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Further analysis of the mechanisms underlying the tracheal relaxant action of SCA40

S.J. Cook, K. Archer, A. Martin, *K.H. Buchheit, *J.R. Fozard, *T. Müller, A.J. Miller, K.R.F. Elliott, R.W. Foster & [1]R.C. Small

School of Biological Sciences, University of Manchester, Oxford Road, Manchester M13 9PT, U.K. and *Preclinical Research Department Sandoz Pharma Ltd., CH-4002 Basle, Switzerland

1 SCA40 (1nM–10 μM), isoprenaline (1–300 nM) and levcromakalim (100 nM–10 μM) each produced concentration-dependent suppression of the spontaneous tone of guinea-pig isolated trachea. Propranolol (1 μM) markedly (approximately 150 fold) antagonized isoprenaline but did not antagonize SCA40. The tracheal relaxant action of SCA40 was unaffected by suramin (100 μM) or 8-(*p*)-sulphophenyltheophylline (8-SPT; 140 μM).

2 An isosmolar, K^+-rich (80 mM) Krebs solution increased tracheal tone, antagonized SCA40 (approximately 60 fold), antagonized isoprenaline (approximately 20 fold) and very profoundly depressed the log concentration-effect curve for levcromakalim. Nifedipine (1 μM) did not itself modify the relaxant actions of SCA40, isoprenaline or levcromakalim. However, nifedipine prevented the rise in tissue tone and the antagonism of SCA40 and isoprenaline induced by the K^+-rich medium. In contrast, nifedipine did not prevent the equivalent antagonism of levcromakalim.

3 Charybdotoxin (100 nM) increased tracheal tone, antagonized SCA40 (approximately 4 fold) and antagonized isoprenaline (approximately 3 fold). Nifedipine (1 μM) prevented the rise in tissue tone and the antagonism of SCA40 and isoprenaline induced by charybdotoxin.

4 Quinine (30 μM) caused little or no change in tissue tone and did not modify the relaxant action of isoprenaline. However, quinine antagonized SCA40 (approximately 2 fold). Nifedipine (1 μM) prevented the antagonism of SCA40 induced by quinine.

5 Tested on spontaneously-beating guinea-pig isolated atria SCA40 (1 nM–10 μM) increased the rate of beating in a concentration-dependent manner. Over the concentration-range 1 μM–10 μM, SCA40 also caused an increase in the force of atrial contraction.

6 Intracellular electrophysiological recording from guinea-pig isolated trachealis showed that the relaxant effects of SCA40 (1 μM) were often accompanied by the suppression of spontaneous electrical slow waves but no change in resting membrane potential. When the concentration of SCA40 was raised to 10 μM, its relaxant activity was accompanied both by slow wave suppression and by plasmalemmal hyperpolarization.

7 SCA40 (10 nM–100 μM) more potently inhibited the activity of cyclic AMP phosphodiesterase (PDE) than that of cyclic GMP PDE derived from homogenates of guinea-pig trachealis. Theophylline (1 μM–10 mM) also inhibited these enzymes but was less potent than SCA40 in each case and did not exhibit selectivity for inhibition of cyclic AMP hydrolysis.

8 Tested against the activity of the isoenzymes of cyclic nucleotide PDE derived from human blood cells and lung tissue, SCA40 proved highly potent against the type III isoenzyme. It was markedly less potent against the type IV and type V isoenzymes and even less potent against the isoenzymes types I and II.

9 It is concluded that the tracheal relaxant action of SCA40 (1 nM–1 μM) does not involve the activation of β-adrenoceptors or P_1 or P_2 purinoceptors. Furthermore, this action is unlikely to depend upon the opening of BK_{Ca} channels with consequent cellular hyperpolarization and voltage-dependent inhibition of Ca^{2+} influx. The tracheal relaxant action of SCA40 (up to 1 μM) is more likely to depend upon its selective inhibition of the type III isoenzyme of cyclic nucleotide PDE. At concentrations above 1 μM, SCA40 exerts more general inhibition of the isoenzymes of cyclic nucleotide PDE and may then promote the opening of BK_{Ca} channels.

Keywords: Trachealis muscle; cardiac atrial muscle; SCA40; isoprenaline; levcromakalim; K^+-rich Krebs solution; charybdotoxin; quinine; nifedipine; cyclic nucleotide phosphodiesterase

Introduction

The synthesis and antibronchospastic properties of SCA40 (6-bromo-8-methylaminoimidazo[1,2-a]pyrazine-2-carbonitrile) were first described by Bonnet *et al.* (1992). Subsequently, the same group of authors reported studies designed to analyse the mechanism by which SCA40 caused relaxation of guinea-pig isolated trachealis muscle (Laurent *et al.*, 1993). On the basis that SCA40 could be antagonized by a K^+-rich (80 mM) medium and by the K^+-channel inhibitors, charybdotoxin and quinine, Laurent *et al.* (1993) concluded that the relaxant activity of this agent resided mainly in its ability to promote the opening of high conductance, Ca^{2+}-dependent K^+-channels (BK_{Ca}) in the plasmalemma of the smooth muscle cells. That BK_{Ca} channel opening might explain the activity of SCA40 (<1 μM) received further support from the observation by the same group of workers (Michel *et al.*, 1993) that SCA40 (1 nM–1 μM) exerts negative chronotropic and inotropic effects in rat isolated atria.

As yet the ability of SCA40 (<1 μM) to promote the opening of BK_{Ca} in airways smooth muscle cells awaits direct demonstration by the technique of patch clamp recording.

[1] Author for correspondence.

Furthermore, the work of Huang *et al.* (1993) has recently called into question the specificity of K^+-rich media and K^+-channel inhibitors as tools for identifying relaxants of airways smooth muscle that act by promoting the opening of plasmalemmal K^+-channels. Huang *et al.* (1993) demonstrated that the relaxant effects of salbutamol acting on guinea-pig isolated trachealis muscle were antagonized by either charybdotoxin or iberiotoxin. However, such antagonism could be offset by nifedipine. This strongly suggests that the antagonism of salbutamol provided by either of the toxins reflects functional antagonism (via toxin-induced promotion of the cellular influx of Ca^{2+}) rather than a toxin-salbutamol interaction at the level of the control of BK_{Ca} channel gating mechanisms. Similar considerations apply to the antagonism of salbutamol caused by a K^+-rich medium (Huang *et al.*, 1993).

The present experiments were therefore performed to shed more light on the mechanisms underlying the tracheal relaxant action of SCA40. A preliminary account of this work has been communicated to the British Pharmacological Society (Cook *et al.*, 1994).

Methods

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

Guinea-pigs (300–500 g) of either sex were killed by stunning and bleeding. The hearts were quickly excised from the animals and transferred to a dish of cold Krebs solution where the pericardium was removed. The bulk of the ventricular tissue was then removed from each heart but care was taken not to destroy conduction of excitation between the right and left atria. Tracheae were also 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 4–5 cartilage rings) of guinea-pig 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 tissue tone. The aminophylline was removed from the tissues (initial wash followed by two further washes at 10 min intervals) and when spontaneous tone subsequently became maximal (40 min after initial wash), study of bronchodilator drugs commenced.

Cumulative log concentration-effect curves were constructed for all relaxant drugs studied. Generally 3.16 fold concentration increments were used. Each concentration of ATP, 5'-N-ethylcarboxamidoadenosine (NECA), isoprenaline, theophylline, SCA40 and levcromakalim was allowed 3, 3, 4, 5, 6 and 8 min tissue contact, respectively. All experiments with ATP were performed in Krebs solution containing S-(4-nitrobenzyl)-6-thioinosine (NBTI; 300 nM). The effects of the various relaxants were expressed in terms of the maximal relaxation induced by aminophylline.

Following construction of initial log concentration-effect curves for the bronchodilator drugs, test tissues were exposed to one of the following modifying agents: charybdotoxin (100 nM), nifedipine (1 μM), propranolol (1 μM), quinine (30 μM), 8-SPT (140 μM), suramin (100 μM) or isosmolar, K^+-rich (80 mM) Krebs solution (see below), and combinations of nifedipine (1 μM) with charybdotoxin (100 nM), with quinine (30 μM) or with the isosmolar K^+-rich (80 mM) medium. Charybdotoxin was allowed to equilibrate with the tissue for 20 min before proceeding with the experiment. All other modifying agents were allowed to equilibrate with the tissue for at least 40 min. Following equilibration of the modifying agent with the test tissues, log concentration-effect curves for the bronchodilator drugs were reconstructed in the presence of the modifying agent. In all experiments time-matched control tissues were treated identically to test tissues but were not exposed to the modifying agents.

Tissue bath studies with isolated atria

Isolated, spontaneously-beating guinea-pig paired atria were set up in Krebs solution (37.5°C) under an initial resting tension of 1 g. Atrial tension changes were recorded with a force displacement transducer coupled to a Grass 7P1 preamplifier mounted in a Grass model 79D polygraph. The tension change signal was used to trigger a Grass model 7P44 tachograph so that recordings of atrial tension and rate of beating were made simultaneously. Once atrial rate had stabilized, test tissues were treated with SCA40 (1 nM–10 μM). The drug was administered cumulatively, ten-fold concentration increments being made at 15 min intervals. Time-matched control tissues were treated similarly to test tissues, but were exposed to vehicle in place of the SCA40.

Intracellular electrophysiological recording from trachealis

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

After impalement of a trachealis cell, several minutes were allowed to elapse to check the stability of the record of electrical activity. SCA40 (1 or 10 μM) was then added to the Krebs solution, superfusing the tissue and the electrical activity of the impaled cell was monitored for 6 min or until the microelectrode became dislodged.

Assessment of the ability of SCA40 and theophylline to inhibit the cyclic nucleotide PDE activity of guinea-pig trachea

Muscle-rich strips of guinea-pig trachea were prepared as previously described (Foster *et al.*, 1983). Tracheal strips from two animals were combined and homogenized with an Ilado laboratory disperser in 20 volumes of cold medium (pH 7.5) containing 100 mM Tris-HCl, 2 mM $MgCl_2$ and 1 mM dithiothreitol. The homogenate was used within 30 min of preparation. Adenosine 3':5'-cyclic monophosphate (cyclic AMP)- and guanosine 3':5'-cyclic monophosphate (cyclic GMP)-PDE activity in the homogenate was measured by a modification of the method of Thomson *et al.*, (1979). The homogenate was diluted 1 in 5 and 25 μl was assayed for enzyme activity in a final volume of 100 μl containing 40 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 3.75 mM mercaptoethanol, 0.1 unit calmodulin (PDE activator), 10 μM $CaCl_2$ and either 1 μM cyclic AMP with 0.2 μCi[^{3}H]-cyclic AMP or 1 μM cyclic GMP with 0.2 μCi[^{3}H]-cyclic GMP. In tests of enzyme inhibition, the reaction mixture contained various concentrations of theophylline (1 μM–10 mM) or SCA40 (10 nM–100 μM).

The reagents and homogenate 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 vessel to a bath of boiling water for 3 min. After cooling on ice, 20 μl of a 1 mg ml^{-1} solution of *Ophiophagus hannah* venom was added to the reaction mixture and the mixture was incubated at 37°C for 10 min. Unreacted [^{3}H]-cyclic AMP or [^{3}H]-cyclic GMP was removed by the addition of 400 μl of a 1 in 3

suspension of Dowex resin (1 × 8-400) and incubation on ice for 30 min. Each tube was then centrifuged for 2 min and 200 μl of the supernatant was removed for liquid scintillation counting. Less than 10% of the tritiated cyclic nucleotide was hydrolysed in any assay.

Assessment of the inhibitory activity of SCA40 against the isoenzymes of cyclic nucleotide PDE isolated from human lung and blood cells

Platelets and neutrophils were obtained from human blood and were used as the sources of cyclic nucleotide PDE isoenzyme types II, III and IV. Human lung was obtained from patients undergoing surgery and was used as the source of isoenzyme types I and V. The isoenzymes were identified according to the classification of Beavo & Reifsnyder (1990). Cells and tissues were suspended in a medium containing sucrose (250 mM), EDTA (1 mM), dithiothreitol (1 mM), leupeptin (1 mg ml^{-1}), pepstatin A (1 mg ml^{-1}) and Tris hydrochloride (19 mM), adjusted to pH 7.4. Phenylmethylsulphonylfluoride (PMSF; 0.17 mg ml^{-1}) was added to this medium immediately prior to sonication/homogenization of the cells or tissue. Suspensions of platelets and neutrophils were sonicated (4 × 15 s) using a Branson probe. Human lung tissue was homogenized (2 bursts of 30 s) using a Polytron homogenizer. Preparations of PDE isoenzyme types III and IV were derived from low speed supernates of the platelet and neutrophil sonicates, respectively. These preparations selectively hydrolysed cyclic AMP (1 μM) with respect to cyclic GMP (1 μM). The enzyme activity in the supernate from platelets was inhibited by cyclic GMP (10 μM) while that from neutrophils was inhibited by rolipram (10 μM). Other PDE isoenzymes were separated from homogenates of human lung (types I and V) or platelets (type II) by anion exchange chromatography (Q-Sepharose) in a medium similar to that used for tissue homogenization but without sucrose and PMSF. A gradient of NaCl (0 to 0.1 M in 2.5 column volumes) was used to provoke elution of the isoenzymes. PDE I was found in the fraction eluting at 0.17–0.18 M NaCl and its hydrolysis of cyclic AMP (1 μM) could be stimulated by a combination of Ca^{2+} (0.5 mM) and calmodulin (125 nM). PDE II was identified in the fraction eluting at 0.31–0.32 M NaCl. Its hydrolytic activity against cyclic AMP (100 μM) was 15–25 fold higher than that observed against cyclic AMP (1 μM), suggesting an apparent K_m value closer to 100 μM than 1 μM. The hydrolytic activity of this enzyme fraction against cyclic AMP (1 μM) was also stimulated (approximately 2 fold) by cyclic GMP (10 μM). PDE V was identified in the fraction eluting at 0.20–0.24 M NaCl and showed selectivity in hydrolysing cyclic GMP (1 μM) as opposed to cyclic AMP (1 μM).

The activity of the isoenzymes of cyclic nucleotide PDE was assayed essentially according to the batch method of Thompson *et al.*, (1979) using a substrate of cyclic AMP (1 μM) for isoenzyme types I, III and IV. In the case of isoenzyme type I, the assay medium also contained Ca^{2+} (0.5 mM) and calmodulin (125 nM). The substrate for isoenzyme type II was cyclic AMP (100 μM) and that for type V was cyclic GMP (1 μM). In each case the assay medium contained 1.25 mg ml^{-1} bovine serum albumin, 50 μl ml^{-1} dimethylsuphoxide and various concentrations of SCA40 as required. The inhibitory potency of SCA40 was determined by fitting concentration-inhibition curves encompassing the IC_{50} to the two-parameter logistic equation (7 data points).

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), the disodium salt of ATP (Sigma), charybdotoxin (Latoxan), 5'-N-ethylcarboxamidoadenosine (NECA; Research Biochemicals Inc.), (−)-isoprenaline hydrochloride (Sigma), levcromakalim (SmithKline Beecham Pharmaceuticals), nifedipine (Bayer), S-(4-nitrobenzyl)-6-thioinosine (NBTI; Research Biochemicals Inc.), (±)-propranolol (ICI), quinine hydrochloride (BDH), SCA40 (6-bromo-8-methylaminoimidazol[1,2-a] pyrazine-2-carbonitrile; Université de Montpellier I), 8-(*p*)-sulphophenyltheophylline (8-SPT; Research Biochemicals Inc.), suramin

Table 1 The effects of some modifying agencies on the potency of SCA40, isoprenaline, theophylline, levcromakalim and 5'-N-ethylcarboxaminodadenosine

	Mean pD_2 value for relaxant agonist				
Medium	*SCA40*	*Isoprenaline*	*Theophylline*	*Levcromakalim*	*NECA*
Control	7.16 ± 0.10	7.68 ± 0.06	–	–	–
Propranolol (1 μM)	7.18 ± 0.07	5.52 ± 0.07	–	–	–
Control	7.11 ± 0.02	8.20 ± 0.02	3.91 ± 0.04	–	5.82 ± 0.14
8-SPT (140 μM)	7.14 ± 0.01	8.15 ± 0.05	3.90 ± 0.04	–	+
Control	7.10 ± 0.02	7.85 ± 0.18	–	–	–
Suramin (100 μM)	7.30 ± 0.14	7.60 ± 0.11	–	–	–
Control	7.11 ± 0.07	8.21 ± 0.12	–	6.17 ± 0.05	–
K^+-rich (80 mM)	5.28 ± 0.04*	6.98 ± 0.11*	–	†	–
Nifedipine (1 μM)	7.01 ± 0.07	8.11 ± 0.21	–	5.93 ± 0.05	–
K^+-rich (80 mM) + nifedipine (1 μM)	7.17 ± 0.19	8.29 ± 0.14	–	†	–
Control	6.58 ± 0.10	8.26 ± 0.11	–	–	–
Charybdotoxin (100 nM)	5.99 ± 0.08*	7.71 ± 0.09*	–	–	–
Nifedipine (1 μM)	6.56 ± 0.13	8.11 ± 0.21	–	–	–
Charybdotoxin (100 nM) + nifedipine (1 μM)	6.52 ± 0.13	8.15 ± 0.06	–	–	–
Control	6.85 ± 0.09	8.21 ± 0.12	–	–	–
Quinine (30 μM)	6.57 ± 0.09*	8.30 ± 0.09	–	–	–
Nifedipine (1 μM)	6.86 ± 0.12	–	–	–	–
Quinine (30 μM) + nifedipine (1 μM)	6.73 ± 0.15	–	–	–	–

Data represent means ± s.e.mean of determinations made on at least 6 tissues. A dash (–) indicates an interaction not studied. *Significant ($P<0.05$) difference from the time-matched control; †indicates a shift in the position (or depression) of the log concentration-effect curve so marked that a pD_2 value was not calculable.

(Bayer), theophylline (Sigma). In the assays of phosphodiesterase activity, radiochemicals were obtained either from Amersham International or from Du Pont NEN. Other reagents were obtained from Fluka AG or from the Sigma Chemical Company.

Stock solutions of most drugs were prepared in twice-distilled water. NBTI was dissolved in dimethylsulphoxide. Stock solutions of levcromakalim and nifedipine were prepared in 70% and absolute ethanol respectively. Stock solutions of isoprenaline and NECA were prepared in 0.1 M HCl. Dilutions of isoprenaline were made using distilled water containing 0.57 mM ascorbic acid.

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. The K^+-rich Krebs solution was prepared by the addition of KCl in order to raise the concentration of K^+ to 80 mM. The concentration of NaCl was reduced to maintain osmolality. All experiments involving nifedipine were conducted under illumination provided by a sodium vapour lamp to minimize photolysis of the drug.

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

Tissue bath studies with isolated trachea

ATP (3 μM–1 mM), isoprenaline (1–300 nM), levcromakalim (100 nM–10 μM), NECA (100 nM–100 μM), SCA40 (1 nM–10 μM) and theophylline (1 μM–1 mM) each caused concentration-dependent suppression of the spontaneous tone of the trachea. The maximal relaxant effects of isoprenaline, SCA40 and theophylline were equivalent to that of aminophylline. In contrast, the maximal effects of ATP, levcromakalim and NECA were approximately 75%–85% of that of aminophylline.

Propranolol (1 μM) or 8-SPT (140 μM) or suramin (100 μM) did not themselves alter tracheal tone. However, propranolol (1 μM) markedly (150 fold) antagonized isoprenaline without modifying the relaxant action of SCA40. 8-SPT (140 μM) had no effect against the tracheal relaxant actions of isoprenaline, SCA40 or theophylline but very markedly depressed the log concentration-effect curve of NECA. Suramin (100 μM) failed to modify the relaxant activity of either isoprenaline or SCA40 (Table 1). Suramin (100 μM) also failed to modify the tracheal relaxant action of ATP (data not shown).

Exposure of tracheal segments to an isosmolar, K^+-rich (80 mM) Krebs solution increased tissue tone by 49.5 ± 12.4% (mean ± s.e.mean of values from 12 tissues). In the presence of the K^+-rich medium, the log concentration-effect curves of isoprenaline and SCA40 were shifted markedly (20 fold and 60 fold respectively) to the right and the log concentration-effect curve for levcromakalim was very profoundly depressed (Figure 1 and Table 1). Nifedipine (1 μM) caused little or no change in tracheal tone and did not significantly alter the tracheal relaxant potencies of isoprenaline, levcromakalim or SCA40. Nifedipine (1 μM) prevented the increase in tissue tone evoked by the K^+-rich (80 mM) Krebs solution. In the presence of a combination of nifedipine (1 μM) and the K^+-rich medium, the log concentration-effect curves of isoprenaline and SCA40 each lay close to that observed in the control conditions. In other words nifedipine prevented the antagonism of isoprenaline and SCA40 induced by the K^+-rich medium. In contrast nifedipine did not prevent the equivalent antagonism of levcromakalim (Figure 1 and Table 1).

Charybdotoxin (100 nM) increased the tone of tracheal segments by 15.7 ± 4.9% (mean ± s.e.mean of values from 12 tissues). The toxin caused approximately 3 fold antagonism of isoprenaline and 4 fold antagonism of SCA40. Nifedipine (1 μM) prevented the increase in tissue tone induced by charybdotoxin. In the presence of a combination of charybdotoxin (100 nM) and nifedipine (1 μM) the log concentration-effect curves of isoprenaline and SCA40 each lay close to that observed under the control circumstance. Nifedipine (1 μM) therefore prevented the antagonism of isoprenaline and SCA40 induced by charybdotoxin (Figure 2 and Table 1).

Quinine (30 μM) caused little or no change in tissue tone and did not modify the relaxant action of isoprenaline. In contrast, quinine (30 μM) caused approximately 2 fold antagonism of SCA40. In the presence of a combination of

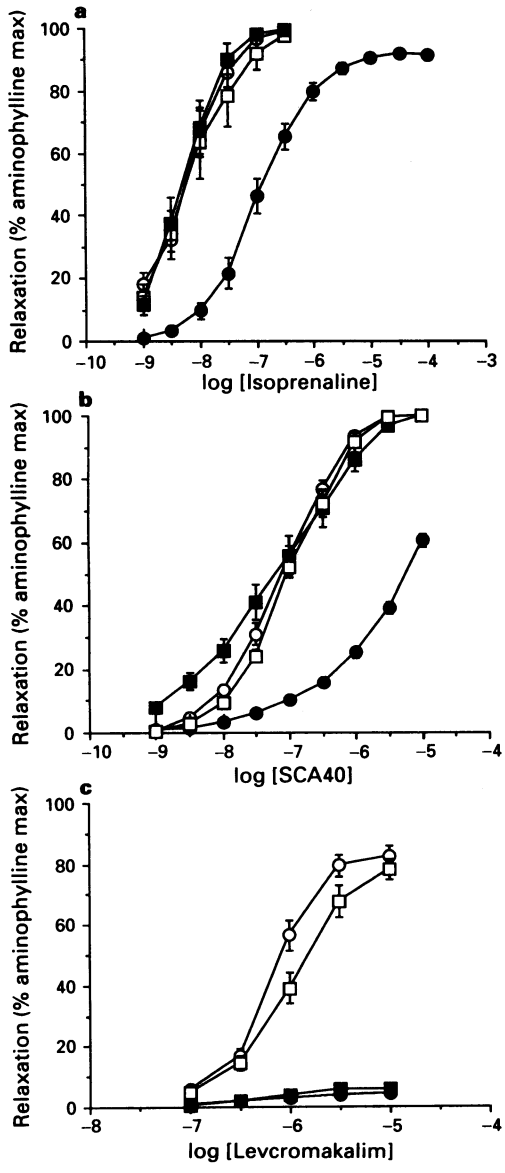

Figure 1 Guinea-pig isolated trachealis muscle: the effects of nifedipine (1 μM) on the ability of an isosmolar K^+-rich (80 mM) medium to inhibit the relaxant actions of isoprenaline, SCA40 and levcromakalim. Abscissae: $\log_{10}$ molar concentration of isoprenaline (a), SCA40 (b) or levcromakalim (c). Ordinates: relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. In each panel (○) indicates the log concentration-effect curve for the relaxant as observed in normal Krebs solution, (●) the curve obtained in K^+-rich (80 mM) Krebs solution, (□) the curve obtained in the presence of nifedipine (1 μM) and (■) the curve obtained in the presence of a combination of the K^+-rich (80 mM) medium and nifedipine (1 μM). Data points are the means ± s.e.mean of values from at least 6 tissues.

quinine (30 μM) and nifedipine (1 μM) the log concentration-effect curve of SCA40 was indistinguishable from that observed in control circumstances. Nifedipine therefore prevented the antagonism of SCA40 induced by quinine (Table 1).

Tissue bath studies with isolated atria

At the outset of the experiments the atrial preparations contracted at the rate of approximately 160 beats per min and the force of contraction was generally close to 350 mg. The beating rate of the vehicle-treated control tissues remained relatively constant for the duration (90 min) of the experiment. However, the force of atrial contraction showed some tendency to decline (Figure 3).

Treatment of test tissues with SCA40 (1 nM–10 μM) caused a concentration-dependent increase in the rate of atrial contraction. At concentrations of 100 nM and above, SCA40 significantly increased the rate of atrial beating compared with that observed in the control tissues. Furthermore, at concentrations of 1 and 10 μM, SCA40 clearly and significantly increased the force of atrial contraction (Figure 3). This effect reached a peak approximately 2 min after the appropriate drug concentration increment and thereafter showed some decline.

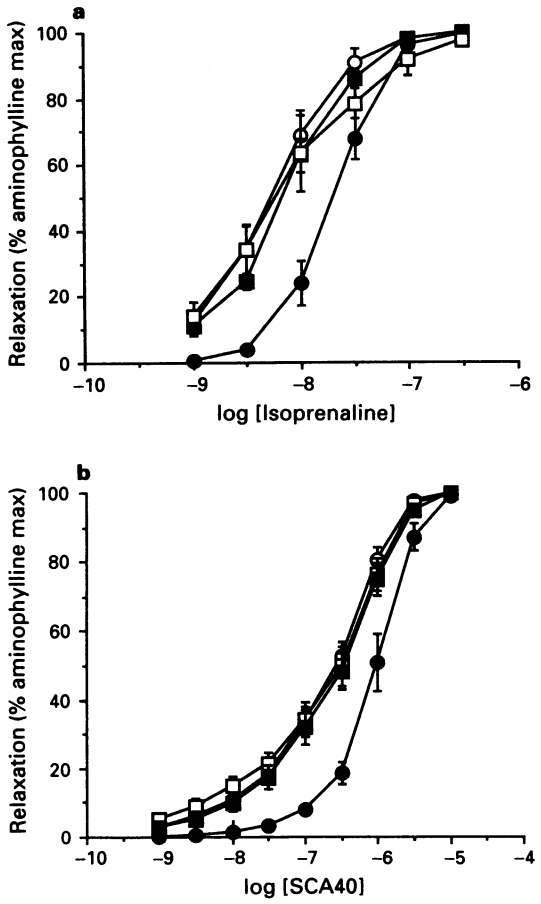

Figure 2 Guinea-pig isolated trachealis muscle: the effects of nifedipine (1 μM) on the ability of charybdotoxin to inhibit the relaxant actions of isoprenaline and SCA40. Abscissae: $\log_{10}$ molar concentration of isoprenaline (a) or SCA40 (b). Ordinates: relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. In each panel (○) indicates the log concentration-effect curve for the relaxant as observed in normal Krebs solution, (●) the curve obtained the presence of charybdotoxin (100 nM), (□) the curve obtained in the presence of nifedipine (1 μM) and (■) the curve obtained in the presence of a combination of the charybdotoxin (100 nM) and nifedipine (1 μM). Data points are the means ± s.e.means of values from at least 6 tissues.

Intracellular electrophysiological recording from trachealis

Microelectrode impalement of trachealis cells revealed that, while some cells were electrically quiescent, others exhibited spontaneous oscillations (slow waves) of membrane potential. The addition of SCA40 (1 μM) to the Krebs solution superfusing the tissue caused full suppression of the mechanical tone of the tissue. This mechanical effect was often accompanied by suppression of the electrical slow waves (Figure 4). However, no significant change in resting membrane potential was observed (Table 2). Tested at a concentration of 10 μM, SCA40 suppressed mechanical tone, suppressed slow wave activity and evoked cellular hyperpolarization (Figure 4 and Table 2).

Assessment of the ability of SCA40 and theophylline to inhibit the cyclic nucleotide PDE activity of guinea-pig trachea

SCA40 (10 nM–100 μM) and theophylline (1 μM–10 mM) each caused concentration-dependent inhibition of the hydrolysis of cyclic AMP and cyclic GMP by homogenates of muscle-rich strips of guinea-pig trachea. SCA40 was the more potent PDE inhibitor in each case and, in contrast to theophylline, exhibited selectivity as an inhibitor of cyclic AMP-PDE as opposed to cyclic GMP-PDE (Table 3).

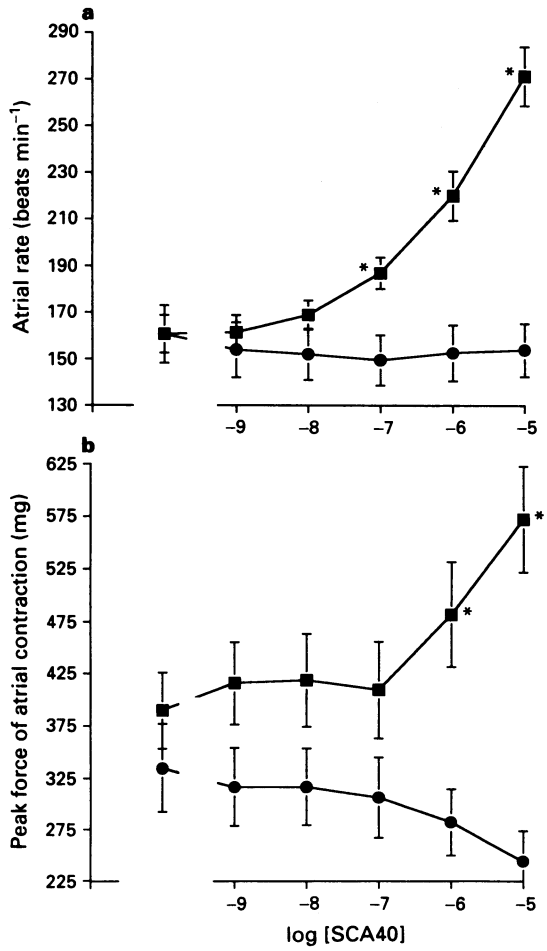

Figure 3 The effects of SCA40 on the rate (a) and force (b) of beating of the guinea-pig isolated atrial preparation. Abscissae: $\log_{10}$ molar concentration of SCA40. Ordinate scales: rate of beating (a) and peak force of contraction (b). In both (a) and (b), (■) indicates test tissue and (●) time-matched control tissues. The plotted data points represent the means ± s.e.means of values from at least 6 tissues. Significant ($P<0.05$) difference from the corresponding point for the control tissues.

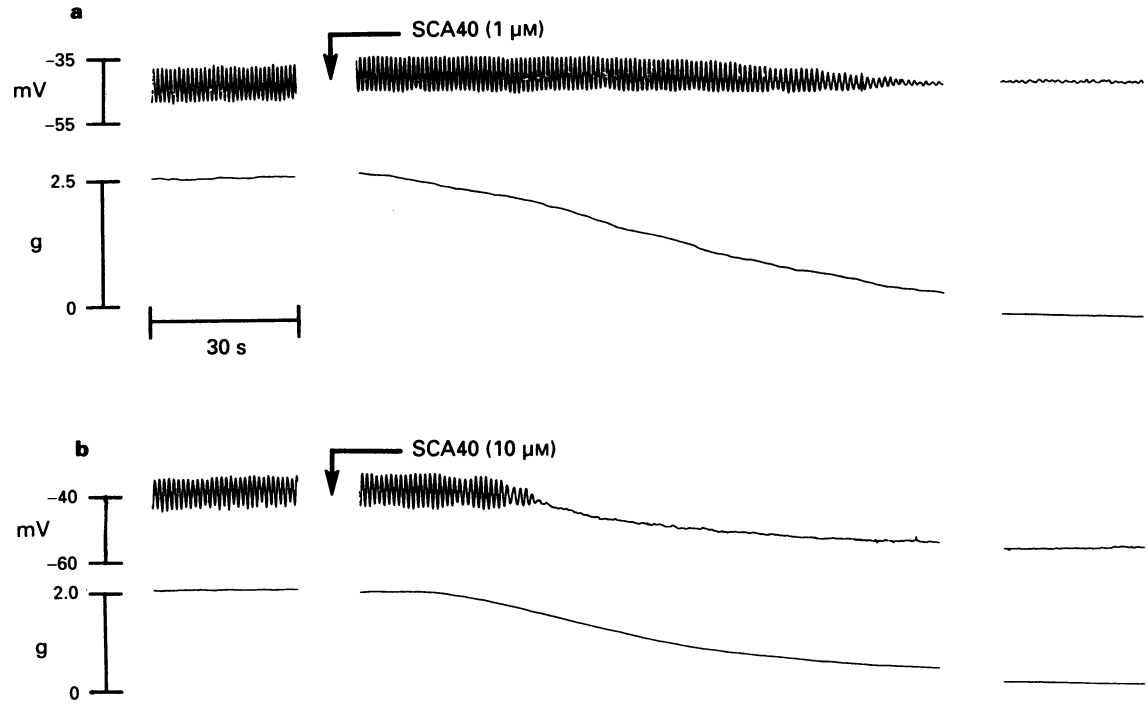

Figure 4 The effects of SCA40 on the spontaneous electrical and mechanical activity of guinea-pig isolated trachealis. In each row of recordings the upper trace represents membrane potential changes recorded from a single cell while the lower trace represents tension changes recorded from a contiguous segment of trachea. In each row the left hand panel represents control activity recorded immediately prior to the administration of SCA40 (1 μM in (a); 10 μM in (b)). The centre panels show activity recorded 1 min and the right hand panels activity recorded 6 min after the administration of the drug.

Table 2 The effects of SCA40 on the electrical and mechanical activity of guinea-pig isolated trachealis muscle

Concentration of SCA40	*Membrane hyperpolarization* (mV)	*Relaxation* (g)
1 μM	−0.6 ± 0.9†	1.8 ± 0.39 (6)
10 μM	19.0 ± 2.7	2.3 ± 0.35 (4)

Data are means ± s.e.mean. The figures in parentheses indicate the number of tissues contributing to the mean. †indicates a value not significantly different from zero.

Table 3 Inhibition of cyclic AMP (cAMP-PDE) and cyclic GMP (cGMP-PDE) phosphodiesterase activity of homogenates of muscle-rich strips of guinea-pig trachea by SCA40 and by theophylline

Inhibitor	*cAMP-PDE*	*cGMP-PDE*
SCA40	4.67 ± 0.19 (4)	4.03 ± 0.11* (4)
Theophylline	3.02 ± 0.07 (8)	2.83 ± 0.10 (7)

Data indicate mean (± s.e.mean) values of $-\log IC_{50}$ for each inhibitor. The figures in parentheses indicate the number of experiments. *indicates a significant difference from the corresponding value for cAMP-PDE.

Table 4 Inhibition by SCA40 of the activity of the isoenzymes of cyclic nucleotide phosphodiesterase isolated from human blood cells and lung tissue

Isoenzyme type				
I	*II*	*III*	*IV*	*V*
3.37	3.30	6.61	4.68	4.34
3.54–3.15	3.49–3.06	6.79–6.43	4.74–4.62	4.52–4.15

Data indicate $-\log IC_{50}$ values for SCA40 together with their upper and lower 95% confidence limits.

Assessment of the inhibitory activity of SCA40 against the isoenzymes of cyclic nucleotide PDE isolated from human lung and blood cells

SCA40 inhibited each of the isoenzymes of human cyclic nucleotide PDE in a concentration-dependent manner. However, among the various isoenzymes, the drug exhibited differing potencies as an inhibitor. SCA40 was most potent in inhibiting the type III isoenzyme. It was approximately equipotent in inhibiting the type IV and type V isoenzymes and very weak as an inhibitor of the isoenzyme types I and II (Table 4).

Discussion

Does SCA40 cause trachealis muscle relaxation by activating β-adrenoceptors or P_1 or P_2 purinoceptors?

The present experiments with propranolol were performed in order to determine whether SCA40 could directly or indirectly activate β-adrenoceptors. Propranolol (1 μM) profoundly antagonized isoprenaline in relaxing guinea-pig isolated trachea but did not modify the relaxant potency of SCA40 (Table 1).

The present experiments with 8-SPT and suramin were prompted by the notion that the molecular structure of SCA40 bears some resemblance to that of the xanthine nucleus. It therefore seemed possible that SCA40 might have some affinity for purinoceptors. 8-SPT (> 1 μM) acts as an antagonist selective for P_1 purinoceptors (Gustaffson, 1984; Collis *et al.*, 1987). In the present study, 8-SPT (140 μM) failed to antagonize the tracheal relaxant actions of isoprenaline, theophylline or SCA40 but markedly antagonized NECA. Suramin, at concentrations greater than 10 μM, acts as an antagonist selective for P_2 purinoceptors (Dunn & Blakeley, 1988; Hoyle *et al.*, 1990). In the present study suramin (100 μM) did not antagonize isoprenaline, SCA40 (Table 1) or ATP. The failure of suramin to

antagonize the tracheal relaxant action of ATP confirms the findings of Kelley & Hollingsworth (1994) and indicates that P_2 purinoceptors may not be expressed in guinea-pig trachealis muscle.

In summary, the present experiments using propranolol, 8-SPT and suramin collectively suggest that the relaxant action of SCA40 in guinea-pig trachealis muscle does not involve the activation of β-adrenoceptors, P_1 purinoceptors of P_2 purinoceptors.

Role of BK_{Ca} channel opening in the tracheal relaxant action of SCA40

The rationale behind using an isosmolar, K^+-rich (>40 mM) medium to identify smooth muscle relaxants that act by opening plasmalemmal K^+-channels is that the K^+-rich medium moves the K^+ equilibrium potential to a value that closely approaches the membrane potential. In this circumstance, K^+-channel opening cannot evoke sufficient hyperpolarization to ensure the closure of voltage-dependent Ca^{2+}-channels. Voltage-dependent inhibition of Ca^{2+} influx does not therefore occur and the mechano-inhibitory effects of the K^+-channel opener are markedly reduced or abolished (Small *et al.*, 1992). However, the K^+-rich medium itself promotes Ca^{2+} influx and increases tissue tone. It may therefore functionally antagonize relaxant agents that have no component of action attributable to K^+-channel opening (Huang *et al.*, 1993; Small *et al.*, 1993).

The present observations (Figure 1 and Table 1) that an isosmolar K^+-rich medium antagonizes the tracheal relaxant action of an agonist at β-adrenoceptors (isoprenaline), an opener of ATP-sensitive K^+-channels (levcromakalim) and SCA40 confirm the results of earlier studies (Allen *et al.*, 1985; 1986; Huang *et al.*, 1993; Laurent *et al.*, 1993). In the case of agonists at β-adrenoceptors such antagonism is prevented by the presence of nifedipine (Huang *et al.*, 1993; present study). Nifedipine also prevented the antagonism of SCA40 provided by a K^+-rich medium (present study). In contrast, nifedipine did not affect the antagonism of levcromakalim provided by a K^+-rich medium (Figure 1 and Table 1). These findings suggest that the interaction between the K^+-rich medium and agonists at β-adrenoceptors or SCA40 represents functional antagonism attributable to the effect of the K^+-rich medium in promoting Ca^{2+} influx through nifedipine-sensitive channels. The failure of nifedipine to prevent the antagonism of levcromakalim provided by the K^+-rich medium suggests that the action of levcromakalim depends upon its ability to hyperpolarize the plasmalemma but not on an ability to cause voltage-dependent inhibition of Ca^{2+} influx through nifedipine-sensitive channels. How membrane hyperpolarization is transduced into the mechano-inhibitory effects of levcromakalim remains a challenge for further experimentation.

The present observations (Figure 2; Table 1) that charybdotoxin (an inhibitor of BK_{Ca} channels) antagonizes the tracheal relaxant actions of an agonist at β-adrenoceptors and that of SCA40 confirm the results of earlier studies (Jones *et al.*, 1990; Murray *et al.*, 1991; Huang *et al.*, 1993; Laurent *et al.*, 1993). Since charybdotoxin converts the spontaneous electrical slow waves of guinea-pig trachealis into regenerative action potentials and increases the mechanical tone of the tissue (Murray *et al.*, 1991), it is likely that this agent promotes the cellular influx of Ca^{2+}. The finding that nifedipine can offset the antagonism of agonists at β-adrenoceptors and that of SCA40 provided by charybdotoxin (Huang *et al.*, 1993; present study) suggests that such antagonism represents not an interaction between the toxin and the relaxant drugs at the level of BK_{Ca} channel gating but rather functional antagonism attributable to the toxin promoting Ca^{2+} influx.

Quinine is a relatively non-selective inhibitor among Ca^{2+}-dependent K^+-channels (Haylett & Jenkinson, 1990). Laurent *et al.*, (1993) showed that quinine could antagonize SCA40 in relaxing guinea-pig trachea bathed by a K^+-rich (20 mM) medium. Similar antagonism was demonstrated in the present experiments (Table 1), but this could be offset by nifedipine. It therefore seems likely that, as for charybdotoxin, the antagonism of SCA40 provided by quinine represents functional antagonism resulting from quinine-induced promotion of Ca^{2+} influx.

The abilities of a K^+-rich medium, charybdotoxin and quinine each to antagonize SCA40 prompted Laurent *et al.*, (1993) to suggest that the opening of BK_{Ca} channels underlies the tracheal relaxant action of this agent. However, the present observation that nifedipine can, in each case, prevent the antagonism casts doubt on the usefulness of each of the three tools for identifying agents that act to promote K^+-channel opening in trachealis muscle. That K^+-channel opening is not an important feature of the tracheal relaxant action of SCA40 is also suggested by the present electrophysiological experiments in which a concentration (1 μM) of SCA40 causing full suppression of the spontaneous tone of the trachea failed to hyperpolarize the trachealis cells (Figure 4; Table 2). Further support for this idea comes from patch clamp experiments performed on bovine trachealis where SCA40 (0.1–10 μM) failed to modify the activity of BK_{Ca} channels recorded from either inside-out, outside-out or cell-attached plasmalemmal patches (MacMillan *et al.*, 1994).

That K^+-channel opening is not an important feature of the action of SCA40 is also suggested by the present experiments with guinea-pig isolated atrial preparations. Were the promotion of K^+-channel opening to be a fundamental action of SCA40 (1 nM–1 μM), we would have expected this agent to have exerted negative chronotropic and negative inotropic effects. However, this proved not to be the case (Figure 3). The present failure of SCA40 (1 nM–1 μM) to reduce the rate and force of atrial beating contrasts with the observations of Michel *et al.* (1993) in atria isolated from the rat. The reasons for this difference are currently unclear. It is possible that rat and guinea-pig atrial tissues may exhibit species differences with respect to their sensitivity to SCA40. However, the report of Michel *et al.* (1993) did not include data from vehicle-treated, time-matched control tissues. Hence, it is currently difficult to assess whether the reductions in atrial rate and force observed (Michel *et al.*, 1993) in rat tissue represented time-dependent changes in the activity of the tissue rather than effects attributable to the action of SCA40.

We conclude that the suggestion of Laurent *et al.* (1992) that SCA40 (<10 μM) relaxes guinea-pig trachea by opening BK_{Ca} channels is unlikely to be correct. As discussed below, this agent is more likely to promote tracheal relaxation by virtue of its activity as an inhibitor of cyclic AMP-PDE.

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

Using an enzyme preparation derived from bovine heart (Sigma PO134), Bonnet *et al.* (1992) demonstrated that SCA40 was able to inhibit cyclic nucleotide PDE with an IC_{50} of 70 μM. In the present study SCA40 inhibited the total cyclic AMP-PDE and the total cyclic GMP-PDE of guinea-pig trachea with IC_{50} values of approximately 20 μM and 100 μM respectively (Table 3). At first sight the relatively low potency of SCA40 as an inhibitor of total cyclic AMP-PDE in guinea-pig trachea might suggest that inhibition of cyclic AMP-PDE cannot explain the tracheal relaxant activity of SCA40, for the relaxant effects of this agent are observed over the concentration-range 10 nM–8 μM (Laurent *et al.*, 1993; present study). However, in guinea-pig trachea, the cyclic AMP hydrolysing isoenzyme that mainly regulates mechanical tone is the type III isoenzyme with the type IV isoenzyme perhaps playing a supportive role (Harris *et al.*, 1989; Berry *et al.*, 1991). Since these isoenzymes account for only a fraction of the total cyclic AMP hydrolytic activity of

a homogenate of the trachea (Berry *et al.*, 1991), experiments involving the measurement of total cyclic AMP-PDE may mask the true potency of any selective inhibitor of the isoenzymes that are the principal regulators of the mechanical tone of the tissue.

The fact that, in homogenates of guinea-pig trachea, SCA40 proved more potent as an inhibitor of total cyclic AMP-PDE than as an inhibitor of total cyclic GMP-PDE suggests that it may selectively inhibit one or more of the isoenzymes (i.e. types III andIV) that preferentially hydrolyse cyclic AMP. The cyclic nucleotide PDE isoenzyme that principally regulates the mechanical activity of guinea-pig cardiac muscle is the isoenzyme type III (Gristwood *et al.*, 1985; Gristwood & Owen, 1986; Reeves *et al.*, 1987; Berry *et al.*, 1991; Elliott *et al.*, 1991). In view of this, the ability of SCA40 (1 nM–10 μM; Figure 3) to increase the beating rate of guinea-pig isolated atria suggests that SCA40 selectively inhibits this isoenzyme of cyclic nucleotide PDE. This suggestion receives direct support from the present studies of SCA40 as an inhibitor of the isoenzymes of cyclic nucleotide PDE derived from human blood cells and lung tissue (Table 4).

Since the IC_{50} (approximately 250 nM; Table 4) of SCA40 as an inhibitor of isoenzyme type III lies within the effective concentration range of SCA40 in relaxing guinea-pig trachea, we conclude that inhibition of this isoenzyme represents the principal mechanism by which SCA40 relaxes the trachea. Towards the upper end of the effective concentration-range of SCA40, the inhibition of isoenzyme types IV and/or V may also have a role to play. Plasmalemmal K^+-channel opening with consequential cellular hyperpolarization (Figure 4) is only observed in guinea-pig trachealis with concentrations of SCA40 in excess of 1 μM and may perhaps be mediated by marked inhibition of various of the isoenzymes of cyclic nucleotide PDE and therefore an elevation in the cellular content of cyclic AMP (Small *et al.*, 1993).

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References

ALLEN, S.L., BEECH, D.J., FOSTER, R.W., MORGAN, G.P. & SMALL, R.C. (1985). Electrophysiological and other aspects of the relaxant action of isoprenaline in guinea-pig isolated trachealis. *Br. J. Pharmacol.*, **86**, 843–854.

ALLEN, S.L., BOYLE, J.P., CORTIJO, J., FOSTER, R.W., MORGAN, G.P. & SMALL, R.C. (1986). Electrical and mechanical effects of BRL 34915 in guinea-pig isolated trachealis. *Br. J. Pharmacol.*, **89**, 395–405.

BEAVO, J.A. & REIFSNYDER, D.H. (1990). Primary sequence of cyclic nucleotide phosphodiesterase isoenzymes and the design of selective inhibitors. *Trends Pharmacol. Sci.*, **11**, 150–155.

BERRY, J.L., ELLIOTT, K.R.F., FOSTER, R.W. & SMALL, R.C. (1991). Cyclic nucleotide phosphodiesterase isoenzymes (PDE) in guinea-pig tracheal smooth muscle. *Br. J. Pharmacol.*, **104**, 206P.

BONNET, P.A., MICHEL, A., LAURENT, F., SABLAYROLLES, C., RECHENCQ, R., MANII, J.C., BOUCARD, M. & CHAPAT, J.P. (1992). Synthesis and antibronchospastic activity of 8-alkoxy and 8-(alkylamino)imidaxo[1,2-a]pyrazines. *J. Med. Chem.*, **35**, 3353–3358.

COLLIS, M.G., JACOBSON, K.A. & TOMKINS, D.M. (1987). Apparent affinity of some 8-phenyl-substituted xanthines at adenosine receptors in guinea-pig aorta and atria. *Br. J. Pharmacol.*, **92**, 69–75.

COOK, S.J., ARCHER, K., MARTIN, A., BUCHHEIT, K.H., FOZARD, J.R. & SMALL, R.C. (1994). Evidence that K^+-channel opening is not important in the tracheal relaxant action of SCA40. *Br. J. Pharmacol.*, **112**, 469P.

DIXON, J.S. & SMALL, R.C. (1983). Evidence of poor conduction of muscle excitation in the longitudinal axis of guinea-pig isolated trachea. *Br. J. Pharmacol.*, **79**, 75–83.

DUNN, P.M. & BLAKELEY, A.G.H. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

ELLIOTT, K.R.F., BERRY, J.L., BATE, A.J., FOSTER, R.W. & SMALL, R.C. (1991). The isoenzyme selectivity of AH 21-132 as an inhibitor of cyclic nucleotide phosphodiesterase activity. *J. Enzyme Inhibition*, **4**, 245–251.

FOSTER, R.W., SMALL, R.C. & WESTON, A.H. (1983). Evidence that the spasmogenic action of tetraethylammonium in guinea-pig trachealis is both direct and dependent on the cellular influx of Ca^{2+} ion. *Br. J. Pharmacol.*, **79**, 255–263.

GRISTWOOD, R.W. & OWEN, D.A.A. (1986). Effects of rolipram on guinea-pig cardiac ventricles in vitro: evidence of an unexpected synergism with SK&F 94120. *Br. J. Pharmacol.*, **87**, 91P.

GRISTWOOD, R.W., OWEN, D.A.A. & REEVES, M.L. (1985). Phosphodiesterase in the guinea-pig cardiac ventricle; specific inhibition of type III activity by SK&F 94120. *Br. J. Pharmacol.*, **85**, 224P.

GUSTAFFSON, L.E. (1984). Adenosine antagonism and related effects of theophylline derivatives in guinea-pig ileum longitudinal muscle. *Acta. Physiol. Scand.*, **122**, 191–198.

HARRIS, A.L., CONNELL, M.J., FERGUSON, E.W., WALLACE, A.M., GORDON, R.J., PAGANI, E.D. & SILVER, P.J. (1989). Role of low K_m cyclic AMP phosphodiesterase inhibition in tracheal relaxation and bronchodilation in the guinea-pig. *J. Pharmacol. Exp. Ther.*, **251**, 199–206.

HAYLETT, D.G. & JENKINSON, D.H. (1990). Calcium-activated potassium channels. In *Potassium Channels: Structure, Classification, Function and Therapeutic Potential*, ed. COOK, N.S. pp. 70–95, Chichester: Ellis Horwood Limited.

HOYLE, C.H.V., KNIGHT, G.E. & BURNSTOCK, G. (1990). Suramin antagonises responses to P_2-purinceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *Br. J. Pharmacol.*, **99**, 617–621.

HUANG, J.C., GARCIA, M.L., REUBEN, J.P. & KACZOROWSKI, G.J. (1993). Inhibition of β-adrenoceptor agonist relaxation of airway smooth muscle by Ca^{2+}-activated K^+-channel blockers. *Eur. J. Pharmacol.*, **235**, 37–43.

JONES, T.R., CHARETTE, L., GARCIA, M.L. & KACZOROWSKI, G.J. (1990). Selective inhibition of relaxation of guinea-pig trachea by charybdotoxin, a potent Ca^{2+}-activated K^+-channel inhibitor. *J. Pharmacol. Exp. Ther.*, **255**, 697–706.

KELLEY, A.S. & HOLLINGSWORTH, M. (1994). Evidence that ATP and β,γ-methylene ATP produce relaxation of guinea-pig trachealis by an action involving P_1 purinoceptors. *Br. J. Pharmacol.*, **112**, 503P.

LAURENT, F., MICHEL, A., BONNET, P.A., CHAPAT, J.P. & BOUCARD, M. (1993). Evaluation of the relaxant effects of SCA40, a novel charybdotoxin-sensitive potassium channel opener, in guinea-pig isolated trachealis. *Br. J. Pharmacol.*, **108**, 622–626.

MACMILLAN, S., SHERIDAN, R.D. & PATMORE, L. (1994). Comparison of the effects of NS-004 and SCA40 on the activity of single maxi-K^+ channels in bovine tracheal smooth muscle cells. *Br. J. Pharmacol.*, **112**, 463P.

MICHEL, A., LAURENT, F., BOMPART, J., HADJ-KADDOUR, K., CHAPAT, J.P., BOUCARD, M. & BONNET, P.A. (1993). Cardiovascular effects of SCA40, a novel potassium channel opener, in rats. *Br. J. Pharmacol.*, **110**, 1031–1036.

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

REEVES, M.L., LEIGH, B.K. & ENGLAND, P.J. (1987). The identification of a new cyclic nucleotide phosphodiesterase activity in human and guinea-pig cardiac ventricle. *Biochem. J.*, **241**, 535–541.

SMALL, R.C., BERRY, J.L., BURKA, J.F., COOK, S.J., FOSTER, R.W., GREEN, K.A. & MURRAY, M.A. (1992). Potassium channel activators and bronchial asthma. *Clin. Exp. Allergy*, **22**, 11–18.

SMALL, R.C., CHIU, P., COOK, S.J., FOSTER, R.W. & ISAAC, L. (1993). β-Adrenoceptor agonists in bronchial asthma: role of K^+-channel opening in mediating their bronchodilator effects. *Clin. Exp. Allergy*, **23**, 802–811.

THOMSON, W.J., TERASAKI, W.J., EPSTEIN, P.M. & STRADA, S.J. (1979). Assay of cyclic nucleotide phosphodiesterase and resolution of multiple molecular forms of the enzyme. *Adv. Cyclic Nucleotide Res.*, **10**, 69–92.

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