A VOLTAGE-DEPENDENT OUTWARD CURRENT WITH FAST KINETICS IN SINGLE SMOOTH MUSCLE CELLS ISOLATED FROM RABBIT PORTAL VEIN

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SUMMARY

1. Single smooth muscle cells were isolated enzymatically from the rabbit portal vein. They were voltage-clamped at room temperature using the whole-cell configuration of the patch-clamp technique.

2. When cells were bathed in physiological salt solution, depolarization from a holding potential of -70 mV elicited a time-dependent outward current which reached a maximum within $0.2-0.5$ s, but when a more negative holding potential was used, an additional outward current could be activated. The current (I_{fo}) developed rapidly, was transient and seemed to be carried by potassium ions (K^+).

3. The steady-state inactivation plot for I_{fo} was steeply voltage-dependent between -90 and -60 mV, current being 50 % inactivated at -78 mV. The activation threshold was around -65 mV. The activation and inactivation kinetics were fast and voltage-dependent. When the test potential was -35 mV, peak current occurred after about 15 ms and the decay was complete within 250 ms. Recovery from inactivation was maximal after 1 s at -100 mV but was about five times slower at -70 mV.

4. The outward current I_{fo} was blocked completely by 4-aminopyridine (5 mM) or phencyclidine (0.1 mM), but was insensitive to tetraethylammonium ions (32 mM), apamin (0.1 μM), charybdotoxin from the venom of *Leiurus quinquestriatus* (0.1 μM), toxin-I from the venom of *Dendroaspis polylepis* (1 μM) or the putative K^+ channel opener, cromakalim (10 μM).

5. The steady-state inactivation range and activation threshold, kinetics of activation and inactivation all showed a marked dependence on the concentration of divalent cations in the bathing solution. This effect was consistent with the hypothesis that I_{fo} was affected by membrane surface potential. The current did not seem to be Ca^{2+}-activated.

6. I_{fo} closely resembled the A-current which has been described previously in neurones but not in smooth muscle.

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<center>INTRODUCTION</center>

Spike frequency in smooth muscle is related directly to tone (Bülbring, 1955) and shows a strong inverse relationship to the membrane potential (e.g. Bülbring, 1967). The upstroke of the spike is thought to be due to the voltage activation of Ca^{2+} channels and the downstroke to the inactivation of the Ca^{2+} channels and the activation of Ca^{2+}- and/or voltage-activated K^+ channels. The K^+ channels may also generate an after-hyperpolarization. This paper describes an early transient outward current that was activated upon depolarization of single rabbit portal vein smooth muscle cells. These cells exhibit spike activity on slow waves of depolarization in the whole tissue (Holman, Kasby, Suthers & Wilson, 1968). The current resembled the A-current as characterized in snail neurones (Connor & Stevens, 1971; Neher, 1971) and first observed by Hagiwara, Kusano & Saito (1961), and seemed to indicate an additional type of K^+ channel that might be important for the regulation of spike activity in smooth muscle.

A-currents are usually activated only by depolarization from negative of the resting potential, and thus it has been suggested that the role of this current is during the inter-spike interval when the membrane can become hyperpolarized relative to the resting potential. Sufficient evidence has now accrued to establish its importance for the repetitive firing of neuronal cells (e.g. see Connor, 1978). A-currents have been defined on the basis of their negative steady-state inactivation curve and threshold for activation, their sensitivity to block by 4-aminopyridine and the fast kinetics of their activation and inactivation (for reviews see Adams, Smith & Thompson, 1980; Thompson & Aldrich, 1980; Rogawski, 1985). However, some currents that show very similar time dependence and pharmacology, such as those found in rat ventricular cells (Josephson, Sanchez-Chapula & Brown, 1984), may be activated from less negative voltages. These currents may be important for fast spike repolarization (see also Belluzzi, Sacchi & Wanke, 1985). The identification of an A-current may be complicated if a Ca^{2+}-activated current occurs simultaneously (e.g. see Salkoff, 1983; Brown, Constanti & Adams, 1983) or if a sustained outward current is also made available by the negative holding potential (see Dolly, Stansfield, Breeze, Pelchen-Matthews, Marsh & Brown, 1987). This paper describes a study of the properties of the early transient outward current of portal vein smooth muscle using the patch-clamp technique as a voltage-clamp method. An abstract of the work has been presented (Beech & Bolton, 1988).

<center>METHODS</center>

Cell dispersion

Adult male New Zealand White rabbits (2–2·5 kg) were killed by injection of a lethal dose of sodium pentobarbitone (May & Baker Ltd). The main branch of the portal-mesenteric vein was removed and placed in physiological salt solution (PSS) where it was dissected free of fat and connective tissue and cut into small pieces ($\sim 2 \times 3$ mm). Six pieces were incubated at 36 °C in low-Ca^{2+} (20–30 μM) PSS for 10 min and then resuspended in a mixture of papain (5 mg/ml), dithiothreitol (3–5 mM) and bovine serum albumin (2–4 mg/ml) in the low-Ca^{2+} solution for 20–30 min at 36 °C. The pieces were then removed from the mixture and mildly agitated in low-Ca^{2+} solution. The isolated cells were centrifuged (100 g for 1·5 min, the pellet resuspended in PSS (containing 0·8 mM-Ca^{2+}) and the suspension stored at 4 °C on glass cover-slips. Cells were used between 2 and 12 h after isolation.

Solutions and reagents

Physiological salt solution (PSS) in the bath had the following composition (mM): Na^+, 126; K^+, 6; Ca^{2+}, 1·7; Mg^{2+}, 1·2; Cl^-, 138; glucose, 14; HEPES, 10·5; and was titrated to pH 7·2 with NaOH ($\sim$ 4 mM). Pipette (intracellular) solution had the following composition (mM): K^+, 134; Mg^{2+}, 1·2; Cl^-, 136; EGTA, 0·8; glucose, 14; HEPES, 10·5; and was titrated to pH 7·2 with NaOH. When ionic concentrations were adjusted the osmolarity of the solution was maintained, except that when TEA-Cl was added the osmolarity of the solution was increased (see text). For all solutions a contamination of 10 μM-Ca^{2+} was assumed. As the pipette solution contained EGTA (0·8 mM), the concentration of free Ca^{2+} was estimated to be 1 nM. Solutions containing 4-AP (> 1 mM) were slightly alkaline and were titrated to pH 7·2 with HCl.

Papain (type 4), DL-dithiothreitol, bovine serum albumin, tetraethylammonium chloride (TEA) 4-aminopyridine (4-AP), phencyclidine hydrochloride, ethyleneglycol-bis-(β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA) and N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) were all obtained from Sigma. The charybdotoxin, from the venom of the Israeli scorpion *Leiurus quinquestriatus* (for the separation procedure see Castle & Strong, 1986) and the apamin, from *Apis mellificus*, were generously donated by Dr P. N. Strong. Toxin-I, from the venom of the Eastern green mamba snake *Dendroaspis polylepis*, was a gift from Dr J. O. Dolly. The ($\pm$)6-cyano-3,4-dihydro-2,2-dimethyl-trans-4-(2-oxo-1-pyrrollidyl)-2H-benzo[β]pyrano-3-ol (cromakalim, BRL 34915) was from Beecham Pharmaceuticals.

Current recording and analysis

Single cells (dimensions $\approx 20 \times 50$ μm, surface area = 8–10 000 μm^2) were voltage-clamped by using the whole-cell configuration of the patch-clamp technique (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) at room temperature (20–23 °C). Patch pipettes were of borosilicate glass (Corning; o.d. 1·4–1·6 mm, i.d. 0·6–0·8 mm) and had a resistance of 1–4 MΩ (access resistance = 5–10 MΩ). The current amplifier was a List EPC-7 (List-Electronic) and data were recorded on FM tape (Racal) at tape speeds of 3·75 or 7·5 in/s (filtering specifications were 1·25 and 2·5 kHz respectively).

Data were digitized off-line by using a CED 1401 analog-to-digital interface (Cambridge Electronic Design), stored on floppy diskettes and then analysed using a BBC microcomputer in conjunction with the CED 1401. Whole-cell currents were low-pass filtered at 1 kHz (8-pole Bessel; attenuation rate 48 dB/octave) and digitized at 3 kHz, except where indicated. In one instance a digital filter was applied (Fig. 2C). This took the mean of each consecutive group of five points, giving about 50 % attenuation at 350 Hz. Functions (see text) were fitted by using the iterative algorithm of Marquardt & Levenberg (Marquardt, 1963) with the criterion of minimizing the unweighted sum of the squares of the deviations. The goodness of fit was evaluated by eye, except for the straight line where the correlation coefficient was used as a validation procedure. Leakage current was estimated and subtracted in some instances (see legends). It was determined by measuring the current required to depolarize the cell from the holding potential to six different pre-threshold levels (0·5 s step length). Hyperpolarizing steps were not used as the holding potentials used were very negative; steps negative to these tended to generate noisy and variable current. Points were plotted and a straight line was fitted (see Fig. 2B) using a least-squares method. The correlation coefficient was between 0·95 and 0·99 and the gradient and ordinate-intercept were used to estimate the leakage component for that holding potential. The appropriate component was subtracted from the current activated by steps positive to threshold.

RESULTS

Depolarization of cells bathed in physiological salt solution (PSS) to -10 mV from a holding potential (HP) of -70 mV generated a time-dependent outward current that reached a maximum after about 0·4 s (Fig. 1Aa). This current (I_{dK}) seems to represent a delayed rectifier (D. J. Beech & T. B. Bolton, unpublished). Depolarization from more negative HPs revealed an outward current with much faster kinetics of activation that appeared to be carried by a separate channel population. For example, when stepping to -10 mV from a HP of -90 mV, outward

current reached a maximum about twenty times faster than when stepping from −70 mV (Fig. 1*Aa*). Subtraction of the current elicited from the −70 mV HP from that elicited from more negative HPs gave difference currents (Fig. 1*Ab*) that showed clearly that the additional outward current had a transient time course. This paper shows an investigation of the characteristics of this additional current, which will be termed I_{fo}.
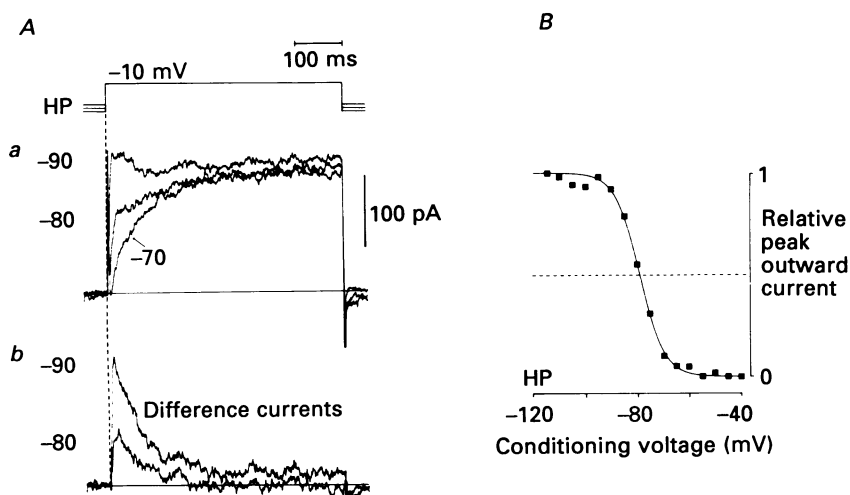

Fig. 1. Current elicited by depolarization from holding potentials (HPs) more negative than −70 mV in single portal vein smooth muscle cells bathed in physiological salt solution (PSS). *Aa*, currents elicited upon stepping to −10 mV for 0·5 s from three HPs (−70, −80 and −90 mV). Leakage current, evaluated for each HP, was subtracted and the horizontal line marks the current level at −70 mV. *Ab*, current elicited from the −70 mV HP subtracted by a digital method from that elicited from the −80 and −90 mV HPs such that the current evoked from −70 mV became the zero line (continuous line). The transient difference currents indicate I_{fo}. *B*, in another cell and using a different voltage protocol, the steady-state inactivation curve for peak I_{fo} on a relative scale. Leakage current was estimated at the HP (−120 mV). The curve was established by applying conditioning voltage steps (duration, 5 s) prior to testing the availability of current at −40 mV every 15 s. The curve represents a fitted Boltzmann equation (see text).

Voltage and time dependence

It will be described below that I_{fo} was strongly affected by changes in the extracellular divalent cation concentration. Thus, I_{fo} was studied in normal Ca^{2+}-containing PSS. Organic Ca^{2+} channel blockers were not used as they are not potent blockers of Ca^{2+} current observed at early times (e.g. see Fox, Nowycky & Tsien, 1987) and show block of K^+ current at high concentrations (Terada, Kitamura & Kuriyama, 1987). The extent to which Ca^{2+} current may have contributed to the net current is discussed for each experiment, although the contamination appeared to be small.

The steady-state inactivation curve for I_{fo} was investigated by using a test potential of −40 mV. This activated I_{fo} but produced little contamination from I_{dK} or from voltage-activated Ca^{2+} current (Ohya, Kitamura & Kuriyama, 1988) as the threshold for activation of these currents is around −40 mV, and thus they should
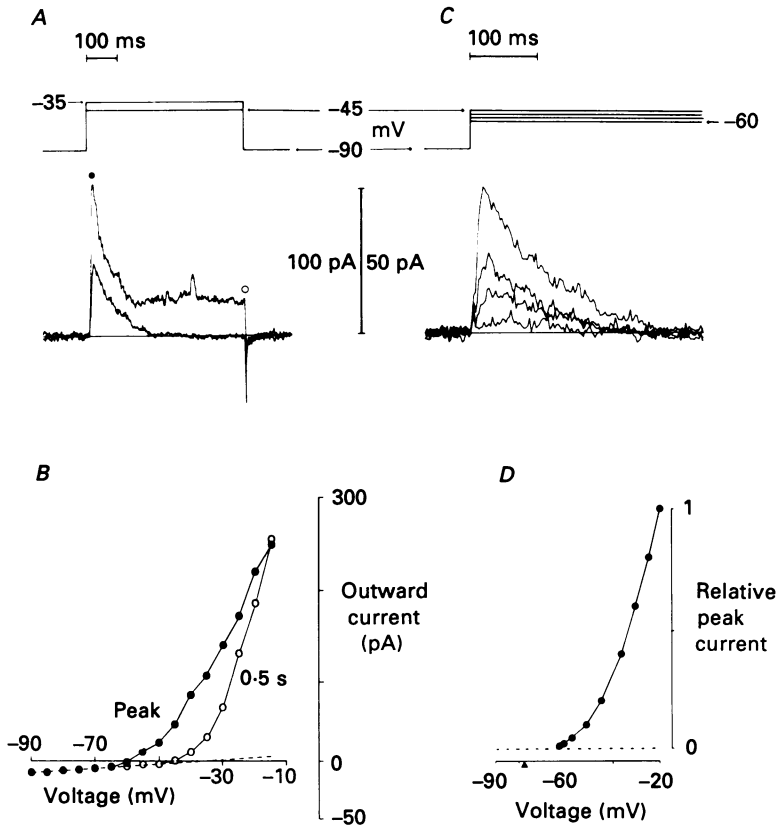
Fig. 2. Threshold for the activation of I_{fo} when in PSS and using a HP of -90 mV. A, leakage subtracted current elicited upon stepping to -45 mV for 0·5 s was mono-phasic and transient but that elicited by stepping to -35 mV was bi-phasic and showed a considerable sustained component. B, threshold for peak current ($\bullet$) was at about -65 mV and for the sustained current at the end of the step ($\bigcirc$, 0·5 s) about -45 mV. Pre-threshold currents, presumably leakage, were fitted by a linear function (dashed line) and were plotted with respect to zero current. C, threshold for I_{fo}. Leakage subtracted currents after a five-point digital filter. Test potentials were -60, -55, -50 and -45 mV and the continuous line marks the current level at the HP. The capacity current (not evident) was complete with 2 ms after the beginning of the command voltage step. D, summary of the threshold for I_{fo} (ten cells). Current was plotted relative to the peak elicited upon stepping to -20 mV. Points were interpolated so that the variation in the voltage to elicit a given current could be exresed as a mean $\pm$ S.E.M. (error bars are within the points). I_{fo} was first appreciable at -65 mV.

be negligible upon stepping to this potential. Long conditioning voltage steps of 5 s were used and the cells were returned to the HP after each test step to ensure that a steady state was established at each potential. I_{fo} was completely inactivated positive of -50 to -60 mV and was maximally available negative of about -90 mV (Fig. 1B). Peak current (I) was plotted against conditioning voltage (V) and the points were fitted by the Boltzmann equation

$$I = (C_1 - C_2)/[1 + \exp((V - V_h)/V_s)] + C_2,$$

where C_1 and C_2 are the maximum and minimum of the curve, V_h the conditioning voltage for 50% inactivation and V_s the slope factor. For Fig. 1B, $C_1 = 1$, $C_2 = 0$, V_h $-79\cdot0$ mV and V_s $4\cdot7$ mV. For four experiments in different cells the mean values ($\pm$ S.E.M.) for V_h and V_s were $-76\cdot8 \pm 2\cdot1$ and $5\cdot1 \pm 0\cdot2$ mV respectively. Thus the steady-state inactivation curve for I_{fo} was almost 50 mV more negative than that for I_{dK} (D. J. Beech & T. B. Bolton, unpublished). Voltage-activated Ca^{2+} current was probably negligible as the current level was almost constant between -60 and -40 mV.

The threshold for activation of I_{fo} was close to -65 mV (Fig. 2C and D). The current was transient upon stepping to -45 mV, exhibiting *no* sustained component. However, stepping to -35 mV elicited a marked sustained outward current at the end of the $0\cdot5$ s step and the shape of the total current was bi-phasic (Fig. 2A). This second component appeared to represent I_{dK} as the threshold was around -40 mV (Fig. 2B). For large voltage steps, positive of about -10 mV, I_{fo} was not distinguishable as a separate phase (not shown). The analysis of the threshold showed that *all* sustained current had a threshold around -40 mV or more positive.

The outward current I_{fo} developed rapidly, the time-to-peak decreasing as the amplitude of the voltage step was increased (e.g. see Fig. 2C). The actual time was variable between cells but the peak occurred after about 50 ms upon stepping to -60 mV and 15 ms when stepping to -35 mV (HP -90 mV). As the time-to-peak was short, the settling time of the membrane voltage was of considerable significance. For an instantaneous command voltage step the settling time course of the membrane voltage was assumed to follow the decay of the capacity current, which was complete within 4 ms or less. Thus, a precise study of the rate of activation of I_{fo} was not attempted. However, I_{fo} clearly activated faster than I_{dK} (Fig. 1A) or the Ca^{2+}-activated K^+ current of intestinal smooth muscle cells (Ohya, Kitamura & Kuriyama, 1987).

The rate and voltage dependence of the decay of I_{fo} was studied after the subtraction of current that was available from -60 mV (see Fig. 3Aa). The rate of decay was quantified by fitting a single exponential to the decay for currents elicited by steps negative to -10 mV. The time constant of the exponential decreased as the amplitude of the voltage step increased (Fig. 3B) and complete inactivation always occurred within about $0\cdot3$ s. For currents elicited by steps positive of -10 mV, single exponentials (time constants shown as stray points in Fig. 3B) were not always a good fit and these currents were described better by bi-exponential functions. The current elicited by the $+20$ mV test step is shown fitted with a bi-exponential and the time constant of the first exponential is shown as a filled symbol (Fig. 3B).

The subtraction procedure described in Fig. 3Aa indicated that a small sustained current component could be activated by large depolarizing steps from -100 mV but not -60 mV (Fig. 3Ab). This current seemed to have a threshold at about -10 mV (not shown). Although I_{dK} is almost 100% available at -60 mV (D. J. Beech & T. B. Bolton, unpublished) some inactivation may occur when holding at this potential for long periods; this would be removed upon hyperpolarization to -100 mV. Thus, although the threshold for the sustained component seemed to be more negative than that for I_{dK} it was not possible to be certain that this current indicated a separate channel type. Peak I_{fo} recovered completely from inactivation
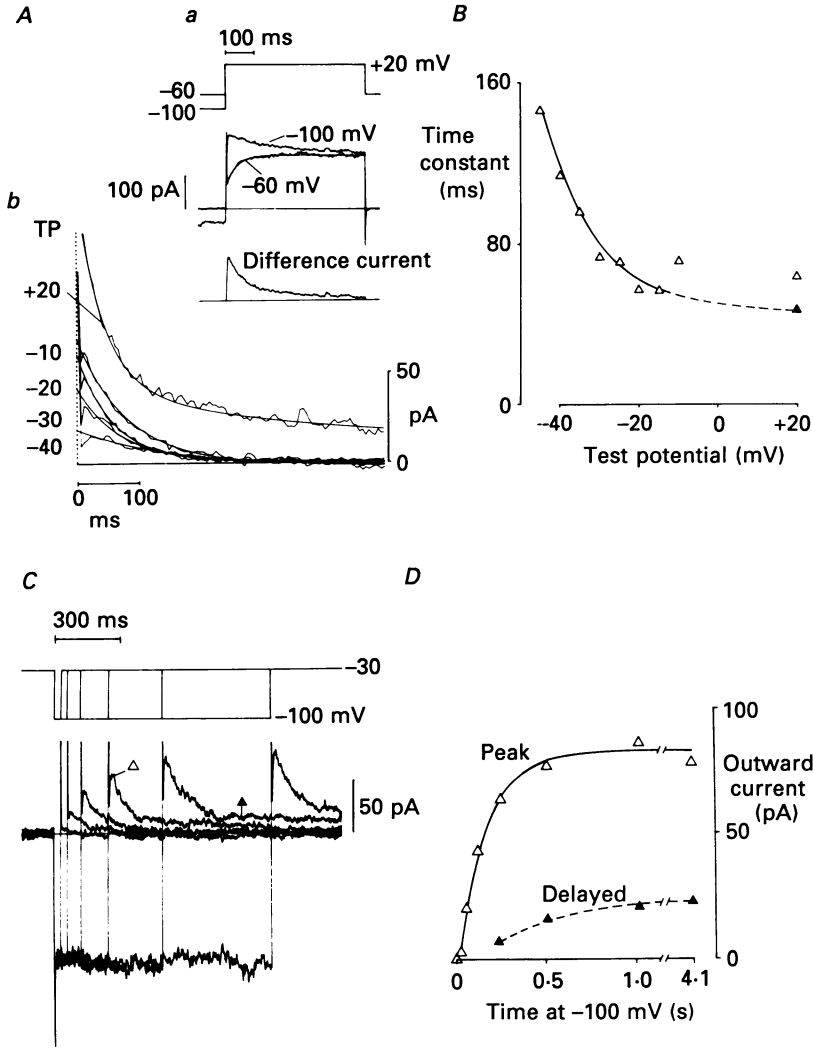
Fig. 3. The time and voltage dependence of the inactivation of I_{fo} when in PSS. *Aa*, subtraction of a single exponential fitted to the current available from -60 mV (shown) from the current available after a conditioning step (duration, 0·3 s) to -100 mV gave the difference current, I_{fo}. Leakage current was not subtracted and the horizontal line marks the current level for the -60 mV HP. *Ab*, difference currents for various test potentials (TP) fitted by single exponentials (TPs -40, -30, -20, -10 mV) or by a bi-exponential (TP $+20$ mV). Current is shown from time zero of the command voltage step. *B*, time constants of single exponentials used to describe the decay of I_{fo} plotted against test potential ($\triangle$). The first time constant of the bi-exponential (TP $+20$ mV) is marked ($\blacktriangle$) and the curve was drawn by eye. *C*, time dependence of the recovery of I_{fo} at -100 mV from complete inactivation at -30 mV. Current activated upon returning to -30 mV is shown for step lengths to -100 mV of 32, 64, 128, 256, 512 and 1024 ms (leakage current not subtracted). *D*, for the same experiment, a single exponential (time constant $= 0·16$ s) fitted to the amplitude of peak I_{fo} plotted with respect to time spent at -100 mV ($\triangle$). Shown also are points for the recovery of the delayed current ($\blacktriangle$) evident in *C*, again fitted by a single exponential (time constant $= 0·32$ s).

within about 1 s at -100 mV (Fig. $3C$ and D). The rate of recovery was described by a single exponential, which had a time constant of 0·16 s (for five cells it was $0·15\pm0·04$ ms, mean$\pm$s.e.m.). Experiments also indicated that the rate was voltage-dependent, being approximately five times slower at -70 mV. Extrapolation of the exponential fitted to the recovery back to zero I_{fo} suggested an initial lag phase of about 30 ms. This could not be due to the settling time of the membrane voltage (see above) and thus seemed to be a characteristic of the recovery process. The sustained (delayed) current component, probably I_{dK}, recovered more slowly than did I_{fo} (compare with Numann, Wadman & Wong, 1987).

Pharmacology

TEA$^+$ has been used commonly to differentiate K$^+$ channel populations (Stanfield, 1983). In smooth muscle cells from the rabbit portal vein, extracellular TEA$^+$ (4 mM) blocks large conductance Ca^{2+}-activated K$^+$ channels completely while leaving I_{dK} almost unaffected (Beech & Bolton, 1987). Although I_{dK} is reduced by higher concentrations of TEA$^+$, I_{fo} showed an even greater lack of sensitivity to block. When current was elicited by stepping to -10 mV from a HP of -90 mV, TEA$^+$ (32 mM) had less effect on the initial peak current than on the sustained current at the end of the 0·5 s test step (Fig. $4A$). However, for a step to -40 mV, which presumably elicited mainly I_{fo}, a larger proportion of peak current was blocked. This may reflect a voltage dependence of TEA$^+$ action on I_{fo}, but the voltage–current relationship (Fig. $4Ab$) indicated that the activation curve for I_{fo} was shifted positively. As the TEA$^+$ solution was hyperosmotic, it seemed likely that the apparent block of I_{fo} was in fact due to a decrease in surface potential (see below). This was supported by the observation that the kinetics of the current at -40 mV were slowed and the steady-state inactivation curve for I_{fo} (not shown) was also shifted positively by 32 mM-TEA$^+$.

4-Aminopyridine (5 mM) induces a marked block of I_{dK} but has little effect on large conductance Ca^{2+}-activated K$^+$ channels (Beech & Bolton, 1987), thus differentiating two K$^+$ channel populations. Both I_{fo} and I_{dK} were reduced similarly by 4-AP with complete block at about 5 mM (Fig. $4B$). However, a separation of the two currents did occur upon wash-out as it was a common observation that block of I_{dK} by 4-AP was variable and difficult to reverse by washing but that block of I_{fo} was consistent and readily reversed by washing. This supported the hypothesis that two distinct channel populations were responsible for I_{fo} and I_{dK}. In the presence of 5 mM-4-AP or 0·1 mM-phencyclidine, which blocks I_{fo} completely (not shown), a net inward current or residual transient outward current was not observed.

The outward current I_{fo} appeared unaffected by the putative K$^+$ channel opener cromakalim (10 μM, Beech & Bolton, 1989), 1 μM-toxin-I (a congener of dendrotoxin) which blocks some types of A-current (Dolly *et al.* 1987), 0·1 μM-charybdotoxin which blocks the large Ca^{2+}-activated K$^+$ channels of smooth muscle cells (Beech, Bolton, Castle & Strong, 1987) or apamin (0·1 μM).

Ion selectivity

The current I_{fo} was outward at all test potentials investigated (-65 to $+20$ mV) which suggested that the main charge carrier was K$^+$. Tail currents for I_{fo} could not
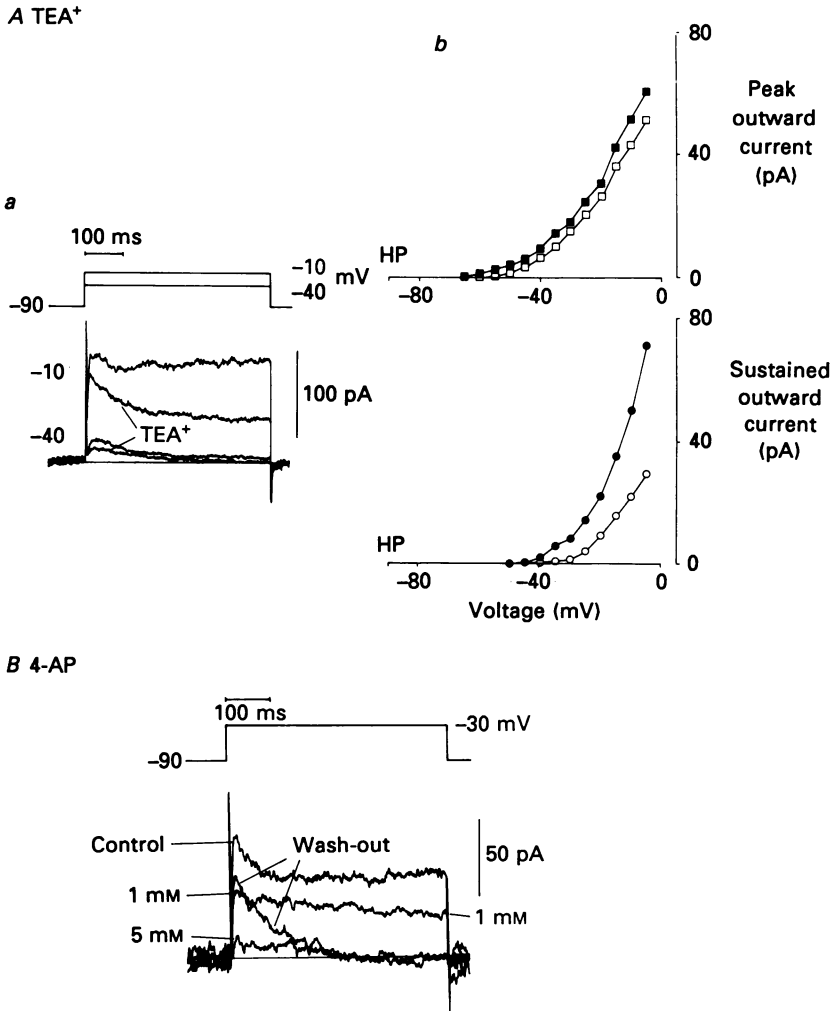
Fig. 4. The effects of bath-applied TEA$^+$ or 4-AP on outward current available from −90 mV for cells bathed in PSS. *Aa*, TEA$^+$, currents elicited by stepping to −40 or −10 mV for 0·5 s, showing the control and when in the presence of TEA$^+$ (32 mM) (marked). *Ab*, in the same cell, voltage–current relationships for initial peak current (control, ■; TEA$^+$, □) and for sustained current at the end of the test step (control, ●; TEA$^+$, ○). *B*, 4-AP, current elicited by stepping to −30 mV for 0·5 s, showing the control, in the presence of 1 and 5 mM-4-AP and after wash-out (marked). Leakage currents were subtracted.

be identified clearly, probably because of their small amplitude; thus, attempts were made to reverse I_{fo} by moving the K$^+$ equilibrium potential (E_K) to a potential positive of the threshold for I_{fo} but where appreciable I_{dK} or Ca^{2+} current would not be activated. When E_K was moved to −45 mV by raising [K$^+$]$_o$ to 22·5 mM and maintaining the same osmolarity of PSS by decreasing [Na$^+$]$_o$, the transient current was much reduced at −35 mV, apparently inward at −55 mV and normal I_{fo} was readily recoverable upon returning E_K to −78 mV (Fig. 5A). Plotting peak current
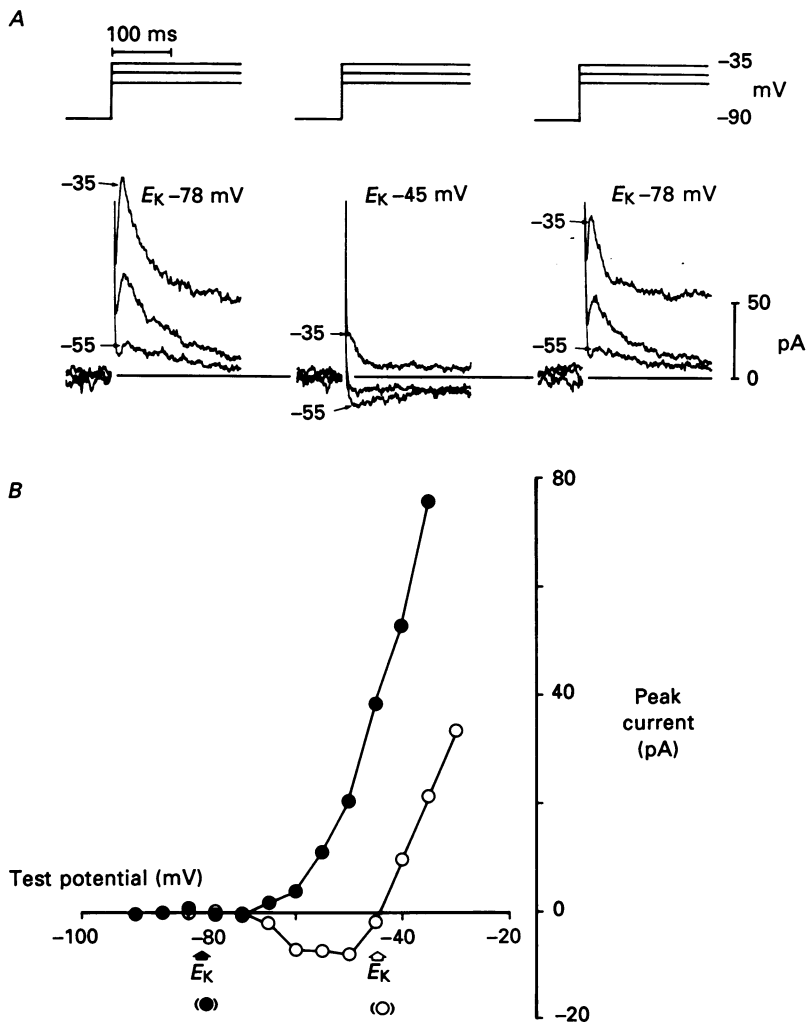
Fig. 5. Dependence of I_{fo} on $[K^+]_o$. *A*, currents elicited by stepping from a HP of -90 mV to -55, -35 (marked) and -45 mV from a cell in PSS (6 mM $[K^+]_o$, E_K -78 mV), in raised K^+–PSS (22·5 mM $[K^+]_o$, E_K -45 mV) and after returning to PSS. The horizontal line marks the current level at the HP. *B*, peak current (mean for three experiments) plotted against test potential for E_K at -78 mV (●) and at -45 mV (○). Values are for peak I_{fo} in the control and the estimated equivalent when E_K was -45 mV. Leakage currents were subtracted.

for the mean of three experiments (Fig. 5*B*) showed clearly that inward current activated with a threshold at -65 mV when E_K was at -45 mV. This current became outward just positive of -45 mV, indicating a reversal potential at -43 mV. The results suggested that I_{fo} was strongly dependent on $[K^+]_o$ and that the main charge carrier was K^+. A Cl^- permeability change or a contamination by voltage-activated Ca^{2+} current would make the reversal potential for I_{fo} appear positive of -45 mV. Thus the data showed that the contribution of Cl^- or Ca^{2+} flux to I_{fo} was minimal.
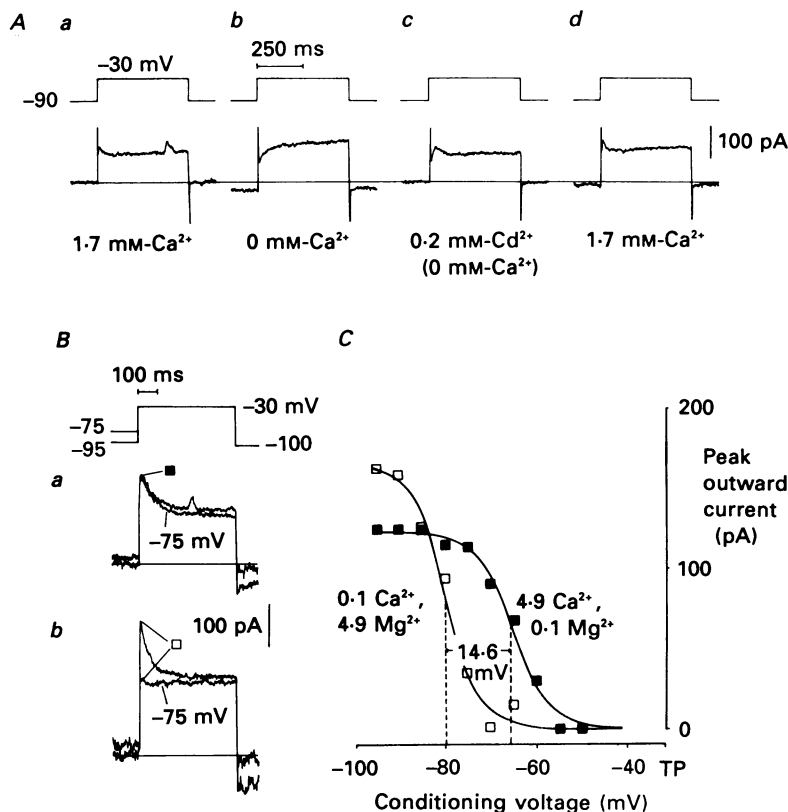
Fig. 6. Effect of $[Ca^{2+}]_o$ on I_{fo}. *A*, outward current elicited by stepping to -30 mV for 0·5 s from a HP of -90 mV. The bathing media were: *a*, PSS (1·7 mM-Ca^{2+}), *b*, Ca^{2+}-free PSS, *c*, after the addition of Cd^{2+} (0·2 mM) to Ca^{2+}-free PSS and *d*, upon returning to PSS (marked). *B* and *C*, the effect of decreasing Ca^{2+} while retaining a constant divalent cation concentration with Mg^{2+}. The HP was -100 mV, the test potential -30 mV, the condition step duration 5 s and the cell was returned to the HP for 9·5 s after each test step. The bathing solutions in *B* were PSS, containing either (mM): Ca^{2+}, 4·9; Mg^{2+}, 0·1 (*Ba*), or Ca^{2+}, 0·1; Mg^{2+}, 4·9 (*Bb*). *B*, currents are shown for the -75 and -95 mV conditioning steps; the horizontal line marks the current level at -95 mV. *C*, the steady-state inactivation curve for peak I_{fo} in the two solutions (as marked, concentrations in mM). Zero current is that which did not inactivate. The fitted function is the Boltzmann equation (see text). V_h and V_s values (in millivolts) were -65 and 4 (■) and $-79·6$ and 3·6 (□) respectively. Leakage current was not subtracted.

Influence of $[Ca^{2+}]_o$

When Ca^{2+} in the bathing solution was omitted and replaced by an equimolar amount of Na^+, I_{fo} seemed to be abolished (Fig. 6*Ab*). The increase in time-independent current (leakage) was probably due to a decrease in seal resistance, which is dependent on the presence of divalent cations. However, I_{fo} did not appear to depend on Ca^{2+} influx, as the addition of 0·2 mM-Cd^{2+} to the Ca^{2+}-free PSS, a procedure used to block completely voltage-activated Ca^{2+} channels (e.g. see Fox *et al.* 1987), caused I_{fo} to return, albeit with slower kinetics. Upon returning to
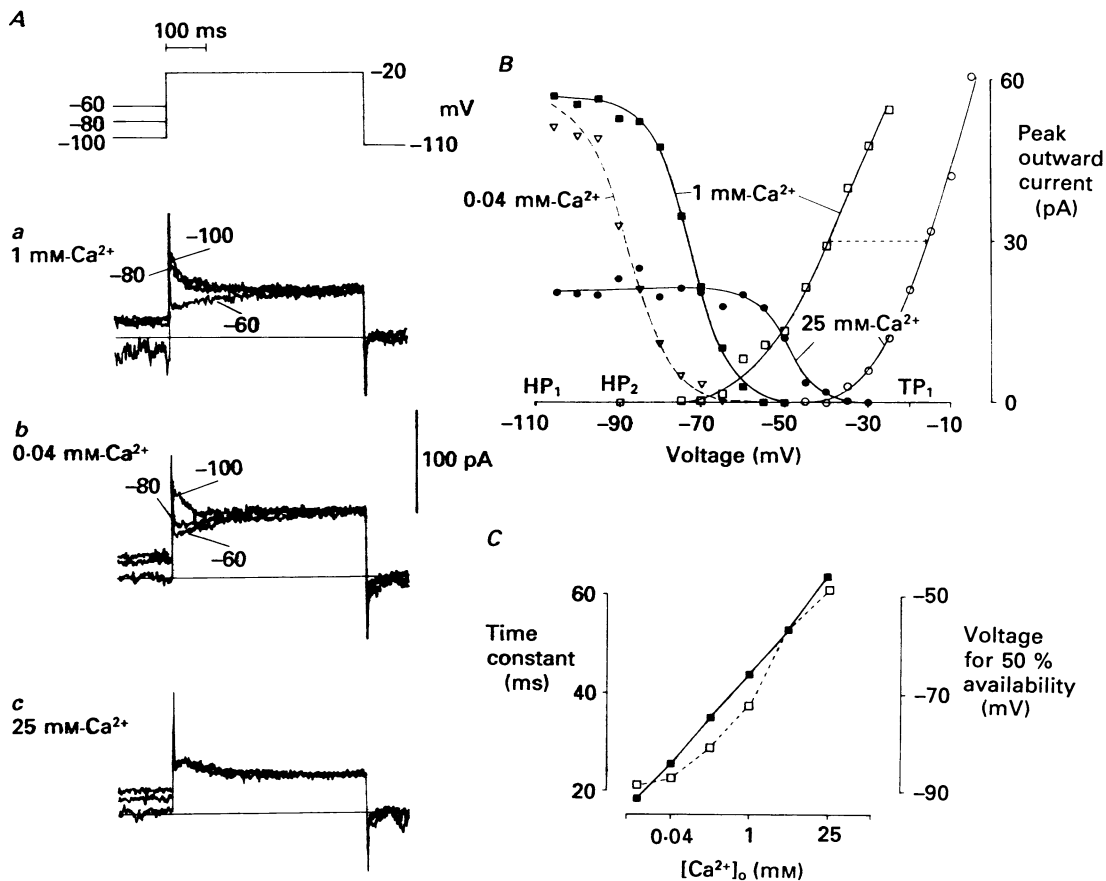
Fig. 7. Effects of $[Ca^{2+}]_o$ on voltage-dependent parameters of I_{fo}. The experiments were on one cell, HP_1 was -110 mV (step duration, 9·5 s), the test potential (TP_1) -20 mV (step duration, 0·5 s) and the conditioning voltage step length 5 s. A, currents elicited at -20 mV after the -100, -80 and -60 mV conditioning steps (marked). $[Ca^{2+}]_o$ was 1 mM (a), 0·04 mM (b) and 25 mM (c). Leakage currents were not subtracted, the horizontal lines mark the current levels upon returning to -110 mV and the digital capture frequency was 667 Hz. B, steady-state inactivation curves (fitted Boltzmann equations, see text) for peak I_{fo} when $[Ca^{2+}]_o$ was 1 mM (■), 0·04 mM (▽) and 25 mM (●). Zero current indicates that which was not inactivated by the conditioning step. The threshold for activation of peak I_{fo} was assessed (HP_2, -90 mV) for $[Ca^{2+}]_o$ 1 mM (□) and 25 mM (○). The curves were drawn by eye and leakage currents were subtracted. C, for the same experiment, the time constants (■) of single exponentials fitted to the decay of I_{fo} (see Fig. 3Aa) and the voltages for 50 % steady-state inactivation (□), plotted against the natural logarithm of $[Ca^{2+}]_o$.

Ca^{2+}-containing PSS, I_{fo} was similar to the control. In addition, if the divalent cation concentration was maintained constant by replacing Ca^{2+} with Mg^{2+}, maximum I_{fo} was increased as Ca^{2+} was reduced, although the steady-state inactivation curve was shifted to a more negative potential range (Fig. 6B and C). This suggested that I_{fo} was not generated by the influx of Ca^{2+} but that it was influenced by the presence of extracellular Ca^{2+} or Cd^{2+}. A common explanation for this kind of effect is that

screening of or binding to surface charges by Ca^{2+} leads to a change in the surface potential and that this changes the recorded voltage dependence of the channels.

A surface potential effect?

Huxley (see Frankenhaeuser & Hodgkin, 1957) proposed that changes in the extracellular Ca^{2+} concentration would affect the surface potential at the membrane–aqueous phase interface and that this potential would be sensed by voltage-dependent gating mechanisms (reviewed by Hille, 1984). Changes in the surface potential are independent of the clamp potential but can be studied by measuring shifts in the voltage-(clamp) dependence of the channels. In the present experiments this was investigated by varying $[Ca^{2+}]_o$ with respect to an equimolar amount of Na^+. Calcium was used as the divalent cation because it is physiological. It seems not to induce direct block of K^+ channels and further data could be generated to determine whether or not any of I_{fo} was Ca^{2+}-activated. Figure 7 describes the effects of varying $[Ca^{2+}]_o$ between 10 μM (none added) and 25 mM in five-fold increments, on the steady-state inactivation curve, the activation curve and the rate of decay of I_{fo}. When -20 mV was used for testing the amount of inactivation (Fig. 7), to enable the complete curve to be established in 25 mM $[Ca^{2+}]_o$, voltage-activated Ca^{2+} current may have introduced some error.

Changes in $[Ca^{2+}]_o$ had a marked effect on the voltage dependence of I_{fo}. Lowering $[Ca^{2+}]_o$ from 1 mM to 0·04 mM shifted the steady-state inactivation curve to a more negative voltage range (Fig. 7A and B) but I_{fo} could still be obtained in 0·04 mM-Ca^{2+} solution or in Ca^{2+}-free PSS (not shown) if the cell was held at a sufficiently negative potential. Raising $[Ca^{2+}]_o$ from 1 to 25 mM shifted the inactivation and activation curves by $+23·5$ and $+26$ mV respectively (Fig. 7A and B), a greater shift than for experiments where Mg^{2+}, as opposed to Na^+, was used to substitute for Ca^{2+} (Fig. 6C). The reduction in the amplitude of I_{fo} elicited at -20 mV when in high Ca^{2+} (e.g. 25 mM) could be explained by the shift in the activation curve. However, on this basis it was anticipated that in low-Ca^{2+} maximum I_{fo} would be increased. Although this was observed for 0·2 mM $[Ca^{2+}]_o$ (not shown) it was not for 0·04 mM $[Ca^{2+}]_o$ (Fig. 7B). This may be explained if the activation curve for I_{fo} approached a maximum at -20 mV ($[Ca^{2+}]_o$ 1 mM) or if a small component of I_{fo} was Ca^{2+}-activated.

Figure 7C summarizes the effect of $[Ca^{2+}]_o$ on the rate of decay of I_{fo} and on its steady-state inactivation curve. The rate of decay at -20 mV was determined by the method described in Fig. 3Aa (thus current that was not inactivated by the conditioning step was subtracted) and quantified by the time constant of a fitted single exponential. The time constants were directly related to the natural logarithm of $[Ca^{2+}]_o$. The conditioning potential for 50 % inactivation was inversely related to the natural logarithm of $[Ca^{2+}]_o$. The time constant for the decay increased by about 1·5 × for a twenty-five-fold increase in $[Ca^{2+}]_o$ and the voltage for 50 % inactivation shifted by about $+20$ mV. In addition, the rate of development of I_{fo} seemed to be slowed as $[Ca^{2+}]_o$ was increased. Thus evidence was obtained that four parameters of I_{fo} showed a dependence on $[Ca^{2+}]_o$. This could be explained if Ca^{2+} affected a surface potential that was detected by the voltage sensors of the channels underlying the current. The experiments provided little evidence that any of I_{fo} was Ca^{2+}-activated.

An outward current (I_{fo}) has been described in single vascular smooth muscle cells that was clearly carried by a population of channels distinct from those previously known to exist in these cells. The steady-state inactivation plot for the current was steeply voltage-dependent between -90 and -60 mV, current being 50 % available at -78 mV. The activation threshold was around -65 mV and the kinetics were fast and voltage-dependent. The main charge carrier seemed to be K^+ and the current could be blocked completely by 4-AP and did not appear to be Ca^{2+}-activated.

Other 4-AP-sensitive transient outward currents

The current I_{fo} had many similarities to the voltage-activated transient outward current of neuronal cells that is usually called the A-current (e.g. Thompson, 1977; Gustafsson, Galvan, Grafe & Wigström, 1982; Belluzzi et al. 1985; Numann et al. 1987). The general similarities are: a threshold and almost complete inactivation at -60 mV, 50 % inactivation at about -80 mV, rapid recovery from inactivation at negative potentials (faster than for the delayed rectifier, see Fig. 3C and D and Numann et al. 1987, their Fig. 6), a transient time course and being carried mainly by K^+ (see also Taylor, 1987). In addition, the A-current is commonly blocked by 4-AP, a property used to distinguish it from transient currents activated by Ca^{2+} (e.g. see Siegelbaum & Tsien, 1980; Salkoff, 1983; Josephson et al. 1984; but see also Adams, Brown & Constanti, 1982). Some A-currents (see Dolly et al. 1987) also seem to be blocked by dendrotoxin, and the more potent congener toxin-I (e.g. see Halliwell, Othman, Pelchen-Matthews & Dolly, 1986). However, a high concentration of toxin-I (1 μM) had little effect on I_{fo}.

A-currents are usually considered to be transient, but similar currents in hippocampal CA3 pyramidal cells (Gustafsson et al. 1982; test step -40 mV) or *Drosophila* muscle (ShB1 mRNA-induced A-current, Timpe, Schwarz, Tempel, Papazian, Jan & Jan, 1988; test step $+40$ mV) show a sustained current component. The current I_{fo} also had a sustained component (Figs 1 and 3), but *only* at test steps positive of about -10 mV (see Fig. 3A). This may reflect a sustained component of I_{fo}, or a separate current also available from more negative holding potentials. Such a current has been reported in type-A rat visceral sensory neurones (Stansfeld, Marsh, Halliwell & Brown, 1986; Dolly et al. 1987) and this current seemed to be particularly sensitive to dendrotoxin. Preliminary experiments in portal vein cells have shown block of a sustained current by a high concentration (1 μM) of toxin-I for test potentials negative of -10 mV (D. J. Beech & T. B. Bolton, unpublished observation). At present this must be interpreted as a block of I_{dK}.

The role of Ca^{2+}

A number of transient outward currents have been suggested to be Ca^{2+}-activated (e.g. see Siegelbaum & Tsien, 1980; Salkoff, 1983). The current I_{fo} did not seem to be Ca^{2+}-activated because it could be elicited when voltage-activated Ca^{2+} current was blocked or not activated, its amplitude and rate of decay were inversely related to $[Ca^{2+}]_o$ and its pharmacology was different to that of the known Ca^{2+}-activated K^+ currents of these cells. However, I_{fo} clearly did depend on $[Ca^{2+}]_o$. The most plausible

explanation for this effect was that the local potential at the membrane surface was changed as $[Ca^{2+}]_o$ was changed and that the channels carrying I_{fo} experienced this potential independently of the clamp potential.

The observed changes in four voltage-dependent parameters of I_{fo} upon changing $[Ca^{2+}]_o$ were supportive of a surface potential hypothesis. However, some divalent cations had much stronger effects than others, which seemed to indicate some binding of ions as opposed to a simple shielding effect. For example, replacement of Ca^{2+} by Mg^{2+} produced a -14.6 mV shift in the steady-state inactivation curve (Fig. 6C) and 0·2 mM-Cd^{2+} in Ca^{2+}-free PSS affected I_{fo} in a manner comparable to 25 mM-Ca^{2+} solution (cf. Figs 6Ac and 7Ac), which suggested that Cd^{2+} had the greatest ability to shift the voltage dependence of I_{fo} and Mg^{2+} the least (cf. Mayer & Sugiyama, 1988). The strong effect of Cd^{2+} could explain why 0·2 mM-Cd^{2+} in Ca^{2+}-containing PSS reduced I_{fo} elicited from -90 mV (D. J. Beech & T. B. Bolton, unpublished observation), which has also been observed for the A-current in sympathetic neurones (Galvan & Sedlmeir, 1984). It seems unlikely that this effect of Cd^{2+}, an inorganic Ca^{2+} channel blocker (e.g. see Fox *et al.* 1987), reflected block of Ca^{2+} influx.

The current I_{fo} appeared to be more strongly affected by $[Ca^{2+}]_o$ than other voltage-activated channels in these cells. For example, the activation and inactivation curves for I_{dK} are little affected by the removal of $[Ca^{2+}]_o$ (D. J. Beech & T. B. Bolton, unpublished), which agrees with observations in dorsal root ganglion cells (Mayer & Sugiyama, 1988). This may be explained if the channels carrying I_{fo} are orientated in the membrane in a particular way that makes them very susceptible to the surface potential or if these channels have binding sites for certain ions near their voltage sensors. The steepness of the inactivation curve seems also to be an important factor when considering the effect of extracellular divalent cations on I_{fo} as the current was strongly affected by Ca^{2+}-free solution. Although neuronal A-currents also depend on the extracellular concentration of divalent cations (e.g. Thompson, 1977; Numann *et al.* 1987), and more strongly so than other currents in these cells (Mayer & Sugiyama, 1988). Ca^{2+}-free solution has little effect on them. These A-currents seem to have a shallower steady-state inactivation curve ($V_s = 7$–8 mV) than I_{fo} ($V_s = 5$ mV), which will confer less dependence on the membrane and surface potentials. The strong voltage dependence of I_{fo} is also an important factor when considering the role of this current in the electrical activity of these cells.

What is the role of an A-current in smooth muscle?

It is clear from this study that a current that is very similar to the neuronal A-current also exists in portal vein smooth muscle cells. The role of this kind of current in neurones seems to be to regulate spike frequency by activating during the depolarizing phase of the after-hyperpolarization (Connor & Stevens, 1971; reviewed by Hille, 1984). However, microelectrode recordings suggest that a significant after-hyperpolarization does not occur in portal vein smooth muscle and the membrane potential is quite low, at about -50 mV (e.g. see Kuriyama, Ohshima & Sakamoto, 1971; but see Hamilton, Weir & Weston, 1986), and thus the A-current would never become available for activation. However, as I_{fo} is strongly affected by the concentration of extracellular, and presumably intracellular, ions, their absolute

values will be critical in determining whether the A-current has any role to play in these cells. This effect may come into play in smooth muscle of the taenia caecum, where spike activity was reported to become regular and pacemaker-like in high-Na^+ and/or Ca^{2+} solutions (Bülbring & Kuriyama, 1963), perhaps indicating the introduction of a regulatory current. In smooth muscle types where an after-hyperpolarization does occur, e.g. in guinea-pig ileum (Nakao, Inoue, Hamanaka & Kitamura, 1986), an A-current would presumably have a role analogous to that described for neurones, even under normal conditions, or, if an inhibitory junction potential occurred, it would affect its duration. Whether or not such a current exists in other types of smooth muscle has not yet been established.

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