Evaluation of the bronchodilator properties of Ro 31-6930, a novel potassium channel opener, in the guinea-pig

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1 Ro 31-6930 (0.001–0.3 μM), cromakalim (0.03–3.0 μM), salbutamol (0.001–0.3 μM) and theophylline (0.3–100 μM) evoked dose-related reductions in guinea-pig spontaneous tracheal tone with IC_{50} values of 0.044, 0.20, 0.021 and 21.0 μM respectively. All four agents also relaxed tone supported by betahistine, carbachol, 5-hydroxytryptamine (5-HT), leukotriene D_4 (LTD_4), U46619 and prostaglandin D_2 (PGD_2). The order of potency of tracheal relaxants was always salbutamol > Ro 31-6930 > cromakalim > theophylline.

2 All four agents evoked dose-related reductions in 5-HT- and histamine-induced bronchoconstriction in pithed vagotomised guinea-pigs. The dose of Ro 31-6930 producing 50% inhibition of a 5-HT bronchoconstriction was 11.6 $\mu\text{g kg}^{-1}$ and the dose producing 50% inhibition of a histamine bronchoconstriction was 4.4 $\mu\text{g kg}^{-1}$. Salbutamol was approximately 4–5 times more potent than Ro 31-6930 whilst cromakalim was approximately 10 times less potent than Ro 31-6930 as a bronchodilator. Theophylline was markedly less potent than any of the other agents.

3 Ro 31-6930, cromakalim, salbutamol and theophylline each protected conscious guinea-pigs from histamine-induced respiratory distress. Ro 31-6930 and salbutamol were each effective at oral doses of 1.0 and 3.0 mg kg^{-1} whilst cromakalim was effective at oral doses of 3.0 and 10.0 mg kg^{-1} . Theophylline showed activity only at 300 mg kg^{-1} p.o.

4 Ro 31-6930 is a novel potassium channel opener which is a potent relaxant of guinea-pig tracheal smooth muscle *in vitro* and a bronchodilator *in vivo*.

Introduction

Ro 31-6930, 2-(6-cyano-2,2-dimethyl-2H-1-benzopyran-4-yl)-pyridine 1-oxide is a novel pyridine N-oxide derivative possessing smooth muscle relaxant properties. The mechanism of action of Ro 31-6930 is believed to be through the opening of potassium channels, thus hyperpolarizing the plasma membrane (Paciorek *et al.*, 1989a). In some smooth muscle membrane hyperpolarization reduces the entry of calcium through dihydropyridine-sensitive voltage-operated channels, e.g. rabbit aorta (Cook *et al.*, 1988), and may also have an action on ion transport systems such as $\text{Na}^+/\text{Ca}^{2+}$ exchange (Quast & Cook, 1988), resulting in active Ca^{2+} extrusion, or an inhibition of refilling of intracellular Ca^{2+} stores (Bray *et al.*, 1988; Cowlrick *et al.*, 1988). The result of any of these events would be to reduce the availability of Ca^{2+} for contraction of smooth muscle.

It has previously been reported that Ro 31-6930 is a potent relaxant of vascular smooth muscle (Paciorek *et al.*, 1989a). In addition Ro 31-6930 is a potent relaxant of tracheal smooth muscle (Paciorek *et al.*, 1989b) both *in vitro* and *in vivo* thus suggesting it may be of use as a bronchodilator in the treatment of asthma. The aim of this study was to evaluate the bronchodilator properties of Ro 31-6930 in comparison with two standard anti-asthmatic drugs, salbutamol and theophylline, and the potassium channel opener cromakalim, which has been shown to be an effective bronchodilator both in experimental animals (Arch *et al.*, 1988) and man (Williams *et al.*, 1988). A preliminary account of some of these findings has been given previously (Paciorek *et al.*, 1989b).

Methods

In vitro inhibition of spontaneous and agonist-induced tone

Female Dunkin-Hartley guinea-pigs (400–500 g) were killed by a blow to the head and exsanguinated. Segments of trachea (≈ 4 rings) were removed, cut diametrically opposite the trachealis muscle, opened out and placed in organ baths contain-

ing Krebs solution of the following composition: (mm) NaCl 118, NaHCO_3 25, D-glucose 10.5, KCl 4.7, MgSO_4 0.49, KH_2PO_4 1.2 and CaCl_2 2.5. The Krebs solution was maintained at 37°C and aerated with 95% O_2 /5% CO_2 . Tissues were placed under 1 g tension and were connected to Grass FTO3 force displacement transducers for the isometric recording of tone. For the assessment of the inhibition of spontaneous tone, tracheae were left to equilibrate for 90 min before test agents were applied. In the case of agonist-induced tone, tracheae were left for 90 min and were then incubated with indomethacin (3 μM) for 60 min to inhibit prostaglandin-mediated spontaneous tone (Farmer *et al.*, 1974). Indomethacin remained in the bathing solution for the remainder of the experiment. Following the 60 min incubation period tracheae were exposed to $\approx \text{EC}_{75}$ concentrations of betahistine (10 μM), carbachol (0.3 μM), 5-hydroxytryptamine (5-HT, 0.6 μM), leukotriene D_4 (LTD_4) (0.02 μM), U46619 (0.1 μM) or prostaglandin D_2 (PGD_2) (2.25 μM). When stable increases in tracheal tone were achieved, inhibitory dose-response curves were constructed to cumulative doses of Ro 31-6930, cromakalim, salbutamol, theophylline or appropriate dilutions of vehicle (<1.0% ethanol in 0.9% w/v NaCl). Drug-induced relaxation of tone was expressed as a percentage of the maximum relaxation to 1 mM aminophylline given at the end of each experiment.

In vivo inhibition of agonist-induced bronchoconstriction in anaesthetized guinea-pigs

Female guinea-pigs (400–500 g) were anaesthetized with urethane (1.75 g kg^{-1} , i.p.). The right jugular vein was cannulated for the administration of test compounds and the left carotid artery for measurement of blood pressure. The trachea was cannulated and animals were subsequently bilaterally vagotomised and pithed. Guinea-pigs were then artificially ventilated with a Palmer pump set at 10 ml kg^{-1} stroke volume and a respiratory rate of 55–60 inflations min^{-1} . Lung resistance was measured by a modified air overflow technique of Konzett & Rössler (1940) where the volume of air not entering the lungs on inspiration was measured by a Gould model 0000 flow transducer connected to a Gould pneumotachograph. Following an initial dose-response curve, $\approx \text{ED}_{65}$ doses

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of 5-HT ($1.5\text{--}8.0\ \mu\text{g kg}^{-1}$ i.v., as base) or histamine ($1.0\text{--}4.0\ \mu\text{g kg}^{-1}$ i.v., as base) were administered every 5 min until reproducible bronchospasm was established. Ro 31-6930, cromakalim, salbutamol, theophylline or appropriate dilutions of vehicle ($<1.0\%$ ethanol in 0.9% w/v NaCl), were then administered i.v. in a cumulative fashion 10 min before each agonist challenge. Inhibition of the bronchoconstriction was expressed as a percentage of the pretreatment air overflow.

In vivo histamine-induced bronchoconstriction in conscious guinea-pigs

Female guinea-pigs ($400\text{--}500\text{ g}$) were placed in a clear perspex container of 2.5 l capacity and challenged with a histamine aerosol generated from a 0.5 mg ml^{-1} solution of histamine diphosphate using a Mistogen E 145 electronic nebulizer. The duration of the period of exposure to the aerosol resulting in respiratory distress, the 'preconvulsive time' (PCT) (Herxheimer, 1952) was recorded. The first sign of respiratory distress was taken as deep abdominal respiration. Guinea-pigs were removed from the perspex container at this time and were fully recovered within 5 min. Animals were then dosed orally with Ro 31-6930, cromakalim, salbutamol, theophylline or vehicle ($<1.0\%$ ethanol in 0.5% carboxymethyl cellulose dissolved in 0.9% w/v NaCl) and the PCT reassessed at 30 min intervals for up to 240 min.

Drugs

Betahistine dihydrochloride (Aldrich), carbachol (Koch-Light), aminophylline, 5-HT hydrochloride, histamine diphosphate, indomethacin, leukotriene D_4 , prostaglandin D_2 , salbutamol hemisulphate, theophylline, 11,9 epoxymethano-PGH $_2$ (U46619, Sigma). Ro 31-6930 and cromakalim were synthesized at Roche Products. For *in vitro* studies and for i.v. administration Ro 31-6930 and cromakalim were dissolved in absolute ethanol and diluted to the required concentrations in 0.9% w/v NaCl. Indomethacin was dissolved in absolute ethanol. All other substances were dissolved in 0.9% w/v NaCl. For oral administration Ro 31-6930 and cromakalim were dissolved in absolute ethanol and diluted to the required concentration in 0.5% carboxymethyl cellulose dissolved in 0.9% w/v NaCl. Salbutamol and theophylline were suspended in 0.5% carboxymethyl cellulose dissolved in 0.9% w/v NaCl.

Statistical analysis

Results are expressed as mean $\pm$ s.e.mean. Statistical evaluation of changes in PCT were performed by a split plot analysis of variance and significance accepted where $P < 0.05$.

Results

In vitro inhibition of spontaneous and agonist-induced tone

Figure 1a shows an original trace of the effects of Ro 31-6930 on spontaneous tracheal tone and also the maximal relaxant effects to 1 mM aminophylline. Ro 31-6930 ($0.001\text{--}0.3\ \mu\text{M}$), cromakalim ($0.03\text{--}3.0\ \mu\text{M}$), salbutamol ($0.001\text{--}0.3\ \mu\text{M}$) and theophylline ($0.3\text{--}100\ \mu\text{M}$) evoked dose-related reductions in spontaneous tracheal tone with IC_{50} values of 0.044 ± 0.019 , 0.2 ± 0.006 , 0.021 ± 0.001 and $21.0 \pm 2.0\ \mu\text{M}$ ($n = 4$) respectively (Figure 1). In each case the relaxation obtained was between $85\text{--}95\%$ of the maximum relaxation obtained with 1 mM aminophylline. This dose of aminophylline produced a complete relaxation of spontaneous and agonist-induced tracheal tone, irrespective of the agonist employed. Over a similar concentration range all four agents evoked dose-related reductions in betahistine, 5-HT, LTD $_4$, U46619 and PGD $_2$ -induced tone. The IC_{50} values obtained are presented in Table 1. The degree of relaxation was between $85\text{--}100\%$ of the maximum relaxation obtained with 1 mM aminophylline. The concentration range over which agents relaxed carbachol-induced tone was approximately 10 fold higher than that needed for the other agonists (Table 1). On carbachol-induced tone, salbutamol and theophylline evoked a relaxation of between $85\text{--}100\%$ of the maximum obtained with aminophylline whilst the potassium channel openers only managed between $60\text{--}70\%$ of the aminophylline maximum. In time-matched control tissues administration of vehicle evoked $<20\%$ reduction in tone.

Irrespective of the agonist employed, salbutamol was consistently the most potent bronchodilator, closely followed by Ro 31-6930. Cromakalim was between $5\text{--}28$ times less potent than Ro 31-6930, whilst theophylline was consistently the least potent agent tested.

Figure 1 (a) An original trace showing the effects of Ro 31-6930 on guinea-pig spontaneous tracheal tone, and also the maximal relaxant effects of 1 mM aminophylline. (b) The effect of Ro 31-6930 $0.001\text{--}0.3\ \mu\text{M}$ (■), cromakalim $0.03\text{--}3.0\ \mu\text{M}$ (●), salbutamol $0.001\text{--}0.3\ \mu\text{M}$ (▲) and theophylline $0.3\text{--}100\ \mu\text{M}$ (▼) on guinea-pig spontaneous tracheal tone. Each data point represents the mean value derived from four experiments, vertical bars show s.e.mean.

Table 1 The effect of Ro 31-6930, cromakalim, salbutamol and theophylline on agonist-induced guinea-pig tracheal tone

Tone	Ro 31-6930	Tracheal relaxant (μM)		Theophylline
		Cromakalim	Salbutamol	
Bethahistine (10 μM)	0.053 ± 0.013	0.35 ± 0.12	0.006 ± 0.001	7.6 ± 2.4
Carbachol (0.3 μM)	0.63 ± 0.32	17.0 ± 3.0	0.12 ± 0.02	53.0 ± 8.0
5-HT (0.6 μM)	0.051 ± 0.009	0.13 ± 0.02	0.011 ± 0.008	9.0 ± 2.0
LTD ₄ (0.02 μM)	0.043 ± 0.009	1.20 ± 0.34	0.012 ± 0.004	7.2 ± 3.0
U46619 (0.1 μM)	0.091 ± 0.023	0.61 ± 0.13	0.016 ± 0.001	53.5 ± 7.8
PGD ₂ (2.25 μM)	0.11 ± 0.04	1.30 ± 0.47	0.008 ± 0.002	44.0 ± 4.0

IC₅₀ values presented are mean ± s.e.mean, n = 4.
In the case of carbachol-induced tone, the maximal relaxation obtained with Ro 31-6930 and cromakalim was only 60–70% of the maximum relaxation to 1 mM aminophylline. In all other cases the relaxation obtained was between 85–100% of that obtained with aminophylline.

In vivo inhibition of agonist-induced bronchoconstriction in anaesthetized guinea-pigs

Ro 31-6930 (1.0–100.0 μg kg⁻¹, i.v.), cromakalim (10.0–1000 μg kg⁻¹, i.v.), salbutamol (1.0–10.0 μg kg⁻¹, i.v.) and theophylline (1000–30 000 μg kg⁻¹, i.v.) evoked dose-related reductions in the bronchoconstriction evoked by 5-HT (Figure 2) with ID₅₀ values of 11.6 ± 2.6, 123 ± 28.0, 4.3 ± 1.1 and 15 600 ± 2300 μg kg⁻¹ (n = 4) respectively. Salbutamol was the most potent bronchodilator with Ro 31-6930 being some 4 times less potent. Cromakalim was approximately 10 times less potent than Ro 31-6930 whilst theophylline was more than 1000 times less potent than Ro 31-6930. Ro 31-6930 (1.0–30.0 μg kg⁻¹, i.v.), cromakalim (10.0–300 μg kg⁻¹, i.v.), salbutamol (0.1–3.0 μg kg⁻¹, i.v.) and theophylline (300–30 000 μg kg⁻¹, i.v.) evoked dose-related reductions in the bronchoconstriction evoked by histamine (Figure 3) with ID₅₀ values of 4.4 ± 0.98, 56.1 ± 15.7, 0.80 ± 0.38 and 8750 ± 1160 μg kg⁻¹ (n = 4) respectively. The rank order of potency was similar to that observed for 5-HT-induced bronchoconstriction. Vehicle-treated animals exhibited <20% reduction in air overflow, throughout the time course of the experiment (data not shown).

The mean pre-dose diastolic blood pressure of pithed, vagotomised guinea-pigs was 13.8 ± 0.8 mmHg (n = 5). Drug treatment had little or no effect on this parameter.

In vivo histamine-induced bronchoconstriction in conscious guinea-pigs

The mean pre-dose preconvulsive time (PCT) was 68.2 ± 3.2 s (n = 54). Ro 31-6930 1.0 and 3.0 mg kg⁻¹ p.o. evoked maximal dose-related increases in the PCT of 68.2 ± 14.8 s and 92.0 ± 21.3 s (n = 6) respectively (Figure 4). A significant

Figure 3 The effect of Ro 31-6930 1.0–30.0 μg kg⁻¹, i.v. (■), cromakalim 10.0–300 μg kg⁻¹, i.v. (●), salbutamol 0.1–3.0 μg kg⁻¹, i.v. (▲) and theophylline 300–30 000 μg kg⁻¹, i.v. (▼) on intravenous histamine-induced bronchoconstriction in pithed vagotomised guinea-pigs. Each data point represents the mean value derived from 4 experiments, vertical bars show s.e.mean.

increase in PCT was observed for up to 90 min following the highest dose employed. Cromakalim 3.0 and 10.0 mg kg⁻¹ p.o. evoked dose-related increases in the PCT with peak increases of 38.5 ± 13.8 s and 74.7 ± 20.7 s (n = 6) respectively (Figure 5). Significant increases in the PCT were observed for 120 min following drug administration. Following the highest doses of both potassium channel openers, guinea-pigs appeared subdued. This may have been due to the vasodilator properties of these agents. Salbutamol 1.0 and 3.0 mg kg⁻¹ p.o. evoked dose-related increases in the PCT with peak increases of 72.7 ± 32.7 s and 155.2 ± 11.4 s (n = 6) respec-

Figure 2 The effect of Ro 31-6930 1.0–100 μg kg⁻¹, i.v. (■), cromakalim 10.0–1000 μg kg⁻¹, i.v. (●), salbutamol 1.0–10.0 μg kg⁻¹, i.v. (▲) and theophylline 1000–30 000 μg kg⁻¹, i.v. (▼) on intravenous 5-hydroxytryptamine-induced bronchoconstriction in pithed vagotomised guinea-pigs. Each data point represents the mean value derived from 4 experiments, vertical bars show s.e.mean.

Figure 4 The effect of vehicle 10.0 ml kg⁻¹, p.o. (●), Ro 31-6930 1.0 mg kg⁻¹, p.o. (■) and 3.0 mg kg⁻¹, p.o. (▲) on preconvulsive time (PCT) in the conscious guinea-pig. Each data point represents the mean value derived from 6 experiments, vertical bars represent s.e.mean. *P < 0.05 split plot ANOVA.

Figure 5 The effect of vehicle 10.0 ml kg^{-1} , p.o. (●), cromakalim 3.0 mg kg^{-1} , p.o. (■) and 10.0 mg kg^{-1} , p.o. (▲) on preconvulsive time (PCT) in the conscious guinea-pig. Each data point represents the mean value derived from 6 experiments, vertical bars show s.e.mean. * $P < 0.05$ split plot ANOVA.

Figure 7 The effect of vehicle 10.0 ml kg^{-1} , p.o. (●), theophylline 100 mg kg^{-1} , p.o. (■) and 300 mg kg^{-1} , p.o. (▲) on preconvulsive time (PCT) in the conscious guinea-pig. Each data point represents the mean value derived from 6 experiments, vertical bars show s.e.mean. * $P < 0.05$ split plot ANOVA.

tively (Figure 6). The duration of action was 150 min following the highest dose administered. Theophylline prolonged the PCT only following a dose of 300 mg kg^{-1} p.o. with a peak increase of $108.7 \pm 27.6 \text{ s}$ ($n = 6$). Significant protection was observed for approximately 150 min following drug administration (Figure 7). All theophylline experiments were terminated early due to the side effects observed, which were consistent with excessive CNS stimulation. Vehicle-treated animals exhibited no change in the PCT over a 4 h observation period.

Discussion

A number of studies have shown potassium channel openers to be effective in reducing agonist-induced contractions in guinea-pig tracheal preparations (Allen *et al.*, 1986; Arch *et al.*, 1988; Nielsen-Kudsk *et al.*, 1988; Paciorek *et al.*, 1989b). In this study Ro 31-6930 and cromakalim, like the currently employed bronchodilators salbutamol and theophylline, were effective in relaxing tracheal tone supported by a variety of agonists. Since a number of spasmogens are implicated in asthma, these data suggest that potassium channel openers may be of particular value as bronchodilators.

Both Ro 31-6930 and salbutamol were potent relaxants of spontaneous tracheal tone with cromakalim being some 10 times less potent than salbutamol. Theophylline was consistently the least potent agent tested. As spontaneous tracheal

tone is believed to be maintained by an interaction of endogenous cyclo-oxygenase products (Coleman & Kennedy, 1980), experiments on the effects of tracheal relaxants on individual agonist-induced tone were performed in the presence of indomethacin.

Cholinergic mechanisms are of great importance in mammalian airways, mediating airway tone and bronchial responsiveness (Olsen *et al.*, 1965; Nadel, 1980). It was observed that both Ro 31-6930 and cromakalim were less potent relaxants of carbachol-induced tone than of any other agonists tested. This potency difference could not be accounted for by experimental errors in the estimation of agonist EC_{75} values although these errors may have contributed to the small variations in IC_{50} values seen for the potassium channel openers with the other agonists. It is believed that a major source of Ca^{2+} for muscarinic receptor-mediated tracheal smooth muscle contraction is intracellular stores (Creese & Denborough 1981). As a result, potassium channel openers, which are believed to impede the movement of Ca^{2+} into the cell are likely to have reduced efficacy against carbachol-induced contractions. The lack of effect of cromakalim against muscarinic responses in the guinea-pig has been previously documented (Allen *et al.*, 1986; Taylor *et al.*, 1988). However, this is not the case in man where cromakalim relaxes carbachol-induced tension in human bronchioles (Taylor *et al.*, 1988). Theophylline did not show such marked differences in potency against carbachol-induced tone. This is perhaps not surprising as its mechanism of action is believed to involve directly intracellular secondary messengers.

The potassium channel openers were potent relaxants of betahistine (Brown *et al.*, 1986)-induced tone. Betahistine is a histamine H_1 receptor agonist which evokes a more sustained contraction of the guinea-pig trachea than histamine. Responses to histamine are more dependent on entry of extracellular calcium into smooth muscle than responses to carbachol (Kirkpatrick *et al.*, 1975; Creese & Denborough, 1981), hence the greater potency of potassium channel openers against this spasmogen.

The potassium channel openers possessed the same order of potency as seen with betahistine-induced tone when tracheal tone was maintained by 5-HT, LTD_4 , U46619, a thromboxane A_2 (TxA_2)-mimetic (Coleman *et al.*, 1981) and PGD_2 . Although 5-HT induces bronchoconstriction in experimental animals, it is not thought to be important in human asthma (Barnes *et al.*, 1988). However, leukotrienes, thromboxanes and prostaglandins are important in asthma (Coleman & Kennedy, 1980). TxA_2 is a potent bronchoconstrictor *in vitro* and may be involved in the potentiation of cholinergic reflexes (Chung *et al.*, 1985), and has been implicated in the bronchoconstriction and airway hyperreactivity induced by PAF (Joseph *et al.*, 1983). LTD_4 is a potent constrictor of human

Figure 6 The effect of vehicle 10.0 ml kg^{-1} , p.o. (●), salbutamol 1.0 mg kg^{-1} , p.o. (■) and 3.0 mg kg^{-1} , p.o. (▲) on preconvulsive time (PCT) in the conscious guinea-pig. Each data point represents the mean value derived from 6 experiments, vertical bars show s.e.mean. * $P < 0.05$ split plot ANOVA.

airways (Weiss *et al.*, 1983) and is a potent stimulant of mucus secretion (Coles *et al.*, 1983). PGD₂ contracts human airway smooth muscle *in vitro* (Gardiner & Collier, 1980) and is a pro-inflammatory agent mediating vasodilatation and histamine release from basophils (Peters *et al.*, 1984).

As observed with histamine, the tracheal contractions produced by the prostanoids are dependent to a large extent upon the influx of extracellular Ca²⁺ (Creese & Denborough, 1981), and hence a greater efficacy against these responses than those obtained with carbachol would be expected. However there is substantial evidence that the mechanism of action of potassium channel openers is not simply a result of the prevention of Ca²⁺ entry via voltage-operated calcium channels, as Arch *et al.* (1988) could show only a partial inhibition of spontaneous tracheal tone with nifedipine whereas cromakalim was highly effective. We have confirmed these findings with nitrendipine (unpublished observations). Additional theories for the mechanism(s) of action of potassium channel openers have been speculated on by Arch *et al.* (1988) and others (see introduction).

In vivo Ro 31-6930 and cromakalim inhibited both 5-HT- and histamine-induced bronchoconstriction in the pithed vagotomised guinea-pig. The results obtained for cromakalim are in broad agreement with those reported by Arch *et al.* (1988), who in addition demonstrated nifedipine to be inactive in this *in vivo* model of bronchoconstriction. The standard anti-asthma drugs, salbutamol and theophylline were also effective in this model. Salbutamol was some 3–5 times more potent than Ro 31-6930 whilst theophylline was markedly less potent.

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