Guinea-pig isolated trachealis: the effects of charybdotoxin on mechanical activity, membrane potential changes and the activity of plasmalemmal K⁺-channels

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1 A study has been made, in guinea-pig isolated trachealis, of the effects of charybdotoxin in modulating (a) the activity of large conductance K⁺-channels, (b) the spontaneous electrical activity of intact cells and (c) the mechanical effects of some bronchodilator drugs.

2 Single smooth muscle cells were isolated from guinea-pig trachealis by enzymic digestion and were studied by the patch clamp recording technique. Recordings were made from outside-out plasmalemmal patches when the medium bathing the external surface of the patches contained 1.2 mM Ca²⁺ and 6 mM K⁺ while that bathing the cytosolic surface contained 0.1 μM Ca²⁺ and 140 mM K⁺. Charybdotoxin (100 nM), applied to the external surface of patches held at 0 mV, abolished the unitary currents associated with the opening of large conductance K⁺-channels.

3 Opened segments of guinea-pig trachea were used for the simultaneous recording of membrane potential and tension changes. In these experiments charybdotoxin (100 nM) caused the conversion of spontaneous electrical slow waves into spike-like action potentials. This effect was accompanied by a very small reduction in resting membrane potential.

4 Tissue bath recording showed that charybdotoxin (100 nM) increased the spontaneous mechanical tone of the tissue, antagonized (2.8 fold in each case) the relaxant actions of isoprenaline and theophylline but did not antagonize the relaxant actions of cromakalim or RP 49356.

5 It is concluded that charybdotoxin is an effective inhibitor of large conductance K⁺-channels in guinea-pig trachealis cells. The ability of charybdotoxin to convert spontaneous slow waves into spike-like action potentials suggests that the large, charybdotoxin-sensitive, K⁺-channels play an important role in determining the strong outward rectifying behaviour of the cells. The ability of charybdotoxin to antagonize isoprenaline and theophylline, but not to antagonize cromakalim and RP 49356, suggests that opening of the large conductance, charybdotoxin-sensitive K⁺-channel is implicated in the action of the former but not the latter pair of bronchodilator drugs.

Keywords: Trachealis; charybdotoxin; cromakalim; RP 49356; isoprenaline; theophylline; membrane potential recording; patch clamp recording; large conductance K⁺-channels

Introduction

Patch clamp recording techniques have shown that the plasmalemma of trachealis muscle from the dog (McCann & Welsh, 1986), pig (Huang *et al.*, 1987), rabbit (Kume *et al.*, 1990), guinea-pig (Hisada *et al.*, 1990; Small *et al.*, 1990) and ox (Berry *et al.*, 1991; Green *et al.*, 1991) is richly endowed with large, Ca²⁺-dependent K⁺-channels.

Trachealis muscle cells exhibit strong outward rectifying behaviour but this can be suppressed by the K⁺-channel inhibitor tetraethylammonium (TEA) (for reviews see Small & Foster, 1988; Small *et al.*, 1990). The relative abundance of the large, Ca²⁺-dependent K⁺-channels, their high specific conductance and their susceptibility to inhibition by externally-applied TEA were among factors which led McCann & Welsh (1986) to suggest that such channels might play a major role in determining the outward rectifying behaviour of the trachealis cell. However, Kotlikoff (1989) has demonstrated, in canine tracheal myocytes, that major components of the outward current induced by a depolarizing voltage step are resistant to charybdotoxin, an agent known to block large, Ca²⁺-dependent K⁺-channels in smooth muscle (Cook & Quast, 1990) including that of the airways (Green *et al.*, 1991). At present, therefore, the role of the large, Ca²⁺-dependent K⁺-channel in determining the strong outward rectifying behaviour of the trachealis cell membrane remains unclear.

Kume *et al.* (1989) have suggested that these channels are activated by isoprenaline acting on rabbit tracheal myocytes. These authors proposed that the action of isoprenaline involves the activation of protein kinase A. By phosphorylating the channel or a channel-related protein this enzyme promotes opening of the channel. While the opening of such channels may be implicated in the tracheal relaxant actions of agonists at β-adrenoceptors it does not seem to be necessary for the actions of potassium channel opening drugs such as cromakalim and RP 49356. Tested in bovine trachealis, these two potassium channel opening drugs did not modify P_{open} of the large, Ca²⁺-dependent K⁺-channel (Berry *et al.*, 1991). Furthermore, a recent brief report (Jones & Charette, 1990) has indicated that, in guinea-pig trachealis, charybdotoxin can antagonize the relaxant action of isoprenaline without antagonizing cromakalim or pinacidil. If it can be assumed that charybdotoxin acts specifically to inhibit the large Ca²⁺-dependent K⁺-channel then the work of Jones & Charette (1990) lends support to the idea that the opening of these channels is implicated in the actions of agonists at β-adrenoceptors but not in the actions of cromakalim-like K⁺-channel opening drugs.

The experiments of the present study were designed to shed more light on the role of the large Ca²⁺-dependent K⁺-channel in determining the outward rectifying behaviour of the trachealis cell and in mediating the tracheal relaxant actions of agonists at β-adrenoceptors, alkylxanthines and K⁺-channel opening drugs such as cromakalim and RP

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49356. Charybdotoxin has been used as an analytical tool but the great expense of this agent has necessarily placed some restrictions on the scope of the experimental work.

Methods

Tissue bath studies of mechanical activity

Small segments of trachea were set up for the isometric recording of tension changes essentially as described by Foster *et al.* (1983). At the outset of each experiment, tissues were subjected to an imposed tension of 1.5 g. Approximately 20 min later aminophylline (1 mM) was added in order to determine the recorder pen position at zero tone. The aminophylline was removed from the tissues (initial wash followed by two further washes at 10 min intervals) and when tone subsequently became maximal (40 min after initial wash), study of bronchodilator drugs started.

Cumulative log concentration-effect curves were constructed for all relaxant drugs examined. Half-log₁₀ unit concentration increments were used. Each concentration increment of cromakalim or RP 49356 was allowed 8 min tissue contact. For theophylline the contact time was 5 min and for isoprenaline 4 min. In order to minimize desensitization to cromakalim or RP 49356, a concentration-effect curve for theophylline was always interposed between successive concentration-effect curves of the potassium channel openers. The effects of the various relaxants were measured in terms of the maximal relaxation induced by aminophylline.

Following construction of the initial log concentration-effect curve of the relaxant drug under investigation, test tissues were exposed to Krebs solution containing charybdotoxin (100 nM); 20 min later the log concentration-effect curve of the relaxant drug was reconstructed in the presence of the toxin. In all experiments time-matched control tissues were treated identically to test tissues but were not exposed to charybdotoxin.

The effects of charybdotoxin on spontaneous tone were measured by comparison of the maximal tone observed prior to and ~20 min after toxin administration. In both cases zero tone was identified by the position of the maximal relaxation induced by aminophylline.

Intracellular electrophysiological recording from guinea-pig trachealis

Simultaneous recording of intracellular electrical activity and mechanical changes of a contiguous segment of trachea was performed by use of the technique and tissue holder described by Dixon & Small (1983). In brief, part of the trachealis was immobilized to permit long-term electrical recording while mechanical activity of contiguous muscle bundles was measured under an initial, imposed tension of 1.5 g. The recording microelectrodes were filled with 3 M KCl and were of resistance greater than 40 MΩ.

In these experiments microelectrode impalements of several trachealis cells were attempted prior to tissue treatment with charybdotoxin. Subsequently a trachealis cell was impaled and, during the impalement, charybdotoxin (100 nM) was added to the Krebs solution superfusing the tissue. In this way the onset of the effects of the toxin on electrical and mechanical activity could be recorded. The electrical activity was monitored for as long as the microelectrode tip remained within the impaled cell. Thereafter attempts were made to record the electrical activity of several more trachealis cells in the continued presence of the toxin. The great expense of the toxin made it necessary at this stage in the experiment for the charybdotoxin-containing Krebs solution to be re-cycled through the recording chamber.

Patch clamp recording

Muscle-rich strips of guinea-pig trachea were prepared as previously described (Foster *et al.*, 1983). Each tissue strip was

then incubated for 15 min in a Ca²⁺-free physiological salt solution (Ca²⁺-free PSS) of the following composition (mM): NaCl 126, KCl 6, Na₂HPO₄ 1.2, MgCl₂ 1.2, glucose 11, HEPES 10, adjusted to pH 7.4 with NaOH. The tissue was then cut into small segments and subjected to two periods of incubation (30 min at 35°C) in 2 ml Ca²⁺-free PSS to which had been added 4 mg bovine serum albumin, 1400 μg collagenase (Worthington type 1) and 60 μg elastase (Sigma type IIa). After its removal from the enzymic digestion medium, the tissue was resuspended in 2 ml Ca²⁺-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 Ca²⁺-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 the PSS described above but with the addition of CaCl₂ (1.2 mM). The cells were plated onto cover slips coated with poly-L-lysine (molecular wt. 500,000 Da). The cells were kept at 4°C and used within 8 h.

Recordings were made (at 22°C) from outside-out patches. The recording pipette contained K⁺-rich PSS of the following composition (mM): KCl 126, NaCl 6, MgCl₂ 1.2, EGTA 2, glucose 11 and HEPES 10. CaCl₂ was added to achieve a free [Ca²⁺] of 0.1 μM. The pH of this solution was adjusted to 7.4 with KOH solution. The external surface of the patches was bathed by the Ca²⁺-containing PSS used for plating out the isolated cells.

Recording was performed by use of an Axopatch 1B amplifier (Axon Instruments Inc.). Data were stored on magnetic tape and analysed with a microcomputer and pCLAMP Software (Axon Instruments Inc.). Unitary current amplitudes were measured with a Gould DSO 400 digital storage oscilloscope. Once unitary current recording was established from an outside-out plasmalemmal patch, charybdotoxin (100 nM) was applied to the external surface of the patch by superfusion from a second micropipette positioned in close proximity to the recording pipette.

Drugs and solutions/statistical analysis of results

Drug concentrations are expressed in terms of the molar concentration of the active species. The following substances were used: aminophylline (BDH), purified charybdotoxin (Latoxan), cromakalim (SmithKline Beecham Pharmaceuticals), (–)-isoprenaline hydrochloride (Sigma), RP 49356 ((±)-N-methyl-2-(3-pyridinyl)-tetrahydrothiopyran-2-carbothioamide-1-oxide; Rhone Poulenc-Rorer), theophylline (Sigma).

Stock solutions of most drugs were prepared in twice-distilled water. Those of cromakalim and RP 49356 were prepared in 70% ethanol. Isoprenaline stock solutions were prepared in 0.1 M HCl and dilutions were made with distilled water containing 0.57 mM ascorbic acid. Solutions of charybdotoxin were stored at 4°C and used within 48 h of preparation.

The Krebs solution used in the tissue bath experiments and for the microelectrode recording of membrane potential changes had the following composition (mM): Na⁺ 143.5, K⁺ 5.9, Ca²⁺ 2.6, Mg²⁺ 1.2, Cl[–] 127.6, HCO₃[–] 25, SO₄^{2–} 1.2, H₂PO₄[–] 1.2 and glucose 11.1. This solution (pH 7.4) was maintained at 37°C and was gassed with a mixture of 95% O₂, 5% CO₂.

The significance of differences between means was assessed by use of a two-tailed, unpaired *t* test. The null hypothesis was rejected when *P* < 0.05.

Results

Tissue bath recording of mechanical activity

Following the construction of the initial log concentration-effect curve of a relaxant drug, test tissues were given two

washes with drug-free Krebs solution before exposure to Krebs solution containing charybdotoxin (100 nM). Exposure of the test tissues to the toxin led to the development (20 min toxin contact) of peak tone greater than that observed in control tissues. The mean ($\pm$ s.e.mean; $n = 26$) increases in peak tone observed in the test and time-matched control tissues were $17.4 \pm 2.8\%$ and $1.2 \pm 2.0\%$ respectively ($P < 0.05$; two-tailed unpaired *t* test). Tone development observed in the presence of the toxin proceeded smoothly (i.e. was not interrupted by phasic tension changes).

Cromakalim ($0.1\text{--}100\text{ }\mu\text{M}$), isoprenaline ($1\text{ nM--}1\text{ }\mu\text{M}$), RP 49356 ($0.1\text{--}100\text{ }\mu\text{M}$) and theophylline ($1\text{ }\mu\text{M--}1\text{ mM}$) each caused concentration-dependent suppression of tracheal tone. The maximal effects of cromakalim and RP 49356 were smaller than those of isoprenaline and theophylline (Figure 1). The use of time-matched control tissues showed that log concentration-effect curves for these agents were relatively constant in shape and position during the experimental period.

Tested in the presence of charybdotoxin, each of the bronchodilator drugs retained its ability to suppress tracheal tone. However, in some tissues, determination of the shape of the log concentration-effect curve for the relaxant drug was rendered difficult by the occurrence of phasic mechanical activity. Such tissues could not be used to measure shifts in the log concentration-effect curves of the bronchodilator drugs. Charybdotoxin (100 nM) caused small (2.8 fold in each case) but significant rightward shifts in the log concentration-effect curves of isoprenaline and theophylline without reducing their maximal relaxant effects. In contrast, charybdotoxin failed to

Table 1 The effects of charybdotoxin (100 nM) on the actions of some relaxant drugs in guinea-pig isolated trachealis muscle

Relaxant	Control tissues	Test tissues
Isoprenaline	8.13 ± 0.21	$7.69 \pm 0.06^*$
Theophylline	4.38 ± 0.08	$3.94 \pm 0.06^*$
Cromakalim	5.89 ± 0.02	6.12 ± 0.07
RP 49356	5.75 ± 0.11	5.84 ± 0.06

Data are mean ($\pm$ s.e.mean; $n = 6$) values of pD_2 for the tabulated relaxant drugs in time-matched control tissues and in test tissues treated with charybdotoxin. * Indicates a significant difference from the corresponding values in control tissues (two-tailed unpaired *t* test). Note that charybdotoxin antagonized isoprenaline and theophylline but did not significantly affect the pD_2 values of cromakalim or RP 49356.

antagonize either cromakalim or RP 49356 (Figure 1 and Table 1).

Simultaneous recording of membrane potential and mechanical activity

Microelectrode impalement of trachealis cells revealed that, while some cells were electrically quiescent, others exhibited spontaneous oscillations (slow waves) of membrane potential (Figure 2). In this respect the present study has confirmed our earlier findings (Small, 1982; Allen *et al.*, 1986). The addition of charybdotoxin (100 nM) to the Krebs solution superfusing the tissue increased slow wave amplitude and eventually led to the discharge of spike-like action potentials. These changes occurred over a period of 10 minutes and were accompanied by very minor depolarization (mean depolarization measured at 5 min after toxin administration = 2.7 mV ; $n = 3$) of the impaled cell and by a small increase in mechanical tone (Figure 2). Charybdotoxin caused changes of this kind in all 7 preparations examined. The very high cost of the toxin precluded more ambitious experimentation.

Figure 1 Suppression of the spontaneous tone of guinea-pig isolated trachealis by (a) isoprenaline, (b) theophylline, (c) cromakalim and (d) RP 49356: the effects of charybdotoxin. In each panel (●) indicates the log concentration-effect curve of the relevant bronchodilator drug obtained in the absence of charybdotoxin (time-matched control). (■) indicates the log concentration-effect curve obtained in the presence of charybdotoxin (100 nM). Each point represents the mean (vertical bars show s.e.mean) of values from at least 6 tissues. Note that charybdotoxin antagonized isoprenaline and theophylline but did not antagonize either cromakalim or RP 49356.

Figure 2 The effects of charybdotoxin (100 nM) on the spontaneous electrical and mechanical activity of guinea-pig isolated trachealis. In each case the upper trace represents membrane potential changes while the lower trace represents the mechanical activity of a contiguous segment of trachea. All electrical recordings are taken from the same cell. Panel (a) indicates control activity recorded immediately prior to the addition of toxin; (b), (c), (d) and (e) activity recorded 2, 4, 6 and 8 min respectively after the addition of charybdotoxin (100 nM) to the Krebs solution superfusing the tissue. Panel (f) is a segment from (e) but run at higher chart speed in order to reveal the waveform of the action potentials. The broken line in panels (b)-(f) indicates the level of mechanical tone prior to toxin administration. Note that charybdotoxin increases slow wave amplitude and eventually causes the discharge of spike-like action potentials. These effects are accompanied by very minor depolarization of the impaled cell and by a small increase in mechanical tone.

Figure 3 Continuous unitary current recording from an outside-out plasmalemmal patch from guinea-pig trachealis. 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 0.1 μM Ca^{2+} and 140 mM K^{+} . The bar underneath the trace indicates the local perfusion of 100 nM charybdotoxin by positioning a separate pipette near the patch. The upper three sweeps are continuous recordings. A 5 min period of superfusion with toxin-free PSS separates the third and fourth sweeps. Note that the toxin abolished the unitary current activity but that recovery occurred following the removal of the charybdotoxin from the superfusate.

Patch clamp recording of ion channel activity

Outside-out plasmalemmal patches were set up for recording under conditions where 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 0.1 μM Ca^{2+} and 140 mM K^{+} . These plasmalemmal patches exhibited large, outwardly-directed unitary currents when held at potentials in the range 0 to +60 mV. Unitary current activity increased (i.e. channel openings occurred more frequently and the incidence of multiple openings increased) as membrane potential was changed from 0 mV to more positive values. The extrapolated reversal potential of the unitary currents was more negative than -40 mV. The slope of the unitary current amplitude/membrane potential relationship measured over the range 0 to +40 mV indicated that the conductance of the ion channels carrying the large unitary currents was 130 ± 7 pS (mean $\pm$ s.e.mean; $n = 4$). The voltage-dependence of the unitary currents, the reversal potential of the unitary currents, the conductance of the channels carrying the currents and the abundance of the channels collectively suggested that we were recording the activity of large Ca^{2+} -dependent K^{+} -channels. This notion was substantiated when we tested the susceptibility of the unitary currents to blockade by charybdotoxin.

Charybdotoxin (100 nM) applied by local superfusion reduced the number of large unitary currents recorded from outside-out plasmalemmal patches held at 0 mV without affecting unitary current amplitude. The inhibitory effects of charybdotoxin were reversible (Figure 3). Charybdotoxin (100 nM) produced similar effects in all preparations ($n = 4$) examined.

Discussion

Role of large, Ca^{2+} -dependent, charybdotoxin-sensitive K^{+} -channels in determining the strong outward rectifying behaviour of trachealis cells

McCann & Welsh (1986) observed that canine trachealis cells were richly endowed with large, Ca^{2+} -dependent K^{+} -channels. The abundance of these channels, their high conductance and their susceptibility to inhibition by

externally-applied TEA were among factors which led these authors to suggest that such channels play an important role in determining the strong outward rectifying behaviour of the trachealis cells.

Charybdotoxin is a relatively specific inhibitor of large Ca^{2+} -dependent K^{+} -channels (Cook & Quast, 1990). Accordingly, it might be expected to suppress the outward rectifying properties of trachealis cells. However, Kotlikoff (1989) measured outward current induced by a depolarizing voltage step in canine tracheal myocytes and observed that it was resistant both to substitution of extracellular Ca^{2+} by Mn^{2+} and to charybdotoxin (40 nM). Kotlikoff (1989) concluded that such current was carried by charybdotoxin-insensitive, Ca^{2+} -independent (delayed rectifier) K^{+} -channels and that the delayed rectifier K^{+} -channels were the major determinant of the outward rectifying behaviour of trachealis cells. In contrast to Kotlikoff's observations in canine tissue, Green *et al.* (1991) observed in bovine trachealis that charybdotoxin (0.01–1 μM) suppressed the greater part of the outward current induced by a depolarizing voltage ramp. The findings of Green *et al.* (1991), together with the present observations in guinea-pig trachealis that charybdotoxin causes an increase in the amplitude of spontaneous slow waves and their conversion into spike-like action potentials (Figure 3), prompt us to endorse the view of McCann & Welsh (1986) that the large Ca^{2+} -dependent K^{+} -channel plays a major role in determining the outward rectifying behaviour of trachealis cells.

Role of the large, Ca^{2+} -dependent K^{+} -channel in the relaxant actions of isoprenaline and theophylline

As mentioned above, Kume *et al.* (1989) have observed that isoprenaline opens large Ca^{2+} -dependent K^{+} -channels in the plasmalemma of trachealis cells by activating protein kinase A. If this action depends on the intracellular accumulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP), then we should expect cyclic nucleotide phosphodiesterase inhibitors such as theophylline to share the ability of isoprenaline to open the large Ca^{2+} -dependent K^{+} -channel. This expectation is borne out by the finding (Jones & Charette, 1990; present study) that charybdotoxin antagonizes the tracheal relaxant actions of both isoprenaline and theophylline.

It must be admitted that the single concentration (100 nM) of charybdotoxin used in the present study provided quite minor (2.8 fold) rightward shifts in the log concentration-effect curves of isoprenaline and theophylline. While Jones & Charette (1990) were able to demonstrate greater antagonism by the use of higher toxin concentrations, considerations of cost prevented similar experimentation in our laboratories.

The antagonism of charybdotoxin to actions of isoprenaline and theophylline was not a simple reflection of the ability of the toxin to raise tracheal tone because the toxin failed to antagonize cromakalim, pinacidil and RP 49356 (Jones & Charette, 1990; present study). Accordingly, it may be suggested that opening of large, Ca^{2+} -dependent K^{+} -channels forms part of the relaxant actions of isoprenaline and theophylline. It should be appreciated, however, that the opening of plasmalemmal K^{+} -channels with the consequential cellular hyperpolarization and inhibition of Ca^{2+} influx is not crucial to the relaxant actions of these drugs. The relaxant activity of β -adrenoceptor agonists and alkylxanthines is largely retained when these agents are tested against the spasm induced by a medium which is K^{+} -rich (100–120 mM) yet of osmolality equal to that of normal Krebs solution (Kumar, 1978; Allen *et al.*, 1985; 1986; Small *et al.*, 1989). In this circumstance the potassium equilibrium potential and the resting membrane potential of the cell are so closely apposed that K^{+} -channel opening cannot evoke sufficient hyperpolarization to ensure closure of voltage-operated Ca^{2+} -channels in the plasmalemma. We conclude, therefore, that the opening of plasmalemmal K^{+} -channels plays a supportive though not a crucial role in the relaxant actions of isoprenaline and theophylline.

Role of the large, Ca^{2+} -dependent K^+ -channel in the relaxant actions of cromakalim-like potassium channel opening drugs

Our observation that charybdotoxin does not antagonize the tracheal relaxant actions of cromakalim and RP 49356 confirms and extends the earlier findings of Jones & Charette (1990) who reported that charybdotoxin failed to antagonize the tracheal relaxant actions of cromakalim and pinacidil. The failure of charybdotoxin to antagonize the cromakalim-like K^+ -channel opening drugs suggests that the tracheal relaxant action of such drugs does not involve the opening of large, Ca^{2+} -dependent K^+ -channels. This suggestion receives

support from the observation (Berry *et al.*, 1991) that P_{open} for the large Ca^{2+} -dependent K^+ -channel of bovine trachealis is unaffected by cromakalim, RP 49356 and putative inhibitors (i.e. glibenclamide and phentolamine; for review see Small *et al.*, 1991) of the K^+ -channel opened by cromakalim-like drugs.

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