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Effects of SCA40 on bovine trachealis muscle and on cyclic nucleotide phosphodiesterases

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Abstract

While UK-93,928 (1-[[3-(6,9-dihydro-6-oxo-9-propyl-1H-purin-2-yl)-4-ethoxyphenyl] sulfonyl]-4-methylpiperazine; 5 nM–5 μM) was devoid of relaxant activity, benzafentrine, isoprenaline, levcromakalim and SCA40 (6-bromo-8-methylaminoimidazo[1,2-a]pyrazine-2-carbonitrile) each relaxed histamine (460 μM)-precontracted bovine isolated trachealis. Each of these relaxants was antagonised by a K^+-rich (80 mM) medium. Except in the case of levcromakalim, nifedipine (1 μM) offset this antagonism. Charybdotoxin (100 nM) antagonised isoprenaline in a nifedipine-sensitive manner but did not antagonise SCA40 or benzafentrine. Iberiotoxin (100 nM) did not antagonise SCA40. Acting on tissue precontracted with carbachol, SCA40 potentiated isoprenaline but did not potentiate sodium nitroprusside. While levcromakalim (1 and 10 μM) induced hyperpolarisation, SCA40 (1 and 10 μM) induced little change in the membrane potential of bovine trachealis. In trachealis preloaded with $^{86}Rb^+$, levcromakalim (1 and 10 μM) promoted efflux of the radiotracer while SCA40 (1 and 10 μM) had no effect. Tested as an inhibitor of isoenzymes of cyclic nucleotide phosphodiesterase, SCA40 was most potent against the type III, less potent against the type IV and least potent against the type I isoenzyme. It is concluded that neither inhibition of phosphodiesterase type V nor the promotion of BK_{Ca} channel opening explains the tracheal smooth muscle relaxant activity of SCA40. This compound relaxes bovine tracheal smooth muscle mainly by inhibiting phosphodiesterase isoenzyme types III and IV. © 1997 Elsevier Science B.V.

Keywords: SCA40; Trachealis, bovine; K^+-rich media; Electrophysiology; $^{86}Rb^+$ efflux; Phosphodiesterase isoenzymes

1. Introduction

SCA40 (Bonnet et al., 1992) is a derivative of imidazo[1,2-*a*]pyrazine that potently relaxes guinea-pig isolated trachealis muscle (Laurent et al., 1993a,b; Cook et al., 1995). Since SCA40 is not antagonised by propranolol, suramin or 8-(*p*)-sulphophenyltheophylline (Cook et al., 1995) it is unlikely to act as an agonist at β-adrenoceptors, an agonist at P_1 purinoceptors or an agonist at P_2 purinoceptors. Laurent et al. (1993b) observed that the tracheal relaxant action of SCA40 could be antagonised by a K^+-rich (80 mM) medium and by the K^+-channel inhibitors charybdotoxin and quinine. These workers therefore proposed that SCA40 acted to promote the opening of large conductance, Ca^{2+}-sensitive (BK_{Ca}) K^+-channels in the plasmalemma of the trachealis cells. However, several findings now cast doubt on this idea.

Electrophysiological studies (Murray et al., 1991; Isaac et al., 1996) and measurements of the lanthanum-resistant Ca^{2+} fraction (Foster et al., 1983; Raeburn and Rodger, 1984) suggest that K^+-rich media and inhibitors of BK_{Ca} channels (e.g. charybdotoxin) promote the influx of Ca^{2+} into trachealis cells through L-type channels. Furthermore, antagonism of the tracheal relaxant action of SCA40 provided by K^+-rich media or by inhibitors of BK_{Ca} channels can be offset by nifedipine (Cook et al., 1995). There are, thus, two possible explanations for the ability of K^+-rich media and inhibitors of BK_{Ca} channels to antagonise SCA40. Firstly, the relaxant action of SCA40 could, indeed, crucially depend on the opening of BK_{Ca} channels and the cellular hyperpolarisation thereby evoked. Alternatively, the K^+-rich media and inhibitors of BK_{Ca} channels

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may simply act (by promotion of Ca^{2+} influx) as functional antagonists of SCA40. Electrophysiological findings currently argue against the former of these two possibilities. For example, SCA40 causes hyperpolarisation of guinea-pig trachealis muscle, but only in concentrations greater than those maximally-effective in evoking relaxation (Cook et al., 1995). Applied to inside-out or outside-out plasmalemmal patches prepared from bovine trachealis cells, SCA40 (in contrast to NS 004) failed to promote BK_{Ca} channel opening (MacMillan et al., 1995).

Bonnet et al. (1992) showed that SCA40 was an inhibitor of cyclic nucleotide phosphodiesterase derived from bovine heart. Using guinea-pig trachea as the enzyme source, Cook et al. (1995) subsequently found that SCA40 was a more potent inhibitor of cAMP-phosphodiesterase than of cGMP-phosphodiesterase. In experiments using isoenzymes of phosphodiesterase derived from human tissue, SCA40 was found to be a relatively selective inhibitor of phosphodiesterase isoenzyme type III, being less potent against isoenzyme type IV and less potent still against isoenzyme type V (Cook et al., 1995; Cortijo et al., 1996). Since the IC_{50} values for SCA40 against phosphodiesterase isoenzyme types III and IV lie within the effective concentration range of this agent in relaxing airways smooth muscle (Cook et al., 1995; Cortijo et al., 1996), it is possible that phosphodiesterase inhibition may underlie its relaxant activity.

The present experiments were performed to examine further the mechanisms underlying the bronchorelaxant effects of SCA40. A preliminary account of this work has been communicated to the British Pharmacological Society (Pocock and Small, 1996).

2. Materials and methods

2.1. Tissue preparation

Bovine tracheae were collected from the local abattoir and transported to the laboratory immersed in cold Krebs' solution. Strips (approximately 1.25 cm in length) of the cleaned trachealis muscle were prepared essentially as described by Chiu et al. (1993).

2.2. Tissue bath studies of mechanical activity

Strips of bovine trachealis were set up in Krebs' solution containing indomethacin (2.8 μM). Isometric recording of tension changes was performed using an initial, imposed tension of 2 g. Following the initial setting up of each tissue, three exchanges of bath fluid were performed at 15 min intervals. Following the first two exchanges of bath fluid, the imposed tension was readjusted to 2 g. One hour after the initial setting up of each tissue, histamine (460 μM; EC_{80}) was added to the bath fluid in order to induce tissue tone. When the contractile response to histamine had equilibrated (20 min) study of a relaxant drug commenced.

The actions of benzafentrine, isoprenaline, levcromakalim, SCA40 and UK-93,928 were each studied by the construction of a cumulative concentration–effect curve. Only one such curve was constructed in each tissue. The tissue contact time for each concentration of benzafentrine and SCA40 was 6 min while for isoprenaline, levcromakalim and UK-93,928 it was 4, 8 and 10 min respectively. Following tissue exposure to the highest concentration of each relaxant drug, aminophylline (1 mM) was added to the bath fluid and all relaxant responses were expressed in terms of the maximal effect of aminophylline.

In experiments where charybdotoxin (100 nM) or iberiotoxin (100 nM) was used as an inhibitor of BK_{Ca} channels, the toxin (test tissues) or its vehicle (time-matched control tissues) was added at the same time as histamine. In experiments where the effects of K^+-rich (80 mM) Krebs' solution were investigated, test tissue exposure to the K^+-rich medium commenced 30 min before the addition of histamine. In experiments where the interactions between nifedipine (1 μM) and charybdotoxin or the K^+-rich medium were studied, the calcium influx inhibitor was added (where appropriate) 30 min before the addition of histamine.

Additional experiments were performed to determine whether SCA40 could potentiate isoprenaline or sodium nitroprusside in relaxing bovine trachealis muscle precontracted with carbachol. In these experiments test tissues were pre-incubated for 30 min in Krebs' solution containing SCA40 (1 μM). Carbachol (1 μM) was then added to induce the development of tone. 40 min later a cumulative concentration/relaxation curve for isoprenaline (0.1 nM–100 μM; threefold concentration increments at 4 min intervals) or sodium nitroprusside (10 nM–300 μM; threefold concentration increments at 5 min intervals) was constructed. Time-matched control tissues were treated similarly but were not exposed to SCA40. All experiments with sodium nitroprusside were performed under reduced lighting conditions in order to minimise drug photolysis.

2.3. Intracellular electrophysiological recording from bovine trachealis

Simultaneous recording of intracellular electrical activity and mechanical changes of bovine trachealis was performed using the 'flat sheet' tissue holder and other equipment described by Small and Weston (1980). Following tissue fixation to the holder, the segment from which the mechanical activity was recorded was subjected to an initial, imposed tension of 2 g. The tissue was then left to equilibrate for 15 min. 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. Following this, SCA40 (1 or 10 μM) or levcromakalim (1 or 10 μM) was added to the Krebs' solution superfusing the tissue and changes in the electrical activity of the cell were monitored for 6 or 8 min, respectively. The electrode was then withdrawn from the cell.

2.4. $^{86}Rb^+$ efflux studies

The efflux of $^{86}Rb^+$ from bovine trachealis was studied essentially as described by Longmore et al. (1991). In brief, strips of bovine trachealis were impaled on hypodermic needles and loaded with $^{86}Rb^+$ by incubation for 90 min in Krebs' solution maintained at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2. This loading medium contained 185 kBq/ml $^{86}Rb^+$ and the Rb concentration was less than 50 μM. Following loading with the radiotracer, each tissue strip was transferred to the first of a series of efflux tubes containing 3 ml of Krebs' solution gassed with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C. Each tissue strip was transferred to the next tube in the series initially at 4 min intervals.

40 min after the start of the efflux period, the efflux rate coefficient had assumed a relatively low and slowly-changing value. At this time test tissues were transferred (at 2 min intervals over a 20 min period) through a series of efflux tubes containing levcromakalim (1 or 10 μM) or SCA40 (1 or 10 μM). Time-matched control tissues were treated similarly but were exposed to vehicle instead of relaxant agonist. After the 20 min period of drug or vehicle treatment, tissues were transferred (at 4 min intervals) through a further series of 3 efflux tubes containing medium that was free from relaxant drugs or vehicle. At the end of the efflux period the content of each efflux tube was assayed for radioactivity in a gamma counter. Each tissue strip was blotted and similarly assayed for radioactivity.

2.5. Measurement of inhibitory activity against cyclic nucleotide phosphodiesterase isoenzymes

Isoenzymes of cyclic nucleotide phosphodiesterase were isolated from guinea-pig cardiac ventricles (types I and III) and bovine trachealis (type IV) as described by Elliott et al. (1991). The activity of each isoenzyme was measured essentially by the method of Thompson and Appleman (1971) as modified by Rutten et al. (1973). Assays of enzyme activity were performed in a final volume of 100 μl comprising 25 μl of the isoenzyme solution, 50 μl of assay buffer and 25 μl of twice-distilled water or phosphodiesterase inhibitor (SCA40 or theophylline) solution. The assay buffer (pH 8.0) contained 2 μCi [^{3}H]cAMP and yielded final concentrations of 1 μM cAMP, 40 mM Tris–HCl, 2.5 mM $MgCl_2$ and 3.75 mM β-mercaptoethanol in the reaction mixture. In tests of enzyme inhibition, the reaction mixture contained concentrations of SCA40 or theophylline in the ranges 10 nM–100 μM and 1 μM–1 mM respectively.

The reagents were mixed on ice and the reaction was initiated by transferring the mixture to a water bath at 37°C. Following 30 min incubation, the reaction was stopped by transferring the reaction tubes to a bath of boiling water for 3 min. After cooling on ice, 20 μl of a 1 mg/ml solution of Ophiophagus hannah venom was added to each tube and the mixture was incubated at 37°C for 10 min. Unreacted [^{3}H]cAMP was removed by the addition of 400 μl of a 1 in 3 suspension of Dowex resin (1X8-400) and incubation on ice for 30 min. Each tube was then centrifuged ($10\,000 \times g$) for 2 min and 200 μl of the supernatant was removed for liquid scintillation counting. Less than 10% of the tritiated [^{3}H]cAMP was hydrolysed in any assay.

2.6. 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 (Sigma), benzafentrine (AH 21-132; Sandoz), carbamoylcholine chloride (carbachol, Sigma) charybdotoxin (Latoxan), histamine dihydrochloride (Sigma), iberiotoxin (Affiniti), (−)-isoprenaline hydrochloride (Sigma), levcromakalim (SmithKline Beecham), nifedipine (Bayer), SCA40 (6-bromo-8-methylaminoimidazo[1,2-a]pyrazine-2-carbonitrile; Syntex Pharmaceuticals), sodium nitroprusside (B.D.H.), theophylline

Table 1
Histamine (460 μM)-precontracted bovine trachealis muscle: the effects of K^+-rich Krebs' solution and nifedipine on the pD_2 values of some relaxant drugs. Data are means (± S.E.M.) of values from at least 6 experiments

Relaxant agent	pD_2 values			
	control	K^+-rich (80 mM) Krebs' solution	nifedipine (1 μM)	K^+-rich (80 mM) Krebs' solution + nifedipine (1 μM)
Benzafentrine	5.15 ± 0.07	4.69 ± 0.10 [a]	5.47 ± 0.13 [a]	5.54 ± 0.16 [ab]
Isoprenaline	8.21 ± 0.13	7.47 ± 0.28 [a]	8.36 ± 0.15	8.46 ± 0.12 [b]
Levcromakalim	6.52 ± 0.09	< 5.00	6.12 ± 0.11 [a]	< 5.00
SCA40	6.58 ± 0.10	6.27 ± 0.09 [a]	6.58 ± 0.10	6.88 ± 0.09 [ab]

[a] Significant ($P < 0.05$) difference from the control value.
[b] Significant difference from K^+-rich (80 mM) Krebs' solution.

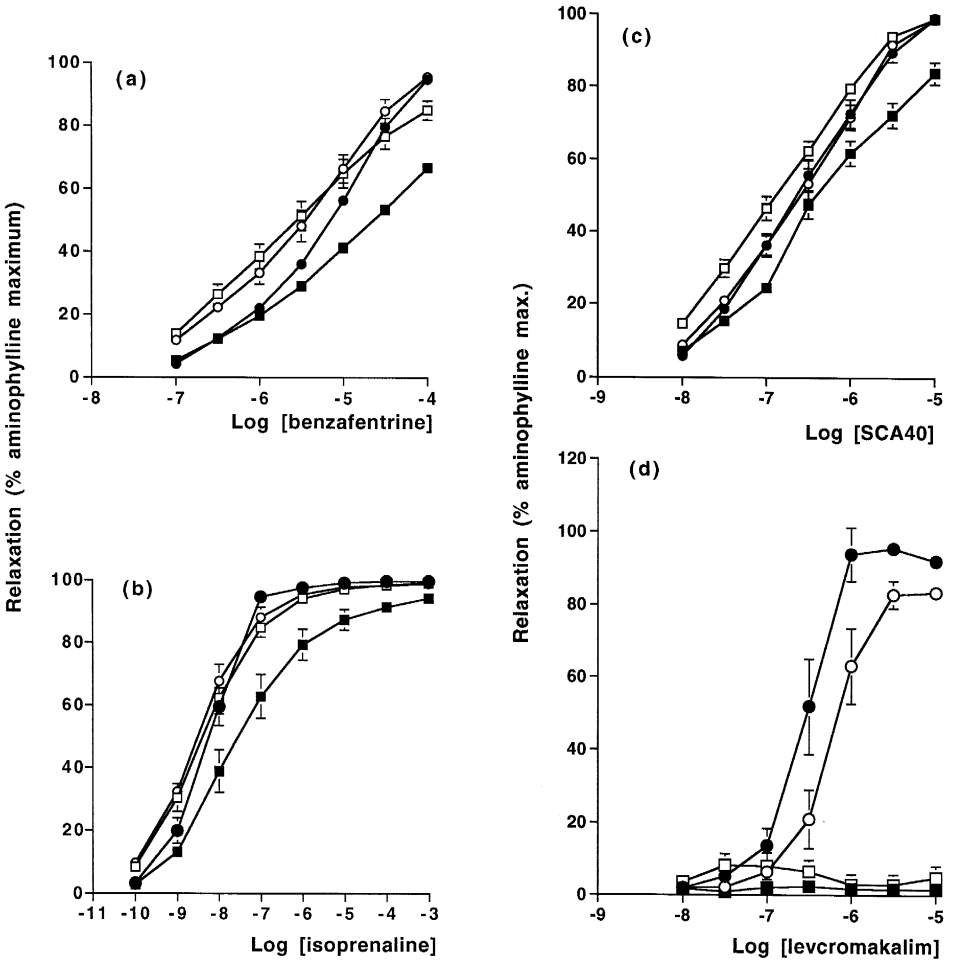

Fig. 1. Histamine (460 μM)-precontracted bovine trachealis muscle: the effects of K^+-rich (80 mM) Krebs' solution and nifedipine (1 μM) alone, and in combination, on the relaxant actions of (a) benzafentrine, (b) isoprenaline, (c) SCA40 and (d) levcromakalim. In each panel (●) indicates the control log concentration–effect curve as observed in normal Krebs' solution, (■) indicates the curve observed in K^+-rich (80 mM) Krebs' solution, (○) the curve observed in the presence of nifedipine (1 μM) and (□) the curve observed in the presence of a combination of K^+-rich (80 mM) Krebs' solution and nifedipine (1 μM). Data points are the means of values from at least 6 tissues. Vertical bars indicate S.E.M.

(Sigma) and UK-93,928 (1-[[3-(6,9-dihydro-6-oxo-9-propyl-1H-purin-2-yl)-4-ethoxyphenyl]sulfonyl]-4-methylpiperazine; Pfizer, a gift from Dr. R.M. Wallis).

Benzafentrine, theophylline and sodium nitroprusside were dissolved in twice-distilled water. Levcromakalim was dissolved in 70% ethanol. All solutions of sodium nitroprusside were protected from light. UK-93,928 was dissolved in dimethylsulphoxide. A stock solution of SCA40 was prepared in 10% ethanol. Dilutions from these stock solutions were prepared using twice-distilled water. Isoprenaline was dissolved in 0.1 M HCl and dilutions were made using twice-distilled water containing 0.57 mM ascorbic acid. Stock solutions of charybdotoxin and iberiotoxin were prepared in normal (0.9%, w/v) saline.

Table 2
Histamine (460 μM)-precontracted bovine trachealis muscle: the effects of charybdotoxin and nifedipine on the pD_2 values of some relaxant drugs. Data are means (±S.E.M.) of values from at least 6 experiments

Relaxant agent	pD_2 values			
	control	ChTx (100 nM)	nifedipine (1 μM)	ChTx (100 nM) + nifedipine (1 μM)
Benzafentrine	4.94 ± 0.15	4.83 ± 0.15	NS	NS
Isoprenaline	8.18 ± 0.19	7.40 ± 0.25 [a]	8.48 ± 0.19	8.42 ± 0.22 [b]
SCA40	6.36 ± 0.27	6.18 ± 0.16	NS	NS

NS indicates an interaction not studied.
[a] Significant ($P < 0.05$) difference from the control value.
[b] Significant difference from charybdotoxin (ChTx; 100 nM).

The Krebs' solution 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. The K^+-rich Krebs' solution was prepared by addition of KCl in order to raise the concentration of K^+ to 80 mM. The concentration of NaCl was reduced to maintain osmolality.

The significance of differences between means was usually assessed by use of a two-tailed, unpaired *t*-test. In the case of the $^{86}Rb^+$ efflux rate coefficient data, one-way analyses of variance were performed at the time point (50 min after the start of efflux) corresponding to 10 min of drug contact with the tissue. Such analyses were followed by Bonferroni multiple comparison tests. The null hypothesis was rejected when $P < 0.05$.

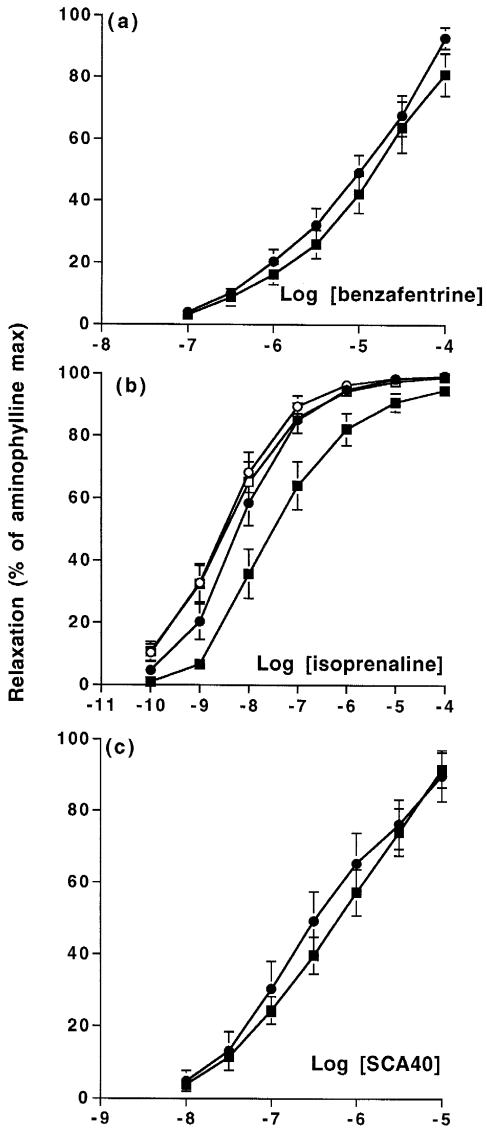

Fig. 2. Histamine (460 μM)-precontracted bovine trachealis muscle: the effects of charybdotoxin on the relaxant actions of (a) benzafentrine, (b) isoprenaline and (c) SCA40. In each panel (●) indicates the control log concentration–effect curve as observed in normal Krebs' solution, while (■) indicates the curve observed in the presence of charybdotoxin (100 nM). In panel (b) (○) indicates the curve observed in the presence of nifedipine (1 μM) and (□) the curve observed in the presence of a combination of charybdotoxin (100 nM) and nifedipine (1 μM). Data points are the means of values from at least 6 tissues. Vertical bars indicate S.E.M.

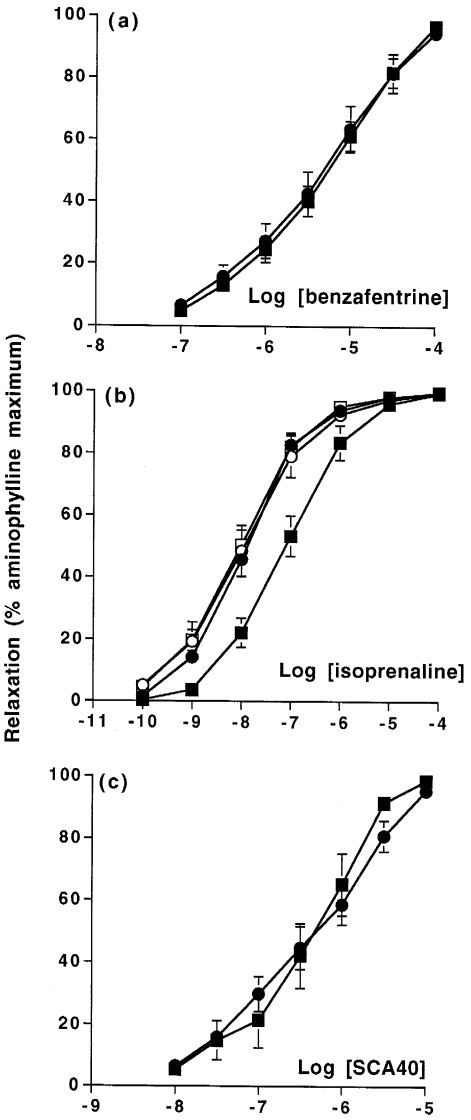

Fig. 3. Histamine (460 μM)-precontracted bovine trachealis muscle: the effects of iberiotoxin on the relaxant actions of (a) benzafentrine, (b) isoprenaline and (c) SCA40. In each panel (●) indicates the control log concentration–effect curve as observed in normal Krebs' solution, while (■) indicates the curve observed in the presence of iberiotoxin (100 nM). In panel (b) (○) indicates the curve observed in the presence of nifedipine (1 μM) and (□) the curve observed in the presence of a combination of iberiotoxin (100 nM) and nifedipine (1 μM). Data points are the means of values from at least 6 tissues. Vertical bars indicate S.E.M.

3. Results

3.1. Tissue bath studies of mechanical activity

Benzafentrine (100 nM–100 μM), isoprenaline (0.1 nM–1 mM), levcromakalim (10 nM–10 μM) and SCA40 (10 nM–10 μM) each caused concentration-dependent

Table 3
Histamine (460 μM)-precontracted bovine trachealis muscle: the effects of iberiotoxin and nifedipine on the pD_2 values of some relaxant drugs. Data are means (±S.E.M.) of values from at least 6 experiments

Relaxant agent	pD_2 values			
	control	IbTx (100 nM)	nifedipine (1 μM)	IbTx (100 nM) + nifedipine (1 μM)
Benzafentrine	5.33 ± 0.17	5.27 ± 0.12	NS	NS
Isoprenaline	7.88 ± 0.13	7.10 ± 0.20 [a]	7.95 ± 0.26	8.03 ± 0.13 [b]
SCA40	6.37 ± 0.18	6.40 ± 0.21	NS	NS

NS indicates an interaction not studied.
[a] Significant ($P < 0.05$) difference from the control value.
[b] Significant difference from iberiotoxin (IbTx; 100 nM).

suppression of the histamine-induced tone of bovine isolated trachea. The maximal relaxant effect of each of these agents exceeded 90% of the maximal relaxant effect of aminophylline (Fig. 1) and their rank order of potency was isoprenaline ($pD_2 = 8.21$) > SCA40 (6.58) ≥ levcromakalim (6.52) > benzafentrine (5.15) (Table 1).

Tissue exposure to K^+-rich (80 mM) Krebs' solution caused tension development that reached a peak after 3–4 min. Thereafter the K^+-induced tension tended to decline. Histamine (460 μM)-induced tension in the presence of the K^+-rich medium (25.7 ± 3.4 g; mean ± S.E.M., $n > 6$) did not significantly differ from that observed in normal Krebs' solution (27.5 ± 3.4 g; mean ± S.E.M.). In the

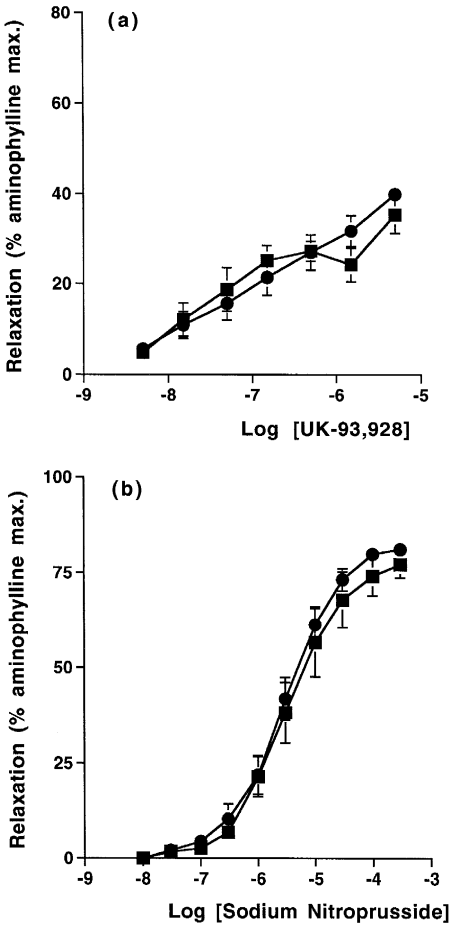

Fig. 4. Histamine (460 μM)-precontracted bovine trachealis muscle: (a) the effects of UK-93,928 (■) and its vehicle (●) on tissue tone. Data points are the means of values from 5 tissues. Vertical bars indicate S.E.M. (b) The effects of SCA40 on the relaxant activity of sodium nitroprusside. Responses to sodium nitroprusside were obtained in the absence (●) or presence (■) of SCA40 (1 μM). Data points are the means of values from at least 8 tissues. Vertical bars indicate S.E.M.

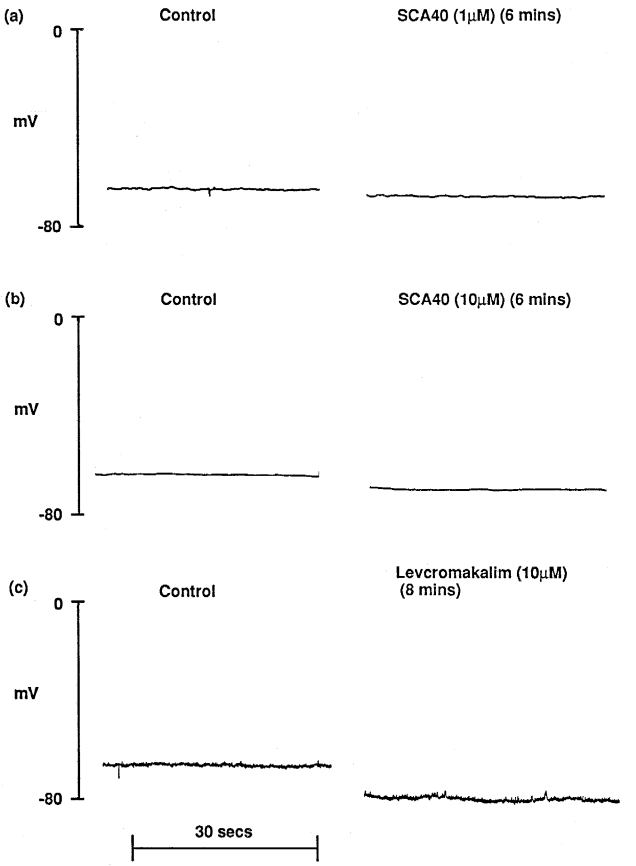

Fig. 5. The effects of levcromakalim (10 μM) and SCA40 (1 and 10 μM) on the membrane potential of bovine isolated trachealis muscle. In each row of traces the two recordings of electrical activity were taken from the same cell. The left hand panel in each case represents membrane potential recorded immediately prior to drug exposure. The right hand panel indicates activity observed after (a) 6 min tissue exposure to 1 μM SCA40, (b) 6 min tissue exposure to 10 μM SCA40 and (c) 8 min tissue exposure to 10 μM levcromakalim.

presence of the K^+-rich medium the log concentration–effect curves of benzafentrine, isoprenaline and SCA40 were shifted rightwards threefold, fivefold and twofold, respectively (Fig. 1 and Table 1). In the presence of the K^+-rich medium the log concentration–effect curve for levcromakalim was very profoundly depressed (Fig. 1 and Table 1).

Nifedipine (1 μM) did not significantly modify the tension evoked by histamine (460 μM) either in tissues bathed by normal Krebs' solution or in those exposed to the K^+-rich Krebs' solution (data not shown). Nifedipine did not alter the relaxant potencies of isoprenaline or SCA40 but caused minor (twofold) potentiation of benzafentrine and minor (approximately twofold) antagonism of levcromakalim (Table 1). The Ca^{2+} influx inhibitor prevented the antagonism of benzafentrine, isoprenaline and SCA40 induced by the K^+-rich medium but not the equivalent antagonism of levcromakalim (Fig. 1 and Table 1).

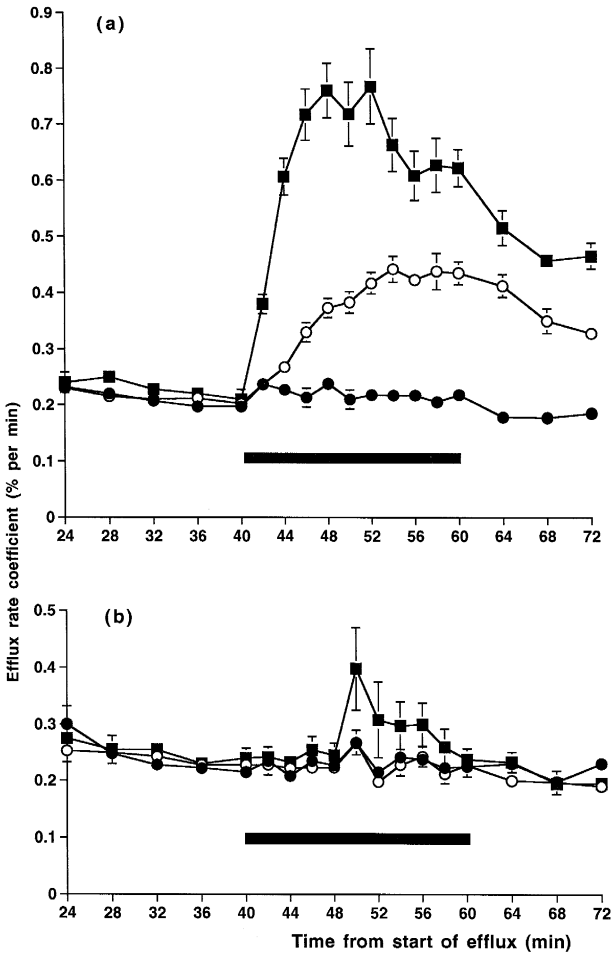

Fig. 6. The effects of (a) levcromakalim and (b) SCA40 on the efflux of $^{86}Rb^+$ from strips of bovine trachealis. The abscissa indicates the time (min) from the start of the efflux period. The ordinate indicates the efflux rate coefficient for $^{86}Rb^+$. The filled horizontal bar indicates the period of time over which the tissues were exposed to vehicle, levcromakalim or SCA40. In both panels (●) = vehicle-treated, time-matched, control tissues. In panel (a) (○) and (■) = test tissues exposed to levcromakalim (1 and 10 μM, respectively). In panel (b) (○) and (■) = test tissues exposed to SCA40 (1 and 10 μM, respectively). Data indicate means of values from at least 6 tissues. Vertical bars indicate S.E.M.

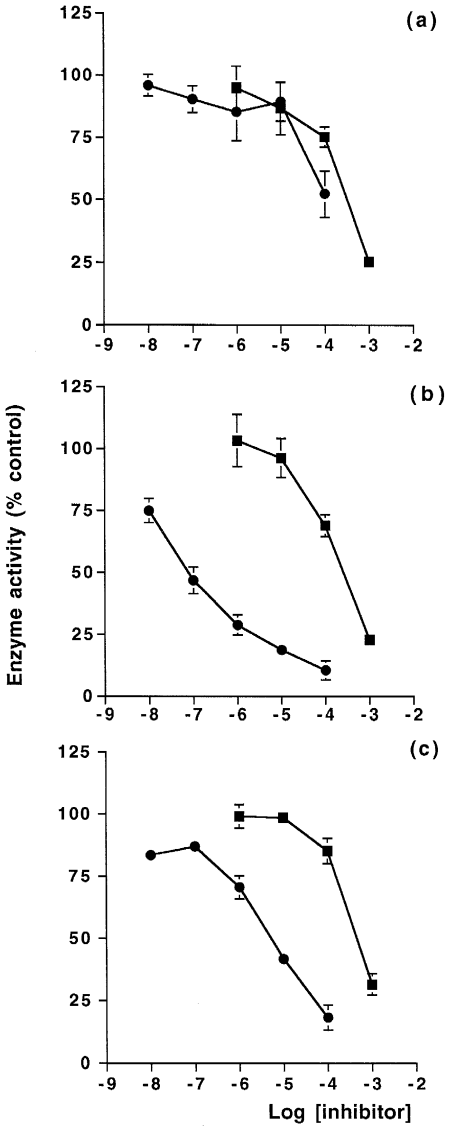

Fig. 7. The effects of SCA40 and theophylline on the activity of cyclic nucleotide phosphodiesterase isoenzyme types I, III and IV. In each case the abscissa indicates $\log_{10}$ molar concentration of inhibitor while the ordinate indicates enzyme activity as a percentage of control. Panels (a), (b) and (c) represent the activity of phosphodiesterase isoenzyme types I, III and IV respectively. (●) = SCA40. (■) = theophylline. Data points indicate means of values from at least 4 experiments. Vertical bars indicate S.E.M.

Charybdotoxin (100 nM) did not significantly modify the tension evoked by histamine (460 μM) (data not shown) nor did it significantly modify the relaxant actions of benzafentrine or SCA40 (Fig. 2 and Table 2). In contrast, charybdotoxin (100 nM) caused a sixfold rightward shift in the log concentration–effect curve of isoprenaline. This antagonism of isoprenaline by charybdotoxin was, however, prevented by nifedipine (1 μM) (Fig. 2 and Table 2).

Iberiotoxin (100 nM) did not significantly modify the tension evoked in strips of bovine trachealis by histamine (460 μM). Iberiotoxin did not modify the relaxant action of benzafentrine or SCA40 (Fig. 3 and Table 3) but it caused sixfold antagonism of isoprenaline. The antagonism of isoprenaline induced by iberiotoxin was prevented by nifedipine (1 μM) (Fig. 3 and Table 3).

Preincubation of bovine trachealis with SCA40 (1 μM) did not significantly modify the tension induced by 1 μM carbachol (control tissues 20.2 ± 0.9 g; test tissues 19.8 ± 1.3 g; mean ± S.E.M., $n = 9$; P = 0.79). Isoprenaline (0.1 nM–100 μM) caused concentration-dependent suppression of carbachol-induced tone with a maximal effect in excess of 90%. In the SCA40-treated test tissues the log concentration–relaxation curve for isoprenaline lay to the left of that observed in the time-matched control tissues. The $-\log EC_{50}$ for isoprenaline in the test tissues (7.08 ± 0.19) was significantly ($P = 0.03$) greater than that observed in the control tissues (6.54 ± 0.13; mean ± S.E.M., $n = 9$), suggesting that SCA40 had potentiated the agonist at β-adrenoceptors.

The vehicle for UK-93,928 caused concentration-dependent relaxation of the histamine-treated trachea. Since virtually identical relaxation was observed in tissues treated with UK-93,928 (5 nM–5 μM) (Fig. 4a) we can conclude that this compound was virtually devoid of relaxant activity. In carbachol-treated trachea, sodium nitroprusside (10 nM–300 μM) caused concentration-dependent relaxation and its maximal effect was more than 70% of that of aminophylline. Pretreatment of the tissue with SCA40 (1 μM) did not alter the shape of the log concentration–effect curve of sodium nitroprusside (Fig. 4b) and did not alter its position on the log concentration axis ($-\log EC_{50}$ for control tissues 5.64 ± 0.13; test tissues 5.44 ± 0.13; $P > 0.05$)

3.2. Intracellular electrophysiological recording

Impalement of bovine trachealis with microelectrodes revealed that the muscle cells were devoid of spontaneous electrical activity but had a resting membrane potential in the range −58 to −75 mV. Addition of levcromakalim (1 and 10 μM) to the superfusate induced hyperpolarisation (14.6 ± 0.9 and 13.3 ± 1.5 mV, respectively; $n \geq 7$) that required 6–8 min to equilibrate (Fig. 5). In contrast, the addition of SCA40 (1 and 10 μM) to the superfusate caused very minor membrane potential change (3.0 ± 0.6 mV and 2.3 ± 0.7 mV hyperpolarisation, respectively; $n = 6$).

3.3. $^{86}Rb^+$ efflux studies

By 40 min after the start of the efflux period, the efflux rate coefficient had assumed a relatively low and slowly-changing value. Addition of the vehicle for levcromakalim or SCA40 at this time failed to evoke any change in the efflux rate coefficient. In contrast, levcromakalim (1 and 10 μM) induced a concentration-dependent increase in the efflux rate coefficient. At time 50 min, for example, the efflux rate coefficient for tissues treated with levcromakalim (10 μM) was significantly greater than that for tissues treated with levcromakalim (1 μM) which, in turn, was significantly greater than that observed in the vehicle-treated control tissues (Fig. 6). In contrast and measured at the same time point, neither the efflux rate coefficient for tissues treated with SCA40 (10 μM) nor that for tissues treated with SCA40 (1 μM) differed from that seen in the vehicle-treated control tissues (Fig. 6).

Table 4
The inhibition of isoenzymes of cyclic nucleotide phosphodiesterase by SCA40 and theophylline. Data are means (± S.E.M.) of values from four experiments

Phosphodiesterase isoenzyme	$-\log_{10} IC_{50}$	
	SCA40	Theophylline
Type I	< 4.00	3.50 ± 0.07
Type III	7.16 ± 0.23	3.59 ± 0.10
Type IV	5.39 ± 0.22	3.34 ± 0.08

3.4. Measurement of inhibitory activity against cyclic nucleotide phosphodiesterase isoenzymes

SCA40 and theophylline each inhibited isoenzyme types I, III and IV of cyclic nucleotide phosphodiesterase in a concentration-dependent manner (Fig. 7). SCA40 was most potent in inhibiting the type III isoenzyme, less potent against the type IV isoenzyme and least potent against isoenzyme type I. As an inhibitor, theophylline exhibited less selectivity among the three isoenzymes and, in each case, was less potent than SCA40 (Fig. 7 and Table 4).

4. Discussion

4.1. Role of BK_{Ca} channel opening in the bronchorelaxant action of SCA40

When the K^+ concentration in a physiological salt solution is increased above 40 mM, the K^+ equilibrium potential for smooth muscle cells immersed in such a medium closely approaches their membrane potential. In this circumstance the opening of plasmalemmal K^+-channels cannot evoke sufficient hyperpolarisation to ensure the closure of voltage-sensitive Ca^{2+} channels (Small et al., 1992). However, antagonism of a smooth muscle relaxant drug by a K^+-rich medium does not necessarily imply that the action of that drug is crucially dependent on K^+-channel opening. The K^+-rich medium itself promotes Ca^{2+} influx and may therefore functionally antagonise a relaxant agent of whatever mechanism of action (Huang et al., 1993; Small et al., 1993). The present observations (Fig. 1 and Table 1) that a K^+-rich medium decreases the

relaxant effects of benzafentrine, isoprenaline, levcromakalim and SCA40 acting on bovine trachealis are consistent with the results of earlier studies of guinea-pig trachea (Small et al., 1989; Laurent et al., 1993b; Cook et al., 1995). In the cases of benzafentrine, isoprenaline and SCA40 such antagonism was prevented by nifedipine. In contrast, nifedipine did not prevent the equivalent antagonism of levcromakalim (Cook et al., 1995; present study). It may therefore be suggested that the interaction between the K^+-rich medium and benzafentrine, isoprenaline or SCA40 represents functional antagonism due to the medium-induced promotion of Ca^{2+} influx. On the other hand the equivalent interaction with levcromakalim may be an index of the profound dependency of levcromakalim's action on K^+-channel opening.

Charybdotoxin and iberiotoxin, agents derived from scorpion venom, are currently employed as pharmacological tools for inhibiting BK_{Ca} channels. In neurones and skeletal muscle cells charybdotoxin has been reported to inhibit K^+-channels other than BK_{Ca} (Galvez et al., 1990; Suarez-Kurtz et al., 1991). However, in the context of smooth muscle, including that of the airways, charybdotoxin is a relatively selective inhibitor of BK_{Ca} (Cook and Quast, 1990; Boyle et al., 1992). In comparison with charybdotoxin, iberiotoxin exhibits greater selectivity for BK_{Ca} (Galvez et al., 1990; Suarez-Kurtz et al., 1991). In guinea-pig trachealis both these agents are spasmogenic. The spasm evoked by the toxins is associated with the conversion of spontaneous electrical slow waves into regenerative action potentials (Murray et al., 1991; Isaac et al., 1996) and can be abolished by nifedipine (Jones et al., 1988; Isaac et al., 1996). This strongly suggests that charybdotoxin and iberiotoxin each promotes the cellular influx of Ca^{2+} through L-type channels.

Laurent et al. (1993a,b) observed that charybdotoxin and iberiotoxin each antagonised SCA40 acting on guinea-pig trachealis and concluded that the relaxant action of SCA40 in this tissue depended on the opening of plasmalemmal BK_{Ca} channels. Cook et al. (1995) subsequently confirmed this antagonism of SCA40 by charybdotoxin, but showed that it could be prevented by nifedipine. This suggests that charybdotoxin-induced antagonism of SCA40 may reflect charybdotoxin-induced promotion of Ca^{2+} influx rather than a specific interaction between SCA40 and charybdotoxin at the level of BK_{Ca} channel gating. The present failure of either charybdotoxin or iberiotoxin to antagonise SCA40 acting on histamine-precontracted bovine trachealis (Figs. 2 and 3 and Tables 2 and 3) is difficult to explain. However, in this tissue and under the experimental conditions employed, it may be that charybdotoxin and iberiotoxin were unable to induce an increment in Ca^{2+} influx sufficiently large to functionally antagonise SCA40. In any event, the failure of the two toxins to antagonise SCA40 provides further support for the notion that the relaxant action of SCA40 does not depend on the opening of plasmalemmal BK_{Ca} channels.

$^{86}Rb^+$ is often used as a marker (albeit imperfect) for the movement of K^+ ions across cell membranes. The present observation (Fig. 6) that levcromakalim (the active enantiomer of cromakalim) promoted the efflux of $^{86}Rb^+$ from bovine trachealis preloaded with the radiotracer is consistent with the results of earlier studies (Longmore et al., 1991; Chiu et al., 1993) of the racemate, cromakalim. Similarly, the present finding (Fig. 5) that levcromakalim caused marked hyperpolarisation of bovine trachealis cells is consistent with earlier observations (Longmore et al., 1991) for the racemate. The ability of cromakalim and its active enantiomer to promote $^{86}Rb^+$ efflux and to hyperpolarise trachealis cells forms part of the evidence that this benzopyran derivative acts to promote the opening of plasmalemmal K^+-channels. In view of this, the present failure of SCA40 both to promote $^{86}Rb^+$ efflux (Fig. 6) and to cause substantial cellular hyperpolarisation (Fig. 5) argue that K^+-channel opening is not a prominent feature of its action in relaxing trachealis muscle. This argument receives support both from membrane potential recordings in guinea-pig trachealis (Cook et al., 1995) and from patch clamp studies of bovine tissue (MacMillan et al., 1995). In the latter studies SCA40 failed to promote BK_{Ca} channel opening when applied either to inside-out or to outside-out plasmalemmal patches.

In summary, the present study of bovine trachealis has employed K^+-rich media, selective inhibitors of BK_{Ca} channels, measurements of $^{86}Rb^+$ efflux and measurements of membrane potential to assess the importance of K^+-channel opening in mediating the relaxant action of SCA40. None of these tools has suggested a crucial role for BK_{Ca} channel opening in the bronchorelaxant action of this imidazo[1,2-*a*]pyrazine derivative.

4.2. Role of inhibition of cyclic nucleotide phosphodiesterase in the tracheal relaxant action of SCA40

The ability of SCA40 to inhibit cyclic nucleotide phosphodiesterase was first reported by Bonnet et al. (1992). Using enzyme derived from guinea-pig trachea, Cook et al. (1995) later showed that SCA40 was a more potent inhibitor of cAMP phosphodiesterase than of cGMP phosphodiesterase. This suggested that SCA40 might exhibit some selectivity as an inhibitor among the various isoenzymes of phosphodiesterase and studies of isoenzymes derived from human tissue (Cook et al., 1995) showed that their susceptibility to inhibition by SCA40 was in the rank order: type III > type IV > type V > types I and II. The present studies of phosphodiesterase isoenzymes derived from guinea-pig cardiac ventricle and bovine trachealis (Fig. 7 and Table 4) have confirmed that SCA40 is a relatively selective inhibitor with greatest potency against isoenzyme type III and next greatest potency against isoenzyme type IV.

The present experiments with UK-93,928 and sodium nitroprusside were carried out in order to determine whether

inhibition of the type V isoenzyme of phosphodiesterase plays an important role in the relaxant activity of SCA40. UK-93,928 is a highly selective inhibitor of the type V isoenzyme of phosphodiesterase with an IC_{50} value of 6.4 nM (R.M. Wallis, personal communication). The failure of UK-93,928 (5 nM–5 μM) to relax histamine-treated bovine trachea (Fig. 4a) suggests that the type V isoenzyme of phosphodiesterase is unlikely to play a major role in moderating the mechanical activity of the tissue and hence in mediating the relaxant action of SCA40. The type V isoenzyme of phosphodiesterase is relatively selective in hydrolysing cGMP rather than cAMP. Furthermore, sodium nitroprusside is believed to relax smooth muscle by increasing the cellular content of cGMP. The present failure of SCA40 to potentiate sodium nitroprusside (Fig. 4b) thus not only confirms observations made in guinea-pig main bronchus (Naline et al., 1996) but also reinforces the notion that inhibition of the type V isoenzyme does not form an important part of the relaxant action of SCA40.

The soluble fraction of homogenates of bovine trachealis has been shown to contain phosphodiesterase isoenzyme types I, II, IV and V but relatively little of the type III isoenzyme (Elliott et al., 1991; Giembycz and Barnes, 1991; Shahid et al., 1991). Furthermore, compared with inhibitors of the type IV isoenzyme, inhibitors of phosphodiesterase type III are relatively ineffective relaxants of bovine trachealis (Shahid et al., 1991). On the basis of such observations it has been suggested that, in bovine trachealis, phosphodiesterase type IV is important but phosphodiesterase type III plays a much smaller role in modulating mechanical activity (Shahid et al., 1991; Giembycz and Barnes, 1991).

In the present study the effective concentration ranges of SCA40 in inhibiting phosphodiesterase type IV or phosphodiesterase type III each exhibited overlap with its effective concentration range in suppressing histamine-induced tension development (Figs. 1 and 7). It is therefore possible that the ability of SCA40 to relax bovine trachealis stems from its ability to inhibit isoenzymes of cyclic nucleotide phosphodiesterase. However, it is difficult to predict the relative importance of isoenzymes III and IV in this respect for, while SCA40 is more potent in inhibiting the type III isoenzyme, the type IV isoenzyme is present at much greater activity and seems the functionally more important.

Benzafentrine (AH 21-132) is a relatively selective inhibitor among the isoenzymes of cyclic nucleotide phosphodiesterase, inhibiting isoenzyme types III and IV with significantly greater potencies than those shown against isoenzyme types I, II and V (Elliott et al., 1991; Giembycz and Barnes, 1991). The pharmacological profile of SCA40 in several respects resembles that of benzafentrine. In the present study of histamine-precontracted bovine trachealis, benzafentrine and SCA40 each evoked relaxation and neither was antagonised by charybdotoxin (Fig. 2) or by iberiotoxin (Fig. 3). Both phosphodiesterase inhibitors were antagonised by a K^+-rich medium but, in each case, the antagonism was offset by nifedipine (Fig. 1 and Table 1). Benzafentrine and SCA40 both cause minor hyperpolarisation of trachealis muscle (Small et al., 1991; Cook et al., 1995; present study) but this membrane potential change is only observed at concentrations close to, or above, the concentration that is maximally effective in evoking relaxation. Benzafentrine and SCA40 are both able to potentiate isoprenaline in relaxing trachealis muscle (Small et al., 1991; present study). Benzafentrine and SCA40 both exert positive chronotropic and inotropic effects on guinea-pig isolated atria (Berry et al., 1989; Cook et al., 1995). Similarities of this kind are consistent with the idea that, like benzafentrine, SCA40 may relax trachealis muscle by inhibiting cyclic nucleotide phosphodiesterase. However, additional experimentation (e.g. concurrent measurements of tissue relaxation and cAMP content) is warranted in order to provide a more critical test of this hypothesis.

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