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Effects of Cromakalim (BRL 34915) in Trachea Isolated from Actively Sensitized Guinea-pigs

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Abstract—The effects of cromakalim were examined in tracheal strips isolated from normal (unsensitized) guinea-pigs and from animals actively sensitized to bovine serum albumin. Sensitized tracheae exhibited hyper-responsiveness to KCl, acetylcholine and histamine. In normal and sensitized tracheae, cromakalim (0·01–10 μM) produced a concentration-related suppression of spontaneous tone. The ability of cromakalim to relax tracheal strips was reduced when tone was raised by KCl (25 mM), acetylcholine (0·1 mM) or histamine (0·1 mM) and lost against KCl (120 mM)-induced spasm. Procaine (5 mM) abolished the relaxant effect of cromakalim whilst tetraethylammonium (8 mM) was without effect. These effects were similar in normal and sensitized tissues. Cromakalim (10 μM) produced minor alterations of the concentration–effect curves of KCl (1–100 mM), acetylcholine (1 nM–1 mM) and histamine (1 nM–1 mM) in normal and sensitized tissues. The results from this pharmacomechanical study do not support the hypothesis that altered properties of cromakalim-sensitive K^+ channels underlie the airway hyper-reactivity induced by active sensitization to bovine serum albumin.

Cromakalim (BRL 34915) is representative of a new class of smooth muscle relaxants called K^+-channel openers, the pharmacology of which has recently been reviewed (Hamilton & Weston 1989; Cook 1990). Asthma is among the potential therapeutic targets for these substances (Williams et al 1990).

Airway hyper-reactivity to physical, chemical, immunological and pharmacological stimuli is a characteristic feature of asthma but the mechanisms underlying non-specific hyper-responsiveness in human asthma and animal models of allergic asthma are poorly understood (Boushey et al 1980).

Small & Foster (1988) suggested that the hyper-responsiveness of airway muscle in asthma might represent altered gating characteristics of membrane K^+ channels. The generation of action potentials in human airway muscle requires reduction of K^+-channel activity (Marthan et al 1989) and an increased discharge of spontaneous action potentials has been reported to occur during asthmatic episodes (Akasaka et al 1975). In that event, K^+-channel openers such as cromakalim could restore responsiveness to normal and are worthy of further investigation.

The immunization of laboratory animals is commonly utilized as a model of allergic asthma. We have previously characterized the existence of hyper-responsiveness to a variety of pharmacological stimuli in airway smooth muscle isolated from actively sensitized guinea-pigs (Morcillo et al 1984; Ortiz et al 1989). The aim of the present study was to determine whether immunization of guinea-pigs influences the action of cromakalim on isolated trachea.

Materials and Methods

Tissue preparation

Guinea-pigs of either sex, 350–400 g, were randomly allocated to one of two groups, normal (unsensitized) and test (sensitized). Animals were sensitized as previously described (Ortiz et al 1989). Briefly, on day 0 the animals were injected subcutaneously with 0·25 mL of Freund's complete adjuvant plus 1·25 mg kg^{-1} bovine serum albumin (BSA) dissolved in 0·25 mL 0·9% NaCl (saline). On days 2 and 4 the animals received the same amount of Freund's complete adjuvant and BSA by the intramuscular route. The animals were used for experiments on days 21 to 25. The normal group was subjected to the same protocol but received only saline.

Animals were killed by stunning and bleeding. Tracheae were excised, cleaned of adhering tissue and divided into 3 mm wide rings. Rings were opened by cutting longitudinally through the cartilage rings diametrically opposite the trachealis and mounted in jacketed 20 mL organ baths containing Krebs buffer (composition in mM: NaCl 118·4, KCl 4·7, $NaHCO_3$ 25·0, $CaCl_2$ 2·5, $MgSO_4$ 0·6, KH_2PO_4 1·2, dextrose 11·1) maintained at 37°C and gassed with 5% CO_2 in oxygen (pH 7·4 ± 0·1). Tension changes were recorded isometrically using Hewlett-Packard FTA 100-1 or Grass FT0·3 C transducers in conjunction with a polygraph. In preliminary experiments, relaxant responses to isoprenaline (1 μM) or cromakalim (10 μM) were tested against spontaneous tone of normal and sensitized strips subjected to a wide range (1–9 g) of resting tension during a 90 min equilibration period. Optimal tension for relaxant responses (data not shown) was found to be 4 g in normal and sensitized tissues. This was the imposed tension in all subsequent experiments.

Effect of cromakalim on spontaneous and stimulated tracheal tone in normal and sensitized tissues

The relaxant effect of cromakalim against spontaneous tone was studied in two ways. One was the challenge of the preparation with a single concentration of cromakalim (10 μM) or isoprenaline (0·1 μM). In other experiments, cumulative (tenfold concentration increments at intervals of 8 min) concentration–effect curves to cromakalim (0·01–10 μM) were constructed. After washing and recovery of

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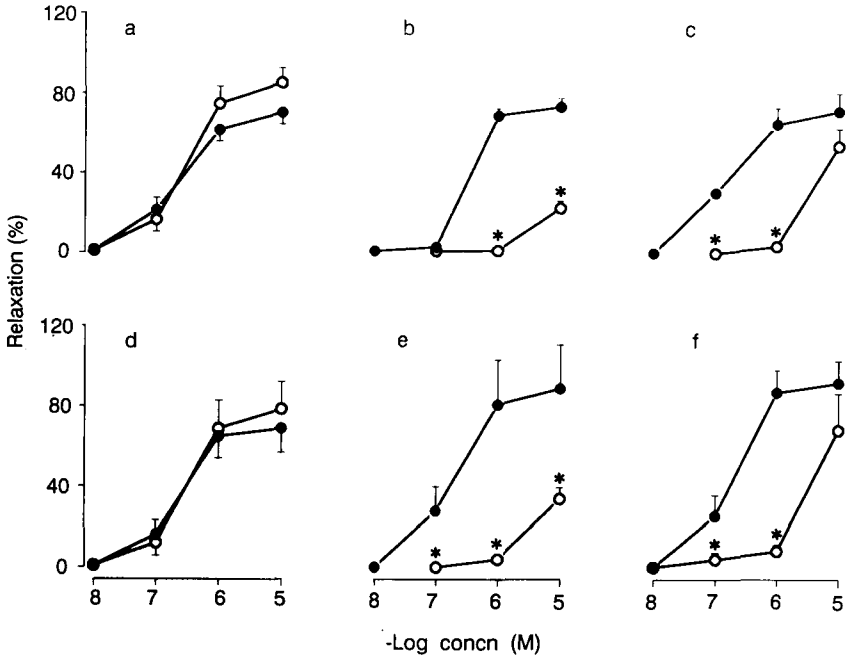

FIG. 1. The effects of cromakalim on the spontaneous (a, d), acetylcholine (b, e)- and histamine (c, f)-induced tone in normal (a, b, c) and sensitized (d, e, f) guinea-pig trachea. The abscissa scales indicate the concentration (−log molar) of cromakalim. The ordinate scales represent relaxation as a percentage of the relaxation to isoprenaline (1 μM). ● Initial log concentration–effect curve in control tissues. ○ Subsequent log concentration–effect curve constructed in control tissues with spontanous tone or in tissues previously contracted with acetylcholine (0·1 mM) or histamine (10·1 mM). Points indicate the means of values from 6 tissues with s.e.m. shown by vertical bars. $*P < 0.05$ compared with the corresponding point in the time-matched control tissues.

resting tension, tissues were exposed to Krebs solution in the absence (no spasmogen) or presence of acetylcholine (0·1 mM) or histamine (0·1 mM) and a second cumulative concentration–effect curve for cromakalim was obtained. The relaxant effects of cromakalim were expressed as the percentage of the relaxation produced by isoprenaline (1 μM) which was taken to represent the maximum relaxation. The molar concentration required to produce 50% of maximum relaxation (IC50) was calculated graphically from plots of individual log concentration–effect curves and then transformed into pD_2 values (−log IC50) for statistical purposes.

Effect of K^+-rich Krebs solution or K^+ channel inhibitors on the relaxant action of cromakalim in normal and sensitized tissues

Following construction of an initial concentration–effect curve for cromakalim, tissues were allocated randomly to test or time-matched control groups. Test tissues were exposed to either Krebs solution containing K^+ (25 or 120 mM in excess of K^+ provided by the Krebs solution) or Krebs solution containing a K^+ channel inhibitor (tetraethylammonium (TEA), 8 mM; procaine, 0·25, 0·5, 1 or 5 mM). These modifying agents were allowed 20 min equilibration before reconstructing the concentration–effect curve of cromakalim.

Effects of cromakalim on concentration–effect curves of KCl, acetylcholine and histamine in normal and sensitized tissues

Cumulative concentration–effect curves were constructed for KCl (1–100 mM), acetylcholine (1 nM–1 mM) and histamine (1 nM–1 mM). After constructing an initial concentration–effect curve for one of these spasmogens, tissues were allocated randomly to test or time-matched control groups. In test tissues, concentration–effect curves for spasmogens were reconstructed following 20 min incubation with, and in the presence of, cromakalim (10 μM). Time-matched control tissues were treated similarly but were not exposed to cromakalim.

Drugs and solutions

Drug concentrations are expressed as the molar concentrations of the active species. The following substances were used: acetylcholine chloride (Sigma, Madrid, Spain), bovine serum albumin (Sigma), cromakalim (BRL 34915, Smith-Kline Beecham Pharmaceuticals), Freund's complete adjuvant (Difco, Detroit, USA), histamine dihydrochloride (Sigma), (−)-isoprenaline hydrochloride (Sigma), procaine hydrochloride (Sigma), tetraethylammonium bromide (Sigma).

Stock solutions of cromakalim and isoprenaline were prepared in 70% v/v ethanol and 0·1 M HCl, those of other agents in twice-distilled water. Dilutions of isoprenaline were prepared in distilled water containing 0·57 mM ascorbic acid as an antioxidant. The final concentrations of vehicles in the bath did not alter either baseline tension or drug-induced responses.

Statistical analysis of results

Results are given as means ± s.e.m. Statistical analysis of the

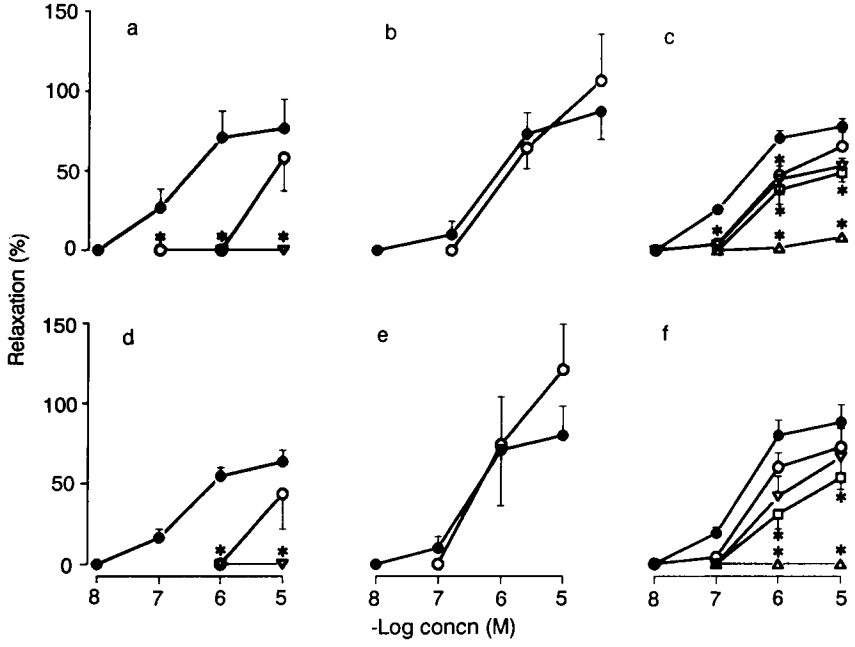

FIG. 2. The effect of K^+-rich Krebs solution (a, d) and K^+-channel inhibitors (b, c, e, f) on the relaxant action of cromakalim in normal (a, b, c) and sensitized (d, e, f) guinea-pigs. The abscissa scales indicate the concentration (−log molar) of cromakalim. The ordinate scales represent relaxation as a percentage of the relaxation to isoprenaline (1 μM). Concentration–effect curves of cromakalim were generated in time-matched control tissues (●), in tissues (a, d) contracted with KCl (25 mM, ○; 120 mM, ▽), or in tissues treated with TEA (b, e; 8 mM, ○), or procaine (c, f; 0·25 mM, ○; 0·5 mM, ▽; 1 mM, □; 5 mM, △). Points indicate the means of values from 6 tissues with s.e.m. shown by vertical bars. *$P<0·05$ compared with the corresponding point in the time-matched control tissues.

results was performed by analysis of variance, followed by Duncan's test (Duncan 1955). Differences were considered significant when $P<0·05$.

Results

Effects of cromakalim on spontaneous and stimulated tone

Cromakalim (10 μM) and isoprenaline (0·1 μM) inhibited spontaneous tone to a similar extent in normal ($-1·57\pm0·36$ and $-1·61\pm0·35$ g, respectively) and sensitized ($-1·62\pm0·19$ and $-2·11\pm0·21$ g, respectively) tissues ($P<0·05$, n=6 in each group). Cromakalim (0·01–10 μM) caused similar, concentration-dependent inhibitions of spontaneous tone in normal and sensitized tracheal strips (Fig. 1). Values for −log IC50 were $6·55\pm0·05$ in normal and $6·70\pm0·13$ in sensitized tissues ($P<0·05$, n=6 in each group). The second concentration–effect curve to cromakalim did not significantly differ from the initial concentration–effect curve (Fig. 1).

When tone had been raised with acetylcholine (0·1 mM), the concentration–effect curve for cromakalim was markedly depressed. When histamine (0·1 mM) was used to increase tone, the concentration–effect curve for cromakalim was moved to the right but the maximal effect of cromakalim was not reduced (Fig. 1).

Effects of K^+-rich Krebs solutions and K^+-channel inhibitors on the relaxant action of cromakalim

The concentration–effect curves of cromakalim against tone produced by KCl (25 mM) were moved to the right but the maximal effect of cromakalim was not reduced (Fig. 2). Relaxation to cromakalim was abolished in tracheal strips contracted by KCl (120 mM).

TEA (8 mM) and procaine (5 mM) each caused tracheal spasm. Contraction to TEA was maintained while that to procaine was followed by relaxation. Although spasm to TEA (8 mM) and procaine (5 mM) tended to be of greater magnitude in sensitized than in normal trachea, the differences did not reach statistical significance (data not shown). In the presence of TEA (8 mM), the concentration–effect curve of cromakalim was not significantly altered. Procaine produced a concentration-related suppression of the concentration–effect curve of cromakalim (Fig. 2). The effects of TEA and procaine on the concentration–effect curve of cromakalim were similar in normal and sensitized tissues.

Effects of cromakalim on concentration–effect curves of KCl, acetylcholine and histamine

KCl (1–100 mM), acetylcholine (1 nM–1 mM) and histamine (1 nM–1 mM) each caused concentration-dependent contraction of tracheal strips in normal and sensitized tissues. Control experiments showed that the concentration–effect curves for these spasmogens, except KCl, underwent a minor rightward shift following further incubation in Krebs solution. Responses to KCl (100 mM), acetylcholine (1 mM) and histamine (1 mM) were greater in sensitized tissues compared with normal tissues (Fig. 3). Treatment of test tissues with cromakalim (10 μM) decreased basal tone in normal ($-1·18\pm0·12$ g; n=18) and sensitized ($-1·12\pm0·15$ g; n=18) tissues. In the presence of cromakalim (10 μM) responses to KCl (100 mM) were enhanced while those to KCl (10 mM) were depressed in normal and sensitized tissues

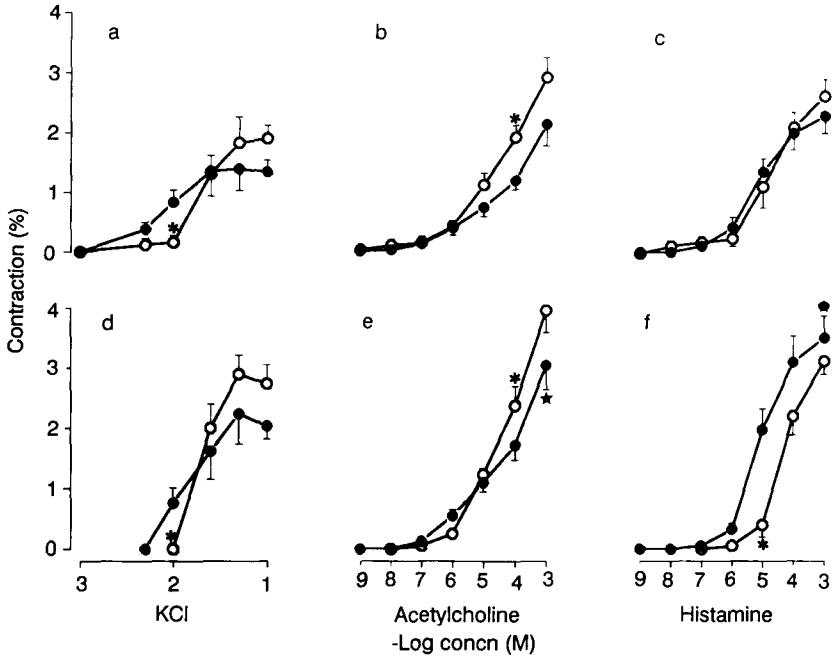

FIG. 3. The effect of cromakalim on the log concentration–effect curves of KCl (a, d), acetylcholine (b, e) and histamine (c, f) in normal (a, b, c) and sensitized (d, e, f) guinea-pig trachea. The abscissa scales represent the concentration ($-\log_{10}$ molar) of the spasmogens. The ordinate scales represent contraction in g. ● Responses to the spasmogens in the absence of cromakalim (time-matched control). ○ Responses to the spasmogens in the presence of cromakalim (10 μM). Points indicate the means of values from 6 experiments and vertical bars are s.e.m. *$P < 0.05$ compared with the corresponding point in the time-matched control tissues. ★ $P < 0.05$ compared with the corresponding point in the normal tissues.

(Fig. 3a, d). Cromakalim (10 μM) produced a minor leftward shift on the concentration–effect curve for acetylcholine but differences only reached statistical significance for acetylcholine (0·1 mM) in normal tissues (Fig. 3b, e). The concentration–effect curve of histamine in normal tissues was not altered in the presence of cromakalim (10 μM) but a rightward shift was observed in sensitized trachea (Fig. 3c, f).

Discussion

In the normal (unsensitized) guinea-pig trachea, cromakalim is a relaxant agent with the pharmacological profile of a K^+-channel opener (Hamilton & Weston 1989). Our results confirm those of Allen et al (1986) in the same preparation and extend the observations to sensitized tissues. Sensitized tracheal strips were hyper-responsive to KCl, acetylcholine and histamine when compared with normal tissues. This is in agreement with previous findings in this model of allergic asthma (Ortiz et al 1989). In the present study, spasmogens were added to tracheal strips with spontaneous tone whilst in the experiments of Allen et al (1986) the spontaneous tone was initially suppressed with indomethacin. The presence of spontaneous tone complicates the analysis of the findings of this study. Although a number of relaxant agents were equally effective as inhibitors of spontaneously developed tone in normal and sensitized tissues (Cortijo et al 1989) it is not precisely known whether spontaneous tone development was similar in normal and sensitized tracheae. However, suppression of spontaneous tone with indomethacin has disadvantages. Indomethacin alters the tracheal responsiveness to spasmogens and eliminates the hyper-responsiveness of sensitized trachea (Cortijo et al 1989).

Cromakalim inhibited basal tone and tone raised by acetylcholine and histamine. Relaxation by cromakalim is attributed to its activity at K^+ channels (Hamilton & Weston 1989). Cromakalim relaxed tone induced by 25 mM KCl but failed to relax the tone generated by 125 mM KCl. The inhibition of spontaneous tone elicited by cromakalim was suppressed by procaine (5 mM) but remained unaffected in the presence of TEA (8 mM) as previously reported by Allen et al (1986) with these two K^+-channel inhibitors. This profile of activity of cromakalim was observed in normal and sensitized tissues. Other actions (inhibition of Ca^{2+} entry, enhanced Ca^{2+} extrusion and alteration of the intracellular Ca^{2+} release), not well characterized (Bray et al 1991), may also contribute to the relaxant effects of cromakalim in the guinea-pig airways.

An antispasmogenic effect of cromakalim was not observed in this study. The lower part of the KCl concentration–effect curve was depressed in normal and sensitized tissues and a rightward shift of the histamine concentration–effect curve was observed only in sensitized trachea. Cromakalim produced minor enhancement of tracheal responses to high concentrations of KCl and acetylcholine. Cromakalim inhibits basal tone and hence concentration–effect curves to the spasmogens were constructed from a lower baseline than those obtained in time-matched control tissues. Therefore, the possibility that the effects of cromakalim on the responsiveness to spasmogens was a reflection of the reduction in the basal tone cannot be excluded. Cromakalim may have opposite effects on different classes of K^+ channels, as shown in vascular smooth muscle (Masuzawa et al 1991), thereby diversely affecting responses to spasmogens. However, the involvement of membrane K^+-channels in the

response to contractile agonists is not completely elucidated. Boyle et al (1988) have demonstrated that, although the spasmogenic effects of acetylcholine and histamine are accompanied by the opening of K^+-channels, TEA (10 mM) and procaine (5 mM) did not augment the tension generated by these agonists. On the other hand, cromakalim hyperpolarizes the airway smooth muscle cell membrane (Allen et al 1986) and hyperpolarization has been shown to increase the amplitude of the depolarization produced by cholinergic excitatory stimuli (McCaig 1987).

The mechanisms underlying in-vitro hyper-responsiveness are basically unknown but an alteration in the handling of Ca^{2+} by airway smooth muscle cells has been suggested (Triggle 1983). Sensitized tracheal preparations were less dependent on extracellular Ca^{2+} (Perpiñá et al 1989; Ortiz et al 1991) and had an augmented uptake and retention of high affinity Ca^{2+} (Perpiñá et al 1991). An enhancement of Na^+, K^+-ATPase and Ca^{2+}-ATPase activities in sensitized trachea has also been suggested (Souhrada et al 1988; Ortiz et al 1991). The hypothesis that a K^+-channel dysfunction underlies hyper-reactivity of airway smooth muscle (Allen et al 1986) is very attractive. A blockade of K^+-channels would produce a partial depolarization of airway smooth muscle cells and the subsequent hyper-responsiveness. The K^+-channel inhibitor, TEA, produces hyper-reactivity to cooling, acetylcholine and 5-hydroxytryptamine in rat isolated trachea, which is predominantly mediated via the influx of extracellular Ca^{2+} (Chand et al 1990). However, the situation appears to be different in the guinea-pig isolated trachea in several aspects: TEA and procaine depressed rather than enhanced spasmogenic responses (Boyle et al 1988); TEA consistently produced a spasm (Allen et al 1986; this study) while rat trachea generally did not contract in response to TEA (Chand et al 1990); the contractions produced by TEA and procaine were not significantly different in normal and sensitized tissues (this study); the relaxant effects of cromakalim were similar in normal and sensitized preparations (this study); and sensitization caused a sustained hyperpolarization of airway smooth muscle cells (Souhrada & Souhrada 1984) instead of the expected depolarization.

In conclusion, there is no experimental evidence in favour of an alteration in cromakalim-sensitive K^+ channels as the mechanism underlying hyper-responsiveness of trachea isolated from sensitized guinea-pigs. However, the involvement of other types of K^+-channels cannot be ruled out.

Acknowledgements

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