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# Effects of some $K^+$-channel inhibitors on the electrical behaviour of guinea-pig isolated trachealis and on its responses to spasmogenic drugs

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**1** A study has been made of the effects of inhibitors selective among plasmalemmal $K^+$-channels on the sensitivity and responsiveness of guinea-pig trachealis muscle to carbachol, histamine and KCl. The effects of the $K^+$-channel inhibitors on the resting membrane potential and spontaneous electrical activity of the trachealis cells have also been examined.

**2** In indomethacin (2.8 $\mu$M)-treated trachealis muscle, dofetilide (1 $\mu$M) and glibenclamide (10 $\mu$M) were each devoid of spasmogenic activity. In contrast 4-aminopyridine (4-AP, 62.5 $\mu$M–8 mM), charybdotoxin (ChTX, 100 nM) and iberiotoxin (IbTX, 100 nM) were each spasmogenic. Spasm evoked by 4-AP, IbTX or ChTX was reduced, though not abolished, by atropine (1 $\mu$M). Spasm evoked by 4-AP (1 mM), ChTX (100 nM) or IbTX (100 nM) was unaffected by tetrodotoxin (TTX; 3.1 $\mu$M) or by tissue pretreatment with capsaicin (1 $\mu$M for 30 min). Spasm evoked by IbTX or ChTX was abolished by nifedipine (1 $\mu$M).

**3** Dofetilide (1 $\mu$M) and glibenclamide (10 $\mu$M) were each without effect on the tracheal sensitivity or responsiveness to carbachol, histamine or KCl. 4-AP (1 mM) antagonized carbachol, potentiated histamine but did not affect tissue sensitivity to KCl. When the effects of 4-AP were examined in the presence of atropine (1 $\mu$M), it potentiated all the spasmogens including carbachol. IbTX and ChTX (each 100 nM) potentiated all three spasmogens. Potentiation of histamine induced by 4-AP (1 mM) or IbTX (100 nM) was also observed in tissues treated with a combination of atropine (1 $\mu$M) and TTX (3.1 $\mu$M).

**4** Dofetilide (1 and 10 $\mu$M) was without effect on the resting membrane potential or spontaneous electrical activity of the trachealis cells. 4-AP (1 mM) evoked depolarization and caused a small increase in the frequency of slow wave discharge. The depolarization evoked by 4-AP was abolished by atropine (1 $\mu$M). IbTX (100 nM) and ChTX (100 nM) each evoked little or no change in resting membrane potential but converted the spontaneous slow waves into spike-like, regenerative action potentials. These electrophysiological effects of IbTX and ChTX were unaffected by atropine (1 $\mu$M).

**5** It is concluded that the dofetilide-sensitive, cardiac, delayed rectifier $K^+$-channel is either not expressed in trachealis muscle or is of no functional importance in that tissue. The ATP-sensitive $K^+$-channel ($K_{ATP}$) does not moderate tracheal sensitivity to spasmogens such as carbachol, histamine and KCl. The 4-AP-sensitive delayed rectifier $K^+$-channel ($K_{dr}$) and the large $Ca^{2+}$-dependent $K^+$-channel ($BK_{Ca}$) each moderate trachealis muscle sensitivity to spasmogens. Neither $K_{dr}$ nor $BK_{Ca}$ plays an important role in determining the resting membrane potential of guinea-pig trachealis cells. However, the $BK_{Ca}$ channel is responsible for limiting the effects of the increase in membrane $Ca^{2+}$ conductance associated with the depolarizing phase of slow waves. It is $BK_{Ca}$ channel opening that prevents the development of a slow wave into a spike-like regenerative action potential.

**Keywords:** Trachealis muscle; 4-AP; ChTX; dofetilide; glibenclamide; IbTX; $K^+$-channels; actions of spasmogens; electrophysiology

## Introduction

Various types of $K^+$-channel have been identified in the plasmalemma of airways smooth muscle cells. These include a 4-AP sensitive, delayed rectifier $K^+$-channel ($K_{dr}$) the large, $Ca^{2+}$-dependent $K^+$-channel ($BK_{Ca}$) and the glibenclamide-sensitive $K^+$-channel ($K_{ATP}$) (Kotlikoff, 1993; Small *et al.*, 1993a). For some years it has been recognised that plasmalemmal $K^+$-channels may help determine the resting membrane potential of airways smooth muscle cells, their relatively low tendency to exhibit spontaneous membrane potential changes and their sensitivity and responsiveness to contractile and relaxant drugs. However, progress in identifying the roles played by individual types of $K^+$-channel, such as $K_{dr}$ or $BK_{Ca}$, has been very dependent upon the development of channel-selective inhibitors.

Recently, the role of $BK_{Ca}$ in determining the sensitivity and responsiveness of airways smooth muscle to relaxant drugs has been investigated with inhibitors selective for $BK_{Ca}$ such as ChTX and IbTX (Jones *et al.*, 1990; 1993; Murray *et al.*, 1991; Huang *et al.*, 1993; Laurent *et al.*, 1993; Cook *et al.*, 1995). Most authors agree that blockade of $BK_{Ca}$ by ChTX or IbTX inhibits the relaxant actions of agonists at $\beta$-adrenoceptors. However, the ability of nifedipine to prevent this antagonism suggests that it results not from a specific interaction between the toxin and the $\beta$-agonist at the level of $BK_{Ca}$ channel gating but, instead, from functional antagonism attributable to the toxin promoting the cellular influx of $Ca^{2+}$ (Huang *et al.*, 1993; Cook *et al.*, 1995). For this, and other reasons, it may be that $BK_{Ca}$ channel opening is not crucial for the relaxant actions of $\beta$-agonists in airways smooth muscle (Small *et al.*, 1993b; Black *et al.*, 1994).

To date there have been relatively few quantitative studies of the effects of selective blockade of $K_{dr}$, $BK_{Ca}$ or $K_{ATP}$ on the

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sensitivity and responsiveness of airways smooth muscle to spasmogenic drugs. Boyle *et al.* (1988) showed that tetraethylammonium (TEA) and procaine each failed to potentiate acetylcholine (ACh) or histamine in contracting guinea-pig isolated trachealis muscle. In contrast, Chand *et al.* (1990) reported that TEA potentiated both ACh and 5-hydroxytryptamine acting on rat trachealis muscle. Since neither TEA nor procaine exhibits great selectivity as an inhibitor among the various types of $K^+$-channel, the studies of Boyle *et al.* (1988) and Chand *et al.* (1990) have done very little to elucidate the roles played by $K_{dr}$, $BK_{Ca}$ or $K_{ATP}$ in regulating the sensitivity or responsiveness of airways smooth muscle to spasmogenic agents. Accordingly, we now describe experiments performed on guinea-pig isolated trachealis muscle in which we have examined the effects of inhibitors selective for $K_{dr}$, $BK_{Ca}$ or $K_{ATP}$ on the actions of spasmogens such as carbachol, histamine and KCl. In addition to measuring the effects of each $K^+$-channel inhibitor on the actions of the spasmogenic drugs, we have also studied the effects of the inhibitors themselves on the mechanical and electrical activity of the trachealis cells. A preliminary account of this work has been communicated to the British Pharmacological Society (Isaac & Small, 1994).

## Methods

### *Isolation and dissection of tissues used in the mechanical and electrophysiological studies*

Guinea-pigs (350–800 g) of either sex were killed by stunning and bleeding. Tracheae were excised from the animals and were cleaned of adhering fat and connective tissue. Each trachea was opened by cutting longitudinally through the cartilage rings diametrically opposite the trachealis muscle.

### *Tissue bath studies with isolated trachea*

Small segments (containing 3–4 cartilage rings) of guinea-pig tracheae 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, the spontaneous tone of the tissues was suppressed by addition of indomethacin (2.8 $\mu$M) to the bathing medium. Full suppression of tone was achieved 60 min after tissue exposure to indomethacin and was facilitated by exchange of the bath fluid at 20 min intervals.

Several types of experiment were performed with indomethacin-treated tissues in order to analyse the spasmogenic effects of $K^+$-channel inhibitors. In the first group of experiments all tissues were first exposed to carbachol (10 $\mu$M). On washout of the carbachol, test tissues were treated (30 min preincubation) with either atropine (1 $\mu$M) or TTX (3.1 $\mu$M) while time-matched control tissues were incubated in Krebs solution containing vehicle. The tracheal segments were then treated with ChTX (100 nM), IbTX (100 nM) or 4-AP (1 mM). The spasmogenic effects of the $K^+$-channel inhibitors were monitored for 20 min (4-AP) or 60 min (IbTX and ChTX). In the experiments where atropine was used to analyse the spasmogenic effects of IbTX and ChTX, nifedipine (1 $\mu$M) was added to both test and control tissues at the end of their 60 min exposure to the relevant toxin.

In the case of 4-AP (62.5 $\mu$M–8 mM), cumulative concentration-effect curves were constructed both in the presence and in the absence of atropine (1 $\mu$M). The tissue contact time for each concentration of 4-AP was 20 min. Following the construction of the cumulative concentration-effect curve to 4-AP, the tissue was challenged with carbachol (1 mM). All contractile responses to 4-AP were subsequently expressed in terms of the (maximal) response to carbachol (1 mM).

In order to examine the role of peptidergic neurotransmitter release in mediating the spasmogenic effects of $K^+$-channel inhibitors, indomethacin-treated test tissues were exposed to capsaicin (1 $\mu$M) for 30 min. At the end of this period the tissue was washed (4 times at 15 min intervals) with capsaicin-free Krebs solution. The tissue was then challenged with carbachol (10 $\mu$M). Following carbachol washout (30 min) the tissue was treated with 4-AP (1 mM), IbTX (100 nM) or ChTX (100 nM). Time-matched control tissues were treated similarly but were exposed to vehicle instead of capsaicin.

The effects of $K^+$-channel inhibitors on the actions of spasmogens (carbachol, histamine or KCl) were studied by the construction of cumulative concentration-effect curves for the spasmogens. Each concentration of carbachol, histamine or KCl was allowed 10, 6 and 12 min tissue contact, respectively, before a concentration increment was made. Following washout (3 exchanges of bath fluid over at least 30 min) of the spasmogen, second and third cumulative concentration-effect curves were constructed. A $K^+$-channel inhibitor [4-AP (1 mM), ChTX (100 nM), dofetilide (1 $\mu$M), glibenclamide (10 $\mu$M) or IbTX (100 nM)] was added to the bathing medium 20 min before construction of the third concentration-effect curve for the spasmogen and remained present for the rest of the experiment. Time-matched control tissues were treated similarly to test tissues but were exposed to vehicle instead of the $K^+$-channel inhibitor. Similar studies of the interaction between 4-AP (1 mM) and each of the spasmogens (carbachol, histamine and KCl) were performed in tissues treated with atropine (1 $\mu$M), the latter agent being administered immediately following construction of the second concentration-effect curve for the spasmogen. Tissues treated with a combination of atropine (1 $\mu$M) and TTX (3.1 $\mu$M) were used to extend analysis of the effects of 4-AP (1 mM) or IbTX (100 nM) on the log concentration-effect curve for histamine. The atropine/TTX mixture was administered 30 min before tissue exposure to the $K^+$-channel inhibitor or its vehicle.

### *Intracellular electrophysiological recording from trachealis*

Simultaneous recording of intracellular electrical activity and mechanical changes of a contiguous segment of guinea-pig trachea was performed using 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.

After impalement of a trachealis cell, several minutes were allowed to elapse to check the stability of the record of electrical activity. A $K^+$-channel inhibitor [4-AP (1 mM), ChTX (100 nM), dofetilide (1 or 10 $\mu$M), or IbTX (100 nM)] was then added to the Krebs solution superfusing the tissue and the electrical activity of the impaled cell was monitored for as long as the microelectrode remained within the cell. Following the deliberate or spontaneous dislodgement of the electrode, other cells were impaled in order to assess further the effects of the $K^+$-channel inhibitor on their electrical activity.

Most of the electrophysiological experiments were carried out in normal Krebs solution. Some of the experiments with 4-AP, ChTX and IbTX were carried out in Krebs solution containing either indomethacin (2.8 $\mu$M) or atropine (1 $\mu$M).

### *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: 4-AP (Sigma), atropine sulphate (Sigma), capsaicin (Sigma), carbamoylcholine chloride (carbachol; Sigma), ChTX (Latoxan), dofetilide (Pfizer Central Research), glibenclamide (Sigma), histamine dihydrochloride (Sigma), IbTX (Affiniti Research Products), indomethacin (Sigma), nifedipine (Bayer), potassium chloride (BDH), tetrodotoxin (Sigma). Stock solutions of ChTX and IbTX were prepared in 0.9% w/v saline.

Stock solutions of capsaicin, glibenclamide, indomethacin and nifedipine were prepared in absolute ethanol. A stock solution of dofetilide was prepared in 70% ethanol. Other drugs were dissolved in twice-distilled water.

The Krebs solution used in the tissue bath experiments and for the microelectrode recording of membrane potential changes had the following composition (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25 and glucose 11.1. When maintained at 37.5°C and gassed with a mixture of 95% $O_2$ and 5% $CO_2$, this medium was of pH 7.4.

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

## Results

### *Tension changes induced by the $K^+$-channel inhibitors*

Neither dofetilide (1 μM) nor glibenclamide (10 μM) caused any spasm of the indomethacin (2.8 μM)-treated trachealis muscle. In contrast, 4-AP (62.5 μM–8 mM) induced concentration-dependent tension development. This action of 4-AP was antagonized, but not abolished, by atropine (1 μM) (Figure 1; Table 1). Tissue treatment with TTX (3.1 μM) profoundly reduced tracheal reponses to electrical field stimulation of intramural excitatory neurones (data not shown) but did not reduce the spasmogenic effect of 4-AP (1 mM) (Table 1). Tracheal exposure to capsaicin (1 μM for 30 min) evoked spasm equivalent to 73.2±1.6% (mean±s.e.mean; $n=18$) of that evoked by carbachol (10 μM). Capsaicin-induced spasm peaked within a few mintues and thereafter exhibited marked decay. Pretreatment of tracheal segments with capsaicin abolished tracheal responses to electrical field stimulation of non-cholinergic, non-adrenergic neurones (data not shown) but did not reduce the spasmogenic response to 4-AP (1 mM) (Table 1).

IbTX and ChTX (each 100 nM) almost invariably caused spasm of the indomethacin (2.8 μM)-treated trachealis muscle. Toxin-induced tension development was sometimes tonic and sometimes phasic in nature and could take up to 60 min to develop fully. The spasm evoked by IbTX or ChTX was not modified by pretreatment of the tissue with capsaicin (1 μM for 30 min) or by TTX (3.1 μM) (Table 1). In contrast, the spasm evoked by the toxins was reduced by atropine (1 μM) (Table 1) Nifedipine (1 μM) abolished the spasm evoked by IbTX (100 nM) or by ChTX (100 nM), both in tissues treated with atropine (1 μM) and in tissues not exposed to the antagonist at muscarinic ACh receptors.

### *Effects of the $K^+$-channel inhibitors on tissue sensitivity and responsiveness to carbachol, histamine and KCl*

Carbachol (10 nM–10 μM), histamine (100 nM–1 mM) and KCl (5–120 mM) each induced concentration-dependent tension development. In the time-matched control tissues it was observed that, for each of these agents, the initial log concentration-effect curve lay to the left of the second and third curves in the series. The position of the third curve, however, did not differ from that of the second curve. It was for this reason that, in the test tissues, the effects of the $K^+$-channel inhibitors were studied by adding them to the Krebs solution between the construction of the second and third log concentration-effect curves in the series constructed for each spasmogen.

Dofetilide (1 μM) and glibenclamide (10 μM) each failed to cause any change in the tissue sensitivity (Table 2) or responsiveness (data not shown) to carbachol, histamine or KCl. 4-AP (1 mM) failed to modify the tissue sensitivity or responsiveness to KCl (Figure 2 and Table 2), caused minor (approximately three fold) antagonism of carbachol, but potentiated (approximately four fold) histamine (Figure 2 and Table 2). When the effects of 4-AP (1 mM) were examined in tissues additionally treated with atropine (1 μM), it was observed to potentiate carbachol, histamine and KCl (Figure 3 and Table 2). 4-AP (1 mM) also potentiated histamine in trachea treated with a combination of atropine (1 μM) and TTX (3.1 μM) (Table 2).

ChTX potentiated carbachol (approximately four fold), histamine and KCl (each between two and three fold) (Table 2).

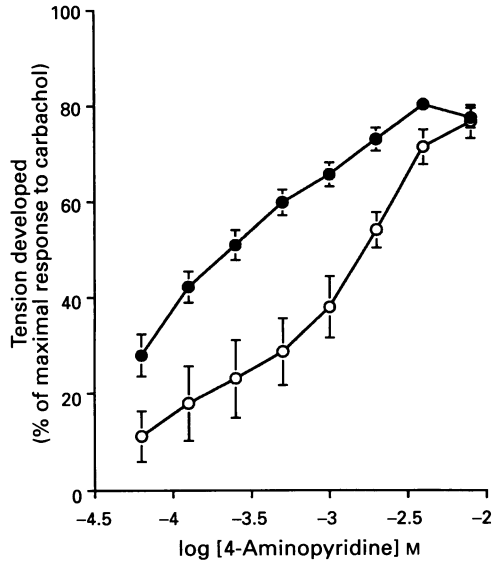


**Figure 1** Guinea-pig isolated trachealis treated with indomethacin (2.8 μM): the effects of atropine (1 μM) on the spasmogenic activity of 4-AP. Abscissa scale: $\log_{10}$ molar concentration of 4-AP. Ordinate scale: contraction expressed as a percentage of the (maximal) contraction induced by carbachol (1 mM). (●) log Concentration-effect curve obtained in vehicle-treated, time-matched control tissues; (○) log concentration-effect curve obtained in test tissues treated with atropine (1 μM). Data points are the means (±s.e.mean) of values from at least 6 tissues.

**Table 1** Analysis of the spasm induced in indomethacin (2.8 μM)-treated trachealis muscle by 4-AP, IbTX and ChTX

| | *Tension induced (% of carbachol maximum) by $K^+$-channel inhibitor* | | |
|---|---|---|---|
| | *4-AP* (1 mM) | *IbTX* (100 nM) | *ChTX* (100 nM) |
| Control | 65.6±2.5 | 83.4±3.5 | 52.6±3.5 |
| Atropine (1 μM) | 38.1±6.4* | 59.7±10.3* | 18.8±5.1* |
| Control | 61.8±4.7 | 56.0±3.6 | 24.6±5.8 |
| TTX (3.1 μM) | 63.5±4.1 | 50.9±7.9 | 36.7±7.1 |
| Control | 55.4±6.7 | 73.8±9.9 | 31.7±8.2 |
| Capsaicin (1 μM for 30 min) | 48.9±6.1 | 67.8±8.0 | 24.6±10.3 |

The spasmogenic response is expressed as a percentage of the response evoked by carbachol (10 μM). Data indicate mean (±s.e.mean) of values from at least 6 tissues. *Significant ($P<0.05$) difference from the control value.

**Table 2** The effects of some $K^+$-channel inhibitors on the spasmogenic potencies of carbachol, histamine and KCl acting on indomethacin (2.8 μM)-treated guinea-pig isolated trachealis muscle

| | *Carbachol* | | *Histamine* | | *KCl* | |
|---|---|---|---|---|---|---|
| | *Control* | *Test* | *Control* | *Test* | *Control* | *Test* |
| 4-AP (1 mM) | 6.64 ± 0.04 | 6.19 ± 0.05* | 4.93 ± 0.07 | 5.54 ± 0.06* | 1.61 ± 0.02 | 1.68 ± 0.03 |
| 4-AP (1 mM) + atropine (1 μM) | 3.40 ± 0.05 | 3.76 ± 0.04* | 5.00 ± 0.13 | 5.76 ± 0.06* | 1.55 ± 0.03 | 1.74 ± 0.04* |
| 4-AP (1 mM) + atropine (1 μM) + TTX (3.1 μM) | | | 4.52 ± 0.06 | 5.43 ± 0.05* | | |
| Dofetilide (1 μM) | 6.53 ± 0.07 | 6.60 ± 0.09 | 5.02 ± 0.19 | 4.98 ± 0.08 | 1.66 ± 0.03 | 1.69 ± 0.01 |
| Glibenclamide (10 μM) | 6.62 ± 0.07 | 6.52 ± 0.07 | 4.70 ± 0.11 | 4.64 ± 0.06 | 1.51 ± 0.02 | 1.48 ± 0.02 |
| ChTX (100 nM) | 6.67 ± 0.04 | 7.26 ± 0.08* | 5.02 ± 0.08 | 5.41 ± 0.07* | 1.61 ± 0.04 | 2.01 ± 0.06* |
| IbTX (100 nM) | 6.48 ± 0.03 | 6.87 ± 0.09* | 4.73 ± 0.09 | 5.40 ± 0.18* | 1.52 ± 0.02 | > 2.30* |
| IbTX (100 nM) + atropine (1 μM) + TTX (3.1 μM) | | | 4.84 ± 0.12 | 5.42 ± 0.09* | | |

The data indicate mean ± s.e.mean of values from at least 6 tissues. *Value significantly ($P < 0.05$) different from the corresponding control.

In the case of carbachol the potentiation was manifest principally as an elevation of the lower portion of the log concentration-effect curve (Figure 4). IbTX (100 nM), too, potentiated carbachol, histamine and KCl. Potentiation of histamine was manifest as a five fold leftward shift in the log concentration-effect curve with a small increase in the maximal response. In contrast, potentiation of carbachol was manifest mainly as an elevation of the lower portion of the log concentration-effect curve without change in the maximal response (Figure 5). Potentiation of KCl by IbTX involved marked increases in the amplitude of responses obtained with low (5–20 mM) concentrations of KCl. However, tested in the presence of IbTX, KCl (40 mM) evoked partial relaxation and further increments in the KCl concentration failed to evoke greater contractile responses (Figure 5). The ability of IbTX to potentiate histamine was retained in tissues treated with a combination of atropine (1 μM) and TTX (3.1 μM) (Table 2).

### *Electrophysiological studies*

Dofetilide (1 and 10 μM) did not modify the resting membrane potential of trachealis cells, nor did it affect the discharge of spontaneous electrical slow waves (Table 3 and Figure 6). In contrast, 4-AP (1 mM) evoked an increase in tissue tone and cellular depolarization (Table 3) that was accompanied by a slight reduction in slow wave amplitude (Figure 6b). In the presence of atropine (1 μM) the spasm evoked by 4-AP was significantly reduced and the depolarization evoked by 4-AP was abolished (Table 3 and Figure 6). IbTX (100 nM) and ChTX (100 nM) each caused increases in mechanical tone that were accompanied by little or no change in resting membrane potential but that were accompanied by the conversion of the spontaneous slow waves into spike-like regenerative action potentials (Table 3 and Figure 7). The ability of the two toxins to induce action potential discharge was not affected by the addition of atropine (1 μM; Figure 7).

## Discussion

### *Analysis of the spasmogenic effects of the $K^+$-channel inhibitors*

The observation (Urquhart & Broadley, 1991) that 4-AP has spasmogenic effects in guinea-pig trachealis muscle has been confirmed in the present work. Treatment of guinea-pig trachea with capsaicin (1 μM for 30 min) evokes spasm, desensitizes the tissue to further challenge with capsaicin and abolishes responses of the tissue to stimulation of intramural, non-adrenergic, non-cholinergic (presumed peptidergic) neurones (Szolcsyani & Bartho, 1982; Ellis & Undem, 1990; 1994; present study). In view of this, the resistance of 4-AP-induced spasm to tissue treatment with capsaicin (Table 1) suggests that such spasm is unlikely to depend on the release of neurotransmitters from intramural peptidergic neurones. Like the spasm evoked by 4-AP in canine trachealis muscle, that observed in guinea-pig tissue is reduced, but not abolished, by atropine (Kannan *et al.*, 1983; present study, Figure 1). Furthermore, work with rat striatal slices (Drukarch *et al.*, 1989) has shown that 4-AP can promote the neural release of ACh. Such findings prompt the notion that ACh release could, at least in part, explain the tracheal spasmogenic effect of 4-AP. However, the resistance of 4-AP-induced tracheal spasm to TTX (3.1 μM) (Table 1) suggests that any promotion of ACh release by 4-AP must involve a process that is independent of neuronal action potential discharge.

Drukarch *et al.* (1989) observed that 4-AP inhibits the specific binding of [$^3$H]-dexetimide to rat striatal muscarinic receptor binding sites. For this reason we should also consider the possibility that 4-AP interacts directly with muscarinic receptors on the smooth muscle cells. The present observation (Figure 2) that 4-AP antagonizes carbachol acting on guinea-pig trachea is consistent with the earlier finding of Urquhart & Broadley (1991) and suggests that 4-AP acts (either directly or indirectly) as a muscarinic agonist with efficacy lower than that of carbachol. Whatever the mechanism underlying the muscarinic agonist action of 4-AP, this action complicates the use of 4-AP as a pharmacological tool for blocking $K_{dr}$ channels. It was for this reason that interactions between 4-AP and various spasmogens were studied both in the presence and absence of atropine. It was argued that the presence of atropine would reveal the $K^+$-channel blocking effects of 4-AP uncontaminated by its direct or indirect activity at muscarinic cholinoceptors.

The atropine-resistant component of the spasmogenic action of 4-AP (Figure 1) presumably reflects 4-AP-induced blockade of $K_{dr}$ channels in the plasmalemma of the trachealis cells. The atropine-resistant spasm is unlikely to reflect depolarization-induced promotion of $Ca^{2+}$ influx for 4-AP did not, in atropine-treated tissues, reduce the resting membrane potential and did not convert spontaneous slow waves into regenerative action potentials (see below). Accordingly, the way in which blockade of 4-AP-sensitive $K_{dr}$ channels induces tension development remains a challenge for further experimentation.

The present finding that ChTX and IbTX have spasmogenic activity in indomethacin-treated guinea-pig trachealis confirms the results of earlier studies (Jones *et al.*, 1988; 1993). As discussed above in the context of 4-AP, the resistance of IbTX- or ChTX-induced spasm to tissue treatment with capsaicin (Table 1) suggests that such spasm is unlikely to depend on the release of neurotransmitters from intramural peptidergic neurones. It

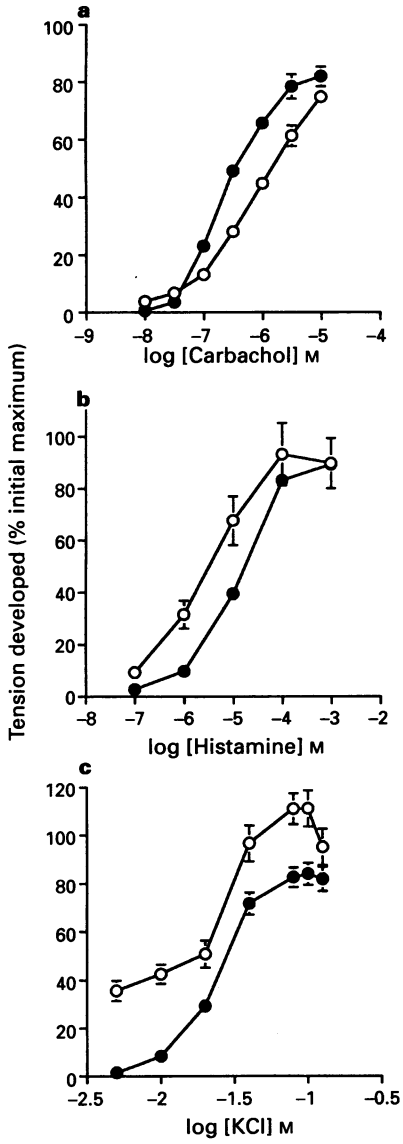


**Figure 2** Guinea-pig isolated trachealis treated with indomethacin (2.8 $\mu$M): the effects of 4-AP (1 mM) on the spasmogenic actions of (a) carbachol, (b) histamine and (c) KCl. In each panel the abscissa scale indicates the $\log_{10}$ molar concentration of spasmogen while the ordinate scale indicates the tension developed as a percentage of the initial maximum. (●) log Concentration-effect curve obtained in vehicle-treated, time-matched control tissues; (○) log concentration-effect curve obtained in test tissues treated with 4-AP (1 mM). Data points are the means (±s.e.mean) of values from at least 6 tissues.

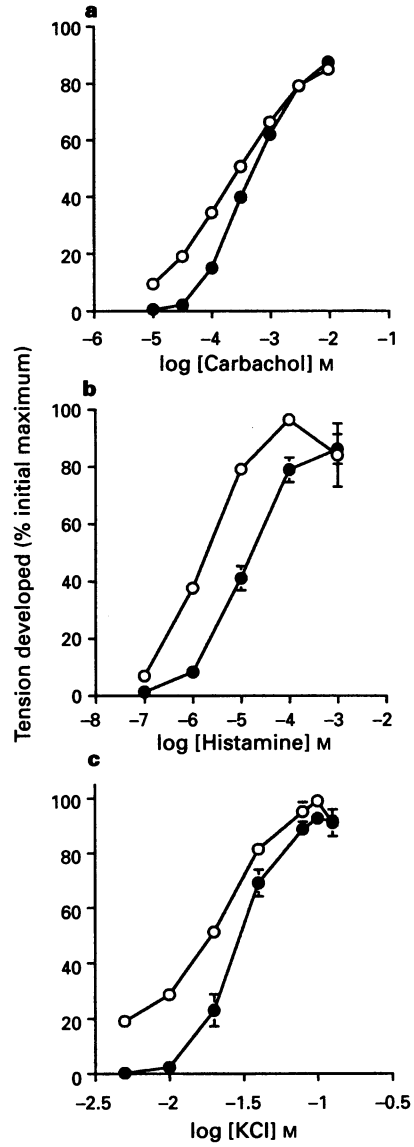


**Figure 3** Guinea-pig isolated trachealis treated with indomethacin (2.8 $\mu$M): the effects of a combination of atropine (1 $\mu$M) and 4-AP (1 mM) on the spasmogenic actions of (a) carbachol, (b) histamine and (c) KCl. In each panel the abscissa scale indicates the $\log_{10}$ molar concentration of spasmogen while the ordinate scale indicates the tension developed as a percentage of the initial maximum. (●) log Concentration-curve effect obtained in vehicle-treated, time-matched control tissues; (○) log concentration-effect curve obtained in test tissues treated with a combination of atropine (1 $\mu$M) and 4-AP (1 mM). Data points are the means (±s.e.mean) of values from at least 6 tissues.

has been reported (unpublished observations of Jones & Charette cited by Jones *et al.*, 1993) that the spasmogenic effects of IbTX and ChTX in guinea-pig trachealis are resistant to atropine. In contrast, the present study has shown that the spasmogenic effects of the two toxins are significantly reduced by atropine (1 $\mu$M). To date there is no published data to suggest that ChTX or IbTX directly activate muscarinic ACh receptors. However, Kotlikoff (1993) has proposed that the effects of inhibitors of $BK_{Ca}$ acting on smooth muscle-containing tissues may involve promotion of transmitter release from intramural nerves. If the atropine-sensitive components of the spasmogenic actions of IbTX and ChTX observed in guinea-pig trachea indeed reflect promotion of ACh release, then the release process is unlikely to involve the discharge of neuronal action potentials for the spasmogenic actions of the two toxins were resistant to TTX (3.1 $\mu$M) (Table 1). Atropine caused a greater reduction of the spasm evoked by ChTX (100 nM) than that evoked by IbTX (100 nM) (Table 1). This finding might be explicable in terms of ChTX having the greater propensity to promote neurotransmitter release. This notion receives support from the work of Galvez *et al.* (1990) who have reported that, in contrast to IbTX, ChTX inhibits several different types of voltage-dependent $K^+$-channel in neuronal tissue.

The finding that the spasmogenic effects of both IbTX and ChTX are abolished by nifedipine (Jones *et al.*, 1988; unpublished observations of Jones & Charette cited by Jones *et al.*, 1993; present study) suggests that the two toxins act to promote $Ca^{2+}$ influx through L-type channels. Such channels may well be activated during the upstroke of spike-like action potentials. ChTX (Murray *et al.*, 1991; present study, Figure 7) and IbTX (present study, Figure 7) are each able to induce

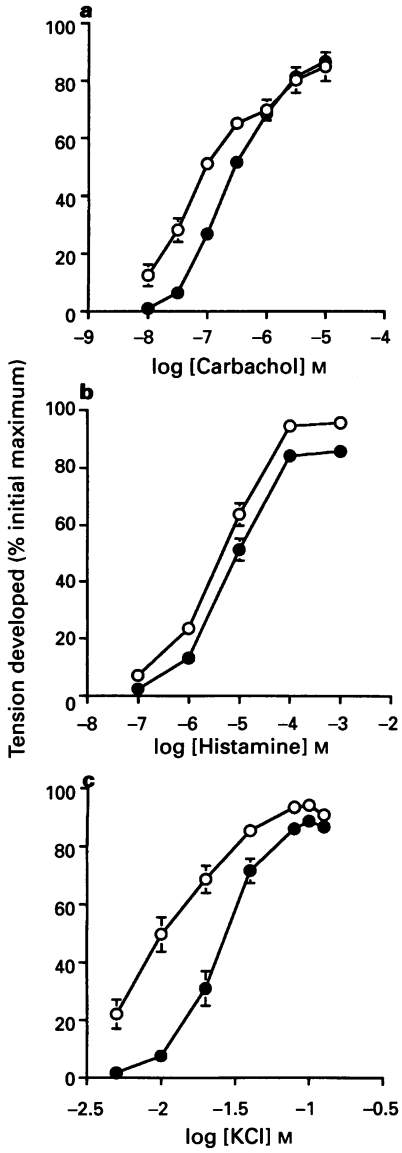


**Figure 4** Guinea-pig isolated trachealis treated with indomethacin (2.8 μM): the effects of ChTX (100 nM) on the spasmogenic actions of (a) carbachol, (b) histamine and (c) KCl. In each panel the abscissa scale indicates the $\log_{10}$ molar concentration of spasmogen while the ordinate scale indicates the tension developed as a percentage of the initial maxium. (●) log Concentration-effect curve obtained in vehicle-treated, time-matched control tissues; (○) log concentration-effect curve obtained in test tissues treated with ChTX (100 nM). Data points are the means (±s.e.mean) of values from at least 6 tissues.

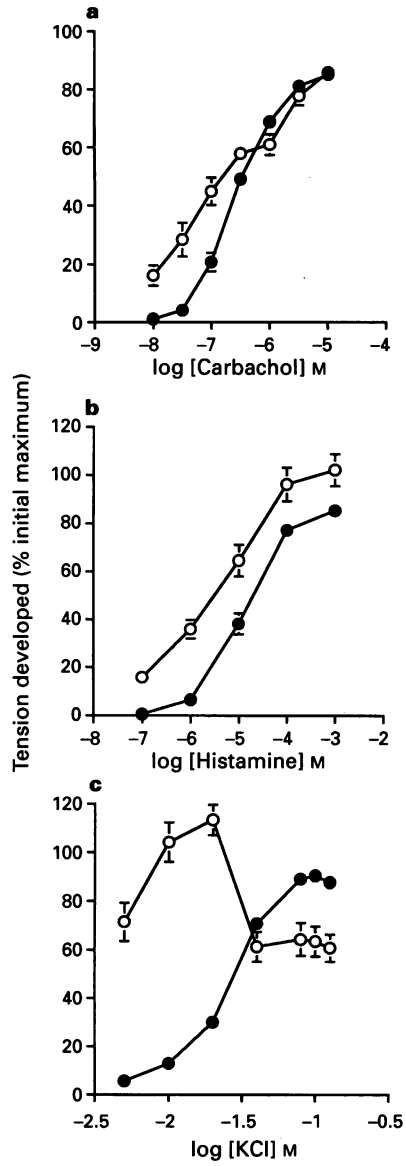


**Figure 5** Guinea-pig isolated trachealis treated with indomethacin (2.8 μM): the effects of IbTX (100 nM) on the spasmogenic actions of (a) carbachol, (b) histamine and (c) KCl. In each panel the abscissa scale indicates the $\log_{10}$ molar concentration of spasmogen while the ordinate scale indicates the tension developed as a percentage of the initial maximum. (●) log Concentration-effect curve obtained in vehicle-treated, time-matched control tissues; (○) log concentration-effect curve obtained in test tissues treated with IbTX (100 nM). Data points are the means (±s.e.mean) of values from at least 6 tissues.

action potentials of this kind and these are events that, in guinea-pig trachealis, are known to be suppressed by $Ca^{2+}$ influx inhibitors such as nifedipine and verapamil (Foster *et al.*, 1984; Ahmed *et al.*, 1985).

### *Roles played by plasmalemmal $K^+$-channels in determining the resting membrane potential of airways smooth muscle cells*

Dofetilide (50 nM–2 μM) inhibits the rapidly activating component of the delayed rectifier $K^+$-current in cardiac muscle. Furthermore, dofetilide exhibits selectivity of action in that it does not simultaneously inhibit the slowly activating component of the delayed rectifier $K^+$-current, the inwardly rectifying $K^+$-current, the fast $Na^+$-current or the L-type $Ca^{2+}$ current (Gwilt *et al.*, 1991; Carmelite, 1992; Jurkiewicz & Sanguinetti, 1993; Kiehn *et al.*, 1994). Tested on guinea-pig trachealis muscle, dofetilide (1 μM) was devoid of mechanical effects *per se*, did not affect the tissue sensitivity or responsiveness to a variety of spasmogenic substances (Table 2) and had no discernible effect on the resting membrane potential (Table 3) or spontaneous electrical activity of the trachealis muscle cells. This may imply that the rapidly-activating delayed rectifier $K^+$-channel of cardiac muscle differs markedly from the delayed rectifier $K^+$-channels that are expressed in smooth muscle. Should this prove to be the case, dofetilide could exhibit great selectivity *in vivo* in controlling cardiac dysrhythmias without disturbing the function of smooth muscle-containing tissues. However, in studies of the latter type of tissue, dofetilide may be of no use as a tool for studying the role played by delayed rectifier channels in determining resting membrane potential or cellular excitability.

Boyle *et al.* (1992) prepared inside-out plasmalemmal patches of canine trachealis under conditions where the activity of $BK_{Ca}$ channels was suppressed by the use of ChTX and TEA. In such circumstances the activity of $K_{dr}$ channels was reduced

**Table 3** The effects of some drugs and combinations of drugs on the resting membrane potential of guinea-pig trachealis muscle cells

| | *Resting membrane potential* (mV) | n |
|---|---|---|
| Control (no drug treatment) | −44.2 ± 0.49 | 57 |
| Atropine (1 μM) | −44.3 ± 0.99 | 16 |
| Dofetilide (1 μM) | −43.3 ± 0.67 | 15 |
| Dofetilide (10 μM) | −46.2 ± 0.97 | 6 |
| 4-AP (1 mM) | −33.4 ± 1.29* | 7 |
| 4-AP (1 mM) + atropine (1 μM) | −44.4 ± 1.37 | 12 |
| IbTX (100 nM) | −44.9 ± 1.03 | 21 |
| IbTX (100 nM) + atropine (1 μM) | −49.7 ± 0.94* | 20 |
| ChTX (100 nM) | −43.2 ± 1.13 | 22 |
| ChTX (100 nM) + atropine (1 μM) | −42.6 ± 1.94 | 19 |

Data are the mean ± s.e.mean of values recorded from *n* cells. *Significant ($P<0.05$) difference from the control value.

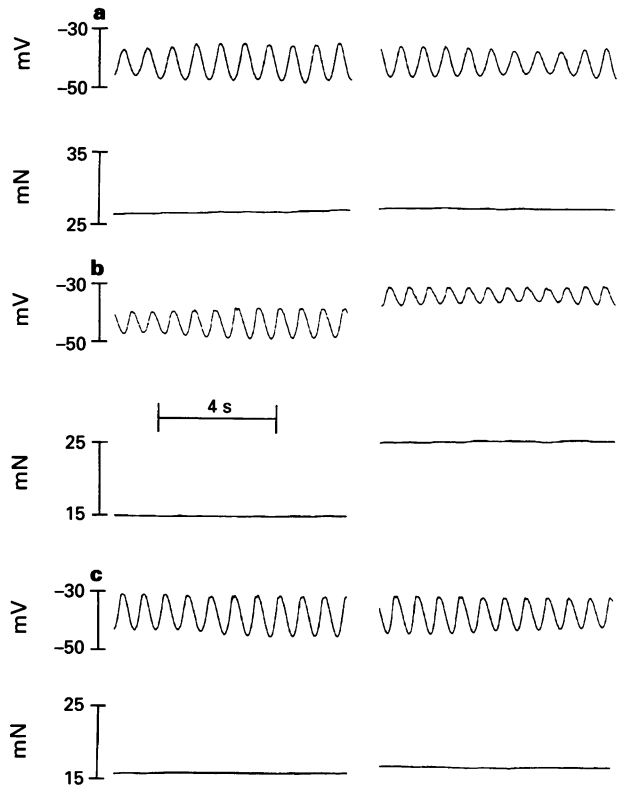


**Figure 6** Guinea-pig isolated trachealis: the effects of dofetilide (10 μM) and 4-AP (1 mM) on spontaneous electrical activity and mechanical tone. In each row of recordings the upper trace indicates the membrane potential and the lower trace represents tension changes. In each row the two recordings of electrical activity were obtained from the same cell. (a) Recordings made before (left hand panel) and 11 min after tissue treatment with dofetilide (10 μM). Note the failure of dofetilide markedly to modify either electrical or mechanical activity. (b) Recordings made before (left hand panel) and 17 min after tissue treatment with 4-AP (1 mM). Note the depolarization and increase in mechanical tone induced by 4-AP. Note also that 4-AP did not cause the conversion of slow waves into spike-like action potentials. (c) Recordings made from an atropine (1 μM)-treated tissue before (left hand panel) and 17 min after tissue treatment with 4-AP (1 mM). Note, by comparison with (b), the ability of atropine to reduce the depolarization and tension rise induced by 4-AP.

by more than 90% following the addition of 4-AP (1 mM) to the bathing medium. In other experiments using excised plasmalemmal patches of canine trachealis, the same concentration of 4-AP was without effect on the activity of $BK_{Ca}$ channels (Muraki *et al.*, 1990; Boyle *et al.*, 1992). This suggests that, in airways smooth muscle, 4-AP exhibits some selectivity as an inhibitor of $K_{dr}$ channels as opposed to $BK_{Ca}$.

Several studies have now been made of the effects of 4-AP on the resting membrane potential of airways smooth muscle cells (Kannan *et al.*, 1983; Daniel *et al.*, 1992; Fleischmann *et al.*, 1993; present study). It seems clear that, in trachealis muscle of the dog and guinea-pig, 4-AP (1–5 mM) induces depolarization of the order of 10–20 mV. However, the depolarization of canine trachealis muscle induced by 4-AP is very markedly reduced in the presence of atropine (Daniel *et al.*, 1992) and the depolarization of guinea-pig trachealis induced by 4-AP is abolished by atropine (present study, Table 3). This suggests that, in these tissues, the depolarization induced by 4-AP is mainly a consequence of its direct or indirect agonist activity at muscarinic cholinoceptors. Unfortunately, Fleischmann *et al.* (1993) did not examine whether 4-AP-induced depolarization (14 mV) of ferret trachealis cells was atropine-sensitive. However, this membrane potential change was accompanied by an increase in input resistance, a finding which might argue against its containing a cholinomimetic component.

Although Fleischmann *et al.* (1993) showed that 4-AP was able to depolarize enzymically-dispersed ferret trachealis cells, these cells had a very low (−33 mV) resting membrane potential compared with that (−58 mV) observed in the intact tissue (Coburn, 1984). Furthermore, $K_{dr}$ channels are unlikely to be open at transmembrane potentials more inside-negative than −35 mV (Noack, 1992). Hence the experimental model of Fleischmann *et al.* (1993) may have provided an over-estimation of the importance of 4-AP-sensitive $K_{dr}$ channels in determining the normal resting membrane potential of airways smooth muscle cells. Support for this idea comes from work with canine and guinea-pig tissue. Tested in the presence of atropine, 4-AP evoked little or no depolarization of canine or guinea-pig trachealis muscle (Daniel *et al.*, 1992; present study). In view of these findings, we conclude that 4-AP-sensitive $K_{dr}$ channels do not play an important role in determining the normal resting membrane potential of the airways smooth muscle cell.

In smooth muscle cells ChTX is a relatively selective inhibitor of $BK_{Ca}$ channels (Cook & Quast, 1990; Boyle *et al.*, 1992). IbTX exhibits greater specificity for such channels (Galvez *et al.*, 1990; Suarez-Kurtz *et al.*, 1991). In canine trachealis muscle ChTX (100 nM) evoked depolarization in excess of 10 mV (Kamei *et al.*, 1994). This finding may indicate that, in canine trachealis muscle, $BK_{Ca}$ channels are open under resting conditions and help to determine the resting membrane potential. However, the experiments of Kamei *et al.* (1994) were not carried out in the presence of an atropine-like drug and it is therefore possible that the depolarizing effect of the toxin was a consequence of the toxin directly or indirectly activating muscarinic cholinoceptors on the smooth muscle cells. In the studies of Fleischmann *et al.* (1993) ChTX (100 nM) failed to depolarize current-clamped, enzymatically-dispersed ferret trachealis cells. As indicated above, these cells had a very low resting membrane potential compared with that observed in the intact tissue. For this reason further elucidation of the role of $BK_{Ca}$ channels in determining the resting membrane potential of ferret trachealis cells awaits examination of the toxin's effect on the membrane potential of cells not subjected to enzymic dispersal. In guinea-pig trachea, ChTX (100 nM) and IbTX (100 nM) each evoked little or no membrane potential change either in the presence or absence of

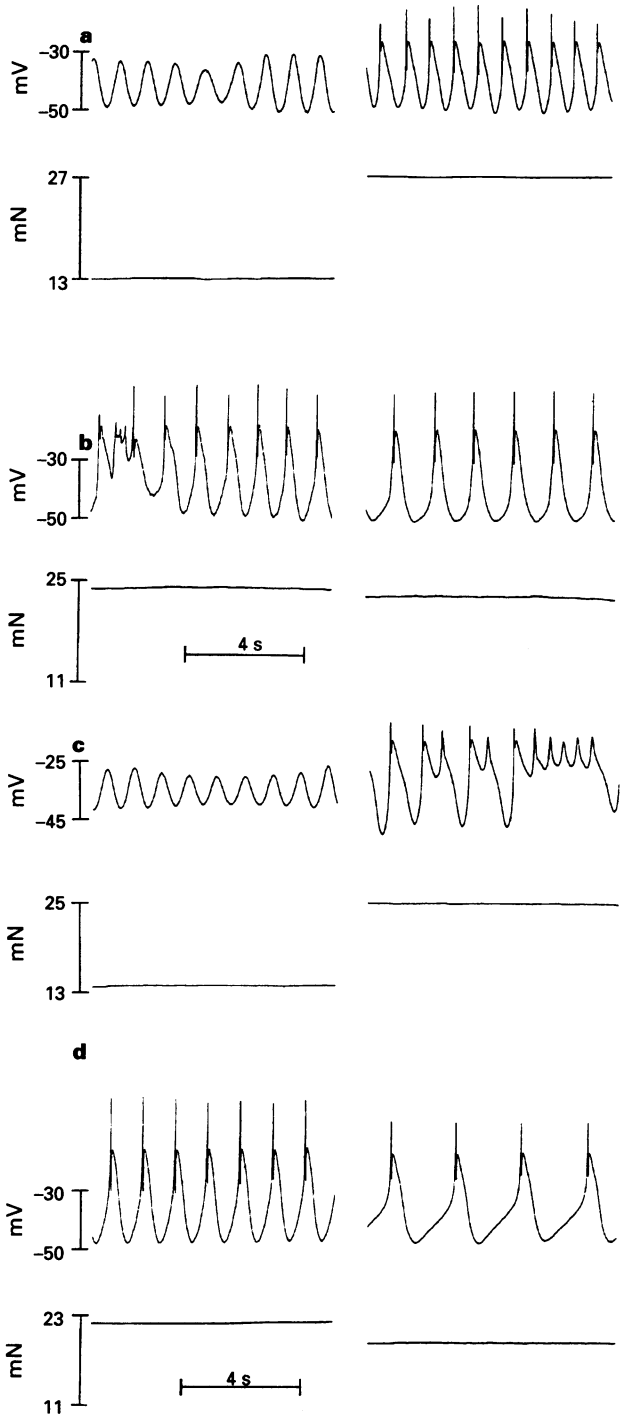


**Figure 7** Guinea-pig isolated trachealis: the effects of ChTX (100 nM) and IbTX (100 nM) on spontaneous electrical activity and mechanical tone. In each row of recordings the upper trace indicates the membrane potential and the lower trace represents tension changes. Unless otherwise stated, the two recordings of electrical activity in each row were obtained from different cells of the same tissue. (a) Recordings made before (left hand panel) and 27 min after tissue treatment with ChTX (100 nM). Note that ChTX converted the spontaneous electrical slow waves into regenerative action potentials. (b) Recordings made from a ChTX (100 nM)-treated tissue before (left hand panel) and 46 min after tissue treatment with atropine (1 $\mu$M). Note that atropine did not prevent the toxin from converting slow waves into spike-like action potentials. (c) Recordings made before (left hand panel) and 16 min after tissue treatment with IbTX (100 nM). Note that both electrical recordings were obtained from the same cell and that IbTX converted the spontaneous electrical slow waves into regenerative action potentials. (d) Recordings made from an IbTX (100 nM)-treated tissue before (left hand panel) and 60 min after tissue treatment with atropine (1 $\mu$M). Note that atropine did not prevent the toxin from converting slow waves into regenerative action potentials.

atropine (Murray *et al.*, 1991; present study Table 3). This strongly suggests that, in this tissue, very few $BK_{Ca}$ channels are open under resting conditions. Such channels thus play a relatively minor role in determining the value of the resting membrane potential. The same is true for $K_{ATP}$ channels, because neither glibenclamide nor phentolamine evoked significant depolarization of the guinea-pig trachealis muscle (Murray *et al.*, 1989). In summary, it seems that the resting membrane potential of the airways smooth muscle cell is unlikely to be determined by the activity of 4-AP-sensitive $K_{dr}$ channels, $BK_{Ca}$ or $K_{ATP}$. Other $K^+$-channels such as the inward rectifier ($K_{ir}$) may more importantly determine the resting plasmalemmal $K^+$ permeability in smooth muscle (Bolton & Beech, 1992).

### *Effects of selective $K^+$-channel inhibitors on the spontaneous electrical slow waves of the trachealis cells*

The slow waves observed in the guinea-pig trachaelis cells are resistant to hyoscine, tetrodotoxin, hexamethonium, guanethidine and propranolol (Boyle *et al.*, 1987). It may therefore be suggested that the slow waves have a myogenic rather than a neurogenic basis. The discharge of slow waves is dependent on the inward movement of $Ca^{2+}$ across the plasmalemma, for slow wave discharge is abolished by $Ca^{2+}$-free media (Foster *et al.*, 1983; McCaig, 1986) or by organic inhibitors of $Ca^{2+}$ influx such as gallopamil, nifedipine and verapamil (Small, 1982; Ahmed *et al.*, 1985; Small & Foster, 1986). The susceptibility of guinea-pig trachealis muscle slow waves to organic inhibitors of $Ca^{2+}$ influx not only suggests that their ionic basis differs from that of slow waves observed in gastrointestinal smooth muscle, but also that they represent aborted action potentials (Small, 1982).

The ability of TEA to convert the slow waves of guinea-pig trachealis muscle into regenerative action potentials (Small, 1982; Foster *et al.*, 1983; Ahmed *et al.*, 1985) suggests that $K^+$-channel opening is responsible for preventing the development of slow waves into regenerative events. However, TEA is poorly selective among the subtypes of plasmalemmal $K^+$-channel. It cannot therefore be used to identify the $K^+$-channel subtype that is primarily responsible for suppressing action potential activity in this tissue.

Glibenclamide and phentolamine have each been shown to act as inhibitors of $K_{ATP}$, yet neither of these agents induce spike-like action potentials in guinea-pig trachealis muscle (Murray *et al.*, 1989). This suggests that $K_{ATP}$ does not play an important role in preventing the development of slow waves into regenerative action potentials. That 4-AP-sensitive $K_{dr}$ channels also play little role in this process is suggested by the present findings (Figure 6) that 4-AP (either alone or in combination with atropine) did not elicit action potential discharge.

The observation (Murray *et al.*, 1991; present study) that ChTX can induce spike-like potentials may indicate that, in guinea-pig trachealis, the development of a slow wave into a regenerative action potential is prevented by the opening of $BK_{Ca}$ channels. To some extent this argument is weakened by reports that, in tissue other than smooth muscle, ChTX may inhibit $K^+$-channels other than $BK_{Ca}$ (Galvez *et al.*, 1990; Suarez-Kurtz *et al.*, 1991). However, as indicated above, the specificity of IbTX for $BK_{Ca}$ channels is greater than that of ChTX and IbTX, too, converted the tracheal slow waves into spike-like action potentials (Figure 7).

Kotlikoff (1993) has suggested that the actions of inhibitors of $BK_{Ca}$ in smooth muscle-containing tissues may be complicated by the promotion of neurotransmitter release. Since the electrical changes evoked by ChTX and IbTX were also observed in the presence of atropine (1 $\mu$M) (Figure 7) it is unlikely that such changes involved the activation of muscarinic cholinoceptors. The results of the present study thus provide compelling evidence that, in guinea-pig trachealis, the spontaneous electrical slow waves are normally prevented from developing into regenerative action potentials by the opening

of $BK_{Ca}$ channels. Presumably the depolarizing phase of the slow wave is associated with the opening of L-type $Ca^{2+}$ channels and it is the resultant cellular influx of $Ca^{2+}$ that triggers the $BK_{Ca}$ channels to open at transmembrane potentials close to the resting value.

### *Role of $K^+$-channels in modulating trachealis sensitivity and responsiveness to spasmogens*

In the present study, glibenclamide (10 $\mu$M) did not increase the maximal response of the trachea to carbachol, histamine or KCl and did not potentiate these spasmogens (Table 2). Glibenclamide does not evoke depolarization of guinea-pig trachealis muscle (Murray *et al.*, 1989) suggesting that $K_{ATP}$ channels are not open under resting conditions. Furthermore, the work of Collier *et al.* (1991) has suggested that the opening of $K_{ATP}$ in airways smooth muscle is only weakly voltage-dependent. Accordingly, depolarization evoked by carbachol, histamine or KCl may not be an effective stimulus for promoting the opening of $K_{ATP}$. It is perhaps for this reason that $K_{ATP}$ does not seem to exert a modulating influence on trachealis muscle sensitivity and responsiveness to spasmogens.

When 4-AP was tested in the presence of atropine, it potentiated carbachol, histamine and KCl in causing tracheal contraction (Table 2). IbTX and ChTX also increased tissue sensitivity to the three spasmogens (Table 2). It might be argued that the effects of 4-AP, IbTX and ChTX in increasing tissue sensitivity to spasmogens reflects their ability to promote the release of ACh and other transmitters from intramural neurones. However, the ability of 4-AP and IbTX to increase tissue sensitivity to histamine was retained in a medium containing a combination of atropine (1 $\mu$M) and TTX (3.1 $\mu$M). It therefore seems likely that the effects of 4-AP, IbTX and ChTX in increasing trachealis sensitivity to spasmogens reflects their direct interaction with $K^+$-channels in the airways smooth muscle cell membrane.

As mentioned above, we have previously reported (Boyle *et al.*, 1988) that TEA failed to potentiate either ACh or histamine in contracting indomethacin-treated, guinea-pig trachealis muscle. Since TEA is known to inhibit $BK_{Ca}$, this observation contrasts with the present findings that IbTX and ChTX were each able to potentiate carbachol and histamine. It is not easy to account for this difference. However, TEA exhibits poor selectivity as an inhibitor among $K^+$-channels (Cook, 1988) and, in the experiments of Boyle *et al.* (1988), is likely to have blocked several types of $K^+$-channel simultaneously. Since the experiments of Boyle *et al.* (1988) were carried out in the absence of atropine or TTX, the release of neurotransmitters may have contributed to the effects of the TEA. As observed at present for some of the effects of 4-AP in blocking $K_{dr}$, it is possible that TEA-induced neurotransmitter release could have masked the effects of blockade of $BK_{Ca}$ in the plasmalemma of the muscle cells. In summary the present findings suggest that closure of either the 4-AP-sensitive $K_{dr}$ channel or the $BK_{Ca}$ channel can increase trachealis muscle sensitivity to spasmogenic substances.

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