ANOXIA-EVOKED CHANGES IN INTRACELLULAR pH IN ACUTELY DISSOCIATED ADULT RAT HIPPOCAMPAL NEURONS

by

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ABSTRACT

The ratiometric fluorophore, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was utilized to measure pH_i in acutely dissociated adult rat hippocampal CA1 neurons in response to periods of anoxia of 3, 5 and 10 min duration. At 37°C, in the presence or absence of HCO_3^-, the pH_i response to anoxia was characterized by three phases: i) during the anoxic period, there was an intracellular acidification; ii) in the continued absence of oxygen, there was a rise in pH_i toward pre-anoxic pH_i values; and iii) in the immediate post-anoxic period, there was an intracellular alkalinization above pre-anoxic pH_i values. These changes were not secondary to anoxia-evoked changes in intracellular free Ca^{2+} concentration.

Next, the mechanisms underlying the anoxia-evoked changes in pH_i were examined. In addition to supporting the established roles of an increased rate of ATP hydrolysis and intracellular lactate accumulation, evidence is presented that a decreased activity of the Na^+/H^+ exchanger contributed to the production of the intracellular acidification observed during an anoxic period. The rise in pH_i observed in the continued absence of oxygen was attributed, at least in part, to activation of a Zn^{2+}-sensitive, voltage-dependent proton conductance. Finally, an increased activity of the Na^+/H^+ exchanger contributed to the production of the post-anoxic alkalinization. Reductions in extracellular pH and ambient temperature were found to attenuate the post-anoxic activation of Na^+/H^+ exchange, as was an inhibitor of PKA.

The pH_i changes characterized in the present study may determine, at least in part, neuronal viability following an anoxic insult.

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INTRODUCTION

1 Anoxia and Neuronal Viability

Due to a lack of energy storage and a high metabolic rate, neurons within the central nervous system are extremely sensitive to oxygen and/or glucose deprivation. Several seconds following the induction of hypoxia and/or ischemia, there is a slowing of cortical electroencephalographic activity and a loss of consciousness and higher brain functions (Hansen, 1985; Haddad and Jiang, 1993; Fortuna *et al*, 1997). On a cellular level, a transient period of ischemia of as little as 2.5 - 5.0 minutes can cause severe neuronal damage to the hippocampal CA1 region, the substantia nigra, the parietal cortex and the thalamic nuclei (Diemer and Siemkowicz, 1981; Li *et al*, 1996; Fortuna *et al*, 1997). Histological examination reveals regions of extensive neuronal necrosis, with neurons exhibiting characteristically shrunken cytoplasm and nuclei (Diemer and Siemkowicz, 1981; Fortuna *et al*, 1997) surrounded by transition areas of incomplete ischemia which also contain cellular damage (penumbral zones; Choi, 1990).

It has been suggested that it is the reduction in oxygen availability, and not the reduction in blood flow (and, therefore, glucose supply), which is the important determinant of the neuronal response to an anoxic/ischemic insult (Astrup *et al*, 1981). As the partial pressure of oxygen is reduced, progressing from an hypoxic to an anoxic insult, neuronal function is more severely compromised and there is a decreased likelihood of functional recovery upon reoxgenation (Siesjö *et al*, 1975; O'Reilly *et al*, 1995). Thus, the reduction in oxygen availability is the major factor which determines the neuronal response to a transient period of ischemia.

2 Neuronal Response to a Transient Anoxic Period

In response to a transient period of anoxia, neurons undergo a progressive series of intracellular events which determine their excitability and viability during and following the anoxic period. These changes, while more exaggerated and more rapid to their onset, also underlie the neuronal response to a transient ischemic period. Moreover, this neuronal response has been confirmed in hippocampal, brainstem, and neocortical neurons (Cowan and Martin, 1992; Donnelly *et al*, 1992; Jiang and Haddad, 1992; O'Reilly *et al*, 1995; Tanaka *et al*, 1997).

The primary event during an ischemic or anoxic period is a compromised cellular energy metabolism. Immediately upon the induction of a period of energy imbalance, there is a rapid decline in phosphocreatine (PCr) levels, which acts to maintain intracellular ATP levels for a short period of time (~ 30 - 90 seconds; Whittingham *et al*, 1984; Katsura *et al*, 1992b). During this period, there are only minor changes in the cellular membrane potential, intracellular pH (pH_i) and/or extracellular pH (pH_o; Martin *et al*, 1994). Once the PCr supply is depleted, ATP levels begin to fall along with parallel increases in ADP and AMP levels, which, in turn, stimulate the glycolytic rate-controlling enzyme, phosphofructokinase (Erecinska and Silver, 1989). As a result of an increased rate of anaerobic glycolysis, pH_i, and then pH_o, begin to decrease (Dennis *et al*, 1991; Katsura *et al*, 1992b; Smith *et al*, 1993). Approximately 2 minutes following the onset of anoxia, a now significant decrease in tissue ATP levels initiates electrophysiological failure (Lipton and Whittingham, 1982), which is signaled by an initial, slow decrease in membrane potential (Santos *et al*, 1996). In some cases, prior to

membrane depolarization, a slight hyperpolarization, attributed to an activation of ATP-sensitive K^+ currents (I_{KATP}) and Ca^{2+}-dependent K^+ currents ($I_{K(Ca^{2+})}$; Leblond and Krnjevic, 1989; Jiang and Haddad, 1991; Yamamoto *et al*, 1997) may be observed (Cummins *et al*, 1991). With a further decline in intracellular ATP levels, neurons undergo anoxic depolarization and there is a rapid redistribution of all major intracellular ions (Silver and Erecinska, 1990; Donnelly *et al*, 1992; Balestrino, 1995). Anoxic depolarization coincides with a rapid increase in intracellular Na^+ ($[Na^+]_i$), intracellular Ca^{2+} ($[Ca^{2+}]_i$) and intracellular Cl^- ($[Cl^-]_i$), a decrease in intracellular K^+ ($[K^+]_i$), a loss of cell volume regulation and a further reduction in intracellular ATP levels (Jiang and Haddad, 1991; Katsura *et al*, 1992b; Tanaka *et al*, 1997; Fowler and Li, 1998). As a result of the severe intracellular ATP depletion and membrane depolarization, anoxia and ischemia cause a significant release of glutamate and monoamine neurotransmitters (Santos *et al*, 1996). In the continued absence of oxygen, neither the cellular energy status nor the membrane potential are able to recover. If the insult is transient in nature, the recovery of ATP levels to ~ 30% of pre-anoxic levels is sufficient to fuel Na^+/K^+ ATPase activity and restore transmembrane ion gradients (Ekholm *et al*, 1993). However, the restoration of transmembrane ion gradients following a transient period anoxia/ischemia does not occur consistently and the normalization of extracellular glucose and/or oxygen levels do not necessarily result in the restoration of ion homeostasis and membrane repolarization (O'Reilly *et al*, 1995; Tanaka *et al*, 1997). In fact, in peripheral cell types, cellular damage is initiated during the reperfusion period (Currin *et al*, 1991; Harper *et al*, 1993; Maddaford and Pierce, 1997).

3 Mechanisms Contributing to Anoxia-Induced Neuronal Damage

In mammalian central neurons, a great deal of experimental work has addressed the relationship between the cellular response to a transient period of anoxia or ischemia and the events that may mediate neuronal damage.

3.1 Glutamate excitotoxicity

One theory proposed to explain anoxia/ischemia-induced neuronal damage centers around the concept of excitotoxicity. As a result of a significant membrane depolarization, an increase in $[Na^+]_i$, $[Ca^{2+}]_i$, and a decrease in intracellular ATP, anoxia/ischemia induces the release of excitatory neurotransmitters (most notably glutamate) into the synaptic cleft (Santos *et al*, 1996). The resulting excessive stimulation of *N*-methyl-D-aspartate (NMDA) and possibly other subtypes of glutamate receptors causes a dramatic rise in $[Ca^{2+}]_i$, which then initiates neuronal damage by aggravating the intracellular decline in ATP levels and stimulating a plethora of intracellular degradative enzymes (Choi, 1990; Herman *et al*, 1990; Mitani *et al*, 1994). However, a number of studies have suggested that glutamate-mediated excitotoxicity may not be a completely valid model for the detrimental effects of anoxia on neuronal viability. Firstly, while glutamate receptor antagonists are neuroprotective following a period of combined oxygen and glucose deprivation, they fail to influence neuronal survival following a period of severe oxygen deprivation (Newell *et al*, 1995). Secondly, the time course of glutamate-induced neuronal dysfunction is distinct from that induced by anoxia (Friedman and Haddad, 1993; Haddad and Jiang, 1993). Thirdly, an increase

in $[Ca^{2+}]_i$ does not necessarily proceed to neuronal death (Friedman and Haddad, 1993; Li *et al*, 1996). Finally, it is well-established not only that the NMDA receptor-channel complex is inhibited by reductions in pH_o (*e.g.* Vyklicky *et al*, 1990) but also that marked falls in pH_o occur during anoxia and/or ischemia *in vivo* (*e.g.* Silver and Erecinska, 1992). These facts have lead some investigators to question the contribution of NMDA receptor-mediated excitotoxicity to neuronal death evoked by anoxia/ischemia *in vivo* (Tombaugh and Sapolsky, 1993).

3.2 The role of extracellular pH

Rapid shifts in pH_o occur in response to both physiological and pathophysiological events (Hansen, 1985) and these shifts are able to modulate neuronal function under both normoxic and anoxic conditions.

Increased neuronal activity causes an initial extracellular alkalinization often followed by an extracellular acidification (Jarolimek *et al*, 1989; Rose and Deitmer, 1995). Moreover, during such pathological events as cardiac arrest and spreading depression, cerebral pH_o transiently declines followed by an alkaline shift that gives way to a prolonged acidosis (Mutch and Hansen, 1984; Chesler, 1990). These pH shifts observed in the cerebral interstitial space range in magnitude from 0.1 to 1.0 pH units (Hansen, 1985) and, in turn, pH_o changes of corresponding magnitudes are able to influence neuronal function (Balestrino and Somjen, 1988; Church and McLennan, 1989; Tombaugh, 1994; Tombaugh and Somjen, 1996). For example, seizure activity induces a decrease in pH_o which may, in turn, act to limit or even terminate the seizure activity

itself (*e.g.* Aram and Lodge, 1987). In a similar manner, mild extracellular acidosis, which occurs during and following an anoxic insult, exerts a neuroprotective effect, while mild extracellular alkalosis is detrimental to neuronal survival. The neuroprotective ability of mild extracellular acidosis has been largely attributed to the ability of extracellular protons to inhibit Ca^{2+}-influx through both NMDA and voltage-activated Ca^{2+} channels (Church and McLennan, 1989; Giffard *et al*, 1990; Traynelis and Cull-Candy, 1991; Tombaugh and Somjen, 1996; Church *et al*, 1998). In addition, mild extracellular acidosis suppresses neuronal excitability and reduces neuronal energy demand by decreasing voltage-gated Na^+ and Ca^{2+} currents and augmenting $GABA_A$-mediated Cl^- current (Krnjevic and Walz, 1990; Cummins *et al*, 1991; Robello *et al*, 1994; Tombaugh and Somjen, 1996). These observations indicate that reductions in pH_o may exert a neuroprotective effect which can exceed that produced by glutamate receptor antagonists alone, a possibility supported by direct experimental evidence (Tombaugh and Sapolsky, 1990; Kaku *et al*, 1993). In contrast to the neuroprotective effects of mild reductions in pH_o, marked falls in pH_o may kill neurons (reviewed by Siesjö *et al*, 1993; Tombaugh and Sapolsky, 1993; Siesjö *et al*, 1996; see also Nedergaard *et al*, 1991). Excessive and/or prolonged extracellular acidosis rapidly depletes tissue ATP levels and, following an anoxic insult, stimulates lipid peroxidation and protein denaturation as well as the accumulation of free radicals (Tombaugh and Sapolsky, 1993; Nedergaard and Goldman, 1993; Siesjö *et al*, 1996; Raley-Susman and Barnes, 1998). Thus, pH_o is a key determinant of both neuronal function and viability during and following an ischemic/anoxic insult.

3.3 The role of intracellular pH

The great majority of studies which have examined the effects of changes in pH_o on neuronal function, whether under physiological or pathophysiological conditions, have not assessed the possible contribution of changes in pH_i that may occur coincident with a change in pH_o. In the large majority of peripheral cell types (*e.g.* myocytes, neutrophils, and cardiac Purkinje fibers), pH_o has only a minor influence on pH_i (the slope of linear regression line relating pH_i to pH_o, ΔpH_i:ΔpH_o, = 0.35 - 0.40; see Aickin, 1984; Wilding *et al*, 1992). In contrast, in cultured post-natal hippocampal neurons, neocortical neurons, and rat brain synaptosomes, pH_o is a critical determinant of pH_i (ΔpH_i:ΔpH_o = 0.75; Ou-Yang *et al*, 1993; Sanchez-Armass *et al*, 1994; Church *et al*, 1998). The influence of pH_o on neuronal function under normoxic and anoxic conditions may then, at least in part, be secondary to a change in pH_i. Indeed, pH_i is known to influence the activities of a large number of intracellular processes and the shifts in pH_i which occur in response to an anoxic or ischemic insult have been suggested to be a critical determinant of the neuronal response to anoxia (see below).

Thus, pH_i regulates the activities of intracellular metabolic pathways and intracellular second messenger systems, both of which are critical to the genesis of anoxic/ischemic neuronal damage (Siesjö *et al*, 1996; Wieloch *et al*, 1996). The activity of phosphofructokinase, for example, is markedly pH_i dependent with a change as small as 0.1 pH unit being able to completely activate or inactivate the glycolytic pathway (Busa and Nuccitelli, 1984). Similarly, adenylyl cyclase and cyclic nucleotide phosphodiesterase, those enzymes responsible for cAMP synthesis and hydrolysis,

respectively, are regulated by pH_i such that an intracellular alkalinization causes an elevation in intracellular cAMP levels (Busa and Nuccitelli, 1984). Additional intracellular second messengers, including Ca^{2+}, Ca^{2+}/calmodulin (Busa and Nuccitelli, 1984), and inositol trisphosphate (Vignes et al, 1996), are similarly sensitive to changes in pH_i. In turn, both in vivo and in vitro studies have suggested that anoxia/ischemia-induced changes in membrane potential and $[Ca^{2+}]_i$, for example, reflect, at least in part, a change in pH_i (Silver and Erecinska, 1990 and 1992; Cowan and Martin, 1995). In addition, Krnjevic and Walz (1990) observed a strong correlation between anoxia-induced falls in pH_o and the degree of depression of the population spike in the CA1 region of hippocampal slices, and they attributed this effect on a concomitant fall in pH_i.

Reductions in pH_i evoked by anoxia or ischemia have been suggested to exert a neuroprotective effect. Thus, reductions in pH_i can act to inhibit Ca^{2+} influx via voltage-activated Ca^{2+} channels (Takahashi and Copenhagen, 1996; Tombaugh and Somjen, 1997); reduce neurotransmitter release (Nachshen and Drapeau, 1988); and reduce gap junctional conductance, thereby inhibiting neuronal synchrony and (possibly) anoxia-induced epileptiform activity (Spray et al, 1985; Church and Baimbridge, 1991; Perez-Velazquez et al, 1994). On the other hand, the neurotoxicity associated with exposure to highly acidic media ($pH_o < 6.5$) appears to be a function of the degree and duration of the intracellular acidification produced consequent upon the fall in pH_o (Nedergaard et al, 1991). Thus, a marked decrease in pH_i is capable of inducing cellular dysfunction by initiating DNA damage and the production of free radicals (Siesjö et al, 1996), as well as stimulating cellular swelling (Jakubovicz and Klip, 1989), all of which have been

implicated in anoxia/ischemia-induced neuronal damage (Choi, 1990; Tombaugh and Sapolsky, 1993).

The studies cited above indicate that changes in intracellular pH can modulate not only neuronal activity under normoxic conditions but also neuronal viability in response to excitotoxic, anoxic, or ischemic insults. Indeed, differences in the magnitudes of the pH_i changes evoked by anoxia have been suggested to underlie the increased resistance of fetal neurons to anoxia-induced injury (Roberts, Jr. and Chih, 1997), the neurotoxic nature of pre-anoxic hyperglycemia compared with hypoglycemia (LeBlanc et al, 1993; Tyson et al, 1993), and the neuroprotective nature of mild hypothermia during and following an anoxic insult (Laptook et al, 1995a). Thus, the changes in pH_i which occur following an anoxic or ischemic insult may participate in determining cellular viability. In cardiac myocytes and hepatocytes, for example, the return to physiological pH_i values in the immediate post-anoxic period, rather than the fall in pH_i which is observed during anoxia, initiates an intracellular cascade of events which results in cellular damage (Currin et al, 1991; Bond et al, 1993; Miyame et al, 1996). The relevance of the "pH paradox" has not been established in central neurons, although a similar sequence of events may occur, given the recent demonstration that neuronal damage in cultured fetal neocortical neurons evoked by metabolic inhibition (a combination of 2-deoxy-D-glucose and cyanide) can be reduced by inhibiting the rate of restoration of pH_i to normal values in the immediate post-anoxic period (Vornov et al, 1996).

4 Intracellular pH Regulating Mechanisms

As the intracellular compartment is significantly more alkaline than predicted if protons were passively distributed across the plasma membrane, mechanisms must be in place to extrude intracellular protons. However, compared to that of invertebrate neurons, there are relatively few studies investigating pH_i regulatory mechanisms in vertebrate, and, in particular, mammalian central neurons, even under normoxic conditions (reviewed by Chesler, 1990). This is surprising given that the dysfunction of specific pH_i regulating mechanisms has been linked to such pathophysiological disorders as slow-wave epilepsy (Cox et al, 1997), Alzheimer's dementia (Bosman et al, 1997), glutamate excitotoxicity (Hartley and Dubinsky, 1993) and, indeed, in peripheral cell types, anoxia/ischemia-induced cellular damage (Scholz and Albus, 1993; Bugge et al, 1996; Piper et al, 1996). Thus, given the importance of pH_i in regulating neuronal excitability and viability, the characteristics of the pH_i regulating mechanisms involved in maintaining pH_i are of critical importance.

To date, two major classes of pH_i regulatory mechanisms have been found to be present in the central nervous system: i) a HCO_3^--independent, Na^+/H^+ exchanger, and ii) HCO_3^--dependent exchangers. However, given the large number of additional mechanisms which have been found to participate in pH_i regulation in invertebrate neurons and vertebrate non-neuronal cells, it is unlikely that the aforementioned exchangers are the only mechanisms acting to regulate pH_i in mammalian central neurons.

4.1 Na$^+$/H$^+$ exchange

Under normoxic conditions at 37°C, the resting pH$_i$ of rat hippocampal CA1 neurons, rat neocortical neurons, and rat brain synaptosomes is largely dependent on the activity of the Na$^+$/H$^+$ exchanger (Raley-Susman *et al*, 1991; Ou-Yang *et al*, 1993; Sanchez-Armass *et al*, 1994; Baxter and Church, 1996; Bevensee *et al*, 1996; Smith *et al*, 1998; but see Schwiening and Boron, 1994). To date, six isoforms of the mammalian Na$^+$/H$^+$ exchanger (NHE1-6) have been cloned, each demonstrating varying tissue distributions with a notable scarcity in the brain (Sardet *et al*, 1989; Counillon and Pouysségur, 1995; Yun *et al*, 1995; Wakabayashi *et al*, 1997; Numata *et al*, 1998). The primary function of Na$^+$/H$^+$ exchange in all cell types studied to date is to extrude acid that accumulates intracellularly due to metabolism and the large inward transmembrane gradient for protons. In addition, Na$^+$/H$^+$ exchange activity serves to regulate steady-state intracellular Na$^+$ (Frelin *et al*, 1984; Pike *et al*, 1993), and, in a variety of specialized cell types, Na$^+$ reabsorption across epithelia, as well as cellular growth and proliferation (reviewed by Mahnensmith and Aronson, 1985; Noël and Pouysségur, 1995; Yun *et al*, 1995). Common properties of the Na$^+$/H$^+$ exchanger present in both peripheral cell types and mammalian central neurons are that this antiport extrudes one intracellular H$^+$ in exchange for one extracellular Na$^+$. In addition, extracellular Li$^+$ is an effective external Na$^+$ substitute (Kinsella and Aronson, 1981; Raley-Susman *et al*, 1991). Thus, this system is electroneutral, although the reversal of NHE1 activity has been associated with an outward proton conductance (Demaureux *et al*, 1995; see Section 4.3.2). An additional common property which, at least in part, underlies the ability of the Na$^+$/H$^+$

exchanger to respond to intracellular and extracellular signals is the presence of an intracellular proton modifier site, which, when protonated, increases the affinity of the Na^+/H^+ exchanger for intracellular protons (Aronson *et al*, 1982). Thus, in all cell types, both the rapidity of Na^+/H^+ exchange and its sensitivity for substrates (i.e. intracellular protons) can be tightly controlled.

In contrast to NHE1-4 and the Na^+/H^+ exchangers present in other mammalian central neurons and glial cells (see Vornov *et al*, 1996; Bevensee *et al*, 1997a; Pedersen *et al*, 1998), all of which can be pharmacologically inhibited by amiloride, amiloride analogues and benzoyl guanidinium compounds (Tse *et al*, 1993; Yun *et al*, 1995), the Na^+/H^+ exchanger present in hippocampal neurons is completely insensitive to these compounds with concentrations as large as 1 mM having no effect on exchange activity (Raley-Susman *et al*, 1991; Schwiening and Boron, 1994; Baxter and Church, 1996). Furthermore, hippocampal Na^+/H^+ exchange activity is regulated by a number of intracellular and extracellular factors. Thus, the activity of the Na^+/H^+ exchanger present in hippocampal neurons is stimulated by a decrease in pH_i (Raley-Susman *et al*, 1991; Baxter and Church, 1996; Smith *et al*, 1998), activation of β-adrenoceptors and/or the cAMP/protein kinase A (PKA) pathway (Smith *et al*, 1998), an increase in ambient temperature (Baxter and Church, 1996), and in response to hyperosmotic cell shrinkage (Bookstein *et al*, 1996). Although it is established that noradrenaline and the cAMP/PKA second messenger pathway can modulate the activity of the Na^+/H^+ present in hippocampal neurons (Smith *et al*, 1998), the activity of the Na^+/H^+ exchanger present in a variety of peripheral cell types can be controlled by a wide variety of external agents

and intracellular second messenger systems. These agents are not active in all cell types and may differ between neuronal preparations. For example, in contrast to acutely dissociated hippocampal neurons, in rat brain synaptosomes, Na^+/H^+ exchange activity is not regulated by either PKA or PKC and is primarily influenced by the levels of intracellular Ca^{2+} (Sanchez-Armass *et al*, 1994). In addition, the intracellular pathways involved in the control of the activity of Na^+/H^+ exchangers in peripheral cell types are not mutually exclusive and the activity of a given Na^+/H^+ exchanger in a given cell type may be modulated concomitantly by more than one intracellular signaling pathway. It therefore appears likely that the activity of the Na^+/H^+ exchanger in rat hippocampal neurons may be controlled by pathways other than the cAMP/PKA pathway.

Therefore, there is a distinct variant of the Na^+/H^+ exchanger present in hippocampal CA1 neurons. In recent years, isoforms NHE4 and NHE6 have been localized to the rat brain hippocampus and human brain tissue, respectively (Bookstein *et al*, 1996; Chambrey *et al*, 1997a; Numata *et al*, 1998). While NHE4 demonstrates a partial sensitivity to amiloride (K_d = 0.8 mM; Chambrey *et al*, 1997b), NHE6 has been localized to the mitochondrial inner membrane and, as a result, its role in cytosolic pH_i regulation is unclear. As a result, it is unlikely that either isoform represents the Na^+/H^+ exchange activity observed by our laboratory (Baxter and Church, 1996; Smith *et al*, 1998) and the laboratory of others (Raley-Susman *et al*, 1991; Bevensee *et al*, 1996).

In parallel to its role under normoxic conditions, during and following either cardiac or cerebral ischemia, the Na^+/H^+ exchanger has been implicated in the regulation of pH_i, $[Na^+]_i$, and intracellular volume (Jakubovicz and Klip, 1989; Friedman and

Haddad, 1993; Askenasy *et al*, 1996). In fact, just as pH_i is a key factor in determining cellular viability (see Section 3.3), overactivity of the Na^+/H^+ exchanger appears to be detrimental to functional recovery and survival following an anoxic or ischemic insult, although this relationship has been supported primarily by data collected in peripheral cell types, such as cardiac myocytes and hepatocytes (reviewed by Scholz and Albus, 1993; Piper *et al*, 1996; Bugge *et al*, 1996). Recently, in cortical neurons, Vornov *et al* (1996), demonstrated that Na^+/H^+ exchange inhibition, which blocked the pH_i recovery following a period of metabolic inhibition, improved neuronal survival. Furthermore, given the facts that Na^+/H^+ exchange activity may contribute to anoxia-evoked increase in $[Na^+]_i$ (Friedman and Haddad, 1994b; Gleitz *et al*, 1996) and that an increase in $[Na^+]_i$ may be involved in anoxia-evoked neuronal damage (Friedman and Haddad, 1994b; Taylor *et al*, 1995), it becomes increasingly possible that changes in the activity of the Na^+/H^+ exchanger may be related to subsequent neuronal viability following a transient anoxic insult. However although anoxia is known to evoke changes in pH_i *in vivo*, the role of changes in the activity of the neuronal Na^+/H^+ exchanger in the production of these changes is unknown.

4.2 HCO_3^--dependent pH_i regulating mechanisms

In both peripheral cell types and central neurons, Na^+/H^+ exchange often co-exists with HCO_3^--dependent pH_i regulatory mechanisms (Chesler, 1987; Boyarsky *et al*, 1988; Raley-Susman *et al*, 1991; Ou-Yang *et al*, 1993; Raley-Susman *et al*, 1993; Schweining and Boron, 1994; Baxter and Church, 1996; Smith *et al*, 1998). In hippocampal neurons,

two HCO_3^--dependent pH_i regulatory mechanisms have been identified to date: i) a Na^+-dependent HCO_3^-/Cl^- antiporter, which transports HCO_3^- into the cell in exchange for external Cl^-, thereby functioning as an acid extruder; and ii) a Na^+-independent HCO_3^-/Cl^- antiporter which transports HCO_3^- out of the cell at physiological pH_i values, thereby acting as an alkali extruder. This latter mechanism can reverse under conditions of extreme intracellular acidosis to participate in acid extrusion (Baxter and Church, 1996). Both HCO_3^-/Cl^- exchangers are sensitive to inhibition by 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS).

The regulation of the activity of HCO_3^-/Cl^- exchangers in hippocampal neurons has not been investigated. However, in a manner similar to the Na^+/H^+ exchanger, HCO_3^-/Cl^- exchangers in peripheral cell types respond to a variety of intracellular and extracellular factors, such as changes in pH_o and the activities of various intracellular second messenger systems (Boron *et al*, 1979; Vigne *et al*, 1988; Ludt *et al*, 1991). Importantly, Na^+/H^+ exchange and HCO_3^-/Cl^- exchange activity are often independently regulated. In osteoblasts, for example, a rise in $[Ca^{2+}]_i$ stimulates Na^+-independent HCO_3^-/Cl^- exchange but has no effect on Na^+/H^+ exchange activity whereas a rise in intracellular cAMP inhibits both exchangers (Green and Kleeman, 1992). Furthermore, while the mineralocorticoid, aldosterone, stimulates both Na^+/H^+ exchange and Na^+-independent HCO_3^-/Cl^- exchange in cardiac myocytes, the relative pattern of stimulation differs during cellular development (Korichneva *et al*, 1995). Similar to the Na^+/H^+ exchanger, changes in the activities of HCO_3^-/Cl^- exchangers may play a role in the neuronal response to anoxia/ischemia. Thus, on the basis of NMR-based pH_i

measurements in hippocampal slices, Pirttilä and Kauppinen (1994a) suggested that anoxia causes changes in the activities of HCO_3^--dependent pH_i regulatory mechanisms, although this study was unable to differentiate between the possible role of neuronal vs. glial elements in the effects observed. In addition, inhibition of HCO_3^-/Cl^- exchange protects against excitotoxic damage in chick retinal cells (Zeevalk *et al*, 1989). Furthermore, in some cell types, forward Na^+-independent HCO_3^-/Cl^- exchange participates in the maintenance of $[Cl^-]_i$ and, thus, a particular value of the Cl^- equilibrium potential (*e.g.* Aickin and Brading, 1984; Vaughan-Jones, 1979). Given the importance of $[H^+]_i$, $[HCO_3^-]_i$, and $[Cl^-]_i$ for the control of neuronal excitability (*e.g.* Kaila, 1994; Staley *et al*, 1995), it is possible (though untested) that HCO_3^-/Cl^- exchange may be able to modulate excitability in mammalian central neurons. Thus, it is becoming increasingly recognized that, independent of Na^+/H^+ exchange, HCO_3^-/Cl^- exchange regulates both pH_i and neuronal excitability under a variety of physiological and pathophysiological conditions.

4.3 Additional pH_i regulating mechanisms

In the majority of cell types, a large number of mechanisms act in concert to regulate pH_i. Under conditions which inhibit both Na^+/H^+ exchange and HCO_3^-/Cl^- exchange, the demonstration of pH_i regulation (i.e. recovery from an imposed intracellular acid load) supports the presence of alternative pH_i regulating mechanisms in a variety of cell types (see Peral *et al*, 1995; Bevensee *et al*, 1996; Gibb *et al*, 1997). Outlined below are several significant pH_i regulating mechanisms that can function in the

absence of Na^+/H^+ exchange and HCO_3^-/Cl^- exchange to regulate pH_i under normoxic conditions and which may participate in the production of pH_i changes during anoxia/ischemia.

4.3.1 Ca^{2+}/H^+ exchange

There is a close, interdependent relationship between pH_i and $[Ca^{2+}]_i$. In addition to H^+ and Ca^{2+} ions competing for common intracellular binding sites, pH_i regulates the influx of extracellular Ca^{2+} through voltage-gated channels (Tombaugh and Somjen, 1997), the release of Ca^{2+} from intracellular stores (Ou-Yang et al, 1994b), and the affinity of intracellular binding proteins (e.g. calmodulin) for Ca^{2+} (Busa and Nuccitelli, 1984). In turn, Ca^{2+}, both directly and via intracellular second messenger cascades, can regulate the activity of pH_i regulating mechanisms (see Vaughan-Jones and Wu, 1990; Sanchez-Armass et al, 1994) in addition to influencing proton flux through voltage-dependent H^+ channels (Gordienko et al, 1996). Given the relationship between H^+ and Ca^{2+} ions, it is perhaps not surprising that mechanism(s) exist to regulate in concert the internal concentrations of both ions. One such mechanism is the Ca^{2+}/H^+ ATPase, initially described in snail neurons which extrudes one intracellular Ca^{2+} ion in exchange for an extracellular H^+ (Schweining et al, 1993). This mechanism may also exist in rat hippocampal neurons (Trapp et al, 1996). Ischemia or anoxia may affect the activity of this ATPase, given the facts that these insults lead a rise in $[Ca^{2+}]_i$, and an external acidosis, both of which act to enhance to activity of the Ca^{2+}/H^+ ATPase (see Schweining et al, 1993; Ou-Yang et al, 1994b). Others have suggested the existence of a reversible

Ca^{2+}/H^+ exchange mechanism which is not directly ATP-dependent (Ou-Yang *et al*, 1994a).

4.3.2 Voltage-activated proton conductance

In a variety of cell types, a voltage-activated proton conductance (g_{H^+}) contributes to the recovery of pH_i following intracellular acid loads imposed during membrane depolarization (Meech and Thomas, 1987; Byerly and Suen, 1989; Kapus *et al*, 1993; Gordienko *et al*, 1996). g_{H^+}s demonstrate an extremely high selectivity for H^+ and, upon activation, are capable of producing dramatic shifts in pH_i (~ 5.0 pH units min^{-1}), without demonstrating rapid current inactivation or desensitization (Meech and Thomas, 1987; Kapus *et al*, 1993; Lukacs *et al*, 1993; DeCoursey and Cherny, 1994). Although the existence of g_{H^+} in mammalian central neurons has not been investigated, it is possible that it could be an important mechanism of pH_i regulation during ischemic or anoxic insults which are associated with a prolonged membrane depolarization. In addition, evidence indicates that g_{H^+}s can couple to Na^+/H^+ exchange (Demaurex *et al*, 1995) such that activation of the electroneutral Na^+/H^+ exchanger can reduce the transmembrane H^+ current.

4.3.3 Proton ATPases

P-type and V-type ATPases represent an integrated family of ATP-driven cation transporters. P-type ATPases are primarily found in the stomach, colon, and kidney, where they contribute to pH_i regulation and K^+ homeostasis (van Driel and Callaghan, 1995). The V-type ATPase has been characterized in cardiac myocytes, renal epithelia and osteoclasts (Kurtz, 1987; Nelson and Klionsky, 1996). During and following periods

of metabolic inhibition, V-type ATPase activity provides a cardioprotective effect by attenuating the production of intracellular acidosis during the metabolic insult as well as decreasing Na^+/H^+ exchange-induced Ca^{2+} overload (Karwatowska-Prokopcauk et al, 1998).

4.3.4 Lactate/H^+ cotransport

Although neurons are primarily metabolically aerobic, during periods of intense neuronal activity or periods of ATP depletion, anaerobic metabolism becomes the key energy-producing pathway and, as a result, lactic acid accumulates intracellularly (Ljunggren et al, 1974; Hope et al, 1988; Jarolimek et al, 1989). However, given a pK_a of ~ 3.9, at physiological pH_i values, lactic acid will exist primarily in its undissociated form and, thus, be unable to cross the lipid membrane (but see Dringen et al, 1995). In both peripheral cell types and mammalian central neurons, a lactate/H^+ cotransport mechanism removes intracellular lactate and, in this way, may contribute to pH_i regulation under normoxic conditions (Assaf et al, 1990; Nedergaard and Goldman, 1993; Pirttilä and Kauppinen, 1994b; Juel, 1997). While some studies in peripheral cell types support a role for this mechanism in contributing to the recovery of pH_i following transient periods of anoxia (Vandenberg et al, 1993), studies in mammalian central neurons have failed to document a similar role (Pirttilä and Kauppinen, 1994b).

4.3.5 K^+-dependent transporters

A fifth class of pH_i regulatory mechanisms are those that are K^+-dependent, namely the K^+/H^+ exchanger and K^+/HCO_3^- exchanger (Hofer and Machen, 1992; Hogan et al, 1995a and 1995b; Zhao et al, 1995). These mechanisms have been shown to

participate in the recovery of pH_i from imposed intracellular alkali or acid loads in squid giant axons (Hogan *et al*, 1995a and 1995b). Although these transporters have not been identified in mammalian central neurons, they may participate in pH_i regulation in hippocampal neurons and, furthermore, their role may become increasingly important in pH_i regulation during periods of intracellular acidosis and/or a change in $[K^+]_o$ (*e.g.* membrane depolarization, periods of anoxia/ischemia).

5 Summary

Although anoxia and ischemia have long been known to elicit changes in pH_o and pH_i both *in vivo* and in slice preparations *in vitro* (*e.g.* Fujiwara *et al*, 1992; Pirttilä and Kauppinen, 1992, 1994a, 1994b; Melzian *et al*, 1996; Siesjö *et al*, 1996), it is difficult to separate the contribution of various cell types (including glia) to the effects observed under these experimental conditions. In addition, although changes in neuronal pH_i associated with glutamate receptor-mediated excitotoxicity and metabolic inhibition have been described (*e.g.* Hartley and Dubinsky, 1993; Vornov *et al*, 1996), these insults are not a completely valid model for the direct action of anoxia on central neurons (see Friedman and Haddad, 1993). Furthermore, the mechanism(s) responsible for the pH_i shifts observed in response to either anoxia, ischemia or excitotoxin exposure have not been rigorously characterized.

Recently, data concerning the mechanisms which participate in pH_i regulation in mammalian central neurons under normoxic conditions have become available and support a role for both Na^+/H^+ exchange and HCO_3^-/Cl^- exchange. However, there is

precedence for alternative pH_i regulatory mechanisms which may function, as they do in peripheral cell types, to regulate pH_i during both normoxic and anoxic conditions. Because changes in pH_i may play an important role in the pathophysiology of neuronal death following anoxia and/or ischemia, a knowledge of the pH_i changes evoked by anoxia in central neurons and the mechanisms involved in the regulation of neuronal pH_i both during and following anoxia may provide insights into the pathogenesis of neurodegenerative phenomena. The aim of the present study, therefore, is to characterize the pH_i changes which occur in response to transient anoxia in acutely dissociated adult rat CA1 hippocampal neurons, an experimental preparation in which the pH_i regulating mechanisms operating under normoxic conditions have been extensively characterized (Schweining and Boron, 1994; Bevensee *et al*, 1996, Smith *et al*, 1998) and in which changes in pH_i can be reliably quantified and their underlying mechanisms investigated.

MATERIALS AND METHODS

1 Experimental Preparations

1.1 Acutely dissociated adult rat hippocampal CA1 neurons

Acutely dissociated adult rat hippocampal CA1 neurons were utilized for the majority of the studies. Adult rat hippocampal CA1 neurons were isolated using a modified version of the procedure described by Kohr and Mody (1991). Male Wistar rats approximately 45 days old (180 - 200 g) were obtained from the Animal Care Center (University of British Columbia) and housed under conditions of controlled temperature (20°C - 22°C) and lighting (lights on 0600 - 1800). Food (Lab Diet, PMI Feeds Inc., St. Louis, MO) and water were available *ad libitum*.

The animals were anesthetized with 3% halothane in air, decapitated, and their brains were rapidly removed and placed in ice-cold (4°C - 8°C) HCO_3^-/CO_2-buffered saline (Table 2, solution 6) previously equilibrated with 5% CO_2/95% O_2. One of the hippocampi was separated from the surrounding tissue as described by Tyler (1980) and transverse hippocampal slices, 450 μm thick, were obtained with a McIlwain tissue chopper and collected in ice-cold (4°C - 8°C) HCO_3^-/CO_2-buffered media. The slices were then transferred to an incubation chamber containing HCO_3^-/CO_2-buffered saline and allowed to recover for at least one hour. Three hippocampal slices were then enzymatically digested at 32°C in 2.0 ml of HCO_3^-/CO_2-buffered saline containing 1.5 mg ml^{-1} of pronase (protease type XIV bacterial from *Streptomyces griseus*; Sigma Chemical Co., St. Louis, MO). After 30 min, the CA1 region of each slice was then microdissected with a dissecting chisel and triturated with fire-polished Pasteur pipettes of diminishing

tip diameters (0.7, 0.5, 0.3, and 0.2 mm) in 0.5 ml of loading medium, consisting of HEPES-buffered saline (Table 1, solution 1), pH 7.35 at room temperature, with the iso-osmotic addition of 3.0 mM $NaHCO_3$ replacing NaCl. The triturated mixture was then deposited onto an 18 mm poly-D-lysine- (20-40 μg ml^{-1}; Sigma Chemical Co.) coated glass coverslip. The neurons were allowed to adhere to the substrate for 15 min at room temperature exposed to a 100% O_2 atmosphere prior to the start of an experiment.

Freshly isolated hippocampal CA1 neurons were chosen for study based on morphological criteria established by Schwiening and Boron (1994), *i.e.* a smooth, non-granular appearance; 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 the presence of two or more smaller processes (basal dendrites) at the opposite pole (Fig. 1).

1.2 Cultured post-natal rat hippocampal neurons

Cultured post-natal rat hippocampal neurons were employed only to derive parameters for the calibration of the BCECF signal (see Section 3.3). We have previously determined that the parameters derived from *in situ* calibration experiments using cultured neurons are identical to those obtained from *in situ* calibrations using acutely dissociated cells.

Cultured post-natal rat hippocampal neurons were prepared from 4 - 5 day old Wistar rat pups according to Zorumski *et al* (1992). Transverse hippocampal slices (< 1 mm thick) were collected in ice-cold Leibovitz L-15 medium (pH 7.2 - 7.3 at 37°C;

Gibco, Grand Island, NY) and then incubated for 30 min at 37°C in L-15 medium containing 0.2 mg ml^{-1} bovine serum albumin, 1.0 mg ml^{-1} papain (type IV; Sigma Chemical Co.) and 25 μg ml^{-1} DNAase (type II; Sigma Chemical Co.). Afterwards, the L-15 medium was discarded and replaced with a medium containing Eagles's minimum essential medium (EMEM; Gibco), 22 mM NaHCO$_3$, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), 17 mM glucose, 400 μM glutamine and 25 μg ml^{-1} DNAase dissolved in tissue culture grade water. The slices were mechanically dissociated immediately with fire-polished Pasteur pipettes of decreasing tip diameters, underlayed with fetal bovine serum (FBS) and then cold centrifuged at 150 x g at 4°C for 5 - 6 minutes. The supernatant was discarded and the cell pellet was re-suspended in EMEM. To determine total viable cell concentration, a sample of the cell suspension was removed and mixed with trypan blue, a cell viability indicator. A hemocytometer (Neubauer) chamber was used to count the number of viable hippocampal neurons within the sample and a dilution factor was calculated so that the cell suspension could be diluted with EMEM, supplemented with 5% FBS and 5% horse serum (5/5 serum EMEM), to obtain a final density of 3.0 x 10^5 cells ml^{-1}. The cell suspension was then dispersed onto 18 mm glass coverslips coated with poly-D-lysine and laminin and allowed to adhere for 1- 2 hours in a 5% CO$_2$ atmosphere at 37°C. The coverslips were transferred to six well plates and incubated in 5/5 serum EMEM for 24 h in a 5% CO$_2$ atmosphere at 37°C. The growth medium was then switched to a serum-free, N2-supplemented EMEM (containing 5 μg ml^{-1} insulin, 20 nM progesterone, 100 μM putrescine and 30 nM sodium selenite) and the cell cultures were treated with 10 μM

cytosine-β-D-arabinofuranoside hydrochloride to suppress glial cell proliferation. The serum-free, N2-supplemented EMEM was half-changed with fresh medium every 3 - 4 days. Each coverslip consisted primarily of hippocampal CA1 and CA3 neurons with a maximum of 15% of cells being glial. The cultured neurons were used in experiments from 4 - 10 days after plating.

All neuronal cultures were generously provided by Ms. Stella Atmadja, Department of Physiology, University of British Columbia.

2 Solutions and Chemicals

Unless otherwise noted, all experiments were performed at 37°C. The HEPES-buffered and HCO_3^-/CO_2-buffered media employed in experiments utilizing acutely dissociated neurons are listed in Tables 1 and 2, respectively. For experiments involving cultured post-natal hippocampal neurons, the solutions were identical to those listed in Table 1 except for the concentration of glucose which was reduced to 10.0 mM from 17.5 mM. The reduced Na^+, HEPES-buffered medium in which Li^+ was substituted for Na^+ was prepared by replacement of 134.5 mM NaCl and 1.5 mM NaH_2PO_4 (Table 1, solution 1) with 136 mM LiCl. All low glucose solutions were prepared by reducing the concentration of glucose from 17.5 mM to 5.0 mM with the addition of 12.5 mM D-mannitol to maintain osmolarity (see Cendoroglo *et al*, 1998; Wang *et al*, 1998). The omolarities of these solutions were measured with an μOsmette osmometer (Precision Systems, Inc., Natick, MA), calibrated daily before use.

All solutions were prepared at room temperature (22°C). Accordingly, HEPES-buffered solutions were titrated to pH 7.48 - 7.50 (at room temperature) in order to achieve a final pH of pH 7.35 - 7.36 at 37°C. The pH to which the HEPES-buffered media were titrated, at room temperature, was determined by the equation

$$pH_{37} = 0.1809 + (0.9673 \times pH_{22}), \qquad \text{(Equation 1)}$$

where pH_{37} denotes the final pH of the HEPES-buffered saline at 37°C and pH_{22} represents the pH to which the HEPES-buffered medium was titrated at room temperature (Baxter, 1995). To vary the pH of HCO_3^-/CO_2-buffered solutions, the concentration of $NaHCO_3$ was adjusted by equimolar substitution for NaCl (at a constant P_{CO_2}) according to the equation

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

where pH_{37} represents the pH of a HCO_3^-/CO_2-buffered solution at 37°C following equilibration with 5% CO_2 (Baxter, 1995). The pH dependence of HCO_3^-/CO_2-buffered media varies with temperature. For experiments performed at 22°C (room temperature), the appropriate concentration of $NaHCO_3$ was calculated according to the equation

$$pH_{22} = 5.833 + (1.038 \times \log[HCO_3^-]) \qquad \text{(Equation 3)}$$

All HEPES-buffered solutions were titrated to the appropriate temperature-corrected pH value with 10 M NaOH, except for the high-K^+ calibration medium (Table 1, solution 5) and the reduced-Na^+, HEPES-buffered medium substituted with $NMDG^+$ (Table 1, solution 2) or Li^+, which were titrated with 10 M KOH, 1.0 M HCl and 2.0 M LiOH, respectively. A Corning 240 pH meter (Corning Inc., Corning, NY), calibrated daily, was utilized to measure the pH of all solutions.

A list of pharmacological agents used in these studies is presented in Table 3. Unless otherwise noted, all agents were supplied by Sigma Chemical Co. Bafilomycin A_1 was a generous gift from Dr. V. Palatý (Department of Anatomy, University of British Columbia). Table 3 provides the stock concentrations and the solvent used to prepare the stock solution for each compound. Where applicable, the storage temperature is listed; however, if not provided, the compound was prepared fresh immediately prior to an experiment. The final concentration of dimethylsulphoxide (DMSO) in the test solutions never exceeded 0.1%, which, in control experiments, fails to influence either steady-state pH_i or the activities of the pH_i regulating mechanisms in rat hippocampal neurons (Baxter and Church, 1996; Smith et al, 1998). Stock solutions were diluted to the appropriate test concentration in physiological saline. Solutions containing 500 μM $ZnCl_2$ did not contain NaH_2PO_4, and $MgCl_2$ iso-osmotically replaced $MgSO_4$.

3.0 Microspectrofluorimetry

3.1 Cell loading and BCECF

The microspectrofluorimetric technique used to measure pH_i employed the intracellular fluorescent hydrogen ion indicator, 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF; Molecular Probes Inc., Eugene, OR; Rink et al, 1982; Nett and Deitmer, 1996). The acetoxymethyl ester of BCECF (BCECF-AM) was prepared initially as a 1.0 mM stock solution in anhydrous dimethylsulphoxide (DMSO) and stored in 60 μL aliquots at -60°C. BCECF-AM is hydrophobic and uncharged, allowing it to permeate through the plasma membrane; however, upon entry into the neurons, BCECF-AM is hydrolyzed by intracellular esterases to produce the hydrophilic, polyanionic BCECF free acid which becomes trapped within the neuron.

For in situ calibration experiments employing cultured post-natal hippocampal neurons, a loading medium was prepared consisting of HEPES-buffered saline, pH 7.35 at room temperature (Table 1, solution 1), with the iso-osmotic addition of 3.0 mM $NaHCO_3$ replacing NaCl. When needed, 5.0 μl of the 1.0 mM BCECF-AM stock solution was added to 2.5 ml of the loading medium to achieve a final concentration of 2 μM BCECF-AM. A coverslip with post-natal neurons attached was placed face-up in the 2 μM BCECF-AM-containing loading medium for 30 minutes at room temperature. The coverslip was then transferred onto an insert of the perfusion/recording chamber, supported by a 22 mm glass coverslip and an O-ring rimmed with high vacuum silicone grease (Dow Corning, Midland, MI) so as to form the floor of the chamber.

For each experiment involving acutely dissociated adult hippocampal CA1 neurons in which pH_i was measured, 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; see Section 1.1) and the neurons were allowed to load with dye for 15 minutes at room temperature in a 100% O_2 atmosphere.

Changes in intracellular free Ca^{2+} concentration were measured in experiments utilizing acutely dissociated adult rat hippocampal CA1 neurons. To measure changes in $[Ca^{2+}]_i$, the fluorescent indicator fura-2-AM (7 µM; Grynkiewicz et al., 1985; Molecular Probes, Inc.) was loaded into the neurons in the same manner as BCECF, except the loading occurred at 37°C for 30 minutes.

The prepared chamber (containing neurons loaded with BCECF or fura-2) was placed onto the stage of an inverted microscope. The neurons were then superfused continuously with either HEPES-buffered saline (Table 1, solution 1) or HCO_3^-/CO_2-buffered saline (Table 2, solution 6), pH 7.35 at 37°C, at a rate of 2.0 ml min^{-1} for 15 minutes prior to the start of each experiment to ensure full de-esterification of the dye. The temperature-controlled perfusion chamber allowed for uninterrupted switching of perfusion solutions, e.g. normoxic to anoxic medium. When perfusing with HCO_3^-/CO_2-buffered media, the atmosphere in the perfusion chamber contained 5% CO_2 in balance air (normoxia) or 5% CO_2 in balance Ar or N_2 (anoxia).

3.2 Experimental setup and the ratiometric method

The dual-excitation ratio method for estimating pH_i with BCECF is based upon the relationship between pH and the ratio of emitted fluorescence intensities at alternating wavelengths of excitation at 488 nm and 452 nm (*i.e.* I_{488}/I_{452}). The intensity of the fluorescence emission during excitation at 488 nm is pH sensitive whereas emission during excitation at 452 nm is relatively pH insensitive. For fura-2, also a dual-excitation fluorophore, the excitation wavelengths employed were 334 nm and 380 nm, both of which are sensitive to changes in $[Ca^{2+}]_i$, although I_{334} increases with an increase in $[Ca^{2+}]_i$ and I_{380} decreases with an elevation in $[Ca^{2+}]$. In either case, because the intensities of fluorescence emissions at both excitation wavelengths are from the same cell volume, a ratio of intensities emitted at two different excitation wavelengths will, in principle, not be susceptible to artifacts caused by variations in optical path length, local probe concentrations, illumination intensity and photobleaching (Bright *et al*, 1987; Bright *et al*, 1989; Silver *et al*, 1992).

Throughout an experiment in which pH_i was being measured, the viability of the neuron was assessed by monitoring the stability of the fluorescence emission intensity during excitation at 452 nm (I_{452}; pH-insensitive). Bevensee *et al* (1995) monitored rates of BCECF loss and morphological deterioration of acutely isolated post-natal rat hippocampal CA1 neurons and determined that the rate of change of I_{452} is a sensitive indicator of cell membrane integrity. In addition, studies from the same laboratory indicate that acutely dissociated rat hippocampal neurons which retain BCECF also exclude propidium iodide, a widely-employed indicator of cell membrane integrity

(Bevensee *et al*, 1995). Consequently, neurons in which the rate of change of I_{452} was greater than 5% min^{-1} were excluded from analysis.

The experimental apparatus consisted of a Zeiss Axiovert 10 microscope (Carl Zeiss Canada Ltd., Don Mills, ON) in conjunction with an Attofluor Digital Fluorescence Ratio Imaging System (Atto Instruments Inc., Rockville, MD). UV light, emitted from a 100 W mercury arc lamp, was alternately passed through one of two 10 nm interference filters, centered at 488 nm and 452 nm for BCECF, and was then reflected by a dichroic mirror (FT > 495 nm) into the objective (Zeiss LD Achroplan, n.a. 0.60, 40x) to excite the BCECF loaded into the neurons. Fluorescence emitted by the neurons was collected at 520 nm and measured by an intensified charge-coupled device camera. The camera gains at each excitation wavelength were set to maximize the image intensity, minimize the possibility of camera saturation, and were held constant throughout an experiment. The images were digitized to an 8 bit resolution with a 512 x 480 pixel frame size. Data were obtained from multiple neuronal cell bodies simultaneously, with each neuron delineated as a region of interest (ROI).

In order to minimize photobleaching of the dye and UV-induced damage to the neurons, neutral density filters were placed in the UV light path to reduce the intensity of the incident light at each excitation wavelength. In addition, a computer actuated high speed shutter restricted the exposure of the neurons to the UV light to periods when emitted fluorescence intensities at each excitation wavelength were being measured (usually once every 2 to 15 seconds). Finally, when using BCECF, a variable intensity

lamp control (Attoarc, Carl Zeiss Canada Ltd.) was employed to reduce the intensity of the 100 W mercury arc lamp by 50%.

3.3 Calculation of pH_i and $[Ca^{2+}]_i$

At the beginning of each experiment, background readings of autofluorescence at each excitation wavelength were measured in an area devoid of cellular elements. Using Visual Basic macros running in Microsoft Excel 5.0 (Microsoft Corp., Redmond, WA), the respective background fluorescence intensities were subtracted from the raw emission intensities at each excitation wavelength from each ROI throughout the experiment to yield background-corrected ratios of emission intensities (BI_{488}/BI_{452} in the case of BCECF; BI_{334}/BI_{380} in the case of fura-2). Calibration of fura-2 signals was not attempted (see Church *et al*, 1998) and changes in $[Ca^{2+}]_i$ evoked by experimental maneuvers are presented as changes in background-corrected I_{334}/I_{380} ratio values.

At the end of each experiment in which BCECF was employed, a one-point calibration was performed where the neurons were exposed to an high-K^+ HEPES-buffered solution, pH 7.00 at 37°C (Table 1, solution 5) that contained 10 μM nigericin, a K^+/H^+ ionophore (Thomas *et al*, 1979). Nigericin, a charged electron carrier, balances cytoplasmic and extracellular $[K^+]$ and, in doing so, equilibrates pH_o to pH_i (Chaillet and Boron, 1985). Since external pH was titrated to pH 7.00, pH_i was eventually clamped at pH 7.00 after 5 - 10 minutes of exposure to the calibrating solution. It has been suggested that the high K^+/nigericin technique may introduce errors when employed to measure absolute pH_i (Boyarsky *et al*, 1996a and 1996b). In our hands, however, correction

factors provided by these authors failed to significantly affect steady-state pH_i values estimated without correction. Furthermore, it is important to note that relative pH_i changes evoked by an experimental maneuver are not affected by the application of the aforementioned correction factors (see Nett and Deitmer, 1996). For each experiment, the background-corrected ratio of emission intensities during the calibration period represented the BI_{488}/BI_{452} ratio corresponding to pH 7.00.

The experimentally-derived background-corrected ratios (BI_{488}/BI_{452}) were then divided by the ratio corresponding to pH 7.00, obtaining normalized ratios which were then converted to corresponding values of pH_i using Equation 4, which is based on the Henderson-Hasselbalch expression 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 denotes the background-corrected ratio normalized to pH 7.00, $R_{n(max)}$ and $R_{n(min)}$ represent the maximum and minimum obtainable values for the normalized ratio, and pK_a is the -log of the dissociation constant of BCECF (for the derivation of Eq. 4 see Baxter, 1995). $R_{n(max)}$, $R_{n(min)}$ and pK_a were derived from *in situ* calibration experiments (Fig. 2). In such experiments, cultured post-natal hippocampal neurons were exposed to a series of high-K^+ HEPES-buffered solutions, each containing 10 μM nigericin, titrated to pH values ranging from pH 5.5 to pH 8.5 in 0.5 pH increments using 10 KOH or 1 M HCl. The average background-corrected ratios (BI_{488}/BI_{452}) for all neurons at each pH were normalized to the average ratio of background-corrected emission intensities at pH 7.00

and a pH titration curve was produced (Fig. 2B). The upper and lower asymptotes of this curve represent the $R_{n(max)}$ and $R_{n(min)}$, respectively, while the cytosolic pH on the abscissa corresponding to the point of inflection of the curve represents the measured pK_a of BCECF (see Boyarsky *et al*, 1988; Baxter and Church, 1996). The advantage of this normalization procedure is that it allows for a one-point calibration for each neuron or coverslip (*i.e.* population of neurons) examined. The calibration parameters were not dependent upon the temperature of the perfusate or the age of hippocampal neurons and were reassessed whenever the mercury arc burner was replaced or the optical setup of the microscope was altered. For the 13 full calibration experiments used in analyzing all experiments, the values for $R_{n(max)}$, $R_{n(min)}$ and pK_a (mean ± S.E.M.) were 1.89 ± 0.04, 0.43 ± 0.02 and 7.19 ± 0.02, respectively.

4 Experimental Procedures

4.1 Induction of anoxia

In most cases, anoxia was induced by the addition of 2 mM sodium dithionite, $Na_2S_2O_4$, an oxygen scavenger, to the superfusing medium (see Friedman and Haddad, 1993). Anoxic media was prepared immediately prior to use and the pH was adjusted as necessary. The partial pressure of oxygen was measured with a Radiometer ABL 500 blood gas analyzer calibrated for low Po_2. In a solution containing 2 mM $Na_2S_2O_4$, the Po_2 was < 1 mm Hg ($n=4$). In some experiments, anoxia was induced by equilibrating solutions for 24 hours with 100% high purity Ar in HCO_3^--free, HEPES-buffered medium or with 5% CO_2-95% high purity Ar in HCO_3^-/CO_2-buffered medium. In this case, Po_2

was also reduced to < 1 mM Hg. Given that the pH_i response was not different when anoxia was induced either by the addition of 2 mM $Na_2S_2O_4$ (Fig. 7A) or the equilibration of the perfusing media with Ar for 24 hours (see Results, Section 4, Fig. 11), these data confirm that the pH_i response to anoxia occurs in response to a reduction in Po_2 and is not secondary to an additional property of 2 mM $Na_2S_2O_4$.

In order to induce hypoxia, a HCO_3^-/CO_2-free, HEPES-buffered medium was equilibrated with 100% high purity Argon gas for one hour. The Po_2 measured was 25 ± 1 mm Hg ($n = 4$). To induce a similar insult, a HCO_3^-/CO_2-free, HEPES-buffered medium containing 0.5 mM $Na_2S_2O_4$ reduced the Po_2 to 30 ± 3 mm Hg ($n = 6$).

4.2 Changes in steady-state pH_i and rates of recovery of pH_i

The effects of anoxia, alone or in combination with a pharmacological treatment or extracellular ion manipulation, on steady-state pH_i were examined. In a HCO_3^-/CO_2 buffered medium, all known pH_i regulating mechanisms present in hippocampal neurons are active, i.e. the Na^+-dependent and Na^+-independent HCO_3^-/Cl^- exchangers and the Na^+/H^+ exchanger. In a nominally HCO_3^--free, HEPES buffered medium, both HCO_3^-/Cl^- exchangers are inactive and Na^+/H^+ exchange is the dominant pH_i regulating mechanism (Raley-Susman *et al*, 1991; Schweining and Boron, 1994; Baxter and Church, 1996; Bevensee *et al*, 1996; Smith *et al*, 1998). By comparing results obtained in the presence or absence of HCO_3^-, the relative roles of HCO_3^--dependent and HCO_3^--independent pH_i regulating mechanisms in mediating any change in steady-state pH_i evoked by an experimental maneuver could be assessed.

At resting (physiological) pH_i, pH_i regulating mechanisms present may or may not be active. For example, in cultured fetal rat hippocampal neurons, it has been shown that the Na^+-independent HCO_3^-/Cl^- exchanger is operational as an acid extrusion mechanism only at very low levels of pH_i ($pH_i < 6.4$) and not at "normal" resting pH_i values (Baxter and Church, 1996). Furthermore, some agents (*e.g.* mitogens) can affect the activities of both acid-extruding and alkali-extruding pH_i regulatory mechanisms to a similar extent, leaving steady-state pH_i unchanged (*e.g.* Ganz *et al*, 1989). Therefore, the effects of anoxia were also assessed on rates of recovery of pH_i after imposed intracellular acid loads. Intracellular acidifications were induced by the NH_4^+ prepulse technique (Boron and De Weer, 1976). This involves exposing the neurons to a medium containing 40 mM NH_4Cl (Table 1, solution 4), a maneuver which causes an intracellular alkalinization. Upon washout of the NH_4^+-containing medium, pH_i undershoots its initial steady-state value. From this acidified level of pH_i, the neuron utilizes acid extrusion mechanisms to recover to the initial resting level of pH_i (Roos and Boron, 1981; Boron, 1989).

In each experiment in which rates of pH_i recovery from imposed internal acid loads were assessed, a given neuron was subjected to two intracellular acid loads. The initial acid load was used to calculate the control rates of pH_i recovery for a given neuron while the second acid load, and subsequent recovery of pH_i, was performed either during or following a transient anoxic period. The control rates of pH_i recovery were compared to the rates of pH_i recovery under a given test condition at the same corresponding absolute values of pH_i. The rationale for such a comparison is that the activities of acid

extrusion mechanisms are dependent upon the absolute pH_i (see Boyarsky *et al*, 1988). Since the activities of acid extrusion mechanisms are determined, at least in part, by the absolute level of pH_i, by comparing rates of pH_i recoveries at the same absolute values of pH_i, the influence of pH_i on the activities of acid extrusion mechanisms (and, thus, rates of recovery of pH_i) are accounted for.

5 Analysis of Data

5.1 Analysis of steady-state changes in pH_i

In order to compare the steady-state pH_i response to anoxia under varying conditions, a variety of parameters were measured (Fig. 3). The time required for the initiation of the acidic shift (A) is the time between the beginning of the anoxic period and the point at which pH_i begins to decrease. The time required for the maximum acidic shift (B) is the time between the beginning of the anoxic period and the point at which the lowest pH_i value occurred. The time required for the rise of pH_i during the anoxic period (C) is the time between the beginning of the anoxic period and the point at which pH_i begins to increase in the continued absence of O_2. The time required for the alkaline shift (D) is the time between the end of the anoxic period and the point at which the highest pH_i value first occurred. The magnitude of the acidic shift (E) is the difference between the pre-anoxic steady-state pH_i value and the lowest pH_i value observed during the anoxic period. The magnitude of the recovery during anoxia (F) is the difference between the pH_i value observed immediately prior to the return to normoxic medium and the lowest pH_i value observed during the anoxic period. This measurement was employed to

calculate the percentage recovery of pH_i towards pre-anoxic resting levels in the absence of O_2 according to the equation: % pH_i recovery = 100*(F/E). The magnitude of the alkaline shift (G) is the difference between the highest pH_i value observed after the anoxic period and the pre-anoxic pH_i value.

In order to inhibit the activity of the Na^+/H^+ exchanger, reduced-$[Na^+]_o$ media were employed, in which all but 2 mM extracellular Na^+ was iso-osmotically replaced with the membrane-impermeant cation, N-methyl-D-glucamine. In acutely dissociated hippocampal neurons, this maneuver produces a rapid intracellular acidification followed by a slow increase in pH_i towards resting pH_i values (Fig. 4A; see Bevensee *et al*, 1996). In order to allow an accurate assessment of the pH_i response to anoxia under reduced-Na^+ conditions, this slow pH_i recovery was fit, utilizing the least squares method (TableCurve 2D, Jandel Scientific, San Rafael, CA), to a double exponential curve having the equation,

$$y = a + b(1-e^{(-cx)})+d(1-e^{(-ex)}) \hspace{3cm} (Equation\ 5)$$

where b and c are exponential parameters of the first exponential portion, and d and e are exponential parameters of the second exponential portion. All data points from the point of maximum acidification in the reduced-Na^+ solution until the induction of anoxia were fit with this equation. The curve was extrapolated through the time points over which anoxia was superimposed, giving a baseline from which the necessary parameters (identified above) could be measured (Fig. 4A). The double exponential curve was

deemed a sufficient fit with r^2 values of ≥ 0.80 and a composite residuals graph showed points that are uniformly distributed around the zero line (Fig. 4B).

Experimentally-evoked changes in any of the steady-state pH_i parameters measured in response to anoxia were assessed with unpaired, two-tailed Student's t tests. Errors are expressed as S.E.M and n values refer to the number of neurons from which data were obtained. Any net difference was deemed to be significant if the resultant p value was <0.05.

Periodically, the 100 W mercury arc burner produced brief fluctuations in the intensity of the UV light output which resulted in variations in the emission intensities and ultimately, the values of pH_i. In order to smooth the graphical representation of the pH_i vs. time records, a moving average (period = 5) was applied to all traces shown (see Boyarsky *et al*, 1988; Baxter and Church, 1996).

5.2 Analysis of the rates of recovery of pH$_i$ from imposed intracellular acid loads

In analyzing the recovery of pH_i from an imposed intracellular acid load, the recovery portion of the experiment was fitted, utilizing the least squares method (SigmaPlot v. 3.02, Jandel Scientific), to a single exponential function of time having the format

$$pH_i = a + b(1 - e^{ct}), \hspace{3cm} (\textit{Equation 6})$$

where a, b, and c are the exponential parameters. The differentiated form of Equation 6 yields the rate of change of pH_i

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

from which the instantaneous rates of pH_i recovery (dpH_i/dt) were calculated at 0.05 unit intervals of pH_i from the point of maximum acidification following an NH_4^+ prepulse until recovery was ~80% complete (see Baxter and Church, 1996; Smith *et al*, 1998).

For each neuron, a paired experiment, consisting of two consecutive intracellular acid loads, was performed (see Section 4.2). At each corresponding value of pH_i, the percentage difference between the control rate of pH_i recovery and the rate of pH_i recovery either during or following a transient period of anoxia was determined. An average of the resultant percentage differences, over the range of pH_i values, was calculated and utilized for description of the data.

To determine the intrinsic percentage differences in rates of pH_i recovery between two consecutive acid loads, paired experiments were performed under control conditions, *i.e.* in the absence of any experimental treatment (Fig. 5A). The instantaneous rates of pH_i recovery following the first acid load were grouped separately from the instantaneous rates of pH_i recovery following the second acid load. The percentage differences between the average instantaneous rates of pH_i recovery from the first and second acid loads at corresponding pH_i values was calculated and the mean percentage difference over the range of pH_i values was obtained. Based on data from 18 neurons, the average difference

observed in the rates of pH_i recovery from the second acid load compared to the first acid load was a -1.20 ± 21.3% increase (mean ± S.D.; Fig. 5B). Therefore, rates of pH_i recovery obtained either during or following an anoxic insult were considered different from the control rates of pH_i recovery only if they displayed either an average increase or decrease of 43% (i.e. ~ 2 S.D.) or more as compared to the control rates of pH_i recovery. Only neurons which exceeded these criteria underwent further analysis.

The instantaneous rates of pH_i recovery under control conditions were grouped separately from the instantaneous rates of pH_i recovery under the influence of a test condition and a formal statistical comparison between these two groups was performed using an unpaired, two-tailed Student's t-test. If the resultant p value was < 0.05, then the rates of pH_i recovery from an imposed intracellular acid load were considered to have been altered significantly in response to the test maneuver.

In summary, data obtained from each neuron were initially assessed on the basis of the average percentage difference between "control" and "test" rates of pH_i recovery to determine if the criteria established for inclusion were satisfied. Control and test data from each accepted neuron were then grouped separately and compared at absolute pH_i values. If the Student's t-test returned a p value < 0.05 at a given pH_i value, then the changes in the rates of pH_i recovery are most likely due to an alteration in the rate of acid extrusion.

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

	Solution				
	1 Standard	2 Low Na^+	3 Ca^{2+} free	4 NH_4Cl	5 High K^+
NaCl	136.5	2.0	136.5	96.5	-
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	3.0	1.5	1.5
Na Glu	-	-	-	-	10.0
K Glu	-	-	-	-	130.5
D-glucose	17.5	17.5	17.5	17.5	17.5
$NMDG^+$	-	136.0	-	-	-
NH_4Cl	-	-	-	40.0	-
HEPES	10.0	10.0	10.0	10.0	10.0
Titrated with:	10 M NaOH	1.0 M HCl	10 M NaOH	10 M NaOH	10 M KOH

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

Abbreviations: Na Glu, sodium gluconate; K Glu, potassium gluconate; $NMDG^+$, *N*-methyl-D-glucamine

Table 2: Composition of HCO_3^-/CO_2-buffered experimental solutions
(all concentrations in mM)

	Solution	
	6	7
	Standard pH 7.35 at 37°C	Standard pH 7.35 at 22°C
NaCl	127.0	117.5
NaHCO$_3$	19.5	29.0
KCl	3.0	3.0
CaCl$_2$	2.0	2.0
NaH$_2$PO$_4$	1.5	1.5
MgSO$_4$	1.5	1.5
D-glucose	17.5	17.5

All HCO_3^--containing solutions were equilibrated with 5% CO_2 in balance air (normoxia) or balance argon or nitrogen (anoxia).

Table 3: List of pharmacological agents employed

	Stock Concentration	Solvent	Test Concentration	Storage
Bafilomycin A$_1$	2 mM	DMSO	1 - 2 µM	-20 °C
4-α-5-hydroxycinnamate	500 mM	4 DMSO: 1 methanol (v:v)	5 - 10 µM	N/A
DIDS	100 mM	DMSO	200 µM	N/A
Isoproterenol	50 mM	ultra-pure water	10 µM	-60 °C
Nigericin	10 mM	ethanol	10 µM	-60 °C
Noradrenaline	50 mM	ultra-pure water	10 µM	-60 °C
Omeprazole	25 mM	DMSO	50 µM	N/A
Rp-cAMPS	50 mM	ultra-pure water	50 µM	-20 °C
Tetrodotoxin	100 µM	ultra-pure water	0.5 - 1 µM	-2 °C

All agents were supplied by Sigma Chemical Company, with the exception of Bafilomycin A$_1$ (a generous gift from Dr. V. Palatý, Department of Anatomy, UBC) and Rp-cAMPS (Biolog Life Science Institute, La Jolla, CA).
Abbreviations: DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid; DMSO, dimethylsulphoxide.

Figure 1. Acutely dissociated adult rat hippocampal CA1 neurons

Neurons were isolated according to procedures described in Section 1.1 and were chosen for study based on morphological criteria established by Schwiening and Boron (1994), *i.e.* a smooth, non-granular appearance; 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 the presence of two or more smaller processes (basal dendrites) at the opposite pole.
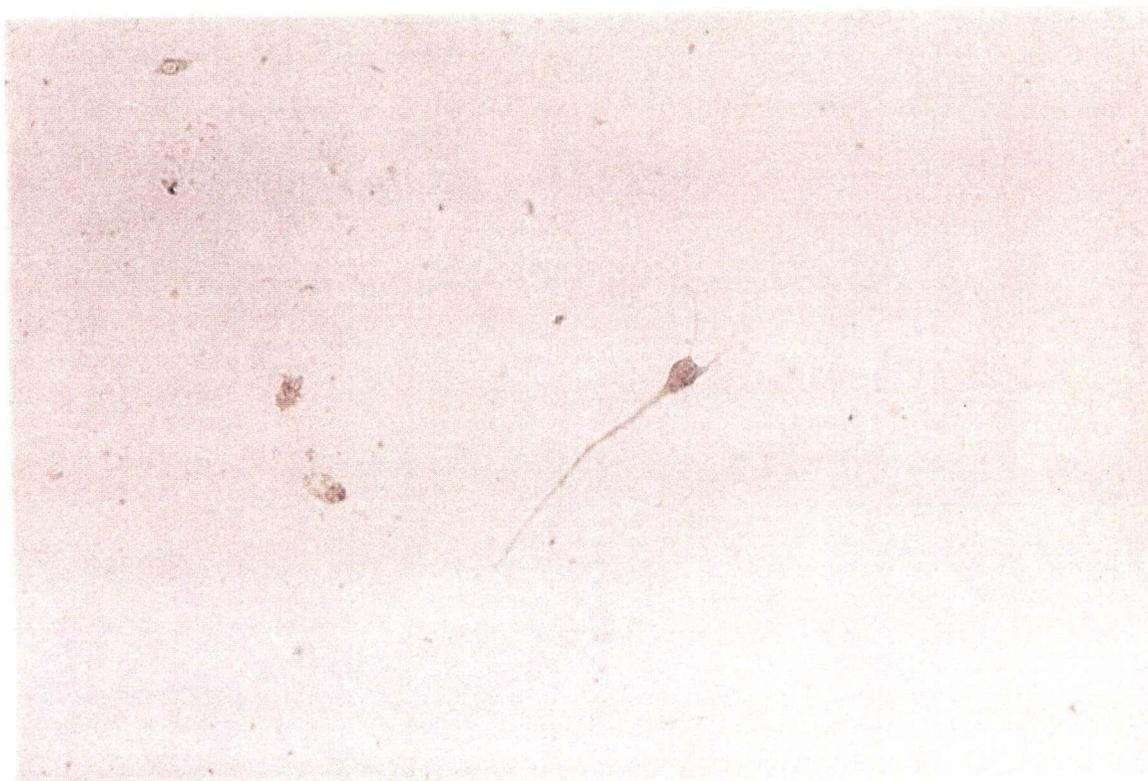
Figure 2. Sample calibration plot for BCECF

A. Cells were exposed to HCO_3^-/CO_2-free, HEPES-buffered solutions containing 10 μM nigericin at pH_o (and therefore pH_i) 5.54, 6.04, 6.51, 7.00, 7.51, 7.98, and 8.48. The duration of each exposure is indicated by the bars above the trace, which is the mean of data obtained from 17 cultured post-natal rat neurons recorded on a single coverslip. **B.** Plot of pH_i against the resulting normalized ratio (R_n). Standard error bars are indicated (n=3 coverslips). Where absent, error bars lie within the symbol area. The curve is a result of a non-linear least squares regression fit to Equation 4. For this particular calibration, the values of $R_{n(max)}$, $R_{n(min)}$, and pK_a were 2.0115, 0.4346, 7.2526, respectively.

A.

| 7.00 | 6.04 | 7.51 | 6.51 | 7.98 | 7.00 | 8.48 | 5.54 |

Time (min)

B.

pH_i
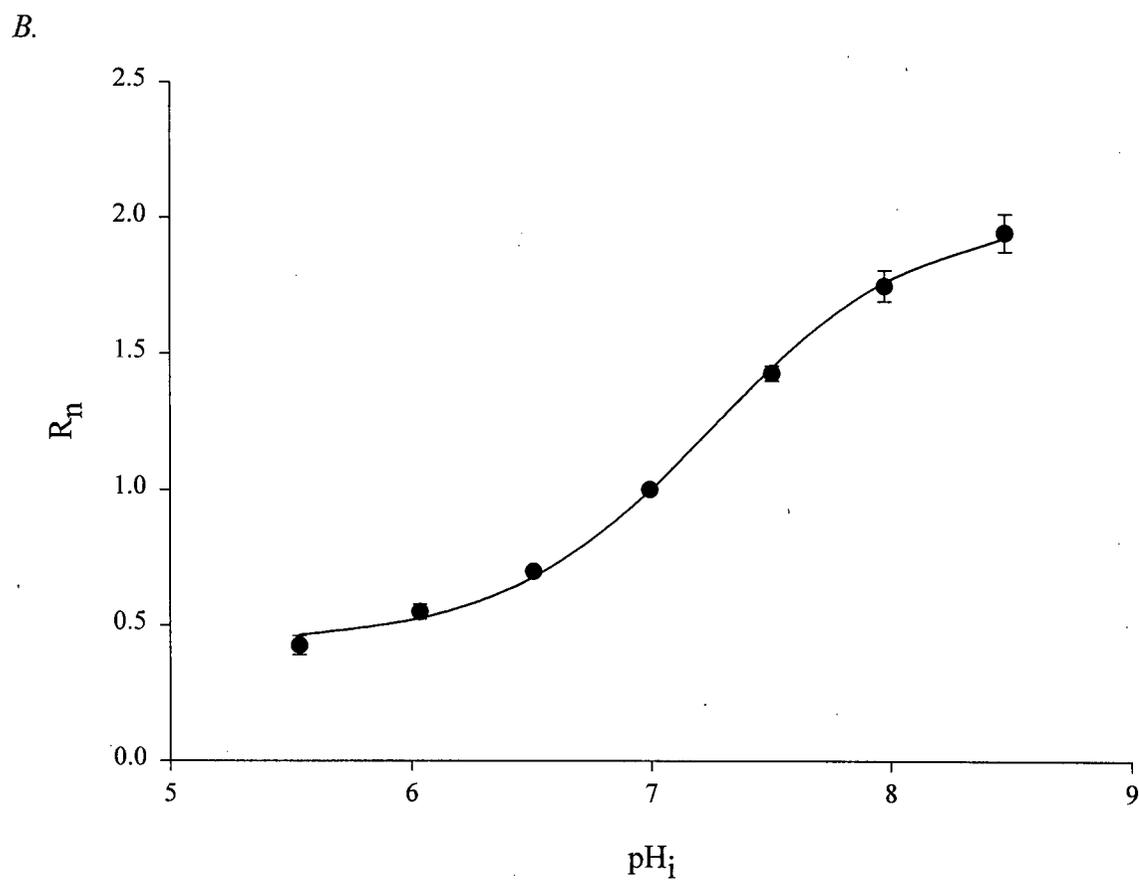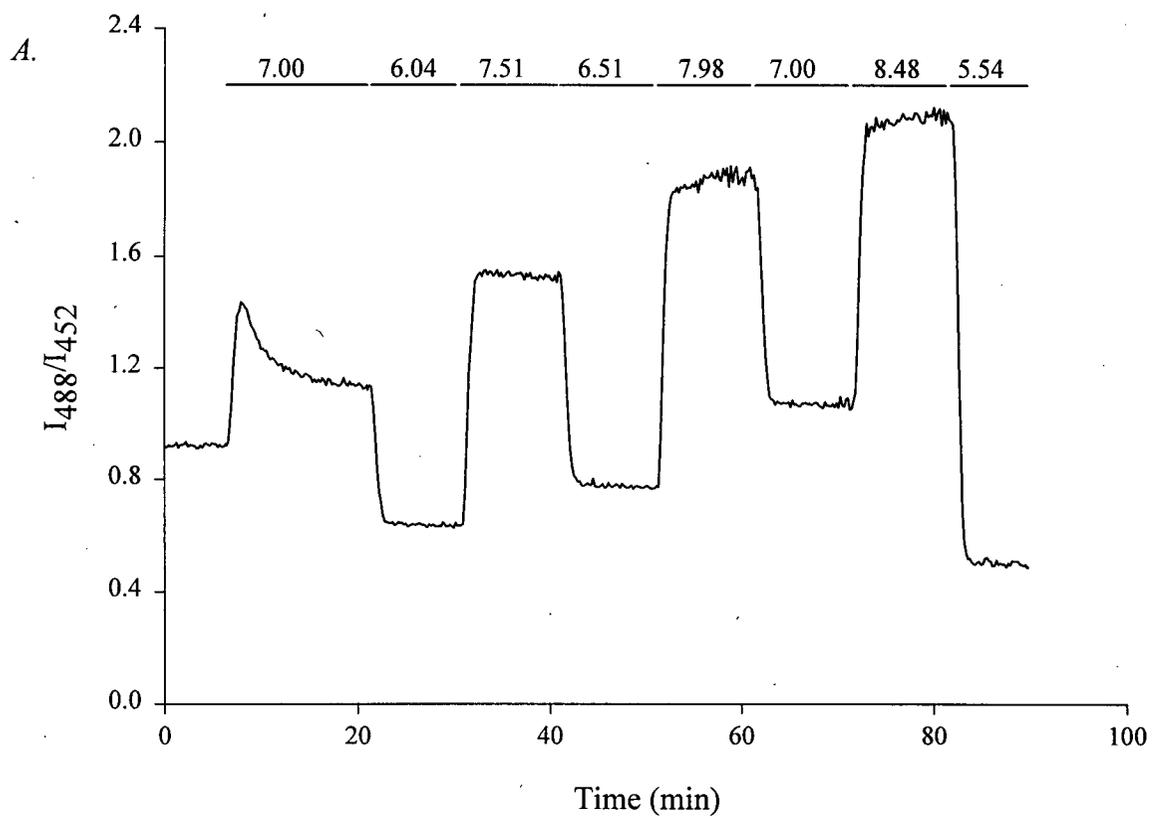
Figure 3. **Experimental parameters measured to assess the steady-state pH_i response to a transient period of anoxia**

The pH_i response of an acutely dissociated adult rat hippocampal CA1 neuron to a 5 min period of anoxia is illustrated. The time required for the initiation of the acidic shift (A) is the time between the beginning of the anoxic period and the point at which pH_i began to decrease. The time required for the maximum acidic shift (B) is the time between the beginning of the anoxic period and the point at which the lowest pH_i value occurred. The time required for the initiation of the rise of pH_i (C) during the anoxic period is the time between the beginning of the anoxic period and the point at which pH_i began to increase in the continued absence of O_2. The time required for the alkaline shift (D) is the time between the end of the anoxic period and the point at which the highest pH_i value first occurred. The magnitude of the acidic shift (E) is the difference between the pre-anoxic steady-state pH_i value and the lowest pH_i value observed in the anoxic period. The magnitude of the recovery during anoxia (F) is the difference between the pH_i value observed immediately prior to the return to normoxic medium and the lowest pH_i value observed during the anoxic period. The magnitude of the alkaline shift (G) is the difference between the highest pH_i value observed after the anoxic period and the pre-anoxic steady-state pH_i value.
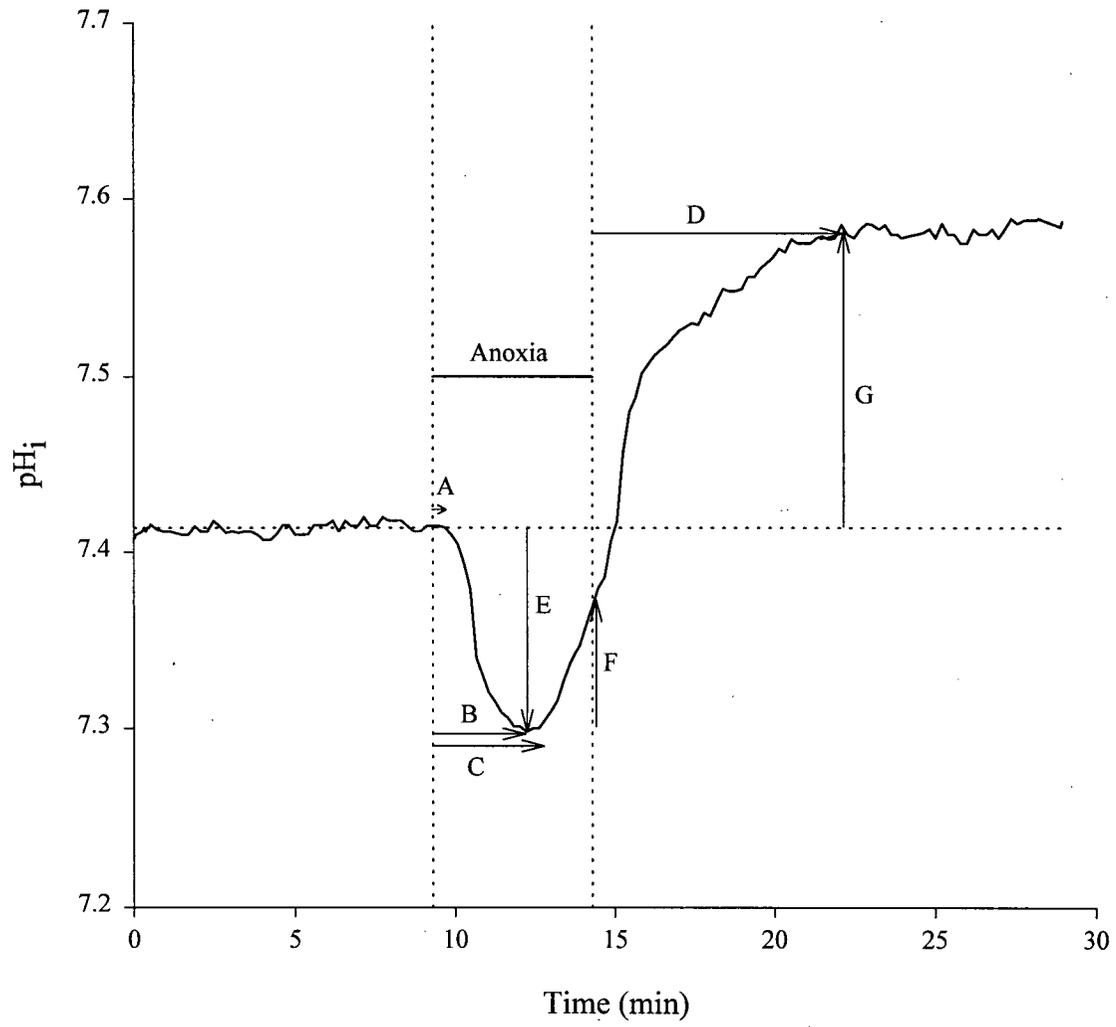
Figure 4. The pH$_i$ response upon exposure to a reduced-[Na$^+$]$_o$ medium

A. Upon exposure to a 2 mM [Na$^+$]$_o$, NMDG$^+$-substituted, HEPES-buffered medium, the initial, steady-state pH$_i$ acidified by ~ 0.50 pH units and gradually recovered. pH$_i$ reached a plateau value of ~ 7.40 after ~ 20 min during the continued perfusion with a reduced-Na$^+$ medium. All data points from the point of the maximum acidification were curve fit to a double exponential curve (solid line). The raw data points are overlying the extrapolated curve (r^2=0.99). **B.** Plotted are the average residual values (the magnitude of the difference between a raw data point and the curve fitted data point) against time from the point of peak acidification upon exposure to 2 mM [Na$^+$]$_o$, NMDG$^+$-substituted, HEPES-buffered media until the end of the curve-fitted data points.

A.

2 mM [Na$^+$]$_o$, NMDG$^+$-substituted

pH$_i$

Time (min)

B.

Residuals (pH units)

Time (min)

point of peak acidification
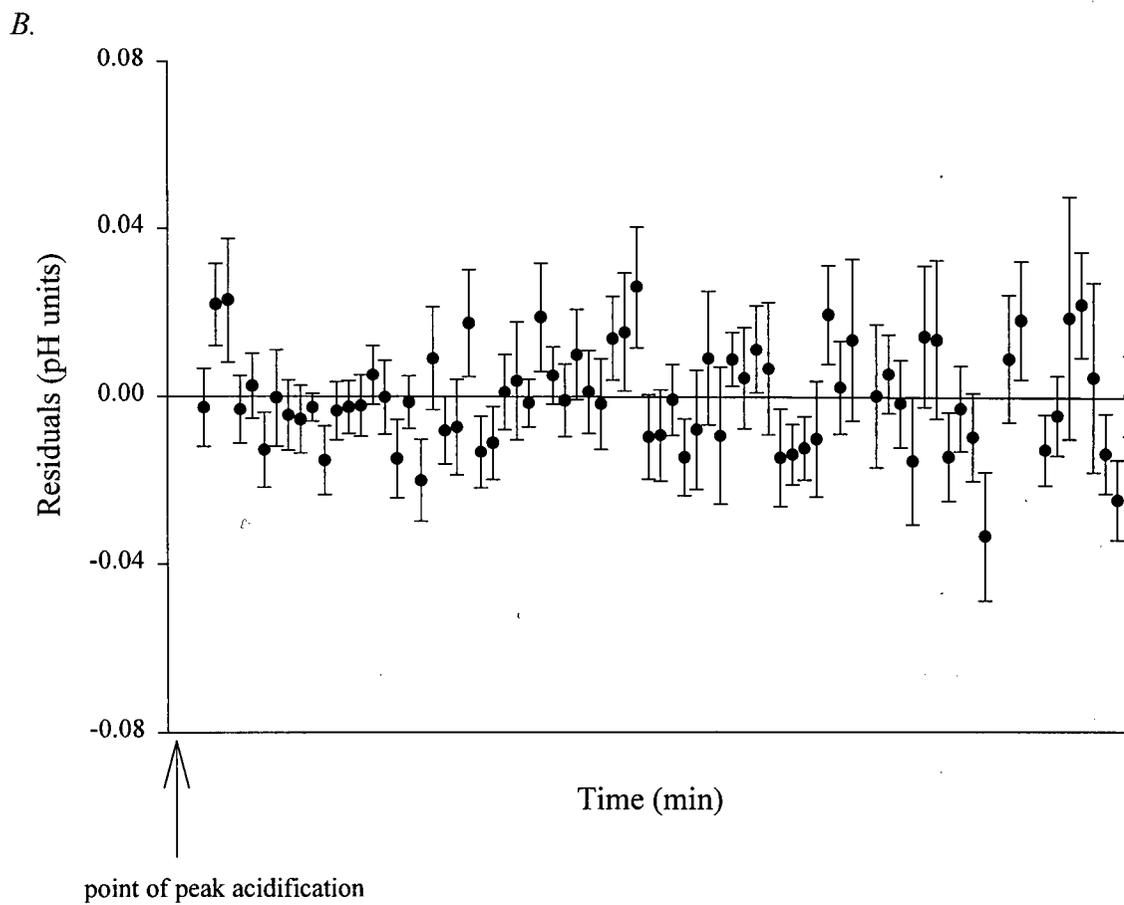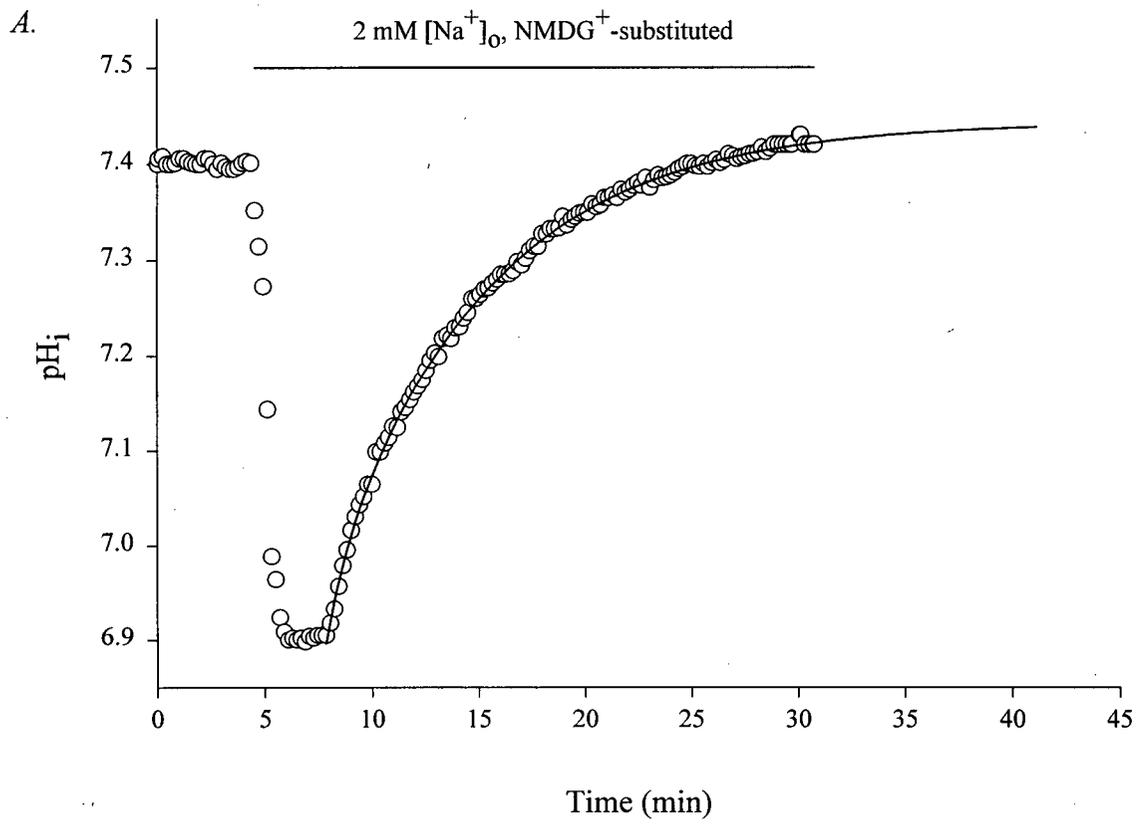
Figure 5. Control acid load recoveries during perfusion with HCO_3^-/CO_2-free, HEPES-buffered medium

A. In order to determine whether anoxia influences the rates of pH_i recovery from an imposed intracellular acid load as compared to the control rates of pH_i recovery, the intrinsic differences between the rates of pH_i recoveries of two consecutive, control acid loads must be accounted for. In the example shown, an initial acid load was performed and, after pH_i had recovered to a new steady-state value, a second acid load was conducted. The percentage differences between the rates of pH_i recovery following the initial acid load and the rates of pH_i recovery following the second acid load at each corresponding absolute value of pH_i were calculated. An average of the percentage differences at each pH_i value was then determined. In this example shown, the rates of pH_i recovery from the second acid load were on average 27% faster than the rates of pH_i recovery from the initial acid load. **B.** The graph represents a comparison between the rates of pH_i recovery from an initial (O) and from a second (●) acid load, both obtained under control conditions, at the same absolute values of pH_i (n=18). The rates of pH_i recovery were evaluated at 0.05 pH unit intervals of pH_i and error bars represent S.E.M. Both sets of data were independently fitted with a linear least-squares regression line. The average percent difference, calculated over the range of pH_i values shown, was -1.20 ± 21.3% (mean ± S.D.).
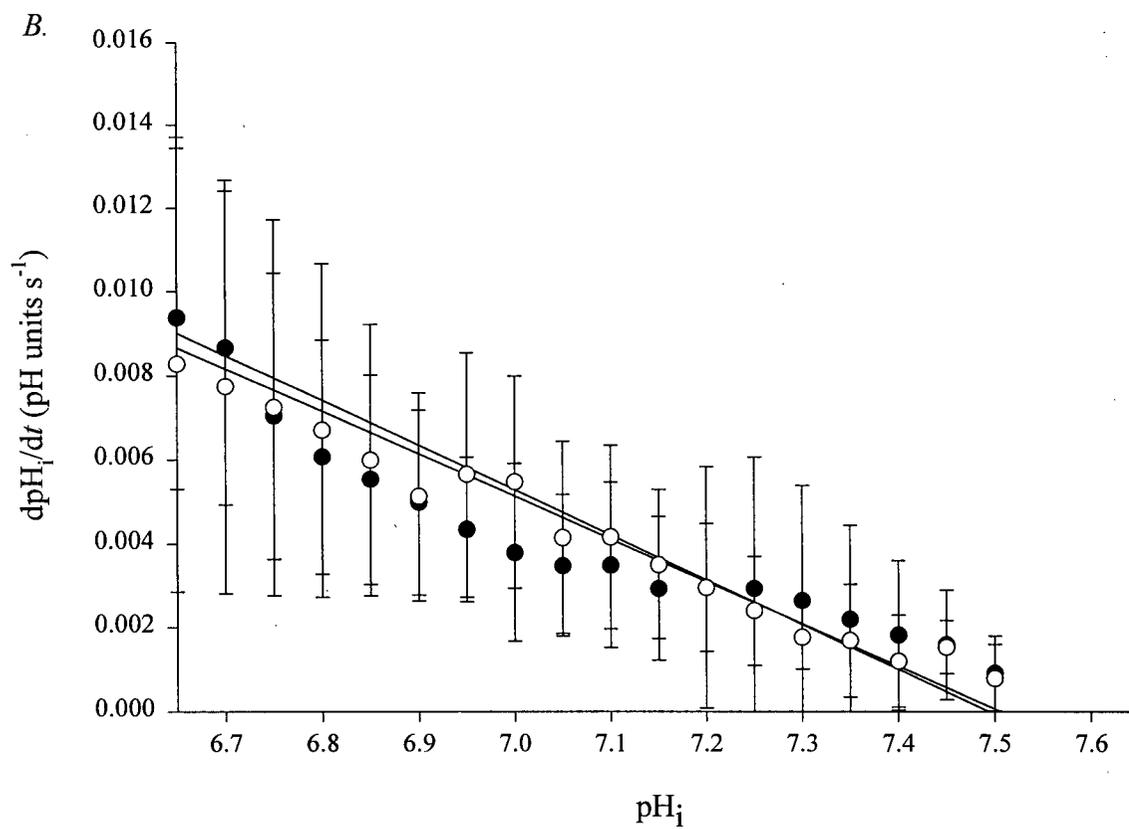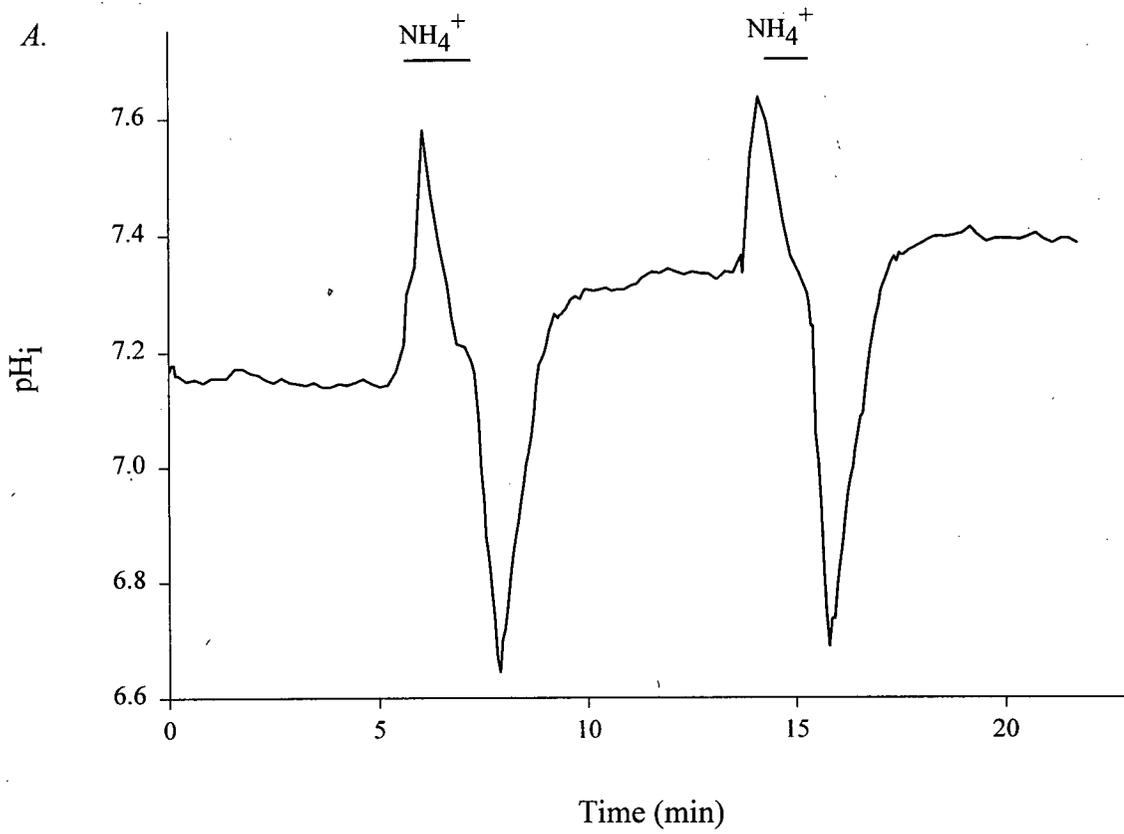
RESULTS

Using acutely dissociated adult rat hippocampal CA1 neurons, I initially characterized the changes in steady-state pH_i which occur in response to a transient period of anoxia. Next, the underlying mechanisms responsible for the production of the anoxia-evoked changes in pH_i were examined, with particular emphasis on the role of changes in the activity of the Na^+/H^+ exchanger. Finally, the effects of such variables as ambient temperature, extracellular pH, extracellular glucose concentration and the activity of the cAMP/PKA second messenger system on the pH_i response to anoxia were examined. Each of these variables have previously been shown to affect neuronal viability following a transient anoxic insult.

1 Distribution of steady-state intracellular pH under normoxic conditions

In HCO_3^-/CO_2-free, HEPES-buffered media at pH 7.35, the mean steady-state pH_i value was 7.30 ± 0.21 (range pH_i 6.40 - 7.80; $n=694$; Fig. 6A). The distribution was best fitted by a bimodal Gaussian distribution with means at pH_i 6.93 ± 0.17 and 7.37 ± 0.14. In HCO_3^-/CO_2-buffered media, pH_i was distributed, in a Gaussian manner, around a mean of 7.30 ± 0.17 (range pH_i 6.90 - 7.60; $n=83$; Fig. 6B). The mean steady-state pH_i values, in the absence and presence of HCO_3^-, were not statistically different, suggesting that the predominant mechanism involved in the maintenance of steady-state pH_i in acutely dissociated adult rat hippocampal CA1 neurons at 37°C is HCO_3^--independent and likely to be the Na^+/H^+ exchanger, as previously described (Baxter and Church, 1996; Bevensee *et al*, 1996; Smith *et al*, 1998).

2 Characterization of the intracellular pH response to anoxia

2.1 Steady-state changes in intracellular pH evoked by anoxia under HCO_3^-/CO_2-free, HEPES-buffered conditions

The pH_i changes evoked by a 5 min period of anoxia were characterized by three phases (Fig. 7; Table 4). In a HCO_3^-/CO_2-free, HEPES-buffered medium (pH 7.35), a 5 min period of anoxia induced an initial acidic shift in pH_i of 0.16 ± 0.01 pH units ($n=28$), with the peak acidification occurring 2.5 ± 0.2 min following the start of anoxia. In the continued absence of oxygen, pH_i recovered by 0.05 ± 0.01 pH units, a $40 \pm 5\%$ recovery. Upon the return to normoxia, there was a further internal alkaline shift in pH_i of 0.20 ± 0.02 pH units above the initial pre-anoxic steady-state pH_i value which reached a maximum at 9.5 ± 1.1 min after the end of anoxia. The magnitude of the internal acidification observed during a 5 min period of anoxia showed a positive correlation to the initial pre-anoxic steady-state pH_i of the cell ($r^2 = 0.70$). No similar correlation was observed between the pre-anoxic steady-state pH_i value and the magnitude of the post-anoxic alkalinization, nor was there any apparent correlation between the magnitude of the anoxic acidification and the magnitude of the post-anoxic alkalinization ($r^2 = 0.25$ and 0.20, respectively).

A change in the duration of the anoxic period from 5 min to 3 or 10 min did not influence significantly the magnitude of the intracellular acidification observed during anoxia (Fig. 8; Table 4). However, the magnitudes of the rise in pH_i observed during anoxia and the post-anoxic alkalinization increased with an increasing duration of anoxia,

being larger during/following a 10 min period than compared to during/following a 3 min period of anoxia (Table 4; $p < 0.05$ in each case).

2.2 Steady state changes in intracellular pH evoked by anoxia under HCO_3^-/CO_2-buffered conditions

Transient anoxia of 3, 5, or 10 min duration induced a qualitatively similar triphasic pattern of pH_i changes under HCO_3^-/CO_2-buffered conditions as was observed under HCO_3^-/CO_2-free conditions. A 5 min period of anoxia induced an intracellular acidification that partially recovered in the continued absence of oxygen and, upon the return to normoxia, there was an intracellular alkalinization above pre-anoxic steady-state pH_i values (Fig. 9B; Table 5). Under HCO_3^-/CO_2-buffered conditions, the magnitudes of the anoxic acidification and the post-anoxic alkalinization were not correlated with either the initial resting pH_i observed prior to anoxia or with each other ($r^2 = 0.61$, 0.29, 0.35, respectively). In a manner similar to that observed under HEPES-buffered conditions (see Section 2.1), the magnitude of the per-anoxic rise in pH_i increased as the duration of the anoxic period increased from 3 to 5 to 10 min (Table 5).

In response to a 5 min period of anoxia, none of the parameters measured were influenced by the presence or absence of HCO_3^-. In response to a 3 min period of anoxia, although the magnitude of the intracellular acidification seen during the anoxic period was smaller in the presence of HCO_3^- (Table 5; $p<0.05$), all other aspects of the pH_i response were similar in the presence and absence of HCO_3^-. However, following a 10 min period of anoxia, the magnitude of the post-anoxic alkalinization was statistically

larger under HCO_3^-/CO_2-free, HEPES-buffered conditions compared to that observed under HCO_3^-/CO_2-buffered conditions ($p<0.05$). In HCO_3^-/CO_2-buffered media, the addition of 200 μM DIDS failed to affect this difference (the rise in pH_i observed following a 10 min period of anoxia in the presence of DIDS was 0.23 ± 0.03 pH units ($n=12$) vs. 0.19 ± 0.02 pH units for the magnitude of the post-anoxic alkalinization in the absence of DIDS; $p>0.1$). All other aspects of the pH_i response to a 10 min period of anoxia (with the exception of the time required to reach the maximum post-anoxic internal alkalinization; Table 5) were not influenced by the presence or absence of extracellular HCO_3^-. Therefore, potential changes in the activities of HCO_3^--dependent pH_i regulating mechanisms appear to contribute little, if at all, to the pH_i response to anoxia. Consequently, all subsequent experiments were performed under HCO_3^-/CO_2-free, HEPES-buffered conditions.

BI_{452} remained stable during and following transient anoxic periods, indicating that the pH_i shifts observed were not an artifact caused by changes in BI_{452} consequent upon a deterioration in membrane integrity (Fig. 7B; see Bevensee *et al*, 1995; Smith *et al*, 1998). However, the morphology of the cells did show visible changes during the course of an experiment. In agreement with previous reports (Florine-Castel *et al*, 1991; Friedman and Haddad, 1994a), anoxia induced the formation of membrane blebs on the surface of the soma, and along the length of the apical and basal dendrites. In more severe cases, the membrane blebs either fused together, causing the cell body to swell, or the blebs would detach from the plasma membrane. Under phase-contrast microscopy, following a transient period of anoxia, the cytosol of the cell appeared granular.

Although the present study did not evaluate the mechanisms initiating the observed anoxia-induced neuronal damage, possibilities include an increase in $[Na^+]_i$, a stimulation of cytoskeletal proteases, phospholipases, and/or a change in plasma membrane structure (Herman *et al*, 1990; Arai *et al*, 1991; Florine-Casteel *et al*, 1991; Friedman and Haddad, 1994b).

3 Characterization of the intracellular calcium response to anoxia

Intracellular calcium increased in response to a transient period of anoxia. Experiments were performed in HCO_3^-/CO_2-free, HEPES-buffered media at pH_o 7.35. The average resting BI_{334}/BI_{380} value prior to the induction of anoxia was 0.7 ± 0.15 (Fig. 10; $n=3$). This value increased sharply beginning 1.6 ± 0.4 min after the induction of anoxia and reached a maximum value of 2.74 ± 0.43 at 3.1 ± 0.5 min following the induction of anoxia. After the return to normoxia, the ratio remained elevated for up to 15 min (the maximum period recorded). A 3 min period of anoxia induced a similar $[Ca^{2+}]_i$ response, the time course and magnitude of which were statistically not different to the respective values measured during and after a 5 min period of anoxia ($n=3$; data not shown).

4 Validation of the use of $Na_2S_2O_4$ to induce anoxia

In order to control for any properties of sodium dithionite unrelated to its ability to reduce the Po_2 of a given media, the effects on pH_i of exposing neurons to physiological media equilibrated with high-purity Argon gas were investigated.

Initially, a HCO_3^-/CO_2-free, HEPES-buffered medium was bubbled vigorously with 100% high purity Argon gas for one hour, a maneuver which reduced Po_2 to 25 ± 1 mm Hg ($n = 4$). This procedure is often employed to induce "anoxia" (*e.g.* Krnjevic and Walz, 1990; Donnelly *et al*, 1992; Pirttilä and Kauppinen, 1992; O'Reilly *et al*, 1995); however, the present data suggest that exposure to this solution induces an hypoxic insult (see Arai *et al*, 1991; Fujiwara *et al*, 1992). Out of 14 cells examined, 6 cells responded to a 5 min period of hypoxia with an acidification during the hypoxic period of 0.07 ± 0.03 pH units followed by an alkalinization of 0.25 ± 0.11 pH units above pre-hypoxic resting levels upon the return to normoxia. The remaining 8 cells did not show any change in pH_i in response to perfusion with the hypoxic medium. A 0.5 mM solution of $Na_2S_2O_4$ in a HEPES-buffered medium, in the absence of equilibration with Argon, reduced the Po_2 in the medium to 30 ± 3 mm Hg ($n = 6$), a level not statistically different from that reached after equilibration with 100% high purity Argon gas for one hour. The pH_i response to a 5 min period of hypoxia induced with the 0.5 mM $Na_2S_2O_4$-containing medium was not statistically different in any respect from that induced by perfusion with $Na_2S_2O_4$-free medium equilibrated with Argon for one hour (0.03 ± 0.01 pH units and 0.29 ± 0.04 pH units for the magnitudes of the acidification and alkalinization, respectively, evoked by a 5 min period of anoxia induced with 0.5 mM $Na_2S_2O_4$; $n=6$).

When the Po_2 was reduced to < 1 mM Hg by equilibration of a HCO_3^-/CO_2-buffered solution with 95% Argon/5% CO_2 for ≥ 18 h, a 5 min period of anoxia produced a pH_i response which was identical to that observed when the Po_2 was reduced to < 1 mm Hg by the addition of 2 mM $Na_2S_2O_4$ (compare Fig. 9B and Fig. 11; 0.12 ± 0.01 pH units

vs. 0.18 ± 0.02 pH units, respectively, for the acidification seen during the anoxic period, and 0.18 ± 0.07 pH units vs. 0.16 ± 0.05 pH units, respectively, for the post-anoxic alkalinization; $n \geq 4$ in each case). Thus, the pH_i response to anoxia is not an artifact associated with the use of $Na_2S_2O_4$ to induce anoxia but rather, is determined by the reduction in the Po_2 in the experimental media. This latter conclusion is reflected by the fact that both the acidification observed during anoxia and the post-anoxic alkalinization were significantly different when the hypoxic ($Po_2 \cong 25\text{-}30$ mm Hg) and the anoxic ($Po_2 \cong 0$ mm Hg) responses were compared. While the per-anoxic acidification was smaller, the post-anoxic alkalinization was larger under hypoxic conditions compared to anoxic conditions (0.04 ± 0.01 pH units vs. 0.17 ± 0.01 pH units under hypoxic and anoxic conditions, respectively, for the per-anoxic acidification; 0.28 ± 0.04 pH units vs. 0.16 ± 0.03 pH units under hypoxic and anoxic conditions, respectively, for the post-anoxic alkalinization; $p < 0.05$ in each case).

A second concern regarding the method of inducing anoxia with $Na_2S_2O_4$ was that the addition of $Na_2S_2O_4$ may alter the pH of the anoxic test media, even after retitration. Arguing against this possibility, however, is that the pH of the anoxic media measured at the end of an experiment decreased by 0.021 ± 0.003 pH units ($n = 24$) from the pH value measured prior to its use, a reduction which cannot account for the magnitude of the cellular pH_i shifts seen (see Friedman and Haddad, 1993; Diarra *et al*, 1998). Thus, the use of 2 mM $Na_2S_2O_4$ provides an effective anoxic insult and, therefore, the addition of 2 mM $Na_2S_2O_4$ was employed to induce anoxic insults in all subsequent experiments.

5 Mechanisms underlying the intracellular pH response to transient anoxia

Having characterized the pH_i and $[Ca^{2+}]_i$ responses of isolated CA1 hippocampal neurons to transient anoxic periods of varying durations, the next series of experiments examined possible changes in the activities of pH_i regulating mechanisms that may be involved in the production of the observed steady-state pH_i response.

5.1 Role of Ca^{2+}

Given the close interrelationship between $[Ca^{2+}]_i$ and $[H^+]_i$ (Meech and Thomas, 1977; Ives and Daniel, 1987; Ou-Yang *et al*, 1994a and 1995), it was necessary to assess the possible role of changes in $[Ca^{2+}]_i$ to the changes in pH_i evoked by anoxia. Initially, the effect of the removal of extracellular Ca^{2+} on the pH_i response to anoxia was examined. Exposure to a HCO_3^-/CO_2-free, HEPES-buffered, Ca^{2+}-free solution containing 200 µM EGTA caused an average increase in steady-state pH_i of 0.11 ± 0.05 pH units (Fig. 12A; $n=6$). Once a new steady-state pH_i value was reached, a 5 min period of anoxia imposed under Ca^{2+}-free conditions induced an intracellular acidification of 0.21 ± 0.04 pH units, reaching this maximum at 2.0 ± 0.4 min into the anoxic period (Fig. 12B). In the continued absence of oxygen, pH_i recovered by 0.06 ± 0.01 pH units, a $40 \pm 11\%$ recovery. Upon the return to normoxia, there was an intracellular alkalinization of 0.18 ± 0.04 pH units above pre-anoxic resting pH_i levels, which reached a maximum value at 6.4 ± 1.3 min following the return to normoxia. Neither the time course nor the magnitude of any phases of the pH_i response to anoxia were significantly different from those seen in the presence of Ca^{2+} under identical buffering conditions (see Fig. 7A;

Table 4). Thus, the pH_i response to a 5 min period of anoxia is independent of Ca^{2+} influx. This conclusion is supported by the fact that the internal acidification observed during anoxia began prior to the start of the anoxia-evoked rise in $[Ca^{2+}]_i$ (1.0 ± 0.1 min vs. 1.6 ± 0.4 min, respectively; $p<0.05$).

The effect of the removal of extracellular Ca^{2+} on the $[Ca^{2+}]_i$ response to a 5 min period of anoxia was also examined. The average resting BI_{334}/BI_{380} value prior to the removal of extracellular Ca^{2+} was 0.56 ± 0.04 (n=6). Exposure to a HCO_3^-/CO_2-free, HEPES-buffered, Ca^{2+}-free solution caused a 0.30 ± 0.02 decrease in this value (Fig. 13). Under Ca^{2+}-free conditions, a 5 min period of anoxia induced a 0.02 ± 0.01 increase in the ratio value, an increase which was significantly smaller than that observed in the presence of extracellular Ca^{2+} under identical buffering conditions (see Fig. 10, Section 3; $p<0.05$). Interestingly, in 4/6 cells examined, there was a small post-anoxic increase in the BI_{334}/BI_{380} ratio value of 0.09 ± 0.02 which may reflect Ca^{2+} release from intracellular stores, although this was not investigated. Thus, the $[Ca^{2+}]_i$ response to transient anoxia is dependent on the presence of extracellular Ca^{2+}; however, the pH_i response to anoxia appears to be independent of any changes in $[Ca^{2+}]_i$.

5.2 Role of the Na^+/H^+ exchanger

5.2.1 Technique for isolation of Na^+/H^+ exchange activity

The isoform of the Na^+/H^+ exchanger present in hippocampal neurons is insensitive to amiloride, amiloride derivatives (*e.g.* dimethylamiloride, 5-(N-ethyl-N-isopropyl)amiloride), and benzoyl guanidinium compounds (*e.g.* HOE 694), agents which

inhibit Na^+/H^+ exchange activity in peripheral cell types (Raley-Susman *et al*, 1991; Baxter and Church, 1996; Bevensee *et al*, 1996; Smith *et al*, 1998). Thus, in order to assess the role of the Na^+/H^+ exchanger in the production of the steady-state pH_i response to anoxia, all but 2 mM extracellular Na^+ was iso-osmotically replaced with *N*-methyl-D-glucamine ($NMDG^+$). Upon exposure to this reduced-Na^+ solution, pH_i acidified but then gradually recovered to a value approximating the initial steady-state pH_i observed in the presence of normal extracellular Na^+ (see Bevensee *et al*, 1996). In 7 cells, pH_i acidified by 0.50 ± 0.04 pH units, followed by a $99.4 \pm 0.5\%$ recovery of pH_i towards the initial pH_i value after 30 ± 3 min in the presence of reduced-Na^+ media (Fig. 14A). The influence of the residual 2 mM extracellular Na^+ on the alkalinization process was assessed. Under external Na^+-free conditions ($NMDG^+$-substitution), pH_i initially fell by 0.45 ± 0.10 pH units and recovered to $102 \pm 2\%$ of the initial resting pH_i value over 24 ± 6 min (*n*=5). There was no statistical difference between either the magnitude of the internal acidification nor the degree of alkalinization observed under the different conditions of $[Na^+]_o$, a finding which suggests that the Na^+/H^+ exchanger was inactive even in the presence of 2 mM external Na^+. This finding is consistent with the previous report that the apparent K_m of the Na^+/H^+ exchanger in rat hippocampal neurons for extracellular Na^+ is 23-26 mM (Raley-Susman *et al*, 1991). Possible mechanisms mediating the rise in pH_i under reduced or zero-$[Na^+]_o$ conditions are investigated in Section 11.

5.2.2 Effects of Na⁺/H⁺ exchange inhibition on steady-state pH$_i$ changes evoked by anoxia

In the analysis of experiments that included a 5 min period of anoxia imposed under reduced-$[Na^+]_o$ conditions, the pH$_i$ recovery in the reduced-Na^+, $NMDG^+$-substituted medium was curve-fit with a double-exponential equation (see Materials and Methods, Section 5.1) and extrapolated through the anoxic period to estimate the baseline pH$_i$ from which the effects of the anoxic insult on pH$_i$ could be derived. A 5 min period of anoxia induced an intracellular acidification of 0.11 ± 0.01 pH units followed by a per-anoxic rise in pH$_i$ of 0.04 ± 0.01 pH units, a $34 \pm 10\%$ recovery (*n*=20; Fig. 14B; Table 6). Upon the return to normoxia, there was an intracellular alkalinization of 0.12 ± 0.02 pH units above the steady-state pH$_i$ value observed prior to the induction of anoxia. The magnitudes of the per-anoxic acidification and the post-anoxic alkalinization, as well as the time required to reach the maximum post-anoxic alkalinization, were significantly reduced from values obtained in the presence of normal external Na^+ under identical buffering conditions (*p*<0.05 in each case). As a result of the prolonged duration of these experiments, the time over which recordings could be maintained in the post-anoxic period was often shorter than seen in the presence of normal extracellular Na^+. Thus, to ensure that the reduction in the magnitude of the post-anoxic alkalinization was not secondary to a change in its time course, the magnitude of the post-anoxic alkalinization was measured at fixed time points following an anoxic period in the presence or absence of normal extracellular Na^+, and this possibility was found not to be the case (data not shown). Taken together, the above findings indicate that the reduction of extracellular

Na^+ by substitution with $NMDG^+$, a maneuver that inhibits the Na^+/H^+ exchanger, attenuates the magnitudes of the per-anoxic intracellular acidification and the post-anoxic alkalinization.

Unlike N-methyl-D-glucamine, extracellular Li^+ can act as a substrate for the Na^+/H^+ exchanger (Kinsella and Aronson, 1981). Thus, experiments were performed with 2 mM Na^+, Li^+-substituted media. Exposure to this solution produced an intracellular acidification of 0.34 ± 0.02 pH units (n=13) that, over a period of 11 ± 2 min, recovered to $100 \pm 0.3\%$ of the initial steady-state pH_i value observed prior to the reduction in $[Na^+]_o$ (see Baxter and Church, 1996; Smith et al, 1998). After pH_i reached a new steady-state plateau level, a 5 min period of anoxia induced an intracellular acidification which recovered slightly during the anoxic period and, following the return to normoxia, there was an intracellular alkalinization (Fig. 15; Table 6). Under these conditions, the magnitudes of both the per-anoxic acidification and the post-anoxic alkalinization were statistically larger than those seen when $NMDG^+$ was employed as the Na^+ substitute but were not significantly different from those values measured in the presence of a normal concentration of extracellular Na^+. Together, these findings strongly support a role for the Na^+/H^+ exchanger in the production of the pH_i response to a 5 min period of anoxia.

5.2.3 Anoxia-evoked changes in the activity of the Na^+/H^+ exchanger

The differences observed between the effects of anoxia on steady-state pH_i under $NMDG^+$ vs. Li^+-substituted conditions suggest strongly that changes in the activity of the

Na^+/H^+ exchanger contribute to the steady-state pH_i response to anoxia in acutely dissociated adult rat hippocampal CA1 neurons. Therefore, in the next series of experiments, I assessed directly whether there was a change in the activity of the Na^+/H^+ exchanger either during or following a transient anoxic period. To do so, paired intracellular acid loads were performed in HCO_3^-/CO_2-free, HEPES-buffered media, under which conditions the recovery of pH_i is primarily mediated by the Na^+/H^+ exchanger (Raley-Susman et al, 1991; Baxter and Church, 1996; Bevensee et al, 1996; Smith et al, 1998). Following an initial, control acid load, a second acid load was performed either during or following a 5 min period of anoxia, at a time when the steady-state pH_i was decreased or increased, respectively.

In the first set of experiments, the second acid load was performed during the anoxic period. In 8 cells, there was an overall $44 \pm 3\%$ decrease in the average rate of pH_i recovery from intracellular acid loads imposed during anoxia compared to the pre-anoxic control rates of pH_i recovery established in the same cells (Fig. 16). The rates of pH_i recovery were plotted against the absolute pH_i at which they were measured. At the same absolute pH_i values, the rates of recovery of pH_i were significantly slower during the anoxic period compared to the corresponding rates under normoxic conditions (Fig. 17; $p<0.05$ at all pH_i values except at pH_i 6.95). The slope of the linear regression line relating rates of pH_i recovery to absolute pH_i, an indication of transporter activity, was reduced from -21.88×10^{-3} s^{-1} under normoxic conditions to -13.90×10^{-3} s^{-1} under anoxic conditions, a 36% reduction in slope. There was also an acidic shift in the pH_i dependence of pH_i recovery, with the x-intercept of the regression line shifting from pH_i

$\cong 7.50$ under normoxic conditions to $pH_i \cong 7.43$ under anoxic conditions. These results support the possibility that, during a transient anoxic period, there is both a decrease in the rate of activity of the Na^+/H^+ exchanger and an acidic shift in the pH_i sensitivity of the antiport.

In the next set of experiments, the second acid load was performed following a 5 min period of anoxia. In 17 cells, there was a $111 \pm 18\%$ increase in the overall rate of pH_i recovery from intracellular acid loads imposed following a 5 min period of anoxia (Fig. 18). Rates of pH_i recovery were plotted against the pH_i values at which they were measured, and linear regression lines were obtained relating the rates of pH_i recovery to absolute values of pH_i under pre-anoxic and post-anoxic conditions. At every absolute value of pH_i, the rate of recovery of pH_i was significantly faster following a 5 min period anoxia when compared to the corresponding pre-anoxic control rate of recovery established in the same cells (Fig. 19; $p<0.05$ at all pH_i values). The slope of the linear regression line was increased from -17.74×10^{-3} s^{-1} under pre-anoxic control conditions to -21.43×10^{-3} s^{-1} following a 5 min period of anoxia, a 121% increase in slope, indicating an increase in the activity of the Na^+/H^+ exchanger. In addition, there was a shift in the x-intercept of the regression line from $pH_i \cong 7.45$ under pre-anoxic conditions to $pH_i \cong 7.60$ under post-anoxic conditions, indicating an alkaline shift in the pH_i sensitivity of the exchanger. Thus, following a transient anoxic period, there is both an increase in rate of the activity, and an alkaline shift in the pH_i sensitivity, of the Na^+/H^+ exchanger.

5.3 The effects of tetrodotoxin (TTX)

During anoxia, there is a dramatic increase in intracellular Na^+ which may reduce the activity of the Na^+/H^+ exchanger (Friedman and Haddad, 1994b). As Na^+ influx via TTX-sensitive, voltage-activated Na^+ channels has been reported to mediate, at least in part, this rise in $[Na^+]_i$ (Haddad and Jiang, 1993; Balestrino, 1995; Gleitz *et al*, 1996; Fung and Haddad, 1997), the influence of 0.5 - 1 μM TTX on the steady-state pH_i response to anoxia and the rates of pH_i recovery following intracellular acid loads imposed during an anoxic period were examined. Experiments were performed in HCO_3^- /CO_2-free, HEPES-buffered media. The addition of 0.5 - 1 μM TTX had a minimal effect on resting pH_i (pH_i 7.40 $\pm$ 0.03 in the presence of TTX, n=17; compared to pH_i 7.44 $\pm$ 0.03 in the absence of TTX, n=28). In the presence of TTX, a 5 min period of anoxia induced an acidification of 0.10 $\pm$ 0.01 pH units that recovered by 0.02 $\pm$ 0.01 pH units, a 29 $\pm$ 8% recovery, in the continued absence of oxygen, followed by a post-anoxic alkalinization of 0.17 $\pm$ 0.02 pH units (Fig. 20; Table 7). The magnitude of the anoxic acidification was reduced in the presence of TTX (n=17; p<0.05). In addition, the absolute magnitude of the per-anoxic rise in pH_i was reduced in the presence of TTX although the relative magnitude of the per-anoxic recovery was not significantly different than observed in the absence of TTX (29 $\pm$ 3% vs. 40 $\pm$ 5% recovery in the presence or absence of TTX, respectively)

The fact that TTX was able to reduce the absolute magnitude of the intracellular acidification seen during anoxia suggested the possibility that TTX may help to maintain Na^+/H^+ exchange activity during an anoxic period at a higher level than observed in the

absence of TTX. To investigate this possibility, in the presence of 0.5 - 1 μM TTX, intracellular acid loads were performed both before and during a transient anoxic period. In 7 cells examined, there was an overall $49 \pm 3\%$ decrease in the rate of pH_i recovery from acid loads imposed during anoxia in the presence of TTX compared to the rate of pH_i recovery established in the presence of TTX prior to anoxia in the same neurons (Fig. 21). The rates of recovery of pH_i were plotted against the absolute pH_i values at which they were measured and linear regressions were obtained relating the rates of pH_i recovery to absolute pH_i values under both normoxic (with TTX) and anoxic (with TTX) conditions. As previously seen in the parallel set of experiments conducted in the absence of TTX (see Section 5.2.3), in the presence of TTX, at every absolute value of pH_i, the rate of pH_i recovery was significantly slower during anoxia when compared to the corresponding pre-anoxic control rate of pH_i recovery established in the same cells (Fig. 22; $p<0.05$ at every pH_i value). The slope of the linear regression line was reduced from $-27.96 \times 10^{-3} \text{ s}^{-1}$ under normoxic conditions to $-18.59 \times 10^{-3} \text{ s}^{-1}$ under anoxic conditions, a 34% reduction in slope. In the absence of TTX, there was a similar 36% reduction in the slope of the linear regression line when comparing the normoxic and anoxic conditions. Moreover, in the presence of TTX, there was a shift of the x-intercept of the regression line in the acidic direction, from $pH_i \cong 7.50$ under normoxic conditions to $pH_i \cong 7.42$ under anoxic conditions. Thus, TTX failed to affect the reduction in the activity of the Na^+/H^+ exchanger evoked by a 5 min anoxic insult. Thus, while TTX reduced the magnitude of the intracellular acidification evoked by anoxia, it did not attenuate the degree of inhibition of Na^+/H^+ exchange evoked by anoxia.

5.4 The potential involvement of a voltage-activated H^+ conductance

Anoxic depolarization occurs within the first few minutes of an anoxic insult (Balestrino, 1995; Roberts Jr. and Chih, 1997; Tanaka *et al*, 1997; Yamamoto *et al*, 1997). Given the contribution of voltage-sensitive proton conductances (g_{H^+}) to acid extrusion in a variety of cell types (reviewed by Lukacs *et al*, 1993; see Introduction, Section 4.3.2), the activation of a g_{H^+} consequent upon anoxic depolarization may participate in the pH_i response to anoxia. To examine the potential role of a g_{H^+} in the pH_i response to anoxia, experiments were performed in HCO_3^-/CO_2-free, HEPES-buffered media containing 500 μM $ZnCl_2$ to block g_{H^+}. 500 μM $ZnCl_2$ had a minimal effect on steady-state pH_i (pH_i 7.36 ± 0.03 in the presence of $ZnCl_2$, $n=17$; compared with pH_i 7.30 ± 0.04 in the absence of $ZnCl_2$; $n=15$; $p>0.1$). In response to a 10 min period of anoxia, the presence of 500 μM $ZnCl_2$ did not influence the magnitude of the internal acidification; however, the rise in pH_i observed during anoxia was reduced from 0.08 ± 0.02 pH units (a 57 ± 11% recovery) under control conditions to 0.03 ± 0.01 pH units (a 26 ± 8% recovery) in the presence of $ZnCl_2$ (Fig. 23; Table 7; $p<0.05$). The presence of $ZnCl_2$ also significantly attenuated the magnitude of the post-anoxic alkalinization, as well as decreasing the time required to reach the maximum alkalinization ($p<0.05$ in each case; Table 7). These experiments suggest a role for a Zn^{2+}-sensitive, g_{H^+} in the production of the per-anoxic rise in pH_i and the post-anoxic internal alkalinization observed in response to a 10 min period of anoxia.

6 The effect of changing ambient temperature

The activity of the Na^+/H^+ exchanger in rat hippocampal neurons is temperature-sensitive (Baxter and Church, 1996; also see Bookstein *et al*, 1997; Marjanvoic *et al*, 1998 for evidence in other NHE isoforms). Furthermore, reductions in temperature are known to exert a neuroprotective action (Young *et al*, 1983; Kurihara *et al*, 1996; Kozlowski *et al*, 1997). To investigate whether the pH_i response to anoxia was influenced by a decrease in temperature, 5 min periods of anoxia were imposed at 22°C rather than at 37°C.

Prior to anoxia, in HCO_3^-/CO_2-free, HEPES-buffered media at pH 7.35, steady-state pH_i values were distributed, in a Gaussian manner, around a mean of pH_i 7.23 ± 0.02 (range pH_i 7.00 - 7.45; n=26; Fig. 24A). In HCO_3^-/CO_2-buffered media, steady-state pH_i values were similarly distributed, around a mean of pH_i 7.24 ± 0.02 (range pH_i 7.08 - 7.35; n=16; Fig. 24B). Under both buffering conditions, the mean steady-state pH_i values were statistically lower at 22°C than the respective values observed at 37°C, suggesting a decrease in the relative importance of the Na^+/H^+ exchanger to the maintenance of steady-state pH_i at 22°C (see Baxter and Church, 1996).

6.1 Steady state changes in intracellular pH evoked by anoxia at 22°C under HCO_3^-/CO_2-free, HEPES-buffered conditions

In HCO_3^-/CO_2-free, HEPES-buffered media at 22°C, a 5 min period of anoxia induced an intracellular acidification of 0.10 ± 0.01 pH units (n=9), that reached its maximum at 2.8 ± 0.3 min following the induction of anoxia (Fig. 25A; Table 8). In the

continued absence of oxygen, there was a 0.005 ± 0.002 pH unit increase of pH_i, a 4 ± 3% recovery. Upon the return to normoxia, an intracellular alkalinization of 0.04 ± 0.02 pH_i units occurred, which reached a maximum value after 7.2 ± 1.0 min (Table 8). Thus, the magnitude of each phase of the pH_i response to anoxia was significantly reduced at 22°C when compared to the pH_i response to 5 min periods of anoxia imposed under identical buffering conditions at 37°C ($p<0.05$ in each case).

6.2 Steady state changes in intracellular pH evoked by anoxia at 22°C under HCO_3^-/CO_2-buffered conditions

Parallel experiments to those reported in Section 6.1 were performed under HCO_3^- /CO_2-buffered conditions at 22°C (Fig. 25B; Table 8). Under these conditions, a 5 min period of anoxia induced a maximum intracellular acidification of 0.057 ± 0.005 pH units ($n=13$). During the continued absence of oxygen, pH_i increased by 0.03 ± 0.01 pH units, a 53 ± 15% recovery. Upon the return to normoxia, a maximum intracellular alkalinization of 0.06 ± 0.01 pH units occurred, which was reached after 3.6 ± 0.6 min following the return to normoxia. As was the case under HCO_3^-/CO_2-free, HEPES-buffered conditions, the magnitudes of all three phases of the pH_i response to anoxia were significantly reduced when compared to the corresponding values obtained at 37°C ($p<0.05$ in each case).

At 22°C, the magnitudes of the intracellular acidification observed during the anoxic period and the per-anoxic rise in pH_i were significantly smaller and larger, respectively, under HCO_3^-/CO_2-buffered conditions when compared with HCO_3^-/CO_2-

free, HEPES-buffered conditions (Table 8). However, the magnitude of the post-anoxic alkalinization was not influenced by the presence or absence of HCO_3^- at 22°C.

6.3 Anoxia-evoked changes in the activity of the Na^+/H^+ exchanger at 22°C

In HCO_3^-/CO_2-free, HEPES-buffered media, intracellular acid loads were performed to assess whether the decrease in the magnitude of the post-anoxic alkalinization observed at 22°C reflected a relative reduction in the activity of the Na^+/H^+ exchanger following a 5 min period of anoxia at 22°C compared to 37°C. In 11 cells, there was a 102 ± 11% increase in the overall rate of pH_i recovery from intracellular acid loads imposed following anoxia at 22°C (Fig. 26). Rates of pH_i recovery were plotted against absolute pH_i values and linear regression lines relating rates of pH_i recovery to absolute pH_i values were obtained under both pre-anoxic and post-anoxic conditions at 22°C. In a manner similar to that observed at 37°C (see Section 5.2.3), at every pH_i value, the rate of pH_i recovery was significantly faster after a 5 min period of anoxia compared to the pre-anoxic control rate of pH_i recovery established in the same cells (Fig. 27; $p < 0.05$ at every pH_i value). The slope of the linear regression line relating pH_i to rates of pH_i recovery was increased from -0.76×10^{-3} s^{-1} under normoxic conditions at 22°C to -1.47×10^{-3} s^{-1} under post-anoxic conditions, indicating that there was a post-anoxic stimulation of Na^+/H^+ exchange activity. Most importantly, at every absolute value of pH_i, under both normoxic and post-anoxic conditions, rates of recovery of pH_i from imposed acid loads were significantly faster at 37°C than at 22°C (Fig. 27; $p < 0.05$ at every pH_i value under both normoxic and post-anoxic conditions). The results suggest

that while the activity of the Na^+/H^+ exchanger is stimulated following transient periods of anoxia at both 22°C and at 37°C, the absolute level of Na^+/H^+ exchange activity is lower at 22°C compared to 37°C. Thus, the reduced activity of the Na^+/H^+ exchanger likely underlies the reduced magnitude of the post-anoxic steady-state rise in pH_i observed at 22°C.

7 The effects of changing extracellular pH

In vivo ischemia and anoxia produce changes in extracellular pH (pH_o) (Mutch and Hansen, 1984; Silver and Erecinska, 1992). In turn, pH_o is known to influence pH_i in rat hippocampal neurons (Church *et al*, 1998), possibly by altering the activity of the Na^+/H^+ exchanger (Aronson *et al*, 1983; Jean *et al*, 1985; Vaughan-Jones and Wu, 1990). Thus, the effects of changing pH_o from 7.35 to either 6.60 or 7.60 on the pH_i response to a 5 min period of anoxia were examined.

7.1 The effects of reducing extracellular pH

Exposure to a HCO_3^-/CO_2-free, HEPES-buffered solution at pH_o 6.60 caused a rapid 0.49 ± 0.03 pH unit fall in pH_i ($n=26$). Once the new steady-state pH_i level was reached, a 5 min period of anoxia induced an acidification of 0.15 ± 0.01 pH units ($n=10$) that recovered by 0.02 ± 0.01 pH units, a 17 ± 9% recovery, within the anoxic period (Fig. 28; Table 9). Upon the return to normoxia, there was an internal alkalinization of 0.027 ± 0.005 pH units above the pre-anoxic steady-state level that reached its maximum after 4.0 ± 0.4 min. Compared to the results obtained at pH_o 7.35, the magnitude of the

anoxic acidification was not influenced by the reduction in pH_o; however, the magnitudes of the per-anoxic rise in pH_i and the post-anoxic alkalinization were significantly smaller at pH_o 6.60 than observed at pH_o 7.35 ($p<0.05$ in each case). In addition, the time required to reach the maximum of the post-anoxic alkalinization was significantly reduced at pH_o 6.60 compared to pH_o 7.35.

In many cell types, a reduction in pH_o inhibits the activity of the Na^+/H^+ exchanger (Jean *et al*, 1985; Vaughan-Jones and Wu, 1990). To investigate whether the attenuation of the post-anoxic alkalinization observed under pH_o 6.60 conditions was due to a relative decrease in the activity of the Na^+/H^+ exchanger, intracellular acid loads were performed both before and after a 5 min period of anoxia at pH_o 6.60. A 5 min period of anoxia evoked a 173 ± 16% increase in the overall rate of pH_i recovery in the immediate post-anoxic period (Fig. 29; *n*=9). Rates of pH_i recovery were plotted against absolute pH_i values and linear regression lines relating the rates of pH_i recovery and absolute pH_i values were obtained for the pre- and post-anoxic conditions. As was the case at pH_o 7.35, at pH_o 6.60 the rate of recovery of pH_i was significantly faster following a 5 min period of anoxia when compared with the corresponding pre-anoxic control rate of pH_i recovery (Fig. 30; $p<0.05$ at all pH_i values except pH_i 6.70). The slope of the linear regression line relating pH_i to rates of pH_i recovery was increased from -6.00 x 10^{-3} s^{-1} under pre-anoxic conditions to -19.21 x 10^{-3} s^{-1} in the post-anoxic period. indicating that an increase in the activity of the Na^+/H^+ exchanger occurs following anoxia under pH_o 6.60 conditions. On the other hand, anoxia did not influence the x-intercept of the linear regression lines relating rates of pH_i recovery to absolute values of pH_i. Thus, following

an anoxic period imposed at pH_o 6.60, Na^+/H^+ exchange activity is stimulated, but there is no apparent change in the pH_i sensitivity of the exchanger.

At every pH_i value, under both pre- and post-anoxic conditions, the rates of pH_i recovery from imposed acid loads were significantly slower at pH_o 6.60 when compared with the corresponding rates of pH_i recovery obtained prior to and following anoxia at pH_o 7.35 (Fig. 30). Thus, although Na^+/H^+ exchange activity is stimulated following anoxia at pH_o 6.60, the reduced magnitude of the intracellular post-anoxic alkalinization at pH_o 6.60 can be attributed to the reduced absolute level of Na^+/H^+ exchange activity in the presence of an external acidosis.

7.2 The effects of increasing extracellular pH

The next series of experiments were performed at pH_o 7.60. Exposure to a pH_o 7.60, HCO_3^-/CO_2-free, HEPES-buffered solution caused a rapid 0.32 ± 0.01 pH unit increase in pH_i ($n=29$). Once pH_i had reached a new steady-state level, a 5 min period of anoxia induced an intracellular acidification of 0.03 ± 0.05 pH units ($n=10$) that recovered by 0.08 ± 0.02 pH units, a 269 ± 80% recovery, in the continued absence of oxygen (Fig. 31; Table 9). Upon the return to normoxia, there was a maximum intracellular alkalinization of 0.28 ± 0.04 pH units which was attained after 3.8 ± 0.2 min upon the return to normoxia. The magnitude of the intracellular per-anoxic acidification was significantly smaller, whereas the magnitudes of the per-anoxic recovery of pH_i and the post-anoxic alkalinization were significantly larger, than those observed at pH_o 7.35 ($p<0.05$ in each case). The time required to reach the maximum post-anoxic

alkalinization was significantly reduced at pH_o 7.60 compared with pH_o 7.35 ($p<0.05$; Table 9).

To investigate whether the increase in the magnitude of the post-anoxic alkalinization observed under pH_o 7.60, compared to pH_o 7.35, conditions reflected a relative increase in the activity of the Na^+/H^+ exchanger, intracellular acid loads were performed before and after a 5 min period of anoxia at pH_o 7.60. Following anoxia, in 7 cells, there was a 127 ± 22% increase in the overall rate of pH_i recovery from acid loads imposed at pH_o 7.60 (Fig. 32). Rates of recovery of pH_i were plotted against the pH_i values at which they were measured, and linear regression lines relating the rates of pH_i recovery and absolute pH_i values were obtained for the normoxic and post-anoxic conditions. At pH_o 7.60, the rate of recovery of pH_i was significantly faster following a 5 min period of anoxia when compared to the corresponding pre-anoxic control rates of pH_i recovery (Fig. 33; $p<0.05$ at all pH_i values except pH_i 7.20). The slope of the linear regression line relating absolute values of pH_i to rates of pH_i recovery was increased from -20.12 x 10^{-3} s^{-1} under pre-anoxic control conditions to -22.01 x 10^{-3} s^{-1} following a 5 min period of anoxia. In addition, there was an alkaline shift in the pH_i sensitivity of the Na^+/H^+ exchanger, as indicated by a shift in the x-intercept of the regression line from pH_i $\cong$ 7.94 to pH_i $\cong$ 8.39 under post-anoxic conditions.

As seen at pH_o 7.35 and at pH_o 6.60, under pH_o 7.60 conditions, the activity of the Na^+/H^+ exchanger is stimulated following a transient anoxic period. Furthermore, the rates of pH_i recovery were significantly faster at pH_o 7.60 when compared to the corresponding rates of pH_i recovery obtained both prior to and following an anoxic period

at pH_o 7.35 (Fig. 33). Thus, the relatively greater activity of the Na^+/H^+ exchanger after anoxia at pH_o 7.60 compared to pH_o 7.35 likely underlies the increased magnitude of the post-anoxic alkalinization observed following an anoxic period at pH_o 7.60.

7.3 The dependence of intracellular pH on extracellular pH under normoxic conditions

In the absence of an anoxic insult, rates of pH_i recovery following intracellular acid loads imposed at pH_o 6.60 were significantly slower, and the rates of pH_i recovery at pH_o 7.60 significantly faster, when compared to the corresponding rates of pH_i recovery observed at pH_o 7.35. Thus, as pH_o increases, there was a progressive increase in the slope of the linear regression lines relating rates of pH_i recovery to absolute pH_i values (the slopes being -6.00×10^{-3} s^{-1}, -17.73×10^{-3} s^{-1} and -20.12×10^{-3} s^{-1} at pH_o 6.60, 7.35 and 7.60, respectively). Similarly, there was a progressive alkaline shift in the x-intercept of the linear regression lines as pH_o increased (the x-intercepts being 6.91, 7.42, and 7.94 at pH_o 6.60, 7.35 and 7.60, respectively). Taken together, these data indicate that Na^+/H^+ exchange in rat hippocampal neurons is inhibited at acidic pH_o values and stimulated at alkaline pH_o values.

The dependence of Na^+/H^+ exchange activity on pH_o may contribute to the overall pH_o dependence of pH_i in post-natal rat hippocampal neurons (see Church et al, 1998). To examine the relationship between pH_o and pH_i in adult rat hippocampal neurons, cells were exposed to HCO_3^-/CO_2-free, HEPES-buffered media at pH 7.75, 7.60, 7.35, 6.80 or 6.60. Exposure to pH 7.75 and pH 7.60 media caused 0.40 ± 0.03 (n=5) and 0.32 ± 0.01 (n=29) pH unit increases, respectively, in steady-state pH_i. Exposure to pH 6.80 and 6.60

media caused 0.42 ± 0.04 ($n=2$) and 0.49 ± 0.03 ($n=26$) pH unit decreases, respectively, in steady-state pH_i. The new steady-state pH_i was then plotted against pH_o. Thus, in contrast to peripheral cell types but in agreement with data obtained from cultured post-natal rat hippocampal neurons, in adult rat hippocampal neurons there was a significant dependence of pH_i on pH_o (Fig. 34; $\Delta pH_i{:}\Delta pH_o = 0.74$; $r^2 = 0.98$). Taken together, the above data support the notion that pH_o is capable of influencing pH_i in adult rat hippocampal CA1 neurons by altering Na^+/H^+ exchange activity.

8 The potential contribution of changes in intracellular buffering power

The results presented above indicate that transient anoxic insults lead to changes in the activity of the Na^+/H^+ exchanger in rat hippocampal neurons. However, it is established that changes in intracellular buffering power may contribute to changes in the rate of acid extrusion (Boron, 1989). For example, exposure of cardiac Purkinje fibres to the aerobic inhibitor, cyanide, caused a 47% decrease in the rate of pH_i recovery from imposed intracellular acid loads that was largely attributed to an increase in intracellular buffering power (Wu and Vaughan-Jones, 1994).

Exposure to the weak base, NH_4Cl, causes an intracellular alkalinization, the magnitude of which, in the absence of CO_2, provides a reasonable estimation of the intrinsic buffering power (β_i) of the cell (Amos and Richards, 1996; Baxter and Church, 1996; Smith *et al*, 1998). The magnitudes of the increase in pH_i evoked by 40 mM NH_4Cl were similar under pre-anoxic, anoxic and post-anoxic conditions (0.27 ± 0.05 pH units, 0.21 ± 0.04 pH units, and 0.21 ± 0.02 pH units, respectively ; $n \geq 9$ in each case). In

addition, the presence of TTX failed to affect the magnitude of this alkalinization both under normoxic and anoxic conditions (a 0.27 ± 0.08 pH unit vs. 0.25 ± 0.03 pH unit increase under normoxic and anoxic conditions, respectively; $n=7$); thus, the presence of TTX failed to affect significantly β_i. Moreover, it has been shown in hippocampal neurons that perfusion with a Na^+-free solution does not alter β_i (Amos and Richards, 1996). Therefore, at pH_o 7.35, a change in β_i appears unlikely to be able to account for the marked changes in the rates of acid extrusion seen either during or following an anoxic period.

It is established that β_i in rat hippocampal neurons increases with a decrease in pH_i (Bevensee et al, 1996). In the present study, the magnitudes of the pre- and post-anoxic alkalinizations produced upon exposure to NH_4Cl were significantly smaller at pH_o 6.60 and significantly larger at pH_o 7.60 than the increases in pH_i observed at pH_o 7.35. Thus, under pre-anoxic conditions, the increase in pH_i evoked by 40 mM NH_4Cl was 0.17 ± 0.02 pH units at pH_o 6.60 and 0.43 ± 0.04 pH units at pH_o 7.60 ($n \geq 11$ in each case; $p<0.05$ for corresponding change measured under pH_o 7.35 conditions). In the post-anoxic period, the increase in pH_i was 0.14 ± 0.02 pH units at pH_o 6.60 and 0.45 ± 0.08 pH units at pH_o 7.60 ($n \geq 11$ in each case; $p<0.05$ for corresponding change measured at pH_o 7.35). These data confirm the inverse relationship between β_i and pH_i in rat hippocampal neurons and suggest the possibility that changes in β_i may contribute to the differences in the rates of acid extrusion observed at pH_o 6.60 and 7.60, when compared to the rates of acid extrusion at pH_o 7.35. However, at a given pH_o value, there was no change in the magnitude of the increase in pH_i when comparing the pre- and post-anoxic

values. Thus, at each of the pH_o conditions tested, a change in β_i in the post-anoxic period cannot account for the increase in acid extrusion rate observed following a transient period of anoxia.

9 The Role of Extracellular Glucose Concentration

Acutely dissociated neurons are commonly prepared in and exposed to solutions containing 17.5 mM glucose, a concentration which, while preserving the integrity of these isolated cells *in vitro* (*e.g.* Fan and Wong, 1996; Dzoljic *et al*, 1998), is considered hyperglycemic *in vivo* (Ibayashi *et al*, 1986, Silver and Erecinska, 1994). In addition, it is established that extracellular [glucose] affects the magnitude of the internal acidification seen during *in vivo* anoxia/ischemia, at least in part by determining the internal lactate and ATP levels during and following the insult (Ljunggren *et al*, 1974; Katsura *et al*, 1992a; but see Hope *et al*, 1988; Tyson *et al*, 1993). Thus, the pH_i response to a 5 min period of anoxia was assessed after reducing the extracellular glucose concentration from 17.5 mM to 5.0 mM, while maintaining the osmolality of the solution with the addition of 12.5 mM D-mannitol (Morimoto *et al*, 1994; Cendoroglo *et al*, 1998; Wang *et al*, 1998). The osmolality of the HCO_3^-/CO_2-free, HEPES-buffered media containing 17.5 mM glucose was 295 ± 2 mOsm/kg H_2O ($n=4$) and the osmolality of the HEPES-buffered solution containing 5.0 mM glucose was 296 ± 2 mOsm/kg H_2O ($n=4$), a value not statistically different from the former.

In the presence of 5.0 mM glucose, a 5 min anoxic period induced an intracellular acidification of 0.12 ± 0.01 pH units that reached its maximum 2.3 ± 0.2 min following

the start of anoxia (Fig. 35B; Table 10; $n=22$). In the continued absence of oxygen, pH_i recovered by 0.021 ± 0.005 pH units, a $21 \pm 6\%$ recovery. Upon the return to normoxia, there was an intracellular alkaline shift of 0.18 ± 0.02 pH units, reaching a maximum after 8.9 ± 0.5 min after the end of the anoxic period. As was observed under 17.5 mM glucose conditions, the initial steady-state pH_i was positively correlated with the magnitude of the anoxic acidification ($r^2=0.73$).

Changing the duration of the anoxic from 5 min to either 3 or 10 min did not significantly influence the magnitudes of the intracellular acidification seen during anoxia or the intracellular alkalinization observed following anoxia (Figs. 35A and 35C; Table 10). However, the magnitude of the per-anoxic rise in pH_i was significantly larger during a 10 min period of anoxia than during a 3 min period of anoxia ($p<0.05$).

The pH_i response to an anoxic period imposed during perfusion with media containing 5.0 mM glucose was qualitatively similar to that observed in the presence of 17.5 mM glucose. However, various phases of the pH_i response were influenced by the change in extracellular glucose concentration. Firstly, the magnitudes of the anoxic acidifications evoked by a 5 or 10 min period of anoxia were statistically smaller in the presence of 5.0 mM glucose when compared to those observed in the presence of 17.5 mM glucose ($p<0.05$ in each case). Secondly, the magnitude of the per-anoxic rise in pH_i was statistically smaller during 3 or 5 min periods of anoxia imposed in the presence of 5.0 mM glucose, despite the fact that there were no differences in the times to the start of these increases in pH_i (which occurred at 3.6 ± 0.2 min and 3.1 ± 0.2 min following the start of anoxia in 5.0 and 17.5 mM glucose, respectively; $n \geq 11$; $p<0.05$ in each case).

Finally, the magnitude of the post-anoxic alkalinization following a 10 min period of anoxia was significantly larger in the presence of 17.5 mM glucose compared to 5.0 mM glucose.

9.1 Role of the Na^+/H^+ exchanger at a reduced extracellular glucose concentration

To investigate the potential role of the Na^+/H^+ exchanger in the presence of 5.0 mM glucose, a 5 min period of anoxia was induced under reduced-Na^+, $NMDG^+$-substituted conditions. Exposure to this solution caused a rapid intracellular acidification of 0.45 ± 0.04 pH units that recovered to a plateau value of $101 \pm 1\%$ of the initial pH_i value after 15 ± 2 min ($n=17$; Fig. 36A). All parameters of this recovery of pH_i were not statistically different than found during perfusion with a reduced-Na^+, 17.5 mM glucose solution (see Section 5.2.1).

During perfusion with a reduced-Na^+, 5.0 mM glucose-containing medium, a 5 min anoxic period evoked an intracellular acidification of 0.09 ± 0.02 pH units ($n=17$) that recovered by 0.02 ± 0.01 pH units, a $17 \pm 8\%$ recovery, in the continued absence of oxygen (Fig. 36A; Table 11). Upon the return to normoxia, there was an intracellular alkalinization of 0.10 ± 0.03 pH units. While the magnitudes of the per-anoxic acidification and the per-anoxic rise in pH_i were not influenced by reduced-Na^+, $NMDG^+$-substituted conditions, the magnitude of the post-anoxic alkalinization was significantly reduced. This finding parallels the observation made with 17.5 mM glucose (Table 6) and is consistent with the possibility that an increase in the activity of the Na^+/H^+

exchanger is responsible for the production of the post-anoxic alkalinization in the presence of 5 mM as well as 17.5 mM glucose.

Experiments were then performed in a reduced-Na^+, Li^+-substituted medium containing 5.0 mM glucose. The exposure to this solution caused an intracellular acidification of 0.30 ± 0.03 pH units that recovered to $102 \pm 1\%$ of the initial steady-state pH_i value over 18 ± 1 min (n=15; Fig. 37B). A 5 min period of anoxia induced an intracellular acidification that recovered slightly in the continued absence of oxygen, and upon the return to normoxia, there was a rapid intracellular alkalinization (Fig. 36B; Table 11). Under Li^+-substituted conditions, the magnitude of the post-anoxic alkalinization was not significantly different than observed in the presence of normal extracellular Na^+ and was significantly larger than seen under $NMDG^+$-substituted conditions (Table 11; p<0.05 in each case). In addition, as observed in the presence of 17.5 mM glucose, the time required to reach the maximum of the post-anoxic alkalinization was significantly faster under reduced-Na^+, Li^+-substituted conditions than observed in the presence of normal extracellular Na^+. However, analogous to the situation in the presence of 17.5 mM glucose, the restoration of the post-anoxic alkalinization was not secondary to a change in its time course, as the magnitude of the post-anoxic alkalinization was measured at fixed time points following a 5 min period of anoxia and this possibility was found not to be the case (data not shown). Taken together, these experiments suggest that, in the presence of 5.0 mM glucose, the Na^+/H^+ exchanger is involved in the production of the post-anoxic alkalinization. Thus, the activity of the Na^+/H^+ exchanger appears to be influenced to only a minor degree by

relatively limited changes in extracellular glucose concentration. On the other hand, at both 5.0 mM and 17.5 mM glucose, a reduction in Po_2 has a significant influence on Na^+/H^+ exchange activity in the post-anoxic period.

10 The potential role of intracellular second messenger systems

These studies described above establish that a change in the activity of the Na^+/H^+ exchanger contributes to the pH_i response to a transient period of anoxia in adult rat hippocampal neurons. In peripheral cell types, the post-anoxic increase in Na^+/H^+ exchange activity is consequent upon the normalization of pH_o. The fall in pH_o during anoxia/ischemia is suggested to inhibit Na^+/H^+ exchange, which can only respond to the fall in pH_i induced by ischemia once pH_o returns to normal. In contrast to these data, in the present study, given that pH_o was held constant throughout the anoxic and post-anoxic periods, alternate factors must contribute to the increase in the activity of the Na^+/H^+ exchanger following transient periods of anoxia. Thus, preliminary experiments investigated the potential role of anoxia-evoked changes in second messenger systems in producing the observed changes in Na^+/H^+ exchange activity. The present focus was on the involvement of the cAMP/PKA pathway because: i) it has been shown to be involved in the regulation of Na^+/H^+ exchange activity in acutely dissociated adult rat hippocampal CA1 neurons (Smith et al, 1998); and ii) its activity is altered during and following transient periods of anoxia (Kobayashi et al, 1977; Whittingham et al, 1984; Blomqvist et al, 1985; Domanska-Janik and Pylova, 1989).

The application of 50 µM Rp-cAMPS, a selective inhibitor of PKA (Parker-Botelho *et al*, 1988), did not influence pH_i. In the presence of Rp-cAMPS, a 5 min period of anoxia induced an intracellular acidification which was statistically smaller than seen in the absence of Rp-cAMPS ($p<0.05$; $n=4$; Table 12). In addition, upon the return to normoxia, the presence of Rp-cAMPS reduced the magnitude post-anoxic alkalinization ($p<0.05$), suggesting that the increase in $[cAMP]_i$ known to occur in the immediate post-anoxic period in rat hippocampal neurons may act via PKA to promote an increase in the activity of the Na^+/H^+ exchanger following a transient period of anoxia.

Both 10 µM noradrenaline and 10 µM isoproterenol stimulate Na^+/H^+ exchange activity in rat hippocampal neurons via β-adrenoceptors and the cAMP/PKA pathway (Smith *et al*, 1998). In the present study, application of 10 µM noradrenaline increased steady-state pH_i by 0.15 ± 0.03 pH units ($n=5$). Similarly, the application of 10 µM isoproterenol increased steady-state pH_i by 0.22 ± 0.09 pH units ($n=5$). Given that these treatments activate Na^+/H^+ exchange to a similar extent through the same intracellular pathway (Smith *et al*, 1998), their effects on the pH_i response to transient anoxic periods were pooled. Thus, once the new steady-state pH_i was reached in the presence of a β-adrenoceptor agonist, a 5 min period of anoxia induced an intracellular acidification that recovered slightly in the continued absence of oxygen (Table 12). Upon the return to normoxia, there was an intracellular alkalinization, the magnitude of which was significantly larger in the presence of a β-adrenoceptor agonist than observed in its absence ($n=10$; $p<0.05$). The time required to reach the maximum post-anoxic alkalinization was significantly faster in the presence of a β-adrenoceptor agonist than in

its absence. Thus, inhibiting the cAMP/PKA pathway reduces, and stimulating the cAMP/PKA pathway increases, the magnitude of the post-anoxic alkalinization, a result consistent with the involvement of the Na^+/H^+ exchanger in the production of the post-anoxic increase in pH_i.

11 Intracellular pH regulation in the absence of extracellular sodium

An observation made during the course of this study was the recovery of pH_i from the intracellular acidification produced upon exposure to zero-Na^+ or reduced-Na^+, HCO_3^- /CO_2-free, HEPES-buffered solutions, media which block the Na^+/H^+ exchanger as well as all HCO_3^--dependent pH_i regulating mechanisms. A similar finding has been reported by others in hippocampal neurons (Bevensee *et al*, 1996).

In a concerted effort to undercover the mechanism(s) underlying this response, the effects of a number of pharmacological treatments and ionic substitutions on this alkalinizing process were tested (see Table 13). Pharmacological treatments included 50 µM omeprazole (an inhibitor of P-type ATPases), 1-2 µM bafilomycin A_1 (an inhibitor of V-type ATPases), 300 µM eosin Y (an inhibitor of Ca^{2+}/H^+ ATPases), 500 µM $ZnCl_2$ (an inhibitor of g_{H^+}), and 5 mM 4-α-5-hydroxycinnamate (an inhibitor of lactate/H^+ cotransport). Ionic manipulations included the removal of extracellular K^+, Ca^{2+}, Cl^- (with NaCl, KCl, and $CaCl_2$ being substituted with their respective gluconate salts), or Mg^{2+}, which act to inhibit K^+/H^+ exchange, Ca^{2+}/H^+ exchange, Na^+-independent H^+/Cl^- exchange, and ATPase activity. The parameters measured to assess the influence of these maneuvers on the recovery of pH_i in the absence of extracellular Na^+ were: i) the

magnitude of the initial fall in pH_i upon exposure to zero-Na^+ media; ii) the time, from the point of exposure to zero-Na^+ media, to the initiation of the rise in pH_i; and iii) the time required, from the point of exposure to zero-Na^+ media, to reach a plateau during the continued perfusion with zero-Na^+ media.

None of these manipulations influenced significantly steady-state initial pH_i (see Table 13). Moreover, none of these manipulations completely blocked the pH_i recovery observed during perfusion with zero-Na^+ media. However, perfusion with 500 μM $ZnCl_2$, a $[K^+]_o$-free, or a $[Ca^{2+}]_o$-free solution significantly delayed the onset of pH_i recovery. While these experiments suggest the presence of (an) alternate pH_i regulating mechanism(s) in acutely dissociated adult rat hippocampal CA1 neurons, the identity of the mechanism(s) which participate in the observed internal alkalinization produced under conditions where the Na^+/H^+ exchanger and HCO_3^--dependent mechanisms are blocked remains unknown. The possibility also remains that this mechanism(s) is not functional under resting conditions and is activated only during perfusion with a zero-Na^+ solution.

Table 4: Magnitude and time course of anoxia-evoked changes in intracellular pH under HCO_3^--free, HEPES-buffered conditions

	Duration of Anoxia		
	3 minutes (n=13)	5 minutes (n=28)	10 minutes (n=15)
Magnitude of (pH_i units):			
Acidic Shift	0.17 ± 0.02^c	0.16 ± 0.01	0.15 ± 0.01
Recovery	$0.015 \pm 0.004^{a,b}$	0.05 ± 0.01	0.08 ± 0.02^a
Alkaline Shift	0.19 ± 0.05^b	0.20 ± 0.02	$0.27 \pm 0.04^{a,c}$
Time to Reach (minutes):			
Start of Acidification	0.9 ± 0.1	1.0 ± 0.1	1.1 ± 0.2
Peak Acidification	$1.9 \pm 0.2^{a,b}$	2.5 ± 0.2	2.6 ± 0.3
Peak Alkalinization	11.6 ± 1.9	9.5 ± 1.1	9.2 ± 1.5^c

[a] indicates statistical significance when compared to the equivalent parameter after 5 min of anoxia in a HCO_3^--free, HEPES-buffered solution.

[b] indicates statistical significance when compared to the equivalent parameter after 10 min of anoxia in a HCO_3^--free, HEPES-buffered solution.

[c] indicates statistical significance when compared to the equivalent parameter in a HCO_3^-/CO_2-buffered medium

Table 5: Magnitude and time course of anoxia-evoked changes in intracellular pH under HCO_3^-/CO_2-buffered conditions

	Duration of Anoxia		
	3 minutes (n=17)	5 minutes (n=14)	10 minutes (n=12)
Magnitude of (pH$_i$ units):			
Acidic Shift	0.12± 0.01[a,c]	0.18± 0.02	0.15 ± 0.02
Recovery	0.02 ± 0.01[a,b]	0.07 ± 0.01[b]	0.11 ± 0.02
Alkaline Shift	0.13± 0.02	0.16 ±0.05	0.19 ± 0.02[c]
Time to Reach (minutes):			
Start of Acidification	0.9 ± 0.1	1.1 ± 0.1	0.9 ± 0.1
Peak Acidification	1.9 ± 0.1[b]	2.2 ± 0.2	2.5 ± 0.3
Peak Alkalinization	11.9 ± 1.6	10.8 ± 1.3	13.0 ± 0.8[c]

[a] indicates statistical significance when compared to the equivalent parameter after 5 min of anoxia in a HCO_3^-/CO_2-buffered medium

[b] indicates statistical significance when compared to the equivalent parameter after 10 min of anoxia in a HCO_3^-/CO_2-buffered medium

[c] indicates statistical significance when compared to the equivalent parameter in a HEPES-buffered medium

Table 6: The influence of the reduction of extracellular Na^+ on the pH_i response to anoxia under HCO_3^--free, HEPES-buffered conditions

	Ionic Substitution		
	Control	Low Na^+ (NMDG$^+$)	Low Na^+ (Li$^+$)
	(n=28)	(n=20)	(n=13)
Magnitude of (pH_i units):			
Acidic Shift	0.16 ± 0.01	0.11 ± 0.01[a]	0.18 ± 0.02[b]
Recovery	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01
Alkaline Shift	0.20 ± 0.02	0.12 ± 0.02[a]	0.20 ± 0.03[b]
Time to Reach (minutes):			
Start of Acidification	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
Peak Acidification	2.5 ± 0.2	2.8 ± 0.2	2.6 ± 0.3
Peak Alkalinization	9.5 ± 1.1	6.4 ± 0.8[a]	6.5 ± 0.5[a]

[a] indicates statistical significance when compared to the equivalent parameter after 5 min of anoxia in a HCO_3^--free, HEPES-buffered solution containing normal extracellular Na^+.
[b] indicates statistical significance when compared to the equivalent parameter after 5 min of anoxia in a low-Na^+, NMDG-substituted, HCO_3^--free, HEPES-buffered solution

Table 7: Magnitude and time course of anoxia-evoked changes in pH_i in the presence of TTX or $ZnCl_2$

	Control (*n*=28)	0.5 - 1 µM TTX (*n*=17)	Control (*n*=15)	500 µM $ZnCl_2$ (*n*=17)
Duration of Anoxia: Buffer:	5 min HEPES	5 min HEPES	10 min HEPES	10 min HEPES
Magnitude of (pH_i units):				
Acidic Shift	0.16 ± 0.01	0.10 ± 0.01^a	0.15 ± 0.01	0.16 ± 0.02
Recovery	0.05 ± 0.01	0.02 ± 0.01^a	0.08 ± 0.02	0.03 ± 0.01^b
Alkaline Shift	0.20 ± 0.02	0.17 ± 0.02	0.27 ± 0.04	0.13 ± 0.02^b
Time to Reach (min):				
Start of Acidification	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	0.9 ± 0.1
Peak Acidification	2.5 ± 0.2	2.1 ± 0.1	2.6 ± 0.3	3.3 ± 0.5
Peak Alkalinization	9.5 ± 1.1	8.3 ± 1.0	9.2 ± 1.5	4.6 ± 0.5^b

[a] indicates statistical significance when compared to the equivalent parameter after 5 min of anoxia in a HCO_3^--free, HEPES-buffered solution in the absence of any pharmacological treatment.

[b] indicates statistical significance when compared to the equivalent parameter after 10 min of anoxia in a HCO_3^--free, HEPES-buffered solution in the absence of any pharmacological treatment.

Table 8: The influence of temperature on the magnitude and time course of anoxia-evoked changes in pH_i

	$37°C$ $(n=28)$	$22°C$ $(n=9)$	$37°C$ $(n=14)$	$22°C$ $(n=13)$
Buffer	HEPES	HEPES	HCO_3^-/CO_2	HCO_3^-/CO_2
Magnitude of (pH_i units):				
Acidic Shift	0.16 ± 0.01	0.10 ± 0.01^a	0.18 ± 0.02	$0.057 \pm 0.005^{b,c}$
Recovery	0.05 ± 0.01	0.005 ± 0.002^a	0.07 ± 0.01	$0.03 \pm 0.01^{b,c}$
Alkaline Shift	0.20 ± 0.02	0.04 ± 0.02^a	0.16 ± 0.05	0.06 ± 0.01^b
Time to Reach (minutes):				
Start of Acidification	1.0 ± 0.1	1.0 ± 0.2	1.1 ± 0.1	$0.5 \pm 0.1^{b,c}$
Peak Acidification	2.5 ± 0.2	2.8 ± 0.3	2.2 ± 0.2	$1.4 \pm 0.1^{b,c}$
Peak Alkalinization	9.5 ± 1.1	7.2 ± 1.0	10.8 ± 1.3	$3.6 \pm 0.6^{b,c}$

[a] indicates statistical significance when compared to the equivalent parameter after 5 min of anoxia in a HCO_3^-/CO_2-free, HEPES-buffered solution at $37°C$.

[b] indicates statistical significance when compared to the equivalent parameter after 5 min of anoxia in a HCO_3^-/CO_2-buffered solution at $37°C$.

[c] indicates statistical significance when compared to the equivalent parameter after 5 min of anoxia in a HCO_3^--free, HEPES-buffered solution at $22°C$

Table 9: The influence of pH_o on the magnitude and time course of anoxia-evoked changes in pH_i

	Extracellular pH		
	7.35 (n=28)	6.60 (n=10)	7.60 (n=10)
Buffer	HEPES	HEPES	HEPES
Magnitude of (pH_i units):			
Acidic Shift	0.16 ± 0.01	0.15 ± 0.01	0.03 ± 0.05[a]
Recovery	0.05 ± 0.01	0.02 ± 0.01[a]	0.08 ± 0.02[a]
Alkaline Shift	0.20 ± 0.02	0.027 ± 0.005[a]	0.28 ± 0.04[a]
Time to Reach (minutes):			
Start of Acidification	1.0 ± 0.1	0.8 ± 0.2	2.0 ± 0.2[a]
Peak Acidification	2.5 ± 0.2	2.8 ± 0.3	3.1 ± 0.2
Peak Alkalinization	9.5 ± 1.1	4.0 ± 0.4[a]	3.8 ± 0.2[a]

[a] indicates statistical significance when compared to the equivalent parameter after 5 min of anoxia in a HCO_3^--free, HEPES-buffered solution at pH_o 7.35.

Table 10: Magnitude and time course of anoxia-evoked changes in pH_i under HCO_3^--free, HEPES-buffered conditions in the presence of 5.0 mM glucose

	Duration of Anoxia		
	3 minutes (n=10)	5 minutes (n=22)	10 minutes (n=8)
Magnitude of (pH_i units):			
Acidic Shift	0.14 ± 0.02	0.12 ± 0.01[b]	0.11 ± 0.01[b]
Recovery	0.004 ± 0.002[a,b]	0.021 ± 0.005[b]	0.05 ± 0.02
Alkaline Shift	0.10 ± 0.03	0.18 ± 0.02	0.13 ± 0.03[b]
Time to Reach (minutes):			
Start of Acidification	0.9 ± 0.1	0.8 ± 0.1	1.0 ± 0.2
Peak Acidification	2.0 ± 0.2	2.3 ± 0.2	2.4 ± 0.2
Peak Alkalinization	6.3 ± 0.8[a,b]	8.9 ± 0.5	4.7 ± 0.8[a,b]

[a] indicates statistical significance when compared to the equivalent parameter in response to a 5 min period of anoxia in a HCO_3^--free, HEPES-buffered medium containing 5.0 mM Glucose

[b] indicates statistical significance when compared to the equivalent parameter in a HCO_3^--free, HEPES-buffered medium containing 17.5 mM Glucose (see Table 4)

Table 11: The influence of the reduction of extracellular Na^+ on the anoxia-evoked changes in pH_i under HCO_3^--free, HEPES-buffered conditions in the presence of 5.0 mM glucose

	Ionic Substitution		
	Control	Low Na^+ (NMDG$^+$)	Low Na^+ (Li$^+$)
	(n=22)	(n=17)	(n=15)
Magnitude of (pH_i units):			
Acidic Shift	0.12 ± 0.01	0.09 ± 0.02	0.14 ± 0.02
Recovery	0.021 ± 0.005	0.02 ± 0.01	0.02 ± 0.01
Alkaline Shift	0.18 ± 0.02	0.10 ± 0.03^a	0.20 ± 0.02^b
Time to Reach (minutes):			
Start of Acidification	0.8 ± 0.1	1.5 ± 0.3^a	0.9 ± 0.1^b
Peak Acidification	2.3 ± 0.2	3.1 ± 0.2^a	1.8 ± 0.2
Peak Alkalinization	8.9 ± 0.5	6.2 ± 0.8^a	5.8 ± 0.6^a

[a] indicates statistical significance when compared to the equivalent parameter in a HCO_3^--free, HEPES-buffered medium containing 5.0 mM Glucose and normal extracellular Na^+.

[b] indicates statistical significance when compared to the equivalent parameter in a low Na^+, NMDG$^+$-substituted HCO_3^--free, HEPES buffered medium containing 5.0 mM Glucose.

Table 12: Magnitude and time course of anoxia-evoked changes in pH_i in the presence of agents that influence the activity of the cAMP/PKA pathway

	Control (*n*=28)	50 µM Rp-cAMPS (*n*=12)	10 µM NA/ISO (*n*=10)
Buffer:	HEPES	HEPES	HEPES
Magnitude of (pH_i units):			
Acidic Shift	0.16 ± 0.01	0.07 ± 0.01[a]	0.10 ± 0.02[a]
Recovery	0.05 ± 0.01	0.03 ± 0.01[a]	0.04 ± 0.02
Alkaline Shift	0.20 ± 0.02	0.11 ± 0.03[a]	0.27 ± 0.03[a]
Time to Reach (minutes):			
Start of Acidification	1.0 ± 0.1	1.1 ± 0.2	0.8 ± 0.2
Peak Acidification	2.5 ± 0.2	2.3 ± 0.3[a]	2.2 ± 0.5
Peak Alkalinization	9.5 ± 1.1	4.5 ± 0.5[a]	4.0 ± 0.5[a]

[a] indicates statistical significance when compared to the equivalent parameter after 5 min of anoxia in a HCO_3^--free, HEPES-buffered solution in the absence of any pharmacological treatment.
Abbreviations: NA, Noradrenaline; ISO, Isoproterenol

Table 13: The influence of various pharmacological treatments on the recovery of pH_i in the continued absence of extracellular Na^+

		Parameters Measured in Na^+-free Media			
	Resting pH_i	Drop in pH_i	Time to start of pH_i recovery	Time to reach a plateau	n
Control	7.29 ± 0.29	0.46 ± 0.03	4.5 ± 0.4	25.6 ± 2.1	36
2 μM Bafilomycin	7.30 ± 0.03	0.44 ± 0.04	3.0 ± 1.5	25.3 ± 3.8	8
0 Ca^{2+}	7.15 ± 0.06	0.46 ± 0.04	7.6 ± 1.7^a	46.6 ± 18.3^a	10
5 mM α-CHC	7.20 ± 0.10	0.46 ± 0.07	3.0 ± 0.5	11.2 ± 1.6^a	6
0 Cl^-	7.08 ± 0.15	0.42 ± 0.13	6.5 ± 1.1	21.7 ± 5.8	5
300 μM Eosin Y	7.08 ± 0.08^a	0.39 ± 0.06	5.7 ± 1.2	14.3 ± 1.5^a	10
5.0 mM Glucose	7.23 ± 0.14	0.41 ± 0.03	5.1 ± 3.0	16.1 ± 2.1^a	3
0 K^+	7.15 ± 0.08	0.66 ± 0.07^a	7.1 ± 0.8^a	26.9 ± 2.1	21
0 Mg^{2+}	7.2 ± 0.34	0.38 ± 0.04	4.7 ± 0.7	52.0 ± 13.2^a	11
50 μM Omeprazole	7.16 ± 0.3	0.32 ± 0.04	4.9 ± 0.7	27.5 ± 1.2	3
500 μM $ZnCl_2$	7.20 ± 0.07	0.79 ± 0.05^a	7.9 ± 0.6^a	28.4 ± 4.2	10

[a] indicates statistical significance when compared to the equivalent parameter observed in a Na^+-free, $NMDG^+$-substituted, HEPES-buffered solution in the absence of any pharmacological treatment

Figure 6. Distributions of steady-state pH$_i$ values for all neurons during perfusion with HCO$_3$$^-$/CO$_2$-free, HEPES-buffered and HCO$_3$$^-$/CO$_2$-buffered media at 37°C

A. The distribution of the resting pH$_i$ values for 694 adult rat hippocampal CA1 neurons during perfusion with HCO$_3$$^-$/CO$_2$-free, HEPES-buffered media at pH 7.35 (bin width = 0.05 pH units). The data were best fitted by a bimodal Gaussian distribution with means at pH$_i$ 6.93 ± 0.17 and 7.37 ± 0.14. **B.** The distribution of resting pH$_i$ values for 83 neurons during perfusion with HCO$_3$$^-$/CO$_2$-buffered media (bin width = 0.05 pH units) was fitted with a Gaussian distribution around a mean of 7.30 ± 0.17.

A.
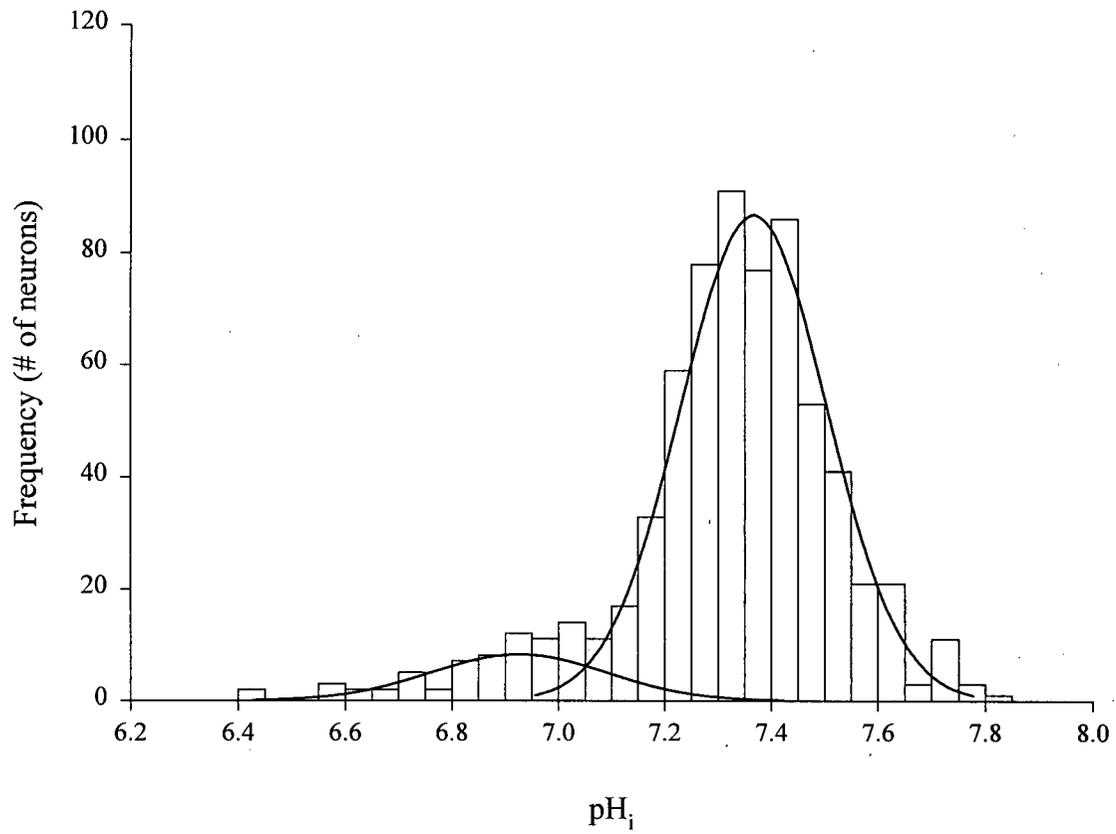
B.
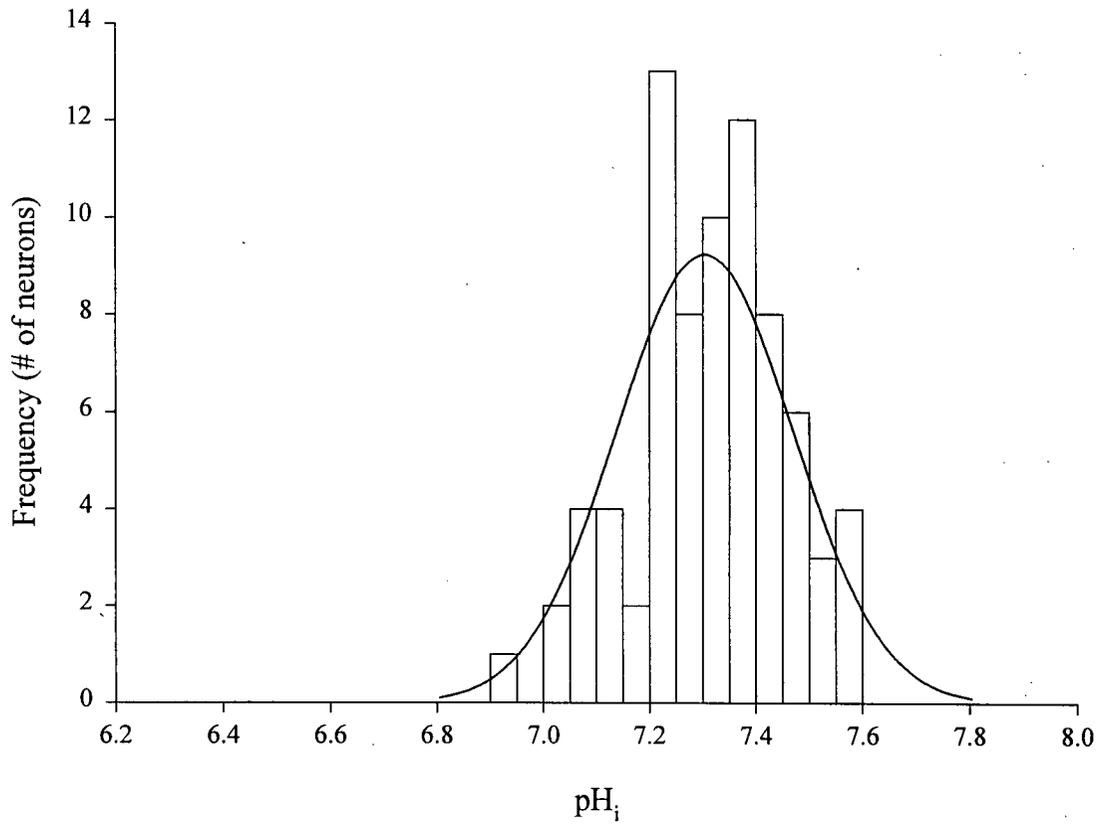
Figure 7. Effects of a 5 min period of anoxia on steady-state pH_i during perfusion with HCO_3^-/CO_2-free, HEPES-buffered medium

A. Upon exposure to a 5 min period of anoxia, the initial resting pH_i value of ~ 7.26 fell ~ 0.14 pH units, reaching its maximum ~ 1 min following the induction of anoxia. pH_i recovered ~ 0.11 pH units (79%) in the continued absence of oxygen. Upon the return to normoxia, there was an intracellular alkalinization of ~ 0.24 pH units above the initial resting pH_i value, which reached its maximum ~ 7 min following the return to normoxia. **B.** The stability of the corresponding BI_{452} value, during the experiment depicted in Fig. 7A, indicates that the membrane integrity of the neuron was not compromised (see Materials and Methods, Section 3.2).

A.
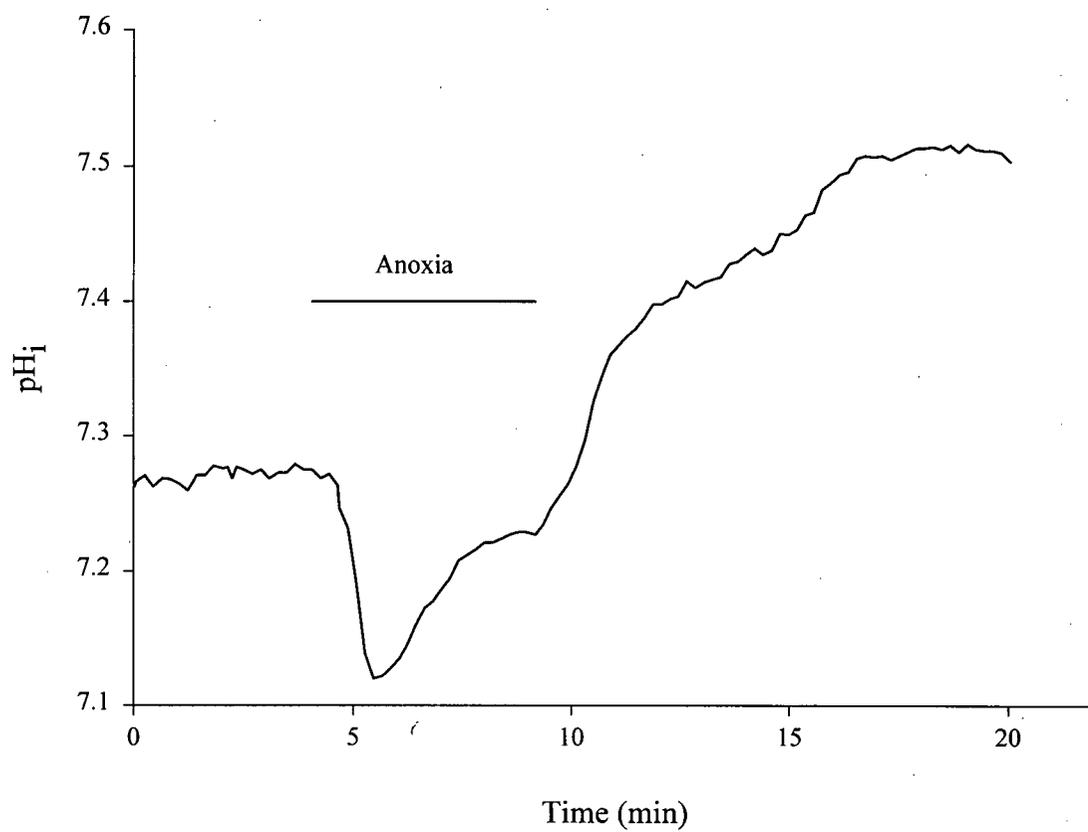
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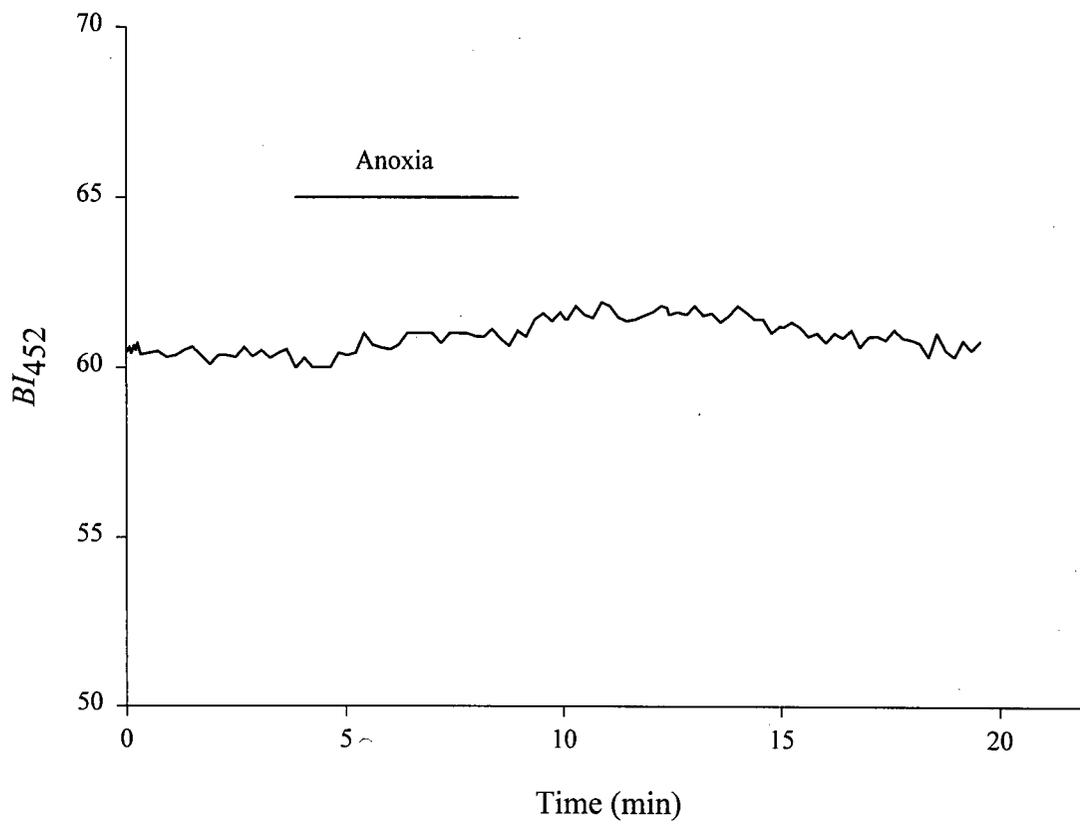
Figure 8. Effects of a 3 and 10 min period of anoxia on steady-state pH_i during perfusion with HCO_3^-/CO_2-free, HEPES-buffered medium

A. Upon exposure to a 3 min period of anoxia, the initial resting pH_i value of ~ 7.26 fell ~ 0.10 pH units, reaching its maximum ~ 1.7 min following the induction of anoxia. pH_i recovered by ~ 0.02 pH units (20%) in the continued absence of oxygen. Upon the return to normoxia, there was an intracellular alkalinization of ~ 0.16 pH units above the initial resting pH_i value, which reached its maximum at ~ 8.7 min following the return to normoxia. **B.** Upon exposure to a 10 min period of anoxia, the initial resting pH_i value of ~ 7.10 fell ~ 0.18 pH units, reaching its maximum ~ 4.4 min following the induction of anoxia. pH_i recovered ~ 0.19 pH units (106%) in the continued absence of oxygen. Upon the return to normoxia, there was an intracellular alkalinization of ~ 0.38 pH units above the initial resting pH_i value, which reached its maximum at ~ 9.1 min following the return to normoxia. Each trace represents data collected from a different neuron.

A.

B.
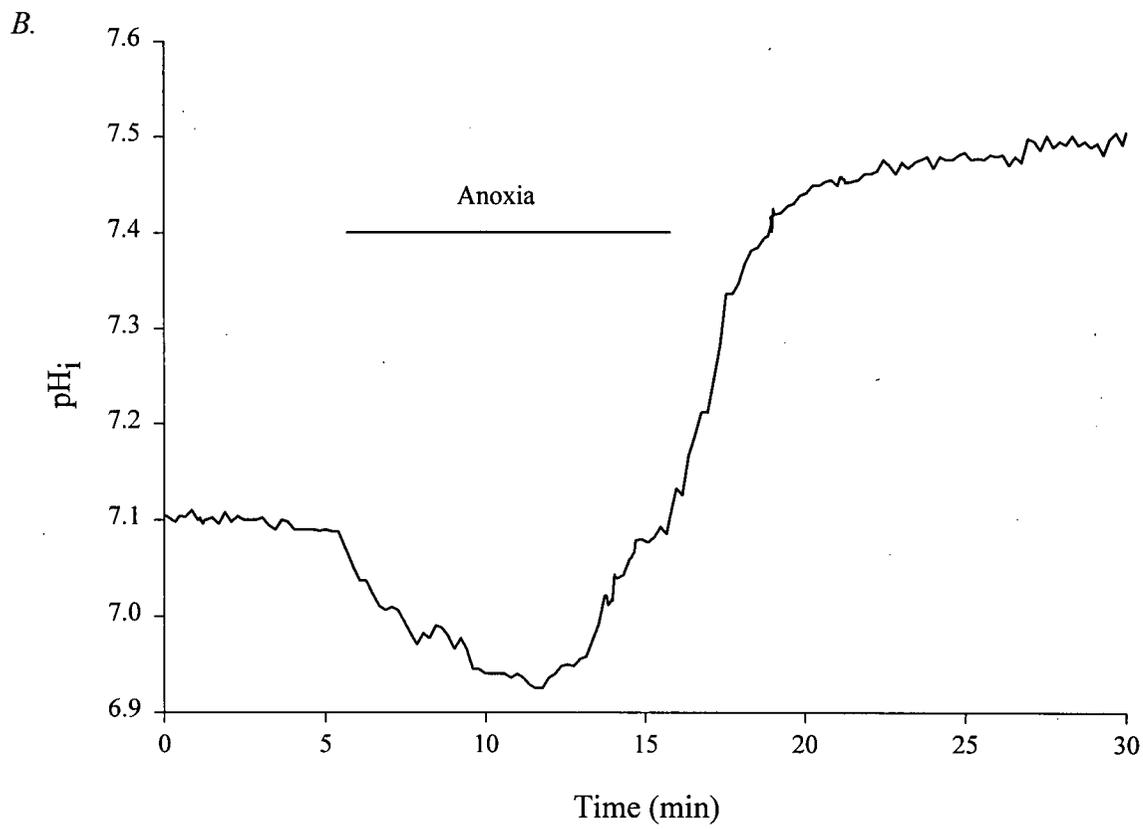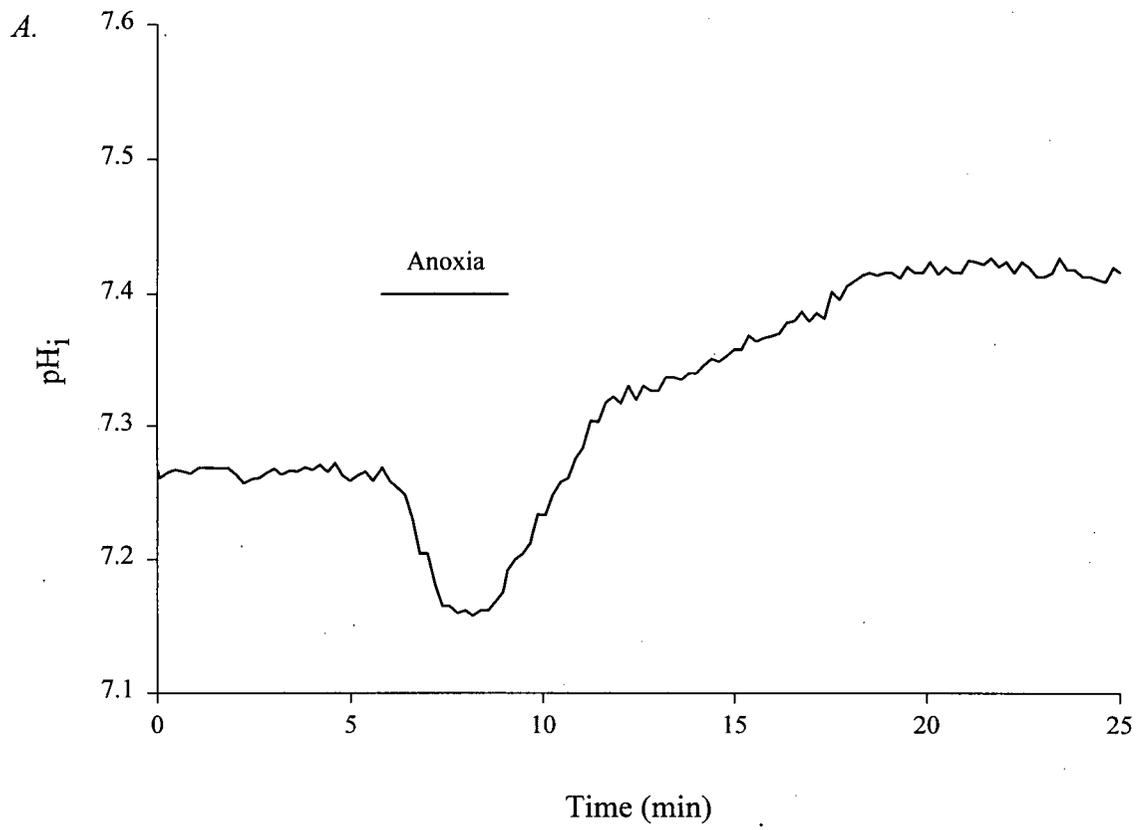
Figure 9. Effects of a 3, 5 and 10 min period of anoxia on steady-state pH_i during perfusion with HCO_3^-/CO_2-buffered medium

A. Upon exposure to a 3 min period of anoxia, the initial resting pH_i value of ~ 7.35 fell ~ 0.14 pH units, reaching its maximum at ~ 1.8 min following the induction of anoxia. pH_i did not rise during the anoxic period. Upon the return to normoxia, there was an intracellular alkalinization of ~ 0.12 pH units above the initial resting pH_i value, which reached its maximum at ~ 7.6 min following the return to normoxia. **B.** Upon exposure to a 5 min period of anoxia, the initial resting pH_i value of ~ 7.14 fell ~ 0.14 pH units, reaching its maximum at ~ 1.9 min following the induction of anoxia. pH_i recovered by ~ 0.08 pH units (57%) in the continued absence of oxygen. Upon the return to normoxia, there was an intracellular alkalinization of ~ 0.22 pH units above the initial resting pH_i value, which reached its maximum at ~ 7.5 min following the return to normoxia. **C.** Upon exposure to a 10 min period of anoxia, the initial resting pH_i value of ~ 7.30 fell ~ 0.15 pH units, reaching its maximum at ~ 1.2 min following the induction of anoxia. pH_i recovered ~ 0.15 pH units (100%) in the continued absence of oxygen. Upon the return to normoxia, there was an intracellular alkalinization of ~ 0.21 pH units above the initial resting pH_i value, which reached its maximum at ~ 4.5 min following the return to normoxia. Each trace represents data collected from a different neuron.
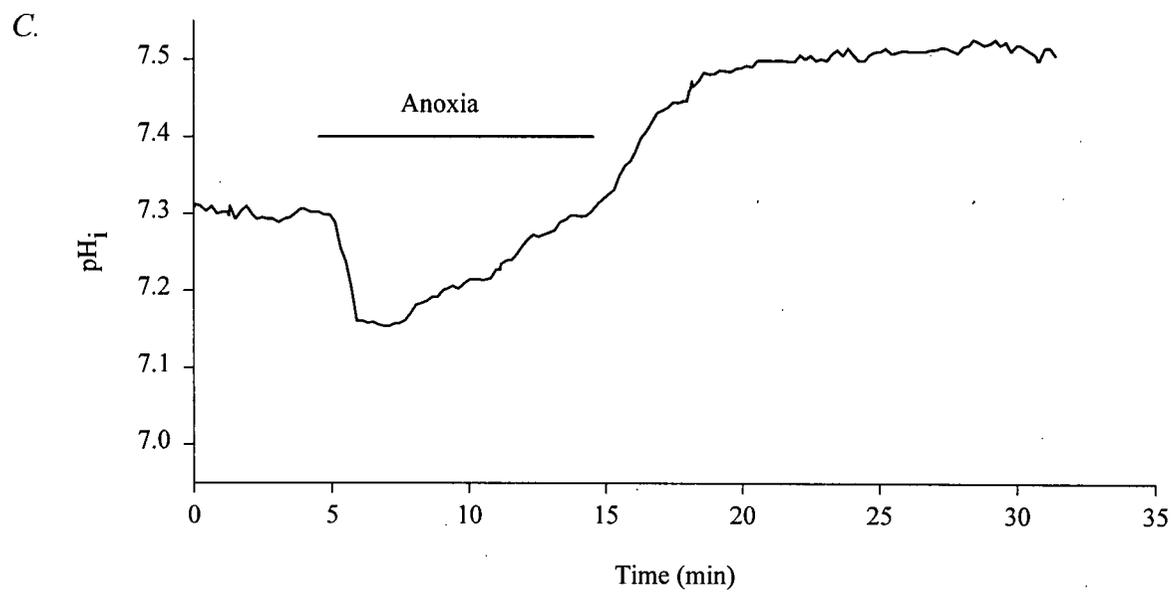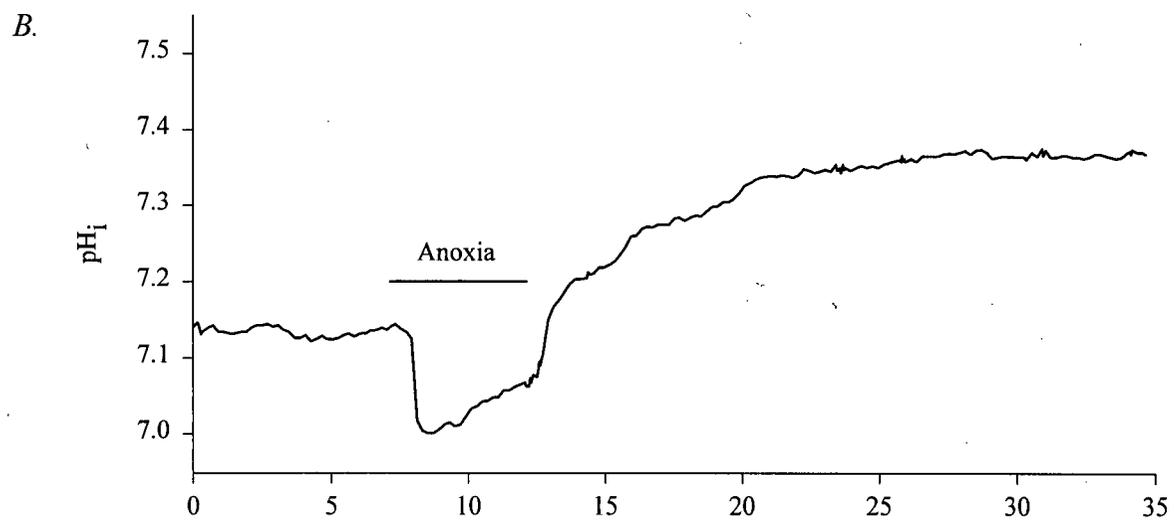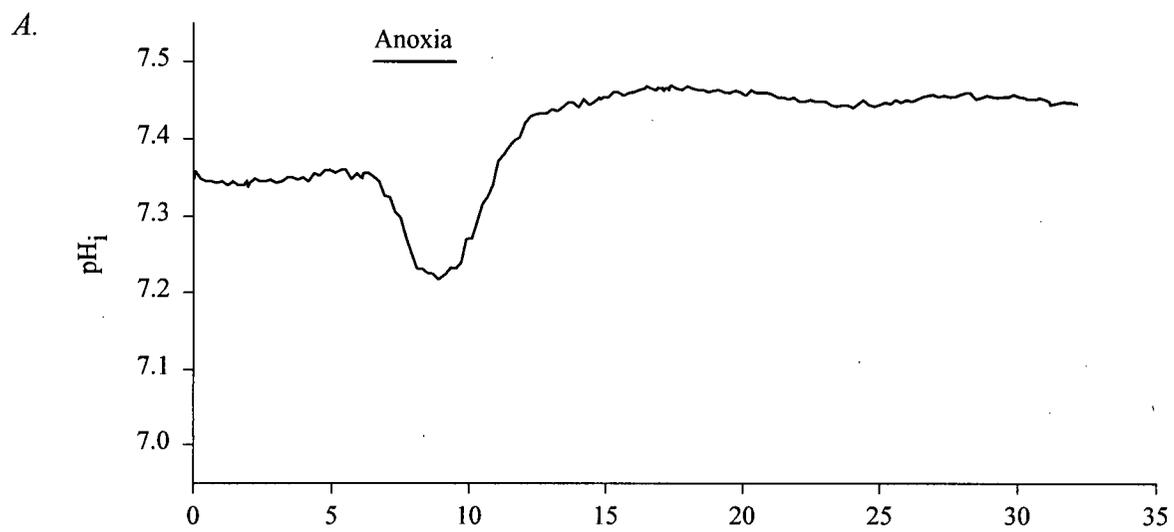
Figure 10. Effects of a 5 min period of anoxia on BI_{334}/BI_{380} ratio values

The experiment was performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. The resting BI_{334}/BI_{380} ratio value (the ratio of the background-corrected I_{334} and I_{380}) was ~ 0.5. Approximately 2 min following the start of a 5 min period of anoxia, the ratio value increased to ~ 2.5, reaching its maximum value ~ 3.5 min following the induction of anoxia. Upon the return to normoxia, the BI_{334}/BI_{380} ratio value remained elevated.
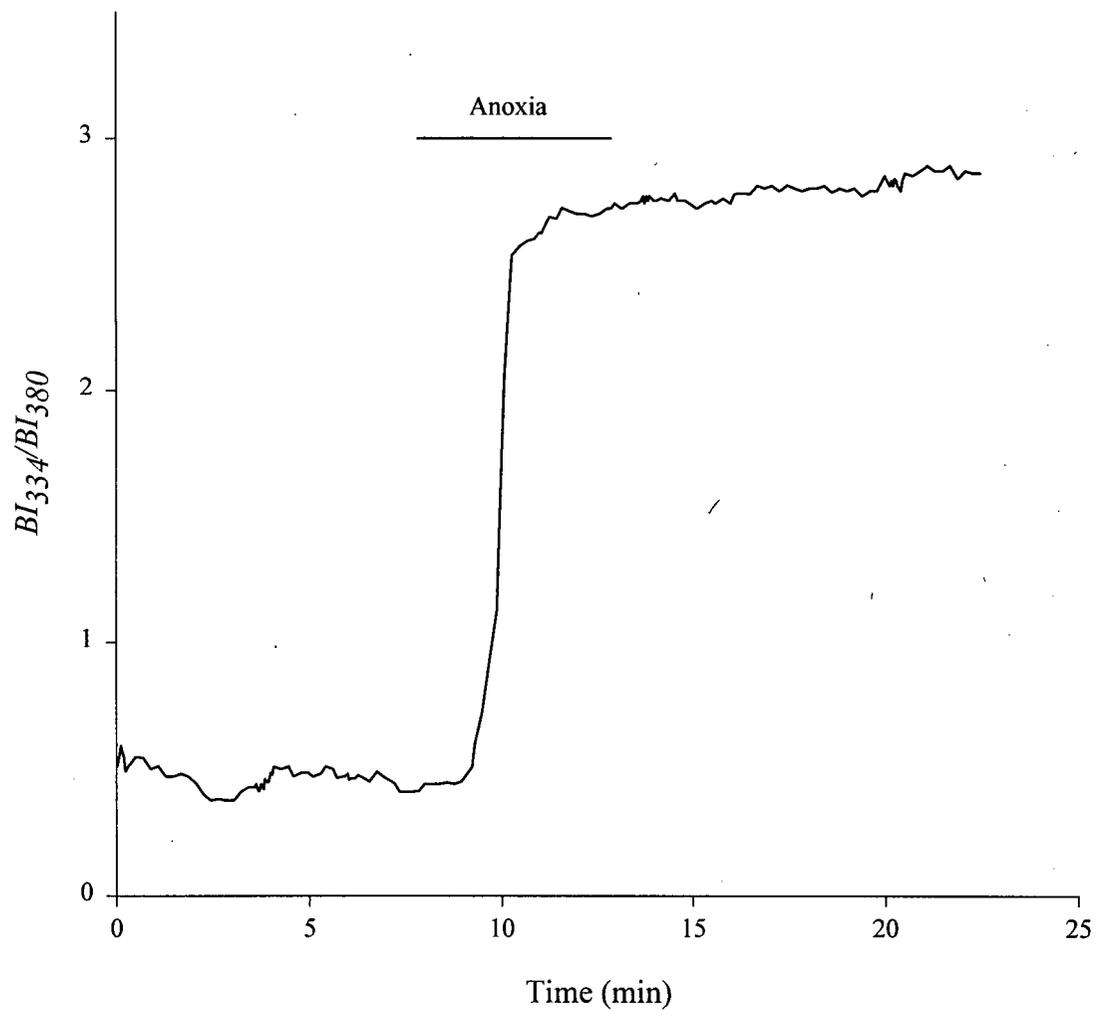
Figure 11. Effects of a 5 min exposure to HCO_3^-/CO_2-buffered medium equilibrated with 95% Ar/ 5% CO_2 for > 18 hours on steady-state pH_i

Upon exposure to a 5 min period of anoxia, the initial resting pH_i value of ~ 7.38 fell ~ 0.13 pH units, reaching its maximum ~ 2 min following the induction of anoxia. pH_i recovered by ~ 0.07 pH units (54%) in the continued absence of oxygen. Upon the return to normoxia, there was an intracellular alkalinization of ~ 0.24 pH units above the initial resting pH_i value, which reached its maximum ~ 5.4 min following the return to normoxia. The pH_i response to anoxia induced by equilibrating HCO_3^-/CO_2-buffered media with 95% Ar/5% CO_2 for > 18 hours was not statistically different from the pH_i response to anoxia induced with media containing 2 mM $Na_2S_2O_4$ (compare with Figure 9B).
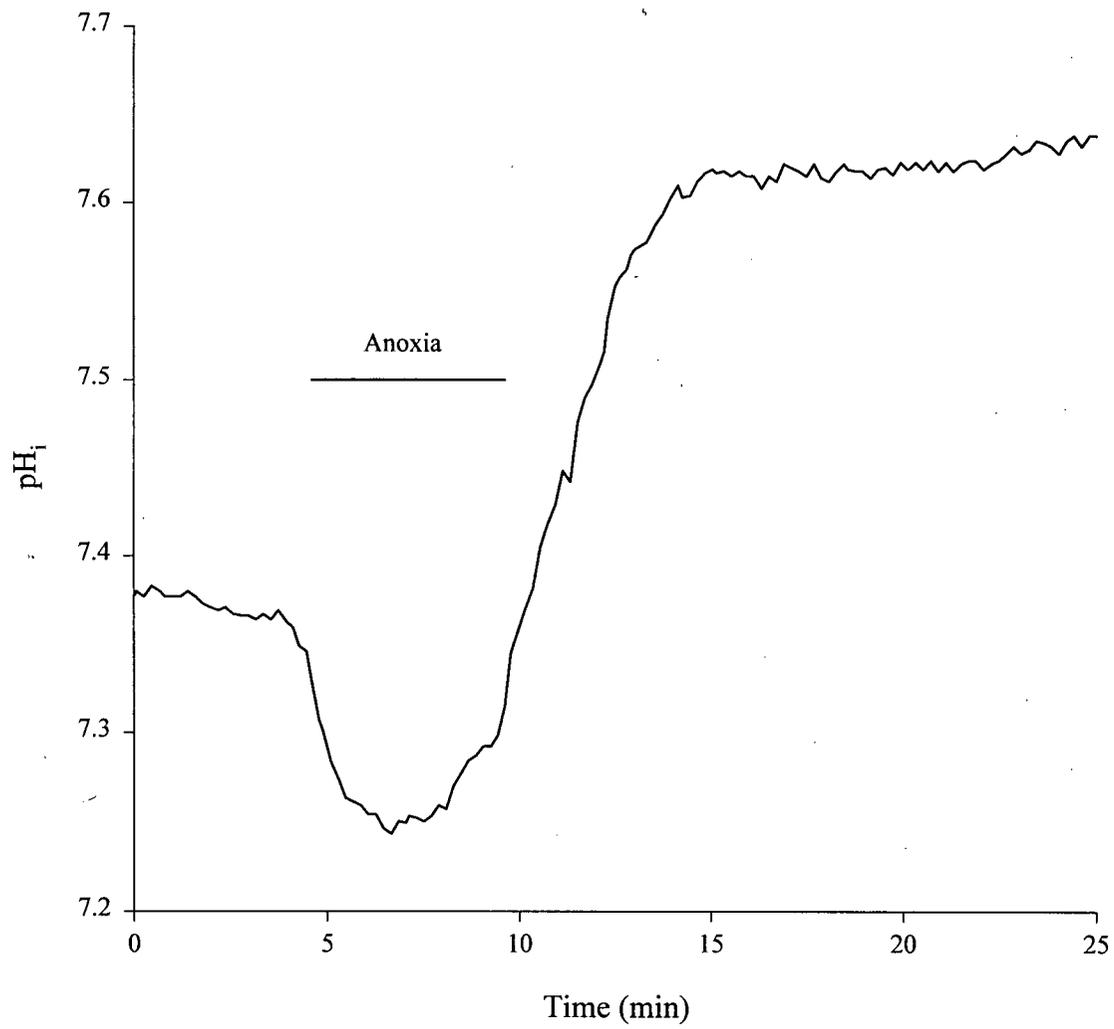
Figure 12. Effects of the removal of extracellular Ca^{2+} on the pH_i response to a 5 min period of anoxia

Experiments were performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. **A.** Upon exposure to a Ca^{2+}-free solution containing 200 μM EGTA, steady-state pH_i increased ~ 0.11 pH units and reached a plateau after ~ 7.2 min. pH_i remained stable during the following 25 min. **B.** Upon exposure to a Ca^{2+}-free solution containing 200 μM EGTA, steady-state pH_i increased. Once a new steady-state pH_i of ~ 7.24 was reached, a 5 min period of anoxia induced an intracellular acidification that recovered slightly in the continued absence of oxygen. Upon the return to normoxia, there was an intracellular alkalinization that rose above the pre-anoxic steady-state pH_i. The magnitude and the time course of the pH_i response to a 5 min period of anoxia was not influenced by the presence or absence of extracellular Ca^{2+} (compare with Fig. 7A, the same experiment performed in the presence of extracellular Ca^{2+}). Each trace represents data collected from a different neuron.

A.
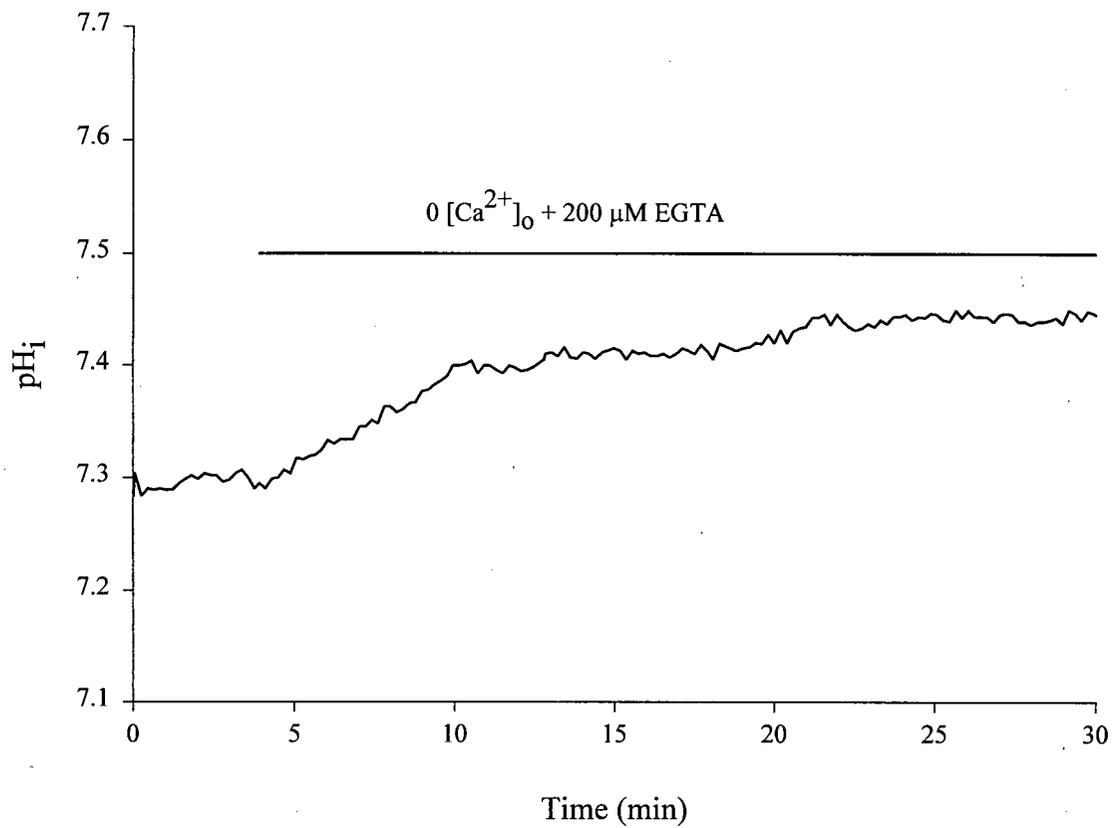
B.
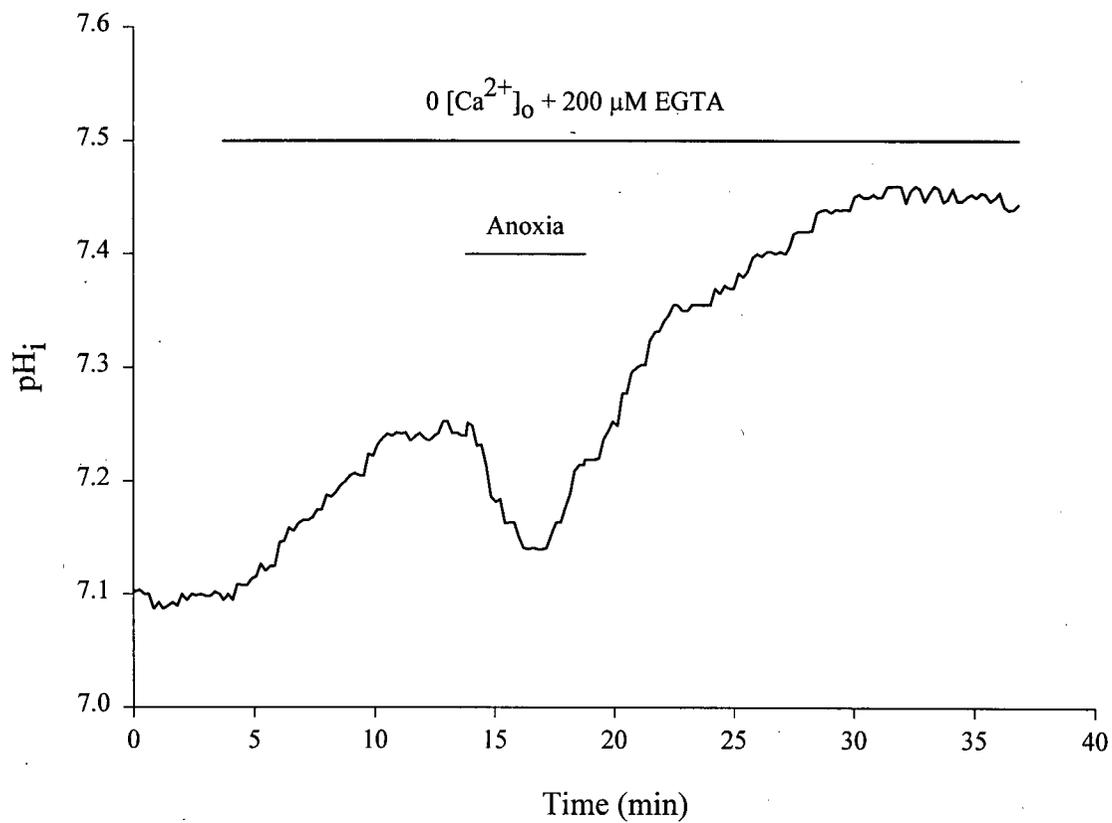
Figure 13. Effects of the removal of extracellular Ca^{2+} on the Ca^{2+} response to a 5 min period of anoxia

The experiment was performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. The resting BI_{334}/BI_{380} ratio value (the ratio of the background-corrected I_{334} and I_{380}) was ~ 0.45. Upon exposure to a Ca^{2+}-free solution containing 200 µM EGTA, the BI_{334}/BI_{380} ratio value decreased. During a 5 min period of anoxia, there was a small increase in the BI_{334}/BI_{380} ratio value. Upon the return to normoxia, this cell displayed a further post-anoxic rise in the BI_{334}/BI_{380} ratio value. Thus, the Ca^{2+} response to a 5 min period of anoxia is dependent on the presence of extracellular Ca^{2+} (compare with Fig. 10, the same experiment performed in the presence of extracellular Ca^{2+}).
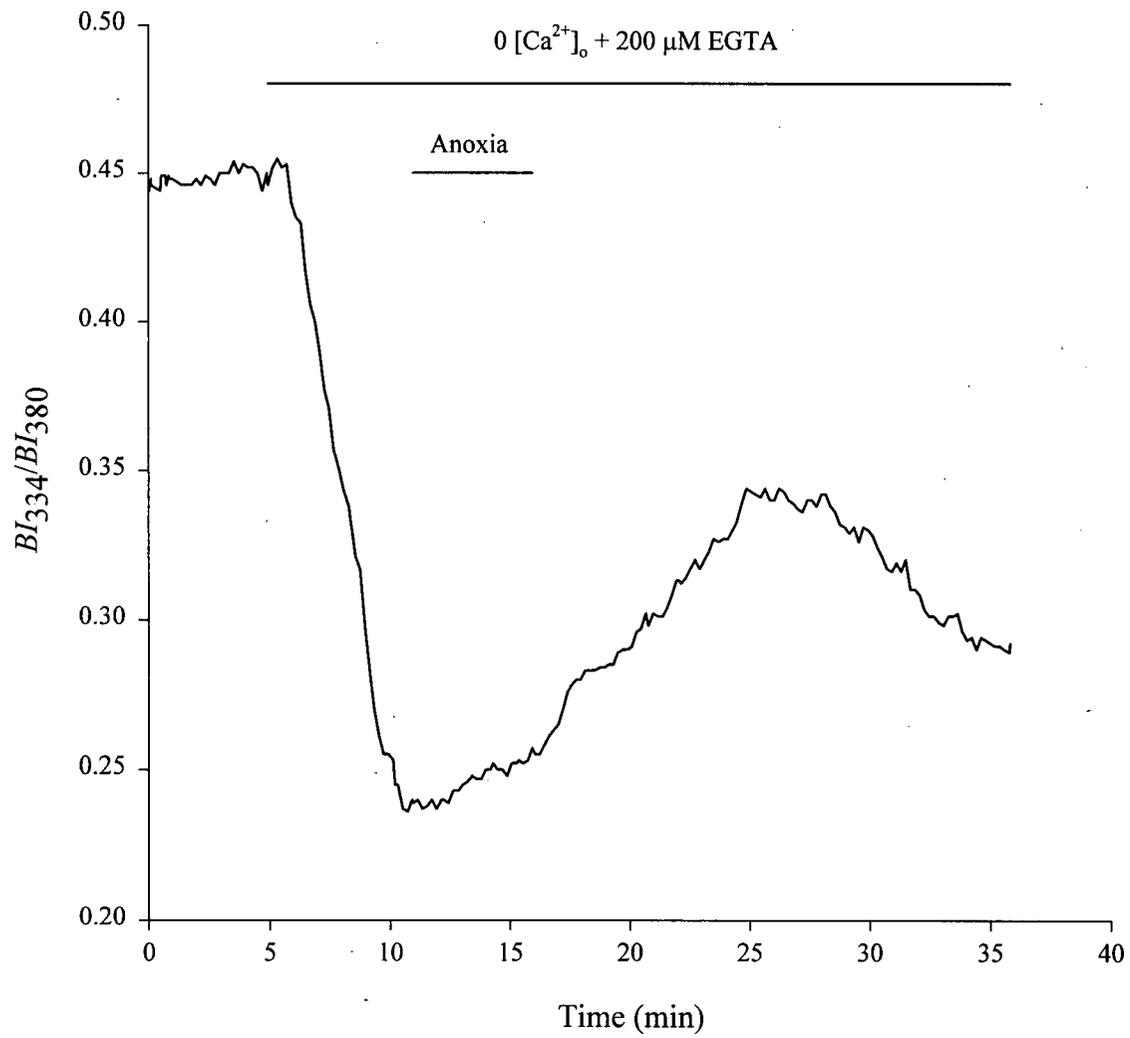
Figure 14. Effects of exposure to reduced Na$^+$, NMDG$^+$-substituted medium on the pH$_i$ response to a 5 min period of anoxia

Experiments were performed under HCO$_3^-$/CO$_2$-free, HEPES-buffered conditions. **A.** With a steady-state pH$_i$ of ~ 7.37, exposure to a 2 mM Na$^+$, NMDG$^+$-substituted medium caused a fall in pH$_i$. During the continued perfusion with this reduced-Na$^+$ medium, pH$_i$ gradually recovered and, over ~ 22 min, reached a new steady-state pH$_i$ value that approximated the pH$_i$ value observed prior to exposure to reduced-Na$^+$ medium. **B.** With a steady-state pH$_i$ of ~ 7.22, exposure to a 2 mM Na$^+$, NMDG$^+$-substituted medium caused a fall in pH$_i$. pH$_i$ then recovered to ~ 7.40. A 5 min period of anoxia induced an intracellular acidification of ~ 0.10 pH units, the magnitude of which was smaller than observed in the presence of normal external Na$^+$. pH$_i$ rose slightly in the continued absence of oxygen. Upon the return to normoxia, there was an intracellular alkalinization of ~ 0.11 pH units, a magnitude which was smaller than observed in the presence of normal extracellular Na$^+$ (compare with Fig. 7A, the same experiment performed in the presence of normal extracellular Na$^+$; Table 6). Each trace represents data collected from a different neuron.

A.
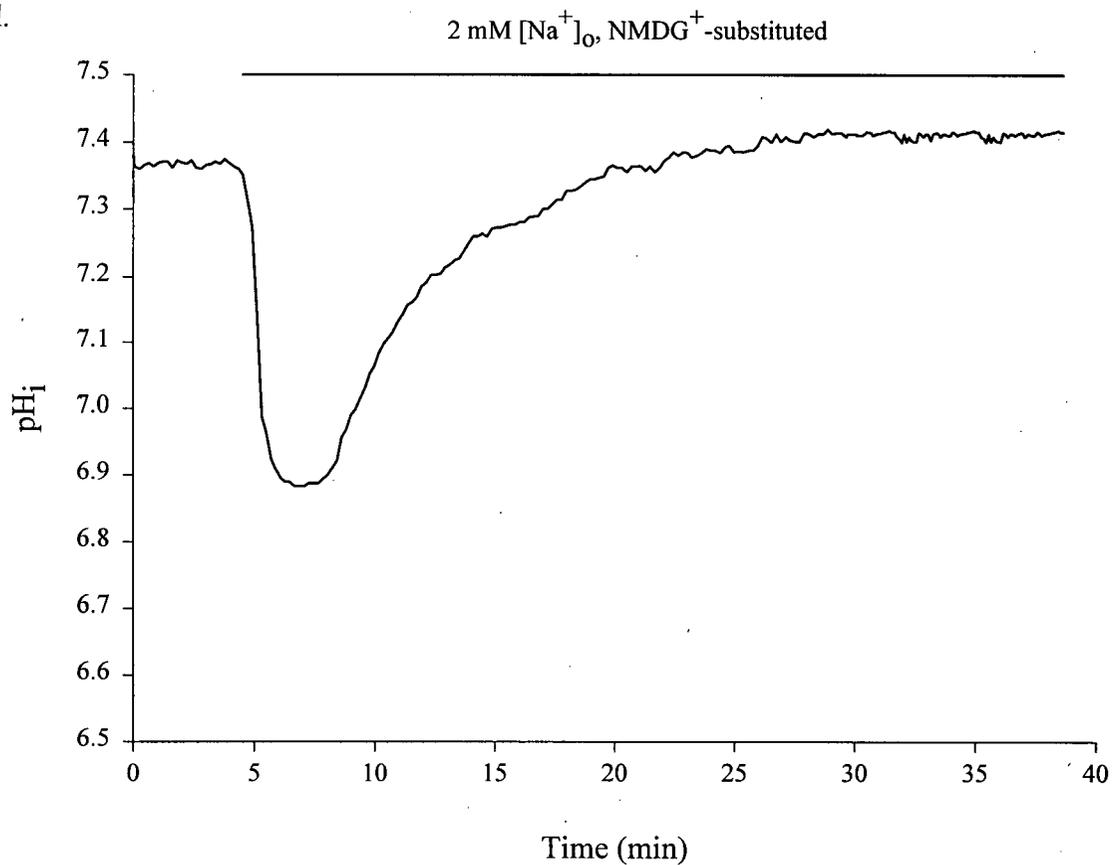
B.
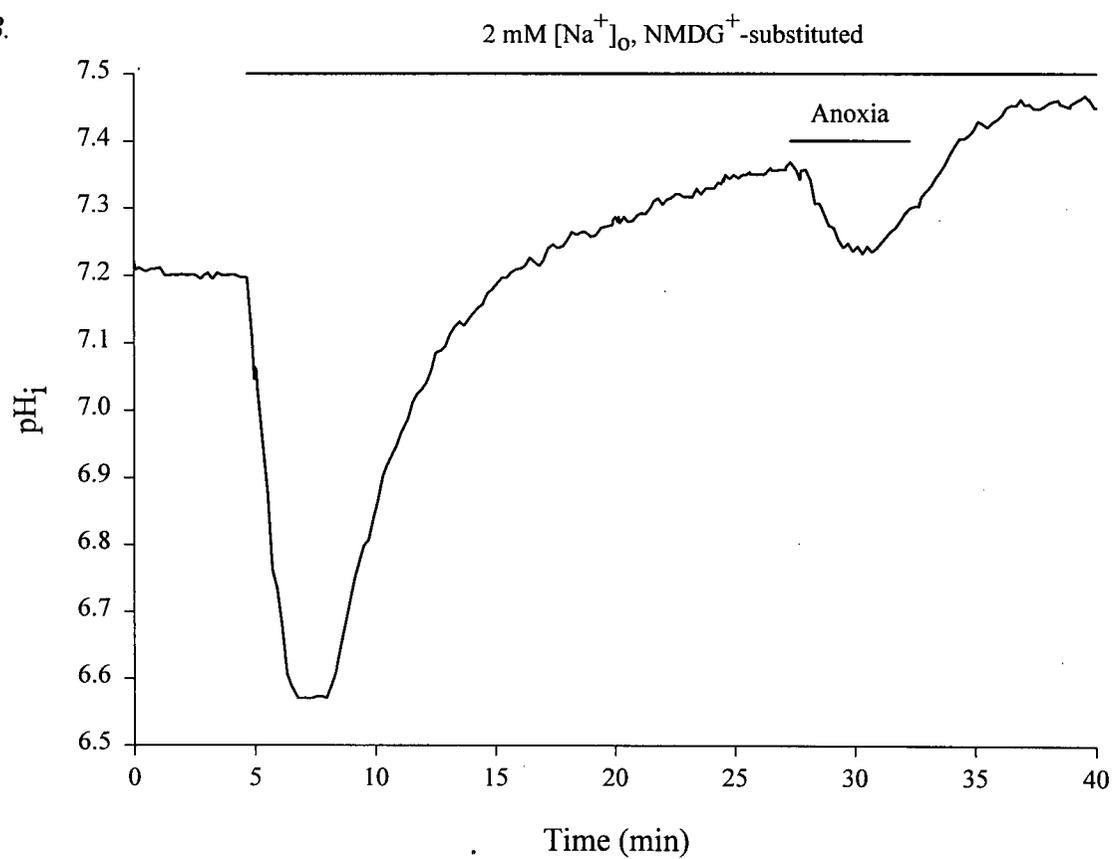
Figure 15. Effects of exposure to reduced Na$^+$, Li$^+$-substituted medium on the pH$_i$ response to a 5 min period of anoxia

The experiment was performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. Exposure to a 2 mM Na$^+$, Li$^+$-substituted medium caused pH$_i$ to fall. Over ~ 12 min, pH$_i$ recovered and reached a new steady-state pH$_i$ value. A 5 min period of anoxia then induced an intracellular acidification that recovered slightly in the continued absence of oxygen. Upon the return to normoxia, there was an intracellular alkalinization. Under these conditions, the magnitudes of all three phases of the pH$_i$ response to anoxia were not different than those observed in the presence of normal external Na$^+$ (compare with Fig. 7A, the same experiment performed in the presence of normal external Na$^+$; Table 6). In contrast, the magnitudes of the per-anoxic acidification and per-anoxic alkalinization were significantly larger than those observed in the presence of reduced Na$^+$, NMDG$^+$-substituted media (compare with Fig. 14, the same experiment performed under reduced-Na$^+$, NMDG$^+$-substituted conditions; Table 6).
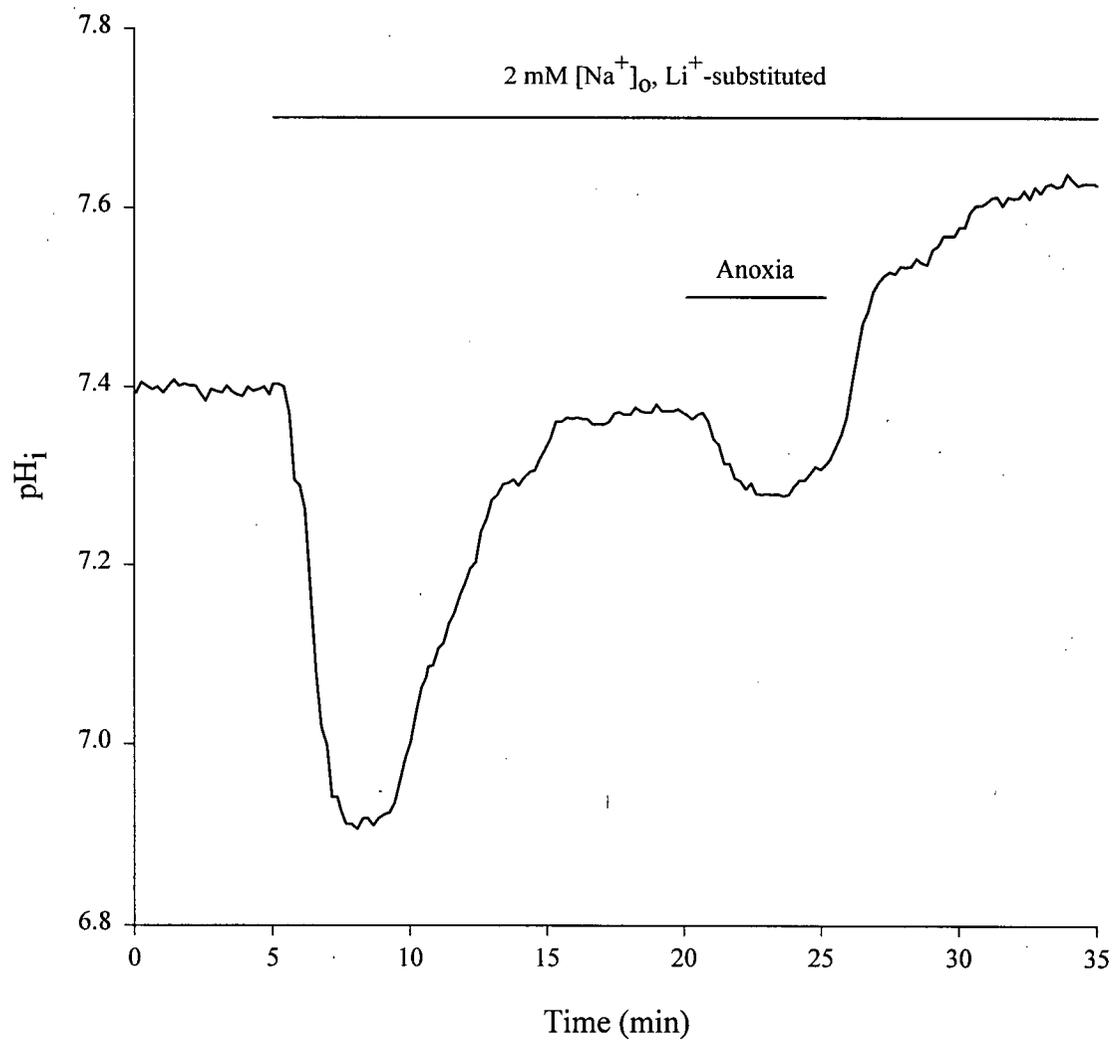
Figure 16. Effect of an anoxic period on the rate of pH_i recovery from an intracellular acid load imposed during an anoxic period

The experiment was performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. An initial acid load and subsequent recovery of pH_i was conducted under control conditions. A second acid load was then performed during an anoxic period. The rate at which pH_i recovered after the second intracellular acid load was decreased when compared to the control rate of pH_i recovery.
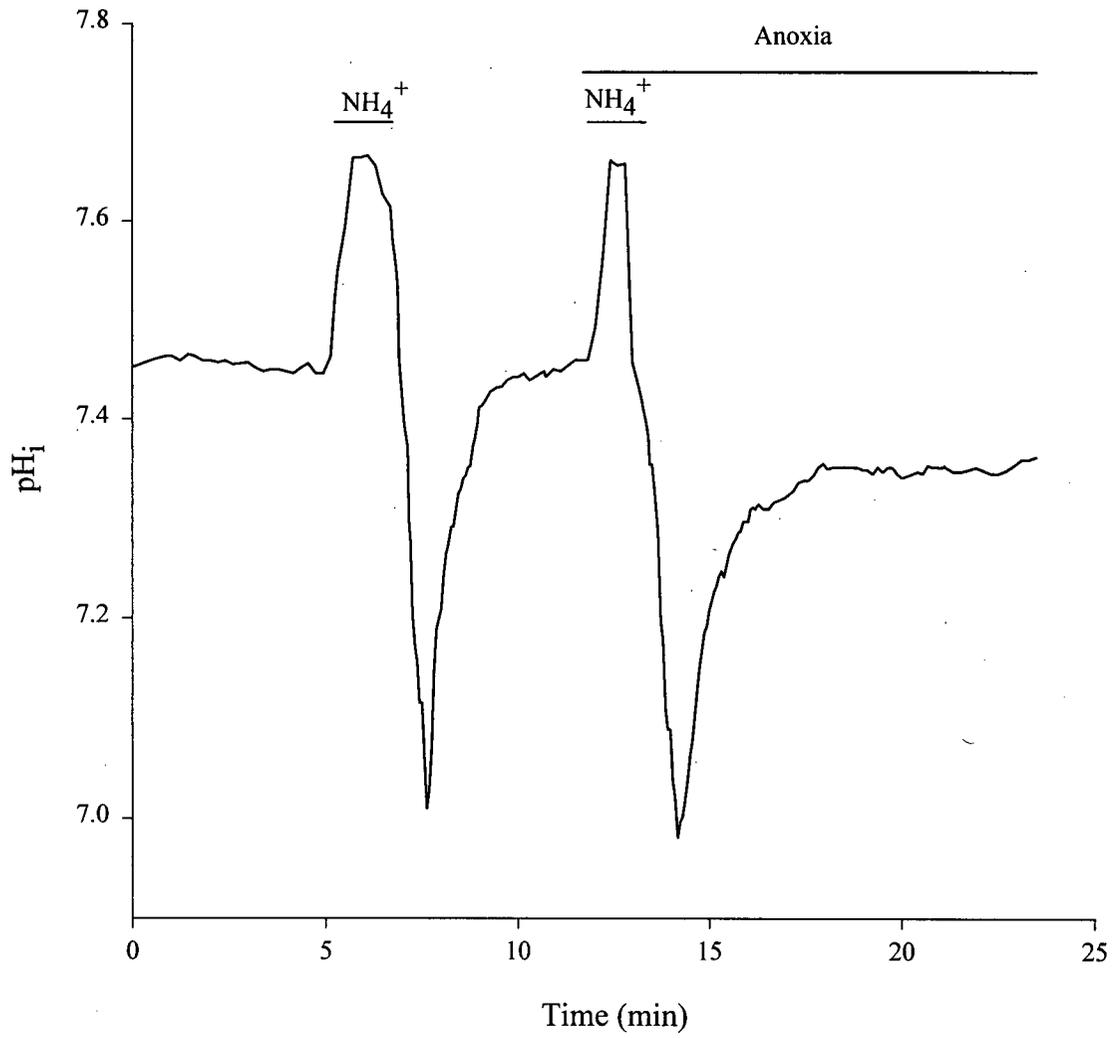
Figure 17. Comparison of the rates of pH_i recovery from intracellular acid loads imposed prior to and during an anoxic period

Experiments were performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. Plotted are the mean rates of pH_i recoveries from intracellular acid loads conducted prior to (●) and during (○) an anoxic period, at pH_i values denoted on the x-axis. The rates of pH_i recovery were evaluated at 0.05 unit intervals of pH_i and error bars represent S.E.M. Continuous lines represent the least squares linear regression fits to the data points indicated for each experimental condition. Data were obtained from 8 paired experiments of the type shown in Fig. 16. Except at pH_i 6.95, at every absolute level of pH_i, the rates of pH_i recovery were significantly slowed during an anoxic period as compared to the control rates of pH_i recovery ($p<0.05$ at each absolute value of pH_i, except at pH_i 6.95).
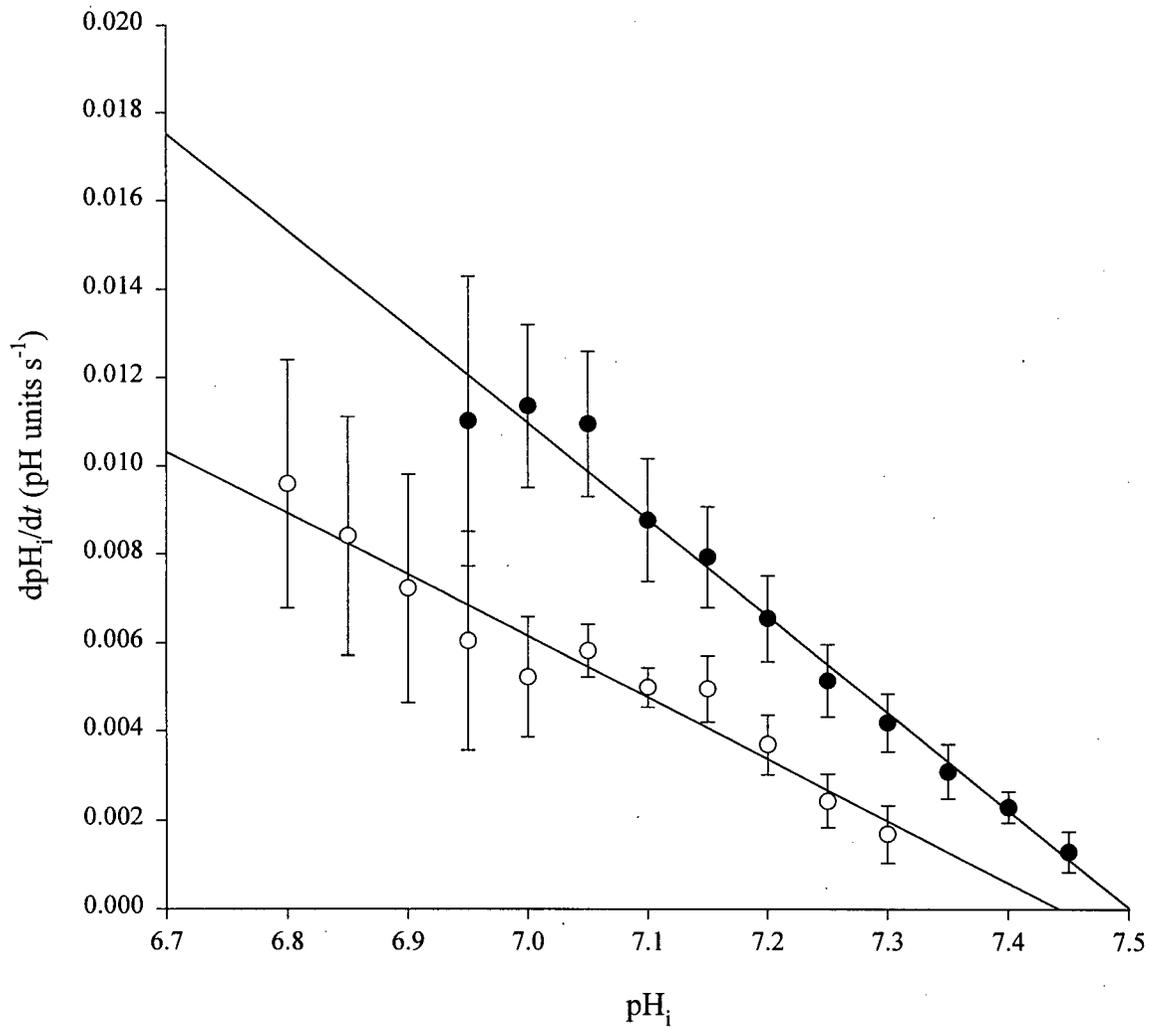
Figure 18. Effect of a transient anoxic period on the rate of pH$_i$ recovery from an intracellular acid load

The experiment was performed under HCO$_3^-$/CO$_2$-free, HEPES-buffered conditions. An initial acid load and subsequent recovery of pH$_i$ was conducted under control conditions. A second acid load was then performed following a 5 min period of anoxia, at a time when steady-state pH$_i$ was increased. The rate at which pH$_i$ recovered after the second intracellular acid load was increased when compared to the control rate of pH$_i$ recovery.
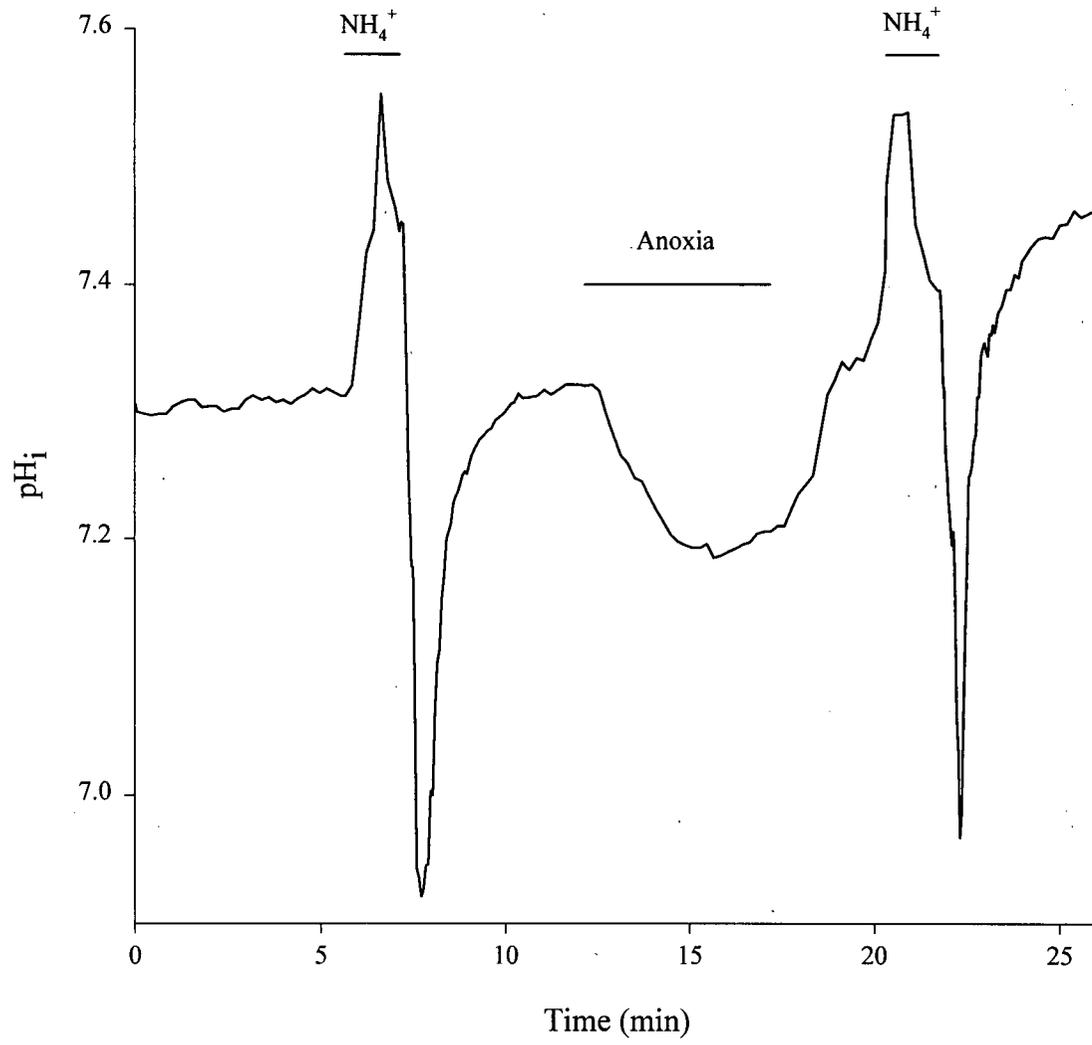
Figure 19. Comparison of the rates of pH_i recovery from intracellular acid loads imposed prior to and following a 5 min period of anoxia

Experiments were performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. Plotted are the mean rates of pH_i recoveries from intracellular acid loads conducted prior to (●) and following (○) a 5 min period of anoxia, at pH_i values denoted on the x-axis. The rates of pH_i recovery were evaluated at 0.05 unit intervals of pH_i and error bars represent S.E.M. Continuous lines represent the least squares linear regression fits to the data points indicated for each experimental condition. Data were obtained from 17 paired experiments of the type shown in Fig. 18. At each absolute level of pH_i, the rates of pH_i recovery were significantly faster following an anoxic period as compared to the control rates of pH_i recovery ($p < 0.05$ at each absolute value of pH_i).
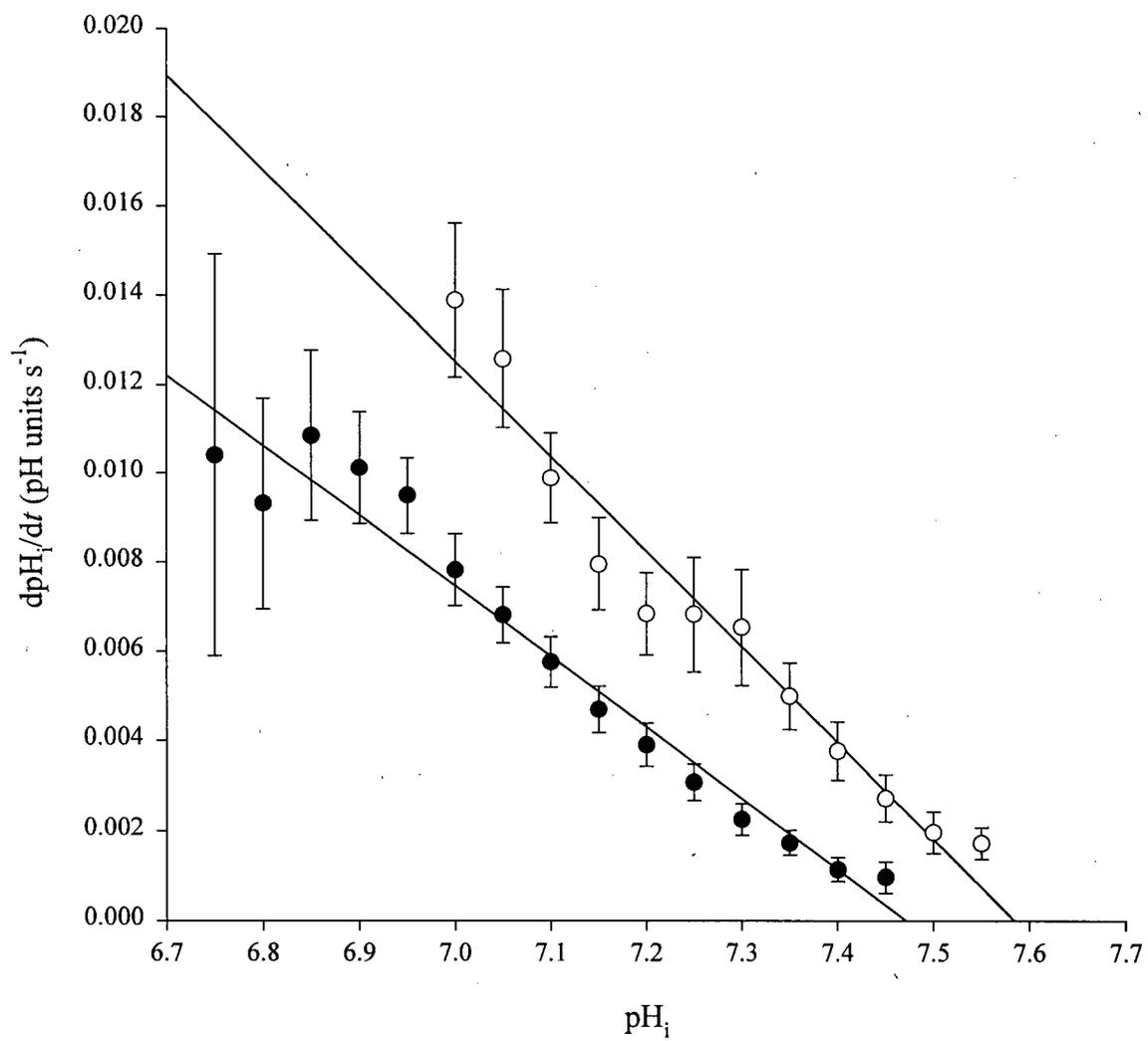
Figure 20. Effects of 0.5 µM tetrodotoxin (TTX) on the steady-state pH_i response to a 5 min period of anoxia

The experiment was performed under HCO_3^-/CO_2-free, HEPES-buffered conditions in the presence of 0.5 µM TTX. Upon exposure to a 5 min period of anoxia, the initial resting pH_i value decreased by ~ 0.07 pH units and recovered by ~ 0.03 pH units in the continued absence of oxygen. The absolute magnitudes of both of these phases were smaller than observed in the absence of TTX. Upon the return to normoxia, there was an intracellular alkalinization of ~ 0.21 pH units above the initial resting pH_i value. The magnitude of the post-anoxic alkalinization was not statistically different than observed in the absence of TTX (compare with Fig. 7A, the same experiment performed in the absence of TTX; Table 7).
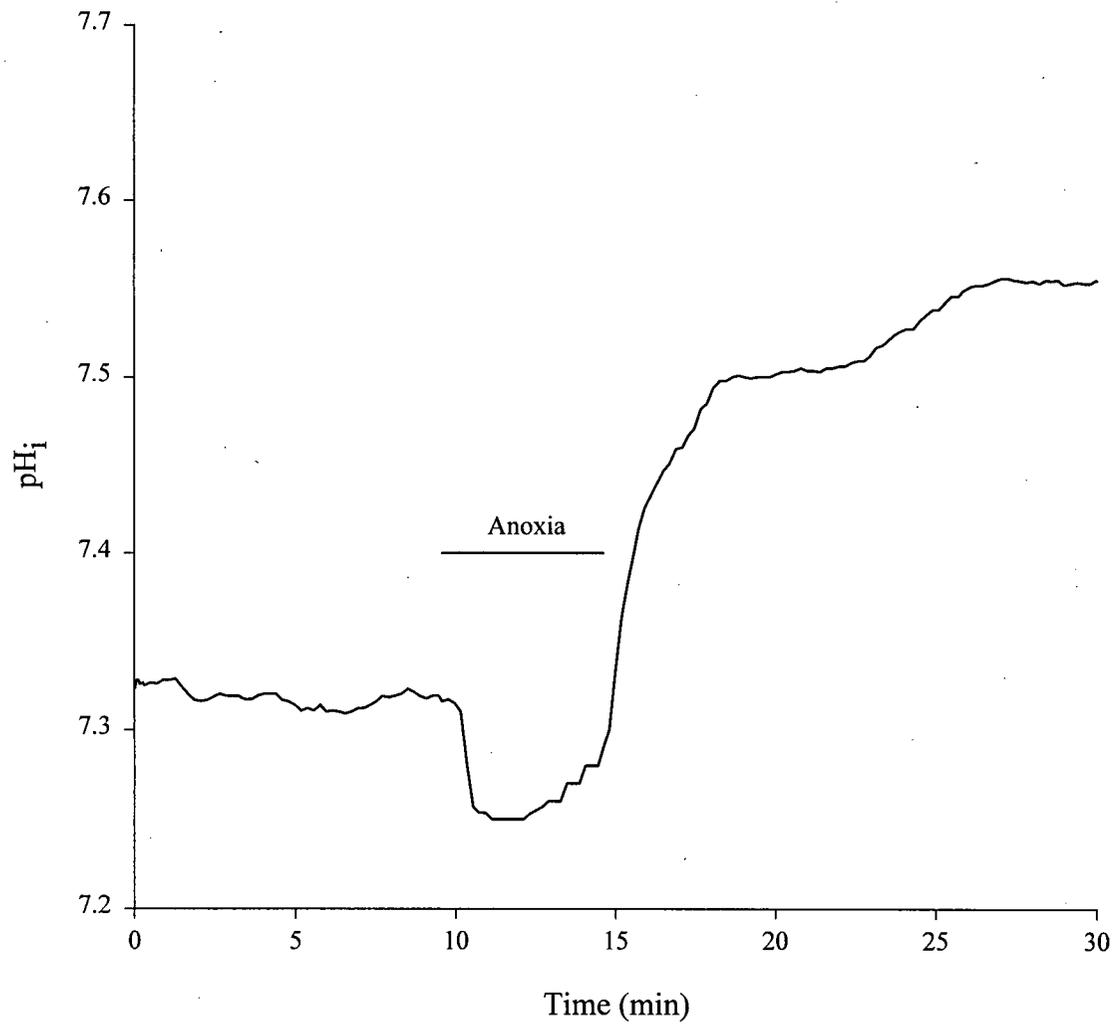
Figure 21. Effect of 0.5 μM TTX on the rate of pH_i recovery from intracellular acid loads imposed prior to and during the anoxic period

The experiment was performed under HCO_3^-/CO_2-free, HEPES-buffered conditions and 0.5 μM TTX was present throughout the course of the experiment. An initial acid load and subsequent recovery of pH_i was conducted under normoxic conditions. A second acid load was then performed during an anoxic period at a time when steady-state pH_i was decreased. The rate at which pH_i recovered after the second intracellular acid load was decreased when compared to the control rate of pH_i recovery at the same absolute values of pH_i (compare with Fig. 16, the same experiment performed in the absence of TTX).
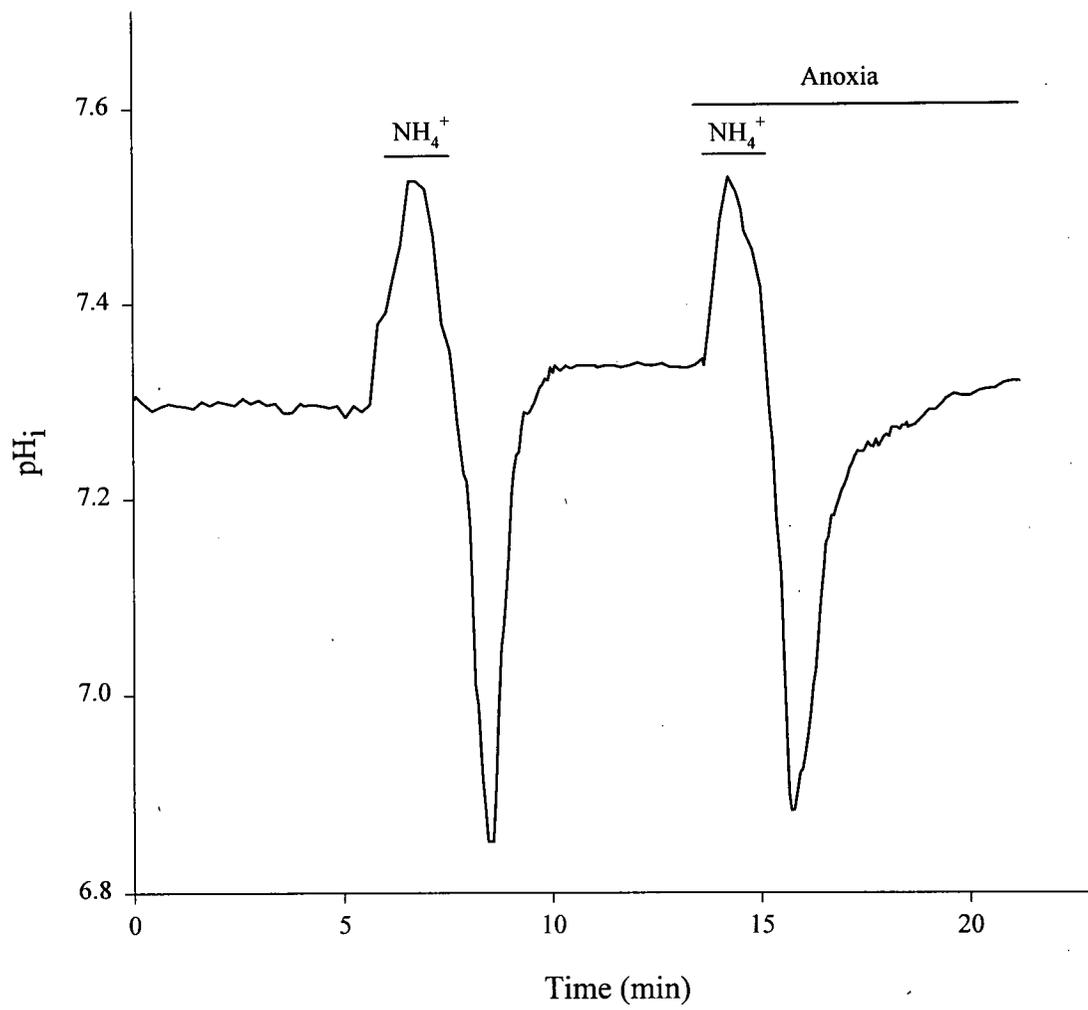
Figure 22. Comparison of the rates of pH_i recovery from intracellular acid loads imposed prior to and during an anoxic period in the presence or absence of TTX

Experiments were performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. Plotted are the mean rates of pH_i recoveries from intracellular acid loads conducted prior to (●) and during (○) an anoxic period in the presence of 0.5 μM TTX, at pH_i values denoted on the x-axis. In addition, plotted are the mean rates of pH_i recoveries from intracellular acid loads conducted prior to (■) and during (□) an anoxic period in the absence of TTX (see Fig. 17). The rates of pH_i recovery were evaluated at 0.05 unit intervals of pH_i and error bars represent S.E.M. Continuous lines represent the least squares linear regression fits to the data points indicated for each experimental condition. Data in the presence of TTX were obtained from 7 paired experiments of the type shown in Fig. 21. Both prior to and during an anoxic period, at each absolute level of pH_i, the rates of pH_i recovery were not statistically different in the presence or absence of TTX.
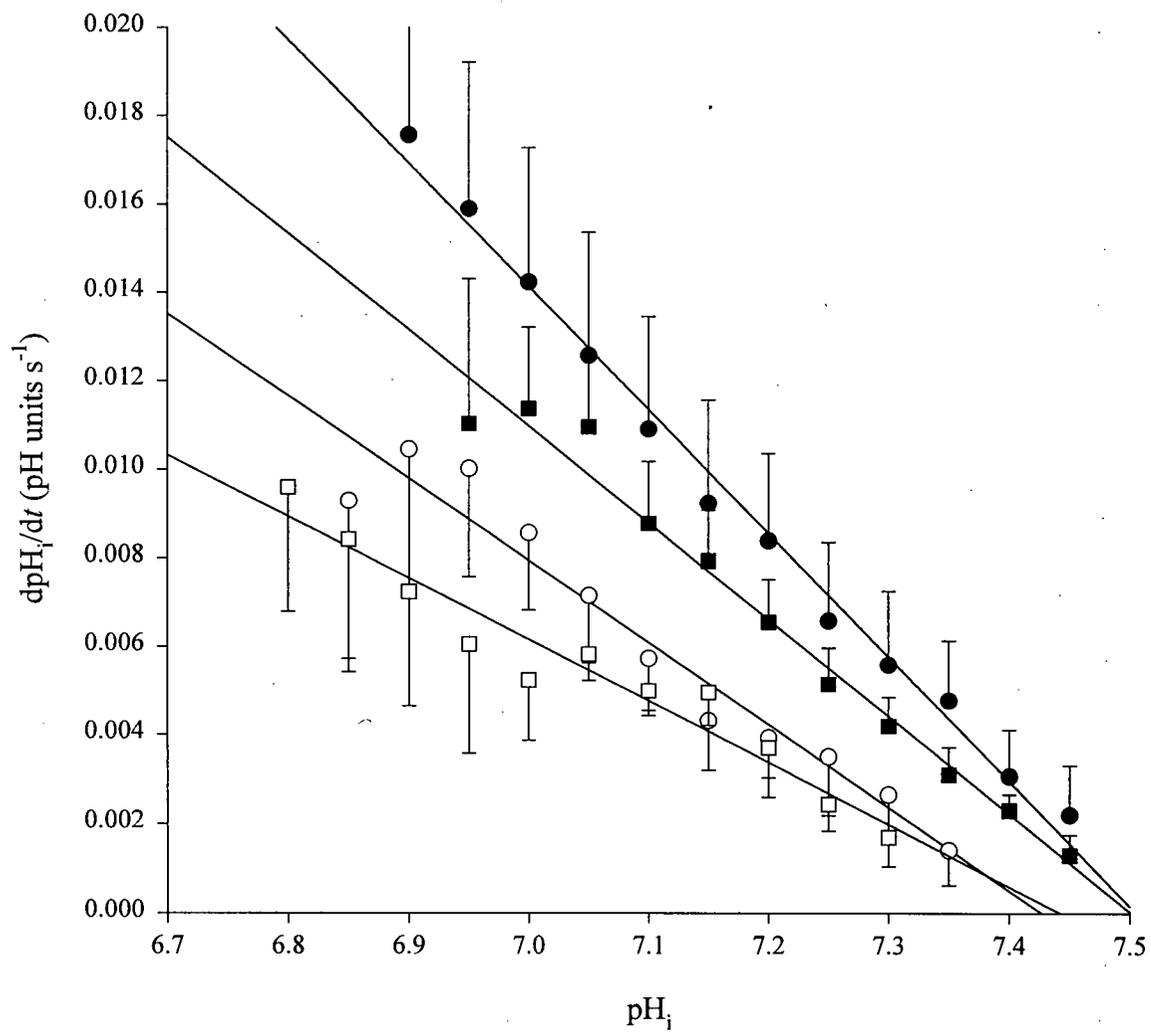
Figure 23. Effect of 500 μM ZnCl$_2$ on the steady-state pH$_i$ response to a 10 min period of anoxia

The experiment was performed under HCO$_3^-$/CO$_2$-free, HEPES-buffered conditions. The application of 500 μM ZnCl$_2$ did not influence steady-state pH$_i$. A 10 min period of anoxia induced an intracellular acidification that was not significantly different than observed in the absence of ZnCl$_2$. In the continued absence of oxygen, pH$_i$ recovered by ~ 0.04 pH units (29%) and, upon the return to normoxia, there was an intracellular alkalinization of ~ 0.13 pH units above the initial resting pH$_i$ value. The magnitudes of both the per-anoxic rise in pH$_i$ and the post-anoxic alkalinization were smaller in the presence of ZnCl$_2$ than observed in the absence of ZnCl$_2$ (compare with Fig. 8B, the same experiment performed in the absence of 500 μM ZnCl$_2$; Table 7).
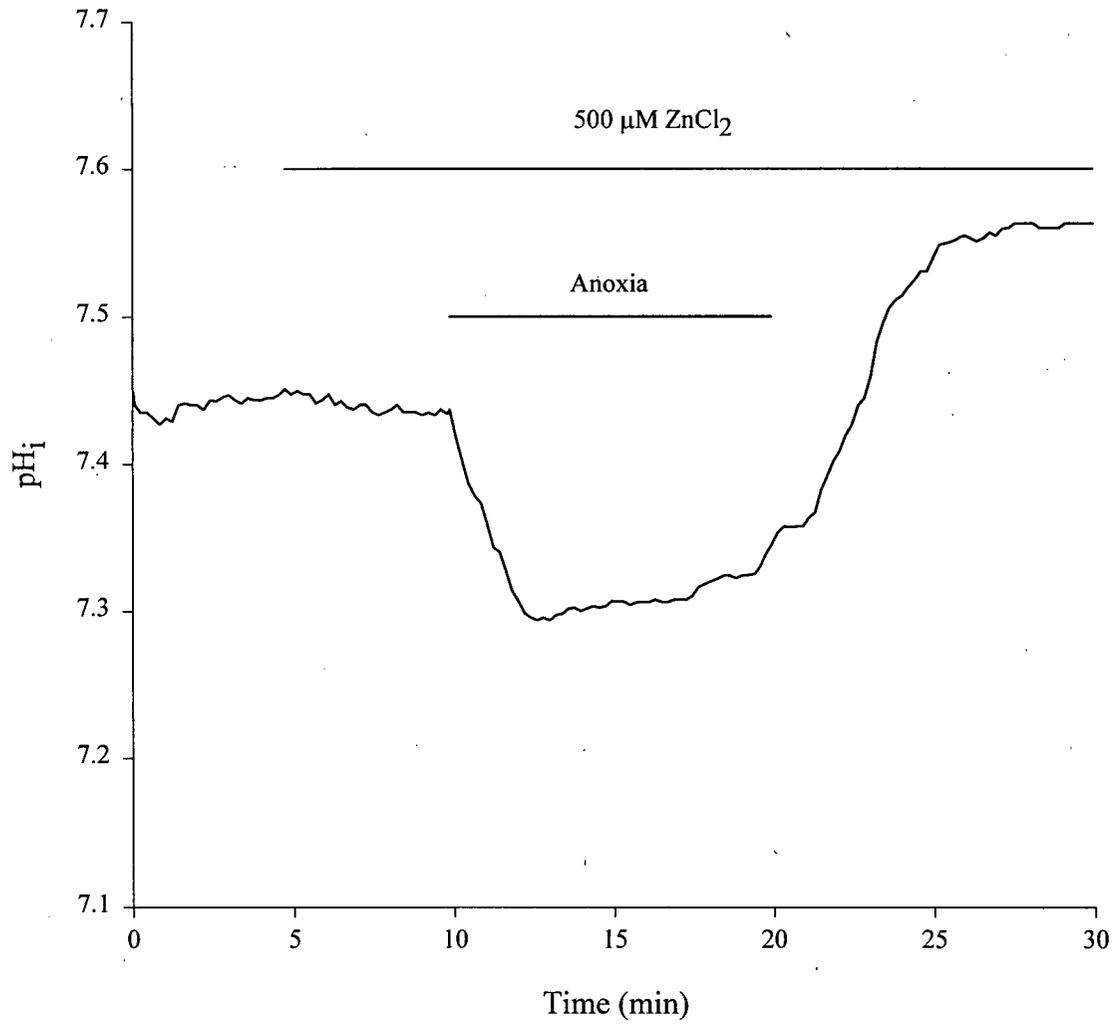
Figure 24. Distributions of steady-state pH_i values for all neurons during perfusion with HCO_3^-/CO_2-free, HEPES-buffered and HCO_3^-/CO_2-buffered media at 22°C

A. The distribution of the resting pH_i values for 26 adult rat hippocampal CA1 neurons during perfusion with HCO_3^-/CO_2-free, HEPES-buffered media at 22°C (bin width = 0.05 pH units). The data were best fitted by a Gaussian distribution with a mean at pH_i 7.23 ± 0.02. **B.** The distribution of resting pH_i values for 16 neurons during perfusion with HCO_3^-/CO_2-buffered media at 22°C (bin width = 0.05 pH units) was fitted with a Gaussian distribution, around a mean of 7.24 ± 0.02.

A.
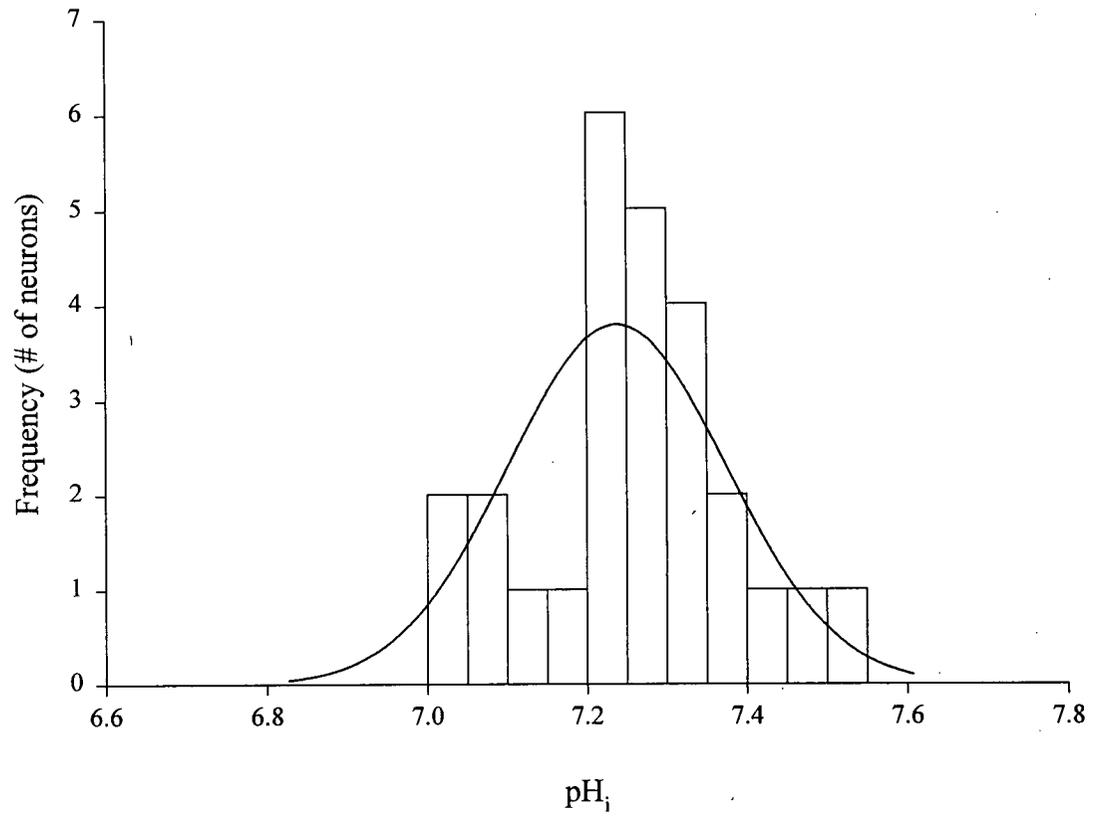
B.
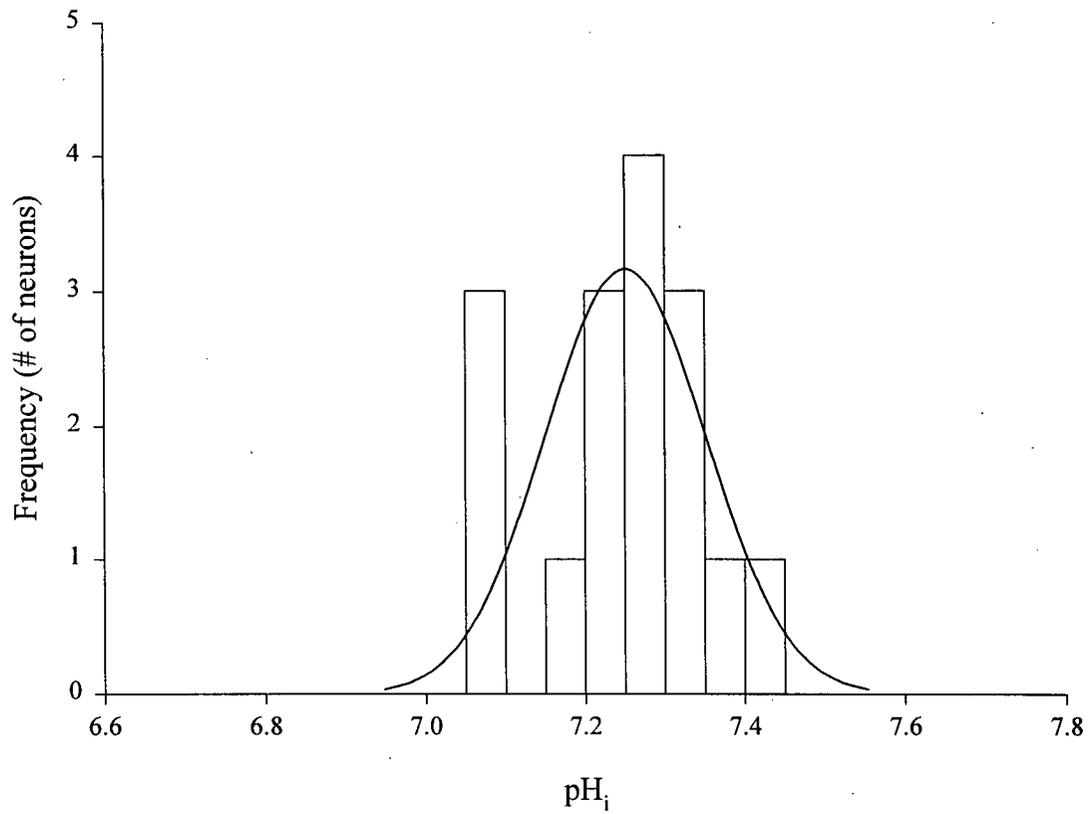
Figure 25. Effects of a 5 min period of anoxia on steady-state pH$_i$ at 22°C

A. Prior to an anoxic period in HCO$_3$$^-$/CO$_2$-free, HEPES-buffered media at 22°C, the initial resting pH$_i$ value was ~ 7.15. A 5 min period of anoxia induced an intracellular acidification that did not show any recovery in the continued absence of oxygen. Upon the return to normoxia, there was a small intracellular alkalinization. The magnitudes of all three phases of the pH$_i$ response to anoxia were smaller at 22°C when compared to 37°C under identical buffering conditions (compare with Fig. 7A, the same experiment performed at 37°C; Table 8). **B.** Upon exposure to a 5 min period of anoxia in HCO$_3$$^-$ /CO$_2$-buffered media at 22°C, the initial resting pH$_i$ value of ~ 7.18 decreased. In the continued absence of oxygen, pH$_i$ recovered and, upon the return to normoxia, there was an intracellular alkalinization. Again, the magnitudes of all three phases of the pH$_i$ response to anoxia were smaller at 22°C when compared to 37°C, under identical buffering conditions (compare with Fig. 9B, the same experiment performed at 37°C; Table 8). Each trace represents data collected from a different neuron.

A.

Anoxia

Time (min)

B.

Anoxia

Time (min)
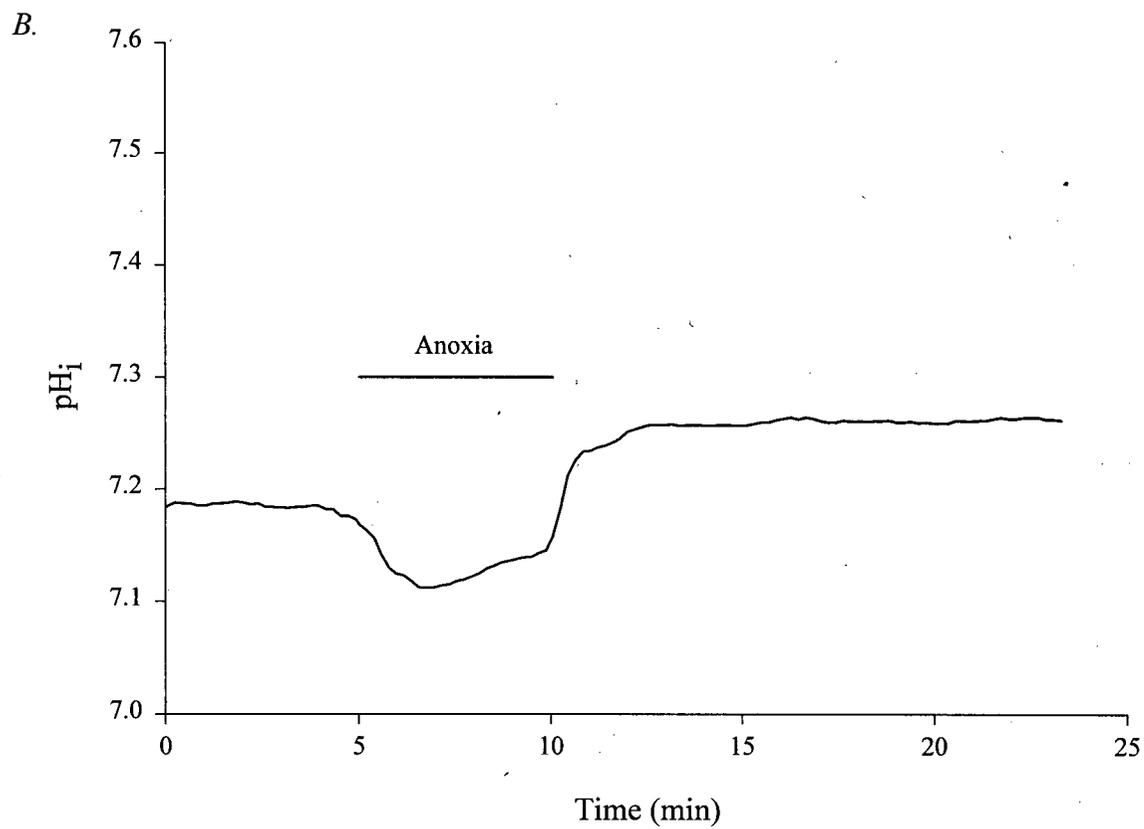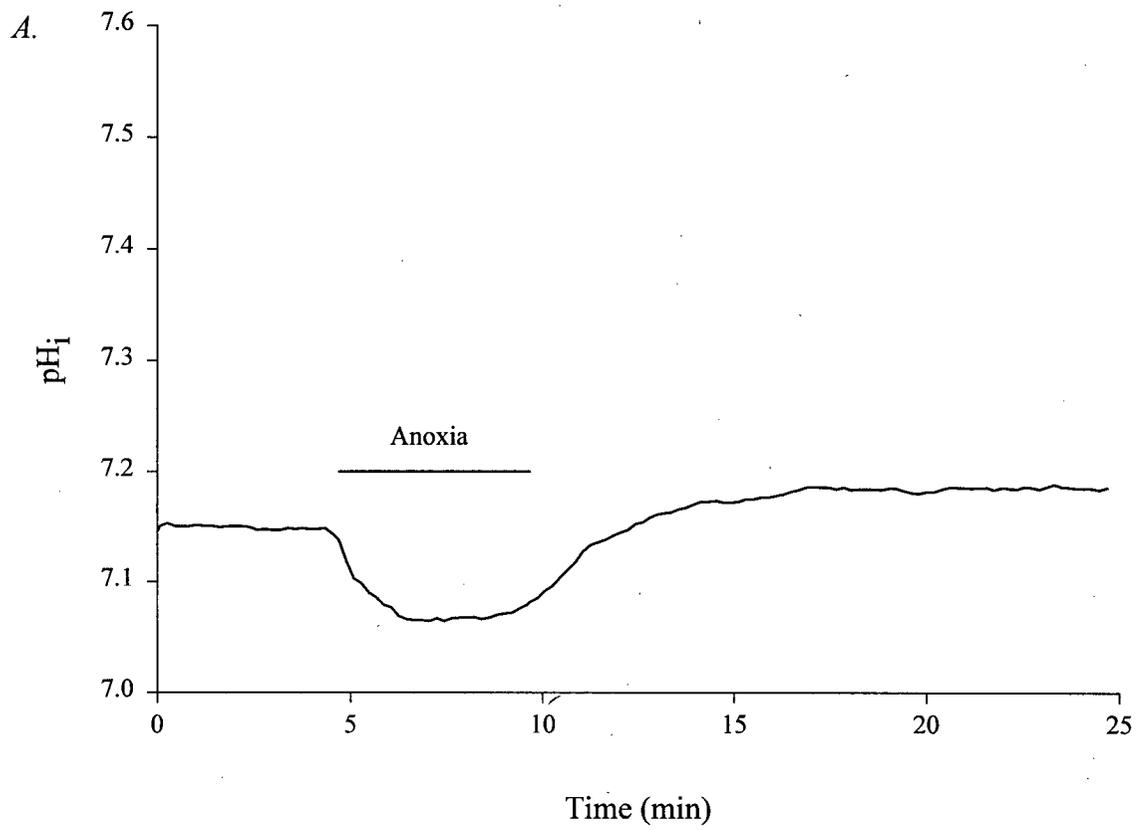
Figure 26. Effect of a transient period of anoxia on the rate of pH$_i$ recovery from an intracellular acid load at 22°C

The experiment was performed under HCO$_3^-$/CO$_2$-free, HEPES-buffered conditions. An initial acid load and subsequent recovery of pH$_i$ was conducted under control conditions at 22°C. A second acid load was then performed following a 5 min period of anoxia. The rate at which pH$_i$ recovered after the second intracellular acid load was increased when compared to the control rate of pH$_i$ recovery at 22°C.
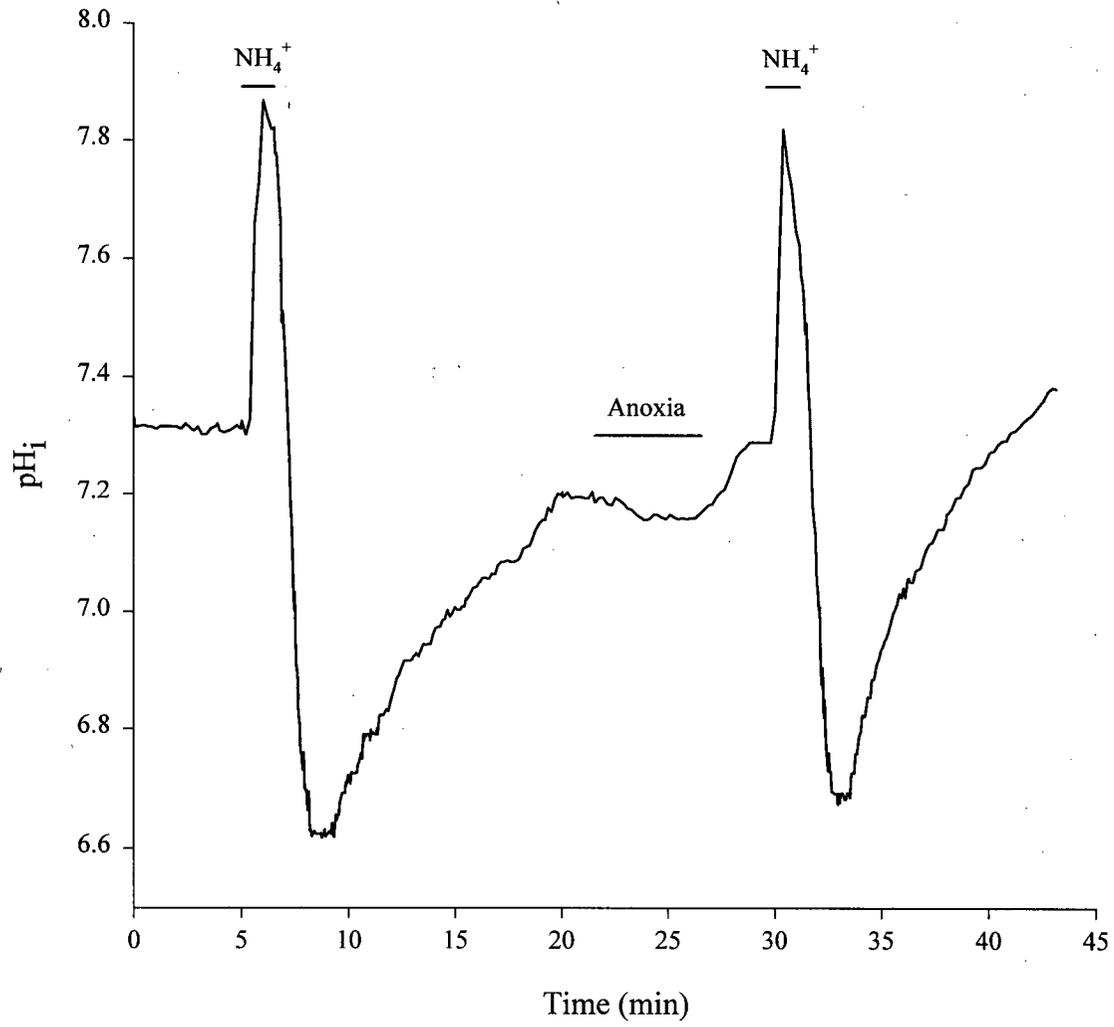
Figure 27. Comparison of the rates of pH$_i$ recovery from intracellular acid loads imposed prior to and following 5 min periods of anoxia at 22°C and 37°C

Experiments were performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. Plotted are the mean rates of pH$_i$ recoveries from intracellular acid loads conducted prior to (●) and following (○) a 5 min period of anoxia at 22°C, at pH$_i$ values denoted on the x-axis. Also plotted are the mean rates of pH$_i$ recoveries from intracellular acid loads conducted prior (■) and following (□) a 5 min period of anoxia at 37°C, at pH$_i$ values denoted on the x-axis (also see Fig. 19). The rates of pH$_i$ recovery were evaluated at 0.05 unit intervals of pH$_i$ and error bars represent S.E.M. Continuous lines represent the least squares linear regression fits to the data points indicated for each experimental condition. Data collected at 22°C were obtained from 11 paired experiments of the type shown in Fig. 26. At 22°C, at each absolute level of pH$_i$, the rates of pH$_i$ recovery were significantly faster following an anoxic period compared to the control rates of pH$_i$ recovery ($p<0.05$ at each absolute value of pH$_i$). Both prior to and following a 5 min period of anoxia, at each absolute level of pH$_i$, the rates of pH$_i$ recovery were significantly faster at 37°C as compared to the corresponding rates of pH$_i$ recovery at 22°C ($p<0.05$ at each absolute value of pH$_i$).
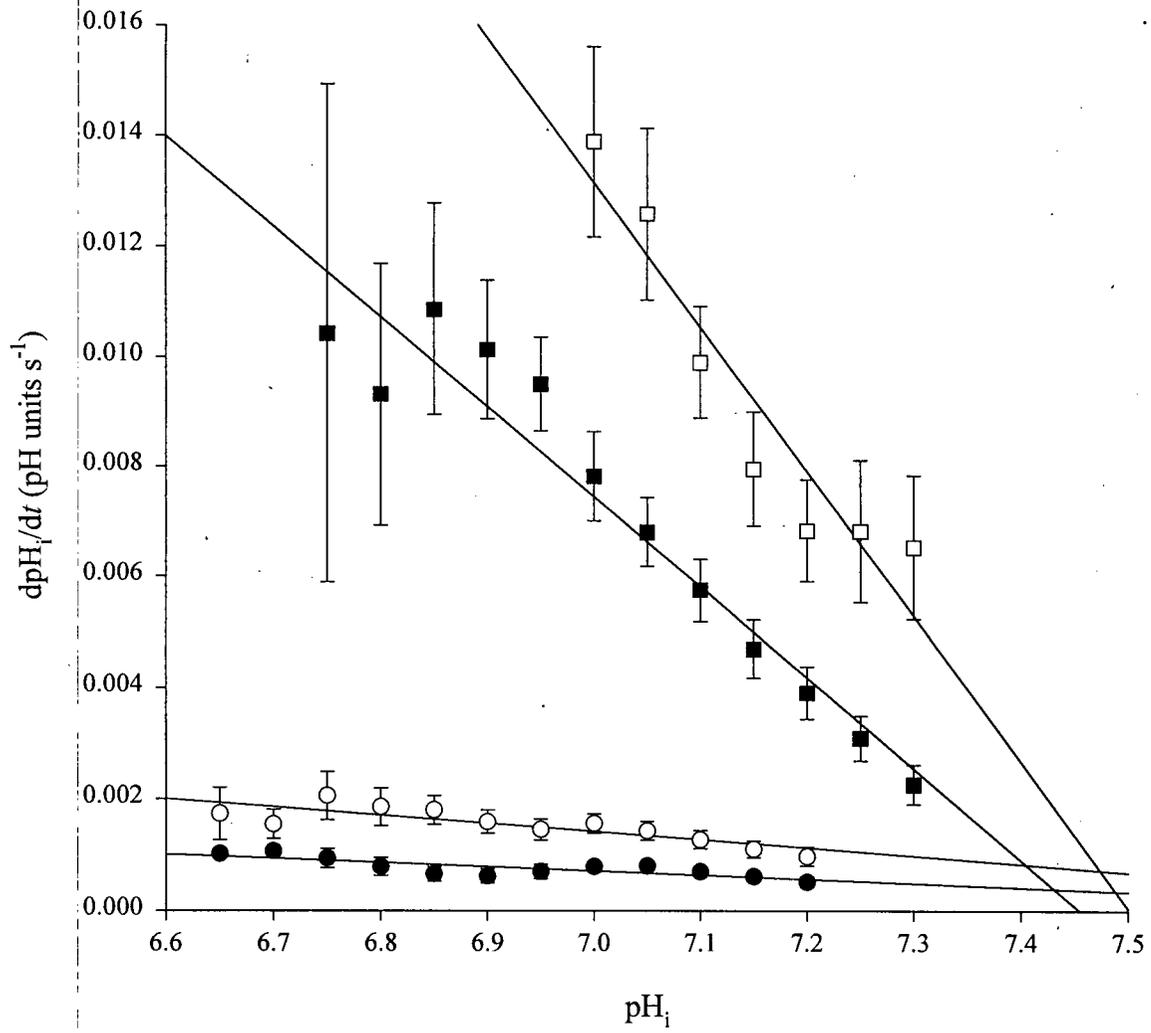
Figure 28. Effects of reducing pH_o to 6.60 on the pH_i response to a 5 min period of anoxia

The experiment was performed under HCO_3^-/CO_2-free, HÉPES-buffered conditions. Upon exposure to a pH 6.60 solution, the initial steady-state pH_i declined and a new steady-state pH_i of ~ 6.85 was reached. A 5 min period of anoxia induced an intracellular acidification which was not different from that observed under control conditions at pH_o 7.35. During the anoxic period, pH_i recovered by ~ 0.03 pH units (21%), and, upon the return to normoxia, there was an intracellular alkalinization of ~ 0.02 pH units. The magnitudes of the per-anoxic rise in pH_i and the post-anoxic alkalinization were smaller than observed under control conditions at pH_o 7.35 (compare with Fig. 7A, the same experiment performed at pH_o 7.35; Table 9).
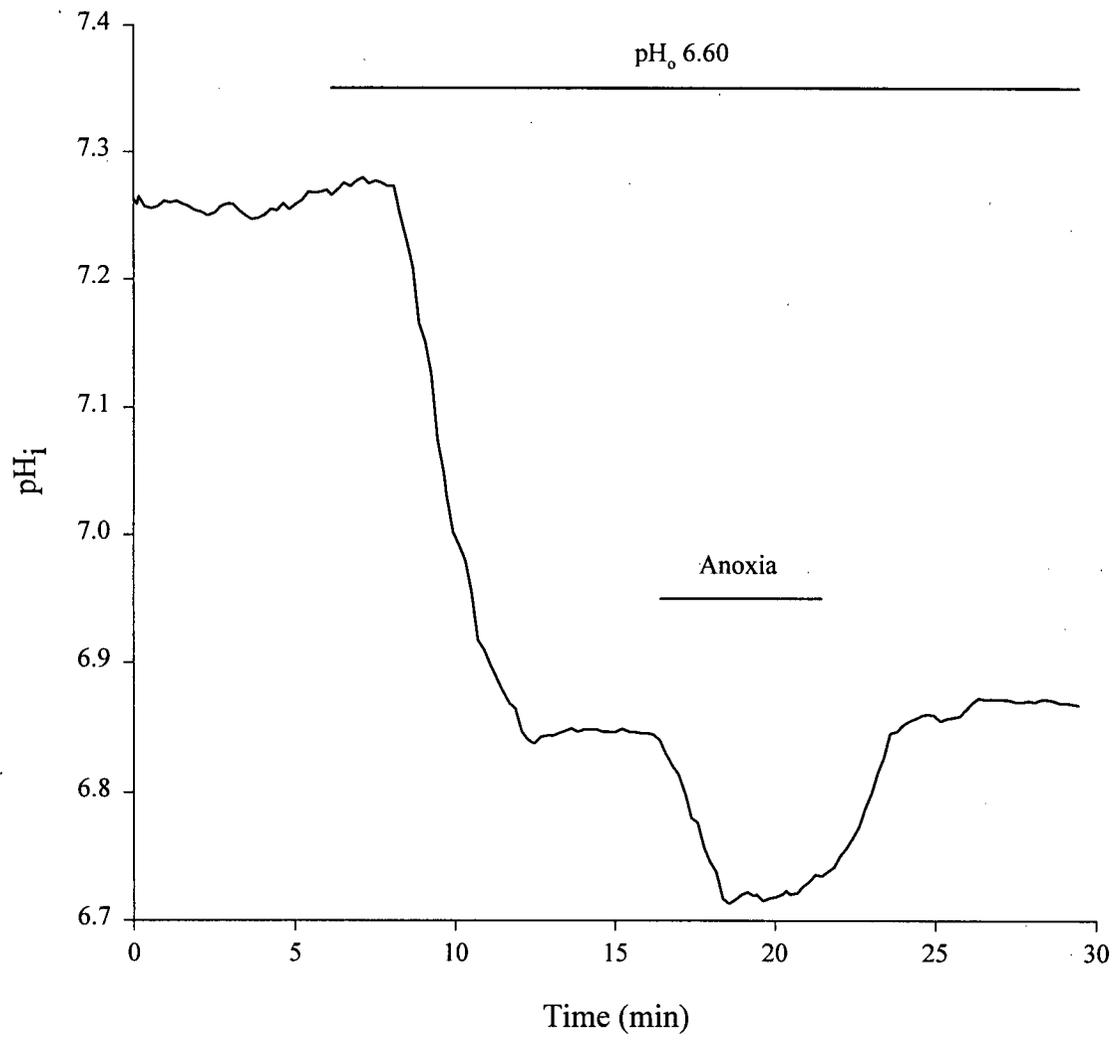
Figure 29. Effect of a transient period of anoxia on the rate of pH_i recovery from an intracellular acid load imposed at pH_o 6.60

The experiment was performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. An initial acid load and subsequent recovery of pH_i was conducted prior to an anoxic period at pH_o 6.60. A second acid load was then performed following a 5 min period of anoxia at pH_o 6.60. The rate at which pH_i recovered after the second intracellular acid load was increased when compared to the rates of pH_i recovery observed prior to the induction of anoxia at pH_o 6.60.
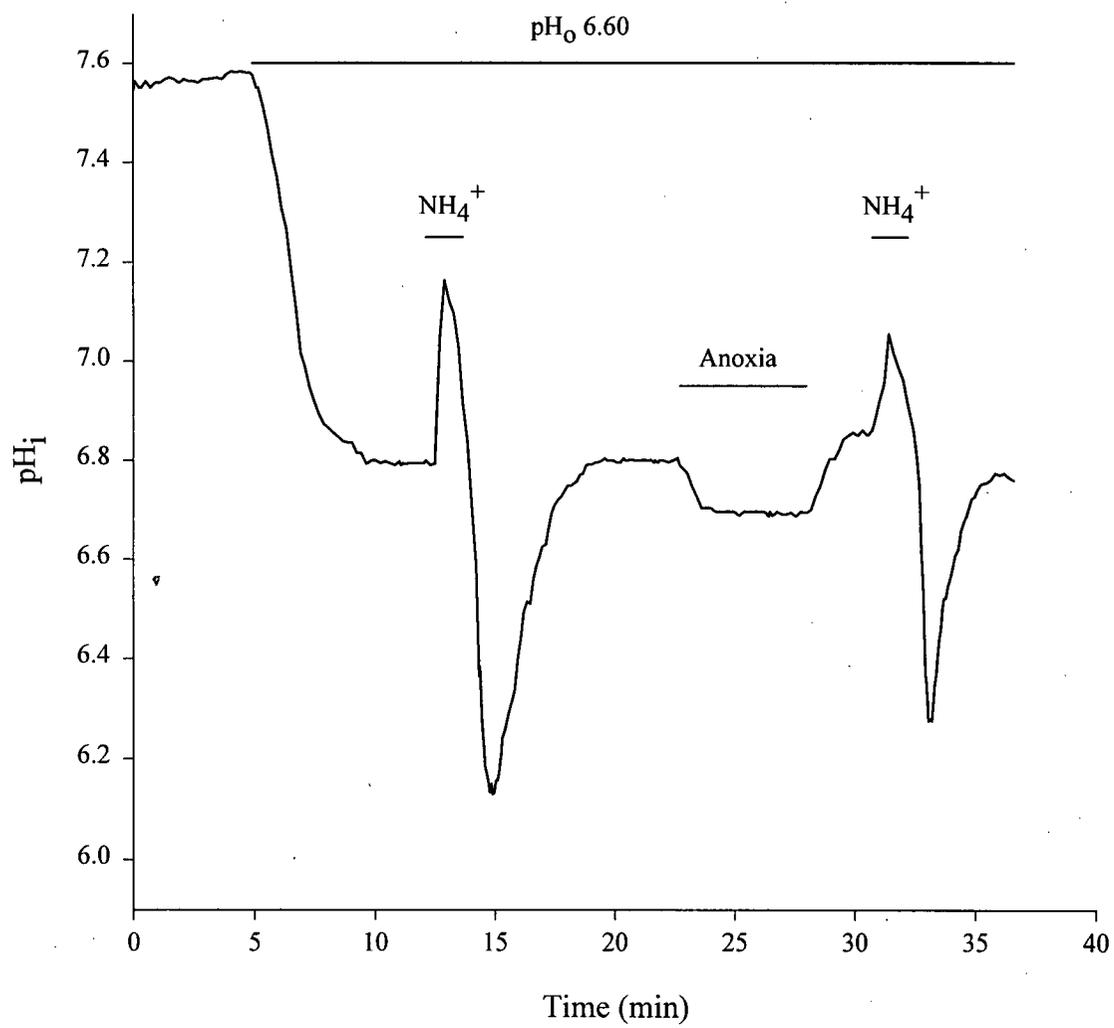
Figure 30. Comparison of the rates of pH_i recovery from intracellular acid loads imposed prior to and following a 5 min period of anoxia at pH_o 6.60 and pH_o 7.35

Experiments were performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. Plotted are the mean rates of pH_i recoveries from intracellular acid loads conducted prior to (●) and following (○) a 5 min period of anoxia at pH_o 6.60, at pH_i values denoted on the x-axis. Also plotted are the mean rates of pH_i recoveries from intracellular acid loads conducted prior to (■) and following (□) a 5 min period of anoxia at pH_o 7.35 (also see Fig. 19). The rates of pH_i recovery were evaluated at 0.05 unit intervals of pH_i and error bars represent S.E.M. Continuous lines represent the least squares linear regression fits to the data points indicated for each experimental condition. Data collected at pH_o 6.60 were obtained from 9 paired experiments of the type shown in Fig. 29. At pH_o 6.60, at each absolute level of pH_i except pH_i 6.70, the rates of pH_i recovery were significantly faster following a 5 min period of anoxia as compared to the control rates of pH_i recovery at pH_o 6.60 ($p<0.05$ at each absolute value of pH_i, except at pH_i 6.70). Both prior to and following a 5 min period of anoxia, at each absolute level of pH_i, the rates of pH_i recovery were significantly faster at pH_o 7.35 as compared to the corresponding rates of pH_i recovery observed at pH_o 6.60 ($p<0.05$ at each absolute value of pH_i).
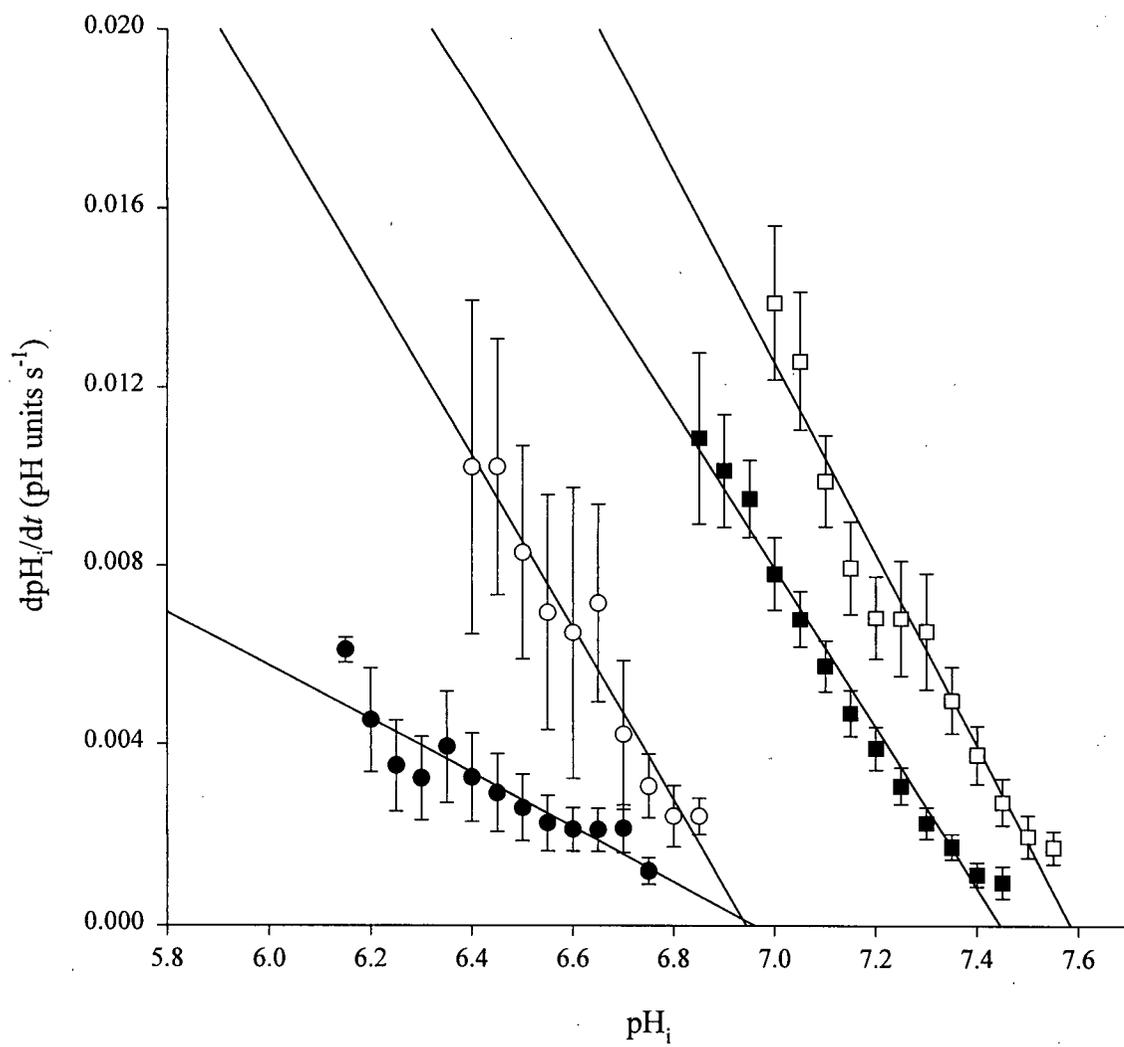
Figure 31. Effects of increasing pH$_o$ to 7.60 on the pH$_i$ response to a 5 min period of anoxia

Upon exposure to a HCO$_3^-$/CO$_2$-free, HEPES-buffered solution, pH 7.60, the initial steady-state pH$_i$ increased to reach a new steady-state pH$_i$ of ~ 7.92. A 5 min period of anoxia caused an intracellular acidification that was smaller than observed at pH$_o$ 7.35. In the continued absence of oxygen, pH$_i$ recovered and, upon the return to normoxia, there was a large intracellular alkalinization. The magnitudes of the per-anoxic rise in pH$_i$ and the post-anoxic alkalinization were larger than observed under control conditions at pH$_o$ 7.35 (compare with Fig. 7A, the same experiment performed at pH$_o$ 7.35; Table 9).
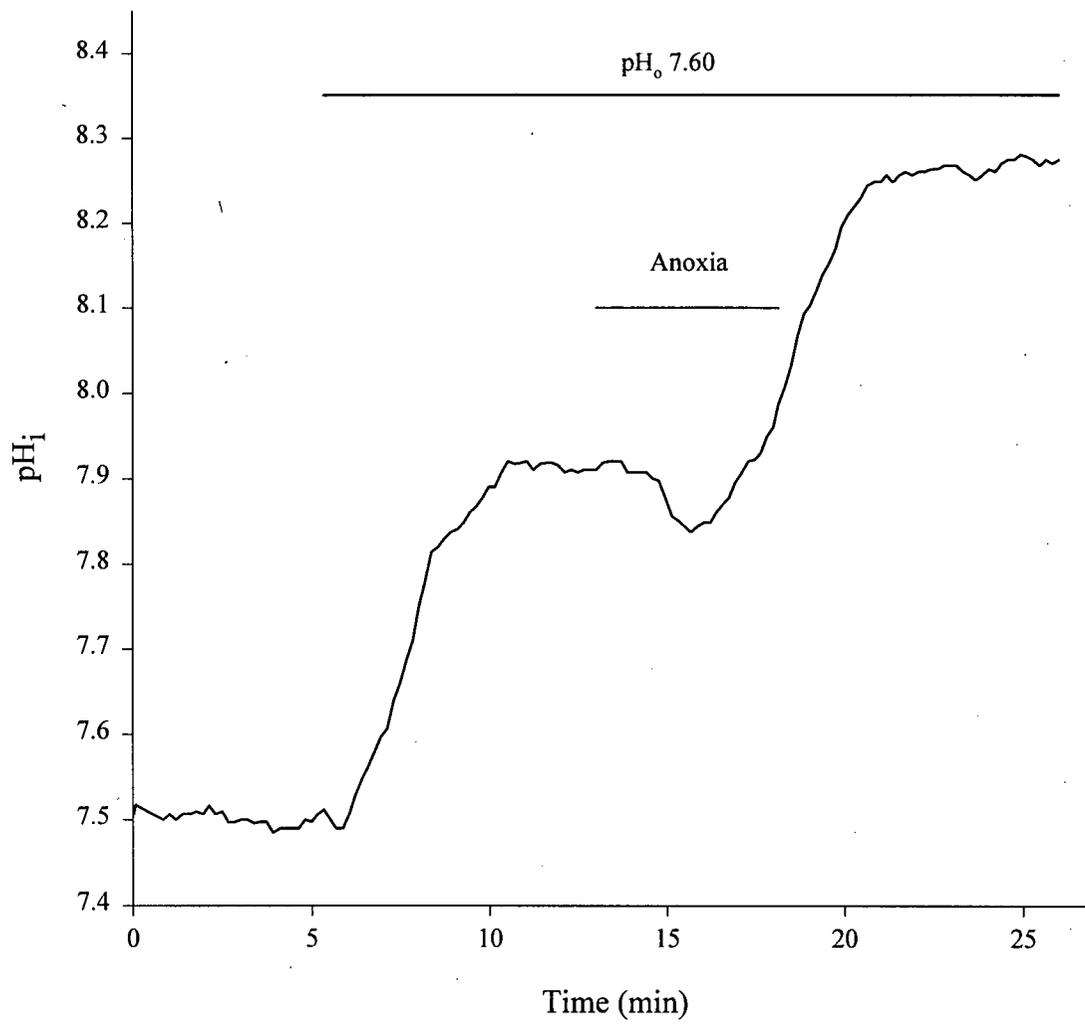
Figure 32. Effect of a transient anoxic period on the rate of pH_i recovery from an intracellular acid load at pH_o 7.60

The experiment was performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. An initial acid load and subsequent recovery of pH_i was conducted prior to an anoxic period at pH_o 7.60. A second acid load was then performed following a 5 min period of anoxia at pH_o 7.60. The rate at which pH_i recovered after the second intracellular acid load was increased when compared to the control rate of pH_i recovery at pH_o 7.60.
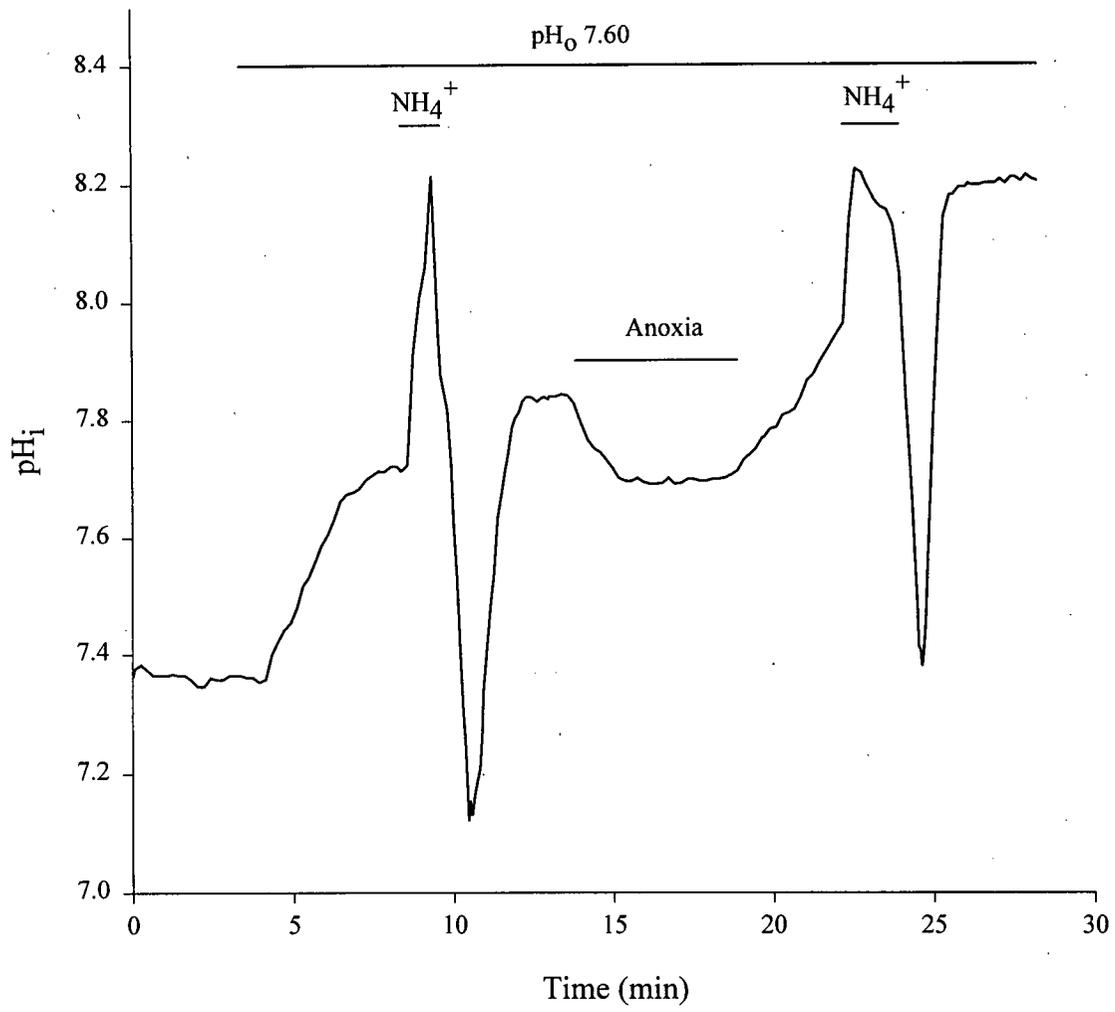
Figure 33. Comparison of the rates of pH_i recovery from intracellular acid loads imposed prior to and following a 5 min period of anoxia at pH_o 7.60 and pH_o 7.35

Experiments were performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. Plotted are the mean rates of pH_i recoveries from intracellular acid loads conducted prior to ($\bullet$) and following (O) a 5 min period of anoxia at pH_o 7.60, at pH_i values denoted on the x-axis. Also plotted are the mean rates of pH_i recoveries from intracellular acid loads conducted prior to ($\blacksquare$) and following ($\square$) a 5 min period of anoxia at pH_o 7.35, at pH_i values denoted on the x-axis (see also Fig. 19). The rates of pH_i recovery were evaluated at 0.05 unit intervals of pH_i and error bars represent S.E.M. Continuous lines represent the least squares linear regression fits to the data points indicated for each experimental condition. Data collected at pH_o 7.60 were obtained from 7 paired experiments of the type shown in Fig. 32. At pH_o 7.60, the rates of pH_i recovery were significantly faster following a 5 min period of anoxia compared to the corresponding control rates of pH_i recovery at pH_o 7.60 ($p<0.05$ at each absolute value of pH_i except at pH_i 7.20). Both prior to and following a 5 min period of anoxia, at every absolute level of pH_i, the rates of pH_i recovery were significantly faster at pH_o 7.60 when compared to the corresponding rates of pH_i recovery observed at pH_o 7.35 ($p<0.05$ at each absolute value of pH_i).
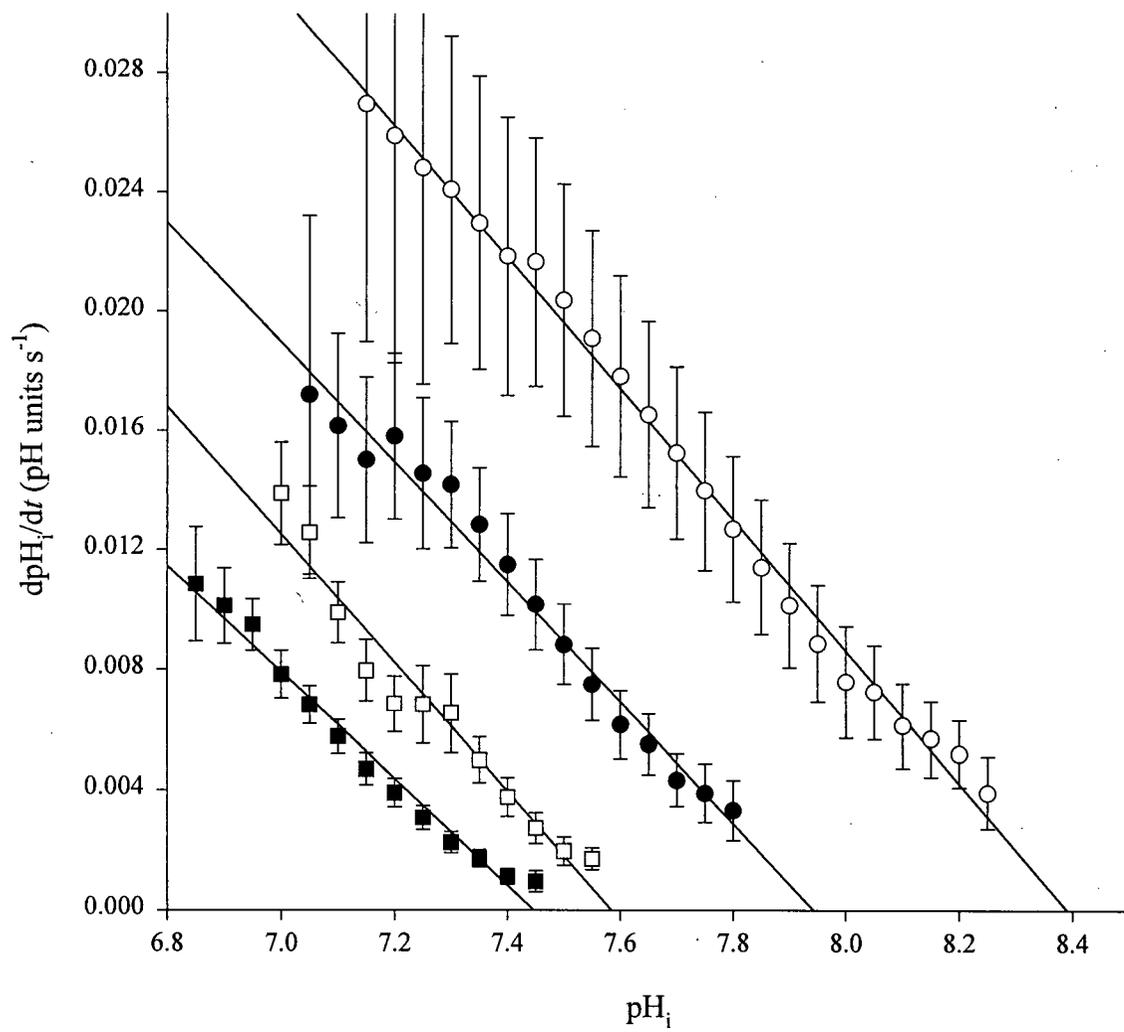
Figure 34. Relationship between pH_o and pH_i in HCO_3^-/CO_2-free, HEPES-buffered media under normoxic conditions

Plotted are the pH values of the superfusing media (pH_o) against the resulting mean steady-state pH_i values. Error bars represent S.E.M. The continuous line represents the least squares linear regression fit to the data points. The numbers in parentheses above or below the data point indicate the number of individual neurons from which the observation was made. The slope of the linear regression line is ~ 0.74.
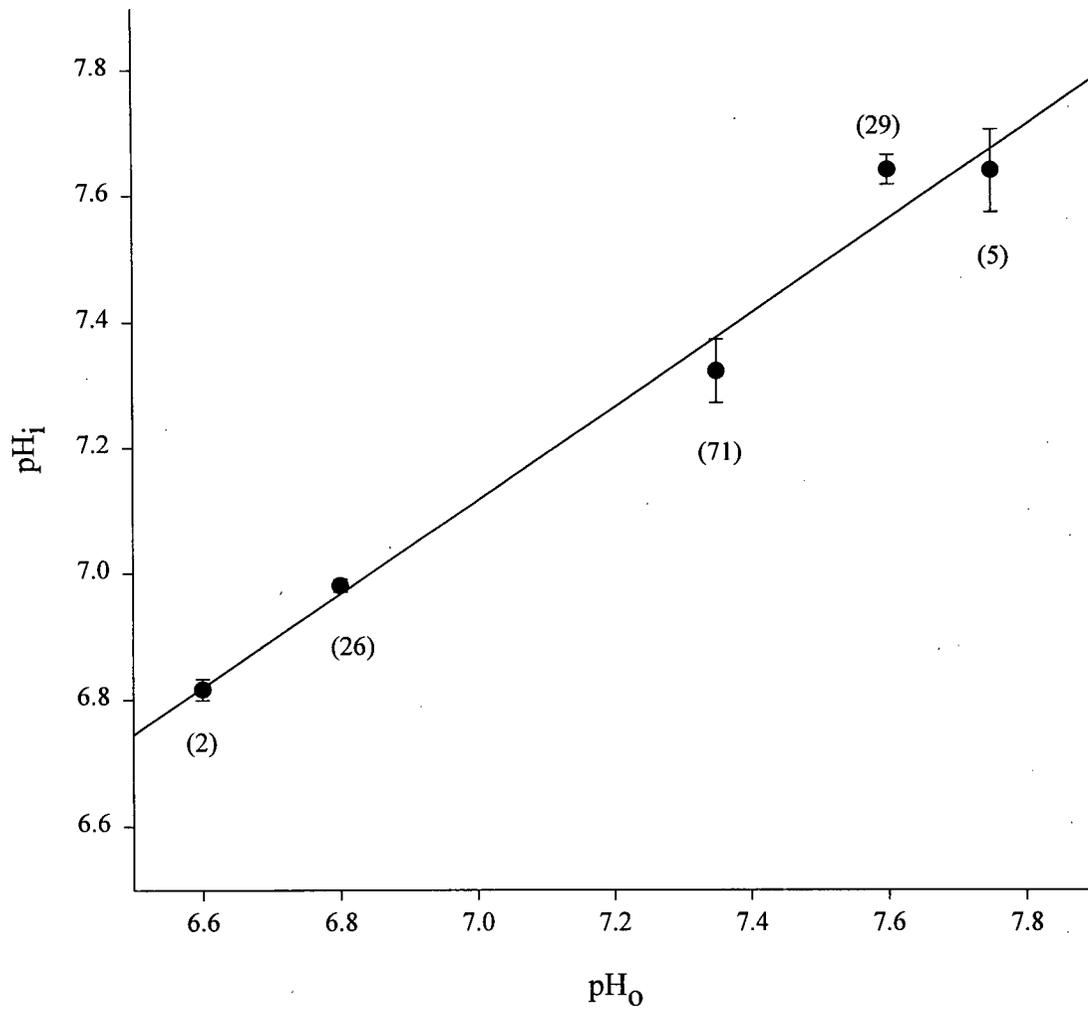
Figure 35. Effects of 3, 5 and 10 min periods of anoxia on steady-state pH$_i$ during perfusion with media containing 5.0 mM glucose

Experiments were performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. **A.** Upon the induction of a 3 min period of anoxia, there was an intracellular acidification. pH$_i$ did not recover in the continued absence of oxygen. The magnitude of the per-anoxic rise in pH$_i$ was smaller than observed in the presence of 17.5 mM (compare with Fig. 8A, the same experiment performed in the presence of 17.5 mM glucose; Table 10). Upon the return to normoxia, there was an intracellular alkalinization above the initial resting pH$_i$ value which was not different than observed in the presence of 17.5 mM glucose. **B.** Upon the induction of a 5 min period of anoxia, the initial resting pH$_i$ value of ~ 7.26 decreased and, in the continued absence of oxygen, recovered slightly. The magnitudes of the per-anoxic acidification and per-anoxic rise in pH$_i$ were smaller than observed in the presence of 17.5 mM glucose (compare with Fig. 7A, the same experiment performed in the presence of 17.5 mM glucose; Table 10). Upon the return to normoxia, there was an intracellular alkalinization that was not different than observed in the presence of 17.5 mM glucose. **C.** Upon exposure to a 10 min period of anoxia, there was an intracellular acidification, the magnitude of which was smaller than observed in the presence of 17.5 mM glucose (compare with Fig. 8B, the same experiment performed in the presence of 17.5 mM glucose; Table 10). In the continued absence of oxygen, pH$_i$ recovered, and upon the return to normoxia, there was an intracellular alkalinization, neither of which were different than observed under identical buffering conditions in the presence of 17.5 mM glucose. Each trace represents data collected from a different neuron.

A.

B.

C.
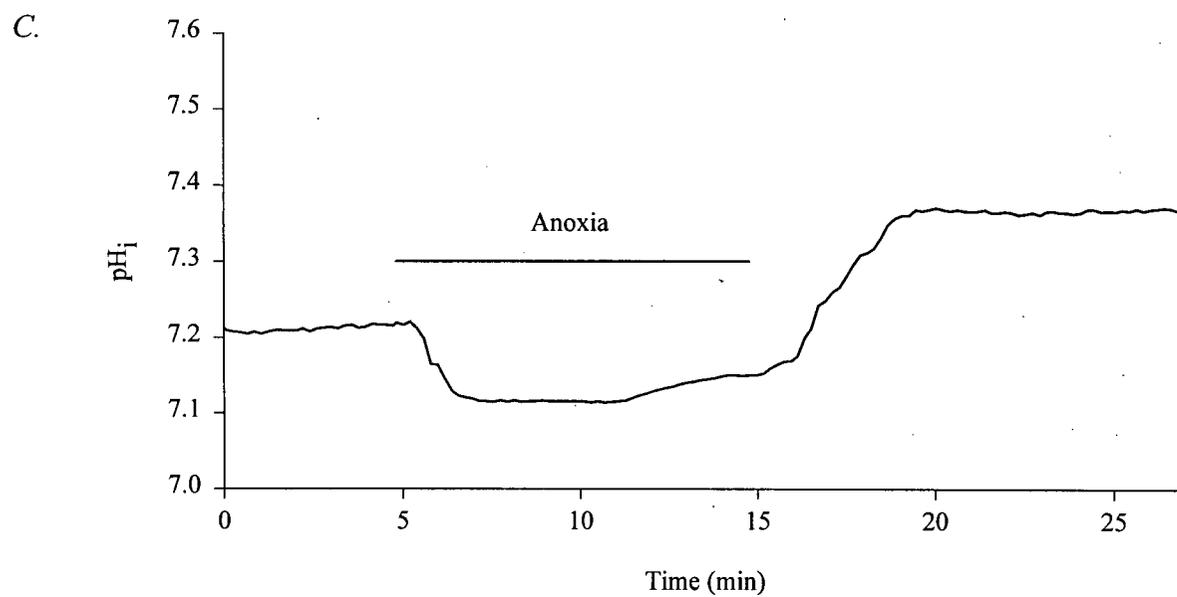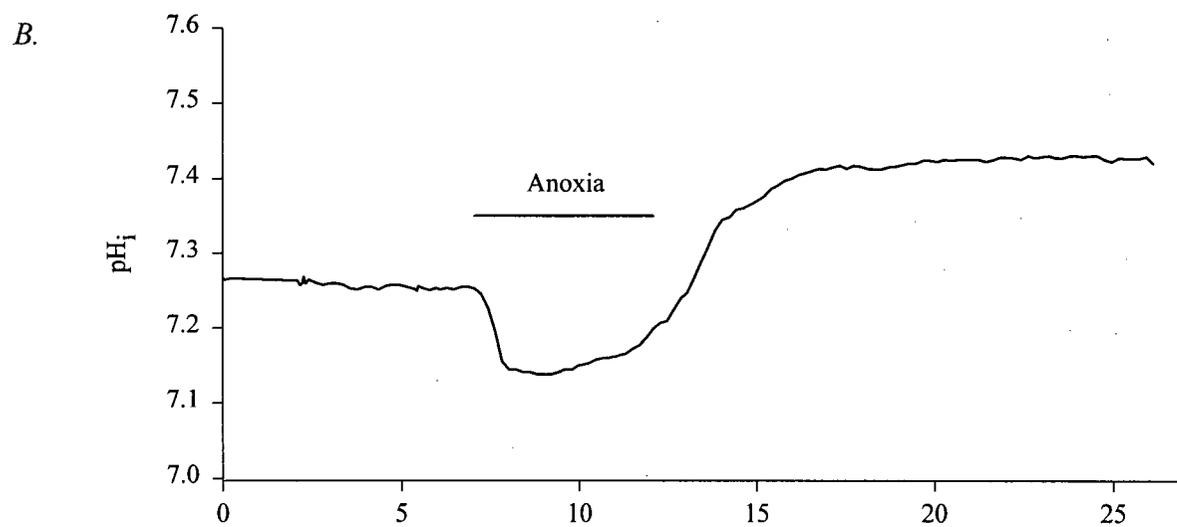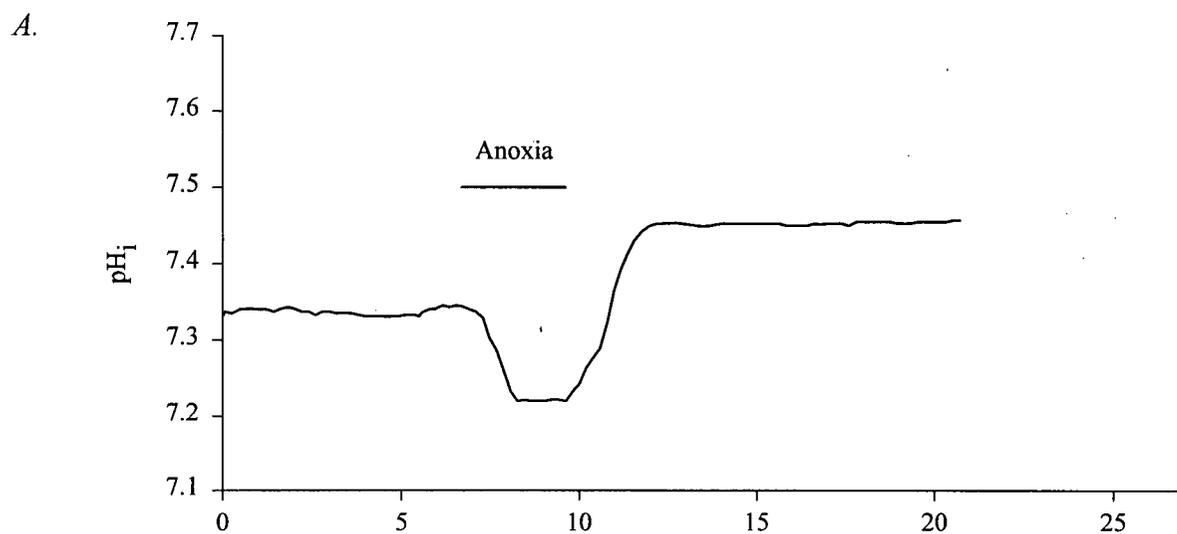
Figure 36. Effects of exposure to reduced Na$^+$ medium on the pH$_i$ response to a 5 min period of anoxia in media containing 5.0 mM glucose

Experiments were performed under HCO$_3$$^-$/CO$_2$-free, HEPES-buffered conditions. **A.** Upon exposure to a 2 mM Na$^+$, NMDG$^+$ substituted medium, pH$_i$ fell from an initial steady-state pH$_i$ of ~ 7.44. Over ~ 12 min, pH$_i$ recovered and reached a new steady-state pH$_i$ value. A 5 min period of anoxia caused an intracellular acidification that recovered slightly in the continued absence of oxygen. Upon the return to normoxia, there was an intracellular alkalinization that was smaller than observed in the presence of normal extracellular Na$^+$ and 5.0 mM glucose (compare with Fig. 35B, the same experiment performed in the presence of normal extracellular Na$^+$; Table 11). **B.** Upon exposure to 2 mM Na$^+$, Li$^+$ substituted media, pH$_i$ decreased and gradually recovered to a new steady-state pH$_i$ value of ~ 7.22. A 5 min period of anoxia induced an intracellular acidification that recovered slightly in the continued absence of oxygen. Upon the return to normoxia, pH$_i$ increased ~ 0.22 pH units above the initial steady-state pH$_i$. The magnitude of the post-anoxic alkalinization was not different than observed in the presence of normal extracellular Na$^+$ (compare with Fig. 35B, the same experiment performed in the presence of normal extracellular Na$^+$; Table 11). Each trace represents data collected obtained from a different neuron.

A.

2 mM [Na$^+$]$_o$, NMDG$^+$-substituted

Anoxia

pH$_i$

Time (min)

B.

2 mM [Na$^+$]$_o$, Li$^+$-substituted

Anoxia

pH$_i$

Time (min)
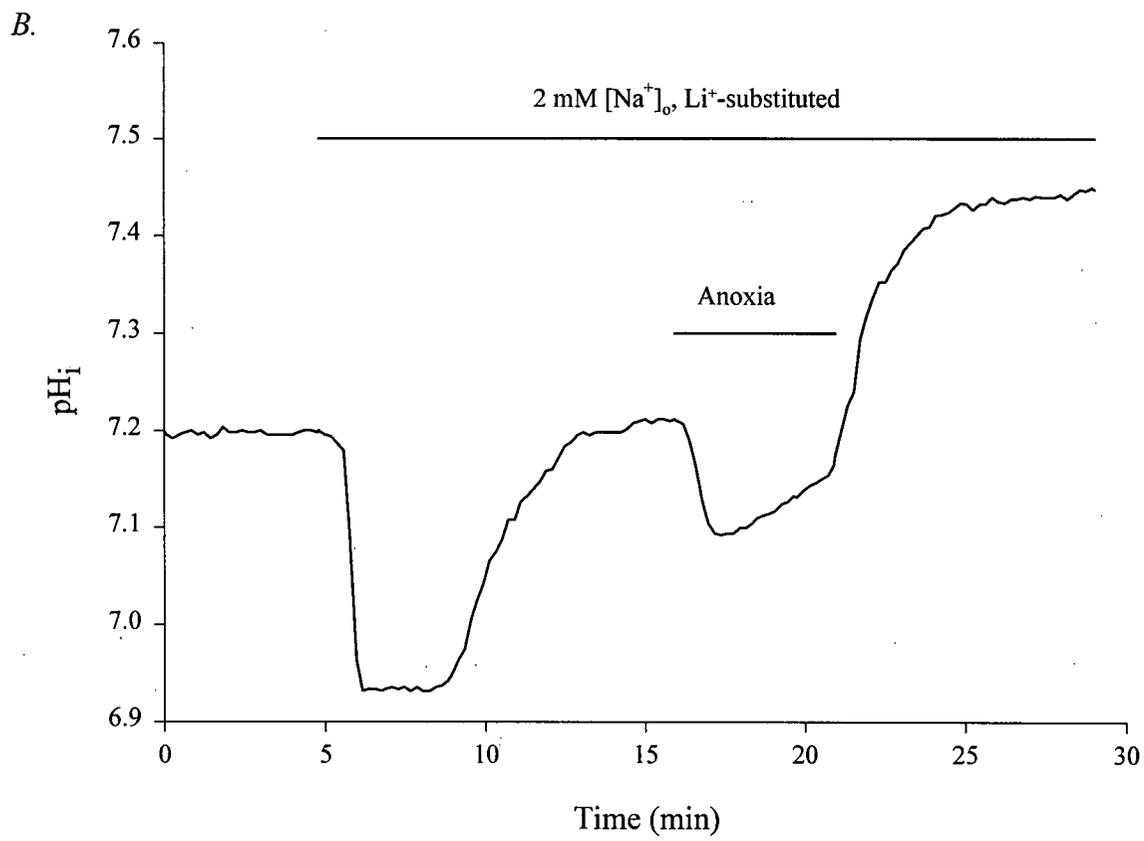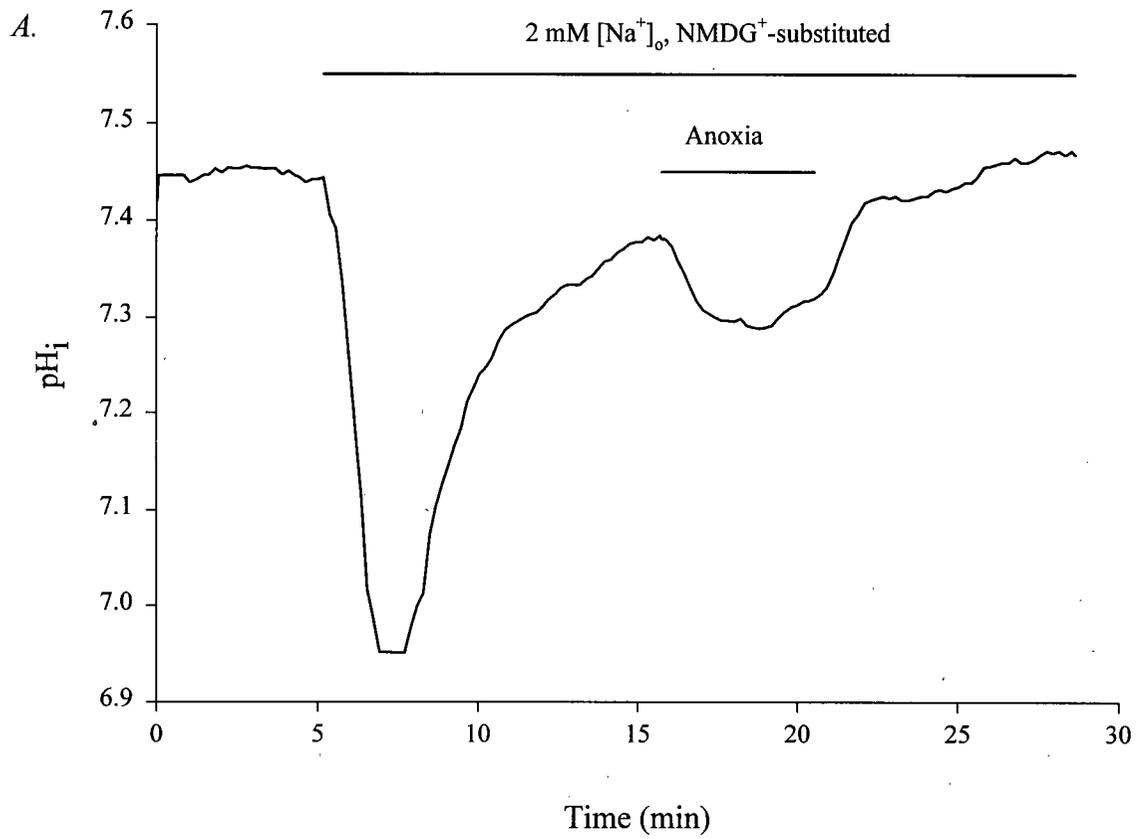
Figure 37.   Effects of 50 μM Rp-cAMPS and 10 μM Noradrenaline on the pH$_i$ response to a 5 min period of anoxia

Experiments were performed under HCO_3^-/CO_2-free, HEPES-buffered conditions. In the absence of any pharmacological treatments (●), a 5 min period of anoxia induced a fall in pH$_i$ of ~ 0.12 pH units that recovered by ~ 0.05 pH units (42%) in the continued absence of oxygen. Upon the return to normoxia, pH$_i$ increased ~ 0.16 pH units above the initial steady-state pH$_i$. In the presence of 50 μM Rp-cAMPS (an inhibitor of PKA; △), the magnitude of all three phases of the pH$_i$ response to a 5 min period of anoxia were reduced (Table 12). In the presence of 10 μM noradrenaline (an activator of PKA; □), the magnitude of the post-anoxic alkalinization was larger than observed under control conditions (Table 12). Each trace represents data collected from a different neuron.
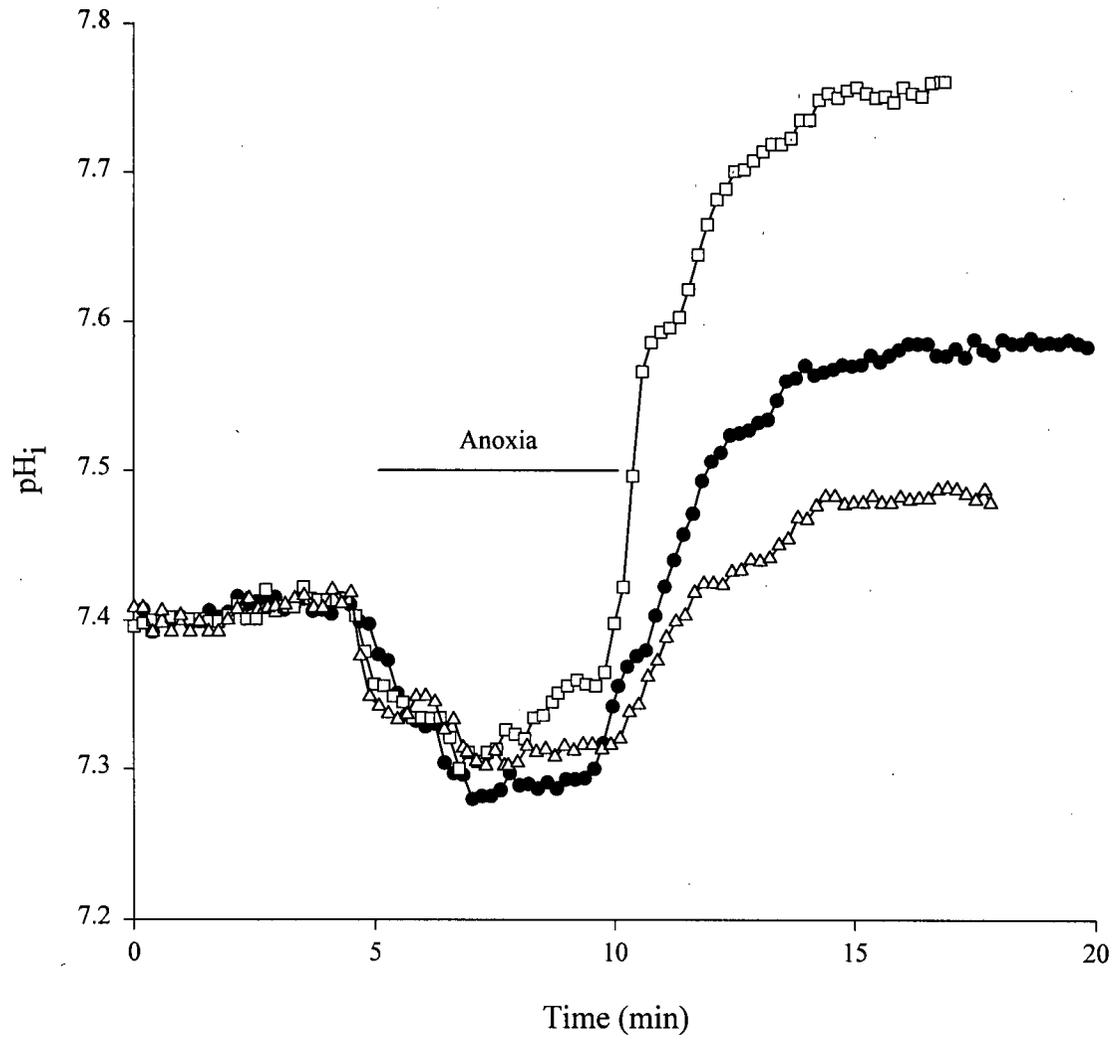
DISCUSSION

The results presented in this thesis are the first detailed description of the effects of anoxia on intracellular pH in adult mammalian central neurons, under conditions where the pH_i response to anoxia can be attributed to mechanisms intrinsic to the neurons under study. Although previous studies have documented the effects of anoxia on pH_i both *in vivo* and in slice preparations *in vitro* (*e.g.* Whittingham *et al*, 1984; Silver and Erecinska, 1990; Eleff *et al*, 1991; Fujiwara *et al*, 1992; Vornov *et al*, 1996), it is difficult (if not impossible) to separate the contribution of various cell types (including glia) to the effects observed under these experimental conditions. In addition to characterizing anoxia-evoked changes in steady-state pH_i, the mechanisms underlying these changes were examined, with particular reference to the role of changes in the activity of the Na^+/H^+ exchanger. Not only is the latter transport mechanism the dominant acid extrusion pathway in rat hippocampal neurons (Raley-Susman *et al*, 1991; Baxter and Church, 1996; Bevensee *et al*, 1996; Smith *et al*, 1998), but also changes in Na^+/H^+ exchange activity have been shown to be critically important for determining cellular viability in both peripheral cell types and in central neurons following ischemia-like insults (Bond *et al*, 1993; Harper *et al*, 1993; Vornov *et al*, 1996). Finally, results presented in the thesis also contribute to our understanding of the control of pH_i in hippocampal neurons under normoxic conditions.

1 Intracellular pH Regulation Under Normoxic Conditions

1.1 Intracellular pH regulating mechanisms present in rat hippocampal CA1 neurons

Under physiological conditions, three mechanisms have been found, to date, to contribute to pH_i regulation in rat hippocampal neurons; an acid-extruding Na^+/H^+ exchanger, an acid-extruding Na^+-dependent HCO_3^-/Cl^- exchanger, and an acid-loading Na^+-independent HCO_3^-/Cl^- exchanger (Raley-Susman *et al*, 1991 and 1993; Schwiening and Boron, 1994; Baxter and Church, 1996; Bevensee *et al*, 1996; Smith *et al*, 1998). Although it has been suggested that the Na^+-dependent HCO_3^-/Cl^- exchanger is the most important acid extrusion mechanism present in this cell type (Schwiening and Boron, 1994), this laboratory (Baxter and Church, 1996; Smith *et al*, 1998) and the laboratories of others (Raley-Susman *et al*, 1991; Bevensee *et al*, 1996) have provided evidence that Na^+/H^+ exchange is, in fact, the dominant acid extrusion mechanism. In support, in the present study, the mean steady-state pH_i was not influenced by the presence or absence of extracellular HCO_3^-.

Evidence has been found in the present study for the existence of additional mechanism(s) which contribute to pH_i regulation in rat hippocampal neurons. Thus, pH_i regulation was observed even in the absence of extracellular Na^+ under HCO_3^-/CO_2-free, HEPES-buffered conditions, suggesting the presence of a Na^+- and HCO_3^--independent pH_i regulatory mechanism(s) in this cell type (also see Bevensee *et al*, 1996). This is perhaps not surprising, given the very large number of Na^+- and HCO_3^--independent mechanisms which have been found to participate in pH_i regulation in a variety of

peripheral cell types (*e.g.* Kurtz, 1987; Blatter and McGuigan, 1990; Gibb *et al*, 1997; Karwatowska-Prokopczuk *et al*, 1998). These include, for example, voltage-dependent proton conductances (g_{H^+}; Byerly and Suen, 1989; Kapus *et al*, 1993; Gordienko *et al*, 1996), lactate/H^+ cotransport (Poole and Halestrap, 1993; Gibb *et al*, 1997), Ca^{2+}/H^+ ATPase (Schwiening *et al*, 1993), V- and P-type H^+ ATPases (Nelson and Klionsky, 1996; Karwatowska-Prokopczuk *et al*, 1998), and K^+/H^+ exchange (Sachs *et al*, 1976; Hofer and Machen, 1992; Wilding *et al*, 1992; Hogan *et al*, 1995a and 1995b). Preliminary evidence is presented in this thesis that Ca^{2+}/H^+ ATPase, lactate/H^+ cotransport, g_{H^+}, and/or K^+/H^+ exchange activity may exist in hippocampal neurons, given the fact that maneuvers which influence the activities of each of these mechanisms are able to affect the recovery of pH_i during exposure to Na^+-free, $NMDG^+$-substituted media. However, additional experiments are required to assess formally the potential roles of these mechanisms in pH_i regulation in rat hippocampal neurons. The possibility remains that these mechanisms may be dormant under physiological conditions and be activated under particular (i.e. $[Na^+]_o$-free) conditions (see Kurtz, 1987; Peral *et al*, 1995).

1.2 The role of extracellular pH in determining intracellular pH

In contrast to many peripheral cell types in which pH_i is not markedly affected by changes in pH_o (*e.g.* Aickin, 1984; Wilding *et al*, 1992), pH_i in mammalian central neurons appears to be steeply dependent upon pH_o. Thus, the ratio $\Delta pH_i : \Delta pH_o$ (the slope of the linear regression line relating pH_i to pH_o) is approximately 0.7 in rat brain synaptosomes (Sanchez-Armass *et al*, 1994), cultured fetal rat neocortical neurons (Ou-

Yang *et al*, 1993) and cultured fetal rat hippocampal neurons (Church *et al*, 1998). The present study confirms that the steep dependency of pH_i on pH_o also exists in adult rat hippocampal neurons, a finding which suggests that changes in pH_i may account for some of the known effects of changes in pH_o on hippocampal neuron function (see; Church and McLennan, 1989; Church, 1992; Tombaugh and Somjen, 1996 and 1997; Balestrino and Somjen, 1998; Church *et al*, 1998).

In both peripheral cell types (Vaughan-Jones and Wu, 1990; Peral *et al*, 1995) and in rat brain synaptosomes (Jean *et al*, 1985), Na^+/H^+ exchange activity is influenced by changes in pH_o and thereby provides a mechanism to link changes in pH_o to changes in pH_i. While Jean *et al* (1985) suggested that, in rat brain synaptosomes, the transmembrane proton gradient determines the rate of activity of the exchanger, Vaughan-Jones and Wu (1990), using cardiac Purkinje fibers, found that the absolute magnitudes of both pH_o and pH_i determine Na^+/H^+ activity. In both cases, however, an increase in pH_o shifted the pH_i dependence of the exchanger in the alkaline direction, thereby increasing the activity of the exchanger. Conversely, a decrease in pH_o caused a decrease in pH_i-mediated stimulation of acid extrusion (Jean *et al*, 1985; Vaughan-Jones and Wu, 1990).

In the present study, changes in pH_o affected the activity of the Na^+/H^+ exchanger as judged by changes in the slopes of the regression lines relating pH_i to the rates of change of pH_i following internal acid loads imposed under HCO_3^--free conditions. Thus, Na^+/H^+ exchange activity in adult rat hippocampal neurons is inhibited by a reduction in pH_o whereas its activity is stimulated with an increase in pH_o. In addition to influencing

the rate of Na^+/H^+ exchange activity, changes in pH_o evoked shifts in the pH_i dependence of Na^+/H^+ exchange, as there was a progressive alkaline shift in the x-intercept values of the linear regression lines relating pH_i to rates of pH_i recovery from imposed intracellular acid loads under pre-anoxic conditions as pH_o was increased. Thus, at pH_o 6.60, the increased concentration of extracellular protons appears not only to directly inhibit Na^+/H^+ exchange but also decrease the intracellular affinity of the exchange mechanism for protons, thus further reducing the rate of acid extrusion. Conversely, at pH_o 7.60, there was a rapid rate of acid extrusion. Therefore, in rat hippocampal neurons, pH_o appears to be able to influence pH_i by influencing the activity of the Na^+/H^+ exchanger. The relevance of these findings to the neuronal response to anoxia is discussed in Section 6.3.1.

1.3 The role of ambient temperature in determining intracellular pH

It is well known that steady-state pH varies with temperature; in HCO_3^-/CO_2-buffered media, pH rises as temperature rises whereas, for HCO_3^-/CO_2-free media, pH falls as temperature rises. In the present experiments, under conditions where pH_o was maintained at a constant value (pH 7.35), as temperature was reduced from 37°C to 22°C, steady-state pH_i declined both in the presence and absence of HCO_3^-/CO_2. This result contrasts with those of Marjanvoic *et al* (1998) and Laptook *et al* (1995a and 1995b) who observed an inverse relationship between brain temperature and cerebral pH_i *in vivo*. The results of the present study suggest that the reduced steady-state pH_i values observed in isolated adult hippocampal neurons at 22°C may, at least in part, reflect reduced Na^+/H^+

exchange activity, as evidenced by the reduced slope of the relationship between pH_i vs. rate of pH_i recovery from imposed acid loads as temperature is reduced. A similar, steep dependence of Na^+/H^+ exchange activity on ambient temperature has previously been demonstrated for NHE1, NHE3, and for the NHE isoform present in hippocampal neurons (Baxter and Church, 1996; Bookstein *et al*, 1997) and has been suggested to reflect a change in the relative protonation of α-imidazole sites on histidine residues present on the Na^+/H^+ exchanger (Hauge and Kofstad, 1995). The relevance of the temperature-dependence of Na^+/H^+ exchange activity to anoxia is discussed in Section 6.3.2.

2 The Intracellular pH Response to a Transient Anoxic Period

pH_i transients are observed under both physiological and pathophysiological conditions and play a role in regulating neuronal function (see Introduction, Section 3.3). In acutely dissociated adult rat hippocampal CA1 neurons, a transient anoxic period induced an intracellular acidification that recovered slightly in the continued absence of oxygen, followed by a post-anoxic internal alkalinization. A similar pattern of an anoxic acidification followed by a post-anoxic alkalinization has been observed in cortical brain slices *in vitro* and in rat whole brain following ischemia, although, as noted above, the contribution of neuronal elements to the pH_i changes observed in these preparations could not be assessed (Mabe *et al*, 1983; Pirttilä and Kauppinen, 1992). The pH_i response to a transient period of anoxia observed in the present study is also similar, in many respects,

to the neuronal pH_i changes induced by seizure activity or the application of exogenous glutamate (Siesjö *et al*, 1985; Hartley and Dubinsky, 1993; Ou-Yang *et al*, 1995).

2.1 Studies of the intracellular pH response to anoxia in vivo and in slice preparations in vitro

The majority of studies have investigated the pH_i response to anoxia/ischemia using *in vitro* slice preparations and have reported an intracellular acidification that, upon reoxygenation, recovers to pre-anoxic steady-state pH_i levels; however, with the exceptions noted above, per-anoxic rises in pH_i and marked post-anoxic internal alkalinizations have not been commonly observed (Whittingham *et al*, 1984; Eleff *et al*, 1991; Fujiwara *et al*, 1992). A similar pattern of pH_i changes has also been observed when measuring the pH_i changes of CA1 neurons in response to a transient period of ischemia *in vivo* (Silver and Erecinska, 1992). The pH_i responses observed in the *in vitro* preparations are a composite average of pH_i changes occurring in numerous cell types which may respond differentially to anoxia/ischemia. Indeed, this has been suggested to underlie the heterogeneity of cellular pH_i changes observed in response to HCO_3^-/CO_2 removal in cortical brain slices (Pirttilä and Kauppinen, 1994b). Moreover, Kraig *et al* (1986) suggested that during anoxia, protons are heterogeneously distributed between separate neuronal and glial compartments, each contributing to the overall pH_i response to ischemia/anoxia (but see Katsura *et al*, 1992a). In addition, in *in vivo* studies and in studies using brain slices, extracellular pH (pH_o), a variable that directly influences pH_i and the activities of pH_i regulating mechanisms (see Section 1.2), is not controlled, and,

in fact in the latter preparation, varies along the depth of the slice (Krnjevic and Walz, 1990). Each of these potentially confounding variables are minimized when utilizing acutely dissociated hippocampal neurons and, thus, the results of this study illustrate the inherent neuronal pH_i response to a transient anoxic insult. If pH_o is reduced during and immediately following an anoxic insult, as occurs *in vivo*, the pH_i response observed in acutely dissociated neurons resembles the pH_i response expected to occur both in slice preparations and *in vivo* (see Section 6.3.1). This observation strongly supports both the validity of the neuronal pH_i response to anoxia observed in the present study as well as the method used to induce anoxia.

2.2 The role of extracellular HCO_3^- in the production of the intracellular pH response to anoxia

While an extensive literature exists concerning changes in the activities of Na^+/H^+ exchangers during and following a transient period of anoxia (see Sections 4.3 and 6.2), the potential role of HCO_3^--dependent pH_i regulatory mechanisms in the pH_i response to anoxia remains largely unknown. HCO_3^--dependent pH_i regulating mechanisms have been shown to be involved in the regulation of pH_o during spreading depression and cerebral ischemia (Mutch and Hansen, 1984), and have been shown to contribute to the recovery of pH_i following ischemia in cardiac tissue (Vandenberg *et al*, 1993) and following glutamate excitotoxicity in cultured hippocampal neurons (Hartley and Dubinsky, 1993). Conversely, in cortical brain slices, DIDS fails to influence the pH_i response to hypoxia (Pirttilä and Kauppinen, 1994a). In further support for the lack of

involvement of changes in the activities of HCO_3^--dependent pH_i regulating mechanisms, in the present study the pH_i response to anoxic periods of various durations was statistically similar in all respects in the presence or absence of extracellular HCO_3^-. Thus, HCO_3^--dependent pH_i regulating mechanisms are unlikely to be major contributors to the pH_i response to anoxia in adult rat hippocampal CA1 neurons, suggesting that the Na^+/H^+ exchanger may be central to the production of the pH_i response to transient periods of anoxia in this cell type.

Despite the present results which suggest that changes in the activities of HCO_3^--dependent pH_i regulatory mechanisms are not involved in the production of the pH_i response to transient periods of anoxia, their possible contribution cannot be unequivocally ruled out. The presence of extracellular HCO_3^- has been shown to both stimulate Na^+/H^+ exchange in renal epithelia (Chen and Boron, 1995) and inhibit Na^+/H^+ exchange in a mouse embryo cell line (Gillies and Martinez-Zaguilan, 1991). Furthermore, the presence of extracellular HCO_3^- increases extracellular buffering capacity, an effect which, independent of HCO_3^--dependent transport mechanisms, inhibits the magnitude of the pH_i alkalinization observed following an hypoxic insult in cortical neurons (Pirttilä and Kauppinen, 1993).

3 The Intracellular Calcium Response to a Transient Anoxic Period

A profound and rapid increase in intracellular $[Ca^{2+}]$ is consistently observed during periods of anoxia/ischemia (Lipton and Lobner, 1990; Mitani *et al*, 1994), excitotoxicity (Wang *et al*, 1994; Budd and Nicholls, 1996), and spreading depression

(Erecinska and Silver, 1996). In fact, the size and duration of these internal Ca^{2+} transients, as well as their origin, may directly determine subsequent neuronal survival (Tymianski *et al*, 1993; Kristiane and Siesjö, 1997). Among the most sensitive regions of the brain to anoxia-induced neuronal damage is the hippocampal CA1 region and, in the present study, there was a rapid increase in $[Ca^{2+}]_i$ within two minutes of the initiation of an anoxic insult and $[Ca^{2+}]_i$ remained elevated following the return to normoxia. These data confirm those of Friedman and Haddad (1993), whose laboratory used an identical tissue preparation and method of inducing anoxia.

Considering the dispersed nature of these dissociated cells, along with the constant flow of perfusate, an anoxia-evoked release of glutamate and subsequent activation of *N*-methyl-D-aspartate receptors appears unlikely to be able to account for the rapid influx of extracellular Ca^{2+}, although this possibility was not directly examined. Rather, the initial increase in $[Ca^{2+}]_i$ is likely due to the activation of voltage-sensitive calcium channels consequent upon membrane depolarization and the subsequent influx of extracellular Ca^{2+} (also see Friedman and Haddad, 1993). Interestingly, $[Ca^{2+}]_i$ did not recover even upon the return to normoxia (also see Friedman and Haddad, 1993), suggesting that the neurons are unable to repolarize in the immediate post-anoxic period (Tanaka *et al*, 1997; Yamamoto *et al*, 1997). The combination of membrane depolarization and an anoxia-evoked elevation in $[Na^+]_i$ (see Section 4.3) may lead to the reversal of the Na^+/Ca^{2+} exchanger which could also contribute to maintaining an elevated $[Ca^{2+}]_i$ (Stys, 1998).

In the absence of extracellular Ca^{2+}, a small and transient increase in $[Ca^{2+}]_i$ occurred in the post-anoxic period which may tentatively be attributed to the release of Ca^{2+} from intracellular stores. Inositol 1,4,5-trisphosphate- ($InsP_3$) -sensitive, rather than Ca^{2+}-sensitive, stores have been described in hippocampal neurons and have been shown to be involved in the anoxic response of these cells (Belousov et al, 1995; Erecinska and Silver, 1996). Thus, intracellular and extracellular Ca^{2+} both likely contribute to the production of the anoxia-evoked increase in $[Ca^{2+}]_i$ in acutely dissociated adult rat CA1 hippocampal neurons.

3.1 Role of calcium in the production of the intracellular pH response to anoxia

An increase in $[Ca^{2+}]_i$ can induce an intracellular acidification at least in part by competing with protons for shared intracellular binding sites (see Ives and Daniel, 1987). Influx of Ca^{2+} can also stimulate Ca^{2+}/H^+ ATPase activity both in snail neurons (e.g. Schwiening et al, 1993) and, possibly, in rat hippocampal neurons (Trapp et al, 1996), an effect which would also promote an internal acidification. However, in the present study, neither the fall in pH_i evoked by anoxia (nor any of the other phases of the pH_i response to anoxia) were influenced by the removal of extracellular Ca^{2+}. In addition, the time course of the pH_i response to anoxia was not influenced by the removal of extracellular Ca^{2+}. Thus, anoxia-evoked changes in pH_i are not secondary to anoxia-evoked changes in $[Ca^{2+}]_i$ in acutely dissociated adult rat hippocampal CA1 neurons.

4 Factors Contributing to the Anoxic Acidification

A decrease in pH_i is often the first observed event in the neuronal response to an anoxic or ischemic insult and, as such, may initiate and/or regulate subsequent changes in neuronal function (Silver and Erecinska, 1990; Martin *et al*, 1994). In the present study, anoxic periods of either 3, 5 or 10 min duration consistently produced an initial intracellular acidification. Previous studies have attributed the intracellular acidification to either the failure to regenerate ATP or to the increased production of metabolic acids (*e.g.* lactic acid). In addition, the present study suggests that a reduction in Na^+/H^+ exchange activity during anoxia may also contribute to the fall in pH_i observed.

4.1 Intracellular ATP during anoxia

Within the central nervous system, under normoxic conditions, ATP is primarily generated through oxidative phosphorylation; however, this process is inhibited during anoxia and glycolysis becomes the sole source of ATP. Unlike oxidative phosphorylation, glycolysis does not recycle the protons that are liberated during ATP hydrolysis (Dennis *et al*, 1991; Katsura *et al*, 1992b). Thus, even in the absence of lactate accumulation (see Section 4.2), protons accumulate due to an increasing dependence on anaerobic metabolism (Smith *et al*, 1993; Espanol *et al*, 1998). Given the profound reduction in intracellular ATP observed during anoxia (Lipton and Lobner, 1990; Mitani *et al*, 1994), the release of protons during ATP hydrolysis likely contributes to the intracellular acidosis evoked by anoxia in the present study.

The Na^+/K^+ ATPase is the primary consumer of ATP in neurons (Erecinska and Silver, 1989); thus, ATP levels may be preserved during periods of anoxia by maintaining the transmembrane Na^+ gradient, thereby reducing Na^+/K^+ ATPase activity. Indeed, in rat hippocampal slices, inhibition of Na^+ influx during anoxia attenuates anoxia-evoked falls in ATP (Hansen, 1985; Boening et al, 1989; Fried et al, 1995; Fowler and Li, 1998). Given that ATP hydrolysis contributes to the intracellular acidification observed during anoxia (see above), it follows that either the application of TTX or the removal of extracellular Na^+ should attenuate the magnitude of the anoxic acidification. Indeed, this is what was observed in the present study. Alternatively, it has been suggested that TTX may inhibit the anoxic acidification by reducing the anoxia-evoked Ca^{2+} influx mediated by reverse Na^+/Ca^{2+} exchange (Yamaguchi et al, 1997). However, the present study does not support this possibility in that neither the magnitude of the acidification, nor its time course, was affected by the removal of extracellular Ca^{2+}.

4.2 Intracellular lactate during anoxia

The end-products of anaerobic glycolysis are two molecules of lactic acid per molecule of glucose metabolized. It has been suggested that anoxia-evoked reductions in pH_i are directly proportional to intracellular lactate accumulation, which is, in turn, determined by the pre-anoxic/ischemic glucose concentration (Ljunggren et al, 1974; Smith et al, 1986; White et al, 1989; Katsura et al, 1992a; Roberts Jr. et al, 1998). However, others have suggested that a discontinuous relationship exists between internal lactate concentrations and pH_i (Kraig et al, 1986). In support of a continuous relationship

between lactate accumulation and pH$_i$, in the present study a significantly smaller intracellular acidification was evoked by anoxia in the presence of 5.0 mM glucose compared with 17.5 mM glucose. Indeed, the absolute magnitudes of the anoxia-evoked intracellular acidifications produced in the present study in the presence of 5.0 mM or 17.5 mM glucose are similar to those reported in rat hippocampal slices when the pre-anoxic glucose concentration was varied from 5 - 15 mM (Roberts Jr. *et al*, 1998). The ability of lactate to produce an intracellular acidification is reduced under reduced-Na$^+$ conditions (Pirttilä and Kauppinen, 1992), a finding which may contribute to the attenuation of the anoxic acidification observed under reduced-Na$^+$ conditions in the presence of 17.5 mM glucose. The absence of a similar effect in the presence of 5.0 mM glucose may be secondary to the reduced accumulation of lactate.

4.3 Changes in Na$^+$/H$^+$ exchange activity

In cardiac tissue, Na$^+$/H$^+$ exchange inhibition does not influence the magnitude of the intracellular acidification observed in response to ischemia (Askenasy *et al*, 1996; Piper *et al*, 1996). These results suggest that either the exchanger is not involved in pH$_i$ regulation during anoxia (Pike *et al*, 1993; Askenasy *et al*, 1996) or that the exchanger is inhibited during anoxia (Piper *et al*, 1996; Maddaford and Pierce, 1997). In support of the latter possibility, the present data indicate for the first time that, in rat hippocampal neurons, the Na$^+$/H$^+$ exchanger is inhibited during an anoxic period and may contribute to the production of the observed intracellular acidification. Thus, the activity of the Na$^+$/H$^+$ exchanger was significantly slowed under anoxic conditions and there was an acidic shift

in its pH_i sensitivity. A similar inhibition of Na^+/H^+ exchange activity has been demonstrated during perfusion with the metabolic inhibitor, 2-deoxyglucose, in cardiac myocytes (Wu and Vaughan-Jones, 1994). However, despite its reduced activity, the Na^+/H^+ exchanger in hippocampal neurons is still partially active during anoxia. As has been demonstrated in peripheral cell types (Pike *et al*, 1993; Satoh *et al*, 1995; but see Tani and Neely, 1989; Ruβ *et al*, 1996), this residual activity may contribute to anoxia-evoked increases in $[Na^+]_i$ which occur in acutely dissociated adult rat hippocampal CA1 neurons (Fig 38; also see Friedman and Haddad, 1994b). In addition, this residual Na^+/H^+ exchange activity may contribute to the reduction in pH_o seen during anoxia *in vivo*, as well as the production of anoxia-induced cellular swelling (Jakubovicz and Klip, 1989; Obrenovich *et al*, 1990).

Thus, the present data indicate that the Na^+/H^+ exchanger present in rat hippocampal neurons is inhibited during an anoxic period. What is the identity of the factor(s) causing the reduction in Na^+/H^+ exchange activity? Firstly, during an anoxic period, increased phosphocreatine (PCr) and ATP hydrolysis produce a rise in the intracellular concentrations of inorganic phosphates, which are important physiological intracellular buffers (Burton, 1978; Wu and Vaughan-Jones, 1994). An increase in β_i could potentially contribute to the observed decrease in the rate of acid extrusion. Arguing against this possibility, however, are the present findings that β_i does not change dramatically during an anoxic period. Secondly, in peripheral cell types *in vivo*, anoxia-induced inhibition of Na^+/H^+ exchange is a result of a decrease in pH_o (see Section 1.2);

however, in the present study, pH_o was maintained at 7.35 throughout the anoxic period and yet a reduction in Na^+/H^+ exchange activity was still observed.

An alternate explanation for the inhibition of Na^+/H^+ exchange activity observed during anoxia in the present study is that the activity of the Na^+/H^+ exchanger in rat hippocampal neurons may be dependent upon intracellular ATP levels. In rat hippocampal slices, there is an ~ 50% reduction in intracellular ATP levels after 5 min of anoxia (Boening et al, 1989; Lipton and Lobner, 1990). For Na^+/H^+ exchange isoforms 1, 2 and 3, ATP depletion causes both a decrease in the V_{max} and a reduced sensitivity of the antiporters for internal protons (Cassel et al, 1986; Little et al, 1988; Levine et al, 1993; Kapus et al, 1994). These changes in Na^+/H^+ exchange activity are analogous to those changes observed in the present study during an anoxic period. ATP depletion inhibits Na^+/H^+ exchange activity independent of changes in the phosphorylation levels of the exchangers and in the presence of maintained ionic gradients (Goss et al, 1994). In a manner similar to β_2-adrenoceptor stimulation of Na^+/H^+ exchange activity (Hall et al, 1998), an associated regulatory protein may sense intracellular ATP levels and, in turn, influence Na^+/H^+ exchange activity (Goss et al, 1994). Although the internal ATP-dependence of the Na^+/H^+ exchanger present in rat hippocampal neurons has not been formally assessed, the possibility remains that the reduction in Na^+/H^+ exchange activity observed during anoxia in the present study may reflect anoxia-evoked reductions in intracellular ATP concentration. Nevertheless, it should be noted that TTX would be expected to preserve ATP levels during anoxia (see Section 4.1), however, Na^+/H^+ exchange activity during anoxia was reduced to a similar extent in the presence or

absence of TTX. This incongruity may imply that: i) TTX maintains internal ATP at a level such that the activities of secondary active transport mechanisms (i.e. Na^+/H^+ exchange) are reduced while, given its preferential sensitivity for ATP derived from glycolysis as well as its low K_m for ATP, the Na^+/K^+ ATPase is functional, albeit at a slower rate (Erecinska and Silver, 1989); or ii) alternate mechanisms, *e.g.* anoxia-evoked changes in second messenger systems, are acting to inhibit Na^+/H^+ exchange activity during anoxia.

As opposed to the reduced levels of intracellular ATP inhibiting Na^+/H^+ exchange activity *during* an anoxic period, the rapid restoration of intracellular ATP levels following an anoxic period may contribute to the post-anoxic activation of Na^+/H^+ exchange (see Section 6.2). Indeed, in cardiac myocytes, the application of exogenous ATP produces an intracellular alkalinization as a result of enhancing Na^+/H^+ exchange activity and shifting the sensitivity of the antiporter to more alkaline values (Puceat and Vassort, 1995).

While the roles of ATP hydrolysis and lactate accumulation are further supported by the present data, this study suggests that inhibition of Na^+/H^+ exchange may also play a role in the production of the intracellular acidification seen during a transient anoxic insult. Residual Na^+/H^+ exchange activity may be linked to the anoxia-evoked increase in $[Na^+]_i$ and decrease in pH_o evoked by anoxia *in vivo*.

4.4 Physiological relevance of the anoxic acidification

The physiological importance of the intracellular acidification seen during an anoxic insult is confused by conflicting data which indicate that, on the one hand, acidosis is neuroprotective and, on the other hand, that it is detrimental to neuronal survival (see Introduction, Section 3.3). However, the relationship between a decrease in pH_i and neuronal viability may be governed by a step function (Tombaugh and Sapolsky, 1993; Li and Siesjö, 1997). Thus, on the one hand, mild intracellular acidosis improves the functional recovery of hippocampal slices following anoxia (Roberts Jr. *et al*, 1998) and inhibits anoxia-induced neuronal damage (Giffard *et al*, 1990), possibly by stimulating glycolysis and/or reducing anoxia-induced intracellular Ca^{2+} overload (Tombaugh and Somjen, 1997). On the other hand, excessive and/or prolonged acidosis is detrimental to neuronal survival following anoxia (Nedergaard *et al*, 1991). For example, a large intracellular acidification of approximately 1.0 pH units increases the size of the infarcted core following ischemia/anoxia (Kraig *et al*, 1987). In the present study, the reduced intracellular acidification observed in response to an hypoxic/anoxic insult in the presence of TTX or mild hypothermia may reflect the increased likelihood of cellular recovery under these conditions.

Although conflicting results have been reported under *in vivo* and *in vitro* preparations (Li and Siesjö, 1997), a similar step function may also explain the ability of variations in pre-ischemic extracellular glucose concentrations and lactate accumulation during an anoxic period to influence neuronal viability following an anoxic period. Thus, while increasing glucose concentrations slightly, from 5 to 15 mM, improves the

recovery of synaptic function following anoxia (Roberts Jr., *et al*, 1998), a significant increase in pre-ischemic glucose concentration, to ~ 25 mM, inhibits the recovery of ATP and PCr levels in the post-anoxic period and augments neuronal damage (LeBlanc *et al*, 1993; Tyson *et al*, 1993; Li and Siesjö, 1997). Similarly, while intracellular lactate has been suggested to be the preferred neuronal energy source in the post-anoxic period (Schurr *et al*, 1988; Schurr *et al*, 1997), a number of studies have documented the neurotoxic effects of marked lactic acidosis (Kraig *et al*, 1997; Siesjö *et al*, 1996).

5 Factors Contributing to the Per-Anoxic Rise in Intracellular pH

In the continued absence of oxygen, pH_i slowly recovered from the maximum intracellular acidification induced by anoxia at a rate of ~ 0.04 pH units min^{-1}. A similar per-anoxic recovery of pH_i has previously been observed during a transient period of glucose and oxygen deprivation in mouse hippocampal slices (Fuijwara *et al*, 1992; Melzian *et al*, 1996).

5.1 Role of a voltage-activated proton conductance

In response to anoxia/ischemia, hippocampal neurons transiently hyperpolarize. This hyperpolarization is quickly followed by a slow depolarization, which concludes in a rapid depolarization (Tanaka *et al*, 1997; Yamamoto *et al*, 1997). This rapid anoxic depolarization occurs within the first few minutes following the induction of anoxia (Donnelly *et al*, 1992; Roberts Jr. *et al*, 1998). During anoxia, this dramatic membrane depolarization may activate a voltage-sensitive proton conductance (g_{H^+}), which produces

an outward proton current and a rise in pH_i, as has been described in snail neurons and a variety of peripheral cell types (Meech and Thomas, 1987; Gordienko et al, 1996; Kapus et al, 1993). In addition, the fall in pH_i induced by anoxia would act to lower the threshold voltage for activation of g_{H^+} (Lukacs et al, 1993). Furthermore, the anoxia-evoked increase in $[Ca^{2+}]_i$ observed in the present study is likely consequent upon depolarization (Friedman and Haddad, 1993) and an elevation in $[Ca^{2+}]_i$ has been shown to potentate g_{H^+} in eosinophils, suggesting that the anoxia-evoked increase in $[Ca^{2+}]_i$ may also contribute to a significant activation of g_{H^+} (Gordienko et al, 1996). Finally, Na^+/H^+ exchange can couple to H^+ conductances, such that inhibition of Na^+/H^+ exchange, as occurs during anoxia, can potentiate the outward proton conductance (Demaurex et al, 1995). Thus, activation of g_{H^+} is entirely likely to occur during an anoxic period.

Although the possible presence of a g_{H^+} in rat hippocampal neurons has not been investigated, in all cell types studied to date sub-millimolar concentrations of $ZnCl_2$ effectively inhibit g_{H^+} (DeCoursey and Cherny, 1994). In the present study, the addition of 500 μM $ZnCl_2$ reduced the magnitude of the pH_i rise seen during a 10 min period of anoxia, suggesting the possibility that an outward H^+ flux may underlie the rise in pH_i observed during anoxia. Furthermore, g_{H^+} is regulated by pH_o; an increase in pH_o stimulates, while a decrease in pH_o inhibits, current flow through these channels (Lukacs et al, 1993). This sensitivity to pH_o may reflect a change in the concentration gradient for protons and charge screening, although the binding of protons to an allosteric extracellular site is also postulated to modify the activation properties of this conductive pathway (Lukacs et al, 1993; DeCoursey and Cherny, 1994). In the present study, there

was a greater rise in pH_i during anoxia under extracellular alkaline conditions and a smaller rise in pH_i under extracellular acidic conditions, compared to the results obtained at pH_o 7.35. These results once again support the potential involvement of a g_{H^+} in mediating the rise in pH_i observed during anoxia, although the role of changes in pH_i consequent upon changing pH_o in the regulation of g_{H^+} is unknown. The decreased magnitude of the rise in pH_i observed during anoxia at 22°C compared to 37°C may reflect the high temperature sensitivity of g_{H^+}, which produces a larger current at more elevated temperatures (Byerly and Suen, 1989), although a corresponding reduction in cerebral metabolic rate and, thus, an increase in the latency to anoxic depolarization cannot be excluded (Bart et al, 1998). Physiologically, the activation of g_{H^+} may allow for the rapid alleviation of intracellular acidosis evoked by anoxia (DeCoursey and Cherny, 1994) and, thus, may play a role in pH_i regulation during an anoxic insult.

Arguing against an involvement of g_{H^+} in the rise in pH_i during anoxia are the facts that neither the removal of extracellular Na^+ nor the addition of 1 μM TTX influenced the relative magnitude of the per-anoxic recovery. Although these maneuvers are able to delay the onset of and/or reduce the magnitude of the rapid depolarization evoked by anoxia in rat hippocampal slices (Tanaka et al, 1997; Yamamoto et al, 1997), in the present study, neither influenced the time to onset of the rise in pH_i observed during the anoxic period (data not shown). However, during an hypoxic insult in carotid body chemoreceptor cells, the ability of TTX to inhibit voltage-dependent Na^+ channels is inversely proportional to the severity of the membrane depolarization induced by hypoxia (Rocher et al, 1994). A similar situation may occur under reduced-Na^+

conditions. Thus, the possibility remains that voltage-sensitive proton channels are activated during anoxia and may contribute to the rise in pH_i observed in adult rat hippocampal neurons.

5.2 Role of lactate transport during anoxia

Secondary to lactate accumulation during an anoxic period, the efflux of lactate contributes to the extracellular acidification observed during an ischemic/anoxic insult (Siesjö et al, 1985). Moreover, carrier-mediated lactate transport has been described in hippocampal neurons, and the extrusion of lactate is associated with an intracellular alkalinization (Assaf et al, 1990; Kauppinen and Williams, 1990; Nedergaard and Goldman, 1993; Pirttilä and Kauppinen, 1994b). Thus, with lactate accumulating intracellularly during an anoxic period (see Section 4.2), extrusion of lactate via lactate/H^+ cotransport may contribute to the rise of pH_i observed during an anoxic period. In support of this possibility, in the present study, there was a larger rise in pH_i during anoxia in the presence of 17.5 mM glucose than in the presence of 5.0 mM glucose, a finding consistent with the fact that lactate accumulation, and thus lactate efflux, varies with pre-ischemic glucose concentrations (Ljunggren et al, 1974).

Studies examining neuronal lactate/H^+ cotransport have failed to observe any significant effect of 4α-5-hydroxycinnamate (α-CHC), an effective lactate/H^+ cotransport inhibitor in peripheral cell types, on either the rate of lactate uptake or its extrusion in mammalian central neurons and glia (Walz and Mukerji, 1988; Nedergaard and Goldman, 1993; Pirttilä and Kauppinen, 1994b; but see Dringen et al, 1993). Similarly, in

preliminary studies, 5 mM α-CHC fails to affect resting pH_i in acutely dissociated adult rat hippocampal neurons (data not shown). While previous reports in neurons have discounted the contribution of lactate/H^+ cotransport to pH_i regulation *during* anoxia (Kauppinen and Williams, 1990; Prittilä and Kauppinen, 1992), the present study is unable to do so.

Following an ischemic period, the removal of accumulated intracellular lactate is central to the recovery of pH_i in cardiac tissue. (Vandenberg *et al*, 1993). In the present study, given that the magnitude of the post-anoxic alkalinization was not influenced by reducing extracellular glucose concentration from 17.5 to 5 mM glucose, lactate/H^+ cotransport is unlikely to play a major role, if any, in the recovery of pH_i following a transient period of anoxia in rat hippocampal neurons. Indeed, in cortical brain slices, lactate transport proceeded at a significantly slower rate than pH_i recovery following anoxia (Pirttilä and Kauppinen, 1992).

In summary, the results of the present study suggest, albeit indirectly, the presence of two additional pH_i regulating mechanisms in adult rat hippocampal neurons, namely, a voltage-dependent proton conductance and/or a lactate/H^+ cotransporter. Furthermore, these mechanisms may contribute to the rise in pH_i observed during anoxia and, in doing so, may help to counteract a potentially harmful profound decline in pH_i.

6 Factors Contributing to the Post-Anoxic Alkalinization

Following a transient anoxic period, there was an intracellular alkalinization above the initial pre-anoxic, steady-state pH_i from which the majority of cells did not

show any recovery. Intracellular pH_i "overshoots" have been observed following ischemia (both *in vivo* and in slice preparations *in vitro*), acute glutamate application, and induced seizure activity (Mabe *et al*, 1983; Siesjö *et al*, 1985; Yoshida *et al*, 1985; Pirttilä and Kauppinen, 1992; Hartley and Dubinsky, 1993; Ou-Yang *et al*, 1995), although in none of these cases have the underlying mechanism(s) been identified.

6.1 Role of a voltage-activated proton conductance

One possible factor which may contribute to this phase of the pH_i response to transient anoxia is a persistent neuronal depolarization which, in turn, activates proton efflux through voltage-dependent H^+ channels. Once anoxic depolarization occurs, the return to normoxia is not necessarily associated with membrane repolarization, and, in fact, a further depolarization may be seen (Tanaka *et al*, 1997; Yamamoto *et al*, 1997). Increasing the duration of anoxia not only increases the likelihood of anoxic depolarization, but also decreases the likelihood of repolarization upon the return to normoxia (O'Reilly *et al*, 1995).

In the present study, $[Ca^{2+}]_i$ remained elevated in the post-anoxic period, suggesting that repolarization does not occur (*e.g.* Silver and Erecinska, 1990; Tanaka *et al*, 1997; Pisani *et al*, 1998). Furthermore, 500 μM Zn^{2+} reduced the magnitude of the post-anoxic alkalinization following a 10 min period of anoxia. Therefore, a g_{H^+} active in the immediate post-anoxic period may contribute to the intracellular alkalosis observed following a transient anoxic insult in adult rat hippocampal CA1 neurons.

6.2 Changes in Na$^+$/H$^+$ exchange activity

In cardiac myocytes, the Na$^+$/H$^+$ exchanger is activated immediately upon reperfusion following an ischemic/anoxic insult (Maddaford and Pierce, 1997) and is primarily responsible for both the post-anoxic increase in [Na$^+$]$_i$ and the recovery of pH$_i$ from the intracellular acidosis produced during the anoxic period (Tani and Neely, 1989; Docherty *et al*, 1997). In the present study, substituting extracellular Na$^+$ with NMDG$^+$ (a maneuver which inhibits the Na$^+$/H$^+$ exchanger) attenuated the magnitude of the post-anoxic alkalinization, whereas, when Li$^+$ was employed as the extracellular Na$^+$ substitute, this phase of the pH$_i$ response was restored. These findings suggest that the Na$^+$/H$^+$ exchanger in rat hippocampal neurons is activated immediately following the return to normoxia and contributes to the rise in pH$_i$ observed after the anoxic period. This possibility was confirmed directly by comparing the rates of pH$_i$ recovery from acid loads imposed under HCO$_3^-$-free conditions both prior to and following anoxia. Following a transient anoxic period, there was an increase in Na$^+$/H$^+$ exchange activity, along with an alkaline shift in its pH$_i$ sensitivity.

The present results are consistent with previous reports which have suggested that activation of Na$^+$/H$^+$ exchange activity may participate in the restoration of pH$_i$ following the intracellular acidosis evoked by anoxia. Thus, in hippocampal slices, Pirttilä and Kauppinen (1992) suggested that while the Na$^+$/H$^+$ exchanger was not functional during an anoxic period, its activity was central to the production of the post-anoxic overshoot in pH$_i$. Furthermore, Obrenovitch *et al* (1990) suggested that the activation of Na$^+$/H$^+$ exchange contributes to the extracellular acidosis observed following an anoxic period.

And, in cultured fetal cortical neurons, Vornov et al (1996) employed pharmacological inhibitors to similarly conclude that inhibition of Na^+/H^+ exchange decreases the rate of recovery of pH_i following a transient period of metabolic inhibition. However, none of these studies directly assessed Na^+/H^+ exchange activity in the post-anoxic period.

6.2.1 Possible factors contributing to the increase in Na^+/H^+ exchange activity

Given that there is a significant activation of Na^+/H^+ exchange activity in the post-anoxic period, the relevant issue then becomes the identity of the underlying activating factor(s).

One factor that could potentially contribute to the increased Na^+/H^+ exchange activity following an anoxic insult is a reduction in intrinsic buffering power (β_i). However, present estimates of β_i under normoxic and post-anoxic conditions, at pH_o 7.35 and over a similar pH_i range, do not support this possibility. Moreover, over a range of pH_o values and temperatures, there was no change in β_i prior to or following an anoxic period. Thus, the observed increase in the rate of pH_i recovery following an anoxic period appears to be unlikely to reflect a decrease in β_i.

Secondly, in cardiac myocytes *in vivo*, the intracellular acidification produced during anoxia and the rapid normalization of pH_o which occurs in the immediate post-anoxic period act to stimulate Na^+/H^+ exchange activity (Tani and Neely, 1989; Maddaford and Pierce, 1997). However, corresponding changes in pH_i and pH_o are not the only factors which promote the post-anoxic activation of the Na^+/H^+ exchanger in rat hippocampal neurons because: i) pH_o was kept constant throughout the experiment and

yet Na^+/H^+ exchange activation still occurred upon the return to normoxia; and ii) in some cases, pH_i recovered to pre-anoxic steady-state levels in the continued absence of oxygen (*e.g.* Fig. 8B), but, once again, post-anoxic activation of the Na^+/H^+ exchanger occurred.

Thirdly, the sustained increase in lactate levels which occurs in the immediate post-anoxic period (Kobayashi *et al*, 1977; Hope *et al*, 1988; Kauppinen and Williams, 1990) may stimulate Na^+/H^+ exchange activity. The metabolism of lactate generates protons, whose intracellular accumulation activates Na^+/H^+ exchange, thereby contributing to the production of the post-anoxic alkalinization (Mabe *et al*, 1983; Katsura *et al*, 1992). Indeed, the detrimental effect of lactate accumulation on cellular recovery following an ischemic period in cardiac myocytes is reportedly mediated through a post-anoxic stimulation of Na^+/H^+ exchange (Karmazyn *et al*, 1993). However, in the present study, there was no difference in the magnitude of the post-anoxic alkalinization produced in media containing 17.5 or 5.0 mM glucose, conditions in which the intracellular levels of lactate will vary.

Finally, changes in cellular energy state and the activities of intracellular second messenger systems which act to control the activity of the Na^+/H^+ exchanger may contribute to the activation of the exchanger in the immediate post-anoxic period. The possibility that a rapid restoration of intracellular ATP may trigger or permit Na^+/H^+ exchange activation has already been discussed (see Section 4.3). An additional possibility is that anoxia-evoked changes in the activities of intracellular second messenger systems may produce the observed increase in Na^+/H^+ exchange activity. Thus, in hippocampal neurons, stimulation of the cAMP/PKA pathway causes both an

increase in the slope and a large alkaline shift in the x-intercept of the linear regression line relating rates of pH_i recovery to absolute pH_i (Smith et al, 1998). These changes in Na^+/H^+ exchange activity are analogous to those observed in the present study following a transient period of anoxia, raising the possibility that the increase in the activity of the Na^+/H^+ exchange activity in the post-anoxic period may be consequent upon stimulation of the cAMP/PKA pathway. Indeed, during recovery from an ischemic insult, cAMP levels rise rapidly (Kobayashi et al, 1977; Domanska-Janik and Pylova, 1989; Blomqvist et al, 1985) and stimulus-evoked accumulations of $[cAMP]_i$ are enhanced (Domanska-Janik and Pylova, 1989). Furthermore, in the present study, the addition of Rp-cAMPS, a PKA inhibitor, significantly decreased the magnitude of the post-anoxic alkalinization. On the other hand, the addition of β-adrenoceptor agonists, which stimulate the cAMP/PKA pathway, significantly increased the magnitude of the post-anoxic alkalinization. Thus, a post-anoxic increase in $[cAMP]_i$ may contribute to the post-anoxic activation of Na^+/H^+ exchange in rat hippocampal neurons.

As will be discussed in the following section (Section 6.3), just as an increase in Na^+/H^+ exchange activity following anoxia promotes cellular dysfunction, an increase in $[cAMP]_i$ is also apparently correlated with an increased likelihood of neuronal damage following anoxia/ischemia (Domanska-Janik and Pylova, 1989; Shibata et al, 1992). Thus, the intracellular cascade of increased Na^+/H^+ exchange activity in the post-anoxic period promoting neuronal damage may be due to an increased activity of the cAMP/PKA second messenger system.

6.3 Potential physiological relevance of the post-anoxic activation of Na^+/H^+ exchange

In peripheral cell types, for example cardiac myocytes, it is well established that Na^+/H^+ exchange inhibition effectively protects against reperfusion-induced cellular injury following ischemia (reviewed by Scholz and Albus, 1993; Piper *et al*, 1996; Bugge *et al*, 1996). In addition, in cultured fetal neocortical neurons, it has recently been reported that neuronal survival following metabolic inhibition (2-deoxy-D-glucose and cyanide) was improved when the Na^+/H^+ exchanger was inhibited (Vornov *et al*, 1996). However, the precise nature of the relationship between Na^+/H^+ exchange activity in the post-anoxic period and cellular viability remains unknown.

On the one hand, Na^+/H^+ exchange activity following anoxia may cause an increase in intracellular Na^+, which, in combination with membrane depolarization, could reverse the activity of the Na^+/Ca^{2+} exchanger. Through activation of various proteases, endonucleases, and phospholipases, the resulting Ca^{2+} overload may ultimately initiate cell death (Choi, 1990; Herman *et al*, 1990; Arai *et al*, 1991). Thus, inhibition of Na^+/H^+ exchange in the immediate post-anoxic period may reduce potentially harmful increases in $[Na^+]_i$ and $[Ca^{2+}]_i$ following anoxia (Tani and Neely, 1989). On the other hand, recent studies have suggested a role of Na^+/H^+ exchange, independent of changes in $[Ca^{2+}]_i$, in the regulation of cellular viability, indicating that the normalization of pH_i following a transient anoxic period *per se* promotes cell death (Bond *et al*, 1993), and that cytoprotection does not necessarily require the attenuation of a post-anoxic increase in $[Ca^{2+}]_i$ (Harper *et al*, 1993). In support of a dissociation between Na^+/H^+ and reverse

Na^+/Ca^{2+} exchange activity following anoxia, inhibition of Na^+/H^+ exchange in isolated cardiac myocytes blocks the apparently deleterious restoration of pH_i in the post-anoxic period while not influencing the rise in $[Ca^{2+}]_i$ (Ruβ *et al*, 1996). A role for Na^+/H^+ exchange in *neuro*protection, independent of changes in $[Ca^{2+}]_i$, has also been suggested in spinal cord axons, where Na^+/H^+ exchange inhibitors improved, and Na^+/Ca^{2+} exchange inhibitors did not improve, functional recovery following spinal cord compression (Agrawal and Fehlings, 1996). Similarly, in the present study, the anoxia-induced cellular injury that occurred during and following a transient anoxic period (as indicated by membrane bleb formation and swelling) continued to occur in the absence of extracellular Ca^{2+}, a condition which attenuated the anoxia-evoked increases in $[Ca^{2+}]_i$ (also see Friedman and Haddad, 1994a), suggesting that an increase in $[Ca^{2+}]_i$ is not an absolute prerequisite for acute damage in response to anoxia in acutely dissociated hippocampal neurons. Thus, the post-anoxic increase in Na^+/H^+ exchange activity observed in the present study may promote neurodegeneration following anoxia, not by modulating $[Ca^{2+}]_i$, but by increasing in $[Na^+]_i$ and decreasing $[H^+]_i$. A decrease in $[H^+]_i$ activates the majority of degredative enzymes (Herman *et al*, 1990). Additionally, overactivity of Na^+/H^+ exchange, and influx of Na^+ through voltage-sensitive Na^+ channels, will increase $[Na^+]_i$ and may contribute to a worsening of cellular energy state (Erecinska and Dagani, 1990), a reduction in Ca^{2+} extrusion, and an initiation of early osmotic injury (Jakubovicz and Klip, 1997).

6.3.1 The modulatory effects of changes in extracellular pH

Profound shifts in extracellular pH occur during and following transient ischemic periods *in vivo* and in slice preparations *in vitro* (Mutch and Hansen, 1984; Siesjö *et al*, 1985; Erecinska and Silver, 1994). As noted in the Introduction (see Section 3.2), these changes in pH_o may have a direct influence on neuronal survival. Indeed, following an ischemic insult, an increase in pH_o increases the susceptibility of neurons within the penumbral area to damage (Choi, 1990). Conversely, mild extracellular acidosis exerts a neuroprotective effect attributed to reduced NMDA-mediated excitotoxicity, reduced intracellular Ca^{2+} and Na^+ overload, and the suppression of neuronal excitability and a consequent reduction in metabolic energy demand (Giffard *et al*, 1990; Krnjevic and Walz, 1990; Tombaugh and Sapolsky, 1990; Cummins *et al*, 1991; Ebine *et al*, 1994; Ou-Yang *et al*, 1994a; Tombaugh and Somjen, 1996; Vornov *et al*, 1996). Given the relationship between the post-anoxic activation of Na^+/H^+ exchange and neuronal survival (see Section 6.3), the results of the present study indicate that the established neuroprotective effects of extracellular acidosis and the detrimental effects of extracellular alkalosis may also reflect their influence on the absolute level of Na^+/H^+ exchange activity in the immediate post-anoxic period. Thus, the rate of Na^+/H^+ exchange activity in the post-anoxic period was significantly faster at pH_o 7.60 and significantly reduced at pH_o 6.60. As pH_o was increased, there was a progressive increase in the slope, and alkaline shift in the x-intercept, of the linear regression lines relating pH_i and rates of pH_i recovery from intracellular acid loads imposed following an anoxic period. Thus, the

ability of changes in pH_o to influence neuronal viability following a transient anoxic period may be mediated, at least in part, by changes in the degree of post-anoxic activation of Na^+/H^+ exchange.

6.3.2 The modulatory effects of changes in ambient temperature

Mild hypothermia prevents neuronal loss of function that occurs in response to anoxia (Kurihara *et al*, 1996; Taylor and Weber, 1993). In fact, hypothermia may be a behavioral and/or physiological response to oxygen deprivation (Wood and Gonzales, 1996). The neuroprotection afforded by mild hypothermia has been suggested to be related to a reduction in cerebral energy utilization rate and a corresponding attenuation of anoxia-induced reductions in ATP levels (Kozlowski *et al*, 1997; Bart *et al*, 1998). The results of the present study suggest an additional mechanism whereby hypothermia may exert a neuroprotective effect, i.e. a reduced activity of the Na^+/H^+ exchanger in the immediate post-anoxic period. Thus, the present data not only support the role of the Na^+/H^+ exchanger in the production of the post-anoxic alkalinization, but also uphold the relationship between Na^+/H^+ exchange and neuronal viability.

7 Summary

This study detailed the pH_i response of acutely isolated adult rat hippocampal CA1 neurons to transient anoxic periods of varying durations, and assessed the mechanisms which contribute to the production of each of the pH_i shifts which occurred. As supported by evidence presented in this study, Figure 39 outlines the pH_i regulating

mechanisms that may underlie the pH_i response to a transient period of anoxia in this cell types. While supporting the role of ATP hydrolysis and lactate accumulation in the production of the fall in pH_i during anoxia, the present study suggests that inhibition of the acid-extruding Na^+/H^+ exchanger during anoxia may also contribute to the production of the intracellular acidification produced during the anoxic period. The gradual rise in pH_i seen in the continued absence of oxygen was tentatively ascribed to the activation of Zn^{2+}-sensitive, voltage-dependent proton conductance and/or lactate/H^+ cotransport. Finally, a rapid and significant activation of the Na^+/H^+ exchanger was found to be responsible, in large part, for the post-anoxic alkalinization, although membrane depolarization may also contribute to this phase of the pH_i response. Given the ability of pH_i to influence many facets of neuronal function, the anoxia-evoked shifts in pH_i and changes in the activities of pH_i regulating mechanisms may determine, at least in part, neuronal viability following a transient period of oxygen deprivation.

Figure 38. Effect of a 5 min period of anoxia on [Na$^+$]$_i$

The experiment was performed under HCO$_3^-$/CO$_2$-free, HEPES-buffered conditions. Prior to the induction of anoxia, [Na$^+$]$_i$ was ~ 25 mM. Upon the induction of anoxia, [Na$^+$]$_i$ began to increase after ~ 0.80 min, reaching a maximum value of ~ 70 mM at the end of the anoxic period. Upon the return to normoxia, [Na$^+$]$_i$ increased further to 100 mM. Changes in [Na$^+$]$_i$ were measured using SBFI (sodium-binding benzofuran phthalate), a sodium-sensitive ratiometric fluorophore. Acutely dissociated hippocampal CA1 neurons were loaded with 30 μM of the AM-ester form of SBFI for one hour at 37°C. SBFI was excited at wavelengths 334 nm and 380 nm and the emitted wavelengths were measured at 520 nm. In an analogous situation to that performed for BCECF, a one-point calibration was performed at the completion of the experiment. This one-point calibration solution contained 10 mM Na$^+$ and 5 μM gramicidin, a Na$^+$ ionophore. A full calibration curve was obtained during experiments in which cells were exposed to [Na$^+$]$_o$ ranging between 0 mM to 130 mM.
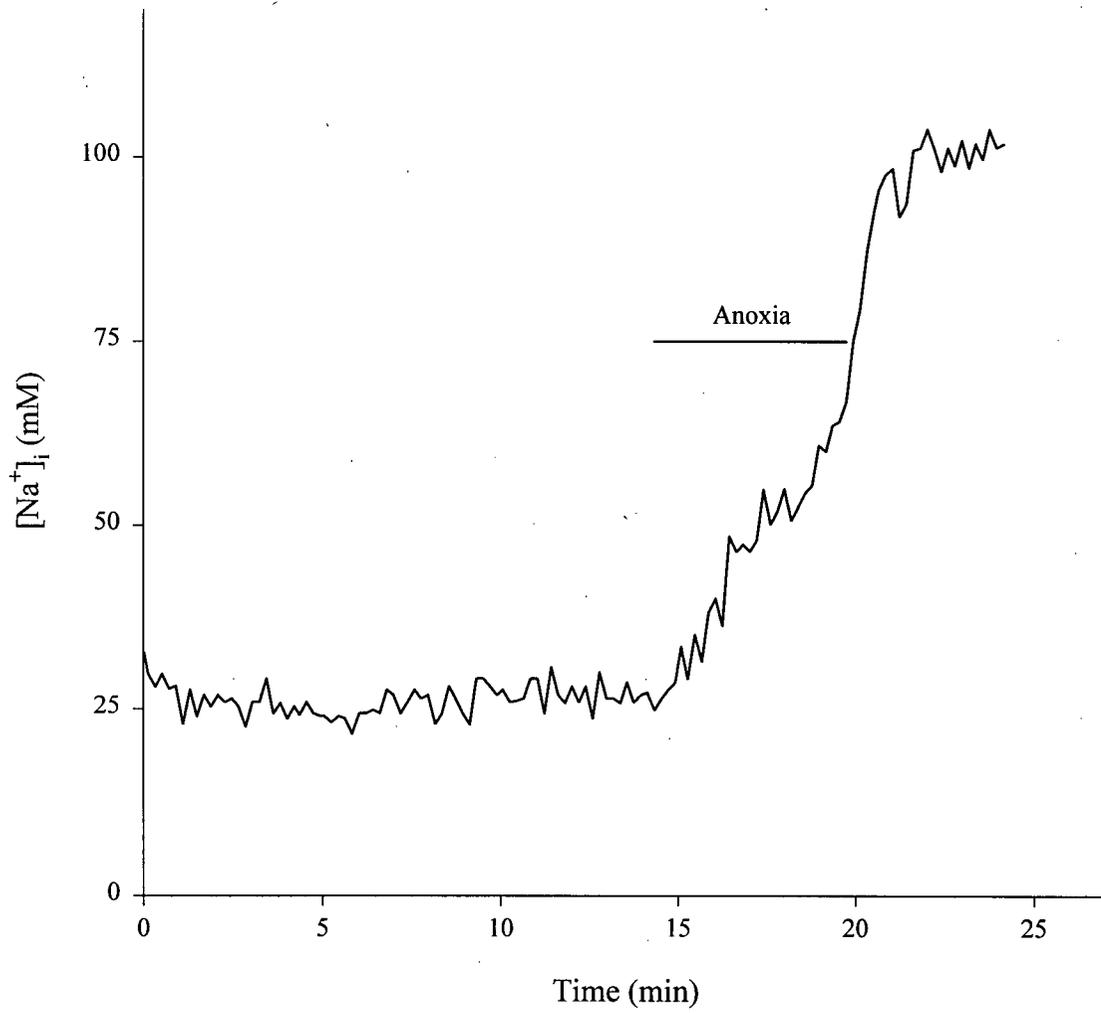
Figure 39. pH$_i$ regulating mechanisms that may contribute to the anoxia-evoked changes in pH$_i$ observed in acutely dissociated adult rat hippocampal CA1 neurons in response to transient periods of anoxia

This diagram outlines the mechanisms discussed in the present study that may contribute to the characterized pH$_i$ response to transient periods of anoxia. The study supports the contribution of ATP hydrolysis and lactate accumulation in the production of the intracellular acidification observed during an anoxic period. In addition, this study provides direct evidence for inhibition of Na$^+$/H$^+$ exchange activity during anoxia which may also contribute to this acidification. Anoxic depolarization may activate a voltage-sensitive proton conductance which, in turn, may contribute to the rise in pH$_i$ observed in the continued absence of oxygen. Lactate/H$^+$ cotransport may also contribute to this phase of the pH$_i$ response. An increase in the activity of the Na$^+$/H$^+$ exchanger is central to the production of the intracellular alkalinization observed in the post-anoxic period. It is important to acknowledge that other mechanisms may contribute to pH$_i$ regulation mechanism in hippocampal neurons (see Introduction, Section 4).

ATP

ADP + P$_i$ + H$^+$

H$^+$

Lactate

Na$^+$

H$^+$

$\uparrow$ Lactic acid

H$^+$

$\downarrow$ Vm
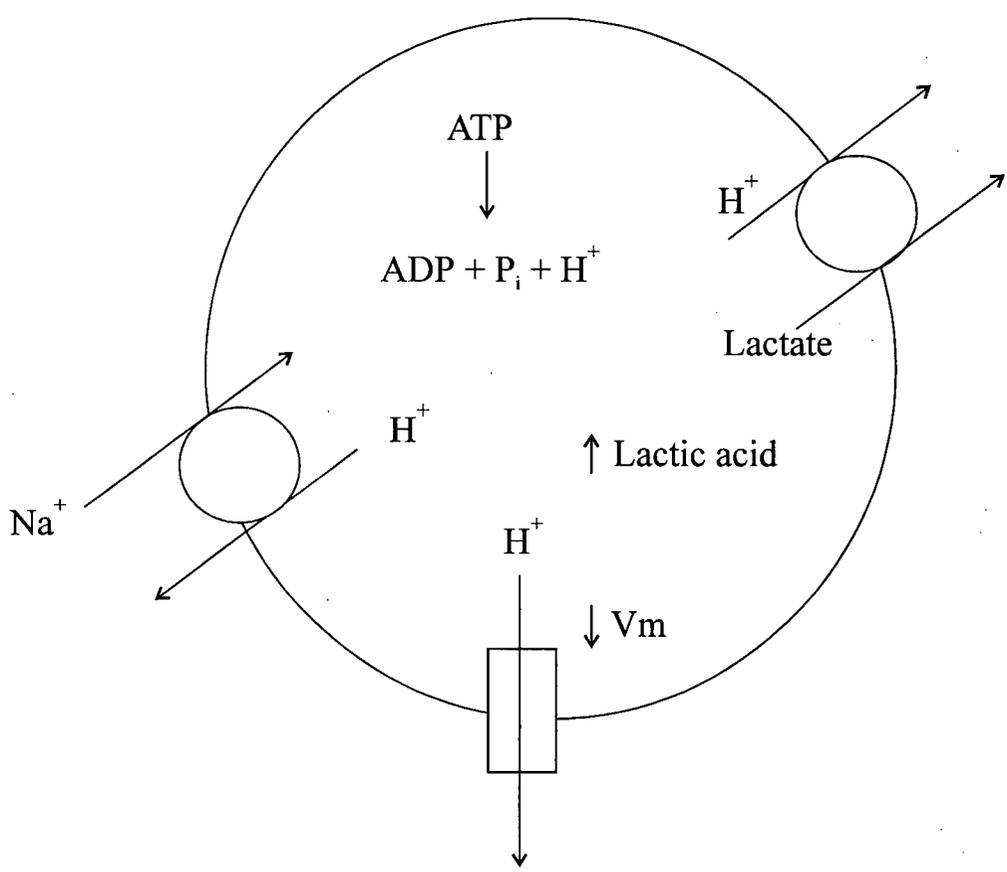
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