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Anti-vasoconstrictor effects of the K^+ channel opener cromakalim on the rabbit aorta — comparison with the calcium antagonist isradipine

[1]Nigel S. Cook, Sheila W. Weir & Max C. Danzeisen

Cardiovascular Department, Preclinical Research, Sandoz Ltd., CH-4002, Basel, Switzerland

1 Contractile responses of rabbit aortic rings elicited by KCl-depolarization, angiotensin II (AII), 5-hydroxytryptamine (5-HT) and noradrenaline (NA) have been investigated in the presence of cromakalim (BRL 34915) and isradipine (PN 200-110).

2 Above 10^{-6} M, cromakalim inhibited contractile responses to low ($\leqslant$32 mM) but not to higher KCl concentrations. The 5-HT and AII concentration-response curves were antagonized non-competitively by cromakalim (10^{-7}–10^{-5} M) and the maximal responses were inhibited by 40 and 55%, respectively.

3 Isradipine caused less inhibition of AII and 5-HT contractile responses than cromakalim, and in the presence of isradipine (10^{-7} M), cromakalim was still able to antagonize further the contractions to AII in this vessel.

4 NA-induced contractions were relatively insensitive to inhibition by cromakalim and isradipine, both drugs causing a small rightward shift of the NA concentration-response curve. This result suggests that NA utilizes different Ca^{2+} pools from those involved in AII- and 5-HT-induced contractions of this vessel.

5 The sustained (tonic) part of the NA response was inhibited in a concentration-dependent manner by cromakalim (10^{-7}–10^{-5} M), but not by isradipine.

6 In aortic rings partially depolarized with 3.5×10^{-2} M KCl, the ability of cromakalim, but not of sodium nitroprusside, atriopeptin III or hydralazine, to inhibit AII- and tonic NA-induced contractions was abolished.

7 Antivasoconstrictor activity of cromakalim on the rabbit aorta appears to involve factors in addition to an indirect inhibition of Ca^{2+} entry through dihydropyridine-sensitive Ca^{2+} channels.

8 The ability of cromakalim to open K^+ channels and thereby modify the membrane potential would appear to underlie these antivasoconstrictor effects. This mechanism of action of cromakalim clearly differs from that of other vasodilators such as sodium nitroprusside and hydralazine.

Introduction

Cromakalim belongs to a new class of vasodilators whose mechanism of action is thought to involve the opening of smooth muscle membrane potassium (K^+) channels. This mechanism has been postulated on the basis of its ability to enhance $^{86}Rb^+$ and $^{42}K^+$ efflux from pre-loaded vascular tissues and to hyperpolarize the cell membrane (Hamilton *et al.*, 1986; Quast, 1987; 1988). Preliminary electrophysiological studies with isolated smooth muscle cells provide evidence in support of this mechanism (Kusano *et al.*, 1987; Beech & Bolton, 1987; Trieschmann *et al.*, 1988). Additional support comes from studies in which K^+ channel blockers such as tetraethylammonium and 3,4-diaminopyridine inhibit the stimulation of $^{86}Rb^+$ and $^{42}K^+$ efflux by cromakalin in vascular tissues (Coldwell & Howlett, 1986; Quast, 1987; 1988). Shifting the membrane potential towards more negative values would oppose the effects of depolarization resulting in the opening of voltage-sensitive ion channels (see Cook, 1988a). In addition, in those tissues which generate spontaneous action potentials the frequency of firing would be reduced; this has been demonstrated for cromakalim in the portal vein (Hamilton *et al.* 1986;

[1] Author for correspondence.

Quast 1987; Hof *et al.*, 1988) and rat uterus (Hollingsworth *et al.*, 1987).

The particular type of K^+ channel(s) responsible for these effects remains poorly understood. Both Kusano *et al.* (1987) and Trieschmann *et al.* (1988) have shown that cromakalim prolongs the open time of large conductance Ca^{2+}-dependent K^+ channels, although, in contrast, Beech & Bolton (1987) showed that cromakalim opened a different K^+ channel which showed little voltage-dependence. The inability of both apamin (Allen *et al.*, 1986; Weir & Weston, 1986; Coldwell & Howlett, 1987; Cook & Hof, 1988) and charybdotoxin (Weir & Strong, 1988) to inhibit the effects of cromakalim, also argues against an action of this drug on Ca^{2+}-dependent K^+ channels. Evidence has been provided that cyclic nucleotides (Coldwell & Howlett, 1987; Quast, 1987), inositol phosphates (Coldwell & Howlett, 1987) and pertussis toxin-sensitive G-proteins (Quast *et al.*, 1988) are not involved in the opening of K^+ channels by cromakalim. In skinned smooth muscle from the trachea and uterus, cromakalim did not modify Ca^{2+}-induced spasm, indicating that it does not interfere with the intracellular action of Ca^{2+} on the contractile proteins (Allen *et al.*, 1986; Hollingsworth *et al.*, 1987).

The present investigation is concerned with the ability of cromakalim to inhibit blood vessel contractions elicited by a variety of different vasoconstrictor agents *in vitro*. We chose the rabbit isolated thoracic aorta for these studies, since it is known that contractions of this vessel can be elicited via mechanisms which are either sensitive (e.g. KCl-depolarization) or relatively insensitive (e.g. contractions by NA) to inhibition by Ca^{2+} antagonists (see Cauvin *et al.*, 1984; Hof *et al.*, 1984). Our results raise important questions concerning the consequences that opening of K^+ channels, with subsequent hyperpolarization of the membrane, might have upon vascular tone under the influence of different pressor substances. A comparison has been made with the dihydropyridine Ca^{2+} antagonist isradipine, since one of the primary consequences of membrane hyperpolarization is likely to be the indirect inhibition of such voltage-sensitive Ca^{2+} channels. Parts of this work have been communicated to the British Pharmacological Society (Cook *et al.*, 1987: Cook, 1988c).

Methods

Mongrel rabbits of either sex weighing 1.8–3 kg were killed by a blow to the base of the skull and the descending thoracic aorta was immediately excised and cleared of connective tissue. In some experiments, the aorta was divided tangentially into equal portions, one half being denuded of endothelial cells by gentle rubbing with a moistened cotton pipe cleaner. The aortae were then cut into rings 2–3 mm wide.

$^{86}Rb^+$ efflux studies

Experiments were performed essentially as described by Hamilton *et al.* (1986). Segments of rabbit thoracic aorta were attached to a gassing manifold (95% O_2/5% CO_2) and incubated in a modified Krebs-Henseleit physiological salt solution (PSS) at 37°C. After 45 min, the tissues were transferred to PSS containing $^{86}Rb^+$ (5 μCi ml^{-1}) for 2 h. The $^{86}Rb^+$ was then allowed to efflux from the tissue into normal PSS for 10 min during which time the tissues were transferred to fresh solution every 2 min. Tissues were then exposed to PSS containing isradipine (5×10^{-7} M) or vehicle for the next 40 min before being transferred to the same solution containing cromakalim (3×10^{-8}–10^{-4} M) or vehicle for 20 min.

Each 2 min PSS sample containing $^{86}Rb^+$ was counted in the Cerenkov mode at 50% counting efficiency (see Quast, 1987). The radioactivity remaining in the aorta segments at the end of the assay was determined by dissolving the tissue in 0.5 ml Lumasolve (Lumac) at 50°C overnight. The sample was then supplemented with 0.5 ml 1N HCl and 10 ml Optifluor (Packard) and counted in the ^{32}P channel at 100% efficiency. The efflux data were expressed in terms of the rate constant (% loss of $^{86}Rb^+$ from the tissue min^{-1}) (see Quast, 1987). Drug effects on the $^{86}Rb^+$ efflux rate coefficient were determined as the area under the curve (AUC, determined by weighing) of the individual rate constant versus time plots.

Contraction experiments

Aortic rings were mounted for recording isometric tension as previously described (Cook *et al.*, 1988). Cumulative concentration-effect curves were constructed for angiotensin II (AII), noradrenaline (NA), 5-hydroxytryptamine (5-HT) or potassium chloride (KCl). A peak (phasic) response to each spasmogen concentration was allowed to develop (contact time of 4–8 min) before progressing to the next concentration. It has previously been shown that release of NA from sympathetic nerve endings by KCl is not a major complicating factor in the rabbit aorta using this protocol (Hof & Vuorela, 1983). We therefore decided not to include an α-adrenoceptor blocking agent during the present investigations with KCl. Following the construction of an initial concentration-effect curve, the tissues were washed several times with PSS and allowed to relax to baseline levels before the substance under investigation

(cromakalim or isradipine) was added to the bath. After an equilibration period of 30 min, any change in tension due to these agents was recorded before responses to the spasmogen were re-examined in the continuing presence of these drugs. A typical concentration-response curve to AII is presented in Figure 6. The response of each aortic ring to a spasmogen was expressed as a percentage of the maximum response to that spasmogen (= 100%) obtained during the first concentration-effect curve. One of every four rings served as a time-matched control, receiving the vehicle of the substance under investigation.

In the experiments designed to study the antivasoconstrictor effects of cromakalim in the presence of isradipine, isradipine + KCl, or various K^+ channel blockers, these agents were added to the organ bath immediately after cromakalim, i.e. 30 min before the spasmogen concentration-response curve. An identical protocol was followed in the experiments with atriopeptin III and sodium nitroprusside (SNP). In the experiments with phenoxybenzamine, tissues were first exposed to phenoxybenzamine (or vehicle) for 30 min. The organ bath was then washed three times with PSS before the addition of cromakalim. The second NA concentration-response curve was carried out 20 min later.

Experiments were also performed to investigate the effects of various vasodilators upon the sustained (tonic) response following the addition of a single (10^{-6} M) NA concentration (see Figure 9). After about 30 min, when a stable plateau response to noradrenaline had developed, the tissues were challenged with increasing concentrations of cromakalim, isradipine, SNP or hydralazine, or with the equivalent vehicle concentration of cromakalim (controls). In some experiments, isradipine (10^{-7} M), followed 10 min later by KCl (3.5×10^{-2} M), was added to the organ bath 20 min before the challenge with NA (10^{-6} M).

Drugs and solutions

The Krebs-Henseleit physiological salt solution (PSS) used had the following composition (mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, EDTA 0.03, glucose 11 and was gassed with 95% O_2 plus 5% CO_2.

The following drugs were used: acetylcholine chloride (Sigma), angiotensin II (AII, Bachem), apamin (Sigma), (−)-ascorbic acid (Fluka), rat atriopeptin III (Bachem), cromakalim (BRL 34915; (±)-6-cyano-3, 4-dihydro-2,2-dimethyl-*trans*-4-(2-oxo-1-pyrrolidyl)-2H-benzo[b]pyran-3-ol; synthesized at Sandoz), 5-hydroxytryptamine (5-HT, Sigma), hydralazine hydrochloride (Sigma), (−)-noradrenaline bitartrate monohydrate (NA; Sigma), isradipine (PN 200-110; 3,5-pyridinedicarboxylic acid, 4-(4-benzofurazanyl)-1, 4-dihydro-2,6-dimethyl-methyl-1-methyl-ethyl ester; Sandoz), sodium nitroprusside (SNP; Sigma), tetraethylammonium chloride (Sigma), toxin I (gift from Dr A.L. Harvey, Strathclyde), (+)-tubocurarine (Serva). A partially purified venom fraction (35% pure) containing a charybdotoxin-like toxin from the Israeli scorpion *Leiurus quinquestriatus hebraeus* was supplied by Dr P.N. Strong (University College London). Stock solutions of all drugs were prepared fresh each day. Stock solutions of cromakalim (10^{-2} M in 50% ethanol:50% polyethylene glycol 400) and isradipine (10^{-3} M in 50% ethanol:50% distilled water) were prepared and diluted further with PSS. NA was prepared in PSS containing ascorbic acid. Other stock solutions were prepared in PSS. Experiments with isradipine were performed under sodium light.

Results

Stimulation of $^{86}Rb^+$ efflux by cromakalim

Figure 1(a and b) shows results from a typical experiment in which $^{86}Rb^+$ efflux from the rabbit aorta was determined in the presence of 10^{-6} and 10^{-4} M cromakalim. Note that with the highest cromakalim concentration, the rate constant for $^{86}Rb^+$ efflux reached a peak and then declined rapidly to a plateau during the continued presence of the drug. The concentration-dependence of the cromakalim-induced $^{86}Rb^+$ efflux is presented in Figure 1c. The maximal effect occurred at concentrations of around 10^{-5} M (possibly as a consequence of the response inactivating during the time cromakalim was applied). A half-maximal increase in the $^{86}Rb^+$ efflux was obtained with approximately 10^{-6} M cromakalim. In the presence of 5×10^{-7} M isradipine, neither the maximum efflux response to cromakalim nor the EC_{50} for this effect were modified (Figure 1c).

Contractions of the rabbit aorta by different spasmogens

The mean maximum contractile responses to the different spasmogens used in this study were: 5.5×10^{-2} M KCl, 3.57 ± 0.22 g ($n = 17$); 10^{-7} M AII, 2.06 ± 0.10 g ($n = 38$); 3×10^{-5} M NA, 3.93 ± 0.41 g ($n = 14$); 3×10^{-5} M 5-hydroxytryptamine, 2.19 ± 0.31 g ($n = 10$). In aortic rings from which the endothelium had been removed, KCl and AII produced maximum responses not significantly different from the responses obtained in matched rings from the same aortae with an intact endothelium.

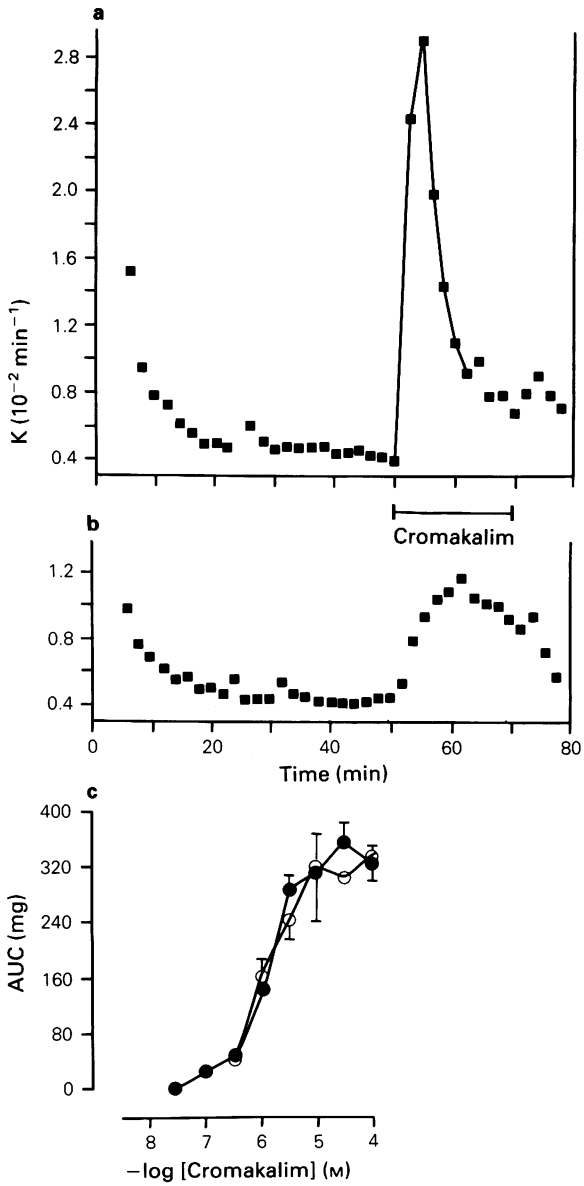

Figure 1 Effect of cromakalim on $^{86}Rb^+$ efflux from the rabbit aorta. The tissues were exposed to (a) 10^{-4} M and (b) 10^{-6} M cromakalim for 20 min during the period indicated by the horizontal bar. $^{86}Rb^+$ efflux is expressed as the rate constant, k in 10^{-2} min^{-1}. (c) −Log concentration-effect curve of cromakalim on the efflux of $^{86}Rb^+$, determined from the area under the curve (AUC, in mg) of k versus time graphs, where 100 mg corresponds to an increase in k of 0.5×10^{-2} min^{-1} for 20 min. Points represent the mean of 3 experiments in the absence (●) or presence (○) of isradipine (5×10^{-7} M, present throughout the experiment). Vertical lines in (c) indicate s.e.mean.

Antivasoconstrictor effects of cromakalim

Cromakalim (10^{-6}–10^{-4} M) inhibited contractions of the aorta elicited by low (<3.2×10^{-2} M) concentrations of KCl, but had little effect on responses elicited by higher KCl concentrations (Figure 2b). With AII as the spasmogen a quite different inhibitory profile for cromakalim was seen (Figure 2a). Above 10^{-7} M cromakalim caused a concentration-dependent inhibition of the maximum response of the aortic rings to AII. At a concentration of 10^{-6} M cromakalim the maximum response to AII was inhibited by 55% and concentrations of cromakalim up to 10^{-4} M caused no further inhibition. The inhibition of responses to 10^{-7} M AII as a function of the concentration of cromakalim is presented in Figure 3a, and the value for the half-maximal inhibition by cromakalim of $2.8 \pm 0.4 \times 10^{-7}$ M was obtained. In aortic rings from which the endothelium had been denuded, essentially the same inhibitory profile of cromakalim was observed as that described above against both KCl- and AII-mediated contractions (not shown). All subsequent studies with cromakalim were therefore performed in aortic rings with an intact endothelium.

The inhibition of 5-HT responses by cromakalim (Figure 2c) was qualitatively similar to the effects seen with AII. At a maximally effective concentration (10^{-6} M), cromakalim inhibited maximal 5-HT responses by approxiamtely 40%. With NA as the agonist (Figure 2d), cromakalim (10^{-7}–10^{-5} M) caused only a rightward shift of the NA concentration-response curve, but had little effect on the maximum tissue response to this agonist.

Antivasoconstrictor effects of isradipine

The non-competitive inhibition of KCl responses of the rabbit aorta by isradipine, using an identical protocol to that described here, has been demonstrated previously by Hof *et al.* (1984). The concentration-dependent inhibition of responses to 5.5×10^{-2} M KCl by isradipine is shown in Figure 3b. A least squares computer fit of this data gave an IC_{50} for isradipine of $2.1 \pm 0.3 \times 10^{-9}$ M.

Effects of isradipine on AII, 5-HT and NA concentration-response curves are presented, respectively, in Figure 4a, b and c. As with cromakalim, isradipine caused a small rightward shift of the AII concentration-response curve and a concomitant decrease in the maximum response. However, at maximally effective concentrations isradipine was only able to inhibit maximum responses of the aorta to AII by around 20%. The concentration-dependence of this inhibition is shown in Figure 3b, half-maximal inhibition occurred with $1.5 \pm 1.1 \times 10^{-9}$ M isradipine. The effects of isradi-

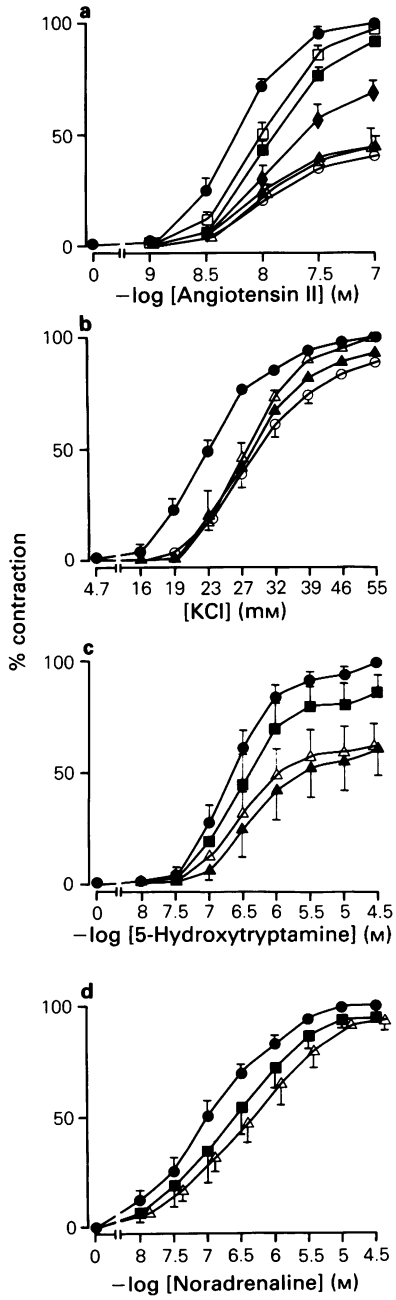

Figure 2 Effects of cromakalim upon contractile responses of rabbit aortic rings elicited by (a) angiotensin II, (b) potassium chloride, (c) 5-hydroxytryptamine and (d) noradrenaline. Endothelium was present. Cromakalim was added 30 min before the start of the concentration-response curves to give the following bath concentrations; 3×10^{-8} M (□), 10^{-7} M (■), 3×10^{-7} M (◆), 10^{-6} M (▲), 10^{-5} M (△) or 10^{-4} M (○). Contractions are expressed as a percentage of the preceding tissue maximum response of each ring to the spasmogen in the absence of cromakalim. Control rings (●) received the vehicle for cromakalim. Symbols represent the mean of 4 to 6 experiments; vertical lines indicate s.e.mean.

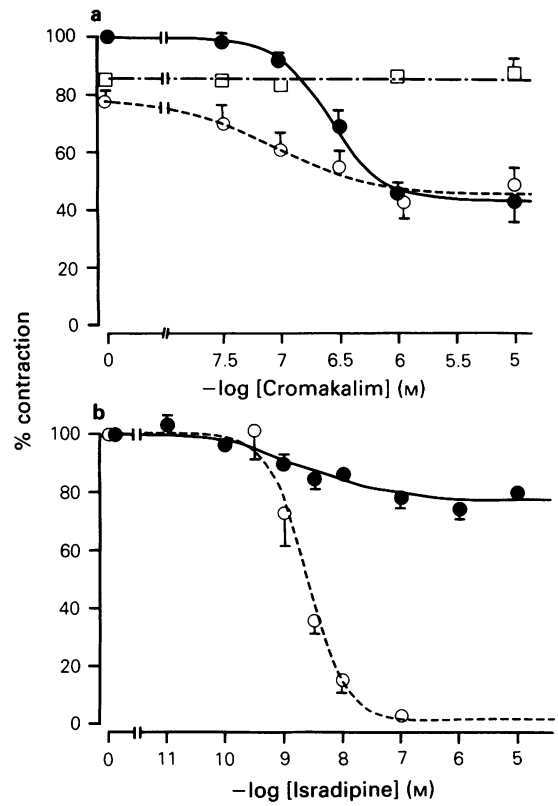

Figure 3 (a) Concentration-dependence of the inhibition of 10^{-7} M angiotensin II contractions of rabbit aortic rings by cromakalim. Endothelium was present. (●) Inhibition by cromakalim alone (see Figure 2a); (○) inhibition by cromakalim in the presence of 10^{-7} M isradipine (see Figure 5); (□) inhibition by cromakalim in the presence of 10^{-7} M isradipine and 3.5×10^{-2} M KCl (see Figure 6). $n = 4$ to 7 experiments. (b) Concentration-dependence of the inhibition by isradipine of aortic contractions elicited by 10^{-7} M angiotensin II (●) or 5.5×10^{-2} M KCl (○). The data are taken from experiments such as those shown in Figure 4; $n = 4$ to 7 experiments. Symbols represent the mean and vertical lines indicate s.e.mean.

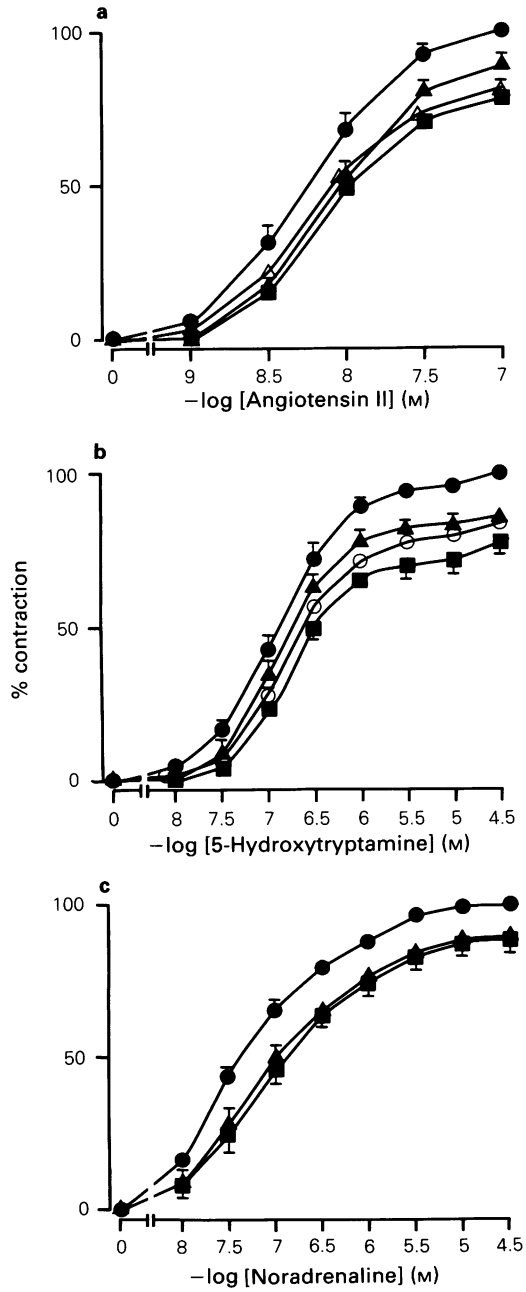

Figure 4 Cumulative concentration-response curves for (a) angiotensin II, (b) 5-hydroxytryptamine and (c) noradrenaline in the presence of various concentrations of isradipine: 10^{-9} M (▲), 10^{-8} M (○), 10^{-7} M (■) and 10^{-5} M(△). Control rings (●) received the vehicle for isradipine. Endothelium was present. Symbols represent the mean of 4 to 7 experiments; vertical lines indicate s.e.mean. For further details see Figure 2.

pine on 5-HT (non-competitive inhibition, maximum response inhibited ≈22%) and NA (predominantly a rightward shift of the agonsit concentration-response curve with only a small inhibition of the tissue maximum response) (Figure 4b,c) were qualitatively similar to the effects seen with cromakalim.

Inhibition of angiotensin II responses by cromakalim in the presenece of isradipine

The significantly greater inhibition of AII responses by cromakalim than by isradipine prompted us to re-investigate the effects of cromakalim in the presence of 10^{-7} M isradipine, a concentration which inhibited maximal AII contractions by 20%. The additional presence of cromakalim (10^{-7} and 10^{-6} M) resulted in a further inhibition of AII responses, as shown in Figure 5. The concentration-dependence of the cromakalim-induced inhibition in the presence of 10^{-7} M isradipine is shown in Figure 3a. Neither the affinity (half-maximal inhibition observed with $9.0 \pm 6.5 \times 10^{-8}$ M) nor efficacy (maximal inhibition of 55%) of cromakalim were greatly modified by the presence of the Ca^{2+} antagonist.

Effects of cromakalim on angiotensin II-evoked responses in partially depolarized aortic rings

These experiments were performed to ascertain whether opening of K^+ channels (to hyperpolarize

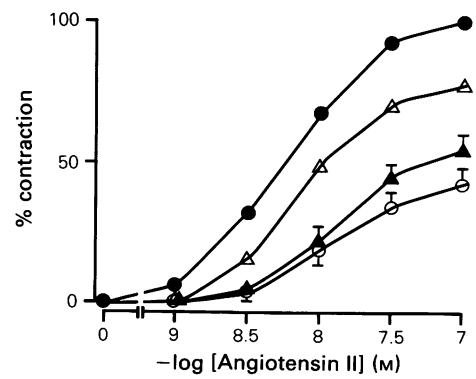

Figure 5 Inhibition by cromakalim 3×10^{-7} M (▲) and 10^{-6} M (○) of angiotensin II-induced contractions of rabbit aortic rings in the presence of a maximally effective concentration of isradipine (10^{-7} M). Endothelium was present. Control rings (●) received the substance vehicles. (△) Inhibition by isradipine (10^{-7} M) alone. The cumulative concentration-response curves in the presence of both drugs should be compared with those in the presence of cromakalim alone, shown in Figure 2a. Symbols represent the mean of 6 to 7 experiments; vertical lines indicate s.e.mean. For further details see Figure 2.

the cell membrane) was likely to be responsible for the antagonism of the AII responses by cromakalim. The previous demonstration that isradipine did not interfere with the inhibitory effects of cromakalim (Figures 3a and 5) allowed experiments to be performed in the presence of 10^{-7} M isradipine to prevent the sustained depolarization-induced contraction which would otherwise ensue. Simultaneous addition of isradipine (10^{-7} M) and KCl (3.5×10^{-2} M) resulted in a transient contractile response, but after 30 min (the normal incubation time of cromakalim) the tissue had relaxed to baseline (Figure 6a). Subsequent AII concentration-response curves in these partially depolarized rings (Figure 6b) were similar to those obtained in the presence of isradipine alone (Figure 4a). However, in the presence of 3.5×10^{-2} M KCl (and isradipine) cromakalim (10^{-6} M) was unable to inhibit further the AII-induced contractions (Figure 6b). Other cromakalim concentrations (3×10^{-8} M–10^{-5} M) were likewise unable to inhibit AII contractions in the partially depolarized vessels (not shown).

To confirm the selectivity of this experimental protocol for cromakalim whose mechanism of action is assumed to involve a modification of the membrane potential, we also looked at the inhibitory effects of atriopeptin III and sodium nitroprusside in normal and in partially depolarized aortic rings. Figure 7

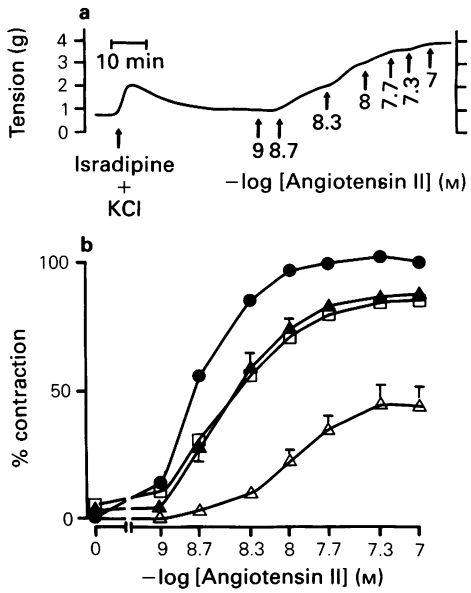

Figure 6 Inhibition by cromakalim of angiotensin II-induced contractions of aortic rings partially depolarized with 3.5×10^{-2} M KCl. (a) Original trace from a typical experiment. Isradipine (10^{-7} M) and KCl (3.5×10^{-2} M) were simultaneously added to the bath 30 min before the start of the angiotensin II concentration-response curve. In other experiments, cromakalim (3×10^{-8} to 10^{-5} M) was also added to the bath, immediately before the isradipine and KCl. Although KCl elicitied a small contraction under these conditions, the tension had returned to basline during the 30 min incubation period. Subsequent responses to angiotensin II were comparable to those seen in the presence of 10^{-7} M isradipine alone (Figure 4a). (b) Inhibition by cromakalim (10^{-6} M) of angiotensin II-induced contractions in the absence (△) or presence (▲) of isradipine (10^{-7} M) and KCl (3.5×10^{-2} M). Control rings (●) received the substance vehicles. (□) Contractions evoked by angiotensin II in the presence of isradipine (10^{-7} M) and KCl (3.5×10^{-2} M) alone. Symbols represent the mean of 4 experiments (depicted in (a)) and vertical lines indicate s.e.mean. Similar experiments have been performed with other cromakalim concentrations, all of which were without effect in the presence of isradipine and KCl (see Figure 3a).

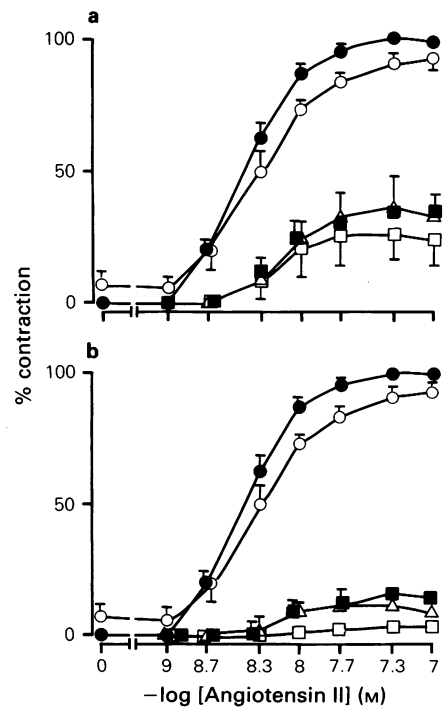

Figure 7 Inhibition by sodium nitroprusside (10^{-7} M, a) and atriopeptin III (10^{-6} M, b) of angiotensin II (AII)-evoked contractions of the rabbit aorta. Matched rings from the same aorta were treated with either one of these vasodilators alone (△), in the presence of 10^{-7} M isradipine (□) or in the presence of 10^{-7} M isradipine and 3.5×10^{-2} M KCl (■), using a similar protocol to that shown in Figure 6. Control rings (●) received the substance vehicle and ○ denotes the responses to AII in the presence of isradipine (10^{-7} M) and KCl (3.5×10^{-2} M) alone. Symbols represent the mean of 4 experiments and vertical lines indicate s.e.mean.

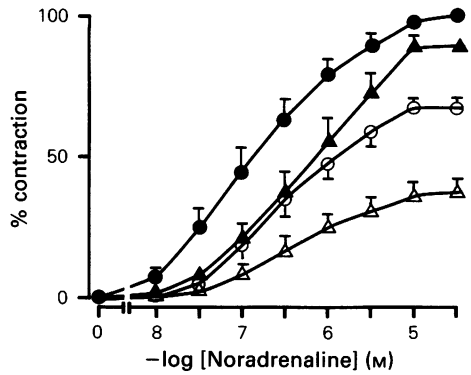

Figure 8 Effect of phenoxybenzamine (Pbz) pretreatment upon the inhibition of noradrenaline contractions by cromakalim. Cumulative concentration-response curves to noradrenaline were constructed in the presence of 3×10^{-6} M cromakalim (▲,△) or vehicle (●,○), following a 20 min incubation period. The open symbols denote aortic rings which had first been exposed to Pbz (10^{-8} M) for 30 min and then washed out before the addition of cromakalim or its vehicle. Solid symbols denote aortic rings treated with the Pbz vehicle. Symbols represent the mean of 4 experiments; vertical lines indicate s.e.mean.

shows that in contrast to cromakalim the anti-vasoconstrictor effects of these two agents were minimally affected in the presence of 3.5×10^{-2} M KCl.

Inhibition of phasic noradrenaline responses by cromakalim in phenoxybenzamine-pretreated rings

Partial blockade of α-adrenoceptors with the irreversible antagonist phenoxybenzamine is known to increase the susceptibility of α_1-adrenoceptor mediated responses to inhibition by Ca^{2+} antagonists (Ruffolo *et al.*, 1984; Timmermans *et al.*, 1985). We have therefore investigated whether a similar increase in susceptibility of NA responses to cromakalim is revealed by this treatment. In preliminary experiments 30 min incubation with 10^{-8} M phenoxybenzamine was found to reduce maximal NA contractions by about 50%. The tissues were then washed three times with PSS before the addition of cromakalim. Phenoxybenzamine was not present in the PSS during the second NA concentration-response curve. Figure 8 shows that cromakalim (3×10^{-6} M) inhibited the maximum NA response of rings which had been treated with phenoxybenzamine by around 50%, in contrast to its relative lack of effect on the maximal NA response in the absence of phenoxybenzamine pretreatment. Similar results were obtained with isradipine (10^{-7} M) in phenoxybenzamine-pretreated aortic rings (not shown).

Inhibition of tonic noradrenaline contractions by cromakalim and other vasodilators

It is known that different sources of Ca^{2+} are responsible for the initial (phasic) and sustained (tonic) tension generated by NA in the rabbit aorta (Lodge & van Breemen, 1985). Hence, the effects of cromakalim and other vasodilators on the tonic response of the aorta to NA (10^{-6} M) have also been investigated. Figure 9a shows typical original traces from such experiments, with the mean results presented in Figure 9b. In the absence of any vehicle additions, the tonic contracture to 10^{-6} M NA in control preparations remained stable throughout the experimental duration (about 2 h; not shown). It can be seen that cromakalim (10^{-7}–10^{-5} M) inhibited the tonic component of the contraction generated by NA (10^{-6} M) in a concentration-dependent manner. At the highest concentration used (10^{-5} M), cromakalim inhibited the response by 70%. In control preparations a small increase in contractility was seen at vehicle concentrations equivalent to 3×10^{-6} M cromakalim and above (Figure 9b).

SNP (10^{-8}–10^{-5} M) and hydralazine (10^{-6}–10^{-5} M) also inhibited this sustained phase of the NA response, but isradipine (10^{-8}–10^{-5} M) was without effect. Whereas effects of SNP were quick to develop (Figure 9a), the relaxation elicited by hydralazine (10^{-6} M) had still not reached a stable plateau after 1 h, at which time the next concentration (10^{-5} M) was applied to the bath. At the highest concentrations tested, SNP (10^{-5} M) produced complete inhibition of the tonic NA contraction (Figure 9a and b).

The ability of the vasodilators to inhibit the sustained component of the NA contracture was also investigated in aortic rings partially depolarized with 3.5×10^{-2} M KCl (in the presence of 10^{-7} M isradipine to prevent KCl itself from causing a marked contraction). Under such conditions, the relaxant activity of cromakalim was completely abolished, whereas sodium nitroprusside and hydralazine produced comparable inhibitory activity to that observed in the non-depolarized tissues (Figure 9).

Effect of various K^+ channel blockers on the inhibition of angiotensin II responses by cromakalim

Apamin (10^{-6} M), toxin I (10^{-6} M) and tubocurarine (10^{-5} M), which themselves were without an effect on basal tone or AII contractions of the rabbit aorta (Cook, 1988b), failed to modify the inhibition of AII contractions by cromakalim (10^{-6}–10^{-5} M) (not shown). Tetraethylammonium (10^{-3} M and 10^{-2} M) and a charybdotoxin-like scorpion toxin (approximately 10 ng ml^{-1}) both augmented AII contractions of the rabbit aorta, increasing the

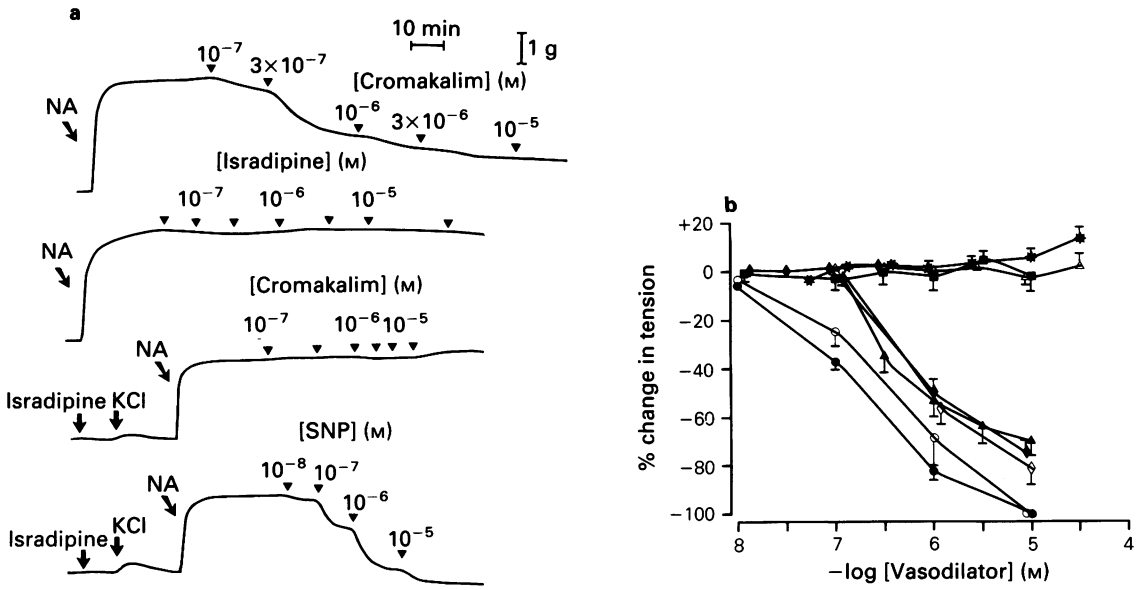

Figure 9 Effects of vasodilators upon the tonic contraction generated by 10^{-6} M noradrenaline (NA) in rabbit aortic rings. (a) Original traces showing (starting at the top) the concentration-inhibitory response curves for cromakalim alone, for isradipine alone, and for cromakalim and sodium nitroprusside (SNP) in the presence of 10^{-7} M isradipine and 3.5×10^{-2} M KCl. The additions to the organ bath of isradipine, KCl and NA are indicated by arrows. Addition of the vasodilators are denoted by ▼, with the following bath concentrations: cromakalim: 10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6} and 10^{-5} M. Isradipine: 10^{-8}, 10^{-7}, 3×10^{-7}, 10^{-6}, 3×10^{-6}, 10^{-5} and 3×10^{-5} M. Cromakalim in the presence of isradipine and KCl: as for cromakalim alone, plus in addition 3×10^{-5} M. SNP in the presence of isradipine and KCl: 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M. For simplicity, only some of these concentrations have been indicated on the figure. Note that the small contractions produced by KCl in the presence of isradipine return to baseline before the addition of NA. (b) Mean results from 4 such experiments with cromakalim (▲△), isradipine (■), SNP (● ○), hydralazine (◆ ◇) or the equivalent vehicle concentration of cromakalim (★). Solid symbols show the inhibition of the tonic noradrenaline response by the vasodilators alone. Open symbols show the effects of these agents in the presence of isradipine (10^{-7} M) and KCl (3.5×10^{-2} M). The mean contraction elicited by 10^{-6} M NA was 2.65 ± 0.31 g in the presence of isradipine + KCl, and 3.04 ± 0.14 g in the absence of these agents. Vehicle concentrations equivalent to 10^{-5} M cromakalim (0.05% ethanol/0.05% polyethylene glycol) and 3×10^{-5} M cromakalim (0.15%, ethanol/0.15% polyethylene glycol) caused a small contraction of the rings, as indicated. In (b) vertical lines indicate s.e.mean.

maximum tissue response to this agonist by up to 40% (see Cook, 1988b). The inhibitory effects of cromakalim appeared to be diminished in the presence of these agents, but it is not possible to say whether this was due to a direct (cromakalim-channel blocking) or indirect (functional) inhibitory effect (results not shown).

Discussion

Receptor-mediated contractions of the rabbit aorta are known to be relatively resistant to inhibition by Ca^{2+} antagonists (Cauvin *et al.*, 1984; Hof *et al.*, 1984). However, we have shown in the present study (Figure 3b) that although maximally effective concentrations of isradipine inhibit only a small (20%) component of AII-induced responses, the IC_{50} of isradipine for this effect (approximately 2×10^{-9} M) is the same as its potency at blocking L-type voltage-sensitive Ca^{2+} channels (Hof *et al.*, 1984). Hence, it is likely that a similar Ca^{2+} channel contributes, albeit to a small extent, to receptor-mediated contractions of this vessel.

Perhaps the most important finding in the present study was that the inhibition of AII responses of the aorta by cromakalim could not be accounted for solely by an indirect inhibition of Ca^{2+} entry through dihydropyridine-sensitive Ca^{2+} channels. Cromakalim caused a much greater (55%) inhibition

of maximal responses to AII than isradipine (20%). In the presence of a maximally effective concentration of isradipine, neither the affinity nor the efficacy of cromakalim at inhibiting AII responses was modified. However, in aortic rings which had been partially depolarized with KCl, cromakalim was unable to inhibit AII contractions, suggesting that this effect of cromakalim was indeed linked to its ability to open K^+ channels and hyperpolarize the membrane. In contrast, the inhibitory action of two vasodilators thought to act via cyclic GMP, SNP and atriopeptin III, was essentially unchanged in partially depolarized aortic rings. Such a protocol would therefore seem to attenuate selectively the action of drugs whose effects are elicited through modification of the cell membrane potential. Since removal of the endothelium did not affect the inhibition of the AII and KCl contractions by cromakalim, we can assume these effects were elicited on the smooth muscle directly.

Stimulation of $^{86}Rb^+$ efflux from the rabbit aorta by cromakalim (which we assume reflects changes in the membrane K^+ permeability) occurred in a concentration-dependent manner between 10^{-7}–10^{-5} M, and was not modified in the presence of 10^{-7} M isradipine. This is comparable with the concentration range over which cromakalim stimulated $^{86}Rb^+$ and $^{42}K^+$ efflux from the guinea-pig portal vein and these effects were also not modified in the presence of isradipine (Quast, 1987; 1988). These results show that the entry of Ca^{2+} through dihydropyridine-sensitive Ca^{2+} channels is not a prerequisite for the ability of cromakalim to elicit its effects on the rabbit aorta.

Another interesting finding of the present study was the greater inhibition by cromakalim of the AII (and to a lesser extent 5-HT) concentration-response curves as compared to NA. Although cromakalim antagonized the initial contractions elicited by low concentrations of NA, the maximum response of the aorta to NA was little affected by cromakalim. Hence, these contractions to NA of the rabbit aorta would appear to utilize different sources of Ca^{2+} from those involved in the response to AII or 5-HT. Following pretreatment with the irreversible α_1-adrenoceptor antagonist phenoxybenzamine, however, a greater sensitivity of the NA responses to inhibition by cromakalim and isradipine was revealed. These results are consistent with previous studies in which the inhibitory effects of Ca^{2+} antagonists against α_1-adrenoceptor-mediated responses were enhanced following phenoxybenzamine treatment (Ruffolo *et al.*, 1984; Timmermans *et al.*, 1985).

The sustained (tonic) phase of NA contractions of the rabbit aorta is associated with Ca^{2+} influx through pathways which are relatively insensitive to Ca^{2+} antagonists (Lukeman & van Breemen, 1985). Consistent with this, isradipine was unable to inhibit the sustained component of the contraction elicited by 10^{-6} M NA (Figure 9). However, cromakalim, SNP and hydralazine were effective at inhibiting these tonic contractions (Figure 9). As with the inhibition of AII-elicited responses, the inhibition of the NA-induced tonic contraction by cromakalim, but not by SNP or hydralazine, was abolished in partially depolarized aortic rings. Hence, this activity of cromakalim is likely to be associated with its ability to open K^+ channels and hyperpolarize the membrane. These results are in agreement with the recent findings of Bray *et al.* (1988).

The results presented here raise important questions concerning the identity of the voltage-sensitive processes, once the dihydropyridine-sensitive Ca^{2+} channels have been excluded, involved in receptor-mediated contractions of blood vessels such as the rabbit aorta. One candidate would be dihydropyridine-insensitive voltage-sensitive Ca^{2+} channels, which have recently been identified in vascular smooth muscle (Bean *et al.*, 1986; Friedman *et al.*, 1986). If, by hyperpolarizing the cell membrane, cromakalim was able to prevent the opening of such channels, this could provide an explanation for the present results. Another possibility is that Ca^{2+} entry through receptor-operated channels (Bolton, 1979) might be modified by changing the cell membrane potential. However, electrophysiological recordings from (dihydropyridine-insensitive) ATP-operated cation channels in isolated cells from the rabbit ear artery (Benham & Tsien, 1987) suggest that these channels are relatively insensitive to changes in membrane voltage.

Electrogenic ion transport systems, such as Na^+/Ca^{2+} exchange (see Brading & Lategan, 1985), might also be affected by hyperpolarization of the cell membrane. We have found that dichlorobenzamil (10^{-5} M), an inhibitor of Na^+/Ca^{2+} exchange (Siegl *et al.*, 1984) can indeed reduce the inhibitory effect of cromakalim on responses to AII of the rabbit aorta (Quast & Cook, 1988). However, in the same study it was shown that dichlorobenzamil also inhibited the cromakalim-stimulated $^{86}Rb^+$ efflux from the rat portal vein and aorta, suggesting that this effect may have been due to a direct (K^+) channel blocking action, rather than to an inhibition of Na^+/Ca^{2+} exchange. The possibility that the anti-vasoconstrictor effects of cromakalim might be indirectly mediated through effects on Na^+/Ca^{2+} exchange therefore requires further investigation.

Lastly, by hyperpolarizing the cell membrane cromakalim might interfere with the release of superficially (membrane) bound Ca^{2+} which might also reduce contractions evoked by agonists (Lodge & van Breemen, 1985). Since certain of the above path-

ways for Ca^{2+} entry into the cell might also contribute to the refilling of intracellular Ca^{2+} stores, it is conceivable that membrane hyperpolarization might (indirectly) interfere with agonist contractions dependent primarily upon the release of intracellularly located Ca^{2+}.

In conclusion, we have shown that cromakalim possesses a quite different antivasoconstrictor profile from the Ca^{2+} antagonist isradipine. This activity occurs over the same concentration range in which cromakalim stimulates $^{86}Rb^{+}$ efflux from this tissue. Taken together with the lack of inhibitory activity seen in depolarized tissues, it is likely that the opening of membrane K^{+} channels by cromakalim is responsible for these effects.

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