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Comparison of the Effects of Cromakalim in Trachea Isolated from Normal and Albumin-sensitive Guinea-pigs*

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Abstract—The effects of the K^+-channel activator, cromakalim, on spontaneous tone and constrictor responses to vagal stimulation or acetylcholine were compared in trachea isolated from groups of guinea-pigs that were: untreated; sensitized and chronically exposed to inhaled albumin; or sham sensitized. Responses were assessed as changes in intraluminal pressure in the isolated, Krebs-filled trachea, increases and decreases in intraluminal pressure directly reflecting constriction and dilatation, respectively. Cromakalim reduced resting intraluminal pressure in normal trachea but in sensitized trachea mixed effects occurred, many preparations exhibiting increases in intraluminal pressure, particularly at lower concentrations of cromakalim. Cromakalim attenuated the frequency-dependent increases in intraluminal pressure evoked by stimulation of the vagus nerve in a concentration-dependent manner and to a similar degree in trachea from each of the three groups tested. The degree of attenuation was similar in the absence and presence of the cyclo-oxygenase inhibitor flurbiprofen. In untreated trachea, responses to a range of concentrations of applied acetylcholine were attenuated by cromakalim. In sensitized trachea the response to the lowest concentration of applied acetylcholine was attenuated by cromakalim but responses to higher concentrations of were unaffected. The results indicate that the direct relaxant effect of cromakalim is altered in sensitized trachea, which may indicate abnormal K^+-channel behaviour in the smooth muscle cell membrane. Attenuation by cromakalim of vagal responses occurs in both normal and sensitized trachea, due chiefly to a pre-junctional effect on cholinergic neurotransmission which is independent of the generation of cyclo-oxygenase products.

The airways of asthmatic patients are highly sensitive to a wide range of bronchoconstricting stimuli which have little or no effect in non-asthmatics, a phenomenon termed airway hyper-reactivity (Empey 1982). It has not yet been established whether airway hyper-reactivity is caused by a fundamental defect in the airway smooth muscle itself or in the neural or humoral control of bronchial tone (Barnes et al 1988). Much of the research into the causes of hyper-reactivity has centred around the use of animal models of asthma, which are based on natural or acquired sensitivity to a particular antigen (Patterson & Kelly 1974; Widdicombe 1977). In two such models, the albumin-sensitive guinea-pig (acquired) and the ragweed-sensitive dog (innate), the isolated trachea has been found to exhibit hyper-responsiveness to stimulation of the vagus nerve, raising the possibility that increased sensitivity to parasympathetic input may contribute to airway hyper-reactivity (McCaig 1987; Mitchell et al 1987). Electrophysiological studies on the trachealis muscle from albumin-sensitive guinea-pigs have indicated that abnormal K^+-channel function might underlie hyper-responsiveness to vagal input (McCaig 1987) and this could contribute to generalized airway hyper-reactivity (Allen et al 1986; McCaig 1987). The abnormality might involve either a decrease in number or in the frequency or duration of opening of K^+-channels in the smooth muscle cell membrane. In recent years drugs such as cromakalim have become available that selectively activate K^+-channels and thereby relax smooth muscle preparations (see Weston 1989). Cromakalim has been shown to relax guinea-pig trachea and to attenuate vagally-mediated constriction in this tissue (McCaig & De Jonckheere 1989). If K^+-channel function were abnormal in sensitized airways then responsiveness to drugs that activate K^+-channels might be altered.

The aim of the present work was to compare the effects of cromakalim on spontaneous tone and vagally-mediated constriction in the trachea isolated from normal and albumin-sensitive guinea-pigs. In addition, since products of the cyclo-oxygenase pathway of arachidonic acid metabolism affect airway smooth muscle contractility (Brink 1988) experiments with cromakalim have been carried out in the absence and presence of the cyclo-oxygenase inhibitor flurbiprofen.

Materials and Methods

Sensitization

Male, Dunkin-Hartley guinea-pigs (n = 32) were sensitized to albumin by injecting 100 mg i.p. and 100 mg s.c. on day 1 and a further 10 mg i.p. on day 8. From day 14, animals were exposed to an aerosol of 4% albumin for 4 min daily for 20 ± 1 days in a closed chamber, dimensions $41 \times 27 \times 15$ cm (albumin-sensitized group, AS). Animals were used the day after the last exposure to albumin. A second group of animals (n = 16, sham-sensitized, SS) was treated identically except that saline vehicle alone was used throughout. A third group of animals (n = 25) received no treatment (untreated controls, UT). All animals were killed by a blow to the head.

Experimental procedure

The first three cartilaginous rings of the trachea below the cricoid cartilage were removed and suspended under 1 g

* A preliminary account of some of the results has been presented to the British Pharmacological Society (McCaig et al 1991).

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tension in an organ bath containing Krebs solution (mM): Na^+ 127, K^+ 5·9, Ca^{2+} 2·5, Mg^{2+} 1·2, Cl^- 121, HPO_4^- 1·2, SO_4^{2-} 1·2, HCO_3^- 25, glucose 11, at 37°C, gassed with 95% O_2–5% CO_2. Tension was recorded with an isometric transducer coupled to a pen recorder (Lectromed). Sensitivity was assessed by adding albumin to the organ bath to give a final concentration of 0·1% albumin and recording the change in tension.

The remainder of the trachea was dissected with or without the right vagus nerve with recurrent laryngeal nerve intact (Blackman & McCaig 1983) and placed horizontally in a chamber at 37°C, volume 10 mL, through which Krebs solution flowed at a rate of 5 mL min^{-1}. The trachea was filled with Krebs solution, then the caudal end was closed and the rostral end attached to a pressure transducer (Statham). Intraluminal pressure (ILP) was recorded continuously on a pen recorder (Lectromed), increases and decreases in ILP reflecting constriction and dilatation, respectively. The vagus nerve was stimulated through a suction electrode with rectangular pulses of 40 V and 1 ms duration for 5 s at 90 s intervals and frequencies of 1–50 Hz.

A frequency/response curve to vagal stimulation was obtained after a 30 min equilibration period. Tissues were then exposed to cromakalim and a second frequency/response curve obtained 20–30 min later. Preparations were then exposed to two higher concentrations of cromakalim. Tissues not exposed to cromakalim served as time-matched controls. In groups of tissues from AS and UT guinea-pigs, responses to exogenous acetylcholine (10^{-7}–10^{-5} M) were assessed before and 20–30 min after exposure to successively higher concentrations of cromakalim. In a second series of experiments an initial frequency/response curve to vagal stimulation was obtained, then the preparation was exposed to flurbiprofen (10^{-6} M) and a second curve generated 30 min later. Tissues were then exposed to cromakalim as before.

Drugs

The following drugs were used: acetylcholine chloride (Sigma, UK); cromakalim (SmithKline Beecham, UK) and flurbiprofen (Boots, UK). All drugs were dissolved in distilled water. Cromakalim and flurbiprofen were added to the reservoir of Krebs solution and acetylcholine was added directly to the tissue bath with the perfusion pump off. Concentrations are expressed as the final bath concentration.

Analysis of data

Results are expressed as the mean ± s.e.m. Frequency/response curves or responses to acetylcholine were compared using two-way analysis of variance followed by *t*-tests for independent or paired data where appropriate. Values were considered significantly different where $P<0·05$.

Results

Effects of albumin on tracheal segments in-vitro

All tracheal segments from AS guinea-pigs contracted on exposure to 0·1% albumin whilst none of the tissues from UT animals responded. In one set of experiments all tissues from SS animals (n = 5) responded to albumin. These animals were caged separately from the AS group, but in the same room. Exposure to albumin or saline aerosol was carried out in a separate room using separate exposure chambers and nebulizers. In a second set of experiments, SS (n = 11) and AS guinea-pigs were housed and exposed to aerosol in separate rooms. Under these conditions, 5 of the SS tracheal segments responded to albumin, indicating that despite extensive precautions some cross-sensitization was occurring.

Effect of cromakalim on resting ILP

The resting ILP was between zero and 20 mm H_2O and there were no significant differences among preparations from AS, SS or UT animals. Cromakalim, 2×10^{-7} M, had no significant effect on ILP in the trachea from UT or SS animals. However, at this concentration, cromakalim induced an increase in ILP, indicative of constriction, in AS animals (Fig. 1). At higher concentrations, cromakalim (10^{-6} and 10^{-5} M) elicited an overall reduction in ILP, indicative of relaxation, in all groups, but a number of AS tissues still exhibited an increase in ILP in response to cromakalim, although the incidence of this effect lessened with increasing concentrations of the drug. It can be seen (Fig. 1) that the mean decrease in ILP with 10^{-6} or 10^{-5} M cromakalim is not significantly different in UT or AS trachea, but when contractile and relaxant responses are differentiated, both the contraction and the relaxation are significantly different from the corresponding relaxation in UT tissues.

In the presence of flurbiprofen, (10^{-6} M) resting ILP was reduced slightly (UT: -5 ± 4 mm H_2O, n = 6; AS: -17 ± 7 mm H_2O, n = 12). Cromakalim elicited a further fall in ILP in UT trachea and in 4 of 5 AS trachea. In the fifth AS trachea a small rise in ILP was observed, suggesting that mixed effects also occur in the presence of flurbiprofen (data not shown).

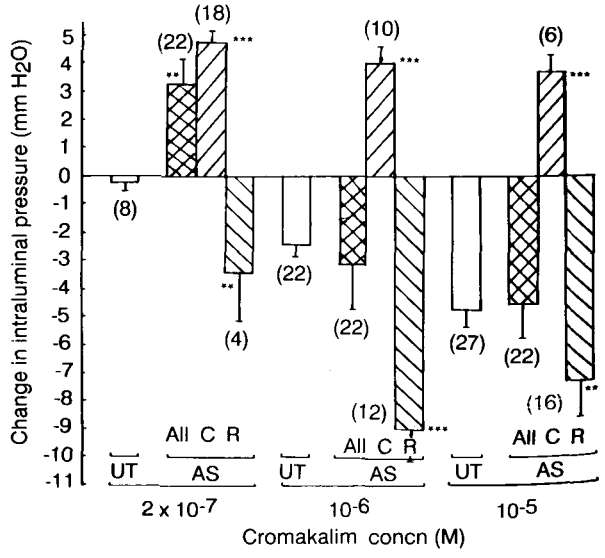

FIG. 1. Histogram showing changes in intraluminal pressure (ILP) induced by cromakalim in trachea isolated from untreated (UT) and albumin-sensitized guinea-pigs (AS; group as a whole, All; tissues responding with an increase in ILP, C; tissues with a decrease in ILP, R). Columns represent the mean and vertical bars the s.e.m. with number of observations shown in parentheses for each column. Asterisks denote values significantly different from the corresponding value in untreated trachea at $^{**}P<0·05$ and $^{***}P<0·001$.

Effect of cromakalim on responses to stimulation of the vagus nerve

Stimulation of the vagus nerve elicited frequency-dependent increases in ILP in the tracheal preparations from each of the 3 groups of animals. The maximum response obtained varied widely between preparations (e.g. UT: 18–250 mm H_2O; AS: 76–260 mm H_2O) but was consistent in any one tissue. Frequency/response curves, plotted either in absolute units or as percentage maximum response (Fig. 2), were almost identical in the 3 groups. In time-matched control tissues from UT and AS groups, response amplitude tended to decrease slightly over time, but significant reductions occurred only at 20 and 50 Hz on the fourth frequency/response curve in the UT group. In the presence of cromakalim, however, vagal response amplitude was decreased in a concentration-dependent manner and to a similar degree in each of the 3 groups of tissues, reductions ranging from approximately 20% at 2×10^{-7} M cromakalim to 80% at 10^{-5} M (Fig. 3). It was noted that responses at low frequencies of vagal stimulation were more susceptible to attenuation by cromakalim than responses at higher frequencies in preparations from all groups of animals. For example, at 10^{-5} M cromakalim, responses at 1 Hz were attenuated by 98 ± 1 and 92 ± 4% in UT and AS groups, respectively and those at 20 Hz by 68 ± 7 and 78 ± 12%, respectively ($P < 0.05$ compared with responses at 1 Hz, paired t-test). There was no evidence, however, that the attenuating effect of cromakalim on vagal responses was altered in sensitized tissues.

Effect of flurbiprofen on responses to vagal stimulation

In the presence of flurbiprofen, 10^{-6} M, the frequency/response curve to vagal stimulation in each group of tracheal preparations was not statistically changed. However, in all groups, cromakalim attenuated vagal responses in flurbiprofen-treated tissues, in a concentration-dependent manner (Fig. 3). This effect was significant at the lowest concentration of cromakalim tested (2×10^{-7} M) in AS and UT groups. However, there were no significant differences in the degree of attenuation in the trachea isolated from AS, SS or UT guinea-pigs. In addition, within each group of tissues the effect of cromakalim was not significantly different in the absence or presence of flurbiprofen.

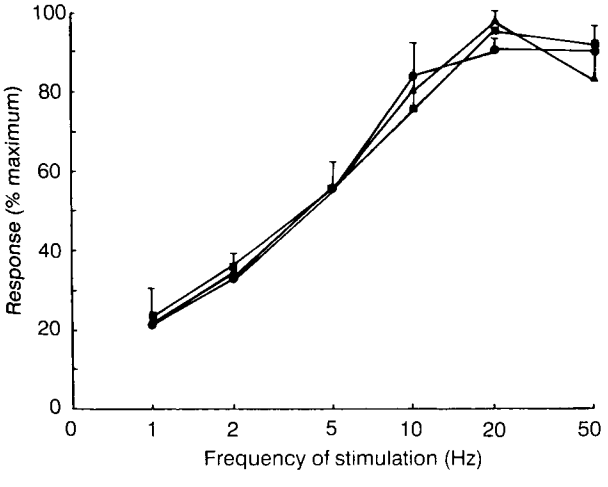

FIG. 2. Relationship between increases in intraluminal pressure (ILP), expressed as percentage maximum response (ordinate) and frequency of stimulation of the vagus nerve (abscissa) in trachea isolated from albumin-sensitized (n = 12, ■), sham-sensitized (n = 11, ▲) and untreated guinea-pigs (n = 21, ●). Values are the mean and vertical bars represent s.e.m.

Effect of cromakalim on the response to acetylcholine

Acetylcholine (10^{-7}–10^{-5} M) elicited concentration-dependent increases in ILP (Fig. 4). In AS trachea, cromakalim at each of the 3 concentrations tested, significantly reduced responses to the lowest concentration of acetylcholine (10^{-7} M) only (-24 ± 11 and -47 ± 7%, n = 12, at 2×10^{-7} and 10^{-5} M cromakalim, respectively, see Fig. 4). In UT trachea, however, cromakalim, 2×10^{-7} or 10^{-6} M, attenuated responses to higher concentrations of acetylcholine by up to 45%. At the highest concentration of cromakalim tested (10^{-5} M) only responses to acetylcholine (10^{-7} M) were attenuated significantly in either AS or UT trachea. These results suggest that the attenuation of responses to applied acetylcholine by cromakalim is not clearly concentration-dependent and that responses in AS trachea are more resistant than those in UT trachea to attenuation by cromakalim.

Discussion

These results indicate that cromakalim attenuates vagally-mediated constriction in isolated trachea from guinea-pigs chronically sensitized to albumin. Cromakalim has been shown previously to relax normal guinea-pig trachea (Allen et al 1986; McCaig & De Jonckheere 1989) by promoting K^+-channel opening in the plasma membrane. In the present study, cromakalim contracted a number of sensitized tissues, but the incidence of this effect decreased with higher concentrations of cromakalim. In the trachea tissue which relaxed in response to cromakalim, the relaxation was considerably greater in AS tissue than in controls. This suggests that sensitivity to cromakalim may be altered in AS tissue, so that at lower doses, K^+-channel opening is augmented in some tissues but inhibited in others. The application of patch-clamp techniques to AS trachea might indicate whether this indeed represents altered K^+-channel function.

Contraction in smooth muscle involves potential-dependent influx of Ca^{2+} ions through voltage-operated Ca^{2+}-channels and also potential-independent influx through receptor-operated Ca^{2+}-channels and release of intracellularly-stored Ca^{2+} (Bolton & Large 1986). Drugs such as cromakalim, hyperpolarize smooth muscle cells and inhibit the potential-dependent component of contraction. The potential-dependent component, however, is thought to be small, except at low concentrations of bronchoactive agents (Farley & Miles 1978; Weiss et al 1985; Baba et al 1986; Bengtsson et al 1987) which may explain the relative ineffectiveness of cromakalim in reducing agonist-induced bronchoconstriction (Allen et al 1986; Hall & Maclagan 1988; McCaig & De Jonckheere 1989). In our previous study, cromakalim (10^{-5} M) had no significant effect on the response to applied acetylcholine in normal guinea-pig trachea (McCaig & De Jonckheere 1989). In the present study, it was

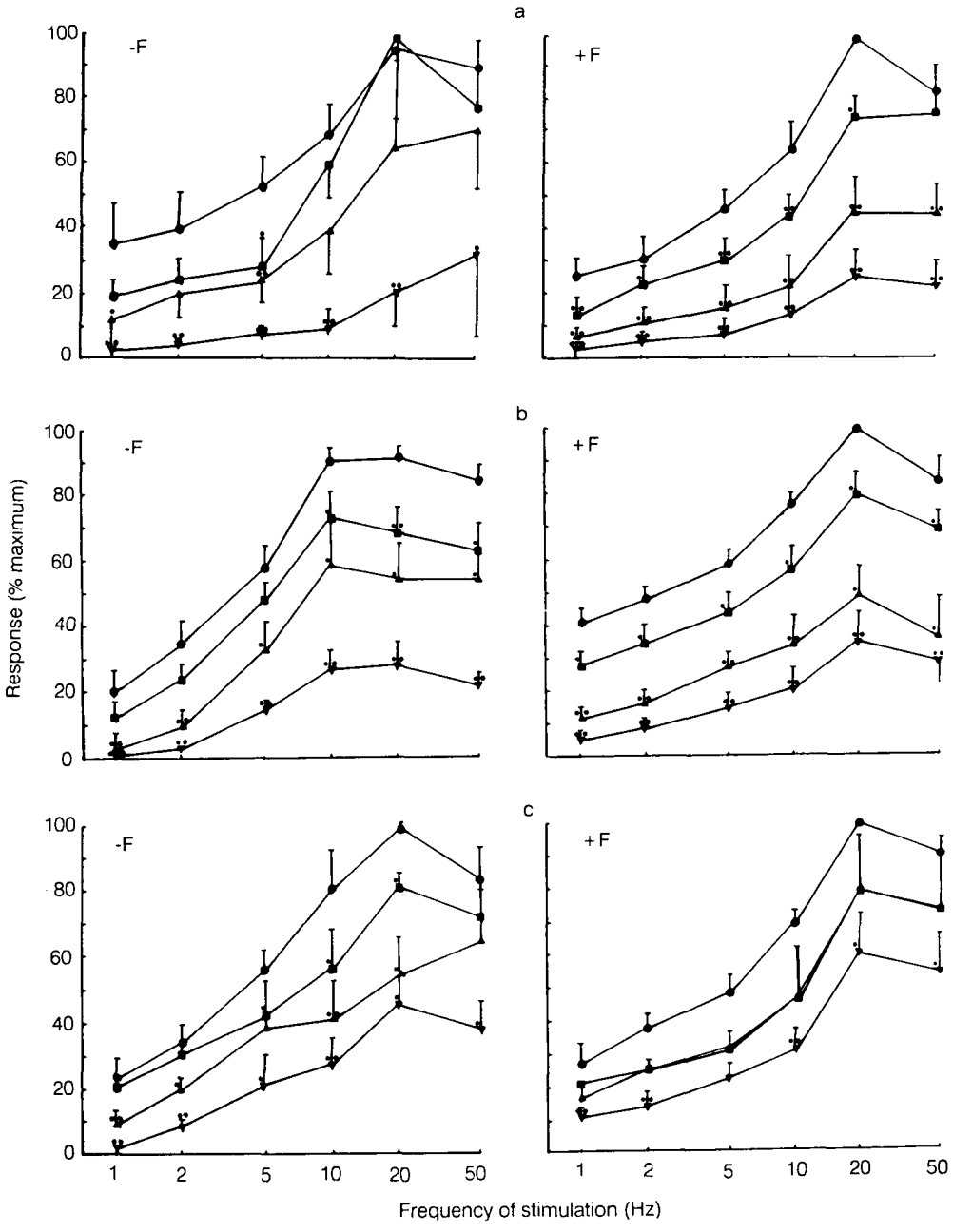

FIG. 3. Relationship between increases in intraluminal pressure, expressed as percentage control maximum response and frequency of stimulation of the vagus nerve (abscissa) in trachea isolated from albumin-sensitized (a), untreated (b) and sham-sensitized guinea-pigs (c) before (●) and 20–30 min after the addition of cromakalim 2×10^{-7} (■), 10^{-6} (▲) and 10^{-5} M (▼) in the absence (−F left hand graphs) and presence of flurbiprofen (+F; 10^{-6} M, right-hand graphs). Values are the mean of 5–8 observations and vertical bars represent s.e.m. Asterisks denote values significantly different from the corresponding value in the absence of cromakalim at $*P < 0{\cdot}005$ and $**P < 0{\cdot}01$.

found that at lower concentrations of cromakalim there was a significant reduction in responses to a range of concentrations of applied acetylcholine in control tissues. In AS tissues, however, only responses to a low concentration of acetylcholine were depressed by cromakalim. This may suggest that the potential-dependent component of contraction is significant only at this low level of acetylcholine. Electrophysiologically, an enhanced depolarizing response to endogenous acetylcholine released by vagal stimulation has been demonstrated in sensitized guinea-pig trachealis which would suggest that the potential-dependent component is augmented in sensitized trachea (McCaig 1987).

Cromakalim attenuated constriction evoked by stimulation of the vagus nerve in normal guinea-pig trachea but because it had little effect on the response to applied acetylcholine it was concluded that attenuation arose through prejunctional modulation of parasympathetic neurotransmission (Hall & Maclagan 1988; McCaig & De

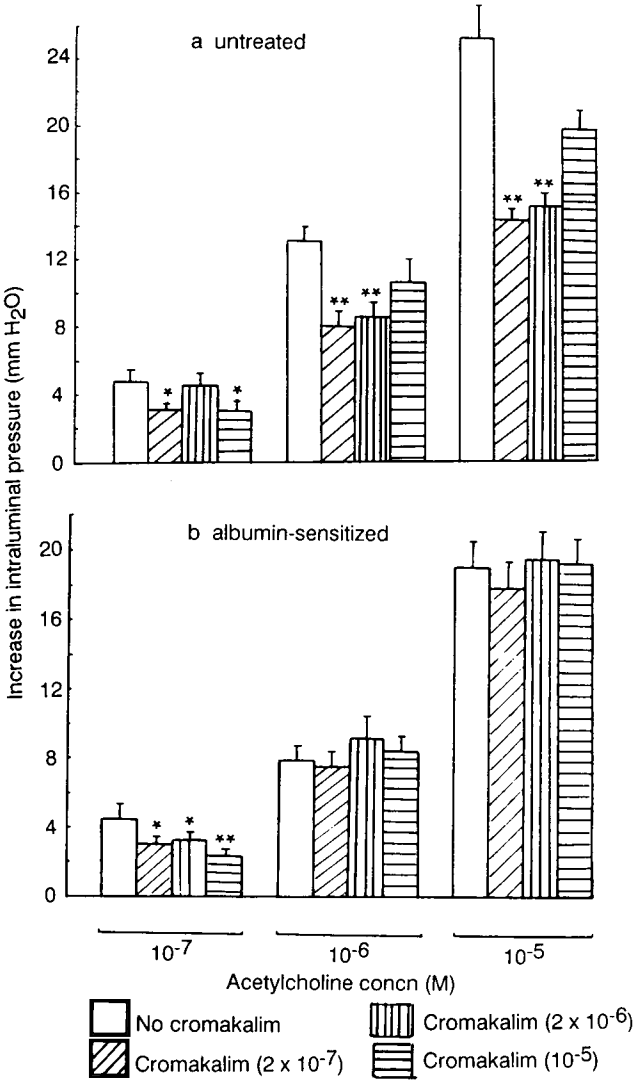

FIG. 4. Histograms showing the increase in intraluminal pressure evoked by acetylcholine (10^{-7}–10^{-5} M) in trachea isolated from untreated guinea-pigs (a) and albumin-sensitized guinea-pigs (b) in the absence and presence of cromakalim, 2×10^{-7}, 10^{-6} and 10^{-5} M. Values are the mean of 6 observations in (a) and 12 observations in (b) and vertical bars represent s.e.m. Asterisks denote values significantly different from the corresponding control value in the absence of cromakalim $^{*}P < 0.05$ and $^{**}P < 0.01$.

Jonckheere 1989). Results from the present study, in which a wider range of concentrations of cromakalim was tested, indicate that both pre- and post-junctional effects contribute to attenuation of vagal responses. The sensitization process alters the electrophysiological characteristics of trachealis cells resulting in a depolarized resting state, increased incidence of spontaneous depolarizations (slow waves) and enhanced depolarization in response to vagal stimulation, suggestive of K^+-channel dysfunction (McCaig 1987). It was necessary to establish, therefore, whether attenuation of vagal constriction by cromakalim in sensitized trachea involved significant post-junctional effects on the smooth muscle cell membrane. Responses to applied acetylcholine (10^{-6} or 10^{-5} M) were unaffected by cromakalim (2×10^{-7}–10^{-5} M) which attenuated vagal responses by up to 98%, strongly suggesting that attenuation arises primarily through pre-junctional effects in AS trachea. The response to a low concentration of acetylcholine (10^{-7} M), however, was reduced by about 50% at the highest concentration of cromakalim tested (10^{-5} M) suggesting that post-junctional effects may contribute to attenuation in AS trachea, especially at lower frequencies of stimulation where the potential-dependent component of the contractile response is likely to be greatest.

The finding that the bronchodilator and hyperpolarizing effects of cromakalim in guinea-pig trachealis can be selectively antagonized by glibenclamide suggests that cromakalim may open ATP-dependent K^+-channels in the smooth muscle cell membrane (Murray et al 1989). These channels are not thought to be open at rest (Murray et al 1989). Whether or not the opening of the same channel type underlies both the relaxant effect and the reduction of response to exogenous acetylcholine is not clear. The fact that the relaxant response is concentration-dependent whereas attenuation of the response to acetylcholine is not, may indicate differences in K^+-channel involvement. In addition the pre-junctional attenuation of responses to endogenously-released acetylcholine may involve yet another K^+-channel subtype or subtypes. It has been shown previously that the direct relaxant effect and depression of vagal responses by cromakalim follow different time-courses in normal trachea supporting this suggestion (McCaig & De Jonckheere 1989). Sensitization could alter the behaviour of one or more types of K^+-channels. The fact that attenuation of vagal responses by cromakalim was no different in sensitized trachea, suggests that the channels involved in pre-junctional modulation are functioning normally. On the other hand, the altered post-junctional effects of cromakalim on spontaneous and acetylcholine-induced tone suggest that the channel or channels mediating these effects may exhibit altered behavioural characteristics.

Excitability of airway smooth muscle is normally kept in equilibrium by efflux of K^+ ions through specific channels in the cell membrane, which prevents depolarization reaching the threshold for action potential generation (Small & Foster 1988). Blockade of these channels, for example by tetraethylammonium (TEA) leads to depolarization, generation of action potentials and contraction (McCaig & Souhrada 1980; Kirkpatrick 1981). A similar pattern of electrical behaviour is seen in the trachea of albumin-sensitive guinea-pigs, leading to the suggestion that K^+-channel dysfunction, such as partial channel blockade might be involved in airway hyper-reactivity (Allen et al 1986; McCaig 1987). It should be noted, however, that treatment of guinea-pig trachea with TEA did not potentiate responses to a variety of spasmogens (Boyle et al 1988). It is clear that there are numerous K^+-channel subtypes present in airway smooth muscle and that TEA is a rather non-specific K^+-channel blocker. The application of drugs with greater specificity for particular K^+-channel subtypes should help to identify which, if any, of these are involved in hyper-reactivity. Drugs that promote K^+-channel opening might be able to restore normal channel function and reverse hyper-reactivity. If this were so, cromakalim might be more effective in reducing vagally-mediated constriction in sensitized trachea. In fact, no

difference was observed in the capacity of cromakalim to attenuate vagal constriction in AS, SS or UT trachea. It should be noted, however, that the mechanical hyper-responsiveness to vagal stimulation demonstrated previously (McCaig 1987) was not observed in this study.

There are two major problems concerning the demonstration of hyper-responsiveness to vagal input in-vitro. Firstly, the contractile response is complex and involves both potential-dependent and -independent processes. Whether an increase in the potential-dependent component would be manifest as an increased contraction is likely to depend on the extent of involvement of potential-independent mechanisms. Further studies of electrical behaviour or measurements of K^+ and Ca^{2+} ion fluxes may resolve this issue.

The second problem in demonstrating hyper-reactivity is the wide variation in amplitude of vagal responses found in both normal and sensitized trachea. This difficulty has been reported by other workers (Watson et al 1991). It has been shown that endogenous cyclo-oxygenase products are closely involved in the regulation of airway smooth muscle contractility. Spontaneous tone, for example, is due, at least in part, to the generation of prostaglandins (Mansour & Daniel 1986). In contrast, cholinergic neurotransmission is depressed by cyclo-oxygenase products, at least in-vitro (Ito & Tajima 1981). Differences in the pattern of eicosanoid release might account for some of the wide variation in responsiveness to vagal stimulation. It was considered, therefore, that responses in the presence of the cyclo-oxygenase inhibitor, flurbiprofen, might be less variable, allowing easier detection of hyper-responsiveness. It was shown, however, that flurbiprofen caused a similar variable shift in the frequency/response curve generated by stimulation of the vagus nerve in sensitized and non-sensitized trachea. In addition, cromakalim was equally effective in attenuating vagal responses in the absence or presence of flurbiprofen in all groups of tissues. It has been shown recently that products of the lipoxygenase pathway are also involved in the regulation of tone in guinea-pig trachea (Yamane & Kobayashi 1990) and it is possible that blockade of the cyclo-oxygenase pathway influences activity in the lipoxygenase pathway. The role of arachidonic acid metabolites, therefore, requires further definition in both normal and sensitized airways.

In conclusion, it has been shown that cromakalim attenuates vagal constriction in isolated trachea from albumin-sensitive guinea-pigs. Attenuation arises primarily through pre-junctional mechanisms, as demonstrated previously in normal trachea (McCaig & De Jonckheere 1989) and is unaffected by inhibition of the enzyme cyclo-oxygenase. At the post-junctional level, cromakalim relaxes non-sensitized trachea but has mixed effects on spontaneous tone in sensitized trachea, suggesting that the behaviour of K^+-channels in the trachealis cell membrane may be changed by the sensitization process. The nature of this change and its significance in relation to airway hyper-reactivity remains to be established.

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