Br. J. Pharmacol. (1989), **98**, 662–668

Effect of cromakalim on bronchoconstriction evoked by cholinergic nerve stimulation in guinea-pig isolated trachea

Dorothy J. McCaig & B. De Jonckheere

School of Pharmacy, Robert Gordon's Institute of Technology, Schoolhill, Aberdeen AB9 1FR

1 Cromakalim reduced intraluminal pressure in the guinea-pig isolated, innervated trachea.

2 Preganglionic stimulation of the cervical vagus nerve elicited a frequency-dependent increase in intraluminal pressure. Cromakalim attenuated responses to vagal stimulation in a concentration-dependent manner at all frequencies tested.

3 Field stimulation caused a frequency-dependent increase in intraluminal pressure mediated by muscarinic cholinoceptors. Cromakalim did not affect the amplitude of responses at any frequency of stimulation, even at high concentrations.

4 Acetylcholine, added to the Krebs solution bathing the adventitial surface of the trachea, evoked a concentration-dependent increase in intraluminal pressure. The concentration-effect curve for acetylcholine was unaltered in the presence of cromakalim.

5 It is concluded that cromakalim modulates cholinergic neuroeffector transmission in the trachea chiefly by a prejunctional mechanism. However, cromakalim probably does not interfere with acetylcholine release from postganglionic cholinergic neurones.

Introduction

Cromakalim is a smooth muscle relaxant which is thought to act by promoting K^+-efflux through specific K^+-channels in the plasma membrane (Hamilton *et al.*, 1986; Weir & Weston, 1986a,b). In guinea-pig trachealis, relaxation is associated with marked hyperpolarisation, close to the K^+-equilibrium potential for these cells (Allen *et al.*, 1986), which would be expected to reduce the general excitability of the tissue. However, cromakalim did not markedly attenuate the bronchoconstriction induced by a variety of agents including potassium chloride (>10 mM), histamine and acetylcholine (ACh) (Allen *et al.*, 1986). Although the effects of applied ACh were relatively unaffected by cromakalim, it seemed possible that cromakalim might be more effective against the bronchoconstriction induced by endogenous ACh released from parasympathetic nerve terminals, perhaps by modulating cholinergic neurotransmission. To test this hypothesis the guinea-pig isolated trachea was used to examine the effects of cromakalim on bronchoconstriction evoked in three different ways: (1) stimulation of the cervical vagus nerve (preganglionic), (2) field stimulation (postganglionic) and (3) applied ACh. Preliminary accounts of these and similar experiments by Hall & Maclagan have been published (Hall & Maclagan, 1988; McCaig & De Jonckheere, 1989).

Methods

Preparation

Guinea-pigs (male, approximately 400 g body weight) were killed by a blow to the head and the trachea rapidly excised with or without the right vagus nerve and its recurrent laryngeal branch, as required (Blackman & McCaig, 1983). The trachea was cannulated at each end and mounted horizontally in an organ bath, maintained at 37°C, through which Krebs solution flowed at a rate of 5 ml min^{-1}. The composition of the Krebs solution was (mM): Na^+ 127, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 121, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, HCO_3^- 25 and glucose 11. The trachea was filled with Krebs solution, then one end was closed and the other attached to a pressure transducer (Statham). Intraluminal pressure (ILP) was recorded continuously on a pen recorder (Devices). Increases and decreases in ILP reflect bronchoconstriction and bronchodilatation, respectively.

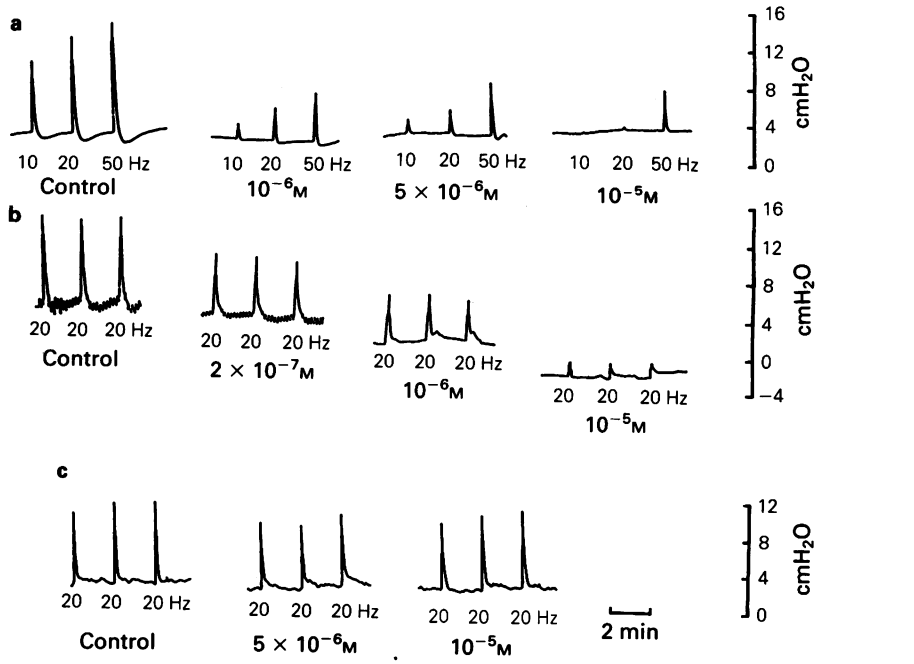

Figure 1 Changes in intraluminal pressure in guinea-pig isolated trachea evoked by (a) stimulation of the vagus nerve at frequencies indicated for 5 s at 2 min intervals, (b) stimulation of the vagus nerve at 20 Hz for 5 s at 2 min intervals and (c) field stimulation at 20 Hz for 5 s at 2 min intervals. Each trace is taken from a single preparation before and 30 min after cumulative additions of cromakalim at concentrations indicated.

Procedure

The cervical vagus nerve was stimulated through a suction or, occasionally, a bipolar electrode (pulses 20 V, 1 ms duration) for 5 s every 2 min at frequencies of 1–50 Hz. Field stimulation was achieved by means of a bipolar electrode placed close to the dorsal aspect of the trachea and the same stimulus parameters. Cumulative concentration-effect curves to applied ACh were constructed by adding ACh directly to the organ bath (with perfusion pump off) in 10 fold increments (10^{-8}–10^{-2} M).

In vagal- or field-stimulated preparations a control frequency-response curve was obtained, then the tissue was stimulated at 20 Hz every 2 min and cromakalim (10^{-7}–10^{-5} M) was added to the reservoir of Krebs solution. (It should be noted that solution from the reservoir was pre-heated by passing it through a coil system (volume approximately 20 ml) in a water bath so that a delay of about 4 min occurred before the tissues were exposed to cromakalim). After 25 min, a second frequency-response curve was obtained. The procedure was then repeated once, or at the most twice, with successively higher concentrations of cromakalim. Cumulative concentration-effect curves to ACh were obtained before and 25 min after addition of cromakalim (10^{-5} M) to the reservoir of Krebs solution. In one group of preparations repeated frequency-response or concentration-effect curves were obtained in the absence of cromakalim to serve as time-matched controls. In another group of preparations frequency-response curves to vagal or field stimulation were obtained in the absence and presence of hexamethonium (2.5×10^{-4} M added to the reservoir of Krebs solution), to determine whether responses were due to pre- or post-ganglionic activation of cholinergic nerve fibres.

Drugs

The following drugs were used: acetylcholine chloride (Sigma), atropine sulphate and hexamethonium bromide (Sigma), cromakalim (Beecham Research Laboratories) and propranolol HCl (ICI).

Statistics

Mean responses were compared by use of t test for independent or paired data as appropriate. Values of $P < 0.05$ were regarded as significant.

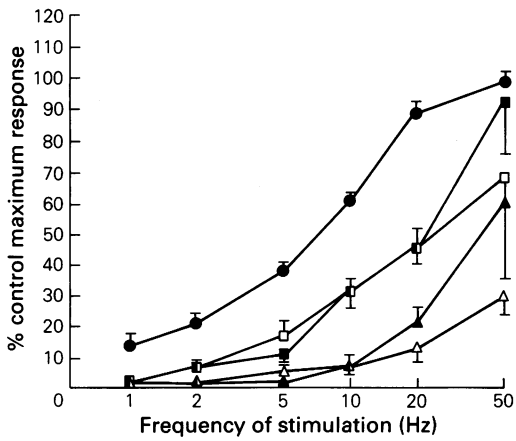

Figure 2 Guinea-pig isolated trachea: relationship between increase in intraluminal pressure, expressed as percentage control maximum response (ordinate scale) and frequency of stimulation (abscissa scale) of the vagus nerve in the absence (●) and presence of cromakalim, 2×10^{-7} M (■), 10^{-6} M (□) 5×10^{-6} M (▲) and 10^{-5} M (△). Values are the mean of 5–8 observations; vertical lines show s.e.mean. All values (except the response at 50 Hz in the presence of 2×10^{-7} M cromakalim) were significantly different from the corresponding control ($P < 0.05$, paired t test).

Results

Relaxant effect of cromakalim

Tracheal preparations had a positive resting ILP of approximately 2–4 cmH_2O, as described previously (Blackman & McCaig, 1983), indicating the presence of spontaneous resting tone in the trachealis muscle. Cromakalim, 10^{-7}–10^{-5} M, evoked a concentration-dependent reduction in ILP which developed gradually, reaching a maximum at 15 ± 1 min (mean ± s.e.mean, $n = 14$). The maximum effect was not maintained in the continued presence of the drug. The amplitude of the response varied considerably between preparations and was probably determined by the initial level of ILP (compare a and b in Figure 1).

Effect of cromakalim on responses to stimulation of the vagus nerve

Stimulation of the vagus nerve elicited frequency-dependent increases in ILP (Figures 1a and 2). When preparations were stimulated at 20 Hz for 5 s at 2 min intervals highly reproducible increases in ILP were obtained. On exposure to cromakalim (10^{-7}–10^{-5} M) the amplitude of this response gradually diminished (see Figure 1b). Maximum attenuation was found to occur after 22 ± 2 min ($n = 17$). Responses were attenuated at all frequencies of stimulation tested (1–50 Hz, Figures 1a and 2), but at 50 Hz the effects of cromakalim were more variable, especially at lower concentrations. The degree of attenuation of vagal responses was concentration-dependent (Figures 1 and 2). At the lowest concentration of cromakalim tested (10^{-7} M) attenuation was already substantial (e.g. at 20 Hz, $50 \pm 15\%$, $n = 3$) and was marked at the highest concentration (10^{-5} M) (at 20 Hz, $86 \pm 5\%$, $n = 5$).

When time-matched control tissues were examined, it was found that the amplitude of vagal responses was well maintained during the first 40 min but declined somewhat thereafter by approximately 20%. Attenuation of vagal responses was significantly greater during exposure to cromakalim at all concentrations tested, than in time-matched controls, as shown in Figure 3a. Cromakalim therefore attenuated markedly responses to stimulation of the vagus nerve.

Responses to vagal stimulation at all frequencies tested were virtually abolished in the presence of hexamethonium (2.5×10^{-4} M, Figure 4a). This confirms that activation of the cholinergic pathway is preganglionic in this preparation.

Effect of cromakalim on responses to field stimulation

In these experiments field stimulation elicited a response which was predominantly an increase in ILP. In some tissues the initial increase in ILP was followed by a reduction in ILP. These responses were blocked respectively by atropine (6×10^{-7} M) and propranolol (3.5×10^{-6} M) indicating muscarinic cholinoceptor-mediated excitation and β-adrenoceptor-mediated inhibition. The amplitude of the initial increase in ILP was unaffected by propranolol pretreatment. This was anticipated since the responses have largely separate time courses, the excitatory response being rapid and brief and the inhibitory response of slower onset and rather longer duration (Blackman & McCaig, 1983). Experiments with cromakalim, therefore, were performed in the absence of an antagonist of β-adrenoceptors.

Field stimulation evoked frequency-dependent increases in ILP. Cromakalim, 2×10^{-7}–10^{-5} M had no demonstrable effect on responses to field stimulation (Figure 1c). Frequency-response curves obtained in the absence and presence of cromakalim were superimposable (Figure 5). In time-matched control tissues there was some reduction in the amplitude of responses with time (Figure 3b). There were no significant differences between responses in time-matched control tissues and cromakalim-treated tissues at any of the concentrations tested. In

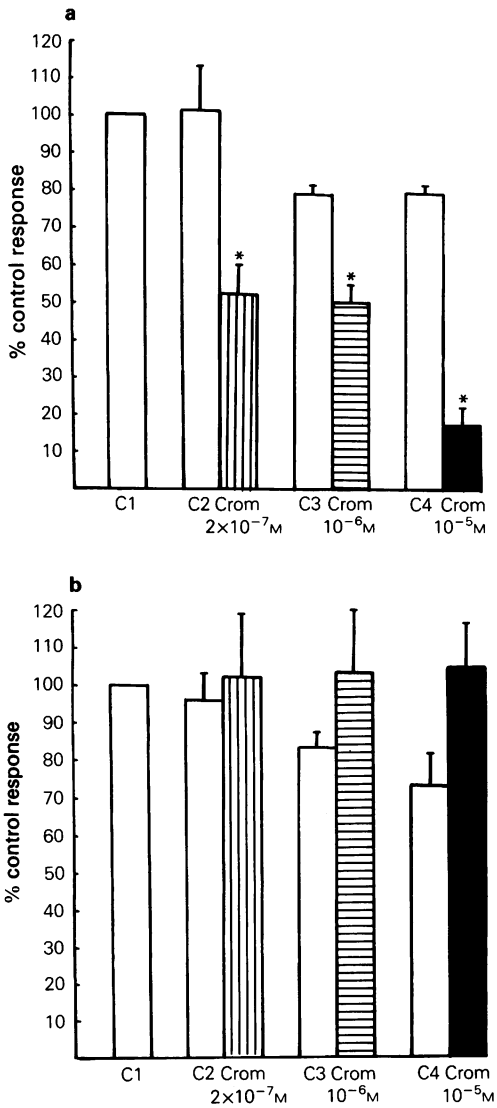

Figure 3 Guinea-pig isolated trachea: increases in intraluminal pressure induced by (a) stimulation of the vagus nerve and (b) field stimulation, each of 20 Hz for 5 s at 2 min intervals, expressed as percentage of the initial control response (C1), in time-matched controls (C2–C4 open columns) and in the presence of cromakalim (Crom) 2×10^{-7} M, 10^{-6} M and 10^{-5} M. Values are the mean of 4–8 observations; vertical bars show s.e.mean. Asterisks denote values significantly different from time-matched controls at $P < 0.01$ (t test).

contrast to the marked attenuation of vagal responses therefore, cromakalim had no effect on cholinergic excitation elicited by field stimulation.

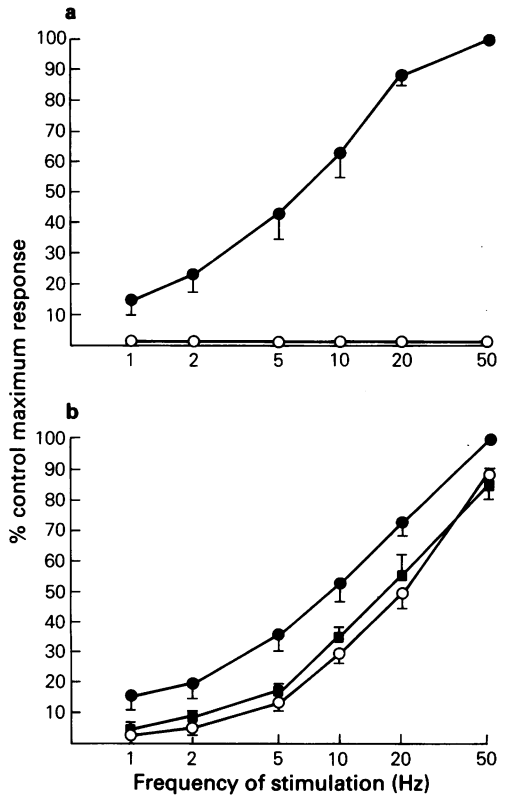

Figure 4 Guinea-pig isolated trachea: relationship between increase in intraluminal pressure, expressed as percentage of the initial control maximum (ordinate scale) and frequency of vagal (a) or field (b) stimulation (abscissa scale). (a) Responses before (●) and 30 min after (○) the addition of hexamethonium, 2.5×10^{-4} M, to the reservoir of Krebs solution. (b) Responses before (●), 30 min after (○) the addition of hexamethonium, 2.5×10^{-4} M, to the reservoir of Krebs solution and in time-matched controls (■). Values are the mean of 5–10 observations; vertical lines show s.e.mean. The response to vagal stimulation was virtually abolished by hexamethonium (a). In (b) responses to field stimulation in time-matched controls were significantly reduced ($P < 0.05$, paired t test) at frequencies of 1 and 2 Hz and in the presence of hexamethonium responses were significantly reduced at 5 and 10 Hz (each as compared with corresponding initial control responses); responses in the presence of hexamethonium were not significantly different from the corresponding time-matched control response at any frequency of stimulation.

When a second frequency-response curve to field stimulation was obtained 30 min after the first curve a small rightward shift was observed, with a significant reduction in response amplitude at low frequencies of stimulation (1 and 2 Hz, Figure 4b). The frequency-response curve obtained in the presence of hexamethonium (2.5×10^{-4} M) also exhibited a

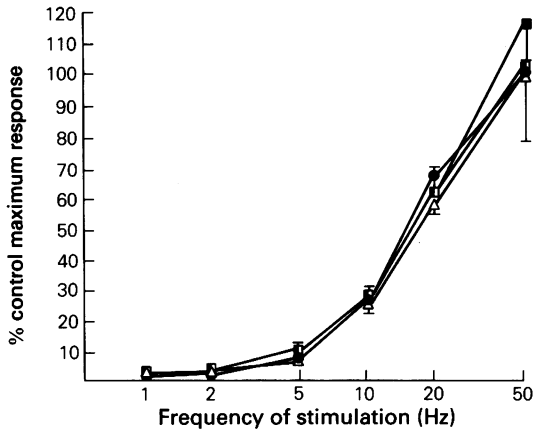

Figure 5 Guinea-pig isolated trachea: relationship between increase in intraluminal pressure, expressed as percentage control maximum (ordinate scale), and frequency of field stimulation (abscissa scale) in the absence (●) and presence of cromakalim 2×10^{-7} M (■), 10^{-6} M (□) and 10^{-5} M (△). Values are the mean of 4–6 observations and none of the values was significantly different from the corresponding control (paired t test). Vertical lines show s.e.mean.

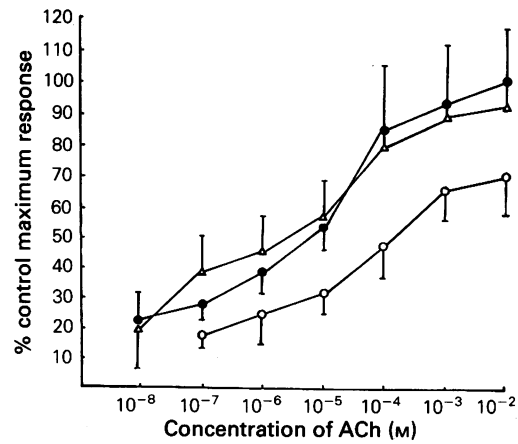

Figure 6 Guinea-pig isolated trachea: relationship between increase in intraluminal pressure, expressed as percentage of the initial control maximum, (ordinate scale) and concentration of applied acetylcholine (ACh) (abscissa scale) in the absence (●) and presence of cromakalim 10^{-5} M (△) and in time-matched controls (○). Values are the mean of 5 observations; vertical lines show s.e.mean. Responses in time-matched controls at ACh 10^{-3} M and 10^{-2} M were significantly reduced ($P < 0.05$, t test) compared with the corresponding responses in the initial curve. Responses in the presence of cromakalim were not significantly different from either the initial control or the time-matched control at any concentration of ACh.

slight rightward shift compared to the initial control curve, but was not significantly different from the time-matched control curve at any frequency of stimulation (Figure 4b). These results indicate that responses to field stimulation are not susceptible to ganglion blockade. Activation of the cholinergic pathway, therefore, is postganglionic in this situation.

Effect of cromakalim on responses to applied ACh

ACh (10^{-8}–10^{-2} M) elicited concentration-dependent increases in ILP. There was a slight rightward shift of the concentration-effect curve with time, with responses at higher concentrations of ACh being significantly smaller than those observed in the initial curve (Figure 6). In the presence of cromakalim, 10^{-5} M, the concentration-effect curve was close to the initial control curve and not significantly different from either this or the time-matched control curve at any concentration of ACh. Cromakalim, therefore, did not attenuate the bronchoconstrictor response to applied ACh.

Discussion

These results indicate that cromakalim can effectively reduce bronchoconstriction mediated by stimulation of the vagus nerve in the isolated guinea-pig trachealis. Hall & Maclagan (1988) obtained a similar finding. Such attenuation could arise pre- and/or post-junctionally and attempts were made, therefore, to localise the effect. Cromakalim had no effect on the response of the trachealis to applied ACh (present study). Allen *et al.* (1986) and Hall & Maclagan (1988) both observed that the concentration-effect curve for ACh in guinea-pig trachea was shifted only slightly to the right when constructed in the presence of cromakalim. Together these results provide strong evidence that the effect of cromakalim against vagal nerve stimulation is predominantly prejunctional. Hall & Maclagan (1988) suggested that this might involve inhibition of ACh release from the cholinergic nerve terminals. However, in the present study, it was found that cromakalim had no effect on bronchoconstriction evoked by field stimulation, suggesting that transmitter output from the postganglionic cholinergic nerve terminals was normal and that the attenuation arises at some earlier point in the cholinergic transmission process. Cromakalim might be acting, for example, to promote K^+-channel opening in tracheal ganglion cells, thus modifying ganglionic transmission, or in the membrane of the pre- or

postganglionic nerve fibres themselves, thus compromising action potential conduction. However, non-specific effects of cromakalim on neuronal function cannot be ruled out.

It is believed that ACh can bring about contraction in smooth muscle through at least three, possibly independent, mechanisms, namely by promoting Ca^{2+}-influx through both receptor-operated Ca^{2+}-channels (ROCs) and voltage-operated Ca^{2+}-channels (VOCs) and by releasing Ca^{2+} from intracellular storage sites (Bolton & Large, 1986). By enhancing K^+-efflux and thus causing hyperpolarisation, cromakalim would be expected to oppose the depolarisation evoked by bronchoconstrictor agents and thereby reduce influx of Ca^{2+} through VOCs. However, it has been suggested that Ca^{2+} influx through VOCs plays a relatively minor role in ACh-induced bronchoconstriction (Farley & Miles, 1977; 1978), which might account for the failure of cromakalim to combat this response in guinea-pig trachealis. It should be noted that it has not yet been shown directly that cromakalim reduces agonist-induced depolarisation. Hyperpolarisation does not always reduce the amplitude of depolarisation by excitatory stimuli. In guinea-pig trachealis, for example, sympathetically-mediated hyperpolarisation can be associated with a reduction, no change or even an increase in amplitude of vagally-mediated depolarisation (McCaig, 1987).

It has been observed that cromakalim is more effective against carbachol-induced bronchoconstriction in human bronchioles than in guinea-pig trachea (Taylor *et al.*, 1988). It is possible that the relative contribution of VOC opening to contraction is greater in human airways than in guinea-pig trachea. Direct determination of the degree of depolarisation induced by carbachol in the absence and presence of cromakalim and/or Ca^{2+}-channel blocking agents would help resolve this question.

Resting tone in guinea-pig trachea is associated with spontaneous fluctuations in membrane potential (slow waves), which are thought to involve cyclical influx of Ca^{2+} through VOCs, since they are abolished in Ca^{2+}-free medium (Foster *et al.*, 1983; McCaig, 1986) or in the presence of Ca^{2+}-channel blockers such as gallopamil and nifedipine (Small, 1982; Ahmed *et al.*, 1985). The hyperpolarising effect of cromakalim would be expected to reduce VOC opening, thus abolishing slow wave activity and resting tone, as demonstrated by Allen *et al.* (1986). It was noted in the present study that the reduction in tone caused by cromakalim was maximal after 15 min, but that maximal attenuation of vagal responses occurred rather later (22 min) at a time when the relaxation was starting to wear off. The different time courses of these two effects would be consistent with the view the relaxation represents a direct effect of cromakalim on the smooth muscle, whereas attenuation of vagal responses occurs independently by a largely prejunctional mechanism.

In conclusion, cromakalim effectively reduces vagally-mediated bronchoconstriction in guinea-pig isolated trachealis, seemingly through prejunctional modulation of cholinergic neurotransmission. Cromakalim, therefore, may be particularly useful in bronchoconstriction where there is a significant reflex component involving vagal efferent pathways.

The financial support of the Scottish Home and Health Department and the SmithKline and Nuffield Foundations is gratefully acknowledged. We thank Beecham Pharmaceuticals for their generous gift of cromakalim.

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(Received March 10, 1989
Revised May 17, 1989
Accepted June 5, 1989)