British Journal of Pharmacology (1995) 115, 1117–1125

Potassium currents in human freshly isolated bronchial smooth muscle cells

[1]V.A. Snetkov, S.J. Hirst, C.H.C. Twort & J.P.T. Ward

Respiratory Research Laboratories, UMDS Smooth Muscle Group, Department of Allergy and Respiratory Medicine, UMDS, London SE1 7EH

1 K^+ currents were studied in smooth muscle cells enzymatically dissociated from human bronchi, by use of the patch-clamp technique.

2 In whole-cell recordings a depolarization-induced, 4-aminopyridine (4-AP)-sensitive current was observed in only 26 of 155 cells, and in 20 of these 26 cells its amplitude at a test potential of 0 mV was less than 100 pA.

3 In the majority of cells depolarization to –40 mV or more positive potentials induced a noisy outward current which activated within milliseconds and showed almost no inactivation even during a 5 s depolarizing voltage step. This current was insensitive to 4-AP (up to 5 mM) but was strongly inhibited in the presence of tetraethylammonium (TEA, 1 mM), charybdotoxin (ChTX, 100 nM) or iberiotoxin (IbTX, 50 nM) in the bath. The same current was also recorded by the nystatin-perforated patch technique.

4 Single channels with a conductance of about 210 pS were recorded in cell-attached patch, inside-out patch, outside-out patch and whole-cell recording configurations. Channel open state probability in inside-out patches was 0.5 at a membrane potential of 4 ± 14 mV (mean $\pm$ s.d., $n = 13$) mV even with a free Ca^{2+} concentration on the cytosolic side of the patch of less than 0.1 nM. Open state probability increased with depolarization and internal Ca^{2+} concentration. Single channels could be reversibly blocked by externally applied TEA, ChTX and IbTX.

5 In current-clamp recordings with 100 nM free Ca^{2+} in the intracellular solution both TEA and ChTX caused substantial concentration-dependent depolarization.

6 These results suggest that in human bronchial smooth muscle cells, in marked contrast to other species, the majority of the outward current induced by depolarization is not due to a delayed rectifier, but to the activity of a large conductance, ChTX-sensitive K^+ channel. The Ca^{2+}- and voltage-dependency of this channel may well allow a sufficiently high open state probability for it to play a part in the regulation of the resting membrane potential.

Keywords: Large conductance potassium channels; delayed rectifier current; 4-aminopyridine; charybdotoxin; iberiotoxin; human bronchial smooth muscle

Introduction

Previous studies of voltage-gated K^+ currents in airway smooth muscle cells (ASMC) from various species have demonstrated a delayed rectifying outward current that is independent of Ca^{2+} influx, and more sensitive to blockade by 4-aminopyridine (4-AP) than tetraethylammonium (TEA). Both whole-cell and single-channel data strongly support the hypothesis that in canine, porcine and ferret tracheal myocytes, the major outward K^+ current is a delayed rectifier (Kotlikoff, 1993). However, an outward current that is charybdotoxin (ChTX)-sensitive and activated by an increase in free cytosolic Ca^{2+} has also been reported and there is evidence that at least in some types of smooth muscle, including that of airways, large conductance, voltage- and Ca^{2+}-dependent K^+ (K_{Ca}) channels may play a role in the maintenance of membrane potential (Kannan *et al.*, 1983; Brayden & Nelson, 1992; Asano *et al.*, 1993). The presence of numerous K_{Ca} channels in canine (McCann & Welsh, 1986), porcine (Saunders & Farley, 1991), bovine (Green *et al.*, 1991) and rabbit (Kume *et al.*, 1990) ASMCs would suggest that these channels are of importance in the regulation of airway smooth muscle function, and the recent demonstration that TEA (1–10 mM) can cause a substantial depolarization in bovine tracheal ASMCs (Nara *et al.*, 1994) implies that these channels have a major influence on the membrane potential. Selective inhibition of K_{Ca} channels also influences the membrane potential in the guinea-pig trachealis, producing either regenerative action potentials (Murray *et al.*, 1991) or an increase in contractility (Suarez-Kurtz *et al.*, 1991).

The relative importance of K_{Ca} channels for outward rectification and resting potential depends on the cytosolic concentration of free Ca^{2+} ions ($[Ca^{2+}]_i$) and on channel Ca-sensitivity. It was found using both bulk fluorometric measurement (Murray & Kotlikoff, 1991) and single cell Ca^{2+} imaging (Murray *et al.*, 1993) that in human cultured airway smooth muscle cells, resting $[Ca^{2+}]_i$ is about 160 nM. Data from experiments with outside-out membrane patches from guinea-pig (Murray *et al.*, 1991) and porcine (Kume & Kotlikoff, 1991) trachealis ASMC show that at $[Ca^{2+}]_i$ of 100 nM and at a membrane potential of 0 mV, K_{Ca} channels could easily be recorded. In inside-out membrane patches from canine trachealis at more depolarized potentials, K_{Ca} channel activity was recorded at $[Ca^{2+}]_i$ of less than 0.1 nM (McCann & Welsh, 1986).

It is likely that smooth muscle tissues differ substantially in the degree to which various K^+ conductances control the resting potential and force generation. There is good evidence that in tracheal ASMC from several animal species, the membrane potential is markedly influenced by the delayed rectifier. There have, however, been very few reports on the electrical behaviour of human airway smooth muscle. Miura *et*

[1]Author for correspondence at: Division of Medicine, United Medical and Dental Schools, St Thomas' Campus, Lambeth Palace Road, London SE1 7EH.

al. (1992) have found that although charybdotoxin (ChTX) pretreatment did not affect spontaneous tone of human bronchial rings, it did influence isoprenaline- and theophylline-induced relaxation, and a brief report from Fleischmann *et al.* (1994) has suggested that a delayed rectifier is the main determinant of resting potential and basal tone in human airways. In direct contrast we have found that in human cultured bronchial smooth muscle cells there was little evidence for any substantive current due to a delayed rectifier. Instead, the major outward current in the presence of physiological intracellular Ca^{2+} was due to ChTX-sensitive K_{Ca} channels (Snetkov *et al.*, 1994). Cells in culture may alter their phenotype, however, and we have therefore investigated ion currents underlying the electrical behaviour of smooth muscle cells freshly isolated from human bronchi obtained following elective surgery.

Methods

Freshly dissociated cells

Human bronchial tissue was obtained from 17 patients of either sex (mean age 63 years, range 36–84 years) following pneumonectomy or lobectomy. Small pieces (2 by 5 mm) of epithelium-denuded smooth muscle from 3rd or 4th generation bronchi were dissected from the cartilage and kept for 30 min in Ca^{2+}-free physiological salt solution (PSS; see below for composition) at 36°C to facilitate relaxation. Tissue was then incubated for 40 min at 36°C in Ca^{2+}-free PSS containing 2 mg ml^{-1} collagenase (Worthington Type I), 2 mg ml^{-1} papain, 5 mg ml^{-1} bovine serum albumin and 20 μM dithiothreitol. Tissue was washed with fresh Ca^{2+}-free PSS and, after 20 min, cells were collected after gentle trituration with a wide-bore flame-polished Pasteur pipette. Five to ten minutes before use, a drop of the cell suspension was placed on a cleaned glass coverslip. This procedure resulted in mixture of long, relaxed cells and cells at different extents of contraction, but no difference was found between data obtained from relaxed and contracted cells. An alternative protocol where collagenase was used together with elastase and soybean trypsin inhibitor was equally successful.

Recording and analysis

Single cells were studied by standard patch-clamp techniques (Hamill *et al.*, 1981) using an Axopatch-1C amplifier (Axon Instruments, U.S.A.). The experiments were carried out at room temperature (20–21°C). The bath (≈ 0.5 ml) was continuously perfused (1–5 ml min^{-1}) with normal PSS during whole-cell experiments, or internal KCl solution during recording from inside-out patches (for solutions see below). When the perforated patch technique was used (Horn & Marty, 1988) in order to minimize changes to the intracellular environment, the pipette solution contained 150 μg ml^{-1} nystatin. A relatively low access resistance (< 30 MOhms) was achieved approximately 15 min after establishing the cell-attached configuration, and decreased further during the course of the experiment. Voltage pulse and ramp commands were generated by software (PATCH v.5, Cambridge Electronic Design, Cambridge, U.K.) to obtain current-voltage ($I-V$) curves. For the ramp protocol the holding potential was maintained at –70 mV, and 1 s voltage ramps from –100 mV to +100 mV were applied every 15 s. For the pulse protocol the holding potential was –80 mV, and 50 ms hyperpolarizing and depolarizing pulses were applied every 10 s. When 5 s pulses were used to study time-dependent inactivation, the inter-pulse interval was increased to 1 min. Current-voltage curves were plotted using current values measured at the end of the 50 ms voltage pulses, or at the respective points of the voltage ramps. Current responses were normalised, when appropriate, to cell size in terms of cell capacitance, which was measured using the built-in circuit of the Axopatch-1C amplifier. Preliminary experiments on a variety of smooth muscle cells that contained substantial delayed rectifier current (freshly isolated from rat pulmonary artery or bronchi, or rabbit trachealis) demonstrated that, during the protocols used in our experiments, there was no significant inactivation of voltage-gated currents.

Drugs used were added to the perfusing solution, and the responses were obtained and averaged from the same cell before, during and after drug application. Any second drug was applied only if the response was restored completely following washout. Recording in the presence of a drug was started following full equilibration, but at least 5 min were allowed if the drug used had no apparent effect.

Single channel currents were filtered at 1 kHz (–3 dB, low pass 80 dB/dec, Bessel type) and digitised with a 5 kHz sampling frequency using a CED 1401 interface (Cambridge Electronic Design, Cambridge, U.K.). The open state probabilities were calculated using PATCH v.5 software, and their voltage-dependence was fitted to the Boltzmann equation using SigmaPlot v.5 software (Jandel Scientific, Inc., U.S.A.). Current-clamp records were sampled at 5 Hz using a custom sampling programme for the CED 1401 interface.

Solutions and reagents

The solution used as internal in whole-cell recording, and both as external and internal in inside-out configuration, contained (mM): KCl 140, $MgCl_2$ 2, ethylene glycol-*bis*-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) 5, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethanesulphonic acid (HEPES) 10, adjusted to pH 7.2 with KOH. Free Ca^{2+} concentration was calculated as described by Fabiato (1988). The bath solution for whole-cell recording (PSS) contained (in mM): NaCl 135, KCl 5, $MgCl_2$ 2, $CaCl_2$ 2, glucose 11, HEPES 10, adjusted to pH 7.4 with NaOH. All solutions were filtered before use (0.22 μm; Millipore, U.S.A.). Charybdotoxin and iberiotoxin were obtained from Peninsula Laboratories, U.S.A., and type I collagenase from Worthington Biochemical Corporation, U.S.A. All other reagents were purchased from Sigma Chemical Company (Poole, Dorset).

The results are expressed as mean ± s.d. when appropriate, and n values refer to the number of cells investigated. Differences between means were tested for significance by Student's t test or analysis of variance as appropriate, and a P value of less than 0.05 was regarded as significant.

This study was approved by the Ethics Committee of the West Lambeth Health Authority.

Results

Whole-cell currents

4-Aminopyridine (4-AP)-sensitive outward current In only 26 of 155 cells did depolarization to –40 mV and more positive potentials result in an outwardly rectifying current that possessed a 4-AP-sensitive component. The 4-AP-sensitive current amplitude distribution in these cells showed a wide range (10–400 pA at 0 mV, mean 94 ± 133 pA, $n = 26$), but with a strong skew towards lower amplitudes, such that in the majority of cells showing this current (20 of 26) the current was less than 100 pA at 0 mV, with 17 cells having currents less than 50 pA. In the few cells where current amplitude was large enough for analysis, (> 100 pA, 6 of 26), the time course of activation was best fitted by second-order exponentials (n^2 kinetics), and was faster at more positive potentials (Figure 1a). Ramp-generated current-voltage relationships in these cells revealed a characteristic 'shoulder' between –20 and +20 mV (Figure 1b and 1c).

Experiments were performed to determine the sensitivity of this current to various K^+ channel blocking agents. The depolarization-induced outward current in these cells was only slightly reduced by 1 mM tetraethylammonium (TEA) (Figure

1b), but was markedly reduced by 2 mM 4-AP (Figures 1b,c). 100 nM ChTX was not effective against this current. Neither the Ca^{2+}-buffering capacity of the intracellular solution, nor the removal of Ca^{2+} from the external solution had any effect on the outward current. In the 6 cells where this current had sufficient amplitude for proper analysis it could be identified as a delayed rectifier on the basis of outward rectification, delayed activation, 4-AP sensitivity and resistance to TEA and ChTX. It is important to note that in the same experimental conditions a large voltage-gated delayed rectifier current could be found in all smooth muscle cells isolated from rat trachealis and bronchi, and rabbit trachealis (unpublished observations). The small amplitude and rare occurrence of this current in human bronchial cells made more detailed study impossible. However, we cannot exclude the possibility that a 4-AP-sensitive current with an amplitude of less than 50 pA was concealed by the relatively large and noisy 4-AP-resistant component seen in the majority of cells studied (see below).

4-AP-resistant outward current In contrast, in the vast majority of cells (129 of 155) depolarization from a holding potential of –80 mV resulted in a noisy, 4-AP- (5 mM) resistant, outward current. The amplitude varied between cells and was dependent on the $[Ca^{2+}]_i$ and was much larger than that of the 4-AP-sensitive current, with a mean value of 560 ± 124 pA ($n = 74$) at 0 mV with $[Ca^{2+}]_i$ less than 0.1 nM. The time-course of activation was best fitted by a first-order exponential (Figure 2a). The current was not sensitive to 4-AP (up to 5 mM), but was substantially reduced by 100 nM ChTX in the bath solution (Figure 2b). TEA (1 mM) and iberiotoxin (50 nM, IbTX) were also very effective inhibitors (Figure 2c). It should be noted that while the effect of ChTX was fast and reversible, that of IbTX developed more slowly, and full recovery was not achieved even after 20 min washout (not shown). The effects of these K^+ channel inhibitors on the current-voltage relationships are summarized in Figure 3. Apamin (1 μM), a blocker of small-conductance Ca^{2+}-dependent K^+ channels ($n = 3$), and glibenclamide (1 μM), a blocker of ATP-dependent K^+ channels ($n = 3$), were without effect upon whole-cell currents under the conditions of the present study. None of the above results was affected by the addition of 2 mM ATP and/or 0.2 mM GTP to the internal solution. The TEA-, ChTX- and IbTX-sensitive current did not show steady-state inactivation (Figure 4a), and did not decrease substantially even during a 5 s depolarization (Figure 4b).

The membrane potential at which a measurable outward

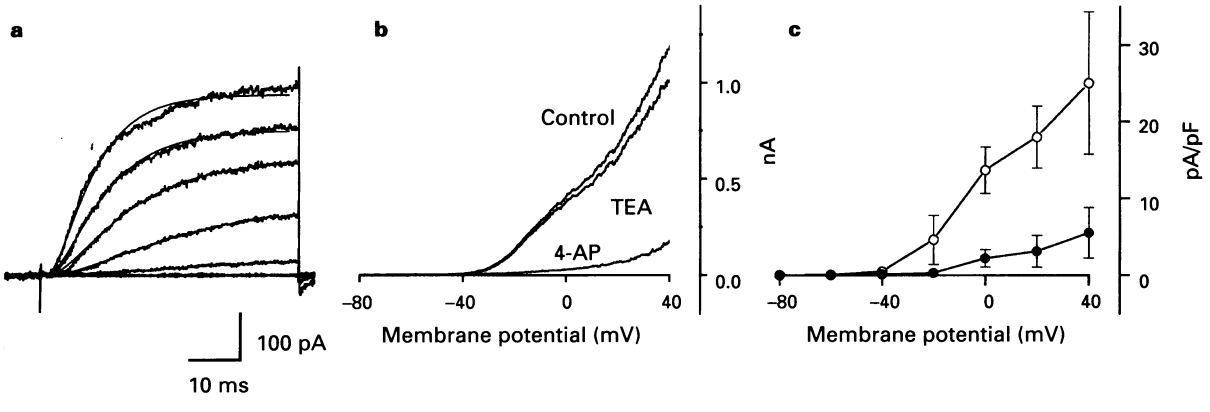

Figure 1 (a) Delayed rectifier whole-cell currents induced in a single freshly dispersed human bronchial smooth muscle cell by 50 ms depolarizing voltage steps from a holding potential of –80 mV to –100, –50, –40, –30, –20, –10 and 0 mV. Thin lines represent fit by second-order exponentials. (b) Current-voltage relationships obtained from a single cell using 1 s voltage ramps from –100 to +50 mV prior to drug application (Control), in the presence of 2 mM 4-aminopyridine (4-AP) and then 1 mM tetraethylammonium (TEA) after complete 4-AP washout. (c) Mean ramp-generated current-voltage relationships (at 20 mV intervals, normalised for cell capacitance) from cells possessing significant (see text) delayed rectifier current in the absence (○) and presence (●) of 2 mM 4-AP. Mean ± s.d., $n = 6$ cells. Internal KCl solution in all figures contained less than 0.1 nM free $[Ca^{2+}]$.

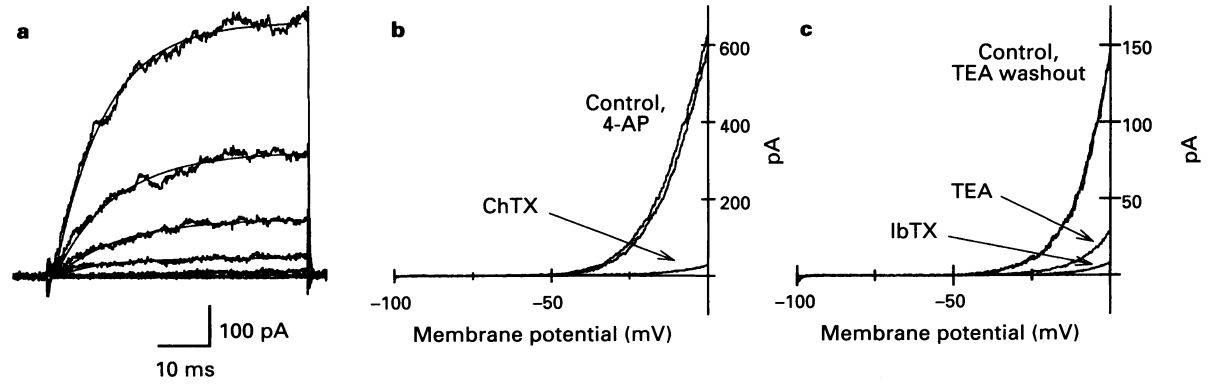

Figure 2 (a) 4-aminopyridine (4-AP)-resistant whole-cell currents induced in a single freshly dispersed human bronchial smooth muscle cell by 50 ms depolarizing voltage steps from a holding potential of –80 mV to –100, –50, –40, –30, –20, –10 and 0 mV. Thin lines represent fit by first-order exponentials. (b) Current-voltage relationships obtained from a single cell using 1 s voltage ramps from –100 mV to 0 mV prior to drug application (Control), in the presence of 5 mM 4-AP and then 100 nM charybdotoxin (ChTX) after complete 4-AP washout. (c) Current-voltage relationships obtained from a single cell using 1 s voltage ramps from –100 mV to 0 mV prior to drug application (control), in the presence of 1 mM tetraethylammonium (TEA) and then 50 nM iberiotoxin (IbTX) after complete TEA washout. Internal KCl solution in all figures contained less than 0.1 nM free $[Ca^{2+}]$.

current could be recorded varied somewhat between cells, being typically in the range –40 to –20 mV with a $[Ca^{2+}]_i$ < 0.1 nM. However, when $[Ca^{2+}]_i$ was increased to 100 nM the activation threshold shifted towards more negative potentials and the current amplitude was strongly increased (Figure 5a). In a few cells (14 out of 155) a measurable outward current was observed only after depolarization to + 50 mV or more positive with a $[Ca^{2+}]_i$ < 0.1 nM.

In 7 cells from 3 patients where whole-cell recording was performed by use of the nystatin permeabilized patch technique, depolarization to –40 mV and more positive potentials elicited an outward current that was sensitive to TEA and ChTX, but was not affected by 4-AP. This current showed characteristics similar to that observed in the majority of cells using conventional whole-cell techniques. In the course of the experiment the whole-cell current was initially recorded in perforated patch configuration with an $[Ca^{2+}]_i$ in the pipette solution of 100 nM. After patch disruption by additional suction, a current could be recorded in the conventional whole-cell configuration for long periods, as previously reported by Zhou & Neher (1993). After cell dialysis the current changed only slightly, which implies that resting $[Ca^{2+}]_i$ in the freshly dispersed cells is close to 100 nM (data not shown).

Single channel currents

Single channel recording revealed at least 4 types of single channel in freshly isolated human bronchial cells with con-

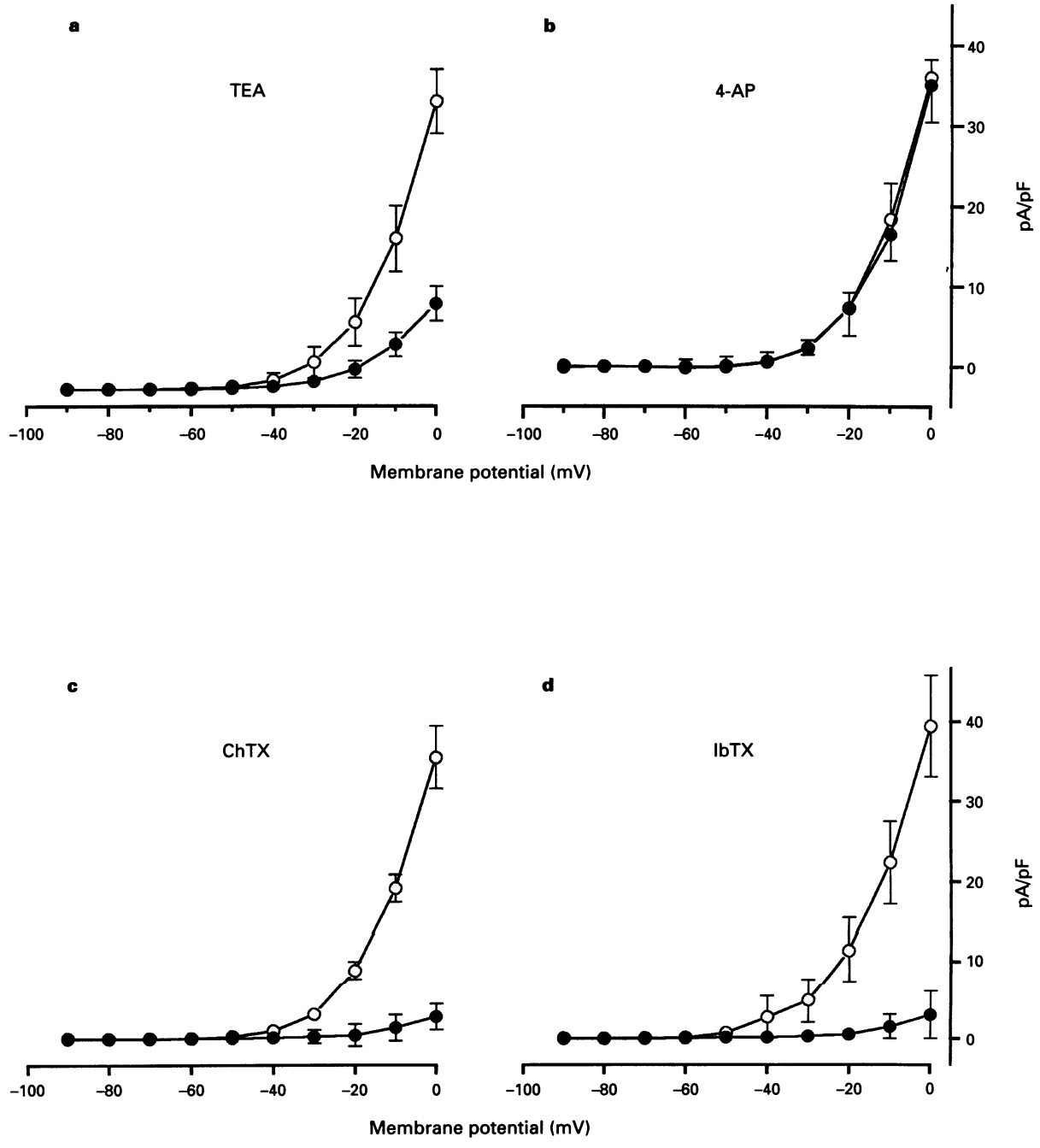

Figure 3 Mean ramp-generated current-voltage relationships (at 10 mV intervals; normalised for cell capacitance) in the absence (○) and presence (●) of various K^+ channel blockers: (a) 1 mM tetraethylammonium (TEA, $n=34$); (b) 5 mM 4-aminopyridine (4-AP, $n=12$); (c) 100 nM charybdotoxin (ChTX, $n=14$); (d) 50 nM iberiotoxin (IbTX, $n=5$). Means ± s.d. Internal KCl solution in all figures contained less than 0.1 nM free $[Ca^{2+}]$.

ductances of around 210, 130, 80 and 20 pS. The most abundant single channels had a conductance of 214±26 pS ($n=34$) with 140 mM KCl solution in the pipette, and 177±23 pS ($n=7$) with external NaCl solution in the pipette, and these were recorded in all cell-attached patches even at hyperpolarizing pipette potentials (Figure 6). Each patch contained up to 6 large conductance channels when pipettes with a resistance of 2–5 MOhm were used. However, with pipettes of 10–15 MOhm it was possible to obtain patches containing only one channel. It should be noted that the large channel currents, recorded from the cell-attached patches, were often distorted by the high input resistance of the cell. Channels with the same conductance (209±14 pS, $n=13$) were also recorded in inside-out patches with symmetrical 140 mM KCl across the membrane. Again, these channels could be recorded easily even at negative membrane potentials (e.g. –80 mV) and with $[Ca^{2+}]_i$ less than 0.1 nM. The voltage-dependence of the open state probability at 0 Ca^{2+} and 5 mM EGTA on the cytosolic side ($[Ca^{2+}]_i$ less than 0.1 nM) was suitably fitted by the Boltzmann equation, giving a mean V_{50} of 4±14 mV ($n=13$). The open state probability was increased further when cytosolic $[Ca^{2+}]_i$ was increased (Figure 5b), with a corresponding shift in the voltage-dependency (Figure 5c).

Channels were not sensitive to 10 mM TEA applied to the cytosolic side, while Ba^{2+} (1 mM) strongly increased the open state probability at negative membrane potentials, and de-

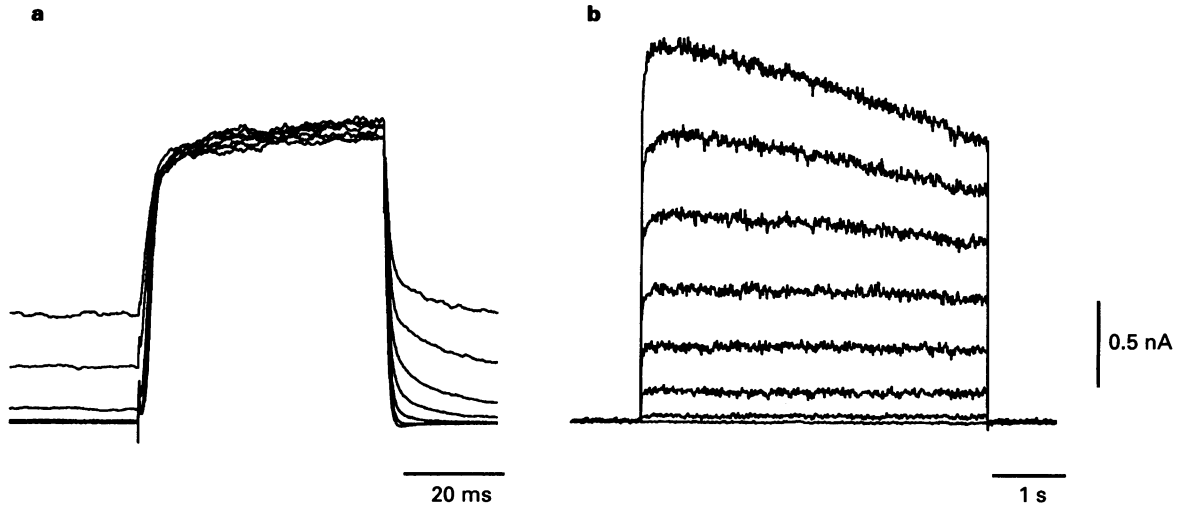

Figure 4 (a) The family of whole-cell currents induced by 50 ms depolarizing pulses to +80 mV from holding potentials of –90, –70, –50, –30, –10, +10 and +30 mV. (b) The family of whole-cell currents induced by 5 s depolarizing pulses to –40, –20, 0 +20, +40, +60, +80 and +100 mV from holding potential of –70 mV. All data obtained from the same cell; less than 0.1 nM free internal $[Ca^{2+}]$.

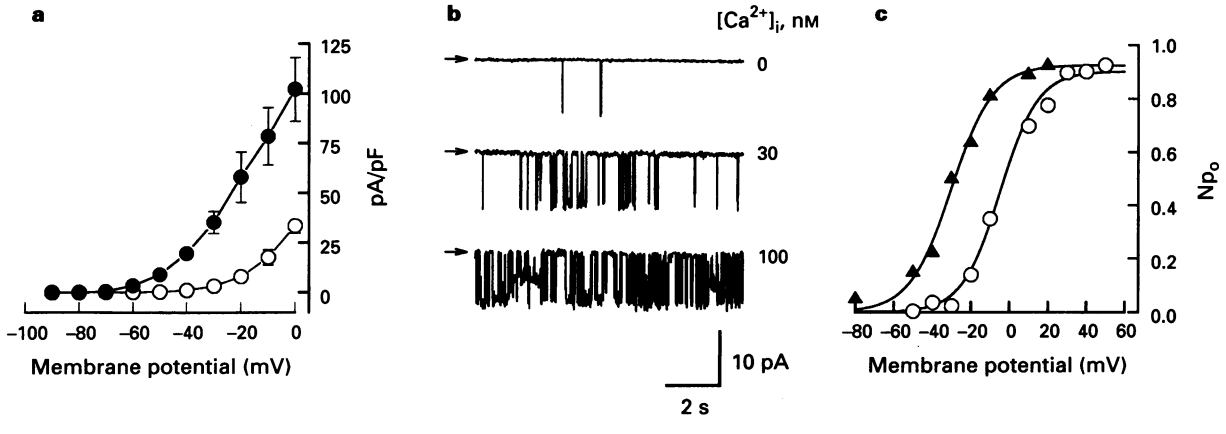

Figure 5 (a) Mean ramp-generated current-voltage relationships (at 10 mV intervals; normalized for cell capacitance) with $[Ca^{2+}]_i$ < 0.1 nM (○; $n=34$) and 100 nM (●, $n=11$); mean±s.d. (b) Large conductance single channel currents recorded from an inside-out patch containing only one channel, at nominally 0 (< 0.1 nM), 30 and 100 nM $[Ca^{2+}]_i$. Symmetrical 140 mM KCl, membrane potential –40 mV. Arrows indicate closed state and all figures follow the normal convention that a downward deflection represents an inward current. (c) Voltage-dependence of the open state probability of large conductance channels recorded from the same patch as (b) with $[Ca^{2+}]_i$ < 0.1 nM (○) and 30 nM $[Ca^{2+}]_i$ (▲). Curves fitted by the Boltzmann equation:

$$\frac{Np_o}{Np_{omax}} = 1 + \exp\left(\frac{V_{50}-V}{k}\right)$$

where Np_o is open state probability with N channels in patch at potential V, Np_{omax} is maximum open state probability for N channels in patch, and k is the slope constant. The half-maximal activation potential V_{50} was calculated as –5 mV and –30 mV for nominally 0 and 30 nM $[Ca^{2+}]_i$ respectively. Experiments performed with symmetrical 140 mM KCl.

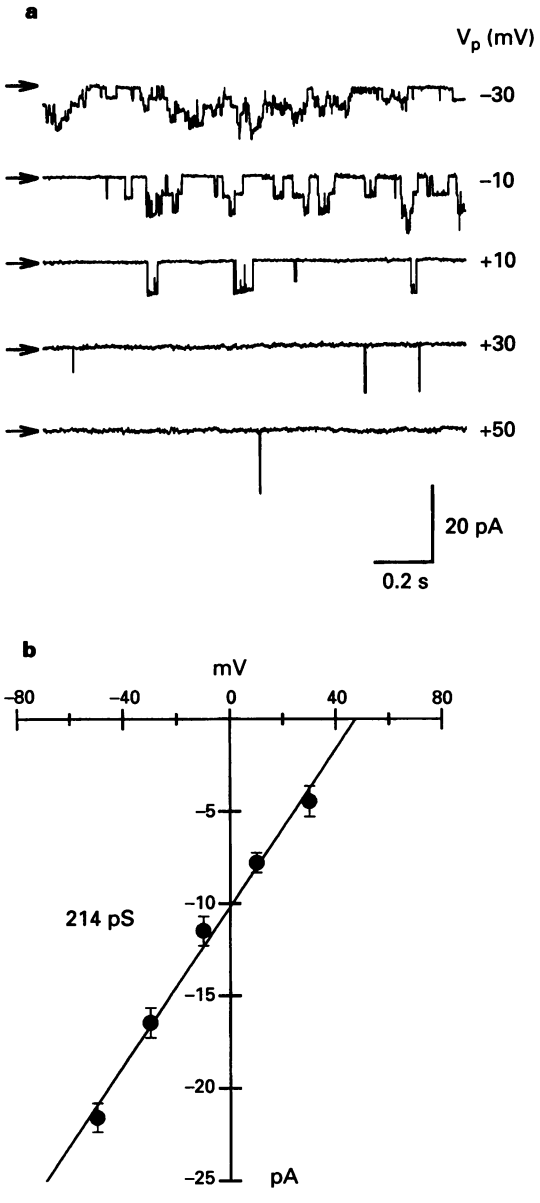

Figure 6 (a) Single channels recorded in cell-attached patch with KCl solution in the recording pipette; bath contained external NaCl solution. Numbers represent pipette potential (V_p) in mV; thus positive potentials refer to hyperpolarization above cell resting potential. Arrows indicate closed state. (b) Mean current-voltage relationship; the straight line corresponds to a slope conductance of 214±26 pS. Mean±s.d., $n=14$ cells.

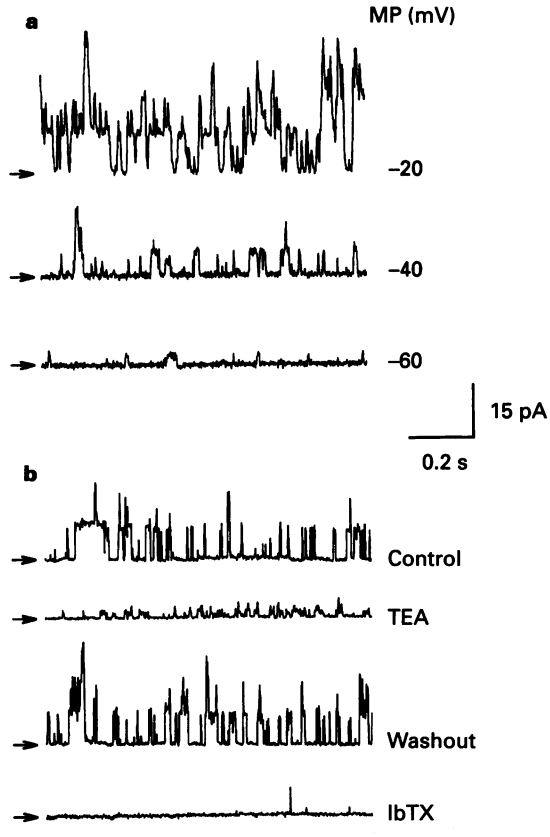

Figure 7 Single channels recorded in whole-cell configuration. Cell input resistance > 20 GOhm. Normal NaCl external solution, and less than 0.1 nM $[Ca^{2+}]_i$ in the internal KCl solution. (a) Voltage-dependence of single channel activity. Numbers indicate membrane potential (MP) in mV. (b) Effects of tetraethylammonium (TEA, 1 mM) and iberiotoxin (IbTX, 50 nM) on single channels recorded in whole-cell configuration. MP, -20 mV. Arrows indicate closed state.

creased it at positive potentials (data not shown). Channels recorded from outside-out patches were sensitive to externally applied TEA (1 mM), which induced strong flickering block of the open channel. External application of ChTX (100 nM) or IbTX (50 nM) resulted in a strong decrease of opening state probability without affecting the amplitude of the single channel current.

The same large conductance single channels were also observed in whole-cell configuration when small cells with an input resistance higher than 20 GOhm were used. In such records rare openings were observed even at a membrane potential of -60 mV and $[Ca^{2+}]_i$ less than 0.1 nM. Depolarization strongly enhanced single channel activity (Figure 7a), resulting in a substantial outward current at potentials positive to -40 mV. As with the corresponding macroscopic current, these single channels could be blocked by externally applied TEA or IbTX (Figure 7b). This observation implies that not only the depolarization-induced whole-cell current, but also the resting membrane potential of human bronchial ASMCs could be influenced by the activity of K_{Ca} channels.

We therefore performed current-clamp recordings to examine the role of K_{Ca} channels in the maintenance of the membrane potential. In current-clamp recordings using an intracellular solution with a low $[Ca^{2+}]_i$ (< 0.1 nM), cells displayed a membrane potential of -41±9 mV ($n=6$) which fluctuated strongly, presumably due to stochastic activity of large conductance channels. Application of 1 mM or 5 mM TEA caused depolarizations of 11±1 mV (1 mM; $n=3$), and 17±3 mV (5 mM; $n=3$), whereas 4-AP (5 mM) had no appreciable effect on membrane potential. In the presence of a more physiological $[Ca^{2+}]_i$ (100 nM) the resting membrane potential was significantly more negative at -58±7 mV ($n=11$; $P<0.01$), and application of TEA again depolarized the cells by 1.23±3.5 mV (1 mM; $n=7$) and 23.5±2.5 mV (5 mM; $n=6$); ChTX also induced a substantial depolarization (Figure 8a). The effect of 4-AP under these conditions was variable; 5 mM 4-AP induced in some cells no response, but in others a rather slowly developing depolarization which was not always reversible (Figure 8b). The mean depolarization induced by 5 mM 4-AP under these conditions was 12.5±11.5 mV, ($n=6$). It should be noted that voltage-clamp experiments on the same cells, performed immediately before going into current clamp, showed as in the majority of other cells used in this study that most of the voltage activated outward current was sensitive to TEA, with only a small component, if any, sensitive to 4-AP.

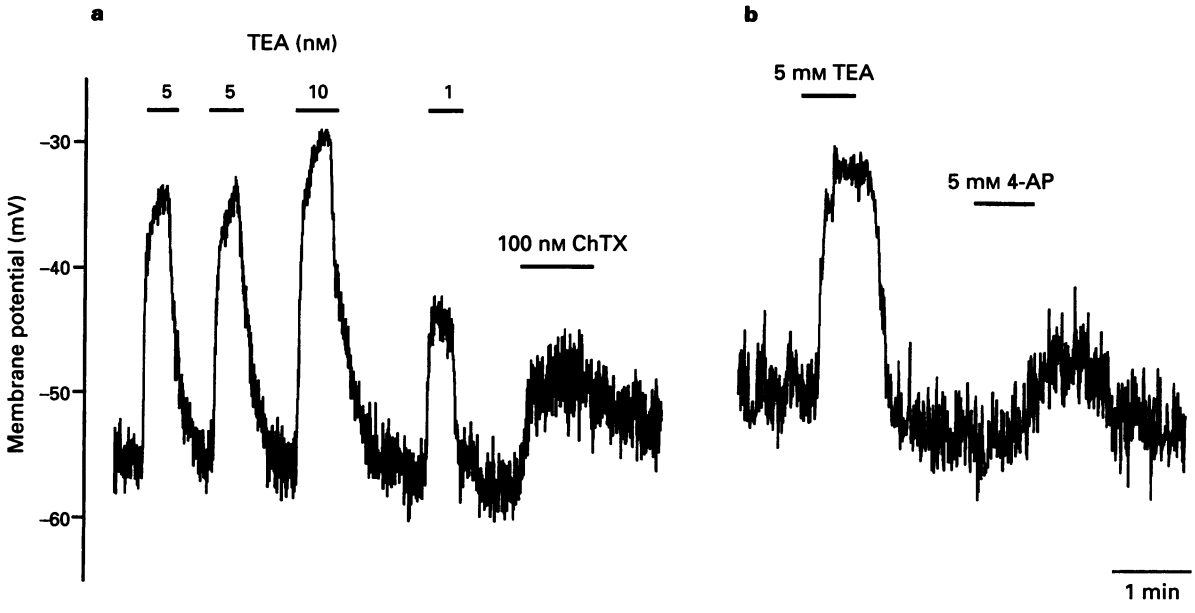

Figure 8 (a) Typical depolarization induced by tetraethylammonium (TEA, 1, 5, 10 mM) and charybdotoxin (ChTX, 100 nM) recorded under whole-cell current-clamp conditions. (b) Effects of TEA (5 mM) and 4-aminopyridine (4-AP, 5 mM) on membrane potential in another cell. Internal solution: 140 mM KCl, $[Ca^{2+}]_i$ = 100 nM. Horizontal bars represent time and duration of application of drug.

Discussion

The ASMC membrane is richly endowed with high-conductance Ca^{2+}-dependent K^+ channels and there is a corresponding macroscopic current which is activated under specific conditions. McCann & Welsh (1986) suggested that such channels may play a major role in determining the outward rectification of ASMC, and Muraki *et al.* (1990) also found a relatively large Ca^{2+}-dependent, TEA-sensitive component of total current in canine tracheal ASMCs. On the other hand, Kotlikoff and co-authors have reported that in freshly dispersed ASMC from several animal species, including dogs, the major voltage-dependent outward current is a 4-AP-sensitive delayed rectifier, and have suggested that the Ca^{2+}-dependent K^+ current may only be important in agonist-induced responses (e.g. Kotlikoff, 1993; Fleischmann *et al.*, 1993).

In contrast, we have found in the present study of freshly dispersed human bronchial smooth muscle cells that a 4-AP-sensitive delayed rectifier was measurable only in a small proportion of cells, and was relatively small. In the majority of cells the major outward current differed substantially from the delayed rectifier previously described for ASMCs in other species, in that: (i) it had first order activation kinetics; (ii) it was sensitive to IbTX, ChTX and TEA but not to 4-AP; and (iii) it did not demonstrate strong steady-state or time-dependent inactivation. These results are consistent with those we have previously reported for human bronchial smooth muscle cells in culture (Snetkov *et al.*, 1994). It should be noted that under identical recording conditions we have found a substantial delayed rectifier current with an amplitude of several hundred pA in ASMCs dispersed from rat, rabbit and bovine airway smooth muscle (Snetkov *et al.*, 1994; and unpublished observations), suggesting that this may be a true species variation. Although ChTX was initially regarded as a specific blocker of the large conductance Ca^{2+}-dependent K^+ channels (Miller *et al.*, 1985), it has recently been shown that some cloned, voltage-gated K^+ channels are also very sensitive to ChTX (Stühmer *et al.*, 1989; Grissmer *et al.*, 1994). As we have shown, however, the major current observed in these cells was also sensitive to IbTX, an agent which does not affect voltage-gated channels (Galvez *et al.*, 1990; Giangiacomo *et al.*, 1993).

Single channel recording revealed the existence of a large conductance K^+ channel which shared the properties of large conductance Ca^{2+}-dependent K^+ channels that have previously been observed in many tissues. These generally have a conductance between 140–250 pS, are sensitive to TEA applied from the extracellular but not cytosolic side, and show a voltage-dependent effect of Ba^{2+} such as that described in smooth muscle cells from canine and bovine trachealis (McCann & Welsh, 1986; Green *et al.*, 1991). The effect of Ba^{2+} in increasing P_o at negative potentials may reflect an ability to substitute for Ca^{2+} in terms of channel activation (Green *et al.*, 1991). The only atypical feature of the large conductance K^+ channels observed in this study seems to be the unusually negative half-activation potential (V_{50} = 4 mV) at an extremely low cytosolic Ca^{2+} concentration. Indeed, Kume *et al.* (1990) found that Ca^{2+}-dependent channels from rabbit trachea only started to open at 0 mV with a cytosolic Ca^{2+} of 0.25–0.4 μM. Murray *et al.* (1991) recorded activity in outside-out patches from guinea-pig trachealis with 100 nM Ca^{2+} on the cytosolic side at a membrane potential between 0 and +60 mV, whereas McCann & Welsh (1986) found a very low open state probability in inside-out patches from canine trachealis at 0.01 μM Ca^{2+} even at +40 mV, although occasional openings could be recorded at +60 mV even when the Ca^{2+}-free cytosolic solution, contained 10 mM EGTA. It is notable that, in our studies on randomly growing human cultured bronchial smooth muscle cells, the large conductance K^+ channels showed a similar sensitivity to Ca^{2+} to those reported in these latter studies, and differed sharply from freshly isolated cells (Snetkov *et al.*, 1994).

The apparently high sensitivity to Ca^{2+} found in these freshly isolated cells could reflect several factors reported elsewhere. For example Sansom & Stockand (1994) have demonstrated two isoforms of K_{Ca} channels isolated from bovine mesenteric artery smooth muscle cells and studied in lipid bilayers. These had the same conductance, but very different V_{50} values of –33 mV and +41 mV at a $[Ca^{2+}]_i$ of 1 μM (Sansom & Stockand, 1994). It is interesting to note that this difference is not dissimilar to that between the V_{50} of K_{Ca} channels found in the present study of freshly isolated human bronchial cells (4 mV), and the V_{50} of channels from cultured cells (+120 mV) that we have reported recently (Snetkov *et al.*, 1994).

It has also been shown that K_{Ca} channel activity in ASMCs

is regulated independently through both phosphorylation-dependent, cyclic AMP-dependent protein kinase-mediated, and membrane-delimited, G_s-protein mediated pathways (Kume *et al.*, 1994). Activation of any of those mechanisms shifts the relationship between voltage and open state probability by several tens of millivolts towards the physiological range of membrane potential. In addition, in cerebral artery smooth muscle cells, a cyclic GMP-dependent protein kinase, in the presence of cyclic GMP and ATP, increases K_{Ca} channel activity approximately eight fold (Robertson *et al.*, 1993). These data indicate that phosphorylation of the channel or of an associated regulatory protein causes a substantial leftward shift in the activation curve. It is therefore possible that the phosphorylation state of K_{Ca} channels in freshly dispersed human ASMCs may differ from that in other species.

On the other hand large conductance Ca^{2+}-independent K^+ channels have been reported in smooth muscle cells from bovine (Green *et al.*, 1991) and porcine (Saunders & Farley, 1991) trachealis, and from rat tail artery (Furspan & Webb, 1990). The channels observed in our study differed in several respects from the apparent Ca^{2+}-independent large conductance channels described in bovine tracheal ASMC by Green *et al.* (1991), which were sensitive to cytosolic TEA, activated at more positive potentials, and were observed exclusively in inside-out patches. Our results strongly suggest that in the large majority of cells the depolarization-induced outward current was due to the activity of large conductance, Ca^{2+}-sensitive K^+ channels, which were present at high density. The role of these channels in the generation of the whole-cell current could be seen particularly well during recordings from very small cells containing small numbers of channels (Figure 7).

Our current-clamp studies clearly demonstrate that the resting membrane potential in these bronchial smooth muscle cells is strongly influenced by intracellular [Ca^{2+}], and that both TEA and ChTX can cause a substantial depolarization. This is fairly good evidence that the K_{Ca} channels that we have described have an important role in the maintenance of resting membrane potential in this tissue. In contrast 4-AP had a less profound effect on membrane potential, which was not observed in all cells, or when intracellular [Ca^{2+}] was low. This might indicate the presence of a small number of delayed rectifier channels, which were not discernible in the voltage clamp experiments. Alternatively 4-AP may affect other conductances such as the K_{ATP} channel, which is known to be blocked by 4-AP (Beech & Bolton, 1989), or have another non-specific effect. Further studies are required to clarify these actions of 4-AP.

One possible source of variation between these experiments and previous studies on ASMCs may derive from the origin of the tissue. In this report all tissue was derived from bronchial sections, whereas most previous work has been performed on trachealis. However in preliminary experiments we have found that tracheal and bronchial smooth muscle cells of the rat were similar in their electrophysiology, and like many other species (excluding man) both exhibited a delayed rectifier as the major outward K^+ current (unpublished observations). Moreover we have recently obtained human trachealis from a single subject, and our preliminary studies suggest that cells from this tissue show identical responses to those described above for human bronchial smooth muscle cells (unpublished observations). We feel that our results are therefore likely to be related to a true species variation, rather than to the origin of the tissue within the airways.

In conclusion, these results suggest that in contrast to most other species, the major outward current in human bronchial smooth muscle is not due to a delayed rectifier, but to a large conductance ChTX-sensitive K^+ channel. The Ca^{2+}- and voltage-dependency of this current may well allow a sufficiently high open state probability for it to play a part in the regulation of the resting membrane potential, and hence airway excitability.

Supported by the Wellcome Trust, grant numbers 035721 (V.A.S.) and 039419 (S.J.H.).

References

ASANO, M., MASUZAWA-ITO, K. & MATSUDA, T. (1993). Charybdotoxin-sensitive K^+ channels regulate the myogenic tone in the resting state of arteries from spontaneously hypertensive rats. *Br. J. Pharmacol.*, **108**, 214–222.

BEECH, D.J. & BOLTON, T.B. (1989). Properties of the cromakalim-induced potassium conductance in smooth muscle cells isolated from the rabbit portal vein. *Br. J. Pharmacol.*, **98**, 851–864.

BRAYDEN, J.E. & NELSON, M.T. (1992). Regulation of arterial tone by activation of calcium-dependent potassium channels. *Science*, **256**, 532–535.

FABIATO, A. (1988). Computer program for calculating total from specified free or free from specified total ionic concentrations in aqueous solutions containing multiple metals and ligands. *Methods Enzymol.*, **157**, 378–417.

FLEISCHMANN, B.K., HAY, D.W.P. & KOTLIKOFF, M.I. (1994). Control of basal tone by delayed rectifier potassium channels in human airways. *Am. J. Resp. Crit. Care Med.*, **149**, A1080.

FLEISCHMANN, B.K., WASHABAU, R.J. & KOTLIKOFF, M.I. (1993). Control of resting membrane potential by delayed rectifier potassium currents in ferret airway smooth muscle cells. *J. Physiol.*, **469**, 625–638.

FURSPAN, P.B. & WEBB, R.C. (1990). Potassium channels and vascular reactivity in genetically hypertensive rats. *Hypertension*, **15**, 687–691.

GALVEZ, A., GIMENEZ-GALLEGO, G., REUBEN, J.P., ROY-CONTANCIN, L., FEIGENBAUM, P. & GARCIA, M.L. (1990). Purification and characterization of a unique, potent, peptidyl probe for the high conductance calcium-activated potassium channel from venom of the scorpion, *Buthus tamulus*. *J. Biol. Chem.*, **265**, 11083–11090.

GIANGIACOMO, K.M., SUGG, E.E., GARCIA-CALVO, M., LEONARD, R.J., MCMANUS, O.B. & GARCIA, M.L. (1993). Synthetic charybdotoxin-iberiotoxin chimeric peptides define toxin binding sites on calcium-activated and voltage-dependent potassium channels. *Biochemistry*, **32**, 2363–2370.

GREEN, K.A., FOSTER, R.W. & SMALL, R.C. (1991). A patch-clamp study of K^+-channel activity in bovine isolated tracheal smooth muscle cells. *Br. J. Pharmacol.*, **102**, 871–878.

GRISSMER, S., NGUYEN, A.N., AIYAR, J., HANSON, D.C., MATHER, R.J., GUTMAN, G.A., KARMILOWICZ, M.J., AUPERIN, D.D. & CHANDY, K.G. (1994). Pharmacological characterization of five cloned voltage-gated K^+ channels, types Kv1.1, 1.2, 1.3, 1.5 and 3.1, stably expressed in mammalian cell lines. *Mol. Pharmacol.*, **45**, 1227–34.

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 Arch.*, **391**, 85–100.

HORN, R. & MARTY, A. (1988). Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J. Gen. Physiol.*, **92**, 145–159.

KANNAN, M.S., JAGER, L.P., DANIEL, E.E. & GARFIELD, R.E. (1983). Effects of 4-aminopyridine and tetraethylammonium chloride on the electrical activity and cable properties of canine tracheal smooth muscle. *J. Pharmacol. Exp. Ther.*, **227**, 706–715.

KOTLIKOFF, M.I. (1993). Potassium channels in airway smooth muscle: a tale of two channels. *Pharmacol. Ther.*, **58**, 1–12.

KUME, H., HALL, I.P., WASHABAU, R.J., TAKAGI, K. & KOTLIKOFF, M.I. (1994). β-Adrenergic agonists regulate K_{Ca} channels in airway smooth muscle by cAMP-dependent and -independent mechanisms. *J. Clin. Invest.*, **93,** 371–379.

KUME, H. & KOTLIKOFF, M.I. (1991). Muscarinic inhibition of single K_{Ca} channels in smooth muscle cells by a pertussis-sensitive G protein. *Am. J. Physiol.*, **261,** C1204–C1209.

KUME, H., TAKAGI, K, SATAKE, T., TOKUNO, H. & TOMITA, T. (1990). Effects of intracellular pH on calcium-activated potassium channels in rabbit tracheal smooth muscle. *J. Physiol.*, **424,** 445–457.

MCCANN, J.D. & WELSH, M.J. (1986). Calcium-activated potassium channels in canine airway muscle. *J. Physiol.*, **372,** 113–127.

MILLER, C., MOCZYDLOWSKI, E., LATORRE, R. & PHILLIPS, M. (1985). Charybdotoxin, a protein inhibitor of single Ca^{2+}-activated K^+ channels from mammalian skeletal muscle. *Nature*, **313,** 316–318.

MIURA, M., BELVISI, M.G., STRETTON, C.D., YACOUB, M.H. & BARNES, P.J. (1992). Role of potassium channels in bronchodilator responses in human airways. *Am. Rev. Resp. Dis.*, **146,** 132–136.

MURAKI, K., IMAIZUMI, Y., KOJIMA, T., KAWAI, T. & WATANABE, M. (1990). Effects of tetraethylammonium and 4-aminopyridine on outward currents and excitability in canine tracheal smooth muscle cells. *Br. J. Pharmacol.*, **100,** 507–515.

MURRAY, R.K., BERRY, J.L., COOK, S.J., FOSTER, R.W., GREEN, K.A. & SMALL, R.C. (1991). Guinea-pig isolated trachealis: the effect of charybdotoxin on mechanical activity, membrane potential changes and the activity of plasmalemmal K^+-channels. *Br. J. Pharmacol.*, **103,** 1814–1818.

MURRAY, R.K., FLEISCHMANN, B.K. & KOTLIKOFF, M.I. (1993). Receptor-activated Ca influx in human airway smooth muscle – use of Ca imaging and perforated patch-clamp techniques. *Am. J. Physiol.*, **264,** C485–C490.

MURRAY, R.K. & KOTLIKOFF, M.I. (1991). Receptor-activated calcium influx in human airway smooth muscle cells. *J. Physiol.*, **435,** 123–144.

NARA, M., SASAKI, T., SHIMURA, S., IROKAWA, T., NAGAKI, M., MIURA, M. & SHIRATO, K. (1994). Endothelin-1 opens a receptor-operated K^+ channel in bovine tracheal smooth muscle cell to modulate its responsiveness to histamine. *Am. J. Resp. Crit. Care Med.*, **149,** A1080.

ROBERTSON, B.E., SCHUBERT, R., HESCHELER, J. & NELSON, M.T. (1993). cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *Am. J. Physiol.*, **265,** C299–C303.

SANSOM, S.C. & STOCKAND, J.D. (1994). Differential Ca^{2+} sensitivities of BK(Ca) isochannels in bovine mesenteric vascular smooth muscle. *Am. J. Physiol.*, **266,** C1182–C1189.

SAUNDERS, H.M. & FARLEY, J.M. (1991). Spontaneous transient outward currents and Ca^{++}-activated K^+ channels in swine tracheal smooth muscle cells. *J. Pharmacol. Exp. Ther.*, **257,** 1114–1120.

SNETKOV, V.A., HIRST, S.J., TWORT, C.H.C. & WARD, J.P.T. (1994). Potassium currents in human bronchial and rabbit tracheal smooth muscle cells in culture. *Am. J. Resp. Crit. Care Med.*, **149,** A1082.

STÜHMER, W., RUPPERSBERG, J.P., SCHRÖTER, K.H., SAKMANN, B., STOCKER, M., GIESE, K.P., PERSCHKE, A., BAUMANN, A. & PONGS, O. (1989). Molecular basis of functional diversity of voltage-gated potassium channels in mammalian brain. *EMBO J.*, **8,** 3235–44.

SUAREZ-KURTZ, G., GARCIA, M.L. & KACZOROWSKI, G.J. (1991). Effects of charybdotoxin and iberiotoxin on the spontaneous motility and tonus of different guinea pig smooth muscle tissues. *J. Pharmacol. Exp. Ther.*, **259,** 439–443.

ZHOU, Z. & NEHER, E. (1993). Mobile and immobile calcium buffers in bovine adrenal chromaffin cells. *J. Physiol.*, **469,** 247–274.

(Received February 13, 1995
Revised April 3, 1995
Accepted April 5, 1995)