Activation of small conductance Ca^{2+}-dependent K^+ channels by purinergic agonists in smooth muscle cells of the mouse ileum

Fivos Vogalis and Raj K. Goyal

Centre for Swallowing and Motility Disorders, Brockton/West Roxbury Veterans Administration Medical Center, West Roxbury, MA 02132 and Harvard Medical School, Boston, MA 02215, USA

1. Whole-cell and single-channel K^+ currents were recorded at room temperature (22–24 °C), from smooth muscle cells enzymatically dispersed from the mouse ileum, using variations of the patch-clamp technique.

2. Net outward K^+ currents recorded through amphotericin-B-perforated patches in response to step depolarizations positive to −50 mV from a holding potential of −80 mV were decreased by up to 70% by external apamin (0·5 μM). Apamin-sensitive whole-cell currents were also recorded from cells perfused internally with 150 nM Ca^{2+} but not from cells perfused internally with 85 nM Ca^{2+}.

3. Three types of non-inactivating Ca^{2+}-sensitive K^+ channels were identified in cell-attached and excised patches under an asymmetrical K^+ gradient: (i) large conductance (BK_{Ca}; ~200 pS) channels blocked by 2 mM external TEA; (ii) intermediate conductance (IK_{Ca}; ~39 pS) channels blocked by 2 mM external TEA and inhibited by external apamin (0·5 μM); and (iii) small conductance (SK_{Ca}; ~10 pS) channels that were not blocked by 5 mM external TEA but were sensitive to extracellular apamin (0·5 μM).

4. The TEA-resistant SK_{Ca} channels were activated by an increase in $[Ca^{2+}]_i$ with an EC_{50} of 1·5 μM and a Hill coefficient of 1·3.

5. P_2 purinoceptor agonists 2-methylthioATP (2-MeSATP), 2-chloroATP and ATP (10–50 μM) increased an apamin-sensitive whole-cell outward K^+ current. Extrapatch application of 2-MeSATP (20–100 μM) stimulated the apamin-sensitive IK_{Ca} and SK_{Ca} channels and activated an apamin-sensitive steady outward current at 0 mV.

6. Smooth muscle cells from the mouse ileum possess two apamin-sensitive K^+ channels (IK_{Ca} and SK_{Ca}); of these, the IK_{Ca} channels are TEA sensitive while the SK_{Ca} channels are TEA resistant. These channels, along with an apamin-sensitive but TEA-resistant steady outward current, may mediate membrane hyperpolarization elicited by purinergic agonists.

Potassium channels that are activated by intracellular calcium (Ca_i^{2+}) are expressed ubiquitously in excitable tissues (Blatz & Magleby, 1987; Latorre, Oberhauser, Labarca & Alvarez, 1989). Large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}; 80–250 pS), which are expressed in all mammalian smooth muscles (Benham, Bolton, Lang & Takewaki, 1985; Singer & Walsh, 1987; Carl & Sanders, 1989), require micromolar concentrations of intracellular Ca^{2+} to open at physiological potentials and undergo rapid activation and deactivation. Therefore BK_{Ca} channels participate principally in the rapid repolarization of Ca^{2+}-dependent action potentials (Blatz & Magleby, 1987). Small conductance Ca^{2+}-activated K^+ channels (SK_{Ca}), in contrast, are typically not voltage dependent and have a higher sensitivity to Ca_i^{2+} than BK_{Ca} channels (Blatz & Magleby, 1986; Latorre *et al.* 1989), allowing them to operate at more negative potentials.

Unitary SK_{Ca} channel currents have been recorded in cultured anterior pituitary GH3 cells (Lang & Ritchie, 1990), mouse neuroblastoma cells (Leinders & Vijverberg, 1992), guinea-pig hepatocytes (Capiod & Ogden, 1989), rat adrenal chromaffin cells (Park, 1994), rat skeletal muscle (Blatz & Magleby, 1986) and rabbit proximal tubular cells (Merot, Bidet, Le Maout, Tauc & Poujeol, 1989) and yield unitary conductances between 5 and 42 pS. Pharmacologically, SK_{Ca} channels are distinguishable from BK_{Ca} channels by their sensitivity to external apamin and by their resistance to millimolar external TEA (Blatz & Magleby, 1986). In smooth muscle strips of the guinea-pig ileum, apamin at submicromolar concentrations causes membrane depolarization and

increases excitability (Bauer & Kuriyama, 1982; Bywater & Taylor, 1986), consistent with inhibition of a resting K^+ conductance. In intrinsically innervated smooth muscle preparations, such as guinea-pig ileum (Bywater & Taylor, 1986) and canine pylorus (Vogalis & Sanders, 1990), apamin also selectively ablates the fast hyperpolarizing component of the inhibitory junction potential (IJP) (Shuba & Vladimirova, 1980) evoked by stimulation of non-adrenergic non-cholinergic (NANC) inhibitory motor nerves (Bennett, Burnstock & Holman, 1966). The K^+ conductance underlying this IJP (Tomita, 1972; Den Hertog & Jager, 1975; Crist, He & Goyal, 1992) is presumed to represent the opening of SK_{Ca} channels. Although non-BK_{Ca} apamin-sensitive K^+ channels have recently been identified in smooth muscle cells of rat renal arterioles (Gebremedhin, Kaldunski, Jacobs, Harder & Roman, 1996), such K^+ channels have not been reported in visceral smooth muscle.

In the present study, we have recorded, in isolated smooth muscle cells from the mouse ileum, an apamin-sensitive whole-cell Ca^{2+}-dependent K^+ current which, at the single-channel level, appears to be generated mainly by the opening of K^+ channels that are also blocked by millimolar external TEA. These channels have a unitary conductance of 39 pS under an asymmetrical K^+ gradient and we have defined them as intermediate conductance K^+ (IK_{Ca}) channels to distinguish them from a population of apamin-sensitive but TEA-resistant, ~10 pS K^+ channels that we have designated as SK_{Ca} channels. In cell-attached patches stimulation of P_{2Y} purinoceptors activated IK_{Ca} and SK_{Ca} channels and also elicited a TEA-resistant, apamin-sensitive steady outward current. Our results suggest that a large proportion of the Ca^{2+}-activated K^+ conductance in smooth muscle cells of the mouse ileum is generated by the opening of channels other than BK_{Ca} that are sensitive to apamin. Moreover, these channels are strongly activated by purinoceptor stimulation. This study has been presented in abstract form (Vogalis & Goyal, 1997).

METHODS

Isolation of smooth muscle cells

Adult mice weighing 25–35 g were killed by inhalation of carbon dioxide and the proximal ileum was removed and placed in modified Hanks' solution (Ca^{2+}-free MHS) of the following composition (mM): NaCl, 120; KCl, 5·4; $NaHCO_3$, 23·8; NaH_2PO_4, 1; Hepes, 15; and dextrose, 5·5. This solution was made fresh each day just before use and the pH was adjusted to 7·4 at room temperature (22–24 °C) with 10 M NaOH. A segment of ileum was sectioned longitudinally and pinned out flat in a dissecting dish, serosal side uppermost. The muscularis was carefully peeled away from the mucosa using fine forceps under a binocular microscope. The muscle strips were cut into smaller pieces, 1–2 mm^2, and incubated in Ca^{2+}-free MHS at 34 °C for 30 min in a glass test-tube. The tissue pieces were then placed in Ca^{2+}-free MHS containing collagenase (Worthington type III; 0·5 mg ml^{-1}) for 10 min at 34 °C, after which the enzyme-containing solution was replaced with enzyme-free Ca^{2+}-free MHS and the tissue pieces were incubated for a further 30 min at 34 °C. After gentle trituration of the tissue pieces through a wide-bore fire-polished Pasteur pipette, single smooth muscle cells were liberated; under the phase contrast microscope they appeared phase bright and contracted in response to puffs of physiological solution (PS; for composition see below) containing elevated $[K^+]$ and, in some cases, cells contracted spontaneously. In general, the cells from which currents were recorded were of a 'stubby' appearance (< 200 μm in length) compared with the more elongate cells. The stubby cells were more numerous and were presumed to be dissociated from the thicker circular muscle layer of the ileum.

Patch-clamp recordings

Aliquots of the cell suspension were placed in the recording chamber (volume, 3 ml) on the stage of an inverted microscope (Olympus, Japan) and allowed to adhere to the glass bottom of the chamber. For whole-cell recordings (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) cells were perfused continuously (0·5 ml min^{-1}) with standard physiological solution (PS) of the following composition (mM): NaCl, 150; KCl, 5·4; $MgCl_2$, 1; $CaCl_2$, 1; Hepes, 10; and dextrose, 5·5. The pH was adjusted to 7·4 at room temperature (22–24 °C) with 10 M NaOH. Whole-cell pipettes were drawn from borosilicate glass (Kimax 51, no. 34500, Kimble, USA) on a programmable puller (P80/PC, Sutter Instruments, Novato, CA, USA) and fire-polished (Narishige MF83, Japan) to have resistances between 2 and 5 MΩ when filled with the standard pipette solution (high-K^+ intracellular solution) which contained (mM): KCl, 150; $MgCl_2$, 1; $CaCl_2$, 1; Hepes, 10; and EGTA, 1·38. The pH of this solution was adjusted to 7·2 with 10 M KOH, yielding a $[Ca^{2+}]$ of ~150 nM. Pipette solutions with lower $[Ca^{2+}]$ were prepared by adjusting the [EGTA], and $[Ca^{2+}]$ was calculated using EQCAL software (Biosoft, Ferguson, MO, USA). In recordings using the perforated patch technique, pipettes were filled with the standard intracellular solution, in which was dissolved 100 μg ml^{-1} of amphotericin B (Sigma). This was made up as a stock solution (3 mg (50 $\mu l)^{-1}$ DMSO) on the day of use and aliquots of stock solution were sonicated in high-K^+ intracellular solution to the final concentration. Whole-cell currents were recorded with an Axopatch 200A amplifier (Axon Instruments) interfaced through an A/D converter (Labmaster, Axon Instruments) to a Pentium-chip Dell computer running pCLAMP 6.02 acquisition software (Axon Instruments). Current recordings were low-pass filtered at 1 kHz on the amplifier and digitized at 2–5 kHz. Data were analysed using the same software. Patch pipettes used in cell-attached and excised patch recordings were drawn from narrow diameter glass tubing (Kimax 51, no. 34502, Kimble). Patch currents were recorded using the capacitor-feedback voltage-clamp circuit of the Axopatch 200A amplifier, low-pass filtered at 200 Hz (Ithaco 4302, DL Instruments, Dryden, NY, USA) and digitized at 1 kHz using the same software. For cell-attached and inside-out patch recordings, pipettes were filled with standard PS and cells or patches were bathed in standard intracellular (high-K^+) solution, thus establishing an asymmetrical K^+ gradient across the recording patch. Under these conditions, the reversal potentials for Cl^- and non-selective cation-conducting channels were near 0 mV, which is the potential at which most recordings were obtained. When required, $[Ca^{2+}]$ of the high-K^+ solution bathing cells or inside-out patches was adjusted by varying [EGTA]. In outside-out excised patch recordings, the pipette was filled with high-K^+ solution with $[Ca^{2+}]$ buffered to 150 nM and the bath was perfused with standard PS. Single-channel data were analysed using Fetchan and pSTAT within pCLAMP6. Open probability (NP_o) was estimated by fitting all-point histograms of digitized 60 s continuous traces with Gaussian distributions, and summing the products of the relative areas of each distribution and the unitary current level they represented. All recordings were obtained at room temperature (22–24 °C). Cell capacitance was measured in perforated patch

recordings using the whole-cell capacitance neutralization circuitry on the patch-clamp amplifier. Access resistance was monitored by adjusting the series resistance measurement dial and recordings were begun when the series resistance was < 25 MΩ. In conventional whole-cell recordings, cell capacitance (in pF) was estimated by integrating the capacitance current with respect to time to measure charge (Q, in pA ms) in response to a 10 mV depolarizing pulse from −80 mV, and dividing Q by the voltage step (10 mV). Series resistance compensation was employed to a level just below ringing.

Drugs

Apamin was purchased from Sigma, dissolved in distilled water and stored in 0·5 ml (0·5 mM) aliquots at −20 °C. Aliquots were thawed on the day of use as required. Other drugs used included scyllatoxin (Peptide Institute, Japan), which was prepared in the same way as apamin, and TEA (Sigma), which was stored as a 1 M stock solution at 4 °C. The purines, ATP (tetrasodium salt, Sigma) and 2-chloroATP (2-ClATP), 2-methylthioATP (2-MeSATP) and α,β-methyleneATP (all three from RBI, Natick, MA, USA), were made up as 10^{-2} M stock solutions in distilled water and stored at −20 °C. Drugs were either dissolved in the pipette-filling solution or in the perfusion buffer to their final concentrations. A23187 (Calbiochem) was dissolved in DMSO at a concentration in the stock solution of 10^{-2} M.

RESULTS

Apamin-sensitive whole-cell K^+ currents

Previous studies on secretory cells have emphasized the dependence of apamin-sensitive K^+ currents on Ca^{2+} entry through voltage-gated Ca^{2+} channels (Park, 1994). To minimize run-down of Ca^{2+} currents in smooth muscle cells freshly dissociated from the mouse ileum, we used the perforated patch technique to record whole-cell currents in response to step depolarizations (340 ms) through a range of potentials (−100 to +40 mV) from a holding potential (V_h) of −80 mV. Typically, net outward currents were generated at potentials positive to −50 mV and consisted of an initial transient outward current followed by a sustained

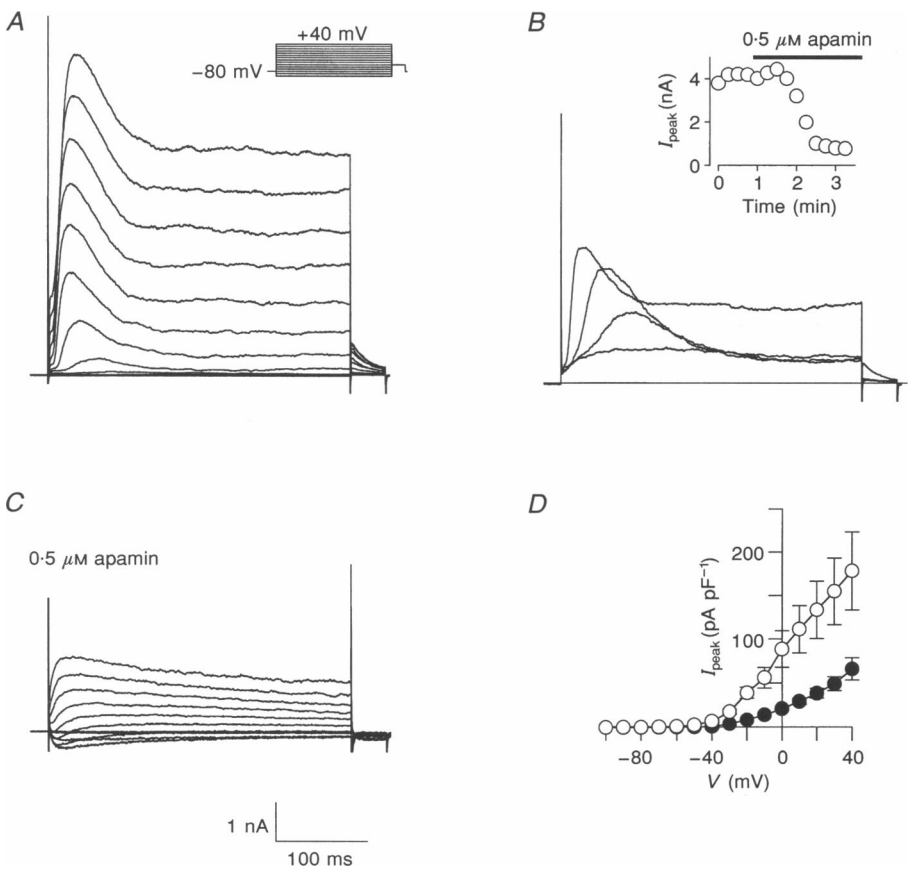

Figure 1. Effect of apamin on whole-cell currents recorded from a smooth muscle cell of the mouse ileum using the perforated patch technique

A, membrane potential was depolarized in steps (340 ms) from a holding potential of −80 mV and repolarized briefly to −50 mV before being returned to −80 mV. Voltage steps were applied every 8 s. *B*, wash-in of apamin (0·5 μM) caused a reduction in the peak and sustained components of outward current evoked by step depolarizations to 0 mV, repeated every 15 s, and abolished the outward tail current. Inset shows time course of the reduction in peak outward current by apamin. *C*, the same stimulus protocol as in *A*, applied in the presence of apamin. Note inward current. *D*, current–voltage relationship of peak outward current normalized to cell capacitance (I_{peak}) in the absence (○) and presence (●) of apamin (0·5 μM), averaged from six cells.

outward current (Fig. 1*A*). The sustained component was followed by a slowly deactivating outward tail current upon repolarization to −50 mV (Fig. 1*A*). Application of a saturating concentration of apamin (0·5 μM) decreased both the transient and sustained components of outward current and abolished the tail current (Fig. 1*B*). Apamin reduced the outward current at all test potentials (Fig. 1*C*). In the cell depicted in Fig. 1*C*, apamin unmasked an inward current that was subsequently blocked by 0·1 mM Cd^{2+} (not shown). The current–voltage (*I–V*) relationship of the peak outward current normalized to cell capacitance averaged from six cells, before and after apamin, revealed that this current accounted for up to 70% of the net outward current at potentials positive to −50 mV (Fig. 1*D*).

We then attempted to demonstrate the Ca^{2+} dependence of the apamin-sensitive current using the conventional whole-cell recording configuration by perfusing cells internally with solutions containing known [Ca^{2+}]. When [Ca^{2+}]$_i$ was clamped to ~150 nM, cells remained relaxed and we were able to faithfully record outward currents in response to the standard stimulus protocol. Typically, test depolarizations positive to −50 mV evoked net outward currents that were sustained throughout the duration of the pulse (Fig. 2*A*) and were followed by slowly deactivating tail currents at −50 mV (Fig. 2*Aa*). Apamin (0·5 μM) suppressed the sustained current and decreased the tail current (Fig. 2*Aa*). The decrease in whole-cell current elicited by apamin was essentially irreversible, with < 10% of the current recovering after 30 min of washout of apamin. Addition of TEA (2 mM) further reduced the sustained current (Fig. 2*Aa*). The *I–V* relationships of the peak current normalized to cell capacitance before and after apamin treatment, averaged from eight cells, revealed the presence of an apamin-sensitive current at all test potentials positive to −60 mV (Fig. 2*Ab*). The net outward current was reduced further by 2 mM TEA, a concentration known to block BK_{Ca} channels (Fig. 2*Ab*) (Latorre *et al.* 1989).

When [Ca^{2+}]$_i$ was clamped to ~85 nM, however, in eight cells studied, test depolarizations failed to activate the characteristically large apamin-sensitive sustained outward currents (Fig. 2*Ba*). The outward current usually consisted of a rapid transient outward current followed by a 'noisy' outward current superimposed on a more slowly inactivating current (Fig. 2*Ba*). The noisy current was abolished by TEA

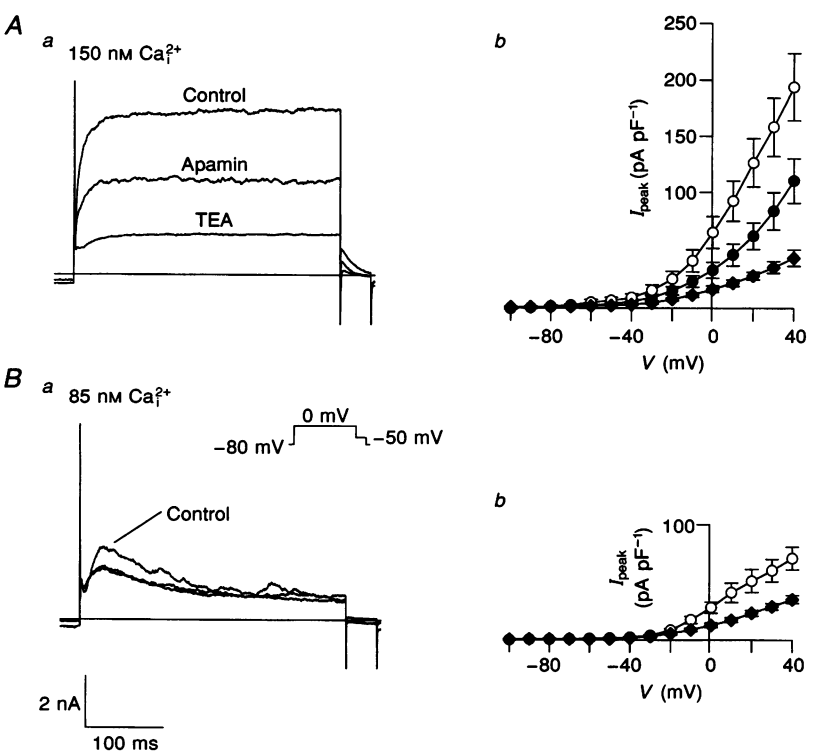

Figure 2. Effect of intracellular [Ca^{2+}] on expression of the apamin-sensitive outward current

Aa, net outward currents evoked by voltage steps to 0 mV in an ileal smooth muscle cell perfused internally with 150 nM [Ca^{2+}] high-K^+ solution, showing suppression of current by apamin (0·5 μM) and by further addition of TEA (2 mM). *Ab*, *I–V* relationship, averaged from eight cells, of the peak current normalized to cell capacitance recorded in 150 nM [Ca^{2+}]-loaded cells under control conditions (○), in the presence of apamin (0·5 μM; ●) and in the presence of both apamin and TEA (2 mM; ◆). *Ba*, typical net outward currents recorded from an ileal smooth muscle cell loaded with 85 nM [Ca^{2+}] high-K^+ solution, showing suppression of noisy current by TEA (2 mM) and lack of further suppression of outward current by addition of apamin (0·5 μM) to the TEA-containing bathing solution. *Bb*, *I–V* relationship averaged from eight cells showing peak current in the absence (○) and in the presence (◆) of TEA (2 mM).

(2 mM) but apamin applied before or after TEA produced no further reduction in the outward current. The peak whole-cell current was reduced by TEA at all potentials positive to −40 mV (Fig. 2*Bb*). In cells internally perfused with 150 nM Ca^{2+}, apamin had a greater effect in suppressing the outward current when applied before (52 ± 10%, $n = 4$) than after 2 mM TEA (4 ± 6%, $n = 3$) at a test potential of 0 mV, indicating that a large portion of the apamin-sensitive outward current is also sensitive to 2 mM TEA.

Leiurustoxin I or scyllatoxin blocks the same population of TEA-resistant Ca^{2+}-activated K^+ channels in neuronal cells as apamin (Auguste *et al.* 1992). Thus we tested the action of scyllatoxin (0·5 μM) on mouse ileal smooth muscle cells and, in two of three cells, the whole-cell outward current was reduced by 71 ± 15% ($n = 2$), further suggesting that such channels are expressed in smooth muscle of the mouse ileum.

Apamin-sensitive single K^+ channel currents

To investigate the nature of the K^+ channels underlying the apamin-sensitive whole-cell currents in smooth muscle cells from the mouse ileum, we obtained single-channel recordings from cell-attached and excised patches. In the cell-attached configuration, pipettes were filled with PS containing 2·5 mM K^+ and cells were bathed in solution containing 150 mM K^+ (high-K^+ solution) with 150 nM Ca^{2+}. In the absence of any blocker in the pipette, at least three non-inactivating K^+ channel types could be discerned. The largest unitary currents (5–7 pA) were usually activated at values of V_h positive to 0 mV (Fig. 3*A*). However, channel openings were seen at more negative potentials, generated by what was clearly a different channel (Fig. 3*A*). The conductance of the negatively activating K^+ channel was estimated by fitting the Goldman–Hodgkin–Katz (GHK) equation (Hille, 1984) to the unitary current–voltage (*i–V*) relationship constructed from averaged data from seven cells and was found to be 39 pS (Fig. 3*B*). We classified these channels as intermediate conductance K^+ (IK_{Ca}) channels. Given that the unitary current through the positively activating large conductance channel was about 5-fold larger, the larger channels were assumed to be BK_{Ca} channels.

In addition to BK_{Ca} and IK_{Ca} channels, in forty-two cell-attached patches K^+ channels with a smaller conductance were also present (Fig. 4*Aa*). Precise measurement of the unitary conductances of these channels was hampered by the presence of contaminating cationic or chloride channel

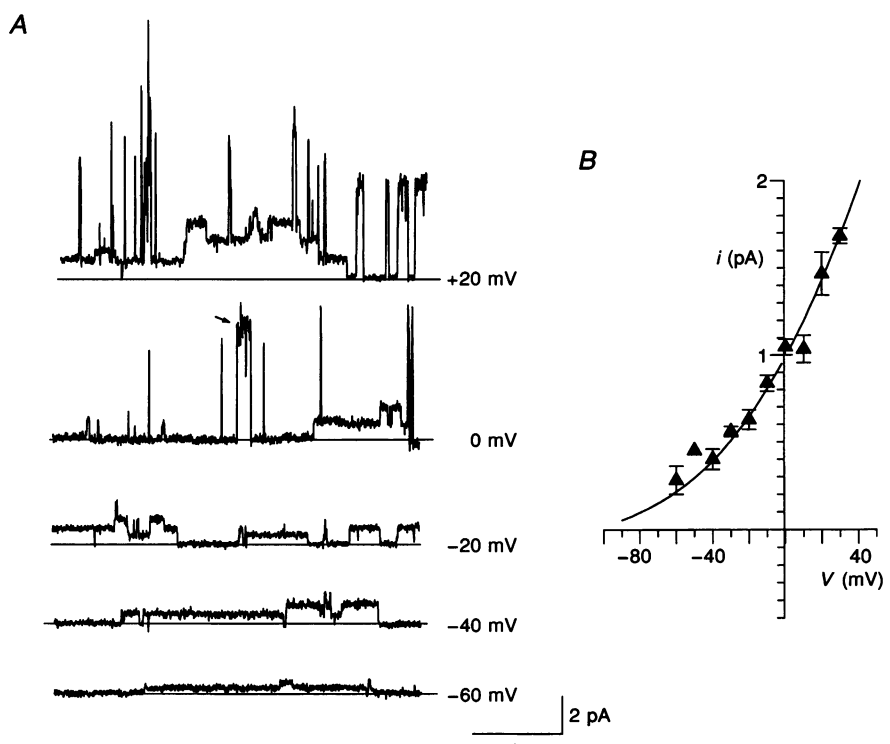

Figure 3. Unitary K^+ channel currents recorded in cell-attached patches from mouse ileal smooth muscle cells under an asymmetrical K^+ gradient (2·5 mM K^+_o : 150 mM K^+_i)

[Ca^{2+}] in the bathing solution was buffered to 150 nM with EGTA and pipettes were filled with PS. In this and subsequent figures, the continuous line through the current traces signifies the closed channel level and channel openings are upwards. *A*, large conductance channels (arrow) opened at holding potentials positive to 0 mV. Openings of smaller conductance channels were discerned at more negative potentials. These channels appeared to have subconductance states. *B*, current–voltage relationship of smaller conductance channels averaged from seven cells was fitted to the GHK equation and yielded a limiting slope conductance at positive potentials of 39 pS.

currents at potentials away from 0 mV. However, based on their unitary current at 0 mV ($0{\cdot}2 \pm 0{\cdot}01$ pA, $n = 6$) (Fig. 4*Ab*), unitary conductance was estimated at ~10 pS and they were classified as small conductance K^+ (SK_{Ca}) channels. The possibility that any of these K^+ channels could be inhibited by apamin was tested by adding apamin ($0{\cdot}5$ μM) to the pipette solution. In eight such patches, both BK_{Ca} and IK_{Ca} channels continued to gate (Fig. 4*Ba*), although the activity of the IK_{Ca} channels appeared to be decreased compared with control patches (Fig. 4*Bb*). Openings of SK_{Ca} channels were also decreased in frequency or abolished by apamin. The suppression of the activity of IK_{Ca} channels by apamin could also be demonstrated in the same cell-attached patches by monitoring channel activity with time after seal formation (Fig. 4*C*). Apamin decreased the average patch current by decreasing the open probability of the IK_{Ca} channels within 2–3 min of gigaseal formation (Fig. 4*D*).

TEA at 1–2 mM is widely used to block BK_{Ca} channel currents in smooth muscle preparations (e.g. Benham *et al.* 1985). We tested whether TEA (2 mM) could also affect the non-inactivating IK_{Ca} and SK_{Ca} channels, given that in whole-cell recordings part of the Ca^{2+}-dependent apamin-sensitive outward current is also blocked by TEA. In eight cell-attached patches tested, when TEA was included in the pipette solution, as expected BK_{Ca} channels were absent but, in addition, openings of IK_{Ca} channels were also attenuated or blocked. The openings of SK_{Ca} channels were little affected by TEA.

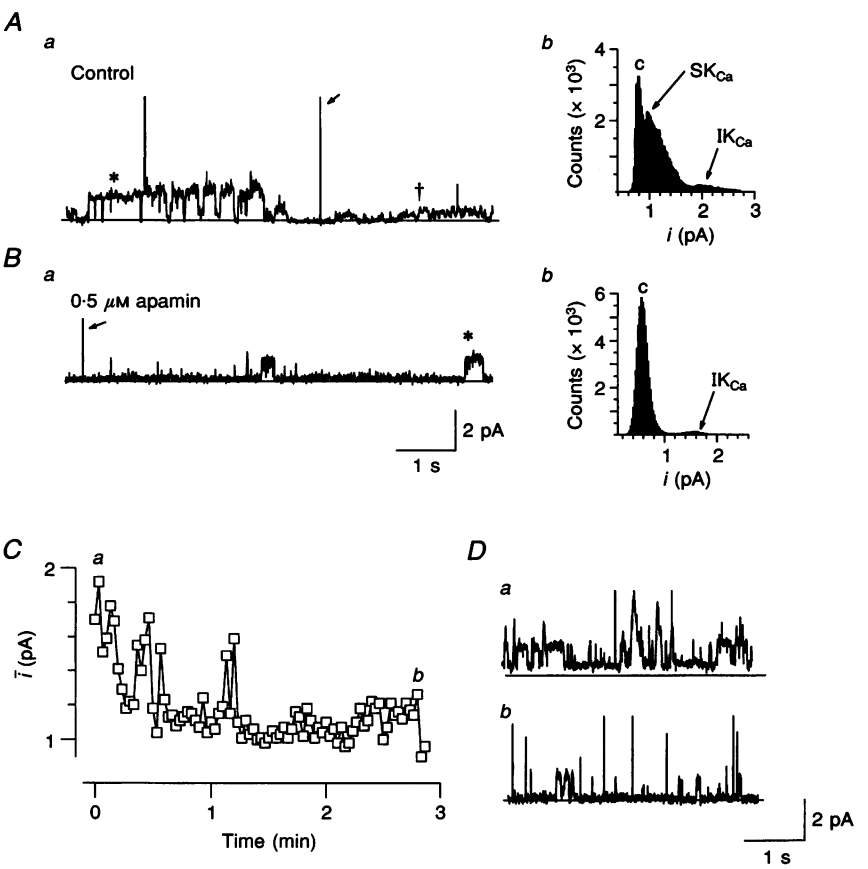

Figure 4. Cell-attached patch recordings under an asymmetrical K^+ gradient at a holding potential of 0 mV

Aa, patch recording showing openings of non-inactivating large conductance K^+ channels (arrow), 39 pS IK_{Ca} channels (*) and small conductance SK_{Ca} channels (†). *Ab*, all-points histogram of 60 s continuous recording from the patch depicted in *Aa* showing closed channel level (c) and the peaks of the Gaussian distribution corresponding to unitary current level of SK_{Ca} and IK_{Ca} channels. Currents were not leak subtracted. *Ba*, unitary currents recorded under the same conditions as in *Aa* but from a different cell and with apamin ($0{\cdot}5$ μM) added to the PS in the pipette. Note openings of large conductance channel (arrow) and IK_{Ca} channel (*) but absence of openings of the small conductance channels. *Bb*, all-points histogram of a 60 s continuous trace from which the trace in *Ba* was extracted showing the distribution corresponding to the closed channel level (c) and the open IK_{Ca} channel current. *C*, time course of the reduction in the average patch current recorded from a cell-attached patch with apamin ($0{\cdot}5$ μM) contained in the pipette solution. Each data point represents the average patch current over 2 s. *D*, representative traces from the recording depicted in *C* corresponding to the period immediately after seal formation (*a*) and approximately 2·5 min later (*b*).

To determine whether these TEA-resistant SK_{Ca} channels were Ca^{2+} dependent, the effect of Ca^{2+} ionophore (A23187, 5 μM) was tested on three outside-out patches. Addition of A23187 to the bathing solution resulted in a tremendous increase in the activity of SK_{Ca} channels (cf. Fig. 5*Aa* and *b*). Their open probability measured from all-points histogram plots (Fig. 5*Ba* and *b*) was increased over 10-fold from 0·067 to 2·402 ($n = 3$) at a V_h of 0 mV. To further test the Ca^{2+} sensitivity of these TEA-resistant SK_{Ca} channels, excised inside-out patches, with 5 mM TEA added to the PS in the pipette to block BK_{Ca} and IK_{Ca} channels, were exposed on the cytoplasmic side to varying $[Ca^{2+}]$ and the average current at +50 mV elicited by ten 7·5 s ramp depolarizations to +50 mV from −100 mV, was measured (Fig. 5*C*). The mean current was plotted as a function of $[Ca^{2+}]_i$ (Fig. 5*D*) normalized to the peak current calculated by fitting the data points with the Hill equation (Hille, 1984). In six such patches, the fitted curves yielded an EC_{50} of 1·8 ± 0·4 μM and a slope factor (n) of 1·6 ± 0·25. These values were similar to the corresponding values obtained by fitting the averaged data points with a single curve using the Hill equation, of 1·5 μM and 1·3, respectively (Fig. 5*D*).

Effect of purinergic agonists on K^+ channels

The actions of purinergic receptor stimulation on K^+ channel currents were recorded in cell-attached patches in response to application of agonists to the membrane outside the patch. Preliminary whole-cell current recordings using the perforated patch technique indicated that P_2 purinoceptor agonists enhanced the outward current (Fig. 6*A*). This current, which persisted despite brief washout of agonist, was largely blocked by subsequent application of apamin (Fig. 6*A* and *B*). On average, the P_{2Y} receptor agonists 2-MeSATP (20 μM) and 2-ClATP (20 μM) increased the peak outward current by 42 ± 19% ($n = 3$) and by 32 ± 25% ($n = 3$), respectively, and ATP (10–30 μM) increased it by 42 ± 19% ($n = 3$). Stimulation with α,β-methyleneATP (10–30 μM) had no effect on net outward current.

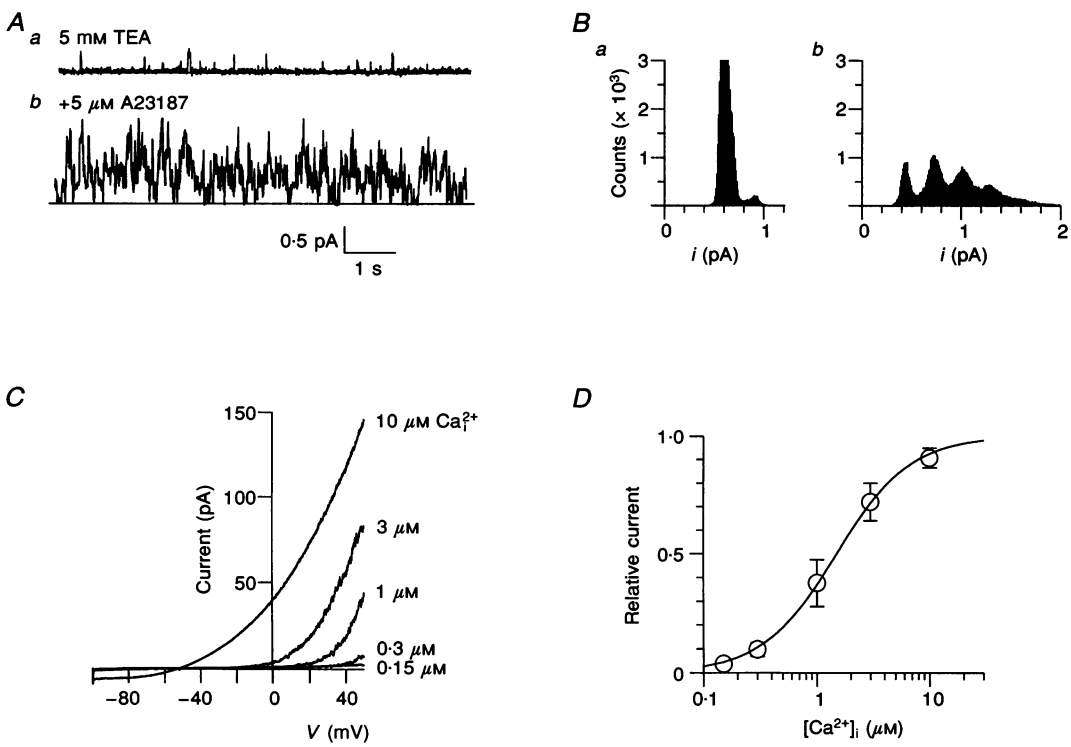

Figure 5. Calcium sensitivity of the TEA-resistant SK_{Ca} channels

A, activation of TEA-resistant small conductance K^+ channels by ionophore-stimulated Ca^{2+} influx in an outside-out patch at a holding potential of 0 mV. *Aa*, representative trace showing sporadic openings of the SK_{Ca} channels in a patch perfused externally with TEA (5 mM) to block BK_{Ca} and IK_{Ca} channels. *Ab*, addition of A23187 (5 μM) to the bathing solution increased the activity of SK_{Ca} channels and a maximum of five channels opened simultaneously. *Ba*, all-points histograms of the extended 60 s recordings revealed that only a single channel was open at any give time in the absence of ionophore. *Bb*, after addition of ionophore the NP_o of the 10 pS channels was increased from 0·127 to 2·022. Data traces were low-pass filtered at 100 Hz. *C*, a family of ramp currents elicited by ramp potentials (−100 to +50 mV over 7·5 s) recorded at different $[Ca^{2+}]_i$ in an inside-out patch with 5 mM TEA added to the pipette solution. *D*, the average normalized patch currents at +50 mV are plotted as a function of $[Ca^{2+}]_i$. The averaged data points fitted with the Hill equation yielded an EC_{50} of 1·5 μM and a slope of 1·3.

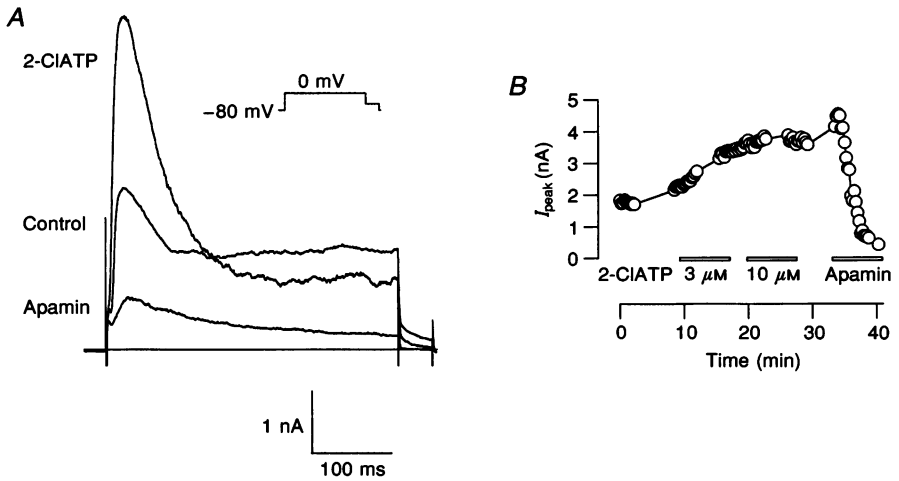

Figure 6. Effect of 2-ClATP on the outward current recorded from an ileal smooth muscle cell using the perforated patch technique

A, the cell was held at −80 mV and the membrane potential was stepped to 0 mV. 2-ClATP (10 μM) increased the peak outward current. Upon the addition of apamin (0·5 μM) to the bathing solution, the peak and sustained outward current components were decreased below the control level. *B*, time course of enhancement of peak outward current by 2-ClATP and subsequent suppression by apamin.

In cell-attached patches, application of 2-MeSATP to the extrapatch membrane consistently increased the activity of K^+ channels in the patch (Fig. 7*A*). The most conspicuous increase occurred with IK_{Ca} channels (Fig. 8*Aa* and *b*), whose open probability (NP_o) was significantly increased ($P < 0{\cdot}05$, paired t test) from 0·16 ± 0·048 to 0·58 ± 0·11 ($n = 10$) by 50 μM 2-MeSATP (e.g. Fig. 8*Ac*). Although, in many patches, the activity of BK_{Ca} channels was also increased upon purinoceptor stimulation, in eight of twenty-one patches little or no effect on the activity of these channels ensued upon purinoceptor stimulation (e.g. Fig. 7*A*). Inclusion of apamin (0·5 μM) in the pipette solution prevented the expected increase in the activity of IK_{Ca} channels upon purinoceptor stimulation (Figs 7*B* and 8*Ba*

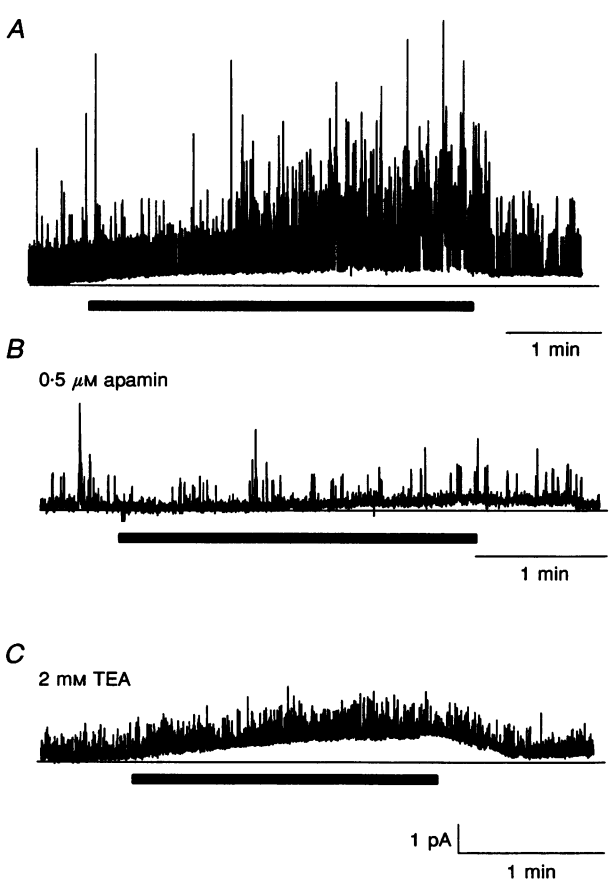

Figure 7. Effect of extra-patch application of 2-MeSATP (50 μM) on K^+ channel activity in cell-attached patches, at a holding potential of 0 mV

A, with PS alone in the pipette, 2-MeSATP increased the activity of IK_{Ca} channels and increased the baseline current. *B*, similar conditions to those in *A* but apamin (0·5 μM) was included in the pipette solution. 2-MeSATP failed to increase the activity of IK_{Ca} channels and elicited a much smaller change in the baseline current. *C*, in the presence of TEA (2 mM) on the outside of the patch, 2-MeSATP increased the activity of SK_{Ca} channels and increased the baseline current. The filled bar below each trace represents the period of application of 2-MeSATP.

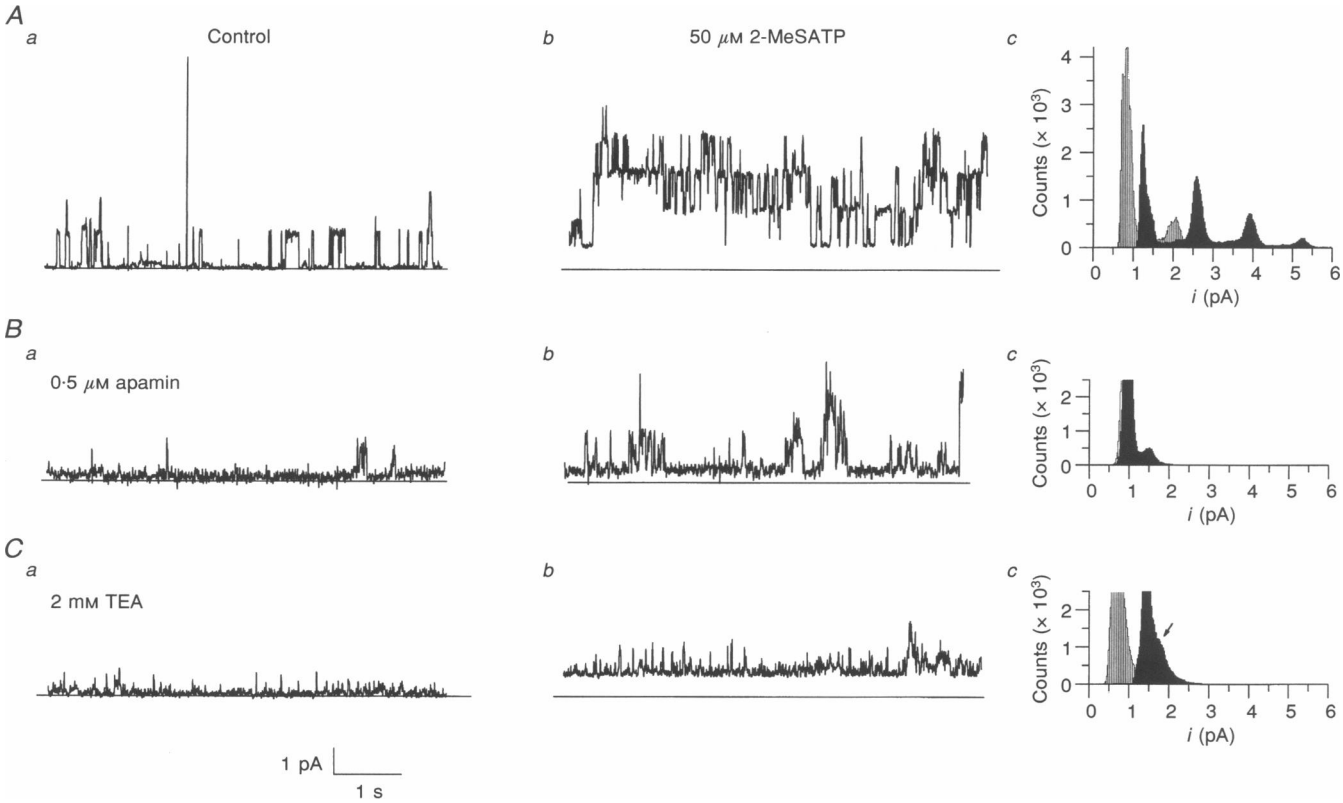

Figure 8. Expanded portions of the traces shown in Fig. 7 and corresponding all-points histograms

Channel activity recorded at a holding potential of 0 mV is shown before application of 2-MeSATP (50 μM) in the absence of blockers in the patch pipette (*Aa*), in the presence of apamin (0·5 μM; *Ba*) and with TEA (2 mM) added to the pipette solution (*Ca*). The corresponding activity recorded during exposure to agonist is shown in *Ab*, *Bb* and *Cb*. *Ac*, *Bc* and *Cc* show superimposed all-points histogram plots of 60 s recordings in the absence (open bars) and presence (filled bars) of 2-MeSATP. 2-MeSATP increased the open probability of IK_{Ca} channels (*Ac*). With apamin in the pipette, no such increase was evident (*Bc*). TEA (2 mM) suppressed the activity of IK_{Ca} channels but the open probability of SK_{Ca} channels was increased, indicated by the appearance of a distinct hump adjacent to the closed channel level (arrow).

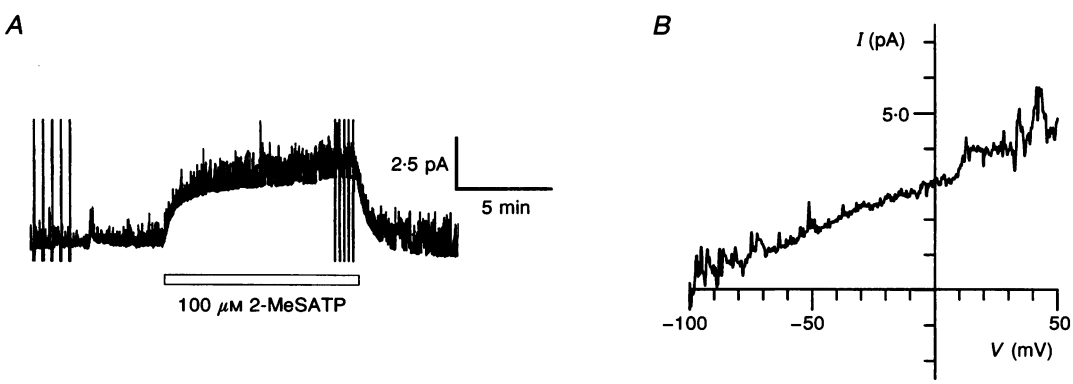

Figure 9. Reversal potential of the baseline current elicited by 2-MeSATP (100 μM) in a cell-attached patch under an asymmetrical K^+ gradient, at a holding potential of 0 mV

A, typical recording showing the increase in baseline current upon wash-in of agonist (open bar below the trace). The vertical lines through the trace represent currents elicited by ramp potentials applied before and during exposure to the agonist. *B*, ramp 'difference' current plotted as a function of ramp potential showing that the agonist-stimulated steady outward current reversed at potentials approaching E_K.

and *b*); the NP_o of these channels averaged $0{\cdot}14 \pm 0{\cdot}04$ before and $0{\cdot}15 \pm 0{\cdot}06$ after the agonist ($n = 8$) (Fig. 8*Bc*). In the presence of 2 mM TEA in the pipette, purinoceptor stimulation (2-MeSATP, 50 μM) increased the NP_o of the activity of SK_{Ca} channels over 4-fold from $0{\cdot}073 \pm 0{\cdot}031$ to $0{\cdot}326 \pm 0{\cdot}121$ ($n = 6$) (Fig. 8*Ca–c*). In addition to stimulating channel activity, purinoceptor stimulation also elicited an increase ($0{\cdot}76 \pm 0{\cdot}2$ pA, $n = 8$) in basal outward current at 0 mV (Fig. 7*A*), which was reduced in the presence of apamin ($0{\cdot}23 \pm 0{\cdot}06$ pA, $n = 7$; Fig. 7*B*) but was unaffected by TEA ($0{\cdot}64 \pm 0{\cdot}2$ pA, $n = 6$; Fig. 7*C*). In some cells, the steady outward current coincided with an increase in the activity of SK_{Ca} channels but in other cells no clear channel openings could be discerned at the resolution of our recordings (Fig. 9*A*). Because this steady outward current reversed close to the equilibrium potential for K^+ (E_K) (Fig. 9*B*), it suggests that purinoceptor stimulation may activate K^+ channel conductances too small to resolve at present.

DISCUSSION

A large fraction of the Ca^{2+}-dependent K^+ conductance in smooth muscle cells is generated by the opening of BK_{Ca} channels. The possibility that K^+ channels other than BK_{Ca} contribute to the Ca^{2+}-dependent K^+ conductance in smooth muscle cells has received less attention. Apamin, a peptide found in bee venom which specifically blocks small conductance Ca^{2+}-activated K^+ channels in non-smooth muscle preparations (Blatz & Magleby, 1986), depolarizes many types of gut smooth muscles and simultaneously eliminates a large portion of the IJP (Shuba & Vladimirova, 1980). This suggests that apamin-sensitive K^+ channels are active at rest and in addition transduce inhibitory neuronal inputs in smooth muscle.

Despite extensive study of K^+ channel currents expressed in visceral smooth muscles, apamin-sensitive K^+ channel currents have largely eluded identification. However, in smooth muscle cells dissociated from rat renal arterioles, Gebremedhin *et al.* (1996) recently reported that apamin selectively blocked a 68 pS Ca^{2+}-activated K^+ channel and in oesophageal smooth muscle cells from the opossum, Jury, Boev & Daniel (1996) reported that apamin decreased outward current at positive potentials. The circular muscle layer of the mouse ileum has an unstable resting potential that oscillates between −45 and −60 mV. Both TEA (2 mM) and apamin (0·3–1 μM) depolarize the resting potential by 10–15 mV but only apamin suppresses a component of the NANC nerve-mediated IJP (He & Goyal, 1993). These data suggest that the K^+ conductance that contributes to the resting potential is different from the conductance underlying the apamin-sensitive IJP.

In the present study we have recorded a Ca^{2+}-dependent whole-cell K^+ current in smooth muscle cells from the mouse ileum which is substantially decreased by apamin over a potential range experienced by cells *in situ* (−60 to 0 mV). In cell-attached patches we identified two major TEA-sensitive conductance levels which we attribute to two different types of K^+ channels: (i) a large conductance (~200 pS) (BK_{Ca}) channel; and (ii) an intermediate conductance (IK_{Ca}) channel with a conductance approximately one-fifth that of BK_{Ca} channels. Of these two major K^+ channel types, only IK_{Ca} channels were sensitive to external apamin. Because the apamin-sensitive whole-cell currents were largely eliminated by pretreatment with TEA (2 mM), these currents are likely to be generated mainly by the opening of IK_{Ca} channels. This conclusion is supported by the observation that, in the same patch, IK_{Ca} channels opened at more negative potentials than BK_{Ca} channels. Moreover, IK_{Ca} channels were characterized by quite long open times, which may account for the slow deactivation of whole-cell tail currents. In rat chromaffin cells, slow tail currents associated with apamin-sensitive currents are attributed to the slow decay in submembrane [Ca^{2+}] (Park, 1994). In the present study, such tail currents were recorded with [Ca^{2+}] clamped to ~150 nM, suggesting that IK_{Ca} channels may be voltage dependent, as evident in single-channel recordings. Voltage dependence has been previously described for an apamin-sensitive, Ca_i^{2+}-dependent delayed rectifier-type K^+ channel in guinea-pig hepatocytes (Koumi, Sato, Horikawa, Aramaki & Okumura, 1994*b*) and in guinea-pig cardiac myocytes (Koumi, Sato & Hayakawa, 1994*a*). However, the participation of SK_{Ca} channels in the whole-cell outward currents in ileal cells cannot be excluded. In a perforated patch recording, TEA (2 mM) alone, which would be expected to block both BK_{Ca} and IK_{Ca} channels, decreased the transient outward current by 52% but the addition of Cd^{2+} (0·1 mM) to block Ca^{2+} channels reduced the residual current by a further 33%, suggesting that TEA-resistant SK_{Ca} channels may be activated by Ca^{2+} influx, for example during an action potential.

The IK_{Ca} channels in mouse ileal smooth muscle cells are similar in pharmacology to the 68 pS channels described by Gebremedhin *et al.* (1996). However, openings of IK_{Ca} channels in the mouse ileum persisted in the presence of a relatively high concentration of apamin (0·5 μM), whereas the 68 pS channels in rat renal arterioles were fully blocked by 50 nM apamin. Moreover, in rat renal arterioles, the 68 pS channels were fully blocked by 0·1 mM TEA, whereas the IK_{Ca} channels required > 2 mM TEA for block, suggesting that, although similar, these channels are not identical. The concentration of apamin required to inhibit outward currents in ileal cells (30 nM produced about 10% inhibition) was in excess of the IC_{50} values reported in rat chromaffin cells (4·4 nM; Park, 1994) and rat hepatocytes (1·4 nM; Yamashita, Ogawa & Akaike, 1996). The low apparent sensitivity to apamin of IK_{Ca} channels in mouse ileal cells may reflect structural differences in the apamin-binding regions. The recent cloning of a family of apamin-sensitive and -insensitive K^+ channels from rat and human brain (see Köhler *et al.* 1996) may elucidate the structural determinants of apamin binding. Confirmation that the low sensitivity of IK_{Ca} channels to apamin is based on structure

will have to await their molecular characterization. Another possible explanation for the low apamin sensitivity that cannot be overlooked is that IK_{Ca} and SK_{Ca} channels may have be modified by proteolytic enzymes used in cell dispersion. Apamin binding to brain synaptosomes is abolished by protease treatment of synaptosomes (Hugues, Duval, Kitabgi, Lazdunski & Vincent, 1982) and, moreover, collagenase-dissociated chromaffin cells require 2–3 days in culture for the recovery of apamin-sensitive currents (Park, 1994).

The NANC neurotransmitter responsible for the apamin-sensitive IJP has been proposed to be ATP (Banks *et al.* 1979; Crist, He & Goyal, 1992). In the present study stimulation of P_{2Y} receptors on ileal smooth muscle cells mildly activated BK_{Ca} channels but strongly activated IK_{Ca} and SK_{Ca} channels and a steady outward current in cell-attached patches, and enhanced an apamin-sensitive whole-cell outward current. Because previous studies have shown that the apamin-sensitive IJP is resistant to millimolar TEA (Bauer & Kuriyama, 1982), our present data suggest that if indeed ATP is the neurotransmitter responsible for the apamin-sensitive IJP, the membrane hyperpolarization may result from the opening of SK_{Ca} channels and/or the steady outward current, and not from IK_{Ca} channels, which are blocked by millimolar TEA. Since we have shown that SK_{Ca} channels are activated by intracellular Ca^{2+} and stimulation of P_{2Y} purinoceptors triggers release of $InsP_3$-sensitive Ca^{2+} stores (Boarder, Weisman, Turner & Wilkinson, 1995; Yamashita *et al.* 1996), the involvement of SK_{Ca} channels in membrane hyperpolarization elicited by purinergic agonists is a distinct possibility. Based on the amplitude of the patch current at 0 mV (~1 pA) and a corresponding unitary current of ~0·2 pA, we estimate that five such channels are activated in a patch by 1 μM [Ca^{2+}]. Assuming a cell surface area of 5000 μm^2, a uniform density of channels and a patch area of 1–2 μm, then a current of greater than 2·5 nA would be expected to be generated by each cell at 0 mV. This magnitude of current is within the range of peak outward currents activated by depolarizing steps to 0 mV during purinoceptor stimulation.

At present, the nature of the steady outward current stimulated by purinoceptor agonists is not clear and deserves a separate study. This current reversed near E_K, but its smooth activation upon application of agonist suggests that it is not generated by SK_{Ca} channels exclusively. Moreover, the rapid activation and deactivation with agonist stimulation, in contrast to the long latency and persistent activity of IK_{Ca} and SK_{Ca} channels after washout of agonist, alludes to different mechanisms of regulation. One possibility is that this current is generated by the recruitment of channels of a smaller conductance than SK_{Ca} by second messengers preceding Ca^{2+} release.

In summary, we have identified two novel apamin-sensitive Ca^{2+}-dependent K^+ channels in smooth muscle cells of the mouse ileum. These two channels can account for the actions of apamin and TEA on the resting potential and on the membrane hyperpolarization elicited by purinergic agonists. It remains to be determined if analogous channels are expressed in other visceral smooth muscles.

Auguste, P., Hugues, M., Borsotto, M., Thibault, J., Romey, G., Coppola, T. & Lazdunski, M. (1992). Characterization and partial purification from pheachromocytoma cells of an endogenous equivalent of scyllatoxin, a scorpion toxin which blocks small conductance Ca^{2+}-activated K^+ channels. *Brain Research* **599**, 230–236.

Banks, B. E. C., Brown, C., Burgess, G. M., Burnstock, G., Claret, M., Cocks, T. M. & Jenkinson, D. H. (1979). Apamin blocks certain neurotransmitter-induced increases in potassium permeability. *Nature* **282**, 415–417.

Bauer, V. & Kuriyama, H. (1982). The nature of non-cholinergic, non-adrenergic transmission in longitudinal and circular muscles of the guinea-pig ileum. *Journal of Physiology* **322**, 375–391.

Benham, C. D., Bolton, T. B., Lang, R. J. & Takewaki, T. (1985). The mechanisms of action of Ba^{2+} and TEA on single Ca^{2+}-activated K^+ channels in arterial and intestinal smooth muscle cell membrane. *Pflügers Archiv* **403**, 120–127.

Bennett, M. R., Burnstock, G. & Holman, M. E. (1966). Transmission from perivascular inhibitory nerves to the smooth muscle of the guinea-pig taenia coli. *Journal of Physiology* **182**, 527–540.

Blatz, A. L. & Magleby, K. L. (1986). Single apamin-blocked Ca-activated K^+ channels of small conductance in cultured rat skeletal muscle. *Nature* **323**, 718–720.

Blatz, A. L. & Magleby, K. L. (1987). Calcium-activated potassium channels. *Trends in Neurosciences* **10**, 463–467.

Boarder, M. R., Weisman, G. A., Turner, J. T. & Wilkinson, G. F. (1995). G protein-coupled P_2 purinoceptors: from molecular biology to functional responses. *Trends in Pharmacological Sciences* **16**, 133–139.

Bywater, R. A. R. & Taylor, G. S. (1986). Non-cholinergic excitatory and inhibitory junction potentials in the circular smooth muscle of the guinea-pig ileum. *Journal of Physiology* **374**, 153–164.

Capiod, T. & Ogden, D. C. (1989). The properties of calcium-activated potassium ion channels in guinea-pig isolated hepatocytes. *Journal of Physiology* **409**, 285–295.

Carl, A. & Sanders, K. M. (1989). Ca^{2+}-activated K^+ channels of canine colonic myocytes. *American Journal of Physiology* **257**, C470–480.

Crist, J. R., He, X. D. & Goyal, R. K. (1992). Both ATP and the peptide VIP are inhibitory neurotransmitters in guinea-pig ileum circular muscle. *Journal of Physiology* **447**, 119–131.

Den Hertog, A. & Jager, L. P. (1975). Ion fluxes during the inhibitory junction potential in the guinea-pig taenia coli. *Journal of Physiology* **250**, 681–691.

Gebremedhin, D., Kaldunski, M., Jacobs, R., Harder, D. R. & Roman, R. (1996). Coexistence of two types of Ca^{2+}-activated K^+ channels in rat renal arterioles. *American Journal of Physiology* **270**, F69–81.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

He, X. D. & Goyal, R. K. (1993). Nitric oxide involvement in the peptide VIP-associated inhibitory junction potential in the guinea-pig ileum. *Journal of Physiology* **461**, 485–499.

Hille, B. (1984). *Ionic Channels in Excitable Membranes.* Sinauer Associates, Inc., Sunderland, MA, USA.

Hugues, M., Duval, D., Kitabgi, P., Lazdunski, M. & Vincent, J.-P. (1982). Preparation of a pure monoiodo derivative of the bee venom neurotoxin apamin and its binding properties to rat brain synaptosomes. *Journal of Biological Chemistry* **257**, 2762–2769.

Jury, J., Boev, K. K. & Daniel, E. E. (1996). Nitric oxide mediates outward potassium current in opossum esophageal circular smooth muscle. *American Journal of Physiology* **270**, G932–938.

Köhler, M., Hirschberg, B., Bond, C. T., Kinzie, J. M., Marrion, N. V., Maylie, J. & Adelman, J. P. (1996). Small-conductance, calcium-activated potassium channels from mammalian brain. *Science* **273**, 1709–1714.

Koumi, S., Sato, R. & Hayakawa, H. (1994*a*). Modulation of the delayed rectifier K^+ current by apamin in guinea-pig heart. *European Journal of Pharmacology* **261**, 213–216.

Koumi, S., Sato, R., Horikawa, T., Aramaki, T. & Okumura, H. (1994*b*). Characterization of the calcium-sensitive voltage-gated delayed rectifier potassium channel in isolated guinea-pig hepatocytes. *Journal of General Physiology* **104**, 147–171.

Lang, D. G. & Ritchie, A. K. (1990). Tetraethylammonium blockade of apamin-sensitive and insensitive Ca^{2+}-activated K^+ channels in a pituitary cell line. *Journal of Physiology* **425**, 117–132.

Latorre, R., Oberhauser, A., Labarca, P. & Alvarez, O. (1989). Varieties of calcium-activated potassium channels. *Annual Review of Physiology* **51**, 385–399.

Leinders, T. & Vijverberg, H. P. M. (1992). Ca^{2+} dependence of small Ca^{2+}-activated K^+ channels in cultured N1E-115 mouse neuroblastoma cells. *Pflügers Archiv* **422**, 223–232.

Merot, J., Bidet, M., Le Maout, S., Tauc, M. & Poujeol, P. (1989). Two types of K^+ channels in the apical membrane of rabbit proximal tubule in primary culture. *Biochimica et Biophysica Acta* **978**, 134–144.

Park, Y. (1994). Ion selectivity and gating of small conductance Ca^{2+}-activated K^+ channels in cultured rat adrenal chromaffin cells. *Journal of Physiology* **481**, 555–570.

Shuba, M. F. & Vladimirova, I. A. (1980). Effect of apamin on the electrical responses of smooth muscle to adenosine 5′-triphosphate and to non-adrenergic, non-cholinergic nerve stimulation. *Neuroscience* **5**, 853–859.

Singer, J. J. & Walsh, J. V. (1987). Characteristics of calcium-activated potassium channels in single smooth muscle cells using the patch-clamp technique. *Pflügers Archiv* **408**, 98–111.

Tomita, T. (1972). Conductance change during the inhibitory potential in the guinea-pig taenia coli. *Journal of Physiology* **225**, 693–703.

Vogalis, F. & Goyal, R. K. (1997). Apamin-sensitive K^+ channels in smooth muscle activated by purinoceptor stimulation. *Biophysical Journal* **72**, A20.

Vogalis, F. & Sanders, K. M. (1990). Excitatory and inhibitory neural regulation of canine pyloric smooth muscle. *American Journal of Physiology* **259**, G125–133.

Yamashita, Y., Ogawa, H. & Akaike, N. (1996). ATP-induced rise in apamin-sensitive Ca^{2+}-dependent K^+-conductance in adult rat hepatocytes. *American Journal of Physiology* **270**, G307–313.

Acknowledgements

The authors wish to thank Dr Hiroshi Mashimo for comments on the manuscript and for discussions and Dr Yong Zhang for technical assistance. This work was funded by NIH grants to F.V. (DK50137) and R.K.G. (DK31092).

Author's email address

F. Vogalis: vogalis@brockton.va.gov

Received 17 February 1997; accepted 25 April 1997.