Biochemical Pharmacology, Vol. 37, No. 21, pp. 4105–4110, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

POTASSIUM EFFLUX ENHANCEMENT BY CROMAKALIM (BRL 34915) IN RABBIT MESENTERIC ARTERY: AN INDIRECT EFFECT INDEPENDENT OF CALCIUM?

MARTIN C. COLDWELL* and DAVID R. HOWLETT
Beecham Pharmaceuticals Research Division, Medicinal Research Centre, Coldharbour Road, The Pinnacles, Harlow, Essex CM19 5AD, U.K.

(*Received* 19 *November* 1987; *accepted* 20 *May* 1988)

Abstract—Experiments have been performed in order to investigate the calcium and exposure time dependency of cromakalim (BRL 34915)-stimulated rubidium efflux in rabbit isolated mesenteric artery.

Removal of calcium from the bathing medium prolonged the effects of cromakalim on rubidium efflux. Lanthanum was without effect on cromakalim-induced efflux whilst high concentrations of nifedipine were required to produce a significant inhibitory effect. Decreasing the exposure time to cromakalim, either in the presence or absence of calcium, led to a progressive loss of the response. However, significant increases in rubidium efflux rate were observed after very short exposures (15 sec) to the drug. In normal medium, exposure to cromakalim resulted in an inhibition of a second response when the drug was reapplied. Blockade by tetraethylammonium of the initial rubidium efflux response to cromakalim did not reverse the inhibition of the second response.

These results suggest that the stimulation by cromakalim of rubidium efflux in rabbit isolated mesenteric artery is independent of calcium influx and requires only a short initial exposure to the drug in order to develop a response. The development and maintenance of the response after the removal of the drug suggest that cromakalim does not directly interact with the potassium channel through which rubidium efflux enhancement is observed.

It has been proposed that the novel antihypertensive drug cromakalim (BRL 34915), exerts its relaxant activity by opening potassium channels in the membrane of vascular and other smooth muscles [1–3]. A study to examine the specificity of this mechanism of action [4] was unable to demonstrate any other effect of the compound at concentrations up to 100 μM. Although there have been reports from isolated tissue studies demonstrating the ability of several potassium channel blockers to inhibit both the relaxant activity of cromakalim [5–7], and its ability to increase rubidium efflux rates [8, 9], very little data have been presented concerning the characteristics of the potassium channels involved, or the site of action of cromakalim. However, it would appear that cromakalim opens potassium channels which are different from those opened by noradrenaline and potassium depolarisation [4].

Previous rubidium efflux studies have used calcium free solutions and inorganic/organic calcium antagonists to investigate the calcium dependency of rubidium efflux in vascular smooth muscle [10–12]. Using these procedures both calcium-dependent [10, 12] and calcium-independent [11] components of rubidium efflux have been described. However, with respect to the calcium dependency of cromakalim-induced rubidium efflux, the published data are apparently contradictory [13, 14]. In an attempt to characterise further the mechanism by which potassium channels are opened by cromakalim, we have therefore used similar techniques to examine the calcium dependency of the rubidium efflux response to cromakalim in rabbit isolated mesenteric artery.

The results from the experiments using "calcium free" solutions suggested that the potassium channels opened by cromakalim remained open for substantial periods of time following removal of cromakalim from the medium. We therefore undertook another series of experiments, designed to determine the effect on the cromakalim response of both the time and number of exposures of the tissue to the drug. These studies suggest that cromakalim may act indirectly to open potassium channels in vascular smooth muscle.

MATERIALS AND METHODS

Materials. Lanthanum chloride, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES), ethyleneglycol-bis-(B-aminoethylether) *N,N,N',N'*-tetraacetic acid (EGTA) and tetraethylammonium chloride (TEA) were supplied by Sigma (St Louis, MO). We thank Bayer (Newbury) for the gift of nifedipine. ^{86}RbCl was obtained from New England Nuclear (Boston, MA). [^{3}H]-cromakalim was prepared at Beecham (Harlow) as described previously [4].

Methods. Sections of mesenteric artery were removed from male sodium pentobarbitone-anaesthetised New Zealand White rabbits and immediately placed in aerated (95/5 O_2/CO_2) HEPES buffer (composition in mM: NaCl 120; KCl 6.0; $CaCl_2$ 2.5; $MgCl_2$ 1.2; HEPES 5.0; glucose 11.4; pH 7.4) at 37°. Arteries were cleaned of connective tissue and cut

* To whom correspondence should be addressed.

into segments of 10–20 mg wet weight. Individual pieces of artery, fixed on stainless steel hooks, were suspended in a tissue bath containing about 200 ml HEPES buffer (37°, slow aeration). After 30 min, 100–200 μCi ^{86}Rb (1–6 mCi/mg) was added to the bath and the tissues left to equilibrate for 90 min. The efflux of rubidium from the tissue was then studied by transferring individual segments (still attached to hooks), at 3 min intervals, through a series of plastic vials containing 3 ml fresh, aerated buffer (but no radioactivity). Three buffers were used during the efflux period: (1) normal HEPES; (2) OCaHEPES (HEPES buffer with no added $CaCl_2$ but 2 mM EGTA added); and (3) CAFH (OCaHEPES buffer with 10 mM $MgCl_2$ added). During the efflux period the vials were agitated gently in a shaking water bath at 37°. After 30 or 45 min of the efflux run (by which time the efflux rate was usually constant), the ability of cromakalim to enhance efflux was tested by exposing the tissue to the drug for the times indicated in the text. Calcium antagonists, when tested for their ability to inhibit stimulation of efflux, were present throughout the efflux period. Radioactive content of the vials was determined by liquid scintillation counting, tissues being solubilised in 1 ml Soluene (Packard Instruments) prior to counting. In some experiments [^{3}H]-cromakalim (c.1 μCi; final concentration <13 nM) was added to the stock drug solution (300 μM cromakalim in ethanol) in order to estimate both the carry over of drug (due to bathing medium adhering to the tissue during the transfer to fresh medium) and the uptake into the tissue.

Results are expressed as rate coefficients, which were calculated as the ^{86}Rb released (counts) during each 3 min period as a percentage of the mean tissue ^{86}Rb content remaining during that period. The average efflux rate over minutes 21–30 or 39–45 (as specified) of the efflux period was taken as the basal efflux rate. Drug stimulation of efflux rate was calculated as the maximum efflux rate observed in the presence of the drug divided by the basal rate and expressed as a percentage.

Statistical analysis was carried out using the Student's *t*-test; an effect was considered to be significant when $P < 0.05$.

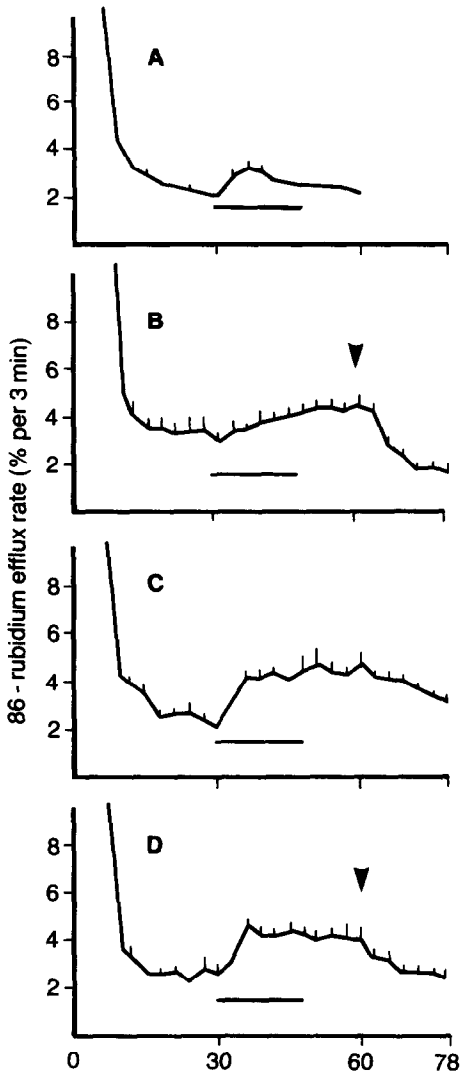

Fig. 1. The effect of different buffers on the stimulation of rubidium efflux caused by an 18 min exposure to cromakalim (10 μM). (A) normal HEPES buffer (N = 101); (B) OCaHEPES (N = 8); (C) CAFH (N = 32) and (D) CAFH (N = 8) (see text for composition of the buffers). Horizontal bars indicate the presence of cromakalim and arrows indicate the addition of 2.5 mM $CaCl_2$ to the medium.

RESULTS

Dependence of efflux response to cromakalim (10 μM) *on extracellular calcium*

In normal calcium-containing HEPES, exposure of tissues to cromakalim significantly increased the rubidium efflux rate by 52% to 3.2 ± 0.2% per 3 min (Fig. 1A). The peak effect occurred within 6 min of exposure to drug but the efflux rate then returned towards basal rates. OCaHEPES increased the basal rate from 2.1 ± 0.1% per 3 min to 3.2 ± 0.3% per 3 min. This was attributed to membrane instability (see [15, 16]) and was reduced by the presence of 10 mM $MgCl_2$. Thus the basal efflux rate in CAFH (OCaHEPES containing $MgCl_2$) was 2.4 ± 0.1% per 3 min. In OCaHEPES, cromakalim caused a mean increase in efflux rate of 28% to 4.1 ± 0.3% per 3 min, an effect which developed, and was maintained, following removal of the drug from the bathing medium (Fig. 1B). The reason for the slower onset of the response to cromakalim in OCaHEPES is unclear. Introduction of the normal buffer concentration of calcium ions (2.5 mM) to the medium during the maintained phase significantly reduced the efflux rate within 6 min to levels similar to those seen in normal HEPES buffer. In CAFH, the mean maximum efflux rate following exposure to cromakalim was increased by 100% to 4.8 ± 0.3% per 3 min (Fig. 1C) within 3 min. There was no significant decrease in the response for up to 30 min after removal of the drug from the medium. Again, the addition of calcium ions to the buffer during this maintained phase (Fig. 1D) significantly reduced the efflux rate within 3 min.

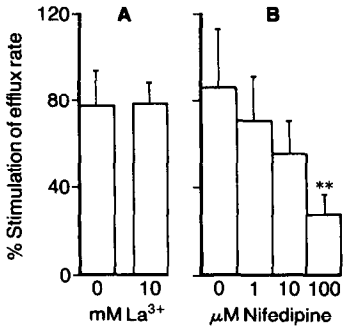

Fig. 2. The effect of (A) lanthanum and (B) nifedipine on the ability of an 18 min exposure to cromakalim (10 μM) to stimulate rubidium efflux (N = 5–9). ** $P < 0.05$.

The presence of lanthanum (10 mM) throughout the efflux period did not significantly decrease the basal efflux rate (2.0 ± 0.4% per 3 min in the absence of lanthanum; 1.5 ± 0.3% per 3 min in the presence of lanthanum; N = 6) and had no effect on the stimulation of rubidium efflux by exposure to cromakalim for 18 min (Fig. 2A). Nifedipine caused a significant decrease in basal efflux rate (2.2 ± 0.4% per 3 min in the absence of the drug, 1.7 ± 0.2% per 3 min in the presence of the nifedipine; N = 6) when present at the highest concentration used (100 μM). At lower concentrations nifedipine had no effect on basal efflux rates. The stimulation of rubidium efflux caused by cromakalim was inhibited in a concentration-dependent manner by nifedipine (Fig. 2B), but again the effect was only significant at the highest concentration used.

Hence concentrations of lanthanum and nifedipine known to block calcium influx have no effect on the cromakalim response, suggesting that the stimulation of rubidium efflux by cromakalim is independent of calcium influx. In the absence of extracellular calcium, the response to cromakalim is maintained for longer periods of time.

Effect of different exposure times to cromakalim (10 μM)

Including [^{3}H]-cromakalim as a marker in the stock drug solution allowed an estimation of the carry-over of drug during tissue transfer (which would be expected due to medium adhering to the tissue). As shown in Table 1, in all cases the bathing medium concentration fell to <100 nM upon the first transfer of the tissue. This concentration of cromakalim does not stimulate rubidium efflux in this tissue (unpublished data and see [4]). There did not appear to be any difference in dilution rate between HEPES and CAFH. In separate experiments, tissues were exposed to efflux medium containing 10 μM cromakalim and <13 nM [^{3}H]-cromakalim for 3 min and were then either immediately blotted or exposed to buffer for two periods of 3 min before blotting. After blotting, the tissues were solubilised. The tissue content of cromakalim was found to be less than 5.1 ± 0.5 pmoles cromakalim/mg tissue (N = 4) after the 3 min exposure period and 0.9 ± 0.2 pmoles cromakalim/mg tissue (N = 4) after two efflux periods. These results agree with our previous studies, where we were unable to show any appreciable [^{3}H]-cromakalim uptake in tissue slices [4].

Hence the removal of cromakalim from the bathing medium immediately reduces the medium concentration to levels of drug below those known to stimulate efflux whilst little cromakalim is retained in the tissue.

In HEPES buffer, decreasing the exposure time to cromakalim produced a progressive decrease in the percentage stimulation of efflux rate and in the duration of the response (Table 2). However, it is clear that only a very short initial exposure to the drug is required in order to produce a significant increase in rubidium efflux (e.g. 56% stimulation of efflux rate, half maximal, by a 30 sec exposure). Furthermore, by the time the maximum stimulation of efflux is achieved, the [^{3}H]-cromakalim dilution studies would suggest that the concentration of cromakalim in the bathing medium is <100 nM, and the efflux rate remains significantly increased even when buffer levels have fallen to <2.4 nM cromakalim. These effects are even more noticeable in CAFH medium. As in HEPES medium, an exposure-time dependent decrease in percentage stimulation and duration of enhanced efflux rate was observed, although the loss of response was more gradual than noted in HEPES buffer (Table 2). Thus, for instance,

Table 1. Concentration of cromakalim in the bathing medium following different times of exposure to the drug

	Concentration (nM) of cromakalim in bathing medium following exposure for				
	HEPES				CAFH
	3 min	30 sec	15 sec	1–2 sec	3 min
Initial	10000	10000	10000	10000	10000
1st Transfer	63.0 ± 6.0	53.0 ± 4.0	50.0 ± 7.0	47.0 ± 9.0	58.9 ± 7.1
2nd Transfer	8.7 ± 1.0	5.9 ± 0.9	2.9 ± 0.3	1.2 ± 0.3	8.1 ± 1.5
3rd Transfer	3.7 ± 0.3	2.4 ± 0.3	1.6 ± 0.2	0.2 ± 0.4	3.5 ± 0.5

Results are mean ± SEM (N = 5–7).
Tissue was exposed to cromakalim for the indicated time (see text for details).

Table 2. The effect of different exposure times on cromakalim-induced rubidium efflux

Buffer	Exposure time	Time to peak response (min)	% Stimulation of efflux rate	Duration of significant increase* (min)
HEPES	18 min	6	112	24
	3 min	6	81	15
	30 sec	3	56	12
	15 sec	3	31	3
	1–2 sec	3	7	0
CAFH	18 min	6	106	>90
	3 min	9	102	60
	30 sec	6	77	>30
	15 sec	6	55	>30
	1–2 sec	3	23	3

% stimulation of efflux rate was obtained by dividing the maximum efflux rate of the mean curve by the basal rate of the mean curves—thus no standard errors can be given.

* Time following cromakalim when efflux rate was significantly increased ($P < 0.05$) over the mean basal efflux rate. N = 7–14.

a 30 sec exposure to cromakalim caused a 77% increase in rubidium efflux rate, which was still significantly above basal efflux rate 30 min after initial exposure (which in this case was the end of the efflux run).

Thus, in both HEPES and CAFH, rubidium efflux responses to cromakalim continue to develop, and are maintained for long periods of time, after removal of the drug from the medium.

Repeated applications of cromakalim (10 μM)

The mean basal efflux rate in tissues exposed to cromakalim between min 45 and 54 of the efflux run was 1.8% per 3 min, and addition of the drug stimulated a 131% increase in rubidium efflux (Fig. 3). When tissues were also exposed to cromakalim for the first 9 min of the efflux period, the initial rapid decrease in efflux rate produced in control tissues was not observed. This suggests that cromakalim is stimulating efflux at the start of the efflux run, but that the effect is masked by the initial rapid release of counts due to radioactive medium adhering to the tissue. It is clear that a second exposure to cromakalim is only able to stimulate a small, non-significant increase in rubidium efflux rate (Fig. 3).

We have shown previously [4] that 30 mM TEA abolishes the increase in rubidium efflux rate caused by cromakalim in rabbit mesenteric artery. In tissues exposed to this concentration of TEA for the first 9 min of the efflux run, the basal rate was 1.9% per 3 min, and cromakalin caused a 100% increase in efflux rate when applied after 45 min of the efflux run (Fig. 4). Following application of both cromakalim and TEA to mesenteric arteries for the first 9 min of the efflux run, a second exposure to cromakalim (in the absence of the TEA) caused only

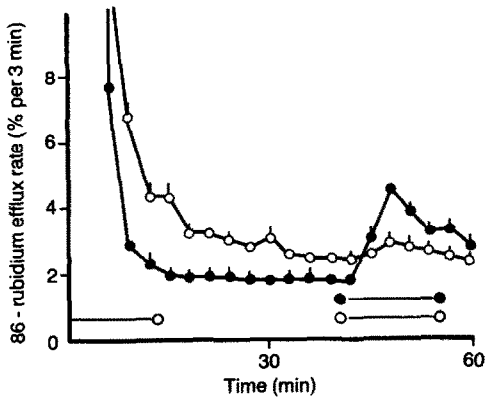

Fig. 3. The effect of prior exposure on cromakalim (10 μM)-stimulated efflux: ●, tissues exposed to cromakalim between 45 and 54 min of the efflux period; ○, tissues exposed to cromakalim between 3 and 9 min and 45 and 54 min of the efflux period (N = 7–8). Horizontal bars represent the presence of cromakalim.

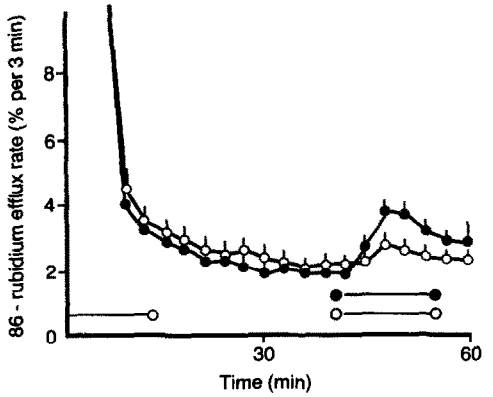

Fig. 4. Inability of TEA (30 mM) to reverse the inhibition of a second cromakalim (10 μM) response by prior cromakalim exposure: ●, tissues exposed to TEA between 3 and 9 min, and cromakalim between 45 and 54 min, of the efflux period; ○, tissues exposed to TEA and cromakalim between 3 and 9 min of the efflux period and cromakalim between 45 and 54 min (N = 7–9). Horizontal bars indicate the presence of cromakalim.

a small, non-significant increase in efflux rate (Fig. 4).

Thus, prior exposure to cromakalim, even in the absence of an efflux response to the compound, largely prevents a later stimulation.

DISCUSSION

The dependence of potassium fluxes in rabbit vascular smooth muscle on extracellular calcium is variable. For noradrenaline- and potassium depolarisation-induced responses, the calcium dependence is related to both the method used (inorganic/organic calcium entry blockers, removal of calcium from the bathing medium) and the tissue [10–12]. Very few data have been published concerning the calcium dependence of cromakalim-induced efflux responses. Kreye and Weston [14] reported that, in rabbit aorta, the rubidium efflux stimulated by cromakalim is calcium-dependent and that the calcium required enters the cell through a channel which can be blocked by both inorganic and organic calcium entry blockers. However, Quast [13] reported that, in guinea-pig portal vein, inhibition of calcium entry by isradipine (a dihydropyridine calcium entry blocker) did not modify cromakalim-stimulated rubidium efflux. In view of these conflicting results, we therefore examined the calcium dependence of the response in rabbit mesenteric artery.

We were unable to show any effect of a high concentration of the inorganic calcium entry blocker, lanthanum, on cromakalim-induced increases in rubidium efflux rates. Nifedipine (1 μM), which abolishes contractions to potassium and has a maximal effect on noradrenaline-induced contractions in rabbit mesenteric artery [17], was also without significant effect on cromakalim-induced rubidium efflux. Only at much higher concentrations of nifedipine, when non-specific effects are apparent [4], was there any significant inhibition of the cromakalim response. Thus it is unlikely that the action of cromakalim is dependent on the influx of calcium in rabbit mesenteric artery.

The apparent discrepancy between our results and those of Kreye and Weston [14] are not readily explained, although our results do concur with the observations of Quast [13]. One possible explanation would be that there is a difference between the channels in rabbit mesenteric artery (and guinea-pig portal vein) and the channels in rabbit aorta. This possibility might also explain the opposite responses to cromakalim observed in the two tissues when calcium was removed from the buffer. Whereas in aorta the response was abolished, we found that removal of extracellular calcium greatly prolonged the response to cromakalim and in many cases appeared to increase the magnitude of the response (this was difficult to show statistically due to the variability of cromakalim reponses—cf. Figs 1 and 3). However, for these experiments, there were several major differences in the methods employed in the two studies—loading of rubidium in the absence/presence of calcium, time of exposure to "calcium free" buffer before addition of cromakalim, presence of magnesium in the efflux buffer etc. Thus further studies would be required in order to clarify fully this situation. However, it is known that this effect is specific to cromakalim-induced responses in mesenteric artery, as the stimulation of rubidium efflux in this tissue by noradrenaline or potassium-depolarisation is much smaller and shorter in CAFH than in normal HEPES [18].

One possible explanation for the maintenance of the efflux to cromakalim in CAFH would be that extracellular calcium or calcium influx is in some way involved in the closing of the channels. In normal media, the mutually antagonistic effects of cromakalim and calcium would result in the apparent curtailed response in the continued presence of the drug. This suggestion is supported by the observation that readdition of calcium during the maintained phase of the response in CAFH rapidly reversed the increased efflux rate.

The [^{3}H]-cromakalim dilution studies showed that, for exposure times of 3 min or less, the buffer concentration of cromakalim fell, upon the first transfer of the tissue, to levels known not to stimulate efflux in this tissue (c 50 nM) and then fell to <4 nM within 9 min. For these exposure times, the full response developed, and was maintained, for up to 60 min after the effective removal of the drug from the medium. One possible explanation of these results is that cromakalim might bind to a high affinity, small capacity site. However, we have been unable previously to demonstrate a specific binding site in this, or any other tissue [4]. An alternative explanation might be that, once opened, the channels require only a small concentration of cromakalim to maintain this status. However, the previously described results, and the low "uptake" of cromakalim into the tissue (at least some of which would be due to incomplete removal of medium by blotting), would appear to contradict this hypothesis. Furthermore we have been unable to show previously [4] displacement of [^{3}H]-cromakalim from tissue slices by unlabelled cromakalim. Thus we consider that removal of the drug from the bathing medium effectively removes it from the tissue altogether and this implies that cromakalim exerts its effects extracellularly.

An alternative explanation for the effects of cromakalim would be to suggest an intermediate step between the cromakalim acceptor and potassium channel gating and would be consistent with the short exposure times required to develop and maintain the response following removal of the drug. This proposal is supported by the observation that prior exposure to cromakalim inhibits a subsequent response. As blockade of the initial efflux response with TEA did not allow a subsequent stimulation with cromakalim, it would seem unlikely that the second response is inhibited due to prior hyperpolarisation of the membrane. Although this self inhibition effect does not manifest itself *in vivo*, or in tissues under tension [13], the phenomenon would appear inconsistent with a direct interaction between the drug and the potassium channel.

The data presented in this paper describe the dependence of cromakalim-induced 86-rubidium efflux on extracellular calcium and time of exposure in rabbit isolated mesenteric artery. Although further work is required to define the precise site of

action of the drug, we would suggest that cromakalim may act indirectly to open potassium channels in blood vessels.

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