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Ca^{2+}-ACTIVATED AND VOLTAGE-GATED K^+ CURRENTS IN SMOOTH MUSCLE CELLS ISOLATED FROM HUMAN MESENTERIC ARTERIES

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SUMMARY

1. Smooth muscle cells were enzymatically isolated from arteries dissected from mesenteric fat removed from patients undergoing routine surgery. The whole-cell patch clamp technique was used to characterize the potassium (K^+) currents and passive electrical properties of these cells, using high-K^+-containing pipette solutions with either 0·2 mM EGTA or 10 mM EGTA and 10 mM BAPTA.

2. Cell capacitance, which is proportional to membrane surface area, was normally distributed around a value of 46 pF, and independent of artery size between 0·4 and 3·6 mm. The mean membrane potential measured under current clamp was $-44{\cdot}1 \pm 1{\cdot}9$ mV ($n = 52$).

3. Cells dialysed with 0·2 mM EGTA in order to weakly buffer intracellular Ca^{2+} demonstrated a noisy outward current with an apparent threshold near −30 mV, upon which were superimposed spontaneous transient outward currents (STOCs). In the presence, but not the absence, of extracellular Ca^{2+}, this current was potentiated if the holding potential was depolarized into the voltage range between −40 and +50 mV. This potentiation had a bell-shaped potential dependency which reflected the activation of voltage-gated Ca^{2+} channels in these cells.

4. The noisy current was blocked by externally applied tetraethylammonium (the dissociation constant, $K_d = 0{\cdot}85$ mM), as were STOCs. This current was also reduced by about 40% by 8 nM charybdotoxin, and was transiently potentiated by 10 mM caffeine. The characteristics of this current therefore suggested that it was carried by large-conductance Ca^{2+}-activated K^+ channels.

5. Dialysis of human mesenteric arterial cells with 10 mM EGTA and 10 mM BATPA was not able to completely suppress the Ca^{2+}-activated current, and reduced by approximately 50% the amplitude of the outward current recorded at positive potentials.

6. Depolarization of strongly Ca^{2+}-buffered cells in the presence of 30 mM TEA to block Ca^{2+}-activated K^+ channels revealed a residual outward current which had both transient and sustained components. These were blocked by 4-aminopyridine (4-AP) with a similar efficiency (K_d was 1·04 and 1·16 mM at +60 mV for transient

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and sustained current, respectively), but the voltage ranges over which they inactivated, and their rates of recovery from inactivation, were significantly different.

7. The transient and sustained currents had different sensitivities to external Ca^{2+} and Cd^{2+} ions. Ca^{2+} (5 mM) significantly reduced the amplitude and shifted the voltage dependency of inactivation of the transient but not the sustained component of the outward current. Cd^{2+} (0·2 mM) reduced the transient current by about 30% without affecting the sustained component amplitude.

8. These data suggest the presence of at least three types of K^+ currents in human mesenteric arterial cells. These include a Ca^{2+}-activated K^+ current probably carried by large conductance channels, a rapidly activating and inactivating A-like current, and a small sustained current which had properties similar to the delayed rectifier described in other smooth muscle cells. Experiments in current-clamped cells suggested that the two 4-AP-sensitive currents are more important in suppressing action potential generation in human mesenteric arterial cells than is the Ca^{2+}-activated K^+ current.

INTRODUCTION

The degree of excitability of smooth muscle cells (SMCs) depends largely upon the characteristic balance between the inward Ca^{2+} and outward K^+ currents present in each type of cell. Most arterial smooth musles respond to excitatory stimuli with a graded depolarization rather than with action potentials. Suppression of K^+ currents, however, has been shown to lead to depolarization and spontaneous action potential activity in many arteries (Bolton, 1979).

Several types of K^+ currents have been characterized in isolated vascular SMCs. In general, it appears that most vascular SMCs have at least two types of K^+ current, one of which is gated by voltage, and the other of which is gated by both voltage and intracellular Ca^{2+}. Voltage-gated currents include the delayed rectifier and the A-current (see for review Rogawski, 1985; Rudy, 1988); both are found to co-exist in some types of SMCs (Beech & Bolton, 1989; Lang, 1989; Imaizumi, Muraki & Watanabe, 1990; Smirnov, Zholos & Shuba, 1992). Currents gated by Ca^{2+} and voltage appear to be ubiquitous, especially those carried by large-conductance or 'BK' channels (Benham, Bolton, Lang & Takewaki; 1986; Ohya, Kitamura & Kuriyama, 1987; Hume & Leblanc, 1989; Beech & Bolton, 1990); smaller conductance Ca^{2+}-activated channels have also been observed (Inoue, Kitamura & Kuriyama, 1985). The activation of a group of Ca^{2+}-activated channels by bursts of Ca^{2+} release from sarcoplasmic reticulum is thought to give rise to spontaneous transient outward currents (STOCs) observed in many types of SMCs (Benham & Bolton, 1986; Benham *et al.* 1986; Ohya *et al.* 1987; Ganitkevich & Shuba, 1988; Bolton & Lim, 1989; Hume & Leblanc, 1989; Zholos, Baidan & Shuba, 1991).

In the present report, we present evidence for three types of K^+ current in vascular SMCs isolated from the human mesenteric artery. These include a TEA-sensitive Ca^{2+}-activated current, an A-like transient current, and a voltage-gated sustained current. Part of this work has been presented in abstract form (Aaronson & Smirnov, 1992).

METHODS

Experiments were performed on single smooth muscle cells isolated from human mesenteric arteries. Arteries (0·4–3·6 mm in outer diameter) were dissected from fat adhering to sections of gastrointestinal tract obtained directly after removal during routine surgery (usually hemicolectomies or gastrectomies) on thirty-one patients of both sexes (age range 23–90 years). This procedure was approved by the St Thomas's Hospital Ethical Committee.

Isolation procedure

Dissected arteries were placed in normal physiological salt solution (PSS) and adhering fat and connective tissue were carefully removed under a binocular microscope. The outer diameter of each artery was measured in the middle part of the vessel by an ocular micrometer. The vessel was then opened along its longitudinal axis and cut transversely into small pieces (1–2 mm wide). These pieces were stored in normal PSS at +4 °C and could be used for cell isolation for up to 5 days when stored in this manner.

Pieces of artery were incubated in nominally Ca^{2+}-free PSS at 37 °C. After a 30 min incubation they were transferred into 2 ml low-Ca^{2+} PSS containing 0·15–0·25 % papain, 0·1–0·15 % collagenase (Type XI), 0·25–0·3 % bovine serum albumin and 1 mM dithiothreitol (all from Sigma) and were incubated at 37 °C for 50–60 min. Following digestion, pieces of artery were placed in nominally Ca^{2+}-free PSS containing no enzymes and gently triturated using a Pasteur pipette to disperse single smooth muscle cells. The cell suspension was stored in low-Ca^{2+} PSS at 4 °C for use within 8 h.

Solutions

The normal PSS contained (mM): 130 NaCl, 5 KCl, 1·2 $MgCl_2$, 1·5 $CaCl_2$, 10 glucose, and 10 *N*-2-hydroxyethylpiperazine-*N'*-ethanesulphonic acid (HEPES). The pH was adjusted to 7·2 with NaOH. The composition of Ca^{2+}-free PSS was identical except that $CaCl_2$ was omitted. Low-Ca^{2+} PSS was prepared from Ca^{2+}-free solution by the addition of 10–15 μl of normal PSS per 1 ml. TEA-containing PSS (TEA-PSS) was prepared from normal PSS by replacing 30 mM NaCl by an equimolar amount of tetraethylammonium chloride (TEA-Cl). All other changes in the external solution are indicated in the text.

Two main pipette solutions, with low and high Ca^{2+} buffer concentrations, were used. Low-EGTA ('weakly Ca^{2+} buffering') pipette solution contained (mM): 135 KCl, 2·5 $MgCl_2$, 10 HEPES, 2 adenosine 5-triphosphate disodium salt (Na_2ATP), 0·2 ethyleneglycol-bis(β-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA). A second pipette solution, designed to strongly buffer intracellular Ca^{2+} ('strongly Ca^{2+} buffering'), contained (mM): 100 KCl, 2·5 $MgCl_2$, 2 Na_2ATP, 10 HEPES, 10 EGTA and 10 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA). The pH of both solutions was adjusted to 7·2 with KOH. Note that the KCl concentration was reduced in order to compensate for the increased osmolarity contributed by the elevated concentrations of EGTA, BAPTA and KOH in the strongly Ca^{2+}-buffering solution.

Eletrophysiological recordings

Voltage clamp experiments were carried out at room temperature using an Axopatch 1A patch clamp Amplifier (Axon Instruments, Foster City, CA, USA). Patch pipettes were fabricated from soft haematocrit glass tubes (Camlab, Cambridge, UK). The pipette tip was coated with Sylgard 170 (Dow Corning, Belgium) to reduce capacitance artifacts. When filled, pipettes had resistances of 1–4 MΩ. An estimate of the series resistance was made following establishment of whole-cell patch clamp in each cell by measuring the peak amplitude of capacitance transients elicited by a 10 mV hyperpolarizing pulse of 5 ms duration, recorded with 20 kHz filtering. The series resistance estimated in this way varied between 3 and 14 MΩ with an average of $7·6 \pm 0·24$ MΩ ($n = 150$), and experiments were performed without series resistance and cell capacitance compensation. The cell membrane capacitance was calculated from the area under the capacitive artifact.

Data was digitized using a Labmaster DMA TL-1-125 interface, stored on a microcomputer, and analysed using the pClamp 5.0 program (Axon Instruments). Figures were made using SigmaPlot 4.01 (Jandel Scientific, California, USA). Theoretical fitting of experimental data by the equations described in the text was performed by a Marquardt–Levenberg algorithm using the 'Curve fit'

program of SigmaPlot 4.01. Values in the text and figures are presented as means ± S.E.M. The significance of an experimental value was determined using Student's t test, with $P < 0.05$ considered to be significant.

TEA-Cl, EGTA, BAPTA and 4-aminopyridine (4-AP) were from Sigma Chemical Company, UK; HEPES was obtained from Calbiochem, USA. Charybdotoxin was kindly provided by Dr Alison Gurney, and was from a lot prepared by Dr Chris Miller. Additional charybdotoxin was acquired from Latoxan, Rosans, France. It is noteworthy that only the former preparation was effective in blocking Ca^{2+}-activated K^+ currents in these cells.

RESULTS

Morphology and passive membrane properties of single human mesenteric arterial cells

The enzymatic dissociation yielded relaxed spindle-shaped cells with an elongated nucleus; the length of cells varied in a range between several tens and about 200 μm. To estimate the membrane surface area a capacitance transient was measured in each cell studied after rupture of the cell membrane under the pipette tip. The decay of the capacitance artifact was monoexponential with a time constant ranging from 0·09 to 1 ms (mean 0·31 ± 0·015 ms, $n = 150$) in human mesenteric arterial cells of different sizes (Fig. 1*A*). It should be pointed out that no correlation between the outer diameter of arteries (ranging from 0·4 to 3·6 mm) and the size of cells dispersed was found; both large and small arteries had cells in which the cell membrane capacitance (C_m) varied widely over a similar range (Fig. 1*B*). The distribution of C_m, measured in cells studied in the presence of both K^+ and Cs^+ (Smirnov & Aaronson, 1992) in the external and pipette solutions, could be described by a normal distribution with a mean value of $C_m = 46$ pF and a standard deviation of 14 pF (Fig. 1*C*). We observed no apparent differences in the properties of potassium and calcium (Smirnov & Aaronson, 1992) currents in human mesenteric arterial cells with widely divergent values of C_m, although a small percentage of cells did not demonstrate Ca^{2+} currents, perhaps owing to damage during the enzymatic treatment. Therefore, these data suggest that human mesenteric arteries were comprised of smooth muscle cells of various sizes belonging to a population which was relatively homogeneous with respect to the particular membrane currents we examined.

Under current clamp the isolated cells had resting potentials between −20 and −70 mV (mean −44·7 ± 1·9 mV, $n = 52$) when the pipette was filled by the low-EGTA solution. The input membrane resistance (r_m) of these cells, estimated with a 10 mV negative voltage step, ranged between one and several tens of GΩ (mean value 14·1 ± 2·1 GΩ, $n = 76$). Multiplying r_m by the cell area obtained from the C_m and assuming a specific membrane capacitance of 1 μF cm^{-2} gives a specific membrane resistance (R_m) of 624 ± 103 kΩ cm^2 ($n = 75$). This value of R_m is comparable to that found in SMCs isolated from rabbit jejunum (Bolton, Lang, Takewaki & Benham, 1985), guinea-pig bladder (Klöckner & Isenberg, 1985), rat aorta (Toro & Stefani, 1987) and guinea-pig ureter (Lang, 1989) and is 5–10 times larger than that found in rabbit ileum and vas deferens (Ohya, Terada, Kitamura & Kuriyama, 1986; Nakazawa, Matsuki, Shigenobu & Kasuya, 1987), rat myometrium (Amédée, Mironneau & Mironneau, 1987) and in rabbit and cat colon (Bielefeld, Hume & Krier, 1990).

Injection through the recording pipette of hyperpolarizing current of between 2·5 and 10 pA resulted in a slow monoexponential decrease of the cell membrane potential with an average time constant of 791 ± 97 ms ($n = 8$, range 447–1165 ms). Changing the polarity of the injecting current produced a much smaller

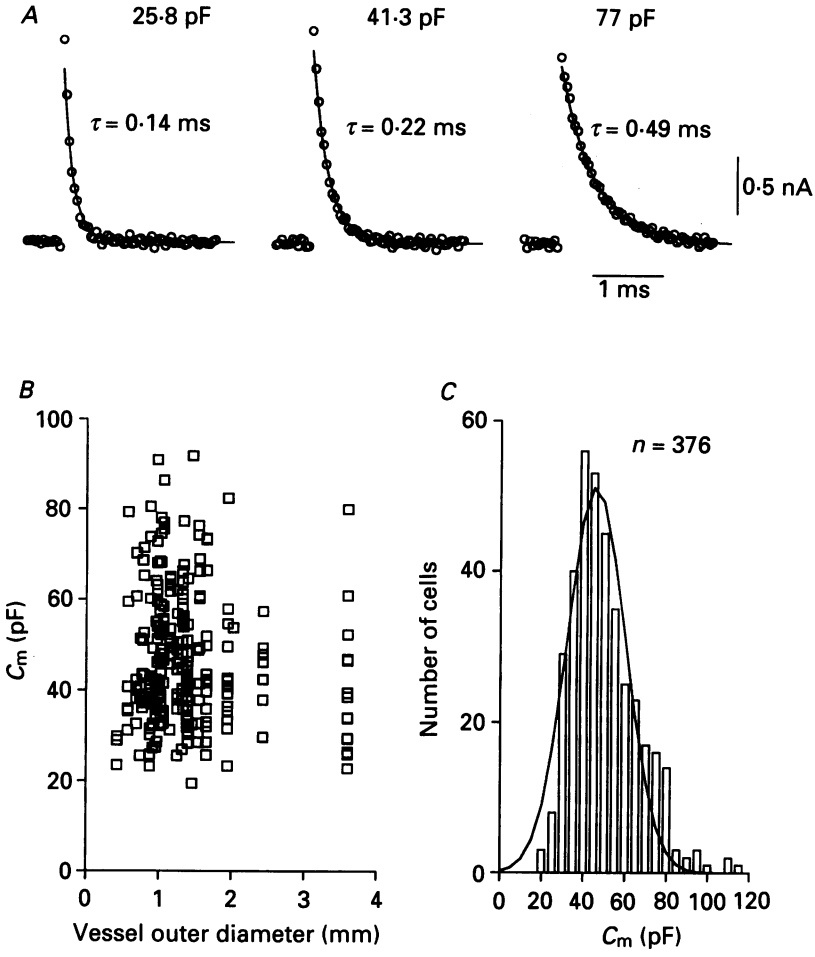

Fig. 1. *A*, Time courses of capacitance artifacts in three different cells isolated from the same artery. Continuous lines represent monoexponential functions with time constants (τ) as indicated. Cell membrane capacitance values (C_m), estimated from areas under capacitance artifacts are shown at the top. *B*, dependence of C_m on vessel outer diameter, estimated as described in the Methods section. *C*, distribution of C_m obtained from 376 cells studied. The continuous line is a normal distribution function with a mean value of 46 pF and a standard deviation of 14 pF.

depolarization with significant fluctuations of the membrane voltage (Fig. 2*A*). The *I–V* relationship was nearly linear for negative current injection but demonstrated strong outward-going rectification for depolarizing current (Fig. 2*B*). It should be noted that no action potential was observed in the presence of normal PSS in eleven cells studied. The mean membrane resistance calculated from the slope of the

negative branch of the current–voltage (I–V) curve was 9·2 GΩ, which is similar to that obtained from voltage clamp experiments (see above). Also, estimates of C_m made from the membrane time constant measured using current clamp, and from the area under the capacitance artifact measured using voltage clamp, were compared in

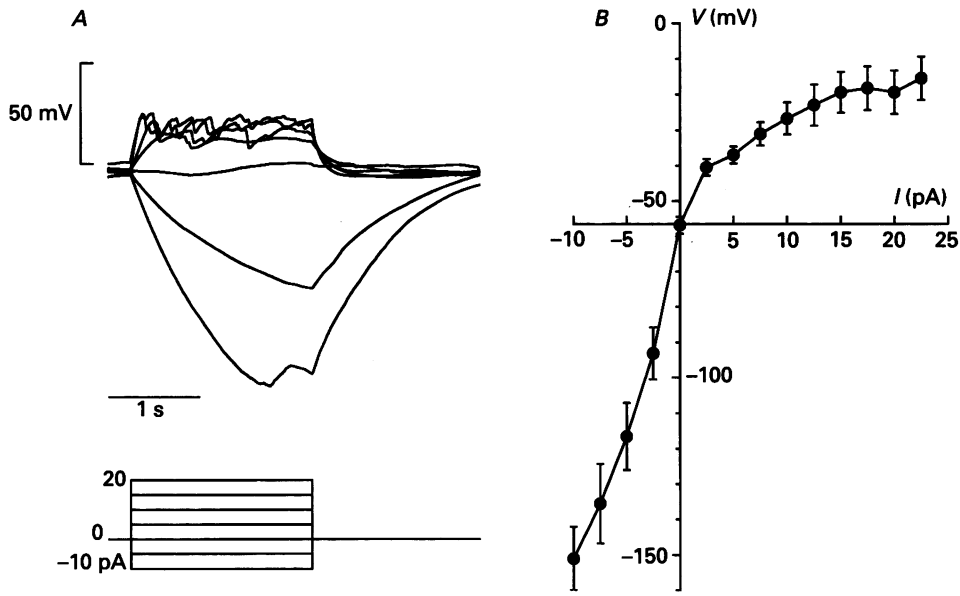

Fig. 2. A, membrane potential changes recorded from a cell under current clamp in response to both hyperpolarizing and depolarizing current injections from −10 to +20 pA with a 5 pA increment. Duration of the current pulse was 2 s. B, the relationship between injected current and membrane potential changes in eight cells studied.

eight cells. Similar values of 58·3 ± 10·3 and 60·9 ± 9·9 pF respectively were obtained, confirming the validity of the approach routinely used for calculation of C_m.

The space constant (λ) of single human mesenteric arterial cells was estimated to be 0·5 cm from the expression $\lambda = \sqrt{(d/4R_m/R_i)}$, where R_i, the specific resistivity of the cytoplasm, was assumed to be 250 Ω cm (see Abe & Tomita, 1968), and the diameter (d) of a completely relaxed cell was taken as 4 μm. This value of λ was comparable to that estimated for single ureter cells (0·34 cm, Lang, 1989). Such a value of the space constant predicts that the entire membrane of a single human mesenteric arterial cell would be virtually isopotential both in the resting state, and during activation of ionic channels sufficient to decrease the cell input resistance by up to 100-fold.

Potassium currents in weakly Ca^{2+} buffered cells

When the pipette was filled by the low (0·2 mM) EGTA solution, depolarization of cells to potentials positive of −30 mV from the holding potential of −60 mV elicited a slowly activating outward current which demonstrated little inactivation in the negative voltage range even during a 5 s depolarization (Fig. 3). Spontaneous transient outward currents (STOCs, Benham & Bolton, 1986), observed in most cell

studied, were usually superimposed on this sustained current with depolarizations of −30 to +20 mV. In some human mesenteric arterial cells, however, STOCs of small amplitude could be observed at membrane potentials of −60 or even −70 mV. STOCs have been described in many SMCs studied and at present are thought to be

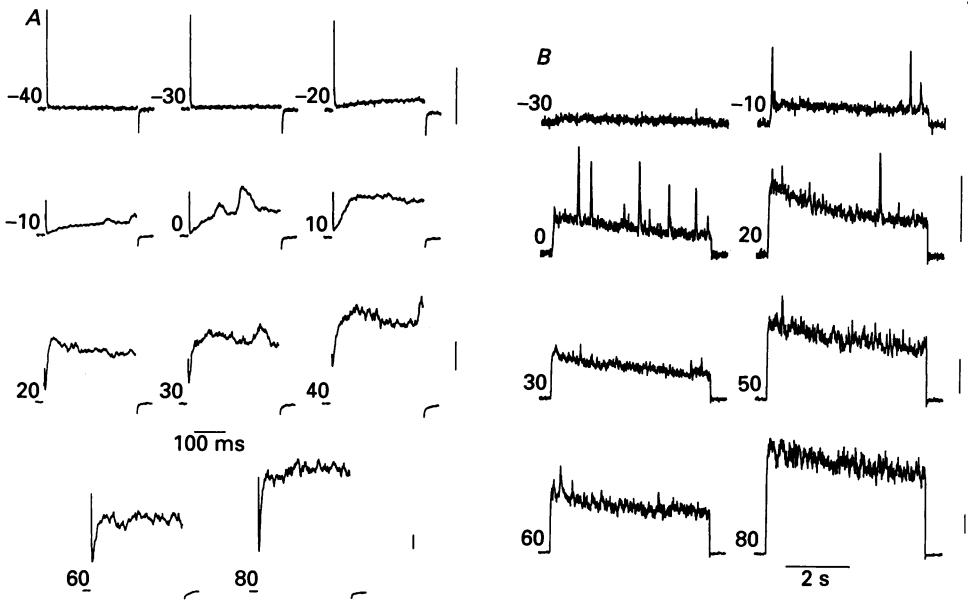

Fig. 3. K^+ currents in weakly Ca^{2+}-buffered cells. *A* and *B*, currents in two different cells recorded with 300 ms and 5 s membrane depolarizations, respectively. Membrane potential indicated in mV near each record. The holding potential was −60 mV. Vertical bars are 100 pA.

due to the activation of clusters of Ca^{2+}-dependent K^+ channels by Ca^{2+} released from the sarcoplasmic reticulum (Benham & Bolton, 1986; Benham *et al.* 1986; Ohya *et al.* 1987; Ganitkevich & Shuba, 1988; Bolton & Lim, 1989; Hume & Leblanc, 1989; Zholos *et al.* 1991). With progressive depolarization of the cell membrane the amplitude of the outward current increased and a slow and incomplete decay of the current occurred. At potentials positive to +50 mV STOCs were not usually observed; however, the outward current become much noisier than that at negative voltages (Fig. 3*B*). Both the sustained outward current and STOCs were blocked in a dose-dependent manner by bath-applied TEA (Fig. 4*A*). The blocking effect of TEA was reversible and observed over the whole voltage ranged studied. The dose–response relationship for the inhibition by externally applied TEA of the outward current measured at the end of a 500 ms step to +80 mV is presented in Fig. 4*B*. The data are well described by the Langmuir equation (see figure legend) with an apparent dissociation constant (K_d) of 0·85 mM, and C, the fraction of TEA-insensitive current, equal to 0·03. A similar blocking effect was found at +50 mV (K_d = 0·56 mM, C = 0·08). It should be noted that at high $[TEA]_o$, a residual component of outward current with a relatively small amplitude was unmasked, which was characterized by an initial rapid decay and low noise (Fig. 4*A*).

Application of 8 nM charybdotoxin caused a marked decrease in the amplitude of the noisy outward current, amounting to 40 and 44% at +80 mV in two cells studied; 4-aminopyridine (4-AP, 5 mM), however, caused only a negligible suppression of this current in two cells tested (data not shown). Brief application by

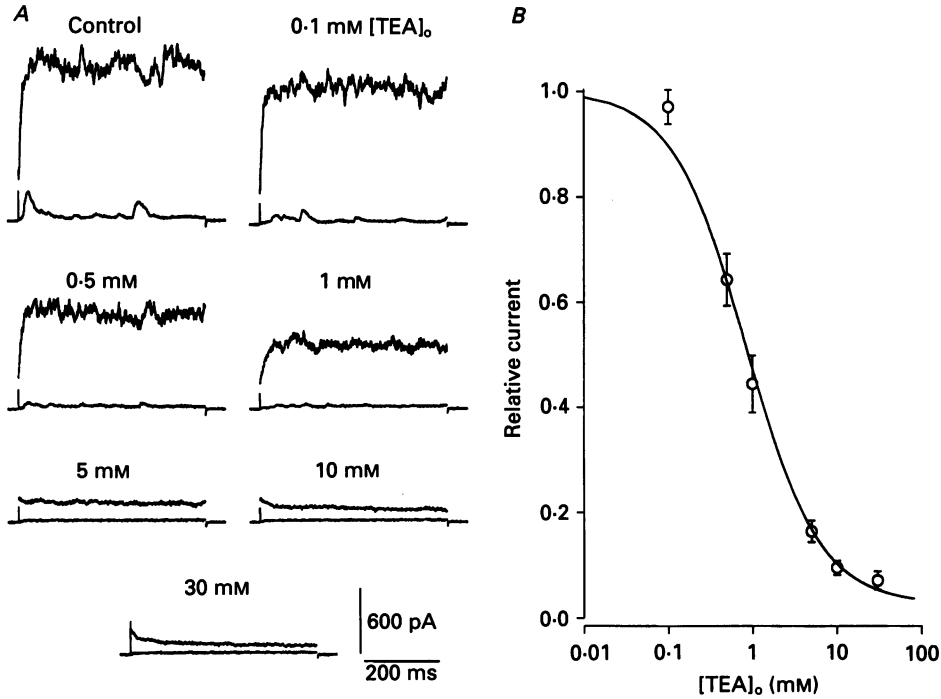

Fig. 4. The effect of different external TEA concentrations ([TEA]$_o$) on K^+ currents. *A*, currents were recorded at test potentials of −10 and +80 mV over the range of [TEA]$_o$ as indicated. The holding potential was −60 mV. *B*, the dose dependence of outward current blockade by [TEA]$_o$. The ordinate represents the ratio of the current at the end of 500 ms depolarizations to +80 mV in the presence and absence of drug. Each point and vertical bar represent the mean ± S.E.M. for eight cells except points at 0·5 and 30 mM TEA obtained from nine and five cells respectively. The continuous line is drawn according to the equation:

$$I = \frac{(1-C)}{1+K_d/[\text{TEA}]_o} + C,$$

where I is the relative amplitude, K_d the apparent dissociation constant, equal to 0·85 mM, and C the fraction of TEA-insensitive current, equal to 0·03. The pipette solution contained 0·2 mM EGTA.

puffer pipette of 10 mM caffeine during a 10 s depolarization to −40, −20 or 0 mV caused an increase in the amplitude of the outward current and/or an increase in STOC frequency (data not shown), suggesting that this current was sensitive to rises in intracellular Ca^{2+}.

Additional evidence that the main component of outward current measured with the low-EGTA pipette solution was increased by a rise in intracellular Ca^{2+} came

from a study of its availability. The outward current was recorded at +80 mV following a 30 s conditioning depolarization which was varied between −60 and +50 mV (Fig. 5*A*). Depolarization of the conditioning potential from −60 mV potentiated rather than suppressed the outward current. This potentiation was

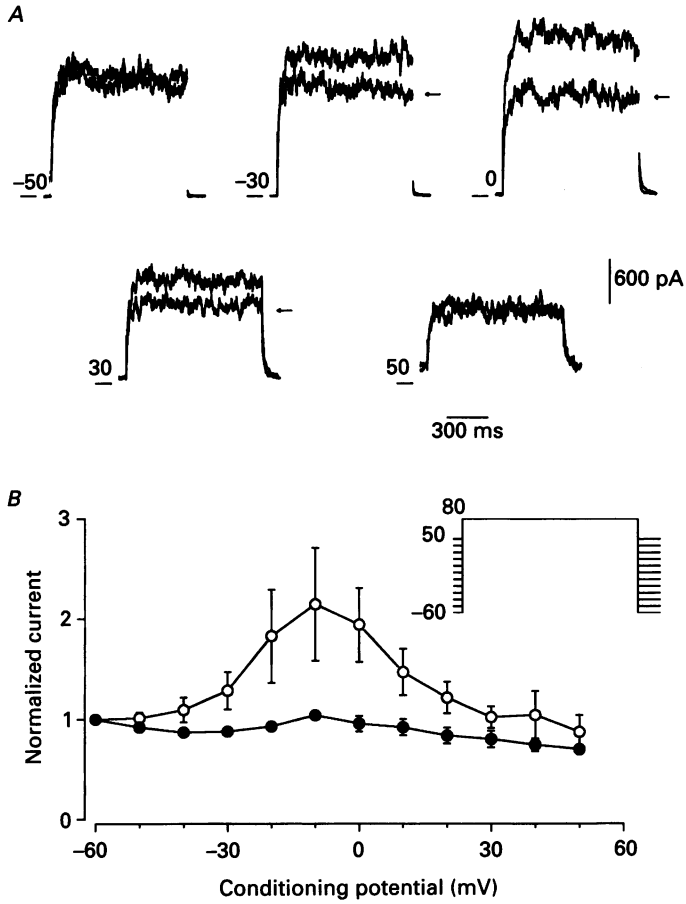

Fig. 5. Measurement of K^+ current availability in weakly Ca^{2+}-buffered cells, measured using 30 s conditioning potentials. *A*, membrane currents recorded in normal and Ca^{2+}-free PSS (1·5 mM Ca^{2+} replaced by Mg^{2+}). The test potential was +80 mV. Conditioning potentials and zero current levels are shown by numbers and horizontal bars respectively, near each pair. Arrows show the current in Ca^{2+}-free PSS. *B*, availability curves in normal (○) and Ca^{2+}-free (●) PSS for four cells. Current amplitude was measured at the end of the 500 ms test pulse to +80 mV and normalized with respect to the current at the conditioning potential of −60 mV in each solution.

evident at −40 to −50 mV and had a bell-shaped potential dependency, decreasing at very positive voltages (Fig. 5*B*, ○). This effect resembled the *I–V* relationship for a non-inactivating component of calcium current described in these cells (Smirnov & Aaronson, 1992) and was completely abolished by removal of Ca^{2+} from the solution. In the absence of external Ca^{2+} (replaced by 1·5 mM Mg^{2+}) the current showed only

a slight decline with progressive membrane depolarization (Fig. 5*B*, ●). An increase of current amplitude, which varied in different cells from 1·4 to 3·8 times the current measured at holding potential −60 mV, was observed in fifteen cells studied in normal PSS. In six other cells no changes in the current amplitude were found. It

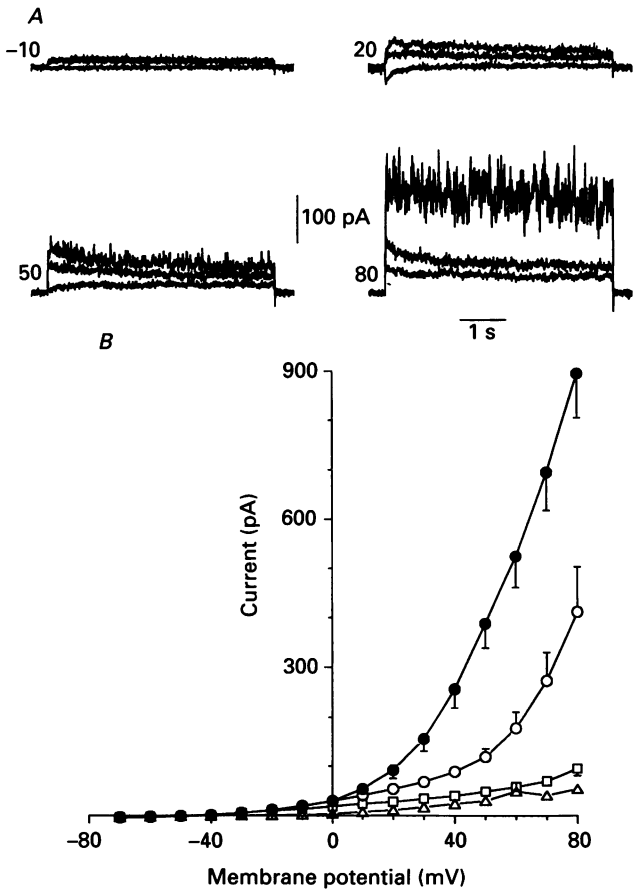

Fig. 6. Membrane currents in strongly Ca^{2+}-buffered cells during prolonged depolarizations. *A*, outward currents elicited by 5 s steps to the potential indicated in normal PSS, TEA-PSS and TEA-PSS containing 5 mM 4-AP (upper, middle and lower traces in each record, respectively). *B*, average *I–V* relationships for the current measured at the end of 5 s depolarizations in normal PSS in twenty-six weakly Ca^{2+}-buffered cells (●) and fourteen strongly Ca^{2+}-buffered cells (○). *I–V* curves for the current in TEA-PSS and TEA-PSS containing 5 mM 4-AP are shown by squares and triangles for ten and six strongly Ca^{2+}-buffered human mesenteric arterial cells, respectively. The holding potential was −60 mV.

should be noted, however, that removal of Ca^{2+} did not diminish the current if cells were not predepolarized, demonstrating that Ca^{2+} was not acting directly on an extracellular site to affect the current. These results suggest strongly that this potentiation was due to an activation of Ca^{2+}-dependent K^+ channels by intracellular Ca^{2+} which had entered cells as a result of the opening of voltage-gated Ca^{2+} channels.

The data presented above showed that in the presence of low EGTA in the pipette solution the K^+ current, especially at positive potentials, was dominated by a Ca^{2+}-activated K^+ current.

Potassium currents in strongly Ca^{2+} buffered cells

In order to characterize outward current components which were not Ca^{2+} sensitive, we used a pipette solution containing 10 mM EGTA and 10 mM BAPTA in an attempt to minimize the basal and stimulated internal Ca^{2+} concentration. Figure 6*A* illustrates the outward current observed with this pipette solution at several potentials, during prolonged membrane depolarizations in normal PSS, TEA-PSS and in the presence of both TEA and 5 mM 4-AP. The current in normal PSS differed from that observed in weakly Ca^{2+}-buffered cells in that STOCs were absent, and also in that the noisy current appeared at more positive potentials and was significantly reduced in amplitude, as shown in Fig. 6*B* (compare also Figs 6*A* and 3*B*). The mean *I–V* curves for the current measured at the end of 5 s depolarizations in twenty-six weakly and fourteen strongly Ca^{2+}-buffered cells (● and ○, respectively) are shown. Increasing the Ca^{2+} buffering capacity of the pipette solution resulted in a diminution in the outward current at all positive potentials, causing in effect an approximately 30 mV positive shift in the activation of the noisy current. TEA (30 mM) suppressed the current by only 51% at +50 mV and 77% at +80 mV in contrast to 90 and 93% at the same potentials in weakly Ca^{2+}-buffered cells (see above). In marked contrast to the overall outward current in weakly Ca^{2+}-buffered cells, which was negligibly affected by 4-AP, the TEA-insensitive current in strongly Ca^{2+}-buffered cells was very much reduced by 5 mM 4-AP (lower traces △).

When examined on a faster timebase, the current in strongly Ca^{2+}-buffered cells demonstrated complex kinetics. Figure 7 illustrates the outward current evoked during 100 ms depolarizations in PSS, TEA-PSS, and TEA-PSS plus 5 mM 4-AP. In PSS (upper traces in each panel), depolarization to between −30 and 0 mV caused a slowly activating current. With depolarizations to between +10 and +30 mV, the current activated rapidly, and then decayed within about 50 ms to a steady level. Increasingly larger current fluctuations were observed with progressively greater depolarizations. The effect of 30 mM TEA (middle traces in each panel), was to abolish the noisy, fluctuating current, clearly revealing an underlying current which at positive potentials demonstrated both transient and sustained components. TEA appeared to have little effect on either transient or sustained components (see, for example, steps to +20 and +50 mV). 4-AP blocked the TEA-insensitive current over the entire potential range studied, to the extent that in its presence a substantial inward calcium current was seen (lower traces in each panel).

It is therefore apparent that the TEA-sensitive current, which we assumed to be carried by Ca^{2+}-activated K^+ channels, was substantially but not completely suppressed in strongly Ca^{2+}-buffered cells. Conversely, the TEA-insensitive current was similar in both weakly and strongly Ca^{2+}-buffered cells (compare Figs 4 and 6). In subsequent studies of this residual outward current, we routinely minimized the Ca^{2+}-activated K^+ current by using the pipette solution with 10 mM EGTA and 10 mM BAPTA and by using TEA-PSS as the bathing solution.

Under these conditions membrane depolarization from the holding potential of

−80 mV to between −30 and 0 mV elicited a slowly activating and sustained outward current (Fig. 8*Aa*). Further depolarization evoked a rapidly activating and inactivating current which appeared to be superimposed upon the sustained current. We refer hereafter to the rapidly activating and inactivating component of the TEA-

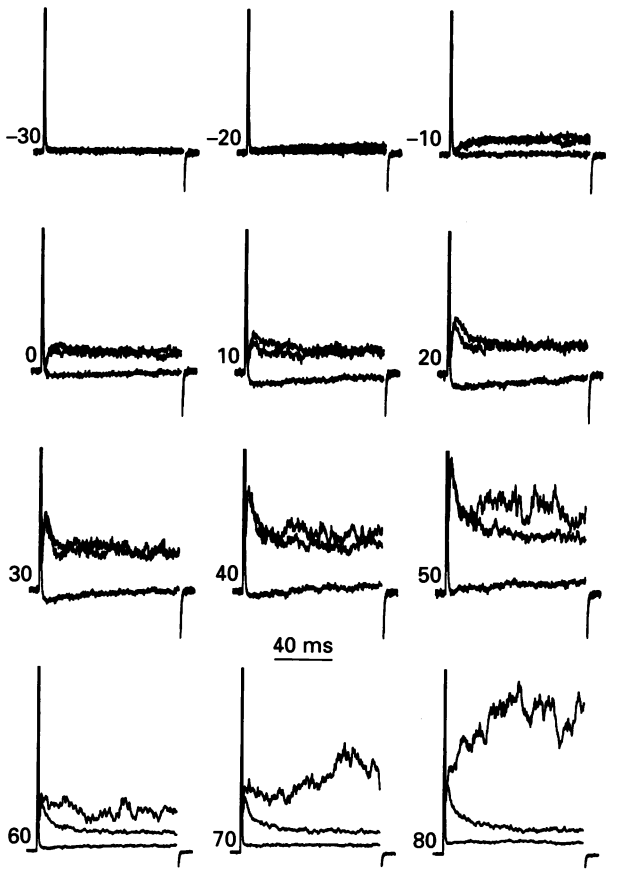

Fig. 7. A family of outward currents recorded at the potentials indicated in normal PSS, TEA-PSS and TEA-PSS containing 5 mM 4-AP (upper, middle and lower traces in each record, respectively). The holding potential was −60 mV. Vertical bars are 100 pA. The pipette solution contained 10 mM EGTA and 10 mM BAPTA.

insensitive outward current as the transient current; the amplitude of this component was always measured at its peak or immediately following the decay of the capacitive artifact. The slowly inactivating component of the outward current which remained following the decay of the transient current, and which appeared to activate negative to the transient current, will be referred to as the sustained current. The amplitude of this current was measured after 300 ms or 2·2 s depending upon the voltage protocol. Average *I–V* curves for the transient and sustained components of current suggest that the sustained component activated in a more negative potential range than did the transient component (Fig. 8*Ab*). It is noteworthy that the amplitude of the transient current increased much more steeply with progressive depolarization

than did that of the sustained current. Both components of current were similar, however, in that they were inactivated at a holding potential of −20 mV (Fig. 8*B*, note the presence at this holding potential of a small residual Ca^{2+} current which was abolished by 0·2 mM Cd^{2+}). Also, both components were markedly blocked by 5 mM

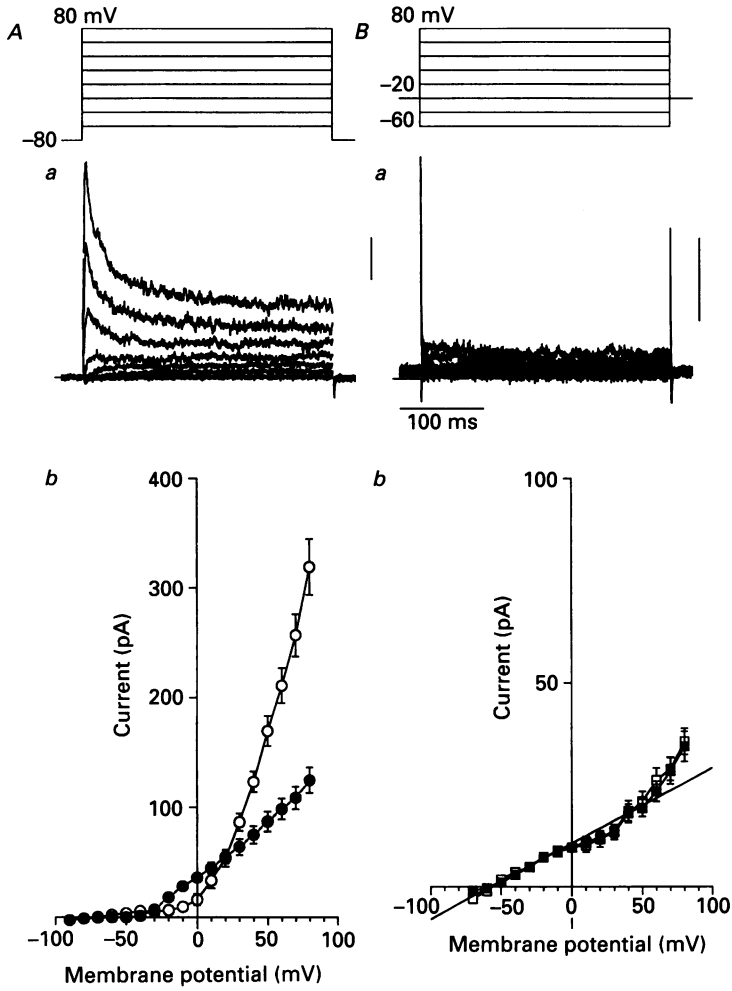

Fig. 8. The TEA-insensitive outward current evoked from holding potentials of −80 and −20 mV. *A*, the membrane potential was stepped by 20 mV increments from a holding potential of −80 mV, giving rise to the current traces shown in *Aa* and the current–voltage relationships for thirteen cells shown in *Ab* for the transient (○) and sustained (●) currents. *B*, currents and *I–V* curves (twelve cells) elicited from a holding potential of −20 mV measured at the same times. Vertical bars are 100 pA, horizontal lines indicate zero current level. *Aa* and *Ba* obtained from the same cell. The pipette solution contained 10 mM EGTA and 10 mM BAPTA.

4-AP (Figs 6 and 7). Therefore, the question arose as to whether these components of outward current reflected the complex kinetic behaviour of a single type of K^+ channel, or the presence of more than one type of channel.

In an attempt to answer this question we analysed the dose dependency of the effect of 4-AP on the TEA-insensitive current using voltage steps during which the digitization rate was varied in order that both the peak of the rapidly inactivating component and the current at the end of 2 s depolarization, where the outward

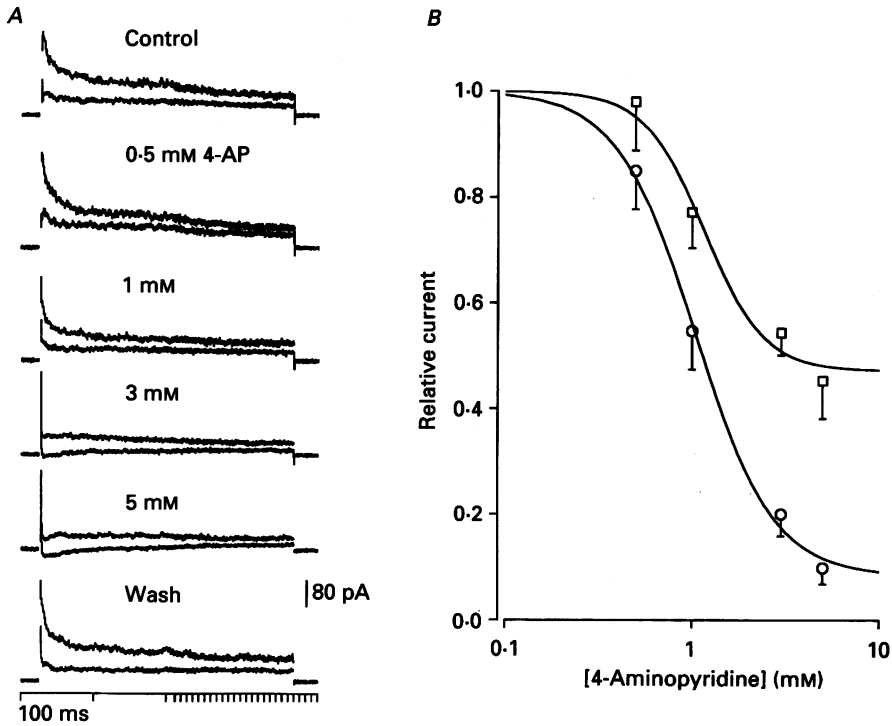

Fig. 9. Effect of 4-AP on outward currents in strongly Ca^{2+}-buffered cells incubated in TEA-PSS. *A*, currents elicited by 2·2 s pulses to +20 and +60 mV from a holding potential of −60 mV in the presence of different concentrations of 4-AP as indicated. Note that the vertical ticks on the time scale below the record always represent 100 ms; the rate of data acquisition was altered after 200 ms. *B*, dose dependence of the blockade of the TEA-insensitive transient (○) and sustained (□, measured after 2·2 s) current by 4-AP, obtained from three to five cells. The ordinate represents the relative current determined as a ratio of the current amplitude recorded in the presence of 4-AP to the amplitude represented in the absence of drug. Continuous lines were drawn according to the following equation:

$$I = \frac{(1-C)}{1+(K_d/[4-\mathrm{AP}])^N} + C,$$

where I is the relative current. The apparent dissociation constants (K_d), and N, the Hill coefficients, were equal to 1·04 and 1·16 mM, and 2·1 and 2·7 for the transient and sustained currents, respectively. The fraction of the current which was insensitive to 4-AP (C) was 0·08 for the peak and 0·47 for the sustained current.

current had decayed almost to a steady level (see Fig. 7*A*), could be measured. Figure 9*A* represents the effect of several concentrations of 4-AP on the transient and sustained current at test potentials of +20 and +60 mV. The normalized

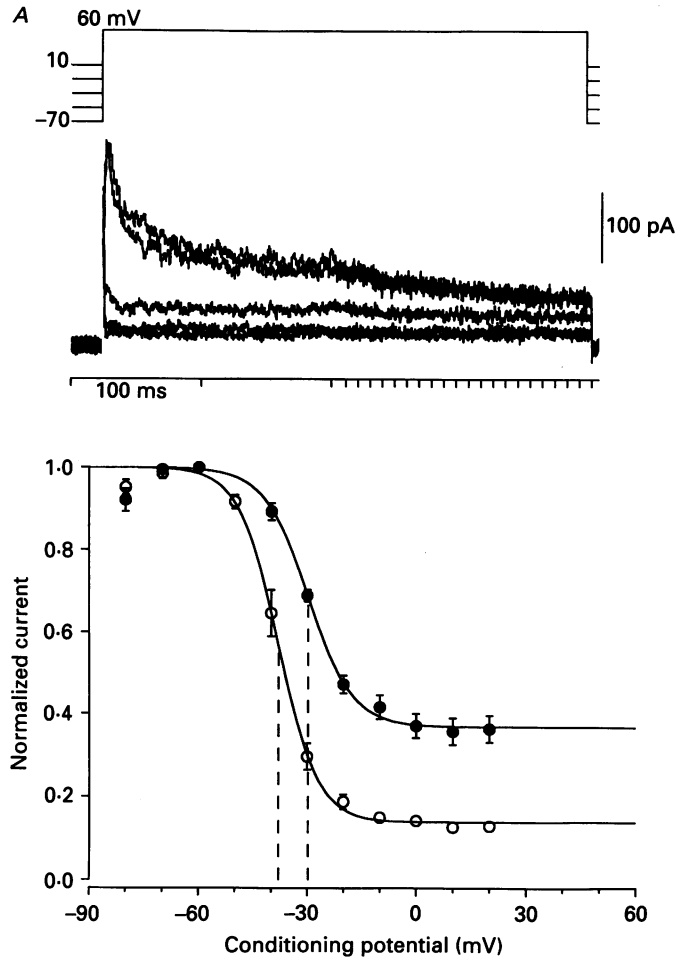

Fig. 10. Steady-state inactivation of transient and sustained currents in strongly Ca^{2+}-buffered cells. *A*, currents recorded using the voltage protocol shown above. Note the time scale is altered at 200 ms. *B*, average availability for the transient (○) and sustained (●) currents in ten cells studied. The test pulse was +60 mV. The current amplitude was normalized with respect to the current at the conditioning potential of −60 mV. Continuous lines are drawn according to the Boltzman function:

$$I = \frac{1-C}{1+\exp((V-V_{0.5})/k)} + C$$

with half-inactivation potentials ($V_{0.5}$) of −38 and −29·7 mV; slope factors (k) of 5·5 and 6·2 mV, and fraction of non-inactivating current (C) of 0·14 and 0·37 for the transient and sustained currents, respectively.

dose–response curves for the effect of 4-AP at a test potential of +60 mV are shown in Fig. 9*B*. 4-AP blocked both components with a similar potency. The apparent K_d values were 1·04 and 1·16, and the Hill coefficients were 2·1 and 2·7, for the peak and sustained currents, respectively, suggesting a co-operative effect of at least two drug molecules in channel blockade in both cases. The effect of 4-AP was completely

reversible (Fig. 9*A*) and was little affected by increasing the test potential to +80 mV (K_d values were 1 and 1·02 mM and the Hill coefficients were 1·7 and 2·7 for the peak and sustained currents).

To study the availability of these currents, cells were stepped to a test potential of +60 mV after being held for 30 s at conditioning potentials between −80 and +20 mV (Fig. 10*A*). Again, the digitization rate was altered during the voltage step. Figure 10*B* illustrates that the availability curve for the peak current was slightly negative of that for the sustained current. The 8 mV difference between the midpoints ($V_{0.5}$) of the curves was significant.

An additional suggestion that these components of the outward current were carried by different sets of channels was provided by experiments in which their recovery from inactivation was examined. Cells were held at −20 mV, which almost completely inactivated both components of the outward current, and then stepped for various periods to −100 mV to relieve inactivation (Fig. 11*A*). The degree to which inactivation had been removed was then assessed during a 300 ms test pulse to +80 mV after a brief (3 ms) step to −20 mV to reduce the size of the capacitive artifact. The recovery from inactivation for both components was monoexponential and almost complete within about 2 s. However, the transient current recovered more quickly than did the sustained current (Fig. 11*A*). The average time dependence of the recoveries of the transient and sustained currents from inactivation in ten cells is illustrated in Fig. 11*B*. The time constants of recovery were 254 and 551 ms, respectively; these values were significantly different.

There is evidence that divalent cations exert a much stronger effect on the transient current (A-current) than on the delayed rectifier current in rat sensory neurones (Mayer & Sugiyama, 1988). Also both Ca^{2+} and Cd^{2+} substantially suppressed a potential-dependent outward current with fast kinetics in smooth muscle cells (Beech & Bolton, 1989; Imaizumi *et al.* 1990; Smirnov *et al.* 1992). Therefore we compared the effect of Ca^{2+}-free TEA-PSS and TEA-PSS with 5 mM Ca^{2+} on the transient and sustained currents. In the presence of 5 mM Ca^{2+} the peak amplitude of the outward current activated by a step to +80 mV from the holding potential of −80 mV was decreased by about 40 % in the cell shown in Fig. 12*Aa* and *Ba* in comparison to that in Ca^{2+}-free solution, while the current at the end of the 300 ms pulse was virtually the same in both solutions. The amplitude of the peak current was suppressed over a range of membrane potentials in high Ca^{2+}; this difference was significant at test potentials of +70 and +80 mV. Conversely, the sustained current was not significantly affected by this alteration of extracellular Ca^{2+} (Fig. 12*Ab* and *Bb*). Figure 12*C* and *D*, shows that the availabilities of the peak and sustained currents were also affected differently by varying extracellular Ca^{2+}. $V_{0.5}$ for the peak current was −43·6 mV in Ca^{2+}-free solution, and was shifted to −30·3 mV in 5 mM Ca^{2+}, while $V_{0.5}$ for the sustained current was −32·8 mV in Ca^{2+}-free solution and was shifted to −26·9 mV in 5 mM Ca^{2+}. This shift was significant for the transient current, but not for the sustained current. Also, the $V_{0.5}$ of the peak current measured at a test potential of +60 mV in 1·5 mM Ca^{2+} (Fig. 10*B*) lay between those obtained in Ca^{2+}-free and 5 mM Ca^{2+} solution, and was significantly different from both, whereas the $V_{0.5}$ for the sustained current was not significantly different from those measured in Ca^{2+}-free and 5 mM Ca^{2+} solution.

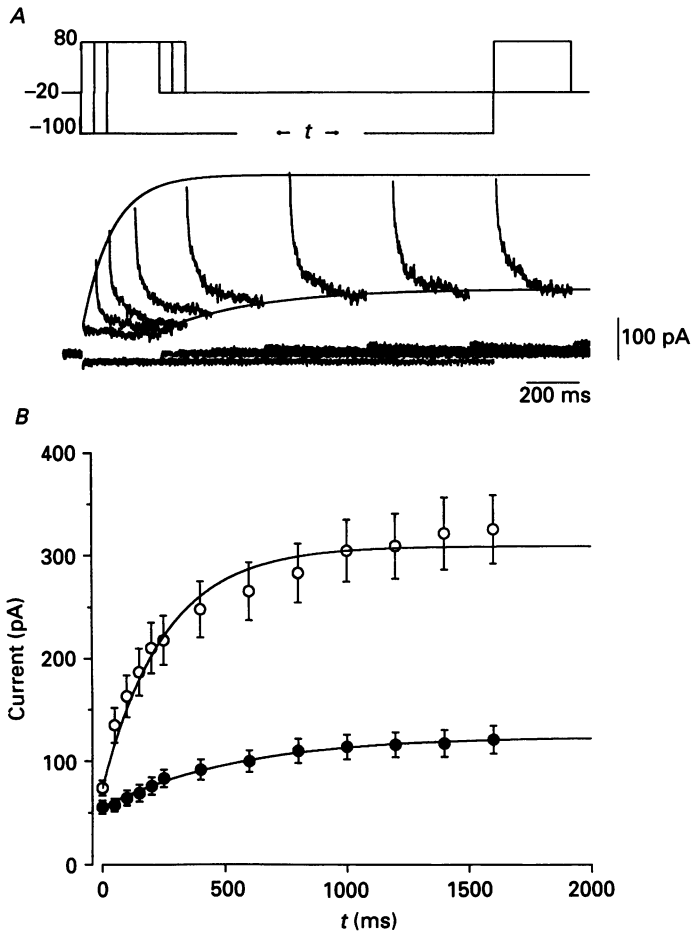

Fig. 11. Recovery from inactivation for transient and sustained currents. The experimental protocol and results obtained from one cell are shown in *A*. The test pulse was to +80 mV and was 300 ms in duration. Conditioning hyperpolarizations illustrated were 0, 50, 100, 200, 400, 800, 1200 and 1600 ms in duration. Continuous lines were drawn according to the equation:

$$I = (A-C)\exp(-t/\tau)+C,$$

with time constants (τ) of 113 and 382 ms for the peak and sustained currents respectively; t represents the duration of the conditioning hyperpolarization. The constant A was determined as the current at the beginning (for the transient current) or at the end (for the sustained one) of the 300 ms test step from the holding potential of −20 mV, without conditioning hyperpolarization. C is the current amplitude at an indefinite time when current recovery is complete. *B*, the averaged data for ten cells studied with the protocol described above. Continuous lines were drawn with mean τ of 254 and 551 ms and C of 310 and 125 pA for the transient and sustained currents, respectively. The pipette solution contained 10 mM EGTA and 10 mM BAPTA.

In three cells tested, 0·2 mM Cd^{2+} caused a diminution of the peak current which amounted to approximately 30% over the voltage range between 0 and +80 mV and which was significant by a paired t test between +50 and +80 mV. This

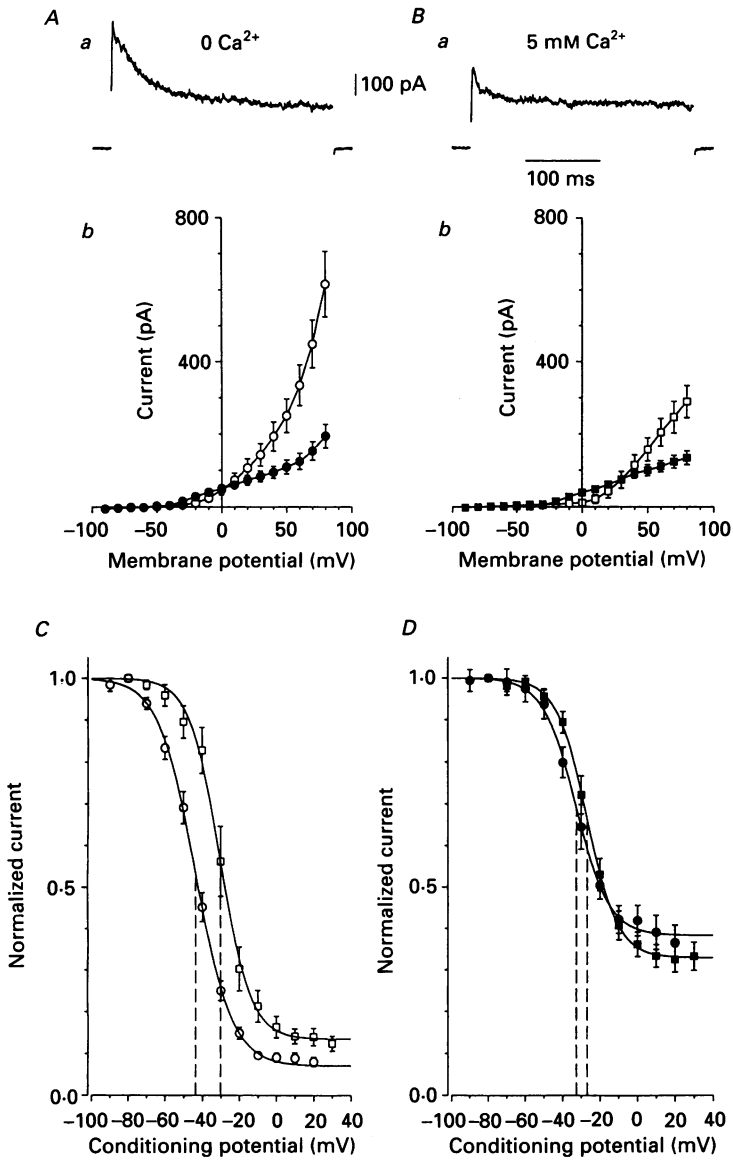

Fig. 12. The effect of Ca^{2+} on the outward current in strongly Ca^{2+}-buffered cells. *A* and *B*, current (*a*) recorded at +80 mV, and average *I–V* relationship (*b*) for the peak current (open symbols) and the current at the end of a 300 ms pulse (filled symbols) obtained for seven cells in Ca^{2+}-free TEA-PSS (*A*) and eight cells in TEA-PSS containing 5 mM Ca^{2+} (*B*). *C* and *D*, normalized availability–potential relationships measured as described in the legend to Fig. 10 in Ca^{2+}-free (circles) and 5 mM Ca^{2+}-containing (squares) TEA-PSS for the transient (*C*) and sustained current (*D*). Continuous lines were drawn according to the equation described in the legend to Fig. 10*B* with $V_{0.5}$ of −43·6 and −32·8 mV, slope factor *k* of 9·8 and 8·9 mV, and a constant value *C* of 0·07 and 0·38 for the transient and sustained current in Ca^{2+}-free solution respectively. In the presence of 5 mM Ca^{2+} values of $V_{0.5}$, *k* and *C* were −30·3 and 8·2 mV, and 0·13 for the transient and −26·9 and 8·3 mV, and 0·33 for the sustained currents correspondingly.

concentration of Cd^{2+} had no effect on the amplitude of the sustained current at any potential (data not shown).

Effects of TEA and 4-AP on the membrane potential under current clamp

Our results show that isolated human mesenteric arterial cells possess a powerful system of K^+ currents which would be responsible for the strong outward-going

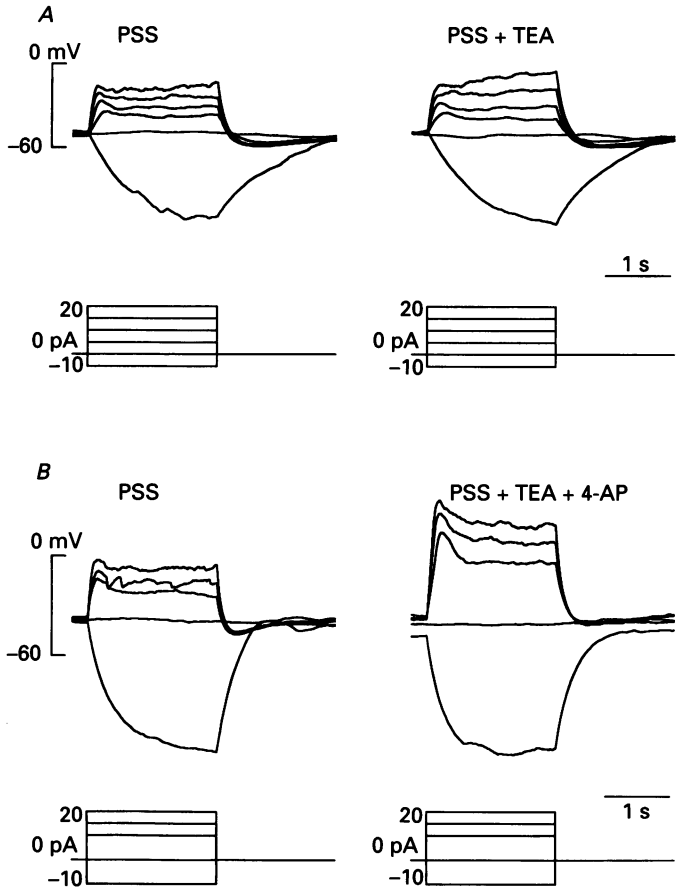

Fig. 13. Effect of TEA (*A*) and TEA+4-AP (*B*) on the membrane potential changes elicited by hyperpolarizing and depolarizing current injection through the recording pipetted filled with 0·2 mM EGTA high-K^+ solution. Current protocol is shown at the bottom of each panel. Both TEA and 4-AP were used at concentrations of 5 mM.

rectification observed in current clamp experiments and which would presumably render these cells non-excitable under physiological conditions. In order to determine whether it was possible to evoke action potentials during suppression of these K^+ currents, we examined the effect of TEA and 4-AP under current clamp. When the pipette was filled by a low-EGTA solution addition of 5 mM TEA to the bath solution partially removed the rectification (Fig. 13*A*) observed during depolarizing current injection. TEA also blocked the fluctuations in the membrane potential observed

during depolarization (not shown), suggesting that these are due to the activation of groups of Ca^{2+}-activated K^+ channels that would, in voltage clamp, be manifested as STOCs. When 5 mM 4-AP was additionally present in the external solution the outward-going rectification was markedly suppressed and action potentials could be recorded (Fig. 13*B*). Four of six cells studied generated action potentials in the presence of TEA and 4-AP. In three of eleven cells studied, a transient hyperpolarization was clearly observed following the end of the depolarizing current injection; this was blocked by 4-AP. These results suggest that the 4-AP-sensitive outward current is mainly responsible for suppression of action potentials under these conditions. This current is also likely to be important in the after-hyperpolarization, which was little affected by TEA but was suppressed in the presence of both K^+ channel blockers.

DISCUSSION

The studies described above distinguish three types of K^+ current in human mesenteric arterial cells. These include a Ca^{2+}-sensitive current, a transient voltage-sensitive current, and a sustained voltage-sensitive current. Separation of these latter types of current was difficult, and must presently be regarded as somewhat tentative.

When cells were dialysed with an intracellular solution containing 0·2 mM EGTA, the dominant current, especially at positive potentials, was a fluctuating current upon which were superimposed spontaneous transient outward currents (STOCs) similar to those previously described in other vascular and intestinal SMCs (Mitra & Morad, 1985; Benham & Bolton, 1986; Benham *et al.* 1986; Ohya *et al.* 1986, 1987; Ganitkevich & Shuba, 1988; Bolton & Lim, 1989; Hume & Leblanc, 1989; Clapp & Gurney, 1991; Volk, Matsuda & Shibata, 1991; Zholos *et al.* 1991). Several properties of this current suggested that it was Ca^{2+}-activated. Firstly, its characteristic high noise, and relatively small tendency to inactivate during either prolonged depolarizing conditioning potentials or at very positive test potentials are reminiscent of Ca^{2+}-activated K^+ currents which have been previously described in other preparations, and which are thought to be carried by large-conductance Ca^{2+}-activated K^+ channels (e.g. Benham *et al.* 1986). Secondly, both TEA and charybdotoxin blocked this current in the concentration ranges expected for this type of channel (Beech & Bolton, 1990). As expected, TEA also inhibited STOCs. 4-AP, which shows some selectivity for purely voltage-gated over Ca^{2+}-activated K^+ channels (Rudy, 1988), had little effect on this current at the relatively high concentration of 5 mM. Thirdly, the current was transiently but markedly potentiated by a brief exposure to caffeine, as were STOCs. This would be caused by the release of Ca^{2+} from the sarcoplasmic reticulum and consequently a rise in the intracellular Ca^{2+} concentration. Fourthly, conditioning potentials expected to give rise to a sustained Ca^{2+} influx through voltage-gated Ca^{2+} channels increased the amplitude of this current measured at +80 mV (at which potential minimal Ca^{2+} influx would be expected to occur during the test pulse itself, see Smirnov & Aaronson, 1992), but only if Ca^{2+} was present in the bathing solution. This effect was not due to an action of Ca^{2+} at an extracellular site, since Ca^{2+} removal did not affect the current evoked from a holding potential of −60 mV. The most likely explanation

for this effect was that Ca^{2+} influx occurring during the conditioning potential step caused a rise in intracellular Ca^{2+}, which then opened the Ca^{2+}-sensitive K^+ channels. These results also suggest that the resting Ca^{2+} influx at -60 mV was too small to increase the intracellular Ca^{2+} concentration enough to stimulate these channels. Also, it is noteworthy that this current was markedly, but not completely, suppressed in cells dialysed with a pipette solution containing 10 mM EGTA and 10 mM BAPTA. This reduction was manifested as a parallel positive shift of approximately 30 mV of the outward current *I–V* curve.

The activation of the Ca^{2+}-sensitive K^+ current in strongly Ca^{2+}-buffered cells occurred in a sufficiently depolarized range to allow us to observe the presence of a much smaller component of outward current which could alternatively be visualized if weakly Ca^{2+}-buffered cells were exposed to a high TEA concentration. It was possible, using strongly Ca^{2+}-buffered cells, to demonstrate that this current component was quite insensitive to TEA (Fig. 7), showing little block even at 30 mM of this drug. We therefore characterized this current in the presence of TEA-PSS and strongly Ca^{2+}-buffered pipette solution.

The TEA-insensitive current activated slowly to a constant level at negative potentials. With depolarization beyond $+10$ mV, a rapidly activating and inactivating component of current was superimposed on the sustained current. The properties of the transient current, including its rapid activation and decay, its sensitivity to 4-AP but not TEA, and its voltage ranges of activation and steady-state inactivation, suggest that it falls into the category of 'A-like' K^+ currents (Rogawski, 1985; Rudy, 1988). Other rapidly activating and inactivating currents have recently been observed in smooth muscle. These have tended to activate and inactivate in a more negative potential range than the transient current described in the present report and have therefore been categorized as true A-currents (Beech & Bolton, 1989; Lang, 1989; Imaizumi *et al.* 1990; Clapp & Gurney, 1991; Smirnov *et al.* 1992). The negative range of inactivation of these transient outward currents, as well as their selective blockade by 4-AP in some types of cell (Smirnov *et al.* 1992), have facilitated the separation of this type of current from other purely voltage-gated K^+ currents such as the delayed rectifier (e.g. Beech & Bolton, 1989; Imaizumi *et al.* 1990; Clapp & Gurney, 1991; Smirnov *et al.* 1992). In human mesenteric arterial cells, however, both the relatively positive voltage range over which the transient current inactivated, and the apparent non-selective action of 4-AP on the transient and sustained components of the TEA-insensitive current, made a separation based on these approaches alone less convincing. Although the kinetic profile of the TEA-insensitive current suggests the presence of more than one K^+ current, it is noteworthy that in experiments in which A-type channels from rat brain have been expressed in oocytes, the current through a single genetically homogeneous channel population can give rise to multiple exponential components of current decay (Pak, Baker, Covarrubias, Butler, Ratcliffe & Salkoff, 1991). We did, however, make several observations which indicated that the transient and sustained currents were carried by different sets of channels. The voltage range of inactivation for the transient current was significantly more negative than that for the sustained current. Also, the transient current recovered more quickly from inactivation than did the sustained current. Ca^{2+} and Cd^{2+} ions exerted significant effects on the amplitude and availability of the peak, but not the sustained, current. These data therefore suggest

strongly that the transient and sustained components of the TEA-insensitive outward current represent current flowing through at least two populations of K^+ channels. The kinetics, sensitivity to 4-AP, and voltage ranges of activation and inactivation of the sustained component of TEA-insensitive current were similar to those described for delayed-rectifier K^+ currents in other vascular smooth muscle cells (Beech & Bolton, 1989; Volk *et al.* 1991), although the amplitude of this current is very small compared to delayed-rectifier K^+ currents in other types of vascular smooth muscle. Conversely, the A-like current in these cells activates and inactivates at more positive potentials than voltage-gated transient currents studied in other smooth muscle cells, although it is of similar amplitude (e.g. Beech & Bolton, 1989; Clapp & Gurney, 1991).

Most of the transient outward currents which have recently been described in smooth muscle cells are sensitive to Ca^{2+}. Beech & Bolton (1989) reported that increasing Ca^{2+} from 1 to 25 mM greatly reduced the amplitude of the A-current in rabbit portal vein cells, while reduction of Ca^{2+} to 40 μM had little effect on amplitude. Smirnov *et al.* (1992) on the other hand reported that decreasing extracellular Ca^{2+} markedly increased the amplitude of the A-current in newborn rat ileum longitudinal muscle. In both studies, lowering Ca^{2+} caused hyperpolarizing shifts of the voltage range of inactivation of this current which were larger than the shift observed in human mesenteric arterial cells. Imaizumi *et al.* (1990) reported suppression by submillimolar concentrations of Cd^{2+} of the transient outward current in guinea-pig ureter cells. The transient outward current in rabbit pulmonary artery was also reduced by a similarly low concentration of Cd^{2+}, leading to the suggestion that it was at least partly dependent upon intracellular Ca^{2+} (Clapp & Gurney, 1991). A unifying hypothesis to explain the suppressive effects of both Cd^{2+} and Ca^{2+} described in these reports and in Fig. 12 of the present report is that these were due to a divalent cation-dependent shift of the activation curve for these A-currents analogous to that described by Mayer & Sugiyama (1988) in cultured rat sensory neurones; an explanation of this type has been invoked by Beech & Bolton (1989) to explain the apparent inhibitory affect of high Ca^{2+} on the transient outward current of rabbit portal vein.

It is noteworthy that BRL 38227, the active enantiomer of the K^+ channel agonist cromakalim, induced a small quasi-instantaneous, non-inactivating K^+ current in human mesenteric arterial cells (Russell, Smirnov & Aaronson, 1992). This suggested, by analogy to other tissues (Standen, Quayle, Davies, Brayden, Huang & Nelson, 1989), that ATP-sensitive K^+ channels may also be present in these cells.

Our results suggest that human mesenteric arterial cells are unlikely to be excitable under basal conditions. We never observed net inward currents or action potentials in the absence of K^+ channel blockers. Our current clamp studies demonstrated that suppression of the Ca^{2+}-activated K^+ channel activity alone was insufficient to allow the firing of action potentials in these isolated cells. Action potential activity could be elicited, however, in the presence of 4-AP and TEA, suggesting that the currents suppressed by the former may play some role in suppressing cell excitability. This may reflect to some extent the observation that the A-like current appears to activate more rapidly than does the Ca^{2+}-dependent K^+ current (compare currents elicited by steps to potentials positive of -20 mV in Figs

3*A* and 6). The relatively positive voltage ranges over which the A-like current activates and inactivates in these cells, compared to A-currents in excitable cells, may underlie a role for this current in suppressing excitability rather than regulating action potential frequency. A full description of the interplay of the various K^+ currents and voltage-gated Ca^{2+} currents (Smirnov & Aaronson, 1992) in these cells during excitation–contraction coupling must await additional electrophysiological studies in intact arteries, and at normal physiological temperatures.

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