CA^{2+}-ACTIVATED POTASSIUM CHANNELS IN SMOOTH MUSCLE CELL FROM CEREBRAL ARTERY OF ADULT RAT

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Abstract

Patch clamp methods were used to study the biophysical properties of Ca^{2+}-activated potassium (K_{Ca}) channels in cerebrovascular smooth muscle cells (CVSMCs) derived from the cerebral arteries of adult rats. Procedures were developed for the enzymatic dissociation of CVSMCs from cerebral arteries. Dissociated cells were maintained at 4°C or cultured at 37°C for 1-3 days prior to use. CVSMCs were identified using a monoclonal antibody specific for smooth muscle α-actin. The calcium-sensitive fluorescent probe fura-2 was employed to measure the resting and serotonin (5-HT) stimulated levels of free intracellular calcium, $[Ca^{2+}]_i$ in these cells.

A resting $[Ca^{2+}]_i$ level of 41 ± 5.6 nM was obtained from a total of 110 CVSMCs in culture. Serotonin (5-HT, 10 nM to 100 μM) induced a transient increase of $[Ca^{2+}]_i$ above resting levels. The effects of three known blockers of voltage-dependent Ca^{2+} channels, nifedipine, La^{3+} and Co^{2+} were tested by coapplication with 5-HT. It was found the neither 10 mM $LaCl_3$ nor 10 mM $CoCl_2$ reduced the rise in $[Ca^{2+}]_i$ evoked by application of 1 μM 5-HT. Nifedipine (10 μM) also failed to significantly reduce the rise in $[Ca^{2+}]_i$ activated by 1 μM 5-HT. The effects of the partially selective 5-HT_2 receptor antagonist, ketanserin (5 nM) reversibly attenuated the 5-HT response in all cells tested. The effect of 5-HT on $[Ca^{2+}]_i$ was clearly dose-dependent and the concentration of 5-HT producing a half-maximal increase in $[Ca^{2+}]_i$ was found to be 10 nM.

During cell-attached patch clamp recordings from CVSMCs kept at 4°C until use, 5-HT (1 - 10 μM) induced the appearance of inwardly directed, 8 - 10 pA single channel currents studied at a pipette potential of 0 mV. 5-HT (10 - 100 μM) also induced the appearance of brief, biphasic current spikes with peak-to-peak amplitude of 10 pA. These observations indicate that isolated CVSMCs retained

electro-responsiveness to 5-HT after 2-3 days *in vitro*. During whole-cell, current clamp recordings from these cells, the resting membrane potential of CVSMCs was found to be -41 $\pm$ 11.7 mV. Application of strong depolarizing stimuli evoked only small (10 - 15 mV) regenerative responses and action potentials were not observed. When measured at zero applied current, the slope membrane resistance, R_m of CVSMCs was 3.2 $\pm$ 0.48 GΩ. The average membrane time constant, τ_m was 78 $\pm$ 26 ms and cell capacitance, C_m was 24 $\pm$ 2.3 pF.

Isolated, inside-out membrane patches excised from CVSMCs displayed two classes of K_{Ca} channel. The first class of channels, designated as $K_{(Ca)L}$ channels, had a mean conductance of 207 $\pm$ 10 pS in symmetrical 140 mM KCl solutions. The permeability of the open channel to potassium was calculated using the Goldman-Hodgkin-Katz constant field equation at $P_K = 3.9 \times 10^{-13}$ cm^3/s. These channels showed a high degree of selectivity for potassium over sodium ions ($P_{Na}/P_K < 0.05$) and for potassium over cesium ions ($P_{Cs}/P_K < 0.05$). Cs^+ caused a voltage-dependent flickering block of open $K_{(Ca)L}$ channels.

The effect of varying $[Ca^{2+}]_i$ on the open probability and open time distributions of $K_{(Ca)L}$ channels was studied. The threshold level of $[Ca^{2+}]_i$ at which channel openings were just detectable was about 0.01 μM. The mean value of $[Ca^{2+}]_i$ at which single $K_{(Ca)L}$ channels were open half of the time, $P_o(0.5)$ was 23 μM when the membrane potential (V) was +40 mV. Over the range of membrane potentials (-80 to +80 mV) and $[Ca^{2+}]_i$ levels (0.01 μM to 1 mM) studied, open time distributions for $K_{(Ca)L}$ channels were well fitted by the sum of two exponentials, indicating the presence of at least two kinetically distinguishable open states for this channel. Raising $[Ca^{2+}]_i$ increased the time constant of the slow component, while having no effect on that of the fast component. The effect of membrane potential on the open probability and open time distribution were also studied. At low $[Ca^{2+}]_i$ (<50 μM), a 14 mV

depolarization induced an e-fold increase in P_o. At $[Ca^{2+}]_i > 50\,\mu M$, P_o approached unity and showed little further increase on membrane depolarization. At $[Ca^{2+}]_i = 10\,\mu M$, the value of the slow time constant derived from open time distributions was significantly increased at positive membrane potentials, while that of the fast time constant was unaffected.

The effect of tetraethylammonium ions (TEA) on current flow in $K_{(Ca)}L$ channels was studied. TEA caused a dose-dependent, reversible reduction in the amplitude of current in the $K_{(Ca)}L$ channels, when applied to the cytoplasmic face of the membrane. This effect was characterized by a dissociation constant, K_d of 0.83 ± 0.09 mM at a membrane potential of +40 mV, and with $[Ca^{2+}]_i = 100\,\mu M$. TEA had no significant effect on the probability of $K_{(Ca)}L$ channels adopting the open state.

The second class of K_{Ca} channels, designated as $K_{(Ca)}I$ channels, was detected on a minority of patches studied. $K_{(Ca)}I$ channels showed a conductance of 92 ± 2.6 pS in symmetrical 140 mM KCl solutions. The potassium permeability of the open channel was $1.75 \pm 0.12 \times 10^{-13}$ cm/s. $K_{(Ca)}I$ channels are highly selective for potassium over sodium ions ($P_{Na}/P_K < 0.05$) and for potassium over cesium ($P_{Cs}/P_K < 0.05$). The open probability, P_o of $K_{(Ca)}I$ channels increased as $[Ca^{2+}]_i$ was elevated over the concentration range $0.1\,\mu M$ to $100\,\mu M$. P_o also increased on depolarization of the membrane patch. TEA caused a reversible, dose-dependent reduction in the amplitude of current in the $K_{(Ca)}I$ channel, when applied to the cytoplasmic membrane face ($K_d = 0.31$ mM $\pm$ 0.03).

The present preparation represents a useful model with which to study the ionic conductances present in cerebrovascular smooth muscle cells. This preparation can also be employed to investigate the effect of vasoconstrictors and vasodilators on calcium mobilization in smooth muscle cells of the cerebral vasculature.

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List of Abbreviations

$[Ca^{2+}]_i$: Concentration of intracellular free calcium

4-AP: 4-aminopyridine

5-HT: 5-hydroxytryptamine, serotonin

ACH: Acetylcholine

ADP: Adenosine diphosphate

ANOVA: Analysis of variance

ANP: Atrial natriuretic peptide

AP: Action potential

ATP: Adenosine triphosphate

ATPases: Enzymes that catalyze the hydrolysis of ATP

cAMP: Cyclic adenosine-3',5'-monophosphate

cGMP: Cyclic guanosine-3',5'-monophosphate

CVSMC: Cerebrovascular smooth muscle cell

CVSMCs: Cerebrovascular smooth muscle cells

DAB: 3,3'-diaminobenzidine

DAG: Diacylglycerol

DMSO: Dimethyl sulfoxide

EBSS: Earl's balanced salt solution

EDRF: Endothelium-derived relaxing factor

EGTA: Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid

FITC: Fluorescein isothiocyanate

G-protein: Guanine nucleotide binding protein.

GIP: Gastric inhibitory peptide

HBSS: Hank's balanced salt solution

HEPES: N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid

IP_3: Inositol-1,4,5-triphosphate

K_{Ca} channel: Calcium-activated potassium channel

K_{Ca} current: Calcium-activated potassium current

$K_{(Ca)I}$ channel: Intermediate conductance calcium-activated potassium channel

$K_{(Ca)L}$ channel: Large conductance calcium-activated potassium channel

KG solution: Potassium glutamate solution

MLCK: Myosin light-chain kinase

MW: Molecular weight

PBS: Phosphate buffer saline

PIP_2: L-α-phosphatidyl inositol diphosphate

PKC: Protein kinase C

PLC: Phospholipase C

P_O: Open probability

SEM: Standard error of the mean

SMC: Smooth muscle cell

SMCs: Smooth muscle cells

SR: Sarcoplasmic reticulum

TEA: Tetraethylammonium

TTX:. Tetrodotoxin

VSMC: Vascular smooth muscle cell

VSMCs: Vascular smooth muscle cells

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Chapter 1

Introduction

Altered contractility of cerebrovascular smooth muscle cells (CVSMCs) is believed to play a role in several cerebrovascular diseases of clinical importance (Michel. et al. 1990). The contractile state of CVSMCs is in turn regulated by the level of free intracellular calcium ($[Ca^{2+}]_i$) and by the flow of ionic current in several types of membrane channels (Johns et al. 1987; Tomita 1988; Hathaway et al. 1991). Among these are the so-called calcium-activated potassium channels (K_{Ca} channels), which are probably important in the modulation of cell resting membrane potential and cell membrane repolarization after excitation (Tomita 1988; Latorre et al. 1989; Edwards and Weston 1990; Kolb 1990). To date, however, most detailed studies on free intracellular calcium and calcium-activated potassium channels in smooth muscle cells have been performed on cells isolated from peripheral vessels or from nonvascular tissues. The goal of this study, therefore was to investigate using patch clamp techniques (Hamill et al. 1981) the properties of K_{Ca} channels in CVSMCs derived from the cerebral vasculature of adult rats. The calcium sensitive dye fura-2 was also used to study the level of free intracellular calcium in these cells.

1.1. The role of vascular smooth muscle cells (VSMCs) in the control of blood flow.

Vascular smooth muscle cells (VSMCs) play a predominant role in regulating the blood flow of the brain and other organs. The contraction and relaxation of smooth muscle cells in the blood vessel wall contribute to the

maintenance of normal vascular function. The following paragraphs summarize current knowledge of the basic contractile mechanisms found in VSMCs.

1.1.1. Contractile mechanisms in VSMCs

The contractility of vascular smooth muscle is dependent on the interaction between two major types of contractile proteins, actin and myosin. Most investigators envisage smooth muscle contraction in terms of the sliding-filament model originally proposed for striated muscle (Huxley 1990). In this model, the heads of myosin molecules, which are bundled together into thick (myosin) filaments, undergo a cycle of high- and low- affinity binding to thin filaments (actin, tropomyosin and other proteins) in a reaction driven by adenosine triphosphate (ATP) hydrolysis. Tension generation occurs as a result of rotation of the heads of myosin, which are bound in cross-bridge structures to actin molecules, from an angle of 90o to 45o with respect to the long axis of actin- and myosin-containing filaments. The direction of force generation is paralleled to the orientation of these filaments.

In VSMCs, contraction mediated by myosin and actin filaments is regulated by Ca^{2+} through a number of different mechanisms (Bolton 1979; Johns et al. 1987; Hathaway et al. 1991). The most important of these involves the phosphorylation of myosin molecules (Adelstein and Sellers 1987). In this mechanism, Ca^{2+} binds to calmodulin and the resulting complex activates the enzyme myosin light-chain kinase (MLCK), which in turn catalyzes myosin phosphorylation and initiates rapid shortening. After dephosphorylation, the actomyosin passes through a "latch" state back to rest (Hai and Murphy 1989). Vascular myosin is composed of two heavy chains (MW, 200,000 and 204,000)

and two sets of light-chain subunits [regulatory, or 20,000 Da, light chains (i.e., LC_{20}) and alkali, or 17,000 Da, light chains]. The serine-19 residue of LC_{20} is the site of phosphorylation by MLCK. Investigations with the fluorescent Ca^{2+} indicator fura-2 or the photoprotein aequorin have demonstrated that the level of free intracellular Ca^{2+} closely regulates the extent of LC_{20} phosphorylation (Rembold et al. 1988; Taylor et al. 1989). Phosphorylation of LC_{20} activates the ATPase activity actomyosin (Sellers 1985) initiating the cycling of cross-bridge formation and, hence, of contraction. The state of phosphorylation in smooth muscle myosin is also regulated by the opposing action of myosin phosphatase and by the modulation of MLCK activity. *In vitro*, MLCK can be phosphorylated by several protein kinases, including cyclic adenosine 3',5'-monophosphate (cAMP)-dependent protein kinase (Payne et al. 1986), cyclic guanosine 3',5'-monophosphate (cGMP)-dependent protein kinase (Nishikawa et al. 1984; Hathaway et al. 1985), Ca^{2+}/calmodulin-dependent protein kinase II (Ikebe and Reardon 1990), and protein kinase C (Nishikawa et al. 1985).

In vascular smooth muscle, tension can be maintained while LC_{20} dephosphorylation and $[Ca^{2+}]$ levels decrease. This mechanical state of tension maintenance with slowly cycling cross-bridges has been called the "latch state" by Hai and Murphy (1989). The latch state is energy efficient because tension is maintained at reduced ATP consumption. The molecular mechanisms that produce the latch state are unknown. Hai and Murphy (1989) hypothesized that dephosphorylation of LC_{20} while in a high-affinity binding conformation might alter the kinetics of cross-bridge detachment. Slowing of cross-bridge detachment would prolong high-affinity binding and tension.

1.1.2. Factors influencing $[Ca^{2+}]_i$ in VSMCs

The generation and maintenance of tension in vascular smooth muscle are dependent on processes that modulate intracellular levels of free Ca^{2+}. Calcium may enter the cytoplasmic compartment of VSMCs in a number of ways, including trans-sarcolemmal entry though voltage-dependent Ca^{2+} channels, Na^{+}-Ca^{2+} exchange, and receptor-operated Ca^{2+} channels. The depolarization of smooth muscle cells by electrical stimulation, increased external potassium, or various neurotransmitters evokes an inward current carried by Ca^{2+} ions through voltage-dependent Ca^{2+} channels which share certain functional characteristics with L-type Ca^{2+} channels of striated muscle (Nelson et al. 1988). Calcium may also be released from internal stores via ryanodine receptor-like Ca^{2+} channels of the sarcoplasmic reticulum (SR) (Ashida 1988) and released from other internal sites via an inositol-1,4,5-triphosphate (IP_3)-activated channel (Nelson et al. 1990).

Several types of K^{+} channels have been detected in vascular muscle cells, including Ca^{2+}-activated K^{+} channels, delayed rectifier K^{+} channels, and ATP-sensitive K^{+} channels. Increases in K^{+} conductance as a result of activation of any of these channels leads to membrane hyperpolarization with subsequent closure of voltage-dependent Ca^{2+} channels (Standen et al. 1989) and enhanced Ca^{2+} extrusion via Na^{+}-Ca^{2+} exchange (Lauger 1987). In all cases, the physiological response is a reduction in vascular smooth muscle tone.

Various hormones acting through membrane-bound guanine nucleotide binding proteins (G-proteins) (Freissmuth et al. 1989) activate phospholipase C (PLC), which converts L-α-phosphatidyl inositol diphosphate (PIP_2) to diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3). IP_3 in turn

stimulates release of Ca^{2+} from the sarcoplasmic reticulum via and IP_3-receptor protein, which is a type of Ca^{2+} channel (Hashimoto et al. 1986; Berridge 1989). DAG activates the enzyme protein kinase C in the sarcolemma which in turn phosphorylates many other membrane proteins, such as receptors and the Na^{+}-K^{+} exchanger (Nishizuka 1988).

Other second messengers, such as cAMP and cGMP regulate intracellular Ca^{2+} and/or change the Ca^{2+}-sensitivity of contractile proteins in smooth muscle cells (Kamm and Stull 1989). cAMP is thought mainly to stimulate Ca^{2+} uptake into the SR, whereas cGMP is thought to act principally by stimulating sarcolemmal ATPases (Lindemann et a. 1983; Vrolix et al. 1988).

1.1.3. Hormonal and neural regulation of contraction in VSMCs

The contractile activity of vascular smooth muscle cells is regulated by a variety of local and systemic mechanisms. Sympathetic and parasympathetic nerves play a major role in the systemic control of vascular smooth muscle tone, while a number of other agents modulate the responsiveness of VSMCs at a local level.

Knowledge concerning these local regulating mechanisms has progressed rapidly in recent years. Local regulation is brought about by the intrinsic contractile response of smooth muscle to stretch, and by the actions of vasodilatory metabolites and local vasoconstrictors. Since Furchgott and Zawadski (1980) first reported that the vasodilatory response of vascular smooth muscle to acetylcholine requires the presence of an intact endothelium, the role of the endothelium in the regulation of vascular tone has attracted

considerable interest. Activation of endothelial receptors by a number of vasoactive substances stored and released by platelets, endothelium, or surrounding tissues stimulates the production of endothelium-derived relaxing or constricting factors (Vanhoutte 1981; De Clerck and David 1981; Burnstock 1990). These subsequently modify vascular tone by contracting or relaxing the vascular smooth muscle (Burnstock 1990).

The endothelium-derived relaxing factor (EDRF) has now been identified as nitric oxide (Ignarro et al. 1986, Palmer et al. 1987). In addition to acetylcholine, endothelium-dependent vasodilation has also been shown to occur in response to other vasoactive substances, including ATP, adenosine diphosphate(ADP), arachidonic acid, substance P, neurokinin A, 5-hydroxytryptamine (5-HT), bradykinin, histamine, neurotensin, vasopressin, angiotensin II and thrombin (Mione et al. 1990). Some of the vasoactive substances which require the presence of endothelium to produce vasodilation also act as vasoconstrictors when released from perivascular nerves. There appears, therefore, to be a resting dynamic balance between endothelium-derived vasodilatory tone and sympathetic vasoconstrictor tone, which is altered under different physiological and pathophysiological circumstances.

Although the nature of the endothelium-dependent contracting factor is still unclear, at least three different classes of endothelial vasoconstrictor substances have been recognized (Luscher 1988). These consist of metabolites of arachidonic acid, polypeptide-like factors, such as endothelin, a 21-residue peptide isolated from porcine aortic endothelial cells (Yanagisawa, 1988) and an unidentified diffusible factor released from anoxic/hypoxic endothelium cells.

Systemic regulation of vascular smooth muscle tone is brought about by circulating substances and by the action of vasomotor nerves. Circulating vasodilatory agents include kinins and atrial natriuretic peptide (ANP). Circulating vasoconstrictors include vasopressin, norepinephrine, epinephrine, and angiotensin II. In the neural regulation of VSMC contraction, sympathetic nerves play a dominant role. With very few exceptions, norepinephrine released from adrenergic nerve terminals activates alpha-adrenoceptors at the membrane of VSMCs (postjunctional alpha-adrenoceptors) (Burnstock 1986; 1990).

1.1.4. Vasoconstrictor action of serotonin on VSMCs

Serotonin is one of the most potent vasoconstrictor agents in the cerebral circulation of mammals (Edvinsson et al. 1984; Young et al. 1986a, 1986b; Chang and Owman 1987). It was first isolated and crystallized by Rapport, Green and Page (1948) and was synthesized by Hamlon and Fisher (1951). Serotonin may play a role in the etiology of several important disorders of the vascular system, including migraine, hypertension, vasospasm associated with subarachnoid haemorrhage and ischemia (Bohr and Webb 1988; Michel et al. 1990; Lance 1982; Vanhoutte 1985; Wilkins 1980). Nerve fibers containing 5-HT have been demonstrated immunohistochemically around the pial arteries of many species (Chang, Owman and Steinbusch 1988). Serotonin receptor subtypes can be categorized into three major families, 5-HT_1, 5-HT_2 and 5-HT_3 receptors, and each family consists of multiple receptor subtypes that share similarities in their molecular biological, pharmacological, biochemical, and/or physiological properties (Schmidt and Peroutka 1989).

In the peripheral vasculature, it is well established that 5-HT induced contraction often involves the binding of serotonin to 5-HT_2 receptors on the membrane of VSMCs. In these vessels, 5-HT_2 receptor occupation triggers phosphoinositide hydrolysis, yielding the second messenger IP_3. IP_3 then initiates contraction by releasing Ca^{2+} from intracellular stores (Peroutka and Snyder 1979; Conn and Saunders-Bush 1987; Peroutka 1987).

In the case of cerebrovascular vessels, however, the mechanisms by which serotonin produces contraction in smooth muscle are less well understood. In conducting cerebral arteries of the rat, serotonin-induced contractions are blocked by nanomolar concentrations of the 5-HT_2 receptor antagonist ketanserin, suggesting the involvement of 5-HT_2 receptors in this tissue (Chang and Owman 1987). However, in similar studies conducted on canine basilar arteries, serotonin effects have been ascribed to activation of 5-HT_1 receptors (Peroutka and Kuhar 1984), 5-HT_2 receptors (Muller-Schweinitzer and Engel 1983), or to an as yet unclassified receptor type (Cohen and Colbert 1986). Contractile responses to 5-HT which cannot be readily ascribed to the activation of 5-HT_2 receptors have also been reported in cerebral arteries from the cat (Young et al. 1986b) and the rabbit (Bradley et al. 1986).

Factors which influence 5-HT_2 receptor mediated changes in intracellular free calcium have been studied in several types of VSMC from peripheral vessels using calcium-sensitive fluorescent dyes (Nabika et al. 1985; Capponi et al. 1987; Takata et al. 1988). There have as yet been no direct measurements on the influence of serotonin on free intracellular Ca^{2+} in

cerebrovascular VSMCs to permit comparison with the data obtained from peripheral tissues.

1.2. Electrophysiological properties of VSMCs

Vascular smooth muscle cells from different preparations exhibit wide variations in their passive and active properties. Intracellular recordings from CVSMCs show resting membrane potentials within the range of -40 mV and -70 mV in intact guinea-pig cerebral artery (Karashima and Kuriyama 1981; Yamamoto and Hotta 1986), dog cerebral artery (Suzuki and Fujiwara 1982; Fujiwara et al. 1982), and cat middle cerebral artery (Harder 1980; Harder et al. 1981). In intact guinea-pig cerebral artery, the membrane of CVSMCs was found to be electrically quiescent, and neither spontaneous action potentials nor miniature excitatory junction potentials were observed. A single, brief stimulus induced a spike potential followed by a depolarizing slow-potential, and these events were associated with muscle contraction. External application of tetraethylammonium (TEA), a K+ channel blocker, enhanced the amplitude of the spike potential (Karashima and Kuriyama 1981; Yamamoto and Hotta 1986). In the intact dog cerebral artery, CVSMCs only generated spikes in response to outward current pulses under conditions of pretreatment with 10 mM TEA. In CVSMCs of intact rabbit basilar artery, the mean resting potential was -61 mV, membrane input resistance and time constant were 5-40 MΩ and 5-30 ms, respectively. Action potentials were readily evoked by depolarizing currents, even in the absence of TEA (Surprenant et al. 1987).

Whole-cell, patch clamp recordings have been made in VSMCs isolated from mesenteric artery (Bolton et al. 1985), aorta (Toro et al. 1986), and rat cerebral artery Steele et al 1991; Zhang et al. 1991). Single aorta VSMCs were found to exhibit input resistances in the 3 GΩ range, much higher than found using conventional intracellular microelectrodes. These cells had a mean capacitance of 17 pF and in many cases could generate Ca^{2+}-dependent action potentials on depolarization (Toro et al. 1986). CVSMCs isolated from rat cerebral arteries exhibited input resistances in the range 4 - 9 GΩ, again much higher than typical of cerebral arteries studied with intracellular microelectrodes (Steele et al. 1991).

In vascular smooth muscle cells, the depolarizing phase of the action potential has been regarded mainly as the result of inward flow of Ca^{2+} through voltage-dependent Ca channels (Bolton 1979; Tomita 1982). Inward membrane currents generating the action potential are little effected by complete replacement of Na with TEA or $Tris^{+}$. Tetrodotoxin, a selective blocker of voltage-sensitive Na channels is also ineffective. In contrast, these inward currents are strongly dependent on the external concentration of Ca^{2+} or Ba^{2+} (Tomita 1988). Two distinct classes of voltage-dependent calcium channels exist in rat vascular smooth muscle cells. One of these channels has a low threshold and a fast inactivation rate ("fast channel"), while the other displays a high threshold and a slow inactivation rate ("slow channel") (Loirand et al. 1986). These "fast" and "slow" Ca channels seem to correspond to the "T (transient)-type" and "L (long-lasting)-type" Ca channels seen in other tissues (McCleskey et al. 1986). A study on guinea-pig thoracic aorta smooth muscle cells, however, only showed the existence of the slow channel type (Caffrey et al. 1986). The inability of some VSMCs to generate action potentials on

membrane depolarization by injected current probably reflects their marked outward rectification due to enhanced K conductance, rather than a lack of Ca channels (Mekata 1976).

1.3. Potassium currents in the membrane of VSMCs

The general role of potassium (K) currents in the modulation of vascular excitability is now well recognized. In smooth muscle cells, potassium currents play a crucial role in the vascular response to endogenous and pharmacological vasodilators by determining the resting membrane potential, and the time course, amplitude and polarity of electrical changes (Brayden et al. 1991). In general, an increase in K conductance raises the membrane potential (hyperpolarization) and depresses electrical excitability (Bulbring and Tomita 1987). The physiological response is relaxation or reduction of vascular tone.

K-currents may be classified into various categories using the criteria of channel gating properties, conductance and pharmacological characteristics. Based on these criteria, K-currents can be divided into the following classes.

1.3.1. The delayed (outward) rectifier K-current

This current contributes to the macroscopic outward K-current which is mainly responsible for the repolarizing phase of the action potential in many cell types. It was first described in the squid giant axon (Hodgkin and Huxley 1952). Single channel recordings conducted in neurones (Conti and Neher 1980) and in skeletal muscle (Standen et al. 1985), have shown an elementary

conductance of 15-20 pS. Whole-cell current flowing in these channels increases sigmoidally on membrane depolarization. Under a constant depolarizing stimulus, a slow, exponential inactivation occurs, requiring up to several seconds for completion. There is evidence that the voltage dependence of activation and inactivation can be modulated by protein phosphorylation (Bezanilla et al. 1985). No selective pharmacological agonists are known for this current. Like most K currents, the delayed rectifier can be blocked by $Cs+$, $Ba2+$ (Wagoner and Oxford 1987), intracellular TEA (Armstrong 1971), and external TEA (Hille 1992).

In smooth muscle cells, delayed rectifier channels have been identified in pulmonary artery (Okabe et al. 1987), rabbit jejunum (Benham and Bolton 1983; Benham et al. 1987), bladder (Klockner and Isenberg 1985) and in intestinal smooth muscle (Ohya et al. 1986). In these tissues, the delayed rectifier channels may open during the repolarization phase of slow electrical waves.

1.3.2. The transient outward current (A-current)

The transient outward current, also known as "the A-current" (I_A) was first described in molluscan neurons (Connor and Stevens 1971). Patch clamp studies have shown that this current is mediated by single channels of conductance 15-20 pS which can be blocked by external application of 4-aminopyridine (4-AP, Taylor 1987). A-current channels activate and inactivate very rapidly during membrane depolarizations. A-current channels are open in the subthreshold region of the membrane potential, and can therefore play a role in determining cell firing frequency. In neurones, this current participates

in the control of neuronal firing rate, spike latency and action potential repolarization (Hille 1984).

In smooth muscle cells, a current with properties similar to neuronal A-currents has been described in pulmonary artery (Ohya et al. 1986). The functional significance of this current in VSMCs remains, however, unclear.

1.3.3. Inward rectifying K-currents

The existence of potassium currents which are activated by membrane hyperpolarization has been reported in various tissues. In general, these currents fall into two broad categories. The first type of current is characterized by a high selectivity for potassium over sodium ions. The voltage range over which this current is activated shifts markedly when the external concentration of potassium is altered. This current is blocked by low (1 mM) concentrations of Ba^{2+} or Cs^{+}. Currents of this type have been classically described in the membrane of starfish eggs (Hagiwara et al. 1976, 1978, 1979), skeletal muscle (Adrian 1969) and cardiac muscle (Noble 1984). Single channel studies using the patch clamp technique show that membrane channels carrying this type of inward rectifier current have a modest conductance (20-30 pS) when measured in physiologically appropriate potassium solutions (Brismar et al. 1989; Burton and Hutter 1990; Clarke et al. 1990). A second class of inward rectifier current has been found in many types of neurone (Halliwell and Adams 1982; Mayer and Westbrook 1983; Crepel et al. 1986) as well as in gut smooth muscle fibres (Benham et al. 1987). This class of ionic conductance shows appreciable permeability to sodium ions as well as to K^{+}. The voltage range for activation of this conductance is insensitive to changes in the concentration of

extracellular potassium. Barium ions are relatively ineffective in blocking this current.

To date, there have been relatively few detailed studies conducted on the inward rectifier channels found in smooth muscle cells. However, in many types of SMC, electrotonic potentials induced by large hyperpolarizing currents reach a peak and then decay with time. This inward rectification has been observed in SMCs isolated from guinea-pig taenia caeci (Tomita 1966) , toad stomach (Sims et al. 1985) and rabbit jejunum (Benham et al. 1987). The inward rectifier current present in rabbit jejunum SMCs has been analyzed using the whole-cell patch clamp technique (Hamill et al. 1981). This current belongs to the second class described above. An inwardly rectifying channel permeable to K^+, Na^+ and Ca^{2+} has also been described in SMCs isolated from the stomach of the toad (Hisada et al. 1991). This channel has a conductance of 64 pS in physiological solutions and is activated by membrane hyperpolarization. In contrast to these findings in gut smooth muscle, evidence indicates that inward rectifiers of the first class are present in CVSMCs the proximal segment of rat cerebral arterioles (Edwards et al. 1988) and in CVSMCs in submucosal arterioles of the guinea-pig ileum (Edwards and Hirst 1988). These currents are unaffected by removal of external Na^+ but are blocked by low concentrations of Ba^{2+}.

1.3.4. ATP-sensitive K-current.

An ATP-sensitive K-current (K_{ATP}) was first identified in cardiac muscle (Noma 1983). This current was first demonstrated at the single channel level in VSMCs by Standen et al. (1989). A primary characteristic of this

current is its activation by the hyperpolarizing vasodilator cromakalim and its inhibition by cytoplasmic ATP, as well as by sulfonylurea compounds, such as glibenclamide and by low concentrations of Ba^{2+}. Most K_{ATP} channels described show no apparent intracellular calcium dependency and only slight voltage sensitivity. Some recent reports, however, have demonstrated a large conductance voltage and Ca^{2+}-sensitive channel in VSMCs from porcine coronary artery (Silberberg and Breemen 1990) and rabbit aorta, rabbit trachea and pig coronary artery (Gelband et al. 1990) which is also sensitive to cytoplasmic ATP and to cromakalim. This channel has been is designated as the BK_{ATP} channel. K_{ATP} channels in various types of SMCs may play a role in protection against ischemic and anoxic insults (Gelband et al. 1990; Silberberg and Breemen 1990).

1.3.5. Ca-activated potassium channels

At present three groups of calcium-dependent K-channels, K_{Ca} have been described in many tissues, based on their single channel conductance, calcium sensitivity, voltage dependence, and pharmacological properties. These three groups of K_{Ca} channels are designated as large conductance calcium-activated channels (BK), intermediate conductance calcium-activated channels (IK), and small conductance calcium-activated channels (SK). A number of reviews about these channels have been published (Tomita 1988; Latorre et al. 1989; Edwards and Weston 1990; Kolb 1990). Properties of these channel types are discussed in the following section.

1.4. Calcium-activated potassium channels

1.4.1. Large conductance calcium-activated potassium channels, BK

BK or maxi-K channels have been extensively studied in many cell types. Elementary currents flowing in BK channels were first observed in bovine adrenal chromaffin cells (Marty, 1981) and later in cells from skeletal muscle (Methfessel and Boheim 1982; Latorre et al. 1982; Vergara and Latorre 1983; Vergara et al. 1984; Blatz and Magleby 1984, 1986; Moczydlowski 1983, 1985), cardiac muscle (Callewaert et al. 1986), endocrine cells (Wong et al. 1982; Marty 1983; Yellen 1984; Wong and Adler 1986), exocrine cells (Maruyama et al. 1983; Cook et al. 1984; Findlay et al. 1985; Gitter et al. 1987; Gray et al. 1990), epithelial cells (Christensen and Zeuthen 1987) and immune cells (Gallin 1984). BK channels have been identified in SMCs derived from the intestine (Benham et al. 1985, 1986; Cecchi et al. 1986; Mayer et al. 1990), stomach (Berger et al. 1984; Carl et al. 1990), peripheral arteries (Benham et al. 1985, 1986; Bolton et al. 1985; Inoue et al. 1985), airway (McCann and Welsh, 1986), ureter (Shuba 1981) and taenia caeci (Inomata and Kao 1979). BK channels exhibit the following properties: (1) large single channel conductance in the range of 150-300 pS at a symmetrical, high K concentrations; (2) high selectivity for K^+ over other monovalent cations; (3) activation by intracellular calcium (1 - 10 μM) and membrane voltage (e-fold increase in current for 9 - 15 mV depolarization); (4) blockade by externally applied TEA (K_d < 1 mM), quinine, Ba^{2+} and, in most cases by the scorpion toxin charybdotoxin.

BK channels are highly selective for K^+ over Na^+ ($P_{Na/K} < 0.05$). In general, the permeability sequence for the monovalent cations in these channels

appears to be substantially independent of the cell type (Tomlins et al. 1984; Benham et al. 1986; Akbaraki et al. 1989; Emeran et al. 1990). This sequence is $Tl^+ > K^+ > Rb^+ > NH_4^+ >> Cs^+ > Na^+ > Li^+$.

The sensitivity of BK channels to $[Ca^{2+}]_i$ varies significantly among different cell types. For example, in rabbit jejunum and guinea-pig mesenteric artery SMC, 10^{-9} M $[Ca^{2+}]_i$ is sufficient to activate BK channels (Benham et al. 1986). However, in canine airway SMC, BK channels require $[Ca^{2+}]_i > 10^{-7}$ M for activation (McCann and Welsh 1986). BK channel open probability is also sensitive to membrane potential. Again, however, the voltage dependency of channel opening varies among different cell types. This voltage dependence seems to result from the potential dependent binding of Ca^{2+} to the channel, rather than from an intrinsic channel dipole (Kolb 1990).

The gating kinetics of BK channels can be very complex. BK channels in cultured rat muscle show four distinct modes of gating, defined as normal, intermediate open, brief open and buzz (McManus and Magleby 1988). The normal gating mode (96% of all transitions) itself displays at least three to four open states and six to eight shut states of the channel, as indicated by the multi-exponential nature of dwell time distributions. In contrast, BK channels in VSMCs isolated from mesenteric artery display apparently simpler kinetics (two open states and three closed states, Benham et al. 1986). However, it is not clear whether this simplicity reflects the use of shorter lengths of data for analysis, which would eliminate infrequently occurring open states and long-duration closed states (McManus and Magleby 1988).

Experiments with BK channels reconstituted into lipid bilayers strongly indicate that Ca^{2+} acts as a simple ligand to promote channel opening, rather than activating a calcium-dependent reaction pathway, such as Ca^{2+}-calmodulin or protein kinase C-dependent phosphorylation (Latorre et al. 1985). Complex state diagrams have been developed for the BK channel in which 1-3 calcium ions bind sequentially to the channel, stabilizing one of a number of open or closed states. Uniliganded channels are presumed to be responsible for the majority of short duration channel openings, while the binding of the second and third calcium ions stabilizes the longer-lived open states (Barrett et al. 1982; Moczydlowski and Latorre 1983: McManus and Magleby 1988). In BK channels of skeletal muscle fibers, intracellular magnesium ions can promote channel opening at physiological concentrations of this cation (0.4 - 3 mM) (Squire and Petersen 1987). The functional significance of this phenomenon is as yet unclear.

BK channels in most, but not all preparations are blocked by nanomolar concentrations of the scorpion toxin charybdotoxin applied to the external membrane face (Miller et al. 1985). This toxin appears to act as an open channel blocker. In most preparations, low concentrations (0.1 - 1 mM) of TEA applied to the external membrane face reversibly block current flow in BK channels in a manner only weakly dependent on membrane voltage. This interaction may be described as a mono molecular reaction with an apparent dissociation constant, K_d = 0.3 mM (Yellen 1984a, 1984b; Vergara and Latorre 1983; Blatz and Magleby 1984).

TEA also blocks current flow in BK channels when applied to the internal membrane face. In cultured myotubes (Blatz and Magleby 1984),

chromaffin cells (Yellen 1984a, 1984b), and in mesenteric artery VSMCs (Benham et al. 1985), the K_d for block by internally applied TEA is 12 mM or higher. In marked contrast, BK channels in clonal pituitary cells (Wong and Adler 1986) and in brain synaptosomal membranes (Farley and Rudy 1988) display a much higher sensitivity to internal TEA, with a K_d in the 0.1 mM range.

BK channels are also blocked by Cs^+ applied to either membrane face. This block is intensified at membrane voltages which drive Cs ions into the open channel. Typically, the block is too fast to be kinetically resolved, and results in an apparent reduction in mean single channel current (Yellen 1984a, 1984b). In neurones, current flow in BK channels serves to repolarize the membrane towards E_K, terminating voltage-dependent calcium entry and restoring firing frequency to resting levels. BK channels apparently mediate the macroscopic membrane current designated as I_C in many types of excitable cell. This current generates the fast after-hyperpolarization seen during action potential firing in these cells (Kaczmarek and Levitan 1987). It has also been proposed that BK channels may play a role in the after-hyperpolarization seen in CVSMCs of cerebral arteries (Hirst et al. 1986; Steele et al. 1991).

1.4.2. Intermediate conductance calcium-activated potassium channels, IK

IK channels have been identified in a great variety of tissues including neurones (Ewald et al. 1985), rat kidney tubules (Frindt and Palmer 1987) and rat brain synaptosomes (Farley and Rudy 1988). These channels sometimes coexist with BK channels (Akbarali et al. 1990). Channel conductance varies

from 30 to 120 pS. Quinine, quinidine, TEA and charybdotoxin act as blockers of IK channels (Kolb 1990; Edwards and Weston 1990).

IK channels of conductance around 100 pS have been detected in VSMCs isolated from rabbit portal vein (Inoue et al. 1985, 1986) and human cystic artery (Akarali et al. 1990). These channels show an increased open probability on cell depolarization or increasing $[Ca^{2+}]_i$, but the open probability is quite low at normal resting potentials of -50 mV to -55 mV. These IK channels may therefore be more important in repolarization after the action potential than in maintaining the resting membrane potential (Inoue et al. 1985).

1.4.3. Small conductance calcium-activated channels, SK.

The SK channels exhibit a conductance in the range of 10-14 pS and were first described in cultured rat skeletal muscle cells (Blatz and Magleby 1986) and in guinea-pig hepatocytes (Cook and Haylett 1985). SK channels exhibit no voltage sensitivity and in general these channels are more sensitive than BK channels to $[Ca^{2+}]_i$. SK channels mediate the slow afterhyperpolarization (AHP) which follows the action potential in many cell types (Pennefather et al. 1985). The slow after-hyperpolarization plays a key role in spike frequency adaptation in neurones (Barrett and Barrett 1976; Barrett et al. 1981; Kawai and Watanabe 1986).

Externally applied TEA at concentrations up to 25 mM has no significant blocking effect on SK channels in skeletal muscle cells (Romey and Lazdunski 1984; Blatz and Magleby 1986) or in the sympathetic neurones

(Pennefather et al. 1986). SK channels are however blocked by nanomolar concentrations of the bee toxin apamin (Kolb 1990). Among smooth muscle cells, SK channels have yet to be studied at the single channel level of resolution. However, apamin is known to block neurotensin induced relaxation in rat ileum smooth muscle, suggesting that SK channels are indeed present in some SMCs (Kullack et al. 1987)

1.4.4. Modulation of the ionic current through K_{Ca} channels

Both single channel and whole-cell studies have suggested that the current through K_{Ca} channels can be modulated by neurotransmitters or intracellular second messengers, and by putative K^+ channel openers, such as cromakalim and penacidil.

The relaxation of guinea-pig taenia coli mediated by α-adrenergic agonists involves activation of K_{Ca} channels, with subsequent membrane hyperpolarization (Bulbring and Tomita 1987). As noted above, neurotensin induced relaxation of rat ileum smooth muscle can be blocked by apamin, suggesting the involvement of SK channels in this process (Kullack et al. 1987). cAMP has been shown to activate K_{Ca} channels in rat aorta VSMCs, suggesting that phosphorylation may play a role in the activation of these channels (Sadoshima et al. 1988). In tracheal myocytes, protein kinase A activates K_{Ca} channels (Kume et al. 1989) while the muscarinic activation of canine colonic smooth muscle appears to involve suppression of K_{Ca} channel activity via a pertussis toxin sensitive G protein (Cole and Sanders 1989). In porcine coronary artery, the newly discovered vasoactive peptide endothelin has been found to enhance the probability of opening of BK channels, while higher doses

of the peptide (> 10 nM) irreversibly inhibited this channel. These actions could play roles in the vasodilator and vasoconstrictor effects of this peptide (Hu et al. 1991). The potent vasodilator nitroprusside appears to relax aortic VSMCs by activating K_{Ca} channels (Williams et al. 1988). The potassium channel opener cromakalim activates BK channels in human mesenteric artery by decreasing the mean shut time of these channels (Klockner et al. 1989).

1.5. Rationale

It is evident from this review that K_{Ca} channels have been well studied in VSMCs from isolated peripheral vessels, as well as in smooth muscle cells from non-vascular tissues (Tomita 1988; Sperelakis and Ohya 1989; Akbaraki et al. 1990; Edwards and Weston 1990). In contrast, however, K_{Ca} channels have been little studied in SMCs derived from the cerebral circulation of mammals. The few papers which have appeared on this topic have clearly demonstrated the presence of K_{Ca} currents in CVSMCs isolated from rat cerebral arteries, but as yet few details of the biophysical properties of these channels are available (Zhang et al. 1991; Steele et al. 1991).

This absence of knowledge is regrettable, in view of the apparent importance of these channels in regulating the contractile state of VSMCs. K_{Ca} channels may also play a important role in the etiology of the vasospasm that occurs in many patients following subarachnoid hemorrhage (Michel et al. 1990). It has been proposed that free radicals may mediate the vasoconstrictor action of oxyhemoglobin on cerebrovascular smooth muscle. Oxyhemoglobin induced contractions are accompanied by an increase in K_{Ca} currents in

CVSMCs, presumably as a result of an elevated level of free intracellular calcium (Steele et al. 1991).

With these considerations in mind, it was decided to perform a study designed to elucidate the biophysical properties of K_{Ca} channels in CVSMCs derived from adult rats. Conventional intracellular recording techniques were not suitable for this project, since these methods cannot resolve current flow in individual membrane channels. For this purpose it was necessary to employ the patch clamp method, devised by Neher and Sakmann (Hamill et al. 1981; Sakmann and Neher 1983). This decision in turn required the use of enzymatically dissociated CVSMCs, since the membrane of smooth muscle cells is invested by a tough connective tissue matrix in intact vessels (Ross and Reith 1985).

The goals of this project may then be stated as follows:

1. To devise methods for the enzymatic dispersion of CVSMCs from the cerebral arteries of adult rats. Isolated cells were found to remain viable *in vitro* for several days after isolation. Histological and immunocytochemical methods were employed to identify dispersed CVSMCs in these preparations.

2. To perform patch clamp recordings in the whole-cell, current clamp mode to determine the basic electrophysiological properties of dispersed CVSMCs.

3. To perform patch clamp recordings in the isolated, inside-out patch mode in order to investigate the biophysical properties of single K_{Ca} channels in the CVSMC membrane. These properties included single channel conductance, channel kinetics, modulation of channel gating by membrane voltage and intracellular free calcium, and the sensitivity of these channels to the potassium channel blockers TEA and Cs+.

4. Since the gating of K_{Ca} channels is critically dependent on the level of intracellular free calcium, it was also decided to estimate the value of this parameter in dispersed CVSMCs, using the calcium-sensitive probe fura-2.

5. As noted previously, there is some uncertainty as to the mode of action of the vasoconstrictor 5-HT on cerebrovascular smooth muscle. It was therefore decided to investigate this matter using the fura-2 assay of intracellular calcium.

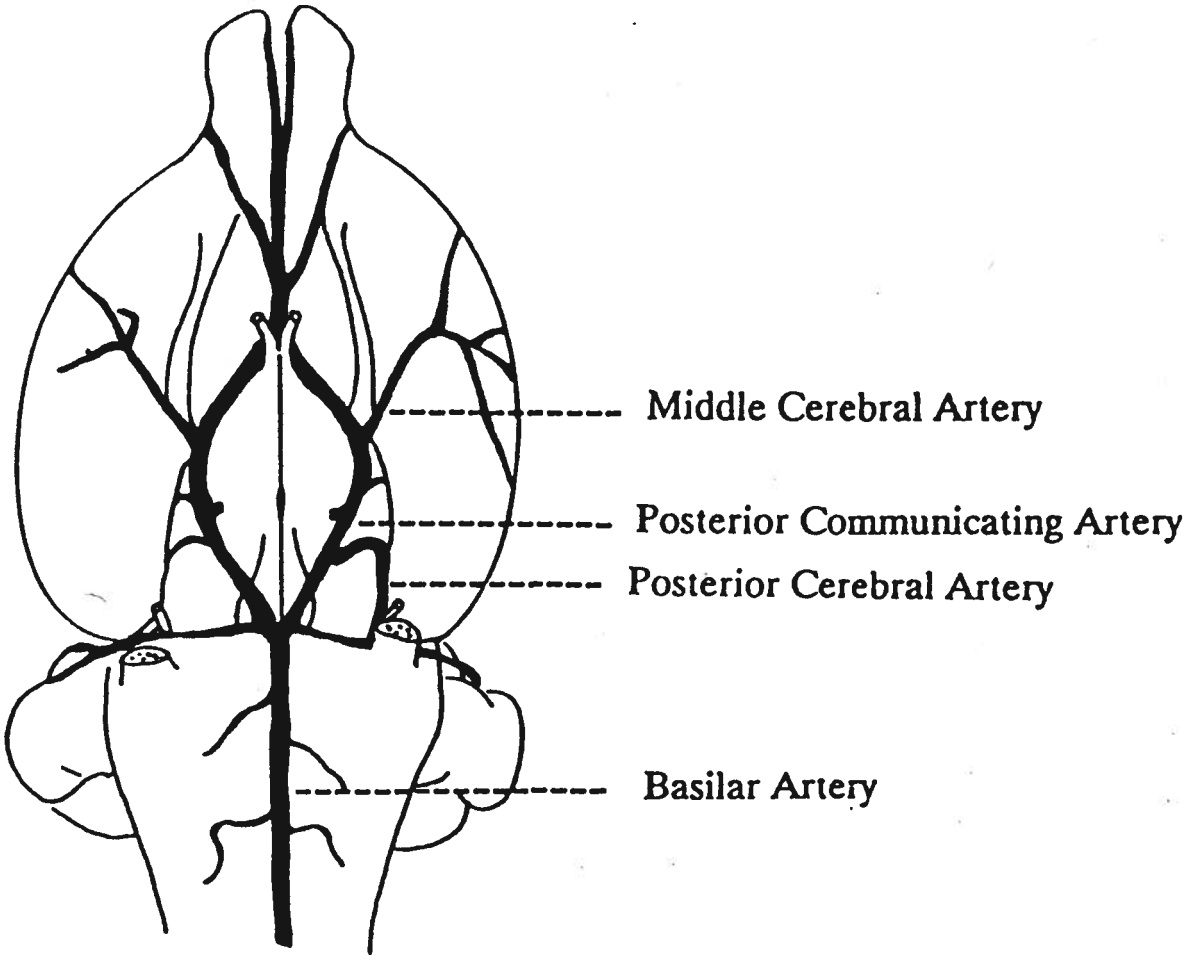
Chapter 2

Experimental Procedures

2.1. Dispersal of cerebrovascular smooth muscle cells

Experiments were performed on cerebrovascular smooth muscle cells (CVSMCs) isolated from the middle, basilar, posterior communicating and posterior cerebral arteries of adult Wistar rats (200-250 g, Charles River, Montreal). Rats were sacrificed by decapitation under sodium pentobarbital anaesthesia (30 mg/kg). The cerebral hemispheres and cerebellum were exposed by removing the parietal bone. The brain was carefully removed under aseptic conditions and placed in a 60 mm culture dish with brain dissecting buffer solution containing Ca^{2+} and Mg^{2+} free Hank's Balanced Salt solution (HBSS, Gibco Laboratories, Grand Island, NY) of the following composition, in mM: 138 NaCl, 5 KCl, 0.3 KH_2PO_4, 0.3 $Na_2HPO_4 \cdot 7H_2O$, 18 Dextrose, 4 $NaHCO_3$, 15.7 HEPES with penicillin 100 U/ml and streptomycin 100 μg/ml (Sigma Chemical Company, St. Louis, MO), pH 7.4. Under the low power of a dissecting light microscope, the basilar, middle, posterior communicating and posterior cerebral arteries and their first order and second order side branches (Fig 1) were removed (Fig 1) using iridectomy scissors and fine forceps and placed in a 65 mm culture dish filled with potassium glutamate (KG) buffer solution containing, in mM: 140 glutamic acid monopotassium, 16 $NaHCO_3$, 0.5 NaH_2PO_4, 16.5 Dextrose and 25 HEPES, pH 7.4. After incubation in KG solution for 10 minutes at 37°C, the vessels were minced with iridectomy scissors into 0.5 mm fragments. The fragments were then transferred into a 15 ml centrifuge tube containing 3 ml of 0.1% trypsin (Type C, Sigma, dissolved in KG solution) and incubated at 37°C for 10 minutes. The tissue suspension was then

Figure 1. Ventral aspect of the adult rat brain showing positions of the basilar, posterior communicating, posterior and middle cerebral arteries used in this study.


Middle Cerebral Artery
Posterior Communicating Artery
Posterior Cerebral Artery
Basilar Artery

centrifuged, and resuspended in a tube containing 3 ml of 0.3% collagenase (Type 1A, Sigma, dissolved in KG solution) and 0.2 ml of 0.5% trypsin inhibitor (Sigma, dissolved in KG solution). Following incubation at 37°C for 15 minutes, the cell suspension was allowed to sediment for 3 minutes and the supernatant was removed. Cells were resuspended in 3 ml of horse serum (heat-inactivated, Gibco) at 4°C in order to inhibit the activity of enzymes. This suspension was centrifuged and the supernatant was discarded. A 6 ml volume of KG solution was placed in the tube and cells were resuspended, washed and centrifuged. A final resuspension in 1 ml of KG solution was made. A 0.2 ml of this cell suspension was pipetted onto a pre-coated glass coverslip (see Section 2.1.1.) and allowed to settle for at least 30 minutes at room temperature (21-23°C). If intended for use in fura-2 determination of intracellular calcium, the coverslip was transferred into a 35 mm culture dish with 2 ml of Minimum Essential Medium (MEM, Gibco) containing, in mM: 4 L-glutamine, 16 $NaHCO_3$, 20 HEPES,and 15% horse serum (Gibco) (Sturek and Hermsmeyer 1986). These cultures were incubated in a 10% CO_2 incubator at 37°C for 2-4 days prior to use. In the case of electrophysiological studies, coverslips were transferred into 35 mm culture dishes with 2 ml of maintenance solution containing, in mM: 133 NaCl, 5 KCl, 0.8 $CaCl_2$, 1.3 $MgCl_2$, 5 Glucose, 10 HEPES with penicillin (100 U/ml, Sigma) and streptomycin (100 μg/ml, Sigma) (Zhang et al. 1991). Cells were maintained at 4°C in a refrigerator and used within 72 hours of plating.

2.1.1. Coverslip preparation

18 mm circle glass coverslips (Fisher Scientific Company, cat.no. 12-545-100) were cleaned in concentrated nitric acid at 60°C for 30 minutes and then

sterilized in an autoclave for 40 minutes. After soaking overnight in 10 μg/ml poly-D-lysine (Sigma) dissolved in 0.15 M borate buffer solution (pH 8.4), coverslips were rinsed in sterile distilled water. 0.2 ml HBSS (Gibco) containing 16.7 μg laminin (Sigma) was added to each coverslip 2-3 hours before use.

2.2. Identification of isolated CVSMCs

2.2.1. Masson trichrome test

Smooth muscle cells can be identified by the use of the Masson trichrome stain (Masson 1929; Spatz et al. 1983). In this method, the cells on the cover slip were first fixed with 2.5% formalin in phosphate buffer solution (PBS) containing, in mM: 149 NaCl, 2 KH_2PO_4, 4.2 Na_2HPO_4, pH 7.4 for 10 minutes. Nuclei were stained with 50% iron hematoxylin (1:1 in H_2O) in 5 minutes followed by differentiation with 1% acid alcohol for 1 second. After washing with distilled water, the cells were treated with 2% Ponceau acid solution (dissolved in 1% acetic acid) for 2.5 minutes. This dye stains the cytoplasm of smooth muscle cells. The cells were washed with distilled water and finally differentiated with 1% phosphotungstic acid for 5 minutes. Stained cells were dehydrated by sequential application of 70%, 95%, and 100% ethanol solutions, cleaned in xylene and mounted on precleaned glass microscope slides (Fisher) with mounting media (Cover bond,Scientific Products, McGraw Park, Illinois). The nuclei of CVSMCs and background cells (endothelial cells, fibroblasts) were stained black. The cytoplasm of CVSMCs was stained red, while the cytoplasm of cells of connective tissue origin was stained blue.

2.2.2. Immunofluorescent and immunoperoxidase staining of CVSMCs

Smooth muscle cells can also be identified by application of a monoclonal antibody specific for smooth muscle α-actin (Franke et al. 1980). In this method, coverslips were rinsed three times with PBS, fixed in 2.5% formalin (dissolved in PBS) for 5 minutes, air dried, and washed three times with PBS. After blocking nonspecific binding with 50% goat serum (Gibco) for 20 minutes at room temperature, coverslips were incubated with the primary antibody, a monoclonal antibody specific for smooth muscle α-actin (CGA7, Sigma, diluted 1:400 with PBS) overnight at 4oC. Coverslips were rinsed three times with PBS and incubated with a 1:5000 dilution in PBS of the secondary antibody, biotin-AP-affinipure goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc., West Grove, PA).

For immunofluorescent studies, the biotinylated IgG was localized by incubation with a 1:5000 dilution of fluorescent conjugated egg-white avidin (Jackson Immunoresearch Lab. Inc.) for two hours at room temperature. Stained coverslips were given a final rinse in PBS and mounted.on glass microscope slides with mounting media (Cover bond). These preparations were visualised and photographed using a fluorescent microscope (Zeiss, West Germany).

For immunoperoxidase staining, the biotinylated IgG was localized by incubating with a peroxidase conjugated egg-white avidin (Vector Lab) for two hours at room temperature. Coverslips were rinsed three times in PBS. The peroxidase reaction was developed by incubating the coverslips with the peroxidase substrate 3,3'-diaminobenzidine (DAB, 10 mg/ml in PBS) for 10

minutes and 0.03% hydrogen peroxide (H_2O_2) for 30 minutes. These coverslips were given a final rinse in PBS. Cells were dehydrated in graded ethanol-water solutions, cleared in xylene and mounted onto microscope slides with mounting media. Immunoperoxidase preparations were visualized and photographed under light microscopy (Olympus CCK-1, x300 magnificaton).

The following controls for specificity of staining were done for both the immunoperoxidase and immunofluorescent procedures described above: (a) replacement of the primary antibody, CGA7, by an irrelevant antibody specific for gastric inhibitory peptide (GIP, MRC peptide group, Dept. Physiology, UBC), (b) omission of the primary antibody, (c) incubation with 50% goat serum (Gibco).

2.3. Determination of free intracellular calcium concentration in isolated CVSMCs

The fluorescent Ca^{2+} indicator fura-2AM (Grynkiewicz et al, 1985) was used to measure the level of free intracellular calcium $[Ca^{2+}]_i$ present in isolated CVSMCs. This method was also applied to determine the sensitivity of the cells to the vasoconstrictor serotonin (5-HT) (Capponi et al. 1987). These experiments were performed on 2-3 day old cultures incubated at 37°C, as described previously. These cultures were used, since the spherical CVSMCs became morphologically distinct from the flat background cells after 2-3 days incubation at 37°C, as revealed by Masson staining and immunocytochemical staining for smooth muscle α-actin (see Results section).

2.3.1. Loading of the fura-2-AM indicator into CVSMCs.

Fura-2AM (Molecular Probes Inc., Eugene, OR) 1 mg was dissolved in 1 ml of chloroform, and 50 μl aliquots of this solution were pipetted into 20 small plastic ampules. These ampules were placed in a dissicator, and vacuum-dried for 3 hours. The dried aliquots were stored at -70°C. For each coverslip used, 50μg of fura-2AM per plastic ampule was dissolved in 50 μl of dimethyl sulfoxide (DMSO, Sigma), producing an 1 mM stock solution. 20 μl of this stock solution were added to 2 ml of Earl's balanced salt solution (EBSS) containing (mM): 5 KCl, 0.8 $MgCl_2$, 1.8 $CaCl_2$, 117 NaCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 5.6 Dextrose, and 10 HEPES, pH 7.4, temperature 37°C. 1 ml of this solution was immediately added to a 35 mm culture dish containing 1 ml EBSS. The coverslip with the smooth muscle cells was placed face-up in the dish. Fura-2AM is hydrophobic and therefore penetrates the plasma membrane without difficulty. Cells were incubated with fura-2AM for 1.5 hours at 37°C to allow the uptake to reach equilibrium. Once inside the cell, cytosolic esterases cleave the acetoxymethyl groups from the indicator to release free fura-2 which is membrane impermeant and is therefore trapped inside the cells. After a rinse with EBSS, a further incubation of 30 minutes at 37°C was carried out in 2 ml of fresh EBSS to wash out excess fura-2AM. These loading conditions were chosen to minimize the risk of overloading the cells with dye, and also served to suppress the formation of partially hydrolyzed fura-2 derivatives (Tsien 1988).

2.3.2. Cell chamber and perfusion system for fura-2 measurements.

After loading, coverslips were mounted face-down into a specially designed laminar flow-through chamber (volume 350 μl) filled with Hank's

balanced salt solution (HBSS) consisting of (mM): 150 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES, pH 7.4. Silicone grease was used to complete a water-tight seal between the coverslip and chamber. The chamber was inserted into a stainless steel holder, and then the entire assembly was mounted onto the stage of a Jenalumar Zeiss fluorescent microscope. Cells were viewed under oil immersion at 100x magnification on the microscope.

During determination of intracellular free calcium, cells were continuously perfused with HBSS at 21-23°C using a flow rate of 4 ml/min. The HBSS contained 1 mM Ca^{2+} in all experiments utlilizing fura-2. This concentration of Ca^{2+} was selected to allow comparison of results with a previous study of this type, conducted on aortic VSMCs (Nabika et al., 1985). Ketanserin (Janssen Biotech, Beerse, Belgium), lanthanum chloride (Sigma), cobalt chloride (Sigma) and nifedipine (Sigma) were dissolved in HBSS and perfused over the cells in place of drug-free HBSS. Serotonin (5-hydroxytryptamine, 5-HT, Sigma) was dissolved in 100 μl HBSS and injected into the inlet tube of the perfusion chamber. When the effects of antagonists were being studied, these agents were also present in the 100 μl volume containing 5-HT, at the same dose as applied to the cell chamber. Application of drug-free HBSS in this way produced a transient increase in $[Ca^{2+}]_i$ in most cells tested. The peak increase in $[Ca^{2+}]_i$ seen during these artifactual responses averaged 23 $\pm$ 5% over baseline levels of free intracellular calcium (n = 39 cells, mean $\pm$ SEM). For quantitative analysis, this artifactual increase was subtracted from the signal induced by serotonin application to yield a more accurate estimate of the effect of 5-HT on $[Ca^{2+}]_i$. To reduce the influence of errors introduced by artifact subtraction, only corrected increases in $[Ca^{2+}]_i$ that exceeded baseline

levels by a total of 50% or more were considered during quantitative analyses of 5-HT responses..

2.3.3. Measurement of $[Ca^{2+}]_i$ in CVSMCs

Free intracellular calcium was determined in individual CVSMCs by using a Zeiss Jenalumar microscope equipped with an epifluorescence detector. The light source was a 200 W mercury arc lamp powered by a DC power supply. The light was first passed through one of three differential interference filters which selèctively passed the following wavelengths: 350 $\pm$ 10, 380 $\pm$ 10 or 365 $\pm$ 10 nm (Fig. 2). These filters were held in a turret which could be rotated by a computer-controlled stepping motor. The light was then passed through a 410 nm dichroic mirror and a 100x apochromatic oil immersion lens with a numerical aperture of 1.4 and an adjustable diaphragm to reduce the light intensity. A field diaphragm in the light path prior to the dichroic mirror was used to reduce the area of illumination to the size of a single CVSMC. All fluorescent light passed back through the dichroic mirror and a 450 nm band pass filter to reduce background fluorescence. The 365 nm filter was employed during cell selection, since both the Ca^{2+}-bound and unbound fura-2 molecules emit a similar intensity of fluorescence at this excitation wavelength (Fig. 3) (Grynkiewicz et al. 1985). The emitted fluorescence taken at 350 nm (indicator fluorescence enhanced by Ca^{2+} binding) and 380 nm (decreased by Ca^{2+} binding) was either observed by deflecting the light to the microscope eyepiece, or quantified by deflecting the light to a photomultiplier tube placed in the camera position of the microscope. The photomultiplier converted the light signal into a DC voltage (Fig. 2). This voltage was then converted to digital form by an analogue to digital converter board in an IBM compatible computer, and stored on the

Figure 2. Schematic illustration of the system for measuring $[Ca^{2+}]_i$ using the fluorescent probe fura-2.

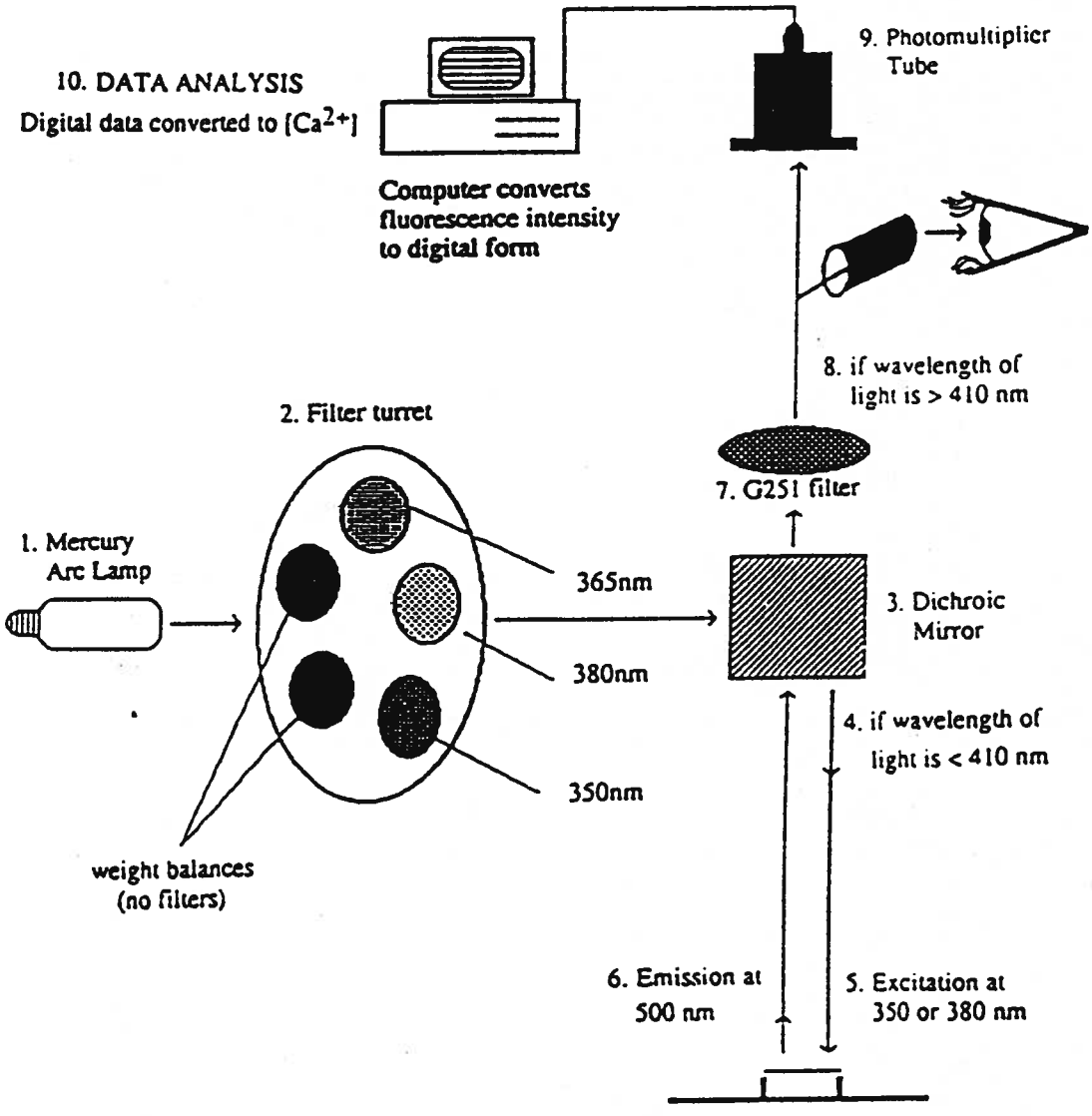
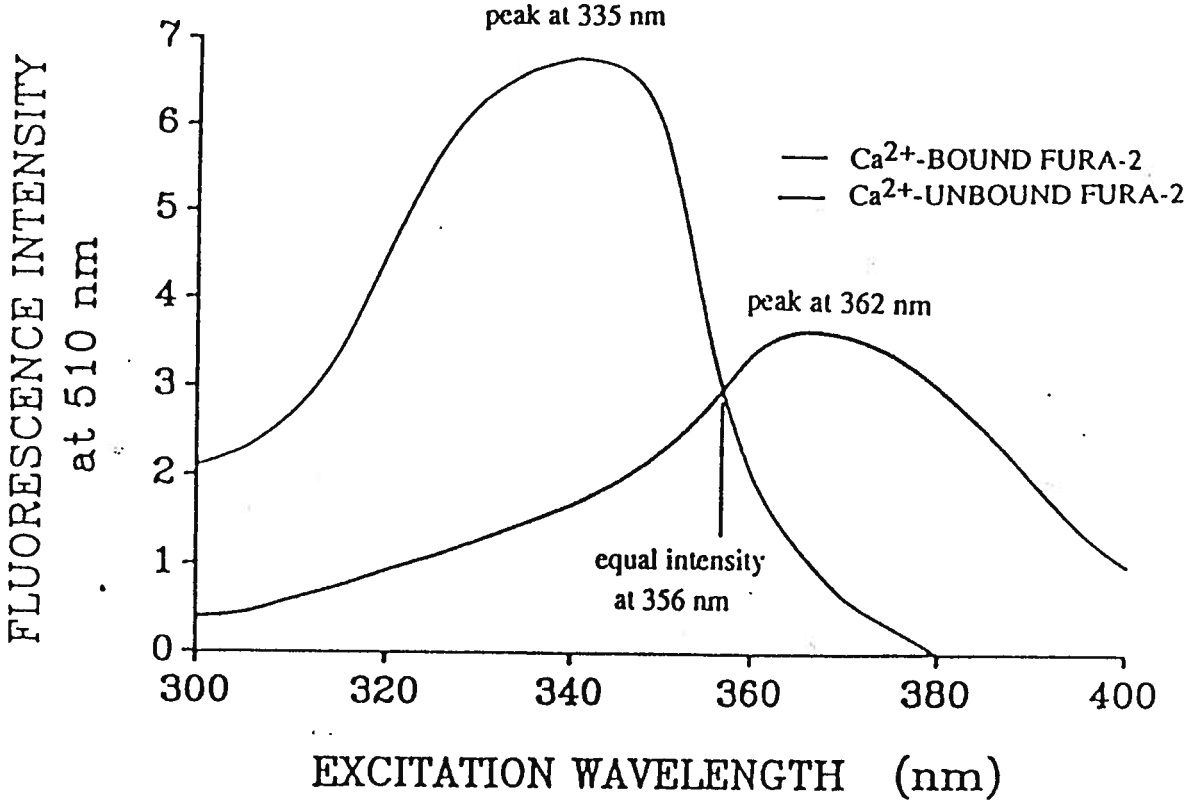

10. DATA ANALYSIS
Digital data converted to [Ca2+]
Computer converts fluorescence intensity to digital form
9. Photomultiplier Tube
8. if wavelength of light is > 410 nm
7. G251 filter
2. Filter turret
1. Mercury Arc Lamp
365nm
380nm
350nm
3. Dichroic Mirror
4. if wavelength of light is < 410 nm
weight balances (no filters)
6. Emission at 500 nm
5. Excitation at 350 or 380 nm

Figure 3. Excitation spectra of 1 mM fura-2. Both the Ca^{2+} bound and the unbound fura-2 emit a similar intensity of fluorescence at an excitation wavelength of 365 nm.



EXCITATION SPECTRA OF 1 mM FURA–2.

Adapted from Grynkiewicz et. al. 1985.

computer hard disk. Measurements of fluorescence ratios were obtained on a 5 second time base. Transient changes in $[Ca^{2+}]_i$ occurring in less than 5 seconds could have gone undetected by this system.

Fura-2 measurements were made from a total of 110 cells in 40 cultures derived from 20 dissections. No significant trends were noted in the baseline or 5-HT stimulated calcium levels as a function of the number of days passed *in vitro*. This observation makes it unlikely that 5-HT sensitivity was being gradually down-regulated by the presence of serotonin in the culture medium. However, cultures derived from different dissections did show appreciable variability in the proportion of cells responsive to 5-HT, and in the degree of responsiveness the cells showed to serotonin. This variability could have resulted in part from the use of proteolytic enzymes, since it was very difficult to ensure that each dissection exposed the cells to the same degree of enzyme activity. However, the possibility that some of the variability noted is also present *in vivo* cannot be discounted.

2.3.4. Calculation of free intracellular calcium in CVSMCs.

The concentration of free intracellular calcium $[Ca^{2+}]_i$ in the cells studied was calculated using the following formula (Grynkiewicz et al., 1985):

$$[Ca^{2+}]_i = K_d \times \beta \times R - R_{min}/R_{max} - R$$

where K_d = the equilibrium dissociation constant for the association of fura-2 with cytosolic free calcium: 224 μM.

β = ratio of values: the fluorescence intensity at 380 nm with zero $[Ca^{2+}]$/380 nm with infinite $[Ca^{2+}]$

R = experimentally determined ratio of the fluorescence intensity at 350 nm/380 nm.

R_{min} = ratio of values: the fluorescence of intensity at 350 nm/380 nm with zero $[Ca^{2+}]$.

R_{max} = ratio of values: the fluorescence of intensity at 350 nm/380 nm with infinite $[Ca^{2+}]$.

The values of β, R_{min} and R_{max} were determined by calibration of the system, as described in the following section. For this study, β = 7.464, R_{min} = 0.584, and R_{max} = 5.549.

2.3.5. Calibration of the fura-2 system for determination of $[Ca^{2+}]_i$ in CVSMCs.

Calibration procedures were carried out using 2-4 day old CVSMC cultures loaded with fura-2AM in the manner previously described. A pair of cultures was first placed in the measuring chamber filled with HBSS, containing 1.0 mM Ca^{2+} and 0.8 mM Mg^{2+}. One culture was then exposed to Ca^{2+} free HBSS containing 5 mM EGTA and 10 μM Br-A23187, a non-fluorescent calcium ionophore (HSC Reseach Development Corporation, Toronto, Canada). After 15 minutes, the ratio of flourescence intensity at 350 nm and 380 nm was measured in 30 cells, under conditions in which $[Ca^{2+}]_i$ was effectively 0 mM. This ratio yielded the value of R_{min}. The second culture was now exposed to standard HBSS containing 1 mM Ca^{2+} and 10 μM Br-A23187. The ratio of fluorescence intensities at 350 nm and 380 nm was measured in 30 cells, as extracellular Ca^{2+} quickly entered the cell and $[Ca^{2+}]_i$ became effectively 1 mM. This ratio yielded the value of R_{max}. The value of β was calculated by

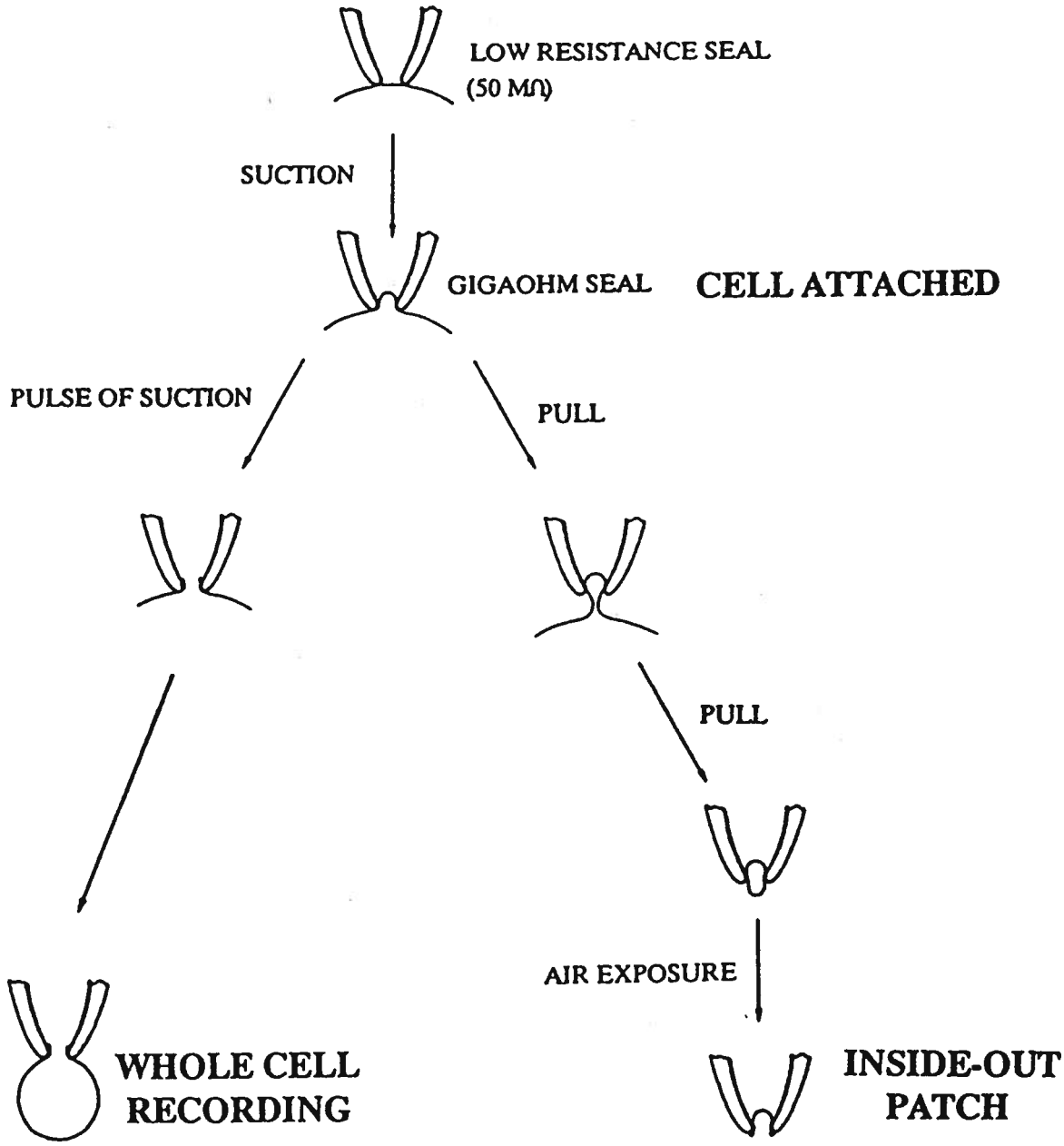
dividing the fluorescence intensity at 380 nm obtained from the first culture by the value obtained from of the second culture. This entire procedure was repeated in two further cultures prepared from a different dissection. The values of R_{min}, R_{max} and β obtained from these cultures did not differ from the original estimates by more than 10%. The average values were used in subsequent calculations of $[Ca^{2+}]_i$.

2.4. Electrophysiological studies on isolated CVSMCs.

Patch clamp recordings were carried out at room temperature (21-23oC). At the time of recording, one culture dish containing cells was taken out of the refrigerator. The maintenance solution was drawn off and replaced with 2 ml of a bathing solution appropriate to the experimental design. The culture dish was then mounted on the stage of an inverted, phase-contrast microscope (Olympus CK, Tokyo, x300 magnification). The microscope, patch clamp amplifier head-stage and the perfusion system were mounted on a vibration damping table (Kinetic Systems Inc., Boston, Massachusetts) and shielded from electromagnetic radiation by a Faraday cage.

Recordings were made using the cell-attached, whole-cell (current-clamp), inside-out patch configurations (Fig. 4) of the patch clamp technique. In this technique, a low resistance seal is produced upon mechanical contact between the electrode and the cell membrane. With gentle suction, the seal increases in resistance by 2-3 orders of magnitude, resulting in a cell-attached gigaohm seal. Background noise is greatly reduced with this seal, leading to improved resolution of recordings. Additional suction applied to the patch created the whole-cell recording configuration for study of basic

Figure 4. Schematic illustration of the procedures used to make the whole-cell and inside-out patch recordings reported in this study.


LOW RESISTANCE SEAL
(50 MΩ)
SUCTION
GIGAOHM SEAL
CELL ATTACHED
PULSE OF SUCTION
PULL
PULL
AIR EXPOSURE
WHOLE CELL
RECORDING
INSIDE-OUT
PATCH

electrophysiological properties of the cells. To form an inside-out patch, the electrode was drawn away from the cell surface, creating a membrane vesicle at the electrode tip. The outer membrane of this vesicle was ruptured by brief passage through the solution/air interface (Hamill et al. 1981).

2.4.1. Preparation of patch electrodes

Patch electrodes were pulled from capillary tubes of borosilicate glass (1.5 mm OD x 0.75 mm ID, Frederick Haer Corp, Brunswick, ME) in two successive steps on a modified David Kopf 700 vertical microelectrode puller. Electrode tip diameters ranged from 1-2 μm. Electrodes were coated to within 50 μm of their tips with 3140 RTV sealant (Dow Corning Corporation, Midland, Michigan) which reduced pipette-bath capacitance by forming a hydrophobic surface and increasing the wall thickness (Hamill et al. 1981). Patch clamp recordings of K_{Ca} channel activity were made from CVSMCs cultured for 2-3 days at 37oC or alternatively from cells maintained at 4oC, as described previously. It was evident that the latter procedure produced a higher probability of obtaining inside-out patches containing functional K_{Ca} channels. It was therefore decided to employ cells maintained at 4oC for all electrophysiological studies reported in this thesis.

A disadvantage of using the cooled cell preparation was that both CVSMCs and other cell types retained a spherical morphology over the incubation period (see Results). For this reason, it was necessary to confirm the identity of each cell by means of the Masson trichrome test performed at the end of the recording session. Recordings were made from several cells in the same field. After completion of recording, the entire plate of cells was stained using

the Masson technique, without changing the field of cells visible in the microscope. Data from cells which failed to react positively to this test, or which dissociated from the culture dish during staining were not used in this study.

At the time of the experiment, the tip of each electrode was fire-polished to produce a clean and smooth tip rim. This facilitated the formation of a large resistance seal between the electrode and the cell membrane (Hamill et al. 1981). To carry out this step, the electrode was placed on the stage of a compound microscope (x480). The tip of electrode was brought to within 160 μm of a platinum filament heated by the passage of DC current for about 1 second. After fire-polishing and filling with physiological saline, electrodes had resistances of 5-10 MΩ.

2.4.2. Gravity perfusion system for application of experimental solutions.

A perfusion system was designed to apply experimental solutions to the cytoplasmic face of isolated membrane patches. The system comprised of eight 60 ml syringe barrels mounted above the stage of the microscope. Plastic taps were connected tightly to the end of each cylinder to allow for selection of the appropriate test solution. A short length of plastic tubing ran from this assembly to an L-shaped glass tube held in a micromanipulator. Following patch isolation, the orifice of this tube was moved to within 200 μm of the patch electrode tip. Tests performed using isolated inside-out patches containing K_{Ca} channels and solutions containing various concentrations of Ca^{2+} showed that membrane patches equilibrated with a new test solution within 25 seconds.

2.4.3. Experimental solutions

Prior to recording, the medium bathing the cells was changed to a standard extracellular solution containing (in mM): 140 NaCl; 4 KCl; 1 $CaCl_2$; 1 $MgCl_2$; 10 HEPES, pH 7.4. In cell-attached and in whole-cell recordings, patch pipettes were filled with a solution of composition (mM): 140 KCl; 3 NaCl; 0.65 $CaCl_2$; 3 EGTA; 10 HEPES, pH 7.4. The free Ca^{2+} concentration in this solution was calculated at 10^{-8} M using procedures devised by Stockbridge (1987). The level of free Ca^{2+} present as a contaminant in other salts was found to be below 10^{-5} M, as measured by a calcium-sensitive electrode (Model 93-20, Orion, Massachusetts). This value was small compared to the total calcium concentration needed in all the EGTA buffered salines. A contamination level of 8×10^{-6} M was assumed when calculating the total concentration of $CaCl_2$ required in experimental solutions. During recordings from inside-out membrane patches, pipettes usually contained a solution of composition (in mM): 140 KCl; 10 HEPES; 3 EGTA; 0.65 $CaCl_2$, pH 7.4. The free calcium concentration in this solution was calculated at 10 nM (Stockbridge 1987). The cytoplasmic face of the isolated membrane patches was normally exposed to a solution containing (in mM): 140 KCl, 10 HEPES, 3 EGTA, pH 7.4, and the following concentrations of total and free Ca^{2+}: 0.36 mM, 5 nM; 0.65 mM, 10 nM; 1.72 mM, 50 nM; 2.19 nM, 0.1 μM; 2.54 mM, 0.2 μM; 2.79 mM, 0.5 μM; 2.7 mM, 1 μM; 2.98 mM, 5 μM; 3 mM, 10 μM and 3.1 mM, 100 μM (Stockbridge 1987).

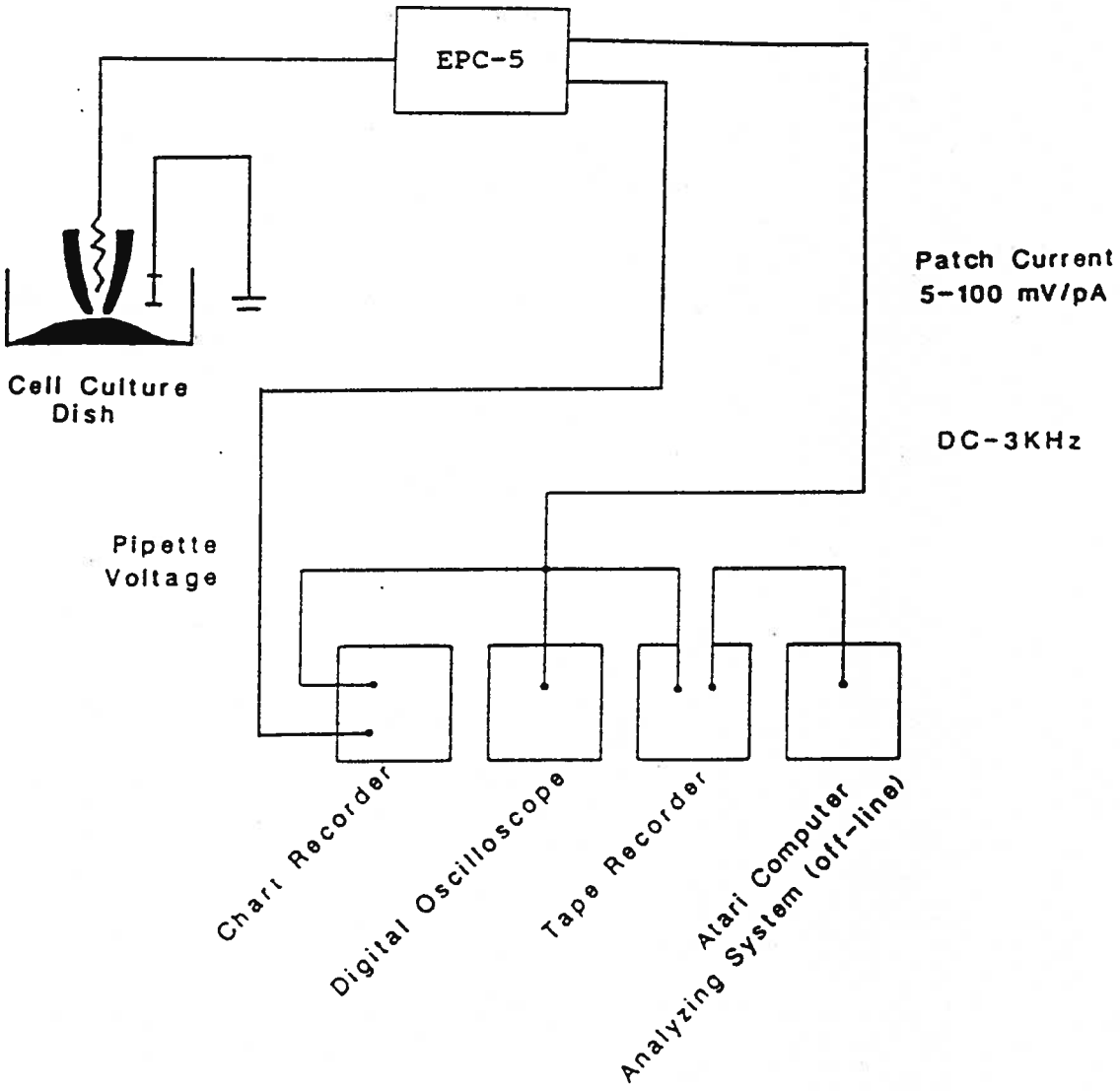
To determine the potassium selectivity of K_{Ca} channels, 80 mM KCl was replaced by an equimolar amount of NaCl or CsCl, in either the pipette solution or in the solution bathing the cytoplasmic membrane face. Replacement of K^+ by ions of different mobility in solution lead to the appearance of liquid junction

potentials (Hubbard et al. 1969). These voltages were measured by flowing experimental salines past the tip of a patch electrode filled with standard extracellular solution. Liquid junction potentials were indicated as the change in zero-current pipette voltage measured by the patch clamp amplifier. Appropriate corrections were applied to the patch potentials measured during experimental solution changes. Correction factors were less than ± 5 mV. The effect of the potassium channel blocker tetraethylammonium (TEA) on K_{Ca} channels was determined by dissolving the chloride salt of the drug (Eastman Kodak, Rochester, New York) in the solution bathing the cytoplasmic face of isolated membrane patches.

2.4.4. Analysis of patch clamp data

The general arrangment of the analysis system is shown in Figure 5. Patch electrode currents and voltages were recorded using a List EPC-5 amplifier (Medical System Corp, New Jersey). Signals were captured on FM wideband tape at a bandwidth of DC-3kHz (-3dB, Bessel) using an instrumentation recorder (Store 4DS, Racal-Decca, England) and displayed on a rectilinear pen recorder (DC-100 Hz, Gould, Ohio). Data analysis proceeded off-line using an Atari Mega 4 computer (Atari, Sunnyvale, California) and software devised by Instrutech Corporation, New York. After analogue-to-digital(A-D) conversion at 8 kHz, current records 8 kBytes in length were displayed on a high-resolution monitor, and obvious artifacts were edited out. Gaussian digital filtering was applied to yield a final corner frequency, $f_c = 2$ kHz (-3dB) using the relation $1/f_c^2 = 1/f_1^2 + 1/f_2^2$ where f_1 is the Gaussian filter cut off and f_2 is that of the tape recorder output filter (Colquhoun and Sigworth, 1983). A threshold for event detection was set at 50% of the mean

Figure 5. Block diagram of the patch clamp recording system.


EPC-5
Patch Current
5-100 mV/pA
DC-3KHz
Cell Culture
Dish
Pipette
Voltage
Chart Recorder
Digital Oscilloscope
Tape Recorder
Atari Computer
Analyzing System (off-line)

single channel current for K_{Ca} channels encountered in the patch under study. Under-estimation of the durations of brief openings and closings barely crossing this threshold was addressed by applying a frequency correction factor. Open time distributions were valid down to the dead-time of the recording system, given as $T_d = 0.179/f_c$ i.e. 90 μs (Colquhoun and Sigworth 1983).

Frequency histograms of channel open times and amplitudes were constructed using bin widths 0.4 % of the total range of the parameter being plotted. Open time distributions were plotted on a logarithmic time axis. This transformed the exponential function $y = A.e^{-t/\tau}$ into a curve with peak amplitude at the time constant τ and an area proportional to the number of events in that component (Sigworth and Sine 1987). Simplex maximization of likelihood was used to fit one, two or three exponential components to the observed open time distributions (Colquhoun and Sigworth 1983). Curve fitting disregarded channel openings briefer than twice the system dead-time. Frequency distributions of current amplitudes were fitted by eye with computer-generated Gaussian functions. Currents and voltages were denoted with respect to the cytoplasmic face of the membrane in all recording configurations used. Reversal potentials for single channel currents were determined as the zero current intercepts of theoretical curves fitted to the data points by linear or non-linear regression, as appropriate.

The probability, P_o of finding a single K_{Ca} channel in the open state during a recording of total duration, T_{tot} was calculated from the relation

$$P_o = (T_1 + T_2 + ... + T_N)/NT_{tot}$$

Here, N is the total number of functional K_{Ca} channels in the patch. T_1, T_2, ... T_N are the times when at least 1, 2 ... N channels were open (Mayer et al. 1990).

N took values in the range 0 to 4, as estimated by depolarizing each patch to +50 mV with 1 mM Ca^{2+} present at the cytoplasmic membrane face. Active patches generally had 1-2 K_{Ca} channels present. As noted earlier CVSMCs which had been cultured at 37°C typically showed more inside-out patches devoid of K_{Ca} activity than was the case in cells maintained at 4°C until use. For this reason, the latter procedure was adapted in all single channel studies on K_{Ca} currents presented in this thesis. Results were expressed as mean $\pm$ S.E.M. The Student's t-test or ANOVA was used to evaluate differences between experimental groups.

Chapter 3

Results

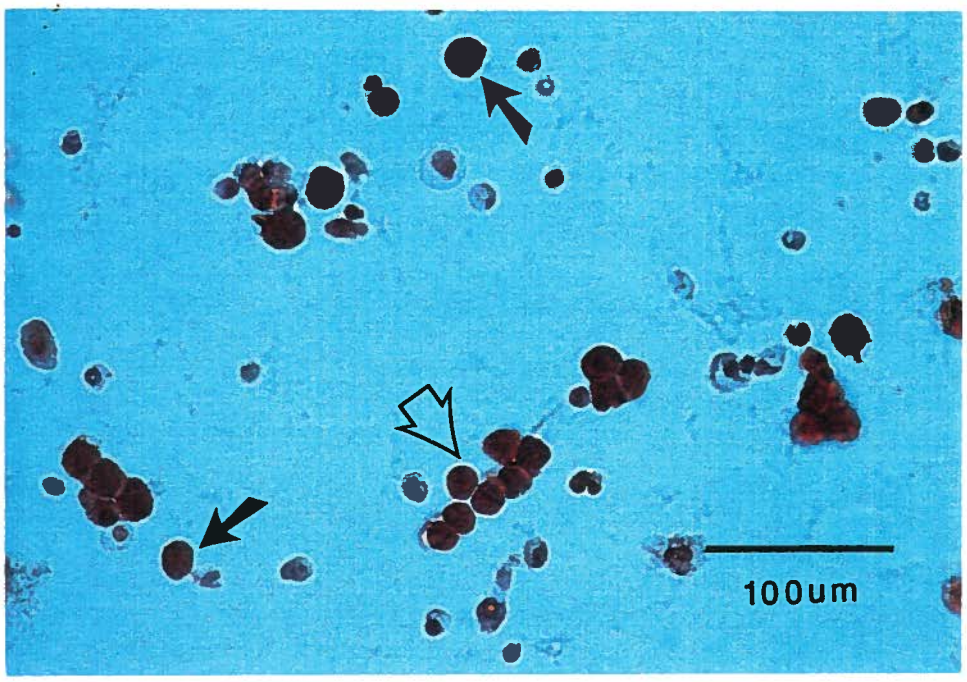
3.1. Morphological characteristics of CVSMCs *in vitro*.

Collagenase-trypsin treatment of isolated vessel fragments yielded a heterogeneous preparation consisting of various cell types and debris. These dispersed CVSMCs did not readily attach to the culture substrate, making it difficult to conduct fura-2 or electrophysiological studies on freshly isolated cells. However, after 2-3 days *in vitro*, many CVSMCs did become firmly attached to the substrate. The appearance of these preparations was dependent on the incubation protocol followed. After 2-3 days maintenance at 4oC , CVSMCs usually appeared as Masson positive cells of spherical shape with an average diameter of $12 \pm 0.6\,\mu m$ (mean $\pm$ SEM, n = 31). Occasionally, CVSMCs were slightly fusiform in shape on these plates. Small clusters of incompletely dissociated CVSMCs were also present in these preparations. These plates contained a number of Masson-negative cells , which were also of spherical shape (Fig. 6A).

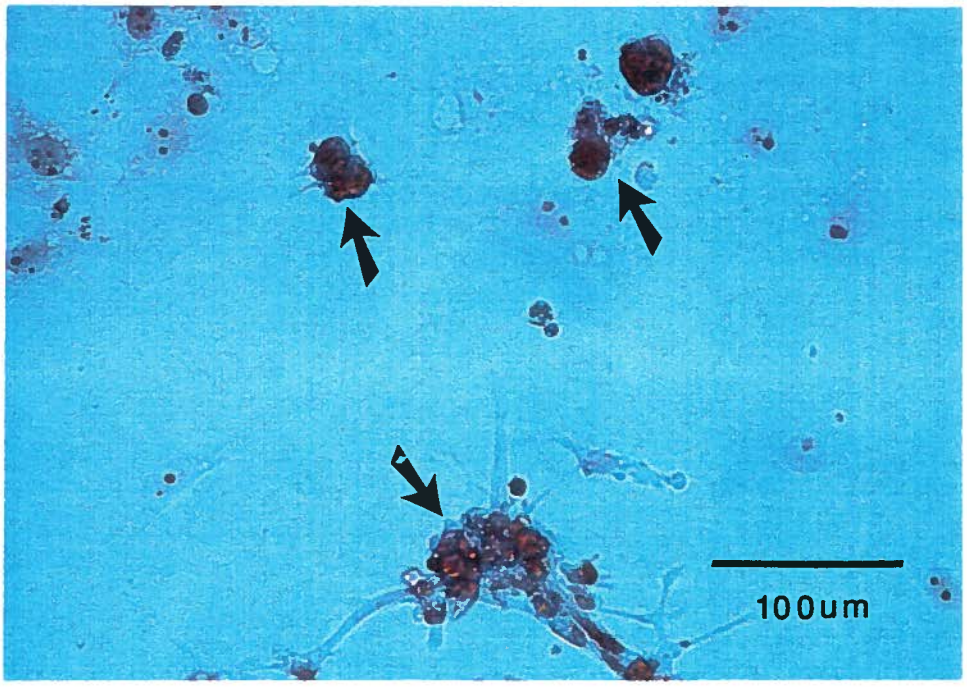
In preparations cultured for 2-3 days at 37oC, Masson positive CVSMCs retained the spherical morphology seen after low temperature incubation (Fig. 6B). However, the Masson negative cells in these cultures adopted flattened, extended morphologies characteristic of fibroblasts (Rose 1970) and polygonal endothelial cells maintained *in vitro* (Warren 1990). The cytoplasm of these background cells stained blue-grey under the Masson trichrome test (Fig. 6B).

Figure 6. Photomicrographs of dispersed rat cerebral artery smooth muscle cells *in vitro*, treated with the Masson trichrome stain. A, Preparation incubated at 4oC for 3 days. Masson positive CVSMCs are stained red and appear as single cells (black arrows) or as small clusters of cells (open arrows). Masson negative cells (white arrows) and debris are also present. B, A second plate incubated at 37oC for 3 days. The Masson positive CVSMCs (red) have retained a spherical shape (black arrows). The Masson negative background cells (blue-grey) have differentiated into flattened, extended shapes (white arrows).

A



B



· CVSMCs incubated either at 4oC (Fig. 7A) or at 37oC (Fig. 7B) for 2-3 days also reacted positively on incubation with anti-α-actin antibody, and were readily visualized using the fluorescent FITC marker. Omission of the primary antibody from this procedure lead to the loss of specific labelling in all preparations tested (Fig. 7C). CVSMCs exposed to the anti-α-actin antibody were also readily visualized using the peroxidase marker (Fig.8). The morphology and size of antibody-positive cells agreed well with results obtained using the Masson test.

3.2. Intracellular free calcium level of CVSMCs in culture

3.2.1. Resting $[Ca^{2+}]_i$ of CVSMCs in culture

$[Ca^{2+}]_i$ was determined using 2-3 day old cultures of CVSMCs incubated at 37°C, since the smooth muscle cells in these plates were morphologically distinct from background cells. Measurements of fluorescence ratios from a total of 31 CVSMCs in the same culture plates used for calibration yielded an average value of resting $[Ca^{2+}]_i$ of 39 ± 3.6 nM. Fluorescence ratios were also measured in 110 additional cells in 28 other cultures. $[Ca^{2+}]_i$ was calculated in these cells using the calibration factors determined from the first group of plates. These cells yielded a resting $[Ca^{2+}]_i$ level of 41 ± 5.6 nM. This value was not significantly different from that determined in the calibration plates ($P > 0.05$, Student's t-test), indicating that fluorescence ratio measurements were stable over the course of this study.

3.2.2. Effect of serotonin on $[Ca^{2+}]_i$ of CVSMCs in culture

Figure 7. Photomicrographs of dispersed CVSMCs after incubation with a monoclonal antibody directed against smooth muscle α-actin and visualized using the fluorescent marker FITC. A, A culture incubated at 4°C for 3 days. Antibody positive CVSMCs appear bright green (white arrows). B. A culture incubated for 3 days at 37°C. Antibody positive CVSMCs again appear bright green (white arrows). Faintly stained background cells exhibited a degree of auto-fluorescence. C. Auto-fluorescence is also present in a negative control culture, incubated at 37°C for 3 days and processed in the absence of the primary antibody. Calibration bar is 100 μm for all micrographs.

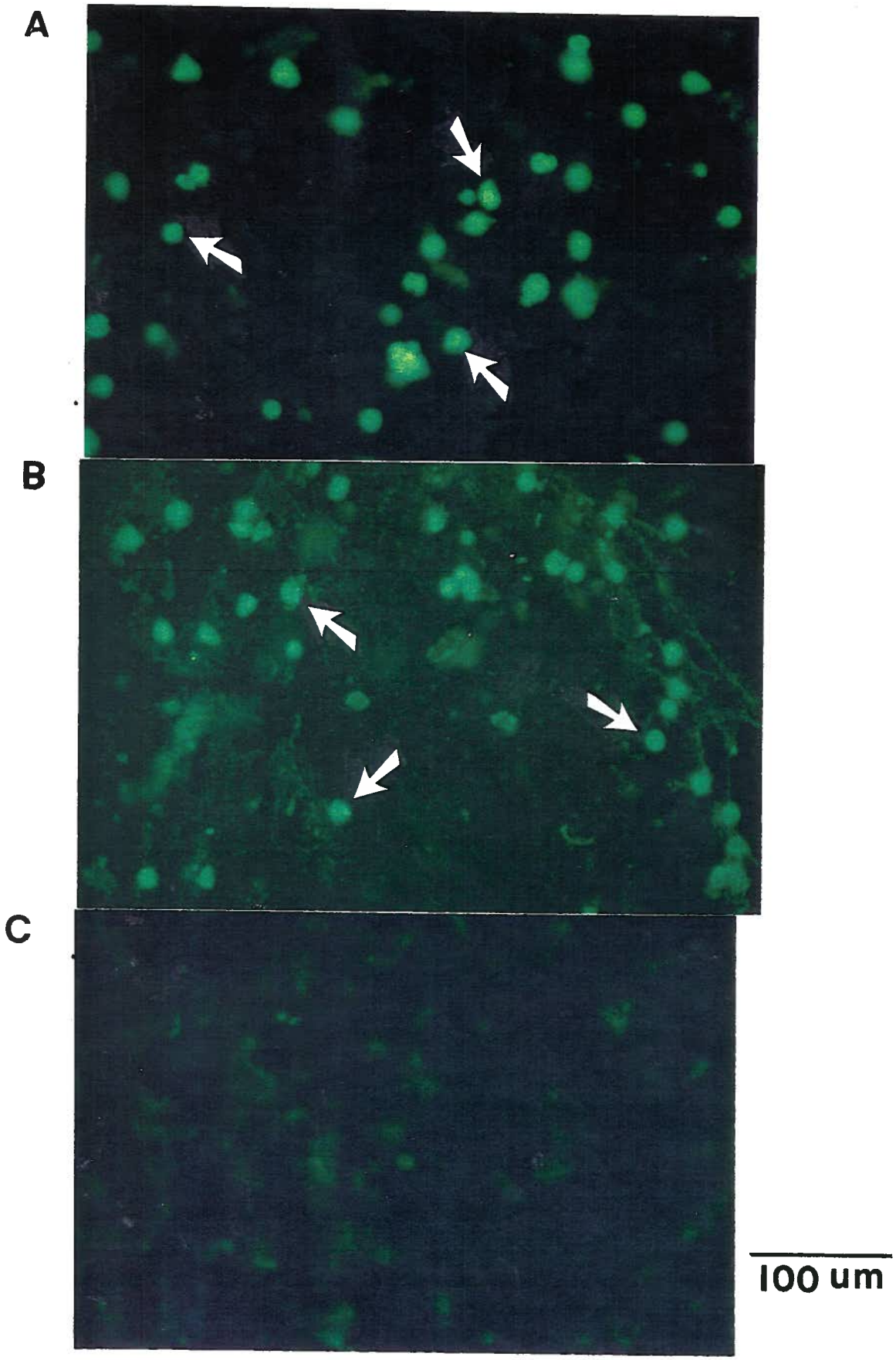
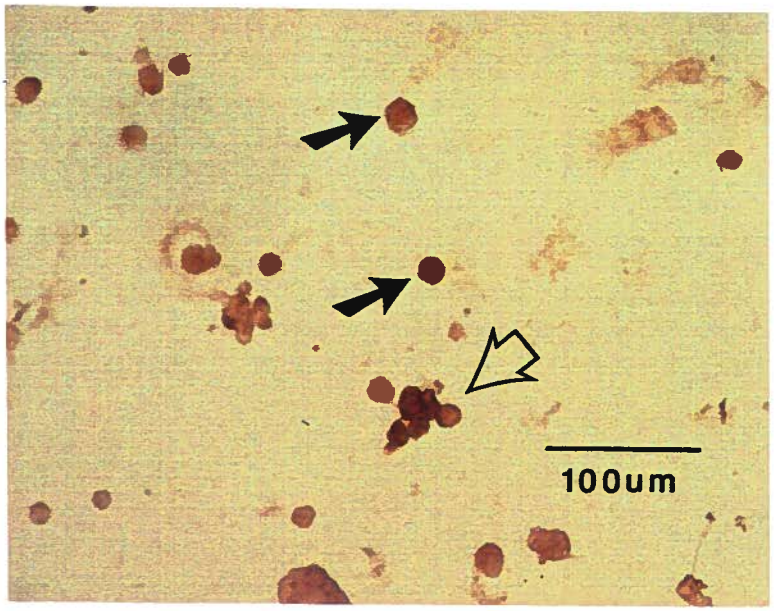
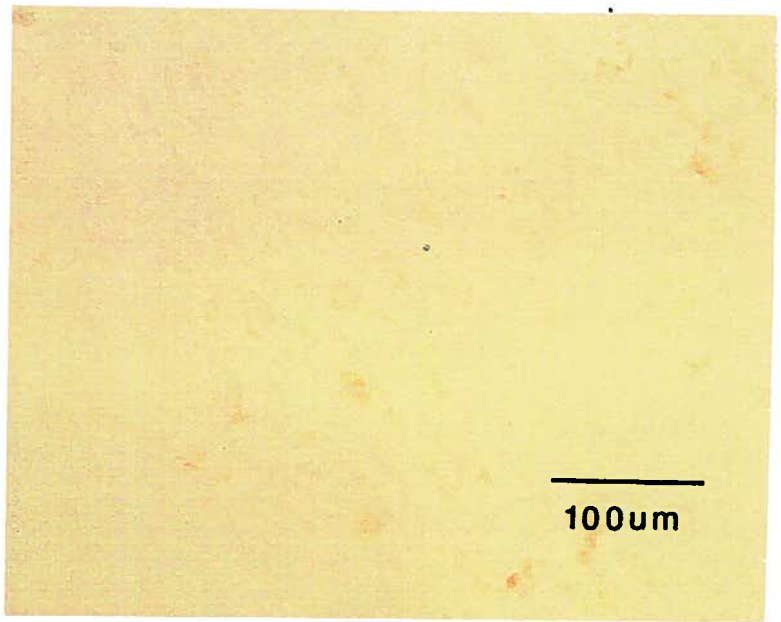

A
B
C
100 um

Figure 8. Photomicrographs of dispersed CVSMCs after incubation with a monoclonal antibody against smooth muscle α-actin and visualized using the peroxidase marker. A, A culture incubated at 37oC for 3 days. Antibody positive CVSMCs appear dark brown and appeared as single cells (black arrows) or small clusters (open arrows). Antibody negative background cells are also present. B, A negative control culture incubated at 37oC for 3 days in the absence of the primary antibody. Calibration bar is 100 μm for both micrographs.

A



B



5-HT (10 nM to 100 μM) was applied by injecting 100 μl volumes of the agonist into the inlet port of the cell chamber. Under these conditions, 5-HT induced a rapid, transient increase of $[Ca^{2+}]_i$ above resting levels from 84 out of 110 cells tested. A typical response of this type, induced by application of 1 μM 5-HT, is shown in Fig. 9. The average time course of the response of 13 cells to 1 μM 5-HT is shown in Fig. 10. No evidence was seen for oscillatory or long-lasting changes of free Ca^{2+} levels following the transient application of serotonin to CVSMCs.

It seemed possible that the 5-HT induced rise in $[Ca^{2+}]_i$ simply reflected an influx of extracellular Ca^{2+} through voltage-sensitive calcium channels in the smooth muscle cell membrane. That such channels were indeed present on CVSMC membranes was indicated by the fact that depolarization by high potassium solutions also evoked a increase in intracellular free calcium (Fig. 11).

To address this possibility, the effect of three known blockers of voltage-dependent Ca^{2+} fluxes, nifedipine, La^{3+} and Co^{2+} (Suprenant et al. 1987) were tested by coapplication with 5-HT. It was found that neither 10 mM $LaCl_3$ (4 out of 4 cells) nor 10 mM $CoCl_2$ (4 out of 4 cells) reduced the rise in $[Ca^{2+}]_i$ evoked by application of 1 μM 5-HT (Fig. 12, Table I). Nifedipine (10 μM) also failed to significantly reduce the rise in $[Ca^{2+}]_i$ activated by 1 μM 5-HT in 4 out of 4 cells tested (Fig. 13, Table I). Increases in $[Ca^{2+}]_i$ were also seen when 5-HT was applied to CVSMCs bathed in a calcium-free medium (0 mM Ca^{2+} + 0.1 mM EGTA). However, under these conditions cell responsiveness was too erratic for quantitative comparisons with normal medium. The Trypan Blue dye exclusion test comfirmed that the viability of CVSMCs cells was greatly reduced on exposure to Ca^{2+}-free medium.

Figure 9. The increase in intracellular free calcium, $[Ca^{2+}]_i$, triggered by application of 5-HT to a single smooth muscle cell cultured for 3 days prior to use. At the time marked by the arrow, 5-HT was applied by rapid injection of a 100 μl volume of HBSS solution containing 1 μM agonist into the inlet tube of the cell chamber. Note the expanded time scale compared with 5-HT responses shown in most subsequent figures. The long latency of the response is largely the result of perfusion delays in the drug delivery system. Temperature 21^{o}C.

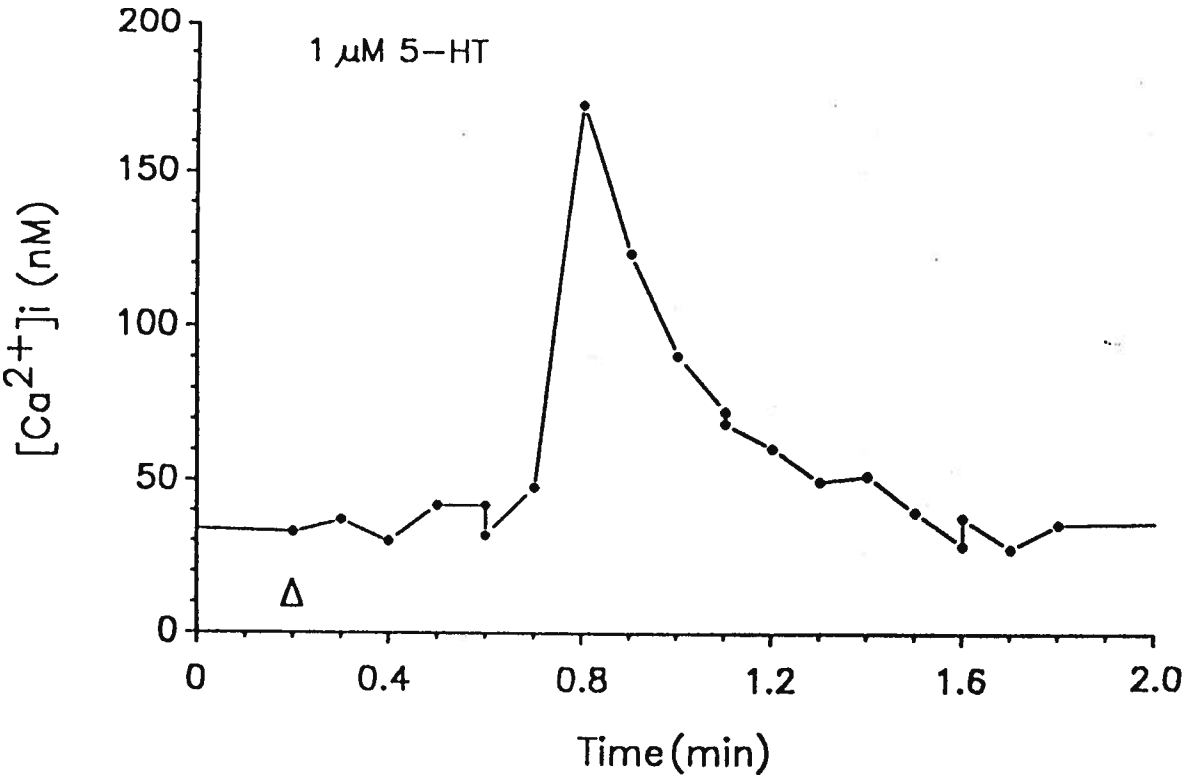

200
150
100
50
0
1 μM 5-HT
[Ca2+]i (nM)
Δ
0
0.4
0.8
1.2
1.6
2.0
Time (min)

Figure 10. Average time course of the increase in $[Ca^{2+}]_i$ triggered by application of 1μM 5-HT to 13 CVSMCs in 12 different culture plates. These responses have been normalized by peak amplitude and aligned temporally by denoting the time at onset of each response as t = 0. Data points represent the mean response of the 13 cells measured at a particular time after response onset.

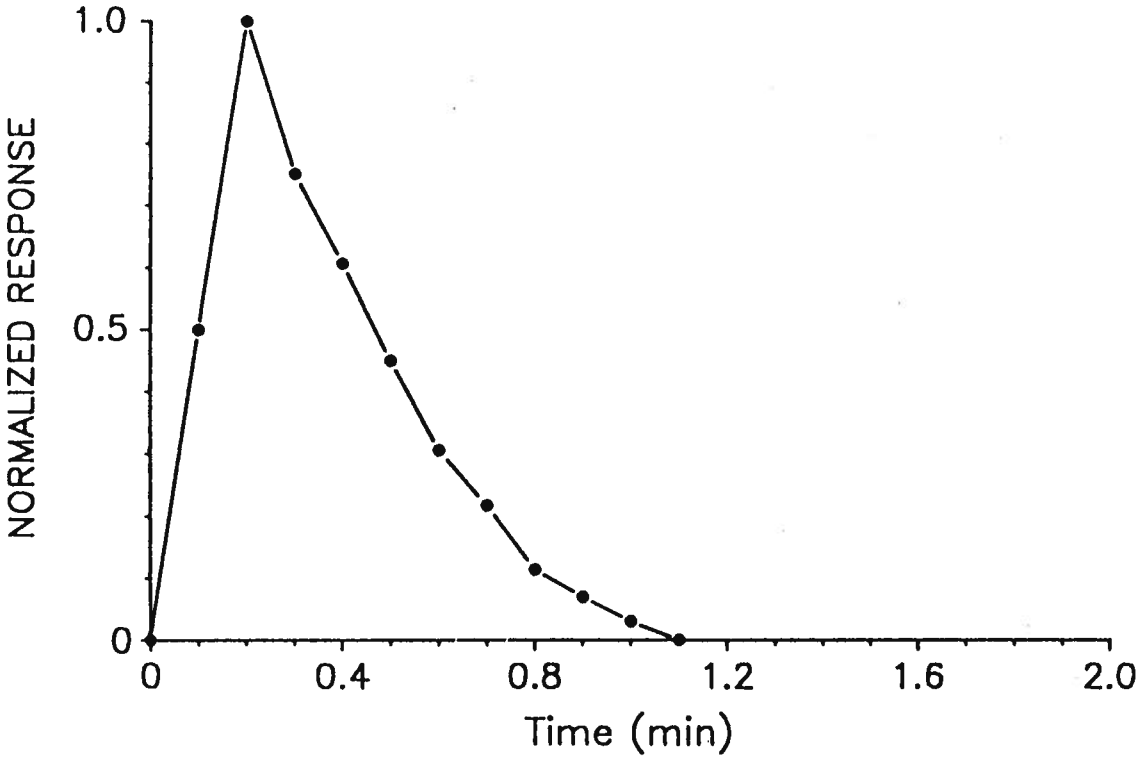

NORMALIZED RESPONSE
1.0
0.5
0
0
0.4
0.8
1.2
1.6
2.0
Time (min)

Figure 11. Increase in $[Ca^{2+}]_i$ triggered by application of 100 mM K+ saline to a cultured smooth muscle cell after 3 day *in vitro*. This solution was prepared by replacing sodium chloride isotonically with potassium chloride. A 100 μl volume of this solution was injected into the inlet tube of the cell chamber at the time indicated by the arrow.

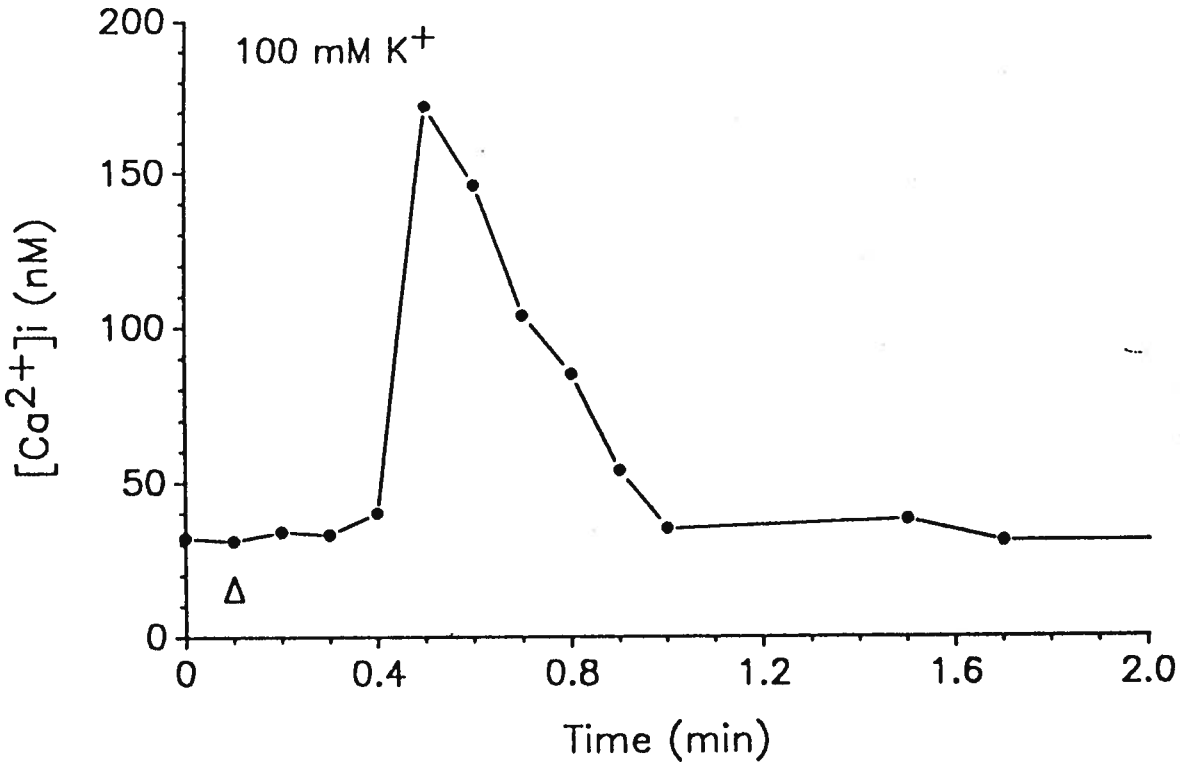

200
150
100
50
0
100 mM K+
[Ca2+]i (nM)
Δ
0
0.4
0.8
1.2
1.6
2.0
Time (min)

Figure 12. Effect of La^{3+} and Co^{2+} on serotonin-induced changes in $[Ca^{2+}]_i$ of a CVSMC cultured for 3 days prior to use. 5-HT (1 μM) was applied by injection of 100 μl volumes of the agent dissolved in HBSS into the inlet tube of the cell chamber. The times of these injections are indicated by the arrows. Application of drug-free HBSS solution in this manner (control) resulted in a small rise in $[Ca^{2+}]_i$ which was below the threshold level required for a response. During the times indicated by the horizontal bar, La^{3+} or Co^{2+} was perfused through the cell chamber as 10 mM solutions of their respective chloride salts.

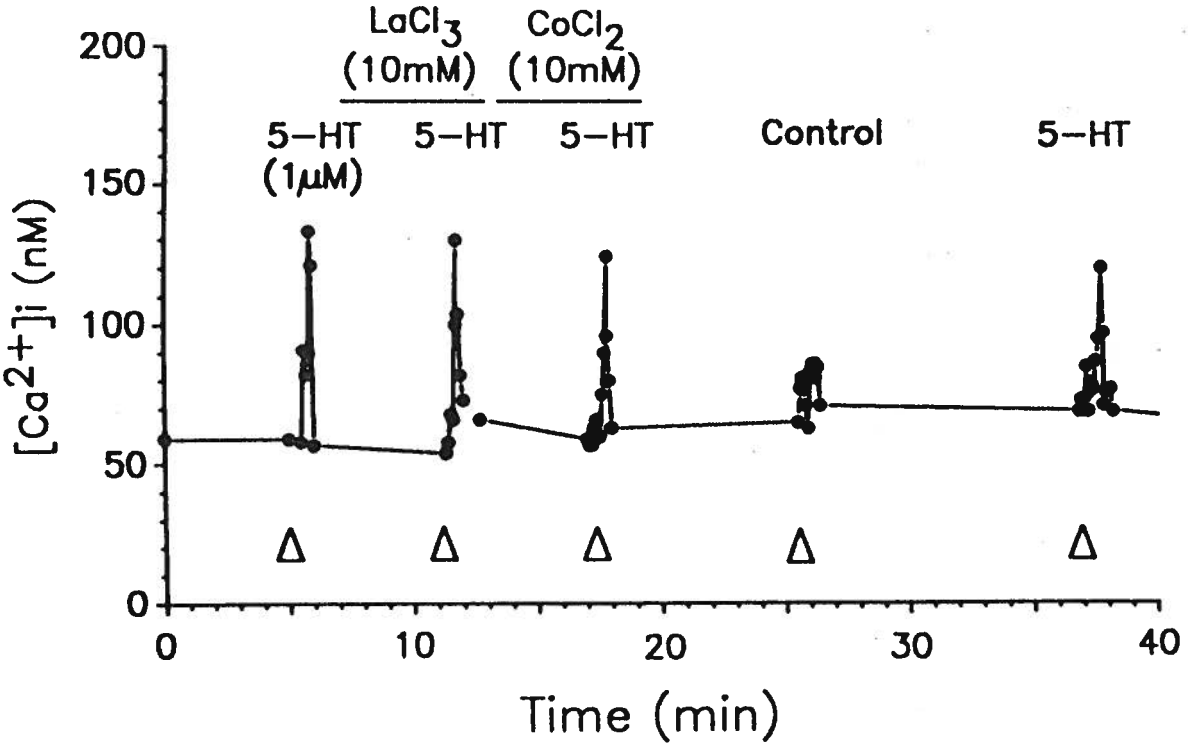

LaCl3
(10mM)
CoCl2
(10mM)
5–HT
(1μM)
5–HT
5–HT
Control
5–HT
200
150
100
50
0
[Ca2+]i (nM)
0
10
20
30
40
Time (min)

Figure 13. Effect of nifedipine (10 μM) on the rise in $[Ca^{2+}]_i$ triggered by 1 μM 5-HT in a CVSMC cultured for 3 days prior to use. Serotonin applications were made as shown by the arrows, using the procedure described for Fig. 12. Nifedipine was dissolved in HBSS and perfused over the cells, as indicated by the horizontal bar.

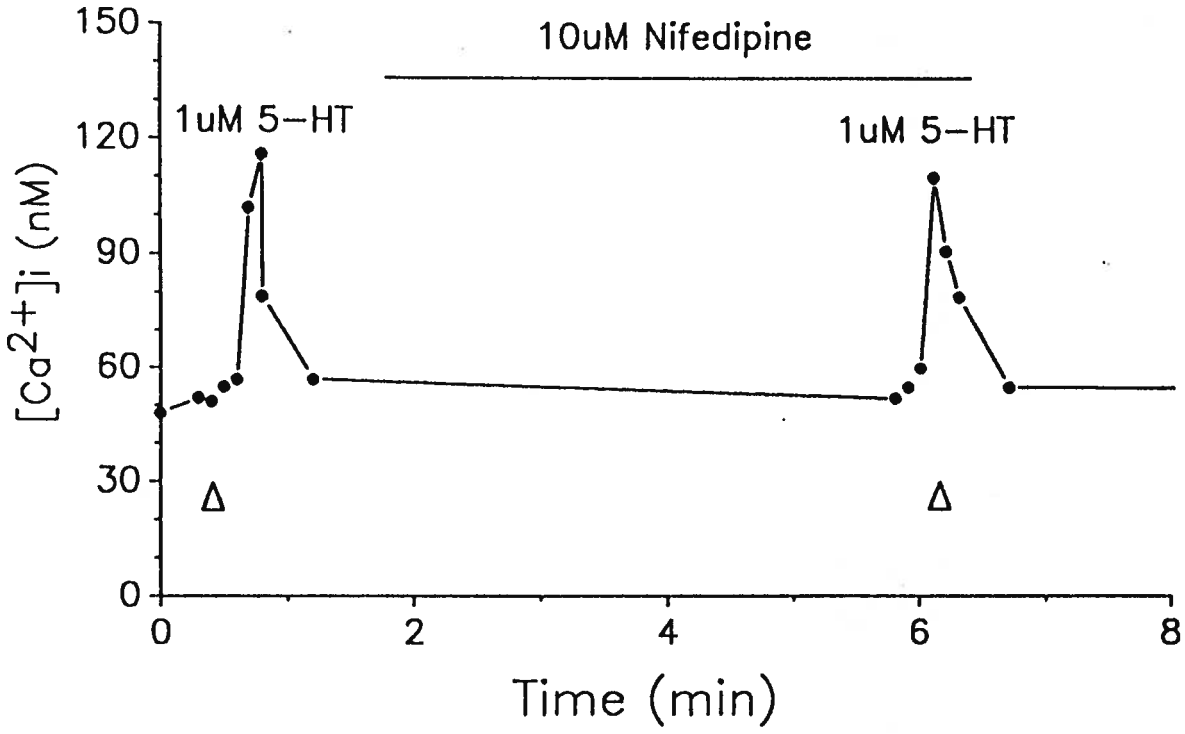

10uM Nifedipine
1uM 5-HT
1uM 5-HT
[Ca2+]i (nM)
150
120
90
60
30
0
Δ
Δ
0
2
4
6
8
Time (min)

Table I The influence of Co^{2+}, La^{3+}, and nifedipine on the percentage increase in $[Ca^{2+}]_i$ induced by 5-HT in cultured rat CVSMCs

Treatment	% increase in $[Ca^{2+}]_i$ over baseline level	Student's t-test
5-HT (1 μM)	115 ± 18	P < 0.01
5-HT + ketanserin (5 nM)	63 ± 9	(n = 5)
5-HT (1 μM)	187 ± 38	P > 0.05
5-HT + Co^{2+} (10 mM)	190 ± 33	(n = 4)
5-HT (1 μM)	162 ± 48	P > 0.05
5-HT + La^{3+} (10 mM)	165 ± 41	(n = 4)
5-HT (1 μM)	141 ± 16	P > 0.05
5-HT + nifedipine (10 μM)	138 ± 27	(n = 4)

The effects of the partially selective 5-HT2 receptor antagonist, ketanserin, were examined in 10 cells. Ketanserin (5 nM) reversibly attenuated the 5-HT response in all 5 cells tested (Fig. 14, Table I). When applied at a concentration of 10 μM, ketanserin produced a complete block of the rise in $[Ca^{2+}]_i$ triggered by 1 μM 5-HT in 5 out of 5 cells tested.

In many cases, repeated exposure of 5-HT reactive cells to a constant dose of serotonin lead to a gradual loss of responsiveness to the agonist. The loss of response seen during repeated application of 5-HT could occur in the absence of any increase in the baseline level of free calcium in the cell (Fig. 15). This decline could not be completely reversed, even by washing the cell under study in serotonin-free saline for a period of 30 minutes. However, robust responses to 5-HT could still be detected in other cells in the same culture, which had not previously been exposed to photoexcitation.

. The effect of 5-HT on $[Ca^{2+}]_i$ was clearly dose-dependent, as is shown in Fig. 16. This log dose-response relation was obtained from 84 CVSMCs exposed to a single, transient application of 5-HT, to minimize the deleterious effects of photoexcitant wavelengths. Quantitative aspects of this log dose-response relation should, however, be interpreted with caution. The 100 μl volumes of agonist used may have been subject to dilution during passage through the cell chamber. In addition, the artificial increase in $[Ca^{2+}]_i$ caused by fluid injection precluded accurate measurements for serotonin doses below 10 nM. Under the conditions used, however, it was observed that the response of most CVSMCs was already maximal at a serotonin dose of 1 μM. The concentration of 5-HT producing a half-maximal increase in $[Ca^{2+}]_i$ was 10 nM (Fig. 16). The

Figure 14. Effect of 5 nM ketanserin on the rise in $[Ca^{2+}]_i$ evoked by 1 μM 5-HT in a CVSMC studied after 2 days *in vitro*. 5-HT was applied by injection of a 100 μl volume of drug, as described in previous figures. During the time indicated by the horizontal bar, the culture was perfused with HBSS solution containing 5 nM ketanserin. The antagonist reduced, but did not abolish the response of this cell to 5-HT. Injection of drug-free HBSS produced only a small, artifactual increase in $[Ca^{2+}]_i$ (control).

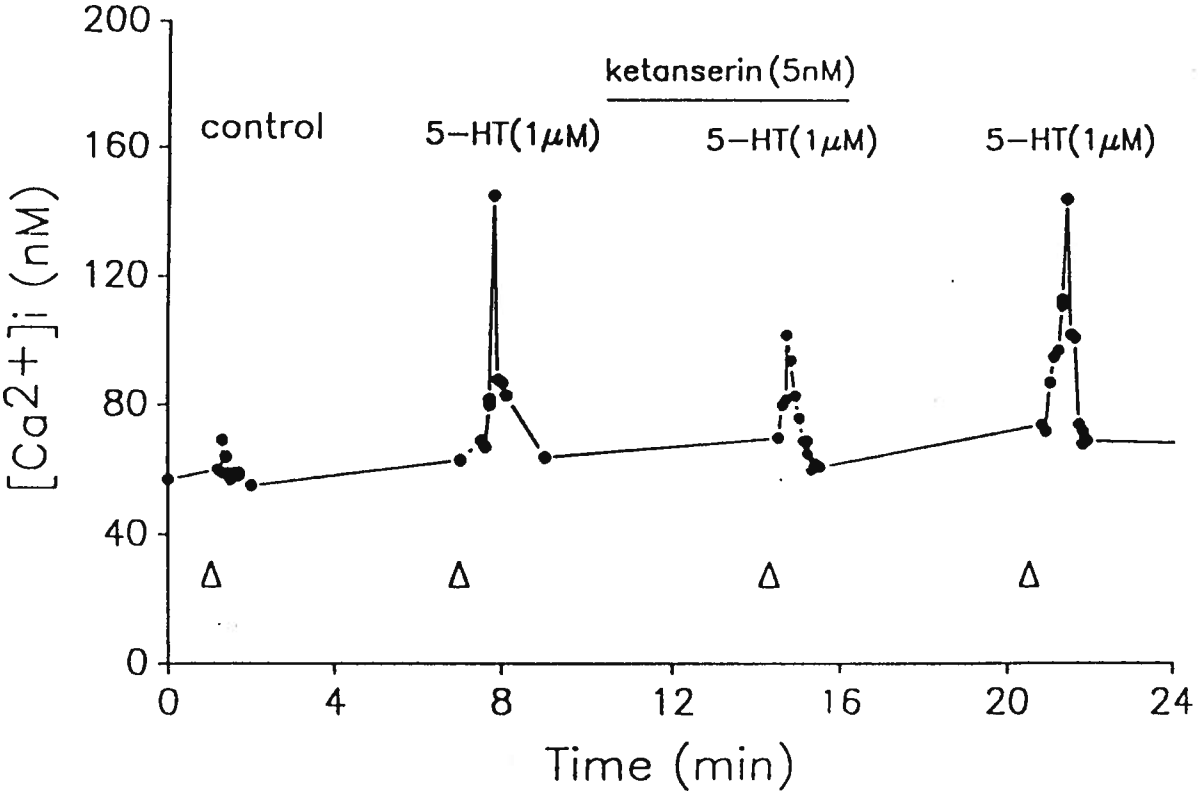

200
160
120
80
40
0
[Ca2+]i (nM)
control
ketanserin (5nM)
5-HT(1μM)
5-HT(1μM)
5-HT(1μM)
Δ
Δ
Δ
Δ
0
4
8
12
16
20
24
Time (min)

Figure 15. Decreasing responsiveness of a CVSMC cultured for 3 days to repeated applications of 0.1 μM 5-HT. Serotonin was applied by injection of 100 μl volumes of the agent at the times indicated by the arrows.

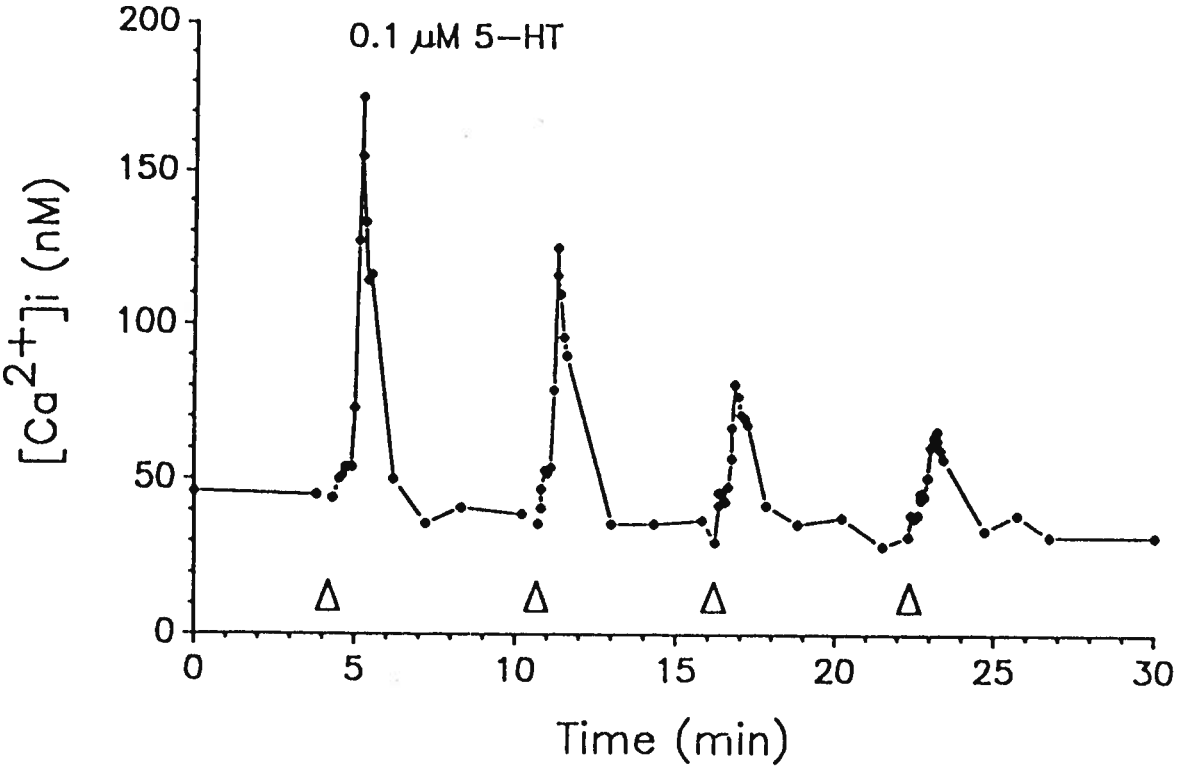
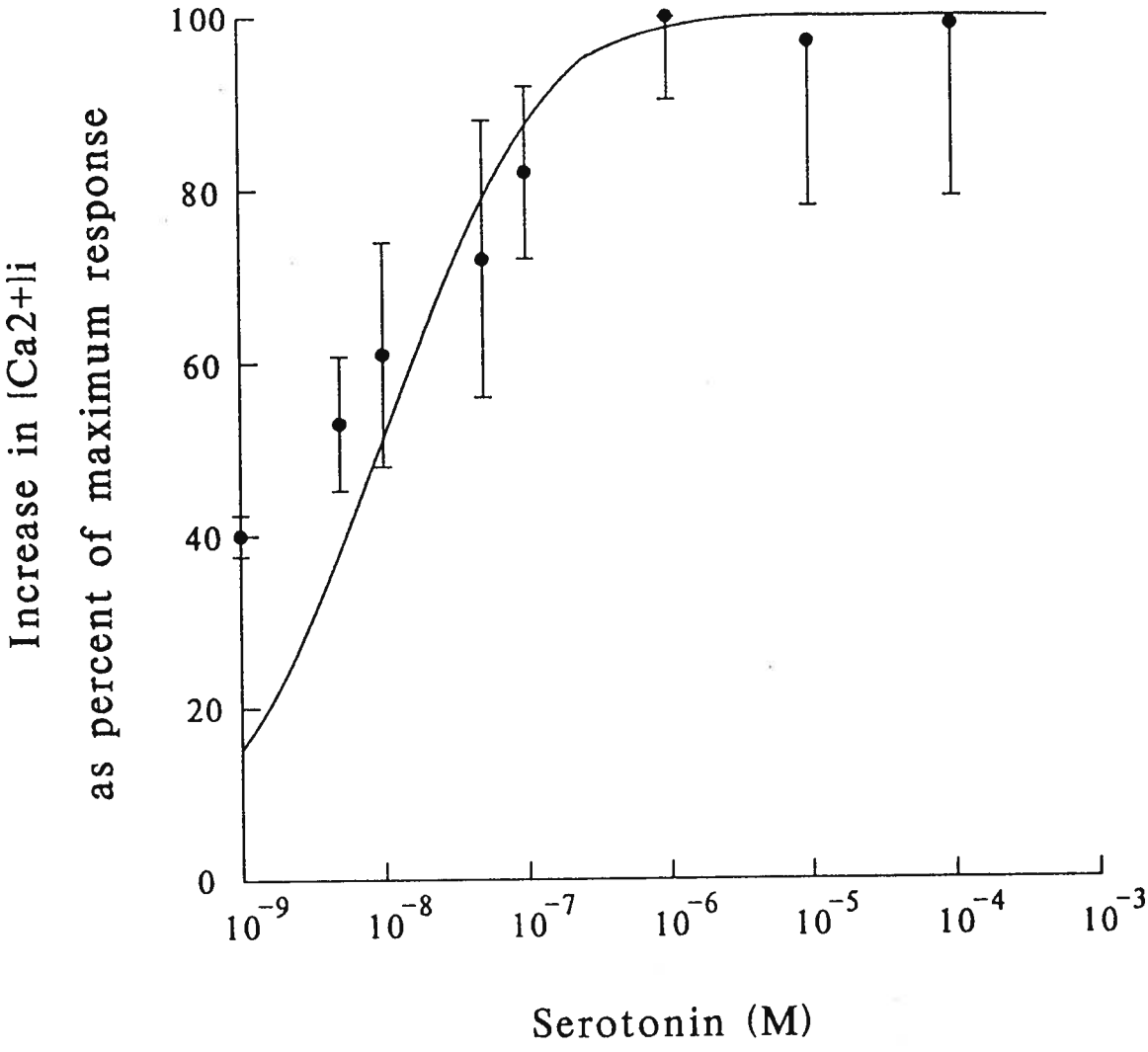

0.1 μM 5–HT
200
150
100
50
0
[Ca2+]i (nM)
0
5
10
15
20
25
30
Time (min)
Δ
Δ
Δ
Δ

Figure 16. Log-dose response relation showing the percentage increase in $[Ca^{2+}]_i$ triggered by various concentrations of 5-HT, tested in a total of 84 CVSMCs after 2-4 days in vitro. Symbols show the mean $\pm$ SEM values for each 5-HT concentration used. The smooth curve was drawn to the equation: Response = 100%/(1 + K/[5-HT]) with K=10 nM.


Increase in [Ca2+]i
as percent of maximum response
100
80
60
40
20
0
10^{-9}
10^{-8}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
Serotonin (M)

maximal level of $[Ca^{2+}]_i$ attained during stimulation by 5-HT was approximately 170 nM.

3.3. Electrical properties of isolated CVSMCs

The electrophysiological studies described in this thesis were conducted on CVSMCs maintained for 2-3 days at 4°C prior to use. It was found that cells treated in this manner consistently yielded a greater percentage of membrane patches having functional K_{Ca} channels than was the case for CVSMCs incubated at 37°C.

3.3.1.Basic electrophysiological properties of isolated CVSMCs studied with cell-attached and whole-cell, current clamp recordings.

During patch clamp recording, patch electrodes formed high resistance (10-50 GΩ) seals on the membrane of CVSMCs in about 70% of attempts. Patch electrodes were filled with a solution of composition (mM): 140 KCl, 3 NaCl, 0.65 $CaCl_2$, 3 EGTA and 10 HEPES, pH 7.4. The pipette voltage was 0 mV. Under these conditions, about 25% of the cell-attached patches showed no discernable single channel activity (Fig. 17A). In the remaining 75% of the patches, inwardly directed small amplitude (1-2 pA) single channel currents were observed (Fig. 17B). Single channel activity could be modified in cell-attached patches by bath application of 5-HT in the standard external saline bathing the cells. 5-HT (1-10 μM) induced the appearance of inwardly directed, 8-10 pA single channel currents in 6/10 cell-attached patches studied at a pipette potential of 0 mV (Fig. 18). The identity of these channels was not investigated

Figure 17. Recordings obtained from two cell-attached patches studied in two CVSMCs incubated at 4^{o}C for 3 days prior to use. Patch electrodes were filled with a solution containing 140 mM KCl. Bandwidth DC-2kHz, pipette potential 0 mV. A. No discernable single channel currents were present in this patch. B. Small amplitude single channel currents were present in this recording. In this and in all subsequent traces, inward membrane current is denoted by downward deflection from baseline.

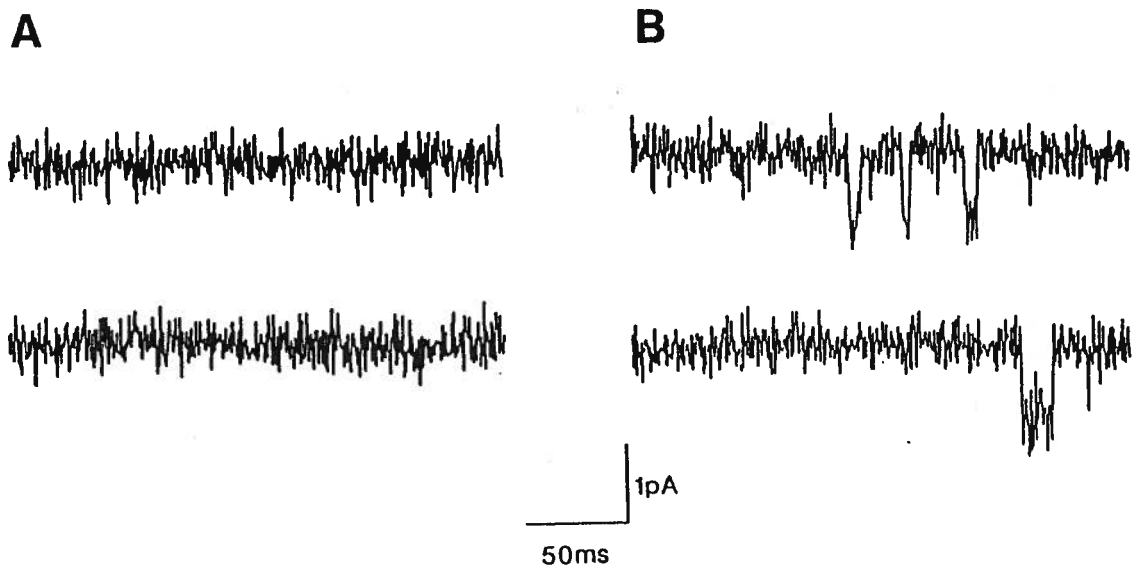
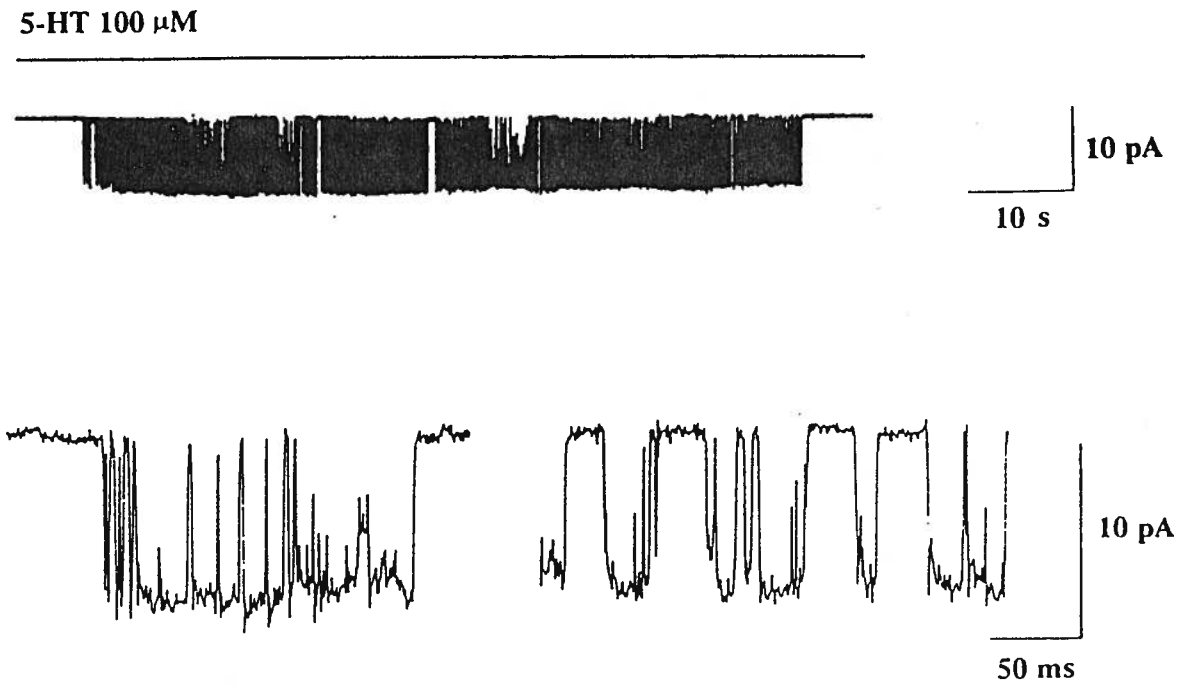

A
B
1pA
50ms

Figure 18. Upper panel. Large amplitude, inwardly directed single channel currents evoked in a cell-attached patch during bath application (bar) of 100 μM 5-HT to the exposed membrane surface of a CVSMC. Bandwidth DC-200 Hz. Lower panel. Part of the recording shown in upper panel at increased time resolution (DC-2 kHz). Pipette potential 0 mV. The patch electrode contained 140 mM KCl.


5-HT 100 μM
10 pA
10 s
10 pA
50 ms

further in this study. When applied at a higher concentration range (10-100 μM), 5-HT also induced the appearance of brief, biphasic current spikes in 6/10 cell-attached patches studied (Fig. 19). These events were about 10 pA in peak-to-peak amplitude, when measured at a pipette potential of 0 mV. These currents were similar in form and amplitude to those associated with action potential discharge in high input impedance cells studied using the cell-attached configuration (Fenwick et al. 1982). These observations indicate that isolated CVSMCs retained electro-responsiveness to 5-HT after 2-3 days *in vitro*.

The resting membrane potential of a large sample of CVSMCs were measured using the whole-cell, current-clamp recording technique. In these studies the patch pipettes were again filled with a saline of composition (mM): 140 KCl, 3 NaCl, 0.65 $CaCl_2$, 3 EGTA, 10 HEPES, pH 7.4. The resting potentials of CVSMCs were unimodally distributed with an average of -41 $\pm$ 11.7 mV (n = 69), and ranged from -18 mV to -69 mV (Fig. 20). The resistance, capacitance and time constant of the CVSMC membrane were estimated by applying small depolarizing and hyperpolarizing currents to the patch electrode (Fig. 21). Fluctuations in membrane potential were noted at all membrane voltages, and these were particularly apparent when the membrane was strongly hyperpolarized or depolarized. Application of strong depolarizing stimuli evoked only small (10-15 mV) regenerative responses and action potentials were not observed. This result is in agreement with previous studies conducted on rat CVSMCs in intact vessels (Hirst et al. 1986).

The current-voltage relations of two typical CVSMCs are shown in Fig. 22. The resting membrane potentials of these cells were -31 mV and -49 mV. It can be seen that the mean slope resistance of these cells decreased markedly at

Figure 19. Biphasic currents recorded fom a CVSMC after 2 days *in vitro* using the cell-attached configuration. The solution bathing the cell contained 100 μM 5-HT. Pipette potential 0 mV. Bandwidth DC-2kHz. The pipette solution contained 140 mM KCl.

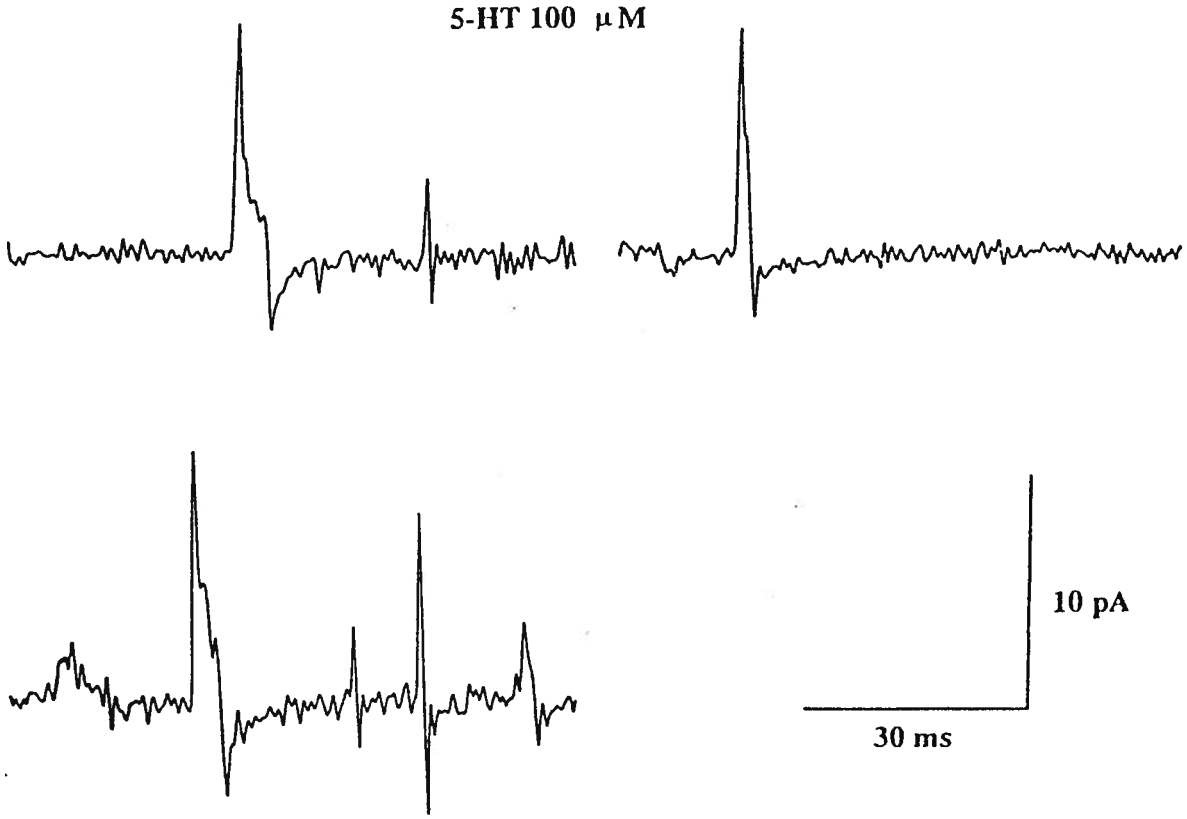

5-HT 100 μM
10 pA
30 ms

Figure 20. Resting membrane potentials recorded from 69 CVSMCs after 2-3 days *in vitro* at 4oC. Whole-cell recording methods were used to obtain these data. Patch pipettes contained 140 mM KCl and 3 mM NaCl.

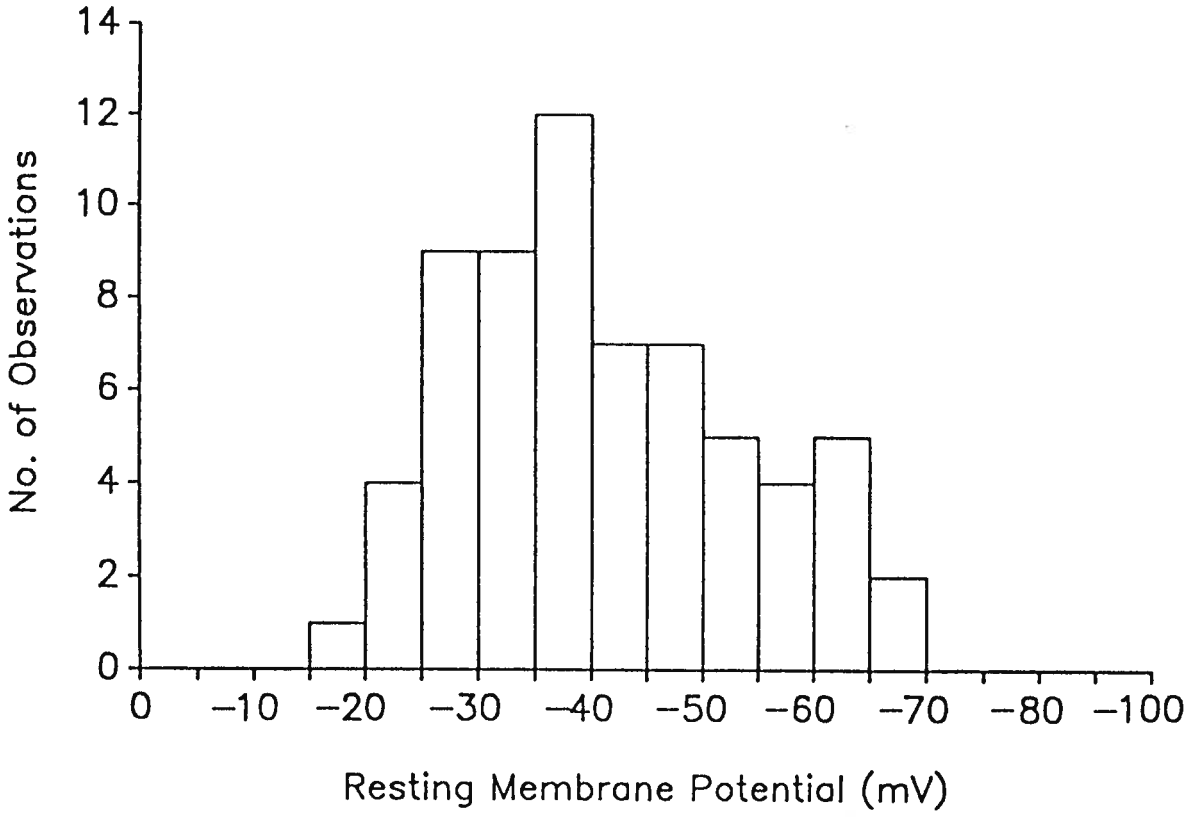

14
12
10
8
6
4
2
0
No. of Observations
0
−10
−20
−30
−40
−50
−60
−70
−80
−100
Resting Membrane Potential (mV)

Figure 21. Voltage changes (lower traces) induced by application of constant current pulses (upper traces) in a CVSMC studied using the whole-cell, current clamp technique. Resting potential -31 mV, cell diameter 12.5 μm. Pipette solution contained 140 mM KCl and 3 mM NaCl. Bandwidth DC-200 Hz.

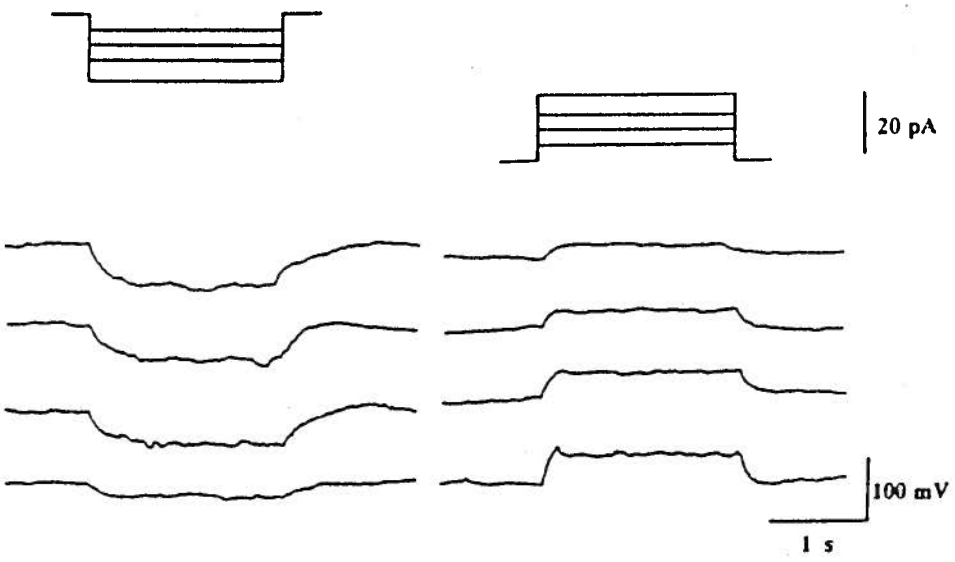
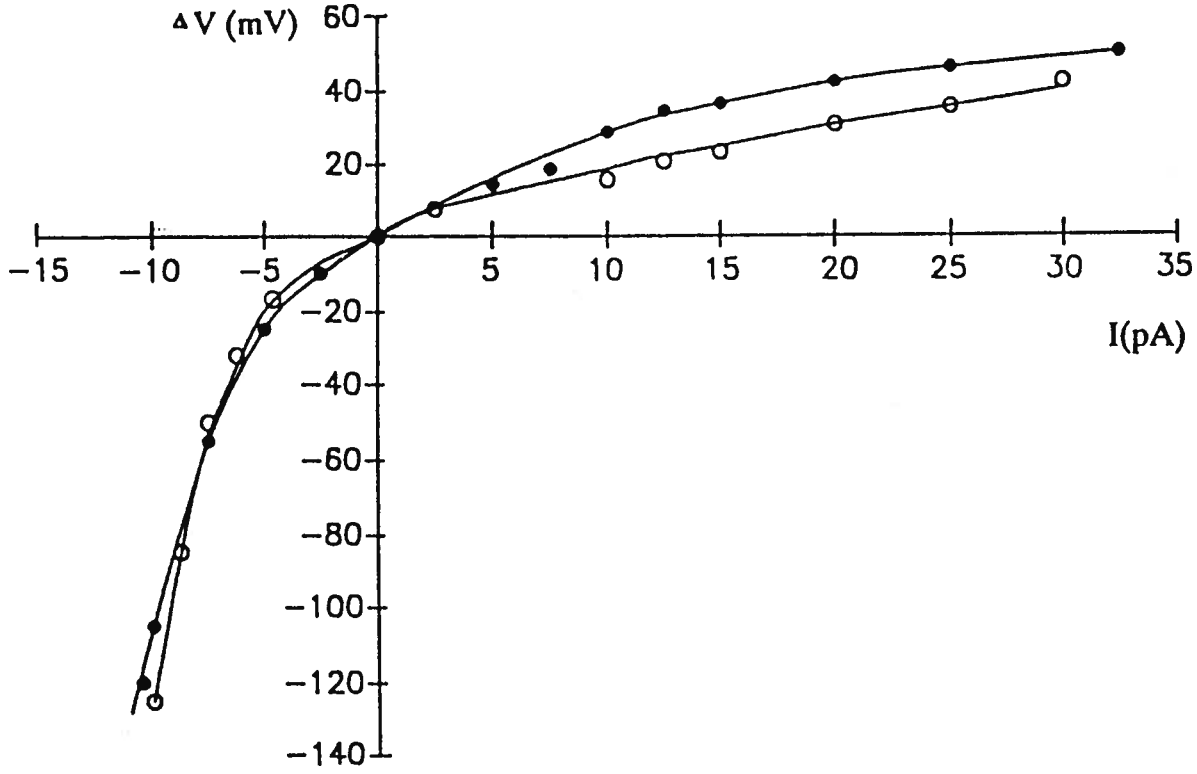

20 pA
100 mV
1 s

Figure 22. Current-voltage relationships for two CVSMCs studied in the whole-cell, current clamp recording mode. The resting membrane potentials of these cells were -31 mV (closed circles) and -49 mV (open circles). The graph shows the change in resting membrane potential, ΔV, evoked by application of constant current pulses of amplitude, I. Lines were fitted to data points by eye. Recording pitettes contained 140 mM KCl and 3 mM NaCl. Temperature 21^{o}C.


ΔV (mV)
60
40
20
−15
−10
−5
5
10
15
20
25
30
35
I(pA)
−20
−40
−60
−80
−100
−120
−140

potentials near to, and depolarized from the resting membrane voltage. The slope of the I-V relationship at the resting potentials indecated a membrane resistance, R_m of 3.2 $\pm$ 0.48 GΩ (n = 20). Cell capacitance and time constant were assessed from the decay of hyperpolarizing electrotonic potentials of about 20 mV amplitude in 7 cells. These decays were well fitted by a single exponential term, yielding an average membrane time constant of τ_m = 78 $\pm$ 26 ms (n = 7) (Fig. 23). Cell capacitance was calculated from the relation $C_m = \tau_m/R_m$. A mean value of 24 $\pm$ 2.3 pF was obtained from 7 cells. This value is larger than that calculated for 12 μm diameter cells, assuming spherical shape and a specific membrane compacitance of 1 $\mu F/cm^2$.

3.4. K_{Ca} channels in inside-out membrane patches excised from CVSMCs

Isolated inside-out membrane patches excised from CVSMCs displayed a variety of single channel currents when exposed to 140 mM KCl solutions at both membrane faces. In some cases, small currents corresponding to single channel conductances in the range 10-25 pS were observed. These events could readily be distinguished from the activity of K_{Ca} channels by their smaller conductance and insensitivity to changes in calcium ion concentration at the cytoplasmic membrane face.

Evidence was obtained for the presence of two classes of K_{Ca} channel in these patches. These were distinguished on the basic of a large difference in single channel conductance, as measured in symmetrical 140 mM K+ solutions (Fig. 24). The first class of channels, designated as K(Ca)L (large conductance) channels, was characterized by a mean single channel conductance of 207 $\pm$ 10 pS (n = 16). The second category, designated as K(Ca)I (intermediate

Figure 23. Semi-logarithmic plot of the decay phase of a hyperpolarizing electrotonic potential evoked in a CVSMC membrane under whole cell, current-clamp conditions. This cell had a resting membrane potential of -39 mV. The graph shows the deviation, ΔV of the membrane potential from this resting level as a function of time since the termination of the stimulating current pulse. Data points were fitted by a straight line using linear regression. The arrow indicates the membrane time constant τ = 98 ms.

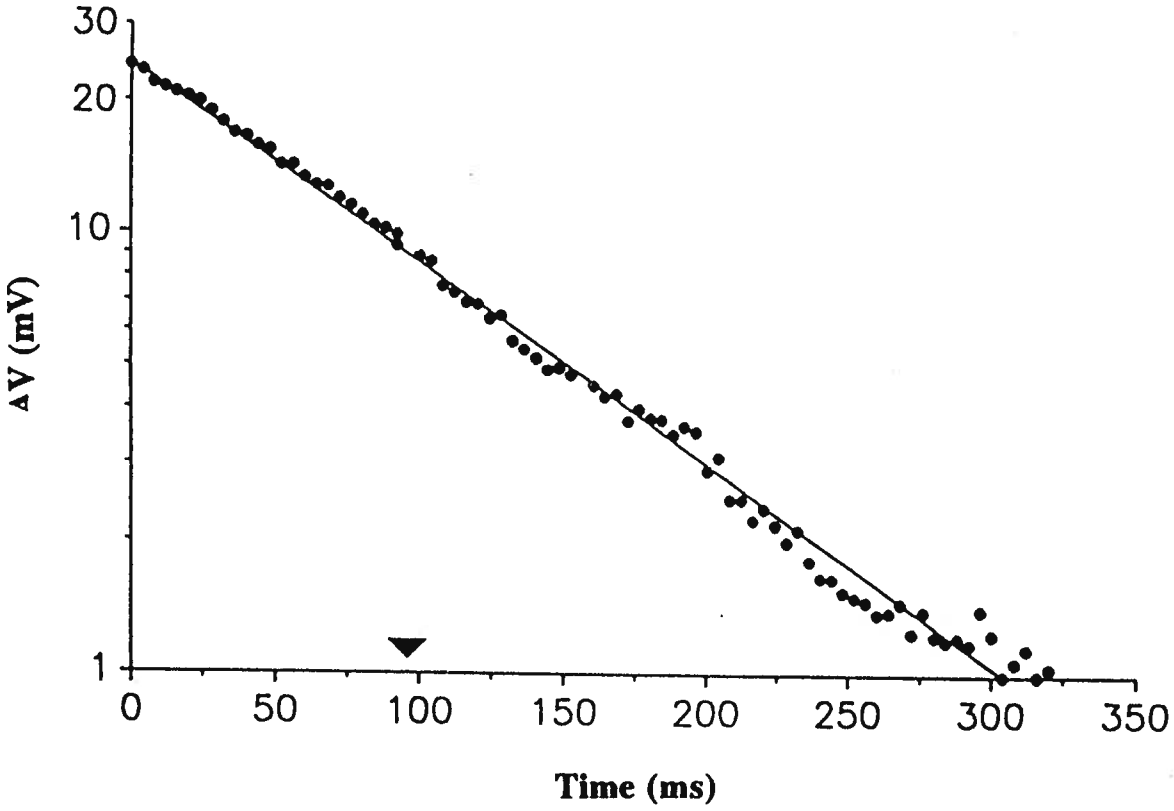
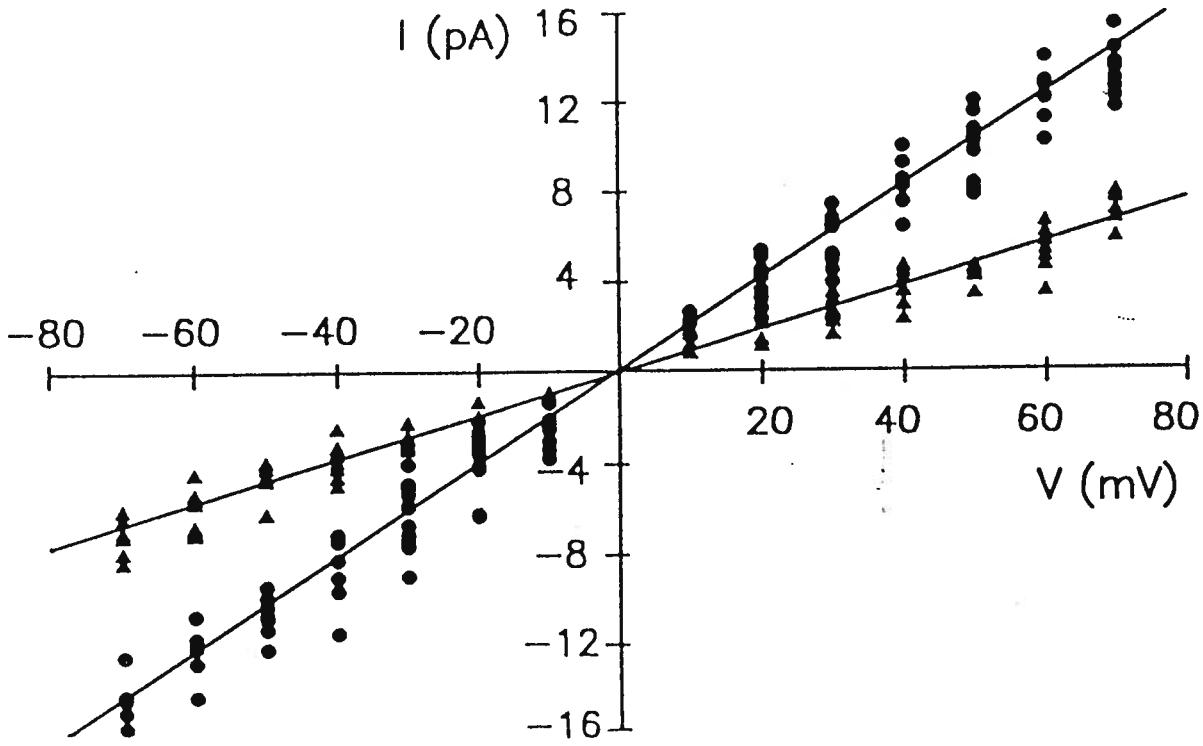

30
20
10
1
ΔV (mV)
0
50
100
150
200
250
300
350
Time (ms)

Figure 24. Current-voltage (I-V) relationships of single K(Ca)L channels (circles, n = 16 patches) and K(Ca)I channels (triangles, n = 10 patches) studied in inside-out membrane patches bathed in symmetrical 140 mM potassium solutions. The straight lines were fitted to the data by linear regression, and represent the mean single channel conductance for K(Ca)L channels (207 $\pm$ 10 pS) and for K(Ca)I channels (92 $\pm$ 2.6 pS).


I (pA)
16
12
8
4
−80
−60
−40
−20
20
40
60
80
V (mV)
−4
−8
−12
−16

conductance channels) displayed a significantly smaller conductance, 92 $\pm$ 2.6 pS (n = 10), P < 0.05 in Student's t-test with K(Ca)L class. As shown in Fig. 24 , there was little overlap in the conductance ranges characterized by these mean values, suggesting that indeed two distinct classes of events were present . It was also noted that the mean K(Ca)L channel conductance was not simply an integer multiple of the K(Ca)I channel conductance, indicating that the former events did not result from the simultaneous opening of two or more K(Ca)I channels. For these reasons, it was decided to treat K(Ca)L and K(Ca)I currents as two distinct classes of event, and to assess their properties with regard to ionic selectivity, Ca^{2+} and voltage dependence and sensitivity to the blocking action of TEA. The properties of the K(Ca)L channel will be described first, since this type was encountered more frequently than the smaller K(Ca)I channel.

3.4.1. Conductance and ionic selectivity of the K(Ca)L channel

Single channel currents ascribed to the opening of K(Ca)L channels were observed in 16/20 inside-out patches studied. Fig. 25 shows currents flowing through a single K(Ca)L channel in an inside-out patch voltage-clamped at various membrane potentials. This patch was bathed in symmetrical 140 mM K^+ solutions and $[Ca^{2+}]_i$ was 10 μM. Fig. 26 shows the amplitude distributions obtained for K(Ca)L channel currents recorded from a patch at membrane potentials -30 mV and +30 mV. At membrane potentials in the range -80 mV to +80 mV, these distributions were well fitted by a single Gaussian term in all 16 patches studied. This result shows that the K(Ca)L channel did not exhibit significant substate conductance behavior when studied in excised membrane patches.

Figure 25. Single-channel currents flowing through a K(Ca)L channel in an inside-out patch voltage-clamped at various membrane potentials, V. This patch was bathed in symmetrical 140 mM K+ solutions, $[Ca^{2+}]_i$ and $[Ca^{2+}]_o$ were 10 μM and 0.01 μM respectively. 0 indicates channel closed and 1 indicates channel open. Bandwidth DC-200 Hz.

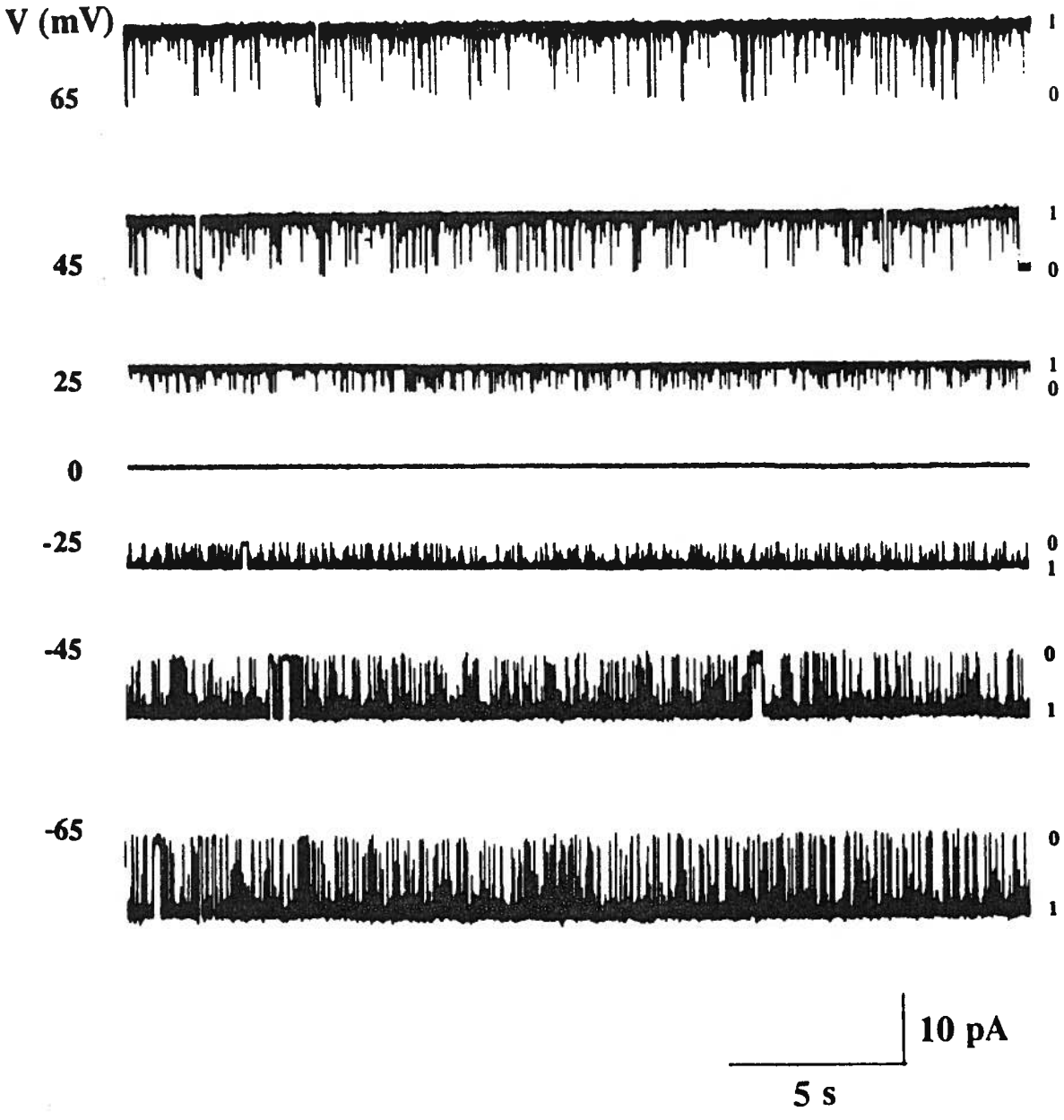
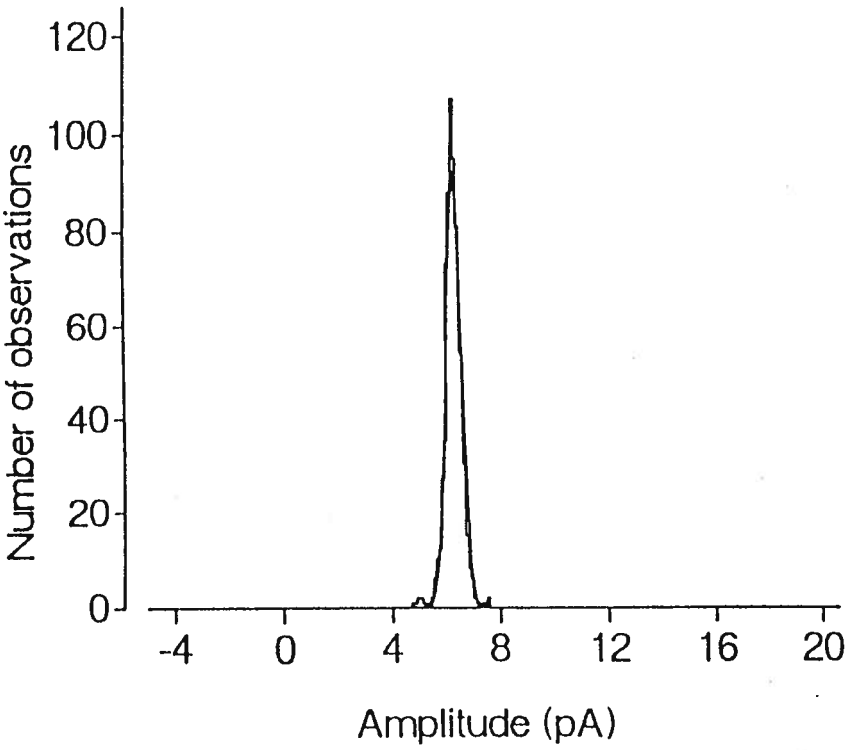
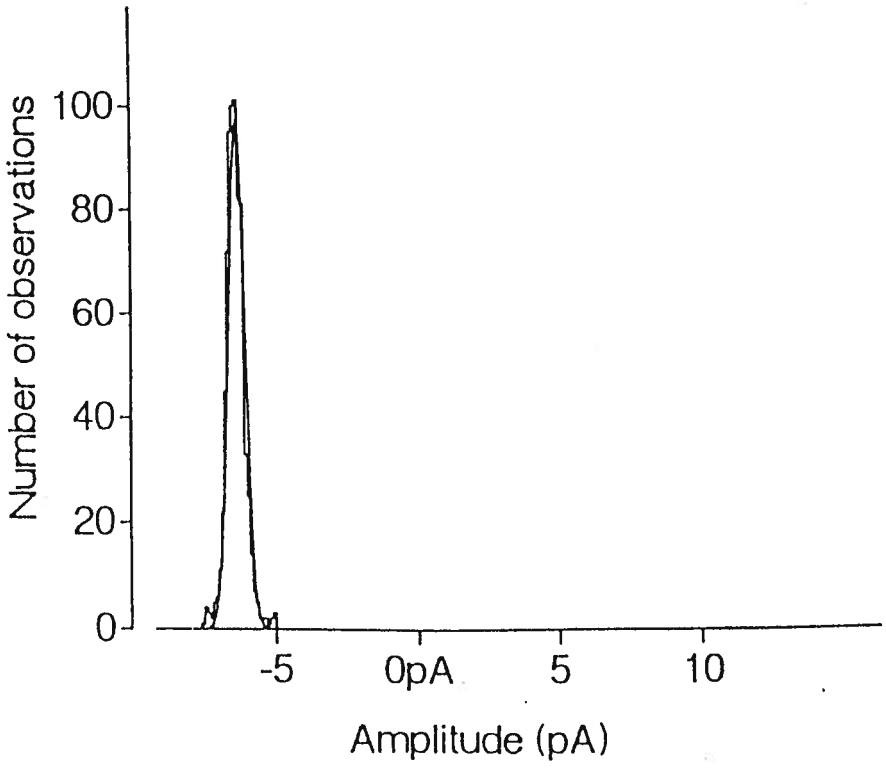

V (mV)
65
45
25
0
-25
-45
-65
1
0
1
0
1
0
0
1
0
1
0
1
10 pA
5 s

Figure 26. Amplitude distributions of K(Ca)L channel currents obtained from a inside-out patch voltage-clamped to membrane potentials of (A) +30 mV, (B) -30 mV. Both distributions were well fitted by single Gaussian terms (smooth curves) with modal values accurring at 6.27 pA in (A) and at -6.48 pA in (B). Patch was bathed in symmetrical 140 mM K+ solutions and $[Ca^{2+}]_i$ was 10 μM. $[Ca^{2+}]_o$ = 0.01 μM.

A


120
100
80
60
40
20
0
Number of observations
-4
0
4
8
12
16
20
Amplitude (pA)

B


100
80
60
40
20
0
Number of observations
-5
0pA
5
10
Amplitude (pA)

In patches bathed in symmetrical 140 mM KCl solutions, the I-V relationship of the K(Ca)L channel was linear over the voltage range -80 mV to + 80 mV, showed a reversal potential of 0 mV and a mean slope conductance of 207 $\pm$ 10 pS (n = 16, Fig. 27). The range of conductance values encountered in different patches was from 170 pS to 267 pS.

The permeability of the open channel to potassium, P_K was calculated from the I-V relation in symmetrical 140 mM K+, using the Goldman-Hodgkin-Katz constant-field equation (Goldman, 1943; Hodgkin & Katz, 1949):

$$I_K = P_K \cdot VF^2/RT\{[K^+]_o - [K^+]_i \exp(VF/RT)\}/\{1-\exp(VF/RT)\} \quad (1)$$

When $[K^+]_o = [K^+]_i$, Equation 1 simplifies to

$$I_K = P_K \cdot VF^2[K^+]/(RT) \quad (2)$$

Here, I_K is the current through an open channel, V is membrane potential, $[K^+]_o = [K^+]_i$ = 140 mM, F is Faraday's constant, R is the gas constant, T is the absolute temperature and o and i indicate outside and inside (cytoplasmic) concentrations of ions respectively (Benham et al. 1986). Using Equation 2, a P_K of 3.9 x 10^{-13} cm/s was calculated from the I-V plots averaged from sixteen patches (Fig. 27).

When 80 mM KCl in the solution bathing the external membrane face was replaced by an equimolar amount of NaCl, currents flowing in single K(Ca)L channels reversed at a potential around -21 mV (Fig. 28A). An average V_{rev} of _21.2 $\pm$ 0.50 mV was obtained from 5 patches (see Fig. 29). This value

Figure 27. Current-voltage (I-V) relationship of single K(Ca)L channel currents averaged from 16 patches bathed in symmetrical 140 mM potassium solutions. It was found that the I-V relationship of the K(Ca)L channel was linear over the voltage range -80 mV to + 80 mV, showed a reversal potential at 0 mV, and a mean slope conductance of 207 $\pm$ 10 pS. Error bars are $\pm$SEM. In this and in all subsequent figures showing standard errors, this parameter is omitted if its value is less than the size of the symbol.

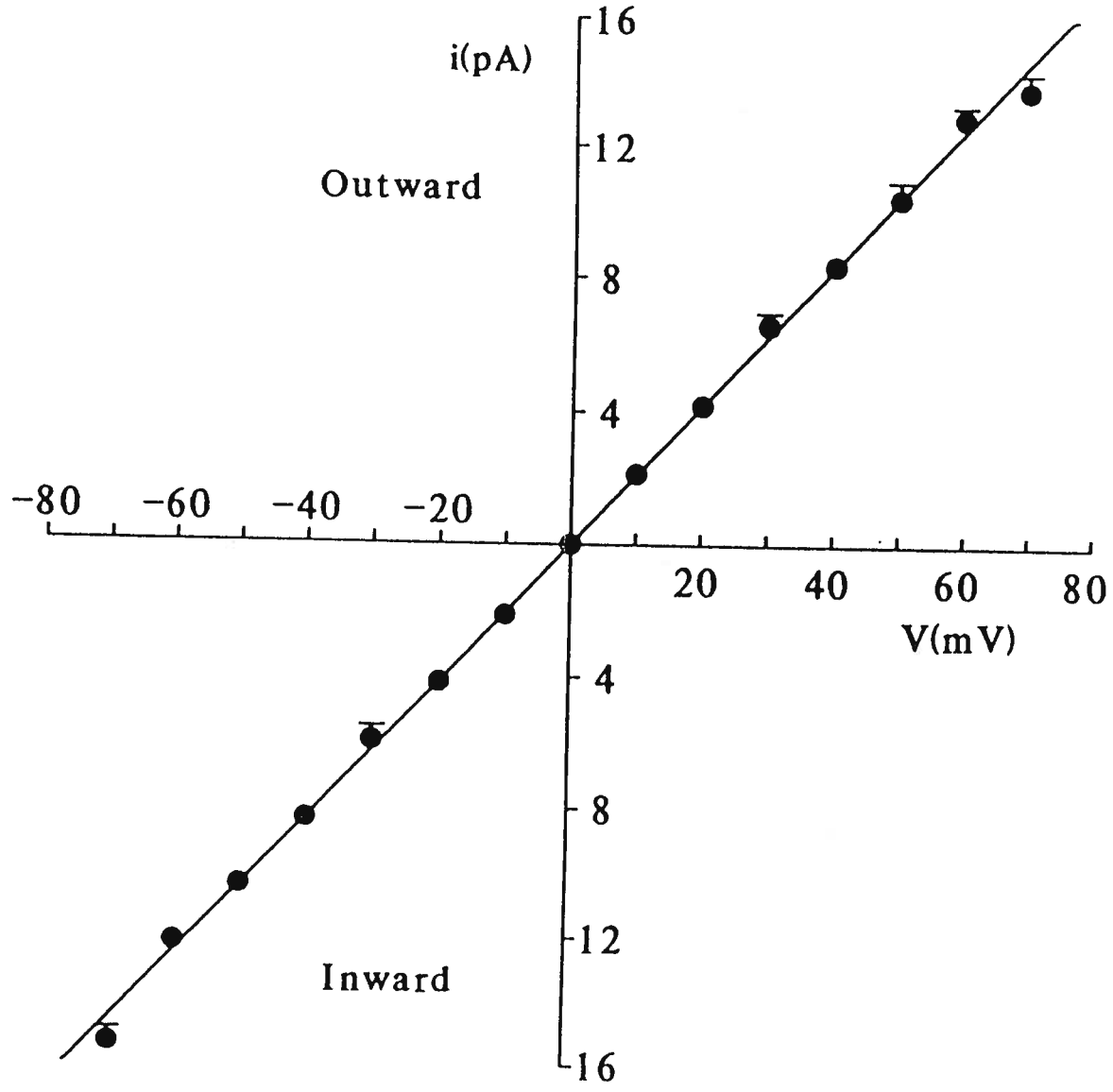

i(pA)
16
12
8
4
Outward
-80
-60
-40
-20
20
40
60
80
V(mV)
4
8
12
16
Inward

Figure 28. Effects of $Na+$ or $Cs+$ replacement of $K+$ on the single channel currents in K(Ca)L channels. Single channel currents at various membrane potentials, V_i were obtained from a patch with one active channel when 80 mM KCl in the pipette was replaced by an equimolar amount of NaCl in A, and of CsCl in B. 0 indicates channel closed and 1 indicates channel open. Bandwidth DC-2 kHz. $[Ca^{2+}]_i$, 100 μM and $[Ca^{2+}]_o$, 0.01 μM. A and B were obtained from different patches.

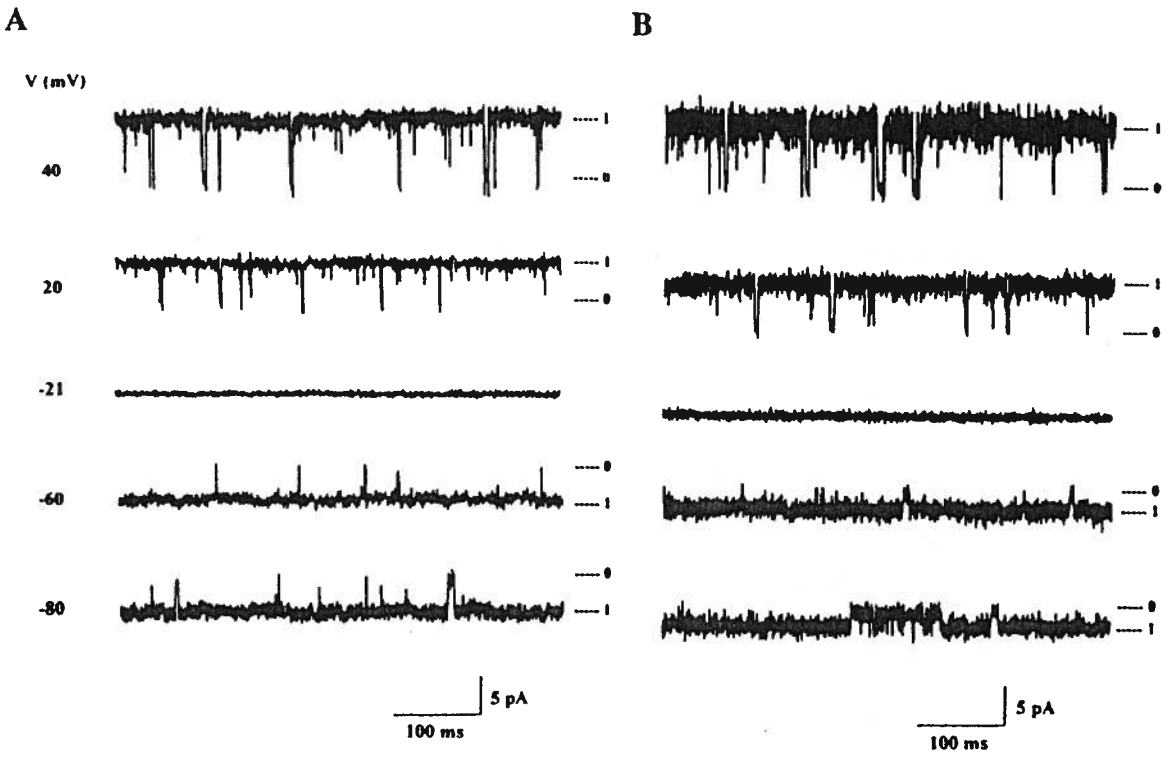
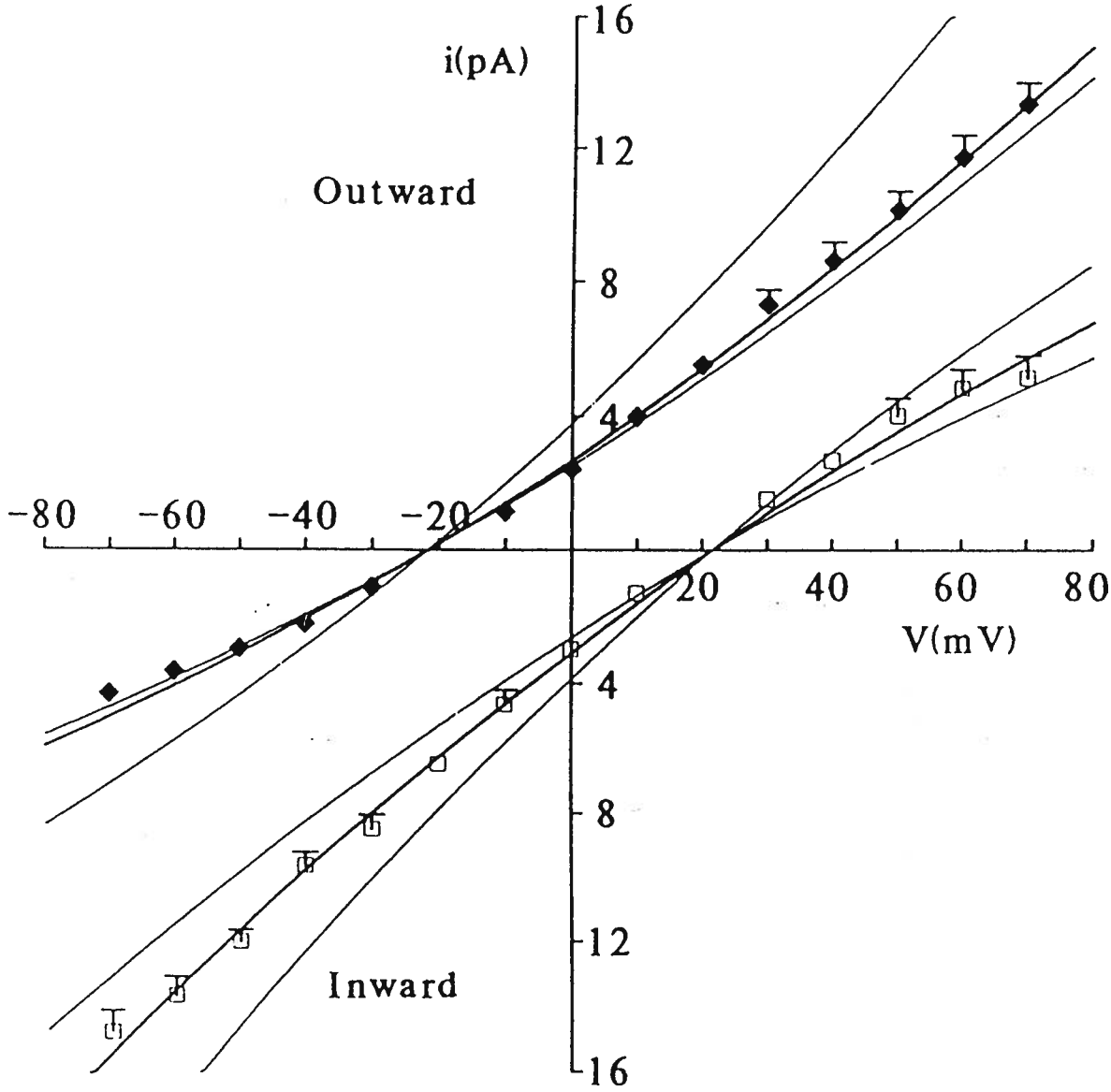

A
B
V (mV)
40
20
-21
-60
-80
5 pA
100 ms
5 pA
100 ms

Figure 29. Current-voltage (I-V) relationship of single K(Ca)L channel currents when 80 mM KCl at the cytoplasmic (open squares) or external membrane face (filled diamonds) was replaced by NaCl. The symbols show mean currents calculated from 5 patches. Standard error bars were omitted if less than the symbol size. The thick solid lines show currents predicted from constant-field theory, assuming P_K = 3.5 x 10^{-13} cm/s (external K^+ replacement) or P_K = 4.0 x 10^{-13} cm/s (cytoplasmic K^+ replacement). All the currents observed fall within the range of values predicted from the constant-field equation, using the extreme values of P_K seen in symmetrical 140 mM solutions (thin lines). $[Ca^{2+}]_i$ = 100 μM. $[Ca^{2+}]_o$ = 0.01 μM.


i(pA)
16
12
8
4
Outward
-80
-60
-40
-20
20
40
60
80
V(mV)
4
8
12
Inward
16

is very close to the reversal potential expected for a potassium selective channel under these conditions (V_{rev} = -21.4 mV). When 80 mM KCl in the solution bathing the cytoplasmic face was replaced by an equimolar amount of NaCl, currents in K(Ca)L channel reversed at a membrane potential of +21.2 $\pm$ 0.37 mV (n = 5, see Fig. 29), again very close to the Nernstian reversal potential value for K+ (+21.4 mV). These reversal potentials were used to calculate the permeability ratio P_{Na}/P_K, employing the relation

$$V_{rev} = (RT/zF)\ln[(P_{Na}[Na]_o + P_K[K]_o)/(P_{Na}[Na]_i + P_K[K]_i)] \qquad (3)$$

(Benham et al. 1986). Here V_{rev} is the observed reversal potential, and z is the valence. A value of $P_{Na}/P_K < 0.05$ was obtained, indicating a high degree of selectivity for potassium over sodium ions in this channel. It was also noted that, since E_{Cl} was 0 mV throughout these experiments, the permeability of the K(Ca)L channel to Cl- must be very low. The potassium selective nature of this channel was later confirmed in experiments with the potassium channel blockers Cs+ and TEA, as will be described in a later section. Under the assumption of negligible permeability to Na+, the constant-field relation (Equation 1) was used to calculate the expected current through K(Ca)L channels when 80 mM K+ was replaced by an equimolar amount of Na+ at the cytoplasmic or at the external membrane face. In calculating these currents, the parameter P_K was allowed to vary between 3.3 x 10^{-13} cm/s and 4.9 x 10^{-13} cm/s, the extreme values noted for K(Ca)L channels studied in symmetrical 140 mM K+ solutions. It can be seen that the mean currents observed in 5 patches were well predicted by constant-field theory when P_K = 3.5 x 10^{-13} cm/s (replacement of 80 mM external K+ by Na+) and P_K = 4.0 x 10^{-13} cm/s (replacement of 80 mM internal K+ by Na+) (Fig. 29). Fig. 29 also shows (as thin solid lines) the

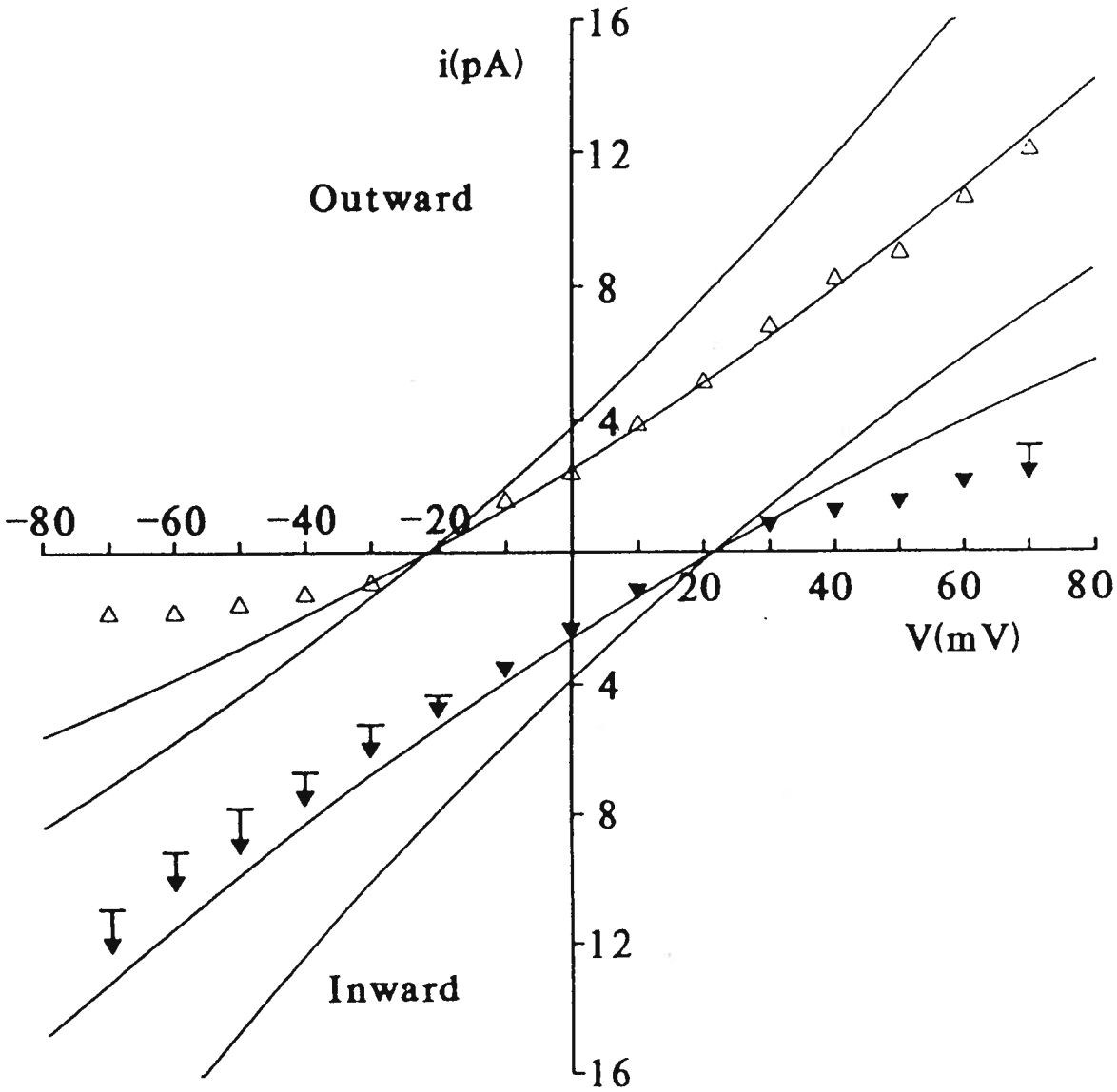
current through the K(Ca)L channel calculated using the extreme value of P_K noted earlier, namely 3.3 x 10^{-13} cm/s and 4.9 x10^{-13} cm/s. It is evident that all the currents observed during Na^+ replacement of K^+ fall within the range of values predicted from the constant-field equation. It was therefore concluded that Na^+ ions do not interfere with the passage of K^+ ions through the K(Ca)L channel, under the conditions used in this study.

3.4.2. Effect of K^+ replacement by Cs^+ on current flow through K(Ca)L channels.

The effects of replacing 80 mM KCl by an equimolar amount of CsCl in either the cytoplasmic or the external bathing solution were each studied in 5 patches. When Cs^+ replaced K^+ at the external membrane face, the single channel currents had a reversal potential close to -21 mV (Fig. 28B). An average value of V_{rev} = -21.3 $\pm$ 0.46 mV was obtained from 5 patches (see Fig. 30). Replacement of 80 mM K^+ in the cytoplasmic bathing solution by equimolar Cs^+ resulted in a reversal potential of +21.2 $\pm$ 0.51 mV (5 patches, see Fig. 30). These reversal potentials were used to calculate the permeability ratio P_{Cs}/P_K, employing Equation 3. A value of $P_{Cs}/P_K < 0.05$ was obtained, indicating that the K(Ca)L channel is very poorly permeable to Cs^+.

It was also noted that currents in single K(Ca)L channels recorded in the presence of Cs^+ exhibited a greater noise variance, when compared with currents recorded in symmetrical K^+ gradients, or during replacement of K^+ by Na^+ (see Figs. 25 and 28). This observation is consistent with a flickering block of open K(Ca)L channels by Cs^+, as has previously been observed in other types

Figure 30. Current-voltage (I-V) relationship of single K(Ca)L channel currents when 80 mM KCl at the cytoplasmic (filled triangles) or external membrane face (open triangles) was replaced by CsCl. Symbols show mean currents obtained from 5 patches. Standard error bars were omitted if smaller than symbol. Solid lines show predicted currents assuming constant-field behavior, calculated for the extreme values of P_K seen in symmetrical 140 mM K+ solutions. Mean currents were smaller than predicted by the constant-field equation, especially under conditions promoting the entry of Cs+ into the K(Ca)L channel (i.e. at negative potentials when Cs+ was present in the external solution and at the positive potentials when Cs+ was applied to the cytoplasmic membrane face. $[Ca^{2+}]_i$ = 100 μM. $[Ca^{2+}]_o$ = 0.01 μM.

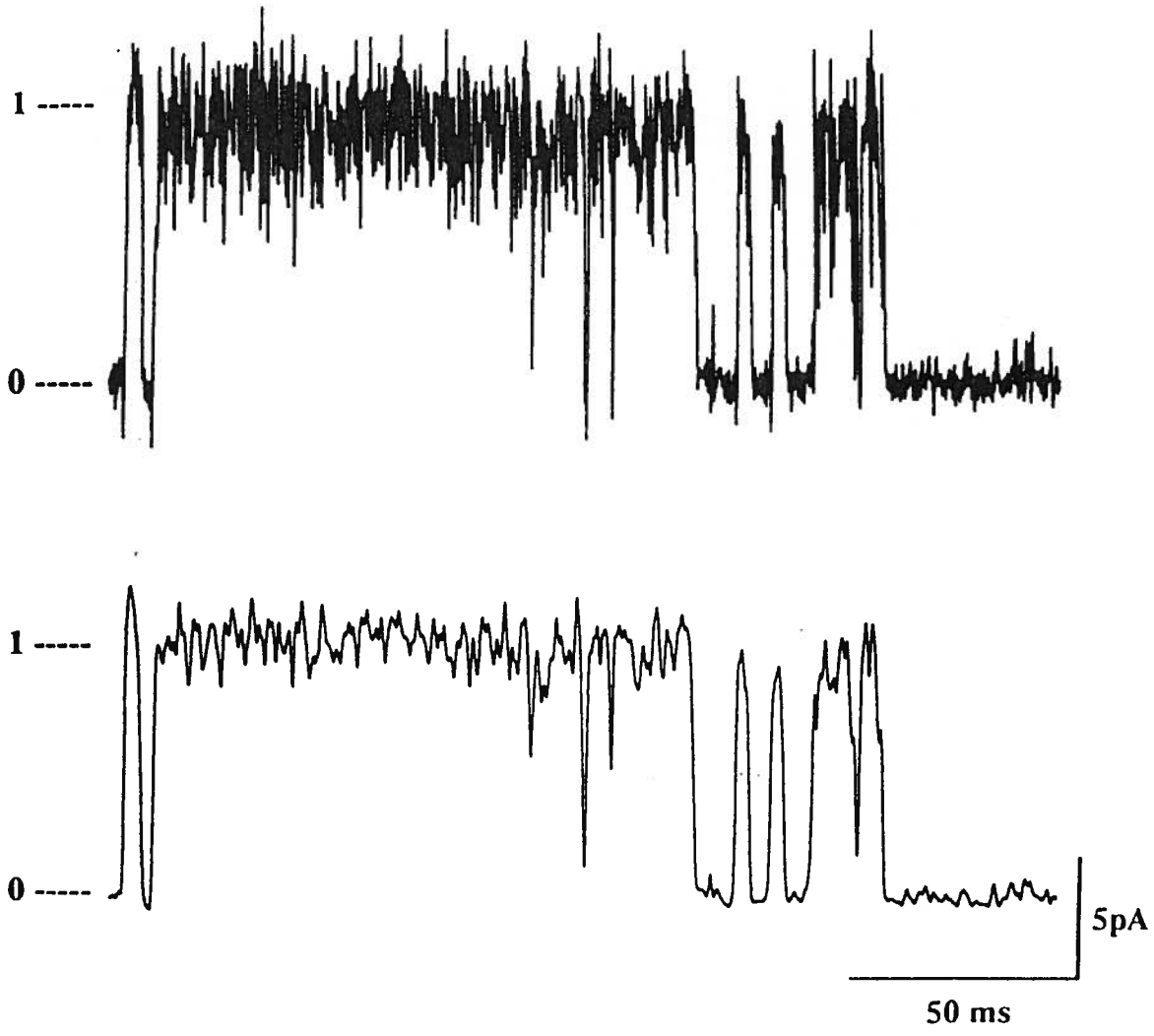

i(pA)
16
12
8
4
Outward
-80
-60
-40
-20
20
40
60
80
V(mV)
4
8
12
16
Inward

of K_{Ca} channel (Benham et al. 1986). Individual blocking and unblocking events were at least partially resolved at a 2 kHz bandwidth. This indicated that the rate constants for channel block and unblock were not very fast, since this would result in a smoothed, time-averaged value of current through the channel (Yellen 1984 a,b).

The occurrence of significant noise during current flow in the presence of Cs+ made it difficult to determine an appropriate mean channel current for use in I-V plots. For this reason, these currents were subjected to further digital filtering to reduce their effective bandwidth to DC-400 Hz. As shown in Fig. 31, this procedure resulted in a smooth, time-averaged current suitable for the construction of I-V plots (Benham et al. 1986). Mean values of such currents obtained from 5 patches tested with cytoplasmic or external substitution of 80 mM K+ by Cs+ are shown in Fig. 30. This Figure also shows the range of expected currents through the K(Ca)L channel, under the assumption that Cs+ is non-permeable but does not block the passage of K+ through the channel. As was the case in Fig. 29, these theoretical currents were calculated from the constant-field relation, using the extreme values of P_K noted in symmetrical 140 mM K+ solutions.

It was noted that the observed currents were smaller than predicted by constant-field theory, especially under conditions promoting the entry of Cs+ into the K(Ca)L channel, that is at negative membrane potentials with Cs+ in the external solution, and at positive membrane potentials with Cs+ at the cytoplasmic membrane face (Fig. 30). Assuming that the flicker block by Cs+ is not influenced by other ions, then the time-averaged current through the blocked channel is given as:

Figure 31. Single channel K(Ca)L current recordings from an inside-out patch bathed in 140 mM K+ solution with 100 μM [Ca^{2+}]i. The patch electrode contained 60 mM K+ and 80 mM Cs+. 0 indicates channel closed and 1 indicates channel open. The same group of single channel currents is seen at a filter bandwidth of DC-2kHz (upper trace), and at a filter bandwidth of DC-400Hz (lower trace). Membrane potential +60 mV.


1 -----
0 -----
1 -----
0 -----
5pA
50 ms

$$\langle i(V) \rangle = i_o (V) [1 + [Cs] / K_{(o)} \exp (F\delta V / RT)]^{-1} \quad (4)$$

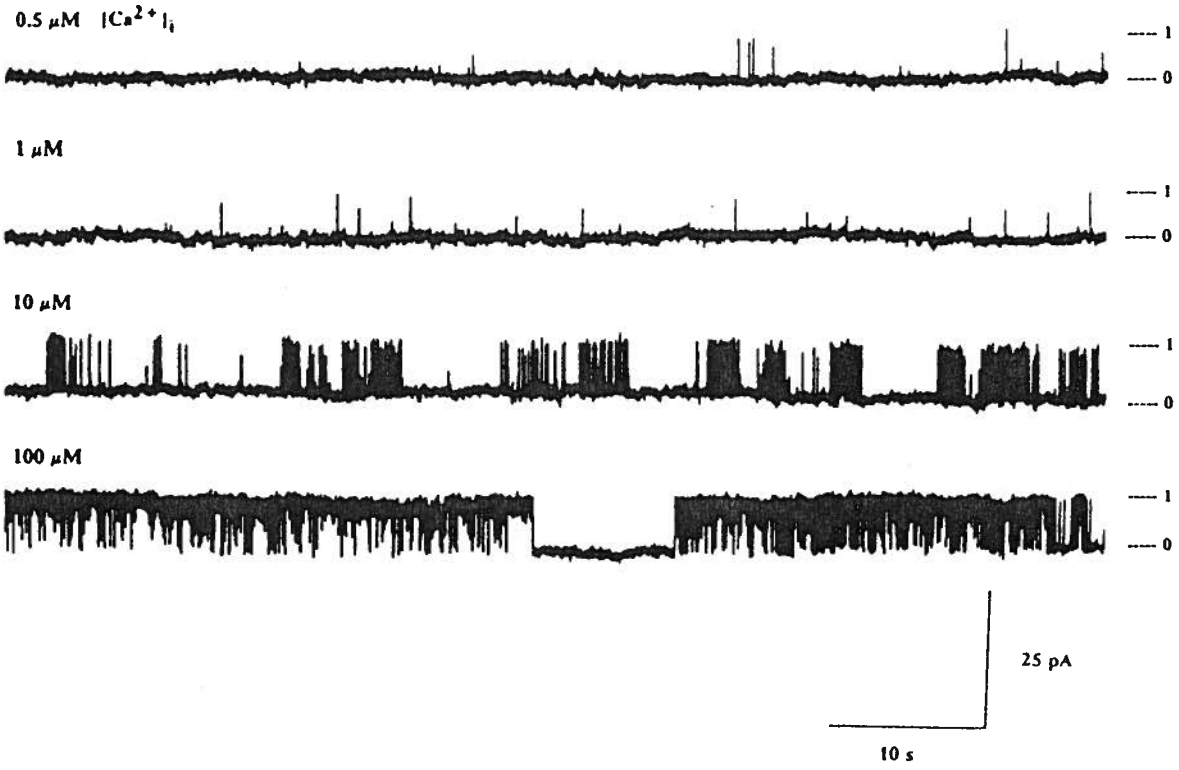
where $i_o(V)$ is the current through the unblocked channel, V is potential, $K_{(o)}$ is the zero voltage equilibrium constant and δ is the fraction of the membrane field felt by Cs+ at its blocking site, sometimes referred to as the "effective valence" (Woodhull 1973; Yellen 1984a). Plots of $\ln(i_o / i - 1)$ versus V should therefore yield a straight line of slope $F\delta/RT$. The value of δ provides a measure of the steepness of voltage dependent blocking. Values of i_o were obtained from the 5 patches in which Na+ replaced K+, since no evidence for Na+ block of the K(Ca)L channel was seen. Using the data shown in Fig. 29 and Fig. 30, plots of this type were found to be approximately linear over the voltage range -70 mV to +70 mV. When Cs+ was present at the cytoplasmic membrane face, a value of δ = 0.07 was obtained, indicating that the cation detects very little of the membrane field at this site. In contrast, externally applied Cs+ yielded a value of δ = -0.66, suggesting that the ion must transverse an appreciable fraction of the membrane to reach its binding site, if applied externally.

3.4.3. Effect of varying $[Ca^{2+}]_i$ on the open probability of K(Ca)L channels

To determine the effect of $[Ca^{2+}]_i$ on the probability of the single K(Ca)L channels being in the open state, recordings were made from isolated inside-out patches exposed to symmetrical 140 mM K+ solutions. $[Ca^{2+}]_i$ was varied while the membrane potential of the patch was voltage-clamped to +40 mV.

Fig. 32 shows single channel currents obtained from a patch containing a single active K(Ca)L channel. When $[Ca^{2+}]_i$ was 0.5 μM, few channel openings

Figure 32. Effect of varying $[Ca^{2+}]_i$ on the opening of K(Ca)L channels. Single channel current recordings were obtained from an isolated inside-out patch containing one active channel and bathed in symmetrical 140 mM K^+ solutions. The recording pipette contained 0.01 μM Ca^{2+}. Membrane potential was +40 mV. 0 indicates channel closed and 1 indicates channel open. Bandwidth DC-200 Hz.


0.5 μM [Ca2+]i
1
0
1 μM
1
0
10 μM
1
0
100 μM
1
0
25 pA
10 s

were seen (P_O = 0.0045) and the mean channel open time was clearly very brief. On increasing $[Ca^{2+}]_i$ to 1, 10 and 100 μM, P_O increased markedly (P_O = 0.60 at 100 μM $[Ca^{2+}]_i$). The mean shut time of the K(Ca)L channel was greatly reduced at $[Ca^{2+}]_i$ = 100 μM, while the mean open time appeared greater than that seen at low cytoplasmic calcium.

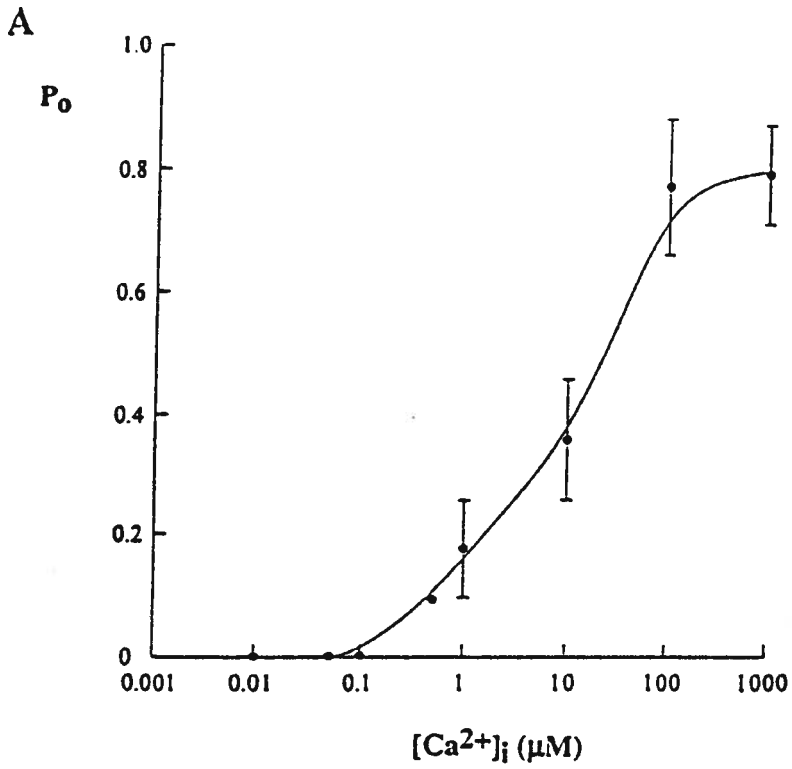
The effect of varying $[Ca^{2+}]_i$ from 0.01 μM to 1 mM on P_O values measured in 5 patches at V = +40 mV is shown in Fig. 33A. The threshold level of $[Ca^{2+}]_i$ at which channel openings were just detectable was about 0.01 μM. The mean value of $[Ca^{2+}]_i$ at which single K(Ca)L channels were open half of the time, $P_O(0.5)$ was 23 μM.

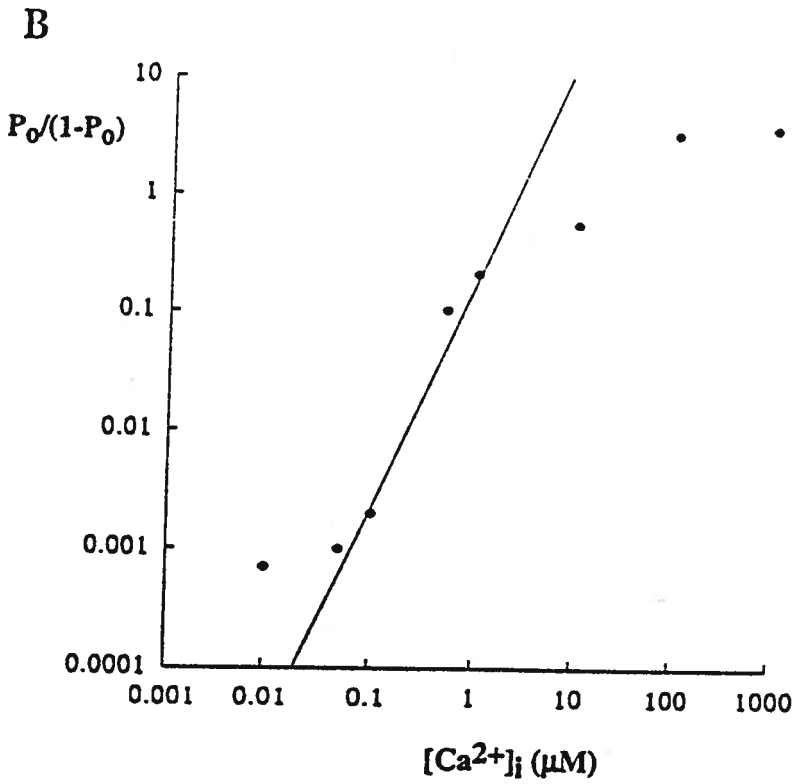
Fig. 33B shows these data presented as a Hill plot, i.e. log $P_O/(1-P_O)$ versus log $[Ca^{2+}]_i$. A linear regression line has been fitted to the data points obtained for $[Ca^{2+}]_i$ values between 0.05 μM and 1 μM, the probable physiological range of this parameter in smooth muscle cells (Klockner et al. 1989). Over this range, this line had a mean slope of 2.00 This result shows that 2 calcium ions must bind to fully stabilize each K(Ca)L channel in the open state (Barrett et al. 1982). At very low and very high $[Ca^{2+}]_i$, the slope factor was greatly reduced, as has previously been noted in K_{Ca} channels studied in rat skeletal muscle fibres (Barrett et al. 1982).

3.4.4. The effect of varying $[Ca^{2+}]_i$ on the open time of the K(Ca)L channel.

Over the range of membrane potentials (-80 to +80 mV) and $[Ca^{2+}]_i$ levels (0.01 μM to 1 mM) used in this study, open time distributions for K(Ca)L channels were well described by the sum of two exponential terms, indicating the

Figure 33. Effect of $[Ca^{2+}]_i$ on the open probability of the $K_{(Ca)}L$ channel. (A) Mean open probability P_o is plotted as a function of $[Ca^{2+}]_i$ on semilogarithmic co-ordinates. The smooth curve was fitted to data points using polynomial regression. (B) Hill plot of these data, i.e. log $P_o/(1-P_o)$ against log $[Ca^{2+}]_i$. Over range of $[Ca^{2+}]_i$ from 0.05 μM to 1 μM, data were well fitted by linear regression line (solid line) of slope n = 2.00. Data were obtained from five patches. Membrane potential was +40 mV during these recordings. $[Ca^{2+}]_o$ = 0.01 μM.


A
P_o
1.0
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
10
100
1000
$[Ca^{2+}]_i$ (μM)


B
$P_o/(1-P_o)$
10
1
0.1
0.01
0.001
0.0001
0.001
0.01
0.1
1
10
100
1000
$[Ca^{2+}]_i$ (μM)

presence of at least two kinetically distinguishable open states for this channel (Fig. 34). These distributions were therefore well fit by an equation of the form

$$y = N_f \cdot e^{-t/\tau_f} + N_S \cdot e^{-t/\tau_S} \quad (5)$$

Here N_f and N_S are the zero time amplitudes of two exponential functions governed, respectively, by a fast time constant τ_f and a slow time constant τ_S. From these parameters, the total number of openings represented by either component was calculated using the relations

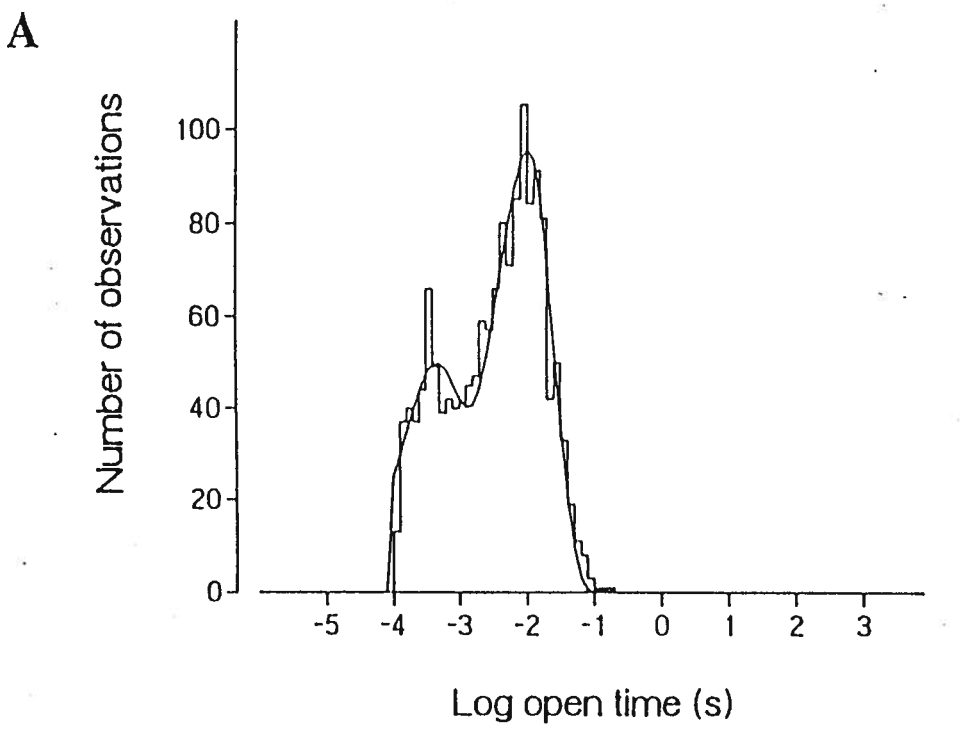
$$A_f = N_f \cdot \tau_f / \text{Bin width} \quad (6)$$

and

$$A_S = N_S \cdot \tau_S / \text{Bin width} \quad (7)$$

Here, A_f and A_S are the numbers of openings in the exponential terms governed by the fast time constant, τ_f and the slow time constant, τ_S respectively. The value of the bin width parameter was obtained directly from the open time histograms. The effect of changing $[Ca^{2+}]_i$ on the open time distributions of K(Ca)L channels was investigated in 5 patches voltage-clamped at a membrane potential of +40 mV (Fig. 35). It was found that increasing $[Ca^{2+}]_i$ had no significant effect on the mean value of the fast time constant τ_f in these patches (Fig. 35A). At $[Ca^{2+}]_i = 0.01\,\mu M$, a mean value of $\tau_f = 0.58 \pm 0.08$ ms was obtained while at $[Ca^{2+}]_i = 10\,\mu M$, $\tau_f = 0.52 \pm 0.15$ ms was seen ($P > 0.05$, ANOVA). In contrast, τ_S was found to increase on elevating $[Ca^{2+}]_i$ (Fig. 35B). At $[Ca^{2+}]_i = 0.01\,\mu M$, a mean value of $\tau_S = 5.9 \pm 0.85$ ms was obtained, while at $[Ca^{2+}]_i = 10\,\mu M$, $\tau_S = 14.6 \pm 2.24$ ms was observed ($P < 0.05$, ANOVA).

Figure 34. Distribution of open times for the K(Ca)L channel studied in one isolated patch containing a single active channel. $[Ca^{2+}]_i = 1\,\mu M$ in A and 1 mM in B. Both distributions were plotted on a logarithmic time axis and fitted by the sum of two exponential terms (smooth curves) using the Simplex algorithm. In A, the fast fit component had a time constant $\tau_f = 0.41$ ms, this component made up 29% of the total openings. The slow fit component was governed by a time constant $\tau_S = 11.4$ ms, and made up the remaining 71% of openings. Corresponding values for the distribution shown in B were $\tau_f = 0.38$ ms (19% of openings) and $\tau_S = 14.3$ ms (81% of openings). Membrane potential was +40 mV in both A and B. $[Ca^{2+}]_o = 0.01\,\mu M$.


A
Number of observations
100
80
60
40
20
0
-5
-4
-3
-2
-1
0
1
2
3
Log open time (s)

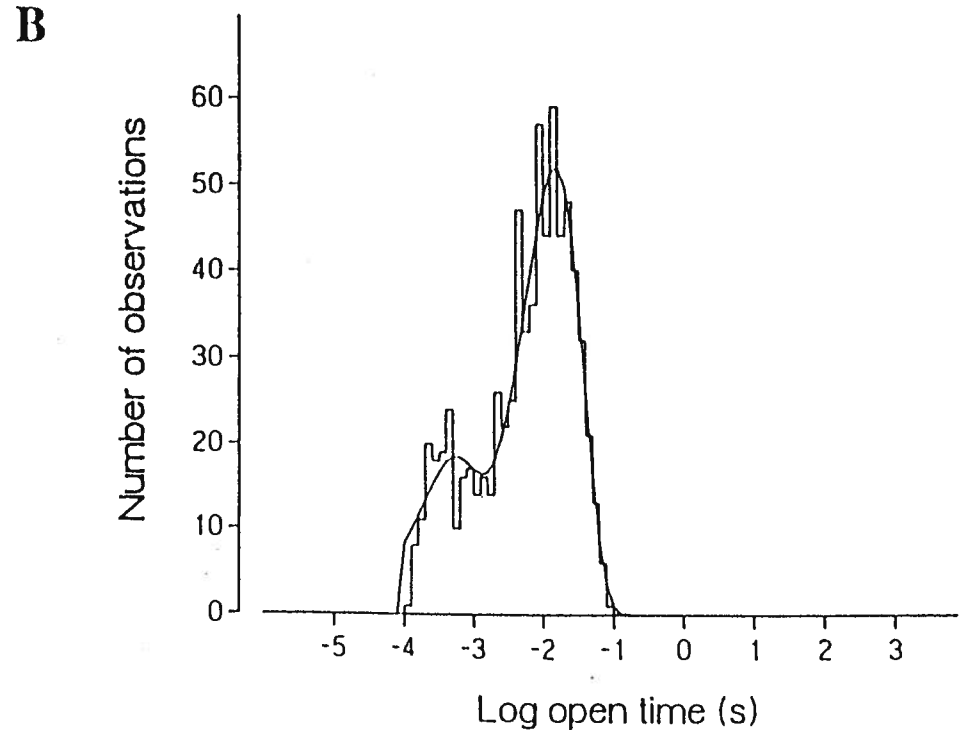
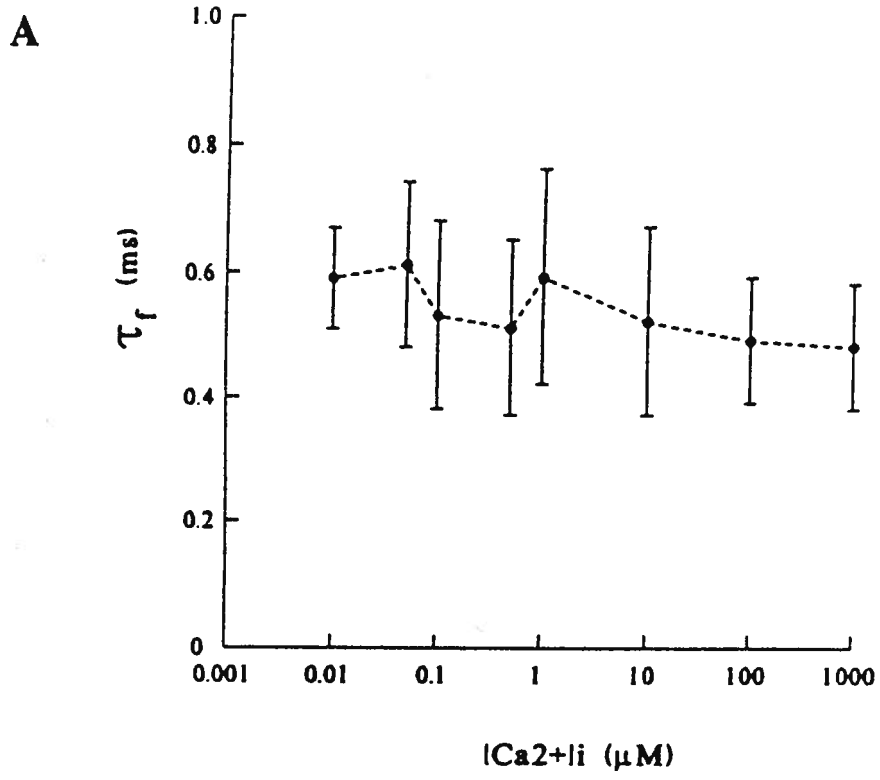
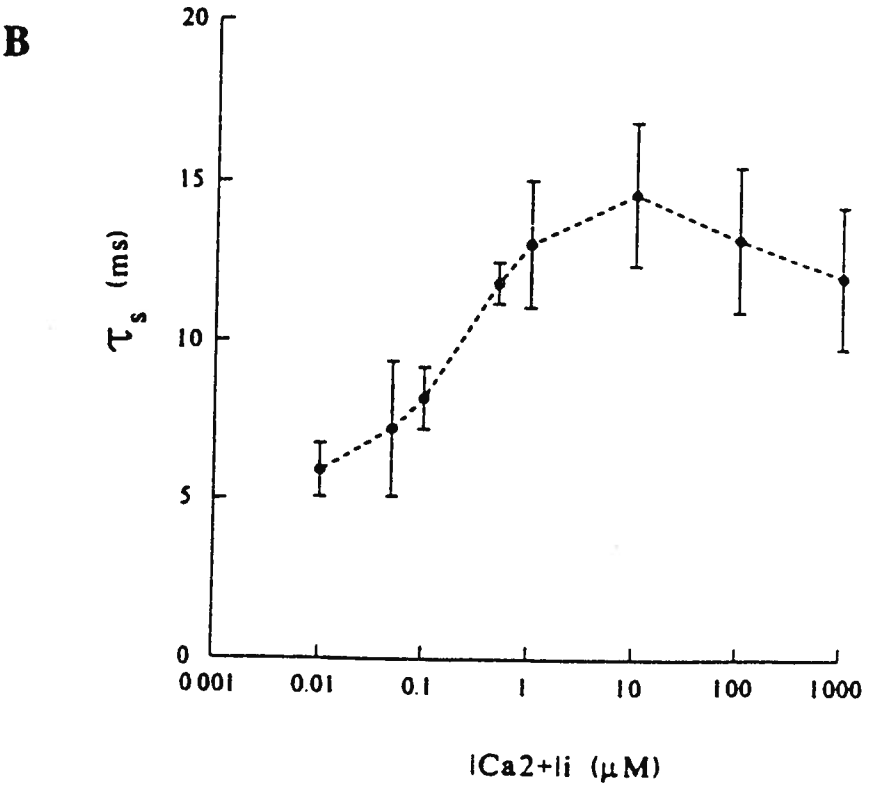

B
Number of observations
60
50
40
30
20
10
0
-5
-4
-3
-2
-1
0
1
2
3
Log open time (s)

Figure 35. Effect of varying $[Ca^{2+}]_i$ on the time constants governing open time distribution of $K_{(Ca)}L$ channels studied in 5 inside-out patches voltage-clamped at +40 mV. A, The fast time constant (τf) versus $[Ca^{2+}]_i$. B, The slow time constant (τs) versus $[Ca^{2+}]_i$. $[Ca^{2+}]_o = 0.01\,\mu M$.


A
1.0
0.8
0.6
0.4
0.2
0
τ_f (ms)
0.001
0.01
0.1
1
10
100
1000
ICa2+Ii (μM)


B
20
15
10
5
0
τ_s (ms)
0.001
0.01
0.1
1
10
100
1000
ICa2+Ii (μM)

Thus, increasing $[Ca^{2+}]_i$ increased the time constant of the slow exponential component, while having no effect on that of the fast exponential component.

Changing $[Ca^{2+}]_i$ also had an effect on the relative numbers of openings governed by the time constants τ_f and τ_s. As shown in Fig. 36, increasing $[Ca^{2+}]_i$ from 0.01 μM to 1 mM lead to a monotonic increase in the value of the ratio $A_s/(A_f+A_s)$ (Fig. 36B), with a corresponding decrease in the ratio $A_f/(A_f+A_s)$ (Fig. 36A). These observations show that increasing $[Ca^{2+}]_i$ preferentially stabilized openings of the K(Ca)L channel governed by the slow time constant τ_s.

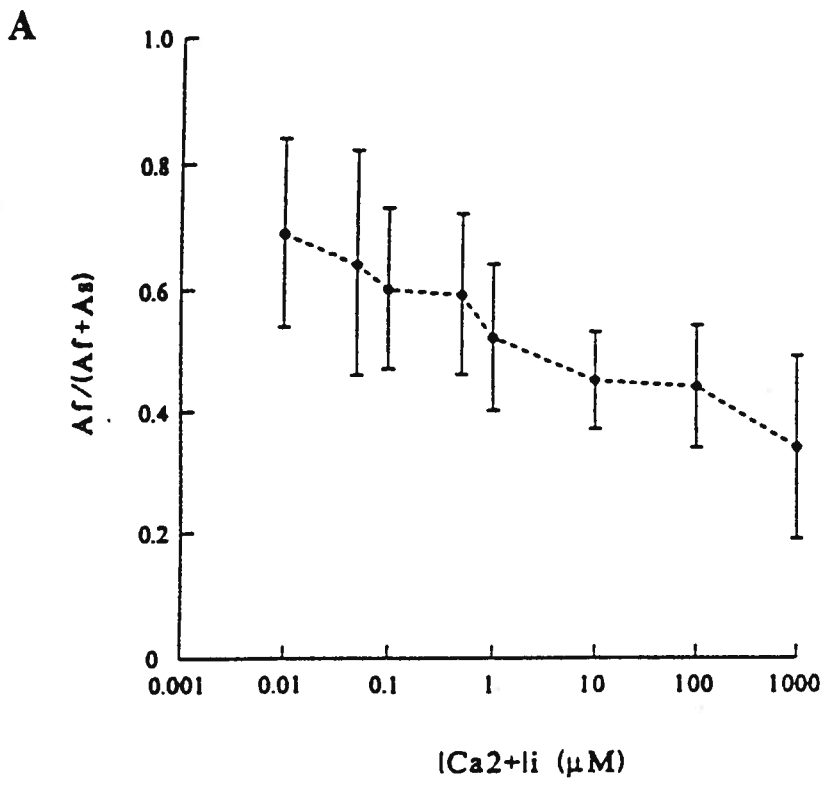
The mean open time, τ_{mean} of the K(Ca)L channel was calculated from the above data using the relation

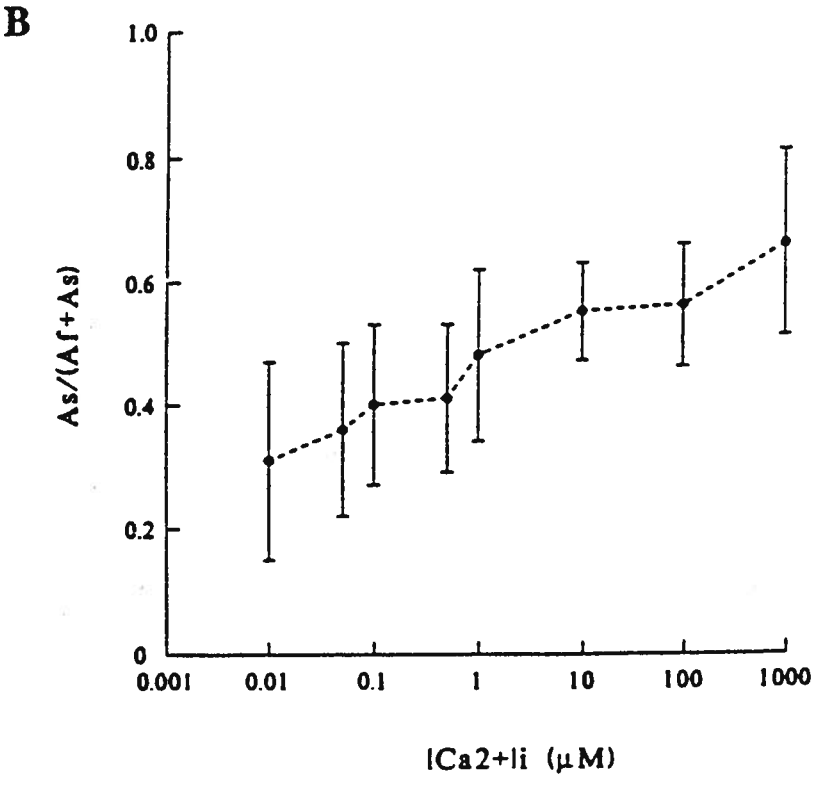
$$\tau_{mean} = A_f/(A_f + A_s)\cdot\tau_f + A_s/(A_f + A_s)\cdot\tau_s \qquad (8)$$

At a membrane potential of +40 mV, τ_{mean} increased from 2.7 $\pm$ 0.23 ms (n=5) at 0.01 μM $[Ca^{2+}]_i$ to 13.5 $\pm$ 0.35 ms at 10 μM $[Ca^{2+}]_i$ ($P < 0.05$, Students' t-test). This 5 fold rise in τ_{mean} was much too small to fully account for the 425 fold increase seen in open probability on elevating $[Ca^{2+}]_i$ from 0.01 μM to 10 μM (see Fig. 33). It was therefore concluded that the major effect of increasing internal free calcium concentration was to decrease the mean shut time of the K(Ca)L channel.

3.4.5. Effect of membrane potential on the open probability of the K(Ca)L channel

Figure 36. Effect of varying $[Ca^{2+}]_i$ on the relative number of openings of K(Ca)L channels governed by the time constants τ_f and τ_s. Data are mean $\pm$ SEM from 3-5 inside-out patches voltage-clamped at +40 mV. In A, the relationship between the ratio $A_f/(A_f + A_s)$ and $[Ca^{2+}]_i$ is illustrated. In B, the effect of $[Ca^{2+}]_i$ on the ratio $A_s/(A_f + A_s)$ is shown. $[Ca^{2+}]_o = 0.01\,\mu M$.


A
1.0
0.8
0.6
0.4
0.2
0
Af/(Af+As)
0.001
0.01
0.1
1
10
100
1000
[Ca2+]i (μM)


B
1.0
0.8
0.6
0.4
0.2
0
As/(Af+As)
0.001
0.01
0.1
1
10
100
1000
[Ca2+]i (μM)

The probability of $K_{(Ca)}L$ channels being in the open state was also a function of the potential applied across the membrane patch as shown in Fig. 37. When $[Ca^{2+}]_i$ was less than 50 μM, the dependence of P_o on membrane potential was well described by the Boltzmann relation (Kolb 1990)

$$P_o = [1+ \exp(-K(V-V_o))]^{-1} \quad (9)$$

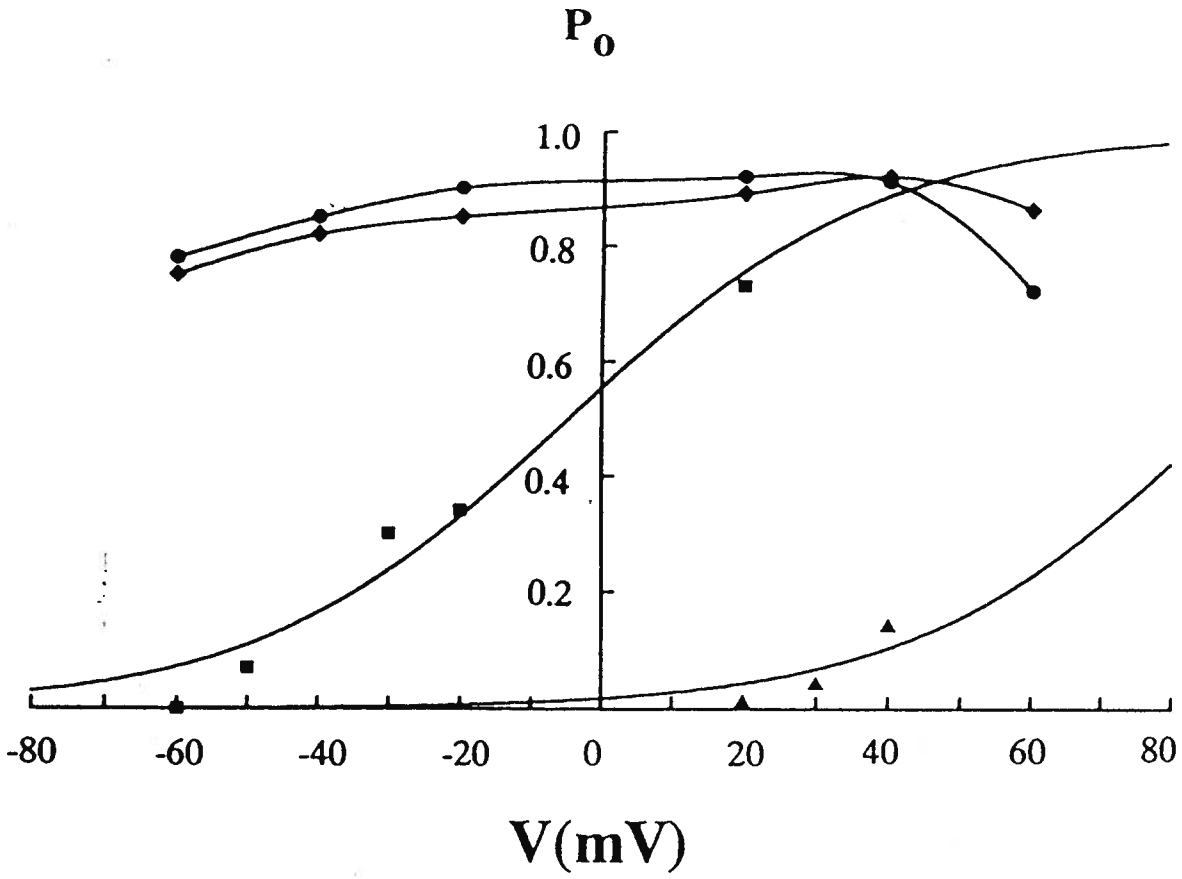
where V_o is the potential at which $P_o = 0.5$ (Fig. 37).

In 5 patches exposed to $[Ca^{2+}]_i < 50\,\mu$M, the mean value of the constant K was 0.045 ± 0.006 mV^{-1}. At $[Ca^{2+}]_i$ levels between 0.01 μM and 50 μM, the principal effect of increasing intracellular free calcium was to shift the Boltzmann relation to the left on the voltage axis. In 4 patches studied in this range of $[Ca^{2+}]_i$, the mean shift of the V_o was 45 mV per decade change in intracellular free calcium.

At $[Ca^{2+}]_i > 50\,\mu$M, P_o approached unity and showed little further increase on membrane depolarization (Fig. 37). Indeed a tendency was noted toward reduced P_o at membrane potentials more positive than +40 mV (Fig. 37). Reduced P_o at positive membrane potentials has previously been noted in K_{Ca} channels studied in vascular smooth muscle cells of the guinea-pig (Benham et al. 1986) and in K_{Ca} channels incorporated into lipid bilayers (Vergara and Latorre 1983).

3.4.6. Effect of membrane potential on the open time of K_{Ca} channels.

Figure 37. Dependence of the open probability, P_o of K(Ca)L channels on membrane potential (V) and $[Ca^{2+}]_i$ measured in 4 inside-out patches. When $[Ca^{2+}]_i$ was 1 mM (circles) and 100 μM (diamonds), fitting lines were drawn by the polynomial regression. When $[Ca^{2+}]_i$ was 50 μM (squares) and 0.5 μM (triangles), the fitting curves were drawn to the Boltzmann equation $P_o = [1+\exp(-K(V-V_o))]^{-1}$ where V_o is the potential at which $P_o = 0.5$. At both 50 μM and 0.5 μM free intracellular calcium, the constant K had the value 0.045 mV^{-1}. At 50 μM free calcium, V_o = -5 mV. at 0.5 μM calcium, V_o = +88 mV.

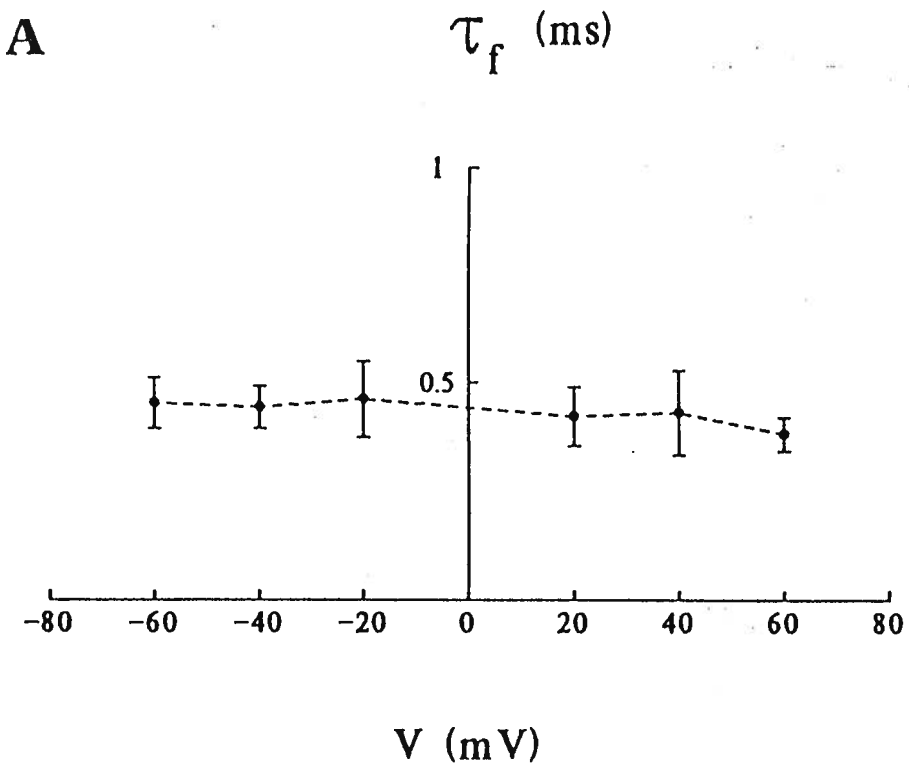

P_o
1.0
0.8
0.6
0.4
0.2
-80
-60
-40
-20
0
20
40
60
80
V(mV)

The effect of varying membrane potential on the distribution of open times for K(Ca)L channels was studied in 5 patches exposed to $[Ca^{2+}]_i$ = 10 μM. These distributions were well described by the sum of two exponential terms, as noted previously. It was found that changes in membrane potential did not significantly alter the value of the fast time constant, τ_f in these patches (Fig. 38A). The mean value of τ_f at V = -60 mV was 0.45 $\pm$ 0.06 ms, not significantly different from the value obtained at V = +60 mV (τ_f = 0.38 $\pm$ 0.04 ms, P > 0.05, ANOVA). In contrast, the slow time constant, τ_s describing open time distributions was significantly altered by membrane potential in these patches (Fig. 38B). τ_s was weakly voltage-dependent, increasing from 8.4 $\pm$ 0.55 ms at V = -60 mV to 16.6 $\pm$ 1.64 ms at V = +60 mV (P < 0.05, ANOVA). It should however be noted that these value of τ_f are close to the deadtime of the recording system. Small changes in τ_f could therefore have gone undetected.

3.4.7. The effect of TEA applied to the cytoplasmic membrane face on current flow in the K(Ca)L channel.

The effect of TEA on the K(Ca)L channel was studied in 6 inside-out membrane patches voltage-clamped at +40 mV with $[Ca^{2+}]_i$ = 100 μM. Fig. 39 shows single channel currents recording during an experiment of this kind in which TEA was applied by bath perfusion to the cytoplasmic membrane face. Under these conditions, TEA caused a dose-dependent, reversible reduction in the amplitude of current in the K(Ca)L channel. It was noted that the blocking action of TEA was not accompanied by a large increase in the variance of current noise in the open channel as observed at a bandwidth of DC-2-kHz (Fig. 39). TEA did not alter the reversal potential of the current through K(Ca)L channels, which was 0 mV in these experiments.

Figure 38. Effect of membrane potential on the time constants governing the open time distribution of K(Ca)L channels. A. Fast time constant (τf) versus membrane potential. B. Slow time constant (τs) versus membrane potential. Data points are mean $\pm$SEM from three to five patches. $[Ca^{2+}]_i = 10\,\mu M$ and $[Ca^{2+}]_o = 0.01\,\mu M$ in both A and B.


A
τ_f (ms)
1
0.5
-80
-60
-40
-20
0
20
40
60
80
V (mV)

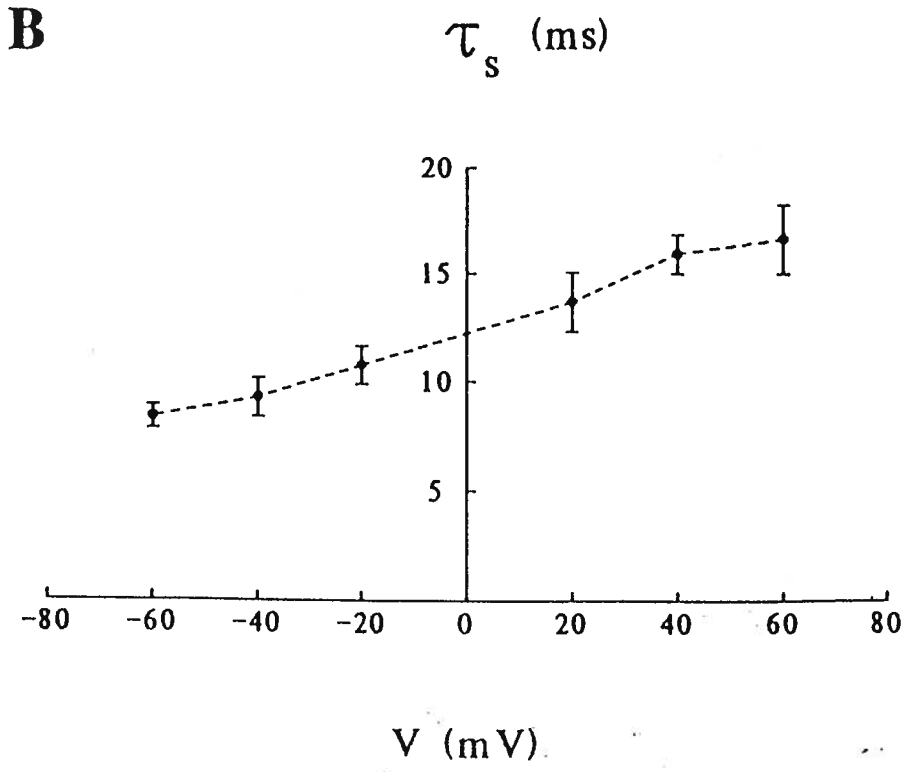
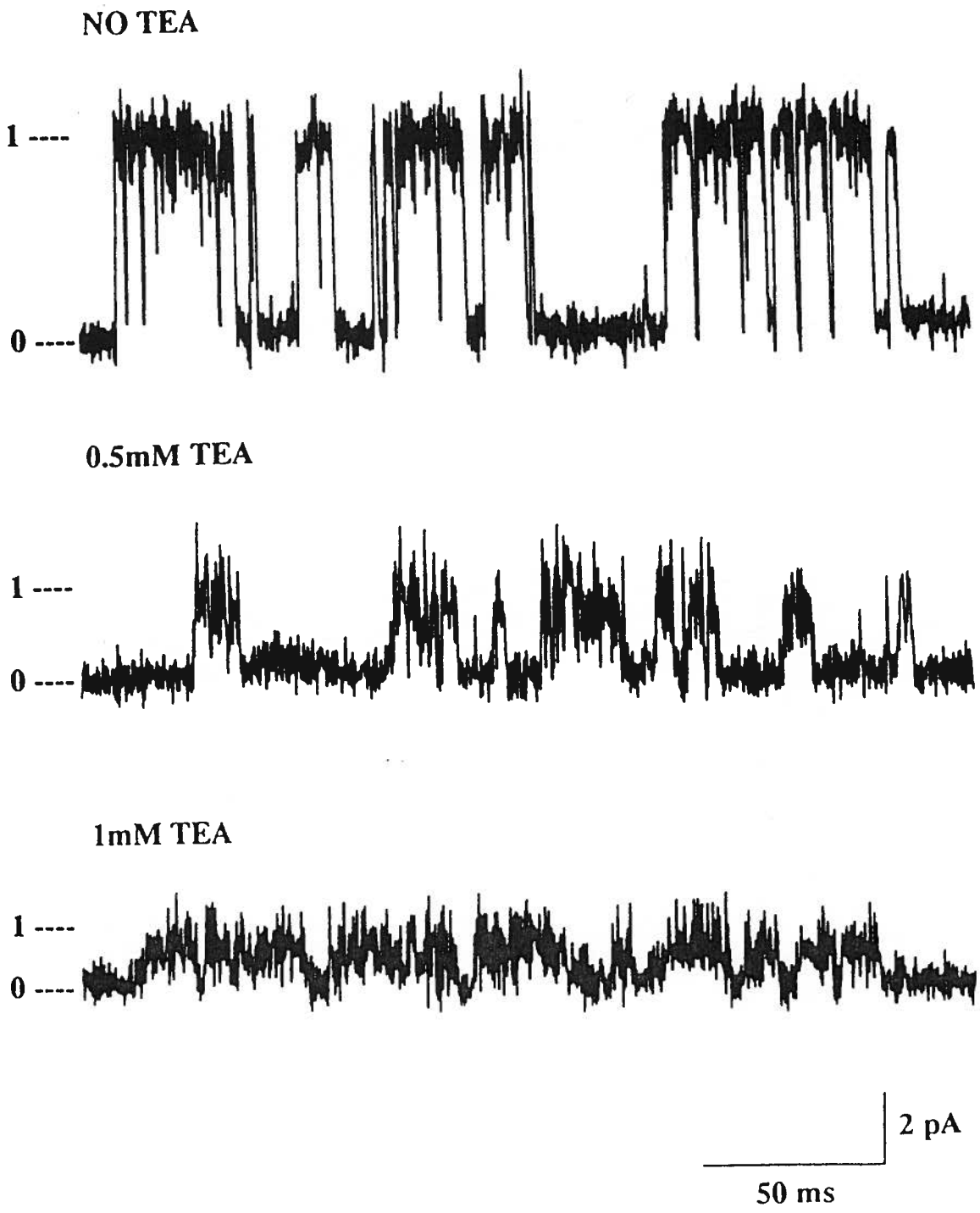

B
τ_s (ms)
20
15
10
5
-80
-60
-40
-20
0
20
40
60
80
V (mV)

Figure 39. Blocking effect of internal TEA on the K(Ca)L channel. Single channel currents were recorded from an inside-out membrane patch bathed in symmetrical 140 mM potassium solutions. TEA was applied to the cytoplasmic face of the membrane at the concentrations indicated. 0 indicates channel closed and 1 indicates channel open. Bandwidth DC-2 kHz, membrane potential +40 mV. $[Ca^{2+}]_o = 0.01\,\mu M$.


NO TEA
1 ----
0 ----
0.5mM TEA
1 ----
0 ----
1mM TEA
1 ----
0 ----
2 pA
50 ms

The percentage reduction, R_i in single channel current caused by various concentrations of TEA applied to the cytoplasmic membrane face is shown in Fig. 40. It was found that the value of R_i was well described by the relation

$$R_i = 100\% / (1 + K_d / [TEA]) \qquad (10)$$

where $K_d = 0.83 \pm 0.09$ mM (n = 5 patches) at a membrane potential of +40 mV and $[Ca^{2+}]_i = 100 \mu M$.

The present data on TEA block were transformed into a Hill plot by replotting as log (R_i/1-R_i) versus log [TEA], where R is the reduction in single channel currents seen in the presence of the drug. This plot yielded a slope of 0.82, suggesting that TEA interacts with the channel in a one-to-one fashion, presumably by transiently entering the channel and blocking movement of K^+.

In these experiments, TEA had no significant effect on the probability of K(Ca)L channels adopting the open state (Fig. 41). Thus $P_O = 0.55 \pm 0.15$ (n = 3) in the absence of TEA, and $P_O = 0.53 \pm 0.17$ (n =3) in the presence of 10 mM TEA at the cytoplasmic membrane face ($P > 0.05$, ANOVA).

3.5. K(Ca)I channels in inside-out membrane patches excised from isolated CVSMCs.

Figure 40. Dose-response curve for the block of current flow in the K(Ca)L channel by internal TEA. The percentage reduction in single channel current is plotted against the concentration of TEA at the cytoplasmic membrane face. The solid line plots the relation $R_i = 100\%/(1 + K_d / [TEA])$ where the apparent dissociation constant K_d was 0.83 mM and [TEA] was the concentration of TEA. Data points are mean $\pm$ SEM from 3-5 patches. The calculated curve was fitted to the data points by non-linear regression. V = +40 mV, $[Ca^{2+}]_i = 100\,\mu M$. $[Ca^{2+}]_o = 0.01\,\mu M$.

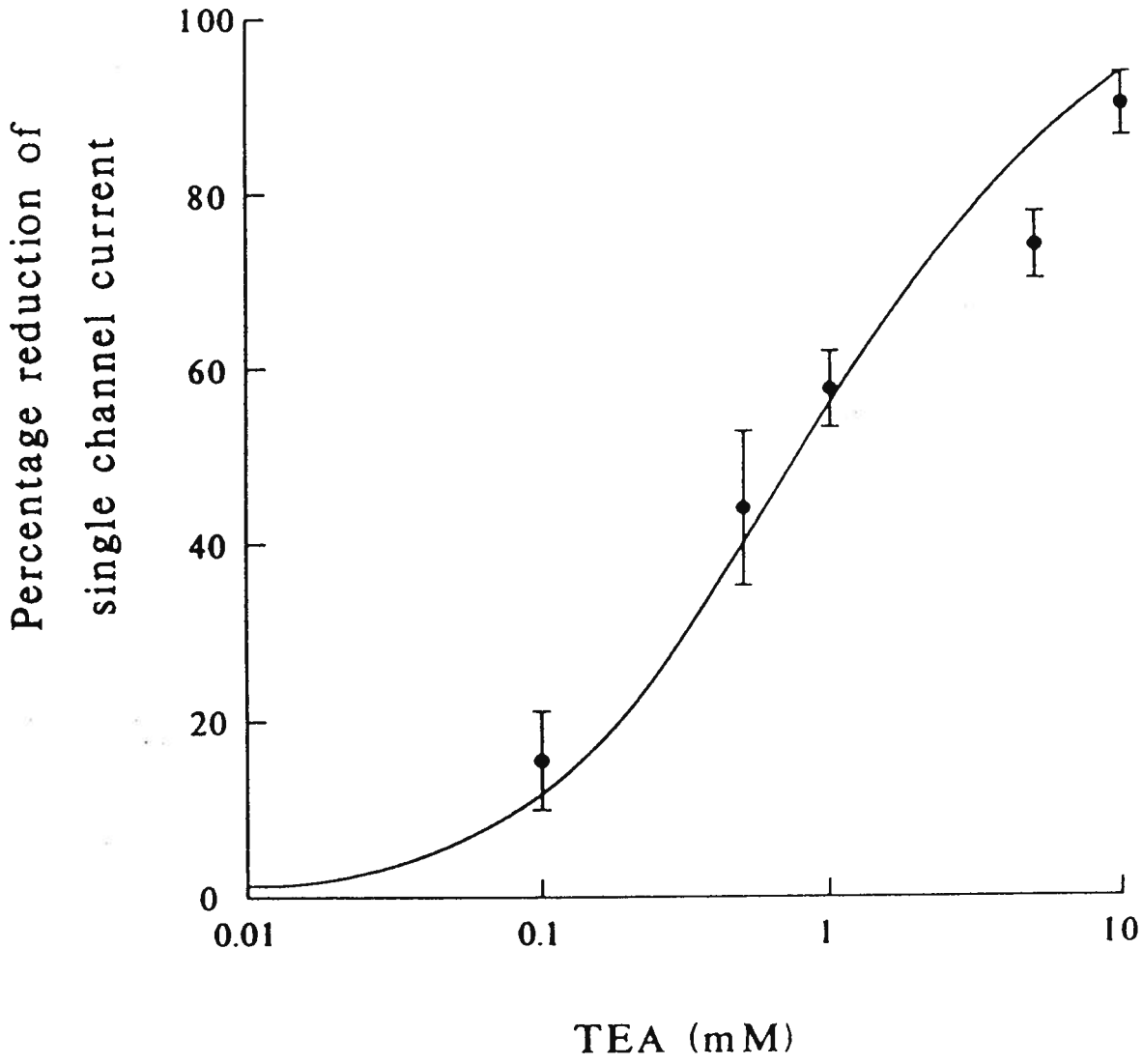
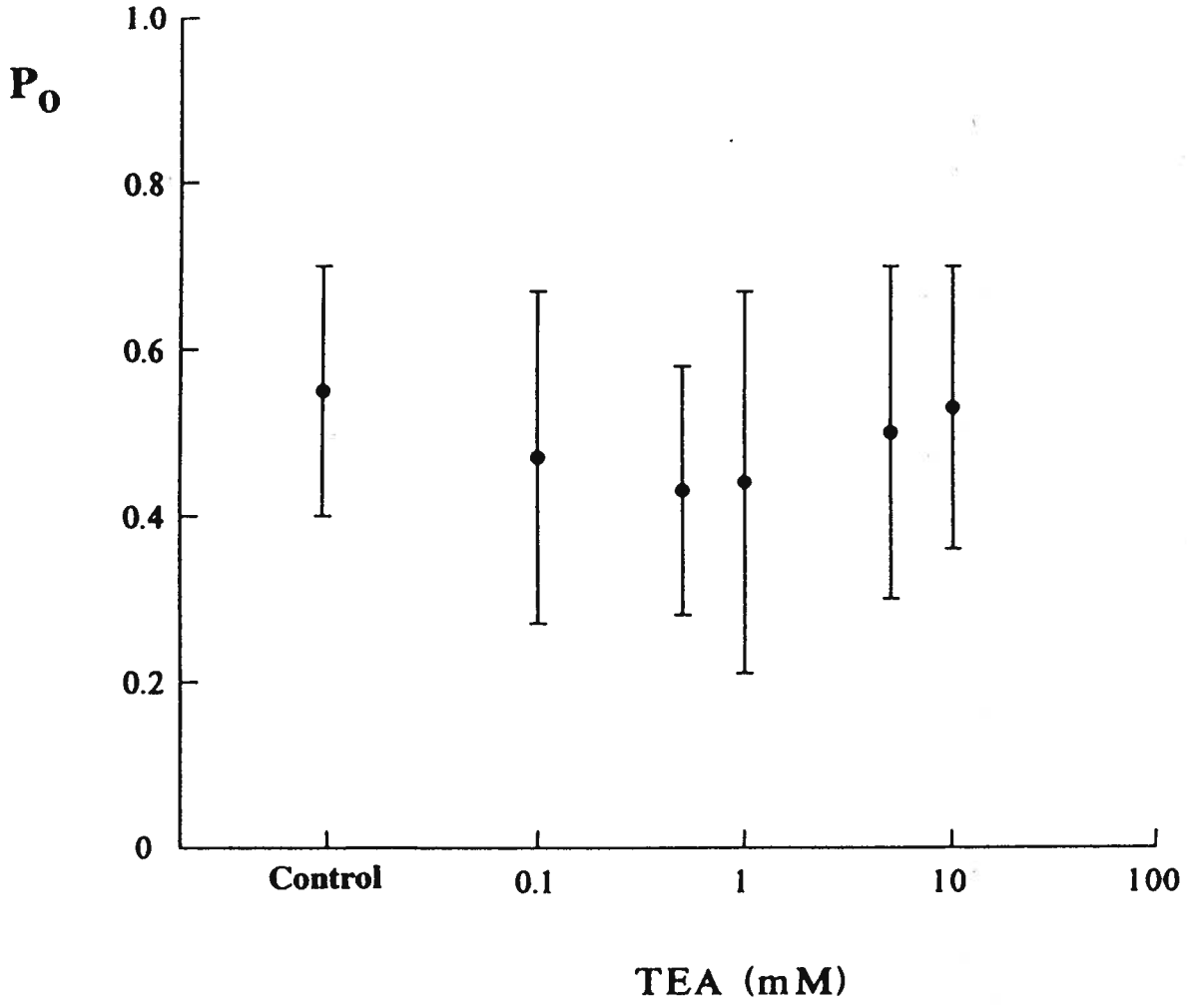

Percentage reduction of
single channel current
100
80
60
40
20
0
0.01
0.1
1
10
TEA (mM)

Figure 41. Effect of TEA on the open probability of the K(Ca)L channel. The open probability of the channel is plotted against the concentration of internal TEA. Data points are mean $\pm$ SEM from 3-5 patches. No significance between the mean values of P_O calculated at each TEA concentration ($P > 0.05$ ANOVA). Membrane potential +40 mV, $[Ca^{2+}]_i$ = 100 μM. $[Ca^{2+}]_i$ = 0.01 μM.


P_o
1.0
0.8
0.6
0.4
0.2
0
Control
0.1
1
10
100
TEA (mM)

$K_{(Ca)}I$ channels were detected in about 25% of the inside-out patches examined in this study. When measured in symmetrical 140 mM K^+ solutions, currents in $K_{(Ca)}L$ channels showed a reversal potential of 0 mV (Fig. 42). Under these conditions, the current-voltage relation of this channel was linear with a mean single channel conductance of 92 ± 2.6 pS (n = 10 patches). Amplitude distributions of currents flowing in $K_{(Ca)}I$ channels were well described by single Gaussian terms, indicating that this channel did not display a significant substate or superstate conductance (Fig. 43). From these data, the potassium permeability of the open channel was calculated using the constant-field relation (Equation 2). A mean value of $P_K = 1.75 \pm 0.12 \times 10^{-13}$ cm/s was obtained from 10 patches.

The infrequent occurrence of $K_{(Ca)}I$ channels in these patches precluded a detailed quantitative examination of the ionic selectivity of this channel or its sensitivity to changes in $[Ca^{2+}]_i$ and membrane potential. However, it was possible to demonstrate qualitatively that $K_{(Ca)}I$ channels are indeed selective for potassium ions, and are modulated by both membrane potential and $[Ca^{2+}]_i$.

When 80 mM KCl was replaced by an equimolar amount of NaCl, currents through single $K_{(Ca)}I$ channels reversed at +21.1 mV (cytoplasmic face KCl replacement) and -21.1 mV (external face KCl replacement, Fig. 44). The ratio P_{Na}/P_K was calculated from these data using Equation 3. A value of $P_{Na}/P_K < 0.05$ was obtained, indicating that $K_{(Ca)}I$ channels are highly selective for potassium over sodium ions. In similar experiments where Cs^+ replaced K^+, the ratio P_{Cs}/P_K was also found to be less than 0.05. In the presence of Cs^+, current flow in the $K_{(Ca)}I$ channel was less than predicted

Figure 42. Single-channel current recordings of the activity of $K_{(Ca)}I$ channels at various membrane potentials (mV). All traces were recorded from a single inside-out patch containing four active channels. This patch was bathed in symmetrical 140 mM K+ solutions. 0 indicates all channels were closed, while the numbers 1, 2, 3 and 4 indicate current levels associated with 1, 2, 3 and 4 channels open, respectively. Bandwidth DC-200 Hz. Calibration bars apply to all traces. The cytoplasmic membrane face was exposed to $[Ca^{2+}]_i = 1\,\mu M$. The pipette contained $[Ca^{2+}]_o = 0.01\,\mu M$.

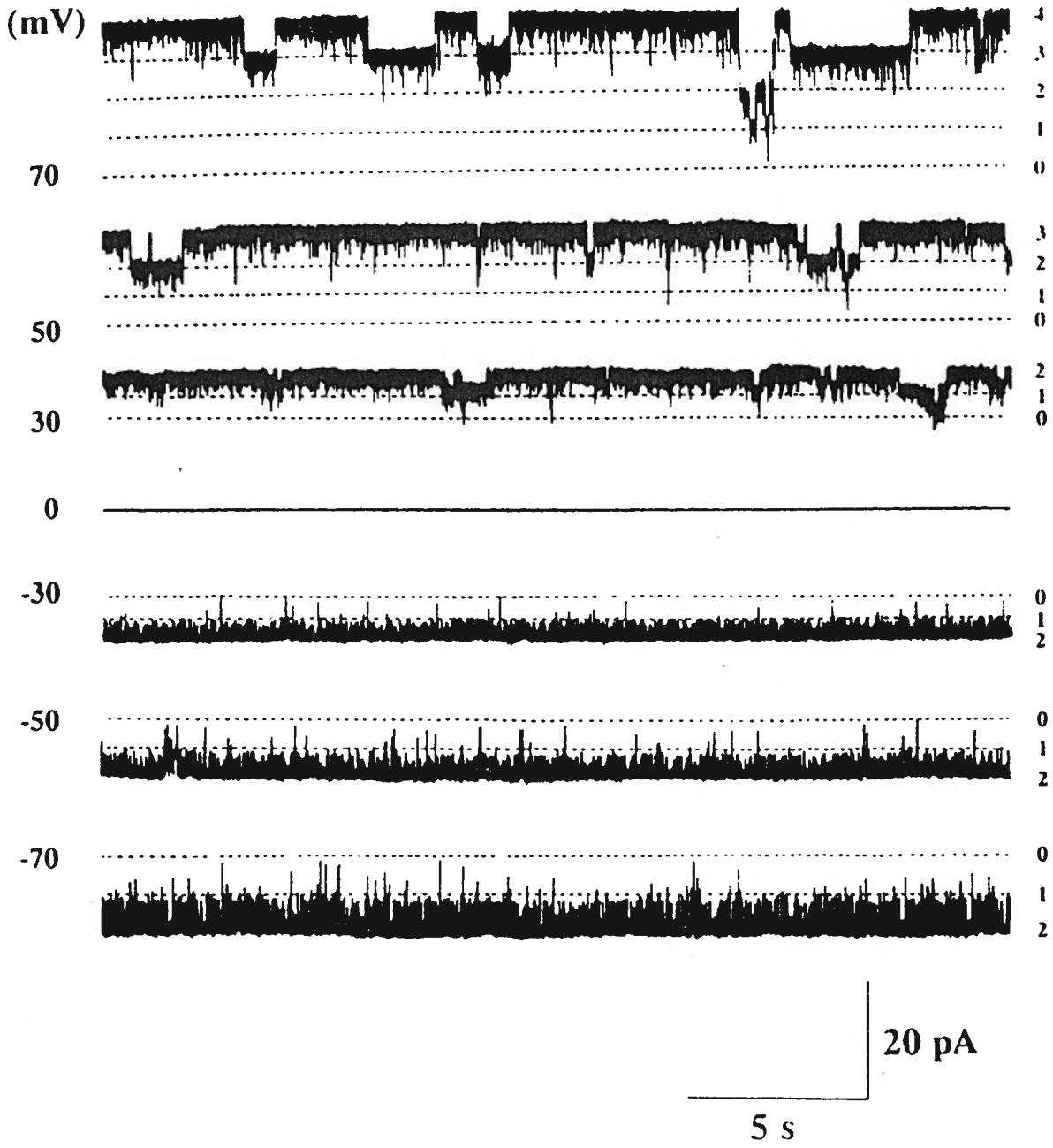

(mV)
70
50
30
0
-30
-50
-70
20 pA
5 s

Figure 43. Amplitude distributions obtained for K(Ca)I channel currents recorded from a single inside-out patch voltage-clamped at a membrane potential of +30 mV in A and -30 mV in B. Both distributions were well fitted by single Gaussian terms (smooth curves) with modal values of 2.44 pA in A and -2.43 pA in B. The patch was bathed in symmetrical 140 mM K+ solutions. $[Ca^{2+}]_i = 10\,\mu M$, $[Ca^{2+}]_o = 0.01\,\mu M$.

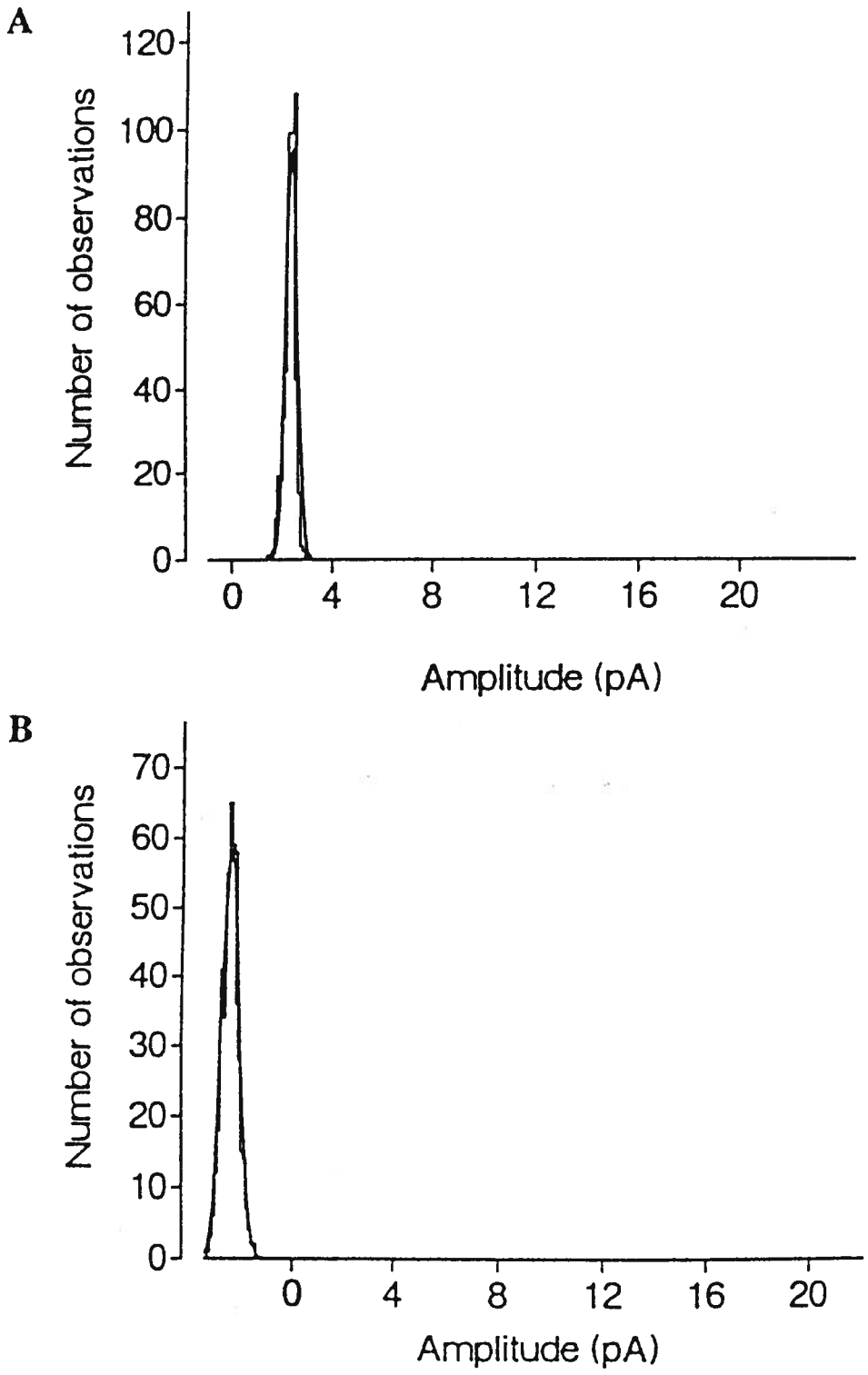
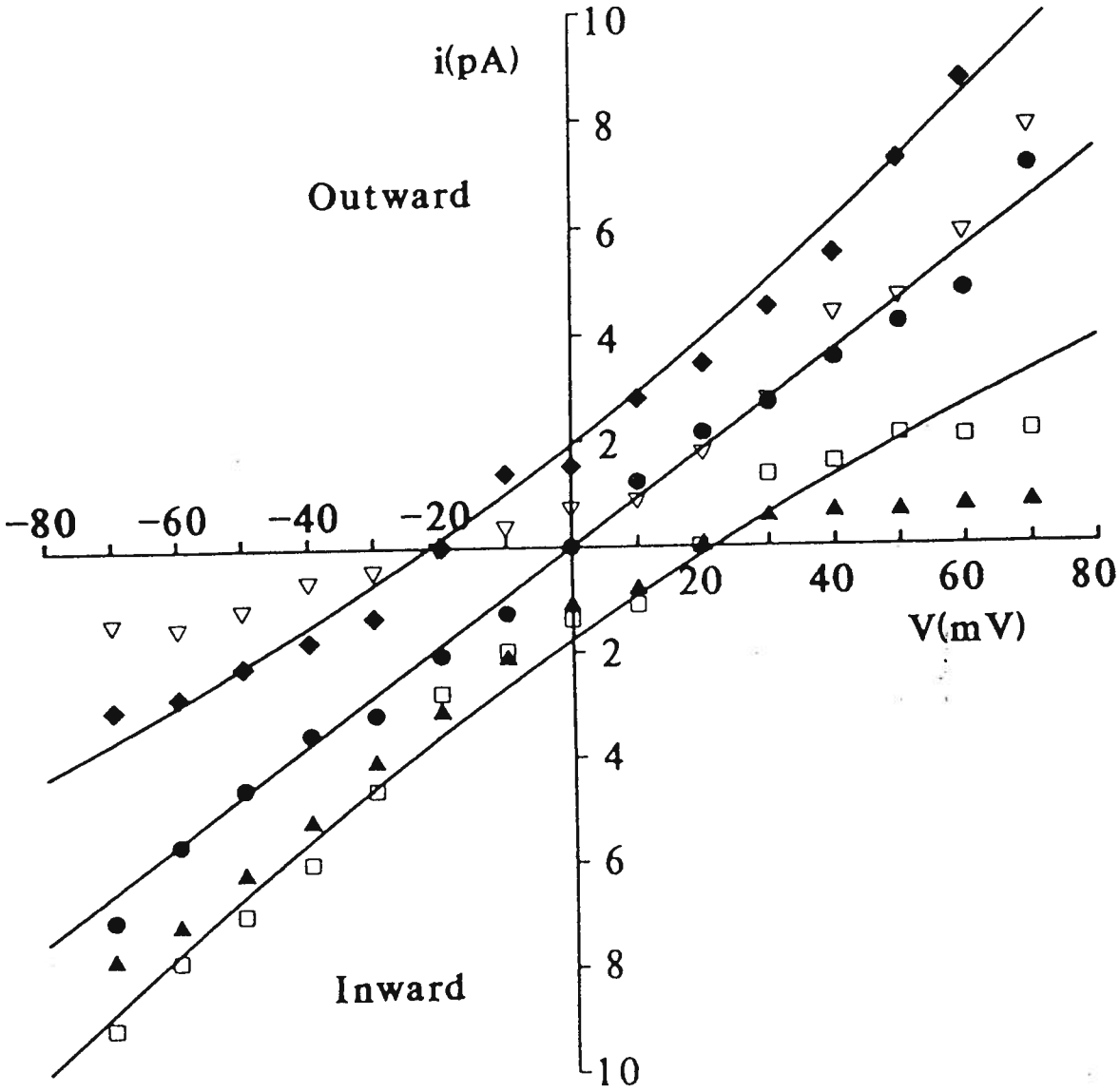

A
120
100
80
60
40
20
0
Number of observations
0
4
8
12
16
20
Amplitude (pA)
B
70
60
50
40
30
20
10
0
Number of observations
0
4
8
12
16
20
Amplitude (pA)

Figure 44. Conductance and ionic selectivity of the K(Ca)I channel. Current-voltage plots of single-channel currents recorded from five inside-out patches in five different ionic gradients: (filled circles) symmetrical 140 mM K+ solutions; (open squares) 80 mM internal K+ replaced by an equimolar amount of Na+; (filled diamonds) 80 mM external K+ replaced by an equimolar amount of Na+; (filled triangles) 80 mM internal K+ replaced by an equimolar amount of Cs+; (open triangles) 80 mM external K+ replaced by an equimolar amount of Cs+. $[Ca^{2+}]_o$ and $[Ca^{2+}]_i$ were 0.01 μM and 100 μM respectively. Continuous lines are theoretical curves fit by linear regression (data obtained in symmetrical potassium solutions) or non-linear regression to the Goldman-Hodgkin-Katz constant-field equation, using the data points obtained during replacement of K+ by Na+.


i(pA)
Outward
Inward
V(mV)
10
8
6
4
2
2
4
6
8
10
-80
-60
-40
-20
20
40
60
80

from the constant-field relation. This effect was especially marked under conditions tending to drive $Cs+$ into the open channel, i.e. at negative membrane potentials with $Cs+$ present in the external solution, and at positive membrane potentials when $Cs+$ was present at the cytoplasmic membrane face (Fig. 44).

The open probability of K(Ca)I channels increased as $[Ca2+]_i$ was elevated over the concentration range 0.1 μM to 100 μM (Fig. 45). Fig. 46A shows the dependence of P_o on $[Ca2+]_i$ for one membrane patch voltage-clamped at V = +40mV. These data were replotted as a Hill plot, i.e. as log $P_o/(1-P_o)$ against log $[Ca2+]_i$ (Fig. 46B). It is evident that P_o was steeply dependent on $[Ca2+]_i$ over the probable physiological range of this parameter (0.05-1 μM). In this range, the slope of the Hill plot was 2.17, suggesting that more than one calcium ion must bind to fully activate each K(Ca)I channel.

The open probability of K(Ca)I channels also increased on depolarization of the membrane patch (Fig. 47). The dependance of P_o on membrane potential was well described by the Boltzmann relation (Equation 9). The effect of increasing $[Ca2+]_i$ was to shift the Boltzmann relation to the left on the voltage axis, by about 40 mV per decade increase in free intracellular calcium (Fig. 47).

The effect of TEA on the K(Ca)I channel was examined quantitatively in 6 inside-out patches voltage-clamped at V = +40 mV with $[Ca2+]_i$ = 100 μM. TEA was applied by bath perfusion to the cytoplasmic membrane face. Under these conditions, TEA caused a reversible, dose-dependent reduction in the amplitude of current in the K(Ca)I channel (Fig. 48). TEA had no effect on the reversal potential of the current in K(Ca)I channels (0 mV).

Figure 45. Effect of varying $[Ca^{2+}]_i$ on the activity of $K_{(Ca)I}$ channels. Single channel current recordings were obtained from an isolated inside-out patch with two active channels bathed in symmetrical 140 mM potassium solutions. Membrane potential was +40 mV. 0 indicates channel closed, while the numerals 1and 2 indicate current levels associated with 1 and 2 channels open, respectively. Bandwidth DC-200 Hz. $[Ca^{2+}]_o = 0.01\,\mu M$.

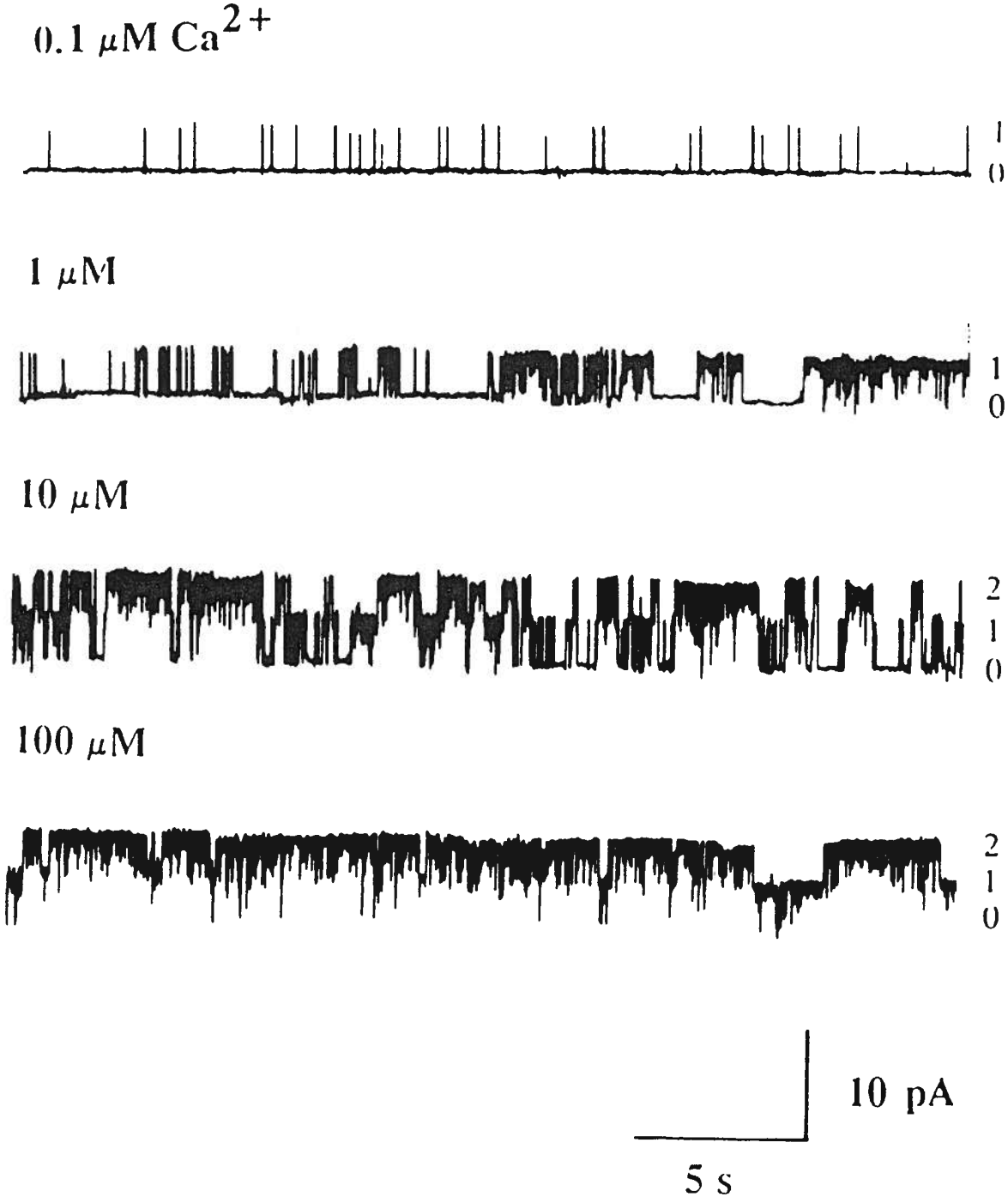
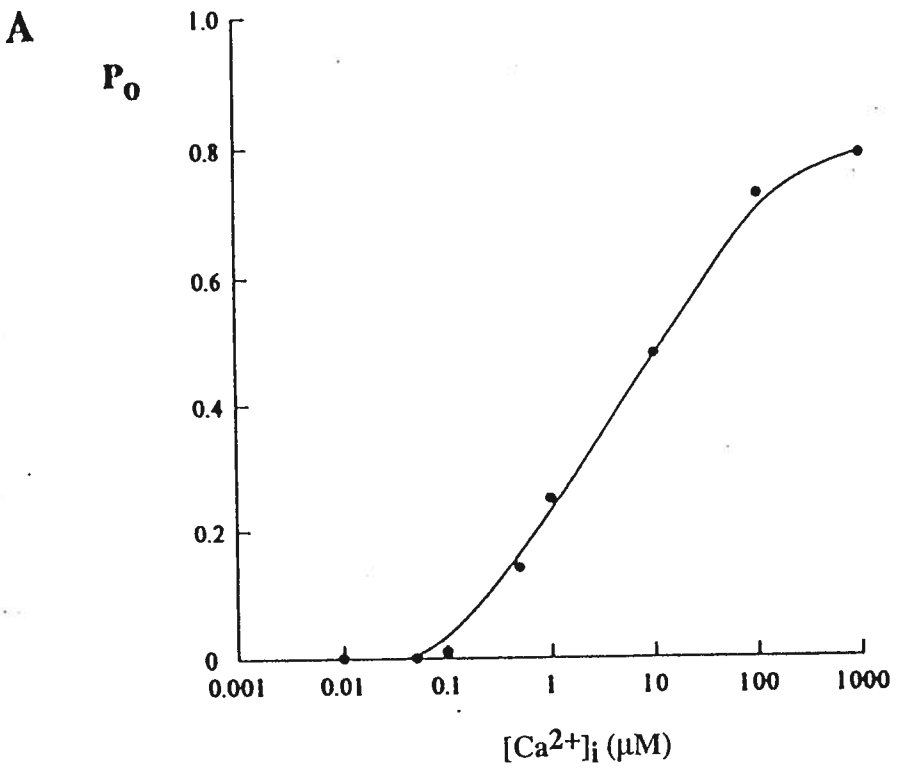

0.1 μM Ca2+
1
0
1 μM
1
0
10 μM
2
1
0
100 μM
2
1
0
10 pA
5 s

Figure 46. Effect of $[Ca^{2+}]_i$ on the open probability of the K(Ca)I channel. (A) Open probability plotted against $[Ca^{2+}]_i$ on semilogarithmic co-ordinates. (B) Hill plot of these data, i.e. log $P_O/(1-P_O)$ against log $[Ca^{2+}]_i$. Over range of $[Ca^{2+}]_i$ from 0.05 μM to 1 μM, data were well fitted by linear regression line (solid line) of slope n = 2.17. Data were obtained from one inside-out patch. V = +40 mV, $[Ca^{2+}]_O$ = 0.01 μM.


A
P_o
1.0
0.8
0.6
0.4
0.2
0
0.001
0.01
0.1
1
10
100
1000
$[Ca^{2+}]_i$ (μM)

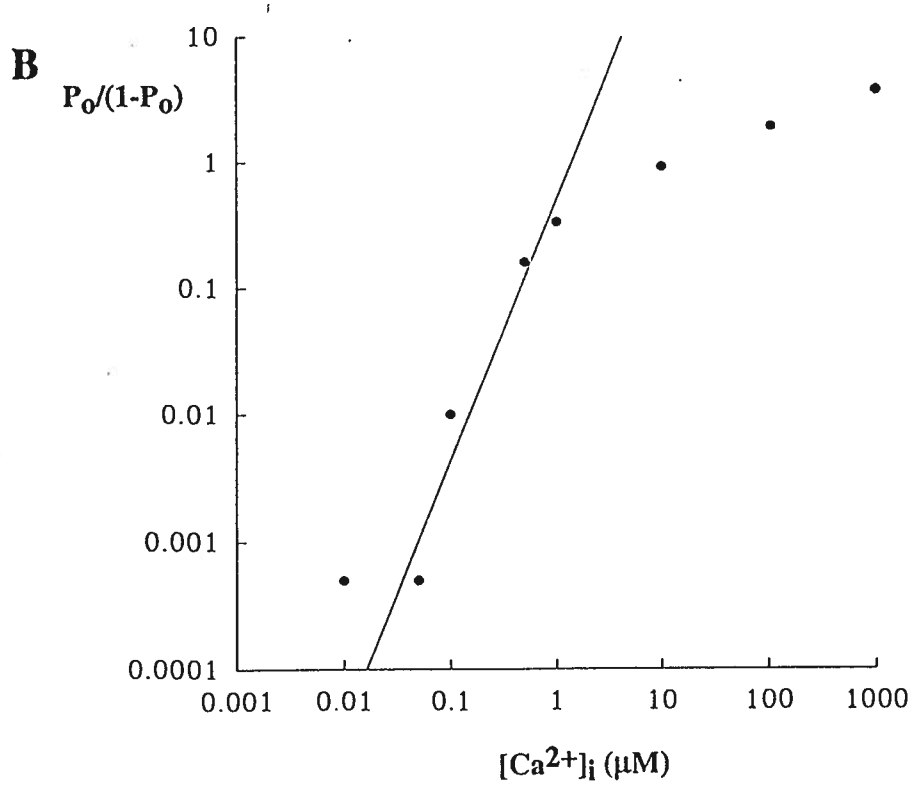

B
$P_o/(1-P_o)$
10
1
0.1
0.01
0.001
0.0001
0.001
0.01
0.1
1
10
100
1000
$[Ca^{2+}]_i$ (μM)

Figure 47. Dependence of the open probability, P_O of K(Ca)I channels on membrane potential V (mV) at different values of $[Ca^{2+}]_i$. The data were obtained from 4 inside-out patches exposed to the following $[Ca^{2+}]_i$: 1 μM (open squares); 10 μM (filled squares); 100 μM (filled triangles); 1 mM (filled diamonds). The fit curves were drawn to the Boltzmann equation $P_O = [1+\exp(-K(V-V_O))]^{-1}$ where V_O is the potential at which $P_O = 0.5$. V_O at 1 μM, 10 μM, 100 μM and 1 mM $[Ca^{2+}]_i$ were +40 mV, +18 mV, 0 mV and -65 mV, respectively. The constant K took the following values: 0.16 mV^{-1} at $[Ca^{2+}]_i$ = 1 μM; 0.096 mV^{-1} at $[Ca^{2+}]_i$ = 10 μM; 0.035 mV^{-1} at $[Ca^{2+}]_i$ = 100 μM and 0.011 mV^{-1} at $[Ca^{2+}]_i$ = 1 mM. $[Ca^{2+}]_O$ = 0.01 μM.

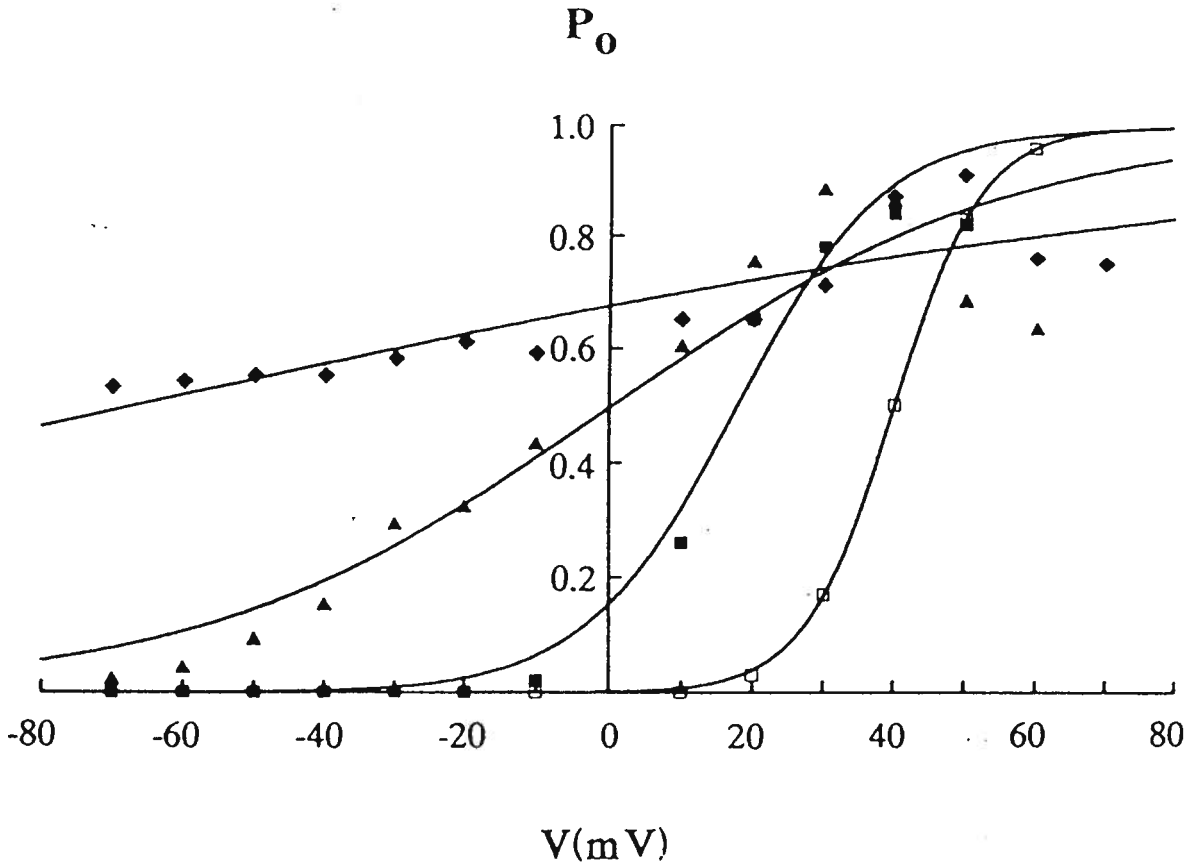
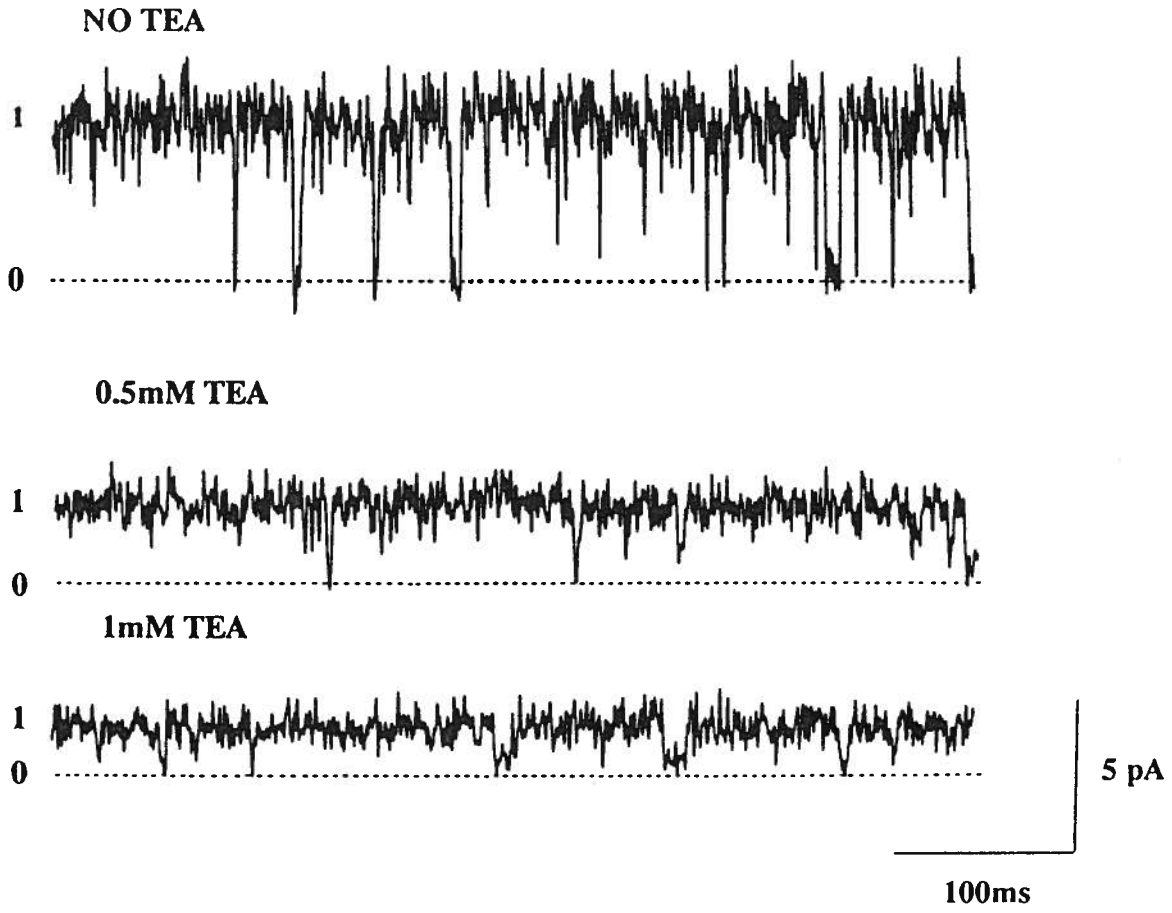

P_o
1.0
0.8
0.6
0.4
0.2
-80
-60
-40
-20
0
20
40
60
80
V(mV)

Figure 48. Blocking effect of TEA on current flow in the $K_{(Ca)I}$ channel. Single channel current recordings from an inside-out patch with one active channel. TEA was applied to the cytoplasmic face of the membrane. 0 indicates channel closed and 1 indicates channel open. Bandwidth DC-2 kHz, V = +40 mV. $[Ca^{2+}]_i$ = 100 μM, $[Ca^{2+}]_o$ = 0.01 μM. The patch was bathed in symmetrical 140 mM K^+ solutions.


NO TEA
1
0
0.5mM TEA
1
0
1mM TEA
1
0
5 pA
100ms

The percentage reduction, R_i in single channel current caused by various concentrations of TEA applied to the cytoplasmic membrane face is shown in Fig. 49. The value of R_i was well predicted by the relation $R_i = 100\% / (1 + K_d / [TEA])$, where the constant K_d had the value 0.31 mM under these conditions. These data were replotted as a Hill plot, log (R_i/1-R_i) versus log [TEA] where R is the reduction in mean single channel current caused by a given concentration of TEA. The slope of this plot was 0.70, suggesting that TEA blocks this channel in a one-to-one fashion, as seen for the K(Ca)L channel. TEA had no significant effect on the probability of K(Ca)I channels adopting an open state (Fig. 50). An ANOVA test was performed to compare these results with comparable data obtained for the K(Ca)L channel. It was found that no significant differences existed between these two data sets ($P > 0.05$). This result indicates that the K(Ca)L and the K(Ca)I channels have comparable sensitivities to internally applied TEA.

Figure 49. Dose-response curve for block of the $K_{(Ca)I}$ channel by TEA. The percentage reduction of single channel current is plotted against the concentration of TEA applied to the cytoplasmic membrane face. Data points are the mean $\pm$ SEM from 3 - 5 patches voltage-clamped at V = +40 mV and exposed to symmetrical 140 mM K+ solutions. The solid line plots the relation $R_i = 100\% / (1 + K_d/[TEA])$. The apparent dissociation constant K_d was 0.31 mM. $[Ca^{2+}]_i = 100\,\mu M$, $[Ca^{2+}]_o = 0.01\,\mu M$.

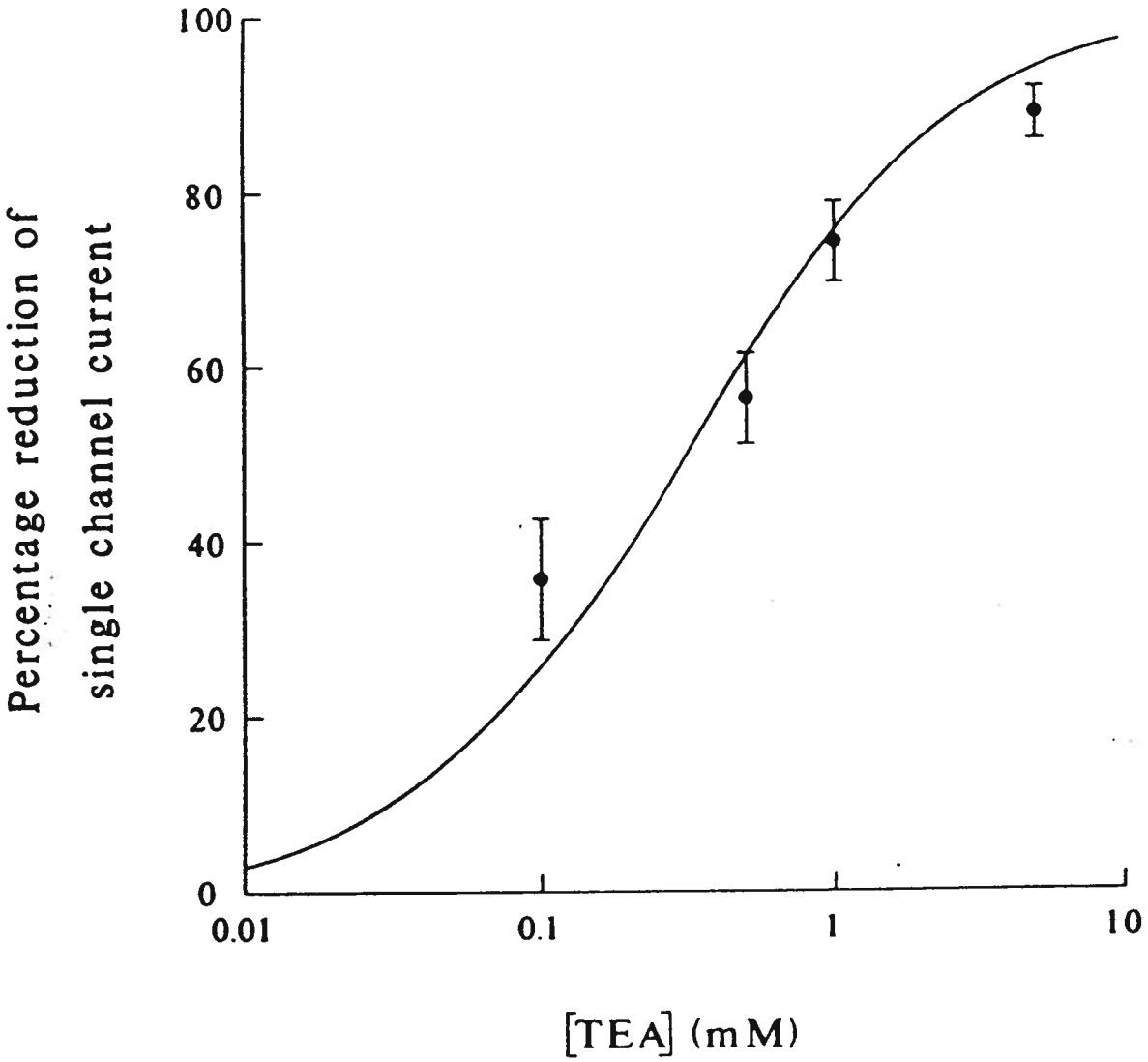
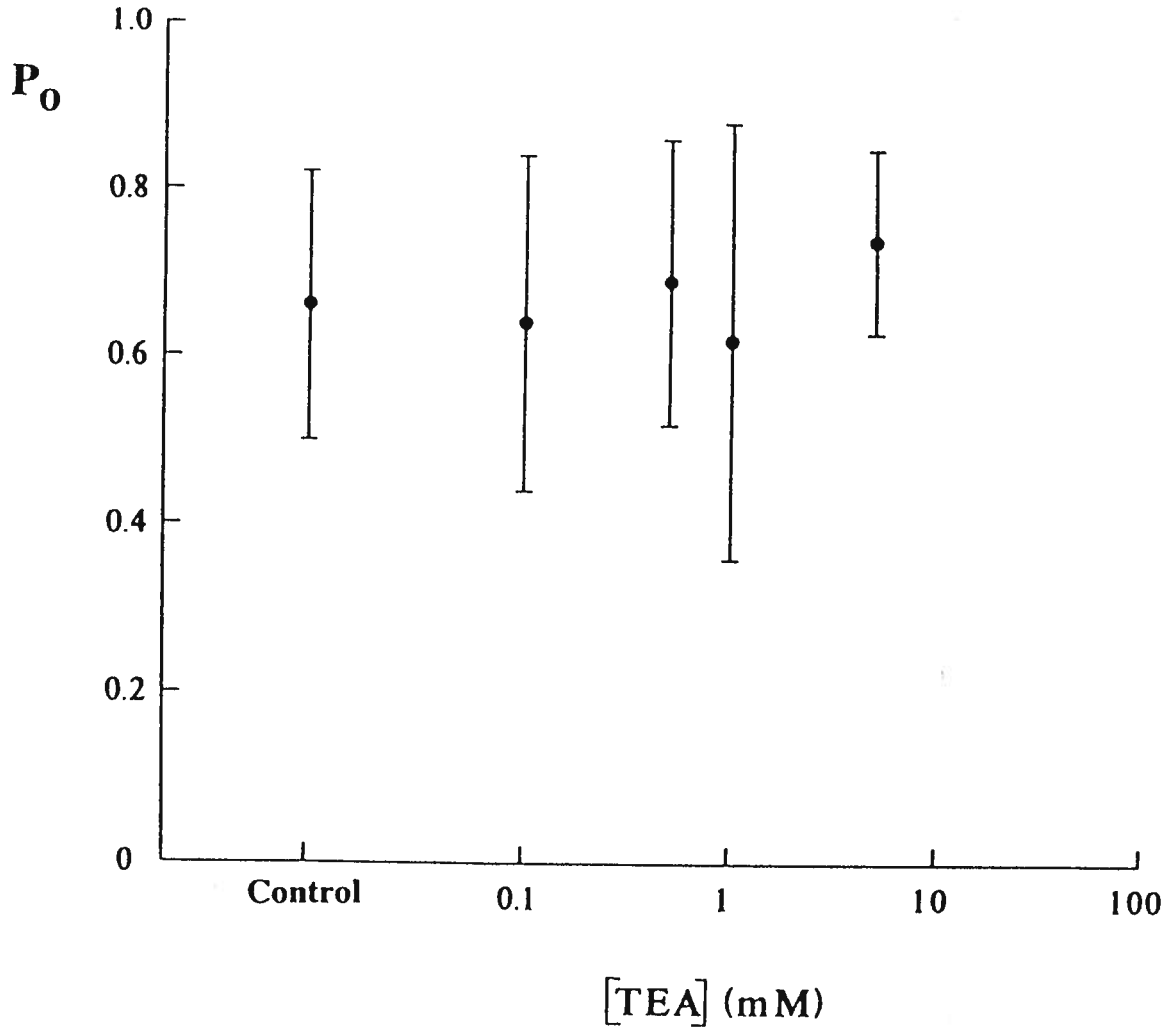

Percentage reduction of single channel current
100
80
60
40
20
0
0.01
0.1
1
10
[TEA] (mM)

Figure 50. Effect of TEA on the open probability of the $K_{(Ca)I}$ channel. Opening probability of the channel, P_O is plotted against the concentration of internal TEA applied to the cytoplasmic membrane face. $[Ca^{2+}]_i = 100\,\mu M$, $[Ca^{2+}]_O = 0.01\,\mu M$. Patches were bathed in symmetrical 140 mM K+ solutions. Data points are mean $\pm$ SEM from 3-5 patches voltage-clamped at +40 mV. No significant differences were found between these means ($P > 0.05$, ANOVA).


P_o
1.0
0.8
0.6
0.4
0.2
0
Control
0.1
1
10
100
[TEA] (mM)

Chapter 4

Discussion

The present study has resulted in the development of a novel *in vitro* preparation of CVSMCs derived from the cerebral arteries of adult rats. These cells were identified using the Masson trichrome test and an antibody specific to smooth muscle α-actin. The calcium-sensitive fluorescent probe fura-2 was employed to measure the resting level of free intracellular calcium in CVSMCs and to study the effect of 5-HT on calcium mobilization in these cells. Patch clamp methods were used to measure the basic electrophysiological properties of CVSMCs *in vitro*. The biophysical properties of two types of K_{Ca} channel were studied in inside-out membrane patches excised from these cells. The significance of these results will now be discussed in relation to previous findings from other laboratories.

4.1. Morphological characteristics of CVSMCs *in vitro*

Several reports have appeared describing the *in vitro* growth of CVSMCs from cerebral resistance vessels (Spatz et al. 1983; Diglio et al. 1986). However, the present study is apparently the first to describe short-term cultures of CVSMCs derived from conducting cerebrovascular arteries of the rat. When examined after 1-4 days *in vitro*, the cells obtained typically displayed a spherical shape. The shape of these cells was the same regardless of whether plates were incubated at 37°C in culture medium or kept at 4°C in a maintenance solution. Similar morphology has also been observed in short-term cultures of CVSMCs derived from penetrating vessels supplying the rat brain, where more than four

days *in vitro* were required for the cells to adopt a fusiform or polygonal shape (Spatz et al. 1983; Diglio et al. 1986).

Cerebral arteries may be classified as the "muscular" type of blood vessel, in which the tunica media contains only a few elastic laminae and relatively little intercellular space, when compared to "elastic" arteries, such as the aorta. CVSMCs are spindle shaped when relaxed, but lack the highly fusiform morphology typical of gastrointestinal smooth muscle, the ratio of maximum to minimum axes being about 9:1 (Rhodin, 1980). Steele et al. (1991) have described a procedure by which CVSMCs approximating this shape can be produced following enzymatic dissociation of rat basilar arteries. The reason why this result was not obtained in the present study remains unclear. It is possible that the dissociation procedures used resulted in cytoskeletal damage, as reported for other CVSMCs following enzymatic treatment and plating onto foreign substrates (Spatz et al. 1983; Diglio et al. 1986). It is also known that CVSMCs dissociated from rat basilar arteries are unable to relax following a brief period of active contraction, presumably because of the absence of elastic elements in these preparations (Steele et al. 1991). The present study indicates that the intracellular free calcium level of CVSMCs was about 40 nM, far below the micromolar levels required to activate muscle contraction (Johns et al. 1987). Therefore, it is probable that the spherical shape of the CVSMCs used in this study was the result of failure of the cells to relax passively, rather than of an actively maintained contraction.

The majority of the spherical cells present in our short term primary cultures were found to react positively to both Masson's trichrome stain and to a monoclonal antibody directed against smooth muscle α-actin. In differentiated

SMCs, smooth muscle α-actin and γ-actin predominate over other, non-SMC specific forms of the protein. In vascular SMCs studied to date, most of the actin present is in the α-isoactin form (Gabbiani et al. 1981; Owens et al. 1986). In addition, the α-isoactin of SMCs is immunologically distinct from that found in skeletal or cardiac muscle cells (Owens et al. 1986). SMC α-actin is therefore considered to be a reliable marker for smooth muscle tissue studied *in vitro* (Moore et al. 1984; Diglio et al. 1986), although certain tumors may also express the antigen (Skalle et al. 1986). These considerations strongly suggest that the cells used in the present study were indeed SMCs of vascular origin.

4.2. Intracellular free calcium in cultured CVSMCs

The resting level of free intracellular calcium in the CVSMCs cultured at 37oC was found to be about 40 nM. This value is comparable with the value of 50 nM cytosolic free [Ca^{2+}] obtained from resting airway smooth muscle cells obtained using fluorescence measurements (Rodger and Small, 1991). However, it is significantly lower than that found in VSMCs dispersed from a peripheral vessel, the rat aorta, in which $[Ca^{2+}]_i$ was found to be 126 nM, (Takata et al. 1988); 153 nM,(Capponi et al. 1987); and 114 nM, (Nabika et al. 1985). In the present study, baseline levels of $[Ca^{2+}]_i$ were determined in the same cultures used to calibrate the measurement of free intracellular calcium. In addition, *in situ* calibration using cells is generally considered to be more reliable than methods employing fluorescence ratios determined in cell-free systems (Ishii et al. 1989). The mean resting level of $[Ca^{2+}]_i$ which was obtained should therefore be reliable.

$[Ca^{2+}]_i$ is known to decline when extracellular calcium is lowered below about 2 mM, at least in the case of SMCs derived from the gastrointestinal tract (Williams et al. 1985; Pritchard and Ashley 1986). In both the present study, and in one of the studies quoted above, 1 mM Ca^{2+} was employed in the external bathing solution (Nabika et al. 1985). It seems unlikely therefore that the smaller value of $[Ca^{2+}]_i$ obtained from cultured cerebrovascular SMCs was due entirely to the use of a relatively low concentration of Ca^{2+} in the external bathing solution.

Binding of the free acid by cellular proteins can lead to a change in affinity of fura-2 for calcium, and to an underestimate of $[Ca^{2+}]_i$ (Konishi et al. 1988). However, comparison of fura-2 data with results obtained using calcium sensitive microelectrodes suggests that the underestimate introduced by fura-2 is no more than two-fold (Somlyo and Himpens 1989). In addition, this error should apply to all studies in which a cuvette calibration of fura-2 calcium affinity was employed.

4.2.1. Modulation of $[Ca^{2+}]_i$ by serotonin in CVSMCs

The results presented in here show that 5-HT increases free intracellular Ca^{2+} levels in CVSMCs derived from rat cerebral arteries. This effect showed an apparent EC_{50} of 10 nM, a value close to that seen in similar studies conducted on an aorta SMC line, EC_{50} = 26 nM, (Doyle et al. 1986). In addition, the effect of 5-HT on calcium mobilization was shown to be maximal at a serotonin dose of 1 uM in all three of these preparations (Doyle et al. 1986; Capponi et al. 1987).

In studies conducted on cultured rat aorta VSMCs, it was found that 5-HT increased $[Ca^{2+}]_i$ to a maximal level of 187 nM, similar to that seen in the present study (Takata et al. 1988). However, Capponi et al. (1988), also using rat aorta VSMCs in culture, found that 5-HT application could elevate $[Ca^{2+}]_i$ to 400 nM in these cells. The reason for this discrepancy is not clear, although these studies used cells after differing numbers of passage in culture (Capponi et al. 1988; Takata et al. 1988).

Simultaneous measurements of $[Ca^{2+}]_i$ and force development in individual vascular smooth muscle cells suggest that the threshold level of $[Ca^{2+}]_i$ for tension production is around 125 nM and that maximal force can be produced at a $[Ca^{2+}]_i$ of 500-600 nM (Yagi et al. 1988). These results suggest that the maximal levels of $[Ca^{2+}]_i$ reached in the present study would be sufficient for tension development in isolated CVSMCs. It should also be noted that the sensitivity of the contractile system to $[Ca^{2+}]_i$ has been found to vary with the manner in which vascular smooth muscle cells are stimulated. Thus, aorta VSMCs stimulated with norepinephrine exhibited half-maximal tension at a $[Ca^{2+}]_i$ of only 100 nM, while cells stimulated by the calcium ionophore ionomycin required 600 nM to reach a comparable tension (Bruschi et al. 1988). It is known that myosin light chain kinase can be phosphorylated by protein kinase C, an enzyme activated by the diacylglycerol liberated on activation of 5-HT_2 receptors. It has been suggested that this phosphorylation process could reduce the calcium required for maintained contraction (Bruschi et al. 1988). These considerations make it difficult to accurately assess the degree of contracture which would be produced by a given rise in $[Ca^{2+}]_i$, following activation of the present cells by 5-HT.

The 5-HT induced increase in $[Ca^{2+}]_i$ was not prevented by the presence of organic or inorganic Ca^{2+} channel blockers. In contrast, it is known that action potential generation in rat basilar artery CVSMCs is powerfully blocked by both Co^{2+} and nifedipine (Suprenant et al. 1987). Taken together, these results suggest that the rise in $[Ca^{2+}]_i$ evoked by 5-HT did not depend on the entry of Ca^{2+} through voltage-sensitive calcium channels, during action potential firing. A similar conclusion was reached in the case of serotonin induced calcium release occurring in VSMCs dissociated from the rat aorta (Capponi et al. 1987).

At doses of 1-50 μM, 5-HT triggers a small depolarization and a brief burst of Ca^{2+} spikes in cerebrovascular SMCs (Suprenant et al. 1987). Calcium entry through voltage-gated Ca channels activated in this way might have been expected to give rise to an enhanced fura-2 signal at 5-HT doses of 1 μM or greater. This signal should be sensitive to calcium channel blockers. No evidence for this effect was seen in the present results. This suggests that Ca^{2+} entry through membrane channels was relatively minor, in comparison to Ca^{2+} released from internal stores. It remains possible, however, that calcium channel blockade would be more effective in reducing the response to 5-HT concentrations above the 1 μM dose used in the present experiments.

In the present study, the effect of 5-HT on $[Ca^{2+}]_i$ was reduced by about 50 % in the presence of 5 nM ketanserin. This concentration is similar to the affinity of ketanserin measured at agonist labelled 5-HT_2 receptor sites in rat brain cortex, which show a K_d = 1.4 nM, (Lyon et al. 1987). 1 nM ketanserin also suppresses what appear to be 5-HT_2 receptor mediated calcium fluxes in the A7r5 smooth muscle cell line by about 50 % (Doyle et al. 1986). Ketanserin

is known to block responses mediated by the 5-HT$_{1C}$ receptor, but the affinity of this site is apparently two orders of magnitude too low to readily account for the present results (Conn and Sanders-Bush 1987).

In the present study, the observed insensitivity of 5-HT action to calcium channel blockers, and the high sensitivity to blockade by ketanserin are compatible with a mechanism in which 5-HT$_2$ receptor activation results in the mobilization of calcium from internal stores. Serotonin induced contractions in intact rat cerebral arteries are suppressed by nanomolar levels of ketanserin (Chang and Owman 1987). However, these contractions are also strongly inhibited by low concentrations of calcium channel blockers (Chang and Owman 1987). It seems likely, therefore, that full activation of the contractile mechanism in cerebrovascular SMCs requires Ca^{2+}-dependent processes over and above the release of calcium from intracellular stores.

The effects of 5-HT on membrane potential and on calcium release from internal stores have been studied in detail in rat mesangial cells, a contractile, smooth muscle-like cell from the kidney glomerulus (Mene et al. 1991). It was found that 5-HT activated a 5-HT$_2$ receptor, which triggered immediated discharge of calcium from internal stores. The elevation of $[Ca^{2+}]_i$ activated a calcium-dependent chloride conductance, producing a small, maintained depolarization of the cell which was dependent on the continued occupation of 5-HT$_2$ receptors by the agonist. This depolarization in turn activated voltage-sensitive Ca channels, leading to tonic entry of calcium into the cell (Mene et al. 1991). It has been suggested that a tonic influx of Ca^{2+} through voltage-sensitive channels may also occur in 5-HT stimulated VSMCs, and that this

influx may be necessary to maintain the contractile state, possibly by facilitating the recycling of internal calcium (Sutter 1990).

In some cells investigated in this study, a marked loss of 5-HT responsiveness was seen on repeated application of the agonist. This phenomenon has also been reported in studies of 5-HT induced calcium mobilization in SMCs from the peripheral vasculature (Capponi et al. 1987; Takata et al. 1988). The progressive decline in response to 5-HT was most marked in the cell being exposed to the photoexcitant wavelengths. It seems likely, therefore, that this phenomenon involved a degree of photodynamic damage, as has been noted in previous studies using fura-2 (Tsien 1988). In the cell being illuminated, photodynamic damage may also have enhanced the deleterious effect of elevated $[Ca^{2+}]_i$, which is known to activate Ca^{2+}-dependent proteases in many tissues (Bond, 1988). The decreased response to 5-HT seen on repeated agonist application occurred in the absence of elevating baseline levels of free calcium, suggesting that membrane integrity was not greatly compromised during this phenomenon. It seems unlikely, therefore, that gross damage to the cell membrane was responsible for the declining responsiveness to 5-HT.

In summary, the present results show that CVSMCs cultured from rat cerebral arteries provide an useful preparation in which to study Ca^{2+} mobilization by serotonin and other agents active in the cerebrovascular circulation of mammals.

4.3. Electrophysiological properties of isolated CVSMCs

In the present study, the mean resting potential of isolated CVSMCs from rat cerebral arteries was found to be about -40 mV. These cells showed an input resistance of about 3 GΩ at the resting potential and a total membrane capacitance of 24 pF. Similar values of input resistance have been reported in a previous patch clamp study of isolated CVSMCs derived from rat basilar artery (Steele et al. 1991). Assuming a specific membrane capacitance of $1\,\mu F/cm^2$, as has been suggested for VSMCs (Toro et al. 1986), a 24 pF cell should possess a surface area of $2400\,\mu m^2$. This is greatly in excess of the surface area calculated for $12\,\mu m$ diameter cells of purely spherical shape ($452\,\mu m^2$). This result indicates that the membrane surface of the present cells is highly involuted, presumably as a consequence of cell contraction and of the presence of numerous caveolae in vascular smooth muscle cells (Mulvany 1986).

Hirst et al. (1986) studied the electrical properties of arteriolar segments of rat middle cerebral artery using intracellular recording techniques. They obtained a mean resting membrane potential of -63 mV. Similar experiments conducted on intact cat basilar arteries (Harder et al. 1981) and on guinea-pig basilar artery (Fujiwara et al. 1982) yielded mean resting potentials of -55 mV and -50 mV respectively. The slightly lower values of resting potential observed in the present study could be the result of several factors. Following formation of the whole-cell recording configuration, dialysis of the cellular contents occurs, leading to the formation of a new equilibrium between the bathing solution and the solution filling the patch pipette (Sakmann and Neher 1983). The latter solution must therefore accurately reflect the ionic composition of the sarcoplasm in cerebrovascular smooth muscle cells, if reliable values of resting potential are to be obtained.

The membrane of vascular smooth muscle cells shows significant resting permeability to Na^+ and Cl^-, as well as to K^+ (Sutter 1990; Fujiwara et al. 1982; Johansson and Somlyo 1980). Electron probe analysis studies conducted on mesenteric portal veins have indicated the following values for intracellular ionic concentrations, in mmole/kg dry weight: Na^+ 167; K^+ 611; Cl^- 278; Mg^{2+} 36 and Ca^{2+} 1.9 (Somlyo et al. 1979). These values indicate that, in intact CVSMCs, $[K^+]_i/[Na^+]_i$ may be appreciably smaller than the value which pertained during whole-cell recordings reported in this study (47:1). Wahlstrom (1971) estimated that the Nernst potential for Na^+ in vascular smooth muscle is probably no more positive than 21 mV, while E_K was -86 mV. The use of low internal Na^+ in whole-cell recordings could therefore have resulted in an underestimate of the true membrane potential in isolated CVSMCs. In addition, a ouabain sensitive Na^+/K^+ pump is known to regulate ionic concentrations in VSMCs and to electrogenically contribute several millivolts to the resting potential of these cells *in vivo* (Sutter 1990; Fujiwara et al. 1982). This pump was presumably inhibited during maintenance of the cells at 4°C and depressed during whole-cell recordings, since experiments were conducted at 21°C and the pipette solution did not contain ATP.

The input resistance of the isolated CVSMCs used in the present study was found to average 3 GΩ, a value comparable to results obtained in patch clamp studies on SMCs isolated from rat caudal vein, 1.4 GΩ (Toro et al. 1986), rabbit jejunum, 2 GΩ (Bolton et al. 1985), and rat basilar artery, 4 GΩ (Steele et al. 1991). The measured value includes the leakage resistance between the pipette and the membrane, which was typically about 10 GΩ. Hence the

measured input resistances slightly underestimated the true value of this parameter.

These resistances in the gigaohm range greatly exceed values determined in isolated VSMCs using intracellular recording methods, which are typically in the range 0.45 - 0.59 GΩ (Toro et al. 1986). This difference probably reflects the presence of appreciable leakage currents around the microelectrode insertion (Sakmann and Neher 1983). Intracellular recordings from VSMCs in intact blood vessels yield yet lower estimates of membrane resistance, in the order of 10 - 100 MΩ. This probably reflects the syncytial nature of smooth muscle preparations in intact vessels (Hirst et al. 1986; Hermsmeyer 1976; Somlyo 1980; Suprenant et al. 1987).

CVSMCs isolated from rat cerebral arteries showed a marked fall in input resistance on membrane depolarization, and only small regenerative responses could be evoked by direct electrical stimulation. These properties were also seen in CVSMCs studied with intracellular microelectrodes in intact cerebral arteries of the rat (Hirst et al. 1986) and guinea-pig (Fugiwara and Kuriyama 1983). In contrast, CVSMCs in intact rabbit basilar artery do generate large action potentials on depolarization in normal bathing media (Suprenant et al. 1987). In CVSMCs studied in intact rat basilar arteries, large action potentials were evoked on direct electrical stimulation, following suppression of outward potassium current by TEA. Inward current during these action potential was carried by calcium ions (Hirst et al. 1986). The enhanced level of intracellular free calcium seen in the present cells on depolarization by high potassium solutions suggests that voltage-sensitive calcium channels were also present in this preparation. It seems likely, however, that the inward current

flowing in these channels was insufficient to overcome the damping effect of rectifier currents, during direct electrical stimulation.

During application of serotonin to the present cells, biphasic currents were observed in cell-attached membrane patches. These currents strongly resembled the capacitance currents recorded in cell-attached patches during action potential activity in other types of high impedance cell (Fenwick et al. 1982). Serotonin (1 - 50 μM) is known to cause a small depolarization in cerebral artery VSMCs, and to initiate a burst of action potentials in these cells (Suprenant et al. 1987). Calcium entry during these action potentials might be expected to activate K_{Ca} channels in the present cells. Large amplitude single channel currents were indeed observed in cell-attached patches during exposure of CVSMCs to serotonin. Further work is needed, however, to identify these currents definitively.

4.4. Ca^{2+}-activated K^+ channels in inside-out membrane patches

In this study, evidence was obtained for two types of voltage and Ca-dependent K-channels in CVSMCs dispersed from rat cerebral arteries. On the basis of a large difference in mean single channel conductance, these were designated as K(Ca)L and K(Ca)I channels.

4.4.1. Properties of the K(Ca)L channel

The K(Ca)L channels described in this paper showed a conductance of 207 $\pm$ 10 pA in symmetrical high potassium solutions, similar to that of BK

channels studied in VSMCs from mesenteric artery, and a variety of other smooth muscle preparations (See Table II). Measured conductance values of K(Ca)L channels varied from 170 pS to 267 pS when measured under constant ionic conditions. Similar variation has been seen in the conductance of BK channels in chromaffin cells, 190 - 330 pS (Marty 1981) and in rat skeletal muscle, 150 - 240 pS (Barrett et al. 1982). The meaning of these variations in terms of channel structure remains, however, unclear. A more definitive assignment of the present channel to the BK category awaits determination of channel sensitivity to charybdotoxin, a selective blocker of BK channels in many tissues (Lang and Ritchie 1990; MacKinnon and Miller 1988).

4.4.1.1. Ionic selectivity of the K(Ca)L channel

K(Ca)L channels were highly selective for K+ over Na+ (P_{Na}/P_K < 0.05), as has been observed for BK channels in other smooth muscle cells (Benham et al. 1986; Inoue et al. 1985; Green et al. 1991; Mayer et al. 1990). Na+ did not significantly interfere with the passage of K+ through open K(Ca)L channels, even when present at a concentration of 80 mM. Similar results have been obtained for the BK channel found in guinea-pig mesenteric artery (Benham et al. 1986). However, current flow in the BK channels of bovine chromaffin cells is subject to a fast flicker block by 5 mM internal Na+, providing K+ is absent from the external medium (Yellen 1984a). Under the ionic conditions used in the present study, a flicker block of the K(Ca)L channel by internal Na+ may have been relieved by the presence of 140 mM K+ in the external solution, and could therefore have gone undetected (Yellen 1984b).

Table II Characteristics of BK channels in various preparations

Preparation	Ionic conditions[a]	Conductance (pS)	Selectivity	Voltage dependence (a)	Calcium dependence (b)	TEA block(c) $Kd(0)_i$	TEA block(c) $Kd(0)_o$	Reference
Bovine chromaffin cell	160 K^+	265	K>Rb>Na,Cs	12-15 mV	10nM	27mM	0.2mM	Marty 1981, 1983, Yellen 1984a
Rat myotubes	140 K^+	240	Tl>K>Rb>NH4>> Na,Li,Cs	11-16 mV	40mV/10-fold V_o(1 μM)=40mV	60mM	0.3mM	Barret et al. 1982, Blatz and Magleby 1984,1986
Rabbit T-tubules[b]	100 K^+	260	Ti>K>Rb>NH4>> Na>Li>Cs	11-13 mV	40mV/10-fold V_o(1 μM)=40mV	45mM	0.29mM	Latorre et al. 1982, Moczydlowski et al.1983,1985, Vergara 1983 et al.1984
Rat anterior pituitary cells (AtT20/D16-16)	140 K^+	200	-	9 mV	60mV/10-fold V_o(1 μM)=-30mV	0.08mM	52.2mM	Wong and Adler 1986, Wong et al. 1982
Rat anterior pituitary (GH_3)	150 K^+	250-300	K>>Na	8 mV	V_o(1 μM)=50mV	$Kd(60)_o$=2mM		Lang and Ritchie 1988
Mouse parotid acini	145 K^+	250	K>>Na	12 mV	1 nM	-		Maruyama et al 1983
Rat panceatic B-cell	140 K^+	244	K>>Na	15 mV	110mV/10-fold V_o(1 μM)=0mV	-		Cook et al.1984, Findlay et al.1985
Rabbit collecting duct cells	140 K^+	180	K>>Na	17 mV	100 nM	-		Gitter et al. 1987
Rat pancreatic duct cells	150 K^+	200	-	yes	60-70mV/100-fold V_o(3 μM)=-4mV	-		Gray et al. 1990
Rabbit intestinal SMC[b]	100 K^+	230	K>Rb>Na,Li,Cs	15 mV	30mV/10-fold V_o(2 μM)=60mV	-		Cecchi et al. 1986
Toad stomach SMC	130 K^+	250	K>>Na	9 mV	80mV/10-fold V_o(1 μM)=10mV	-		Singer and Walsh 1987
Frog and toad stomach SMC	120 K^+	200	K>>Na	yes	10 nM	20 mM for total block externally		Berger et al. 1984
Rabbit intestinal SMC	126 K^+	200	K>>Na	yes	10 nM	12mM	-	Benham et al. 1985
Rabbit mesenteric artery SMC	126 K^+	200	Tl>K>Rb>> Na,Cs	30 mV	30-60mV/10-fold	-		Benham et al. 1986

Rabbit colonic SMC	126 K^+	210	K>>Na	yes	60mV/10-fold V_o(5 μM)=0mV	30mM for total block externally	Mayer et al. 1990
Canine gastric SMC	140 K^+	265	K>>Na	yes	100mV/10-fold	NE 0.18 mM	Carl et al. 1990
Rabbit portal vein SMC	142 K^+	273	K>>Na	-	V_o(1.4 μM)=0 mV	NE 0.1 mM	Inoue et al. 1985
Rat cerebral artery SMC	140 K^+	207	K>>Na,Cs	14 mV	45 mV/10-fold V_o(23 μM)=+40mV	$Kd(+40)_i$=0.83mM	Present study

a = concentration is in mM. Symmetrical solutions were used.

b = parameters determined in planar bilayers. For all other channels, patch-clamp technique was used.

(a) = expressed as e-fold change in P_o (fraction of the time in the open state) per x mV.

(b) = expressed as x mV change in V_o (voltage at which P_o=0.5) per 10-fold increase in $[Ca^{2+}]_i$; V_o (x mM)$_i$ indicates the voltage at which P_o=0.5 at given $[Ca^{2+}]_i$. Otherwise the lowest $[Ca^{2+}]_i$ is given at which channel activity can be seen.

(c) = $Kd(0)_i$ indicates concentration of internally applied TEA causing 50% block of single channel current at 0 mV. $Kd(0)_o$ is corresponding value during external application of TEA. If data were obtained at other membrane voltages, this is indicated in brackets.

SMC = Smooth muscle cell.

NE = No effect of internally applied TEA.

The K(Ca)L channel also showed negligible permeability to Cs+ ($P_{Cs}/P_K < 0.05$). Under conditions which favoured the entry of Cs+ into the channel, the amplitude of potassium currents was significantly depressed. Qualitatively similar observations have been made in the case of BK channels in smooth muscle cells derived from guinea-pig mesenteric artery. In this preparation, however, BK channels exhibit a much higher sensitivity to the blocking effect of Cs+ than was evident in the present study, suggesting that these two channel populations, despite their similar conductance, are probably not identical (Benham et al. 1986).

The action of Cs+ on the present channel can be explained by the theory of ionic blockade (Woodhull 1973; Hille 1991). It is assumed Cs+ binds to a site in the open channel and blocks it. At equilibrium, the channel will fluctuate between closed, open and blocked states. The frequency of blocking events increases at potentials which promote the entry of Cs+ into the channel. If the average lifetime of the blocked state were less than the time resolution of the recording system, then the open channel current would represent a time-averaged current, reflecting the proportion of time the channel spent in the blocked state during an opening (Benham et al. 1986).

The flicker block of K(Ca)L channels by Cs+ showed voltage-dependency. The effective valence for internal Cs+ block was only 0.07, while externally applied Cs+ blocked with an effective valence δ of -0.66. This ten-fold difference indicates that the two sites of Cs+ binding are not located at equal depths in the membrane field. A similar result was obtained by Yellen

(1984a) in a study of Cs+ block of BK channels in chromaffin cells. However. the values of δ were different (0.25 for internal block, -1.0 for external block). Benham et al. (1986) reported a value of δ = -1.4 for external block of BK channels in mesenteric artery VSMCs. These results suggest that the large conductance K_{Ca} channels found in these three preparations exhibit some fine differences with respect to their ionophore structures.

4.4.1.2. Ca^{2+} dependence of K(Ca)L channel opening

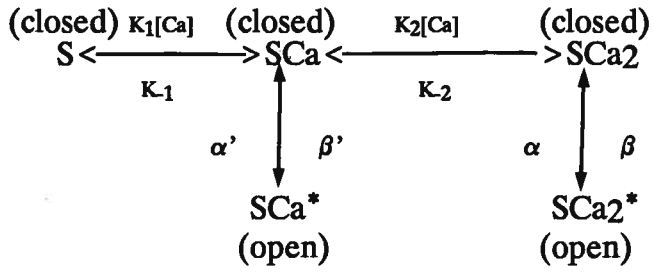
The open probability of K(Ca)L channels was sensitive to the concentration of intracellular free calcium ions. At a membrane potential of V = +40 mV, the K(Ca)L channel was open for half of the time at $[Ca^{2+}]_i$ = 23 μM. As can be seen in Table II, the calcium sensitivity of BK channels varies appreciably among different cell types. BK channels in certain secretory cells, including mouse parotid acinar cells are highly calcium-sensitive, being open 50% of the time at $[Ca^{2+}]_i$ values between 10^{-8} - 10^{-7} M (V = 0 mV) (Maruyama et al 1983). For BK channels found in skeletal muscle fibres, the corresponding value of $[Ca^{2+}]_i$ is around 5 μM (Barrett et al. 1982; Methfessel and Boheim 1982), while for nonvascular and vascular smooth muscle cells, estimates in the range 0.5 - 1 μM have been reported (Carl et al. 1990; Benham et al. 1986). The low apparent calcium sensitivity of the K(Ca)L channel has implications for the functional significance of this channel, as will be discussed later in this thesis.

In the present study, a second power relationship was found between the probability of the K(Ca)L channel opening and the value of $[Ca^{2+}]_i$, when measured over the range of intracellular free calcium likely to pertain under

physiological conditions. This result is in agreement with previous studies conducted on BK channels in other tissues, which suggest that 2-4 calcium ions are required to fully activate each channel (Barrett et al. 1982; Methfessel and Boheim 1982; Moczydlowski and Latorre 1983).

Open time distributions for K(Ca)L channels were well fitted by the sum of two exponential terms, when investigated for $[Ca^{2+}]_i$ values over the range 10^{-8} - 10^{-3} M. Increasing $[Ca^{2+}]_i$ had no significant effect on the value of the fast time constant fitted to these distributions, while the slow time constant was elevated by this procedure. Similar results were reported in the case of BK channels in rat skeletal muscle (Barrett et al. 1982; Magleby and Pallotta 1983a). However, in BK channels studied in smooth muscle cells isolated from mesenteric artery, open time distributions were well fit by a single exponential term at low $[Ca^{2+}]_i$ ($< 10^{-6}$ M). On raising $[Ca^{2+}]_i$, a second, fast fit component appeared in these distributions, and the time constant of the original component increased (Benham et al. 1986).

The present results are compatible with the following minimum state diagram for the gating of the K(Ca)L channel



Here, most of the brief channel openings are to the monoliganded state SCa^*, while the majority of long openings are to state $SCa2^*$, with two calcium ions bound per channel.

This model predicts that the ratio of openings in the fast fit components to the number in the slow component should fall at higher $[Ca^{2+}]_i$, as was seen experimentally. However, this scheme does not account for the observed increase in the time constant of the slow fit component on raising $[Ca^{2+}]_i$. There are a number of possible mechanisms which could produce this effect. Firstly, state $SCa2^*$ could bind a further calcium ion and make a transition to a further open state, $SCa3^*$, increasing the observed open time of the long open distribution (Magleby and Pallotta 1983b). However, this would likely result in open time distributions requiring three exponential fit components, which was not observed in the present study. Neither was the slope of the open probability versus $[Ca^{2+}]_i$ plot steep enough to indicate three calcium ions were needed to fully activate each channel. Alternatively, one may postulate the occurrence of direct transitions between the open states SCa^* and $SCa2^*$, accompanied by the loss or gain of calcium ions as required. However, this model predicts that the time constant of the fast fit component should decrease as $[Ca^{2+}]_i$ is raised. A trend of this type was seen in the present data, but this did not reach statistical significance.

The increase in the slow time constant with $[Ca^{2+}]_i$ could also result from the occurrence of very short lived closed states interposed between states $SCa2$ and $SCa2^*$. At least one of the transitions between these interposed closed states would have to be dependent on $[Ca^{2+}]_i$, and the lifetime of these states would need to be too brief to allow detection with the present recording system. In this scheme, calcium driven reopenings to $SCa2^*$ from these short-lived closed states would lead to an apparent increase in the magnitude of the slow time constant. Finally, a slow time constant dependent on $[Ca^{2+}]_i$ can also

be generated by models which propose that intracellular Ca^{2+} both activates and blocks K_{Ca} channels (Methfessel and Boheim 1982). The present data do not allow a clear choice to be made between this model and that of the short-lived closed states.

BK channels in rat skeletal muscle have been observed occasionally to enter alternative modes of gating in which open probabilities may be very different from the normal condition (McManus and Magleby 1988). When large numbers of transitions are analyzed (>30,000), it is observed that about 96% of these fall into the normal gating mode, the remainder being distributed in the so-called intermediate open (3.2%), brief open (0.5%) and buzz modes (0.1%). The later three modes are all associated with a much briefer mean open time than is seen in the normal gating mode. In addition, analysis of large numbers of transitions (up to 10^6) in the normal mode reveal that open time distributions in this mode contain at least three to four exponential components, not the two detected in earlier studies (McManus and Magleby 1988). In the present study, open time distributions were calculated for up to 2000 transitions, this being limited by the lifetime of excised patches, which rarely exceeded 20 minutes. For these reasons, the possibility that rare modes of gating and additional open states also exist for $K(Ca)L$ channels cannot be excluded.

4.4.1.3. Voltage dependence of opening of $K(Ca)L$ channels

The probability of opening for $K(Ca)L$ channels increased e-fold for a 14 mV membrane depolarization, when $[Ca^{2+}]_i$ was at low levels. A similar voltage dependency (e-fold for 11-16 mV change) has been reported in BK channels studied in rat skeletal muscle (Barrett et al. 1982; Methfessel and

Boheim 1982; Blatz and Magleby 1984), renal epithelial cells (Kolb et al. 1986) and in chromaffin cells (Marty 1981), see Table II. BK channels in anterior pituitary cells and in toad stomach smooth muscle cells show a steeper voltage dependency (e-fold for 8-9 mV change (Wong and Adler 1986; Lang and Ritchie 1988; Singer and Walsh 1987). BK channels in mesenteric artery smooth muscle cells exhibited a lower voltage dependency than seen in $K_{(Ca)}L$ channels (e-fold for 30 mV change) (Benham et al. 1986). Voltage could alter channel kinetics directly by changing the rate constants governing state transitions, or could act indirectly by changing the effective concentration of Ca^{2+} at binding sites located part way through the membrane field (Woodhull 1973; Barrett et al. 1982).

Using BK channels from rat skeletal muscle incorporated into planar lipid bilayers, Moczydloski and Latorre (1983) found that the voltage dependency of channel opening could be entirely accounted for by the potential-sensitive binding of Ca^{2+} to sites in the membrane field. Calculation suggested that calcium ions bind to sites which sense 75-95% of the total membrane field. Such sites could be located in a wide, deep atrium leading to the ionic selectivity filter, or could reside at a part of the channel not directly connected with ion translocation. There is evidence which favours the latter possibility, since Ca^{2+} activation sites are more sensitive to membrane surface potential than is the K^+ conduction process in these channels. Thus, the addition of negatively charged membrane lipids markedly increases the apparent Ca^{2+} sensitivity of BK channel gating while little change is seen in the potassium permeability of the channel (Moczydlowski et al. 1985).

In the present study. membrane depolarization at constant $[Ca^{2+}]_i$ had no effect on the fast time constant of open time distributions, while increasing the value of the slow time constant. Membrane depolarization therefore mimicked the effect of increasing $[Ca^{2+}]_i$ at a constant membrane voltage. This result is consistent with the view that the voltage dependency of $K_{(Ca)}L$ channel gating simply reflects the potential-dependent binding of Ca^{2+} to sites on the channel complex.

4.4.1.4. Blockade of $K_{(Ca)}L$ channels by internally applied TEA

Internally applied TEA reduced the apparent single channel current in $K_{(Ca)}L$ channels with a K_d of 0.83 mM at +40 mV membrane potential. As shown in Table II, BK channels in chromaffin cells (Yellen 1984a), rat skeletal muscle (Blatz and Magleby 1984) and rabbit t-tubule cells (Vergara and Latorre 1983) show a much higher K_d for internally applied TEA, values being in the range 27-60 mM. BK channels in VSMCs from rabbit portal vein were unaffected by internally applied TEA at concentrations up to 10 mM (Inoue et al. 1985). In marked contrast, BK channels in clonal anterior pituitary cells (Wong and Adler 1986) and BK channels from rat brain synaptosomes (Farley and Rudy 1988) both exhibit very high sensitivity to internally applied TEA, with K_d of 0.08 mM and 0.8 mM respectively.

The high sensitivity of $K_{(Ca)}L$ channels to the blocking effect of internal TEA is unlikely to be an artifact caused by enzyme treatment of CVSMCs, since similar dissociation procedures were employed in other studies which did not exhibit this phenomenon (Benham et al. 1985; Inoue et al. 1985). The possibility that the present data resulted from inadvertent formation of outside-out

membrane patches also seems unlikely, since the recording pipettes contained only 0.01 μM Ca^{2+}, insufficient to yield the observed channel open probability, if applied to the cytoplasmic membrane face.

In the case of the A-current K^+ channel coded by the **Shaker** gene in *Drosophila*, a single amino acid residue critically affects the affinity of the channel for internally applied TEA (Yellen et al. 1991). This residue lies at position 441 in the so called SS1-SS2 region of the channel, which is highly conserved among voltage-gated K^+ channels, and may cross the membrane twice to form the ion conducting pore itself (MacKinnon and Yellen 1990). Wild type flies showed channels with a threonine residue at position 441, and exhibited a K_d for internally applied TEA of about 0.7 mM. The mutant T441S possessed a serine residue at position 441 and the K_d for internal TEA increased by ten fold as a result of this single substitution (Yellen et al. 1991). The sensitivity of the channel to externally applied TEA was unaltered by this mutation. Although these studies were not performed on gene coding for a K_{Ca} channel, they do illustrate the extreme sensitivity of TEA affinity to changes in the primary structure of potassium channels. It may be relevant to note that all three of the cell types which exhibit BK channels with high internal sensitivity to TEA are derived from brain tissue, suggesting that a slightly different form of the BK channel may be expressed in this organ.

The present data on TEA block were transformed into a Hill plot by replotting as log (R/1-R) versus log [TEA], where R is the reduction in single channel currents seen in the presence of the drug. This plot yielded a slope of 0.82, suggesting that TEA interacts with the channel in a one-to-one fashion, presumably by transiently entering the channel and blocking movement of K^+.

An approximate value for the dwell time of TEA at its blocking site can be derived from the K_d for TEA, where $K_d = K_{-1}/K_1$ (Benham et al. 1985). The maximum value for the blocking rate constant, K_1 is limited by the drug diffusion rate and is thought to be about 10^8 s^{-1} M^{-1} (Gutfreund 1972). Thus K_{-1} may be estimated at 8.3×10^4 s^{-1} and hence the mean block time was about 12 μs, one order of magnitude too brief to be detected by the present measuring system. This result is consistent with the observation that TEA block was not accompanied by a marked increase in current noise in the channel, as studied at a bandwidth of DC-2 kHz.

The block of K_{Ca} channels by internal TEA may be expected to be voltage-dependent, since positive membrane potentials favour the entry of the TEA cation into the channels. However, the degree of voltage-dependency is governed by the fraction of the membrane field sensed by the TEA molecule at its binding site (Woodhull 1973). Experiments performed on BK channels in several cell types have indicated that block by internal TEA is only weakly voltage-dependent, exhibiting an equivalent valence of 0.06-0.1 (Yellen 1984a; Langton et al. 1991; Yellen et al. 1991). The theoretical maximum value of the equivalent valence for a monovalent blocking ion interacting in a 1:1 fashion with the channel is 1.0 (Benham et al. 1986). This corresponds to an e-fold increase in affinity for a 25 mV depolarization (Yellen et al. 1984a). Let it be assumed the present K_{Ca} channels possess a TEA affinity at 0 mV membrane potential typical of BK channels studied in skeletal muscle, i. e. $K_d(0\ mV) = 30$ mM. If measured at +40 mM, the K_d should still be greater than 4 mM, even of the equivalent valence of TEA is assumed to be 1.0. It seems unlikely, therefore,

that the high sensitivity of $K_{(Ca)}L$ channels to internal TEA is due solely to a very steep voltage dependency of channel block.

4.4.2. Properties of $K_{(Ca)}I$ channels

In addition to the $K_{(Ca)}L$ channel, a K_{Ca} channel of intermediate conductance (IK channel) was detected in the minority of inside-out patches excised from isolated CVSMCs. This $K_{(Ca)}I$ channel showed a single channel conductance of 92 pS and was highly selective for K^+ over Na^+ and Cs^+. This channel was activated by intracellular free calcium and by membrane depolarization. Application of TEA to the cytoplasmic membrane face reduced the current in this channel in a dose-dependent fashion with a K_d of 0.31 mM. These data were replotted as a Hill plot, log (R/1-R) versus log [TEA] where R is the reduction in mean single channel current caused by a given concentration of TEA. The slope of this plot was 0.70, suggesting that TEA blocks this channel in a one-to-one fashion, as seen for the $K_{(Ca)}L$ channel. No significant difference between the K_d values found for block of the $K_{(Ca)}L$ and $K_{(Ca)}I$ channels by internal TEA. This suggests that these channels share a common structure in the region of the TEA binding site. It seems unlikely, however, that the $K_{(Ca)}I$ channel simply represents a conductance substate of the $K_{(Ca)}L$ channel, since amplitude distributions for current flow in both species were well fitted by single Gaussian terms.

K_{Ca} channels of intermediate conductance (30-120 pS) have previously been detected in a wide variety of cell types (Latorre et al. 1989; Edwards and Weston 1990; Kolb 1990). IK channels of conductance 90-100 pS have been reported in VSMCs isolated from rabbit portal vein (Inoue et al. 1985) and from

human cystic artery (Akbarali et al 1990). However, the IK channel reported in rabbit portal vein is unaffected by TEA applied to the cytoplasmic membrane face, in marked contrast to the present results (Inoue et al. 1985). An IK channel has been reported in aortic smooth muscle of the rat, but this channel shows a conductance of only 55 pS, appreciably lower than that of the K(Ca)I channel (Shoemaker and Worrell 1991). Two types of IK channel have been observed following incorporation of rat brain synaptosomal proteins into lipid bilayers (Farley and Rudy 1988). However, both of these channels are little affected by application 10 mM TEA to the cytoplasmic membrane face.

4.4.3. Physiological roles for K(Ca)L and K(Ca)I channels

The low apparent calcium sensitivity of the K(Ca)L and K(Ca)I channels would seem to preclude a significant contribution of these channel to resting potassium conductance, since $[Ca^{2+}]_i$ in unstimulated vascular smooth muscle has been estimated as 40 - 130 nM (Nabika et al. 1985; Takata et al. 1988; Kuriyama et al. 1982; present study). However, during the fully contracted state, vascular smooth muscle cells may show $[Ca^{2+}]_i$ values approaching 10 μM (Kuriyama et al. 1982). Micromolar levels of free intracellular calcium can also be attained during the action of certain vasoconstrictor hormones, including angiotensin II (Capponi et al. 1986). $[Ca^{2+}]_i$ levels in the low micromolar range would suffice to activate a few per cent of the available K(Ca)L channels at depolarized membrane potentials. It should be noted that the opening of even a small fraction of the available K(Ca)L channels would markedly reduce the input resistance of a 3 GΩ cell, as well as providing a strong hyperpolarizing influence on membrane potential. In addition, it is possible that locally very high concentrations of $[Ca^{2+}]_i$ are established near the inner face of voltage-sensitive

Ca^{2+} channels. If K_{Ca} channels were located close to these sites, their contribution to membrane repolarization during the action potential could be greatly enhanced (Benham et al. 1986). The calcium sensitivity of $K(Ca)L$ and $K(Ca)I$ channels may also be enhanced *in vivo*, by the presence of intracellular modulators such as Mg^{2+} (Squire and Petersen 1987). For these reasons, it seems probable the $K(Ca)L$, and possibly also $K(Ca)I$ channels play an important role in the repolarizing phase of the action potential in CVSMCs.

The action potential in CVSMCs of intact rat basilar arteries has been studied following partial blockade of potassium conductances by externally applied TEA (Hirst et al. 1986). Under these conditions, the spike consists of a rapid depolarization of about 56 mV amplitude followed by a fast repolarization. This is followed by an after-depolarization of about 13 mV, lasting 300-1000 ms. In most cells, a slow after-hyperpolarization of a few millivolts is seen at the end of the action potential. Both the initial spike depolarization and the after-depolarization require the presence of Ca^{2+} in the bathing medium, while the slow after-hyperpolarization probably reflects an increase in $K+$ conductance. The latter potential is also abolished by removal of Ca^{2+} from the bathing medium, suggesting that it is probably mediated by a K_{Ca} conductance. This conductance apparently serves to terminate the after-depolarization, which probably reflects the entry of calcium through non-inactivating Ca^{2+} channels. The majority of calcium entry into the fibre takes place during this after-depolarization phase of the action potential (Hirst et al. 1986). These observations strongly suggest that K_{Ca} channels play an important role in the regulation of action potential duration and of calcium entry into cerebrovascular smooth muscle cells.

4.5 Conclusions and significance

At least 50% of neurological patients exhibit signs of cerebrovasular disease, often including the major categories of stroke, aneurysm, migraine or transient ischemic attack (Adams and Victor 1985). In the hypertension often associated with stroke, a number of abnormalities have been detected in cerebrovascular smooth muscle, including increased membrane permeability, altered membrane potential and electrogenic pump activity, and an increased sensitivity to vasoconstrictors, including serotonin (Webb and Bohr 1984; Bohr and Webb 1988; Michel et al. 1990). Similar changes have been found to occur in atheroma and during the normal aging process (Michel et al. 1990; Michel 1987). Atherosclerotic damage to the endothelial layer of cerebral vessels probably exacerbates hypersensitivity of smooth muscle to serotonin. This is because the endothelium normally produces EDRF, which mediates endothelium-dependent vasodilation (Burnstock, 1990) and prostacyclin, which inhibits platelet aggregation (Moncada et al. 1976). Endothelial cells also contribute to the degredation of serotonin by monoamine oxidase (Peach et al. 1985). The enhanced responsiveness of VSMCs to serotonin may contribute to arterial spasm *in vivo* (Beamish et al. 1984).

Cerebral vasospasm develops in most patients following rupture of an intracranial aneurysm. This phenomenon is of great clinical importance, since the resulting ischemia can greatly compound the neurological damage done by the initial hemorrhage, and may prove fatal (Steele et al. 1991). In an animal model of cerebral vasospasm, evidence has been found for depolarization and increased action potential activity in VSMCs of the basilar artery. These

changes appeared to result from decreased potassium conductance in the cells. The observed vasospasm could be partially supressed by application of the potassium channel opener nicorandil (Waters and Harder 1985; Harder et al. 1987). However, the mechanisms underlying vasodilation by potassium channel openers remains a subject to controversy. Cromakalim has been claimed to activate K_{Ca} channels in VSMCs isolated from rabbit aorta (Kreye et al. 1987) and from guinea-pig mesenteric artery (Nakao et al. 1988). However, Standen et al. (1989) attributed the vasodilatory action of cromakalim on the latter preparation to activation of K_{ATP} channels. The relaxation of intact dog cerebral arteries by cromakalim has also been attributed to activation of K_{ATP}, rather than to K_{Ca} channels (Masuzawa et al. 1990).

In view of the great clinical importance of cerebrovascular smooth muscle, and of the urgent need for rational and effective means of preventing arterial spasm, it is imperative that the potassium conductances of CVSMCs be well understood. The present study has contributed to this goal by developing a preparation in which to study the biophysical properties of K_{Ca} channels in cerebrovascular smooth muscle cells. The modulation of these channels by clinically important drugs and by physiological stimuli can also be studied in the present *in vitro* preparation.

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