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Evaluation of the relaxant effects of SCA40, a novel charybdotoxin-sensitive potassium channel opener, in guinea-pig isolated trachealis

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1 Experiments have been performed in order to analyse the mechanism whereby SCA40, a new imidazo[1,2-*a*]pyrazine derivative relaxes airway smooth muscle.

2 SCA40 (0.01–10 μM) caused a complete and concentration-dependent relaxation of guinea-pig isolated trachea contracted with 20 mM KCl but failed to inhibit completely the spasmogenic effects of 80 mM KCl.

3 Quinine (30 μM) antagonized the relaxant activity of SCA40 in 20 mM KCl-contracted guinea-pig isolated trachea. The ATP-sensitive K^+-channel blocker, glibenclamide (3 μM), did not antagonize the relaxant activity of SCA40 in either 20 mM KCl or 1 μM carbachol-contracted isolated trachea.

4 SCA40 (0.01–10 μM) and isoprenaline (0.1 nM–10 μM) caused a complete and concentration-dependent relaxation of guinea-pig isolated trachea contracted with carbachol 1 μM.

5 The large-conductance Ca^{2+}-activated K^+-channel blocker, charybdotoxin (60–180 nM), non-competitively antagonized the relaxant activity of isoprenaline on 1 μM carbachol-contracted trachea. The inhibition was characterized by rightward shifts of the isoprenaline concentration-relaxation curves with depression of their maxima.

6 The relaxant activity of SCA40 in 1 μM carbachol-contracted trachea was antagonized by charybdotoxin (60–600 nM) in an apparently competitive manner. The concentration-relaxation curves to SCA40 were shifted to the right with no significant alteration in the maximum response.

7 It is concluded that SCA40 is a novel potassium channel opener which is a potent relaxant of guinea-pig airway smooth muscle *in vitro*. The relaxant activity of SCA40 does not involve ATP-sensitive K^+-channels but rather large-conductance Ca^{2+}-activated K^+-channels or other charybdotoxin-sensitive K^+-channels.

Keywords: Guinea-pig trachealis; smooth muscle relaxation; SCA40; charybdotoxin; glibenclamide; isoprenaline; potassium channels

Introduction

We have previously described the pharmacological properties of imidazo[1,2-*a*]pyrazine derivatives (Sablayrolles *et al.*, 1984; Michel *et al.*, 1989; Bonnet *et al.*, 1992). These compounds exhibited theophylline-like properties, not blocked by β-adrenoceptor antagonists, potent smooth muscle relaxant activity *in vitro*, potent anti-bronchospasm activity *in vivo* and adenosine 3':5'-cyclic monophosphate (cyclic AMP) phosphodiesterase inhibitory activity.

Among the tested 8-alkylaminoimidazo[1,2-*a*]pyrazines, SCA40, 6-bromo-8-methylaminoimidazo[1,2-*a*]pyrazine-2-carbonitrile, exhibited the highest smooth muscle relaxant activities among the derivatives of the series. Indeed, SCA40 was shown to be 40 to 500 times more active than the reference drug theophylline *in vivo* and *in vitro*, respectively (Michel *et al.*, 1990). This potent smooth muscle relaxant activity, especially in guinea-pig tracheal tissue, could not be linked up to the SCA40 cyclic AMP phosphodiesterase inhibitory properties which are similar to those of theophylline (Bonnet *et al.*, 1992). Therefore other mechanism(s) must account for the potent relaxant activity of SCA40. Many studies have shown that an increase in intracellular cyclic AMP plays an important role in regulating tracheal smooth muscle contraction, but other cellular events may be associated with relaxation in airway smooth muscle. Recent studies have shown that K^+-channel agonists such as cromakalim and pinacidil induced hyperpolarization of the cell membrane which leads to tracheal smooth muscle relaxation (Allen *et al.*, 1986; Mellemkjær *et al.*, 1989). ATP-sensitive K^+-channels have been identified as the targets of these K^+-channel openers since the *in vitro* relaxant activity of cromakalim and pinacidil was blocked in an apparently competitive manner by the sulphonylurea, glibenclamide (Quast & Cook, 1989). ATP-sensitive K^+-channels are not the only type of K^+-channels involved in the regulation of trachealis cell polarization. Patch-clamp studies have shown that the plasmalemma of trachealis smooth muscle cells from ox (Green *et al.*, 1991), dog (McCann & Welsh, 1986) and pig (Huang *et al.*, 1987) is richly endowed with large-conductance, Ca^{2+}-dependent K^+-channels. Charybdotoxin (ChTX), a purified peptide toxin present in *Leiurus quinquestriatus* venom which has been found to block large conductance, Ca^{2+}-dependent K^+-channels in a variety of cells (Castle *et al.*, 1989) is able to block the tracheal relaxant activity of β-adrenoceptor agonists (Murray *et al.*, 1991). These observations imply that large conductance, Ca^{2+}-dependent K^+-channels may be involved in the tracheal relaxant activity of β-adrenoceptor agonists. These results show that hyperpolarization of tracheal smooth muscle induced by the opening of various type of K^+-channels may be involved in tracheal smooth muscle relaxation. As xanthines had been shown to possess K^+-channel opening properties (Jones *et al.*, 1990; Murray *et al.*, 1991), the aim of the present study was to investigate the potential K^+-channel opening properties of the purine-like derivative SCA40 in guinea-pig isolated trachea and to characterize the type of K^+-channel involved.

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Methods

Adult male Dunkin-Hartley guinea-pigs (Iffa Credo, Lyon, France), weighing 400–500 g, fed with UAR A114 diet and fasted 18 h prior the experiment were used. Guinea-pigs were killed by a blow to the head. Tracheae were excised, cleaned of adhering adipose and connective tissue and contractility of tracheal segments (4 tracheal rings in all cases) was measured by adapting the method previously described (Hooker *et al.*, 1977). Tissues were suspended in 20 ml organ baths containing a Chenoweth Koelle buffer. At the outset of each experiment, tissues were subjected to an imposed tension of 0.5 g and allowed to equilibrate for 20 to 30 min during which time they were washed every 4 min. Changes in tension were measured isometrically with a myograph transducer connected to a Physiograph Narco Bio-system.

Effects of SCA40 against tone induced by KCl

KCl (20 mM or 80 mM) induced contractions which reached a stable maximum within 5 min. Cumulative log concentration-response curves to SCA40 were determinated for trachea contracted with KCl 20 or 80 mM, taking the intensity of the initial contraction as 100%. Then, cumulative log concentration-response curves to SCA40 were determinated for trachea contracted with KCl 20 mM in the absence (control) or in the presence of glibenclamide (3 μM) or quinine (30 μM).

Effects of glibenclamide on the relaxant action of SCA in carbachol-contracted trachea

In these experiments, cumulative log concentration-response curves to SCA40 were determinated for trachea contracted with carbachol (1 μM) in the absence (control), or in the presence of 3 μM glibenclamide (20 min pretreatment).

Effects of charybdotoxin on the relaxant actions of SCA40 and isoprenaline

In a second set of experiments, cumulative log concentration-response curves to SCA40 and isoprenaline were determinated for trachea contracted with carbachol (1 μM) in the absence (control), or in the presence of 60, 180 or 600 nM ChTX (20 min pretreatment). In these experiments, tissues were suspended in 2 ml organ baths containing a Krebs buffer. In all the experiments, the cyclo-oxygenase inhibitor, indomethacin (1.4 μM) was added to the buffer in order to prevent spontaneous tone development due to released prostanoids. Half-$\log_{10}$ unit concentration increments were used for all log concentration-response curves. All relaxant responses are expressed as a percentage reduction in response to the appropriate spasmogen. For each relaxant drug, potency is expressed as the negative log EC_{50} where EC_{50} is the concentration producing 50% inhibition of the contraction. The EC_{50} values for control and treatment curves were calculated by linear regression analysis applied to the linear portion of each dose-response curve.

Statistical evaluation of results

Statistical evaluation of the results was assessed by use of a two-tailed, unpaired *t* test. The null hypothesis was rejected when $P<0.05$.

Drugs and solutions

The substances used were obtained from the following sources: Sigma Chemicals (U.S.A.): (±)-isoprenaline, glibenclamide, carbachol, quinine; Latoxan (France): purified charybdotoxin.

For *in vitro* experiments, 10^{-2} M stock solution of SCA40 and 10^{-3} M stock solution of glibenclamide were made up in ethanol. Further dilutions were made up in distilled water. KCl, carbachol, and quinine solutions were prepared in distilled water. Lyophilised ChTX has been reconstituted in saline solution (150 mM NaCl) and stored at −20°C until use. The Chenoweth-Koelle solution used had the following composition (mM): NaCl 120, KCl 5.6, $CaCl_2$ 2.4, $MgCl_2$ 2.2, $NaHCO_3$ 15 and glucose 10. The Krebs solution used had the following composition (mM): NaCl 120, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 10. These solutions were maintained at 37°C and gassed continuously with a mixture of 95% O_2, 5% CO_2.

Results

Effects of SCA40 against tone induced by KCl

Cumulative concentration-response curves to SCA40 on guinea-pig isolated trachea precontracted with KCl 20 and 80 mM are shown in Figure 1. SCA40 produced complete concentration-dependent inhibition of response to KCl 20 mM. During 80 mM KCl-induced contraction, SCA40 produced only partial relaxation, which corresponded to approximately 40% of the maximum relaxation that could be achieved against the 20 mM KCl-induced contraction. Moreover, the relaxation concentration-response curve to SCA40 against KCl 80 mM-induced contraction was shifted to the right approximately 1000 fold compared with SCA40 relaxant activity against KCl 20 mM ($-\log EC_{50} = 7.43 \pm 0.27$ and 4.44 ± 0.33 respectively). In the presence of glibenclamide 3 μM, the relaxation concentration-response curve to SCA40 against KCl 20 mM-induced contraction was not modified (Figure 2a). On the contrary, the relaxation concentration-response curve to SCA40 against KCl 20 mM-induced contraction in the presence of quinine (30 μM) was shifted to the right approximately 5 fold compared with SCA40 relaxant activity against KCl 20 mM alone ($-\log EC_{50} = 7.57 \pm 0.29$ and 6.84 ± 0.08 respectively) with no significant alteration in the maximum response (Figure 2b).

Effects of glibenclamide on the relaxant action of SCA40 in carbachol-contracted trachea

Cumulative concentration-response curves to SCA40 on guinea-pig isolated trachea precontracted with carbachol 1 μM in the presence or absence of glibenclamide 3 μM are shown in Figure 3. The tracheal contraction induced by carbachol

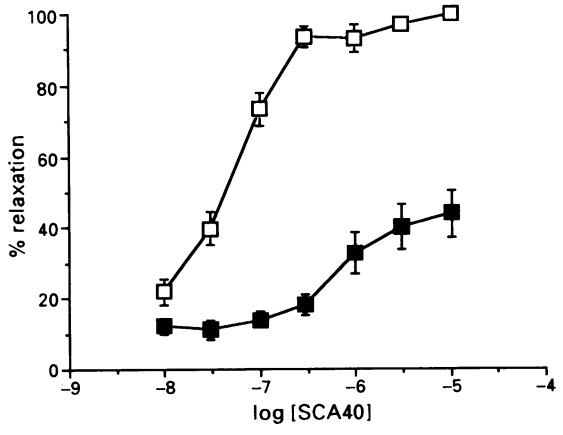

Figure 1 Guinea-pig isolated trachealis: relaxant activity of SCA40 against established contraction to KCl 20 mM (□) and KCl 80 mM (■). Abscissae: − log molar concentration of SCA40. Ordinate scale: percentage reduction in responses to KCl 20 mM or KCl 80 mM as appropriate. Each point is the mean derived from at least 6 experiments, vertical lines show sample s.e.mean values. All experiments were carried out in the presence of 1.4 μM indomethacin.

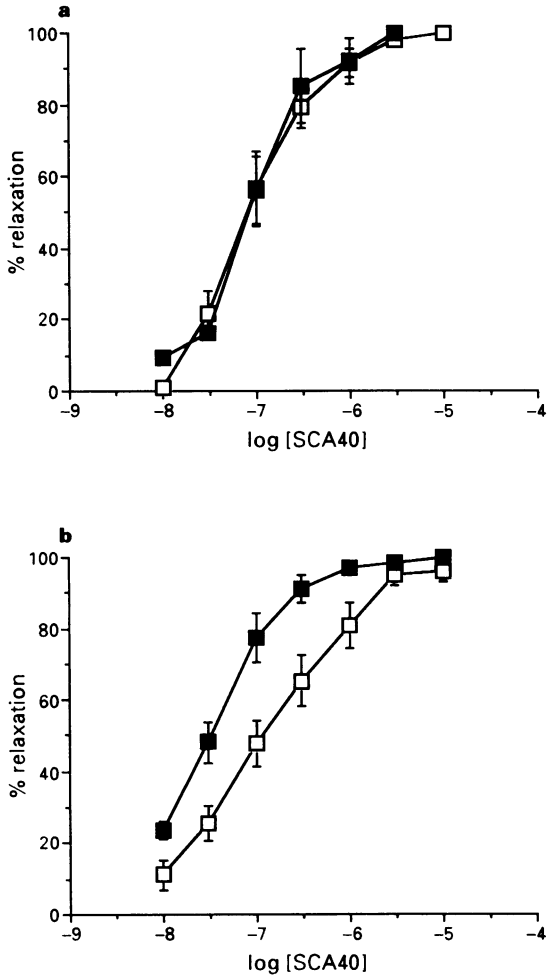

Figure 2 Guinea-pig isolated trachealis: relaxant activity of SCA40 against established contraction to KCl 20 mM in absence (■) or in presence of (a): glibenclamide 3 μM (□) and (b): quinine 30 μM (□). Abscissae: – log molar concentration of SCA40. Ordinate scale: percentage reduction in responses to KCl 20 mM. Each point is the mean derived from at least 6 experiments, vertical lines show sample s.e.mean values. All experiments were carried out in the presence of 1.4 μM indomethacin.

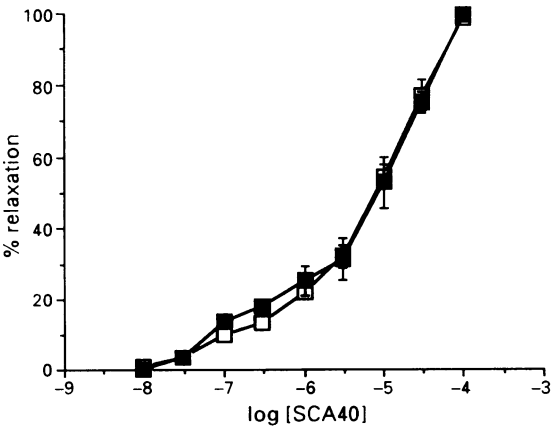

Figure 3 Guinea-pig isolated trachealis: the effects of glibenclamide (3 μM) on the relaxant action of SCA40 against established contraction to carbachol 1 μM. The log concentration-effect curves were obtained in the absence of glibenclamide (■) or in the presence of glibenclamide 3 μM (□). Abscissae: – log molar concentration of SCA40. Ordinate scale: percentage reduction in responses to carbachol 1 μM. Each point is the mean derived from at least 6 experiments, vertical lines show sample s.e.mean values. All experiments were carried out in the presence of 1.4 μM indomethacin.

1 μM represented 85 to 95% of the maximum contraction that could be induced by maximal concentrations of carbachol (10 μM). Under these conditions, SCA40 induced a complete and dose-dependent relaxation of carbachol-induced tracheal contraction. In the presence of glibenclamide 3 μM, the control concentration-response curves to SCA40 was not altered (Figure 3 and Table 1).

Effects of charybdotoxin on the relaxant actions of SCA40 and isoprenaline

Cumulative concentration-response curves to isoprenaline or SCA40 on isolated guinea-pig trachea precontracted with carbachol 1 μM in the presence or absence of ChTX 60, 180 or 600 nM are shown in Figure 4. In these conditions, ChTX 60, 180 and 600 nM did not produce any further increase in the carbachol-induced baseline tone and thus no functional antagonism of relaxation induced by ChTX should be expected. The non-selective β-adrenoceptor agonist, isoprenaline and SCA40 induced a complete relaxation of carbachol-induced tracheal contraction. The negative log EC_{50} for control isoprenaline and control SCA40 were 8.64 ± 0.10 and

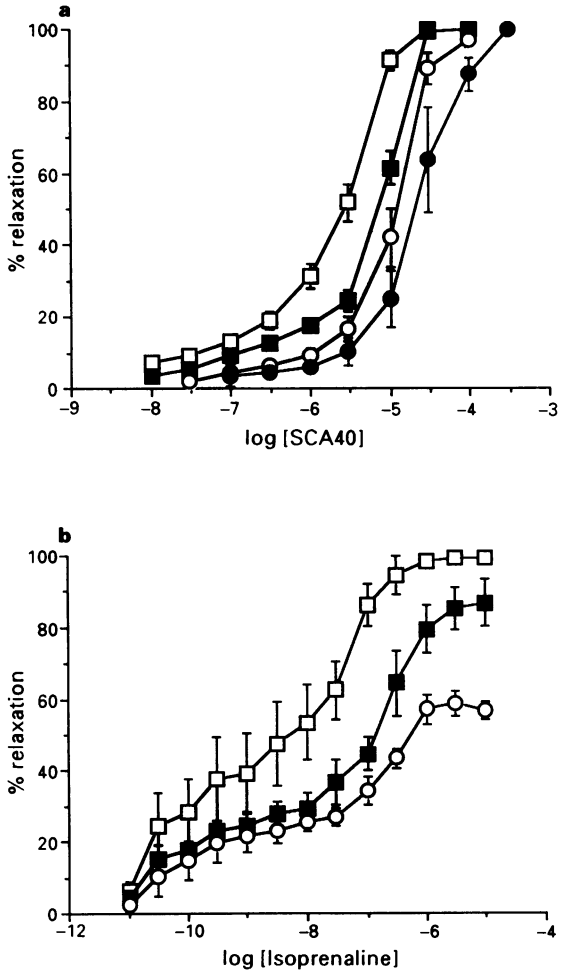

Figure 4 Guinea-pig isolated trachealis: the effects of charybdotoxin (ChTX) on the relaxant action of SCA40 (a) and isoprenaline (b) against established contraction to carbachol 1 μM. The log concentration-effect curves were obtained in the absence of ChTX (□) or in the presence of ChTX 60 mM (■), ChTX 180 mM (○) and ChTX 600 nM (●). The effects of ChTX 600 nM on the relaxant activity of isoprenaline were not tested. Abscissae: (a) – log molar concentration of SCA40, (b) – log molar concentration of isoprenaline. Ordinate scale: percentage reduction in responses to carbachol 1 μM. Each point is the mean derived from at least 6 experiments, vertical lines show sample s.e.mean values. All experiments were carried out in the presence of 1.4 μM indomethacin.

Table 1 Effects of charybdotoxin (ChTX) and glibenclamide on the relaxant activity of SCA40 and isoprenaline in guinea-pig isolated trachea contracted with 1 μM carbachol

Treatment	n	*− log EC_{50}*	*% maximum relaxation*
Isoprenaline			
Control	8	8.64 ± 0.10	99.4 ± 0.6
ChTX 60 nM	6	7.40 ± 0.76*	87.9 ± 7.1*
ChTX 180 nM	6	5.89 ± 0.21*	56.9 ± 2.5*
SCA40			
Control	8	5.92 ± 0.18	100.0 ± 0.0
ChTX 60 nM	6	5.51 ± 0.24	100.0 ± 0.0
ChTX 180 nM	6	5.18 ± 0.31*	97.1 ± 1.9
ChTX 600 nM	6	4.85 ± 0.19*	100.0 ± 0.0
SCA40			
Control	8	5.38 ± 0.12	100.0 ± 0.0
Glibenclamine 3 μM	6	5.40 ± 0.14	100.0 ± 0.0

Data are mean (± s.e.mean) values ($n = 6-8$) of pD_2 (− log EC_{50}) and percentage maximum relaxation.
*Indicates a significant difference from the corresponding values in control tissues (two-tailed unpaired t test). All experiments were carried out in the presence of 1 μM carbachol and 1.4 μM indomethacin.

5.92 ± 0.18 respectively. In the presence of ChTX 60 and 180 nM, the control concentration-response curves to isoprenaline were shifted to the right 17 and 563 fold, respectively, and the extent of relaxation to isoprenaline was reduced to 87.9 ± 0.6 and 56.9 ± 2.5% respectively of the control maximum relaxation (Figure 4b and Table 1). As ChTX 180 nM largely blocked the relaxant activity of isoprenaline, and considering the high cost of the toxin, the effects of ChTX 600 nM on the relaxant activity of isoprenaline have not been tested. In the same manner, the control concentration-response curves to SCA40 in the presence of ChTX 60, 180 and 600 nM were shifted to the right 2.6, 5.5 and 11.7 fold, respectively but the control maximum relaxation was unchanged (Figure 4a and Table 1).

Discussion

SCA40 is a newly synthesized imidazo[1,2-*a*]pyrazine derivative which exhibits potent smooth muscle relaxant properties *in vitro* (Michel *et al.*, 1990), potent anti-bronchospasm activity *in vivo* and a moderate cyclic AMP phosphodiesterase inhibitory activity (Bonnet *et al.*, 1992). It is therefore unlikely that an increase in cyclic AMP formation due to the inhibition of cyclic AMP phosphodiesterase could totally explain the potent SCA40 smooth muscle relaxant activity. Thus, other mechanisms of action have to be considered. In guinea-pig isolated trachea, SCA40 is able to inhibit completely the contractions induced by low concentrations of KCl (20 mM) as opposed to high concentrations (80 mM) of KCl. Such a pharmacological profile has been described for K^+-channel openers (Hamilton *et al.*, 1986; Robertson & Steinberg, 1990) which are able to block smooth muscle contractions induced by low K^+ concentrations (20 mM), but not high depolarizing K^+ concentrations (80 mM). With high depolarizing K^+ concentrations, potassium equilibrium potential and cell membrane potential are so close that hyperpolarization induced by K^+-channel opening is too weak to close voltage-operated Ca^{2+}-channels. Interestingly, SCA40, at high concentrations (10 μM) retained some relaxant activity against the spasm induced by 80 mM KCl (40% of the maximum relaxation that could be achieved against 20 mM KCl-induced contraction). This relaxant activity of SCA40 at high concentrations might be attributed to its cyclic AMP phosphodiesterase inhibitory properties.

In the presence of glibenclamide, a known ATP-sensitive K^+-channel blocker (Quast & Cook, 1989), the relaxant activity of SCA40 against 20 mM KCl-induced contraction was not altered. Similarly, glibenclamide failed to antagonize the tracheal relaxant activity of SCA40 against 1 μM carbachol-induced contraction. These results suggest that, as opposed to the known K^+-channels openers cromakalim, lemakalim or pinacidil, ATP-sensitive K^+-channels are not involved in the pharmacological activity of SCA40. On the contrary, the relaxant activity of SCA40 was antagonized in the presence of quinine, a non-selective blocker of Ca^{2+}-activated K^+-channels (Haylett & Jenkinson, 1990).

The relaxant activity of SCA40 and isoprenaline has been evaluated in presence of ChTX, an agent known to block the large-conductance Ca^{2+}-dependent K^+-channel (Cook & Quast, 1990), especially in guinea-pig isolated trachea (Murray *et al.*, 1991). As potassium is a potent blocker of ChTX binding in smooth muscle (Vasquez *et al.*, 1989), the smooth muscle relaxant activity of SCA40 and isoprenaline, in the presence and absence of ChTX, have not been evaluated in guinea-pig isolated trachea contracted with KCl but with 1 μM carbachol. ChTX (60 and 180 nM) induced a non-competitive inhibition of isoprenaline relaxant activity, i.e; a rightward shift in the concentration-response curves with decrease in the maximum response. These observations are in agreement with previous findings that ChTX was able to produce a non-competitive inhibition of isoprenaline or salbutamol-induced relaxation of guinea-pig isolated trachea (Jones *et al.*, 1990). In contrast, ChTX (60, 180, 600 nM) induced an apparently competitive inhibition of SCA40 relaxant activity (rightward shift in the concentration-response curves) with no significant alteration in the maximum response. These results indicate that ChTX-sensitive K^+-channels are involved in both SCA40 and isoprenaline relaxant activity in guinea-pig trachea.

SCA40 and isoprenaline could activate different ChTX-sensitive K^+-channels since ChTX has been reported to block not only the large conductance Ca^{2+}-dependent K^+-channel but also the intermediate Ca^{2+}-dependent K^+-channel (Haylett & Jenkinson, 1990), an n type voltage-dependent K^+-channel in human T lymphocytes (Attali *et al.*, 1992) and recently, a small conductance Ca^{2+}-dependent K^+-channel unaffected by apamin in rat aortic smooth muscle cells (Van Renterghem & Lazdunski, 1992). However, airway smooth muscle cell membranes from different species (McCann & Welsh, 1986; Huang *et al.*, 1987; Green *et al.*, 1991) are characterized by a dense distribution of large conductance Ca^{2+}-dependent K^+-channels and isoprenaline, a potent bronchodilator, has been reported to activate these K^+-channels in guinea-pig trachealis cell membranes (Jones *et al.*, 1990; Murray *et al.*, 1991). It is possible therefore that SCA40, which is also a potent bronchodilator, might activate the large Ca^{2+}-dependent K^+-channel in guinea-pig trachealis cell membranes.

The β-adrenoceptor agonist, isoprenaline and theophylline, a cyclic AMP phosphodiesterase inhibitor, both produce an elevation of intracellular cyclic AMP and are able to activate the large Ca^{2+}-dependent K^+-channel (Jones *et al.*, 1990; Murray *et al.*, 1991). Kume *et al.* (1989) have reported that isoprenaline opened large-conductance Ca^{2+}-dependent K^+-channel of rabbit trachealis cell membranes by activating protein kinase A and phosphorylation of some regulatory protein(s) on the cytoplasmic side of the membrane. In these conditions, it is easily understood that ChTX, which blocks the Ca^{2+}-dependent K^+-channel at the external pore of the membrane (Gimenez-Gallego *et al.*, 1988), non-competitively inhibited the relaxant activity of isoprenaline. In contrast, ChTX inhibited the relaxant activity of SCA40 in an apparently competitive manner which suggests a different mechanism of action. It could also be mentioned that, if the opening of K^+-channels is involved in the relaxant activity of isoprenaline or theophylline, it is not the only mechanism implicated since isoprenaline (Allen *et al.*, 1985) and theophylline (Small *et al.*, 1989) are able to relax completely guinea-pig trachea in a very K^+-rich (120 mM) medium. In contrast,

SCA40, even at high concentration (10 μM), induced only a partial relaxation in 80 mM KCl-contracted trachea. However, under these conditions the cyclic AMP phosphodiesterase activity of SCA40 seems to play a minor role in its relaxant activity.

All these results indicate that SCA40 may be the first known agent of a new class of K^+-channel openers, involving ChTX-sensitive K^+-channels. Among these ChTX-sensitive K^+-channels, only the large conductance Ca^{2+}-dependent K^+-channel has so far been described in trachealis cells and it might be thought that SCA40 relaxant activity involves this K^+-channel. Patch-clamp studies have to be performed in order to confirm this hypothesis. With the development of such compounds will come a greater understanding of the major role that K^+ channels play in the regulation of smooth muscle excitability. As airway smooth muscle cells are richly endowed with the large conductance Ca^{2+}-dependent K^+-channels, such compounds have potential interest as bronchodilators in the treatment of asthma.

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