2$-buffered conditions, pH$_i$ was allowed to recover after imposing an initial acid load. A second acid load was performed in the presence of 25 µM 1′, 9′-dideoxyforskolin after which pH$_i$ recovered to the resting SS level at a similar rate to that observed under control conditions. **C** From 5 experiments of the type shown in *B*, mean rates of pH$_i$ recovery were plotted against absolute values of pH$_i$ and regression lines were fitted to the data points. No significant differences were found between rates of pH$_i$ recovery observed in the absence (●) and presence (○) of 25 µM 1′, 9′-dideoxyforskolin, at all absolute values of pH$_i$.

A
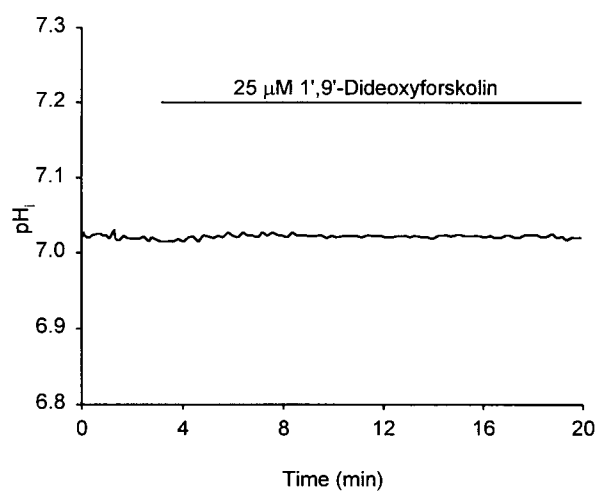
B
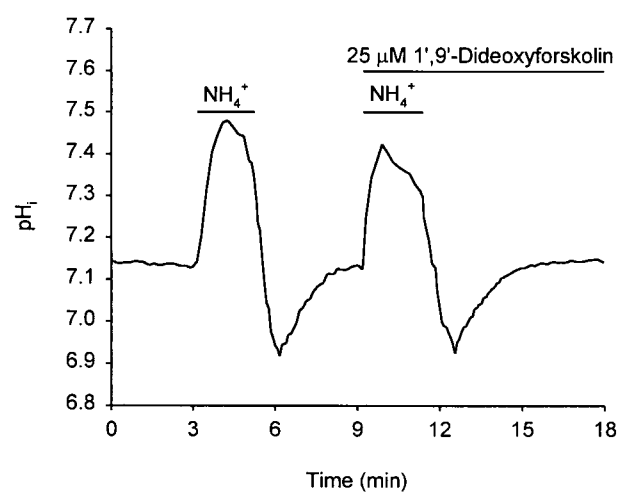
C
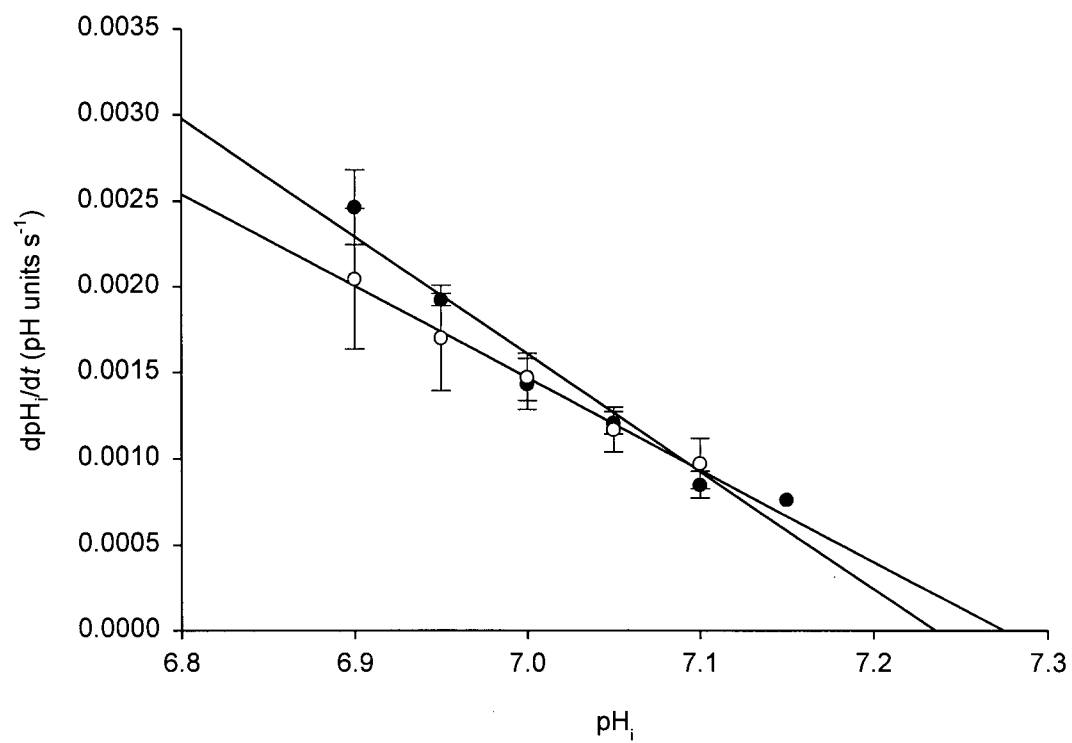
Figure 24. Effect of 2', 5'-dideoxyadenosine on SS pH_i under HCO_3^-/CO_2-buffered conditions.

A A neuron that had a resting pH_i at ~7.29 was exposed to 100 μM 2', 5'-dideoxyadenosine which caused SS pH_i to increase under HCO_3^-/CO_2-buffered conditions. **B** From 9 experiments of the type shown in **A**, the changes in SS pH_i elicited by 100 μM 2', 5'-dideoxyadenosine were plotted against resting values of pH_i observed prior to the addition of DDA and a regression line was fitted to the data points. This regression line had a negative slope and an x-intercept at pH_i = ~ 7.45.

A
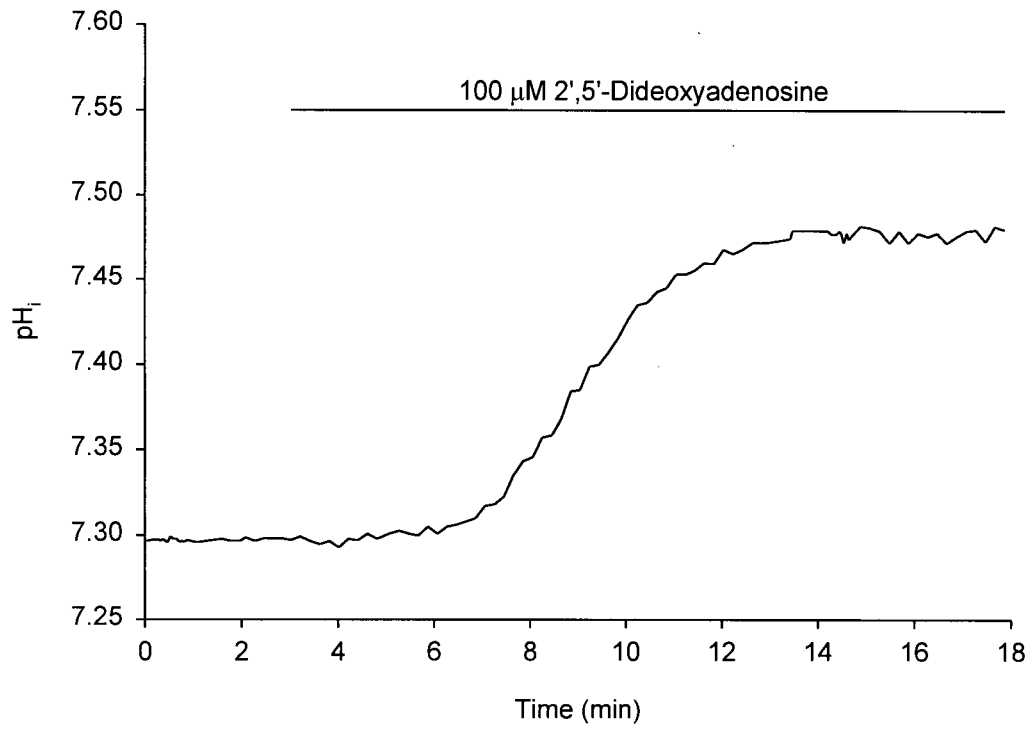
B
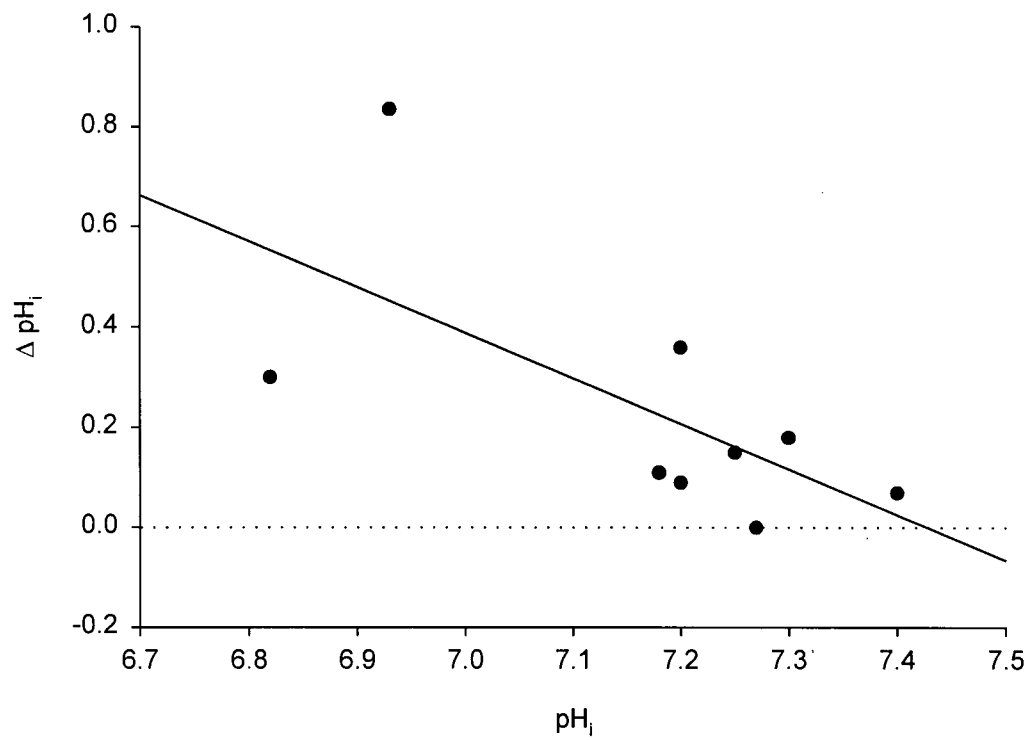
Figure 25. Effect of Rp-cAMPS on SS pH_i under HCO_3^-/CO_2-buffered conditions.

A A rise in SS pH_i was observed with the addition of 50 μM Rp-cAMPS in a neuron that had a resting pH_i at ~7.04 under HCO_3^-/CO_2-buffered conditions. **B** A different neuron with a resting pH_i at ~7.79 showed a decrease in SS pH_i when 50 μM Rp-cAMPS was added to the HCO_3^- /CO_2-buffered perfusion medium. **C** From 16 experiments of the types shown in **A** and **B**, the changes in SS pH_i elicited by 50 μM Rp-cAMPS were plotted against resting values of pH_i and these data points were fitted to a least squares regression line. This line had a negative slope and an x-intercept at pH_i = ~7.4. Compare with Fig. 8C (the effect on SS pH_i of adding HCO_3^-/CO_2 to neurons perfused initially with HEPES-buffered medium).

A
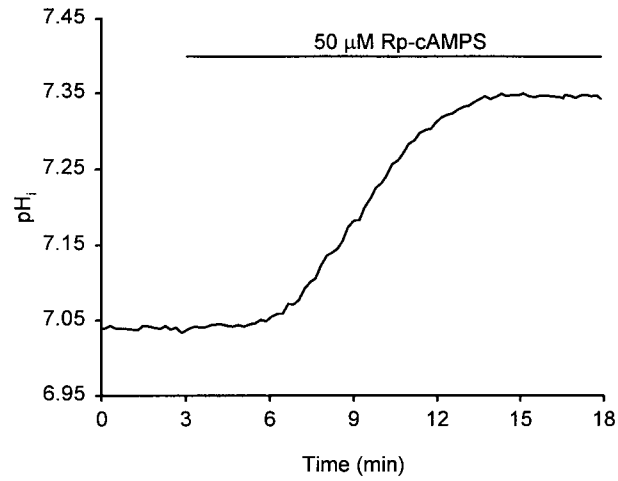
B
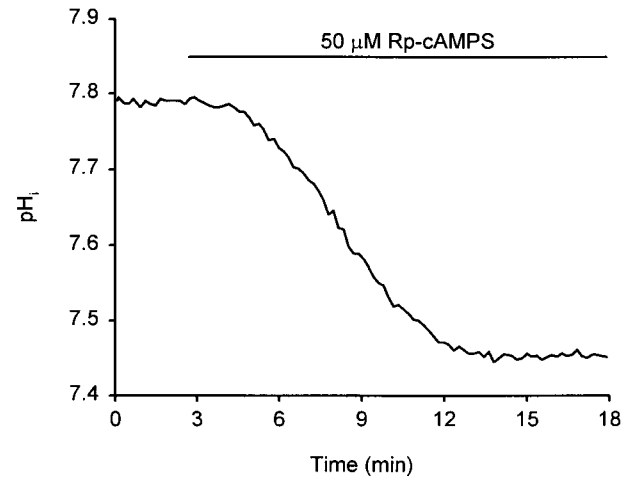
C
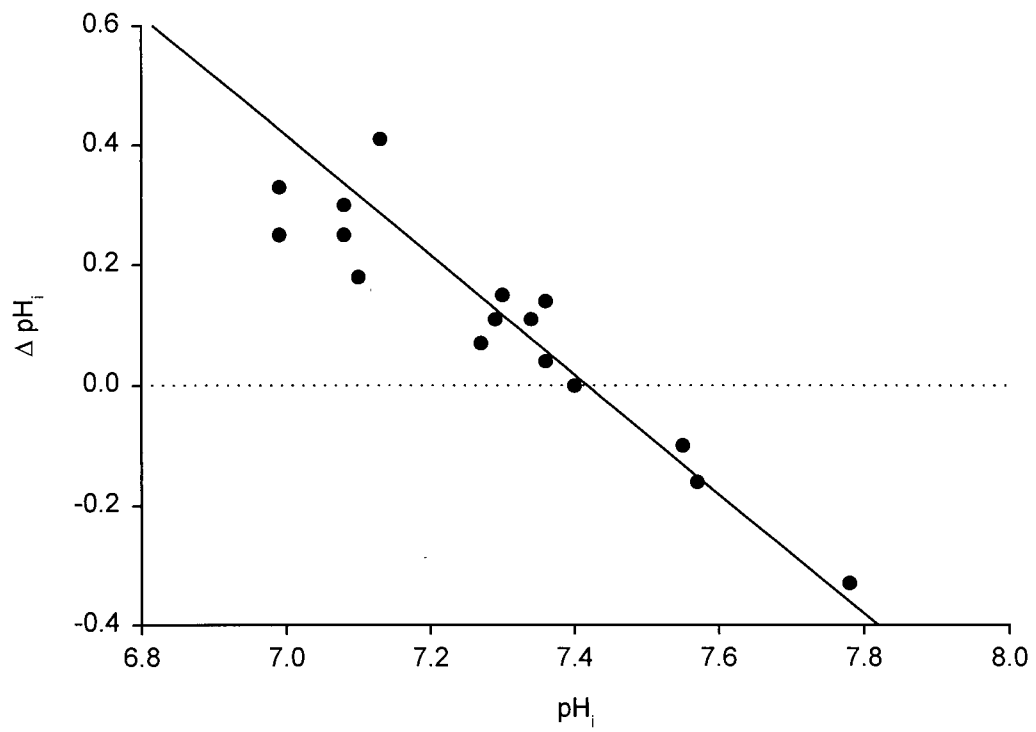
Figure 26. Effect of DIDS on the changes in SS pH_i evoked by Rp-cAMPS under HCO_3^- /CO_2-buffered conditions.

The resting pH_i of this neuron was ~7.05 prior to the addition of 200 μM DIDS to the HCO_3^- /CO_2-buffered perfusion medium. DIDS evoked a fall in SS pH_i and the subsequent addition of 50 μM Rp-cAMPS failed to alter SS pH_i (compare with Fig. 25A; the effect of Rp-cAMPS on SS pH_i in a neuron with a low resting pH_i under HCO_3^-/CO_2-buffered conditions in the absence of DIDS).
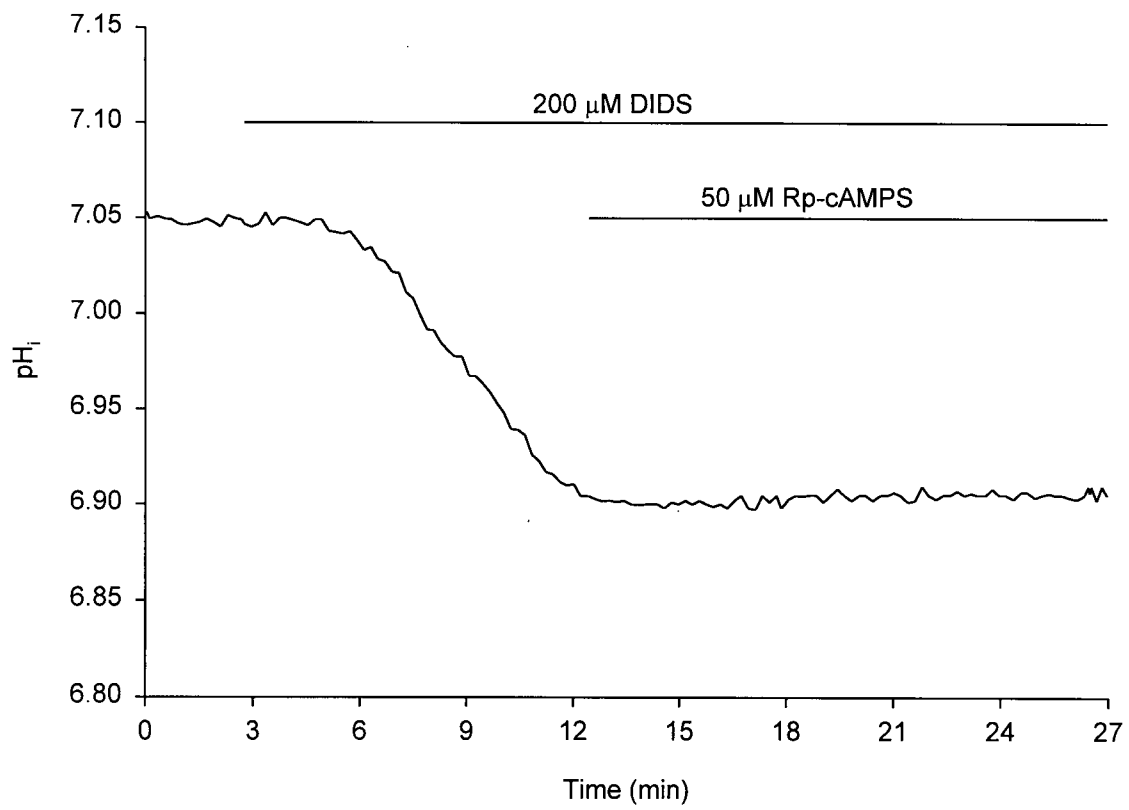
Figure 27. Effects of Sp-cAMPS and okadaic acid on SS pH_i under HCO_3^-/CO_2-buffered conditions.

A A neuron that had a resting pH_i at ~7.26 under HCO_3^-/CO_2-buffered conditions (solid line) was exposed to 25 μM Sp-cAMPS which, after a relatively long delay (~ 8 min), caused SS pH_i to increase by ~0.12 pH units. Also shown is a similar experiment performed on a different neuron under nominally HCO_3^--free, HEPES-buffered conditions (O); under these conditions there was a larger increase in SS pH_i which reached a maximum value after a significantly shorter delay. **B** A neuron that had a resting pH_i at ~7.25 under HCO_3^-/CO_2-buffered conditions (solid line) was exposed to 10 μM okadaic acid which caused SS pH_i to increase by ~0.15 pH units. Also shown is a similar experiment performed on a different neuron under nominally HCO_3^--free, HEPES-buffered conditions (O); under these conditions, okadaic acid evoked a greater increase in SS pH_i.

A
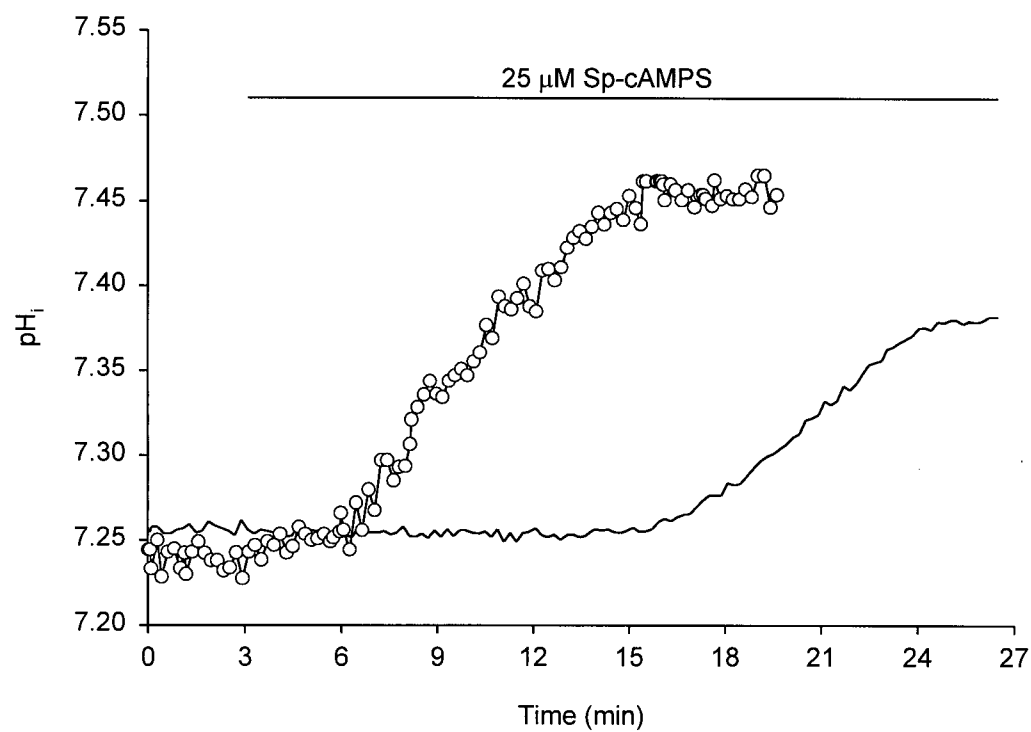
B
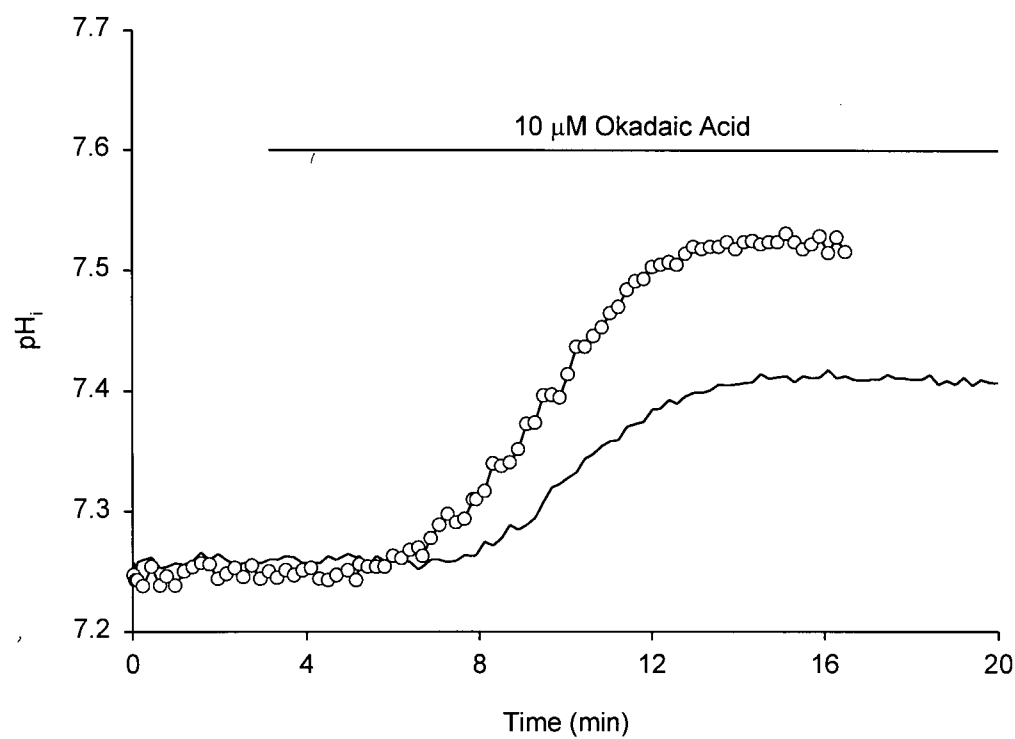
Figure 28. Effect of DIDS on pH$_i$ recovery from internal alkaline loads imposed under HCO$_3^-$/CO$_2$-buffered conditions.

A neuron that had a resting pH$_i$ at ~7.12 was exposed to an alkaline load under HCO$_3^-$/CO$_2$-buffered conditions. pH$_i$ was allowed to recover to the resting SS level and, subsequently, the addition of 200 μM DIDS caused SS pH$_i$ to decrease. A second alkaline load was administered in the continued presence of DIDS, after which the pH$_i$ recovery towards the initial SS level was greatly diminished.
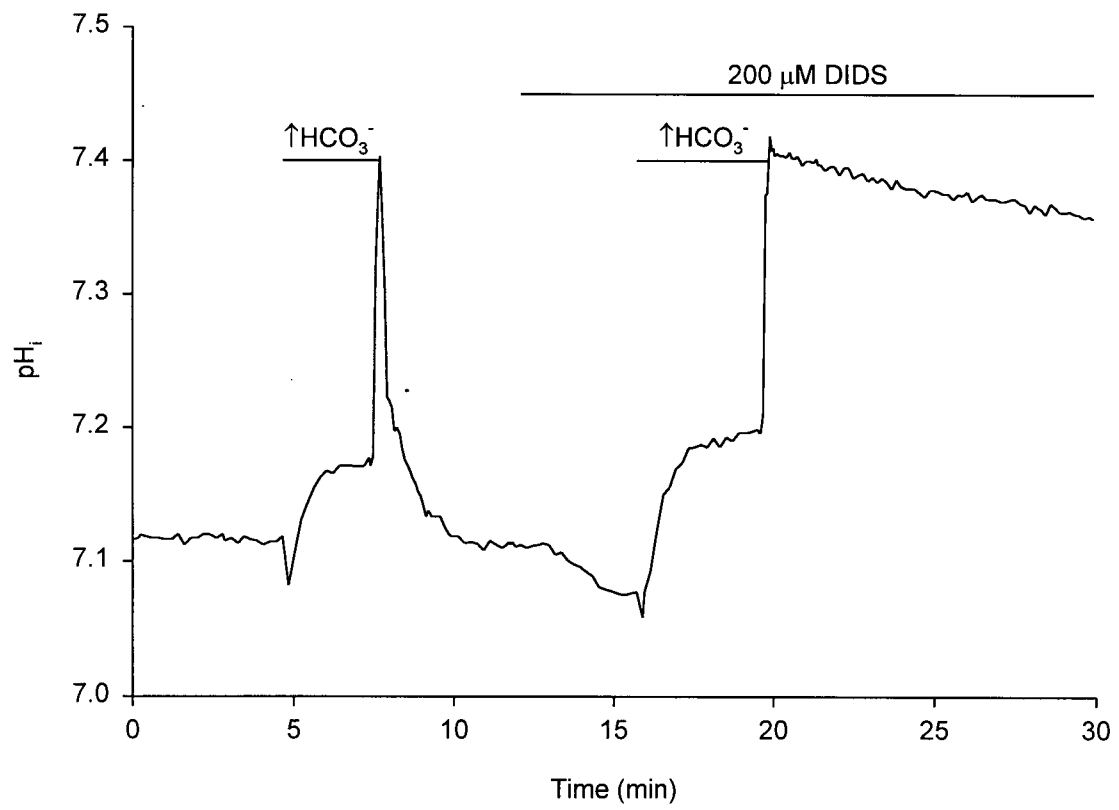
Figure 29. Effects of Rp-cAMPS on pH_i recovery from internal alkaline loads under HCO_3^- /CO_2-buffered conditions in the presence and absence of external Na^+.

A A neuron with a resting pH_i at ~7.24 was exposed to an alkaline load and after pH_i recovered to the resting SS level, 50 μM Rp-cAMPS was added to the perfusion medium. This caused an alkalinization, during which a second alkaline load was performed. The rate of pH_i recovery from the second alkaline load was slower than the rate of pH_i recovery from the first. **B** Mean rates of pH_i recovery obtained in the absence (●) and presence (O) of 50 μM Rp-cAMPS were plotted against absolute values of pH_i and it was found that the rates of pH_i recovery were significantly reduced in the presence than in the absence of Rp-cAMPS at all absolute values of pH_i (n = 14). Regression lines were fitted to the data points and the line representing the data obtained in the presence of Rp-cAMPS had a reduced slope and a more alkaline x-intercept than the line representing control data. **C** Under Na^+_o-free, HCO_3^-/CO_2-buffered conditions, a different neuron with a resting pH_i at ~7.15 was exposed to an alkaline load and after pH_i recovered to the resting SS level, 50 μM Rp-cAMPS was added to the perfusion medium. This caused an alkalinization, during which a second alkaline load was performed. The rate of pH_i recovery from the second alkaline load was slower than the rate of pH_i recovery from the first. **D** In the absence of Na^+_o, mean rates of pH_i recovery obtained in the absence (●) and presence (O) of 50 μM Rp-cAMPS were plotted against absolute values of pH_i and it was found that the rates of pH_i recovery were reduced in the presence of Rp-cAMPS (n = 5). Regression lines were fitted to the data points and the line representing the data obtained in the presence of Rp-cAMPS had a reduced slope and a more alkaline x-intercept than the line representing control data.

A
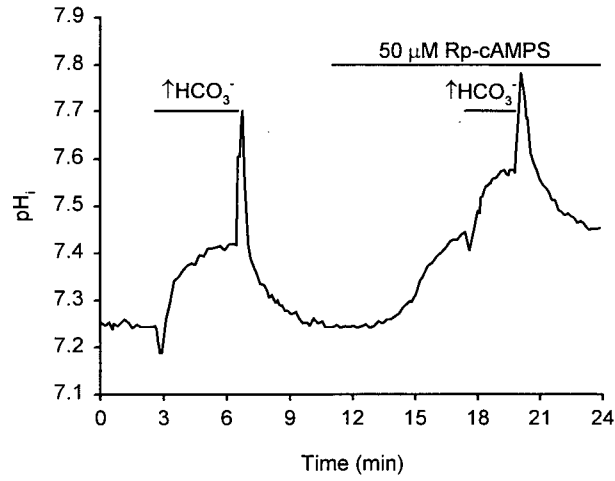
B
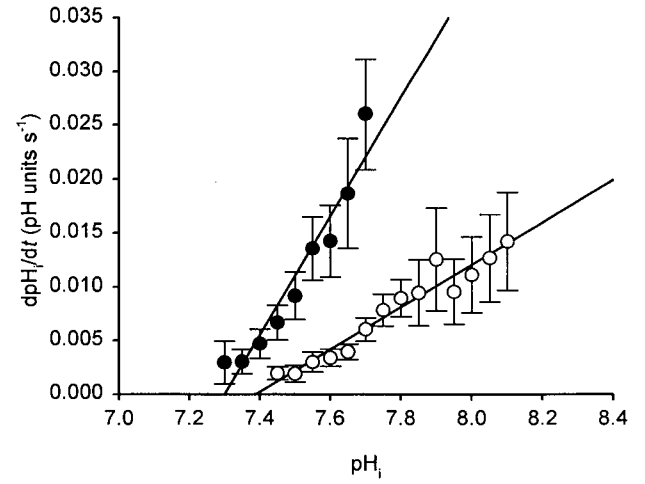
C

D

Figure 30. Effect of Sp-cAMPS on pH_i recovery from imposed alkaline loads under HCO_3^- /CO_2-buffered conditions.

A In a neuron with a resting pH_i at ~7.10, pH_i recovered after an alkaline load performed under control conditions. Subsequently, the addition of 25 μM Sp-cAMPS caused SS pH_i to increase. A second alkali load was then performed and pH_i recovered to the new SS level in the continued presence of Sp-cAMPS. The rate of pH_i recovery from the second alkaline load was faster than the rate of pH_i recovery from the first. **B** From 9 experiments of the type shown in A, mean rates of pH_i recovery obtained in the absence (●) and presence (○) of 25 μM Sp-cAMPS were plotted against absolute values of pH_i. For absolute pH_i values $\leq$ 7.20, rates of pH_i recovery were significantly smaller, and for absolute pH_i values $\geq$ 7.40, rates of pH_i recovery were significantly greater, in the presence than in the absence of Sp-cAMPS. Regression lines were fitted to the data points, the line representing data obtained in the presence of Sp-cAMPS had a greater slope with a more alkaline x-intercept than the line representing control data.

A
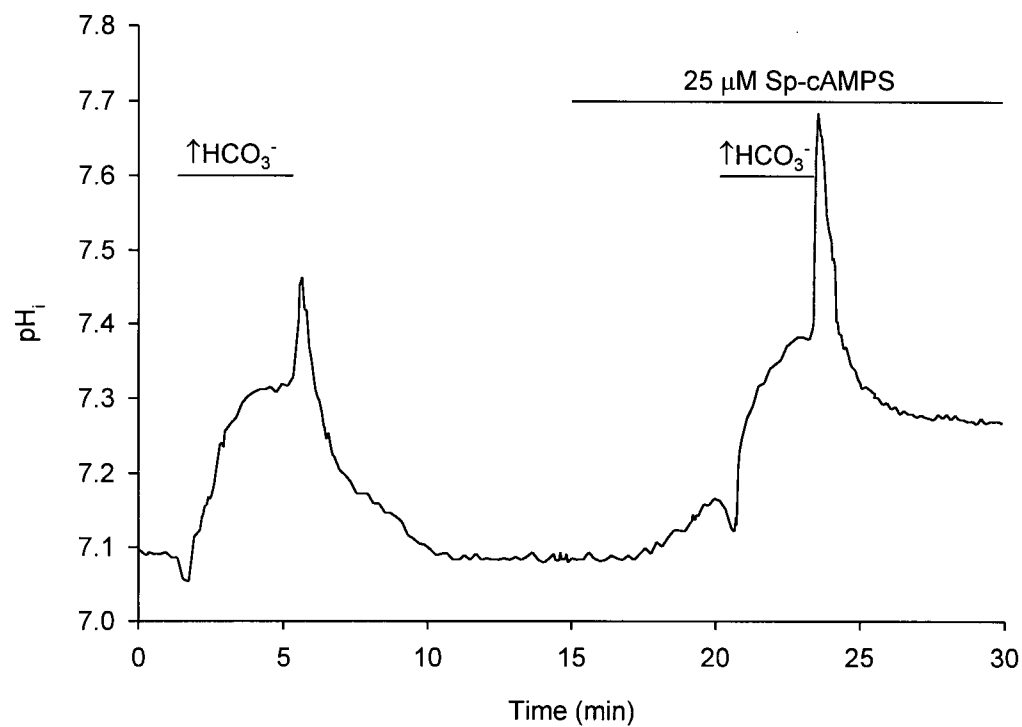
B
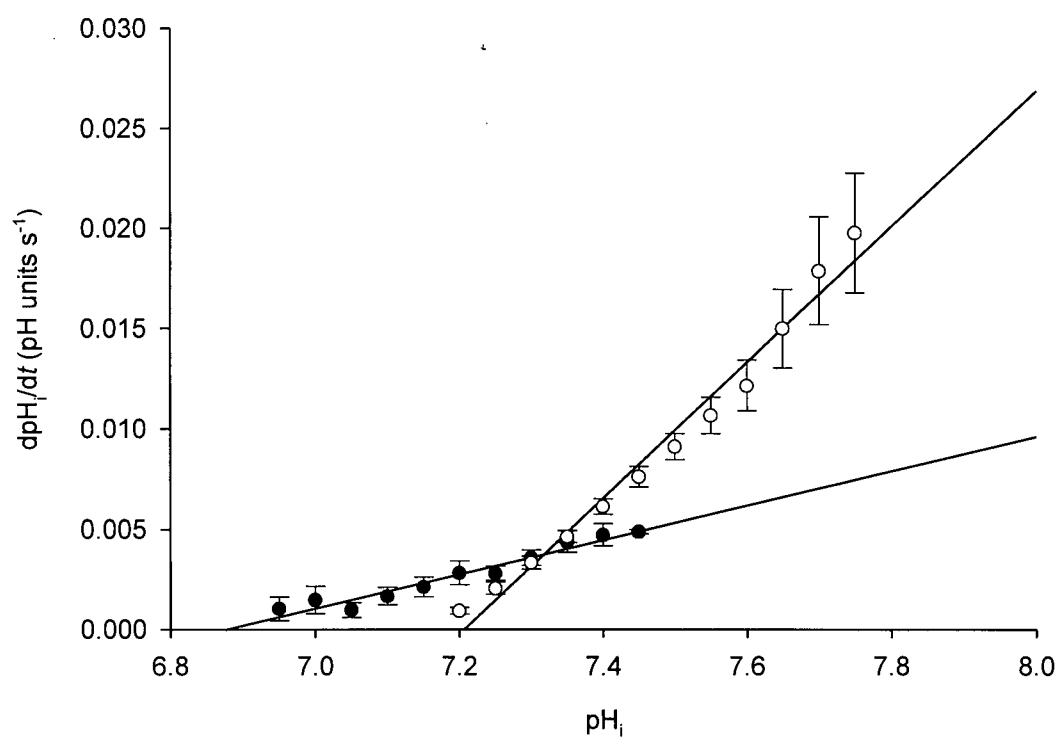
Figure 31. Effects of Rp-cAMPS and Sp-cAMPS on 0 Cl_o-evoked changes in SS pH_i under HCO_3^-/CO_2-buffered conditions.

A The removal of Cl^- from the HCO_3^-/CO_2-buffered perfusion medium caused the SS pH_i of this neuron to increase from ~7.13 to ~7.29. Subsequent reintroduction of Cl_o^- allowed pH_i to recover to the resting value, after which an increase in SS pH_i was observed with the addition of 50 μM Rp-cAMPS. Cl_o^- was then removed from the perfusion medium for a second time, causing an ~0.07 pH unit increase in SS pH_i. In the continued presence of Rp-cAMPS, Cl_o^- was re-introduced and pH_i recovered. The 0 Cl_o^--induced alkalinization was smaller in the presence than in the absence of Rp-cAMPS. *B* A different neuron with a resting pH_i at ~7.05 was exposed to Cl^--free, HCO_3^-/CO_2-buffered medium which caused SS pH_i to increase ~0.18 pH units. Reintroducing Cl_o^- allowed pH_i to recover to the resting SS level, after which a slow increase in pH_i was observed with the addition of 25 μM Sp-cAMPS. Cl^- was then removed from the perfusion medium for a second time causing a ~0.35 pH unit alkalinization. When Cl_o^- was returned to the perfusion medium in the continued presence of Sp-cAMPS, pH_i recovered to a SS level of ~7.40. The 0 Cl_o^--induced alkalinization was greater in the presence than in the absence of Sp-cAMPS. *C* The first time Cl^- was removed from the HCO_3^-/CO_2-buffered perfusion medium, SS pH_i increased and, upon returning Cl_o^-, pH_i returned to the initial SS level. The subsequent addition of 200 μM DIDS and 25 μM Sp-cAMPS initially caused an transient decrease followed by an increase in SS pH_i. In the continued presence of these two agents, the removal of Cl^- from the perfusion medium failed to alter SS pH_i. *D* The SS pH_i responses to the acute removal of Cl_o^- recorded in three different neurons with similar resting pH_i values were overlaid. Removal of Cl^- from the HCO_3^-/CO_2-buffered perfusion medium in the presence of 50 μM Rp-cAMPS (O) elicited a significantly smaller increase in SS pH_i when compared to those obtained in the presence of Sp-cAMPS (∇) or under control conditions (solid line). SS pH_i recovered to the initial value of ~7.15 upon the return of Cl^- to the perfusion medium in all three neurons.

A
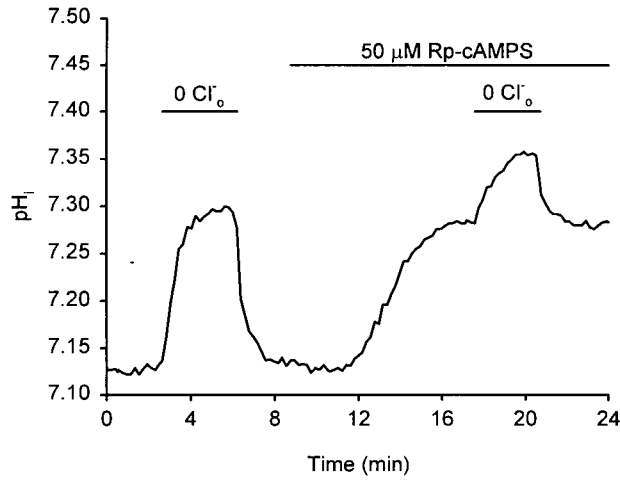
B
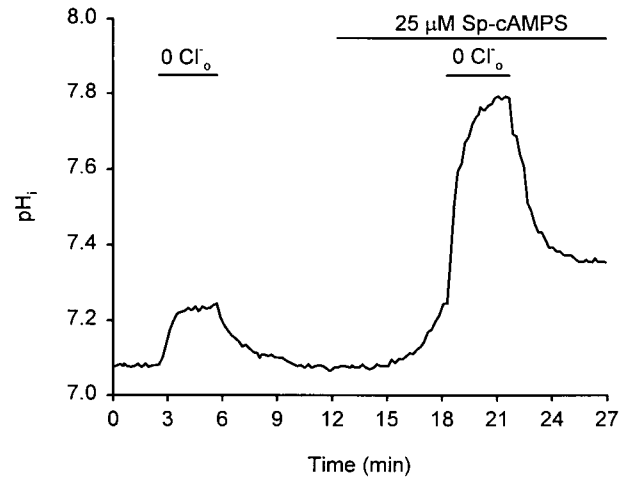
C
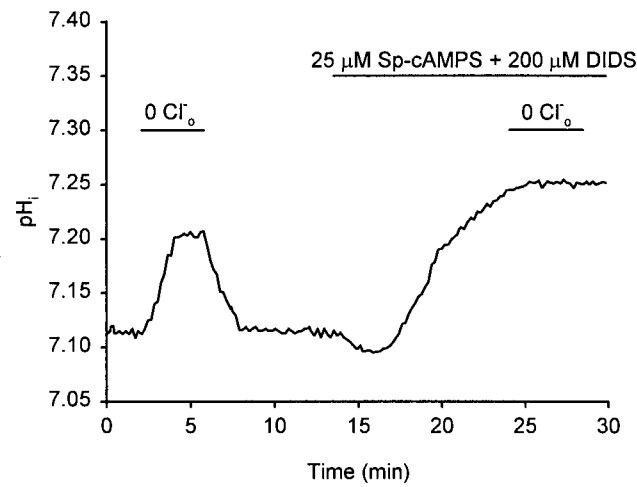
D
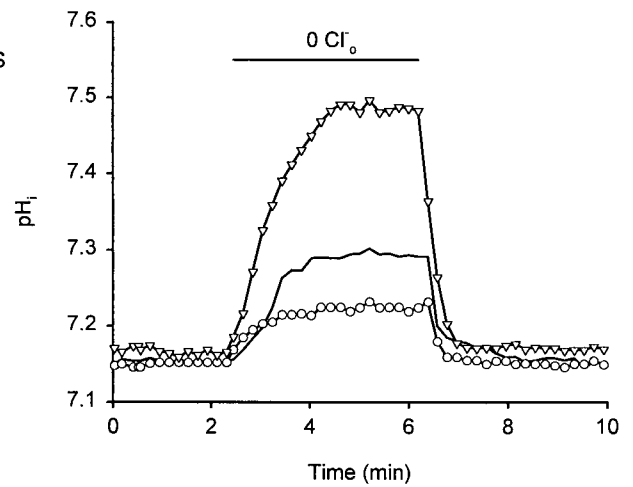
Figure 32. Effect of Sp-cAMPS on SS pH_i under Na^+_o-free, HCO_3^-/CO_2-buffered conditions.

Under Na^+_o-free, HCO_3^-/CO_2-buffered conditions, a neuron had a resting pH_i at ~7.50. Upon the addition of 25 μM Sp-cAMPS, SS pH_i increased by ~0.08 pH units.
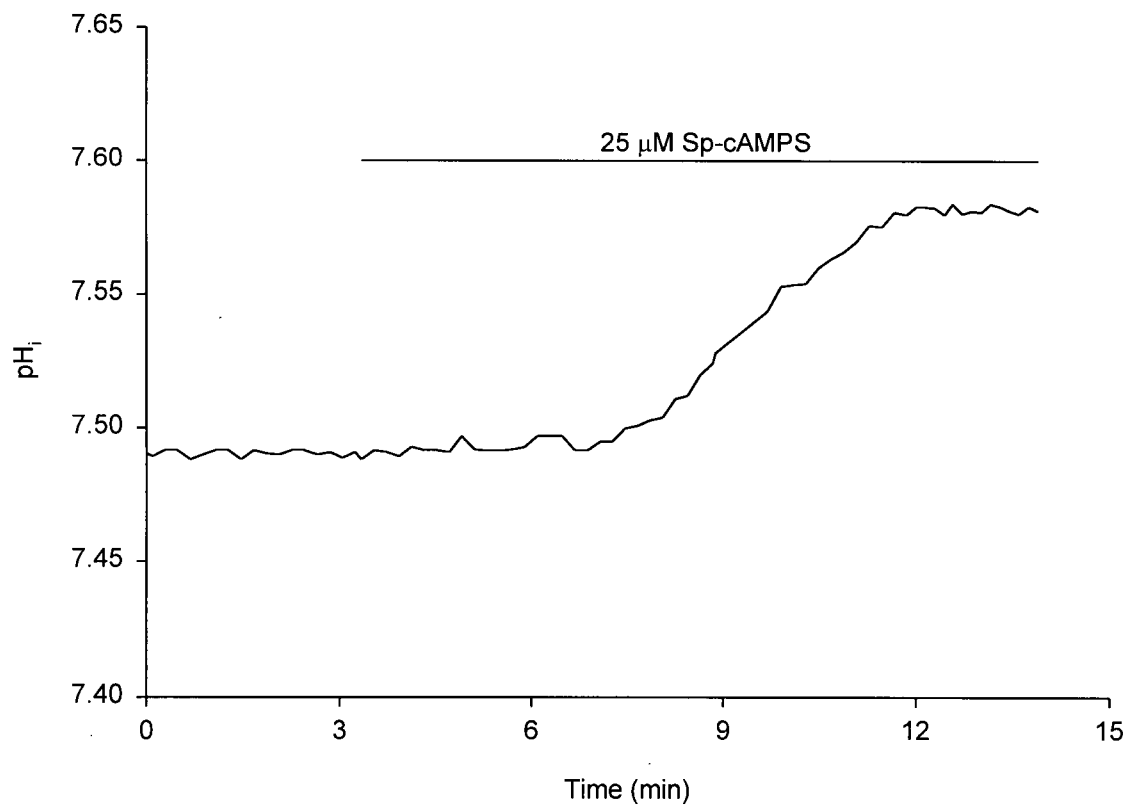
Figure 33. Effect of Rp-cAMPS on SS pH_i under Na^+_o-free, HCO_3^-/CO_2-buffered conditions.

A A neuron perfused with Na^+-free, HCO_3^-/CO_2-buffered medium was allowed to reach a SS pH_i at ~7.25. Addition of 50 µM Rp-cAMPS, in the continued absence of Na^+_o, caused pH_i to increase to a new SS level at ~7.37. **B** A different neuron that had a resting pH_i at ~7.63 under Na^+_o-free, HCO_3^-/CO_2-buffered conditions, was exposed to 50 µM Rp-cAMPS causing SS pH_i to fall. **C** Plotted against resting values of pH_i are changes in SS pH_i elicited by 50 µM Rp-cAMPS under Na^+_o-free, HCO_3^-/CO_2-buffered conditions ($n = 12$). The data points were fitted to a regression line (solid line) which had a negative slope and an x-intercept at $pH_i = ~7.5$. Compare with the line representing the effect of Rp-cAMPS on SS pH_i observed under HCO_3^-/CO_2-buffered conditions in the presence of external Na^+ (dashed line; from Fig. 25C).

A

B

C
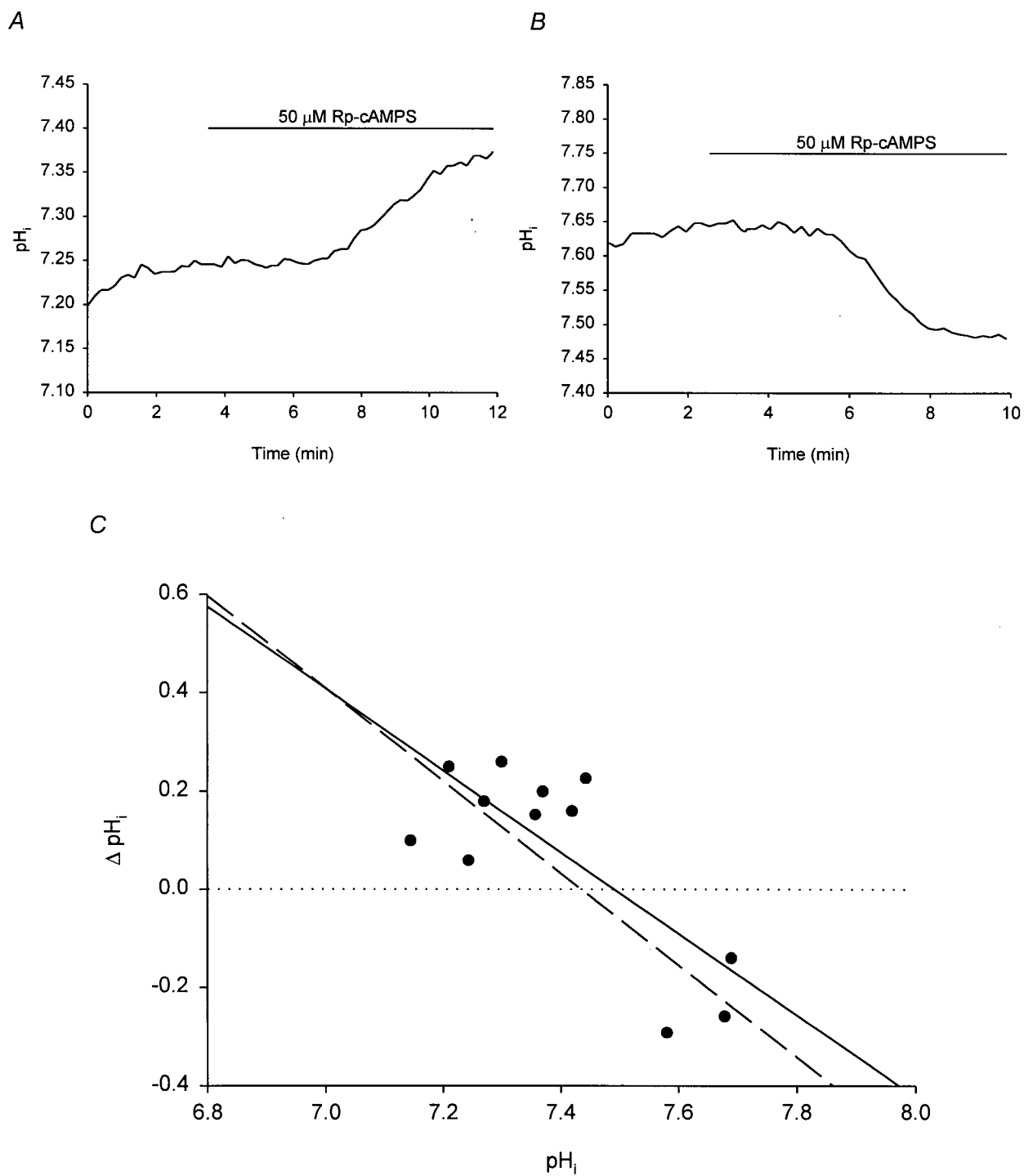
Figure 34. Effect of DIDS on Rp-cAMPS-evoked changes in SS pH_i under Na^+_o-free, HCO_3^- /CO_2-buffered conditions.

The resting pH_i of this neuron was ~7.24 prior to the addition of 200 μM DIDS to the Na^+-free, HCO_3^-/CO_2-buffered perfusion medium. DIDS caused pH_i to fall to a new SS level at ~7.20. The subsequent addition of 50 μM Rp-cAMPS failed to alter SS pH_i.
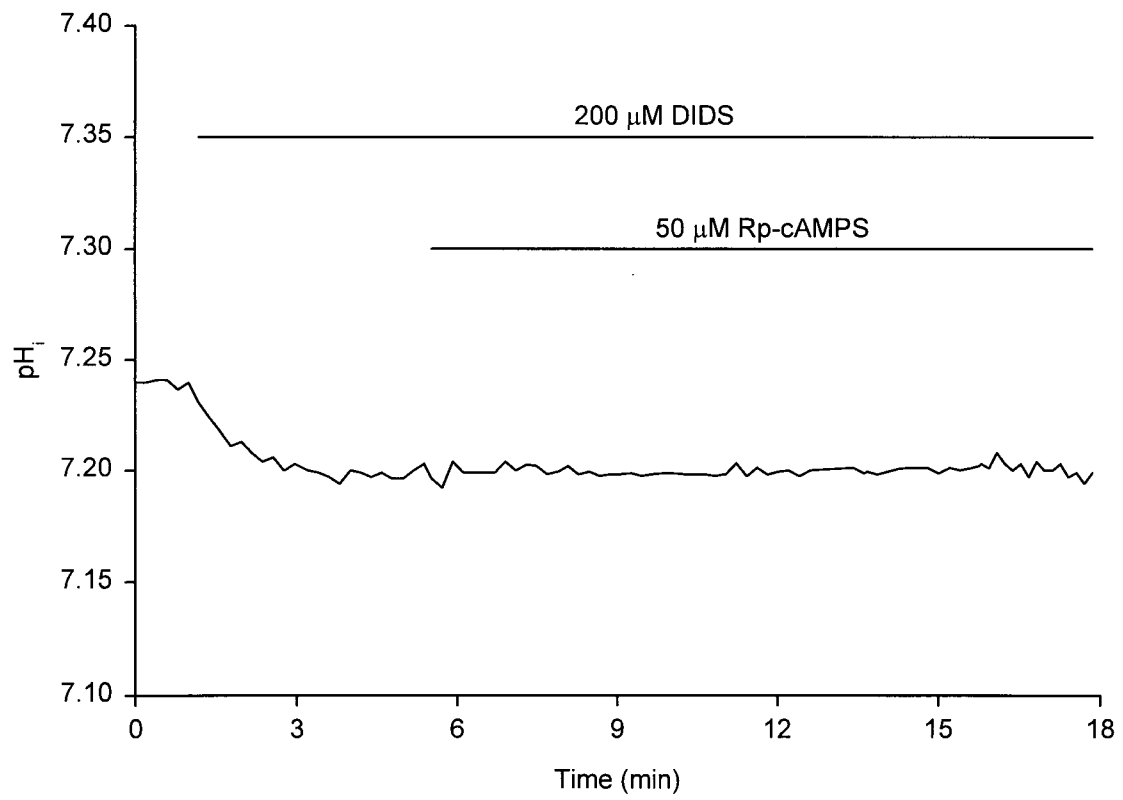
Figure 35. Effect of Sp-cAMPS on pH_i recovery from internal acid loads under HCO_3^- /CO_2-buffered conditions at 37°C.

A A sample paired acid load recovery experiment in which the first acid load recovery was performed under control HCO_3^-/CO_2-buffered conditions and the second was performed in the presence of 25 μM Sp-cAMPS. The rate of pH_i recovery from the second acid load was faster than the rate of pH_i recovery from the first. **B** From 8 experiments of the type shown in A, mean calculated rates of pH_i recovery from acid loads imposed in the absence (●) and presence (O) of 25 μM Sp-cAMPS were plotted against absolute values of pH_i and regression lines were fitted to the data points. The rates of pH_i recovery were significantly faster in the presence than in the absence of Sp-cAMPS at all absolute values of pH_i (except $pH_i = 7.25$). The line representing data obtained in the presence of Sp-cAMPS had a greater slope than the line representing control data.

A
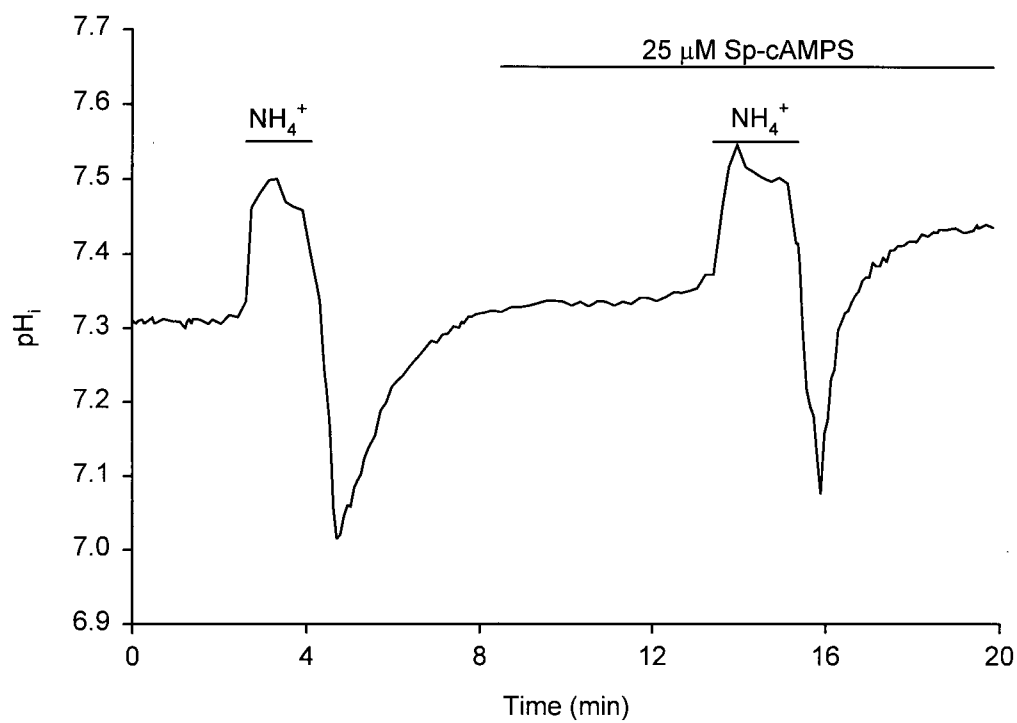
B
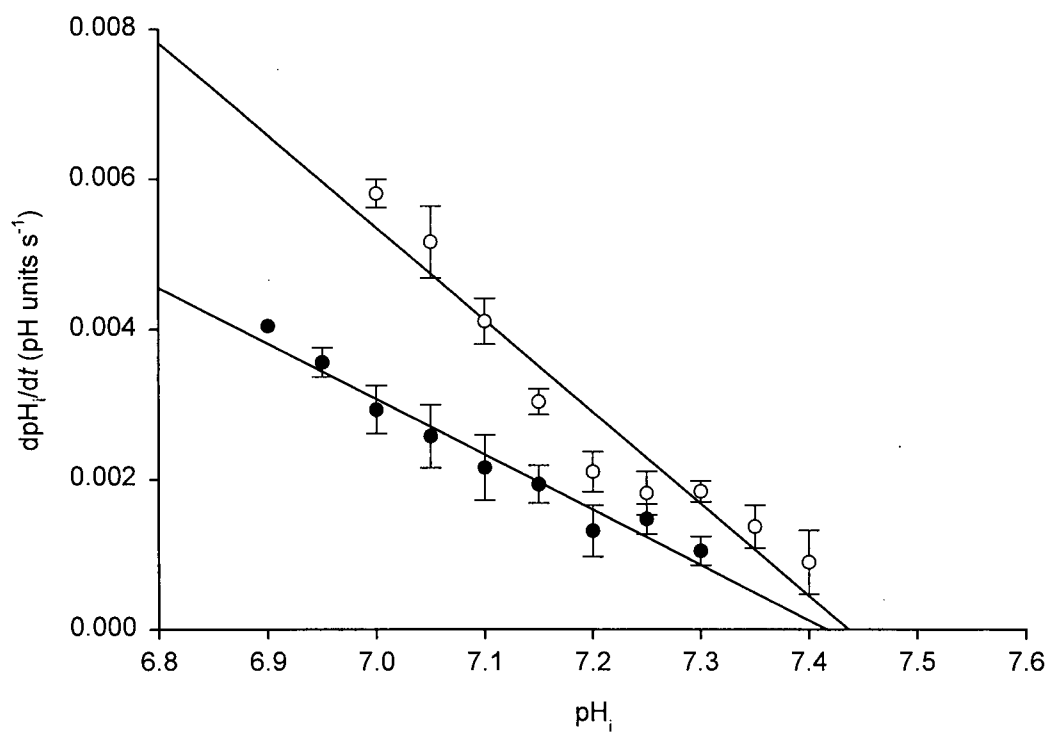
Figure 36. Effects of Sp-cAMPS on pH_i recovery from internal acid loads under HCO_3^- /CO_2 and HEPES -buffered conditions at RT.

A A neuron with a resting pH_i at ~7.10 was administered a control acid load under HCO_3^-/CO_2-buffered conditions at RT. After pH_i recovery to the initial SS value, a second acid load was performed in the presence of 25 μM Sp-cAMPS, after which pH_i recovered at a slower rate than under control conditions. **B** Plotted are mean rates of pH_i recovery from acid loads performed in the absence (●) and presence (O) of 25 μM Sp-cAMPS against absolute values of pH_i under HCO_3^-/CO_2-buffered conditions at RT (n = 5). The rates of pH_i recovery were significantly smaller in the presence than in the absence of Sp-cAMPS at absolute values of pH_i at 7.15 and 7.20. Regression lines were fitted to the data points and the line representing the data obtained in the presence of Sp-cAMPS had a smaller slope than the line representing the control data. **C** After pH_i was allowed to recover from an acid load performed under control HEPES-buffered conditions at RT, the neuron was exposed to 25 μM Sp-cAMPS and a second acid load was performed. The rate of pH_i recovery from the second acid load was similar to the first. **D** Plotted are mean rates of pH_i recovery from acid loads performed in the absence (●) and presence (O) of 25 μM Sp-cAMPS against absolute values of pH_i under HEPES-buffered conditions at RT (n = 9). The rates of pH_i recovery at all absolute values of pH_i were not significantly different in the presence compared to the absence of Sp-cAMPS. Regression lines were fitted to the data points and had a common x-intercept at pH_i = ~7.30.

A

B

C

D
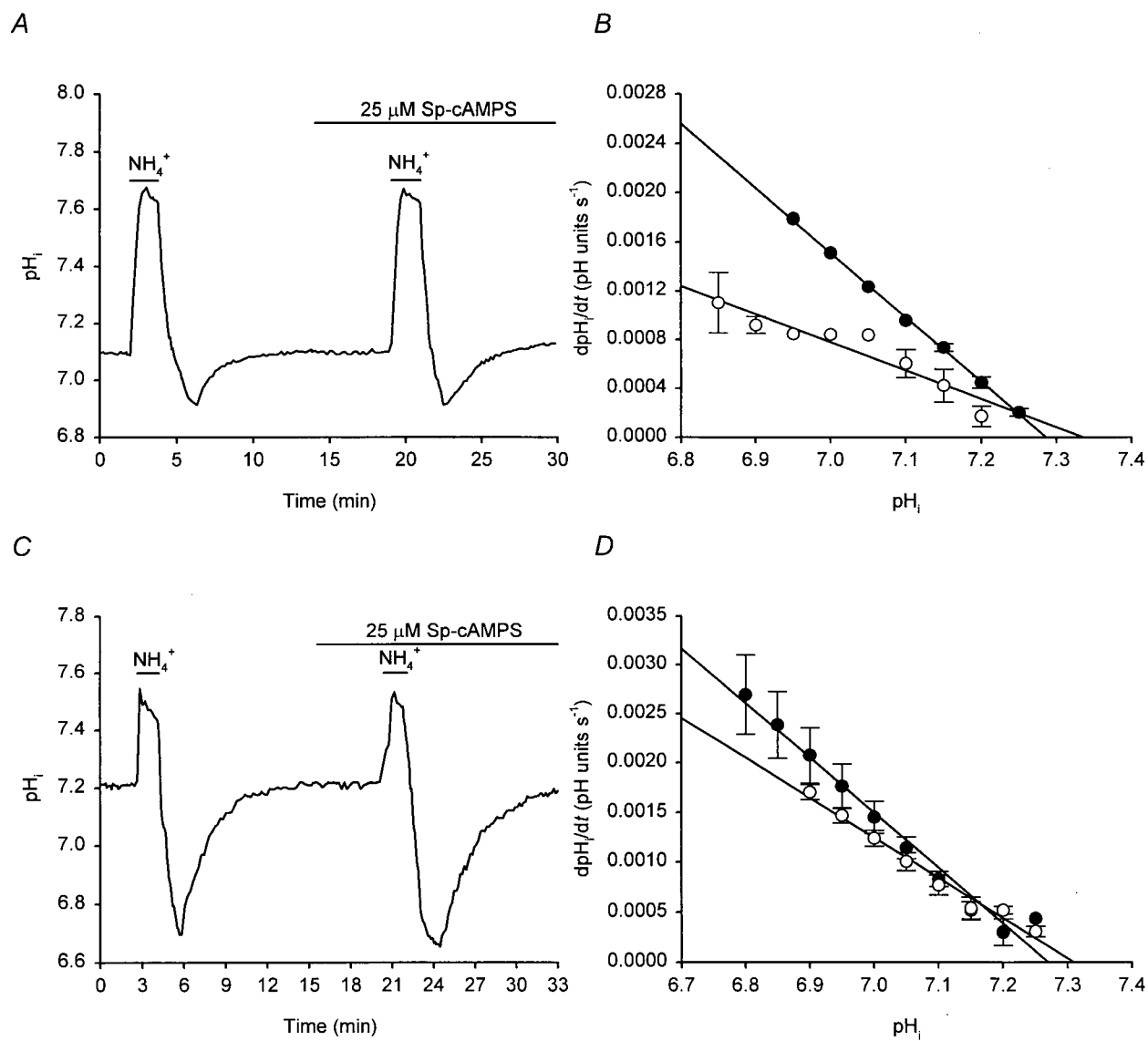
Figure 37. Effects of Rp-cAMPS on pH_i recovery from internal acid loads under HCO_3^- /CO_2-buffered conditions at RT and 37°C.

A A neuron with a resting pH_i at ~7.15 was administered a control acid load under HCO_3^-/CO_2- buffered conditions at RT. After pH_i recovered to the initial SS value, 50 μM Rp-cAMPS was added to the perfusion medium causing SS pH_i to increase, after which a second acid load was performed. In the continued presence of Rp-cAMPS, pH_i recovered at a faster rate than under control conditions. **B** After pH_i recovered from an internal acid load performed under control HCO_3^-/CO_2-buffered conditions at 37°C, the neuron was exposed to 50 μM Rp-cAMPS which elicited a rise in SS pH_i. In the continued presence of Rp-cAMPS, a second acid load was performed after which pH_i recovered at a faster rate than after the first acid load. **C** Plotted are mean rates of pH_i recovery from acid loads performed in the absence (●) and presence (O) of 50 μM Rp-cAMPS against absolute values of pH_i under HCO_3^-/CO_2-buffered conditions at RT (n = 11). The rates of pH_i recovery were significantly greater at all absolute values of pH_i in the presence than in the absence of Rp-cAMPS. Regression lines were fitted to the data points and the line representing data obtained in the presence of Rp-cAMPS had a more alkaline x-intercept than the line representing control data. **D** Plotted are mean rates of pH_i recovery from acid loads performed in the absence (●) and presence (O) of 50 μM Rp-cAMPS against absolute values of pH_i under HCO_3^-/CO_2-buffered conditions at 37°C (n = 4). The rates of pH_i recovery at all absolute values of pH_i were significantly greater in the presence than in the absence of Rp- cAMPS. Regression lines were fitted to the data points and the line representing data obtained in the presence of Rp-cAMPS had a more alkaline x-intercept than the line representing control data.

A
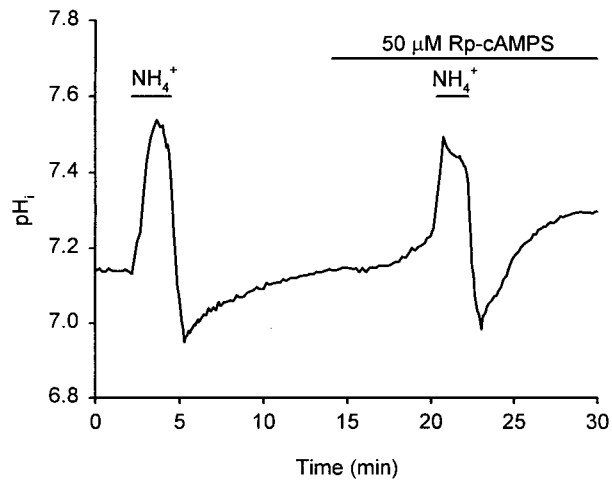
B
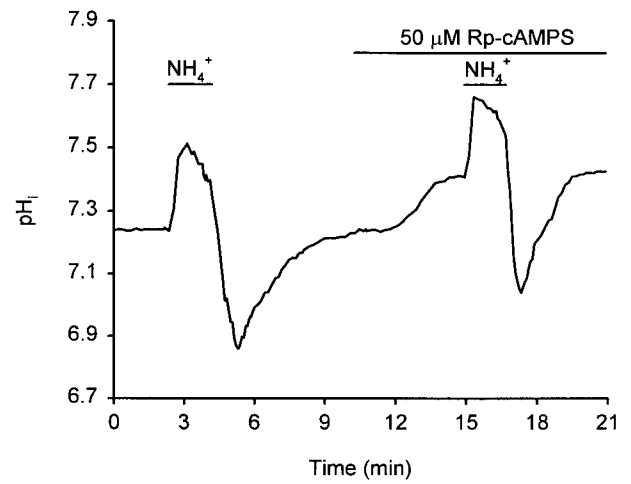
C
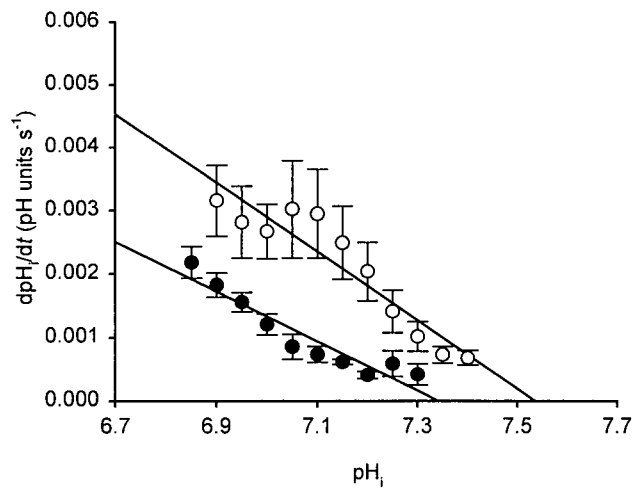
D
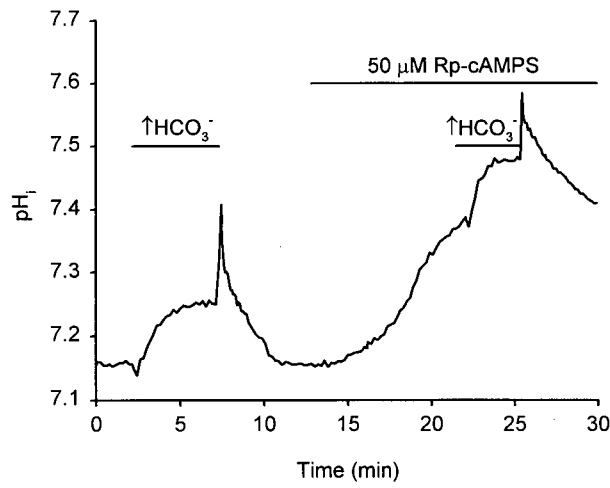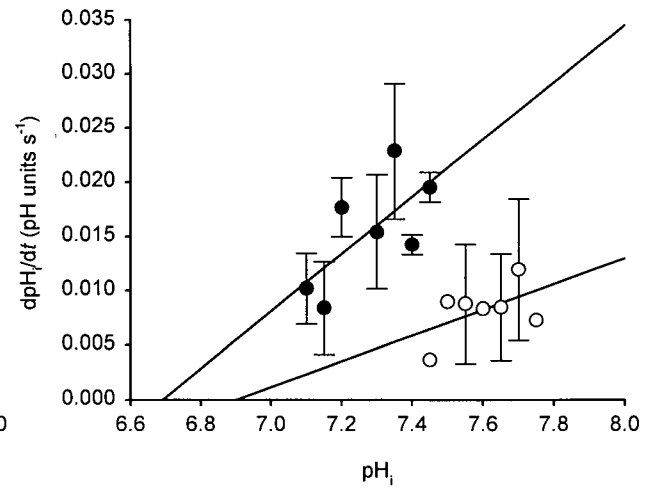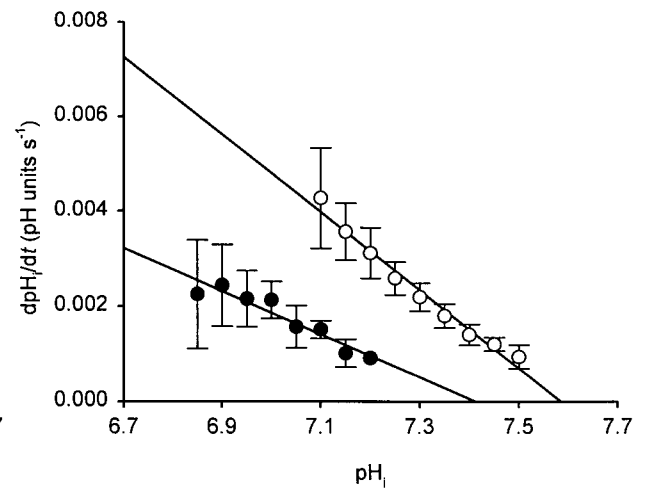
Figure 38. Effects of Rp-cAMPS on pH$_i$ recovery from internal acid loads under DIDS-containing, HCO$_3^-$/CO$_2$-buffered (37°C) and HEPES-buffered (RT) conditions.

A After pH$_i$ was allowed to recover from an acid load performed under control HCO$_3^-$/CO$_2$-buffered conditions at 37°C, the neuron was exposed to 200 µM DIDS and 50 µM Rp-cAMPS and a second acid load was performed. pH$_i$ recovered from the second acid load at a rate that was similar to that observed following the first acid load. **B** Plotted are mean rates of pH$_i$ recovery from acid loads performed in the absence (●) and presence (O) of 50 µM Rp-cAMPS and 200 µM DIDS against absolute values of pH$_i$ under HCO$_3^-$/CO$_2$-buffered conditions at 37°C ($n = 4$). The rates of pH$_i$ recovery were not significantly different in the absence and presence of Rp-cAMPS and DIDS at all absolute values of pH$_i$. Regression lines were fitted to the data points and the lines had a common x-intercept at pH$_i$ = ~7.5. Compare with Fig. 37D (the effect of Rp-cAMPS on rates of pH$_i$ recovery from acid loads performed under HCO$_3^-$/CO$_2$-buffered conditions at 37°C in the absence of DIDS). **C** A different neuron with a resting pH$_i$ at ~7.22 was administered a control acid load under HEPES-buffered conditions at RT. After pH$_i$ recovered to the initial SS value, a second acid load was performed in the presence of 50 µM Rp-cAMPS, after which pH$_i$ recovered to SS at a similar rate to that observed under control conditions. Note that Rp-cAMPS did not change SS pH$_i$ under these HCO$_3^-$-free conditions. **D** Plotted are mean rates of pH$_i$ recovery from acid loads performed in the absence (●) and presence (O) of 50 µM Rp-cAMPS against absolute values of pH$_i$ under HEPES-buffered conditions at RT ($n = 10$). The rates of pH$_i$ recovery were not significantly different in the presence and absence of Rp-cAMPS at all absolute values of pH$_i$ except 6.90, 7.10 and 7.15. Regression lines were fitted to the data points and the lines had a common x-intercept at pH$_i$ = ~ 7.4. Compare with Fig. 37C (the effect of Rp-cAMPS on rates of pH$_i$ recovery from acid loads performed under HCO$_3^-$/CO$_2$-buffered conditions at RT).
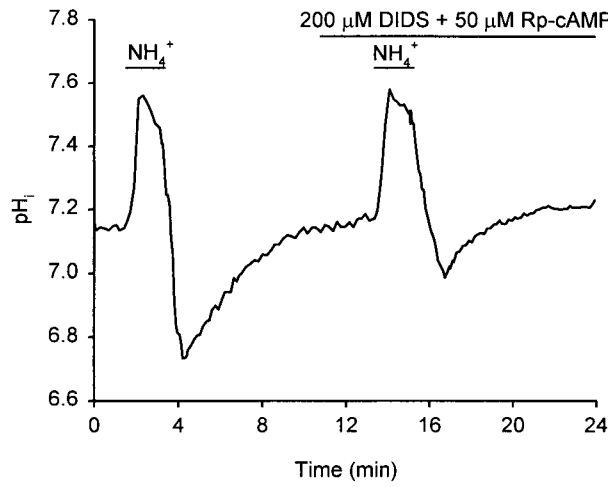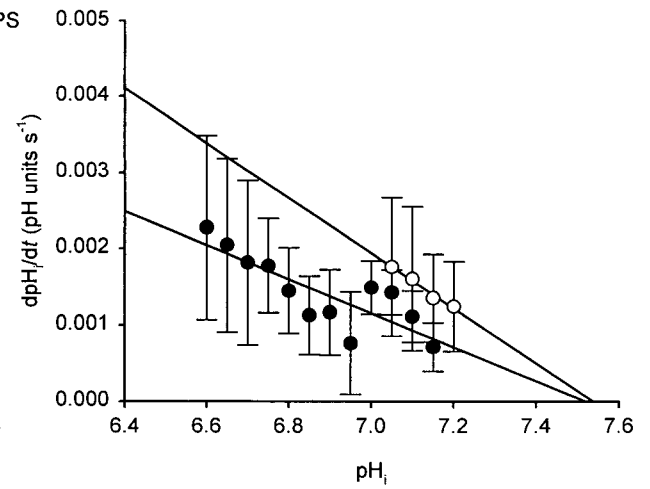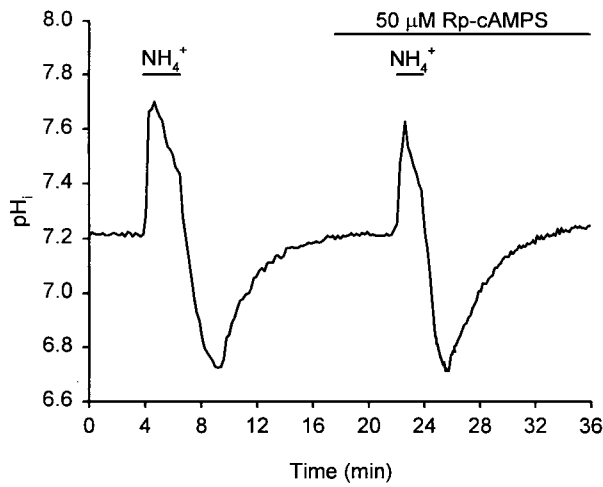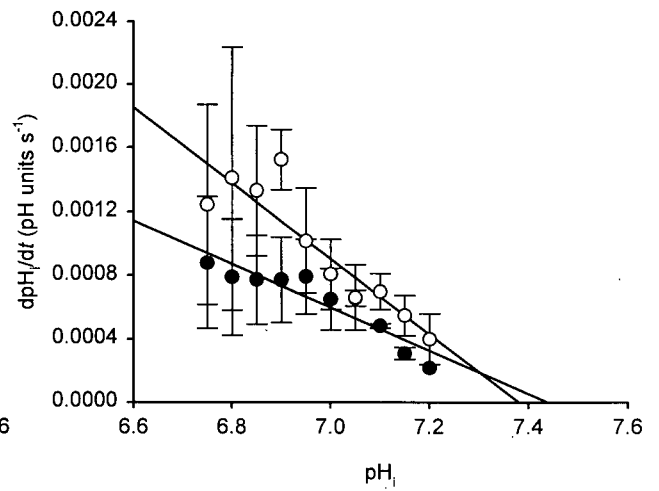
Figure 39. Effects of repeated exposure to HCO_3^-/CO_2 on SS pH_i under Cl_o^--free, HEPES-buffered conditions in the absence and presence of DIDS at RT.

A Initially, the removal of Cl^- from the HEPES-buffered perfusion medium caused the SS pH_i of this neuron to decrease from ~7.20 to ~7.05. Subsequent addition of HCO_3^-/CO_2 in the continued absence of Cl_o^- caused SS pH_i to transiently decrease and then increase to a SS level at pH_i = ~7.22. HCO_3^-/CO_2 was then removed from the perfusion medium causing an alkalinization after which pH_i recovered to the initial SS level at pH_i = ~7.05. Addition and subsequent removal of HCO_3^-/CO_2 was repeated 3 additional times and pH_i followed the same pattern as during the first exposure to HCO_3^-/CO_2, except the SS levels to which pH_i alkalinized in the presence of HCO_3^-/CO_2 diminished with each successive application and, more importantly, the rates at which pH_i reached each new SS level were reduced (see Fig. 40C). **B** A different neuron was *pretreated with 200 μM DIDS* under Cl_o^--free, HEPES-buffered conditions and had a resting pH_i at ~7.19. Subsequent addition of HCO_3^-/CO_2 in the continued absence of Cl_o^- (and presence of DIDS) caused pH_i to transiently decrease and then increase to a SS level at pH_i = ~7.37. HCO_3^-/CO_2 was then removed from the perfusion medium causing an alkalinization after which pH_i recovered to the initial SS level. Addition and subsequent removal of HCO_3^-/CO_2 was repeated 2 additional times and pH_i followed the same pattern as during the first exposure to HCO_3^-/CO_2 (*i.e.* the magnitudes of the alkalinizations and the rates of pH_i increase were similar during each exposure of HCO_3^-/CO_2; see Fig. 40C). Both experiments were performed at RT.

A
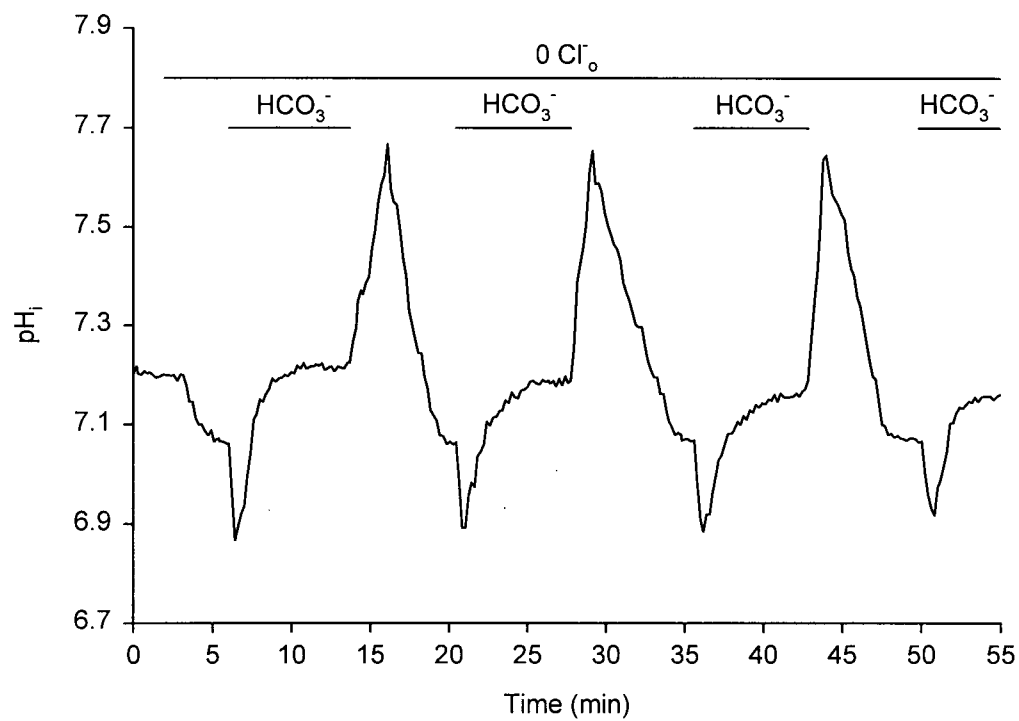
B
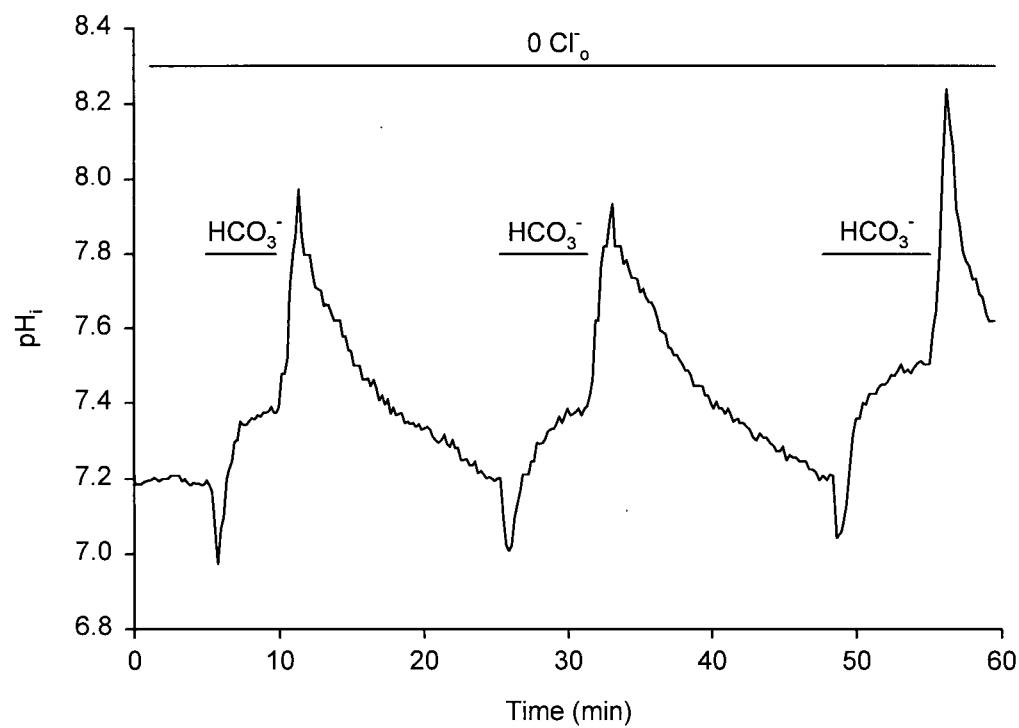
Figure 40. Effects of Rp-cAMPS and Sp-cAMPS on the Cl^-_i-depletion procedure performed at RT.

A Initially, the addition of 50 μM Rp-cAMPS and removal of Cl^- from the HEPES-buffered perfusion medium did not cause the resting pH_i of this neuron to change. Subsequent addition of HCO_3^-/CO_2 in the continued absence of Cl^-_o and presence of Rp-cAMPS caused pH_i to transiently decrease and then increase to a new SS level. HCO_3^-/CO_2 was then removed from the perfusion medium causing an alkalinization after which pH_i recovered to the initial SS level. Addition and subsequent removal of HCO_3^-/CO_2 was repeated 2 additional times and pH_i followed the same pattern as during the first exposure to HCO_3^-/CO_2 except the magnitude of the alkalinization and the rate of pH_i change decreased during the second and third applications of HCO_3^-/CO_2 (see part C). **B** Initially, the addition of 25 μM Sp-cAMPS and removal of Cl^- from the HEPES-buffered perfusion medium caused the resting pH_i of this neuron to decrease ~0.05 pH units. Subsequent addition of HCO_3^-/CO_2 in the continued absence of Cl^-_o and presence of Sp-cAMPS caused pH_i to transiently decrease and then increase to a new SS level. HCO_3^-/CO_2 was then removed from the perfusion medium causing an alkalinization after which pH_i recovered to the initial SS level. Addition and subsequent removal of HCO_3^-/CO_2 was repeated 3 more times and pH_i followed a similar pattern as during the first exposure to HCO_3^-/CO_2. Although the rate of pH_i change was similar in each case (except during the second application of HCO_3^-/CO_2), the magnitude of the alkalinization observed in the presence of HCO_3^-/CO_2 increased with each subsequent application of HCO_3^-/CO_2 (see part C). **C** The mean rates of alkalinization for each application of HCO_3^-/CO_2 were calculated at an absolute pH_i value = 7.10 and were normalized to the rate of alkalinization observed during the first application of HCO_3^-/CO_2. The resulting normalized rates of alkalinization are presented as percentage values under control conditions, in the presence of 200 μM DIDS, in the presence of 50 μM Rp-cAMPS, and in the presence of 25 μM Sp-cAMPS. Under each experimental condition, each bar represents the normalized rate of alkalinization observed during the sequential application of HCO_3^-/CO_2 (four applications under control conditions and in the presence of Sp-cAMPS; three applications in the presence of DIDS or Rp-cAMPS). Note that most of the normalized rates of pH_i increase did not change with each subsequent addition of HCO_3^-/CO_2 in the presence of DIDS or Sp-cAMPS. However, the normalized rates of pH_i increase were reduced during each successive application of HCO_3^-/CO_2 under control conditions (*e.g.* a significant decrease in the normalized rate was observed during the *third* application) and in the presence of Rp-cAMPS (*e.g.* a significant decrease in the normalized rate was observed during the *second* application). Each number in parentheses = number of neurons examined at RT and * represents $P < 0.05$ for the comparison between the marked and first normalized rate of alkalinization values.

A

$0 \, Cl_o^- + 50 \, \mu M \, Rp\text{-cAMPS}$

HCO_3^- HCO_3^- HCO_3^-

pH_i

Time (min)

B

$0 \, Cl_o^- + 25 \, \mu M \, Sp\text{-cAMPS}$

HCO_3^- HCO_3^- HCO_3^- HCO_3^-

pH_i

Time (min)

C

Normalized dpH_i/dt (percentage)

Control (7)   DIDS (3)   Rp-cAMPS (3)   Sp-cAMPS (3)
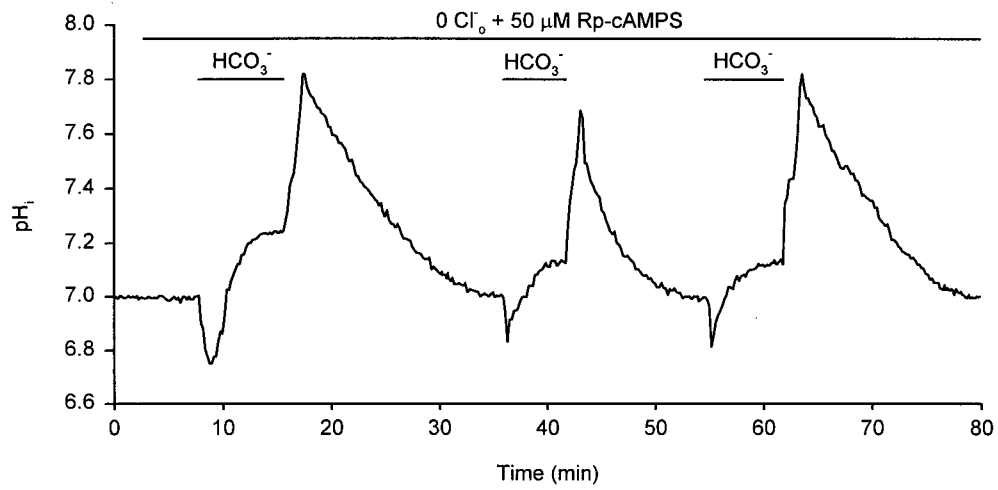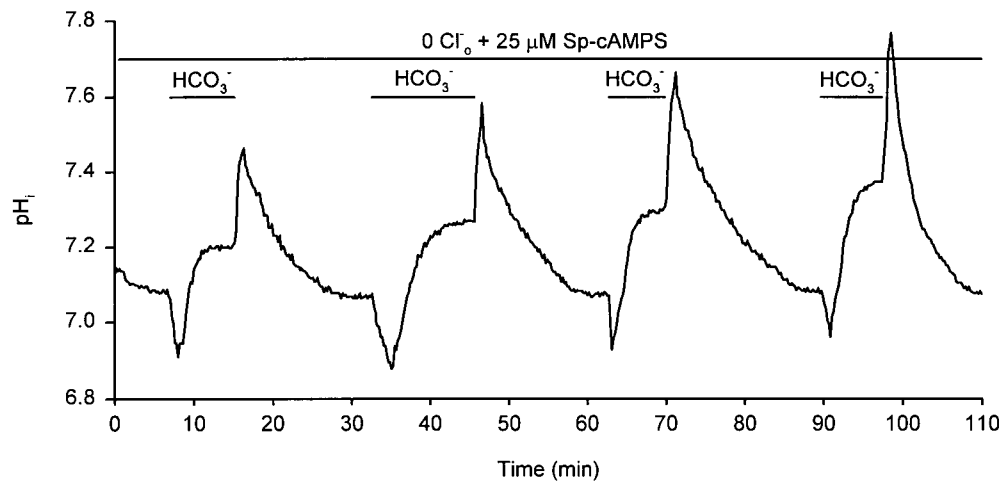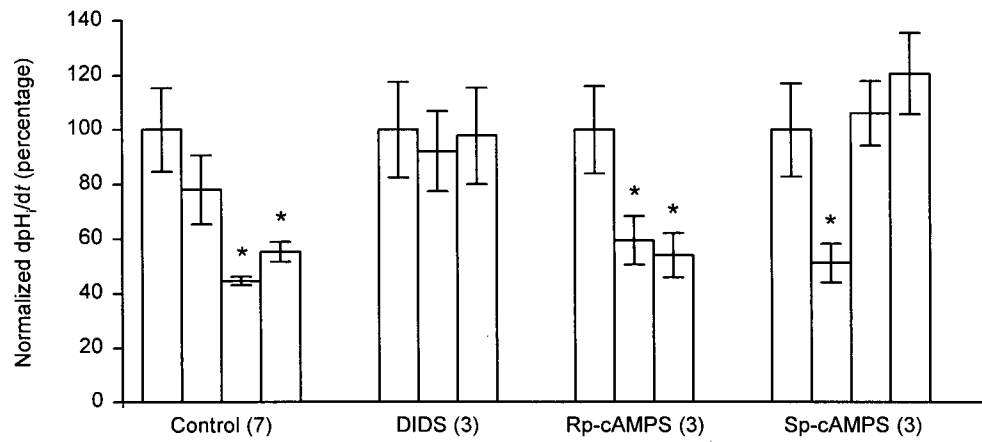
3. Modulation of the activity of the NHE by the CaM/CaMK-II second messenger system

Calmodulin and CaMK-II have been found to be involved in the control of the activities of both HCO_3^--dependent and HCO_3^--independent pH_i regulating mechanisms in a variety of peripheral cell types (see Introduction). In addition, the CaM/CaMK-II pathway has been shown to be important in regulating neuronal function (*e.g.* Mody *et al.*, 1984; Cooper *et al.*, 1994; Ehlers *et al.*, 1996). Therefore, in this third part of the study, the role of the CaM/CaMK-II pathway in the control of the activity of the NHE was assessed in adult rat hippocampal CA1 neurons.

3.1 Inhibition of CaM

3.1.1 Characterization of the SS pH_i response to CaM inhibition

3.1.1.1 HEPES-buffered conditions

Initially, to examine the effect of the inhibition of CaM on SS pH_i, experiments were performed with varying concentrations of W-7, an inhibitor of calmodulin which binds to CaM in a Ca^{2+}-dependent manner (Inagaki *et al.*, 1983) under HEPES-buffered conditions. As shown in Fig. 41A, addition of 5, 20, 50 and 100 μM W-7 elicited 0.12 ± 0.04 ($n = 9/9$), 0.14 ± 0.04 ($n = 9/9$), 0.24 ± 0.04 ($n = 14/15$) and 0.24 ± 0.05 ($n = 7/9$) pH unit increases in SS pH_i, respectively, in neurons with mean resting pH_i values that were not significantly different ($P < 0.5$ between all experimental groups; mean resting pH_i values were 7.13 ± 0.08, 7.16 ± 0.10, 7.18 ± 0.07 and 7.13 ± 0.05, respectively, for neurons exposed to 5, 20, 50 and 100 μM W-7). The remaining 3 neurons did not respond to W-7 treatment. Subsequent washout of W-7 allowed pH_i to fully recover to resting SS levels when low test concentrations were used (5, 20 and 50 μM; Fig. 41B) and to partially recover when 100 μM W-7 was used (data not shown). Thus, W-7 evoked a reversible and concentration-dependent increase in SS pH_i in adult rat hippocampal CA1 neurons under HEPES-buffered conditions. In addition to an increase in the magnitude of the internal alkalinization, decreases in the time of onset of the response (1.9 ± 0.6, 1.5 ± 0.3, 1.4

± 0.1 and 1.0 ± 0.1 min, respectively, for 5, 20, 50 and 100 μM W-7) and the time taken to reach a new elevated SS pH_i (10 ± 3, 6.0 ± 1.5, 6.0 ± 0.6 and 3.7 ± 0.6 min, respectively) were evoked by increasing concentrations of W-7 (*e.g.* Fig. 41A). From these results a test concentration of 50 μM W-7 was chosen for the majority of further experiments, as it was the lowest [W-7] that elicited a maximal SS pH_i response.

The SS pH_i response to W-7 was also dependent on resting values of pH_i measured prior to the application of the compound. For example, the addition of 50 μM W-7 elicited a 0.42 pH unit increase in SS pH_i in a neuron with a resting pH_i at 6.86 (Fig. 42A), and a 0.11 pH unit increase in SS pH_i in a neuron with a resting pH_i at 7.40 (Fig. 42B). When the changes in SS pH_i elicited by 50 μM W-7 were plotted against resting values of pH_i and a regression line was fitted to the data points, the resulting plot (Fig. 42C) showed that as resting pH_i increased, the magnitude of the rise in SS pH_i evoked by 50 μM W-7 decreased.

Similar experiments were performed in the presence of trifluoperazine (TFP), another inhibitor of calmodulin (Levin & Weiss, 1979) to confirm that CaM inhibition was involved in the SS pH_i response to W-7. In a population of neurons with a mean resting value of pH_i at 7.25 ± 0.04, the addition of 50 μM TFP elicited a 0.16 ± 0.02 (*n* = 11, Fig. 43A) pH unit increase in SS pH_i under HEPES-buffered conditions, an increase which was similar to the alkalinization elicited by 50 μM W-7 (= 0.18 pH units at pH_i = 7.25, computed from the regression line presented in Fig. 42C). As observed in the presence of 50 μM W-7, the SS pH_i response to 50 μM TFP was also dependent on resting values of pH_i (Fig. 43B). However, the time required for the onset of the SS pH_i response (5.0 ± 0.6 min) and the time required to reach the final elevated SS pH_i (11.6 ± 0.9 min) following the addition of TFP were significantly greater than the results obtained in the presence of 50 μM W-7 (1.4 ± 0.1 and 6.0 ± 0.6 min, respectively; $P < 0.003$; compare Figs. 41B and 43A). Taken together, these results suggest that inhibition of CaM with TFP or W-7 elicits a pH_i-dependent increase in SS pH_i, which most likely reflects a change in the activity of a HCO_3^--independent pH_i regulating mechanism (*e.g.* the NHE).

3.1.1.2 HCO_3^--buffered conditions

In order to assess the role of HCO_3^- in the SS pH_i response to inhibition of calmodulin, experiments were performed with W-7 and TFP under HCO_3^-/CO_2-buffered conditions. In neurons with a mean resting pH_i value at 7.14 ± 0.05, the addition of 100 μM W-7 elicited a 0.18 ± 0.02 ($n = 10$, Fig. 44A) pH unit rise in SS pH_i. This internal alkalinization observed under HCO_3^-/CO_2-buffered conditions was not statistically different ($P = 0.21$) to the increase in SS pH_i evoked by 100 μM W-7 in the nominal absence of HCO_3^- (a 0.24 ± 0.05 pH unit increase in pH_i observed in neurons with a mean resting pH_i value of 7.13 ± 0.05 which was not statistically different to the resting pH_i values of neurons exposed to 100 μM W-7 under HCO_3^-/CO_2-buffered conditions; $P = 0.93$). Data obtained in the presence of HCO_3^- exhibited a similar dependence on resting pH_i as previously described in the nominal absence of HCO_3^- (data not shown; see Fig. 42C). In neurons with a mean resting pH_i value at 7.23 ± 0.04, the addition of 50 μM TFP under HCO_3^-/CO_2-buffered conditions evoked a 0.16 ± 0.03 ($n = 6/7$, Fig. 44B; the remaining neuron did not respond to TFP treatment) pH unit increase in SS pH_i, an internal alkalinization that was not statistically different ($P = 0.89$) to the increase in SS pH_i evoked by 50 μM TFP in the nominal absence of HCO_3^- (a 0.16 ± 0.02 pH unit increase in pH_i observed in neurons with a mean resting pH_i value of 7.25 ± 0.04; $P = 0.77$ when compared to the mean resting pH_i under HCO_3^-/CO_2-buffered conditions). These internal alkalinizations evoked by 50 μM TFP in the presence of HCO_3^- exhibited a similar pH_i dependence as previously described in the absence of HCO_3^- (data not shown; see Fig. 43B). Thus, the SS pH_i response to inhibition of CaM is not dependent on the presence of HCO_3^-.

Additional experiments were performed with W-7 in the presence of DIDS under HCO_3^-/CO_2-buffered conditions to assess the effect of inhibiting the activities of HCO_3^--dependent pH_i regulating mechanisms on the SS pH_i response to CaM inhibition. In the presence of 200 μM DIDS, neurons had a mean initial SS pH_i at 7.37 ± 0.07 and the addition of 50 μM W-7 elicited a 0.13 ± 0.04 ($n = 8/11$, Fig. 44C; the remaining 3 neurons did not respond to W-7 treatment) pH unit increase in SS pH_i. This internal alkalinization was similar to both the increase in pH_i evoked by 100 μM W-7 in the absence of DIDS (and presence of HCO_3^-/CO_2; a 0.14 pH unit

increase at pH_i = 7.37, computed from the regression line relating pH_i to ΔpH_i evoked by 100 μM W-7 under HCO_3^-/CO_2-buffered conditions; see above) and the increase in pH_i evoked by 50 μM W-7 in the nominal absence of HCO_3^- (a 0.12 pH unit increase at pH_i = 7.37, computed from the regression line presented in Fig. 42C). Again, these alkalinizations elicited by 50 μM W-7 in the presence of DIDS were dependent on the resting pH_i prior to the addition of the CaM inhibitor (data not shown). Thus, the SS pH_i response to CaM inhibition under HCO_3^-/CO_2-buffered conditions is not sensitive to DIDS.

In summary, the inhibition of CaM with W-7 or TFP causes SS pH_i to increase in a manner that is: (1) dependent on the resting value of pH_i; (2) HCO_3^--independent; and (3) insensitive to DIDS. Therefore, it is unlikely that the SS pH_i response to CaM inhibition reflects changes in β_i or the activities of HCO_3^--dependent pH_i regulating mechanisms; rather, the observed alkalinization under both HCO_3^--free and HCO_3^-/CO_2-buffered conditions may reflect an increase in the activity of the NHE.

3.1.2 The effect of CaM inhibition on the activity of the NHE

3.1.2.1 Effect of external Na^+ removal on the SS pH_i response to W-7

To confirm the role of the NHE in the SS pH_i response to W-7, experiments were performed in the absence of external Na^+ (isosmotically replaced by $NMDG^+$) under HEPES-buffered conditions to block the activity of the NHE. Under these conditions and in neurons with a mean resting value of pH_i = 7.30 ± 0.05, the addition of 50 μM W-7 elicited a 0.06 ± 0.02 (n = 9, Fig. 45A) pH unit increase in SS pH_i. A statistical comparison between the magnitudes of the internal alkalinizations evoked by 50 μM W-7 under Na^+_o-free ($NMDG^+$ substitution) and Na^+_o-containing conditions was not made because the mean resting pH_i values of the neurons observed under each condition were significantly different (P = 0.04; mean resting pH_i values were 7.18 ± 0.07, n = 14, and 7.30 ± 0.05, n = 9, under Na^+_o-containing and Na^+_o-free conditions, respectively). However, the increase in SS pH_i evoked by 50 μM W-7 in the absence of Na^+_o ($NMDG^+$ substitution) was much smaller than the increase in SS pH_i evoked by 50 μM W-7

predicted to take place in the presence of Na^{+}_{o} at a resting pH_i value of 7.30 (a 0.16 pH unit increase at pH_i = 7.30, computed from the regression line presented in Fig. 42C). This result indicates that the SS pH_i response to W-7 is, at least partially, dependent on external Na^{+}.

Experiments were then performed in the presence of external Li^{+}(which isosmotically replaced Na^{+}) under HEPES-buffered conditions. Under these conditions, in which the NHE is active (cf NMDG^{+}-substituted conditions), the addition of 50 μM W-7 elicited a 0.25 ± 0.05 (n = 6, Fig. 45B) pH unit increase in SS pH_i in neurons with a mean resting value of pH_i = 7.07 ± 0.09. This W-7-evoked internal alkalinization was not statistically different (P = 0.92) to the increase in SS pH_i evoked by 50 μM W-7 in the presence of external Na^{+} (a 0.24 ± 0.04 pH unit increase in pH_i observed in neurons with a statistically similar mean resting pH_i value of 7.18 ± 0.07, P = 0.29). A formal statistical comparison between the internal alkalinizations evoked by 50 μM W-7 under Na^{+}_{o}-free, Li^{+}-substituted and NMDG^{+}-substituted conditions was not made because the mean resting pH_i values of the neurons observed under each condition were significantly different (P = 0.03; mean resting pH_i values were 7.07 ± 0.09, n = 6, and 7.30 ± 0.05, n = 9, under Na^{+}_{o}-free, Li^{+}-substituted and NMDG^{+}-substituted conditions, respectively). However, the alkalinizations elicited by 50 μM W-7 under 0 Na^{+}_{o} (Li^{+} substitution) conditions were dependent on the resting pH_i prior to the addition of the CaM inhibitor (data not shown). Thus, 50 μM W-7 was predicted to evoke a 0.18 pH unit increase in SS pH_i under Na^{+}_{o}-free (Li^{+} substitution) conditions at a resting pH_i value of 7.30. This predicted alkalinization was greater than the increase in SS pH_i evoked by 50 μM W-7 under Na^{+}_{o}-free (NMDG^{+} substitution) conditions at the same resting pH_i value (= 0.06 ± 0.02 pH units, n = 9; see above). Hence, the attenuated SS pH_i response to W-7 observed in the absence of Na^{+}_{o} (NMDG^{+}-substitution) was completely restored in the presence of Li^{+}, suggesting that a change in the activity of the NHE is involved in the SS pH_i response to CaM inhibitors.

3.1.2.2 Effect of CaM inhibition on the rate of pH_i recovery from acid loads

To further examine effect of CaM inhibition on the activity of the NHE, paired acid load recovery experiments were performed with W-7 and TFP. Under HEPES-buffered conditions

and in the presence of 50 μM W-7, the overall rates of pH$_i$ recovery were increased by 366 ± 41 % (n = 4/5) and 175 ± 18 % (n = 7/8) when compared to control rates of pH$_i$ recovery (Fig. 46A), in neurons with low and high resting pH$_i$ values, respectively. The remaining 2 neurons did not respond to W-7 treatment. As shown in Fig. 46B and C, the calculated net acid flux values following acid loads performed in the absence or presence of 50 μM W-7 were plotted against absolute values of pH$_i$ and regression lines were fitted to the data points. The net acid efflux values were significantly faster in the presence than in the absence of W-7 at all absolute values of pH$_i$ in neurons with both low and high resting pH$_i$ values. Also, W-7 caused alkaline shifts of ~0.3 and ~0.2 pH units in the pH$_i$ dependence of net acid efflux in neurons with low and high resting pH$_i$ values, respectively. Similar experiments were performed under HCO$_3^-$/CO$_2$-buffered conditions and a 284 ± 44% (n = 10, Fig. 47A) increase in the overall rate of pH$_i$ recovery from imposed acid loads was observed in the presence of 50 μM W-7 when compared to the overall rate of pH$_i$ recovery observed in the absence of W-7 (in neurons with resting values of pH$_i$ < 7.4). As shown in Fig. 47B, rates of pH$_i$ recovery from acid loads imposed in the presence or absence of W-7 were plotted against the absolute pH$_i$ values at which they were obtained and regression lines were fitted to the data points. The addition of 50 μM W-7 significantly increased rates of pH$_i$ recovery (P < 0.05) at all absolute values of pH$_i$ but did not cause a marked shift in the pH$_i$ dependence of the pH$_i$ recovery process. With the exception of the absent shift in the pH$_i$-dependence of pH$_i$ recovery observed in the presence of HCO$_3^-$, no significant differences were observed between the changes in the rates of pH$_i$ recovery elicited by 50 μM W-7 in the presence or in the absence of HCO$_3^-$ (P > 0.10 when compared to data obtained from neurons with low resting pH$_i$ values under HEPES-buffered conditions). Thus, the results from the acid load recovery experiments: (1) confirm that the activities of HCO$_3^-$-dependent pH$_i$ regulating mechanisms are most likely unaffected by CaM inhibition; and (2) suggest that CaM inhibition increases the activity of the NHE which, in turn, leads to an increase in SS pH$_i$.

Additional paired acid load recovery experiments were performed with TFP under HEPES-buffered conditions to confirm the effects of CaM inhibition on the activity of the NHE. In the presence of 50 μM TFP, the overall rate of pH_i recovery following acid loads was increased by 125 $\pm$ 41% (n = 7, Fig. 48A) when compared to control rates of pH_i recovery in neurons with low resting pH_i values (pH_i = 7.13 $\pm$ 0.05). No neurons with high resting pH_i values were observed. When the calculated net acid fluxes from acid loads imposed in the absence or presence of 50 μM TFP were plotted against absolute values of pH_i and regression lines were fitted to the data points (Fig. 48B), it was found that the addition of TFP significantly increased net acid efflux at all absolute values of pH_i but did not cause a marked shift in the pH_i dependence of net acid efflux. With the exception of the inability of TFP to shift the pH_i-dependence of net acid efflux, no significant differences were observed between the changes in the net acid efflux values elicited by 50 μM TFP and 50 μM W-7 under HEPES buffered conditions (P = 0.91; for neurons with low resting pH_i values), suggesting that CaM inhibition increases the activity of the NHE.

In summary, these acid load recovery results suggest that the inhibition of CaM, by either W-7 or TFP, increases the activity of the NHE which, in turn, is at least partially responsible for the associated increase in SS pH_i observed in adult rat hippocampal CA1 neurons. Although it has been established that some NHE isoforms possess an 'auto-inhibitory' CaM binding domain (*e.g.* Wakabayashi *et al.*, 1997a, see Discussion), it is possible that the changes in the activity of the NHE evoked by CaM inhibitors may also reflect either changes in $[Ca^{2+}]_i$ (Inagaki *et al.*, 1983; Tornquist, 1993) or changes in the activity of the cAMP/PKA second messenger system (*e.g.* Copper *et al.*, 1994). Thus, further experiments were performed to examine whether similar mechanisms of 'cross-talk' were involved in the SS pH_i response to CaM inhibitors in adult rat hippocampal CA1 neurons. When considering that changes in the activities of HCO_3^--dependent pH_i regulating mechanisms do not appear to contribute to the SS pH_i response to CaM inhibitors (see above), all subsequent experiments were performed under nominally HCO_3^--free, HEPES-buffered conditions.

3.1.3 The potential role of changes in $[Ca^{2+}]_i$ in the SS pH_i response to CaM inhibitors

3.1.3.1 Effect of W-7 on $[Ca^{2+}]_i$

Under normal conditions, CaM has affinity for Ca^{2+} and elevated $[Ca^{2+}]_i$ promotes Ca^{2+} to bind to CaM causing formation of the active Ca^{2+}-CaM-complex and a concomitant reduction in $[Ca^{2+}]_i$. However, when bound to W-7, CaM has a relatively higher affinity for Ca^{2+} even though the activity of calmodulin is inhibited (Inagaki *et al.*, 1983; Tornquist, 1993) and this effect may be associated with a further reduction in $[Ca^{2+}]_i$. This raises the possibility that the effects of W-7 on SS pH_i and the activity of the NHE may reflect, at least in part, concomitant changes in $[Ca^{2+}]_i$ evoked by the CaM inhibitor. Therefore, experiments were performed with W-7 in neurons loaded with Fura-2 to assess the effects of W-7 on $[Ca^{2+}]_i$. The addition of 50 μM W-7 elicited a sustained 0.10 ± 0.05 ($n = 5$, Fig. 49A) ratio unit fall in BI_{334} /BI_{380} values (representing $[Ca^{2+}]_i$) in neurons with a mean resting BI_{334} /BI_{380} ratio at 0.75 ± 0.06. Regardless of the mechanism responsible for the W-7-evoked decrease in $[Ca^{2+}]_i$, this result suggests that at least a portion of the SS pH_i response evoked by W-7 may be secondary to (or dependent on) concomitant changes in $[Ca^{2+}]_i$.

3.1.3.2 Effects of W-7 on $[Ca^{2+}]_i$ and SS pH_i under 0 Ca^{2+}_o conditions

In some neuronal preparations and peripheral cell types, it has been reported that changes in $[Ca^{2+}]_i$ can elicit changes in SS pH_i and the activity of the NHE (Meech & Thomas, 1980; Vicentini & Villereal, 1985; Grinstein & Cohen, 1987; Little *et al.*, 1988; Melvin *et al.*, 1988; Hendey *et al.*, 1989; Ludt *et al.*, 1993; Gupta *et al.*, 1994; Sánchez-Armass *et al.*, 1994; Cooper & Hunter, 1997). To assess the potential role of the W-7-evoked decrease in $[Ca^{2+}]_i$ on the associated SS pH_i response, experiments were performed with W-7 in the absence of external Ca^{2+}. The removal of external Ca^{2+} caused a 0.10 ± 0.02 pH unit rise in SS pH_i ($n = 8$, Fig. 49B). In the continued absence of Ca^{2+}_o, neurons had a mean initial SS pH_i value at 7.32 ± 0.05 and the subsequent addition of 50 μM W-7 elicited a 0.11 ± 0.02 pH unit increase in SS pH_i ($n = 8/11$, Fig. 49B; the 3 remaining neurons did not respond to W-7 treatment). This mean W-7-evoked

alkalinization was smaller than the increase in SS pH_i evoked by 50 μM W-7 in the presence of external Ca^{2+} (a 0.15 pH unit increase at pH_i = 7.32, computed from the regression line presented in Fig. 42C). Thus, the removal of Ca^{2+}_o minimally affects the magnitude of the increase in SS pH_i evoked by 50 μM W-7.

Parallel experiments were performed under Ca^{2+}_o-free conditions in neurons loaded with Fura-2 (Fig. 49C). In 10 of 11 neurons studied, the addition of 50 μM W-7 failed to alter BI_{334} /BI_{380} values (a 0.11 ratio unit fall in BI_{334} /BI_{380} values was observed in the remaining neuron) in the absence of Ca^{2+}_o, which, of itself, elicited a 0.38 ± 0.02 unit fall in BI_{334} /BI_{380} values. It should be noted, however, that SS pH_i was also increased in the presence of W-7 (see Fig. 49B) under Ca^{2+}_o-free conditions, an effect which would lead to a decrease in the K_d of Fura-2 for Ca^{2+}; therefore, although no changes in BI_{334} /BI_{380} values were observed (see Fig. 49C), $[Ca^{2+}]_i$ was most likely reduced with the addition of W-7 under external Ca^{2+}-free conditions. The fall in $[Ca^{2+}]_i$ evoked by W-7 under Ca^{2+}_o-free conditions was estimated (see Methods and Materials, Section 6.2.2) to be 14 nM, assuming a simultaneous 0.11 pH unit increase in SS pH_i under Ca^{2+}_o-free conditions. In the presence of Ca^{2+}_o, the fall in $[Ca^{2+}]_i$ evoked by W-7 was estimated to be 53 nM, assuming a simultaneous 0.15 pH unit increase in SS pH_i. Thus, in the absence of external Ca^{2+}, the $[Ca^{2+}]_i$ response to W-7 was nearly abolished but the concomitant rise in pH_i evoked by this agent was only slightly less than the rise in pH_i evoked by W-7 under external Ca^{2+}-containing conditions. This finding suggests that: (1) the effect of W-7 to increase SS pH_i is, in large part, not secondary to W-7-evoked changes in $[Ca^{2+}]_i$; and (2) the relatively smaller increase in SS pH_i evoked by 50 μM W-7 under Ca^{2+}_o-free as opposed to Ca^{2+}_o-containing conditions may reflect the smaller decrease in $[Ca^{2+}]_i$ evoked by 50 μM W-7 under Ca^{2+}_o-free, as opposed to Ca^{2+}_o-containing conditions. The former possibility was supported by measurements of changes in $[Ca^{2+}]_i$ evoked by W-7 under Na^+_o-free ($NMDG^+$ substitution) conditions. In the absence of Na^+_o ($NMDG^+$ substitution), the rise in pH_i evoked by W-7 was reduced, compared to the rise in pH_i observed in the presence of normal Na^+_o (see above). If the W-7-evoked increase in SS pH_i under normal Na^+_o-containing conditions reflected only a concomitant fall in $[Ca^{2+}]_i$,

the W-7-evoked fall in $[Ca^{2+}]_i$ under Na^+_o-free ($NMDG^+$ substitution) conditions should also be reduced, compared to the fall in $[Ca^{2+}]_i$ evoked by W-7 under Na^+_o-containing conditions. However, contrary to this prediction, the measured fall in $[Ca^{2+}]_i$ evoked by W-7 was significantly larger ($P = 0.03$) under Na^+_o-free ($NMDG^+$ substitution) conditions (a 0.31 ± 0.03 ratio unit fall in BI_{334} $/BI_{380}$ values; $n = 6$, Fig. 50) than under Na^+_o-containing conditions (a 0.10 ± 0.03 ratio unit fall in BI_{334} $/BI_{380}$ values, $n = 5$; see above). Therefore, this result supports the suggestion made earlier that the W-7 evoked increase in SS pH_i under normal Na^+_o-containing conditions does not simply reflect a concomitant fall in $[Ca^{2+}]_i$.

3.1.3.3 Effect of TFP on $[Ca^{2+}]_i$

As shown above, the increase in SS pH_i evoked by W-7 may, in part, reflect a simultaneous decrease in $[Ca^{2+}]_i$. In order to determine if CaM inhibition by TFP causes a similar change in $[Ca^{2+}]_i$, which in turn might contribute to the effect of TFP to increase SS pH_i (see above), experiments were performed in neurons loaded with Fura-2. The addition of 50 μM TFP elicited a 1.17 ± 0.29 ratio unit *increase* in BI_{334} $/BI_{380}$ values ($n = 6$, Fig. 51), an effect which was in the opposite direction to the *decrease* in BI_{334} $/BI_{380}$ values elicited by W-7 under the same conditions. The fact that TFP and W-7 elicited similar changes in SS pH_i (*i.e.* an increase) but opposite effects on $[Ca^{2+}]_i$ further suggests that the effects of the CaM inhibitors to raise SS pH_i are not mediated by changes in $[Ca^{2+}]_i$; rather, they likely reflect activation of the NHE.

3.1.3.4 Effect of W-7 on pH_i recovery from internal acid loads under 0 Ca^{2+}_o conditions

To further examine whether the effects of W-7 on the activity of the NHE were mediated by changes in $[Ca^{2+}]_i$, paired acid load recovery experiments were performed with W-7 in the absence of Ca^{2+}_o, a condition shown previously to markedly reduce the decrease in $[Ca^{2+}]_i$ evoked by W-7, the rise in SS pH_i evoked by the agent only being slightly affected (see Section 3.1.3.2). Under these conditions and in the presence of 50 μM W-7, the overall rates of pH_i recovery from imposed acid loads were increased by 351 ± 112 % ($n = 4$) and 156 ± 28 % ($n = 5$) when compared to the rates of pH_i recovery observed in the absence of W-7 (Fig. 52A) in

neurons with low and high resting pH_i values, respectively. These increases were not significantly different to the increases evoked by 50 μM W-7 under HEPES-buffered conditions in the presence of Ca^{2+}_o (366 ± 41 %, $n = 4$, and 175 ± 18 %, $n = 8$, increases in the overall rates of pH_i recovery in neurons with low and high resting pH_i values, respectively; $P > 0.55$ in each case). The mean rates of pH_i recovery from acid loads imposed in the presence and absence of W-7 were plotted against absolute values of pH_i and regression lines were fitted to the data points (Fig. 52B). The resulting plot showed that the addition of 50 μM W-7 significantly increased rates of pH_i recovery at all absolute values of pH_i and shifted the pH_i-dependence of the pH_i recovery process by ~0.1 and ~0.2 pH units in the alkaline direction (in neurons with low and high resting pH_i values, respectively). It should be noted that mean rates of pH_i recovery were not converted to net acid efflux values because of uncertainty concerning whether the absence of Ca^{2+}_o might have an effect on β_i. These results further support the possibility that the effect of W-7 to activate the NHE in rat hippocampal neurons is independent of changes in $[Ca^{2+}]_i$.

3.1.3.5 Summary

In summary, CaM inhibition by W-7 evokes a fall in $[Ca^{2+}]_i$ in addition to a concomitant rise in SS pH_i. However, the fall in $[Ca^{2+}]_i$ contributes only minimally to the increase in SS pH_i because: (1) under Ca^{2+}_o-free conditions, the W-7-evoked fall in $[Ca^{2+}]_i$ was nearly abolished, although the rise in pH_i was minimally affected; (2) in contrast to W-7, TFP elicited a rise in $[Ca^{2+}]_i$, although CaM inhibition by both agents caused a rise in SS pH_i; and (3) the W-7 evoked increase in the activity of the NHE was unaffected by the removal of external Ca^{2+}, despite the fact that the W-7-evoked change in $[Ca^{2+}]_i$ was almost abolished under Ca^{2+}_o-free conditions.

3.1.4 The potential involvement of the cAMP/PKA pathway

It was previously shown (Section 2) that stimulation of the cAMP/PKA second messenger system in the absence of HCO_3^- elicits a rise in SS pH_i which reflects an increase in the activity of the NHE. As inhibition of calmodulin causes a similar effect on pH_i regulation,

the possibility exists that 'cross-talk' between the calmodulin and cAMP/PKA intracellular signaling pathways may mediate the observed increase in SS pH_i (and the activity of the NHE) evoked by CaM inhibition. Numerous studies have documented interactions between these two pathways (Limbird, 1988; Copper *et al.*, 1994; Taussig & Gilman, 1995; Hanoune *et al.*, 1997; Houslay & Milligan, 1997; Ishikawa & Homcy, 1997; Urushidani & Forte, 1997; Xia & Storm, 1997; Mons *et al.*, 1998). From these studies, three possible interactions between calmodulin and the cAMP/PKA system, that could potentially account for the observed effects of CaM inhibitors on SS pH_i and the activity of the NHE, are suggested: (1) decreases in $[Ca^{2+}]_i$ evoked by W-7 may cause an increase in the activities of two Ca^{2+}_i-inhibited adenylyl cyclase isoforms, AC5 and AC6, leading to an increase in $[cAMP]_i$; (2) decreases in $[Ca^{2+}]_i$ evoked by W-7 may cause a decrease in the activity of a Ca^{2+}-CaM-stimulated phosphodiesterase (PDE1), leading to an increase in $[cAMP]_i$; and (3) inhibition of CaM by W-7 may decrease the activity of calcineurin (Ca^{2+}-CaM-activated protein phosphatase 2B) leading to a decrease in the rate of dephosphorylation of the NHE (or associated regulatory proteins, see Introduction) and/or stimulation of the activity of a calcineurin-inhibited isoform of adenylyl cyclase, AC9. Importantly, all known adenylyl cyclase isoforms have been shown to be present in mammalian hippocampal CA1 neurons (Segal *et al.*, 1981; Choi *et al.*, 1993; Copper *et al.*, 1994; Hanoune *et al.*, 1997).

3.1.4.1 Potential interaction between CaM and adenylyl cyclases

Initially, the role of adenylyl cyclases in the SS pH_i response to W-7 was examined by performing experiments with W-7 in the presence of DDA, a non-selective inhibitor of adenylyl cyclase activity. Under HEPES-buffered conditions, the addition of 50 μM W-7 in the presence of 100 μM DDA caused a 0.10 ± 0.02 (n = 7/11, Fig. 53A; the remaining 4 neurons did not respond to W-7 treatment) pH unit increase in SS pH_i in neurons with a mean resting value of pH_i at 7.44 ± 0.04. From the data presented in Fig. 42C, in a neuron with a resting value of pH_i at 7.44, 50 μM W-7 would be expected to evoke a 0.08 pH unit rise in SS pH_i in the absence of DDA; this rise is similar to the W-7-evoked alkalinization observed in the presence of 100 μM

DDA. Hence, when the pH_i-dependence of the SS pH_i response to W-7 is considered, it is apparent that the increase in SS pH_i evoked by the CaM inhibitor is not dependent on changes in the activities of adenylyl cyclases.

3.1.4.2 Potential interaction between CaM and phosphodiesterases

After eliminating the possible contribution of adenylyl cyclases, the role of phosphodiesterases in the SS pH_i response to W-7 was examined by performing experiments with W-7 in the presence of 100 μM IBMX, a non-selective inhibitor of phosphodiesterase activity. Under these conditions, the addition of 50 μM W-7 elicited a 0.24 ± 0.05 pH unit increase in SS pH_i in neurons with a mean resting value of pH_i at 7.19 ± 0.06 ($n = 7$, Fig. 53B). This alkalinization was similar to the W-7-evoked alkalinization predicted to take place in the absence of IBMX (a 0.22 pH unit increase at $pH_i = 7.19$; see Fig. 42C), suggesting that the increase in SS pH_i evoked by the CaM inhibitor is not dependent on changes in the activities of phosphodiesterases.

3.1.4.3 Potential interaction between CaM and calcineurin

The possible role of calcineurin in the SS pH_i response to W-7 was then examined by performing experiments with W-7 in the presence of FK-506, an inhibitor of calcineurin activity (see Alvaro et al., 1997). In neurons with a mean resting value of pH_i at 7.32 ± 0.04, the addition of 50 μM W-7 induced a 0.14 ± 0.02 pH unit rise in SS pH_i in the presence of 5 μM FK-506 which, in itself, elicited a 0.11 ± 0.03 pH unit increase in SS pH_i ($n = 16/18$, Fig. 53C; the remaining 2 neurons did not respond to treatment with W-7). When considering the pH_i-dependence of SS pH_i response to 50 μM W-7, the alkalinization induced by the CaM inhibitor in the presence of FK-506 was similar to the alkalinization predicted to take place in the absence of FK-506 (a 0.15 pH unit increase at $pH_i = 7.32$; see Fig. 42C). Therefore, the results suggest that changes in the activity of calcineurin do not mediate the increase in SS pH_i evoked by the CaM inhibitor.

3.1.4.4 Potential interaction between CaM and PKA

Finally, as it was shown previously that an increase [cAMP]$_i$ acts to increase the activity of the NHE via stimulation of PKA activity in adult rat hippocampal CA1 neurons (see Section 2.1.3), experiments were performed with W-7 in the presence of Rp-cAMPS, an inhibitor of PKA activity, to examine the potential role of PKA in the SS pH$_i$ response to the CaM inhibitor. Under these conditions, the addition of 50 μM W-7 in the presence of 50 μM Rp-cAMPS elicited a 0.21 ± 0.05 pH unit increase in SS pH$_i$ in a population of neurons that had a mean initial SS pH$_i$ of 7.18 ± 0.10 (n = 6/7, Fig. 53D; the remaining neuron did not respond to W-7 treatment). This alkalinization was similar to the alkalization predicted to be evoked by this agent in the absence of Rp-cAMPS (a 0.22 pH unit increase at pH$_i$ = 7.18; see Fig. 42C), suggesting that the increase in SS pH$_i$ evoked by the CaM inhibitor is not dependent on changes in PKA activity.

Taken together, these results suggest that the effects of calmodulin inhibitors on SS pH$_i$ (and, thus, the activity of the NHE) are not mediated by changes in the activities of the cAMP/PKA second messenger system or the Ca^{2+}-CaM-dependent protein phosphatase 2B (*i.e.* calcineurin).

3.1.5 Summary of the pH$_i$ response to CaM inhibition

In contrast to findings made in other cell types (in which CaM inhibition causes SS pH$_i$ to fall; see Little *et al.*, 1988; Owen *et al.*, 1989; Weissberg *et al.*, 1989; Bertrand *et al.*, 1994; Tanaka *et al.*, 1994; Kawai *et al.*, 1995; Shrode *et al.*, 1995; Cooper & Hunter, 1997; Koren *et al.*, 1997; Le Prigent *et al.*, 1997; Shrode *et al.*, 1997), in rat hippocampal CA1 neurons inhibitors of CaM (*i.e.* W-7 and TFP) cause SS pH$_i$ to increase. The increase in SS pH$_i$ is dependent on the resting value of pH$_i$ and reflects, in large part, stimulation of the activity of the NHE. Although a decrease in [Ca^{2+}]$_i$ appears to mediate a small portion of the SS pH$_i$ response to W-7, the majority of the SS pH$_i$ response to W-7 is independent of changes in [Ca^{2+}]$_i$ and appears to reflect a direct effect of CaM inhibition on the activity of the NHE because the changes in pH$_i$ regulation evoked by CaM inhibitors are not mediated by the cAMP/PKA second messenger pathway or calcineurin.

3.2 Inhibition of CaMK II

It has been shown in peripheral cell types that CaM inhibition evokes a fall in SS pH_i and does so by preventing the binding of CaM to an 'auto-inhibitory' domain on the NHE, thus inhibiting NHE activity (Wakabayashi et al., 1997a; also Discussion). In these cases, the effects of CaM on the activity of the NHE are elicited by a direct interaction and CaMK-II does not play a role in the modulation of NHE activity by CaM. However, in the present study in rat hippocampal CA1 neurons, CaM inhibitors caused SS pH_i to *increase* by stimulating the activity of the NHE. Thus, the CaM-dependent 'auto-inhibitory' mechanism described above can not be the mechanism by which CaM acts to alter the activity of the NHE in adult rat hippocampal CA1 neurons. Rather, the effects of CaM on NHE activity may be mediated by CaMK-II (as suggested in peripheral cell types that show an increase in NHE activity upon inhibition of CaM; see Weinman et al., 1988; Emmer et al., 1989; Cohen et al., 1990b; Fliegel et al., 1992; Chakraborty et al., 1994; Yamada et al., 1996; Le Prigent et al., 1997). Thus, the effects of inhibiting CaMK-II on SS pH_i and NHE activity were examined.

3.2.1 Characterization of the SS pH_i response to CaMK-II inhibition

Initially, experiments were performed with KN-62, an inhibitor of CaMK-II activity, under both HCO_3^-/CO_2-buffered and HCO_3^--free, HEPES-buffered conditions. The addition of 10-20 μM KN-62 for durations of 25-30 min failed to alter SS pH_i under both nominally HCO_3^--free, HEPES-buffered ($n = 10$, Fig. 54A) and HCO_3^-/CO_2-buffered ($n = 6$, not shown) conditions, suggesting that either: (1) CaMK-II inhibition does not affect SS pH_i in adult rat hippocampal CA1 neurons; or (2) KN-62 was not able to enter the neurons. To further investigate the latter possibility, additional experiments were performed under HEPES-buffered conditions with KN-93, a relatively more membrane-permeable inhibitor of CaMK-II (Sumi et al., 1991; Mamiya et al., 1993). The addition of 10-20 μM KN-93, or 10 μM KN-92 (the inactive analogue of KN-93), for durations of 20-25 min failed to alter SS pH_i ($n = 12$, Fig. 54B; $n = 3$, Fig. 54C;

respectively). These results suggest that SS pH_i is not affected by CaMK-II inhibition in adult rat hippocampal CA1 neurons.

3.2.2 Potential effect of CaMK-II inhibition on the activity of the NHE

Although CaMK-II inhibitors had no effect on SS pH_i, the possibility remained that CaMK-II inhibition caused equal changes in the activities of opposing pH_i regulating mechanisms resulting in no net change in SS pH_i. Therefore, to examine the possibility that CaMK-II inhibition may affect the activity of the NHE, paired acid load recovery experiments were performed with 20 μM KN-93 under HEPES-buffered conditions. In the presence of 20 μM KN-93, the overall rates of pH_i recovery from imposed acid loads were not significantly different to rates of recovery observed in the absence of KN-93 in neurons with high ($n = 3$) and low ($n = 4$) resting pH_i values (a 10 ± 21% decrease and 17 ± 20% increase were observed, respectively; Fig. 55). Thus, when also considering that CaMK-II inhibition had no effect on SS pH_i, these results strongly suggest that CaMK-II inhibitors do not change the activity of the NHE.

3.2.3 Increasing $[Ca^{2+}]_i$ to stimulate CaMK-II activity

The results presented above suggest that CaMK-II inhibition has no effect on SS pH_i or the activity of the NHE. However, under the experimental conditions used, it is not clear whether CaMK-II is constitutively active and the possibility remained that CaMK-II activity was not high enough to be significantly reduced in the presence of a CaMK-II inhibitor. Therefore, an attempt was made to stimulate the Ca^{2+}_i/CaM/CaMK-II pathway by elevating $[Ca^{2+}]_i$ before examining the effects of CaMK-II inhibition on SS pH_i. To do so, experiments were performed with 4Br-A23187, a Ca^{2+} ionophore which acts as a Ca^{2+}/H^+ exchanger by importing Ca^{2+} ions in exchange for H^+ ions (Erdahl et al., 1994). Initially, to confirm that 4Br-A23187 does elevate $[Ca^{2+}]_i$, neurons loaded with Fura-2 were exposed to 10 μM 4Br-A23187 under HEPES-buffered conditions. In the presence of 10 μM 4Br-A23187, a 0.59 ± 0.10 ($n = 3$, Fig. 56A) ratio unit

increase in BI_{334}/BI_{380} values was observed. Next, experiments were performed with KN-93 in the presence of 4Br-A23187 under HEPES-buffered conditions. The addition of 20 μM KN-93 failed to change SS pH_i in the presence of 10 μM 4Br-A23187, which, in itself, caused a 0.12 ± 0.07 pH unit increase in SS pH_i ($n = 7$, Fig. 56B).

3.3 Summary

Inhibitors of CaM (W-7 and TFP) cause SS pH_i to increase, in manner dependent on the resting value of pH_i. In contrast to findings made in many other cell types (see above), in adult rat hippocampal CA1 neurons, this increase in SS pH_i evoked by CaM inhibitors reflects stimulation of NHE activity but does not reflect changes in HCO_3^--dependent pH_i regulation. Although a portion of the SS pH_i response to the CaM inhibitor, W-7, appears to be dependent on a concomitant decrease in $[Ca^{2+}]_i$, the majority of the W-7-evoked alkalinization is the result of CaM inhibition. Changes in the activities of the cAMP/PKA second messenger pathway and calcineurin do not mediate the CaM inhibitor-evoked increase in SS pH_i. In addition, CaMK-II inhibitors do not affect SS pH_i or the activity of the NHE even under conditions in which CaMK-II activity was presumably stimulated; therefore, it is unlikely that CaMK-II mediates the SS pH_i response evoked by CaM inhibitors. When considering the CaM-dependent 'auto-inhibitory' mechanism proposed by Wakabayashi *et al.* (1997a; which involves direct binding of CaM to the NHE-1 isoform to stimulate exchange activity; see Discussion), the possibility exists that CaM may be directly binding to the NHE protein at an 'auto-stimulatory' domain to decrease exchange activity in adult rat hippocampal neurons.

Figure 41. Effect of W-7 on SS pH_i under HEPES-buffered conditions.

A Under HEPES-buffered conditions, 4 different neurons (all with resting pH_i values at ~7.23) were exposed to increasing concentrations of W-7. Addition of 10 μM W-7 (▼) elicited a relatively slow and sustained ~0.1 pH unit rise in pH_i and addition of 25 μM W-7 (O) elicited a similar slow increase in pH_i to a slightly higher SS level. Addition of 50 μM W-7 (●) caused pH_i to reach a new SS level at ~7.45 within ~9 min;whereas 100 μM W-7 (∇) elicited a similar change in SS pH_i as 50 μM W-7 but the new SS level was reached in only ~6 min. *B* A different neuron with a resting pH_i value at ~7.35 was exposed to 50 μM W-7 which caused an increase in SS pH_i. Subsequent removal of W-7 from the HEPES-buffered perfusion medium caused pH_i to fully recover to the initial SS level.

A
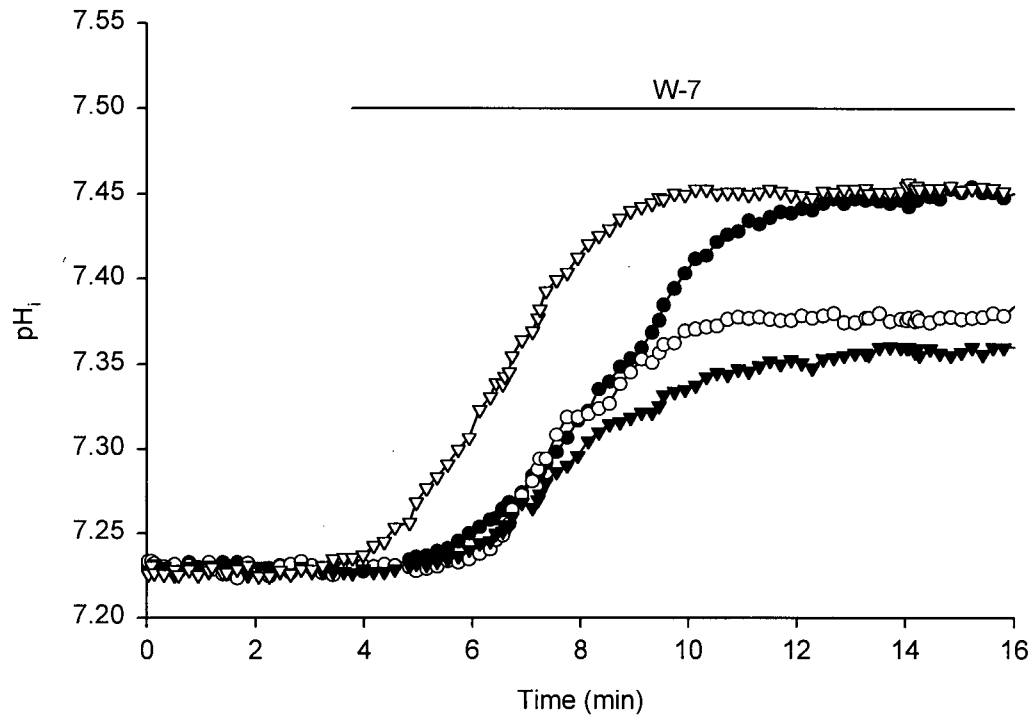
B
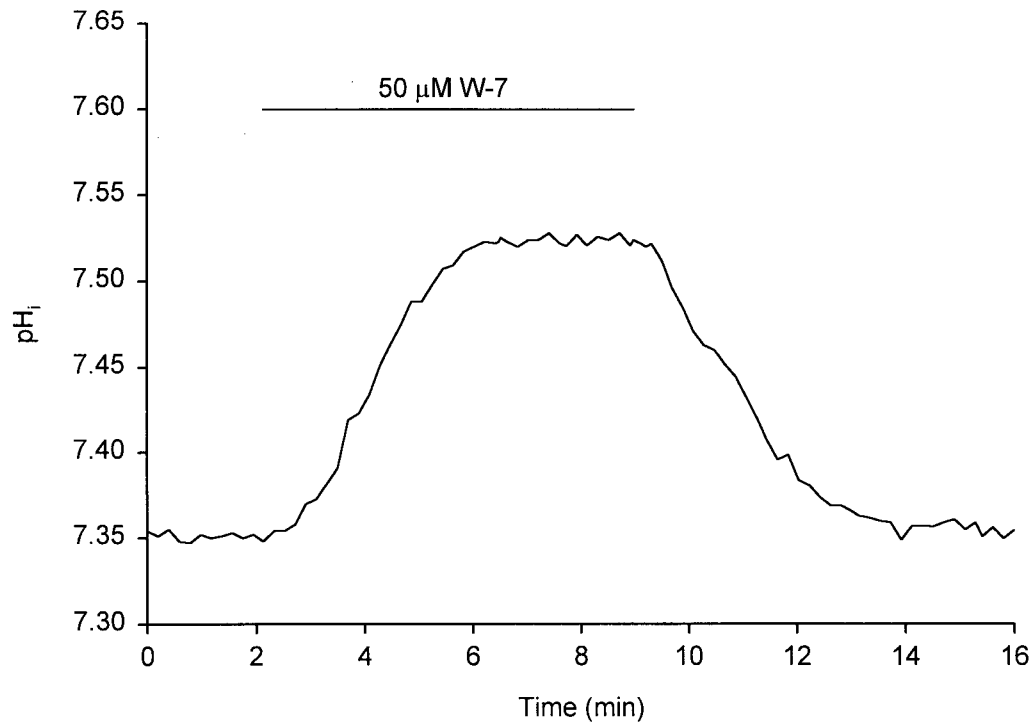
Figure 42. The pH$_i$-dependence of the effect of W-7 on SS pH$_i$ under HEPES-buffered conditions.

A A ~0.43 pH unit rise in SS pH$_i$ was observed with the addition of 50 μM W-7 in a neuron that had a resting pH$_i$ at ~6.86 under HEPES-buffered conditions. **B** A different neuron with a resting pH$_i$ at ~7.40 showed a ~0.10 pH unit increase in SS pH$_i$ when 50 μM W-7 was added to the HEPES-buffered perfusion medium. **C** From 14 experiments of the type shown in *A* and *B*, the changes in SS pH$_i$ elicited by 50 μM W-7 were plotted against resting values of pH$_i$ and the data points were fitted with a least squares regression lines. This line had a negative slope and x-intercept at pH$_i$ = ~7.6. Note that the data point (indicated by the arrow) at pH$_i$ = 7.34 and ΔpH$_i$ = 0.14 represents an *n* = 2.

A
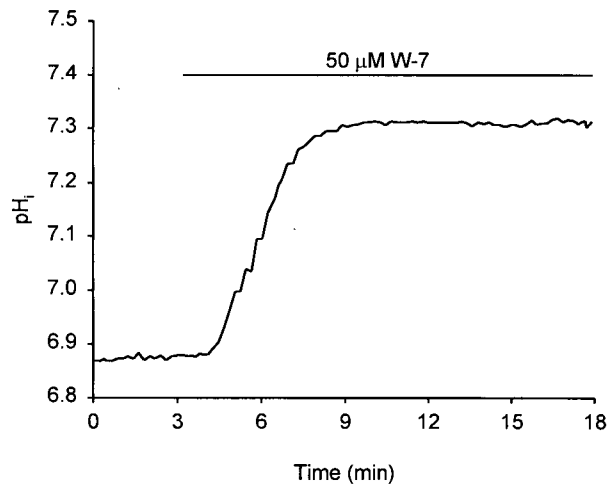
B
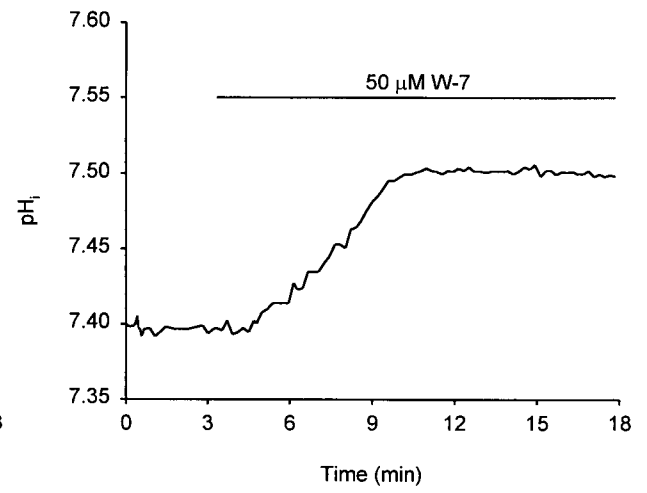
C
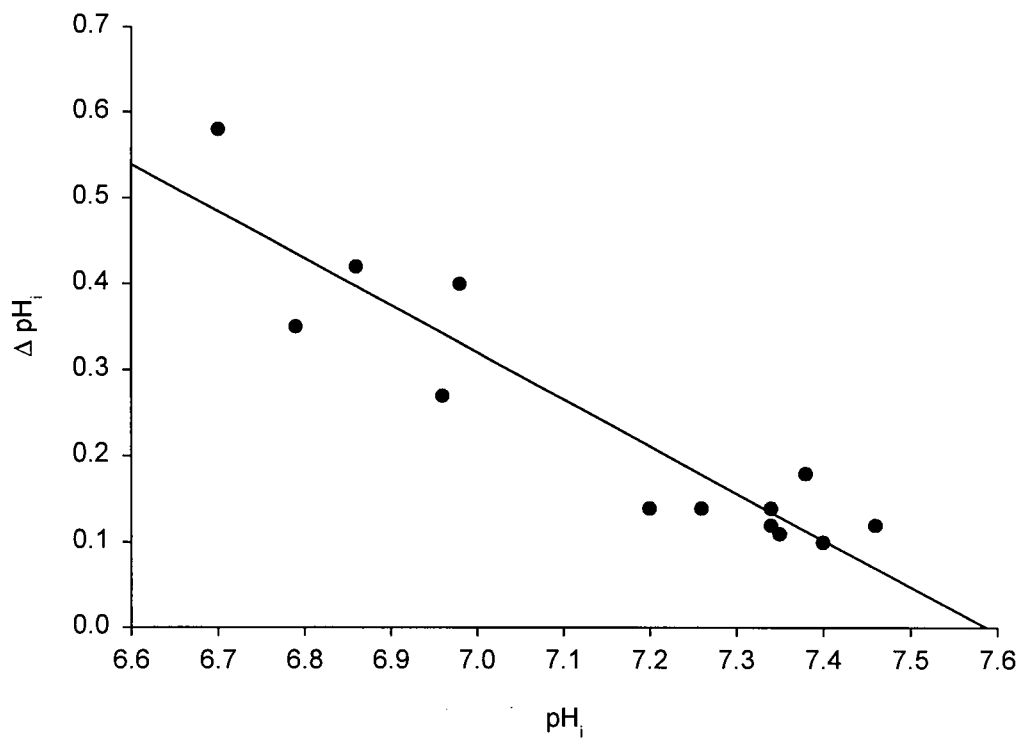
Figure 43. Effect of trifluoperazine on SS pH$_i$ under HEPES-buffered conditions.

A A ~0.18 pH unit rise in SS pH$_i$ was observed with the addition of 50 µM TFP in a neuron that had a resting pH$_i$ at ~7.25 under HEPES-buffered conditions. *B* From 11 experiments of the type shown in *A*, the changes in SS pH$_i$ elicited by 50 µM TFP under HEPES-buffered conditions were plotted against resting values of pH$_i$ and these data points were fitted with a regression line. This line had a negative slope and x-intercept at pH$_i$ = ~ 7.5. Compare with Fig. 42C (a similar regression line observed in the presence of W-7 under HEPES-buffered conditions).

A
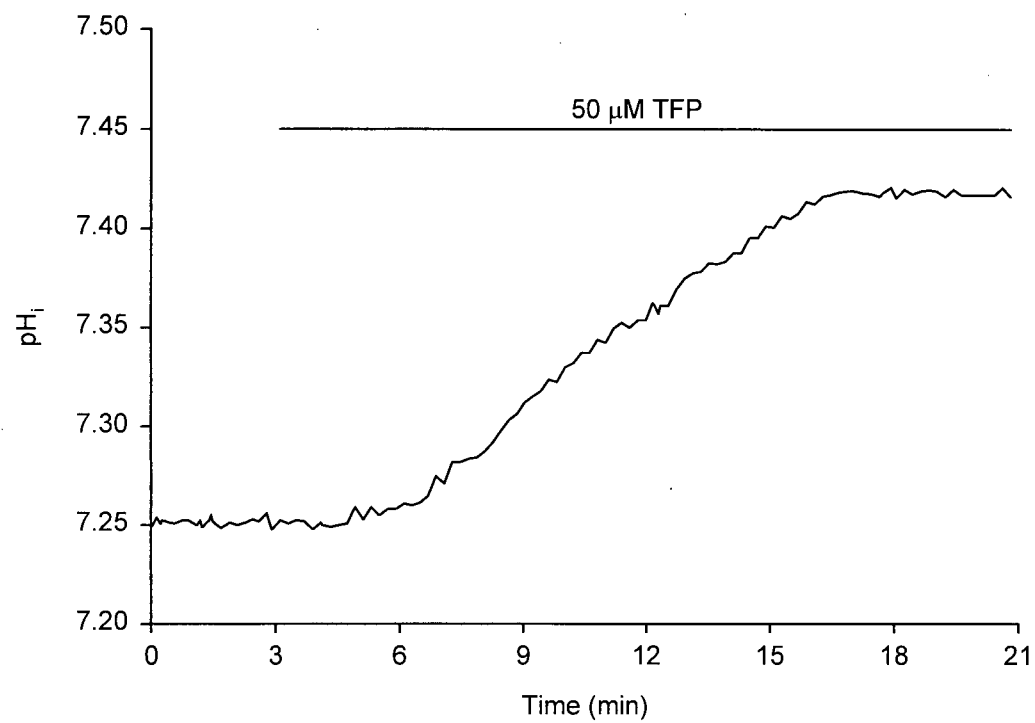
B
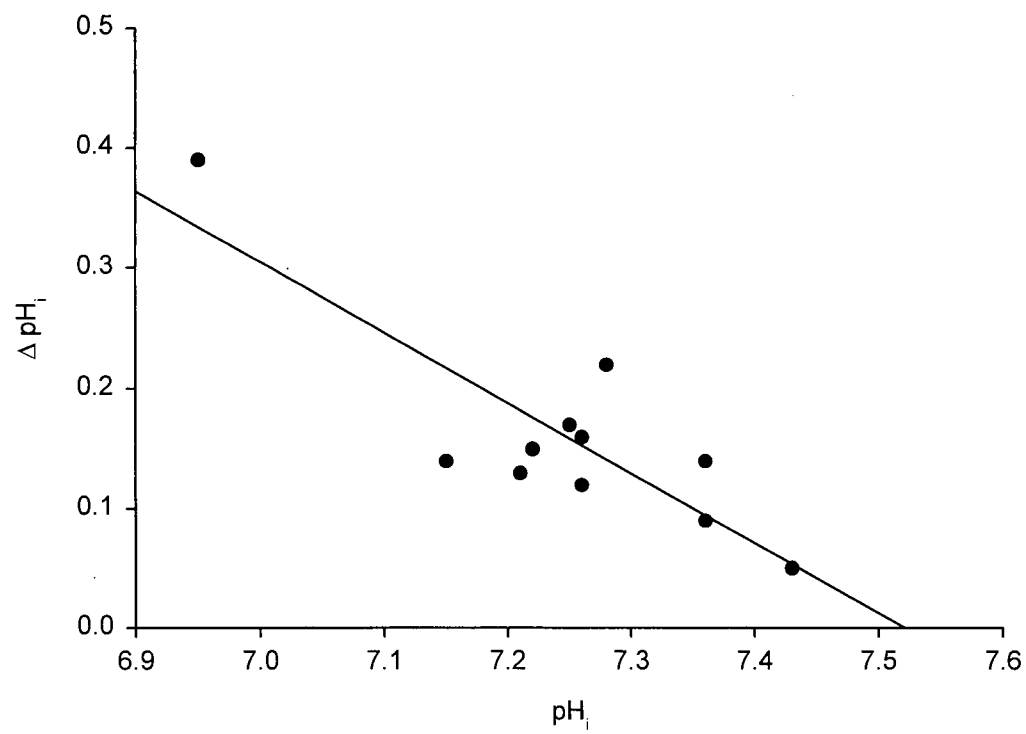
Figure 44. Effects of W-7 (in the presence and absence of DIDS) and trifluoperazine on SS pH$_i$ under HCO$_3^-$/CO$_2$-buffered conditions.

A Addition of 100 µM W-7 caused SS pH$_i$ to increase ~0.20 pH units in a neuron that had a resting pH$_i$ at ~7.10 under HCO$_3^-$/CO$_2$-buffered conditions. **B** A different neuron with a restingl pH$_i$ at ~7.19 showed a ~0.16 pH unit increase in SS pH$_i$ when 50 µM TFP was added to the HCO$_3^-$/CO$_2$-buffered perfusion medium. **C** The SS pH$_i$ of a different neuron, which was initially at ~7.30, remained constant in the presence of 200 µM DIDS under HCO$_3^-$/CO$_2$-buffered conditions. The subsequent addition of 50 µM W-7 caused a ~0.17 pH unit rise in SS pH$_i$.
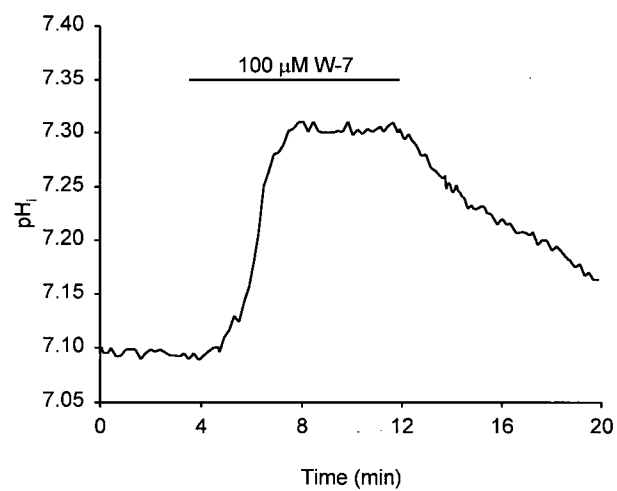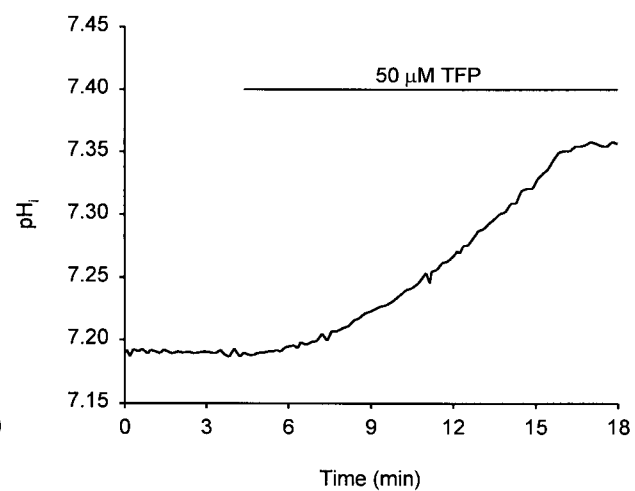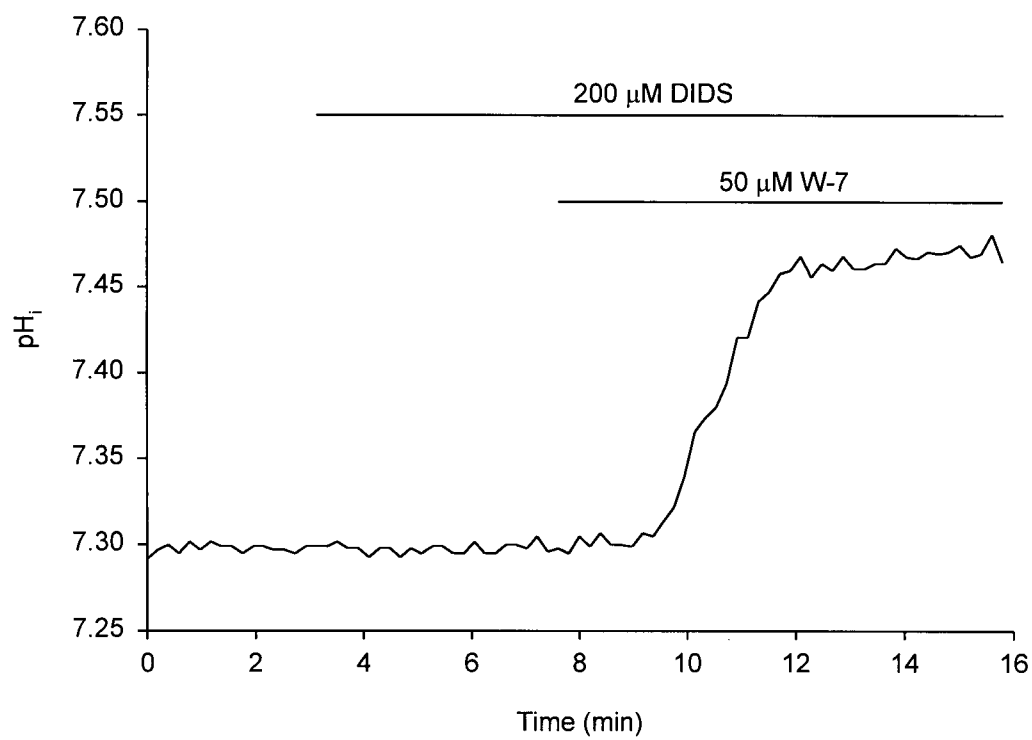
Figure 45. Effects of W-7 on SS pH_i under Na^+_o-free, HEPES-buffered conditions.

A In a neuron with a resting pH_i at ~7.10, removal of Na^+_o (replaced with $NMDG^+$) caused SS pH_i to fall. After pH_i recovered to the initial level, the neuron was exposed to 50 μM W-7 which caused a ~0.07 pH unit increase in SS pH_i. **B** A different neuron with a resting pH_i at ~7.27 was perfused with a Na^+-free (replaced with Li^+) medium which caused SS pH_i to initially fall. After pH_i recovered tothe initial SS level, 50 μM W-7 was added to the perfusion medium and caused SS pH_i to increase to a level at ~7.50. Both experiments shown were performed under HEPES-buffered conditions.

A
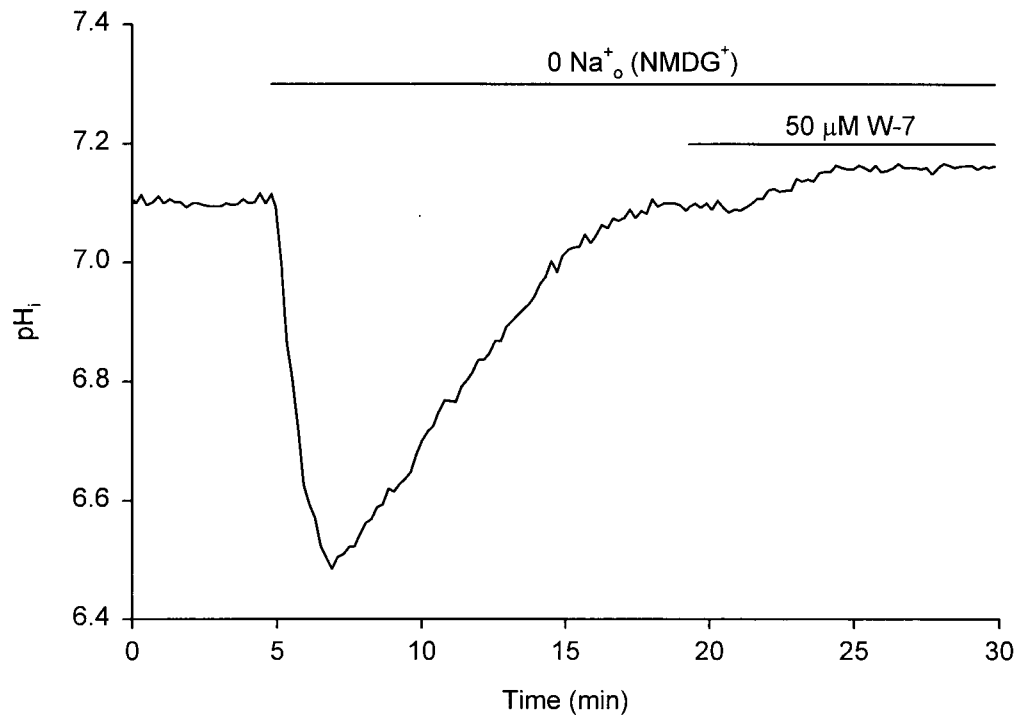
B
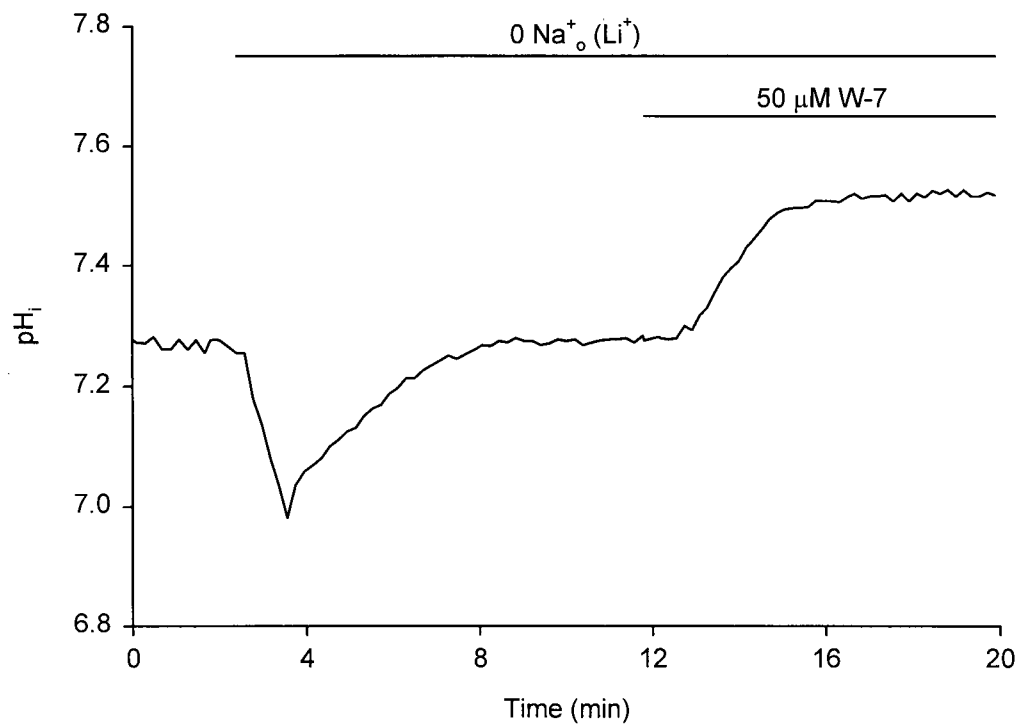
Figure 46. Effect of W-7 on pH$_i$ recovery from internal acid loads under HEPES-buffered conditions.

A A neuron with a resting pH$_i$ at ~7.52 was initially exposed to an internal acid load under HEPES-buffered conditions. After pH$_i$ recovered to the initial SS level, a second acid load was performed in the presence of 50 µM W-7. pH$_i$ recovered faster in the presence of W-7 than under control conditions. **B** The mean net acid fluxes from acid loads imposed in the absence (●) or presence (○) of 50 µM W-7 were plotted against absolute values of pH$_i$ from neurons with low resting pH$_i$ values (n = 4). The net acid efflux values were significantly faster in the presence than absence of W-7 at all absolute values of pH$_i$. When regression lines were fitted to the data points, it was shown that the line representing data obtained in the presence of W-7 had a greater slope and the more alkaline x-intercept than the line representing control data. **C** From 7 experiments of the type shown in A performed in neurons with high resting pH$_i$ values, the mean calculated net acid fluxes from acid loads imposed in the absence (■) or presence (□) of 50 µM W-7 were plotted against values of pH$_i$. The net acid efflux values were significantly faster in the presence than absence of W-7 at all absolute values of pH$_i$. When regression lines were fitted to the data points, it was shown that the line representing data obtained in the presence of W-7 had a greater slope and the more alkaline x-intercept than the line representing control data.

A
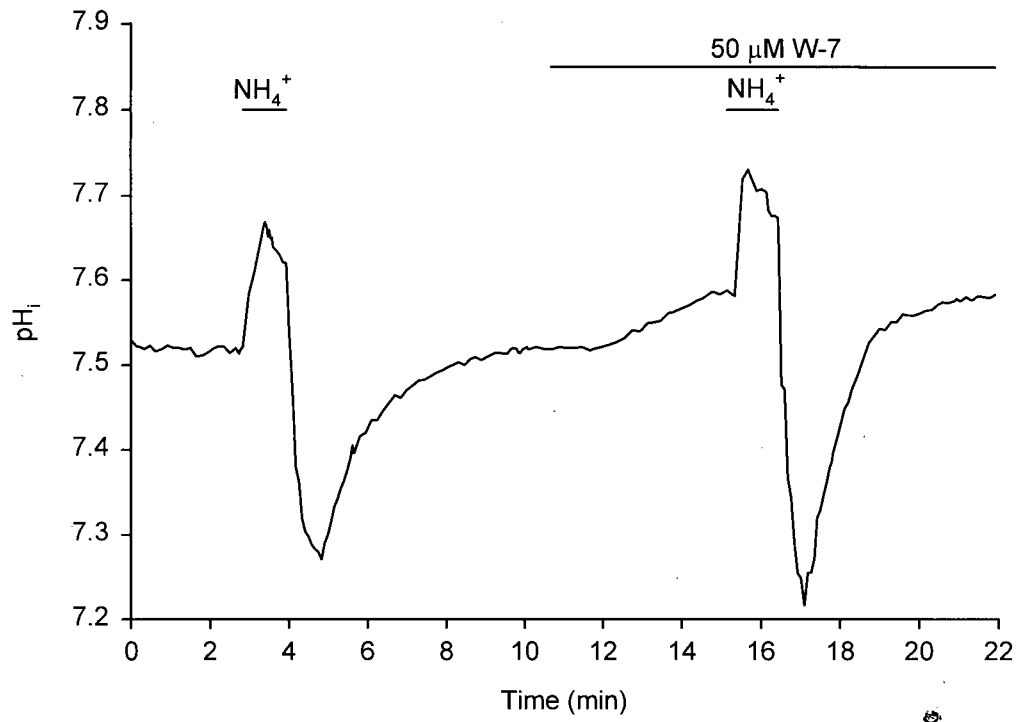
B
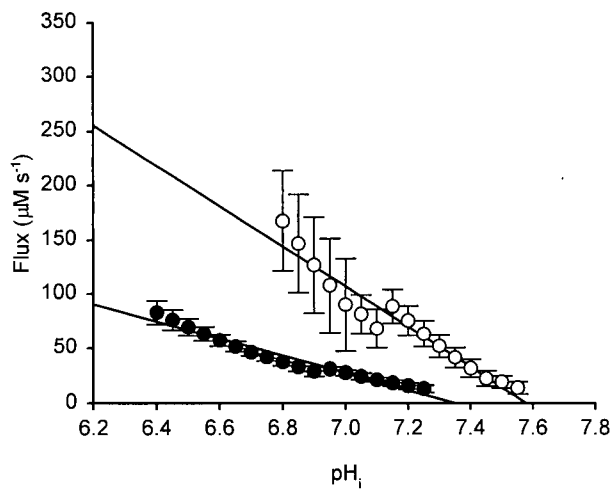
C
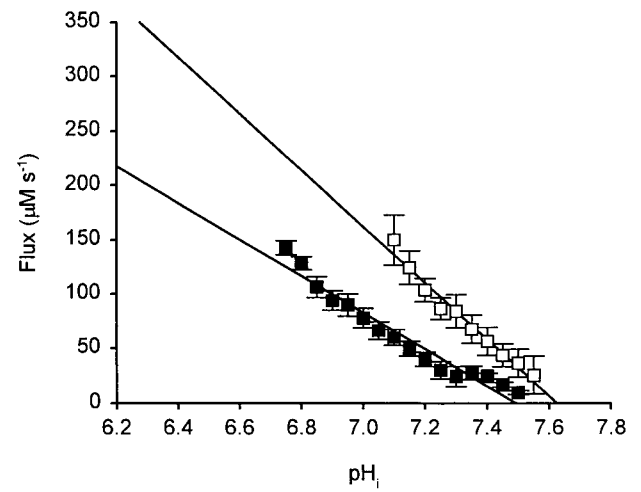
Figure 47. Effects of W-7 on pH$_i$ recovery from internal acid loads under HCO$_3^-$/CO$_2$-buffered conditions.

A In a neuron with a resting pH$_i$ at ~7.15 under HCO$_3^-$/CO$_2$-buffered conditions, pH$_i$ was allowed to recover after an internal acid load. A second acid load was performed in the presence of 50 μM W-7 after which pH$_i$ recovered to an elevated SS level at a faster rate than observed under control conditions. **B** The mean rates of pH$_i$ recovery from acid loads imposed in the absence (●) or presence (O) of 50 μM W-7 were plotted against absolute values of pH$_i$ (n = 10). The rates of pH$_i$ recovery were significantly faster in the presence than absence of W-7 at all absolute values of pH$_i$. When regression lines were fitted to the data points, it was shown that the line representing data obtained in the presence of W-7 had a greater slope than the line representing control data.

A
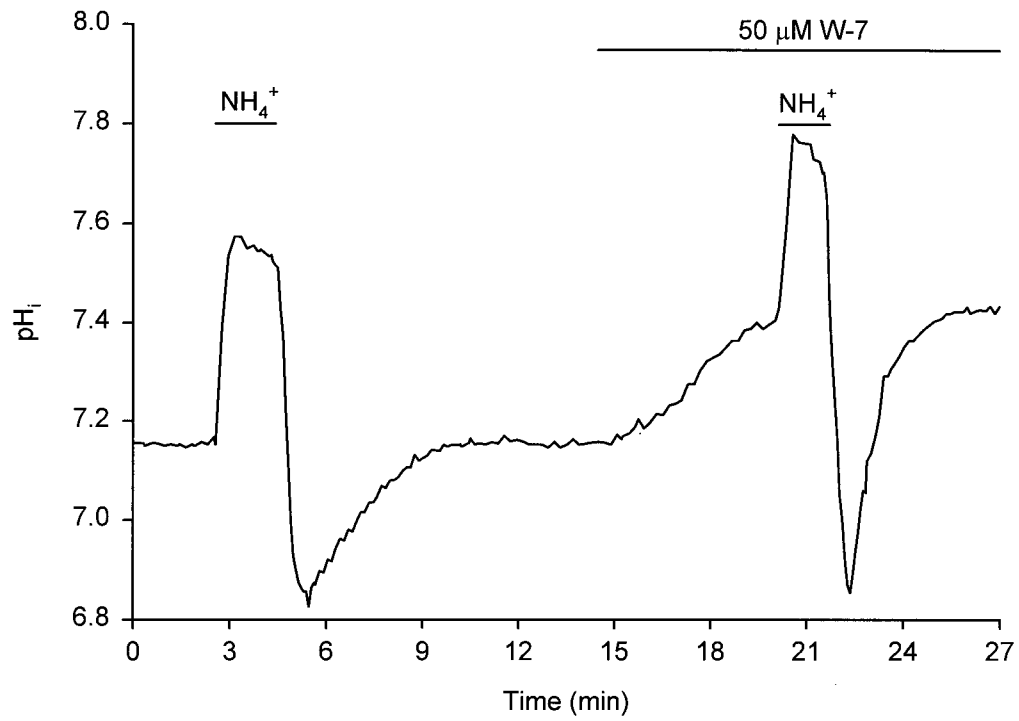
B
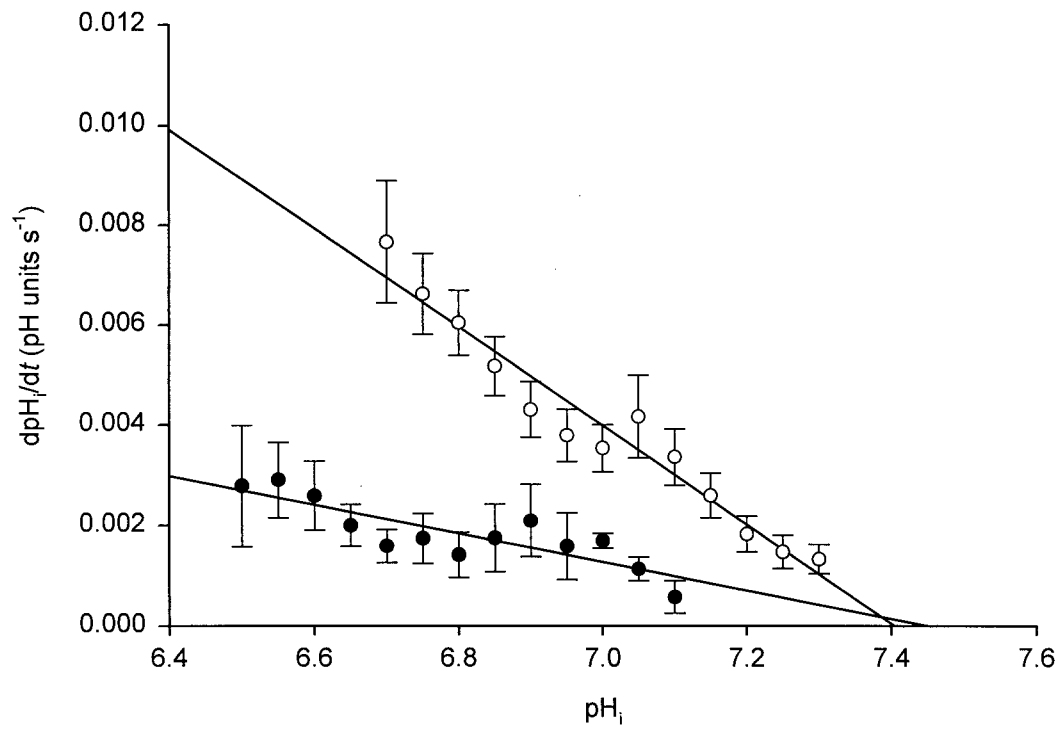
Figure 48. Effects of trifluoperazine on pH$_i$ recovery from internal acid loads under HEPES-buffered conditions.

A A neuron with a resting pH$_i$ at ~ 7.15 was initially exposed to an internal acid load under HEPES-buffered conditions. After pH$_i$ recovered to the initial SS level, a second acid load was performed in the presence of 50 μM TFP. pH$_i$ recovered faster in the presence of TFP when compared to control acid load pH$_i$ recovery. **B** The mean net acid fluxes from acid loads imposed in the absence (●) or presence (○) of 50 μM TFP were plotted against absolute values of pH$_i$ from neurons with low resting pH$_i$ values (n = 7). The net acid efflux values were significantly faster in the presence than absence of TFP at all absolute values of pH$_i$. When regression lines were fitted to the data points, it was shown that the line representing data obtained in the presence of TFP had a greater slope than the line representing control data. No neurons with high resting pH$_i$ values were encountered.

A
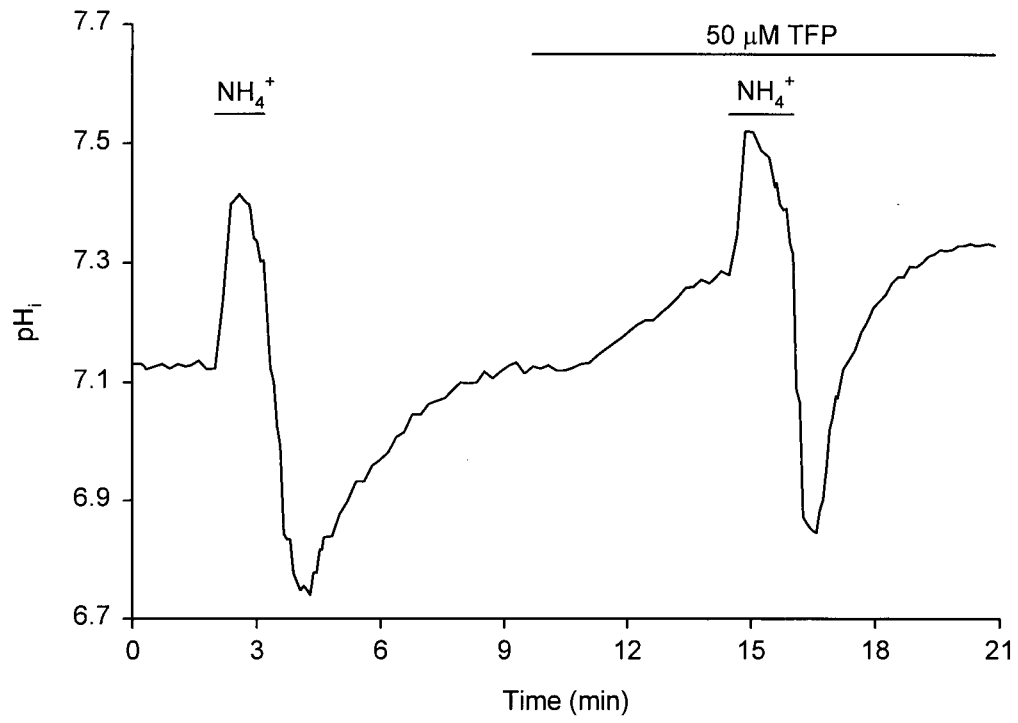
B
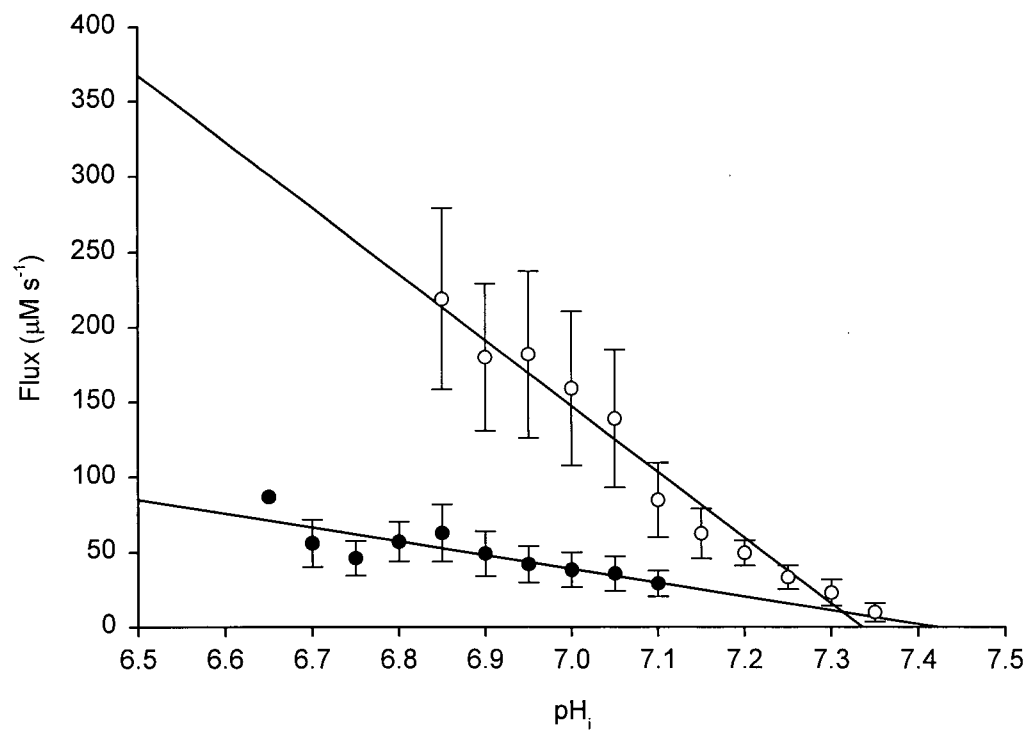
Figure 49. Effects of W-7 on $[Ca^{2+}]_i$ and the effect of Ca^{2+}_o-removal on W-7-induced changes in SS pH_i and $[Ca^{2+}]_i$ under HEPES-buffered conditions.

A The addition of 50 μM W-7 elicited a fall in the background-subtracted intensity ratio values representing $[Ca^{2+}]_i$ in this neuron loaded with Fura-2. **B** Perfusion with a medium free of Ca^{2+} caused SS pH_i to increase in a neuron loaded with BCECF. Subsequent addition of 50 μM W-7 caused SS pH_i to increase a further ~0.11 pH units. **C** A parallel experiment to that shown in **B** was performed with a different neuron loaded with Fura-2 which showed a sustained decrease in BI_{334}/BI_{380} values when Ca^{2+}_o was removed the perfusion medium. Subsequently, 50 μM W-7 was applied but failed to alter BI_{334}/BI_{380} values. All experiments shown were performed under HEPES-buffered conditions.

A
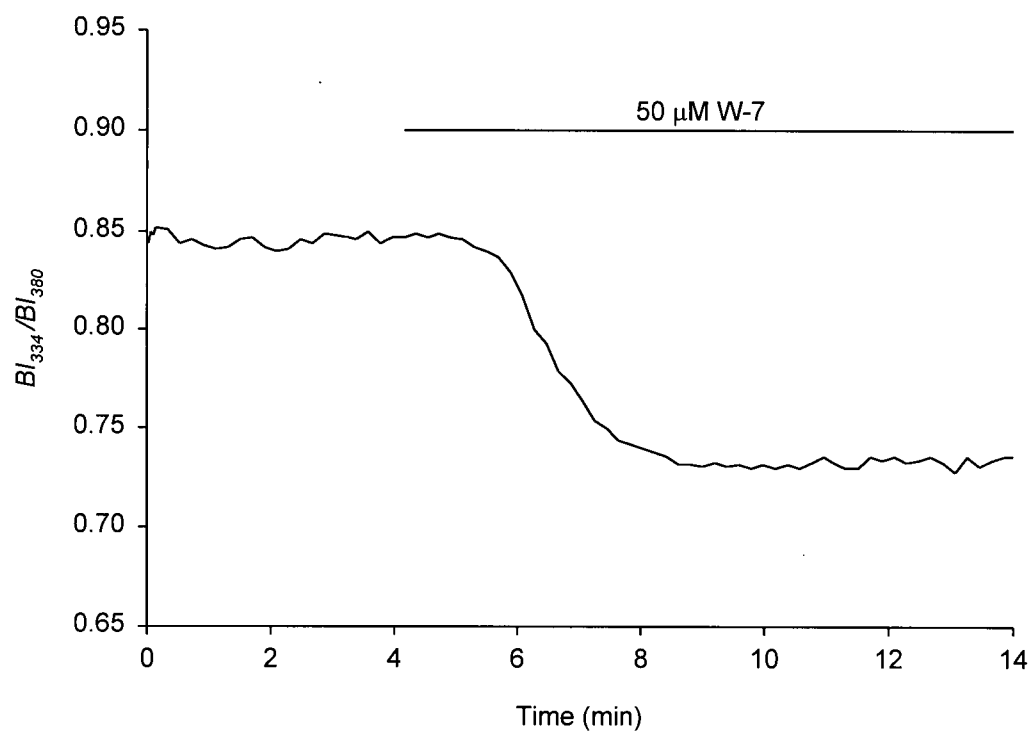
B
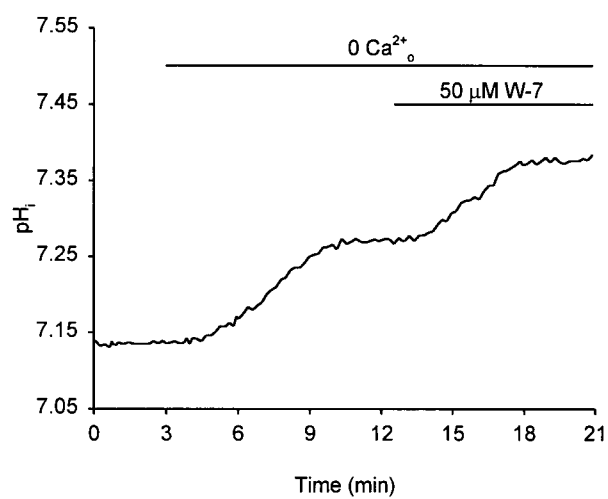
C
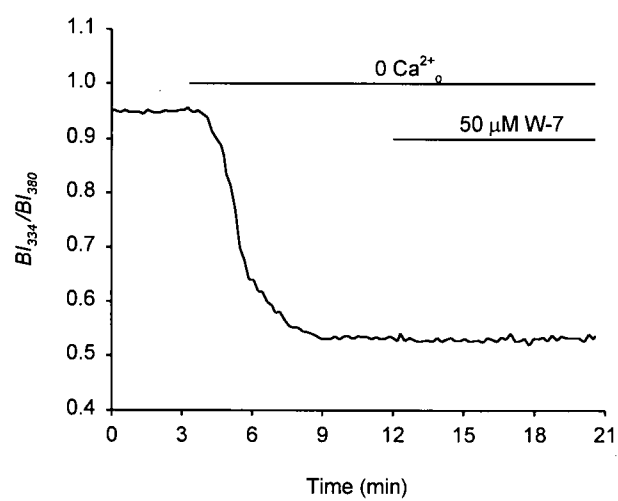
Figure 50. Effect of Na^+_o-removal on W-7-induced changes in $[Ca^{2+}]_i$ under HEPES-buffered conditions.

Under HEPES-buffered conditions, the removal of Na^+_o elicited a rise in BI_{334}/BI_{380} values representing $[Ca^{2+}]_i$ in a neuron loaded with Fura-2. The subsequent addition of 50 µM W-7 caused a sustained decrease in BI_{334}/BI_{380} values.
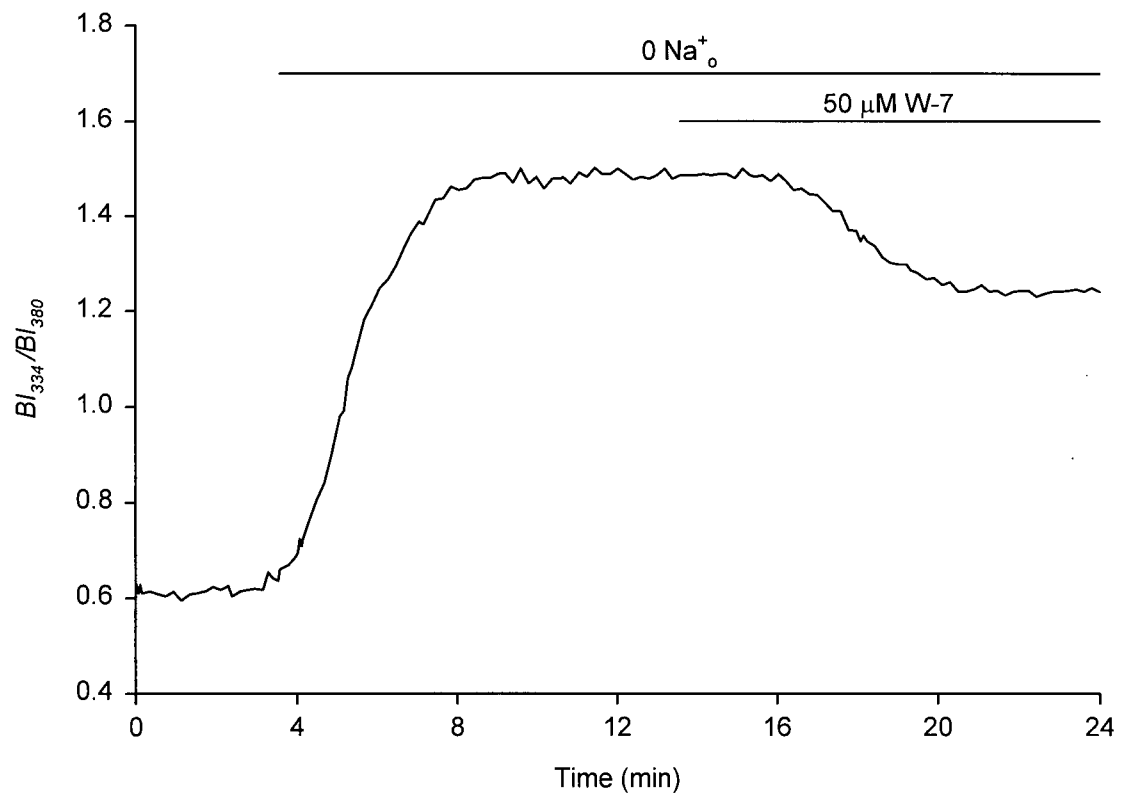
Figure 51. Effects of trifluoperazine on $[Ca^{2+}]_i$ under HEPES-buffered conditions.

A neuron loaded with Fura-2 showed an increase in BI_{334}/BI_{380} values representing $[Ca^{2+}]_i$ when 50 µM TFP was applied under HEPES-buffered conditions.
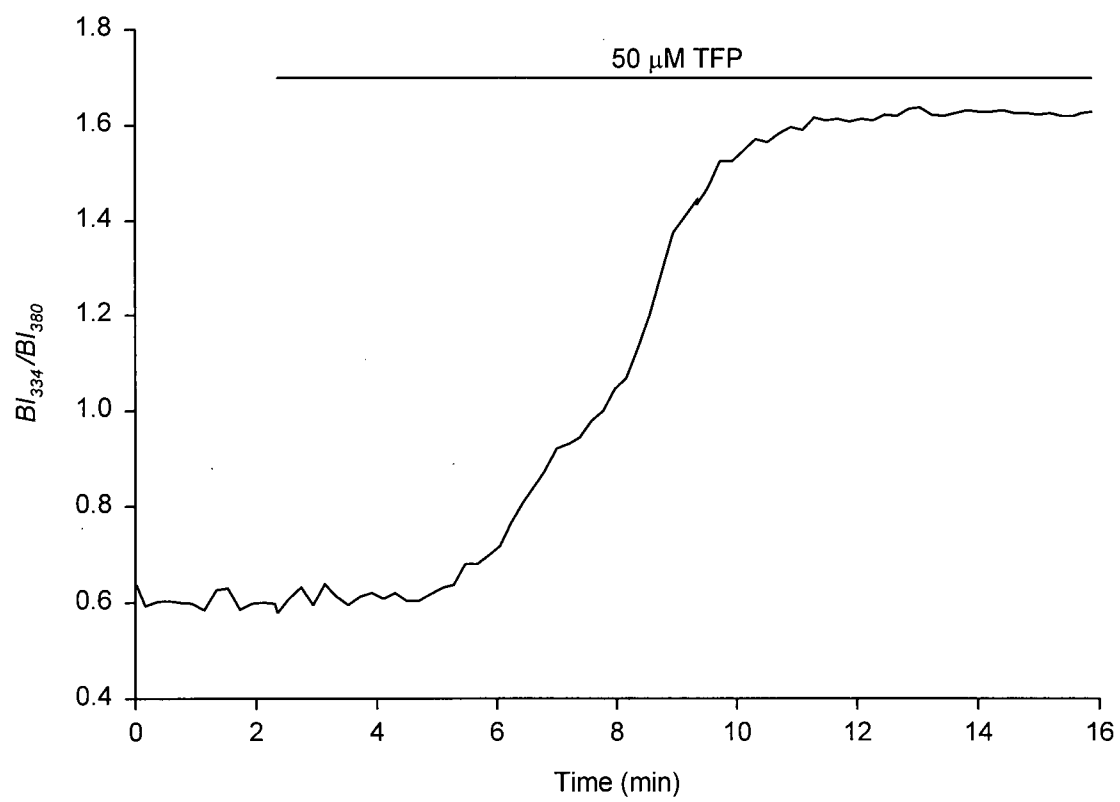
Figure 52. Effects of Ca^{2+}_o-removal on W-7 evoked changes in pH_i recovery from internal acid loads under HEPES-buffered conditions.

A A neuron with a resting pH_i at ~7.28 was initially exposed to an acid load under Ca^{2+}_o-free, HEPES-buffered conditions. After pH_i recovered to the initial SS level in the continued absence of Ca^{2+}_o, a second acid load was performed in the presence of 50 μM W-7. pH_i recovered faster in the presence than in the absence of W-7. **B** Plotted are the mean rates of pH_i recovery against absolute values of pH_i from 9 experiments of the type shown in *A*. The circles and squares represent the data points obtained from neurons with low ($n = 4$) and high ($n = 5$) resting pH_i values, respectively. In all neurons, the rates of pH_i recovery obtained in the presence of 50 μM W-7 (open symbols) were significantly faster than those obtained in its absence (filled symbols) in the continued absence of Ca^{2+}_o. When least squares regression lines were fitted to the data points, it was shown that the lines representing data obtained in the presence of W-7 had greater slopes and more alkaline x-intercepts than the lines representing control data.

A
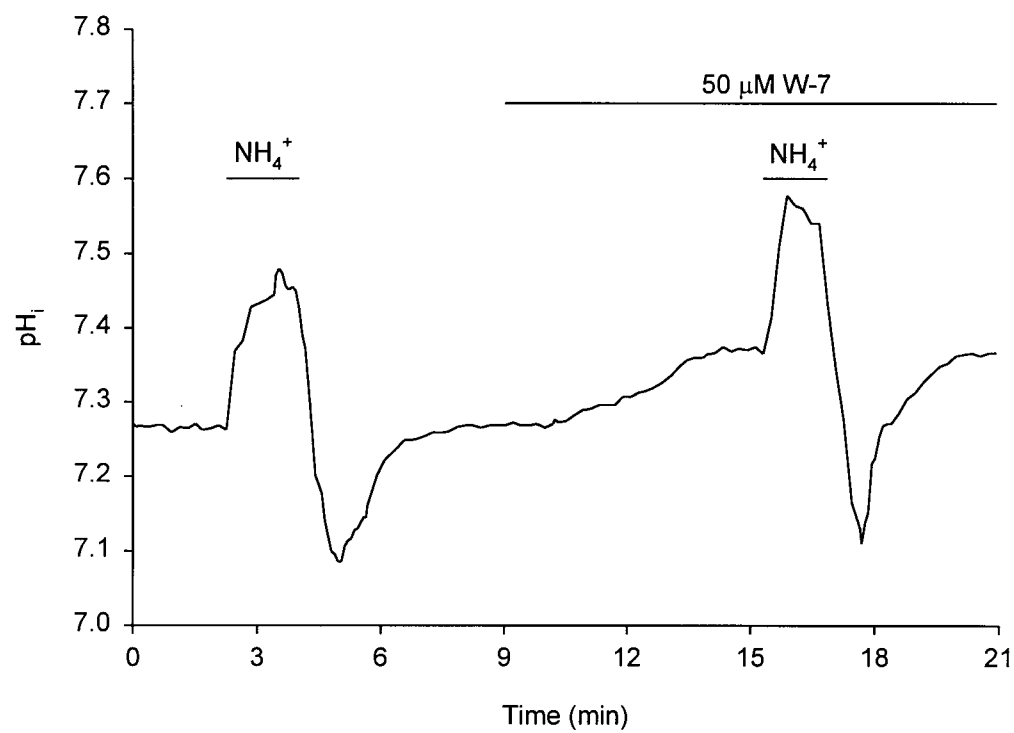
B
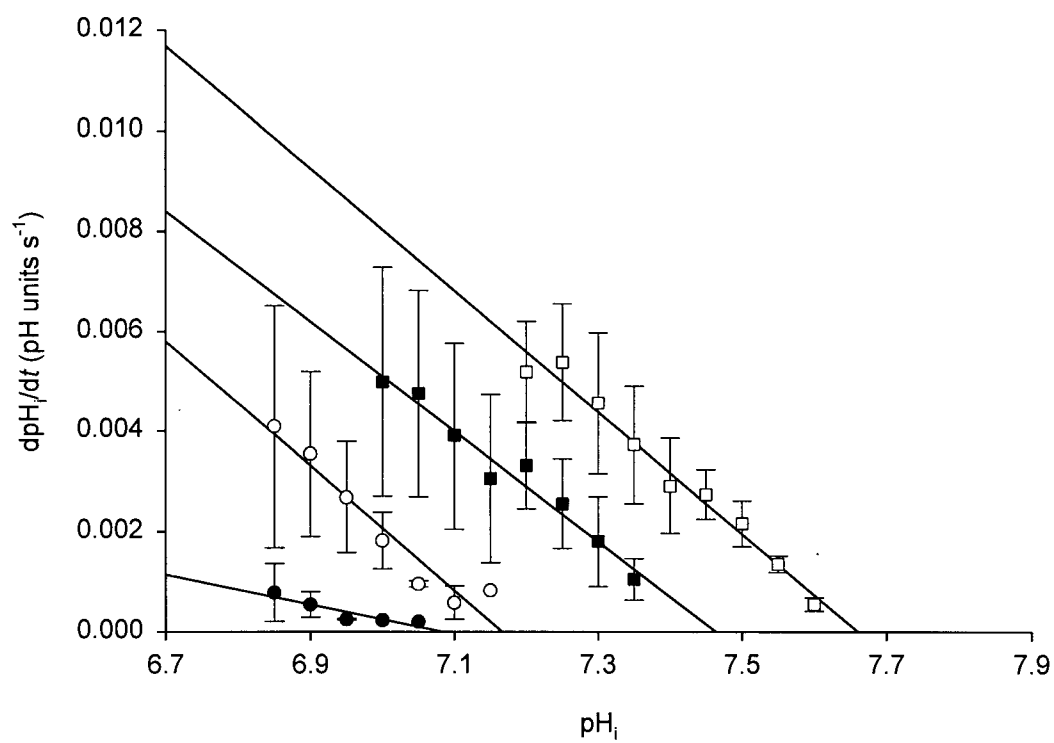
Figure 53. Effects of 2′,5′-dideoxyadenosine, IBMX, FK-506, and Rp-cAMPS on W-7-evoked changes in SS pH$_i$ under HEPES-buffered conditions.

A Under HEPES-buffered conditions, a neuron with a resting pH$_i$ at ~7.37 was exposed to 100 μM 2′, 5′-dideoxyadenosine (DDA) which did not alter SS pH$_i$. In the continued presence of DDA, the addition of 50 μM W-7 caused SS pH$_i$ to increase by ~ 0.13 pH units. *B* A different neuron with a resting value of pH$_i$ at ~7.02 was perfused with HEPES-buffered medium containing 100 μM IBMX which elicited an increase in SS pH$_i$. Subsequently, 50 μM W-7 was added to the perfusion medium causing a further ~0.20 pH unit increase in pH$_i$. *C* A different neuron with an initial pH$_i$ of ~7.23 was perfused with HEPES-buffered medium containing 5 μM FK-506 which elicited an increase in SS pH$_i$. When pH$_i$ reached its new SS level, 50 μM W-7 was added to the perfusion medium causing a further ~0.18 pH unit increase in SS pH$_i$. *D* A different neuron with a resting pH$_i$ at ~7.22 was exposed to 50 μM Rp-cAMPS which did not alter SS pH$_i$ under HEPES-buffered conditions. In the continued presence of Rp-cAMPS, the addition of 50 μM W-7 caused SS pH$_i$ to increase ~0.19 pH units.

A

100 μM 2',5'-DDA

50 μM W-7

B

100 μM IBMX

50 μM W-7

C

5 μM FK-506

50 μM W-7

D

50 μM Rp-cAMPS

50 μM W-7
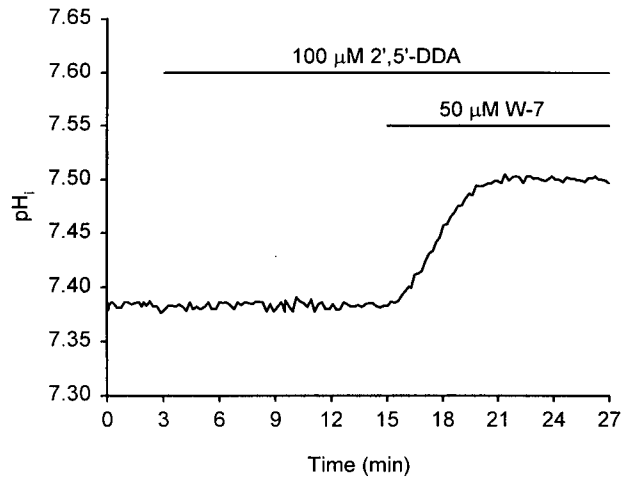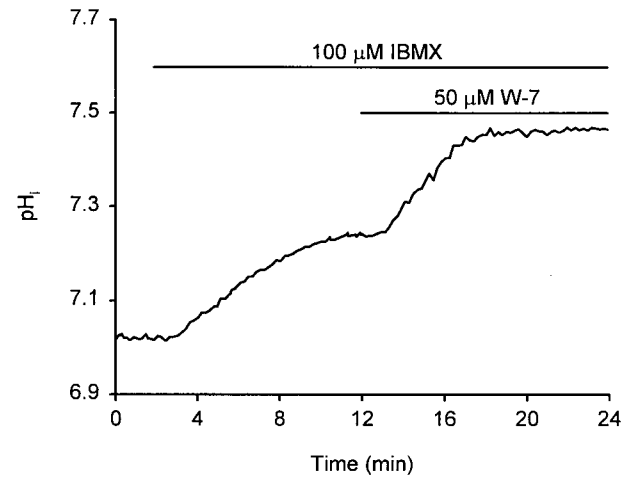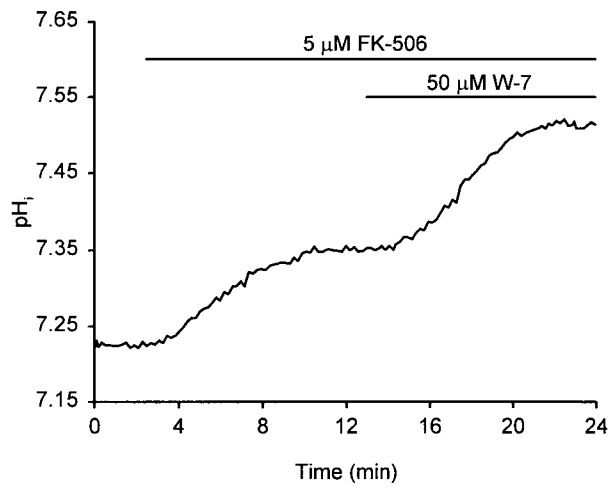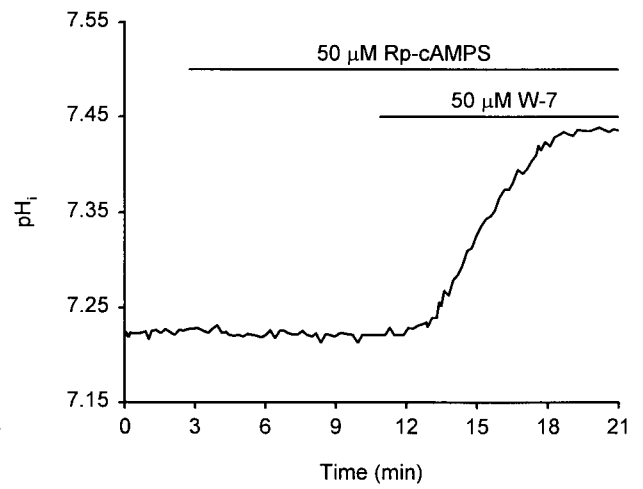
Figure 54. Effects of KN-62, KN-93, and KN-92 on SS pH_i under HEPES-buffered conditions.

Regardless of whether **A** 20 μM KN-62, **B** 10 μM KN-93 or **C** 10 μM KN-92 was added to the HEPES-buffered perfusion medium, the SS pH_i in these 3 different neurons failed to change within ~ 20-30 min.

A
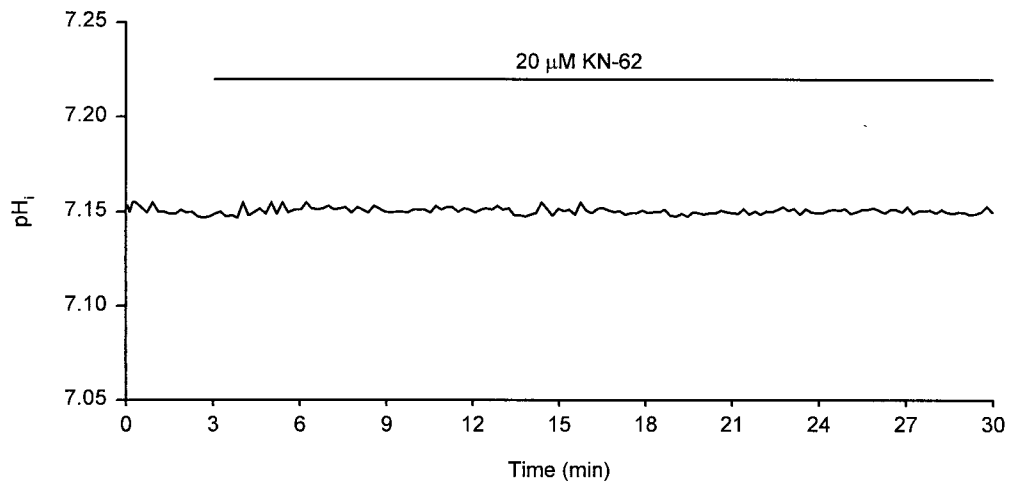
B
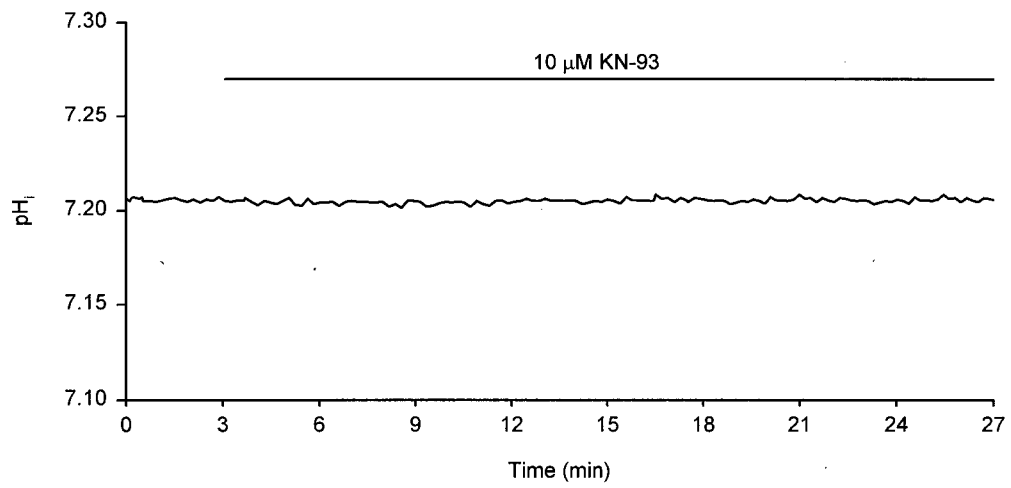
C
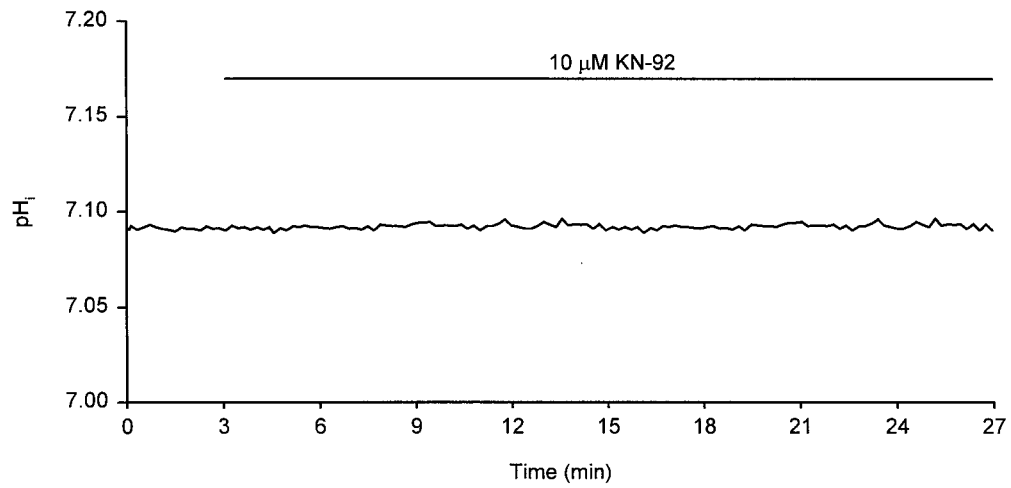
Figure 55. Effect of KN-93 on pH_i recovery from internal acid loads under HEPES-buffered conditions.

A In a neuron with a resting pH_i at ~7.30 under HEPES-buffered conditions, pH_i was allowed to recover after an initial acid load. A second acid load was performed in the presence of 20 µM KN-93 after which pH_i recovered to initial SS levels at a similar rate to that observed under control conditions. **B** Mean calculated net acid fluxes were plotted against absolute values of pH_i from 7 experiments of the type shown in **A**. Circles and squares represent the data points obtained from neurons with low ($n = 4$) and high ($n = 3$) resting pH_i values, respectively. The net acid efflux values obtained in the presence of 20 µM KN-93 (open symbols) were not significantly different than control net acid efflux values (closed symbols) at all values of absolute pH_i. Least squares regression lines were fitted to the data points and the resulting lines had x-intercepts at pH_i = ~7.2 and ~7.5 in neurons with low and high resting pH_i values, respectively.

A
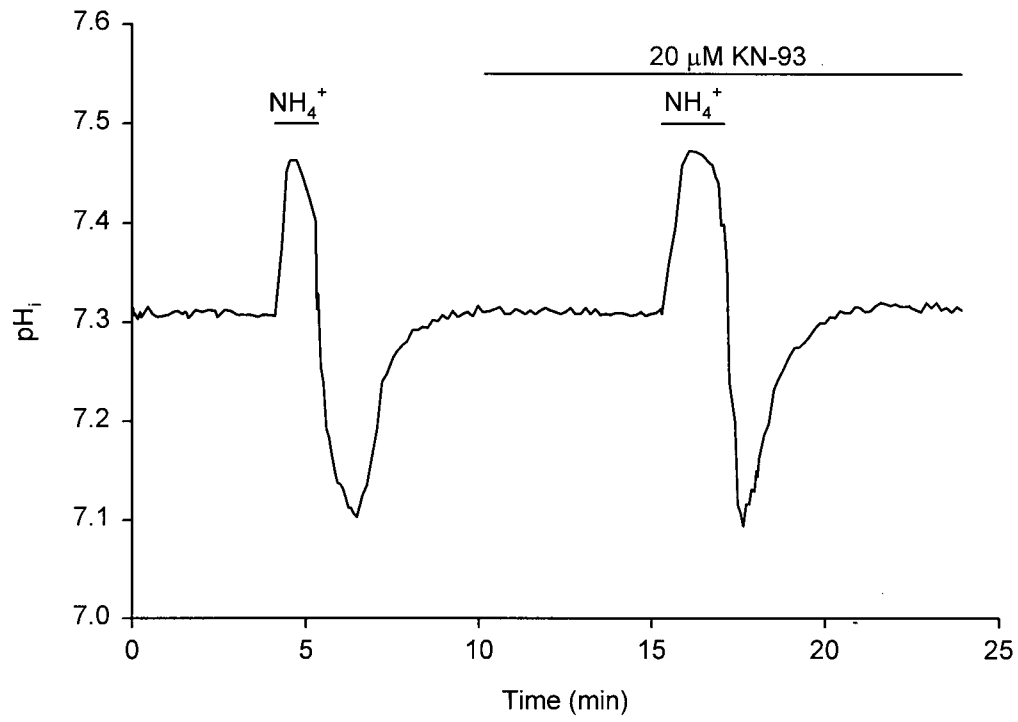
B
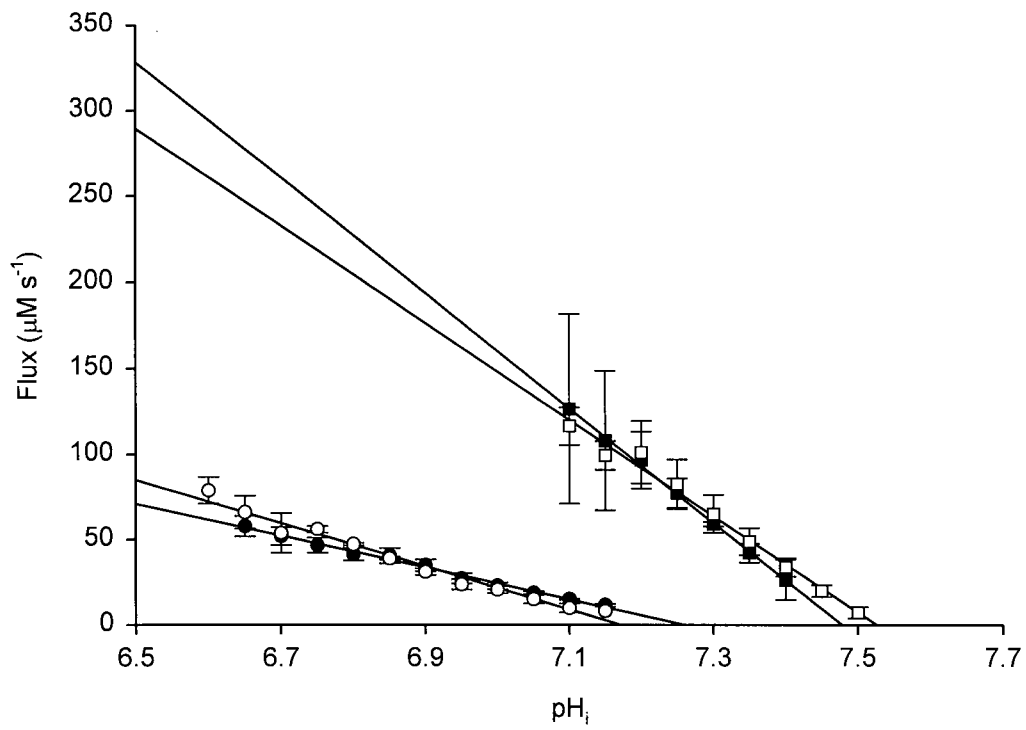
Figure 56. Effects of 4Br-A23187 on $[Ca^{2+}]_i$ and the effect of 4Br-A23187 on the SS pH_i response to KN-93 under HEPES-buffered conditions.

A A neuron loaded with Fura-2 showed a sustained increase in BI_{334}/BI_{380} values (representing $[Ca^{2+}]_i$) upon the addition of 10 µM 4Br-A23187 to the HEPES-buffered perfusion medium. **B** A different neuron loaded with BCECF was exposed to 10 µM 4Br-A23187 which caused SS pH_i to increase ~0.07 pH units. Subsequent addition of 20 µM KN-93 failed to alter SS pH_i.

A
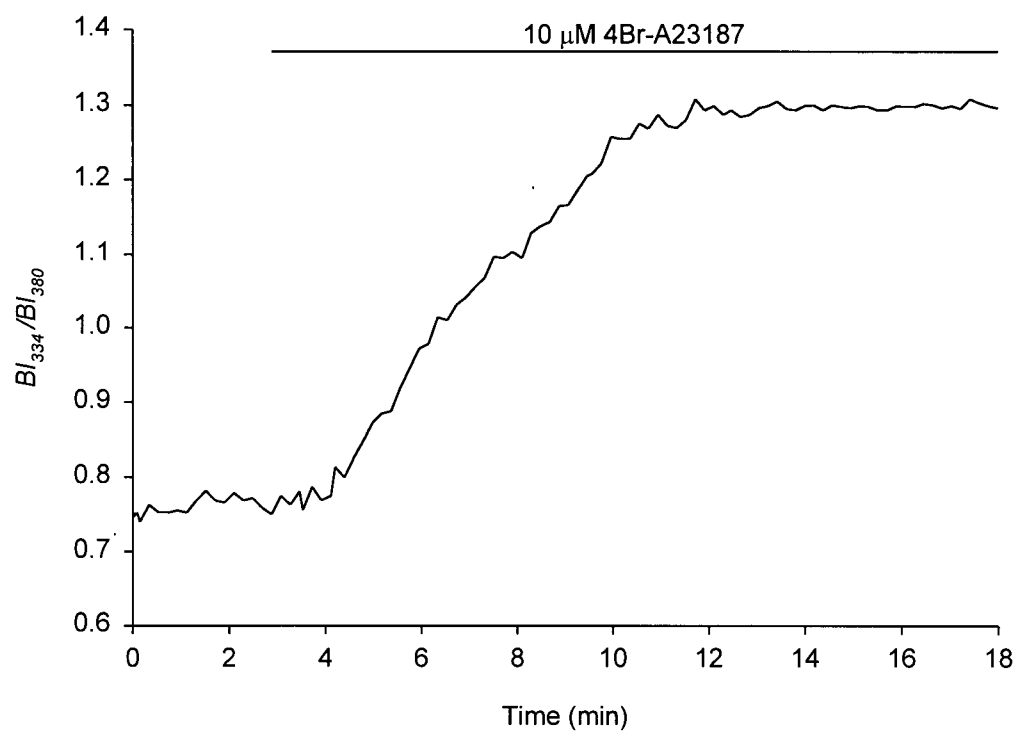
B
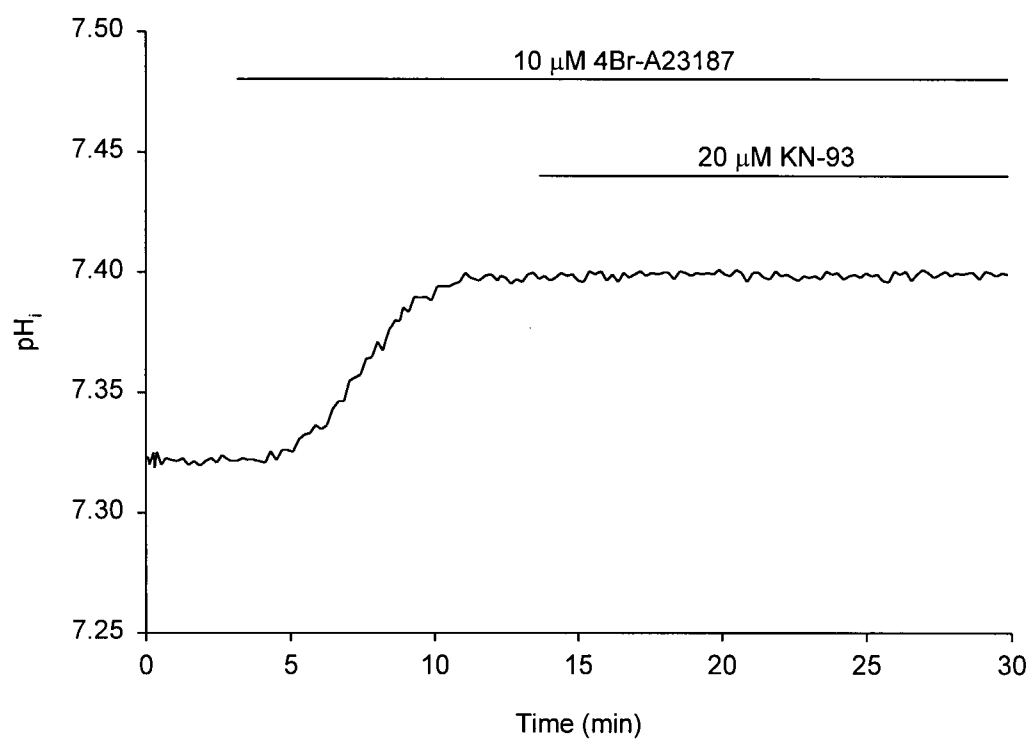
DISCUSSION

Despite the importance of pH_i for the control of neuronal function under both physiological and pathophysiological conditions (see Introduction), relatively little information is available concerning the regulation and modulation of pH_i in mammalian central neurons. In this regard, the principal findings of the present study are: (1) that forward NIAE activity participates in alkali extrusion (or acid loading) and contributes to the maintenance of SS pH_i in adult rat hippocampal CA1 neurons; (2) that the cAMP/PKA second messenger system is involved in the control of the activities of not only the NHE but also the Na^+-dependent and Na^+-independent HCO_3^-/Cl^- exchangers in this cell type; and (3) that calmodulin is also involved in the control of Na^+/H^+ exchange activity. Each of these major findings will be discussed in turn.

1. The control of SS pH_i in adult rat hippocampal CA1 neurons

In acutely dissociated adult rat hippocampal CA1 neurons, both HCO_3^--dependent and HCO_3^--independent pH_i regulating mechanisms, as well as the HCO_3^-/CO_2 open buffering system, contribute to the maintenance of SS pH_i. In the presence of HCO_3^-/CO_2, the HCO_3^-/Cl^- exchangers (*i.e.* the acid loading NIAE and the acid extruding NDAE) act in concert to maintain SS pH_i at a value near pH_o. However, the roles of the two HCO_3^--dependent mechanisms in this regard appeared to be dependent on the relative activity of a HCO_3^--independent pH_i regulating mechanism (most likely, the NHE) which, acting alone, determined whether neurons possessed 'low' or 'high' resting pH_i values.

1.1 Na^+/H^+ exchange

Under HEPES-buffered conditions, the NHE is the dominant acid extrusion mechanism in rat hippocampal neurons and, as such, contributes to the maintenance of SS pH_i in the absence of HCO_3^-. In the present study, the distribution of resting pH_i values from all neurons examined under nominally HCO_3^-/CO_2-free, HEPES-buffered conditions was best fit by the sum of two

Gaussian distributions with means at pH_i = ~6.90 and ~7.35 (Fig. 7B; also see Smith *et al.*, 1998). Those neurons with 'low' resting pH_i values exhibited low rates of net acid equivalent efflux following internal acid loads imposed in the absence of HCO_3^- whereas those neurons with 'high' resting pH_i values exhibited high rates of net acid equivalent efflux (*e.g.* Fig. 5). These findings confirm those of a previous study by Bevensee *et al.* (1996), also conducted in acutely dissociated rat hippocampal CA1 neurons. The latter authors found, in particular, that Na^+/H^+ exchange in 'low' pH_i neurons exhibited a more acidic set-point pH_i than Na^+/H^+ exchange in 'high' pH_i neurons, a finding also confirmed in the present study (see Fig. 5B and C), and suggested that the differences in SS pH_i between neurons with 'low' and 'high' SS pH_i values under nominally HCO_3^-/CO_2-free conditions reflected differences in Na^+/H^+ exchange activity (*i.e.* differences in Na^+/H^+ exchange activity between 'low' and 'high' pH_i neurons gave rise to the differences in SS pH_i observed). Bevensee *et al.* (1996) also suggested that the differences in net acid extrusion between neurons with 'low' and 'high' SS pH_i values might reflect different functional states of the NHE and/or a difference in the number of functional exchangers present in the plasma membranes of neurons with 'low' and 'high' SS pH_i values. The present finding that the functional state of the NHE in rat hippocampal neurons can be altered by modulating the activity of the cAMP/PKA pathway lends support to the former possibility (see Discussion, Section 2).

The observed NHE-dependent heterogeneity of SS pH_i in rat hippocampal CA1 neurons, which likely reflects different functional states of the exchange mechanism in different neurons, is not unique. For example, Bierman *et al.* (1987) reported that the NHE in P19 embryonic carcinoma cells is constitutively active; in consequence, resting pH_i values in these cells were high (~7.50) and the activity of the NHE could not be further stimulated by phorbol esters or growth factors. Conversely, in the differentiated mesodermal derivative (MES-1) of P19 cells, NHE activity was found to be relatively low; consequently, resting pH_i values in these cells were low (~6.95) and NHE activity could be stimulated by phorbol esters and growth factors. From these findings, it was suggested that the high resting pH_i values (reflecting high rates of NHE

activity) found in the undifferentiated P19 cells were most likely required for optimal DNA transcription and protein synthesis during growth (also see Busa & Nuccitelli, 1984). It is an intriguing possibility that differences in NHE activity and/or resting pH_i values in CA1 neurons may contribute to the functional heterogeneity which exists between pyramidal neurons within the CA1 subfield of the hippocampus (*e.g.* see Church & Baimbridge, 1991).

1.2 HCO_3^--dependent pH_i regulating mechanisms

Although the activities of the acid-extruding Na^+/H^+ and Na^+-dependent HCO_3^-/Cl^- exchangers in rat hippocampal neurons have been well characterized (Raley-Susman *et al.*, 1991; Schwiening & Boron, 1994; Baxter & Church, 1996; Bevensee *et al.*, 1996; Smith *et al.*, 1998), internal alkaline shifts also occur in these cells and may have particularly detrimental effects on neuronal function, including the development of epileptiform activity and neurodegeneration (*e.g.* Church & Baimbridge, 1991; Hartley & Dubinsky, 1993; Pirttilä & Kauppinen, 1993; Bonnet *et al.*, 1998; Hoyt & Reynolds, 1998). In many cell types, forward-mode Na^+-independent HCO_3^-/Cl^- exchange mediates alkali extrusion (*e.g.* Boyarsky *et al.*, 1988a & 1988b; Ganz *et al.*, 1989; Tønnessen *et al.*, 1987 & 1990a; Xu & Spitzer, 1994) and, in addition, may act to maintain $[Cl^-]_i$ and, thus, a particular value of the Cl^- equilibrium potential (E_{Cl}; see Vaughan-Jones, 1979; Aickin & Brading, 1984). In contrast, evidence supporting the presence of NIAE in neurons has to date been limited to: (1) a demonstration of AE3 (which functions as a NIAE) mRNA in adult rat brain using *in situ* hybridization (Kopito *et al.*, 1989; Raley-Susman *et al.*, 1993); (2) reports that a HCO_3^--dependent internal alkalinization occurs in rat hippocampal neurons acutely exposed to Cl^--deficient media (Raley-Susman *et al.*, 1993; Schwiening & Boron, 1994; Baxter & Church, 1996); and (3) that a HCO_3^--dependent, Na^+-independent, DIDS-sensitive mechanism contributes to acid extrusion from rat hippocampal neurons at very low values of pH_i (<6.5; Baxter & Church, 1996). Interestingly, in addition to the hippocampus, little evidence has been found to support the existence of NIAE in rat neocortical neurons (see

Ou-yang *et al.*, 1993) although NIAE activity has been documented in cultured rat astrocytes (Mellergård *et al.*, 1993).

Despite the aforementioned reports, the present study is the first to assess the characteristics of NIAE activity and its contribution to alkali extrusion and the maintenance of SS pH_i in a mammalian central neuron. Thus, acute Cl^-_o-removal resulted in a reversible internal alkalinization (see Fig. 31), confirming the presence of reverse-mode NIAE activity in rat hippocampal neurons (also see Raley-Susman *et al.*, 1993; Baxter & Church, 1996). More importantly, however, pH_i was found to recover to normal levels following internal alkaline loads in a DIDS-sensitive (Fig. 28) and Na^+_o-independent (Fig. 30A) manner, suggesting that forward-mode NIAE contributes to alkali extrusion in rat hippocampal neurons. Forward-mode NIAE activity was also suggested by the DIDS-sensitive, pH_i-dependent changes in SS pH_i evoked upon the addition of HCO_3^-/CO_2 in the absence of Na^+_o (Figs. 10 and 11). Thus, the present study confirms reverse-mode (acid-extruding), and is the first to describe forward-mode (acid-loading), NIAE activity in a mammalian central neuron.

The activity of the NIAE in rat hippocampal neurons is dependent on the absolute value of pH_i. Previously, Olsnes *et al.* (1987b) and Zhang *et al.* (1996) characterized the pH_i dependence of the activity of the NIAE in, respectively, Vero cells and *Xenopus* oocytes expressing the AE2 isoform. These authors reported that the exchange mechanism is most active at high absolute values of pH_i and that its activity is highly dependent on pH_i in a manner similar, but opposite, to the NHE and NDAE (also see Boyarsky *et al.*, 1988b; Mugharbil *et al.*, 1990). That Na^+-independent HCO_3^-/Cl^- exchange in rat hippocampal neurons exhibits similar functional properties are suggested by the present findings that: (1) the rate of pH_i recovery from internal alkaline loads is highly dependent on the absolute value of pH_i (as indicated by the slopes of the regression lines for control data presented in Figs. 29B and 30B); and (2) neuronal acid-loading can occur at absolute pH_i values as low as ~6.9 under control conditions, a similar minimum value for forward-mode NIAE activity as that previously reported in Vero cells (pH_i = 7.0 - 7.1; Olsnes *et al.*, 1987b). In addition, it has previously been shown that the AE2 (found in

Vero cells) and AE3 (most likely found in rat hippocampal neurons) isoforms of the NIAE are functionally similar when expressed in HEK 293 cells (Lee *et al.*, 1991).

Finally, evidence was presented that the NIAE, operating in conjunction with the NDAE, contributes to the maintenance of SS pH_i in adult rat hippocampal CA1 neurons. Together, the HCO_3^--dependent pH_i regulating mechanisms appear to act to maintain SS pH_i at a value near pH_o. The evidence supporting this form of pH_i regulation is as follows. First, in contrast to the bimodal distribution of resting pH_i values observed under nominally HCO_3^--free conditions (see Discussion, Section 1.1), the distribution of resting pH_i values from neurons perfused with HCO_3^-/CO_2-buffered medium was unimodal with a mean value at ~7.20 (*i.e.* near pH_o). Second, in neurons initially perfused with HEPES-buffered medium, exposure to HCO_3^-/CO_2 caused pH_i to rise or fall (depending on the initial value of pH_i under HCO_3^--free conditions) and attain a new SS level near pH_o (see Fig. 8C; also see Schwiening & Boron, 1994). Changes in pH_i also occurred upon the addition of HCO_3^-/CO_2 to HEPES-buffered medium in the absence of Na^+_o (see Fig. 10). Third, the addition of DIDS to HCO_3^--containing perfusion media caused SS pH_i to deviate away from the prevailing pH_o value (see Fig. 9).

Taken together, the results of the present study suggest that acutely dissociated adult rat hippocampal CA1 neurons express a NIAE that can function in forward and reverse modes over a wide range of pH_i values in a manner that is dependent upon absolute pH_i. The NIAE is an important contributor to pH_i regulation in hippocampal neurons and, acting in concert with the NDAE, contributes to the maintenance of SS pH_i in this cell type. In addition, by participating in the control of $[Cl^-]_i$, the NIAE might act as an important mediator of neuronal excitability (*e.g.* by modulating $GABA_A$-mediated currents; see Kaila, 1994; Staley *et al.*, 1995).

1.3 The HCO_3^-/CO_2 pH buffering system

ᶦ Although evidence has been presented that both the NIAE and the NDAE act to maintain SS pH_i at a value near pH_o in adult rat hippocampal CA1 neurons, it is also apparent that the open HCO_3^-/CO_2 pH buffering system is an important contributor to this process. Thus, under

external Na^+-free conditions in the presence of DIDS, where the activities of all three known pH_i regulating mechanisms in rat hippocampal neurons are blocked, exposing neurons initially perfused with HEPES-buffered medium to HCO_3^- caused pH_i to stabilize at a SS level slightly above pH_o (see Fig. 11). Although the contribution of HCO_3^-/CO_2-dependent buffering to the regulation of SS pH_i in rat hippocampal neurons has never been formally assessed, in peripheral cell types the HCO_3^-/CO_2 buffering system is essential for pH_i regulation (Frelin *et al.*, 1988), and may contribute to the established pH_i-dependency of the activities of the NHE, NIAE and NDAE (see Reinertsen *et al.*, 1988; Humphreys *et al.*, 1995). Indeed, it has recently been found that carbonic anhydrase (isoform II) binds to the *C* terminus of the NIAE (Vince & Reithmeier, 1998). Interestingly, carbonic anhydrase has been identified in adult rat hippocampal pyramidal neurons (Voipio & Kaila, 1993) and carbonic anhydrase inhibitors possess anticonvulsant activity (*e.g.* Velísek & Veliskova, 1994).

1.4 Net pH_i regulation under physiological conditions

Controversy exists concerning the relative contributions of the pH_i regulating mechanisms discussed above (*i.e.* NHE, NIAE and NDAE) to the maintenance of SS pH_i in rat hippocampal neurons under physiological (*i.e.* HCO_3^-/CO_2-containing) conditions. Initial studies suggested that the NDAE dominates acid extrusion and is the primary determinant of SS pH_i in rat hippocampal neurons (Schwiening & Boron, 1994). However, mean resting pH_i values in the latter study were abnormally low ($pH_i = 7.03$, a condition which favours activation of NDAE) and more recent studies have suggested that the NHE is the dominant acid extrusion mechanism and ultimately determines the resting pH_i of any given hippocampal neuron (Bevensee *et al.*, 1996). However, neither of the aforementioned studies considered the possible contribution of NIAE to pH_i regulation and the control of SS pH_i in rat hippocampal neurons, despite the fact that the importance of this exchange mechanism to both processes has long been established in peripheral cell types (*e.g.* Tønnessen *et al.*, 1987; Boyarsky *et al.*, 1988b; Tønnessen *et al.*, 1990a; Cooper & Hunter, 1997).

Detailed studies by Tønnessen *et al.* (1987 & 1990a) in Vero cells, for example, established not only that the NIAE and NDAE were the major alkali and acid extruding mechanisms, respectively, but also that these mechanisms were the major determinants of SS pH_i; at pH_i values $< pH_o$, the activity of the acid-extruding NDAE dominated the control of SS pH_i whereas at pH_i values $> pH_o$, the activity of the acid-loading NIAE dominated. Thus, when a cell with a resting pH_i value $< pH_o$ in nominally HCO_3^--free, HEPES-buffered medium was exposed to HCO_3^-/CO_2, an increase in SS pH_i occurred, bringing pH_i closer to pH_o (= 7.4); as pH_i increased, Tønnessen *et al.* (1987 & 1990a) suggested that the activity of the NDAE gradually declined whereas the activity of the NIAE gradually increased (also see Reinertsen *et al.*, 1988 for illustrations). In a similar manner, Boyarsky *et al.* (1988b) found that both HCO_3^--dependent anion exchangers, acting together with the NHE, functioned to maintain resting pH_i (~7.25) near pH_o (= 7.4) in rat glomerular mesangial cells. The results of the present study suggest a similar role for the NHE, NDAE and NIAE, acting in combination, in the control of SS pH_i in rat hippocampal neurons. Thus, in neurons with low NHE activity (= low acid-extrusion), both an increase in the activity of the NDAE and a decrease in the activity of the NIAE cause net acid extrusion and SS pH_i is maintained near pH_o (Fig. 57A). Conversely, in neurons with high NHE activity (= high acid-extrusion), the activities of the NDAE and NIAE function in a manner to cause net acid-loading and maintain SS pH_i near pH_o (Fig. 57B). Thus, a dominant role for the maintenance of SS pH_i is not placed on any single one of the three pH_i regulating mechanisms present in acutely dissociated adult rat hippocampal CA1 neurons under physiological (HCO_3^- /CO_2-buffered) conditions.

1.5 Summary

In acutely dissociated adult rat hippocampal CA1 neurons, the NIAE functions in forward (acid-loading) mode over a range of absolute pH_i values and in a manner that is dependent on pH_i. Under physiological (HCO_3^-/CO_2-buffered) conditions, the activities of the NDAE and NIAE contribute to maintain SS pH_i near pH_o in a manner dependent on the activity of the NHE

(which determines whether neurons have 'low' or 'high' resting pH_i values in the absence of HCO_3^-/CO_2) together with a contribution from the HCO_3^-/CO_2 open buffering system.

2. Modulation of the activities of pH_i regulating mechanisms by the cAMP/PKA second messenger system

Modulating the activity of the cAMP/PKA second messenger system in rat hippocampal neurons evoked a variety of effects on SS pH_i which were dependent both on the experimental conditions (presence or absence of HCO_3^-) and the resting pH_i values of the neurons studied. In the nominal absence of HCO_3^-, stimulation of the cAMP/PKA pathway evoked a rise in SS pH_i whereas inhibition of the pathway failed to elicit a change in pH_i. Stimulation of the cAMP/PKA pathway also consistently evoked a rise in SS pH_i under physiological (HCO_3^-/CO_2-buffered) conditions. However, in the presence of HCO_3^-/CO_2, inhibition of the pathway caused SS pH_i to change in HCO_3^-- and pH_i-dependent manners, with the result that pH_i was brought closer to the pH of the external medium. Similar HCO_3^--dependent shifts in the SS pH_i responses resulting from the modulation of various second messenger pathways have been described in rat glomerular mesangial cells, osteoblastic (UMR-106) cells, Vero cells, A10 vascular smooth muscle cells and the rabbit distal convoluted tubule (Ganz et al., 1989; Kikeri et al., 1990; Tønnessen et al., 1990b; Green & Kleeman, 1992; Dagher et al., 1997, respectively). In these previous studies, concomitant changes in the activities of HCO_3^--independent and HCO_3^--dependent pH_i regulating mechanisms were found to be responsible for the diverse changes in SS pH_i. The results of the present study suggest that comparable effects occur in rat hippocampal neurons which, in turn, likely account for the various changes in SS pH_i evoked by modulation of the activity of the cAMP/PKA pathway.

2.1 Na^+/H^+ exchange

In acutely dissociated adult rat hippocampal CA1 neurons, increasing $[cAMP]_i$ activates a Na^+-dependent, HCO_3^--independent acid extrusion mechanism, most likely the NHE, via a

process that is mediated by PKA and is not dependent on changes in $[Ca^{2+}]_i$. Evidence for this pathway includes the findings that, in the nominal absence of HCO_3^-: (1) Forskolin and IBMX evoked increases in SS pH_i and net acid efflux following imposed internal acid loads, effects which were mimicked by the PKA activator, Sp-cAMPS; (2) Forskolin-evoked increases in SS pH_i were almost abolished by Rp-cAMPS, an inhibitor of PKA; (3) In the absence of Na^+_o (replaced with $NMDG^+$), the Sp-cAMPS-induced increase in SS pH_i was abolished; however, when Na^+_o was replaced with Li^+, the Sp-cAMPS-evoked increase in SS pH_i was re-established; (4) The addition of Sp-cAMPS or Rp-cAMPS failed to alter $[Ca^{2+}]_i$, and the Sp-cAMPS- and forskolin-induced increases in SS pH_i were similar under Ca^{2+}_o-free and Ca^{2+}_o-containing conditions. Interestingly, inhibition of the cAMP/PKA pathway failed to affect the activity of the NHE in previously unstimulated neurons, as indicated by the failure of 2',5'-dideoxyadenosine and Rp-cAMPS to affect SS pH_i. However, it should be noted that NHE activity is reduced by these agents in rat hippocampal neurons which have been pretreated with noradrenaline or selective β-adrenoceptor agonists (which act to increase the activity of cAMP/PKA pathway; see Smith *et al.*, 1998).

Although the control of the activities of neuronal NHEs by second messenger systems has not been extensively investigated (a single report suggests that PKC increases the activity of the NHE in cerebellar Purkinje cells; Gaillard & Dupont, 1990), information is available from a large number of non-neuronal cell types (reviewed by Wakabayashi *et al.*, 1997a). These studies indicate that the response of NHEs to modulation of the activity of a given second messenger system varies, depending on the species, cell type and specific NHE isoform studied. In this regard, it is of some interest that the effects of modulating the activity of the cAMP/PKA pathway on the activity of the NHE in rat hippocampal neurons appear to differ from those involved in many other cellular preparations. Thus, elevating $[cAMP]_i$ or activating PKA either has no effect on or inhibits Na^+/H^+ exchange in most cell types studied, including rat brain synaptosomes (reviewed by Wakabayashi *et al.*, 1997a; also see Reuss & Petersen, 1985; Reuss & Stoddard, 1987; Helmle-Kölb *et al.*, 1990; Sasaki & Marumo, 1991; Casavola *et al.*, 1992;

Davis *et al.*, 1992; Green & Kleeman, 1992; Sánchez-Armass *et al.*, 1994; Kandasamy *et al.*, 1995; Weinman *et al.*, 1995; Azarani *et al.*, 1996; Kurashima *et al.*, 1997). For example, in sheep cardiac Purkinje fibres, IBMX evokes a decrease in the activity of the NHE which, interestingly, is mediated via calmodulin (Wu & Vaughan-Jones, 1994). Similarly, in OKP cells over-expressing native NHE-3 and in AP-1 cells transfected with NHE-3 (Zhao *et al.*, 1999), cAMP inhibits the activity of NHE-3, although in these cases the effect is mediated via PKA and direct phosphorylation of the exchange protein.

Although activating the cAMP/PKA pathway has no effect on or inhibits the activity of NHEs in most non-neuronal cell types studied, there are some exceptions (reviewed by Noël & Pouysségur, 1995; also see Gupta *et al.*, 1994; Busch *et al.*, 1995; Kandasamy *et al.*, 1995). Perhaps the best-characterized example of a cAMP-mediated upregulation of NHE activity is seen in the case of the trout red blood cell β-NHE isoform, which can be activated both by β-adrenoceptor agonists and by direct elevation of $[cAMP]_i$. Interestingly, β-NHE contains two potential consensus sites for phosphorylation by PKA in its *C*-terminal cytoplasmic domain (Borgese *et al.*, 1992). Although it is unclear precisely which NHE isoform participates in pH_i regulation in rat hippocampal CA1 neurons, it is tempting to speculate that it might also possess an intrinsic capability to respond to PKA.

Activation of NHEs by external stimuli (*e.g.* mitogens, hormones and transmitters) and second messengers can occur in two ways: (1) an increase in the maximum velocity of ion exchange; and/or (2) an increase in the affinity of the internal allosteric modifier site on the exchange protein for H^+ (see Mahnensmith & Aronson, 1985; Noël & Pouysségur, 1995). The latter mechanism is associated with a change in the conformation of the transporter protein which affects the H^+-sensor site, and is produced by a phosphorylation event which in turn leads to an alkaline shift in the pH_i-dependence of the acid extrusion mechanism (see Wakabayashi *et al.*, 1997a). For example, it has been shown that okadaic acid and protein kinase activators (*e.g.* α-thrombin, phorbol esters, epidermal growth factor) increase the activities of various NHEs via phosphorylation events (reviewed by Tse *et al.*, 1993; Bianchini & Pouysségur, 1996; also see

Sardet *et al.*, 1990; Bianchini *et al.*, 1991; Livne *et al.*, 1991; Sardet *et al.*, 1991; Guizouarn *et al.*, 1993). In addition, as mentioned above, Borgese *et al.* (1992) identified two consensus sites for phosphorylation by PKA in the primary amino acid sequence for the trout red cell β-NHE isoform and, more recently, Zhao *et al.* (1999) identified two consensus sequences for phosphorylation by PKA in the primary amino acid sequence of the mammalian NHE-3 isoform.

The results of the present study suggest that a phosphorylation event may also be involved in the control of the activity of the NHE in rat hippocampal neurons. Thus, similar alkaline shifts in the pH_i-dependence of NHE activity were elicited by forskolin, IBMX and Sp-cAMPS, and the protein phosphatase inhibitor okadaic acid augmented the increase in pH_i evoked Sp-cAMPS. However, the present study provides no evidence to indicate whether activation of the NHE in rat hippocampal neurons by PKA involves direct phosphorylation of the exchange protein itself or of an associated regulatory protein (*e.g.* NHE-RF and/or E3KARP) whose phosphorylation state influences the former's activity (Sardet *et al.* 1991; Weinman *et al.* 1995; Cabado *et al.*, 1996; Lin & Barber, 1996; Yun *et al.*, 1997 & 1998; Hall *et al.*, 1998; Lamprecht *et al.*, 1998). Nevertheless, phosphorylation of NHE regulatory proteins has only been shown to inhibit NHE-3 activity, which contrasts with the finding of the present study that NHE activity increases upon stimulation by PKA. Thus, if a NHE regulatory protein was to exist in rat hippocampal neurons, it would have regulate the activity of the NHE in a manner opposite to that established for NHE-3.

It should also be noted that considerable 'cross-talk' exists between the cAMP/PKA intracellular second messenger system and other signal transduction cascades. For example, PKA may interact with an element in the MAPK cascade downstream of Ras and upstream of Raf-1 (see Kurino *et al.*, 1996). Given that stimulation of the MAPK cascade increases the activities of NHEs in some non-neuronal cell types (*e.g.* Matsuda *et al.*, 1995; Hooley *et al.*, 1996; Bianchini *et al.*, 1997; Tominaga *et al.*, 1998) and that MAPKs may modulate a number of neuronal functions which are also associated with changes in pH_i (Campos-Gonzalez & Kindy, 1992; Pang *et al.*, 1995), it remains possible that other signal transduction pathways may be

involved in the effects of modulating the cAMP/PKA second messenger system on NHE activity in rat hippocampal neurons.

2.2 HCO_3^--dependent pH_i regulating mechanisms

In comparison with the extensive literature which details the regulation of the activities of NHEs in a variety of cell types (see above), relatively few studies have examined the modulation by second messenger systems of the activities of HCO_3^--dependent pH_i regulating mechanisms, even in non-neuronal preparations (to date, there are no reports in neurons). The results of the present study indicate that, in rat hippocampal neurons, the cAMP/PKA pathway is involved in the regulation of the activities of both the NIAE and the NDAE.

2.2.1 Na^+-independent HCO_3^-/Cl^- exchange

One of the principal findings of the present study is that altering PKA activity evokes changes in NIAE activity which are dependent upon the initial resting pH_i of the neuron. Evidence for the involvement of PKA in the control of the activity of the NIAE includes the observations that: (1) Sp-cAMPS, Rp-cAMPS and 2', 5'-dideoxyadenosine exert effects on SS pH_i which are dependent upon the resting pH_i of the neuron prior to the application of a test compound; (2) Sp-cAMPS and Rp-cAMPS exert opposite effects on rates of pH_i recovery following imposed alkaline loads; (3) Sp-cAMPS and Rp-cAMPS exert opposite, pH_i-dependent effects on SS pH_i in the absence of Na^+_o; (4) Rp-cAMPS elicits a decrease in the rate of pH_i recovery following internal alkaline loads imposed in the absence of Na^+_o; (5) Sp-cAMPS increases, whereas Rp-cAMPS decreases, the magnitude of 0 Cl^-_o-evoked internal alkalinizations; and (6) all of the aforementioned effects are sensitive to DIDS and/or are dependent on $HCO_3^-_o$.

In neurons with high resting pH_i values, NIAE activity was regulated by the cAMP/PKA pathway in a manner opposite to that observed in neurons with low resting pH_i values; thus, regulation of NIAE activity by the cAMP/PKA pathway is dependent on resting pH_i values. More specifically, in neurons with resting pH_i values $< \sim 7.4$, activation of PKA increased, and

inhibition of PKA decreased, the activity of the NIAE. In contrast, in neurons with resting pH_i values > ~7.4, activation of PKA decreased, and inhibition of PKA increased, NIAE activity. If the effects of modulating PKA activity on the activity of forward-mode NIAE are considered in isolation (*i.e.* in the absence of any effect of changing PKA activity of the activities of the NHE or NDAE), activation of PKA would act to move SS pH_i away from pH_o because the activity of the NIAE would be decreased in 'high' pH_i neurons (resulting in a further internal alkalinization) and increased in 'low' pH_i neurons (resulting in a further internal acidification). In contrast, inhibition of PKA would act to move SS pH_i towards pH_o, because the activity of the NIAE would be increased in 'high' pH_i neurons (resulting in an internal acidification) and decreased in 'low' pH_i neurons (resulting in an internal alkalinization).

2.2.2 Na^+-dependent HCO_3^-/Cl^- exchange

Altering PKA activity evoked changes in the activity of the NDAE which were dependent upon the initial resting pH_i of the neuron but which were opposite to the effects exerted on the NIAE. Evidence for the involvement of PKA in the control of the activity of the NDAE includes: (1) The Na^+_o- and pH_i-dependent effects of Sp-cAMPS, Rp-cAMPS and 2', 5'-dideoxyadenosine on SS pH_i; (2) The Sp-cAMPS-evoked decrease and Rp-cAMPS-evoked increase in the rates of pH_i recovery following acid loads imposed at RT; (3) The opposing effects of Sp-cAMPS and Rp-cAMPS on the rates of internal alkalinization observed during depletion of Cl^-_i; and (4) The sensitivity to DIDS and/or the dependence on $HCO_3^-_o$ of the aforementioned observations.

In neurons with 'high' resting pH_i values, NDAE activity was regulated by the cAMP/PKA pathway in a manner opposite to that observed in neurons with 'low' resting pH_i values. Thus, as was the case for the NIAE (see above), regulation of NDAE activity by the cAMP/PKA pathway is dependent on resting pH_i. More specifically, in neurons with resting pH_i values < ~7.4, activation of PKA decreased, and inhibition of PKA increased, the activity of the NDAE. Unfortunately, neurons with 'high' (> ~7.4) resting pH_i values were not observed, but by analogy with the results detailed above concerning the control of NIAE activity in 'high' pH_i

neurons, it seems possible that stimulation of PKA might activate the NDAE, whereas inhibition of PKA might inhibit the NDAE, in 'high' pH_i cells. If the effects of modulating PKA activity on the activity of the NDAE are considered in isolation (*i.e.* in the absence of any effect of changing PKA activity on the activities of the NHE or NIAE), activation of PKA would act to move SS pH_i away from pH_o because the activity of the NDAE would be increased in 'high' pH_i neurons (resulting in a further internal alkalinization) and decreased in 'low' pH_i neurons (resulting in a further internal acidification). In contrast, inhibition of PKA would act to move SS pH_i towards pH_o, because the activity of the NDAE would be decreased in 'high' pH_i neurons (resulting in an internal acidification) and increased in 'low' pH_i neurons (resulting in an internal alkalinization).

2.2.3 Comparison with other cell types

As noted above, relatively few studies have examined the control of the activities of HCO_3^--dependent pH_i regulating mechanisms by second messenger systems, even in non-neuronal cell types, although the information which is available suggests that, as in the case of the NHE, considerable diversity exists between cell types (*e.g.* compare Green *et al.*, 1990; Vigne *et al.*, 1988; Alvaro *et al.*, 1995; Strazzabosco *et al.*, 1997).

In the case of the NIAE, it has been reported that the activity of the NIAE is inhibited by elevations in $[cAMP]_i$ in aortic smooth muscle cells (Vigne *et al.*, 1988). In contrast, the addition of glucagon, forskolin or dibutyryl-cAMP (to increase $[cAMP]_i$) increased the activity of the NIAE in isolated rat hepatocytes, and the glucagon-evoked increase in NIAE activity was inhibited by Rp-cAMPS (Alvaro *et al.*, 1995). More recently, the same group showed that Sp-cAMPS and Rp-cAMPS elicited, respectively, increases and decreases in NIAE activity in isolated bile duct units (Alvaro *et al.*, 1997). Similarly, Alpini and colleges (1997) reported that secretin causes an increase in $[cAMP]_i$ which, in turn, increases the activity of the NIAE in large intrahepatic bile ducts. And Clarke & Harline (1998) reported that electroneutral HCO_3^- secretion (representing NIAE activity) was increased following 15 min of forskolin treatment in mouse duodenal cells (also see Niisato & Marunaka, 1996; Nyberg *et al.*, 1998).

In the case of the NDAE, Boron *et al.* (1978), in one of the first studies examining the effect of cAMP on the activities of pH_i regulating mechanisms, found that microinjection of cAMP increases the activity of the NDAE in barnacle muscle fibres. A more recent study performed in the rabbit cortical collecting duct reports that 8Br- and dibutyryl-cAMP increase SS pH_i, an effect which was suggested to reflect an increase in the activity of the NDAE (Emmons & Stokes, 1994).

2.2.4 Potential mechanisms underlying the modulation of NIAE and NDAE activities by PKA

As detailed above, the various mechanisms underlying the regulation of NHE activity by PKA have been studied in considerable detail. In comparison, no information is to date available concerning the molecular mechanism(s) which might be involved in the control of the activities of HCO_3^--dependent pH_i regulating mechanisms by PKA or other intracellular second messengers.

Two possible mechanisms can be suggested for the regulation of the activities of the NIAE and NDAE by PKA. First, by analogy with the NHE, the activities of the NIAE and the NDAE may be regulated by the cAMP/PKA system via a phosphorylation event. Second, the changes in the activities of the NIAE and NDAE may be secondary to changes in SS pH_i evoked by the NHE (see Mason *et al.*, 1989) and also, in the case of the NDAE, by the NIAE. Although the results of the present study do not allow a strict differentiation between these two possibilities, the former potential mechanism of regulation seems most likely in light of the facts that: (1) the activity of the NIAE was altered by the cAMP/PKA pathway independent of changes in the activities of Na^+-dependent acid-extruding mechanisms; (2) alkaline shifts in the pH_i-dependence of NIAE activity were observed in the presence of Rp-cAMPS and Sp-cAMPS; (3) an alkaline shift in the pH_i-dependence of NDAE activity was observed in the presence of Rp-cAMPS; and (4) Sp-cAMPS evoked a relatively smaller increase in SS pH_i under HCO_3^-/CO_2-buffered compared to HEPES-buffered conditions, an effect mimicked by the protein phosphatase inhibitor okadaic acid (see Fig. 27). Taken together these findings in hippocampal neurons are consistent with a report that phosphorylation most likely mediates changes in the

pH_i-sensitivity of NIAE activity in Vero cells (Ludt *et al.*, 1993; Alvaro *et al.*, 1997). In the case of the NDAE, however, the present study provides no evidence to support or refute the second possibility raised above (*i.e.* that the changes in the activity of the NDAE may be secondary to changes in SS pH_i evoked by the NHE and/or the NIAE), as the NIAE and NHE were potentially active in all experiments designed to examine NDAE activity.

Finally, one additional potential mechanism which may be involved in the regulation of the activities of HCO_3^--dependent pH_i regulating mechanisms by the cAMP/PKA pathway needs to be addressed. Using the NHE as a model (see above), increases and decreases in the activities of HCO_3^--dependent pH_i regulating mechanisms may occur either via a change in the pH_i-sensitivity of the exchange processes (*i.e.* a change in set-point pH_i due to a change in the level of phosphorylation of the exchange mechanism or an ancillary protein) or via an increase in the maximum velocity of ion exchange. The latter reflects either an increase in the 'turnover' rate and/or an increase in the number of functional NHEs in the plasma membrane (reviewed by Noël & Pouysségur, 1995). The effect of modulating PKA activity on the activities of the NIAE and NDAE may involve changes in turnover rates because: (1) Sp-cAMPS decreased the slope of the regression line relating absolute pH_i to the rate of pH_i recovery from internal acid loads imposed at RT without markedly affecting the set-point pH_i of the exchange process (Fig. 36B); and (2) Sp-cAMPS increased the slope of the regression line relating absolute pH_i to the rate of pH_i recovery from internal alkaline loads (Fig. 30B; in this case, the change in set-point pH_i can be accounted for by the concomitant effect of Sp-cAMPS on NHE activity). In contrast, the present study provides no evidence to support or refute the possibility that modulation of PKA activity may change the numbers of functional NIAEs or NDAEs present in the neuronal membrane. This may occur either by the up- or down-regulation of protein synthesis (although the time frame of the observed responses make this possibility unlikely; see Wakabayashi *et al.*, 1997a) or by the re-insertion of endosomes containing the exchangers into the membrane (see Benedetti *et al.*, 1994; also see Hensley *et al.*, 1989; Khurana *et al.*, 1996; D'Souza *et al.*, 1998).

2.3 Buffering power

The effects of either increasing or decreasing the activity of the cAMP/PKA pathway on SS pH_i and the activities of pH_i regulating mechanisms did not reflect changes in internal buffering power. Thus, estimates of β_i (made by comparing the magnitudes of NH_4^+-induced alkalinizations observed during internal acid load experiments in the absence and presence of a given cAMP/PKA pathway-targeted treatment; see Aickin & Thomas, 1976; Smith *et al.*, 1998) indicated that the magnitudes of the internal alkalinizations evoked by NH_4^+ under control conditions were similar, in all cases, to the magnitudes of the alkalinizations evoked in the presence of a given pharmacological agent. For example, the mean alkalinization evoked by NH_4^+ under control HEPES-buffered conditions was 0.28 ± 0.04 pH units (n = 29), which was statistically not different (P > 0.5 in each case) to the alkalinizations observed in the presence of Sp-AMPS (0.30 ± 0.05 pH units, n = 7), forskolin (0.26 ± 0.03 pH units, n = 12) or IBMX (0.25 ± 0.04 pH units, n = 10), also under HEPES-buffered conditions. In addition, given the proposed importance of the HCO_3^-/CO_2 open buffering system to the maintenance of SS pH_i in rat hippocampal CA1 neurons (see Discussion, Section 1), it was noteworthy that the NH_4^+-evoked alkalinizations observed in the absence (0.26 ± 0.04 pH units) and presence (0.25 ± 0.04 pH units) of forskolin, IBMX, Sp-cAMPS and Rp-cAMPS were statistically not different (P = 0.88) under HCO_3^-/CO_2-buffered conditions (n = 35), even though it has previously been reported that cAMP increases the activity of carbonic anhydrase in some cell types (*e.g.* red blood cells; Dragon *et al.*, 1996).

2.4 The net effects of modulating the activity of the cAMP/PKA pathway on pH_i in rat hippocampal neurons

Given the fact that changes in the activity of the cAMP/PKA pathway differentially affect the activities of three different pH_i regulating mechanisms in rat hippocampal neurons, it becomes important to attempt assess the effect of activating or inhibiting this second messenger

system on net pH_i regulation and, thus, SS pH_i in this cell type under physiological (*i.e.* HCO_3^- /CO_2-containing) conditions.

As illustrated in Fig. 58, in neurons with low resting pH_i values, *inhibition* of the cAMP/PKA pathway will increase the activity of the NDAE and decrease the activity of the NIAE. Because inhibition of the cAMP/PKA pathway exerts no effect on the activity of the NHE (at least under previously unstimulated conditions), a net internal alkalinization is the result. In addition, rates of pH_i recovery from internal acid, but not alkaline, loads will be increased. Conversely, in neurons with high resting pH_i values, inhibition of the cAMP/PKA pathway will increase the activity of the NIAE and (possibly) decrease the activity of the NDAE. In the absence of any effect on NHE activity, the net result is an internal acidification and an improved ability to recover from internal alkaline, but not acid, loads. Thus, inhibition of the cAMP/PKA pathway increases pH_i in neurons with a low resting pH_i and decreases pH_i in neurons with a high resting pH_i, the overall effect being to move pH_i towards the prevailing value of pH_o.

In contrast, in neurons with low resting pH_i values, stimulation of the cAMP/PKA pathway will increase and decrease, respectively, the activities of the NIAE and NDAE; in the absence of any change in NHE activity, the net effect would be a reduction in SS pH_i. However, because the NHE is activated by cAMP/PKA, the net effect on SS pH_i is an internal alkalinization. In addition, stimulation of the cAMP/PKA pathway in low pH_i neurons will increase rates of pH_i recovery from acid and alkaline loads. Conversely, in neurons with high resting pH_i values, stimulation of the cAMP/PKA pathway will decrease the activity of the NIAE and (possibly) increase the activity of the NDAE; in the absence of any change in NHE activity, the net result would be a rise in SS pH_i. However, the NHE is once again activated under these conditions, and the net result is an internal alkalinization and an improved ability to recover from internal acid, but not alkaline, loads. In the absence of any effect on NHE activity, the effect of stimulating the cAMP/PKA pathway would be to decrease pH_i in neurons with a low resting pH_i and to increase pH_i in neurons with a high resting pH_i, the overall effect being to move pH_i away

from the prevailing value of pH_o. However, because stimulation of the cAMP/PKA pathway concomitantly activates the NHE, the overall effect of stimulating the cAMP/PKA pathway is to increase SS pH_i in neurons with both low and high resting pH_i values.

The schemes detailing the mechanistic basis for the effects of modulating the cAMP/PKA pathway on SS pH_i in rat hippocampal neurons are entirely novel. Of particular interest is the fact that the cAMP/PKA pathway modulates SS pH_i in rat hippocampal neurons in a manner which depends not only on the direction of the change in the activity of the pathway (i.e. inhibition or stimulation) but also on the resting pH_i of the neuron. In these regards, the present findings differ from those reported by others who have attempted to assess the effects of modulating the activities of second messenger pathways on net pH_i regulation in other cell types. For example, increasing $[cAMP]_i$ inhibits both the NHE and the NIAE in Necturus gallbladder epithelial cells and osteosarcoma (UMR-106) cells (Reuss, 1987; Reuss & Stoddard, 1987; Green & Kleeman, 1992). In contrast, in rat cardiomyocytes and human intrahepatic bile duct cells, increasing $[cAMP]_i$ activates the NDAE and the NIAE, but not the NHE, resulting in net HCO_3^--extrusion (Désilets et al., 1994; Strazzabosco et al., 1997). The potential importance of the present findings is highlighted by the study of Ganz and colleagues (1989) in rat glomerular mesangial cells. In this study, arginine vasopressin raised SS pH_i in the absence of HCO_3^- (i.e. under conditions where only NHE can contribute to pH_i regulation) but lowered it when HCO_3^- was present (i.e. under conditions where NHE, NIAE and NDAE may potentially contribute to pH_i regulation). The difference in the SS pH_i response to AVP under HCO_3^--free and HCO_3^--containing conditions was found to reflect the fact that, in the presence of HCO_3^-, AVP stimulated Na^+-independent HCO_3^-/Cl^- exchange more than it stimulated the sum of Na^+/H^+ exchange and Na^+-dependent HCO_3^-/Cl^- exchange. The results of the present study further emphasize the possible dangers of conducting physiological experiments in media lacking HCO_3^- and CO_2.

2.5 Summary

In summary, the cAMP/PKA pathway changes SS pH_i in acutely dissociated adult rat hippocampal CA1 neurons in a manner that is dependent on resting pH_i. Under physiological conditions (*i.e.* in the presence of HCO_3^-/CO_2), stimulation of the cAMP/PKA pathway increases pH_i to a SS level > pH_o, whereas inhibition of the cAMP/PKA pathway 'clamps' pH_i at a value near pH_o. These effects, which are likely mediated by phosphorylation events, reflect concomitant changes in the activities of the acid-extruding NHE and NDAE, and the acid-loading NIAE. The exact relationship between PKA and changes in the activities of the various exchangers, however, remains to be determined.

3. Control of the activity of the NHE by the CaM/CaMK-II second messenger system

In many peripheral cell types, the activities of pH_i regulating mechanisms can be controlled independently by multiple second messenger systems (reviewed by Wakabayashi *et al.*, 1997a). The results of the present study indicate that calmodulin, in addition to the cAMP/PKA pathway, is involved in the regulation of Na^+/H^+ exchange in rat hippocampal CA1 neurons.

3.1 Effect of CaM inhibitors on pH_i regulation

In acutely dissociated adult rat hippocampal CA1 neurons, CaM inhibitors evoked a concentration- and pH_i-dependent increase in SS pH_i, at least in part by increasing the activity of a HCO_3^--independent, Na^+-dependent acid-extruding mechanism, most likely the NHE. I found no evidence to suggest that the effect of CaM inhibitors to increase SS pH_i reflected changes in the activities of HCO_3^--dependent pH_i regulating mechanisms. Nevertheless, as a number studies in non-neuronal cell types indicate that changes in the activities of HCO_3^--dependent pH_i regulating mechanisms can be evoked by changes in $[Ca^{2+}]_i$ (Ganz *et al.*, 1989; Green *et al.*, 1990; Kikeri *et al.*, 1990; Muallem & Loessberg, 1990; Ludt *et al.*, 1993) and, possibly, by changes in the activity of Ca^{2+}/CaM (Green & Kleeman, 1992), it remains to be determined whether CaM inhibitors might modulate equally the activities of the acid-loading NIAE and acid-

extruding NDAE in rat hippocampal neurons, such that the resultant changes in SS pH_i and net acid extrusion following internal acid loads imposed in the presence of HCO_3^- are not different from those observed in the absence of HCO_3^- (see Ganz *et al.*, 1989).

3.2 Mechanisms underlying the changes in pH_i evoked by CaM inhibitors

The effect of CaM inhibitors to increase both SS pH_i and net acid-equivalent extrusion following imposed acid loads was not mediated by CaMK-II, calcineurin or changes in the activity of the cAMP/PKA pathway and for the most part did not reflect changes in $[Ca^{2+}]_i$. The evidence for these findings is as follows.

First, although interactions between CaM and the cAMP/PKA second messenger cascade have been well-documented (reviewed by Hanoune *et al.*, 1997; also see Cooper *et al.*, 1994; Houslay & Milligan, 1997) and have been reported to be involved in the regulation of pH_i in some non-neuronal cell types (*e.g.* Wu & Vaughan-Jones, 1994), in the present study the effect of the CaM inhibitor W-7 to increase SS pH_i was not affected by the adenylyl cyclase inhibitor 2',5'-dideoxyadenosine, the phosphodiesterase inhibitor IBMX, or the PKA inhibitor Rp-cAMPS.

Second, although calcineurin is a Ca^{2+}-CaM-dependent protein phosphatase (2B) which is known to participate in acetylcholine- and Ca^{2+}-evoked pH_i responses in rat cholangiocytes (Alvaro *et al.*, 1997) and is known to play important functional roles in hippocampal CA1 neurons and other cell types (reviewed by Klee *et al.*, 1998; also see Mansuy *et al.*, 1998), in the present study the effects of W-7 on SS pH_i were not altered by the calcineurin inhibitor FK-506.

Third, W-7 decreased $[Ca^{2+}]_i$. However, the majority of the rise in SS pH_i evoked by CaM inhibitors was not secondary to a decrease in $[Ca^{2+}]_i$ because: (1) W-7 and TFP evoked opposite changes in $[Ca^{2+}]_i$, although both increased SS pH_i; and (2) the W-7-evoked increases in SS pH_i and net acid extrusion following internal acid loads persisted in the absence of Ca^{2+}_o, despite the fact that the decrease in $[Ca^{2+}]_i$ evoked by W-7 in the presence of normal $[Ca^{2+}]_o$ was almost abolished under this condition.

Fourth, although CaMK-II is highly concentrated in the hippocampus (~1% of total protein; Erondu & Kennedy, 1985) and has been suggested to be important for hippocampal neuronal function (*e.g.* Waxham *et al.*, 1993), the CaMK-II inhibitors KN-62 and KN-93 did not affect SS pH_i (even when the Ca^{2+}/CaM/CaMK-II pathway was presumably stimulated by elevating $[Ca^{2+}]_i$ with the Ca^{2+}-ionophore 4Br-A23187) or net acid extrusion following internal acid loads. These observations contrast with findings made in a variety of non-neuronal cell types. Thus, Ca^{2+}-induced activation of CaMK-II inhibits NHE activity in intestinal and kidney brush border membranes (*e.g.* Weinman *et al.*, 1988; Emmer *et al.*, 1989; Cohen *et al.*, 1990b), a proximal tubule cell line (LLC-PK1; Chakraborty *et al.*, 1994) and isolated rabbit proximal tubules (Yamada *et al.*, 1996), and the CaMK-II inhibitor KN-62 activates the NHE-3 isoform (Levine *et al.*, 1995).

Finally, the effects of CaM inhibitors on SS pH_i cannot be attributed to changes β_i, as the magnitudes of NH_4^+-induced alkalinizations evoked in the absence (0.25 ± 0.06 pH units) and presence (0.26 ± 0.05 pH units) of W-7 and TFP were not statistically different ($P = 0.86$) under HEPES-buffered conditions ($n = 18$).

3.3 Comparison with other preparations

This is the first study to assess the effects of CaM inhibition on the control of the activity of a NHE in a mammalian central neuron. Nevertheless, a considerable body of data exists concerning the control by CaM of the activities of NHEs in non-neuronal cell types and in cell lines expressing various NHE isoforms. Of particular interest is the fact that the effects of CaM inhibitors on pH_i regulation in rat hippocampal CA1 neurons differ markedly from those observed in most preparations, a finding which reinforces the fact that the effect on NHE activity of modulating the activity of a given intracellular signaling cascade varies dramatically between NHE isoforms and the cell type (or cell line) in which a given exchanger isoform is expressed. Indeed, in the majority of cell types studied to date, Ca^{2+}-CaM activates Na^+/H^+ exchange (in

some cases via CaMK-II; see above) and CaM inhibitors reduce NHE activity and/or block the stimulation of NHE activity evoked by increases in $[Ca^{2+}]_i$.

Thus, the CaM inhibitor TFP abolishes Ca^{2+}_i-dependent, shrinkage-evoked increases in NHE activity in rat astrocytes (Shrode *et al.*, 1995). Similar observations of CaM inhibitors blocking Ca^{2+}_i-mediated effects on NHE activity have been made in human fibroblasts (Owen & Villereal, 1982), rat thymic lymphocytes (Grinstein *et al.*, 1985), NIH-3T3 cells (Owen *et al.*, 1989), rat parotid acinar cells (Manganel & Turner, 1990), hepatocytes (Tanaka *et al.*, 1994), rat brain capillary endothelial cells (Kawai *et al.*, 1995), and in the frog distal tubule (Cooper & Hunter, 1997). In addition, CaM inhibitors have been found to decrease NHE activity under unstimulated conditions in rat thymic lymphocytes (Grinstein *et al.*, 1985), cultured rat aortic smooth muscle cells (Little *et al.*, 1988), spontaneously beating rat heart cells (Weissberg *et al.*, 1989), rat isolated ventricular myocytes (Le Prigent *et al.*, 1997), rat astrocytes (Shrode *et al.*, 1997), and in red blood cells of patients with primary aldosteronism (Koren *et al.*, 1997). In contrast, CaM antagonists have been found to activate NHE-3 in a manner depending on the carboxy-terminal tail; although this result is superficially similar to observations made in the present study in rat hippocampal neurons, in the case of NHE-3 (*cf* hippocampal neurons; see above) the effect of CaM antagonists to activate the exchanger is mimicked by CaMK-II inhibitors (Fliegel *et al.*, 1992; Levine *et al.*, 1995).

In the cases of the NHE-1, NHE-2 and NHE-4 isoforms (*cf* NHE-3; see above), it has been suggested that CaM affects exchange activity by interacting with binding domains in the *C*-terminus of the transport protein. Initially, Bertrand *et al.* (1994) showed that CaM binds strongly to the cytoplasmic domain of NHE-1 at two neighboring regions: a high affinity ($K_d =$ ~20 nM) binding site at amino acids 636-656 (region *A*) and a low affinity ($K_d =$ ~350 nM) binding site at amino acids 664-684 (region *B*). Calmodulin-binding region *A* was found to be responsible for mediating Ca^{2+}_i-evoked changes in the pH$_i$-sensitivity of NHE-1 (Wakabayashi *et al.*, 1994). More recently, in NHE-1/NHE-3 chimeras expressed in PS120 cells, it was found that the CaM-binding *C*-terminus of NHE-1 could interact with the *N*-terminus of the intrinsically

CaM-insensitive NHE-3 to alter the pH_i-sensitivity of exchange activity in a CaM-dependent

manner (Wakabayashi *et al.*, 1995). Further studies identified 4 regulatory subdomains within

the amino acid sequence of the *C*-terminal of NHE-1 (see Wakabayashi *et al.*, 1997a): *domain I*

(a.a. 515-594), which contains potential phosphorylation sites that act to alter the pH_i-sensitivity

of the exchanger; *domain II* (a.a. 595-634), which is a flexible loop that appears to have no role

in the regulation of exchanger activity; *domain III* (a.a. 635-659), a CaM-binding '*auto-*

inhibitory' domain which, when not bound to CaM, acts to inhibit the activity of the NHE; and

domain IV (a.a. 660-815), which currently has no defined role in the regulation of exchange

activity (Ikeda *et al.* 1997). In light of these findings, a model of NHE regulation by CaM was

proposed (see Fig. 59A) in which it was suggested that, under unstimulated conditions, the '*auto-*

inhibitory' CaM-binding domain (III) directly interacts with the pH_i-sensor of NHE-1 such that

protons are prevented from binding to allosteric modifier sites and NHE activity is inhibited.

The formation of Ca^{2+}-CaM causes the '*auto-inhibitory*' domain to bind Ca^{2+}-CaM (at the high

affinity site), an effect which increases the affinity of the pH_i-sensor for H^+ and, in doing so,

relieves auto-inhibition and leads to increased NHE activity (see Robertson *et al.*, 1997;

Siczkowski *et al.*, 1997; Wakabayashi *et al.*, 1997a & 1997b).

It is tempting to speculate that similar mechanisms to those outlined in the preceding

paragraph might mediate the regulation of NHE activity by CaM in rat hippocampal CA1

neurons, in part because all NHE isoforms which have been examined to date are CaM-binding

proteins (Wakabayashi *et al.*, 1997b). Nevertheless, as noted above, the effect of CaM inhibition

to activate the NHE in rat hippocampal neurons is the opposite of the inhibition of NHE activity

evoked by CaM inhibitors in most cell types. Therefore, I suggest that CaM exerts an inhibitory

effect on the activity of the NHE in rat hippocampal CA1 neurons via an '*auto-stimulatory*'

domain (analogous to the '*auto-inhibitory*' domain of the NHE-1; see Fig. 59B). In quiescent

neurons, CaM is bound to the '*auto-stimulatory*' domain, which in turn interacts with the pH_i-

sensor of the exchange mechanism to inhibit NHE activity. The presence of CaM inhibitors

relieves the interaction of CaM with the '*auto-stimulatory*' domain, thereby decreasing the

interaction of the CaM binding domain with the pH_i-sensor. The result is an increased affinity of the NHE for H^+ and an increase in NHE activity.

In summary, the rise in SS pH_i evoked by CaM inhibitors in acutely dissociated adult rat hippocampal CA1 neurons is mediated, in large part, by activation of Na^+/H^+ exchange. Although NHE-3 is also activated by CaM inhibitors, in this case the effect appears to be mediated by CaMK-II. In contrast, CaMK-II inhibitors fail to affect SS pH_i or NHE activity in rat hippocampal neurons, suggesting the possibility that, in a manner analogous to that reported for NHE-1, NHE-2 and NHE-4, the activity of the NHE in rat hippocampal neurons may modulated directly by CaM.

3.4 Effects of CaM inhibitors on $[Ca^{2+}]_i$

Although it has previously been reported that CaM inhibitors may affect $[Ca^{2+}]_i$ (e.g. Inagaki et al., 1983), the present study is the first to assess the potential contribution of these changes in $[Ca^{2+}]_i$ to the changes in SS pH_i evoked by CaM inhibitors. The results indicate that CaM-inhibitor evoked changes in $[Ca^{2+}]_i$ can lead to concomitant changes in SS pH_i; however, this effect is not mediated by changes in the activities of Na^+-dependent pH_i regulating mechanisms, specifically the NHE. Alternative possibilities include: (1) the exchange of Ca^{2+} ions for protons at shared internal binding sites. As H^+ and Ca^{2+} are known to compete for internal binding sites, a fall in $[Ca^{2+}]_i$ might evoke a rise in SS pH_i (see Meech & Thomas, 1977 & 1980). However, using the relationship between changes in $[Ca^{2+}]_i$ and $[H^+]_i$ established by Meech & Thomas (1980) in voltage-clamped snail neurons (and adjusting it to reflect the value of β_i in rat hippocampal CA1 neurons; see Bevensee et al., 1996), I estimate that the ~40 nM decrease in $[Ca^{2+}]_i$ evoked by W-7 in the absence of Na^+_o (replaced with $NMDG^+$) should evoke a ~ 0.0009 nM decrease in $[H^+]_i$ (i.e. a 0.000004 pH unit increase in SS pH_i). These calculations, which are comparable to those made by Mullins et al. (1983) in squid axons, indicate that the exchange of Ca^{2+} ions and protons at internal binding sites is unlikely to be able to account for the rise in pH_i evoked by W-7 under Na^+_o-free, $NMDG^+$-substituted conditions (a 0.06 ± 0.02 pH

unit increase); and (2) alterations in the activity of the plasmalemmal Ca^{2+}/H^+-ATPase which has been identified in rat hippocampal CA1 pyramidal neurons (Trapp *et al.*, 1996). The W-7-evoked fall in $[Ca^{2+}]_i$ could stimulate reverse Ca^{2+}/H^+ exchange (see Paalasmaa & Kaila, 1996; Trapp *et al.*, 1996) such that Ca^{2+} influx is coupled to H^+ efflux, resulting in a rise in SS pH_i. Obviously, additional experiments are required to understand the mechanistic basis of the fall in $[Ca^{2+}]_i$ evoked by W-7.

3.5 Summary

In summary, CaM inhibitors increase SS pH_i in acutely dissociated adult rat hippocampal CA1 neurons in a manner which is dependent upon resting pH_i. This effect reflects an increase in the activity of the acid-extruding NHE and, by analogy with other NHE isoforms, may be mediated by a direct interaction of CaM with the exchange protein. The exact relationship between CaM and the NHE isoform present in hippocampal neurons, however, remains to be determined. The effect of CaM inhibitors to modulate NHE activity is independent of changes in the activity of the cAMP/PKA pathway, indicating that, as in non-neuronal cell types, the NHE in rat hippocampal CA1 neurons can be regulated independently by multiple intracellular second messenger systems.

4. The potential roles of changes in pH_i evoked by modulation of the cAMP/PKA and CaM pathways in neuronal function and dysfunction

The possible functional significance of the present findings remains unclear. However, given the established ability of changes in pH_i to modulate neuronal function (see Introduction), the finding that a NIAE participates in the regulation of pH_i in acutely dissociated adult rat hippocampal CA1 neurons is likely to have physiological implications. In addition, the magnitudes of the changes in pH_i evoked by modulating the activities of the cAMP/PKA and CaM second messenger systems are consistent with the possibility that, as in peripheral cell types, second messenger-mediated modulation of the activities of neuronal pH_i regulating

mechanisms (and, thus, pH_i) may represent a physiologically-relevant transmembrane signalling pathway. Furthermore, because pH_i transients are less buffered than $[Ca^{2+}]_i$ transients, it has been suggested that pH_i changes may affect targets over greater spatial areas within the cell than do $[Ca^{2+}]_i$ changes (Morris *et al.*, 1994). It should also be noted that changes in the activities of pH_i regulating mechanisms will affect not only $[H^+]_i$ and $[HCO_3^-]_i$ but also $[Na^+]_i$ and/or $[Cl^-]_i$. Changes in the concentrations of the latter ionic species may have profound effects on neuronal function (*e.g.* Kimura & Aviv, 1993; Chidekel *et al.*, 1997; Yu & Salter, 1998).

Changes in pH_i consequent upon second messenger-evoked changes in the activities of pH_i regulating mechanisms will also inevitably affect the pH of the microenvironment (*e.g.* Krishtal *et al.*, 1987; Gottfried & Chesler, 1994). The changes in pH_o may, in turn, affect not only the activities of pH_i regulating mechanisms in adjacent cells but also neuronal excitability, given the established sensitivity of neuronal voltage- and ligand-gated ion channels to changes in pH_o (*e.g.* Tang *et al.*, 1990; Traynelis & Cull-Candy, 1990; Vyklický *et al.*, 1990; Tombaugh & Somjen, 1996). Another potential role for this external 'signalling mechanism' includes the regulation of $[HCO_3^-]_o$. $GABA_A$ receptor-mediated excitatory coupling amongst GABAergic interneurons in hippocampal slices has been shown to be largely dependent on the availability of $HCO_3^-{}_i$ (Lamsa & Kaila, 1997). Coupling of interneurons leads to the formation of interneuronal networks which promote long lasting excitatory responses in hippocampal pyramidal neurons and, thus, can accentuate epileptiform activity and enhance long term potentiation (Louvel *et al.*, 1994; Taira *et al.*, 1995; Avoli *et al.*, 1996; Higashima *et al.*, 1996; see below). The present study indicates that, in neurons with low resting pH_i values, stimulation of the cAMP/PKA pathway increases the activity of the NIAE and decreases the activity of the NDAE, events which may promote the accumulation of HCO_3^- in the interstitial space. Such an increase in $[HCO_3^-]_o$ would potentially promote CO_2 entry and a subsequent rise in $[HCO_3^-]_i$ within interneurons, thus promoting the coupling of interneurons.

The roles of the cAMP/PKA and CaM/CaMK-II pathways in the control of neuronal function under both physiological and pathophysiological conditions have been extensively

investigated and the possibility exists that changes in pH_i may contribute to some of the effects of modulating the activity of these second messenger systems on neuronal function.

First, the CA1 subfield of the rat hippocampus expresses many receptor subtypes for various neurotransmitters and neuromodulators that are linked to the cAMP/PKA and CaM/CaMK-II signal transduction cascades (reviewed by Nicoll *et al.*, 1990). For example, stimulation of the cAMP/PKA pathway occurs in response to β-adrenoceptor activation (Segal *et al.*, 1981; Limbird, 1988; Dunwiddie *et al.*, 1992) whereas inhibition of the pathway occurs in response to 5-HT_{1A} (serotonin) receptor activation (Harrington *et al.*, 1988). Although the potential roles of changes in pH_i to neuronal responses mediated by these neurotransmitter and neuromodulator receptor subtypes have not been examined in rat hippocampal CA1 neurons, it is apparent that many of the effects on neuronal excitability and function elicited by their activation can be mimicked by changes in pH_i. Thus, for example, β-adrenoceptor stimulation leads to the activation of L- and N-type high voltage-activated Ca^{2+} channels in mammalian central neurons (*e.g.* Gray & Johnston, 1987; Fisher & Johnston, 1990; Kavalali *et al.*, 1997); interestingly, the same subtypes of high voltage-activated Ca^{2+} channels are activated by increases in pH_i (Tombaugh & Somjen, 1997).

Second, changes in the activity of the cAMP/PKA pathway are known to affect synaptic transmission in the hippocampus. Thus, for example, β-adrenoceptor agonists, forskolin and IBMX increase synaptic strength in the CA1 region of the hippocampal slice (*e.g.* Chavez-Noriega & Stevens, 1992; Dunwiddie *et al.*, 1992; Raman *et al.*, 1996), an effect also observed during increases in pH_i (see Church & McLennan, 1989; Jarolimek *et al.*, 1989). β-adrenoceptor agonists, increases in $[cAMP]_i$ and the activation of PKA also facilitate the induction of LTP (*e.g.* see Audesirk *et al.*, 1997; Bolshakov *et al.*, 1997), whereas inhibition of the cAMP/PKA pathway plays a role in the early maintenance and/or expression of long term depression (LTD) in the CA1 region of the adult hippocampus (Brandon *et al.*, 1995; Thiels *et al.*, 1998). Second messenger-evoked increases in pH_i may play a role in the induction and/or maintenance of LTP because stabilization of long term memory requires the synthesis of new proteins and RNAs

(Davis & Squire, 1984; Matthies, 1989;) and it is generally accepted that elevated pH_i accommodates increased protein synthesis and DNA transcription (reviewed by Busa & Nuccitelli, 1984; Grinstein *et al.*, 1989; also see Bierman *et al.*, 1987); a pH_i value of ~7.45 is optimal for protein synthesis (Aerts *et al.*, 1985). Interestingly, increases in pH_i are also known to increase the activities of adenylyl cyclases. Furthermore, the activity of most phosphodiesterases is optimal near pH 7.2, and acidic or alkaline shifts in pH decrease phosphodiesterase activity. Therefore, an increase in pH_i to >~7.2 would cause a net increase in $[cAMP]_i$ (Hjemdahl & Fredholm, 1977). In rat hippocampal CA1 neurons, stimulation of the cAMP/PKA pathway caused SS pH_i to increase, an effect which might further enhance cAMP production; this potential positive feedback mechanism may contribute to the maintenance of LTP. The binding of Ca^{2+} to CaM is also extremely pH dependent; assuming that $[Ca^{2+}]_i$ is ~100 nM, ~50% of the available Ca^{2+}_i would be bound to CaM at pH_i 7.0, whereas at pH_i 7.5 ~83% would form the Ca^{2+}-CaM complex (Tkachuk & Men'shikov, 1981). Increasing pH_i from 7.0 to 8.0 also increases the ratio of soluble (unbound) CaM to total (Ca-bound) CaM (Wilson & Gillette, 1985). Thus, in rat hippocampal CA1 neurons, the effect of CaM inhibitors to increase SS pH_i would lead to an increase in the affinity of CaM for Ca^{2+}, a potential feedback mechanism which might act to maintain $[Ca^{2+}$-CaM].

Third, increases in pH_i have long been associated with enhanced growth and neoplastic formation in various cell types (reviewed by Harguindey *et al.*, 1995). Recently, Edmonds *et al.* (1995) found that actin filament cross-linking is highly pH_i sensitive, suggesting that pH_i is a potent modulator of the cytoarchitecture. In fact, all isoforms of the NIAE (AE1 - 3) are known to act as a membrane anchors for actin, ankyrin and spectrin cytoskeletal proteins (see Alper, 1994). Similarly, the NHE and NHE-RF have been shown to co-localize and/or bind with cytoskeletal structures such as vinculin, talin and α-actin (reviewed by Grinstein *et al.*, 1992; Orlowski & Grinstein, 1997; Murthy *et al.*, 1998), and have also been shown to have PDZ-binding motifs (suggesting that these proteins may mediate clustering of ion channels and/or junctional proteins; reviewed by Orlowski & Grinstein, 1997). Given the aforementioned facts, it

is tempting to speculate that changes in pH_i may participate in some of the more persistent effects of changing $[cAMP]_i$ on neuronal development.

Fourth, the cAMP/PKA pathway and changes in $[Ca^{2+}]$ are involved in pathophysiological events in the central nervous system. For example, β-adrenoceptor stimulation and the consequent activation of PKA enhances ischemia-induced neuronal damage in the hippocampus (*e.g.* Shibata *et al.*, 1992; also see Wieloch *et al.*, 1996). That increases in pH_i might participate in this effect is suggested by the fact that stimulation of Na^+/H^+ exchange activity and internal alkalinizations are known to be detrimental to neuronal survival following ischemia (*e.g.* Vornov *et al.*, 1996). In addition to changes in pH_i (*e.g.* Wang *et al.*, 1994; Ou-yang *et al.*, 1995), activation of NMDA and AMPA receptors by glutamate (as occurs during ischemia) leads to a potentially detrimental accumulation of $[Cl^-]_i$ and neuronal swelling in the hippocampus (Inglefield & Schwartz-Bloom, 1998). Findings from the present study suggest two possible mechanisms that may contribute to these events: (1) PKA-evoked stimulation of NIAE activity and inhibition of NDAE activity (observed in neurons with low pH_i values) may lead to the accumulation of $[Cl^-]_i$; and (2) stimulation of PKA also increases the activity of the NHE, which would promote the accumulation of Na^+_i and neuronal swelling. Finally, Jefferys & Haas (1982) reported that epileptiform activity can be induced in the CA1 region of hippocampal slices by exposure to calcium-free media, an effect which has recently been found to reflect enhanced gap junctional coupling between CA1 pyramidal neurons (Perez-Velazquez *et al.*, 1994). Given the fact that increases in pH_i are also known to increase gap junctional conductances and promote epileptiform activity in the hippocampus (see Church & Baimbridge, 1991), the increase in pH_i observed during CaM inhibition may be causally related to the genesis of epileptiform activity under Ca^{2+}_o-free conditions.

5. Summary

Although pH_i has been shown to be an important mediator of neuronal function and excitability, relatively few studies have examined either the regulation of the activities of pH_i

regulating mechanisms or the integrated control of SS pH_i by these mechanisms in mammalian neurons. This report is the first to describe the participation of a DIDS-sensitive NIAE in alkali extrusion from rat hippocampal neurons. Acting in concert with acid-extruding mechanisms (*i.e.* the NDAE and NHE), the NIAE also functions to maintain SS pH_i near the external pH value. In addition, the present study is the first to report that: (1) the activities of three pH_i regulating mechanisms (*i.e.* the NIAE, NDAE and NHE) can be altered concomitantly by a single second messenger system (*i.e.* the cAMP/PKA pathway) in a mammalian central neuron; and (2) the activity of the NHE present in rat hippocampal neurons can be modulated by more than one intracellular second messenger system (*i.e.* by the cAMP/PKA pathway and CaM). The exact mechanisms whereby these second messenger systems act to alter the activities of pH_i regulating mechanisms, however, remains to be determined. Activation of surface receptor subtypes linked to the cAMP/PKA and CaM/CaMK-II signal transduction cascades modulates neuronal function and excitability in the CA1 region of the hippocampus. It seems possible that the second messenger control of the activities of pH_i regulating mechanisms (and, thus, SS pH_i) described in the present study may potentially mediate some of the effects of neurotransmitters and neuromodulators on neuronal excitability and function in rat hippocampal CA1 neurons. Further investigation, however, is necessary to directly assess the effects of second messenger mediated changes in pH_i on neuronal function under both physiological and pathophysiological conditions.

Figure 57. Proposed contributions of the NHE, NIAE and NDAE, and the HCO_3^-/CO_2-buffering system, to the maintenance of SS pH_i in acutely dissociated adult rat hippocampal CA1 neurons.

Dark circles represent acid-extruding, Na^+-dependent membrane-bound ion exchange mechanisms that function to increase SS pH_i; conversely, open circles represent acid-loading, Na^+-independent membrane-bound ion exchange mechanisms that function to decrease SS pH_i. The HCO_3^-/CO_2 open buffering system functions both within the neuronal cytosol and in the external medium, although only the passive diffusion of CO_2 is illustrated within the neuron. Thus, HCO_3^--dependent pH_i regulating mechanisms are shown on the right and HCO_3^--independent mechanisms (the NHE) are shown to the left. Arrows that overlay the illustrated exchange mechanism show the relative contribution of the exchanger to the maintenance of SS pH_i under the conditions identified on the Figure. The NDAE is shown as a $NaCO_3^-/Cl^-$-exchanger (see Boron, 1985; Boron & Knakal, 1989). CA, carbonic anhydrase.
A Under physiological, HCO_3^-/CO_2-buffered conditions, in neurons with low NHE activity, the activities of the NHE and NIAE contribute relatively little to the maintenance of SS pH_i. Thus, to maintain SS pH_i near pH_o, the activity of the NDAE is relatively high and the HCO_3^-/CO_2 open buffering system establishes an equilibrium across the neuronal membrane. **B** Conversely, under physiological, HCO_3^-/CO_2-buffered conditions, in neurons with high NHE activity, the activity of the NHE and NIAE strongly contribute to maintenance of SS pH_i, as does the HCO_3^-/CO_2 open buffering system which establishes an equilibrium across the neuronal membrane. The activity of the NDAE, however, contributes little to SS pH_i regulation under these conditions. The net effect of the combined activities of the NHE, NIAE, NDAE and HCO_3^-/CO_2 open buffering system is to maintain pH_i near pH_o.

A

Neurons with low NHE activity
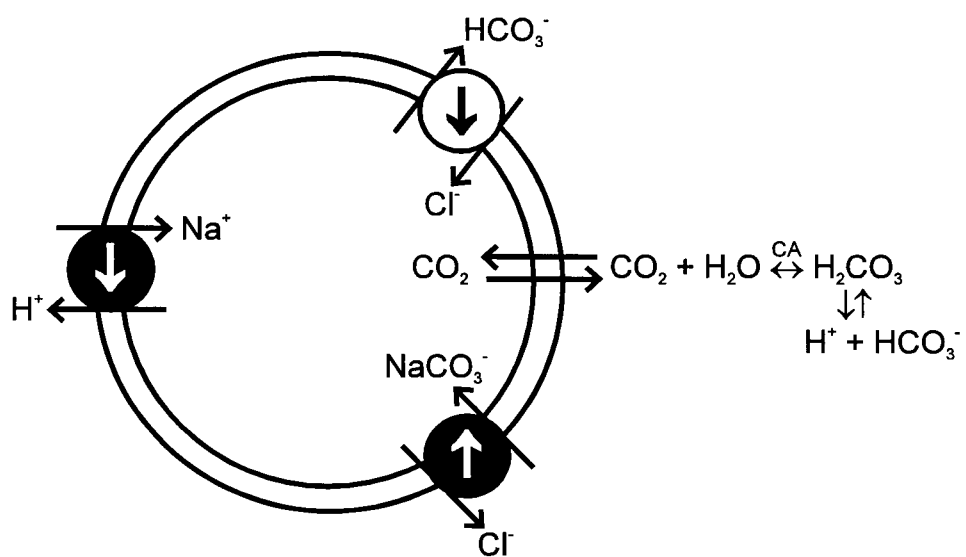
B

Neurons with high NHE activity
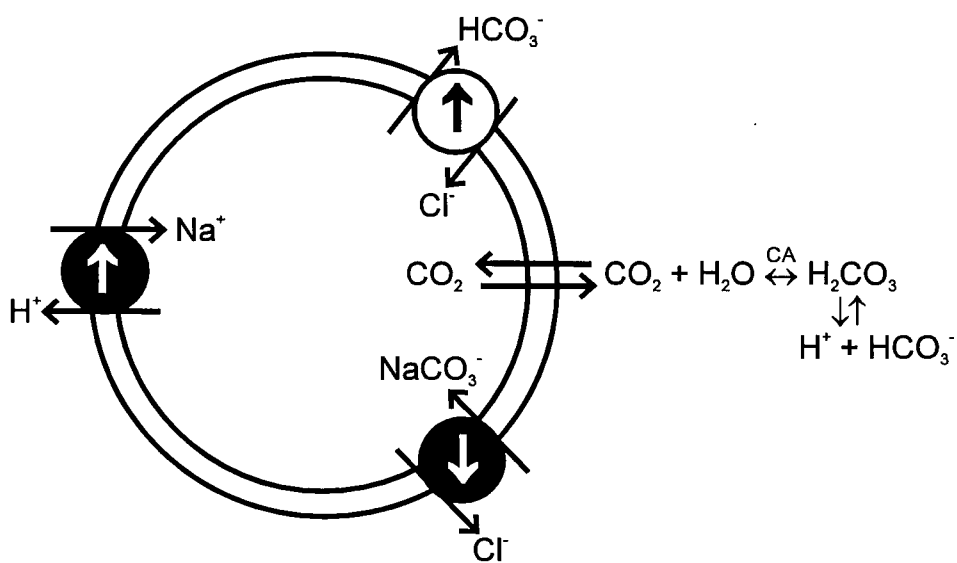
Figure 58. cAMP/PKA-mediated changes in the activities of the NHE, NIAE and NDAE in acutely dissociated adult rat hippocampal CA1 neurons.

Dark circles represent acid-extruding, Na^+-dependent membrane-bound ion exchange mechanisms that function to increase SS pH_i; conversely, open circles represent acid-loading, Na^+-independent membrane-bound ion exchange mechanisms that function to decrease SS pH_i. HCO_3^--dependent pH_i regulating mechanisms are shown to the right and HCO_3^--independent mechanisms (the NHE) are shown to the left. (+) and (-) indicate a cAMP/PKA-evoked increase and decrease in ion exchange activity, respectively. The NDAE is shown as a $NaCO_3^-/Cl^-$-exchanger (see Boron, 1985; Boron & Knakal, 1989).

A In neurons with low resting pH_i values, the activities of the NHE and NIAE are increased upon stimulation of the cAMP/PKA pathway; conversely, the activity of the NDAE is decreased, resulting in a net increase in SS pH_i. *B* In neurons with high resting pH_i values, the activities of the NHE and (potentially) the NDAE are increased upon stimulation of the cAMP/PKA pathway; conversely, the activity of the NIAE is decreased, resulting in a net increase in SS pH_i. *C* In neurons with low resting pH_i values, the activity of the NDAE is increased, the activity of the NIAE is decreased, and the activity of the NHE is unaffected upon inhibition of the cAMP/PKA pathway, resulting in a net increase in SS pH_i. *D* In neurons with high resting pH_i values, the activity of the NIAE is increased, the activity of the NDAE is (potentially) decreased, and the activity of the NHE is unaffected upon inhibition of the cAMP/PKA pathway, resulting in a net decrease in SS pH_i. The net effect of stimulation of the cAMP/PKA pathway on the combined activities of the NHE, NIAE and NDAE is to increase pH_i (to a SS level > pH_o), whereas the net effect of inhibition of the cAMP/PKA pathway on the combined activities of the NHE, NIAE and NDAE is to 'clamp' pH_i at pH_o.

↑cAMP/PKA   ↓cAMP/PKA

↓resting pH$_i$:

A   ↑pH$_i$

C   ↑pH$_i$

↑resting pH$_i$:

B   ↑pH$_i$

D   ↓pH$_i$

Net Effect:

pH$_i$ > pH$_o$ Clamp pH$_i$ to pH$_o$
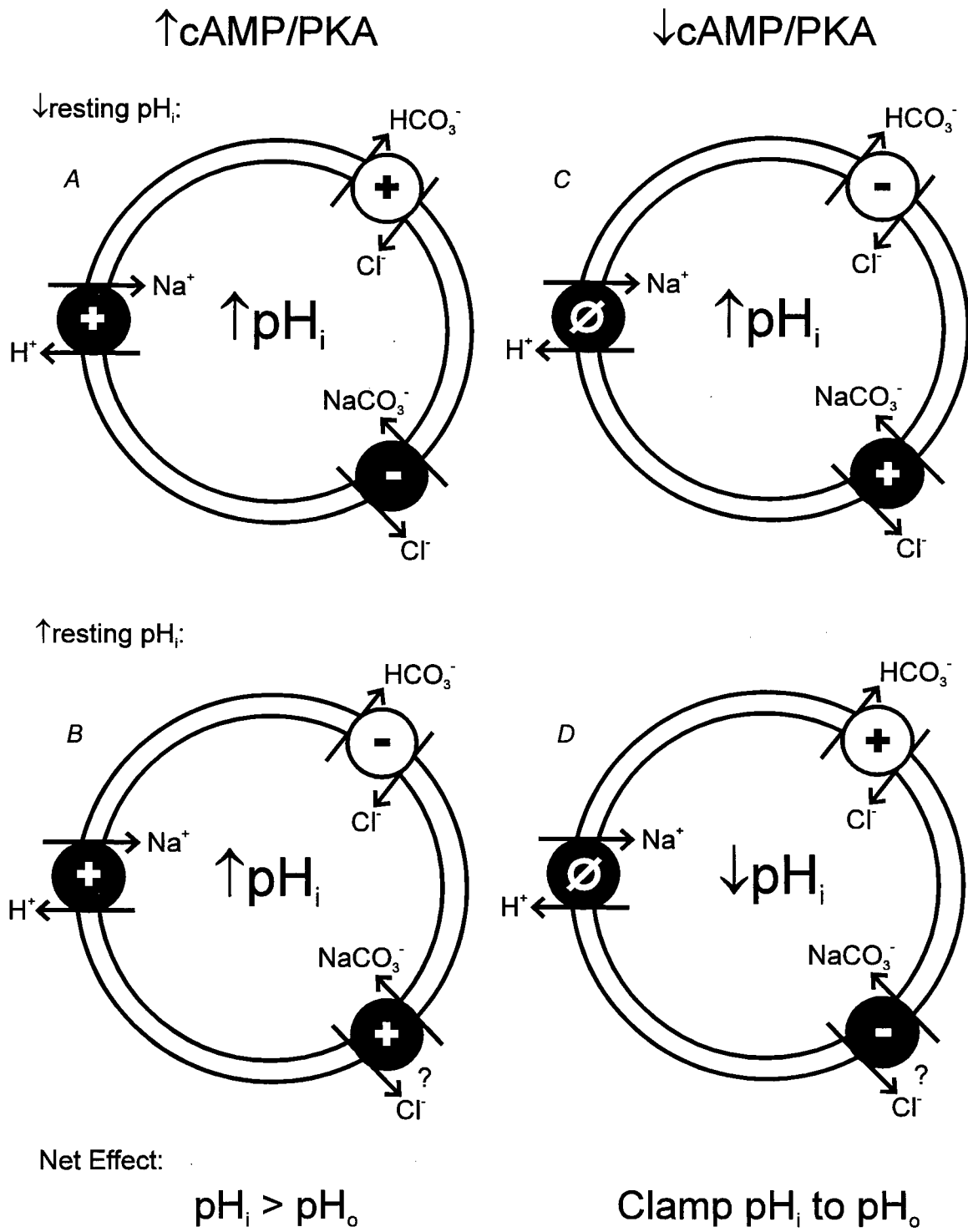
Figure 59. Models of CaM-dependent control of the activities of NHE-1 and the NHE present in rat hippocampal CA1 neurons.

The dark circles represent the ion transporting, transmembrane N-terminal of the NHEs (N_t), which contain a pH_i-sensing domain on the cytosolic surface which binds H^+_i when accessible. The cytosolic, activity regulating C-terminus of the NHE (C_t; a.a. ~500 - 815) contains a high affinity CaM-binding site (CaM BS, shown as a open rectangle) between a.a. 636 - 656.

A Under quiescent conditions, the activity of the NHE-1 is low because the CaM-binding '*auto-inhibitory*' domain interacts with the pH_i sensor preventing H^+-binding. When the formation of Ca^{2+}-CaM is promoted, active Ca^{2+}-CaM binds to the '*auto-inhibitory*' domain, causing a conformational change which exposes the pH_i sensor to the cytosol. This leads to an increase in the affinity of the NHE for H^+_i and an increase in the activity of the NHE-1. This figure was taken and modified from Wakabayashi *et al.* (1997a). **B** Under quiescent conditions in rat hippocampal CA1 neurons, the activity of the NHE may be low because a CaM-binding '*auto-stimulatory*' domain is bound to active Ca^{2+}-CaM and, thus, interacts with the pH_i sensor preventing H^+-binding. In the presence of CaM inhibitors, CaM dissociates from the '*auto-stimulatory*' domain which exposes the pH_i sensor to the cytosol and the affinity of the NHE for H^+_i is increased, leading to an increase in the activity of the NHE in rat hippocampal CA1 neurons.
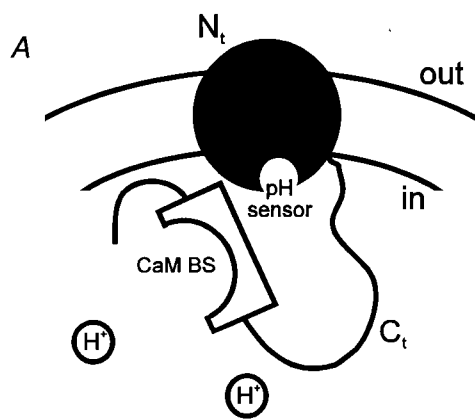
Quiescent
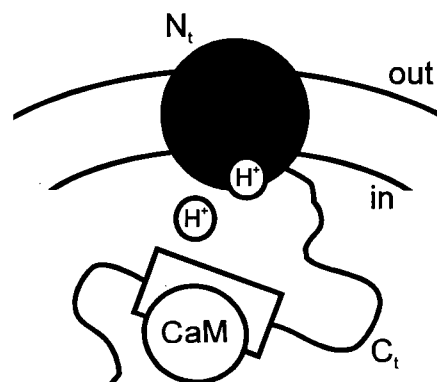
Ca²⁺-stimulated
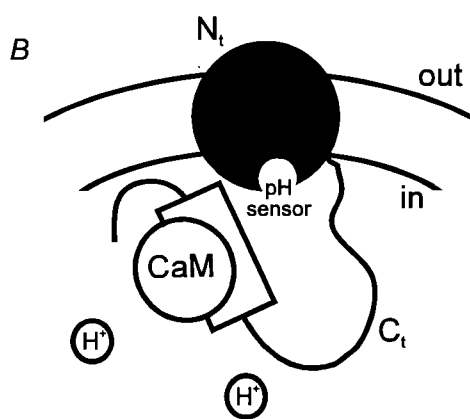
Quiescent
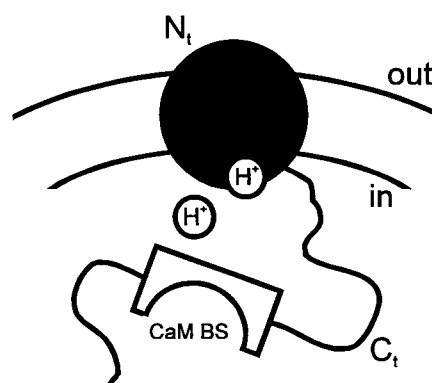
CaM-inhibition

REFERENCES

Abdel-Hamid, K.M. (1994) Ph. D. Thesis. Department of Physiology, University of British Columbia.

Aerts, R.J., Durston, A.J. & Moolenaar, W.H. (1985) Cytoplasmic pH and the regulation of the Dictyostelium cell cycle. *Cell* **43**, 653-657.

Aickin, C.C. (1984) Direct measurement of intracellular pH and buffering power in smooth muscle cells of guinea-pig vas deferens. *Journal of Physiology* **349**, 571-585.

Aickin, C.C. & Brading, A.F. (1984) The role of chloride-bicarbonate exchange in the regulation of intracellular chloride in guinea-pig vas deferens. *Journal of Physiology* **349**, 587-606.

Aickin, C.C. & Thomas, R.C. (1976) Intracellular pH of mouse soleus muscle. *Journal of Physiology* **260**, 25P-26P.

Alper, S.L., Stuart-Tilley, A., Simmons, C.F., Brown, D. & Drenckhahn, D. (1994) The fodrin-ankyrin cytoskeleton of choroid plexus preferentially colocalizes with apical Na^+/K^+-ATPase rather than with basolateral anion exchanger AE2. *Journal of Clinical Investigation* **93**, 1430-1438.

Alpini, G., Glaser, S., Robertson, W., Rodgers, R.E., Phinizy, J.L., Lasater, J. & LeSage, G.D. (1997) Large but not small intrahepatic bile ducts are involved in secretin-regulated ductal bile secretion. *American Journal of Physiology* **272**, G1064-74.

Alvaro, D., Della Guardia, P., Bini, A., Gigliozzi, A., Furfaro, S, La Rosa, T., Piat, C. & Capocaccia, L. (1995) Effect of glucagon on intracellular pH regulation in isolated rat hepatocyte couplets. *Journal of Clinical Investigation* **96**, 665-675.

Alvaro, D., Alpini, G., Jezequel, A.M., Bassotti, C., Francia, C., Fraioli, F., Romeo, R., Marucci, L., Le Sage, G., Glaser, S.S. & Benedetti, A. (1997) Role and mechanisms of action of acetylcholine in the regulation of rat cholangiocyte secretory functions. *Journal of Clinical Investigation* **100**, 1349-1362.

Amos, B.J. & Richards, C.D. (1994) Effect of glutamate receptors on intracellular pH of rat neurons maintained in culture. *Journal of Physiology* **479.P**, 43P.

Amos, B.J., Mathie, A. & Richards, C.D. (1998) Activation of group I metabotropic glutamate receptors elicits pH changes in cultured rat cortical glia and neurons. *Neuroscience* **86**, 1109-1120.

Andreeva, N., Khodorov, B., Stelmashook, E., Sokolova, S., Cragoe, E. Jr. & Victorov, I. (1992) 5-(*N*-ethyl-*N*-isopropyl)-amiloride and mild acidosis protect cultured cerebellar granule cells against glutamate-induced delayed neuronal death. *Neuroscience* **49**, 175-181.

Aniksztejn, L., Catarsi, S. & Drapeau, P. (1997) Channel modulation by tyrosine phosphorylation in an identified leech neuron. *Journal of Physiology* **498**, 135-142.

Aronson, P.S., Suhm, M.A. & Nee, J. (1983) Interaction of external H^+ with the Na^+-H^+ exchanger in renal microvillus membrane vesicles. *Journal of Biological Chemistry* **258**, 6767-6771.

Aronson, P.S. (1985) Kinetic properties of the plasma membrane Na^+-H^+ exchanger. *Annual Review of Physiology* **47**, 545-560.

Aronson, P.S. (1989) The renal proximal tubule: A model for diversity of anion exchangers and stilbene-sensitive anion transporters. *Annual Review of Physiology* **51**, 419-441.

Attaphitaya, S., Park, K. & Melvin, J.E. (1999) Molecular cloning and functional expression of rat Na^+/H^+ exchanger (NHE5) highly expressed in brain. *Journal of Biological Chemistry* **274**, 4383-4388.

Audesirk, G., Cabell, L. & Kern, M. (1997) Modulation of neurite branching by protein phosphorylation in cultured rat hippocampal neurons. *Developmental Brain Research* **86**, 247-260.

Avoli, M., Louvel, J., Kurcewicz, I., Pumain, R. & Barbarosie, M. (1996) Extracellular free potassium and calcium during synchronous activity induced by 4-aminopyridine in the juvenile rat hippocampus. *Journal of Physiology* **493**, 707-717.

Azarani, A., Goltzman, D. & Orlowski, J. (1996) Structurally diverse N-terminal peptides of parathyroid hormone (PTH) and PTH-related peptide (PTHRP) inhibit the Na^+/H^+ exchanger NHE3 isoform by binding to the PTH/PTHRP receptor type I and activating distinct signaling pathways. *Journal of Biological Chemistry* **271**, 14931-14936.

Baird, N.R., Orlowski, J. Szabo, E.Z., Zaun, H.C., Schultheis, P.J., Menon, A.G. & Shull, G.E. (1999) Molecular cloning, genomic organization, and functional expression of Na^+/H^+ exchanger isoform 5 (NHE5) from human brain. *Journal of Biological Chemistry* **274**, 4377-4382.

Balestrino, M. & Somjen, G.G. (1988) Concentration of carbon dioxide, interstitial pH and synaptic transmission in hippocampal formation of the rat. *Journal of Physiology* **396**, 247-266.

Barber, D.L. & Ganz, M.B. (1992) Guanine nucleotides regulate β-adrenergic activation of Na^+-H^+ exchange independently of receptor coupling to G_s. *Journal of Biological Chemistry* **267**, 20607-20612.

Baxter, K.A. (1995) M. Sc. Thesis. Department of Anatomy, University of British Columbia.

Baxter, K.A. & Church, J. (1996) Characterization of acid extrusion mechanisms in cultured fetal rat hippocampal neurones. *Journal of Physiology* **493**, 457-470.

Bazaes, S.E. & Kemp, R.G. (1990) Resistance of brain phosphofructo-1-kinase to pH-dependent inhibition. *Metabolic Brain Disease* **5**, 111-118.

Benedetti, A., Strazzabosco, M., Ng, O.C. & Boyer, J.L. (1994) Regulation of activity and apical targeting of the Cl^-/HCO_3^- exchanger in rat hepatocytes. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 792-796.

Bertrand, B., Wakabayashi, S., Ikeda, T., Pouysségur, J. & Shigekawa, M. (1994) The Na^+/H^+ exchanger isoform 1 (NHE1) is a novel member of the calmodulin-binding proteins. Identification and characterization of calmodulin-binding sites. *Journal of Biological Chemistry* **269**, 13703-13709.

Bevans, C.G. & Harris, A.L. (1999) Regulation of connexin channels by pH. *Journal of Biological Chemistry* **274**, 3711-3719.

Bevensee, M.O., Schwiening, C.J. & Boron, W.F. (1995) Use of BCECF and propidium iodide to assess membrane integrity of acutely isolated CA1 neurons from rat hippocampus. *Journal of Neuroscience Methods* **58**, 61-75.

Bevensee, M.O., Cummins, T.R., Haddad, G.G., Boron, W.F. & Boyarsky, G. (1996) pH regulation in single CA1 neurons acutely isolated from the hippocampi of immature and mature rats. *Journal of Physiology* **494**, 315-328.

Bevensee, M.O., Apkon, M. & Boron, W.F. (1997) Intracellular pH regulation in cultured astrocytes from rat hippocampus. II. Electrogenic Na^+/HCO_3^- cotransport. *Journal of General Physiology* **110**, 467-483.

Bianchini, L., Woodside, M., Sardet, C., Pouysségur, J., Takai, A & Grinstein, S. (1991) Okadaic acid, a phosphatase inhibitor, induces activation and phosphorylation of the Na^+/H^+ antiport. *Journal of Biological Chemistry* **266**, 15406-15413.

Bianchini, L., L'Allemain, G. & Pouysségur, J. (1997) The p42/p44 mitogen-activated protein kinase cascade is determinant in mediating activation of the Na^+/H^+ exchanger (NHE1 isoform) in response to growth factors. *Journal of Biological Chemistry* **272**, 271-279.

Bianchini, L. & Pouysségur, J. (1996) Regulation of the Na^+/H^+ exchanger isoform NHE1: Role of phosphorylation. *Kidney International* **49**, 1038-1041.

Bierman, A.J., Tertoolen, L.G., de Laat, S.W. & Moolenaar, W.H. (1987) The Na^+/H^+ exchanger is constitutively activated in P19 embryonal carcinoma cells, but not in a differentiated

derivative. Responsiveness to growth factors and other stimuli. *Journal of Biological Chemistry* **262**, 9621-9628.

Bolshakov, V.Y., Golan, H., Kandel, E.R. & Siegelbaum, S.A. (1997) Recruitment of new sites of synaptic transmission during the cAMP-dependent late phase of LTP at CA3-CA1 synapses in the hippocampus. *Neuron* **19**, 635-651.

Bonnet, U., Wiemann, M. & Bingmann, D. (1998) CO_2/HCO_3^--withdrawal from the bath medium of hippocampal slices: Biphasic effect on intracellular pH and bioelectric activity of CA3 neurons. *Brain Research* **796**, 161-170.

Bookstein, C., Musch, M.W., DePaoli, A., Xie, Y., Rabenau, K., Villereal, M., Rao, M.C. & Chang, E.B. (1996) Characterization of the rat Na^+/H^+ exchanger isoform NHE4 and localization in rat hippocampus. *American Journal of Physiology* **271**, C1629-38.

Borgese, F., Sardet, C., Cappadoro, M., Pouysségur, J. & Motais, R. (1992) Cloning and expression of a cAMP-activated Na^+/H^+ exchanger: Evidence that the cytoplasmic domain mediates hormonal regulation. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 6765-6769.

Borgese, F., Malapert, M., Fievet, B., Pouysségur, J. & Motais, R. (1994) The cytoplasmic domain of the Na^+/H^+ exchangers (NHEs) dictates the nature of the hormonal response: Behavior of a chimeric human NHE1/trout β-NHE antiporter. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 5431-5435.

Boron, W.F. (1977) Intracellular pH transients in giant barnacle muscle fibers. *American Journal of Physiology* **233**, C61-73.

Boron, W.F. (1989) Cellular buffering and intracellular pH. In: *The Regulation of Acid-Base Balance*, pp. 33-56. Eds. D.W. Seldin & G. Giebisch. Raven Press.

Boron, W.F., Russell, J.M., Brodwick, M.S., Keifer, D.W. & Roos, A. (1978) Influence of cyclic AMP on intracellular pH regulation and chloride fluxes in barnacle muscle fibers. *Nature* **276**, 511-513.

Boron, W.F. (1985) Intracellular pH-regulating mechanism of the squid axon. Relation between the external Na^+- and HCO_3^--dependences. *Journal of General Physiology* **85**, 325-345.

Boron, W.F. & De Weer, P. (1976) Intracellular pH transients in squid giant axons caused by CO_2, NH_3, and metabolic inhibitors. *Journal of General Physiology* **67**, 91-112.

Boron, W.F. & Knakal, R.C. (1989) Intracellular pH-regulating mechanism of the squid axon. Interaction between DNDS and extracellular Na^+ and HCO_3^-. *Journal of General Physiology* **93**, 123-150.

Botelho, L.H., Webster, L.C., Rothermel, J.D., Baraniak, J. & Stec, WJ. (1988) Inhibition of cAMP-dependent protein kinase by adenosine cyclic 3'-, 5'-phosphorodithioate, a second cAMP antagonist. *Journal of Biological Chemistry* **263**, 5301-5305.

Boyarsky, G., Ganz, M.B., Sterzel, R.B. & Boron, W.F. (1988a) pH regulation in single glomerular mesangial cells. I. Acid extrusion in absence and presence of HCO_3^-. *American Journal of Physiology* **255**, C844-56.

Boyarsky, G., Ganz, M.B., Sterzel, R.B. & Boron, W.F. (1988b) pH regulation in single glomerular mesangial cells. II. Na^+-dependent and -independent $Cl^--HCO_3^-$ exchangers. *American Journal of Physiology* **255**, C857-69.

Brandon, E.P., Zhuo, M., Huang, Y.Y., Qi, M., Gerhold, K.A., Burton, KA, Kandel, E.R., McKnight, G.S. & Idzerda, R.L. (1995) Hippocampal long-term depression and depotentiation are defective in mice carrying a targeted disruption of the gene encoding the RI β subunit of cAMP-dependent protein kinase. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 8851-8855.

Bright, G.R., Fisher, G.W., Rogowska, J. & Taylor, D.L. (1987) Fluorescence ratio imaging microscopy: Temporal and spatial measurements of cytoplasmic pH. *Journal of Cell Biology* **104**, 1019-1033.

Bright, G.R., Fisher, G.W., Rogowska, J. & Taylor, D.L. (1989) Fluorescence ratio imaging microscopy. *Methods in Cell Biology* **30**, 157-192.

Busa, W.B. & Nuccitelli, R. (1984) Metabolic regulation via intracellular pH. *American Journal of Physiology* **246**, R409-38.

Busch, S., Wieland, T., Esche, H., Jakobs, K.H. & Siffert, W. (1995) G protein regulation of the Na^+/H^+ antiporter in *Xenopus laevis* oocytes. Involvement of protein kinases A and C. *Journal of Biological Chemistry* **270**, 17898-17901.

Cabado, A.G., Yu, F.H., Kapus, A., Lukacs, G., Grinstein, S. & Orlowski, J. (1996) Distinct structural domains confer cAMP sensitivity and ATP dependence to the Na^+/H^+ exchanger NHE3 isoform. *Journal of Biological Chemistry* **271**, 3590-3599.

Cabantchik, Z.I., Knauf, P.A. & Rothstein, A. (1978) The anion transport system of the red blood cell. The role of membrane protein evaluated by the use of 'probes'. *Biochimica et Biophysica Acta* **515**, 239-302.

Camilión de Hurtado, M.C., Alvarez, B.V., Pérez, NG, Ennis, I.L. & Cingolani, H.E. (1998) Angiotensin II activates Na^+-independent $Cl^--HCO_3^-$ exchange in ventricular myocardium. *Circulation Research* **82**, 473-481.

Campos-Gonzalez, R. & Kindy, M.S. (1992) Tyrosine phosphorylation of microtubule-associated protein kinase after transient ischemia in the gerbil brain. *Journal of Neurochemistry* **59**, 1955-1958.

Carafoli, E. (1987) Intracellular calcium homeostasis. *Annual Review of Biochemistry* **56**, 395-433.

Casavola, V., Reshkin, S.J., Murer, H. & Helmle-Kölb, C. (1992) Polarized expression of Na^+/H^+ exchange activity in LLC-PK1/PKE20 cells: II. Hormonal regulation. *Pflügers Archiv - European Journal of Physiology* **420**, 282-289.

Caspers, H. & Speckmann, E.J. (1972) Cerebral pO_2, pCO_2 and pH: Changes during convulsive activity and their significance for spontaneous arrest of seizures. *Epilepsia* **13**, 699-725.

Cassel, D., Scharf, O., Rotman, M., Cragoe, E.J., Jr. & Katz, M. (1988) Characterization of Na^+-linked and Na^+-independent Cl^-/HCO_3^- exchange systems in Chinese hamster lung fibroblasts. *Journal of Biological Chemistry* **263**, 6122-6127.

Chaillet, J.R. & Boron, W.F. (1985) Intracellular calibration of a pH-sensitive dye in isolated, perfused salamander proximal tubules. *Journal of General Physiology* **86**, 765-794.

Chakraborty, M., Chatterjee, D., Gorelick, F.S. & Baron, R. (1994) Cell cycle-dependent and kinase-specific regulation of the apical Na^+/H^+ exchanger and the Na^+/K^+-ATPase in the kidney cell line LLC-PK1 by calcitonin. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 2115-2119.

Chavez-Noriega, L.E. & Stevens, C.F. (1992) Modulation of synaptic efficacy in field CA1 of the rat hippocampus by forskolin. *Brain Research* **574**, 85-92.

Chen, J.C. & Chesler, M. (1992a) Extracellular alkaline shifts in rat hippocampal slice are mediated by NMDA and non-NMDA receptors. *Journal of Neurophysiology* **68**, 342-344.

Chen, J.C. & Chesler, M. (1992b) Modulation of extracellular pH by glutamate and GABA in rat hippocampal slices. *Journal of Neurophysiology* **67**, 29-36.

Chen, Q., Benson, R.S., Whetton, A.D., Brant, S.R., Donowitz, M., Montrose, M.H., Dive, C. & Watson, A.J. (1997) Role of acid/base homeostasis in the suppression of apoptosis in haemopoietic cells by v-Abl protein tyrosine kinase. *Journal of Cell Science* **110**, 379-387.

Chesler, M. (1990) The regulation and modulation of pH in the nervous system. *Progress in Neurobiology* **34**, 401-427.

Chesler, M. & Kaila, K. (1992) Modulation of pH by neuronal activity. *Trends in Neurosciences* **15**, 396-402.

Chidekel, A.S., Friedman, J.E. & Haddad, G.G. (1997) Anoxia-induced neuronal injury: Role of Na^+ entry and Na^+-dependent transport. *Experimental Neurology* **146**, 403-413.

Chijiwa, T., Mishima, A., Hagiwara, M., Sano, M., Hayashi, K., Inoue, T., Naito, K., Toshioka, T. & Hidaka, H. (1990) Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, *N*-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. *Journal of Biological Chemistry* **265**, 5267-5272.

Choi, E.J., Xia, Z., Villacres, E.C. & Storm, D.R. (1993) The regulatory diversity of the mammalian adenylyl cyclases. *Current Opinion in Cell Biology* **5**, 269-273.

Church, J. (1999) Effects of pH changes on calcium-mediated potentials in rat hippocampal neurons *in vitro*. *Neuroscience* **89**, 731-742.

Church, J. (1992) A change from HCO_3^-/CO_2- to HEPES-buffered medium modifies membrane properties of rat CA1 pyramidal neurones *in vitro*. *Journal of Physiology* **455**, 51-71.

Church, J., Baxter, K.A. & McLarnon, J.G. (1998) pH modulation of Ca^{2+} responses and a Ca^{2+}-dependent K^+ channel in cultured rat hippocampal neurones. *Journal of Physiology* **511**, 119-132.

Church, J. & Baimbridge, K.G. (1991) Exposure to high-pH medium increases the incidence and extent of dye coupling between rat hippocampal CA1 pyramidal neurons *in vitro*. *Journal of Neuroscience* **11**, 3289-3295.

Church, J. & McLennan, H. (1989) Electrophysiological properties of rat CA1 pyramidal neurones *in vitro* modified by changes in extracellular bicarbonate. *Journal of Physiology* **415**, 85-108.

Clark, J.D. & Limbird, L.E. (1991) Na^+-H^+ exchanger subtypes: A predictive review. *American Journal of Physiology* **261**, C945-53.

Clarke, L.L. & Harline, M.C. (1998) Dual role of CFTR in cAMP-stimulated HCO_3^- secretion across murine duodenum. *American Journal of Physiology* **274**, G718-26.

Cohen, P., Holmes, C.F. & Tsukitani, Y. (1990a) Okadaic acid: A new probe for the study of cellular regulation. *Trends in Biochemical Sciences* **15**, 98-102.

Cohen, M.E., Reinlib, L., Watson, A.J., Gorelick, F., Rys-Sikora, K, Tse, M., Rood, R.P., Czernik, A.J., Sharp, G.W. & Donowitz, M. (1990b) Rabbit ileal villus cell brush border Na^+/H^+ exchange is regulated by Ca^{2+}/calmodulin-dependent protein kinase II, a brush border membrane protein. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 8990-8994.

Cooper, D.M., Mons, N. & Fagan, K. (1994) Ca^{2+}-sensitive adenylyl cyclases. *Cellular Signalling* **6**, 823-840.

Cooper, G.J. & Hunter, M. (1994) Na^+-H^+ exchange in frog early distal tubule: Effect of aldosterone on the set-point. *Journal of Physiology* **479**, 423-432.

Cooper, G.J. & Hunter, M. (1997) Intracellular pH and calcium in frog early distal tubule: Effects of transport inhibitors. *Journal of Physiology* **498**, 49-59.

Counillon, L., Noël, J., Reithmeier, R.A. & Pouysségur, J. (1997) Random mutagenesis reveals a novel site involved in inhibitor interaction within the fourth transmembrane segment of the Na^+/H^+ exchanger-1. *Biochemistry* **36**, 2951-2959.

Cowan, A.I. & Martin, R.L. (1996) Ionic basis of the membrane potential responses of rat dorsal vagal motoneurones to HEPES buffer. *Brain Research* **717**, 69-75.

Cox, G.A., Lutz, C.M., Yang, C.L., Biemesderfer, D., Bronson, R.T., Fu, A., Aronson, P.S., Noebels, J.L. & Frankel, W.N. (1997) Sodium/hydrogen exchanger gene defect in slow-wave epilepsy mutant mice. *Cell* **91**, 139-148.

D'Souza, S., Garcia-Cabado, A., Yu, F., Teter, K., Lukacs, G., Skorecki, K., Moore, HP, Orlowski, J. & Grinstein, S. (1998) The epithelial sodium-hydrogen antiporter, Na^+/H^+ exchanger 3, accumulates and is functional in recycling endosomes. *Journal of Biological Chemistry* **273**, 2035-2043.

Dagher, G., Thomas, S.R., Griffiths, N., Siaume-Perez, S. & Sauterey, C. (1997) Calcitonin activates an Na^+-independent HCO_3^--dependent pathway in the rabbit distal convoluted tubule. *American Journal of Physiology* **273**, F97-103.

Daly, J.W., Padgett, W. & Seamon, K.B. (1982) Activation of cyclic AMP-generating systems in brain membranes and slices by the diterpene forskolin: Augmentation of receptor-mediated responses. *Journal of Neurochemistry* **38**, 532-544.

Daumas, P. & Andersen, O.S. (1993) Proton block of rat brain sodium channels. Evidence for two proton binding sites and multiple occupancy. *Journal of General Physiology* **101**, 27-43.

Davis, B.A., Hogan, E.M. & Boron, W.F. (1992) Role of G proteins in stimulation of Na^+-H^+ exchange by cell shrinkage. *American Journal of Physiology* **262**, C533-6.

Davis, H.P. & Squire, L.R. (1984) Protein synthesis and memory: A review. *Psychological Bulletin* **96**, 518-559.

Demaurex, N. & Grinstein, S. (1994) Na^+/H^+ antiport: Modulation by ATP and role in cell volume regulation. *Journal of Experimental Biology* **196**, 389-404.

Désilets, M., Pucéat, M. & Vassort, G. (1994) Chloride dependence of pH modulation by β-adrenergic agonist in rat cardiomyocytes. *Circulation Research* **75**, 862-869.

Dhanasekaran, N., Prasad, M.V., Wadsworth, S.J., Dermott, J.M., & van Rossum, G. (1994) Protein kinase C-dependent and -independent activation of Na^+/H^+ exchanger by $G_{\alpha 12}$ class of G proteins. *Journal of Biological Chemistry* **269**, 11802-11806.

Diarra, A., Sheldon, C.A., Brett, C.L., & Church, J. (1998) Anoxia-evoked intracellular pH changes in cultured rat hippocampal neurons. *Journal of Neurochemistry*, in press.

Dixon, D.B., Takahashi, K. & Copenhagen, D.R. (1993) L-glutamate suppresses HVA calcium current in catfish horizontal cells by raising intracellular proton concentration. *Neuron* **11**, 267-277.

Dixon, D.B., Takahashi, K., Bieda, M. & Copenhagen, D.R. (1996) Quinine, intracellular pH and modulation of hemi-gap junctions in catfish horizontal cells. *Vision Research* **36**, 3925-3931.

Dragon, S., Glombitza, S., Gotz, R. & Baumann, R. (1996) Norepinephrine-mediated hypoxic stimulation of embryonic red cell carbonic anhydrase and 2,3-DPG synthesis. *American Journal of Physiology* **271**, R982-9.

Dudek, F.E., Snow, R.W. & Taylor, C.P. (1986) Role of electrical interactions in synchronization of epileptiform bursts. *Advances in Neurology* **44**, 593-617.

Dunwiddie, T.V., Taylor, M., Heginbotham, L.R. & Proctor, W.R. (1992) Long-term increases in excitability in the CA1 region of rat hippocampus induced by β-adrenergic stimulation: Possible mediation by cAMP. *Journal of Neuroscience* **12**, 506-517.

Ebine, Y., Fujiwara, N. & Shimoji, K. (1994) Mild acidosis inhibits the rise in intracellular Ca^{2+} concentration in response to oxygen-glucose deprivation in rat hippocampal slices. *Neuroscience Letters* **168**, 155-158.

Edmonds, B.T., Murray, J. & Condeelis, J. (1995) pH regulation of the F-actin binding properties of Dictyostelium elongation factor α_1. *Journal of Biological Chemistry* **270**, 15222-15230.

Ehlers, M.D., Zhang, S., Bernhadt, J.P. & Huganir, R.L. (1996) Inactivation of NMDA receptors by direct interaction of calmodulin with the NR1 subunit. *Cell* **84**, 745-755.

Emmer, E., Rood, R.P., Wesolek, J.H., Cohen, M.E., Braithwaite, R.S., Sharp, G.W., Murer, H. & Donowitz, M. (1989) Role of calcium and calmodulin in the regulation of the rabbit ileal brush-border membrane Na^+/H^+ antiporter. *Journal of Membrane Biology* **108**, 207-215.

Emmons, C. & Stokes, J.B. (1994) Cellular actions of cAMP on HCO_3^--secreting cells of rabbit CCD: Dependence on *in vivo* acid-base status. *American Journal of Physiology* **266**, F528-35.

Erdahl, W.L., Chapman, C.J., Taylor, R.W. & Pfeiffer, D.R. (1994) Ca^{2+} transport properties of ionophores A23187, ionomycin, and 4-BrA23187 in a well defined model system. *Biophysical Journal* **66**, 1678-1693.

Erondu, N.E. & Kennedy, M.B. (1985) Regional distribution of type II Ca^{2+}/calmodulin-dependent protein kinase in rat brain. *Journal of Neuroscience* **5**, 3270-3277.

Fafournoux, P., Noël, J. & Pouysségur, J. (1994) Evidence that Na^+/H^+ exchanger isoforms NHE1 and NHE3 exist as stable dimers in membranes with a high degree of specificity for homodimers. *Journal of Biological Chemistry* **269**, 2589-2596.

Felder, C.C., Albrecht, F.E., Campbell, T., Eisner, G.M. & Jose, P.A. (1993) cAMP-independent, G protein-linked inhibition of Na^+/H^+ exchange in renal brush border by D_1 dopamine agonists. *American Journal of Physiology* **264**, F1032-7.

Fisher, R. & Johnston, D. (1990) Differential modulation of single voltage-gated calcium channels by cholinergic and adrenergic agonists in adult hippocampal neurons. *Journal of Neurophysiology* **64**, 1291-1302.

Fliegel, L., Walsh, M.P., Singh, D., Wong, C. & Barr, A. (1992) Phosphorylation of the C-terminal domain of the Na^+/H^+ exchanger by Ca^{2+}/calmodulin-dependent protein kinase II. *Biochemical Journal* **282**, 139-145.

Fliegel, L. & Fröhlich, O. (1993) The Na^+/H^+ exchanger: An update on structure, regulation and cardiac physiology. *Biochemical Journal* **296**, 273-285.

Frelin, C., Vigne, P., Ladoux, A. & Lazdunski, M. (1988) The regulation of the intracellular pH in cells from vertebrates. *European Journal of Biochemistry* **174**, 3-14.

Gaillard, S. & Dupont, J.L. (1990) Ionic control of intracellular pH in rat cerebellar Purkinje cells maintained in culture. *Journal of Physiology* **425**, 71-83.

Ganz, M.B., Boyarsky, G., Sterzel, R.B. & Boron, W.F. (1989) Arginine vasopressin enhances pH_i regulation in the presence of HCO_3^- by stimulating three acid-base transport systems. *Nature* **337**, 648-651.

Giffard, R.G., Monyer, H., Christine, C.W. & Choi, D.W. (1990) Acidosis reduces NMDA receptor activation, glutamate neurotoxicity, and oxygen-glucose deprivation neuronal injury in cortical cultures. *Brain Research* **506**, 339-342.

Goldman, S.A., Pulsinelli, W.A., Clarke, W.Y., Kraig, R.P. & Plum, F. (1989) The effects of extracellular acidosis on neurons and glia *in vitro*. *Journal of Cerebral Blood Flow & Metabolism* **9**, 471-477.

Gottfried, J.A. & Chesler, M. (1994) Endogenous H^+ modulation of NMDA receptor-mediated EPSCs revealed by carbonic anhydrase inhibition in rat hippocampus. *Journal of Physiology* **478 Pt 3**, 373-378.

Gottfried, J.A. & Chesler, M. (1996) Temporal resolution of activity-dependent pH shifts in rat hippocampal slices. *Journal of Neurophysiology* **76**, 2804-2807.

Gray, R. & Johnston, D. (1987) Noradrenaline and β-adrenoceptor agonists increase activity of voltage-dependent calcium channels in hippocampal neurons. *Nature* **327**, 620-622.

Green, J., Yamaguchi, D.T., Kleeman, C.R. & Muallem, S. (1990) Cytosolic pH regulation in osteoblasts. Regulation of anion exchange by intracellular pH and Ca^{2+} ions. *Journal of General Physiology* **95**, 121-145.

Green, J. & Kleeman, C.R. (1992) Role of calcium and cAMP messenger systems in intracellular pH regulation of osteoblastic cells. *American Journal of Physiology* **262**, C111-21.

Grinstein, S., Cohen, S., Goetz, J.D. & Rothstein, A. (1985) Osmotic and phorbol ester-induced activation of Na^+/H^+ exchange: Possible role of protein phosphorylation in lymphocyte volume regulation. *Journal of Cell Biology* **101**, 269-276.

Grinstein, S., Rotin, D. & Mason, M.J. (1989) Na^+/H^+ exchange and growth factor-induced cytosolic pH changes. Role in cellular proliferation. *Biochimica et Biophysica Acta* **988**, 73-97.

Grinstein, S., Woodside, M., Sardet, C., Pouysségur, J. & Rotin, D. (1992) Activation of the Na^+/H^+ antiporter during cell volume regulation. Evidence for a phosphorylation-independent mechanism. *Journal of Biological Chemistry* **267**, 23823-23828.

Grinstein, S., Woodside, M., Waddell, T.K., Downey, G.P., Orlowski, J, Pouyssegur, J., Wong, D.C. & Foskett, J.K. (1993) Focal localization of the NHE-1 isoform of the Na^+/H^+ antiport: Assessment of effects on intracellular pH. *EMBO Journal* **12**, 5209-5218.

Grinstein, S. & Cohen, S. (1987) Cytoplasmic $[Ca^{2+}]$ and intracellular pH in lymphocytes. Role of membrane potential and volume-activated Na^+/H^+ exchange. *Journal of General Physiology* **89**, 185-213.

Grinstein, S. & Rothstein, A. (1986) Mechanisms of regulation of the Na^+/H^+ exchanger. *Journal of Membrane Biology* **90**, 1-12.

Grynkiewicz, G., Poenie, M. & Tsien, R.Y. (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**, 3440-3450.

Guizouarn, H., Borgese, F., Pellissier, B., Garcia-Romeu, F. & Motais, R. (1993) Role of protein phosphorylation and dephosphorylation in activation and desensitization of the cAMP-dependent Na^+/H^+ antiport. *Journal of Biological Chemistry* **268**, 8632-8639.

Gupta, A., Schwiening, C.J. & Boron, W.F. (1994) Effects of CGRP, forskolin, PMA, and ionomycin on pH_i dependence of Na^+-H^+ exchange in UMR-106 cells. *American Journal of Physiology* **266**, C1088-92.

Hall, R.A., Premont, R.T., Chow, C.W., Blitzer, J.T., Pitcher, J.A., Claing, A., Stoffel, R.H., Barak, L.S., Shenolikar, S., Weinman, E.J., Grinstein, S. & Lefkowitz, R.J. (1998) The β_2-adrenergic receptor interacts with the Na^+/H^+-exchanger regulatory factor to control Na^+/H^+ exchange. *Nature* **392**, 626-630.

Hanoune, J., Pouille, Y., Tzavara, E., Shen, T., Lipskaya, L., Miyamoto, N., Suzuki, Y. & Defer, N. (1997) Adenylyl cyclases: Structure, regulation and function in an enzyme superfamily. *Molecular & Cellular Endocrinology* **128**, 179-194.

Harada, H., Kanai, Y., Anzai, M. & Suketa, Y. (1991) cAMP activates Cl^-/HCO_3^- exchange for regulation of intracellular pH in renal epithelial cells. *Biochimica et Biophysica Acta* **1092**, 404-407.

Harguindey, S., Pedraz, J.L., Garcia Canero, R., Perez de Diego, J & Cragoe, E.J., Jr. (1995) Hydrogen ion-dependent oncogenesis and parallel new avenues to cancer prevention and treatment using a H^+-mediated unifying approach: pH-related and pH-unrelated mechanisms. *Critical Reviews in Oncogenesis* **6**, 1-33.

Harrington, M.A., Oksenberg, D. & Peroutka, S.J. (1988) 5-Hydroxytryptamine$_{1A}$ receptors are linked to a G_i-adenylate cyclase complex in rat hippocampus. *European Journal of Pharmacology* **154**, 95-98.

Hartley, Z. & Dubinsky, J.M. (1993) Changes in intracellular pH associated with glutamate excitotoxicity. *Journal of Neuroscience* **13**, 4690-4699.

Helmle-Kölb, C., Montrose, M.H., Stange, G. & Murer, H. (1990) Regulation of Na^+/H^+ exchange in opossum kidney cells by parathyroid hormone, cyclic AMP and phorbol esters. *Pflügers Archiv - European Journal of Physiology* **415**, 461-470.

Hendey, B., Mamrack, M.D. & Putnam, R.W. (1989) Thrombin induces a calcium transient that mediates an activation of the Na^+/H^+ exchanger in human fibroblasts. *Journal of Biological Chemistry* **264**, 19540-19547.

Hensley, C.B., Bradley, M.E. & Mircheff, A.K. (1989) Parathyroid hormone-induced translocation of Na^+-H^+ antiporters in rat proximal tubules. *American Journal of Physiology* **257**, C637-45.

Herman, B., Gores, G.J., Nieminen, A.L., Kawanishi, T., Harman, A. & Lemasters, J.J. (1990) Calcium and pH in anoxic and toxic injury. *Critical Reviews in Toxicology* **21**, 127-148.

Higashima, M., Kinoshita, H., Yamaguchi, N. & Koshino, Y. (1996) Activation of GABA-ergic function necessary for afterdischarge generation in rat hippocampal slices. *Neuroscience Letters* **207**, 101-104.

Hjemdahl, P. & Fredholm, B.B. (1977) Direct antilipolytic effect of acidosis in isolated rat adipocytes. *Acta Physiologica Scandinavica* **101**, 294-301.

Hooley, R., Yu, C.Y., Symons, M. & Barber, D.L. (1996) $G_{\alpha 13}$ stimulates Na^+-H^+ exchange through distinct Cdc42-dependent and RhoA-dependent pathways. *Journal of Biological Chemistry* **271**, 6152-6158.

Horie, S., Moe, O., Miller, R.T. & Alpern, R.J. (1992) Long-term activation of protein kinase C causes chronic Na^+/H^+ antiporter stimulation in cultured proximal tubule cells. *Journal of Clinical Investigation* **89**, 365-372.

Houslay, M.D. & Milligan, G. (1997) Tailoring cAMP-signalling responses through isoform multiplicity. *Trends in Biochemical Sciences* **22**, 217-224.

Hoyt, K.R. & Reynolds, I.J. (1998) Alkalinization prolongs recovery from glutamate-induced increases in intracellular Ca^{2+} concentration by enhancing Ca^{2+} efflux through the mitochondrial Na^+/Ca^{2+} exchanger in cultured rat forebrain neurons. *Journal of Neurochemistry* **71**, 1051-1058.

Huang, S. & Cheung, W.Y. (1994) H^+ is involved in the activation of calcineurin by calmodulin. *Journal of Biological Chemistry* **269**, 22067-22074.

Humphreys, B.D., Jiang, L., Chernova, M.N. & Alper, S.L. (1995) Hypertonic activation of AE2 anion exchanger in *Xenopus* oocytes via NHE-mediated intracellular alkalinization. *American Journal of Physiology* **268**, C201-9.

Ikeda, T., Schmitt, B., Pouyssegur, J., Wakabayashi, S. & Shigekawa, M. (1997) Identification of cytoplasmic subdomains that control pH-sensing of the Na^+/H^+ exchanger (NHE1): pH-maintenance, ATP-sensitive, and flexible loop domains. *Journal of Biochemistry* **121**, 295-303.

Ikeda, U., Arisaka, H., Takayasu, T., Takeda, K., Natsume, T. & Hosoda, S. (1988) Protein kinase C activation aggravates hypoxic myocardial injury by stimulating Na^+/H^+ exchange. *Journal of Molecular & Cellular Cardiology* **20**, 493-500.

Inagaki, M., Tanaka, T. & Hidaka, H. (1983) Calmodulin antagonists enhance calcium binding to calmodulin. *Pharmacology* **27**, 125-129.

Inglefield, J.R. & Schwartz-Bloom, R.D. (1998) Activation of excitatory amino acid receptors in the rat hippocampal slice increases intracellular Cl$^-$ and cell volume. *Journal of Neurochemistry* **71**, 1396-1404.

Irwin, R.P., Lin, S.Z., Long, R.T. & Paul, S.M. (1994) *N*-methyl-D-aspartate induces a rapid, reversible, and calcium-dependent intracellular acidosis in cultured fetal rat hippocampal neurons. *Journal of Neuroscience* **14**, 1352-1357.

Ishikawa, Y. & Homcy, C.J. (1997) The adenylyl cyclases as integrators of transmembrane signal transduction. *Circulation Research* **80**, 297-304.

Jarolimek, W., Misgeld, U. & Lux, H.D. (1989) Activity dependent alkaline and acid transients in guinea pig hippocampal slices. *Brain Research* **505**, 225-232.

Jefferys, J.G. & Haas, H.L. (1982) Synchronized bursting of CA1 hippocampal pyramidal cells in the absence of synaptic transmission. *Nature* **300**, 448-450.

Jiang, L., Stuart-Tilley, A., Parkash, J. & Alper, S.L. (1994) pH$_i$ and serum regulate AE2-mediated Cl$^-$/HCO$_3^-$ exchange in CHOP cells of defined transient transfection status. *American Journal of Physiology* **267**, C845-56.

Johnson, R.A. & Shoshani, I. (1990) Kinetics of "P"-site-mediated inhibition of adenylyl cyclase and the requirements for substrate. *Journal of Biological Chemistry* **265**, 11595-11600.

Johnson, R.A. & Shoshani, I. (1994) Preparation and use of "P"-site-targeted affinity ligands for adenylyl cyclases. *Methods in Enzymology* **238**, 56-71.

Kaila, K., Paalasmaa, P., Taira, T. & Voipio, J. (1992) pH transients due to monosynaptic activation of GABA$_A$ receptors in rat hippocampal slices. *Neuroreport* **3**, 105-108.

Kaila, K., Voipio, J., Paalasmaa, P., Pasternack, M. & Deisz, R.A. (1993) The role of bicarbonate in GABA$_A$ receptor-mediated IPSPs of rat neocortical neurones. *Journal of Physiology* **464**, 273-289.

Kaila, K. (1994) Ionic basis of GABA$_A$ receptor channel function in the nervous system. *Progress in Neurobiology* **42**, 489-537.

Kaila, K. & Voipio, J. (1987) Postsynaptic fall in intracellular pH induced by GABA-activated bicarbonate conductance. *Nature* **330**, 163-165.

Kaku, D.A., Giffard, R.G. & Choi, D.W. (1993) Neuroprotective effects of glutamate antagonists and extracellular acidity. *Science* **260**, 1516-1518.

Kandasamy, R.A., Yu, F.H., Harris, R., Boucher, A., Hanrahan, J.W. & Orlowski, J. (1995) Plasma membrane Na$^+$/H$^+$ exchanger isoforms (NHE-1, -2, and -3) are differentially

responsive to second messenger agonists of the protein kinase A and C pathways. *Journal of Biological Chemistry* **270**, 29209-29216.

Kao, J.P. (1994) Practical aspects of measuring $[Ca^{2+}]$ with fluorescent indicators. *Methods in Cell Biology* **40**, 155-181.

Kashiwagi, K., Fukuchi, J., Chao, J., Igarashi, K. & Williams, K. (1996) An aspartate residue in the extracellular loop of the *N*-methyl-D-aspartate receptor controls sensitivity to spermine and protons. *Molecular Pharmacology* **49**, 1131-1141.

Kavalali, E.T., Hwang, K.S. & Plummer, M.R. (1997) cAMP-dependent enhancement of dihydropyridine-sensitive calcium channel availability in hippocampal neurons. *Journal of Neuroscience* **17**, 5334-5348.

Kawai, N., McCarron, R.M. & Spatz, M. (1995) Endothelins stimulate sodium uptake into rat brain capillary endothelial cells through endothelin A-like receptors. *Neuroscience Letters* **190**, 85-88.

Khurana, S., Nath, S.K., Levine, S.A., Bowser, J.M., Tse, C.M., Cohen, M.E. & Donowitz, M. (1996) Brush border phosphatidylinositol 3-kinase mediates epidermal growth factor stimulation of intestinal NaCl absorption and Na^+/H^+ exchange. *Journal of Biological Chemistry* **271**, 9919-9927.

Kikeri, D., Zeidel, M.L., Ballermann, B.J., Brenner, B.M. & Hebert, S.C. (1990) pH regulation and response to AVP in A10 cells differ markedly in the presence vs. absence of CO_2/HCO_3^-. *American Journal of Physiology* **259**, C471-83.

Kimura, M. & Aviv, A. (1993) Regulation of the cytosolic pH set point for activation of the Na^+/H^+ antiport in human platelets: The roles of the Na^+/Ca^{2+} exchange, the $Na^+/K^+/2Cl^-$ cotransport and cellular volume. *Pflügers Archiv - European Journal of Physiology* **422**, 585-590.

Klanke, C.A., Su, Y.R., Callen, D.F., Wang, Z., Meneton, P., Baird, N, Kandasamy, R.A., Orlowski, J., Otterud, B.E. & Leppert, M. (1995) Molecular cloning and physical and genetic mapping of a novel human Na^+/H^+ exchanger (NHE5/SLC9A5) to chromosome 16q22.1. *Genomics* **25**, 615-622.

Klee, C.B., Ren, H. & Wang, X. (1998) Regulation of the calmodulin-stimulated protein phosphatase, calcineurin. *Journal of Biological Chemistry* **273**, 13367-13370.

Koch, R.A. & Barish, M.E. (1994) Perturbation of intracellular calcium and hydrogen ion regulation in cultured mouse hippocampal neurons by reduction of the sodium ion concentration gradient. *Journal of Neuroscience* **14**, 2585-2593.

Köhr, G. & Mody, I. (1991) Endogenous intracellular calcium buffering and the activation/inactivation of HVA calcium currents in rat dentate gyrus granule cells. *Journal of General Physiology* **98**, 941-967.

Kopito, R.R., Lee, B.S., Simmons, D.M., Lindsey, A.E., Morgans, C.W. & Schneider, K. (1989) Regulation of intracellular pH by a neuronal homolog of the erythrocyte anion exchanger. *Cell* **59**, 927-937.

Koren, W., Postnov, I.Y. & Postnov, Y.V. (1997) Increased Na^+-H^+ exchange in red blood cells of patients with primary aldosteronism. *Hypertension* **29**, 587-591.

Kraig, R.P., Ferreira-Filho, C.R. & Nicholson, C. (1983) Alkaline and acid transients in cerebellar microenvironment. *Journal of Neurophysiology* **49**, 831-850.

Kraig, R.P., Petito, C.K., Plum, F. & Pulsinelli, W.A. (1987) Hydrogen ions kill brain at concentrations reached in ischemia. *Journal of Cerebral Blood Flow & Metabolism* **7**, 379-386.

Kramhoft, B., Hoffmann, E.K. & Simonsen, L.O. (1994) pH_i regulation in Ehrlich mouse ascites tumor cells: Role of sodium-dependent and sodium-independent chloride-bicarbonate exchange. *Journal of Membrane Biology* **138**, 121-132.

Krishtal, O.A., Osipchuk, Y.V., Shelest, T.N. & Smirnoff, S.V. (1987) Rapid extracellular pH transients related to synaptic transmission in rat hippocampal slices. *Brain Research* **436**, 352-356.

Kristián, T., Katsura, K., Gido, G. & Siesjö, B.K. (1994) The influence of pH on cellular calcium influx during ischemia. *Brain Research* **641**, 295-302.

Kruëger, K.M., Daaka, Y., Pitcher, J.A. & Lefkowitz, R.J. (1997) The role of sequestration in G protein-coupled receptor resensitization. Regulation of β_2-adrenergic receptor dephosphorylation by vesicular acidification. *Journal of Biological Chemistry* **272**, 5-8.

Kurashima, K., Yu, F.H., Cabado, A.G., Szabo, E.Z., Grinstein, S. & Orlowski, J. (1997) Identification of sites required for down-regulation of Na^+/H^+ exchanger NHE3 activity by cAMP-dependent protein kinase: Phosphorylation-dependent and -independent mechanisms. *Journal of Biological Chemistry* **272**, 28672-28679.

Kurino, M., Fukunaga, K., Ushio, Y. & Miyamoto, E. (1996) Cyclic AMP inhibits activation of mitogen-activated protein kinase and cell proliferation in response to growth factors in cultured rat cortical astrocytes. *Journal of Neurochemistry* **67**, 2246-2255.

L'Allemain, G., Paris, S. & Pouysségur, J. (1985) Role of a Na^+-dependent Cl^-/HCO_3^- exchange in regulation of intracellular pH in fibroblasts. *Journal of Biological Chemistry* **260**, 4877-4883.

Lamprecht, G., Weinman, E.J. & Yun, C.H. (1998) The role of NHE-RF and E3KARP in the cAMP-mediated inhibition of NHE3. *Journal of Biological Chemistry* **273**, 29972-29978.

Lamsa, K. & Kaila, K. (1997) Ionic mechanisms of spontaneous GABA-ergic events in rat hippocampal slices exposed to 4-aminopyridine. *Journal of Neurophysiology* **78**, 2582-2591.

Le Prigent, K., Lagadic-Gossmann, D., Mongodin, E. & Feuvray, D. (1997) HCO_3^--dependent alkalinizing transporter in adult rat ventricular myocytes: Characterization and modulation. *American Journal of Physiology* **273**, H2596-603.

Lee, B.S., Gunn, R.B. & Kopito, R.R. (1991) Functional differences among nonerythroid anion exchangers expressed in a transfected human cell line. *Journal of Biological Chemistry* **266**, 11448-11454.

Lee, J., Taira, T., Pihlaja, P., Ransom, B.R. & Kaila, K. (1996) Effects of CO_2 on excitatory transmission apparently caused by changes in intracellular pH in the rat hippocampal slice. *Brain Research* **706**, 210-216.

Levin, R.M. & Weiss, B. (1979) Selective binding of antipsychotics and other psychoactive agents to the calcium-dependent activator of cyclic nucleotide phosphodiesterase. *Journal of Pharmacology & Experimental Therapeutics* **208**, 454-459.

Levine, E.S., Dreyfus, C.F., Black, I.B. & Plummer, M.R. (1995) Brain-derived neurotrophic factor rapidly enhances synaptic transmission in hippocampal neurons via postsynaptic tyrosine kinase receptors. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 8074-8077.

Levine, S.A., Montrose, M.H., Tse, C.M. & Donowitz, M. (1993) Kinetics and regulation of three cloned mammalian Na^+/H^+ exchangers stably expressed in a fibroblast cell line. *Journal of Biological Chemistry* **268**, 25527-25535.

Li, J. & Eastman, A. (1995) Apoptosis in an interleukin-2-dependent cytotoxic T lymphocyte cell line is associated with intracellular acidification. Role of the Na^+/H^+-antiport. *Journal of Biological Chemistry* **270**, 3203-3211.

Limbird, L.E. (1988) Receptors linked to inhibition of adenylate cyclase: Additional signaling mechanisms. *FASEB Journal* **2**, 2686-2695.

Lin, H. & Miller, S.S. (1994) pH_i-dependent Cl^-/HCO_3^- exchange at the basolateral membrane of frog retinal pigment epithelium. *American Journal of Physiology* **266**, C935-45.

Lin, X. & Barber, D.L. (1996) A calcineurin homologous protein inhibits GTPase-stimulated Na^+-H^+ exchange. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 12631-12636.

Little, P.J., Weissberg, P.L., Cragoe, E.J.Jr. & Bobik, A. (1988) Dependence of Na$^+$/H$^+$ antiport activation in cultured rat aortic smooth muscle on calmodulin, calcium, and ATP. Evidence for the involvement of calmodulin-dependent kinases. *Journal of Biological Chemistry* **263**, 16780-16786.

Livne, A.A., Sardet, C. & Pouyssegur, J. (1991) The Na$^+$/H$^+$ exchanger is phosphorylated in human platelets in response to activating agents. *FEBS Letters* **284**, 219-222.

Louvel, J., Avoli, M., Kurcewicz, I. & Pumain, R. (1994) Extracellular free potassium during synchronous activity induced by 4-aminopyridine in the juvenile rat hippocampus. *Neuroscience Letters* **167**, 97-100.

Ludt, J., Tønnessen, T.I., Sandvig, K. & Olsnes, S. (1991) Evidence for involvement of protein kinase C in regulation of intracellular pH by Cl$^-$/HCO$_3^-$ antiport. *Journal of Membrane Biology* **119**, 179-186.

Ludt, J., Sandvig, K. & Olsnes, S. (1993) Rapid increase in pH set-point of the Na$^+$-independent chloride/bicarbonate antiporter in Vero cells exposed to heat shock. *Journal of Membrane Biology* **134**, 143-153.

Mahnensmith, R.L. & Aronson, P.S. (1985) The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes. *Circulation Research* **56**, 773-788.

Mamiya, N., Goldenring, J.R., Tsunoda, Y., Modlin, I.M., Yasui, K., Usuda, N., Ishikawa, T., Natsume, A. & Hidaka, H. (1993) Inhibition of acid secretion in gastric parietal cells by the Ca^{2+}/calmodulin-dependent protein kinase II inhibitor KN-93. *Biochemical & Biophysical Research Communications* **195**, 608-615.

Manganel, M. & Turner, R.J. (1990) Agonist-induced activation of Na$^+$/H$^+$ exchange in rat parotid acinar cells is dependent on calcium but not on protein kinase C. *Journal of Biological Chemistry* **265**, 4284-4289.

Mansuy, I.M., Mayford, M., Jacob, B., Kandel, E.R. & Bach, M.E. (1998) Restricted and regulated overexpression reveals calcineurin as a key component in the transition from short-term to long-term memory. *Cell* **92**, 39-49.

Marcus, E.M. & Watson, C.W. (1968) Symmetrical epileptogenic foci in monkey cerebral cortex. Mechanisms of interaction and regional variations in capacity for synchronous discharges. *Archives of Neurology* **19**, 99-116.

Martínez-Zaguilán, R., Martínez, G.M., Lattanzio, F. & Gillies, R.J. (1991) Simultaneous measurement of intracellular pH and Ca^{2+} using the fluorescence of SNARF-1 and fura-2. *American Journal of Physiology* **260**, C297-307.

Mason, M.J., Smith, J.D., Garcia-Soto, J.J. & Grinstein, S. (1989) Internal pH-sensitive site couples Cl^-/HCO_3^- exchange to Na^+/H^+ antiport in lymphocytes. *American Journal of Physiology* **256**, C428-33.

Matsuda, S., Kawasaki, H., Moriguchi, T., Gotoh, Y. & Nishida, E. (1995) Activation of protein kinase cascades by osmotic shock. *Journal of Biological Chemistry* **270**, 12781-12786.

Matthies, H. (1989) In search of cellular mechanisms of memory. *Progress in Neurobiology* **32**, 277-349.

Meech, R.W. & Thomas, R.C. (1977) The effect of calcium injection on the intracellular sodium and pH of snail neurones. *Journal of Physiology* **265**, 867-879.

Meech, R.W. & Thomas, R.C. (1980) Effect of measured calcium chloride injections on the membrane potential and internal pH of snail neurones. *Journal of Physiology* **298**, 111-129.

Mellergård, P., Ou-yang, Y.B. & Siesjö, B.K. (1993) Intracellular pH regulation in cultured rat astrocytes in CO_2/HCO_3^--containing media. *Experimental Brain Research* **95**, 371-380.

Melvin, J.E., Moran, A. & Turner, R.J. (1988) The role of HCO_3^- and Na^+/H^+ exchange in the response of rat parotid acinar cells to muscarinic stimulation. *Journal of Biological Chemistry* **263**, 19564-19569.

Meyer, J.S., Gotoh, F. & Tazaki, Y. (1961). *Electroencephalogical and Clinical Neurophysiology*. **13**, 762-775.

Mironov, S.L. (1995) Plasmalemmal and intracellular Ca^{2+} pumps as main determinants of slow Ca^{2+} buffering in rat hippocampal neurones. *Neuropharmacology* **34**, 1123-1132.

Mironov, S.L. & Lux, H.D. (1991) Cytoplasmic alkalinization increases high-threshold calcium current in chick dorsal root ganglion neurones. *Pflügers Archiv - European Journal of Physiology* **419**, 138-143.

Mody, I., Baimbridge, K.G. & Miller, J.J. (1984) Blockade of tetanic- and calcium-induced long-term potentiation in the hippocampal slice preparation by neuroleptics. *Neuropharmacology* **23**, 625-631.

Mons, N., Decorte, L., Jaffard, R. & Cooper, D.M. (1998) Ca^{2+}-sensitive adenylyl cyclases, key integrators of cellular signalling. *Life Sciences* **62**, 1647-1652.

Moolenaar, W.H., Tsien, R.Y., van der Saag, P.T. & de Laat, S.W. (1983) Na^+/H^+ exchange and cytoplasmic pH in the action of growth factors in human fibroblasts. *Nature* **304**, 645-648.

Moolenaar, W.H. (1986) Effects of growth factors on intracellular pH regulation. *Annual Review of Physiology* **48**, 363-376.

Morimoto, Y. & Kemmotsu, O. (1994) Effect of lactic and CO_2 acidosis on neuronal function following glucose-oxygen deprivation in rat hippocampal slices. *Brain Research* **654**, 273-278.

Muallem, S. & Loessberg, P.A. (1990) Intracellular pH-regulatory mechanisms in pancreatic acinar cells. II. Regulation of H^+ and HCO_3^- transporters by Ca^{2+}-mobilizing agonists. *Journal of Biological Chemistry* **265**, 12813-12819.

Mugharbil, A., Knickelbein, R.G., Aronson, P.S. & Dobbins, J.W. (1990) Rabbit ileal brush-border membrane Cl^-/HCO_3^- exchanger is activated by an internal pH-sensitive modifier site. *American Journal of Physiology* **259**, G666-70.

Mullins, L.J., Tiffert, T., Vassort, G. & Whittembury, J. (1983) Effects of internal sodium and hydrogen ions and of external calcium ions and membrane potential on calcium entry in squid axons. *Journal of Physiology* **338**, 295-319.

Murer, H., Hopfer, U. & Kinne, R. (1976) Sodium/proton antiport in brush-border-membrane vesicles isolated from rat small intestine and kidney. *Biochemical Journal* **154**, 597-604.

Murthy, A., Gonzalez-Agosti, C., Cordero, E., Pinney, D., Candia, C, Solomon, F., Gusella, J. & Ramesh, V. (1998) NHE-RF, a regulatory cofactor for Na^+-H^+ exchange, is a common interactor for merlin and ERM (MERM) proteins. *Journal of Biological Chemistry* **273**, 1273-1276.

Nedergaard, M., Goldman, S.A., Desai, S. & Pulsinelli, W.A. (1991) Acid-induced death in neurons and glia. *Journal of Neuroscience* **11**, 2489-2497.

Nemoto, E.M. & Frinak, S. (1981) Brain tissue pH after global brain ischemia and barbiturate loading in rats. *Stroke* **12**, 77-82.

Nicoll, R.A., Malenka, R.C. & Kauer, J.A. (1990) Functional comparison of neurotransmitter receptor subtypes in mammalian central nervous system. *Physiological Reviews* **70**, 513-565.

Niisato, N. & Marunaka, Y. (1997) Cross talk of bumetanide-sensitive and HCO_3^--dependent transporters activated by IBMX in renal epithelial A6 cells. *Journal of Membrane Biology* **157**, 53-61.

Noël, J. & Pouysségur, J. (1995) Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na^+/H^+ exchanger isoforms. *American Journal of Physiology* **268**, C283-96.

Numata, M., Petrecca, K., Lake, N. & Orlowski, J. (1998) Identification of a mitochondrial Na^+/H^+ exchanger. *Journal of Biological Chemistry* **273**, 6951-6959.

Nyberg, L., Pratha, V., Hogan, D.L., Rapier, R.C., Koss, M.A. & Isenberg, J.I. (1998) Human proximal duodenal alkaline secretion is mediated by Cl^-/HCO_3^- exchange and HCO_3^- conductance. *Digestive Diseases & Sciences* **43**, 1205-1210.

Ober, S.S. & Pardee, A.B. (1987) Both protein kinase C and calcium mediate activation of the Na^+/H^+ antiporter in Chinese hamster embryo fibroblasts. *Journal of Cellular Physiology* **132**, 311-317.

Olsnes, S., Ludt, J., Tønnessen, T.I. & Sandvig, K. (1987a) Bicarbonate/chloride antiport in Vero cells: II. Mechanisms for bicarbonate-dependent regulation of intracellular pH. *Journal of Cellular Physiology* **132**, 192-202.

Olsnes, S., Tønnessen, T.I., Ludt, J. & Sandvig, K. (1987b) Effect of intracellular pH on the rate of chloride uptake and efflux in different mammalian cell lines. *Biochemistry* **26**, 2778-2785.

Orlowski, J. & Grinstein, S. (1997) Na^+/H^+ exchangers of mammalian cells. *Journal of Biological Chemistry* **272**, 22373-22376.

Orlowski, J. & Kandasamy, R.A. (1996) Delineation of transmembrane domains of the Na^+/H^+ exchanger that confer sensitivity to pharmacological antagonists. *Journal of Biological Chemistry* **271**, 19922-19927.

Otsu, K., Kinsella, J., Sacktor, B. & Fröehlich, J.P. (1989) Transient state kinetic evidence for an oligomer in the mechanism of Na^+-H^+ exchange. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 4818-4822.

Ou-yang, Y., Mellergård, P. & Siesjö, B.K. (1993) Regulation of intracellular pH in single rat cortical neurons *in vitro*: A microspectrofluorometric study. *Journal of Cerebral Blood Flow & Metabolism* **13**, 827-840.

Ou-yang, Y.B., Kristián, T., Kristiánova, V., Mellergård, P. & Siesjö, B.K. (1995) The influence of calcium transients on intracellular pH in cortical neurons in primary culture. *Brain Research* **676**, 307-313.

Owen, N.E., Knapik, J., Strebel, F., Tarpley, W.G. & Gorman, R.R. (1989) Regulation of Na^+-H^+ exchange in normal NIH-3T3 cells and in NIH-3T3 cells expressing the *ras* oncogene. *American Journal of Physiology* **256**, C756-63.

Owen, N.E. & Villereal, M.L. (1982) Evidence for a role of calmodulin in serum stimulation of Na^+ influx in human fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America* **79**, 3537-3541.

Paalasmaa, P., Taira, T., Voipio, J. & Kaila, K. (1994) Extracellular alkaline transients mediated by glutamate receptors in the rat hippocampal slice are not due to a proton conductance. *Journal of Neurophysiology* **72**, 2031-2033.

Paalasmaa, P. & Kaila, K. (1996) Role of voltage-gated calcium channels in the generation of activity-induced extracellular pH transients in the rat hippocampal slice. *Journal of Neurophysiology* **75**, 2354-2360.

Pang, L., Sawada, T., Decker, S.J. & Saltiel, A.R. (1995) Inhibition of MAP kinase kinase blocks the differentiation of PC-12 cells induced by nerve growth factor. *Journal of Biological Chemistry* **270**, 13585-13588.

Pappas, C.A., Ullrich, N. & Sontheimer, H. (1994) Reduction of glial proliferation by K^+ channel blockers is mediated by changes in pH_i. *Neuroreport* **6**, 193-196.

Pappas, C.A., Rioult, M.G. & Ransom, B.R. (1996) Octanol, a gap junction uncoupling agent, changes intracellular $[H^+]$ in rat astrocytes. *GLIA* **16**, 7-15.

Paradiso, A.M., Negulescu, P.A. & Machen, T.E. (1986) Na^+/H^+ and Cl^-/OH^- (HCO_3^-) exchange in gastric glands. *American Journal of Physiology* **250**, G524-34.

Paris, S. & Pouysségur, J. (1984) Growth factors activate the Na^+/H^+ antiporter in quiescent fibroblasts by increasing its affinity for intracellular H^+. *Journal of Biological Chemistry* **259**, 10989-10994.

Pasternack, M., Voipio, J. & Kaila, K. (1993) Intracellular carbonic anhydrase activity and its role in GABA-induced acidosis in isolated rat hippocampal pyramidal neurones. *Acta Physiologica Scandinavica* **148**, 229-231.

Pasternack, M., Smirnov, S. & Kaila, K. (1996) Proton modulation of functionally distinct $GABA_A$ receptors in acutely isolated pyramidal neurons of rat hippocampus. *Neuropharmacology* **35**, 1279-1288.

Perez-Velazquez, J.L., Valiante, T.A. & Carlen, P.L. (1994) Modulation of gap junctional mechanisms during calcium-free induced field burst activity: A possible role for electrotonic coupling in epileptogenesis. *Journal of Neuroscience* **14**, 4308-4317.

Pirttilä, T.R. & Kauppinen, R.A. (1993) Extracellular pH and buffering power determine intracellular pH in cortical brain slices during and following hypoxia. *Neuroreport* **5**, 213-216.

Piwnica-Worms, D., Jacob, R., Horres, C.R. & Lieberman, M. (1985) Na^+/H^+ exchange in cultured chick heart cells. pH_i regulation. *Journal of General Physiology* **85**, 43-64.

Pouysségur, J., Franchi, A., L'Allemain, G. & Paris, S. (1985) Cytoplasmic pH, a key determinant of growth factor-induced DNA synthesis in quiescent fibroblasts. *FEBS Letters* **190**, 115-119.

Rajotte, D., Haddad, P., Haman, A., Cragoe, E.J., Jr. & Hoang, T. (1992) Role of protein kinase C and the Na^+/H^+ antiporter in suppression of apoptosis by granulocyte macrophage colony-stimulating factor and interleukin-3. *Journal of Biological Chemistry* **267**, 9980-9987.

Raley-Susman, K.M., Cragoe, E.J., Jr., Sapolsky, RM & Kopito, R.R. (1991) Regulation of intracellular pH in cultured hippocampal neurons by an amiloride-insensitive Na^+/H^+ exchanger. *Journal of Biological Chemistry* **266**, 2739-2745.

Raley-Susman, K.M., Sapolsky, R.M. & Kopito, R.R. (1993) Cl^-/HCO_3^- exchange function differs in adult and fetal rat hippocampal neurons. *Brain Research* **614**, 308-314.

Raman, I.M., Tong, G. & Jahr, C.E. (1996) β-adrenergic regulation of synaptic NMDA receptors by cAMP-dependent protein kinase. *Neuron* **16**, 415-421.

Regula, C.S., Pfeiffer, J.R. & Berlin, R.D. (1981) Microtubule assembly and disassembly at alkaline pH. *Journal of Cell Biology* **89**, 45-53.

Reinertsen, K.V., Tønnessen, T.I., Jacobsen, J., Sandvig, K. & Olsnes, S. (1988) Role of chloride/bicarbonate antiport in the control of cytosolic pH. Cell-line differences in activity and regulation of antiport. *Journal of Biological Chemistry* **263**, 11117-11125.

Reuss, L. (1987) Cyclic AMP inhibits Cl^-/HCO_3^- exchange at the apical membrane of *Necturus* gallbladder epithelium. *Journal of General Physiology* **90**, 173-196.

Reuss, L. & Petersen, K.U. (1985) Cyclic AMP inhibits Na^+/H^+ exchange at the apical membrane of *Necturus* gallbladder epithelium. *Journal of General Physiology* **85**, 409-429.

Reuss, L. & Stoddard, J.S. (1987) Role of H^+ and HCO_3^- in salt transport in gallbladder epithelium. *Annual Review of Physiology* **49**, 35-49.

Rink, T.J., Tsien, R.Y. & Pozzan, T. (1982) Cytoplasmic pH and free Mg^{2+} in lymphocytes. *Journal of Cell Biology* **95**, 189-196.

Robertson, M.A., Woodside, M., Foskett, J.K., Orlowski, J. & Grinstein, S. (1997) Muscarinic agonists induce phosphorylation-independent activation of the NHE-1 isoform of the Na^+/H^+ antiporter in salivary acinar cells. *Journal of Biological Chemistry* **272**, 287-294.

Roos, A. & Boron, W.F. (1981) Intracellular pH. *Physiological Reviews* **61**, 296-434.

Sánchez-Armass, S., Martínez-Zaguilán, R., Martínez, G.M. & Gillies, R.J. (1994) Regulation of pH in rat brain synaptosomes. I. Role of sodium, bicarbonate, and potassium. *Journal of Neurophysiology* **71**, 2236-2248.

Sardet, C., Counillon, L., Franchi, A. & Pouysségur, J. (1990) Growth factors induce phosphorylation of the Na^+/H^+ antiporter, glycoprotein of 110 kD. *Science* **247**, 723-726.

Sardet, C., Fafournoux, P. & Pouysségur, J. (1991) α-thrombin, epidermal growth factor, and okadaic acid activate the Na^+/H^+ exchanger, NHE-1, by phosphorylating a set of common sites. *Journal of Biological Chemistry* **266**, 19166-19171.

Scholz, W.K., Baitinger, C., Schulman, H. & Kelly, P.T. (1988) Developmental changes in Ca^{2+}/calmodulin-dependent protein kinase II in cultures of hippocampal pyramidal neurons and astrocytes. *Journal of Neuroscience* **8**, 1039-1051.

Schwiening, C.J. & Boron, W.F. (1994) Regulation of intracellular pH in pyramidal neurones from the rat hippocampus by Na^+-dependent Cl^--HCO_3^- exchange. *Journal of Physiology* **475**, 59-67.

Schwiening, C.J. Kennedy, H.I. & Thomas, R.C. (1993) Calcium-hydrogen exchange by the plasma membrane Ca-ATPase of voltage-clamped snail neurons. *Proceedings of the Royal Society London B Series* **253**, 285-289.

Segal, M., Greenberger, V. & Hofstein, R. (1981) Cyclic AMP-generating systems in rat hippocampal slices. *Brain Research* **213**, 351-364.

Sekler, I., Lo, R.S. & Kopito, R.R. (1995) A conserved glutamate is responsible for ion selectivity and pH dependence of the mammalian anion exchangers AE1 and AE2. *Journal of Biological Chemistry* **270**, 28751-28758.

Shen, H., Chan, J., Kass, I.S. & Bergold, P.J. (1995) Transient acidosis induces delayed neurotoxicity in cultured hippocampal slices. *Neuroscience Letters* **185**, 115-118.

Shibata, S., Kodama, K., Tominaga, K., Ueki, S. & Watanabe, S. (1992) Assessment of the role of adrenoceptor function in ischemia-induced impairment of 2-deoxyglucose uptake and CA1 field potential in rat hippocampal slices. *European Journal of Pharmacology* **221**, 255-260.

Shirke, A.M. & Malinow, R. (1997) Mechanisms of potentiation by calcium-calmodulin kinase II of postsynaptic sensitivity in rat hippocampal CA1 neurons. *Journal of Neurophysiology* **78**, 2682-2692.

Shrode, L.D., Klein, J.D., O'Neill, W.C. & Putnam, R.W. (1995) Shrinkage-induced activation of Na^+/H^+ exchange in primary rat astrocytes: Role of myosin light-chain kinase. *American Journal of Physiology* **269**, C257-66.

Shrode, L.D., Klein, J.D., Douglas, P.B., O'Neill, W.C. & Putnam, R.W. (1997) Shrinkage-induced activation of Na^+/H^+ exchange: Role of cell density and myosin light chain phosphorylation. *American Journal of Physiology* **272**, C1968-79.

Shrode, L.D., Gan, B.S., D'Souza, S.J., Orlowski, J. & Grinstein, S. (1998) Topological analysis of NHE1, the ubiquitous Na^+/H^+ exchanger using chymotryptic cleavage. *American Journal of Physiology* **275**, C431-9.

Siczkowski, M., Quinn, P.A. & Ng, L.L. (1997) Calcium-induced activation of the rat vascular myocyte Na^+/H^+ exchanger isoform-1. *Metabolism: Clinical & Experimental* **46**, 250-256.

Sidky, A.O. & Baimbridge, K.G. (1997) Calcium homeostatic mechanisms operating in cultured postnatal rat hippocampal neurones following flash photolysis of nitrophenyl-EGTA. *Journal of Physiology* **504**, 579-590.

Siesjö, B.K. (1985) Acid-base homeostasis in the brain: Physiology, chemistry, and neurochemical pathology. *Progress in Brain Research* **63**, 121-154.

Siesjö, B.K. (1988) Historical overview. Calcium, ischemia, and death of brain cells. *Annals of the New York Academy of Sciences* **522**, 638-661.

Silver, R.A., Whitaker, M. & Bolsover, S.R. (1992) Intracellular ion imaging using fluorescent dyes: Artifacts and limits to resolution. *Pflügers Archiv - European Journal of Physiology* **420**, 595-602.

Simon, R.P., Niiro, M. & Gwinn, R. (1993) Prior ischemic stress protects against experimental stroke. *Neuroscience Letters* **163**, 135-137.

Smellie, F.W., Davis, C.W., Daly, J.W. & Wells, J.N. (1979) Alkylxanthines: Inhibition of adenosine-elicited accumulation of cyclic AMP in brain slices and of brain phosphodiesterase activity. *Life Sciences* **24**, 2475-2482.

Smith, G.A. (1996) M. Sc. Thesis. Department of Physiology, University of British Columbia.

Smith, G.A., Brett, C.L. & Church, J. (1998) Effects of noradrenaline on intracellular pH in acutely dissociated adult rat hippocampal CA1 neurones. *Journal of Physiology* **512**, 487-505.

Smith, S.E., Gottfried, J.A., Chen, J.C. & Chesler, M. (1994) Calcium dependence of glutamate receptor-evoked alkaline shifts in hippocampus. *Neuroreport* **5**, 2441-2445.

Somjen, G.G. (1984) Acidification of interstitial fluid in hippocampal formation caused by seizures and by spreading depression. *Brain Research* **311**, 186-188.

Speake, T. & Elliott, A.C. (1998) Modulation of calcium signals by intracellular pH in isolated rat pancreatic acinar cells. *Journal of Physiology* **506**, 415-430.

Spray, D.C. & Bennett, M.V. (1985) Physiology and pharmacology of gap junctions. *Annual Review of Physiology* **47**, 281-303.

Staley, K.J., Soldo, B.L. & Proctor, W.R. (1995) Ionic mechanisms of neuronal excitation by inhibitory GABA$_A$ receptors. *Science* **269**, 977-981.

Strazzabosco, M., Joplin, R., Zsembery, A., Wallace, L., Spirli, C, Fabris, L., Granato, A., Rossanese, A., Poci, C., Neuberger, J.M., Okolicsanyi, L & Crepaldi, G. (1997) Na$^+$-dependent and -independent Cl$^-$/HCO$_3^-$ exchange mediate cellular HCO$_3^-$ transport in cultured human intrahepatic bile duct cells. *Hepatology* **25**, 976-985.

Sumi, M., Kiuchi, K., Ishikawa, T., Ishii, A., Hagiwara, M., Nagatsu, T. & Hidaka, H. (1991) The newly synthesized selective Ca^{2+}/calmodulin dependent protein kinase II inhibitor KN-93 reduces dopamine contents in PC12h cells. *Biochemical & Biophysical Research Communications* **181**, 968-975.

Taira, T., Paalasmaa, P., Voipio, J. & Kaila, K. (1995) Relative contributions of excitatory and inhibitory neuronal activity to alkaline transients evoked by stimulation of Schaffer collaterals in the rat hippocampal slice. *Journal of Neurophysiology* **74**, 643-649.

Takahashi, K.I. & Copenhagen, D.R. (1996) Modulation of neuronal function by intracellular pH. *Neuroscience Research* **24**, 109-116.

Tanaka, Y., Hayashi, N., Kaneko, A., Ito, T., Horimoto, M., Sasaki, Y., Kasahara, A., Fusamoto, H. & Kamada, T. (1994) Characterization of signaling pathways to Na$^+$/H$^+$ exchanger activation with epidermal growth factor in hepatocytes. *Hepatology* **20**, 966-974.

Tang, C.M., Dichter, M. & Morad, M. (1990) Modulation of the *N*-methyl-D-aspartate channel by extracellular H$^+$. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 6445-6449.

Taussig, R. & Gilman, A.G. (1995) Mammalian membrane-bound adenylyl cyclases. *Journal of Biological Chemistry* **270**, 1-4.

Thiels, E., Norman, E.D., Barrionuevo, G. & Klann, E. (1998) Transient and persistent increases in protein phosphatase activity during long-term depression in the adult hippocampus *in vivo*. *Neuroscience* **86**, 1023-1029.

Thomas, R.C. (1979) Recovery of pH$_i$ in snail neurones exposed to high external potassium. *Journal of Physiology* **296**, 77P

Tkachuk, V.A. & Men'shikov, M.I. (1981) Effect of pH on Ca^{2+}-binding properties of calmodulin and on its interaction with Ca^{2+}-dependent phosphodiesterase of cyclic nucleotides. *Biochemistry* **46**, 963-973.

Tombaugh, G.C. (1994) Mild acidosis delays hypoxic spreading depression and improves neuronal recovery in hippocampal slices. *Journal of Neuroscience* **14**, 5635-5643.

Tombaugh, G.C. & Somjen, G.G. (1996) Effects of extracellular pH on voltage-gated Na^+, K^+ and Ca^{2+} currents in isolated rat CA1 neurons. *Journal of Physiology* **493**, 719-732.

Tombaugh, G.C. & Somjen, G.G. (1997) Differential sensitivity to intracellular pH among high- and low-threshold Ca^{2+} currents in isolated rat CA1 neurons. *Journal of Neurophysiology* **77**, 639-653.

Tominaga, T., Ishizaki, T., Narumiya, S. & Barber, D.L. (1998) p160ROCK mediates RhoA activation of Na^+-H^+ exchange. *EMBO Journal* **17**, 4712-4722.

Tønnessen, T.I., Ludt, J., Sandvig, K. & Olsnes, S. (1987) Bicarbonate/chloride antiport in Vero cells: I. Evidence for both sodium-linked and sodium-independent exchange. *Journal of Cellular Physiology* **132**, 183-191.

Tønnessen, T.I., Sandvig, K. & Olsnes, S. (1990a) Role of Na^+-H^+ and Cl^--HCO_3^- antiports in the regulation of cytosolic pH near neutrality. *American Journal of Physiology* **258**, C1117-26.

Tønnessen, T.I., Aas, A.T., Ludt, J., Blomhoff, H.K. & Olsnes, S. (1990b) Regulation of Na^+/H^+ and Cl^-/HCO_3^- antiports in Vero cells. *Journal of Cellular Physiology* **143**, 178-187.

Tornquist, K. (1993) The calmodulin antagonist W-7 depletes intracellular calcium stores in FRTL-5 thyroid cells. *Biochemical & Biophysical Research Communications* **190**, 37-41.

Trapp, S., Lückermann, M., Kaila, K. & Ballanyi, K. (1996) Acidosis of hippocampal neurones mediated by a plasmalemmal Ca^{2+}/H^+ pump. *Neuroreport* **7**, 2000-2004.

Traynelis, S.F., Hartley, M. & Heinemann, S.F. (1995) Control of proton sensitivity of the NMDA receptor by RNA splicing and polyamines. *Science* **268**, 873-876.

Traynelis, S.F. & Cull-Candy, S.G. (1990) Proton inhibition of *N*-methyl-D-aspartate receptors in cerebellar neurons. *Nature* **345**, 347-350.

Traynelis, S.F. & Cull-Candy, S.G. (1991) Pharmacological properties and H^+ sensitivity of excitatory amino acid receptor channels in rat cerebellar granule neurones. *Journal of Physiology* **433**, 727-763.

Tse, M., Levine, S., Yun, C., Brant, S., Counillon, L.T., Pouysségur, J. & Donowitz, M. (1993) Structure/function studies of the epithelial isoforms of the mammalian Na^+/H^+ exchanger gene family. *Journal of Membrane Biology* **135**, 93-108.

Tyler, T.J. (1980) Brain slice preparation: Hippocampus. *Brain Research Bulletin* **5**, 391-403.

Urushidani, T. & Forte, J.G. (1997) Signal transduction and activation of acid secretion in the parietal cell. *Journal of Membrane Biology* **159**, 99-111.

Van Haastert, P.J., van Driel, R., Jastorff, B., Baraniak, J., Stec, W.J. & De Wit, R.J. (1984) Competitive cAMP antagonists for cAMP-receptor proteins. *Journal of Biological Chemistry* **259**, 10020-10024.

Vaughan-Jones, R.D. (1979) Regulation of chloride in quiescent sheep-heart Purkinje fibres studied using intracellular chloride and pH-sensitive micro-electrodes. *Journal of Physiology* **295**, 111-137.

Vaughan-Jones, R.D. & Wu, M.L. (1990) Extracellular H^+ inactivation of Na^+-H^+ exchange in the sheep cardiac Purkinje fibre. *Journal of Physiology* **428**, 441-466.

Velísek, L., Dreier, J.P., Stanton, P.K., Heinemann, U. & Moshe, S.L. (1994) Lowering of extracellular pH suppresses low-Mg^{2+}-induces seizures in combined entorhinal cortex-hippocampal slices. *Experimental Brain Research* **101**, 44-52.

Velísek, L. (1998) Extracellular acidosis and high levels of carbon dioxide suppress synaptic transmission and prevent the induction of long-term potentiation in the CA1 region of rat hippocampal slices. *Hippocampus* **8**, 24-32.

Velísek, L. & Veliskova, J. (1994) Anticonvulsant action of carbonic anhydrase inhibition. *Sbornik Lekarsky* 95, 161-171.

Vicentini, L.M. & Villereal, M.L. (1985) Activation of Na^+/H^+ exchange in cultured fibroblasts: Synergism and antagonism between phorbol ester, Ca^{2+} ionophore, and growth factors. *Proceedings of the National Academy of Sciences of the United States of America* **82**, 8053-8056.

Vigne, P., Breittmayer, J.P., Frelin, C. & Lazdunski, M. (1988) Dual control of the intracellular pH in aortic smooth muscle cells by a cAMP-sensitive HCO_3^-/Cl^- antiporter and a protein kinase C-sensitive Na^+/H^+ antiporter. *Journal of Biological Chemistry* **263**, 18023-18029.

Vignes, M., Blanc, E., Guiramand, J., Gonzalez, E., Sassetti, I. & Recasens, M. (1996) A modulation of glutamate-induced phosphoinositide breakdown by intracellular pH changes. *Neuropharmacology* **35**, 1595-1604.

Vince, J.W. & Reithmeier, R.A. (1998) Carbonic anhydrase II binds to the carboxyl terminus of human band 3, the erythrocyte Cl^-/HCO_3^- exchanger. *Journal of Biological Chemistry* **273**, 28430-28437.

Voipio, J., Paalasmaa, P., Taira, T. & Kaila, K. (1995) Pharmacological characterization of extracellular pH transients evoked by selective synaptic and exogenous activation of AMPA, NMDA, and $GABA_A$ receptors in the rat hippocampal slice. *Journal of Neurophysiology* **74**, 633-642.

Voipio, J. & Kaila, K. (1993) Interstitial pCO_2 and pH in rat hippocampal slices measured by means of a novel fast CO_2/H^+-sensitive microelectrode based on a PVC-gelled membrane. *Pflügers Archiv - European Journal of Physiology* **423**, 193-201.

Vornov, J.J., Thomas, A.G. & Jo, D. (1996) Protective effects of extracellular acidosis and blockade of sodium/hydrogen ion exchange during recovery from metabolic inhibition in neuronal tissue culture. *Journal of Neurochemistry* **67**, 2379-2389.

Voyno-Yasenetskaya, T., Conklin, B.R., Gilbert, RL, Hooley, R., Bourne, H.R. & Barber, D.L. (1994) $G_{\alpha 13}$ stimulates Na^+-H^+ exchange. *Journal of Biological Chemistry* **269**, 4721-4724.

Vyklický, L., Jr., Vlachová, V. & Krušek, J. (1990) The effect of external pH changes on responses to excitatory amino acids in mouse hippocampal neurones. *Journal of Physiology* **430**, 497-517.

Wakabayashi, S., Sardet, C., Fafournoux, P., Counillon, L., Meloche, S., Pages, G. & Pouysségur, J. (1992) Structure function of the growth factor-activatable Na^+/H^+ exchanger (NHE1). *Reviews of Physiology Biochemistry & Pharmacology* **119**, 157-186.

Wakabayashi, S., Bertrand, B., Ikeda, T., Pouysségur, J. & Shigekawa, M. (1994) Mutation of calmodulin-binding site renders the Na^+/H^+ exchanger (NHE1) highly H^+-sensitive and Ca^{2+} regulation-defective. *Journal of Biological Chemistry* **269**, 13710-13715.

Wakabayashi, S., Ikeda, T., Noël, J., Schmitt, B., Orlowski, J., Pouysségur, J. & Shigekawa, M. (1995) Cytoplasmic domain of the ubiquitous Na^+/H^+ exchanger NHE1 can confer Ca^{2+} responsiveness to the apical isoform NHE3. *Journal of Biological Chemistry* **270**, 26460-26465.

Wakabayashi, S., Shigekawa, M. & Pouysségur, J. (1997a) Molecular physiology of vertebrate Na^+/H^+ exchangers. *Physiological Reviews* **77**, 51-74.

Wakabayashi, S., Ikeda, T., Iwamoto, T., Pouysségur, J. & Shigekawa, M. (1997b) Calmodulin-binding autoinhibitory domain controls "pH-sensing" in the Na^+/H^+ exchanger NHE1 through sequence-specific interaction. *Biochemistry* **36**, 12854-12861.

Wang, G.J., Randall, R.D. & Thayer, S.A. (1994) Glutamate-induced intracellular acidification of cultured hippocampal neurons demonstrates altered energy metabolism resulting from Ca^{2+} loads. *Journal of Neurophysiology* **72**, 2563-2569.

Wang, L.Y., Orser, B.A., Brautigan, D.L. & MacDonald, J.F. (1994) Regulation of NMDA receptors in cultured hippocampal neurons by protein phosphatases 1 and 2A. *Nature* **369**, 230-232.

Wang, Z., Orlowski, J. & Shull, G.E. (1993) Primary structure and functional expression of a novel gastrointestinal isoform of the rat Na^+/H^+ exchanger. *Journal of Biological Chemistry* **268**, 11925-11928.

Waxham, M.N., Malenka, R.C., Kelly, P.T. & Mauk, M.D. (1993) Calcium/calmodulin-dependent protein kinase II regulates hippocampal synaptic transmission. *Brain Research* **609**, 1-8.

Weinman, E.J., Shenolikar, S. & Kahn, A.M. (1987) cAMP-associated inhibition of Na^+-H^+ exchanger in rabbit kidney brush-border membranes. *American Journal of Physiology* **252**, F19-25.

Weinman, E.J., Dubinsky, W.P., Fisher, K., Steplock, D., Dinh, Q., Chang, L. & Shenolikar, S. (1988) Regulation of reconstituted renal Na^+/H^+ exchanger by calcium-dependent protein kinases. *Journal of Membrane Biology* **103**, 237-244.

Weinman, E.J., Steplock, D. & Shenolikar, S. (1993) cAMP-mediated inhibition of the renal brush border membrane Na^+-H^+ exchanger requires a dissociable phosphoprotein cofactor. *Journal of Clinical Investigation* **92**, 1781-1786.

Weinman, E.J., Steplock, D., Wang, Y. & Shenolikar, S. (1995) Characterization of a protein cofactor that mediates protein kinase A regulation of the renal brush border membrane Na^+-H^+ exchanger. *Journal of Clinical Investigation* **95**, 2143-2149.

Weinman, E.J. & Shenolikar, S. (1993) Regulation of the renal brush border membrane Na^+-H^+ exchanger. *Annual Review of Physiology* **55**, 289-304.

Weissberg, P.L., Little, P.J., Cragoe, E.J., Jr. & Bobik, A. (1989) The pH of spontaneously beating cultured rat heart cells is regulated by an ATP-calmodulin-dependent Na^+/H^+ antiport. *Circulation Research* **64**, 676-685.

Wielöch, T., Hu, B.R., Boris-Moller, A., Cardell, M., Kamme, F., Kurihara, J. & Sakata, K. (1996) Intracellular signal transduction in the postischemic brain. *Advances in Neurology* **71**, 371-388.

Wilson, M.A. & Gillette, R. (1985) pH sensitivity of calmodulin distribution in nervous tissue fractions. *Brain Research* **331**, 190-193.

Wu, M.L. & Vaughan-Jones, R.D. (1994) Effect of metabolic inhibitors and second messengers upon Na^+-H^+ exchange in the sheep cardiac Purkinje fibre. *Journal of Physiology* **478**, 301-313.

Wu, P.H., Phillis, J.W. & Nye, M.J. (1982) Alkylxanthines as adenosine receptor antagonists and membrane phosphodiesterase inhibitors in central nervous tissue: Evaluation of structure-activity relationships. *Life Sciences* **31**, 2857-2867.

Xia, Z. & Storm, D.R. (1997) Calmodulin-regulated adenylyl cyclases and neuromodulation. *Current Opinion in Neurobiology* 7, 391-396.

Xu, P. & Spitzer, K.W. (1994) Na^+-independent Cl^--HCO_3^- exchange mediates recovery of pH_i from alkalosis in guinea pig ventricular myocytes. *American Journal of Physiology* 267, H85-91.

Yamada, H., Seki, G., Taniguchi, S., Uwatoko, S., Nosaka, K., Suzuki, K. & Kurokawa, K. (1996) Roles of Ca^{2+} and PKC in regulation of acid/base transport in isolated proximal tubules. *American Journal of Physiology* 271, F1068-76.

Yamamoto, M., Kawanishi, T., Kiuchi, T., Ohta, M., Yokota, I., Ohata, H., Momose, K., Inoue, K. & Hayakawa, T. (1998) Discrepant intracellular pH changes following intracellular Ca^{2+} increases induced by glutamate and Ca^{2+} ionophores in rat hippocampal neurons. *Life Sciences* 63, 55-63.

Yasutake, M. & Avkiran, M. (1995) Exacerbation of reperfusion arrhythmias by α_1-adrenergic stimulation: A potential role for receptor mediated activation of sarcolemmal sodium-hydrogen exchange. *Cardiovascular Research* 29, 222-230.

Yu, X.M. & Salter, M.W. (1998) Gain control of NMDA-receptor currents by intracellular sodium. *Nature* 396, 469-474.

Yun, C.H., Tse, C.M., Nath, S.K., Levine, S.A., Brant, S.R., & Donowitz. M. (1995) Mammalian Na^+/H^+ exchanger gene family: Structure and function studies. *American Journal of Physiology* 269, G1-11.

Yun, C.H., Oh, S., Zizak, M., Steplock, D., Tsao, S., Tse, C.M., Weinman, E.J. & Donowitz, M. (1997) cAMP-mediated inhibition of the epithelial brush border Na^+/H^+ exchanger, NHE3, requires an associated regulatory protein. *Proceedings of the National Academy of Sciences of the United States of America* 94, 3010-3015.

Yun, C.H., Lamprecht, G., Forster, D.V. & Sidor, A. (1998) NHE3 kinase A regulatory protein E3KARP binds the epithelial brush border Na^+/H^+ exchanger NHE3 and the cytoskeletal protein ezrin. *Journal of Biological Chemistry* 273, 25856-25863.

Zhang, Y., Chernova, M.N., Stuart-Tilley, A.K., Jiang, L. & Alper, S.L. (1996) The cytoplasmic and transmembrane domains of AE2 both contribute to regulation of anion exchange by pH. *Journal of Biological Chemistry* 271, 5741-5749.

Zhao, H., Wiederkehr, M.R., Fan, L., Collazo, R.L., Crowder, L.A., & Moe, O.W. (1999) Acute inhibition of Na^+/H^+ exchanger NHE-3 by cAMP. *Journal of Biological Chemistry* 274, 3978-3987.