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A patch-clamp study of K^+-channel activity in bovine isolated tracheal smooth muscle cells

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1 Single smooth muscle cells were isolated from bovine trachealis by enzymic digestion. The properties of large conductance plasmalemmal K^+-channels in these cells were studied by the patch-clamp recording technique.

2 Recordings were made from inside-out plasmalemmal patches when $[K^+]$ was symmetrically high (140 mM) and when $[Ca^{2+}]$ on the cytosolic side of the patch was varied from nominally zero to 10 μM. Large unitary currents of both Ca^{2+}-dependent and -independent types were observed. Measured between +20 and +40 mV, the slope conductances of the channels carrying these currents were 249 ± 18 pS and 268 ± 14 pS respectively.

3 Lowering $[K^+]$ on the cytosolic side of the patches from 140 to 6 mM, shifted the reversal potentials of the two types of unitary current from approximately zero to $\gg$ +40 mV, suggesting that both currents were carried by K^+-channels.

4 The Ca^{2+}-dependent and -independent K^+-channels detected in inside-out plasmalemmal patches could also be distinguished on the basis of their sensitivity to inhibitors (tetraethylammonium (TEA), 1–10 mM; Cs^+, 10 mM; Ba^{2+}, 1–10 mM; quinidine, 100 μM) applied to the cytosolic surface of the patches.

5 Recordings were made from outside-out plasmalemmal patches when $[K^+]$ was symmetrically high (140 mM) and when $[Ca^{2+}]$ on the cytosolic side of the patch was varied from nominally zero to 1 μM. Ca^{2+}-dependent unitary currents were observed and the slope conductance of the channel carrying these currents was 229 ± 5 pS.

6 Activity of the Ca^{2+}-dependent K^+-channel detected in outside-out patches could be inhibited by application of TEA (1 mM), Cs^+ (10 mM), Ba^{2+} (10 mM) or quinidine (100 μM) to the external surface of the patch. 4-Aminopyridine (4-AP; 1 mM) was ineffective as an inhibitor.

7 The activity of the Ca^{2+}-dependent K^+-channel recorded from outside-out patches was reversibly inhibited by charybdotoxin (100 nM).

8 When whole-cell recording was performed, the application of a depolarizing voltage ramp evoked outward current which was dependent on the $[Ca^{2+}]$ in the recording pipette and which could be reversibly inhibited by charybdotoxin (50 nM–1 μM) applied to the external surface of the cell.

9 We conclude that bovine trachealis cells are richly endowed with charybdotoxin-sensitive, large conductance, Ca^{2+}-dependent K^+-channels. These channels carry most of the outward current evoked by a depolarizing ramp and could play a major role in determining the outward rectifying properties of the trachealis cells. The role of the large Ca^{2+}-independent K^+-channels remains unclear.

Keywords: Bovine trachealis; patch-clamp recording; Ca^{2+}-dependent and -independent K^+-channels; whole-cell currents; 4-aminopyridine; Cs^+; charybdotoxin; Ba^{2+}; quinidine; tetraethylammonium

Introduction

The plasmalemma of airways smooth muscle cells exhibits such strong outward rectification that regenerative action potentials do not arise spontaneously and cannot be evoked by the transmembrane passage of cathodal current (Small & Foster, 1988; Small *et al.*, 1990). Patch-clamp studies have shown that the plasmalemma of canine and porcine trachealis is richly endowed with Ca^{2+}-dependent K^+-channels of high (266–290 pS) specific conductance (McCann & Welsh, 1986; Huang *et al.*, 1987). The plasmalemma of trachealis muscle from the guinea-pig (Hisada *et al.*, 1990; Small *et al.*, 1990) and rabbit (Kume *et al.*, 1990) also contains such channels.

The K^+-channel inhibitor tetraethylammonium (TEA) has been shown to reduce rectification in airways smooth muscle and therefore to allow spike-like action potentials to be discharged in response to cathodal current pulses (Kroeger & Stephens, 1975; Suzuki *et al.*, 1976; Kannan *et al.*, 1983). The susceptibility of the large Ca^{2+}-dependent K^+-channels to blockade by TEA, their high specific conductance and their relatively high frequency of occurrence in plasmalemmal patches were among factors that led McCann & Welsh (1986) to propose that such channels were responsible for the pronounced outward rectification exhibited by trachealis muscle.

However, there are two reasons why it may be premature, at this stage, to ascribe the outward rectifying behaviour of trachealis muscle solely to the activity of these K^+-channels. Firstly, TEA is non-selective as an inhibitor among the different types of K^+-channel (Cook, 1988). Secondly, the outward current evoked by depolarizing voltage steps in trachealis muscle seems to have several components and these can possibly be attributed to the presence of different types of K^+-channel. For example, in canine and rabbit trachealis cells arranged for whole-cell recording, depolarization to potentials positive to zero evokes a large transient outward current (I_T) followed by a more sustained outward current (I_S) of lower amplitude (Hisada *et al.*, 1990; Muraki *et al.*, 1990). In canine cells, I_T, but not I_S, was abolished by substituting Cd^{2+} for Ca^{2+} in the extracellular medium. In rabbit cells, I_T was inhibited by nifedipine while I_S was resistant to this agent (Hisada *et al.*, 1990). Kotlikoff (1989) has reported that, in canine tracheal myocytes, currents similar to I_T and I_S were both resistant to charybdotoxin, an agent reported specifically to block large Ca^{2+}-dependent K^+-channels (Smith *et al.*,

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1986). These observations collectively suggest that several different types of K^+-channel exist in the plasmalemma of trachealis muscle and that channels other than the large Ca^{2+}-dependent K^+-channel could contribute to its outward rectifying behaviour.

The experiments of the present study were designed to characterize the K^+-channels found in the plasmalemma of bovine trachealis cells and to estimate the contribution of large conductance Ca^{2+}-dependent K^+-channels to the carriage of outward current evoked by depolarizing stimuli.

Methods

Cell separation

Bovine tracheae were transported from the Manchester abattoir in cold Krebs solution. Strips of trachealis muscle were dissected from the organ and incubated for 15 min in a Ca^{2+}-free physiological salt solution (Ca^{2+}-free PSS; composition (mM): NaCl 126, KCl 6, Na_2HPO_4 1.2, $MgCl_2$ 1.2, glucose 11, HEPES 10, adjusted to pH 7.4 with NaOH). The tissue was then finely chopped and subjected to two periods of incubation (30 min at 35°C) in 2 ml Ca^{2+}-free PSS to which had been added 4 mg bovine serum albumin, 1400 μg collagenase (Worthington type I) and 60 μg elastase (Sigma type IIa). After its removal from the enzymic digestion medium, the tissue was resuspended in 2 ml Ca^{2+}-free PSS. The tissue was then repeatedly aspirated (sucked in and out of a blunt Pasteur pipette) to promote cell shedding. The supernatant fluid was drawn off and examined for its content of isolated smooth muscle cells. The tissue was resuspended in a fresh aliquot of Ca^{2+}-free PSS and aspiration was repeated. Working in this way, several samples of supernatant fluid were collected. Samples containing the greatest cell numbers were pooled and the cells were spun down. The cells were resuspended in Ca^{2+}-free PSS and plated onto cover slips coated with poly-L-lysine (molecular wt. 500,000). The cells were kept for up to 8 h at 4°C before use.

Recording media

For recording from inside-out plasmalemmal patches the recording pipette contained 140 mM KCl solution. Cells from which patches were to be prepared were stored in the main body of the recording chamber. This contained the PSS described above but with the addition of 1.2 mM $CaCl_2$. Patches prepared from the stock of cells were taken to a side arm of the recording chamber which was independently perfused with a K^+-rich PSS of composition (mM): KCl 126, NaCl 6, $MgCl_2$ 1.2, EGTA 2, $CaCl_2$ 1.7, glucose 11 and HEPES 10. The pH of this solution was adjusted to 7.4 with KOH solution. Normally the free $[Ca^{2+}]$ in this solution was 1 μM but, when required, this was varied in the range zero to 10 μM, by adjusting the amount of $CaCl_2$ included in the formulation. When inhibitors were added to this solution the concentration of KCl was correspondingly reduced to maintain osmolarity constant.

For recording from outside-out patches the recording pipette contained the K^+-rich PSS described as superfusing the inside-out patches with similar adjustment of free $[Ca^{2+}]$ when required. The extracellular surface of the patches was superfused with the same solution and free $[Ca^{2+}]$ was 1 μM.

For whole-cell recording the solution inside the recording pipette was similar to that used for outside-out patches. The extracellular surface of the cells was superfused with the same solution but free $[Ca^{2+}]$ was 1.2 mM.

Patch-clamp recording

Recordings were made at 22°C using an Axopatch 1B amplifier (Axon Instruments Inc.). Data was stored on magnetic tape and analysed using a microcomputer and pCLAMP Software (Axon Instruments Inc.). Unitary current amplitudes were measured using a Gould DSO 400 digital storage oscilloscope.

The probability of an individual ion channel being open (P_{open}) was calculated as

$$\sum_{j=1}^{N} t_j j/TN$$

where t_j is the time spent with $j = 1,2,\ldots, N$ channels open, N is the maximum number of channels seen and T is the duration of the experiment (Standen *et al.*, 1989). The maximum number of channels present in each patch was estimated from the maximum number of channels simultaneously open at any potential from −60 to +60 mV. In the case of outside-out patches where the pipette solution was nominally Ca^{2+}-free, the activity of individual channels was very low. Accordingly the maximum number of channels present was estimated during additional depolarization to +80 mV.

Whole-cell currents were activated by voltage ramps from −100 to +50 mV over 4 s. These currents were activated and recorded on line with pCLAMP software. Passive ohmic currents across the membrane were subtracted on line by the Axopatch amplifier.

Drugs and solutions

Drug concentrations are expressed in terms of the molar concentration of the active species. The following drugs were used: 4-aminopyridine (Sigma), barium chloride (May & Baker), caesium chloride (Sigma), purified charybdotoxin (Latoxan), quinidine hydrochloride (Sigma), tetraethylammonium bromide (Sigma).

The Krebs solution used for tissue transport to the laboratory had the composition (mM): Na^+ 143.5, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 127.6, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.1.

Results

Recording from inside-out plasmalemmal patches

Patches set up for recording under conditions where K^+ concentrations on either side of the patch were symmetrically high (140 mM) exhibited large, outwardly-directed unitary currents when held at potentials in the range +20 to +60 mV. In the majority of patches these large, unitary currents were all of equal amplitude. However, in some patches, large, unitary currents of two slightly different amplitudes could be observed (Figure 1a). Some patches exhibited much smaller unitary currents but these smaller events were not further investigated in the present study.

The Ca^{2+}-dependency of the ion channels carrying the large unitary currents was examined by altering free $[Ca^{2+}]$ on the cytosolic side of the patches. In 44 out of 70 patches examined, the relationship between P_{open} of the ion channel and membrane potential was strongly Ca^{2+}-dependent (Figure 2a). In these patches containing Ca^{2+}-dependent ion channels, P_{open} assumed a high value (>0.3) and was independent of membrane potential when cytosolic free $[Ca^{2+}]$ was in the range 3–10 μM. When cytosolic free $[Ca^{2+}]$ was 1 μM, P_{open} was close to zero at negative membrane potentials. However, P_{open} increased progressively as membrane potential assumed values more positive than zero. This relationship between P_{open} and membrane potential was shifted to more positive potentials and was depressed when cytosolic free $[Ca^{2+}]$ was reduced below 1 μM (Figure 2a).

In 26 out of the 70 patches examined, the relationship between P_{open} of the ion channel and membrane potential was virtually independent of cytosolic free $[Ca^{2+}]$. In patches containing Ca^{2+}-independent ion channels, P_{open} assumed a very low value at a holding potential of −60 mV but increased progressively as the membrane potential was moved towards

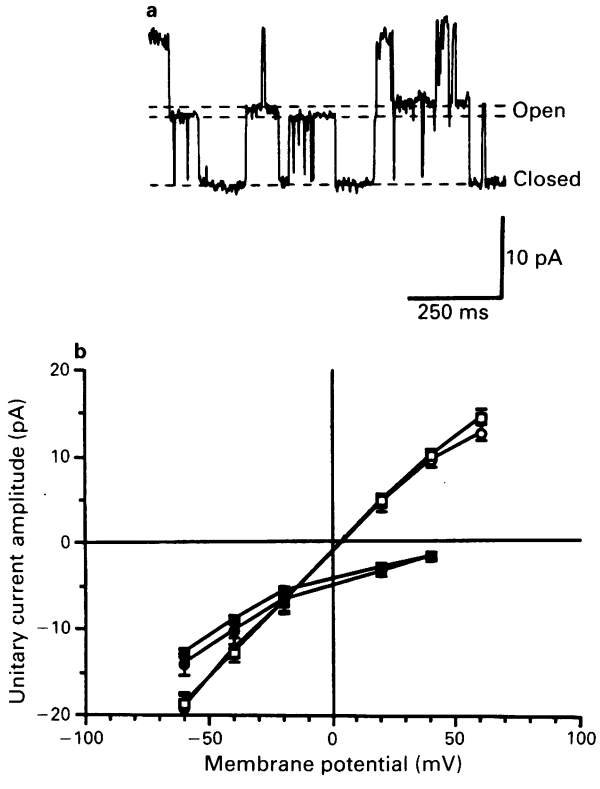

Figure 1 K^+-channel activity recorded from inside-out plasmalemmal patches from bovine trachealis cells. Except where stated otherwise, $[K^+]$ on each side of the patch was 140 mM and $[Ca^{2+}]$ on the cytosolic side of the patch was 1 μM. (a) Recording from patch held at +40 mV. Note that unitary currents of two distinct (albeit large) amplitudes can be discerned. The lowest broken line indicates the position where no channels are open. The upper two broken lines indicate currents corresponding to the opening of a single channel of each type. (b) Unitary current amplitude/voltage relationship for large conductance K^+-channels: (○) Ca^{2+}-dependent channel: symmetrical $[K^+]$; (●) Ca^{2+}-dependent channel: cytosolic $[K^+]$ 6 mM; (□) Ca^{2+}-independent channel: symmetrical $[K^+]$; (■) Ca^{2+}-independent channel: cytosolic $[K^+]$ 6 mM. Points indicate mean of values from at least 11 (symmetrical $[K^+]$) or 3 (6 mM cytosolic $[K^+]$) patches; vertical bars show s.e.mean.

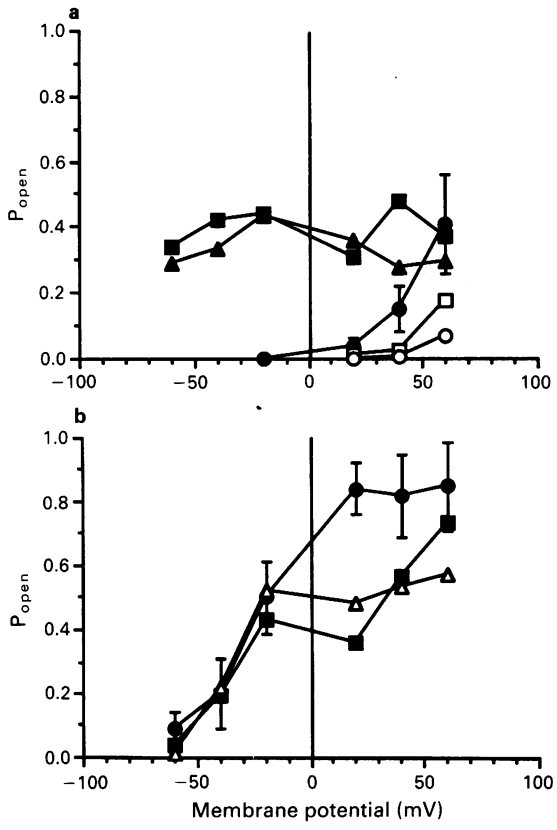

Figure 2 K^+-channel activity recorded from inside-out plasmalemmal patches from bovine isolated trachealis cells: the influence on P_{open} of $[Ca^{2+}]$ on the cytosolic side of the patch. (a) Large, Ca^{2+}-dependent K^+-channels; (b) large, Ca^{2+}-independent K^+-channels. The $[Ca^{2+}]$ on the cytosolic side of the patch was zero (△), 0.01 μM (○), 0.1 μM (□), 1 μM (●), 3 μM (▲) or 10 μM (■). Data for 1 μM Ca^{2+} are means (with s.e.mean) of values from at least 7 patches. Other data are from a single representative patch in each case.

0 mV. At positive holding potentials the channel showed a consistently high P_{open} (frequently in excess of 0.8). This relationship between P_{open} and membrane potential was little altered by increasing cytosolic free $[Ca^{2+}]$ from 1 μM to 10 μM or by reducing cytosolic free $[Ca^{2+}]$ to zero (Figure 2b).

The effects of holding potential on unitary current amplitude were examined both in patches containing Ca^{2+}-dependent channels and in patches containing $[Ca^{2+}]$-independent channels. Cytosolic free $[Ca^{2+}]$ was 1 μM in each case and K^+ concentrations on either side of the patches were symmetrically high (140 mM). The current/voltage relationships for the two types of channel are shown in Figure 1b and were used to calculate the slope conductances of each channel. At negative membrane potentials the slope conductances of the two channels were indistinguishable (306 ± 13 pS). At positive membrane potentials some inward rectification of the unitary currents was manifest as a reduction in the slope of the current/voltage curve. This rectification was more marked in the case of the Ca^{2+}-dependent channels. Hence, when slope conductance was measured over the range +20 to +40 mV, the conductance of the Ca^{2+}-dependent channel (249 ± 18 pS) was slightly lower than that of the Ca^{2+}-independent channel (268 ± 14 pS).

When K^+ concentrations on either side of the patch were symmetrically high (140 mM) the reversal potential of both types of unitary current was approximately zero. However, when $[K^+]$ on the cytosolic side of the patches was reduced to 6 mM the reversal potential of both types of unitary current was shifted to a value in excess of +40 mV (Figure 1b). Extrapolation of the unitary current amplitude/voltage curves in this circumstance indicated a reversal potential close to that expected for K^+ i.e. +80 mV. This suggested that the large, Ca^{2+}-dependent and -independent unitary currents observed in the inside-out plasmalemmal patches were carried by K^+-channels.

The inhibitor sensitivity of the large Ca^{2+}-dependent and large Ca^{2+}-independent K^+-channels recorded in inside-out patches was assessed in experiments where the inhibitors were added to the PSS bathing the cytosolic surface of the patches. TEA (3 mM) had no significant effect on the activity of the large Ca^{2+}-dependent K^+-channel. However, when the concentration of TEA was raised to 10 mM it caused some reduction in unitary current amplitude (Figure 3). The mean reduction in unitary current amplitude measured in 6 patches was 16%.

The Ca^{2+}-independent K^+-channel was much more sensitive to blockade by TEA. At a concentration of 100 μM, TEA caused flickering block of the Ca^{2+}-independent K^+-channel. TEA (1 mM) reduced unitary current amplitude to less than 25% of control values (Figure 4). Block of these channels by TEA (10 mM) was virtually complete (Figure 3).

Cs^+ (10 mM) had no effect on the large Ca^{2+}-dependent K^+-channel, affecting neither the amplitude of unitary currents (Figures 3 and 4) nor P_{open} (5 patches). In contrast Cs^+ (10 mM) caused voltage-dependent blockade of the Ca^{2+}-independent K^+-channel. Cs^+ (10 mM) did not affect the inward unitary currents seen at negative membrane potentials but reduced outward currents observed at positive membrane potentials. At a holding potential of +60 mV, Cs^+ (10 mM)

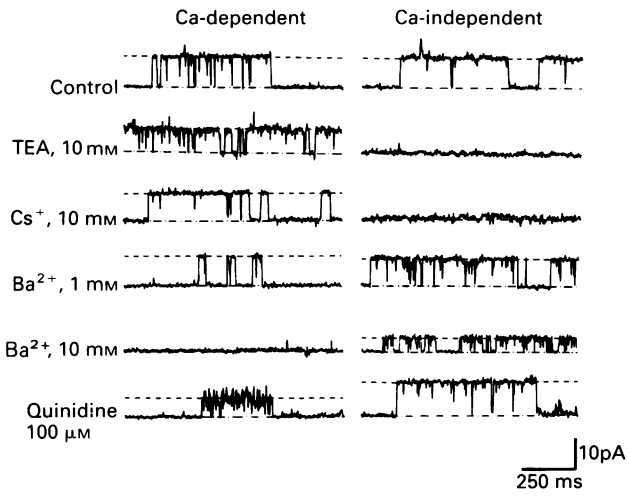

Figure 3 The effects of some inhibitors on the activity of large Ca^{2+}-dependent (left hand panel) and Ca^{2+}-independent (right hand panel) K^+-channels recorded from inside-out plasmalemmal patches from bovine isolated trachealis cells. In each case $[K^+]$ on each side of the patch was 140 mM, $[Ca^{2+}]$ on the cytosolic side of the patch was 1 μM and the holding potential was +40 mV. In each recording the lower broken line indicates the position where no channels are open and the upper broken line indicates the unitary current corresponding to the opening of a single Ca^{2+}-dependent or -independent K^+-channel. All inhibitors were applied to the cytosolic surface of the patches. Note that, compared with the Ca^{2+}-independent channel, the Ca^{2+}-dependent channel is relatively resistant to blockade by tetraethylammonium (TEA, 10 mM) or Cs^+ (10 mM). In contrast, the Ca^{2+}-dependent channel is relatively more susceptible to inhibition by Ba^{2+} (1–10 mM). Quinidine (100 μM) causes flickering block of the Ca^{2+}-dependent channel but has relatively little effect against the Ca^{2+}-independent channel.

reduced the outward unitary currents to 14% (mean value from 5 patches) of control values (Figure 4b). In some patches held at +40 mV, Cs^+ (10 mM) caused total blockade of unitary current activity (Figure 3).

In patches containing large, Ca^{2+}-dependent K^+-channels, Ba^{2+} (1 mM) did not affect unitary current amplitude (Figures 3 and 4). However, this agent decreased P_{open} at positive potentials and increased P_{open} at negative potentials, thereby inverting the relationship between P_{open} and membrane potential for the large Ca^{2+}-dependent K^+-channel (4 patches; Figure 5). Ba^{2+} (10 mM) fully inhibited the opening of the large Ca^{2+}-dependent K^+-channels in patches held at +40 mV (Figure 3).

In the case of the large, Ca^{2+}-independent K^+-channel, Ba^{2+} (1 mM) caused some depression of the relationship between P_{open} and membrane potential (4 patches; Figure 5). This was the result of fast, flickering channel block at positive membrane potentials (Figure 3). At positive, but not at negative membrane potentials, Ba^{2+} (1–10 mM) reduced the amplitude of unitary currents carried by the large Ca^{2+}-independent K^+-channel (Figures 3 and 4).

Quinidine (100 μM) caused flickering block of the large Ca^{2+}-dependent K^+-channels in patches held at +40 mV but did not affect the activity of the large Ca^{2+}-independent K^+-channels (Figure 3). 4-Aminopyridine (1 mM) had little effect on either of the two large K^+-channels. This agent did not modify the unitary current/voltage relationship for either the large Ca^{2+}-dependent K^+-channel or the large Ca^{2+}-independent K^+-channel (Figure 4).

The inside-out patches each contained several large conductance K^+-channels. In most cases these channels were all of the Ca^{2+}-dependent or all of the Ca^{2+}-independent type. As mentioned above, very few of the patches contained both types of channel. This suggests that the two types of large conductance K^+-channel had a clustered distribution in the plasmalemma. We attempted to estimate the relative numbers of the two types of channel in the plasmalemma by counting

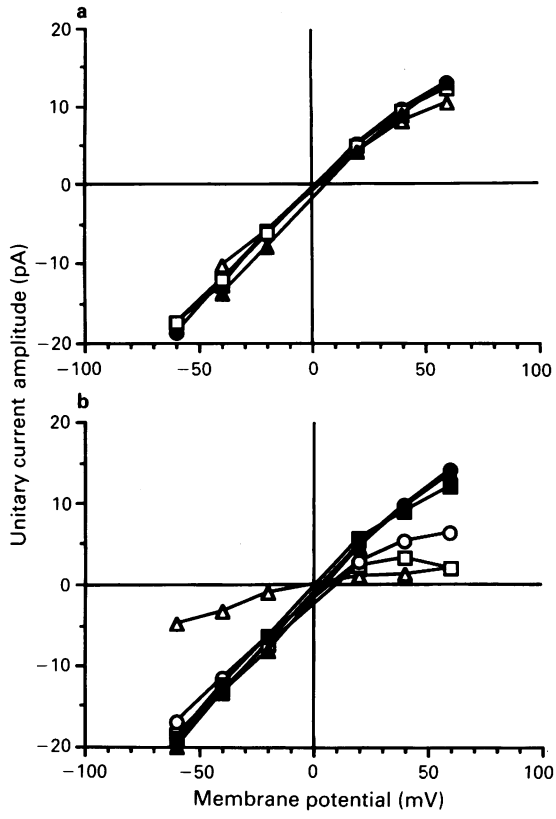

Figure 4 K^+-channel activity recorded from inside-out plasmalemmal patches from bovine isolated trachealis cells: the effects of some inhibitors on the unitary current amplitude/voltage relationship for the large Ca^{2+}-dependent K^+-channel (a) and the large Ca^{2+}-independent K^+-channel (b). All inhibitors were applied to the cytosolic surface of the patch. In each case $[K^+]$ on each side of the patch was 140 mM and $[Ca^{2+}]$ on the cytosolic side of the patch was 1 μM. Data points are means of values from at least 3 patches: (●) control (no inhibitor), (△) TEA, 10 mM in (a), 1 mM in (b); (□) 10 mM Cs^+; (■) 1 mM Ba^{2+}; (○) 10 mM Ba^{2+} and (▲) 1 mM 4-aminopyridine.

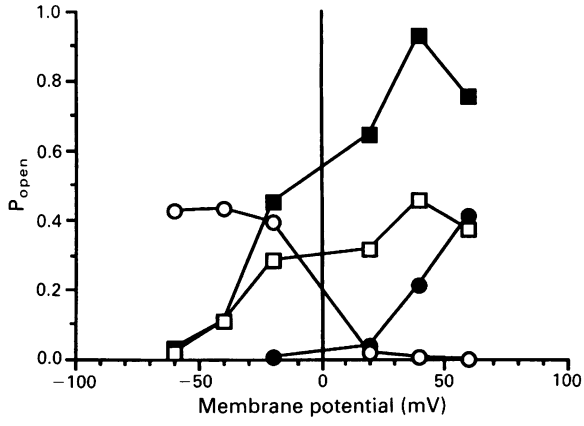

Figure 5 K^+-channel activity recorded from inside-out plasmalemmal patches from bovine isolated trachealis cells: the effect of Ba^{2+} (1 mM applied to the cytosolic surface) on P_{open} for the large Ca^{2+}-dependent and large Ca^{2+}-independent K^+-channels. In each case $[K^+]$ on each side of the patch was 140 mM and $[Ca^{2+}]$ on the cytosolic side of the patch was 1 μM. The data plotted are from two separate patches, one containing Ca^{2+}-dependent channels and one containing Ca^{2+}-independent channels. (●) Ca^{2+}-dependent channel; control; (○) Ca^{2+}-dependent channel; in presence of Ba^{2+} (1 mM); (■) Ca^{2+}-independent channel; control; (□) Ca^{2+}-independent channel; in presence of Ba^{2+} (1 mM). Note the ability of Ba^{2+} to invert the P_{open}/voltage relationship for the Ca^{2+}-dependent K^+-channel. Ba^{2+} depresses but does not invert the corresponding relationship for the Ca^{2+}-independent channel.

the numbers of channels present in the patches. In 21 patches containing Ca^{2+}-dependent channels, the mean (±s.e.mean) number of channels present per patch was 6.1 ± 3.3 while for 18 patches containing Ca^{2+}-independent channels the mean number of channels present per patch was 3.1 ± 1.0. Using these mean values to calculate the total numbers of Ca^{2+}-dependent and Ca^{2+}-independent channels present in the 70 patches examined yielded a ratio of 3.4:1 for the numbers of Ca^{2+}-dependent channels present in the plasmalemma relative to the number of Ca^{2+}-independent channels.

Recording from outside-out plasmalemmal patches

Patches set up for recording under conditions where K^+ concentrations on either side of the patch were symmetrically high (140 mM) exhibited large, outwardly-directed unitary currents when held at potentials in the range +20 to +60 mV.

The Ca^{2+}-dependency of the ion channels carrying the unitary currents was examined by altering the free [Ca^{2+}] inside the recording pipette. When the pipette solution contained 1 μM free Ca^{2+}, P_{open} of the channels carrying the currents was close to zero at a holding potential of −40 mV. However, P_{open} increased to a relatively high value (0.4) as membrane potential was moved through zero to +60 mV (Figure 6a). This relationship between P_{open} and membrane potential was depressed and moved to more positive potentials when the free [Ca^{2+}] inside the pipette was reduced to 0.1 μM or to zero (Figure 6a). The channels carrying the large unitary currents were therefore Ca^{2+}-dependent. Figure 6b shows the unitary current/voltage relationship obtained for the Ca^{2+}-dependent unitary currents of outside-out patches. The slope conductance of the channel carrying these currents was calculated from this relationship and, measured between +20 and +40 mV, had a value of 229 ± 5 pS.

We had anticipated that, when the recording pipette contained 2 mM EGTA but no Ca^{2+}, we would observe unitary currents with a voltage-dependence similar to that of the currents carried by the large Ca^{2+}-independent K^+-channels detected in the inside-out patches. However, currents of this kind were not observed under these conditions in any of 14 patches examined, nor were they seen in a further 6 patches with a solution at the extracellular surface identical to that which was used in the patch pipette for recording from inside-out patches (140 mM KCl). In a few patches large unitary currents of slightly different amplitude were seen but these did not show the same voltage-dependence as the Ca^{2+}-independent channels observed in inside-out patches. Little evidence for the existence of these channels was therefore obtained in the outside-out patches.

The effects of various inhibitors applied to the external surface of outside-out patches were tested against the unitary currents carried by the large Ca^{2+}-dependent K^+-channels. TEA applied to the external surface was a more potent inhibitor of such channels than TEA applied to the cytosolic surface. TEA (1 mM) applied to the external surface of outside-out patches markedly reduced unitary current amplitude at both positive and negative holding potentials (Figure 6b). Cs^+ (10 mM) applied to the external surface of the outside-out patches produced voltage-dependent channel block, reducing unitary current amplitude at negative but not positive potentials. Ba^{2+} (10 mM) had an effect similar to that of Cs^+. In contrast, 4-aminopyridine (2 mM) had little or no effect against unitary current amplitude at any holding potential (Figure 6b).

Charybdotoxin (100 nM) reduced the number of openings of the large Ca^{2+}-dependent K^+-channel without affecting unitary current amplitude (Figure 7). This reduction in channel activity was observed at all potentials tested from 0 to +60 mV.

Whole-cell recording

Recording pipettes of low (3–5 MΩ) resistance were used when recording in whole-cell mode. Current ramps were applied to the cells to achieve depolarization from a holding potential of −100 mV to +50 mV over a 4 s period. In these experiments the solution inside the recording pipette contained 2 mM EGTA and the free [Ca^{2+}] was nominally zero, 0.1 μM or 1 μM. When the recording pipette contained nominally Ca^{2+}-

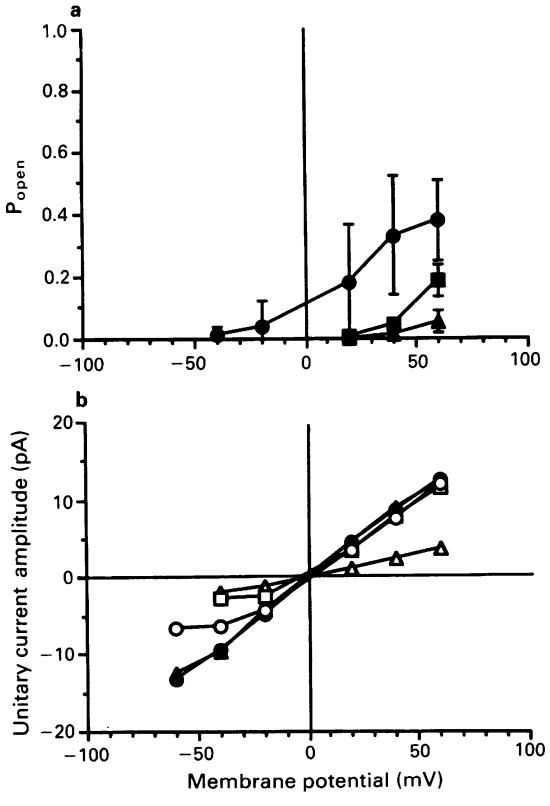

Figure 6 Ca^{2+}-dependent K^+-channel activity recorded from outside-out plasmalemmal patches from isolated bovine trachealis cells. (a) The relationship between P_{open} and [Ca^{2+}] on the cytosolic side of the patch. Data are means of values from 5 patches; s.e.mean shown by vertical bars. The free [Ca^{2+}] on the cytosolic side of the patch was nominally zero (▲), 0.1 μM (■) or 1 μM (●). The [K^+] on each side of the patch was 140 mM. (b) The unitary current amplitude/voltage relationship and its modulation by some inhibitors applied to the external surface of the patch. The free [Ca^{2+}] on the cytosolic and external sides of the patch was 1 μM. The [K^+] on each side of the patch was 14 mM. (●) Control (no inhibitor); (△) 1 mM tetraethylammonium, (□) 10 mM Cs^+, (○) 10 mM Ba^{2+} and (▲) 2 mM 4-aminopyridine. Data are means of values from 5 patches.

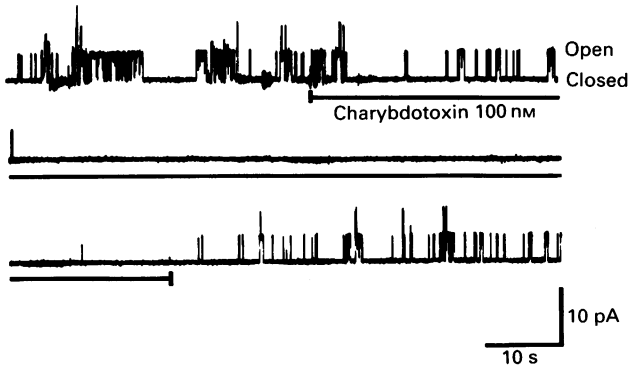

Figure 7 Continuous unitary current recording from an outside-out plasmalemmal patch from bovine trachaelis. The holding potential was 0 mV. The solution bathing the external surface of the patch contained 1.2 mM Ca^{2+} and 6 mM K^+ while that bathing the cytosolic surface contained 1 μM Ca^{2+} and 140 mM K^+. The bar underneath the trace indicates local perfusion of charybdotoxin, 100 nM, by positioning a separate pipette near the patch. Recovery occurred as the charybdotoxin diffused away after the removal of the application pipette.

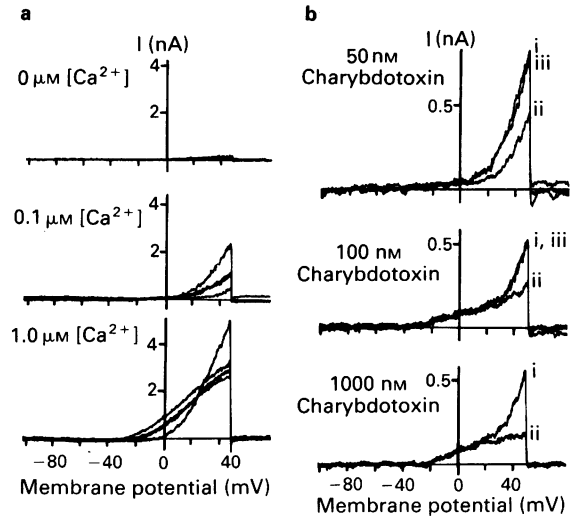

Figure 8 Whole-cell recording from bovine trachealis: outward currents evoked by a ramp depolarization from a holding potential of −100 mV to +50 mV over a period of 4 s. (a) Effects of varying $[Ca^{2+}]$ in the recording pipette solution. Note that the outward current increases in amplitude and shifts leftwards along the voltage axis as $[Ca^{2+}]$ is raised from nominally zero to 1 μM. Each of the plotted currents is from a different cell. (b) Effects of applying charybdotoxin (50 nM–1 μM) to the external surface of three different cells. Note the activation of the second, larger component of current at +10 to +20 mV and the suppression of this current by charybdotoxin. In each case current (i) is the initial control, (ii) was observed after exposure of the cell to charybdotoxin and (iii) was observed after toxin washout. Each superimposed trace is from the same cell. The cell exposed to 1 μM charybdotoxin was lost before full recovery from the toxin was achieved. Note the different vertical scales used in (a) and (b).

free solution, the application of a current ramp induced an outward current. This current was of low (<0.5 nA) amplitude and activated at a threshold of approximately 0 mV. When the free $[Ca^{2+}]$ inside the pipette was raised to 0.1 μM or 1 μM the effect was to increase the amplitude of the evoked outward current and to move the threshold for its activation to more negative potentials (Figure 8a and b). In some recordings it was evident that the outward current could be resolved into two components. The first component activated at approximately −40 mV and current amplitude increased slowly as the transpatch potential was moved to more positive values. The second component activated between −20 and +30 mV and current amplitude increased rapidly as the transpatch potential was moved to more positive potentials.

The effects of externally-applied charybdotoxin were examined in experiments where the free $[Ca^{2+}]$ inside the pipette was 0.1 μM. Charybdotoxin (50 nM–1 μM) selectively inhibited the second of the two components of outward current evoked by the conditioning ramp. The effects of charybdotoxin were concentration-dependent and reversible on washout (Figure 8b).

Discussion

Characteristics of the large, Ca^{2+}-dependent K^+-channel

In many ways the large Ca^{2+}-dependent K^+-channel which we have detected in bovine trachealis resembles that observed in trachealis muscle of other species and in smooth muscle from outside the respiratory tract. For example, the specific conductance of the large, Ca^{2+}-dependent K^+-channel observed in the present study (249 pS) was similar to that of equivalent channels in canine (266 pS; McCann & Welsh, 1986) and porcine (214 pS; Huang *et al.*, 1987) trachealis.

McCann & Welsh (1986) found that the opening of the large, Ca^{2+}-dependent K^+-channel in canine trachealis was dependent both upon $[Ca^{2+}]$ on the cytosolic side of the patch and on the membrane potential. The activity of the large Ca^{2+}-dependent K^+-channels seen in bovine trachealis was similarly sensitive to $[Ca^{2+}]$ and potential (present study). Furthermore, the influence of the cytosolic free $[Ca^{2+}]$ on the relationship between P_{open} and membrane potential (Figure 2) was strikingly similar to that observed for large Ca^{2+}-dependent K^+-channels in the plasmalemma of smooth muscle cells from rabbit jejunum and guinea-pig mesenteric arteries (Benham *et al.*, 1986).

The shape of the P_{open}/membrane potential curve for the large Ca^{2+}-dependent K^+-channels in bovine trachealis suggests that, provided cytosolic free $[Ca^{2+}]$ is 1 μM or less, very few of the large Ca^{2+}-dependent K^+-channels will be open at the normal resting membrane potential of the cell. However, should cytosolic free $[Ca^{2+}]$ rise above 1 μM, then the channels become quite active even at the resting membrane potential.

Fluorescence measurements have indicated that the cytosolic free $[Ca^{2+}]$ in resting airways smooth muscle cells is in the range 0.05–0.35 μM. During cellular excitation this rises to 0.3–1.0 μM (for review see Rodger & Small, 1991). At first sight the cytosolic free $[Ca^{2+}]$ achieved during excitation seems barely adequate to activate the large Ca^{2+}-dependent K^+-channels at potentials close to the resting membrane potential of the cell. However, Benham *et al.* (1986) have pointed out that during cellular excitation, Ca^{2+} entering the cell could achieve high, local concentrations in the area adjacent to its point of entry. If the large, Ca^{2+}-dependent K^+-channels are located close to the internal orifices of Ca^{2+}-channels then the opening of the K^+-channels could well be promoted by very local increases in cytosolic free $[Ca^{2+}]$.

McCann & Welsh (1986) reported that TEA (10–25 mM), applied to the cytosolic surface of plasmalemmal patches from canine trachealis, caused voltage-dependent blockade of large Ca^{2+}-dependent K^+-channels in that unitary current amplitude was reduced at strongly positive potentials. These authors showed, too, that TEA was approximately 10 times more potent when applied to the external surface of the patch. A similar potency difference for externally- and internally-applied TEA was observed in the present study. TEA (10 mM) applied to the cytosolic surface of our patches of bovine trachealis caused minor reduction in unitary current amplitude at both positive and negative potentials (Figure 4a). This indicates that TEA caused weak voltage-independent channel blockade as reported for the large Ca^{2+}-dependent K^+-channels of intestinal and arterial smooth muscle (Benham *et al.*, 1985). When 1 mM TEA was applied to the external surface of patches from bovine trachealis it very markedly reduced unitary current amplitude at both positive and negative potentials. Clearly we have shown that the large Ca^{2+}-dependent K^+-channel of bovine trachealis can be inhibited by TEA and the greater potency of TEA when applied to the external as opposed to the cytosolic surface of the patches is consistent with the suggestion (Benham *et al.*, 1985) that the site of action of TEA is close to the outer end of the channel.

The application of Ba^{2+} (1–10 mM) to the cytosolic surface had no effect on the amplitude of unitary currents carried by large Ca^{2+}-dependent K^+-channels in membrane patches from rabbit jejunum (Benham *et al.*, 1985) but, at negative potentials, caused minor reduction in such currents in patches from canine trachealis (McCann & Welsh, 1986). The results of the present study (Figure 4a) suggest that internally-applied Ba^{2+} has relatively little effect on the amplitude of unitary currents carried by the large Ca^{2+}-dependent K^+-channel in bovine trachealis. A major effect of internally-applied Ba^{2+} was to reduce the probability of channel opening at positive potentials (Figure 5). In this respect we have confirmed similar observations made in rabbit jejunum (Benham *et al.*, 1985). At negative potentials, however, internally-applied Ba^{2+} increased the probability of channel opening. In trachealis muscle of the dog and ox, therefore, the differing effects of Ba^{2+} on channel opening at positive and negative potentials

lead to inversion of the P_{open}/voltage relationship (McCann & Welsh, 1986; present study).

The greater ability of internally- as opposed to externally-applied Ba^{2+} to inhibit channel opening at positive potentials, and the ability of raised extracellular [K^+] to antagonize Ba^{2+}, were among factors which led Benham *et al.* (1985) to propose that Ba^{2+} enters the open channel and causes blockade at a site close to its outer orifice. However, the failure of Ba^{2+} to reduce unitary current amplitude (Figures 3 and 4) may suggest that Ba^{2+} does not act to cause open channel blockade but rather to cause the channel to enter an inactivated state. The ability of Ba^{2+} to increase P_{open} at negative potentials may reflect an ability of Ba^{2+}, under these conditions, to substitute for Ca^{2+} in promoting channel opening.

In the present study, Cs^+ appeared to be a more effective inhibitor of the large Ca^{2+}-dependent K^+-channel when applied to the external as opposed to the cytosolic surface of the patches. In this respect externally-applied Cs^+ (10 mM) markedly reduced the amplitude of unitary currents at negative potentials whereas the same concentration of Cs^+ applied internally did not affect unitary current amplitude (Figures 4a and 6b) at any holding potential. A similar voltage-dependency of the channel blocking action of externally-applied Cs^+ and a similar external: internal inhibitory potency ratio has been reported for the large Ca^{2+}-dependent K^+-channel in smooth muscle cells from rabbit jejunum and guinea-pig mesenteric artery (Benham *et al.*, 1986).

In summary, the large Ca^{2+}-dependent K^+-channel that we have detected in bovine trachealis has many properties (specific conductance, voltage-dependence, Ca^{2+}-dependence, inhibitor sensitivity) in common with the large Ca^{2+}-dependent K^+-channels that have been observed in smooth muscle (airways, vascular and intestinal) from other mammalian species. Berry *et al.* (1991) showed that P_{open} of the large Ca^{2+}-dependent K^+-channel of bovine trachealis was not modified by glibenclamide, phentolamine, RP 49356 or cromakalim. This suggests that opening of the large Ca^{2+}-dependent K^+-channel does not underlie the ability of cromakalim-like drugs to relax airways smooth muscle *in vitro*.

The large Ca^{2+}-independent K^+-channel

Since we recorded large, Ca^{2+}-independent K^+-channel activity in inside-out but not outside-out patches, we considered the possibility that our recordings of such activity were artefacts resulting from the formation at the electrode tip of a plasmalemmal vesicle rather than an inside-out patch. However, two findings argue against this possibility. Firstly, the recorded unitary currents did not exhibit the features of capacity coupling to be expected from the presence of a plasmalemmal vesicle. Secondly, channel activity was readily modified by the addition of inhibitors to the bathing medium, suggesting that drug access to the channels was not impaired by the presence of a membrane barrier.

The sensitivity of the large, Ca^{2+}-independent K^+-channels in inside-out patches to inhibitors applied to the cytosolic surface in many ways resembled the sensitivity of the large, Ca^{2+}-dependent K^+-channels in outside-out patches to the same inhibitors applied to the external surface. This led us to wonder whether our recordings of large Ca^{2+}-independent K^+-channels were taken from patches that had adopted an outside-out rather than an inside-out configuration. However, the voltage-dependency of the opening of the large, Ca^{2+}-independent K^+-channels was in the direction expected for inside-out patches. Furthermore, while quinidine (100 μM) applied to the external surface of outside-out patches inhibited the large Ca^{2+}-dependent K^+-channel, the same agent applied to the cytosolic surface of inside-out patches failed to inhibit the activity of the large Ca^{2+}-independent K^+-channels. We conclude, therefore, that our recordings of the large Ca^{2+}-independent K^+-channels were not artefacts created either as a result of vesicle formation or as a result of the patch adopting an outside-out rather than an inside-out configuration.

The large, Ca^{2+}-independent K^+-channel detected in inside-out patches of bovine trachealis (present study) resembles the slow, potential-sensitive K^+-channels described by Benham & Bolton (1983) in smooth muscle cells from rabbit jejunum. Neither channel was sensitive to [Ca^{2+}] on the cytosolic side of the patch. Both channels were inhibited by relatively low (100 μM–1 mM) concentrations of TEA applied to the cytosolic surface of the patch. The voltage-dependencies of the two channels were similar. Furthermore, the conductance of the Ca^{2+}-independent K^+-channels was similar to that of the slow potential-sensitive K^+-channels observed in rabbit jejunum under comparable ionic gradients (i.e. 62 pS and 50 pS respectively measured at 0 mV with high K^+ concentration on one side of the patch and low K^+ concentration on the opposite side of the patch).

Role of the large Ca^{2+}-dependent and large Ca^{2+}-independent K^+-channels in determining the outward rectifying behaviour of the trachealis cell

We have shown (i) that large Ca^{2+}-dependent K^+-channels are present in great abundance in plasmalemmal patches of bovine trachealis; (ii) that such channels can be inhibited by the external application of TEA (1 mM); (iii) that such channels can be inhibited by the external application of charybdotoxin (100 nM); and that (iv) the larger of the two components of outward current evoked by a depolarizing ramp can be inhibited by charybdotoxin. These four findings are consistent with the proposal of McCann & Welsh (1986) that the large Ca^{2+}-dependent K^+-channel may play an important role in determining the strong outward rectifying behaviour of trachealis cells. This proposal receives further support from the finding that, in guinea-pig trachealis cells, charybdotoxin (100 nM) causes the conversion of spontaneous electrical slow waves into spike-like action potentials (authors' unpublished observations).

The above considerations run contrary to the suggestion of Kotlikoff (1989) that charybdotoxin-insensitive K^+-channels play the more important role in determining the outward rectifying behaviour of the trachealis cell. In support of Kotlikoff's (1989) suggestion, it could be argued that the large Ca^{2+}-dependent K^+-channel is unlikely to be involved in determining outward rectification because (under conditions where cytosolic [Ca^{2+}] is 1 μM) the channel activates only at potentials more inside-positive than −25 mV. However, our measurements of the relationship between P_{open} and membrane potential were made at a temperature of 22°C. It is possible that, at 37°C, the P_{open}/membrane potential relationship may lie to the left of its position at 22°C. Alternatively, local cytosolic Ca^{2+} concentrations might achieve values greater than 1 μM and hence ensure the opening of the large Ca^{2+}-dependent K^+-channel at values of membrane potential much closer to the normal resting value. These possibilities await the test of experiment.

The relationship between P_{open} and membrane potential for the large, Ca^{2+}-independent K^+-channel suggests that channel opening would occur in response to a very small displacement of membrane potential from its resting value in the direction of depolarization. Accordingly, this channel could be expected to play an important role in determining the outward rectifying behaviour of the trachealis cell. Our attempts to assess the charybdotoxin sensitivity of this channel and hence its role in carrying the charybdotoxin-insensitive component of the outward current induced by a depolarized ramp were frustrated by our failure to record the channel activity in outside-out patches.

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