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β-Adrenoceptor subtypes and the opening of plasmalemmal K^+-channels in bovine trachealis muscle: studies of mechanical activity and ion fluxes

P. Chiu, S.J. Cook, [1]R.C. Small, J.L. Berry, J.R. Carpenter, S.J. Downing, R.W. Foster, A.J. Miller & A.M. Small

Smooth Muscle Research Group, Department of Physiological Sciences, University of Manchester, Oxford Road, Manchester, M13 9PT

1 Studies of mechanical activity and $^{86}Rb^+$ efflux have been made in bovine isolated trachealis with the objectives of (a) identifying which of the β-adrenoceptor subtypes mediates the opening of plasmalemmal K^+-channels, (b) gaining further insight into the properties of the novel, long-acting β_2-adrenoceptor agonist, salmeterol and (c) clarifying the role of K^+-channel opening in mediating the mechano-inhibitory actions of agonists at β-adrenoceptors.

2 In bovine trachealis muscle strips precontracted with histamine (460 μM), isoprenaline (0.1 nM–1 μM), procaterol (0.1–10 nM) and salmeterol (0.1–10 nM) each caused concentration-dependent relaxation.

3 ICI 118551 (10 nM–1 μM) antagonized isoprenaline, procaterol and salmeterol in suppressing histamine-induced tone of the isolated trachealis muscle. The antagonism was concentration-dependent. In contrast, CGP 20712A (10 nM–1 μM) failed to antagonize isoprenaline, procaterol or salmeterol.

4 Salmeterol (1– 10 μM) antagonized isoprenaline in relaxing strips of bovine trachea which had been precontracted with carbachol (1 μM).

5 Cromakalim (10 μM), isoprenaline (100 nM–10 μM), procaterol (10 nM–1 μM) and salbutamol (100 nM–10 μM) each promoted the efflux of $^{86}Rb^+$ from strips of bovine trachealis muscle preloaded with the radiotracer. In contrast, salmeterol (100 nM–10 μM) failed to promote $^{86}Rb^+$ efflux.

6 CGP 201712A (1 μM), ICI 118551 (100 nM) and salmeterol (1 μM) did not themselves modify $^{86}Rb^+$ efflux from trachealis muscle strips, nor did they affect the promotion of $^{86}Rb^+$ efflux induced by cromakalim (10 μM). In contrast, CGP 20712A (1 μM) and ICI 118551 (100 nM) were each able to inhibit the promotion of $^{86}Rb^+$ efflux induced by isoprenaline (1 μM) or procaterol (100 nM). Furthermore, salmeterol (10 μM) inhibited isoprenaline (1 μM)-induced promotion of $^{86}Rb^+$ efflux.

7 It is concluded that, in bovine trachealis, activation of either β_1- or β_2-adrenoceptors can promote the opening of $^{86}Rb^+$-permeable K^+-channels in the plasmalemma. The failure of salmeterol to promote plasmalemmal K^+-channel opening may reflect, not its selectivity in activating β_2- as opposed to β_1-adrenoceptors, but rather its low intrinsic efficacy at β_2-adrenoceptors. The opening of plasmalemmal K^+-channels plays a supportive rather than a crucial role in mediating the mechano-inhibitory effects of agonists at β-adrenoceptors acting on trachealis muscle.

Keywords: Trachealis muscle; $^{86}Rb^+$ efflux; β-adrenoceptor subtypes; K^+-channels; isoprenaline; procaterol; salbutamol; salmeterol; CGP 20712A; ICI 118551

Introduction

As background to the experiments described in the preceding paper (Cook *et al.*, 1993) we reviewed the substantial evidence that suggests that agonists at β-adrenoceptors relax airways smooth muscle by mechanisms associated with the opening of plasmalemmal K^+-channels. That such K^+-channel opening is mediated by the activation of β-adrenoceptors is indicated by the ability of propranolol to inhibit the hyperpolarization of airways smooth muscle induced by isoprenaline (Ito & Tajima, 1982; Allen *et al.*, 1985; Honda *et al.*, 1986). However, since propranolol has approximately equal affinity for β_1- and β_2-adrenoceptors, this agent does not help in the identification of the β-adrenoceptor subtype responsible for promoting the K^+-channel opening.

In order to shed more light on (a) the identity of the β-adrenoceptor subtype mediating K^+-channel opening, (b) the importance of K^+-channel opening to the mechano-inhibitory effects of agonists at β-adrenoceptors and (c) the pharmacological properties of salmeterol (Ball *et al.*, 1991; Dougall *et al.*, 1991), we studied mechanical and electrophysiological changes in guinea-pig isolated trachealis (Cook *et al.*, 1993). In these studies of guinea-pig trachealis, the membrane hyperpolarization induced by agonists at β-adrenoceptors was used as an index of plasmalemmal K^+-channel opening. The present work with bovine trachealis muscle was carried out in parallel with the studies of guinea-pig tissue and had identical objectives. In the case of bovine trachealis, we have used drug-induced promotion of $^{86}Rb^+$ efflux as an index of the ability of the agonists at β-adrenoceptors to promote K^+-channel opening. The results of our electrophysiological (Cook *et al.*, 1993) and ion flux measurements (present study) both suggest that (a) activation of either β_1- or β_2-adrenoceptors can promote K^+-channel opening, (b) that K^+-channel opening and the consequential cellular hyperpolarization do not play a crucial role in the process by which agonists at β-adrenoceptors induce relaxation and (c) that salmeterol fails to promote K^+-channel opening by virtue of its low intrinsic efficacy at β_2-adrenoceptors. Some of our findings have been the subject of preliminary reports (Chiu *et al.*, 1992; Cook *et al.*, 1992; Cook & Small, 1992).

[1] Author for correspondence.

Methods

Tissue preparation

Bovine tracheae were collected from the local abattoir and transported to the laboratory immersed in cold Krebs solution. The trachea was opened by cutting longitudinally, diametrically opposite the trachealis muscle. A segment of tracheal wall containing the trachealis muscle was then dissected from the opened organ and pinned out under Krebs solution with the adventitial surface uppermost. Adventitial tissue was carefully dissected from the preparation to expose the dorsal surface of the trachealis muscle. The preparation was then pinned out mucosal surface uppermost and the mucosal and submucosal tissue were carefully removed in order to expose the ventral surface of the trachealis muscle. Strips (approximately 1.25 cm in length) of the cleaned trachealis muscle were prepared by cutting in the longitudinal axis of the muscle bundles.

Mechanical studies of bovine trachealis muscle

Strips of bovine trachealis were set up in Krebs solution containing indomethacin (5.6 μM). Isometric recording of tension changes was performed with an initial, imposed tension of 2 g. Following the initial setting up of each tissue, three exchanges of bath fluid were performed at 15 min intervals. Following the first two exchanges of bath fluid, the imposed tension was readjusted to 2 g. One hour after the initial setting up of each tissue, histamine (460 μM) was added to the bath fluid in order to induce tissue tone. When the contractile response to histamine had equilibrated (20 min) study of a relaxant drug commenced.

The effects of isoprenaline (0.1 nM–1 μM) or procaterol (0.1 nM–10 nM) were studied by constructing, within a single tissue, a cumulative log concentration-effect curve for the relaxant. In both cases, increments in drug concentration were made at 4 min intervals. When the relaxant effect of the highest tested concentration of the agonist at β-adrenoceptors had reached a plateau, aminophylline (1 mM) was added to determine zero tissue tone. All relaxant responses to the agonists at β-adrenoceptors were measured in terms of the maximal response to aminophylline.

In the case of isoprenaline, construction of the initial log concentration-effect curve was followed by washout of the isoprenaline, aminophylline and histamine. An antagonist at β-adrenoceptors (test tissues) or vehicle (time-matched control tissues) was then added to the bath fluid; 45 min later, histamine (460 μM) was again added to the bath fluid and, after a further 20 min, the log concentration-effect curve for isoprenaline was reconstructed in the presence of the antagonist (test tissues) or its vehicle (control tissues). In the case of procaterol only one log concentration-effect curve was constructed in a single tissue. Such tissues were pre-incubated (65 min) with vehicle or an antagonist at β-adrenoceptors before the log concentration-effect curve for procaterol was constructed in the presence of the antagonist or its vehicle. The relaxant effects of salmeterol (0.01–100 nM) were studied in a fashion similar to that described for procaterol. However, only one concentration of salmeterol (tissue contact time 90 min) was tested on each tissue. All relaxant responses to salmeterol were corrected for the tone loss observed over the same period of time in the vehicle-treated control tissues. A further series of experiments was performed in order to assess whether salmeterol acts as a partial agonist at β_2-adrenoceptors. In these experiments salmeterol (1 or 10 μM) was added to the Krebs solution bathing test tissues; 60 min later, carbachol (1 μM) was added in order to induce tissue tone. Once the spasmogenic response to carbachol had reached a plateau (30 min), a cumulative log concentration-effect curve for isoprenaline was constructed (in the presence of salmeterol), essentially as

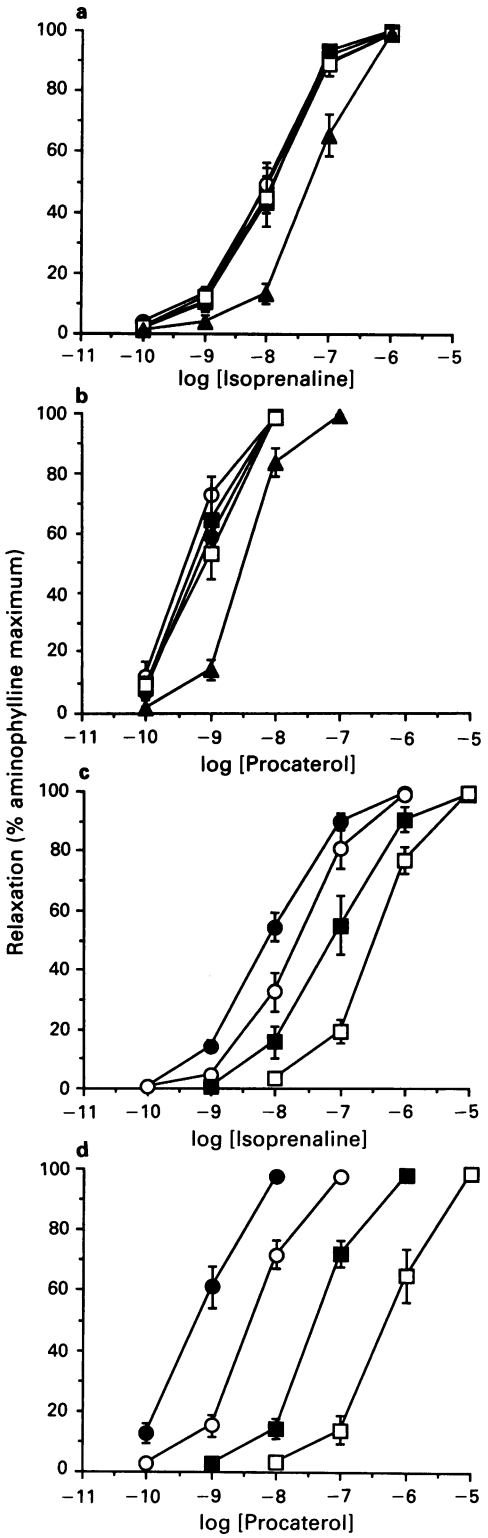

Figure 1 Bovine trachealis pre-contracted with histamine (460 μM): the effects of CGP 20712A and ICI 118551 against the relaxant actions of isoprenaline and procaterol. The abscissae indicate the molar concentration of isoprenaline (panels a and c) or procaterol (panels b and d) on a log scale. The ordinates indicate relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. In each panel, (●) indicates the log concentration-effect curve of the agonist at β-adrenoceptors as observed in the absence of antagonist; (a) and (b) show the effects of isoprenaline and procaterol, respectively, as observed in the presence of CGP 20712A 10 nM (○), 100 nM (■), 1 μM (□) and 10 μM (▲); (c) and (d) show the effects of isoprenaline and procaterol, respectively as observed in the presence of ICI 118551 10 nM (○), 100 nM (■) and 1 μM (□). Data points indicate the means of values from tissue strips from at least 6 tracheae. Where not contained within the plot symbol, vertical bars indicate s.e.mean.

described above. Time-matched control tissues were treated similarly to test tissues but were not exposed to salmeterol.

Studies of $^{86}Rb^+$ efflux from bovine trachealis

The efflux of $^{86}Rb^+$ from bovine trachealis was studied essentially as described by Longmore *et al.* (1991). In brief, strips of bovine trachealis were impaled on hypodermic needles and loaded with $^{86}Rb^+$ by incubation for 90 min in Krebs solution maintained at 37°C and gassed with a mixture of 95% O_2 and 5% CO_2. This loading medium contained 185 kBq ml^{-1} $^{86}Rb^+$ and the Rb concentration was less than 50 μM. Following loading with the radiotracer, each tissue strip was transferred to the first of a series of efflux tubes containing 3 ml of Krebs solution gassed with a mixture of 95% O_2 and 5% CO_2 and maintained at 37°C. Each tissue strip was transferred to the next tube in the series at 4 or 2 min intervals. At the end of the efflux period the contents of each efflux tube was assayed for radioactivity in a gamma counter. Each tissue strip was blotted and similarly assayed for radioactivity. In the case of test tissues, cromakalim (10 μM) or an agonist at β-adrenoceptors was present in the efflux medium for a period of 20 (50 in the case of salmeterol) min. This period of tissue exposure to cromakalim or an agonist at β-adrenoceptors started at least 28 (and generally 40) min after the beginning of the efflux period, at which time the efflux rate coefficient had assumed a relatively low and slowly-changing value. The β-adrenoceptor agonists tested were isoprenaline (100 nM, 1 μM or 10 μM), procaterol (10 nM, 100 nM or 1 μM), salbutamol (100 nM, 1 μM or 10 μM) and salmeterol (100 nM, 1 μM or 10 μM). In the case of time-matched control tissues, the efflux medium, over the same time period, contained the appropriate vehicle for the agonist at β-adrenoceptors. In experiments where CGP 20712A (1 μM), ICI 118551 (100 nM) or salmeterol (1 or 10 μM) were tested as potential inhibitors of the $^{86}Rb^+$ efflux induced by cromakalim or one of the tested agonists at β-adrenoceptors, the inhibitor under test (test tissues) or its vehicle (control tissues) was present in the efflux medium for the whole of the efflux period. For both test and control tissues, the inducer of $^{86}Rb^+$ efflux was added to the bathing medium for a period of 20 min commencing 40 min after the start of the efflux period.

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), CGP 20712A (2-hydroxy-5-(2-((2-hydroxy-3-(4-((1-methyl-4-trifluoro-methyl)-1H-imidazole-2-yl)-phenoxy)propyl)amino)ethoxy)-benzamide monomethane sulphonate; Ciba-Geigy Pharmaceuticals), carbachol chloride (Sigma), cromakalim (SmithKline Beecham Laboratories), histamine hydrochloride (Sigma), ICI 118551 (erytho-DL-1-(7-methylindan-4-yloxyl)-3-isopropylaminobutan-2-ol hydrochloride; ICI Pharmaceuticals), indomethacin (Sigma), (−)-isoprenaline hydrochloride (Sigma), procaterol (Sigma), salbutamol (Glaxo Group Research), salmeterol (Glaxo Group Research).

Stock solutions of most drugs were prepared in twice-distilled water. A stock solution of cromakalim was prepared in 70% ethanol. Isoprenaline, procaterol, salbutamol and salmeterol stock solutions were prepared in 0.1 M HCl. Dilutions of the isoprenaline stock solution were made in distilled water containing 0.57 mM ascorbic acid. The Krebs solution used in the tissue bath experiments and in the studies of $^{86}Rb^+$ efflux had the following composition (mM): Na^+ 143.5, K^+ 5.9, Ca^{2+} 2.6, Mg^{2+} 1.2, Cl^- 127.6, HCO_3^- 25, SO_4^{2-} 1.2, $H_2PO_4^-$ 1.2 and glucose 11.1. This solution (pH 7.4) was maintained at 37°C and was gassed with a mixture of 95% O_2:5% CO_2. The significance of differences between means was assessed by use of a two-tailed, unpaired t test. The null hypothesis was rejected when $P<0.05$.

Results

Mechanical studies of bovine trachealis

The addition of histamine (460 μM) to the indomethacin (5.6 μM)-containing Krebs solution evoked tension development which reached its peak value approximately 15 min after histamine addition. Thereafter the developed tension fell slightly to a plateau, which was either well-sustained or which exhibited a slow decline.

Isoprenaline (0.1 nM–1 μM), procaterol (0.1 nM–10 nM) and salmeterol (0.01 nM–100 nM) each produced concentration-dependent suppression of histamine-induced tone in the trachealis strips. The maximal effects of each of the three agonists at β-adrenoceptors were equivalent to those of aminophylline (Figures 1 and 2). CGP 20712A (10 nM–1 μM) did not itself induce tension development, nor did it modify histamine-induced tone. Furthermore, CGP 20712A (10 nM–1 μM) did not antagonize isoprenaline, procaterol or salmeterol in suppressing histamine-induced tone (Table 1 and Figures 1 and 2). When the concentration of CGP 20712A was raised to 10 μM, it caused minor (five fold, three fold and seven fold, respectively) antagonism of isoprenaline, procaterol and salmeterol (Table 1).

Like CGP 20712A, ICI 118551 (1 nM–1 μM) did not itself induce tension development by the trachealis muscle, nor did it modify histamine-induced tone. However, ICI 118551 produced concentration-dependent antagonism of isoprenaline,

Table 1 The effects of CGP 20712A and ICI 118551 against the actions of some agonists at β-adrenoceptors in suppressing histamine (460 μM)-induced tone of bovine isolated trachealis

	Time-matched control		*CGP 20712A*		
Agonist	(no antagonist)	10 nM	100 nM	1 μM	10 μM
Isoprenaline	7.95 ± 0.07	7.98 ± 0.14	7.88 ± 0.16	7.90 ± 0.16	7.26 ± 0.11*
Procaterol	9.01 ± 0.04	9.28 ± 0.05	9.20 ± 0.13	9.03 ± 0.12	8.48 ± 0.05*
Salmeterol	9.28 (9.13–9.44)	–	–	9.29 (9.04–9.61)	8.42* (8.27 ± 8.59)
	Time-matched control		*ICI 118551*		
Agonist	(no antagonist)	1 nM	10 nM	100 nM	1 μM
Isoprenaline	8.08 ± 0.10	8.19 ± 0.12	7.64 ± 0.14*	7.10 ± 0.19*	6.46 ± 0.07*
Procaterol	9.19 ± 0.11	9.15 ± 0.11	8.37 ± 0.08*	7.37 ± 0.07*	6.33 ± 0.11*
Salmeterol	9.28 (9.13–9.44)	9.23 (9.01–9.48)	8.19* (8.07–8.32)	7.39* (7.24–7.57)	6.34* (6.08–6.68)

For isoprenaline and procaterol the data indicate mean (± s.e.mean) values of $-\log_{10} EC_{50}$. For salmeterol the mean $-\log_{10} EC_{50}$ and its lower and upper 95% confidence limits are shown. For all agents, data from at least 6 tissues contribute to the mean. A dash indicates a measurement not performed.
*indicates a value significantly ($P<0.05$) smaller than that observed in the absence of the relevant antagonist.

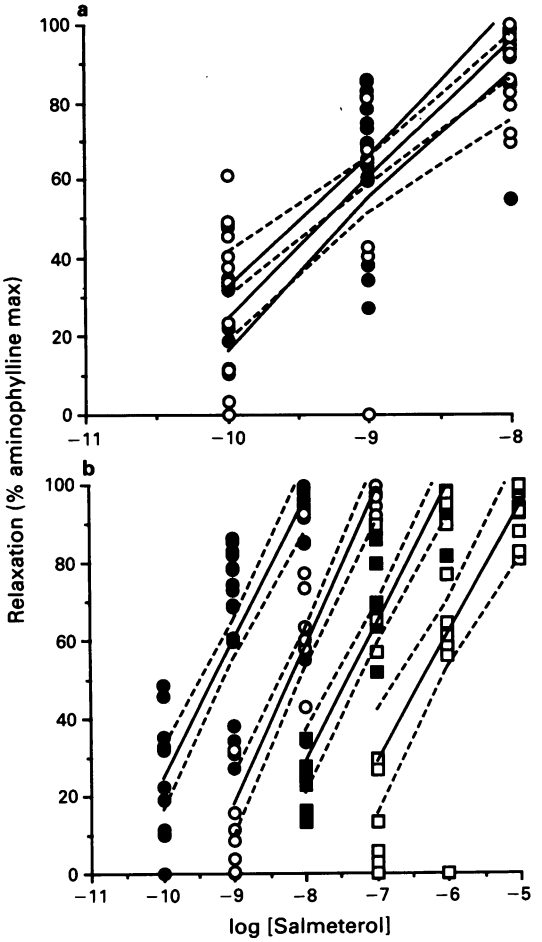

Figure 2 Bovine trachealis pre-contracted with histamine (460 μM): the effects of CGP 20712A and ICI 118551 against the relaxant action of salmeterol. The abscissae indicate the molar concentration of salmeterol on a log scale. The ordinates indicate relaxation expressed as a percentage of the maximal relaxation induced by aminophylline. In (a) the data points indicate responses to salmeterol as observed in the absence (●) and presence (○) of CGP 20712A (1 μM). The linear regression of response upon log molar concentration of salmeterol as observed in the absence of CGP 20712A (1 μM), and its 95% confidence limits are represented by the solid lines. The equivalent regression line and 95% confidence limits observed in the presence of CGP 20712A (1 μM) are represented by the broken lines. In (b) the data points indicate responses to salmeterol as observed in the absence of (●) and presence of ICI 118551 10 nM (○), 100 nM (■), 1 μM (□). In (b) the solid lines indicate the linear regression of response against log molar concentration of salmeterol as observed in the absence and presence of the various concentrations of ICI 118551 tested. The broken lines indicate the upper and lower 95% confidence limits of each regression line.

procaterol and salmeterol (Table 1 and Figures 1 and 2).

The application of carbachol (1 μM) to indomethacin (5.6 μM)-treated strips of bovine trachealis evoked spasm which required 30 min to approach its peak value. In contrast to our observations in equivalent experiments on guinea-pig trachealis (Cook *et al.*, 1993), pretreatment of test strips of bovine trachealis with salmeterol (1 or 10 μM) did not significantly reduce the spasm evoked by carbachol. In control tissues, isoprenaline (1 nM–1 mM) caused concentration-dependent suppression of the spasm evoked by carbachol. Isoprenaline produced similar effects in the salmeterol-treated test tissues. In the presence of salmeterol, however, the log concentration-effect curve for isoprenaline was shifted rightward without change in the maximal response. The mean (± s.e.mean; $n = 6$) $\log_{10}$ concentration ratios for isoprenaline (presence of salmeterol: absence of salmeterol) measured at the level of the half-maximal response were 0.416 ± 0.12 (salmeterol 1 μM) and 0.670 ± 0.15 (salmeterol 10 μM).

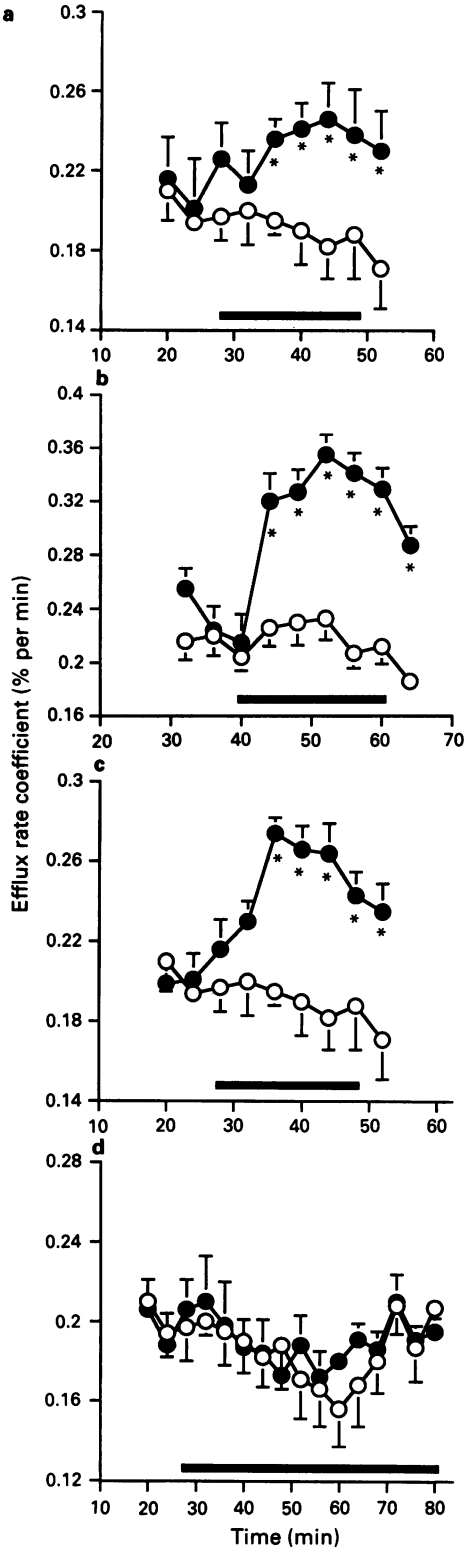

Figure 3 The effects of isoprenaline, procaterol, salbutamol and salmeterol on the efflux of $^{86}Rb^+$ from strips of bovine trachealis muscle preloaded with the radiotracer. In each panel the abscissa scale indicates the time (min) measured from the start of the efflux period. The ordinate scale indicates the efflux rate coefficient (% per min). In each panel the horizontal bar indicates the period for which an agonist at β-adrenoceptors or its vehicle was present in the efflux medium. In each panel (○) indicates time-matched, vehicle-treated control tissues while (●) indicates test tissues treated with 1 μM isoprenaline (a), 100 nM procaterol (b), 10 μM salbutamol (c) or 10 μM salmeterol (d). Data points indicate the mean ± s.e.mean of values from tissue strips from at least 6 tracheae. *indicates a significant ($P < 0.05$) difference from the corresponding data point in the vehicle-treated control tissues. Note that, in contrast to the other agonists at β-adrenoceptors, salmeterol did not modify the efflux of the radiotracer from the trachealis muscle.

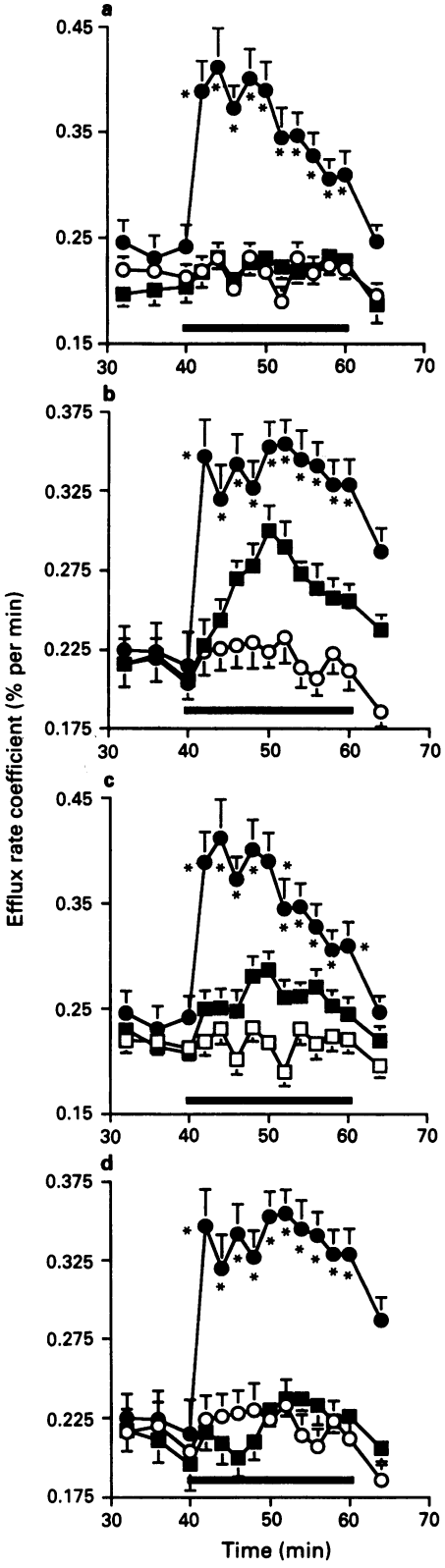

Figure 4 The effects of CGP 20712A (1 μM; a and b) and ICI 118551 (100 nM; c and d) on isoprenaline- or procaterol-induced promotion of $^{86}Rb^+$ efflux from strips of bovine trachealis muscle. In each panel the abscissa scale indicates the time (min) measured from the start of the efflux period. The ordinate scale indicates the efflux rate coefficient (% per min). Time-matched control tissues (○) were not exposed to an agonist at β-adrenoceptors. All other tissues were exposed to either isoprenaline (1 μM; a and c) or procaterol (100 nM; b and d) for the period indicated by the horizontal bar. (●) Indicates tissues exposed to an agonist, but not to an antagonist, at β-adrenoceptors; (■) indicates tissues exposed both to an agonist and to an antagonist at β-adrenoceptors. Where used, the antagonist at β-adrenoceptors was present throughout the efflux period. Data points indicate the mean of values ± s.e.mean from tissue strips from at least 6 tracheae. *indicates a significant ($P<0.05$) difference from the corresponding data point for tissues treated with both the agonist and the antagonist at β-adrenoceptors.

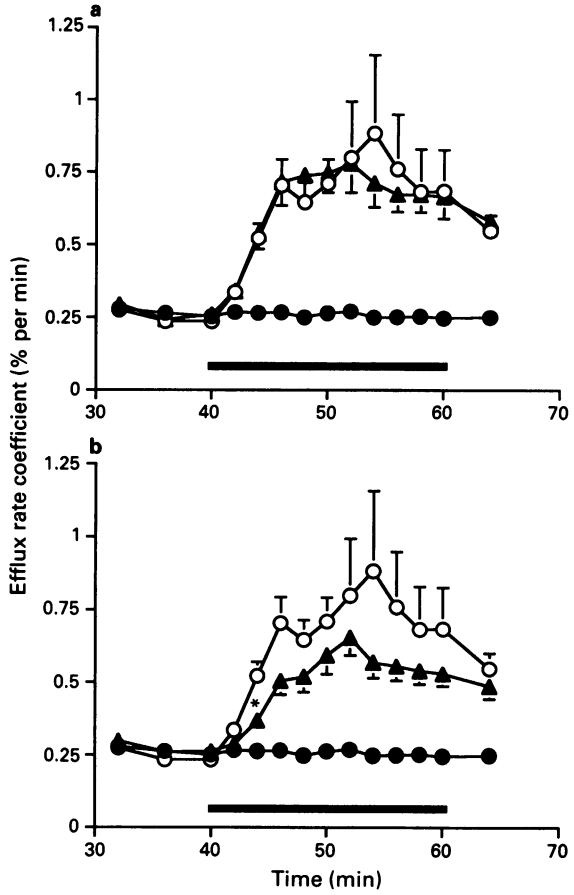

Figure 5 The effects of CGP 20712A (1 μM) and ICI 118551 (100 nM) on cromakalim-induced promotion of $^{86}Rb^+$ efflux from strips of bovine trachealis muscle. In each panel the abscissa scale indicates the time (min) measured from the start of the efflux period. The ordinate scale indicates the efflux rate coefficient (% per min). The horizontal bar indicates the period during which cromakalim (10 μM) was present in the efflux medium. In the case of test tissues (▲), CGP 20712A (1 μM; a) or ICI 118551 (100 nM; b) was present throughout the efflux period. Time-matched control tissues (○) were challenged with cromakalim but were exposed neither to CGP 20712A nor to ICI 118551. A further group of control tissues (●) were not challenged with cromakalim and were exposed neither to CGP 20712A nor to ICI 118551. In the case of this latter group of control tissues, the vehicle for the antagonist at β-adrenoceptors was present throughout the efflux period and the vehicle for cromakalim was present for the period indicated by the horizontal bar. Data points indicate the mean of values ± s.e.mean from tissue strips from at least 6 tracheae. *indicates a significant ($P<0.05$) difference between the mean efflux rate coefficient for tissues treated with a combination of cromakalim and ICI 118551 and that for tissues treated with cromakalim alone.

Studies of $^{86}Rb^+$ efflux from bovine trachealis

Fifteen min after the start of the efflux period, the $^{86}Rb^+$ efflux rate coefficient had declined to a relatively low (0.2–0.3% min^{-1}) value, suggesting that the rapidly-exchanging compartment for $^{86}Rb^+$ had cleared. This was true for both test and control tissues. In the case of the control tissues, the $^{86}Rb^+$ efflux rate coefficient thereafter fell slowly throughout the remainder of the efflux period.

Isoprenaline (100 nM, 1 μM and 10 μM), procaterol (10 nM, 100 nM and 1 μM) and salbutamol (100 nM, 1 μM and 10 μM)

each increased the $^{86}Rb^+$ efflux rate coefficient. In the case of procaterol and salbutamol, concentration-dependency of this effect was suggested by the peak amplitude of the increase in $^{86}Rb^+$ efflux rate coefficient evoked by each of the tested concentrations. In contrast to isoprenaline, procaterol and salbutamol, salmeterol (100 nM, 1 μM and 10 μM) failed to increase the $^{86}Rb^+$ efflux rate coefficient significantly (Figure 3).

CGP 20712A (1 μM) did not itself modify the $^{86}Rb^+$ efflux rate coefficient. However, CGP 20712A (1 μM) ablated the increase in $^{86}Rb^+$ efflux rate coefficient induced by isoprenaline (1 μM). It also attenuated the increase in $^{86}Rb^+$ efflux rate coefficient induced by procaterol (100 nM) (Figure 4). Like CGP 20712A, ICI 118551 (100 nM) did not itself modify the $^{86}Rb^+$ efflux rate coefficient. However, ICI 118551 (100 nM) ablated the increase in $^{86}Rb^+$ efflux rate coefficient induced by procaterol (100 nM) and markedly attenuated that induced by isoprenaline (1 μM) (Figure 4).

Cromakalim (10 μM) induced an increase in the $^{86}Rb^+$ efflux rate coefficient, thereby supporting our earlier findings with the active enantiomer of this agent, BRL 38227 (Berry *et al.*, 1992). The cromakalim-induced increase in the $^{86}Rb^+$ efflux rate coefficient was greater than that evoked by isoprenaline, procaterol or salbutamol (compare Figure 5 with Figures 3 and 4). CGP 20712A (1 μM) did not modify the promotion of $^{86}Rb^+$ efflux induced by cromakalim (10 μM) (Figure 5). In the presence of ICI 118551 (100 nM) the increase in $^{86}Rb^+$ efflux rate coefficient induced by cromakalim (10 μM) appeared slightly smaller than that evoked by cromakalim in the absence of antagonist. However, a significant difference in the $^{86}Rb^+$ efflux rate coefficient was only observed for one of the collection periods (42–44 min from the start of efflux) (Figure 5).

Pretreatment of strips of bovine trachealis muscle with salmeterol (1 μM) did not modify cromakalim (10 μM)-induced promotion of $^{86}Rb^+$ efflux (data not shown). Salmeterol (1 μM) caused an apparent reduction in the promotion of $^{86}Rb^+$ efflux induced by isoprenaline (1 μM). However, no significant differences were observed between mean values of efflux rate coefficient for tissues treated with isoprenaline alone compared with tissues treated with a combination of isoprenaline and salmeterol (Figure 6a). When the concentration of salmeterol was raised to 10 μM, it reduced the ability of isoprenaline to promote $^{86}Rb^+$ efflux (Figure 6b).

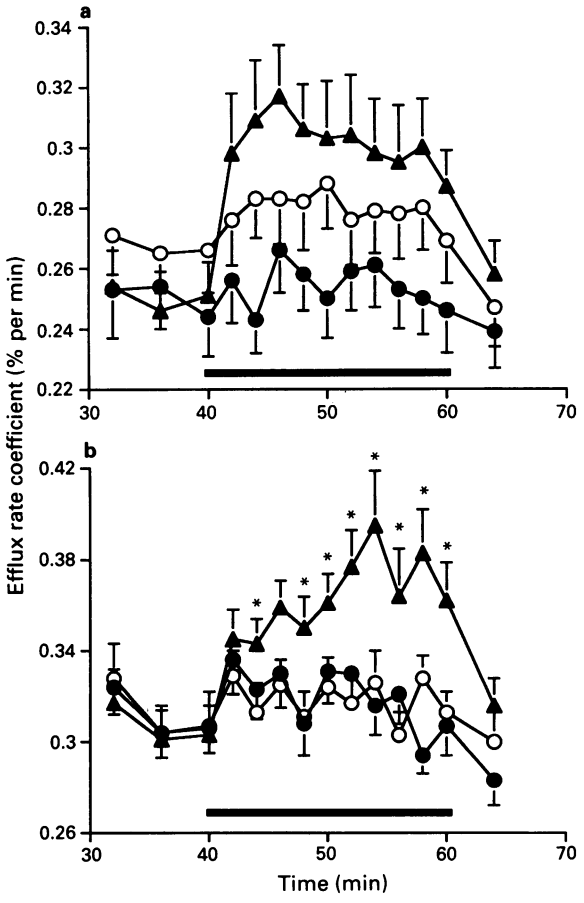

Figure 6 The effects of salmeterol (1 and 10 μM) on isoprenaline (1 μM)-induced promotion of $^{86}Rb^+$ efflux from strips of bovine trachealis muscle. In each panel the abscissa scale indicates the time (min) measured from the start of the efflux period. The ordinate scale indicates the efflux rate coefficient (% per min). The horizontal bar indicates the period during which isoprenaline (1 μM) was present in the efflux medium. Where appropriate, salmeterol 1 μM (a) or 10 μM (b) was present throughout the efflux period; (▲) indicates tissues treated with isoprenaline alone, (○) tissues treated with isoprenaline in combination with salmeterol and (●) tissues treated with salmeterol alone. Each data point indicates the mean of values ± s.e.mean from at least 6 tissues. *indicates a significant ($P<0.05$) difference from the corresponding data point for tissues treated with a combination of salmeterol and isoprenaline. Note the ability of salmeterol (10 μM) to reduce the efflux of $^{86}Rb^+$ promoted by isoprenaline.

Discussion

Properties of CGP 20712A and ICI 118551

In guinea-pig trachealis muscle, CGP 20712A (100 nM–10 μM) did not modify the spontaneous mechanical tone or electrical activity of the tissue. Furthermore, CGP 20712A (1 μM) did not inhibit the tracheal relaxation induced by theophylline or cromakalim or the hyperpolarization of guinea-pig trachealis cells induced by cromakalim (10 μM) (Cook *et al.*, 1993). In the present study, CGP 20712A (100 nM–1 μM) did not induce tension development by bovine trachealis muscle nor did it, *per se*, modify the efflux of $^{86}Rb^+$ from tissue pre-loaded with the radiotracer. CGP 20712A (1 μM) also failed to attenuate the promotion of $^{86}Rb^+$ efflux induced by cromakalim (10 μM) (Figure 5). These findings all support the assertion (Cook *et al.*, 1993) that CGP 20712A does not directly inhibit the plasmalemmal K^+-channels that regulate the excitability of the trachealis cells and does not directly inhibit the K^+-channel (K_{KCO}) involved in the action of cromakalim. Furthermore, the results of the present mechanical studies of bovine trachealis and of studies (Cook *et al.*, 1993) of guinea-pig atria and trachealis are consistent with reports (Dooley & Bittinger, 1984; Lemoine *et al.*, 1985) that CGP 20712A is an antagonist having much greater affinity for β_1- than β_2-adrenoceptors.

In guinea-pig trachealis, ICI 118551 (10–100 nM) did not modify the spontaneous mechanical tone or electrical activity of the muscle cells. ICI 118551 (100 nM) did not inhibit the tracheal relaxant actions of theophylline or cromakalim, or the cellular hyperpolarization induced by cromakalim (10 μM) (Cook *et al.*, 1993). In the present study, ICI 118551 (10 nM–1 μM) did not induce tension development by bovine trachealis muscle not did it, *per se*, modify the efflux of $^{86}Rb^+$ from tissue preloaded with the radiotracer. ICI 118551 (100 nM) also had little or no effect against the promotion of $^{86}Rb^+$ efflux induced by cromakalim (10 μM) (Figure 5). These findings all support the assertion (Cook *et al.*, 1993) that, like CGP 20712A, ICI 118551 does not directly inhibit the plasmalemmal K^+-channels that control the excitability of the trachealis cells not does it inhibit K_{KCO}. Furthermore, reports (O'Donnell & Wanstall, 1980; Bilski *et al.*, 1983; Rimele *et al.*, 1988) that ICI 118551 is an antagonist having greater affinity for β_2- than for β_1-adrenoceptors, have been substantiated by the present mechanical studies of bovine trachealis and by studies (Cook *et al.*, 1993) of guinea-pig atria and trachealis.

In view of these considerations we propose that CGP

20712A (1 μM) and ICI 118551 (100 nM) produce selective antagonism at β_1- and β_2-adrenoceptors respectively and that their blockade of the relevant β-adrenoceptor is not complicated by their acting directly to inhibit the opening of plasmalemmal K^+-channels.

Agonists at β-adrenoceptors and the opening of plasmalemmal K^+-channels in trachealis muscle

In guinea-pig trachealis, isoprenaline, procaterol and salbutamol each cause hyperpolarization (Allen *et al.*, 1985; Honda *et al.*, 1986; Cook *et al.*, 1993). The present observations (Figures 3 and 4) that isoprenaline, procaterol and salbutamol each promote the efflux of $^{86}Rb^+$ from bovine trachealis preloaded with the radiotracer, provides supportive and more direct evidence that these three agonists at β-adrenoceptors open plasmalemmal K^+-channels. The failure of salmeterol to evoke significant hyperpolarization of guinea-pig trachealis muscle (Cook *et al.*, 1993) was paralleled in the present study by its failure to promote $^{86}Rb^+$ efflux from bovine trachealis (Figure 3). These observations provide very strong evidence to suggest that, in contrast to isoprenaline, procaterol and salbutamol, salmeterol is ineffective in provoking the opening of plasmalemmal K^+-channels in trachealis muscle. Studies of guinea-pig trachealis pre-contracted with carbachol (Ball *et al.*, 1991; Dougall *et al.*, 1991; Waldeck & Kallstrom, 1991; Cook *et al.*, 1993) have indicated that salmeterol is a partial rather than a full agonist at β_2-adrenoceptors. The partial agonist activity of salmeterol was not revealed by comparing the maximal relaxant effects of isoprenaline and salmeterol in histamine-precontracted bovine trachealis (Figure 1 and 2). However, the present observation that salmeterol can antagonise isoprenaline in relaxing bovine trachealis precontracted with carbachol lends support to the results of the earlier studies of guinea-pig tissue. In the present experiments, salmeterol (100 nM–10 μM) itself failed to promote the efflux of $^{86}Rb^+$ from bovine trachealis muscle (Figure 3) yet, in a concentration of 10 μM, it attenuated the increase in $^{86}Rb^+$ efflux induced by isoprenaline. That salmeterol (1 μM) did not exert a non-specific effect in inhibiting plasmalemmal K^+-channels is suggested by its failure to modify $^{86}Rb^+$ efflux induced by cromakalim (10 μM). Equivalent experiments with salmeterol (10 μM) were not performed. However, that salmeterol (10 μM) does not act as a non-specific inhibitor of plasmalemmal K^+-channels is suggested by its failure to evoke tracheal spasm. That salmeterol (10 μM) does not exert a non-specific action in airways smooth muscle is also suggested by the susceptibility of its relaxant effect to ICI 118551 (1 μM) (Cook *et al.*, 1993; present study).

The present findings concerning the effects of salmeterol on $^{86}Rb^+$ efflux, parallel the electrophysiological observations (Cook *et al.*, 1993) in guinea-pig trachealis that salmeterol itself fails to cause cellular hyperpolarization but can inhibit the hyperpolarization induced by isoprenaline. We suggest that the weakness of the effects of salmeterol on $^{86}Rb^+$ efflux from trachealis muscle and on membrane potential, combined with its ability to antagonize a full agonist, may be explicable in terms of its reported (Dougall *et al.*, 1991) low intrinsic efficacy at β_2-adrenoceptors.

Subtypes of β-adrenoceptor and the activation of plasmalemmal K^+-channels

The reported high selectivity of salmeterol as an agonist at β_2-adrenoceptors (Ball *et al.*, 1991) and the failure of salmeterol to evoke hyperpolarization of guinea-pig trachealis prompted Cook & Small (1992) to propose that activation of β_1- but not β_2-adrenoceptors might promote the opening of plasmalemmal K^+-channels. However, Cook *et al.* (1993) subsequently showed that (a) isoprenaline-induced hyperpolarization of guinea-pig trachealis could be inhibited either by CGP 20712A (1 μM) or by ICI 118551 (100 nM), (b) that other agonists (salbutamol and procaterol) that selectively activate β_2-adrenoceptors could evoke tracheal hyperpolarization and (c) that the failure of salmeterol to induce hyperpolarization was attributable not to its selectivity for β_2- as opposed to β_1-adrenoceptors but, instead to its low intrinsic efficacy at β_2-adrenoceptors. In view of these findings, Cook *et al.* (1993) rejected the hypothesis that activation of β_1- but not β_2-adrenoceptors could cause K^+-channel opening. Instead, they proposed that activation of either β-adrenoceptor subtype could lead to K^+-channel opening. This new proposal receives support from the present findings that β_2-selective agonists such as procaterol and salbutamol were able to promote the efflux of $^{86}Rb^+$ from bovine trachealis muscle (Figure 3) and that CGP 20712A (1 μM) and ICI 118551 (100 nM) were each able to inhibit the efflux of $^{86}Rb^+$ induced by isoprenaline.

Since procaterol is an agonist exhibiting selectivity for β_2- as opposed to β_1-adrenoceptors (Yabuuchi, 1977; O'Donnell & Wanstall, 1985), we were surprised to observe (Figure 4) that CGP 20712A could significantly attenuate the $^{86}Rb^+$ efflux induced by this agent. It is difficult to decide whether this reflects a limitation of the β_2:β_1 selectivity of procaterol or the β_1:β_2 selectivity of CGP 20712A. However, CGP 20712A is reported (Dooley & Bittinger, 1984; Lemoine *et al.*, 1985) to have a very much greater (at least 10,000 times) affinity for β_1- than β_2-adrenoceptors and, at a concentration of 1 μM, CGP 20712A failed to antagonize isoprenaline, procaterol, salbutamol or salmeterol in relaxing either guinea-pig (Cook *et al.*, 1993) or bovine (present study) trachealis. Procaterol is reported to have 600–850 fold greater affinity for β_2-adrenoceptors than for β_1-adrenoceptors (Yabuuchi, 1977; O'Donnell & Wanstall, 1985). In view of the poorer selectivity ratio of procaterol between the two β-adrenoceptor subtypes and the fact that the concentration of procaterol chosen for use in the $^{86}Rb^+$ efflux studies was more than 100 times greater than its EC_{50} in suppressing histamine-induced tone of bovine trachealis, we ascribe the effectiveness of CGP 20712A against procaterol-induced $^{86}Rb^+$ efflux to this concentration (100 nM) of procaterol being high enough to cause some activation of β_1-adrenoceptors.

Role of plasmalemmal K^+-channel opening in mediating the relaxation of airways smooth muscle induced by agonists at β-adrenoceptors

Opening of K^+-channels could cause plasmalemmal hyperpolarization and hence a voltage-dependent inhibition of Ca^{2+} influx into trachealis cells. However, in the preceding paper (Cook *et al.*, 1993) we have reviewed the substantial evidence that now suggests that such a mechanism does not play a crucial role in mediating the mechano-inhibitory effects of the agonists at β-adrenoceptors. The ability of salmeterol to relax airways smooth muscle without hyperpolarizing the tissue (Cook & Small, 1992; Cook *et al.*, 1993) forms a novel and important part of this evidence. The present observation that salmeterol, in contrast to other agonists at β-adrenoceptors, fails to promote $^{86}Rb^+$ efflux from bovine trachealis is consistent with its lack of effect on membrane potential and provides a strong indication that the relaxant action of salmeterol does not involve the opening of plasmalemmal K^+-channels. Of course, measurements of membrane potential and $^{86}Rb^+$ efflux are somewhat indirect indices of K^+-channel activity and the outcome of patch-clamp studies with salmeterol is awaited with interest.

In conclusion we suggest that, in bovine trachealis, activation of either β_1- or β_2-adrenoceptors can promote the opening of $^{86}Rb^+$-permeable K^+-channels in the plasmalemma. The failure of salmeterol to promote plasmalemmal K^+-channel opening reflects not its selectivity in activating β_2- as opposed to β_1-adrenoceptors, but rather its low intrinsic efficacy at β_2-adrenoceptors. The opening of plasmalemmal K^+-channels plays, if anything, a supportive rather than a

crucial role in mediating the mechano-inhibitory effects of agonists at β-adrenoceptors.

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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.

BALL, D.I., BRITTAIN, R.T., COLEMAN, R.A., DENYER, L.H., JACK, D., JOHNSON, M., LUNTS, L.H.C., NIALS, A.T., SHELDRICK, K.E. & SKIDMORE, I.F. (1991). Salmeterol, a novel, long-acting, β_2-adrenoceptor agonist: characterization of pharmacological activity *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **104**, 665–671.

BERRY, J.L., SMALL, R.C. & FOSTER, R.W. (1992). Tracheal relaxation induced by potassium channel opening drugs: its antagonism by adrenergic neurone blocking agents. *Br. J. Pharmacol.*, **106**, 813–818.

BILSKI, A.J., HALLIDAY, S.E., FITZGERALD, J.D. & WALE, J.L. (1983). The pharmacology of a β_2-selective adrenoceptor antagonist (ICI 118,551). *J. Cardiovasc. Pharmacol.*, **5**, 430–437.

CHIU, P., COOK, S.J., BERRY, J.L., CARPENTER, J.R., DOWNING, S.J., SMALL, A.M. & SMALL, R.C. (1992). Activation of β_1- or β_2-adrenoceptors opens plasmalemmal K^+-channels in airways smooth muscle: evidence from $^{86}Rb^+$ efflux studies in bovine trachealis. *Br. J. Pharmacol.*, **107**, 250P.

COOK, S.J., CHIU, P., BERRY, J.L., CARPENTER, J.R., DOWNING, S.J. & SMALL, R.C. (1992). Activation of β_1 or β_2-adrenoceptors opens plasmalemmal K^+-channels in airways smooth muscle: evidence from mechanical and electrophysiological studies in guinea-pig trachealis. *Br. J. Pharmacol.*, **107**, 379P.

COOK, S.J. & SMALL, R.C. (1992). Role of K^+-channel opening in salmeterol-induced relaxation of trachealis muscle. *Br. J. Pharmacol.*, **106**, 12P.

COOK, S.J., SMALL, R.C., BERRY, J.L., CHIU, P., DOWNING, S.J. & FOSTER, R.W. (1993). β-Adrenoceptor subtypes and the opening of plasmalemmal K^+-channels in trachealis muscle: electrophysiological and mechanical studies in guinea-pig tissues. *Br. J. Pharmacol.*, **109**, 1140–1148.

DOOLEY, D.J. & BITTINGER, H. (1984). CGP 20712A: a selective β_1-adrenoceptor antagonist and useful tool for quantitating β_1 or β_2-adrenoceptors. In *Proceedings of the IUPHAR 19th International Congress of Pharmacology*, 1009P London: Macmillan.

DOUGALL, I.G., HARPER, D., JACKSON, D.M. & LEFF, P. (1991). Estimation of the efficacy and affinity of the β_2-adrenoceptor agonist salmeterol in guinea-pig trachea. *Br. J. Pharmacol.*, **104**, 1057–1061.

HONDA, K., SATAKE, T., TAKAGI, K. & TOMITA, T. (1986). Effects of relaxants on electrical and mechanical activities in the guinea-pig tracheal muscle. *Br. J. Pharmacol.*, **87**, 665–671.

ITO, Y. & TAJIMA, K. (1982). Dual effects of catecholamines on pre- and postjunctional membranes in the dog trachea. *Br. J. Pharmacol.*, **75**, 433–440.

LEMOINE, H., EHLE, B. & KAUMANN, A.J. (1985). On the participation of β_1- or β_2-adrenoceptors in the relaxation of calf trachea caused by (−)-noradrenaline, (−)-adrenaline and (±)-fenoterol. *Naunyn-Schmied. Arch. Pharmacol.*, **329**, R81.

LONGMORE, J., BRAY, K.M. & WESTON, A.H. (1991). The contribution of ^{86}Rb-permeable potassium channels to the relaxant and membrane hyperpolarizing actions of cromakalim, RP 49356 and diazoxide in bovine tracheal smooth muscle. *Br. J. Pharmacol.*, **102**, 979–985.

O'DONNELL, S.R. & WANSTALL, J.C. (1980). Evidence that ICI 118551 is a potent, highly beta$_2$-selective adrenoceptor antagonist and can be used to characterize beta-adrenoceptor populations in tissues. *Life Sci.*, **27**, 671–677.

O'DONNELL, S.R. & WANSTALL, J.C. (1985). Responses to the β_2-selective agonist procaterol of vascular and atrial preparations with different functional β-adrenoceptor populations. *Br. J. Pharmacol.*, **84**, 227–235.

RIMELE, T.J., HENRY, D.E., GIESA, F.R., BUCKLEY, S.K., GEIGER, G., HEASLIP, R.J., LEE, D.K.H. & GRIMES, D. (1988). Comparison of the β-adrenoceptor affinity and selectivity of cetamolol, atenolol, betaxolol and ICI 118551. *J. Cardiovasc, Pharmacol.*, **12**, 208–217.

WALDECK, B. & KALLSTROM, B.L. (1991). Salmeterol inhibits the relaxing effect of formoterol on guinea-pig tracheal smooth muscle: evidence for partial agonism. *Br. J. Pharmacol.*, **104**, 402P.

YABUUCHI, Y. (1977). The β-adrenoceptor stimulant properties of OPC-2009 on guinea-pig isolated tracheal, right atrial and left atrial preparations. *Br. J. Pharmacol.*, **61**, 513–521.

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