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Effects of SCA40 on human isolated bronchus and human polymorphonuclear leukocytes: comparison with rolipram, SKF94120 and levcromakalim

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1 SCA40 (0.1 nM-0.1 mM) produced concentration-dependent suppression of the spontaneous tone of human isolated bronchus ($-\log EC_{50} = 6.85 \pm 0.09$; $n = 10$) and reached a maximal relaxation similar to that of theophylline (3 mM). The potency ($-\log EC_{50}$ values) of SCA40 compared to other relaxants was rolipram (7.44 ± 0.12; $n = 9$) > SCA40 ≥ levcromakalim (6.49 ± 0.04; $n = 6$) > SKF94120 (5.87 ± 0.10; $n = 9$).

2 When tested against the activity of the isoenzymes of cyclic nucleotide phosphodiesterase (PDE) isolated from human bronchus, SCA40 proved highly potent against PDE III ($-\log IC_{50} = 6.47 \pm 0.16$; $n = 4$). It was markedly less potent against PDE IV (4.82 ± 0.18; $n = 4$) and PDE V (4.32 ± 0.11; $n = 4$).

3 Human polymorphonuclear leukocytes (PMNs) stimulated with *N*-formylmethionyl-leucyl-phenylalanine (FMLP) produced a concentration-dependent superoxide anion generation and elastase release. SCA40 (1 nM-10 μM) produced a concentration-related inhibition of FMLP (30 nM ~ EC_{50})-induced superoxide production ($-\log IC_{50} = 5.48 \pm 0.10$; $n = 6$) and elastase release ($-\log IC_{50} = 5.50 \pm 0.26$; $n = 6$). Rolipram was an effective inhibitor of superoxide generation and elastase release ($-\log IC_{50}$ values ~8) while SKF94120 and levcromakalim were scarcely effective.

4 FMLP (30 nM) and thimerosal (20 μM) induced leukotriene B_4 production and elevation of intracellular calcium concentration in human PMNs. The production of leukotriene B_4 was inhibited by SCA40 in a concentration-related manner ($-\log IC_{50} = 5.94 \pm 0.22$; $n = 6$) but SCA40 was less effective against the elevation of intracellular calcium. Rolipram was an effective inhibitor of leukotriene B_4 synthesis ($-\log IC_{50}$ ~7) and intracellular calcium elevation ($-\log IC_{50}$ ~6) while SKF94120 and levcromakalim were scarcely effective.

5 It is concluded that SCA40 is an effective inhibitor of the inherent tone of human isolated bronchus. The bronchodilatation produced by SCA40 appears mainly related to PDE inhibition since the potency of SCA40 as a relaxant of human isolated bronchus was found to be close to its potency as inhibitor of PDE III activity isolated from human bronchus. In addition, SCA40 exhibited inhibitory effects on human PMN function stimulated by FMLP. These effects may be related to the ability of SCA40 to inhibit PDE IV from human PMNs while the contribution of PDE V inhibition is uncertain. We found no evidence of a role for levcromakalim-sensitive plasmalemmal K^+-channels in human PMNs.

Keywords: Human isolated bronchus; airway relaxation; SCA40; rolipram; SKF94120; levcromakalim; human polymorphonuclear leukocytes; FMLP; superoxide generation; elastase release; leukotriene B_4 production; intracellular Ca^{2+} changes.

Introduction

Inhibitors of cyclic nucleotide phosphodiesterase (PDE) isoenzymes and openers of plasmalemmal K^+-channels are amongst other selective compounds being investigated as potential anti-asthma drugs (Buckle, 1993; Morley, 1994; Nicholson & Shahid, 1994). The possibility that some of these selective compounds may have both bronchodilator and anti-inflammatory properties is particularly attractive for the treatment of asthma.

A series of imidazopyrazine derivatives have been recently synthesized which possess smooth muscle relaxant activity (Bonnet *et al.*, 1992). The most potent of these compounds, SCA40, was initially suggested, on the basis of functional studies in guinea-pig trachea, to exert its relaxant effects by opening large conductance, Ca^{2+}-dependent K^+-channels (BK_{Ca}) of the airway smooth muscle cells (Laurent *et al.*, 1993; Michel *et al.*, 1993). Subsequently, Macmillan *et al.* (1995) demonstrated in patch clamp studies that SCA140 (1-10 μM) was not opening BK_{Ca}. Cook *et al.* (1995) showed that SCA40 is a potent inhibitor of type III isoenzyme of cyclic nucleotide PDE (IC_{50} of 0.24 μM) but it was markedly less potent against other PDE isoenzymes. Therefore, an elevation in the cellular content of adenosine 3':5'-cyclic monophosphate (cyclic AMP) may be underlying plasmalemmal K^+-channel opening with consequential cellular hyperpolarization and relaxation of guinea-pig trachea (Small *et al.*, 1993).

In the present study we have compared the bronchodilator and anti-inflammatory effects of SCA40 with those of the selective PDE III inhibitor, SK94120 (Reeves *et al.*, 1987). In addition, we have used rolipram and levcromakalim as reference drugs. Rolipram is a selective PDE IV inhibitor (Reeves *et al.*, 1987) endowed with bronchodilator and anti-inflammatory properties (Nicholson & Shahid, 1994). Levcromakalim is an ATP-sensitive K^+-channel (K_{ATP}) opener (Hamilton & Weston, 1989) that relaxes airway smooth muscle but little is known on its anti-inflammatory activity (Buckle, 1993). In the present study, bronchodilatation by test compounds was examined on the inherent tone of human isolated bronchus. Polymorphonuclear leukocytes (PMNs) are involved in inflammatory reactions and may have a role in asthma pathogenesis (Giembycz, 1992). The anti-inflammatory activity of test compounds was examined in this study in human polymorphonuclear leukocytes (PMNs) stimulated by the synthetic chemotactic peptide N-formyl-L-methionyl-L-leucyl-

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L-phenylalanine (FMLP) to produce superoxide anion generation, elastase release, and leukotriene (LT) B_4 synthesis. The intracellular levels of Ca^{2+} were also determined in human PMNs.

Methods

Relaxant activity in human isolated bronchus

Experiments were performed as previously outlined (Cortijo *et al.*, 1993). Lung tissue was obtained from patients who were undergoing surgery for lung carcinoma. None of the patients had a history of asthma. After the resection of one or more lung lobes, a piece of macroscopically normal tissue was cut free and submerged in Krebs solution (composition in mM: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.6, $NaHPO_4$ 1.2, $NaHCO_3$ 25 and glucose 11) at 4°C for transport to the laboratory. Once in the laboratory, parts of the bronchus were dissected free from parenchymal lung tissue and preparations cut (3–4 mm length × 2–3 mm internal diameter), and then stored in Krebs solution equilibrated with 5% CO_2 in O_2 at 4°C, until used. Experiments were routinely completed within 24 h of the initiation of storage.

For experiments, the bronchial rings were suspended on tissue hooks in 10 ml organ baths containing Krebs solution, gassed with 5% CO_2 in O_2 at 37°C (pH 7.4). Each preparation was connected to a force displacement transducer (Grass FT03) and isometric tension changes recorded on a Grass polygraph (model 7P). The preparations were equilibrated for 60–90 min with changes in bath Krebs solution every 20 min before the addition of drugs. A load of 2 g was maintained throughout the equilibration period and a stable resting level of tone was present immediately before drug administration.

The effects of test compounds (SCA40, SKF94120, rolipram, and levcromakalim) were investigated by adding cumulative concentrations of these to the baths. Responses were allowed time to stabilize before the bath drug concentration was increased (usually within 15 min of addition). Only one concentration-effect curve was constructed with each preparation. Experiments were terminated by the addition of theophylline (3 mM), the effect of which was taken to represent the maximum relaxation in the tissue. Changes in force were measured from isometric recordings and expressed in g. The maximum response (E_{max}) induced with each relaxant agent was expressed as a percentage of the response to theophylline (3 mM). The molar concentration required to produce 50% (EC_{50}) of maximal relaxation was calculated from individual concentration-response curves and transformed into $-\log$ values for statistical purposes. E_{max} and $-\log EC_{50}$ values were considered as estimates of the efficacy and potency of the relaxants.

Inhibition of PDE activity in human bronchus

Individual human bronchi were homogenized in 5 volumes of ice-cold buffer A (composition in mM: Bis Tris 20, sodium acetate 50, benzamidine 2, EDTA 2, β-mercaptoethanol 5, and phenylmethylsulphonylfluoride (PMSF) 50; pH = 6.5). The homogenate was centrifuged at 15000 × *g* for 10 min and the clear supernatant was filtered through 0.22 mm Millex filters. The samples were injected into a MONO-Q HR 5/5 column (1 ml of gel bed, Pharmacia) attached to an FPLC chromatography system and equilibrated in the same buffer. After washing with 15 ml of buffer A, the PDEs were eluted by developing a 20 ml linear sodium acetate gradient from 50 to 1000 mM in buffer A. Flow rate was 1 ml min^{-1} throughout. Fractions of 0.5 ml were collected, analysed and stored as previously described (Gristwood *et al.*, 1992; Cortijo *et al.*, 1993). The cyclic nucleotide PDE isoenzymes were identified according to the nomenclature adopted by Beavo & Reifsnyder (1990). Routinely, samples were analysed for cyclic nucleotide PDE activity within 3–4 h after homogenization.

In the present study we were interested only in the isoenzymes types III, IV and V from human bronchial tissue. We found a major peak in all samples, eluting at 0.9 M sodium acetate, which accounted for >50% of total cyclic AMP PDE activity. This peak was identified in a previous study as mainly containing PDE IV activity (Cortijo *et al.*, 1993). To assure the presence of PDE III activity, we selected two patients in which evidence was obtained of inhibition by cyclic GMP (5 μM) of the cyclic AMP hydrolytic activity of this fraction. Co-elution of PDE III and PDE IV activities has been previously obtained in human airways (De Boer *et al.*, 1992). To reduce the contribution of the contaminating isoenzyme, PDE III activity was assessed in the presence of 10 μM rolipram whereas PDE IV activity was evaluated in the presence of 10 μM SKF94120. These concentrations of SKF94120 and rolipram were shown previously to be selective for PDE III and PDE IV, respectively (Nicholson *et al.*, 1989; Shahid *et al.*, 1991). PDE V activity was identified in the fraction eluting at 0.2 M sodium acetate according to our previous work (Cortijo *et al.*, 1993). PDE activity from this fraction showed selectivity in hydrolysing cyclic GMP (1 μM).

The activity of the isoenzymes of cyclic nucleotide PDE was determined according to the radioisotope method of Thompson & Strada (1984). The standard incubation mixture contained, in a final volume of 400 μl, 40 mM Tris-HCl, 5 mM $MgCl_2$, 3.75 mM β-mercaptoethanol, 1 μM ^{3}H-labelled/unlabelled cyclic nucleotide (~200,000 d.p.m.) and SCA40. Substrate was cyclic AMP for PDE III and IV and cyclic GMP for PDE V. In addition, the standard mixture contained rolipram for PDE III or SKF94210 for PDE IV (each at 10 μM). The standard incubation mixture and the enzyme solution were separately preincubated at 30°C for 2 min. Then, the assay was initiated by adding 100 μl of the enzyme solution to the standard incubation mixture and the reaction was carried out at 30°C for 20 min. Inhibition assays were run in duplicate in two different enzyme preparations. The inhibitory potency of SCA40 was determined from different concentrations of SCA40 encompassing the IC_{50}. Appropriate controls with drug vehicle, at the concentrations employed, were carried out to see whether any alteration of enzyme activities was present.

Isolation of human polymorphonuclear leukocytes

Human blood from healthy donors was obtained in heparin. PMNs were separated by standard laboratory procedures including dextransedimentation, subsequent centrifugation on a Ficoll-Paque. (Histopaque 1077, Sigma) density gradient and hypotonic lysis of contaminating red blood cells (Böyum, 1968). The purity of PMN was about 95% and the viability as measured by trypan blue exclusion was >95%. After isolation, cells were stored at 4°C in Krebs-HEPES buffer (composition in mM: NaCl 140, KCl 5, $MgCl_2$ 1, HEPES 10, pH = 7.4; and 0.5 mg ml^{-1} bovine serum albumin) for chemiluminescence measurements. For elastase release studies, cells were stored at 4°C in a buffer which consisted of (mM): NaCl 147, KCl 5, KH_2PO_4 1.9, and Na_2HPO_4 1.1 (pH = 7.4). For determination of leukotriene B_4 and intracellular calcium levels, cells were stored at 4°C in NaCl/P_i (composition in mM: NaCl 137, KCl 2.7, Na_2HPO_4 8.1, KH_2PO_4 1.5, pH 7.4).

Measurement of superoxide anion generation from human PMN

Superoxide anion generation by PMNs was measured by a chemiluminescence method (Schudt *et al.*, 1991a) by a Perkin Elmer LS50 as chemiluminometer; 10^5 PMN were suspended in an assay volume of 0.5 ml Krebs-HEPES buffer containing 1 mM $CaCl_2$, 5.6 mM glucose, 2 μM microperoxidase and 5 μM luminol. In initial experiments, the concentration-response curve to FMLP was determined. FMLP (3 nM, 10 nM, 20 nM, 30 nM, 100 nM or 1 μM) was added to cell suspension preincubated for 5 min at 37°C, and chemiluminescence was recorded by 5 min. The area under the curve was integrated and

the effect of FMLP was expressed as a percentage of the oxidative burst obtained for FMLP (1 μM). We confirmed that the chemiluminescence signal produced by FMLP (30 nM), a concentration close to its EC_{50} value, was abolished by 50 units of superoxide dismutase indicating the preferential involvement of superoxide. This concentration of FMLP was selected for further experiments in the presence of the drugs studied. In these experiments, cells were preincubated for 5 min at 37°C in the presence of SCA40, SKF94120, rolipram or levcromakalim (each at 1 nM, 10 nM, 100 nM, 1 μM or 10 μM), then FMLP (30 nM) was added and chemiluminescence recorded for 5 min. Experiments with the appropriate drug vehicles were also carried out. The area under the curve was integrated over 5 min and drug-induced reduction was expressed as % inhibition. Inhibitory concentration 50% (IC_{50}) values were then calculated from the concentration-inhibition curves by non-linear regression analysis. Any direct interaction of the compounds with superoxide or the detecting reaction with luminol was excluded for concentrations $\leqslant 10$ μM by measuring superoxide production by the hypoxanthine/xanthine oxidase reaction (Schudt *et al.*, 1991a).

Measurement of elastase release from human PMN

Release of elastase from PMN was measured by a spectrofluorometric method as described by Sklar *et al.* (1982) and De Vries *et al.* (1990). Cells (2×10^6 ml^{-1}) were suspended in a buffer (composition in mM: NaCl 147, KCl 5, $CaCl_2$ 1.5, $MgSO_4$ 0.3, $MgCl_2$ 1, KH_2PO_4 1.9, Na_2HPO_4 1.1, and glucose 5.5) containing MeO-Suc-Ala-Ala-Pro-Val-MCA (substrate) 20 μM, cytochalasin B 1 μg ml^{-1}, and either vehicle or drug. The cell suspension was equilibrated for 5 min at 37°C before addition of stimulus. Fluorescence was recorded by a Perkin Elmer LS50 fluorescence spectrophotometer with the excitation and emission wavelengths at 380 and 460 nm, respectively. After the incubation period, either vehicle or FMLP (3 nM, 10 nM, 30 nM, 100 nM or 1 μM) was added. The initial rate of signal increase was determined, and the enzyme rate was calculated by use of methylcoumarin amide (MCA) as a standard. The enzyme rate was a reflection of the amount of elastase released from the cells. The total elastase content of cells was determined by lysis of the cells in 0.1% Triton X-100 as outlined by Sklar *et al.* (1982). Enzyme release was quantified as a percentage of the total cellular enzyme content measured in cell lysates. A concentration of FMLP of 30 nM, close to its EC_{50} value, was chosen in subsequent experiments in the presence of drugs. In these experiments, cells were preincubated for 5 min at 37°C in the presence of SCA40, SKF94120, rolipram or levcromakalim (each at 1 nM, 10 nM, 100 nM, 1 μM or 10 μM), then FMLP (30 nM) was added and fluorescence recorded. Experiments with the appropriate drug vehicles were also carried out. Drug-induced reduction was expressed as % inhibition. IC_{50} values were then calculated from the concentration-inhibition curves by non-linear regression analysis. To confirm that the drugs under examination were having a direct effect on enzyme activity, samples were stimulated with FMLP (30 nM), and the cells removed by centrifugation, then maximal concentrations of the studied drugs were added to the supernatants. Under these conditions, the drugs did not inhibit elastase activity (data not shown).

Quantitation of leukotriene B_4 in PMN

PMN were resuspended at 10^7 cells ml^{-1} in NaCl/P_i/1 mM Ca^{2+}. Aliquots (1 ml) were preincubated in sterile polypropylene tubes in a shaking water-bath at 37°C for 15 min before the addition of stimulants. Drugs (i.e. SCA40, SKF94120, rolipram or levcromakalim) or their vehicles were added after the first 5 min of preincubation and remained for the rest of the experiment. After 7 min of incubation with drugs thimerosal (20 μM) was added for 3 min followed by addition of FMLP (30 nM) for 5 min. This protocol was derived from Hatzelmann *et al.* (1990) who demonstrated that the addition of thimerosal enhances the response of PMN *in vitro* towards FMLP. Incubations were terminated by immersion of the tubes in ice and the addition of 3 vol of ice-cold methanol. Cells were pelleted by centrifugation at 1500 $\times$ *g* for 20 min at 4°C. The methanolic supernatants (containing leukotrienes released by cells) and extracts of cell pellets treated with 100% methanol for 18 h at 4°C (containing leukotrienes retained intracellularly) were evaporated to dryness in a speed vacuum concentrator, and stored at −80°C before enzyme-immunoassay (EIA). Samples were reconstituted to original volume with ice-cold EIA buffer and leukotriene B_4 was quantitated by EIA as described by the manufacturer of the kit (Biotrak, RPN 223, Amersham Int., UK). Absorbances were measured at 405 nm with a microtitre plate photometer (Multiscan MKII, Labsystems). The assay uses horseradish peroxidase labelled LTB_4 and a rabbit anti-LTB_4. The sensitivity of the assay was 0.3 pg/well (equivalent to 6 pg/ml). Cross-reactivity for 6-trans-LTB_4 and 20-hydroxy-LTB_4 was 16.6% and 2.5%, respectively; cross-reactivity for other related compounds was negligible (<0.004%). Unknown LTB_4 values were quantitated by interpolation on the standard curve (0.3–40 pg/well). Data from EIA were processed by using ELEASY software (Herraez, 1993). Drug-induced reduction was expressed as % inhibition. IC_{50} values were then calculated from the concentration-inhibition curves by non-linear regression analysis.

Measurements of intracellular Ca^{2+}-levels

Measurements of intracellular Ca^{2+}-levels were performed with the fura-2 method as described by Schudt *et al.* (1991a) with modifications. Cells (10^7 ml^{-1}) were suspended in NaCl/P_i containing Ca^{2+} 1 mM, glucose 1 mM, bovine serum albumin 0.5%, and Fura-2/AM 2 μM, and incubated for 45 min at 37°C. After being loaded, PMNs were washed in prewarmed NaCl/P_i (containing Ca^{2+} 1 mM), resuspended as 10^7 cells ml^{-1} and equilibrated in a cuvette for 5 min at 37°C. Then, PMNs were incubated at 37°C for 7 min with a given concentration of test compounds or their vehicles; thimerosal (20 μM) was added for 3 min followed by addition of FMLP (30 nM) for 5 min. The fluorescence intensity of PMNs was monitored (excitation wavelengths 340 and 380 nm, emission wavelength 510 nm) by a spectrofluorometer (Perking Elmer LS50) with thermally controlled cuvette holder and magnetic stirrer. Intracellular Ca^{2+}-concentrations were calculated according to Grynkiewicz *et al.* (1985). Maximal fluorescence was achieved by completely lysing the cells with 0.1% Triton X-100 and minimal fluorescence by complexing calcium with 10 mM EGTA. The fluorescence signal produced by FMLP in the presence of thimerosal is biphasic, with an initial rapid increase followed by a sustained elevation. This later phase is difficult to quantitate due to oscillations of signal (Schudt *et al.*, 1991a). Therefore, instead of calcium levels being measured as a given time point, the area under the curve was integrated over 5 min and drug-induced reduction was expressed as % inhibition. IC_{50} values were then calculated from the concentration-inhibition curves by non-linear regression analysis.

Drugs and solutions/statistical analysis of results

Drug concentrations are expressed in terms of the molar concentration of the active species. The following drugs were used: SKF94120 (5-(4-acetimidophenyl)pyrazin-(1H)-one) and rolipram (synthesized at the Department of Chemistry, Laboratorios Almirall, Barcelona, Spain), SCA40 (6-bromo-8-methylaminoimidazol[1,2-a] pyrazine-2-carbonitrile; Université de Montpellier I), levcromakalim (a gift from SmithKline Beecham Pharmaceuticals, U.K.), theophylline (Sigma-Aldrich Química, S.A., Madrid, Spain). Benzamidine, PMSF (phenylmethylsulphonylfluoride), cyclic AMP and cyclic GMP were from Sigma. [8-^{3}H]-adenosine 3′:5′-cyclic monophosphate and [8-^{3}H]- guanosine 3′:5′-cyclic monophosphate were from Amersham International (U.K.). Fura-2/AM was obtained from Boehringer Mannheim (Germany).

All other chemicals were from standard commercial sources. Water purified on a Milli-Q (Millipore Iberica, Madrid, Spain) system was used throughout. Stock solutions of SKF94120 and rolipram were prepared in 20% polyethyleneglycol 300, and those of SCA40 and levcromakalim in ethanol 30% and 70% respectively.

Data are presented as mean ± s.e.mean. Statistical analysis of results was carried out by analysis of variance (ANOVA) followed by Bonferroni test or by Student's t test as appropriate (InStat, GraphPad software). Significance was accepted when $P<0.05$.

Results

Relaxant activity in human bronchus

SCA40 (0.1 nM–0.1 mM), rolipram (0.01 nM–0.3 mM), SKF94120 (1 nM–0.3 mM) and levcromakalim (1 nM–0.1 mM) each caused concentration-dependent inhibition of the spontaneous tone of human bronchi as shown in Figure 1. SCA40 and the selective PDE inhibitors produced full relaxation (i.e. their maximal relaxation was not significantly different from that obtained with theophylline 3 mM) whereas levcromakalim produced ~80% of relaxation to theophylline (3 mM; Table 1). Rolipram produced a biphasic concentration-response curve whereas the curves of the other relaxants were monophasic (Figure 1). With respect to EC_{50} values, the potency order for relaxation was rolipram > SCA40 ≥ levcromakalim > SKF94120.

Effects of SCA40 on the human bronchial phosphodiesterases

The effects of SCA40 on human bronchial PDE III, IV and V activities are shown in Table 2. SCA40 displayed selectivity for PDE III but it was also an effective inhibitor of PDE IV and V activities.

Influence of drugs on FMLP-induced superoxide release

Stimulation of PMNs with FMLP elicited a concentration-related release of reactive oxygen species (Figure 2a). The chemiluminescence signal increased rapidly after addition of FMLP, reached a peak in 30–60 s, and declined to baseline in 3–5 min. The $-\log EC_{50}$ of FMLP was 7.58 ± 0.09 ($n=6$). FMLP (30 nM ~EC_{50}) was selected in subsequent experiments.

The drugs tested reduced the superoxide anion generation produced by FMLP (30 nM) in a concentration-dependent manner as shown in Figure 2b. IC_{50} values were 3.3 μM for SCA40 and 12.0 nM for rolipram (see Table 3 for $-\log IC_{50}$ values). SKF94120 reached a 50% inhibition with the highest concentration tested (10 μM) while levcromakalim (10 μM) produced ~12% inhibition.

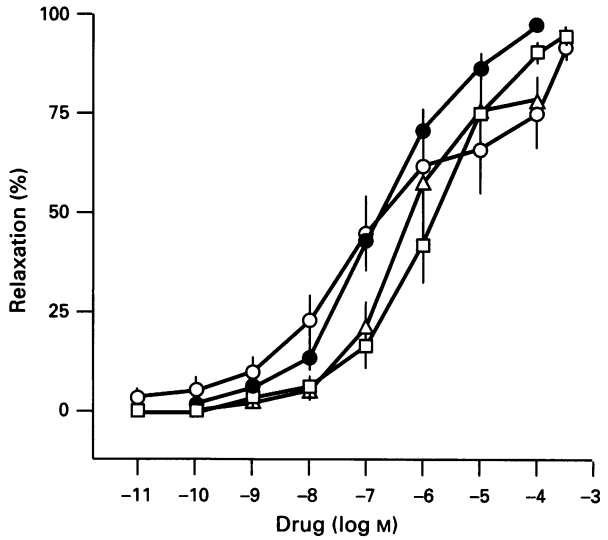

Figure 1 Relaxant effects of SCA40 (●), SKF94120 (□), rolipram (○), and levcromakalim (△) in human isolated bronchi with spontaneous tone. Abscissa scale: $\log_{10}$ molar concentration of drugs. Ordinate scale: relaxation expressed as a percentage of the maximal relaxation produced by theophylline (3 mM). Points are means and vertical lines show s.e.mean; n values are 6–10 from 3 patients as indicated in Table 1.

Influence of drugs on FMLP-induced elastase release

Incubation of human PMN in the presence of FMLP led to a concentration-dependent release of elastase with a $-\log EC_{50}$ value of 7.72 ± 0.08 (Figure 3a). About 85% of the total cellular elastase activity could be released by FMLP (1 μM). The drugs tested reduced FMLP (30 nM ~EC_{50})-induced elastase release in a concentration-dependent manner as shown in Figure 3b. IC_{50} values were 3.2 μM for SCA-40 and 11.5 nM for rolipram (see Table 3 for $-\log IC_{50}$ values). SKF94120 and levcromakalim (each at 10 μM) produced ~30% and ~6% inhibition, respectively.

Influence of drugs on FMLP-induced leukotriene B_4 production

PMNs stimulated by FMLP (1 nM to 1 μM) in the presence of thimerosal (20 μM) produced a concentration-dependent increase in LTB_4 levels (the values in ng/10^7 cells were 56.2 ± 28.1, 256.4 ± 41.9, 430.1 ± 34.1, and 564.8 ± 84.9 for FMLP 1 nM, 10 nM, 100 nM and 1 μM, respectively; $n=4$). FMLP (30 nM) was selected for subsequent experiments in which the inhibitory effects of drugs were tested. The production of LTB_4 promoted by FMLP (30 nM) plus thimerosal (20 μM) was sensitive to the addition of SCA-40 and rolipram in a concentration-dependent manner (IC_{50} values for SCA40

Table 1 Relaxant effects of SCA40, SKF94120, rolipram, and levcromakalim on spontaneous tone of human bronchial preparations

	n	*–log EC_{50}* (M)	*E_{max} (% of theophylline 3 mM)*	*Maximal relaxation* (g) *produced by theophylline* (3 mM)
SCA 40	10	6.85 ± 0.09	98.2 ± 0.5	1.54 ± 0.30
SKF94120	9	5.87 ± 0.10	95.1 ± 2.0	1.55 ± 0.19
Rolipram (first phase)	9	7.44 ± 0.12	66.0 ± 9.6*	–
Rolipram (0.3 mM)	9	–	91.8 ± 3.0	1.59 ± 0.18
Levcromakalim	6	6.49 ± 0.04	78.9 ± 6.0*	1.67 ± 0.13

The concentration-response curve for rolipram was biphasic (see Figure 1) with the first phase producing a plateau taken as the maximal effect from which the EC_{50} value was derived. Values are means ± s.e.mean; n represents the number of preparations obtained from three different patients. ANOVA followed by Bonferroni showed a potency ($-\log IC_{50}$ values) order of 7.44 > 6.85 ≥ 6.49 > 5.87. *$P<0.05$ compared to E_{max} values of the other compounds.

Table 2 Inhibition by SCA40 of the activity of the isoenzymes of cyclic nucleotide phosphodiesterase types III, IV, and V isolated from human bronchus

Type III	*Type IV*	*Type V*
6.47±0.16*	4.82±0.18	4.32±0.11
(5.95–7.00)	(4.25–5.40)	(3.97–4.67)

Data are shown as $-\log IC_{50}$ values (mean±s.e.mean of 4 experiments from 2 patients; the upper and lower 95% confidence limits are given below in parentheses). *$P<0.05$ compared to values for PDE IV and V.

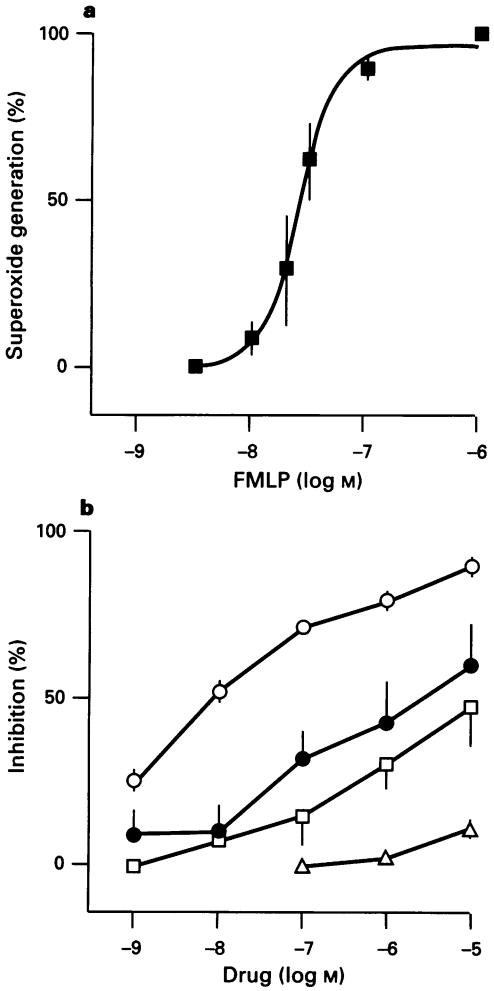

Figure 2 FMLP-stimulated superoxide anion generation in human neutrophils. Cells 10^5 (0.5 ml) were exposed to FMLP and chemiluminescence recorded for 5 min at 37°C. (a) Concentration-response curve for FMLP. (b) Inhibition by SCA40 (●), SKF94120 (□), rolipram (○), and levcromakalim (△) of FMLP (30 nM ~EC_{50})-induced chemiluminescence. Data are derived from 6 different PMN preparations and given as mean±s.e.mean (vertical lines).

and rolipram were 5.75 μM and 0.30 μM, respectively; see Table 3). SKF94120 and levcromakalim produced inhibitions of less than 10% for the highest concentrations tested (Figure 4a).

Influence of drugs on FMLP-induced increase of intracellular Ca^{2+} levels

Changes in intracellular Ca^{2+} levels were monitored in Fura-2 loaded PMNs. Baseline values of $[Ca^{2+}]_i$ were 82.3±5.1 nM ($n=24$). Addition of thimerosal (20 μM) alone caused a slow and progressive increase in intracellular Ca^{2+} to levels below 150 nM. Addition of FMLP (30 nM) in the presence of thimerosal (20 μM) resulted in a rapid initial increase in intracellular Ca^{2+} concentration (462.7±34.6; $n=6$) followed by a sustained elevation. The value of the initial peak of intracellular Ca^{2+} was not affected by the drugs tested (data not shown). SCA40 ($IC_{50}>10$ μM) and rolipram ($IC_{50}=0.32$ μM) depressed the later phase of sustained elevation of intracellular Ca^{2+} (assessed as area under the curve over 5 min) while SKF94120 and levcromakalim had minor effects (Figure 4b, Table 3).

Discussion

Relaxation of human isolated bronchus by SCA40 and the role of cyclic nucleotide phosphodiesterase inhibition

We have shown in this study that SCA40, a drug recently characterized as a relaxant of guinea-pig trachea (Laurent *et al.*, 1993; Cook *et al.*, 1995), also has relaxant properties in human isolated bronchus. SCA40 inhibited, in a concentration-related manner, the inherent tone of human isolated bronchus and reached a maximal relaxation similar to that of theophylline (3 mM). In addition, we have confirmed the relaxant activity of selective PDE III (SKF94120) and PDE IV (rolipram) inhibitors (De Boer *et al.*, 1992; Cortijo *et al.*, 1993; Qian *et al.*, 1993; Rabe *et al.*, 1993), as well as that of the potassium channel opener levcromakalim (Black *et al.*, 1990; Cortijo *et al.*, 1992) in human respiratory muscle *in vitro*.

SCA40 was a potent inhibitor of PDE III isolated from human bronchus while its potency as inhibitor of PDE IV and PDE V was one to two orders of magnitude below (this study). These results are remarkably consistent with those obtained for SCA40 as inhibitor of PDEs, III, IV and V separated from human platelets, human neutrophils, and human lung homogenates, respectively (Cook *et al.*, 1995). The potency of SCA40 as an inhibitor of spontaneous bronchial tone ($-\log IC_{50}=6.85\pm0.09$) was close to its potency as inhibitor of PDE III activity ($-\log IC_{50}=6.47\pm0.16$). A number of selective PDE III inhibitors have been consistently shown to inhibit the spontaneous and stimulated tone of human bronchial preparations with $-\log IC_{50}$ values in the range of those obtained for inhibition of PDE III activity (De Boer *et al.*, 1992; Rabe *et al.*, 1993; Torphy *et al.*, 1993; this study). These findings indicate that PDE III inhibition may contribute to the relaxant activity of SCA40 in human isolated bronchus as previously suggested for relaxation in guinea-pig trachea (Cook *et al.*, 1995).

Table 3 Influence of SCA40, SKF94120, rolipram, and levcromakalim on FMLP-stimulated PMN

	Superoxide	*Elastase*	*LTB_4*	*Ca_i*
SCA40	5.48±0.10	5.50±0.26	5.24±0.22	<5
SKF94120	~5	<5	<5	<5
Rolipram	7.92±0.17	7.94±0.16	6.52±0.26	6.49±0.16
Levcromakalim	<5	<5	<5	<5

$-\log IC_{50}$ values for inhibition of superoxide release, elastase release, production of LTB_4 and Ca_i levels (measured as area under the curve during 3 min after FMLP addition) are summarized. Data are mean±s.e.mean of 6 experiments.

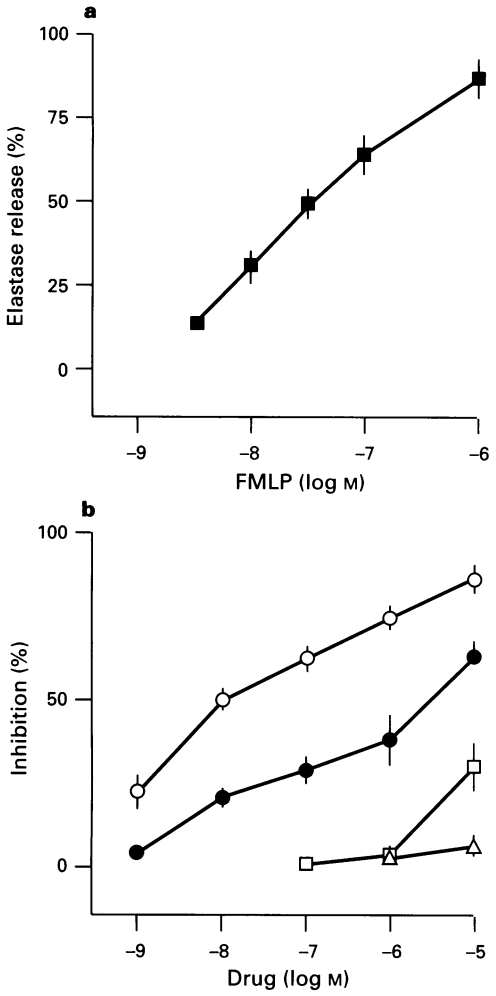

Figure 3 Elastase release from human neutrophils in response to FMLP. The data are presented as an increase in fluorescence resulting in cleavage of the substrate MeO-Suc-Ala-Ala-Pro-Val-MCA. Cell suspensions (0.7×10^6 cells ml^{-1}) were equilibrated for 5 min at 37°C before addition of vehicle or FMLP. (a) Concentration-dependent activation of human PMNs by FMLP expressed as elastase release (%). (b) Inhibition by SCA40 (●), SKF94120 (□), rolipram (○), and levcromakalim (△) of FMLP (30 nM ~EC_{50})-induced fluorescence. Data are derived from 6 different PMN preparations and given as mean ± s.e.mean (vertical lines).

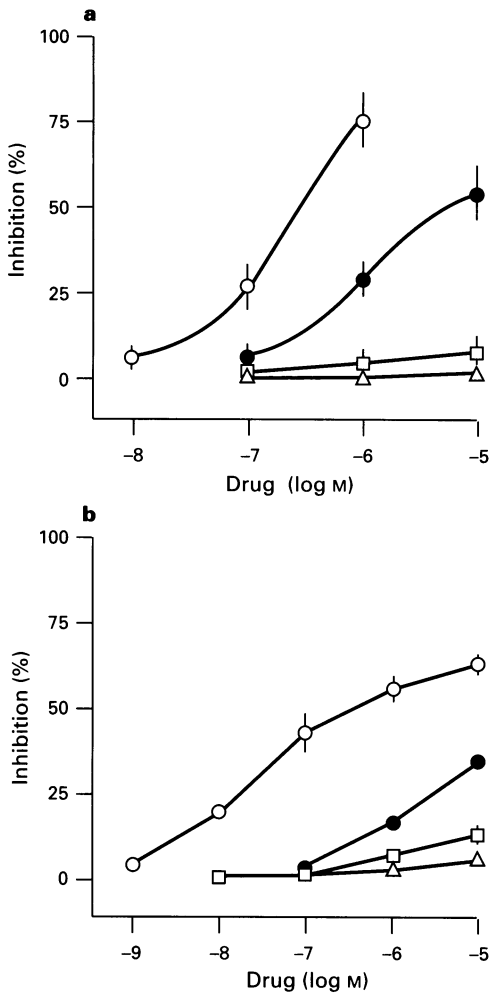

Figure 4 Influence of SCA40 (●), SKF94120 (□), rolipram (○), and levcromakalim (△) on LTB_4 production (a) and intracellular Ca^{2+} levels (b) in stimulated PMNs (FMLP 30 nM in the presence of thimerosal 20 μM). Data are derived from 6 different PMN preparations and given as means ± s.e.mean (vertical lines).

However, the role of PDE III in the maintenance of human bronchial tone is uncertain. Unlike guinea-pig trachea (Harris *et al.*, 1989; Berry *et al.*, 1991), the cyclic AMP hydrolysing isoenzyme that mainly regulates human bronchial tone is the type IV with the type III playing a supportive role (De Boer *et al.*, 1992; Rabe *et al.*, 1993; Torphy *et al.*, 1993). Thus, PDE III activity is not always separated from human bronchus (De Boer *et al.*, 1992; Cortijo *et al.*, 1993), and when isolated represents only a small percentage (15–20%) of total cyclic AMP PDE activity (De Boer 1992). Furthermore, selective PDE III inhibitors failed to induce increases in intracellular cyclic AMP content in human cultured tracheal smooth muscle cells (Hall *et al.*, 1992). It seems unlikely therefore that SCA40 exerts its relaxant effects primarily via inhibition of PDE III activity in human bronchus.

The potency of SCA40 as an inhibitor of PDE IV ($-\log IC_{50} = 4.82 \pm 0.18$) was considerably less than that for relaxation of bronchial tone (this study). Similarly, the potency of rolipram as an inhibitor of PDE IV ($-\log IC_{50} = 6.2$; Nicholson & Shahid, 1994) is less than that for inhibition of resting tone ($-\log EC_{50} = 7.4-7.7$; Quian *et al.*, 1993; this study). The existence of this difference in inhibitory drug potencies for isolated enzyme vs. isolated bronchus and the importance of PDE IV for the regulation of airway tone are consistent with a role for PDE IV inhibition in the relaxant effect of SCA40 in human bronchus. In addition to PDE III and IV, a role for PDE V in the regulation of mechanical tone in human airways has been suggested (Nicholson & Shahid, 1994). Since SCA40 inhibits PDE V activity as well, it cannot be ruled out that the inhibition of this isoenzyme contributed also to the *in vitro* bronchorelaxant activity of SCA40 in man.

Inhibitory effects of SCA40 on human polymorphonuclear leukocytes stimulated by FMLP

Human PMNs are activated *in vitro* when exposed to chemotactic factors such as FMLP. In this study we have examined FMLP-induced superoxide anion generation and elastase release. FMLP is not sufficient by itself to trigger leukotriene-biosynthesis but addition of the SH-blocker thimerosal results in LTB_4 production by human PMNs (Hatzelmann *et al.*, 1990); This protocol was adopted in the present work. The functional responses examined in this study are considered mediated by intracellular Ca^{2+} mobilisation after FMLP receptor activation. Therefore, we examined also changes in intracellular Ca^{2+} levels in human PMNs loaded with fura-2.

Human PMNs contain in their cytosol two major isoenzymes, the types IV and V, while the presence of PDE III was not detected (Schudt *et al.*, 1991a). Inhibition of the cyclic AMP-specific PDE IV leads to elevation of cyclic AMP levels and the subsequent inhibition of a number of functional responses of PMNs, presumably through cyclic AMP-dependent

protein kinase-mediated phosphorylation of specific proteins (Mueller & Sklar, 1989). Human PMNs stimulated by FMLP show an exceptional sensitivity towards PDE IV inhibiting drugs while PDE III inhibitors are scarcely effective (Schudt *et al.*, 1991a). By contrast, the consequences of inhibiting the cyclic GMP specific PDE V are not well documented (Giembycz, 1992).

In the present study we have found that SCA40 was capable of inhibiting, in a concentration-dependent way, FMLP(30 nM $\sim EC_{50}$)-dependent superoxide generation, elastase release, and LTB_4 production. Since PDE III was not present in human PMNs (Schudt *et al.*, 1991a), these inhibitory effects cannot be attributed to the inhibition of the activity of this isoenzyme. However, SCA40 is also an inhibitor of PDE IV ($-\log IC_{50} = 4.82$, this study; see also Cook *et al.*, 1995). The $-\log IC_{50}$ values of SCA40 as an inhibitor of PMN functions lies between 5.24 and 5.50 (see Table 3). These results are consistent with a role for PDE IV inhibition in the effects of SCA40 on human PMN since Schudt *et al.* (1991a) demonstrated that the potency of PDE IV inhibitors against FMLP-induced functions is higher than that determined for enzyme inhibition.

Previous studies have shown that selective PDE III inhibitors have inhibitory effects on human PMN functions when added in concentrations sufficient to inhibit PDE IV activity. Thus, motapizone ($-\log IC_{50}$ as PDE III inhibitor is ~ 7 while against PDE IV is ~ 4; Schudt *et al.*, 1991b; Rabe *et al.*, 1993) inhibits superoxide release and production of 5-lipoxygenase metabolites with $-\log IC_{50}$ values ~ 5 (Schudt *et al.*, 1991a). SKF94120 inhibits PDE III activity with $-\log IC_{50}$ values ~ 6 but its potency against PDE IV ($IC_{50} > 30\ \mu M$; Torphy *et al.*, 1993) was lower than that found in this study for SCA40 ($IC_{50} = 15\ \mu M$). Consistent with this finding, we showed that SKF94120 was less effective than SCA40 in inhibiting FMLP-stimulated human PMN functions.

SCA40 also inhibited PDE V with $-\log IC_{50}$ values lower but close to those found against PDE IV (see Table 2; Cook *et al.*, 1995). A role for cyclic GMP in regulating PMN neutrophil activity is suggested by the finding that dibutyryl cyclic GMP, a cell-permeant analogue of cyclic GMP, and nitric oxide donors which activate soluble guanylyl cyclase inhibit superoxide anion generation by human neutrophils (Ervens *et al.*, 1991; Morikawa *et al.*, 1995). However, a number of selective PDE V inhibitors failed to modify PMN function (Giembycz, 1992; Morikawa *et al.*, 1995). Therefore, the contribution of PDE V inhibition to the effects of SCA40 on human PMN is uncertain.

Changes in intracellular Ca^{2+} levels of fura-2 loaded human PMNs were monitored in this study under conditions (stimulation with FMLP in the presence of thimerosal) similar to those used in experiments in which LTB_4 production was measured (Schudt *et al.*, 1991a). We found that inhibition of FMLP-dependent intracellular Ca^{2+} mobilization by SCA40 required concentrations higher than those producing similar inhibitions of LTB_4 formation. FMLP-induced superoxide generation and elastase release were also more sensitive to SCA40 compared to intracellular Ca^{2+} mobilization. The reason for this difference is uncertain. In the human neutrophil the concentration-response curve for FMLP-stimulated Ca^{2+} mobilization lies to the left of those for other FMLP-dependent effects (Rossi *et al.*, 1985; Thompson *et al.*, 1991). Thus, FMLP (30 nM) produces maximal mobilization of intracellular Ca^{2+} (Thompson *et al.*, 1991) but represents only the EC_{50} for a number of functional responses (this study; Hatzelman *et al.*, 1990). This difference in sensitivity may explain the finding that SCA40 was more effective against responses at the functional level than against full mobilization of intracellular Ca^{2+}

There is little evidence of a role for plasmalemmal K^+-channels in the function of inflammatory cells (Buckle, 1993). K_{ATP} channels appear to be involved in the neutrophil oxidative burst (Pieper & Gross, 1992). In this study we showed that levcromakalim, in concentrations fully effective as a relaxant of human bronchus, produced minor effects on FMLP-dependent superoxide generation, elastase release, LTB_4 production and intracellular Ca^{2+} mobilization. Cellular hyperpolarization due to plasmalemmal K^+-channel opening has only been observed in guinea-pig trachealis for concentrations of SCA40 in excess of 1 μM and may be mediated by inhibition of PDE activity and elevation in the cellular content of cyclic AMP (Cook *et al.*, 1995). The contribution of plasmalemmal K^+-channel opening to the inhibitory effects of SCA40 ($\geqslant 1\ \mu M$) on human PMN functions cannot be ruled out but probably is of minor importance.

In conclusion, SCA40 is an effective inhibitor of the inherent tone of human isolated bronchus. The bronchodilatation produced by SCA40 appears to be mainly due to PDE inhibition since the potency of SCA40 as a relaxant of human isolated bronchus was close to its potency as inhibitor of PDE III activity isolated from human bronchus. In addition, SCA40 exhibited inhibitory effects on human PMN function stimulated by FMLP. These effects may be related to the ability of SCA40 to inhibit also PDE IV from human PMNs, while the contribution of PDE V inhibition is uncertain. Mixed inhibitors (PDE III and PDE IV, or PDE IV and PDE V; Giembycz, 1992) have been suggested as potential anti-asthma drugs due to the combination of bronchodilator and anti-inflammatory activities. However, further studies are necessary to ascertain the clinical relevance of these observations.

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