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Patch and whole-cell voltage clamp of single mammalian visceral and vascular smooth muscle cells

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Summary. Dispersal of the constituent cells of mammalian visceral and vascular smooth muscles has permitted recordings both of membrane currents under whole-cell voltage clamp, and of currents through single ionic channels using the patch-clamp technique. A rectangular depolarizing step applied to a single cell under voltage clamp yielded a net inward current followed by a net outward current in normal physiological solution. In isolated, 'inside-out' patches of cell membrane a calcium- and potential-sensitive K channel (100 pS conductance) and a calcium-insensitive, potential-sensitive K^+ channel (50 pS conductance) with slow kinetics have so far been identified and characterized.

Key words. smooth muscle; K channels; patch clamp.

Experientia 41 (1985), Birkhäuser Verlag, CH-4010 Basel/Switzerland

Introduction

The diversity of the electrical properties of smooth muscles takes several forms. Some muscles, such as the guinea-pig vas deferens, are normally electrically quiescent but can be galvanized into action by nerve discharge or other stimuli. Others continuously contract, for example, the longitudinal muscle of rabbit jejunum. Both the vas deferens and longitudinal intestinal muscle discharge electrical transients. These may be of two main types: briefer actions potentials and longer slow waves of potential. Longitudinal intestinal muscle and other visceral muscles such as uterus show both types, whereas the vas deferens generally shows only action potential discharge. Some smooth muscles seem reluctant to discharge electrical transients such as action potentials, and may do so only under special conditions. However, it is significant that many smooth muscles not normally generating action potentials (e.g. some blood vessels, the anococcygeus, and tracheal smooth muscle) can usually be induced to do so upon application of tetraethylammonium (TEA) a known blocker of potassium channels[14].

In order to understand the diverse potential activities of smooth muscle, attempts were made in the late 1960's and early 1970's by Anderson[1] and others[21,22,24] to study the properties of the ionic channels of the smooth muscle cell membrane by means of a voltage-clamp technique applied to small strips of smooth muscles. Even as the technique was being optimistically taken up by other groups[11,13,15,23,30] caveats were being sounded about the interpretation of current records obtained from multi-unit preparations[20]. Disquiet arose over the influence of distributed resistances in series with the cell membrane, and about the accumulation of ions in extracellular crevices with restricted diffusion, which created great quantitative uncertainty about the ionic currents recorded. Some checks that were made of the voltage uniformity in smooth muscle strips under voltage clamp were not encouraging[7] and it became clear to most workers that the voltage-clamp technique as applied to multi-unit preparations of smooth muscle had considerable complexities and uncertainties which have been summarized elsewhere[8].

To avoid the difficulties created by the use of multi-unit preparations it was obvious that single cells had to be used. Pioneering work on the dispersal of the constituent cells of smooth muscle tissues was done by Bagby et al.[2] and then by Fay and Delise[16]. Later, a two-microelectrode voltage-clamp technique was applied to single isolated smooth muscle cells from toad stomach[31] and from *Amphiuma* stomach[12]. In this paper we describe how we apply a voltage-clamp technique to freshly-isolated single mammalian smooth muscle cells and also how the properties of single ionic channels in the membrane can be studied by means of the patch-clamp technique[3-5].

Methods

Patch and voltage clamp of single smooth muscle cells

To voltage clamp a single isolated smooth muscle cell, or to voltage clamp a small patch of its membrane, experiments were begun in the same way. A glass pipette with a tip diameter of less than 1 μm was formed; this has a resistance of about 5 MΩ when filled with the appropriate physiological salt solution. The pipette was applied to the surface of a single smooth muscle cell. It is important that the cell separation procedure using collagenase also 'cleans' the cell surface which makes it possible by means of mild negative suction applied to the pipette to form a high-resistance (about 10^9 Ω) seal between polished tip and cell surface as can be achieved in other excitable cells[19,25].

Preparation of single cells from both visceral and vascular muscle involves incubation in a collagenase containing solution. However, variation of the protocol was necessary to suit each tissue. Strips (2–3 mm wide; 3–5 mm long) were cut from lengths of longitudinal muscle from rabbit or guinea pig jejunum or longitudinally-cut lengths of rabbit or guinea pig mesenteric artery were used. Strips of jejunal muscle were first incubated (at 35 °C) in a calcium-free physiological saline for at least 10 min. They were then bathed in a calcium-free saline solution containing collagenase (0.1%), trypsin inhibitor (0.1%) and bovine serum albumin (0.2%). Jejunal muscle strips remained in the dispersal solution for 10 min and were then mildly agitated. The dispersal solution was then replaced with new solution; the procedure was repeated twice more. During the final incubation the strips were mildly agitated for several minutes until single cells could be detected under the microscope. Arterial strips were incubated in a calcium-free saline solution containing collagenase (0.05–0.1%) alone, for half an hour and then mildly agitated. This procedure was repeated several times until single cells could be detected. Collagenase treatment resulted in a population of single smooth muscle cells of differing appearance. Numerous, elongated, and presumably relaxed single cells were present as well as cells in various degrees of contracture, and lysed cells[3]. Cells whose membrane had a feature-less 'smooth' appearance under differential interference microscopy, when bathed in a bicarbonate- or Hepes-buffered physiological saline solution, readily attached themselves to the glass bottom of the organ bath. These cells were used for both patch clamping and for whole cell voltage clamping.

Recordings were made from patches of membrane of an intact cell, or after the pipette has been withdrawn tearing a small patch of membrane from the cell (isolated, 'inside-out' patches). Isolation of these patches allowed rapid and repeated exchange of the solution bathing the internal surface of the membrane and unequivocal control of membrane potential. The concentration of potassium in the solutions on both sides of the patch were either symmetrical (e.g. 126 mM K^+ on both sides) or such that an approximate physiological gradient was established (6 mM:126 mM). The temperature of the experiments was 20–25 °C.

For whole-cell voltage clamping, when a high-resistance seal of pipette to cell membrane had been established, the diaphragm of cell membrane separating the interiors of the pipette and single cell was ruptured by applying sharp pulses of suction to the pipette. After the interiors became contiguous the seal of pipette to membrane remained intact.

Results

Voltage clamp of single isolated smooth muscle cells

Using a single pipette placed roughly centrally on a smooth muscle cell, the currents necessary to clamp the interior of the cell at various potentials with respect to the bathing solution could be measured i.e. voltage clamp could be achieved. Access to the cell interior is via a low resistance path, the pipette, of resistance about 5 MΩ compared to the cell input resistance of 1–10 GΩ (1 GΩ = 10^9 Ω). Even during the flow of inward current, input resistance does not fall much below 100 MΩ, so that resistances in series with the cell membrane are negligible. By choosing partially contracted cells, not longer than 100 μm, and placing the pipette centrally, it is hoped that uniform potential can be achieved even during the flow of inward current.

In good cells, the injection of a rectangular depolarizing pulse of current could elicit one or more regenerative action potentials when the cell was in normal physiological salt solution (2.5 mM Ca) (fig. 1A). Under voltage-clamp conditions with the cell held at the resting membrane potential, usually about −50 mV, a rectangular depolarizing voltage-clamp command resulted in excellent control of the recorded membrane potential. A large net inward current flowed after the initial capacity current. This was followed by outward current which showed an initial peak in some cells (fig. 1C).

In solutions containing barium (20 mM) instead of calcium, large net inward currents could be more reliably and consistently evoked. In this condition, outward current, including the initial peak, was much reduced whereas net inward current was increased (fig. 1B, C).

The measured input resistance in the cell shown in figure 1A slightly exceeded 2 GΩ This value includes the leakage resistance between pipette seal and membrane so the true input resistance will be greater than this. For example, if the seal resistance was 10 GΩ, then the measured input resistance will be about 25% less than the true value of the cell input resistance. The time constant of this cell τ (τ = RC) was 0.32 s giving a capacitance of about 0.1 nF. This gives an effective surface area, assuming a capacity of 1 μF·cm², of 10,000 μm² and a specific membrane resistance of about 200–300 kΩcm². This value is surprisingly high compared to Tomita's[29] calculations based on the space constant of strips of smooth muscle tissues.

The cell from which these records was taken was quite strongly contracted which probably results in considerable infolding of cell membrane making estimates of cell

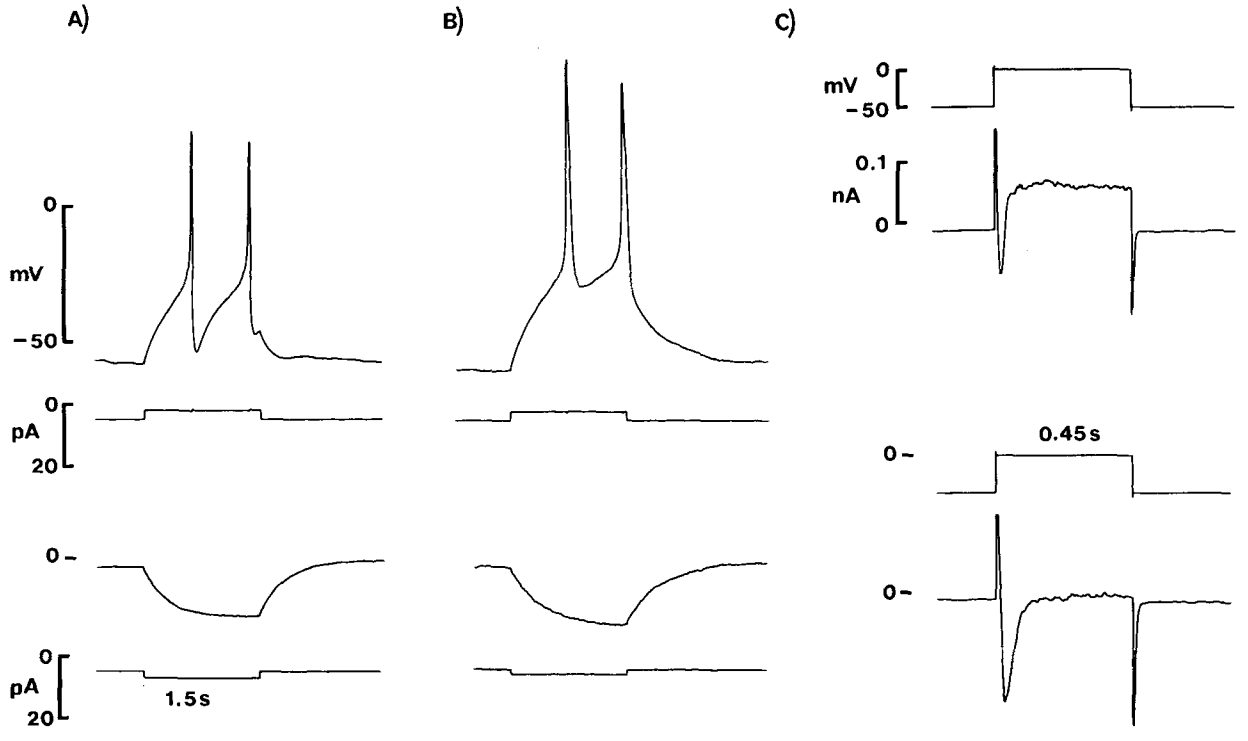


Figure 1. Potential and current recordings from a single isolated smooth muscle cell of rabbit jejunum. A pipette containing Ca-free, 126 mMK, 0.77 mM EGTA solution was first sealed to the membrane. The patch membrane was then ruptured while maintaining the seal to the pipette so that recording of potential and current could be made using a current-to-voltage converter (Neher et al. 1978; Hamill et al. 1981). *A* In normal physiological solution (2.5 mM Ca, 6 mM K, 137 mM Na) a rectangular depolarizing pulse evoked overshooting action potentials and a small hyperpolarizing pulse elicited an electrotonic potential. The steady state size of this and its time course can be used to estimate input resistance. *B* In a Ca-free 20 mM Ba solution, action potential size and duration was increased; repolarization was to a less negative potential following the spike than was observed in normal solution. The hyperpolarizing electrotonic potential, evoked with a smaller current than before, shows that input resistance has increased. In *A* and *B* a small continuous current was passed hyperpolarizing the membrane by 12 mV and so inhibiting spontaneous action potential discharge. *C* Above: Under voltage clamp a rectangular depolarizing step to −0 mV produced a net inward current (after the initial capacitive transient) and then a maintained outward current in normal solution. Below: In Ca-free 20 mM Ba solution inward current was increased while outward current was decreased so that in fact a small inward current flowed during the latter part of the pulse. The holding potential was −50 mV. Temperature 20–25 °C.

membrane surface area from the cell outline gross underestimates. In a large, fully relaxed, elongated cell the cell length may approach or even exceed 500 μm. Assuming such a cell can be approximately represented by two cones base-to-base, then the surface area may approach about 3200 μm^2. This is only about a third of the value obtained from the membrane time constant assuming a specific membrane capacity of 1 $\mu F \cdot cm^{-2}$. Two possible explanations for the difference are that the specific membrane capacity is slightly greater than assumed, and that caveolae[18] and fine processes[3] increase surface area by a factor of two or more.

The space constant λ (mm) in a fully relaxed cell is best calculated by considering a short segment of uniform cross section and unit length. Assuming a radius (a) of 2 μm we can apply the formula $\lambda = \sqrt{[a/2]}\ \sqrt{[R_m/R_i]}$[29] where R_m is the apparent specific membrane resistance calculated from the result that there is three times as much membrane as would appear from simple geometrical calculations in each unit length i.e. 67–100 $k\Omega cm^2$. R_i is the specific resistivity of the cytoplasm and has a value of about 200–300 Ωcm[29]. These values give a resting space constant of 0.14 to 0.17 cm. It is well known that in a short cable less than 0.5 λ long the decline of potential is about 10% – the short cable here is measured from the position of the pipette approxiately in the centre of the cell to one end. This is clearly much less than 0.5 λ so that the cell membrane would be expected to be virtually isopotential in the resting state[28].

The cells used for clamping were seldom greater than 100 μm long or 50 μm half length. For isopotential conditions 0.5 λ must exceed 50 μm i.e. $\lambda > 100$ μm. For λ to decline from about 0.15 cm to 0.01 cm, Rm must decrease about 225-fold. It seems possible therefore that space clamp may be achieved even during the flow of inward current, since conductance at this time does not seem to exceed ten times resting conductance (fig. 1).

Patch clamp on single isolated smooth muscle cells

The formation of a pipette-membrane seal of greater than 10^9 Ω resistance produces effctive electrical isolation of the patch of membrane within the pipette from the surrounding cell membrane. If the patch is torn from the cell, the isolated patch so formed remains attached and sealed to the pipette so that the internal surface of the patch membrane is exposed to the solution in the bath; the external surface is exposed to the solution filling the pipette. In isolated patches, therefore, the composition of solutions bathing both sides of the membrane, and the membrane potential can be closely controlled. In patches which remain attached to the cell, the ionic composition of the solution bathing the internal surface of the membrane is not accurately known. More important still, perhaps, is the fact that in cell-attached patches the patch potential is determined by the cell membrane potential, which may of course vary either spontaneously or in response to ionic changes or to drugs applied in the bath. Hence, unless cell membrane potential is simultaneously measured in some way (which is a difficult procedure we have not yet attempted) exact patch potential is not known[17].

Types of ionic channels. We have so far been able to identify three types of ionic channel in isolated and cell-attached patches from isolated single smooth muscle cells of rabbit and guinea pig jejunum, and anterior mesenteric artery. Two of these are K-selective but can be distinguished by their other properties – the third seems not to be K-selective and may pass other ions. We have found it only infrequently and have only been able to study it when the K channels have been blocked by application of tetraethylammonium (TEA). With a K gradient (pipette:intracellular) of 6 mM:126 mM and in the presence of 10 mM TEA the reversal potential of this channel was about +13 mV (patch membrane potential will be described as if it were attached to a cell) when E_{Ca} was zero (2.5 mM:2.5 mM) and $E_{Na} = +17$ (135 mM:70 mM). Its conductance was about 70 pS.

The two K selective channels will be referred to as 'small' and 'large' since their conductances (6 mM:126 mM K) were about 50 and 100 pS respectively. Both were equally sensitive to, and blocked by, TEA or barium ions. In potential ranges where these channels opened, openings occurred in bursts generally separated by longer closed periods unless the probability of being open was very high. Typical records are shown in figures 2, 4 and 5. From the zero current level the channel abruptly opened at a rate which seems limited only by the frequency response of the recording system. A steady level of current

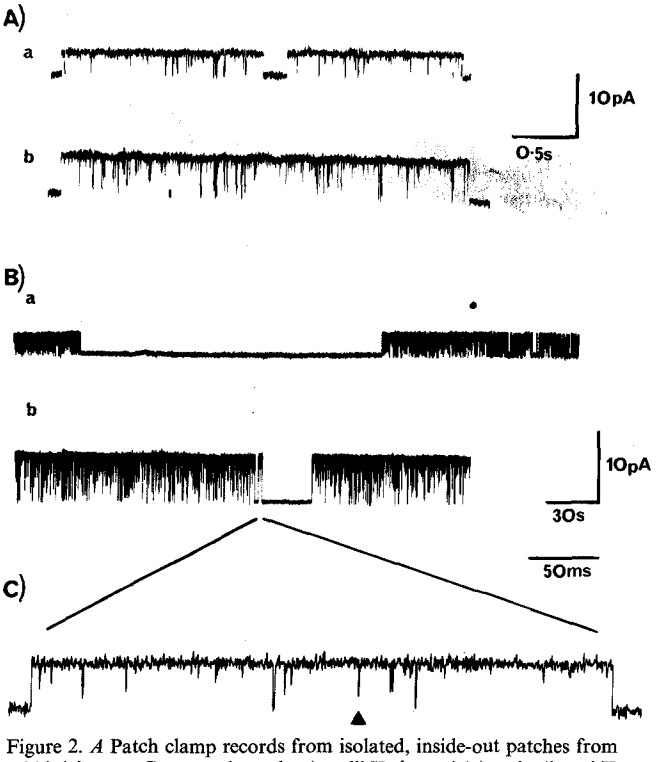


Figure 2. *A* Patch clamp records from isolated, inside-out patches from rabbit jejunum. Currents through a 'small' K channel (*a*) and a 'large' K channel (*b*) at 0 mV. Pipette 6 mM K; bathing solution 126 mM K. Record filtered at 1 kHz. Temperature 20–25 °C. *B* Current through a 'large' potassium channel with a conductance of 127 pS. Long bursts of openings separated by rapid closings are terminated by longer closings (*a, b*). In *C*, a short burst of openings is shown on an expanded time scale. Notice the complete and apparently incomplete (▲) rapid closings to the zero current level. Potential was zero in *Ba* and +20 mV in *Bc* and *C*. Pipette contained 47 mM K, 2.5 mM Ca; the bathing solution 126 mM K, 2.5 mM Ca. Records in *B* filtered at 1 kHz, those in *C* at 2 kHz.

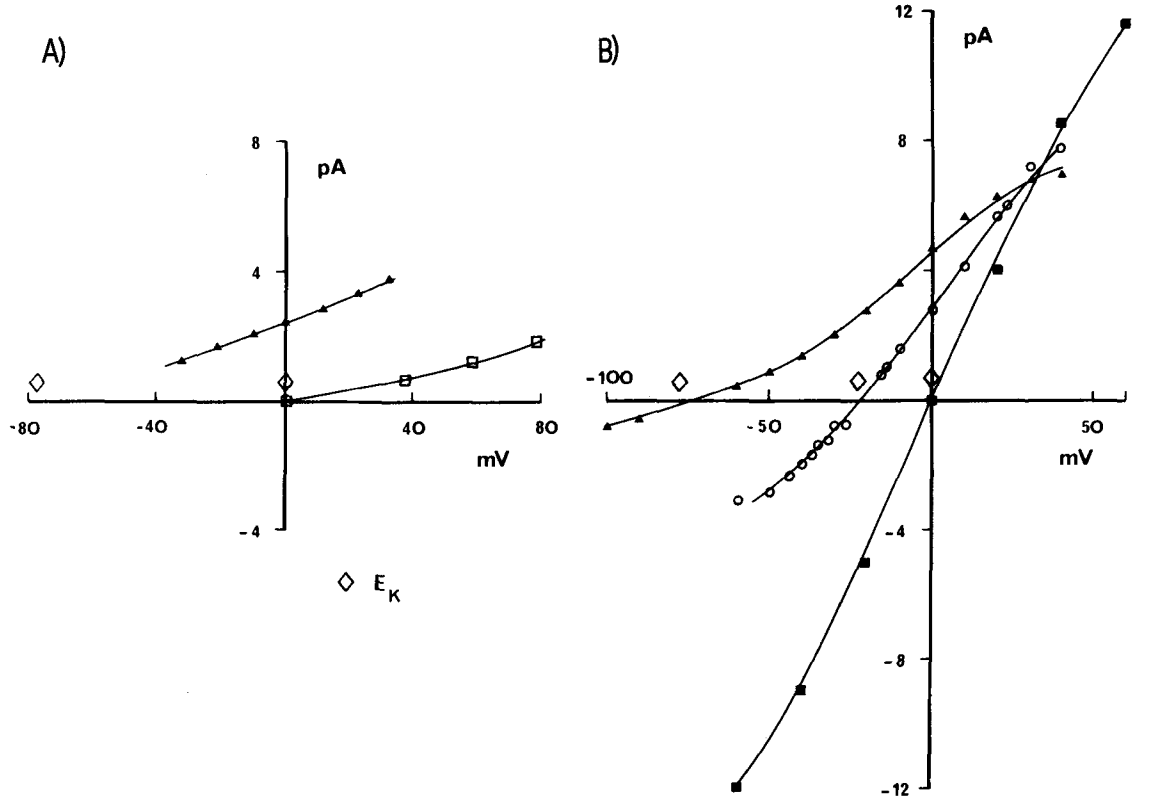


Figure 3. Current voltage relationships of unit K currents from rabbit jejunal cells. *A* shows the effect of a 6 mM:126 mM K gradient (▲) and of changing to a 6 mM:6 mM K gradient (□) on 'small' K channel current. Conductance is reduced from about 40 pS to 20 pS. Notice the failure to reverse in the latter gradient. In the former reversal could not be tested because openings did not occur negative to −40 mV. *B* shows the effect of the K gradient on 'large' K channel current. There is current reversal close to calculated E_K in 6 mM:126 mM K (▲), 47 mM:126 mM K (○), and 126 mM:126 mM K (■) in three separate patches. The conductances were 85, 127 and 250 pS respectively.

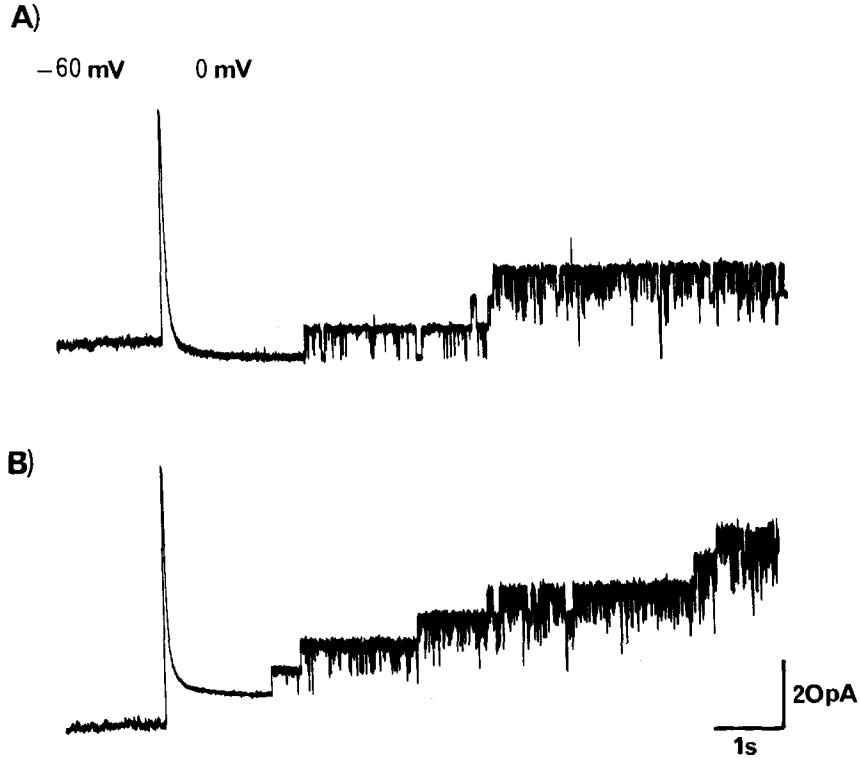
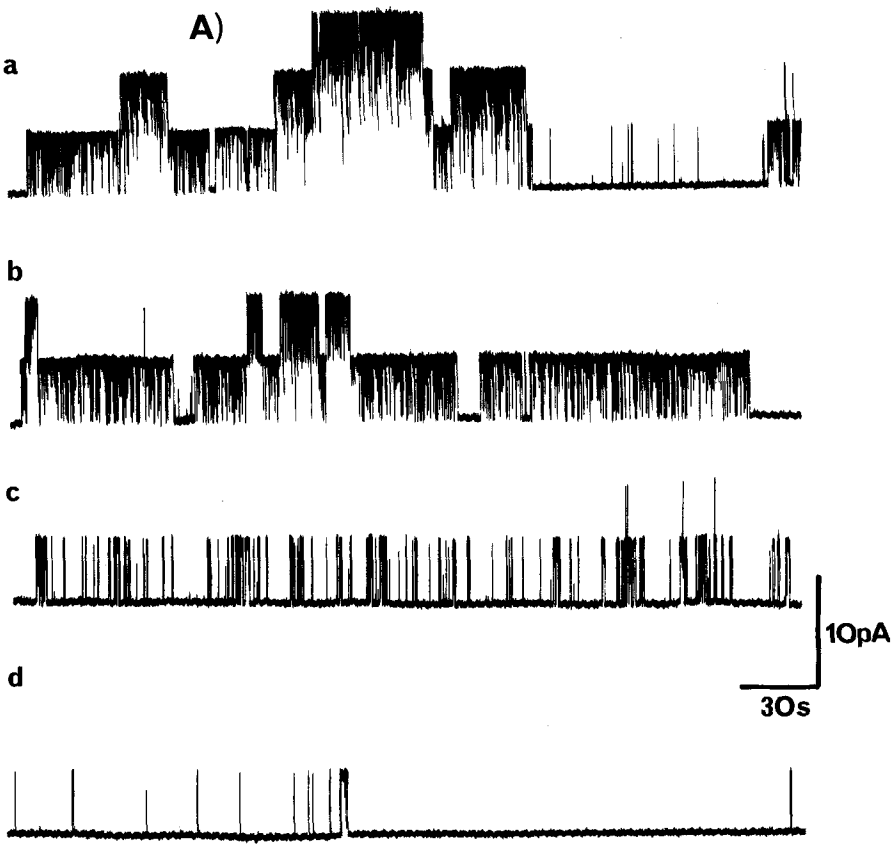


Figure 4. Activation of 'small' K channels upon depolarization in isolated patches from cells of rabbit jejunum (*A*) and mesenteric artery (*B*). Patches were held at −60 mV for 5 s. Upon returning to zero potential there was a delay of about 2 s in each case before the first channel opened. Subsequently several more channels opened in each case. In the steady state an average ot three channels were open in the jejunal patch, six in the artery patch. 6 mM:126 mM K. Records filtered at 1 kHz.



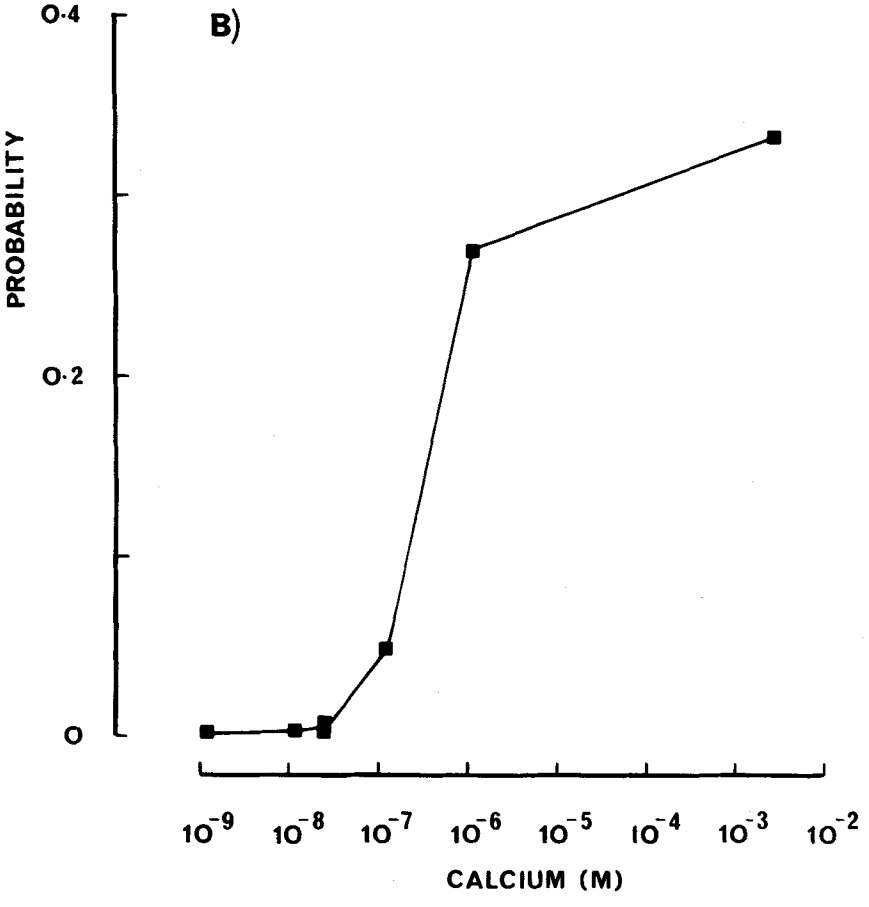


Figure 5. Calcium activation of 'large' K channels. Record from an isolated patch from a cell of the guinea pig mesenteric artery *A* (*a*). In a 2.5 mM Ca^{2+} physiological solution up to three channels were open. *b* In 10^{-6} M Ca^{2+} up to two channels were open. In 10^{-7} M (*c*) and 10^{-8} M Ca^{2+} (*d*) generally only one channel was open and openings were brief. Records from the same patch pipette containing 2.5 mM Ca^{2+}. The bathing solution except for *a* was buffered for Ca^{2+} with 0.77 mM EGTA. *B* Effect of $[Ca^{2+}]$ on probability of channel being open in the same experiment, 6 mM:126 mM K. Patch potential was zero. Records filtered at 1 kHz.

upon which random fluctuations (noise) were superimposed was then achieved. Noise when the channel was open generally exceeded noise when the channel was closed. During a burst of openings, abrupt, rapid, and short-lived closings ('rapid' closings) occurred repeatedly (figs 2, 4 and 5) until a longer closing occurred which terminated the burst. The level of current while the channel was open was affected by the patch potential: if the potassium equilibrium potential was about −78 mV (6 mM:126 mM K) then currents were outward in the range positive to this and increased in size as the patch potential was shifted positively away from E_K (figs 3A, B).

The openings and closings of the K channel were examined in some detail. The durations of individual open and closed periods could be measured in patches where only one channel was active at any time. The distribution of open times was fitted well by a single exponential. However, at least three exponentials were needed to fit the distribution of closed times[3,5]. In the case of the 'small' channel, varying potential had a strong effect on the probability of being open which increased as patch potential was made more positive. This was reflected by a large decrease in long closed periods and a small increase in mean open time. Short closings seemed to be little affected[3]. When only a single channel was active in a patch then the probability of being open, p, can be very simply defined as the fraction of the period of observation the channel spends in the open state (= fractional open time). When more than one channel is active in a patch then the definition of the probability of a channel being open can be defined as p = I/iN where I is the mean current during the period of observation, i is the single channel current and N is the number of channels active in the patch[27].

The conductances of these two channel types were about 50 and 100 pS using a near 'physiological' potassium gradient. Reducing $[K^+]$ (by increasing $[Na^+]$) reduced the conductance of these channels (fig. 3A) and increasing $[K^+]$ when there was 126 mM in the pipette increased their conductance. At the same time, of course, E_K was shifted in a predictable way (fig. 3B).

'Small' K channel. The 'small' K channel had a number of interesting characteristics in addition to those which it shared with the 'large' K channel. Positive to −40 mV, as alluded to, its probability of being in the open state increased considerably. However, negative to −40 mV (6 mM:126 mM K; 2.5 mM:2.5 mM Ca) openings were rarely seen. Even if E_K was shifted positive to −40 mV (6 mM:6 mM K; fig. 3A) inward current due to openings in the range zero to −40 mV was not seen. Under these conditions the 'small' K channel rectifies strongly and inward current seems not to be passed. However, with 126 mM K in the pipette, reversal of the current was sometimes seen. We have not been able to detect much effect of varying $[Ca^{2+}]$ on the behavior of this channel[3]. The speed with which this channel could adapt its opening probability to a change in potential could be studied by applying abrupt step changes in potential. A hyperpolarizing pulse from zero potential could be applied to close the channels: upon stepping back to some potential positive to −40 mV channels were initially closed, but over a period of a few seconds (temperature 20–25 °C) several channels opened until 4–6 channels were active in this patch. This type of channel has been identified in both visceral and vascular smooth muscle cells (figs 4A, B). If the patch potential was held constant after stepping from a negative potential, the level of channel activity achieved after 10 s or so was maintained i.e. there was no sign of inactivation.

'Large' K channel. The 'large' K channel was affected by varying $[Ca^{2+}]$ on the inside of the membrane. If $[Ca^{2+}]$ was high (2.5 mM:2.5 mM Ca) then the channel was mainly in the open state and showed numerous brief closings (figs 2 and 5). If $[Ca^{2+}]$ in the pipette was reduced there was no notable effect. However, if $[Ca^{2+}]$ on the inside of the membrane (in the bath) was reduced to 10^{-8} M, openings were brief and infrequent. At 10^{-7} M they were more frequent and at 10^{-6} M the channel was mainly open showing frequent brief closings as with higher concentrations of Ca. The relationship between the probability of being in the open state and the calcium concentrations is shown in figure 5B. A steep increase in the probability of being open occurred above about 5×10^{-8} M, levelling off above 10^{-6} M. It seems possible that this channel may be activated by calcium entering the cell (or released within it) during physiological activity.

In 2.5 mM:2.5 mM Ca conditions the probability of opening was slightly decreased by depolarization or positive polarization. In 10^{-7} to 10^{-6} M:2.5 mM Ca conditions the probability of opening was strongly increased with a decrease in the negativity of the patch potential. Thus, potential sensitivity was seen in the intermediate (physiological?) range of Ca concentrations but was absent or small at very high or very low Ca concentrations.

Discussion

The whole cell recordings indicate that single jejunal smooth muscle cells can freely generate action potentials. Under voltage clamp a pronounced net inward current is observed when the cells are bathed in physiological salt solution of normal composition (2.5 mM Ca). Probably this current is mainly carried by calcium ions although the extent to which sodium ions may contribute is at present uncertain. However, in the smooth muscle cells of the taenia of the guinea pig, Brading, Bülbring and Tomita[10] found action potentials recorded from the whole tissue with microelectrodes were reduced as $[Ca^{2+}]_o$ was reduced but not if $[Na^+]_o$ was reduced. Inward current in our experiments was reduced in nominally Ca-free conditions and increased if barium was added to Ca-free solution, suggesting that Ba^{2+} ions can replace Ca^{2+} ions in carrying the inward current.

In Ca-free, Ba-containing, solution the outward current was very small or even slightly inward. Patch clamp experiments not described here which we have done indicate that Ba^{2+} ions substantially reduce K channel openings so explaining the reduction in outward current seen under whole-cell voltage clamp. TEA also reduces single channel currents as recorded by patch clamp[3,6] but by a different mechanism. It has been observed to have similar effects to Ba^{2+} ions on the outward current recorded under whole cell voltage clamp.

We have not yet observed single channel currents carried by Ca^{2+} under patch clamp, which would correspond to the inward current and spike upstroke of whole cell

recordings. This is partly because we have not yet looked carefully at early times for these channels which probably have a small conductance[26] but also because probably they are only present in cell-attached patches which we use relatively infrequently, and disappear in isolated patches as in other cell types[17].

The calcium which we are presuming to enter during the upstroke of the action potential in whole cells almost certainly has a number of important functions. Contraction is one of these: also it seems likely that the rise in $[Ca^{2+}]_i$ close to the membrane brought about by this calcium ingress may open the large calcium-dependent K channels studied in the patch-clamp experiments. The depolarization during the spike might also reinforce this effect. Bearing in mind that these experiments were done at 20–25 °C, $[Ca^{2+}]_i$ may be rather slowly restored to its former level after the action potential: at 37 °C this would be expected to occur more quickly. It would seem that the 'large' K channels are likely to be those important for the rapidity of the downstroke of the action potential (in combination with inactivation of the inward current) and restoration of the resting membrane potential. The 'small' K channels are activated only by relatively prolonged periods of depolarization so that it would not be expected that they would normally contribute to repolarization following a single action potential, although in rabbit jejunum a prolonged burst of spikes or a longer-lasting drug-induced depolarization may result in their opening.

A final point is that although the patch studies suggest similar K channels are present in visceral and vascular smooth muscle cells, nevertheless, vascular muscle from guinea pig mesenteric artery much less readily generates action potentials[9]. We have not yet begun serious whole-cell voltage-clamp studies on single vascular smooth muscle cells but it seems possible that the explanation is that it is the relative numbers of these various channels, their conductances and, above all, their kinetics, which determine the ease with which action potentials are generated. These studies are barely in their infancy, but the techniques are now available which should enable us to settle such problems.

This work was supported by the Medical Research Council.

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