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Effects of cromakalim on neurally-mediated responses of guinea-pig tracheal smooth muscle

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1 The ability of cromakalim to modulate several different types of neuroeffector transmission has been assessed in guinea-pig isolated trachea.

2 In trachea treated with propranolol (10^{-6} M) and indomethacin (2.8×10^{-6} M), stimulation of the extrinsic vagal nerves evoked contractions which were blocked by hexamethonium (5×10^{-4} M) or by tetrodotoxin (TTX; 10^{-6} M). Cromakalim (10^{-5} M) caused a two fold rightward shift of the frequency-response curve.

3 In carinal trachea treated with propranolol and indomethacin, transmural stimulation evoked an initial, rapid contraction followed by a more sustained secondary contraction. The initial, rapid contractile response was virtually ablated by atropine (10^{-6} M) or by TTX but was resistant to hexamethonium. Cromakalim (10^{-8}–10^{-5} M) caused a concentration-dependent rightward shift of the frequency-response curve for the initial contraction.

4 In carinal trachea treated with atropine, propranolol and indomethacin, transmural stimulation evoked only the secondary (non-adrenergic, non-cholinergic (NANC)) contractile responses. These were markedly reduced by TTX but were resistant to hexamethonium. Cromakalim (10^{-8}–10^{-5} M) suppressed the NANC contractile responses in a concentration-dependent manner. This action could be offset by glibenclamide (10^{-6} M).

5 In trachea treated with atropine, histamine (10^{-4} M), propranolol and indomethacin, transmural stimulation evoked NANC relaxant responses. Cromakalim (up to 10^{-5} M) was without effect on the frequency-response curve for the stimulation of NANC inhibitory nerves.

6 Tested on trachea bathed by drug-free Krebs solution, cromakalim (10^{-7}–10^{-5} M) caused concentration-dependent suppression of tracheal tone. In trachea treated with propranolol and indomethacin, cromakalim (10^{-7}–10^{-5} M) caused concentration-dependent antagonism of acetylcholine (ACh). In trachea treated with atropine, propranolol and indomethacin, cromakalim (up to 10^{-5} M) failed to antagonize effects of either histamine or substance P.

7 It is concluded that cromakalim can inhibit cholinergic (excitatory) neuroeffector transmission in the trachea but only at a concentration having demonstrable inhibitory activity against the action of exogenous ACh and the spontaneous tone of the airways smooth muscle. In contrast, cromakalim may depress NANC excitatory (putative peptidergic) neuroeffector transmission at a concentration below that exerting inhibitory activity on airways smooth muscle. Cromakalim does not concurrently depress NANC inhibitory neuroeffector transmission. Depression of NANC excitatory neuroeffector transmission could explain the ability of cromakalim to suppress airway hyperreactivity or bronchial asthma at doses lacking direct relaxant effect on airways smooth muscle.

Keywords: Cromakalim; trachealis; extrinsic vagal nerves; NANC excitatory nerves; NANC inhibitory nerves; acetylcholine; histamine; substance P

Introduction

The effects of potassium channel openers in suppressing the tone of airway smooth muscle *in vitro* are well established (Allen *et al.*, 1986; Bray *et al.*, 1987; Arch *et al.*, 1988; Nielsen-Kudsk *et al.*, 1988; Paciorek *et al.*, 1990; Berry *et al.*, 1991; Raeburn & Brown, 1991). The effects of potassium channel openers on neurally-mediated responses in the airways have also attracted interest. For example, Hall & Maclagan (1988) observed that cromakalim reduced responses to preganglionic vagal stimulation of guinea-pig isolated trachea in a preparation where intraluminal pressure changes were monitored. Since cromakalim had a relatively greater effect on responses to vagal stimulation than on responses to exogenous acetylcholine, these authors concluded that cromakalim was inhibiting neurotransmitter release.

Subsequent work has suggested that cromakalim may act on the vagal pathway at a site proximal to the postganglionic nerve terminals. McCaig & De Jonckheere (1989), also using the technique of measuring intraluminal pressure of the guinea-pig isolated trachea, demonstrated that cromakalim reduced pressor responses to preganglionic vagal stimulation without reducing those to acetylcholine (ACh) or postganglionic (field) vagal stimulation. An inhibitory effect of cromakalim on cholinergic neuroeffector transmission has also been demonstrated *in vivo* (Ichinose & Barnes, 1990) but these authors, like Hall & Maclagan (1988), concluded that the drug had both pre- and postjunctional inhibitory activity.

Evidence is emerging that potassium channel openers can also depress the activity of excitatory peptidergic nerves supplying the lung. Potassium channel openers have been reported to inhibit contractions to NANC excitatory nerve stimulation of guinea-pig airways *in vivo* (Ichinose & Barnes, 1990) and bronchial smooth muscle *in vitro* (Good & Hamilton, 1991).

The present study was carried out to compare the effects of cromakalim on contractile and relaxant responses of both NANC and cholinergic neural origin in guinea-pig tracheal smooth muscle *in vitro*. In each case we have attempted to determine whether the effect of cromakalim is mediated at a prejunctional (neural) or postjunctional (airway smooth

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muscle) site. We have also sought to determine whether the effects of cromakalim on neuronal function are observed at concentrations similar to, or lower than, those causing direct relaxation of airway smooth muscle.

Methods

Tissue preparation

Guinea-pigs (300–500 g) of either sex were killed by stunning and bleeding. Tracheae were excised, cleaned of adhering fat and connective tissue and opened by cutting longitudinally through the cartilage rings diametrically opposite the trachealis. The opened trachea was cut into small segments each containing 4–5 cartilage rings. In experiments involving stimulation of the extrinsic vagus nerve, both the right and left vagus nerves next to the trachea were carefully ligated, cut, and removed with the respective recurrent laryngeal nerves attached to the trachea as described by Clark *et al.* (1981).

The tracheal segments were set up for the isometric recording of tension changes (Coburn & Tomita, 1973) in Krebs solution maintained at 37°C and gassed with 95% O_2: 5% CO_2. In most experiments the Krebs solution contained propranolol (10^{-6} M) and indomethacin (2.8×10^{-6} M). The tissues were subjected to an initial imposed tension of 1.5 g. Thereafter, a period of 60 min was allowed for the dissipation of spontaneous tone, a process facilitated by repeated changes of the bath fluid.

Experiments involving transmural stimulation

Transmural stimulation of tracheal segments was carried out by mounting the tissues between stainless steel electrodes (interelectrode distance 1 cm) connected to a stimulator (S-88, Grass Instruments, Quincy, MA, U.S.A.). A cumulative concentration-response curve was first constructed for ACh (10^{-7} M–10^{-2} M) with a contact time of 3 min for each applied concentration. All subsequent responses to drugs or electrical stimulation were related to the initial E_{max} for ACh identified in this curve. Following repeated washing of the tissue and its return to baseline tone, a frequency-response curve was constructed by use of single pulses (0.5 ms duration) of supramaximal strength (45 V) delivered in trains of 10 s duration every 3 min. Pulse frequency was increased in successive trains in two fold steps from 0.5 Hz to 64 Hz. These procedures were repeated in the absence (i.e. time-matched control) or presence of cromakalim (10^{-7}, 10^{-6} and 10^{-5} M) which was administered 8 min before the reapplication of ACh and transmural stimulation. In similarly-designed experiments, hexamethonium (5×10^{-4} M) or TTX (10^{-6} M) were administered 30 min before the reapplication of ACh or transmural stimulation.

In experiments where responses to NANC nerve stimulation were studied in isolation from cholinergic responses, the Krebs solution contained atropine (10^{-6} M), indomethacin (2.8×10^{-6} M), and propranolol (10^{-6} M). A cumulative concentration-response curve was first constructed for histamine (10^{-6}–10^{-3} M). Transmural stimulation was then performed with a pulse duration of 1 ms in order to achieve optimal activation of NANC neurones (Ellis & Undem, 1990). Frequency-response curves for the stimulation of NANC excitatory and NANC inhibitory nerves were constructed, the former against a background of baseline tone and the latter against a background of submaximal tone induced by histamine (10^{-4} M). Pulse frequency was increased in successive trains (10 s duration) in two fold steps from 0.5 Hz to 32 Hz for NANC excitatory responses and from 0.5 Hz to 64 Hz for NANC inhibitory responses. The time between successive pulse trains was determined by the time required for tissue tone to regain the pre-stimulation value. After initial control responses were established, tissues were washed to restore baseline tone. Cromakalim (0, 10^{-7}, 10^{-6}, or 10^{-5} M) was added for 8 min before reconstructing the concentration-effect curve for histamine and repeating the transmural stimulation.

The initial experiments with NANC excitatory nerve stimulation outlined above revealed that the frequency-response curve for NANC excitatory nerves was markedly depressed when attempts were made to reconstruct it in the same tissue. Accordingly, drug effects against NANC excitatory nerve stimulation were mainly studied by use of pulse trains of 10 s duration repeated at 20 min intervals. Pulse frequency was fixed at 4 Hz. This technique yielded NANC contractile responses that remained constant in amplitude for more than 1 h. Following the administration of several control pulse trains, hexamethonium (5×10^{-4} M), TTX (10^{-6} M) or cromakalim (10^{-8}–10^{-6} M) was added to the bath fluid. Cromakalim was added cumulatively, concentration increments occurring 8 min before each pulse train. Time-matched control tissues were treated identically but were not exposed to hexamethonium, TTX or cromakalim. Following stimulation in the presence of the highest concentration of cromakalim (10^{-6} M), glibenclamide (10^{-6} M) was added to the tissue bath for 30 min and the electrical stimulation was repeated.

In a separate series of experiments involving Krebs solution containing atropine (10^{-6} M), propranolol (10^{-6} M) and indomethacin (2.8×10^{-6} M), cumulative concentration-response curves for substance P (10^{-8}–3×10^{-6} M) and histamine (10^{-6}–10^{-3} M) were constructed before and after the application of cromakalim (0, 10^{-7}, 10^{-6} and 10^{-5} M).

Experiments involving stimulation of the extrinsic vagus nerve

A segment of trachea from the carinal or laryngeal end of the trachea was used with the left or right vagi and recurrent laryngeal nerves attached respectively. The vagus nerve was placed across a bipolar electrode for stimulation with single pulses (0.5 ms) at supramaximal strength (30 V) delivered in trains of 10 s duration every 3 min. The tissue was first exposed to ACh (10^{-3} M) to induce a reference contraction. All subsequent response sizes were measured as a percentage of this standard. Following repeated washing of the tissue and its return to baseline tone, a frequency-response curve was constructed by increasing pulse frequency in successive trains in two fold steps from 0.5 Hz to 64 Hz. Test tissues were then exposed to cromakalim (10^{-5} M) 8 min before reconstruction of the frequency-response curve. Control tissues were treated similarly but were not exposed to cromakalim. Tissues were discarded if the maximal contraction obtained with the initial nerve stimulation was smaller than 20% of that obtained with ACh.

Cromakalim and suppression of the spontaneous tone of the trachea

In these experiments small segments of trachea were set up in drug-free Krebs solution; 20 min later aminophylline (1 mM) was added to the bath fluid in order to determine the recorder pen position at zero tone. The tissue was then washed (bath fluid changes at time 0, 10 min and 20 min) and tone was allowed to recover (40 min). A cumulative concentration-effect curve for cromakalim (10^{-7}–10^{-5} M) was then constructed for which half $\log_{10}$ unit concentration increments were used with a tissue contact time of 8 min for each concentration tested.

Drugs and solutions

Drug concentrations are expressed in terms of the molar concentration of the active species. The following substances were obtained from Sigma: acetylcholine chloride, aminophylline, atropine sulphate, glibenclamide, hexamethonium bromide, histamine dihydrochloride, indomethacin, substance P and tetrodotoxin. Propranolol was obtained from ICI and cromakalim was a gift from SmithKline Beecham Research Laboratories. Stock solutions of most drugs were prepared in

twice-distilled water. Stock solutions of cromakalim and glibenclamide were prepared in 70% w/v ethanol and that of indomethacin in absolute ethanol. A stock solution of substance P was made up immediately before use in 0.1% acetic acid. The Krebs solution 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.

Statistical analysis of results

All log concentration-effect and log stimulation frequency-response results were analysed with MEANCURV (Carpenter, 1986). Of the values obtained, the log EC_{20}, log EC_{50}, log EF_{20} and log EF_{50} values and E_{max} relative to initial control values were used for statistical comparison. Analysis of data from protocols employing response curves obtained in succession was carried out with repeated measures of analysis of variance comparing time and dose on a SPSS computer programme (MANOVA; Norusis, 1988). A paired Student's *t* test was used when data from paired tissues were compared. The null hypothesis was rejected when $P < 0.05$.

Results

Transmural stimulation of trachea

In Krebs solution containing propranolol (10^{-6} M) and indomethacin (2.8×10^{-6} M) the response of tracheal segments to transmural stimulation consisted of an initial, rapid, transient contraction that was often followed by a slower, longer-lasting contraction. As reported by Ellis & Undem (1990), the slow secondary contraction was always observed in tissue segments taken from the carinal end of the organ. In contrast, the secondary slow contraction was observed only in 75% (12/16) of tissue segments taken from the laryngeal end of the trachea.

Both phases of the tracheal response to transmural stimulation were frequency-dependent. When tracheal segments were subjected to repeated stimulation in order to assess the reproducibility of the frequency-response relationship, the frequency-response curve for the initial rapid contraction proved relatively constant in shape and position. However, the frequency-response curve for the secondary slow contraction was markedly depressed on stimulating the tracheal segments for a second time.

The log frequency-response curve for the initial rapid response to transmural stimulation was of relatively shallow slope for frequencies up to 8 Hz. Thereafter the slope of the curve became steeper (Figure 1). The log frequency-response curve for the initial rapid response to transmural stimulation was virtually ablated by atropine (10^{-6} M) or by tetrodotoxin (10^{-6} M). In contrast, it was unaffected by hexamethonium (5×10^{-4} M) (Figure 2). Cromakalim (10^{-8}–10^{-5} M) appeared to induce a concentration-dependent rightward shift of the frequency-response curve. However, only at a concentration of 10^{-5} M did cromakalim significantly increase the log EF_{20} and log EF_{50} values. Cromakalim did not affect the maximum contraction evoked by transmural stimulation (Figure 1).

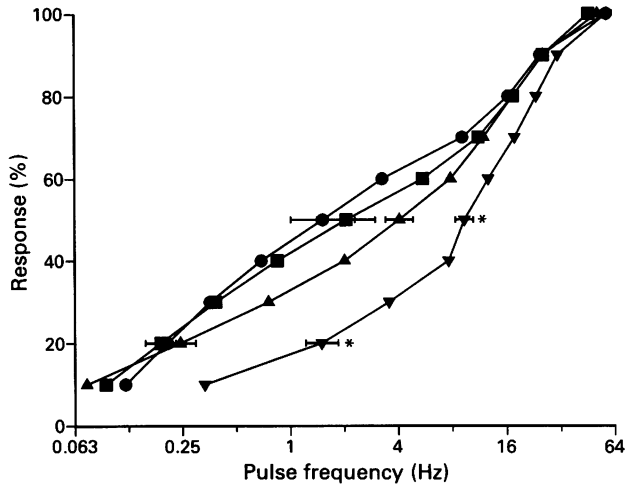

Figure 1 Effects of cromakalim on the initial, rapid contraction evoked by transmural stimulation of guinea-pig isolated trachea. Abscissa scale: pulse frequency (Hz) on a log scale. Ordinate scale: tension developed as a percentage of the response to acetylcholine (10^{-2} M). Pulses (45 V, 0.5 ms duration) were delivered in trains of 10 s duration every 3 min. Propranolol (10^{-6} M) and indomethacin (2.8×10^{-6} M) were present throughout. (●) = log frequency-response curve for time-matched control tissues; (■), (▲), (▼) = log frequency-response curves obtained in tissues treated with 10^{-7}, 10^{-6} and 10^{-5} M cromakalim, respectively. * Indicates a significant ($P < 0.05$) increase in log EF_{20} or log EF_{50} values. Data are means of values from at least 6 tissues; horizontal bars show s.e.mean.

Studies of the slow, secondary (NANC) contractile response of the trachea to transmural stimulation were carried out in Krebs solution containing atropine (10^{-6} M), propranolol

Table 1 Comparison of responses of laryngeal and carinal segments of guinea-pig trachea to transmural stimulation of NANC neurones or to stimulation of the extrinsic vagal (cholinergic) nerves

	NANC inhibitory neurones	*NANC excitatory neurones*	*Vagal cholinergic neurones*
log EF_{20}			
Laryngeal	0.35 ± 0.12 (5)	0.02 ± 0.10 (12)	0.49 ± 0.42 (7)
Carinal	0.34 ± 0.45 (5)	0.15 ± 0.10 (12)	−0.26 ± 0.52* (7)
log EF_{50}			
Laryngeal	0.75 ± 0.16 (5)	0.50 ± 0.10 (12)	0.97 ± 0.40 (7)
Carinal	0.96 ± 0.26 (5)	0.54 ± 0.10 (12)	0.66 ± 0.17* (7)
Maximum response			
	% suppression of response to histamine (10^{-4} M)	*% response to histamine* (10^{-3} M)	*% response to ACh* (10^{-2} M)
Laryngeal	50.0 ± 5.4 (5)	10.6 ± 2.0 (12)	50.6 ± 18.9 (7)
Carinal	24.2 ± 12.0* (5)	17.4 ± 2.9* (12)	48.0 ± 9.3 (7)

Data are means (±s.e.mean) of values from the tabulated (*n*) number of tissues. * Indicates a value significantly ($P < 0.05$) different from the corresponding value from laryngeal segments. Responses to vagal stimulation were recorded in the presence of propranolol (10^{-6} M) and indomethacin (2.8×10^{-6} M). NANC responses to transmural stimulation were recorded in the additional presence of atropine (10^{-6} M). Histamine (10^{-4} M) was also present in the case of NANC relaxant responses.

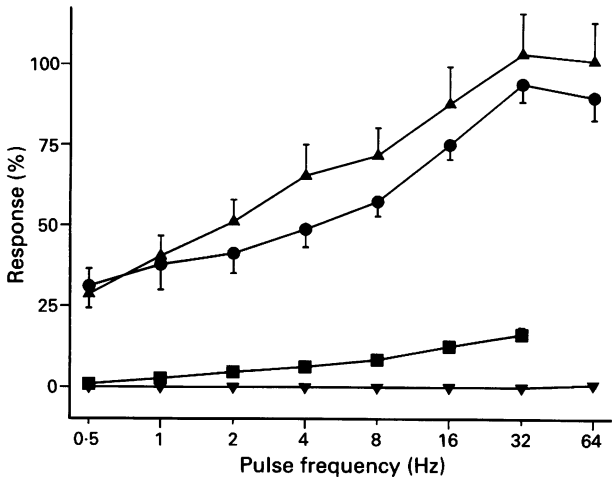

Figure 2 Effects of atropine, tetrodotoxin, and hexamethonium on the initial rapid contraction evoked by transmural stimulation of guinea-pig isolated trachea. Abscissa scale: pulse frequency (Hz) on a log scale. Ordinate scale: tension developed as a percentage of the response to acetylcholine (10^{-2} M). Pulses (45 V, 0.5 ms duration) were delivered in trains of 10 s duration every 3 min. Propranolol (10^{-6} M) and indomethacin (2.8×10^{-6} M) were present throughout. (●) = log frequency-response curve for time matched control tissues; (■), (▲), (▼) = log frequency-response curves obtained in tissues treated with atropine (10^{-6} M), hexamethonium (5×10^{-4} M), or tetrodotoxin (10^{-6} M), respectively. Data are means of values from at least 6 tissues; vertical bars show s.e.mean.

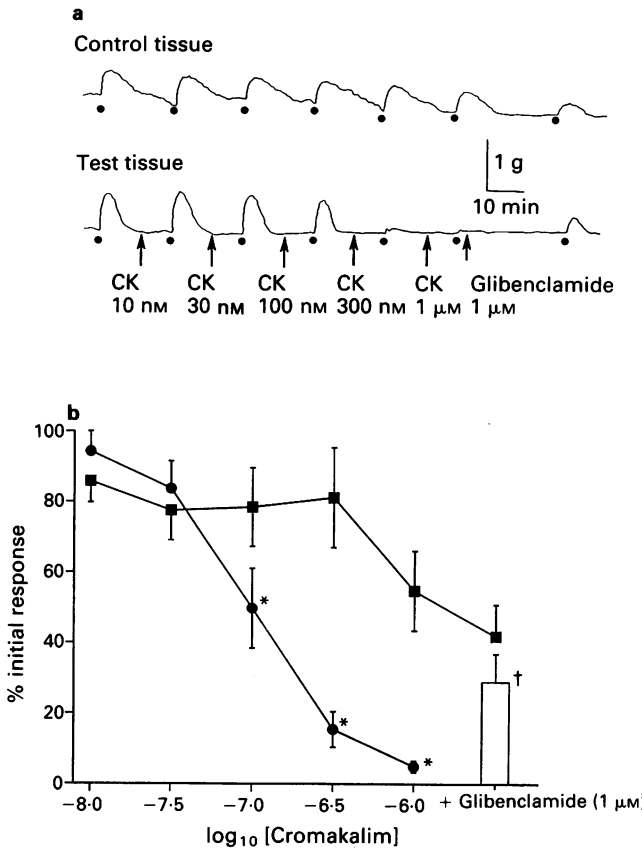

Figure 3 The effects of cromakalim (CK) on the NANC contractile response of carinal segments of guinea-pig isolated trachea to transmural stimulation. (a) Experimental tracings showing the inhibitory effect of cromakalim (10^{-8} M–10^{-6} M, cumulatively applied) on the NANC contractile response to transmural stimulation (●) (45 V pulses of 1 ms duration and 4 Hz frequency delivered in trains of 10 s duration every 20 min). The upper trace represents a time-matched control tissue, the lower the test tissue. Atropine (10^{-6} M), propranolol (10^{-6} M) and indomethacin (2.8×10^{-6} M) were present throughout. Note how glibenclamide (10^{-6} M) applied to the test tissue, was able to offset the inhibitory effect of cromakalim. (b) Log concentration-effect curve for the inhibitory effect of cromakalim against NANC contractile responses evoked by transmural stimulation of carinal segments of guinea-pig trachea (45 V pulses of 1 ms duration and 4 Hz frequency delivered in trains of 10 s duration every 20 min). Atropine (10^{-6} M), propranolol (10^{-6} M), and indomethacin (2.8×10^{-6} M) were present throughout. (■) = response of time-matched control tissues; (●) = response of test tissues. * Indicates a significant ($P < 0.05$) difference between values for the test and time-matched control tissues; † indicates a significant ($P < 0.05$) difference between values of the test tissues in the presence of cromakalim (10^{-6} M) in the presence and absence of glibenclamide (10^{-6} M). Data are means of values from at least 6 tissues; vertical bars show s.e.mean.

(10^{-6} M) and indomethacin (2.8×10^{-6} M). Initially NANC contractile responses of carinal and laryngeal segments of trachea were compared by constructing frequency-response curves. The log EF_{50} values for NANC contractile responses were similar in carinal and laryngeal trachea. However, the maximal NANC contractile response to transmural stimulation was greater in carinal than in laryngeal segments (Table 1).

All further studies of NANC contractile responses were carried out on carinal segments of trachea only. When this tissue was stimulated repetitively at a pulse frequency of 4 Hz, the NANC contractile responses remained relatively constant in amplitude for up to 80 min. Thereafter the responses progressively declined in amplitude (Figure 3a,b). The NANC contractile responses to transmural stimulation were unaffected by hexamethonium (5×10^{-4} M) but were virtually abolished by tetrodotoxin (10^{-6} M) (Figure 4). Cromakalim (10^{-8}–10^{-6} M) applied cumulatively caused concentration-dependent depression of the NANC contractile response to transmural stimulation (log $IC_{50} - 7.06 \pm 0.11$; mean ± s.e.mean, $n = 10$) (Figure 3a,b). Cromakalim (10^{-6} M) inhibited the NANC contractile response by more than 90%. Once the summit of the concentration-effect curve for cromakalim had been reached, the administration of glibenclamide (10^{-6} M; 30 min preincubation) caused the inhibitory action of cromakalim to be offset (Figure 3b).

Studies of NANC relaxant responses to transmural stimulation were carried out in Krebs solution containing atropine (10^{-6} M), histamine (10^{-4} M), propranolol (10^{-6} M) and indomethacin (2.8×10^{-6} M). Comparison of tissue from the laryngeal and carinal ends of the trachea showed that while log EF_{50} values were similar, the maximal relaxant response obtained in carinal tissue was smaller than that obtained in laryngeal tissue (Table 1). NANC relaxant responses to transmural stimulation were unaffected by cromakalim (up to 10^{-5} M) (data not shown).

Effects of cromakalim on vagal stimulation

For anatomical reasons, stimulation of the left vagus nerve required use of a carinal segment of trachea and stimulation of the right vagus nerve required a laryngeal tracheal segment. The log EF_{20} and log EF_{50} values for stimulation of carinal tissue via the left vagus nerve were significantly smaller than the equivalent values for the right vagus nerve and laryngeal segment of trachea. However, the maximum contraction obtainable by nerve stimulation did not differ with location (Table 1). The effects of stimulating the left or right extrinsic vagal nerves were blocked both by hexamethonium (5×10^{-4} M) and by TTX (10^{-6} M). Cromakalim (10^{-5} M) induced a rightward shift of the frequency-response curve for stimulation of the extrinsic vagal nerves, causing a two fold increase in the EF_{50} (Figure 5). When the maximal response to vagal stimulation was measured as a percentage of the contraction evoked by ACh (10^{-3} M), it became evident that cromakalim had additional inhibitory effect. Cromakalim (10^{-5} M) reduced the maximal response from 52.7 ± 5.8% to 37.4 ± 4.7% of the ACh standard (mean ± s.e.mean, $n = 6$ and 9 respectively; $P < 0.05$).

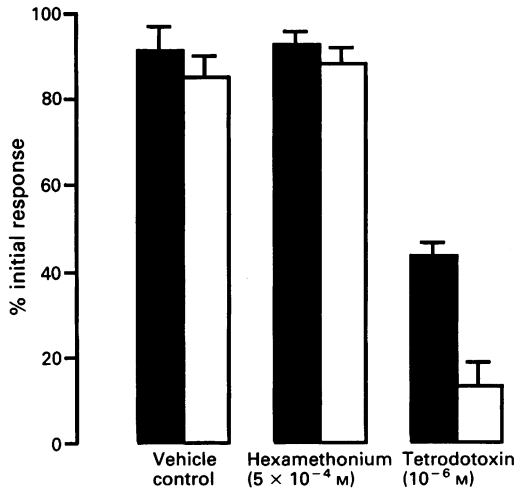

Figure 4 Effects of tetrodotoxin and hexamethonium on the NANC contractile response to transmural stimulation (1 ms pulses of 4 Hz frequency and 45 V strength delivered in trains of 10 s duration every 20 min) of carinal segments of guinea-pig isolated trachea. Atropine (10^{-6} M), propranolol (10^{-6} M) and indomethacin (2.8×10^{-6} M) were present throughout. In each case the solid and open columns represent response height (as a percentage of the initial control) 20 min (solid columns) and 40 min (open columns) after the administration of vehicle, hexamethonium (5×10^{-4} M) or tetrodotoxin (10^{-6} M).

Effects of cromakalim on the spontaneous tone of the trachea and on the actions of exogenous spasmogens

In trachea bathed by normal Krebs solution, cromakalim (10^{-7}–10^{-5} M) produced concentration-dependent suppression of the spontaneous tone of the tissue. The mean (±s.e.mean; $n = 18$) log EC_{50} for the tone suppressant action of cromakalim was -6.35 ± 0.05, a figure significantly ($P < 0.05$; two-tailed unpaired t test) smaller than the corresponding mean log IC_{50} for suppression of contractile responses to transmural stimulation of NANC neurones (see above). The maximal relaxant effect of cromakalim was 73.2 ± 2.5% of that of aminophylline.

Tested in tracheal segments treated with propranolol (10^{-6} M) and indomethacin (2.8×10^{-6} M), cromakalim induced a rightward shift of the ACh concentration-response curve. The EC_{20} and EC_{50} values for ACh were significantly increased (1.8 and 2.5 fold respectively) by cromakalim (10^{-5} M). The maximal contraction induced by ACh was unaffected (Figure 6a).

Tested in trachea treated with atropine (10^{-6} M), propranolol (10^{-6} M) and indomethacin (2.8×10^{-6} M), cromakalim (up to 10^{-5} M) had no effect on contractions induced by histamine

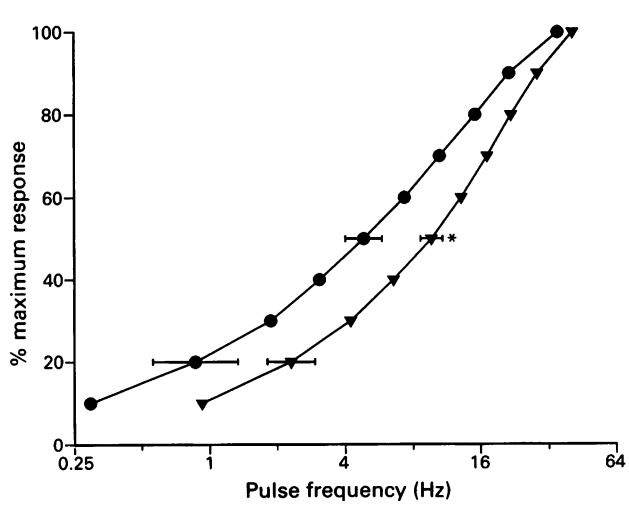

Figure 5 The effects of cromakalim on the contractile response of guinea-pig isolated trachealis to stimulation of its extrinsic vagal nerve supply. Abscissa scale: pulse frequency on a log scale. Ordinate scale: tension developed as a percentage of maximum. (●) = log frequency-response curve obtained in time-matched control tissues; (▼) = log frequency-response curve obtained in test tissues treated with cromakalim (10^{-5} M); 30 V pulses of 0.5 ms duration were delivered in trains of 10 s duration every 3 min. Propranolol (10^{-6} M) and indomethacin (2.8×10^{-6} M) were present throughout. Data are means of values from 9 tissues (carinal trachea with left vagus nerve attached and laryngeal trachea with right vagus nerve attached); horizontal bars show s.e.mean. * Indicates a significant difference between the log EF_{50} values of test and control tissues.

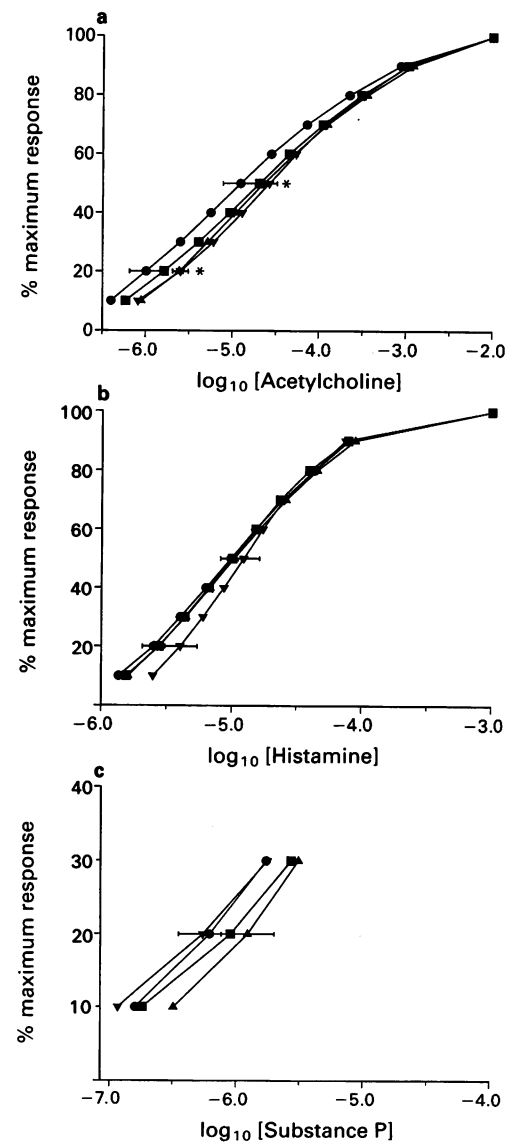

Figure 6 Effects of cromakalim on the contraction of guinea-pig isolated trachealis muscle to (a) acetylcholine, (b) histamine, and (c) substance P. In each panel the abscissa scale indicates the concentration of spasmogen on a log scale. The ordinates represent the tension developed as a % of the E_{max} obtained with acetylcholine (10^{-2} M; a) or histamine (10^{-3} M; b and c). (●) = log concentration-effect curve for the spasmogen as observed in time-matched control tissues; (■), (▲), (▼) = log concentration-effect curves for the spasmogen observed in test tissues treated with 10^{-7} M, 10^{-6} M, and 10^{-5} M cromakalim, respectively. Propranolol (10^{-6} M) and indomethacin (2.8×10^{-6} M) were present throughout the experiments. Atropine (10^{-6} M) was present in the experiments involving histamine and substance P (b and c). Data represent means of values from at least 6 tissues.

or substance P (Figure 6b and c). None of the exogenous spasmogens was affected by hexamethonium (5×10^{-4} M) or TTX (10^{-6} M).

Discussion

Cromakalim and cholinergic neuroeffector transmission in guinea-pig trachea

Stimulation of the extrinsic vagal nerves supplying guinea-pig isolated trachea evokes contractile responses that are suppressed by atropine (Clark *et al.*, 1981), hexamethonium (Clark *et al.*, 1981; present study) or by tetrodotoxin (present study). Such responses are therefore mediated by the preganglionic stimulation of vagal cholinergic neural pathways innervating the trachealis. When carinal segments of guinea-pig trachea are subjected to transmural stimulation in the presence of propranolol and indomethacin, the contractile response is diphasic (Ellis & Undem, 1990; present study). The initial, rapid contractile response is suppressed by atropine and tetrodotoxin (Ellis & Undem, 1990; present study) but is resistant to hexamethonium (present study). The initial contractile response of carinal trachea to transmural stimulation is therefore mediated by the postganglionic activation of vagal cholinergic pathways supplying the trachealis muscle.

That cromakalim inhibits excitatory, cholinergic neuroeffector transmission in guinea-pig trachea is suggested by its ability (at a concentration of 10^{-5} M) to cause rightward shifts in the frequency-response curves to both pre- and postganglionic vagal nerve stimulation (Figure 1). Our finding that cromakalim can inhibit responses to stimulation of preganglionic cholinergic nerves confirms earlier studies (Hall & Maclagan, 1988; McCaig & De Jonckheere, 1989). However, our finding that cromakalim inhibits responses to the postganglionic stimulation of cholinergic pathways contrasts with the observations of McCaig & De Jonckheere (1989). Since inhibitory effects of cromakalim on postganglionic nerve stimulation were observed at a concentration (10^{-5} M) causing marked suppression of the spontaneous tone of the trachea and some antagonism of exogenous acetylcholine (Figure 6a) we must assume that the ability of cromakalim to depress cholinergic neuroeffector transmission includes a postjunctional (on trachealis cells) site of action. In this respect our findings lend support to the *in vivo* observations of Ichinose & Barnes (1990). Assay of neurotransmitter release is clearly required to determine whether a prejunctional action of cromakalim is of any importance in depressing cholinergic neuroeffector transmission in the airways.

Cromakalim and NANC inhibitory neuroeffector transmission in guinea-pig trachea

When guinea-pig trachea is treated with antagonists at muscarinic cholinoceptors and sympatholytic drugs, transmural stimulation elicits relaxant responses attributable to the activation of intramural NANC inhibitory neurones (Coburn & Tomita, 1973; Boyle *et al.*, 1987). The transmitter used by such neurones remains the subject of debate but there is evidence to suggest that it may be vasoactive intestinal polypeptide and/or nitric oxide (Ellis & Farmer, 1989a,b; Li & Rand, 1991). In human airways, NANC inhibitory neurones comprise the principal neural bronchodilator pathway (Richardson & Beland, 1976). The present finding that cromakalim does not attenuate NANC inhibitory neuroeffector transmission in the airways is therefore a good feature of its pharmacological profile bearing in mind its potential use in the chemotherapy of bronchial asthma.

Cromakalim and NANC excitatory neuroeffector transmission in guinea-pig trachea

When guinea-pig trachea is subjected to transmural stimulation in the presence of sympatholytic drugs and an antagonist at muscarinic cholinoceptors, contractile responses are obtained which are attributable to the activation of intramural nerves the transmitter for which may be substance P (Szolcsanyi & Bartho, 1982; Andersson & Grundstrom, 1983; Grundemar *et al.*, 1990; Ellis & Undem, 1990). In the present study such responses were tetrodotoxin-sensitive but hexamethonium-resistant (Figure 4) indicating that the responses were of neural origin but that transmission across cholinergic ganglionic synapses was not involved.

Our demonstration that cromakalim can depress NANC excitatory neuroeffector transmission in guinea-pig trachea is consistent with findings in guinea-pig isolated bronchus (Good & Hamilton, 1991). The IC_{50} for cromakalim-induced suppression of NANC excitatory neuroeffector transmission was significantly smaller than that for cromakalim-induced suppression of spontaneous tone. Furthermore, cromakalim failed to antagonize the contractile effects of exogenous substance P (Figure 6c). These observations suggest that the action of cromakalim in suppressing NANC excitatory neuroeffector transmission may involve inhibition of neurotransmitter release. A similar conclusion has been drawn from the results of *in vivo* studies (Ichinose & Barnes, 1990).

Intramural peptidergic nerves may have pro-inflammatory roles in the airways (Barnes, 1991). Accordingly it may be that cromakalim has important actions on pulmonary peptidergic neurones which may reduce the phenomenon of airway hyperreactivity and relieve the symptoms of bronchial asthma. This idea receives support from the observations that doses of cromakalim too low to cause direct relaxation of airways smooth muscle can suppress airway hyperreactivity in laboratory animals (Chapman *et al.*, 1991) and improve the early morning dip in lung function in patients with nocturnal asthma (Williams *et al.*, 1990).

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