Br. J. Pharmacol. (1991), **102**, 585–594

Differences in the K^+-channels opened by cromakalim, acetylcholine and substance P in rat aorta and porcine coronary artery

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1 The effects of acetylcholine and substance P on the efflux of $^{86}Rb^+$ and $^{42}K^+$ from rat aorta and pig coronary artery, respectively, were compared with those of the K^+ channel opening agent, cromakalim.

2 In rat aorta preloaded with $^{86}Rb^+$ and/or $^{42}K^+$, acetylcholine produced transient, concentration-dependent increases in the efflux rate coefficients of these tracers (maximum $\approx$ 35%). These effects were abolished by endothelial cell removal.

3 Donor/acceptor experiments with rat aorta suggested that at least some of the efflux of $^{86}Rb^+$ seen in the presence of acetylcholine was not derived from the endothelium, but came from the smooth muscle itself.

4 Acetylcholine (10 μM)-induced $^{86}Rb^+$ efflux was reduced by tetraethylammonium (TEA, 10 mM) to 33% and ouabain (300 μM) to 54% of control. Preincubation with Ba^{2+} (100 μM) did not significantly inhibit acetylcholine-induced efflux.

5 Acetylcholine-induced $^{42}K^+/^{86}Rb^+$ efflux was unaffected by preincubation with glibenclamide (10 μM). In contrast, the $^{42}K^+/^{86}Rb^+$ efflux induced by cromakalim was inhibited by glibenclamide (50 nM) by 50%.

6 Acetylcholine (0.3–10 μM)-induced inhibition of phenylephrine (1 μM)-induced tone was abolished by endothelial cell removal but unaffected by glibenclamide. Cromakalim-induced relaxations were endothelium-independent and were inhibited by glibenclamide in a concentration-dependent manner.

7 L^G-monomethyl L-arginine (L-NMMA, 250 μM) produced a significant (37 $\pm$ 14%) inhibition of acetylcholine-induced $^{86}Rb^+$ efflux whereas D^G-monomethyl L-arginine was without effect. In the tissue bath L-NMMA inhibited relaxations produced by acetylcholine (0.3–10 μM), but was without effect on responses to cromakalim.

8 In the pig coronary artery, substance P induced an endothelium-dependent efflux of $^{86}Rb^+$ and $^{42}K^+$, which was unaffected by preincubation with glibenclamide (10 μM) or L-NMMA (250 μM).

9 The present study shows that acetylcholine and substance P each open K^+-channels in arterial smooth muscle. However, the insensitivity of the stimulated $^{86}Rb^+/^{42}K^+$ efflux to inhibition by glibenclamide suggests that the K^+-channel opened by these agents is different from the K^+-channel opened by cromakalim. In addition, the inability of L-NMMA to inhibit fully the acetylcholine- and substance P-stimulated $^{86}Rb^+$ efflux suggests that in rat aorta and pig coronary artery the endothelium-derived hyperpolarizing factor(s) (EDHF) is different from endothelium-derived relaxing factor (EDRF).

Introduction

The existence of channels which can be modulated by the internal adenosine 5'-triphosphate (ATP) concentration of cells was first described in cardiac muscle (Trube & Hescheler, 1983; Noma, 1983) and has since been demonstrated in skeletal muscle (Spruce *et al.*, 1985; 1987), pancreatic β-cells (Cook & Hales, 1984) and cortical neurones (Ashford *et al.*, 1988). More recently, ATP-sensitive K^+-channels (K_{ATP}) have been identified in arterial smooth muscle, where they have been suggested to be the site of action of the K^+-channel opening drugs (Standen *et al.*, 1989), typified by cromakalim (Hamilton *et al.*, 1986).

In inside-out patches derived from rabbit mesenteric arteries, cromakalim opened K^+-channels which had been closed by applying a high concentration of ATP to the cytoplasmic side of the membrane (Standen *et al.*, 1989). The effects of cromakalim were reversed by the sulphonylurea glibenclamide, a potent and selective blocker of K_{ATP} in pancreatic β-cells and cardiac cells (Sturgess *et al.*, 1985; Schmid-Antomarchi *et al.*, 1987). Thus, the effects of cromakalim and other K^+-channel openers may be exerted via the opening of a K^+-channel similar to K_{ATP} in pancreatic β-cells and cardiac muscle. This is supported by a number of reports in which glibenclamide has been shown to inhibit the effects of cromakalim, both *in vitro* and *in vivo* (Buckingham *et al.*, 1989; Cavero *et al.*, 1989; Quast & Cook, 1989a). However, considerable evidence also suggests that other channel types such as large conductance Ca^{2+}-activated (Gelband *et al.*, 1988; Hu *et al.*, 1990) and delayed outward rectifier K^+-channels (Beech & Bolton, 1989) may be involved in the response to cromakalim.

Stimulation of the endothelium by acetylcholine and substance P leads to the release of factors which relax (EDRF) and hyperpolarize (EDHF) vascular smooth muscle (Furchgott & Zawadski, 1980; Zawadski *et al.*, 1983; Taylor *et al.*, 1988). In rat aorta, acetylcholine-induced hyperpolarization was accompanied by an increase in $^{86}Rb^+$ efflux from the tissue, reflecting an increase in the permeability of the cell membrane to K^+ (Taylor *et al.*, 1988). In the rabbit middle cerebral artery the hyperpolarizing effects of acetylcholine were reversed by glibenclamide, suggesting that the effects of acetylcholine in these arterial vessels were mediated through ATP-dependent K^+-channels (Standen *et al.*, 1989; Brayden, 1990).

The aim of the present study was to investigate the type of K^+-channel associated with the effects of two endothelium-dependent vasodilators, acetylcholine and substance P and that associated with the K^+-channel opening drug, cromakalim. The glibenclamide sensitivity of each response and the

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effects of the inhibitor of EDRF synthesis, N^G-monomethyl L-arginine (L-NMMA) (Rees *et al.*, 1989) were assessed and compared. Preliminary accounts of some of the data have already been presented (Quast & Baumlin, 1990; Quast & Bray, 1990).

Methods

Preparation of rat aorta

Male rats (Sprague-Dawley $\approx$ 150 g) were anaesthetized with ether and exsanguinated. Sections of the descending thoracic aorta ($\approx$15 mm long) were removed, carefully placed onto stainless steel tissue holders and bathed in a HEPES-buffered physiological salt solution (PSS) at 37°C, pH 7.4. In some experiments the endothelium was removed by rubbing the intimal surface of the aorta with a small wooden stick. Small rings of aorta (2–3 mm) were then cut from some of the preparations to examine in separate tissue bath experiments, the contractile and relaxant properties of the tissue following this rubbing procedure.

Measurement of $^{86}Rb^+$ and $^{42}K^+$ efflux

For the measurement of tracer efflux the tissues were preincubated for 120 min in PSS to which had been added $^{86}Rb^+$ (5 μCi ml^{-1}) or $^{42}K^+$ (5 μCi ml^{-1}) or both. The tissues were then transferred to thermostatic perfusion chambers containing PSS (perfused at 3 ml min^{-1}). The perfusate was collected at 1 min intervals and counted for radioactivity in the Čerenkov mode at an efficiency of 50% ($^{86}Rb^+$) or 65% ($^{42}K^+$). The radioactivity remaining at the end of the experiment was determined by dissolving the tissue in Lumasolve (at 50°C) overnight. The sample was then supplemented with 500 μl of 1 N HCL and 10 ml scintillant (OPTIFLUOR, Packard) and counted. The efflux data were expressed in terms of the efflux rate coefficient of $^{86}Rb^+$ or $^{42}K^+$ efflux (k, 10^{-2} min^{-1}).

In general, drug effects were expressed as the area under the curve (AUC) of the k vs time plots. In some experiments the peak drug effect was also calculated as percentage change (ΔK%) of the basal value of k, determined during the 10 min period prior to drug application. Results obtained from dual isotope experiments were evaluated as previously described (Quast & Baumlin, 1988).

Inhibition of efflux was generally studied by a double pulse protocol. After a period of equilibration the tissues were exposed to the test substance for 5–20 min (see respective Figures) followed by a period of washout. The blocker (inhibitor) was then added for 20 min before re-exposure to the test substance in the continuing presence of this agent. AUCs were calculated for each exposure period and the results expressed as a ratio of the second to the first peak (AUC_2/AUC_1). In experiments using L-NMMA or D-NMMA, a single pulse protocol was used. Tissues were exposed to L-NMMA (250 μM) or D-NMMA (250 μM) during the $^{86}Rb^+$ loading period and were washed for 25 min with PSS containing L-NMMA/D-NMMA. The tissues were then exposed to acetylcholine (10 μM) for 5 min. Direct comparison of AUCs was made between tissues exposed to L-NMMA/D-NMMA and those exposed only to vehicle.

Donor/acceptor experiments

Rings of rat aorta with the endothelium intact or rubbed (control experiments) were placed on tissue holders. After a period of equilibration, a piece of aorta of similar size, from a randomized group of rings which had been pre-labelled with $^{86}Rb^+$ and rubbed to remove the endothelium (acceptor), was placed next to (and above) the unlabelled (donor) ring. The tissues were then perfused with PSS from the bottom of the chamber upwards and exposed to relaxants following a double pulse protocol, being first exposed to acetylcholine (10 μM) followed by cromakalim (1 μM).

Tissue bath studies

Aortic rings were mounted in conventional tissue baths containing PSS, bubbled with 95% O_2, 5% CO_2 and maintained at 37°C, pH 7.4. Tissues were placed under an initial tension of 1 g. After a period of equilibration the rings were precontracted with phenylephrine (1 μM) and once the response was maintained, were exposed to a single concentration of acetylcholine (0.3–10 μM) or cromakalim (1–10 μM). Following washout, glibenclamide (1 or 10 μM) or L-NMMA (250 μM) was added for 40 min before exposure to phenylephrine and relaxant drug in the continuing presence of glibenclamide or L-NMMA. The relaxation to acetylcholine/cromakalim after exposure to glibenclamide or L-NMMA was expressed as a fraction of that obtained before exposure to these antagonists.

Pig coronary arteries

Pig hearts were obtained from the local slaughter house within 30 min of death and placed in PSS at room temperature. The left and right circumflex coronary arteries were carefully dissected and divided into sections 15 mm (for efflux studies) and 2–3 mm in length (for tissue bath studies).

The rings were mounted for isometric tension recordings under an initial tension of 4 g. After a period of equilibration (30 min) the tissues were retensioned and left for a further hour. After this time the PSS was replaced and the rings retensioned every 30 min for a further 60–90 min. Tissues were then precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10 μM) until a maintained contraction was achieved, followed by addition of a single contraction of susbtance P (30 nM) or cromakalim (0.1–10 μM). Following washout, the tissue was exposed to vehicle (dimethylsulphoxide (DMSO)), glibenclamide (1 or 10 μM) or L-NMMA (250 μM) for 40 min before re-exposure to $PGF_{2\alpha}$ and substance P/cromakalim in the continuing presence of test substance.

For efflux studies the protocol used for pig coronary arteries was identical to that employed in rat aorta experiments. In these experiments a 5 min exposure period to substance P was used.

Drugs and solutions

The following drugs were used: acetylcholine chloride (Fluka), stock solution in twice distilled water; (±)-cromakalim (synthesized at Sandoz), stock solution in DMSO; substance P (Bachem), stock solution in 150 mM acetic acid; (−)-noradrenaline L-hydrogentartrate (Fluka), stock solution in ascorbic acid (1 mM); $PGF_{2\alpha}$ (Upjohn), stock solution in 9% benzylic alcohol; barium chloride, stock solution in twice distilled water; tetraethylammonium (TEA, Janssen), stock solution in PSS; ouabain (Fluka), stock solution in twice distilled water. All dilutions were made in twice distilled water. Final concentrations of solvents did not exceed 0.1% and had no effect on the basal levels of either $^{86}Rb^+$ or $^{42}K^+$ efflux or on basal tension levels. $^{86}Rb^+$ was obtained from Amersham International (U.K.). $^{42}K^+$ was obtained as $^{42}K_2CO_3$ (Eidgenössisches Institut für Reaktorforschung, Würenlingen, Switzerland). The composition of the physiological salt solution was (mM): NaCl 120, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1.2, glucose 11, HEPES 20, pH 7.4 at 37°C, gassed with 95%O_2/5%CO_2.

Statistics

Results are expressed as the mean ± s.e.mean. Difference in tissue responses was assessed by a two-tailed unpaired Student's t test. Inhibitory effects of agents on tracer efflux stimulated by cromakalim, acetylcholine or substance P were assessed by measuring AUCs in the presence and absence of inhibitory substance and by expressing these as a ratio (AUC_2/AUC_1).

Results

Studies in rat aorta

Effects of acetylcholine Figure 1a shows the effects of exposure to acetylcholine (10 μM) followed by cromakalim (1 μM) on the $^{86}Rb^+$ efflux from unrubbed (endothelium intact) and rubbed (endothelium-denuded) aortic rings. Clearly, the effects of acetylcholine were fully dependent on the presence of an intact endothelium. Although in endothelium-denuded preparations, the time taken to reach maximum cromakalim-induced $^{86}Rb^+$ efflux was increased, the peak change in efflux was not significantly different in the two experimental groups ($P > 0.05$, $n = 6$–8). In intact tissues, preloaded with $^{86}Rb^+$, acetylcholine (0.3–30 μM) produced a concentration-dependent increase in the efflux of $^{86}Rb^+$ (Figure 1b) which reached a peak after 3 min and thereafter rapidly declined to baseline levels during the 20 min exposure time to acetylcholine (Figure 1a). In contrast, in the presence of cromakalim (1 μM) peak changes in k were observed only after 10 min (unrubbed tissues) and 15 min (rubbed tissues) (Figure 1a). Although there was some decline in the efflux coefficient after this time, the response to cromakalim was significantly maintained above control levels during the 20 min exposure period (Figure 1a).

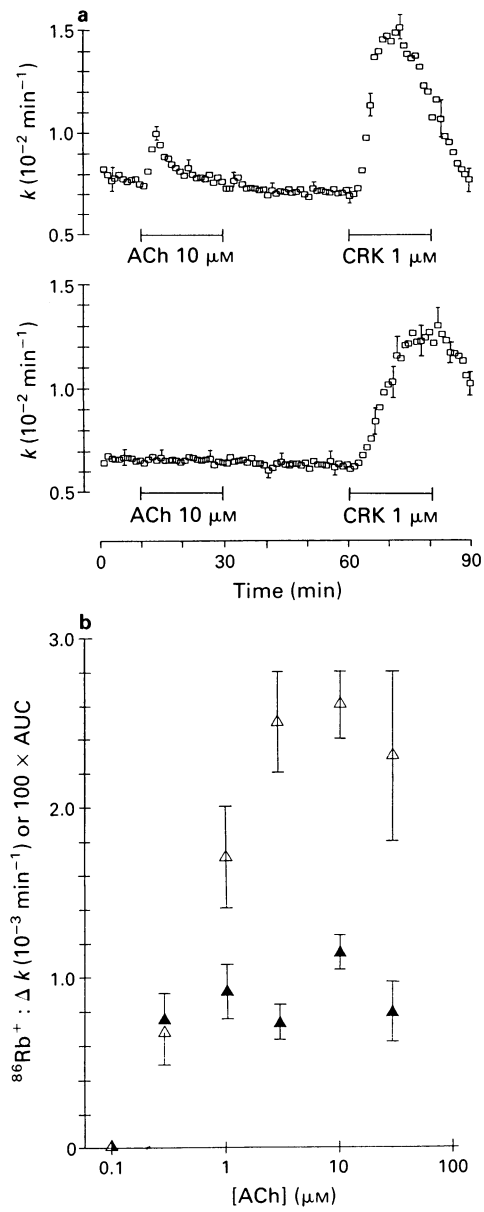

Figure 1 Effects of acetylcholine (ACh) and cromakalim (CRK) on the rate constant, k, of $^{86}Rb^+$ efflux from rat aorta. (a) Effects of ACh (10 μM) and CRK (1 μM) in; Upper trace, endothelium-intact preparations; Lower trace, rubbed tissues. At times indicated, ACh and CRK were each perfused for 20 min. (b) Dependence of $^{86}Rb^+$ efflux on ACh concentration as assessed by the peak increase in k (Δ) and the area under curve of the k vs time plot (AUC, ▲). Values are mean of 6–8 observations; vertical bars show s.e.mean. Some error bars have been omitted for clarity.

The effects of acetylcholine and cromakalim in the above flux experiments correlated with data obtained in tissue bath studies. In these experiments both acetylcholine (0.3 and 10 μM) and cromakalim (1 and 10 μM) inhibited phenylephrine (1 μM)-induced tone (Figure 2, Table 1). However, relaxations to acetylcholine were transient in nature, whereas cromakalim-induced responses were maintained. Of the two, only acetylcholine-induced relaxation was inhibited by endothelial cell removal (data not shown).

Donor/acceptor experiments Figure 3 shows the efflux of $^{86}Rb^+$ from 'acceptor' rings of rat aorta which were denuded of endothelium. In the presence of a 'donor' tissue with an intact endothelium, acetylcholine produced a small increase in the efflux of $^{86}Rb^+$ from the 'acceptor' tissue (Figure 3a). The efflux observed was 25 ± 5% of that observed for acetylcholine under the conditions described for Figure 1. In contrast, if the endothelium was removed from the 'donor' no efflux of $^{86}Rb^+$ from the acceptor tissue was observed (Figure 3b). In control and test conditions the responses to cromakalim were of similar magnitude (Figure 3a,b).

Inhibition studies

Glibenclamide: Figure 4a shows the effects of acetylcholine (10 μM) on the tracer efflux from intact aortic rings labelled, in separate experiments, with $^{86}Rb^+$ or $^{42}K^+$. Acetylcholine (10 μM) induced a transient increase in the efflux of both $^{86}Rb^+$ and $^{42}K^+$ (Figure 4a). Following a washout period of 30 min a second exposure to acetylcholine produced a second increase in flux of both tracers, which was in the case of $^{86}Rb^+$, 87 ± 7% and for $^{42}K^+$, 104 ± 6% of initial control response ($n = 6$). Pretreatment of tissues with glibenclamide (10 μM) alone, had no effect on the basal efflux of either $^{86}Rb^+$ or $^{42}K^+$. In addition, this concentration of glibenclamide was without effect on acetylcholine-stimulated increases in both $^{86}Rb^+$ and $^{42}K^+$ efflux (Figure 4b, Table 2).

In dual-labelling experiments cromakalim (1 μM) induced a relatively maintained efflux of $^{86}Rb^+$ and $^{42}K^+$ from rat aorta (Figure 4c). In contrast to the lack of effect of glibenclamide on acetylcholine-induced efflux, the effects of cromakalim were inhibited by glibenclamide (50 nM–10 μM) in a concentration-dependent manner (Figure 4d, Table 2). In the tissue bath, glibenclamide inhibited cromakalim-induced relaxation in rings precontracted with phenylephrine (1 μM) but was without effect on acetylcholine-induced vasorelaxations (Figure 2, Table 1).

L-NMMA: Preincubation with L-NMMA (250 μM) during the $^{86}Rb^+$-loading period (120 min) produced a significant (37 ± 14%) reduction in the efflux of $^{86}Rb^+$ produced by subsequent exposure to acetylcholine (10 μM), but was without significant effect on cromakalim-induced fluxes (Table 2). In contrast, exposure to D-NMMA (250 μM) was without effect on responses to acetylcholine (Table 2).

In tissue bath experiments, the application of L-NMMA sometimes produced a slowly developing contraction of the aorta and a potentiation of the maximum tension developed to phenylephrine. In the continuing presence of L-NMMA (250 μM) the relaxant effects of acetylcholine (0.3–10 μM) were inhibited (Table 1). This effect could be overcome to some extent by further increasing the concentration of acetylcholine (Table 1).

Tetraethylammonium: Figure 5a shows control responses to acetylcholine when a double pulse protocol was used. Following washout of acetylcholine, pretreatment with TEA (10 mM)

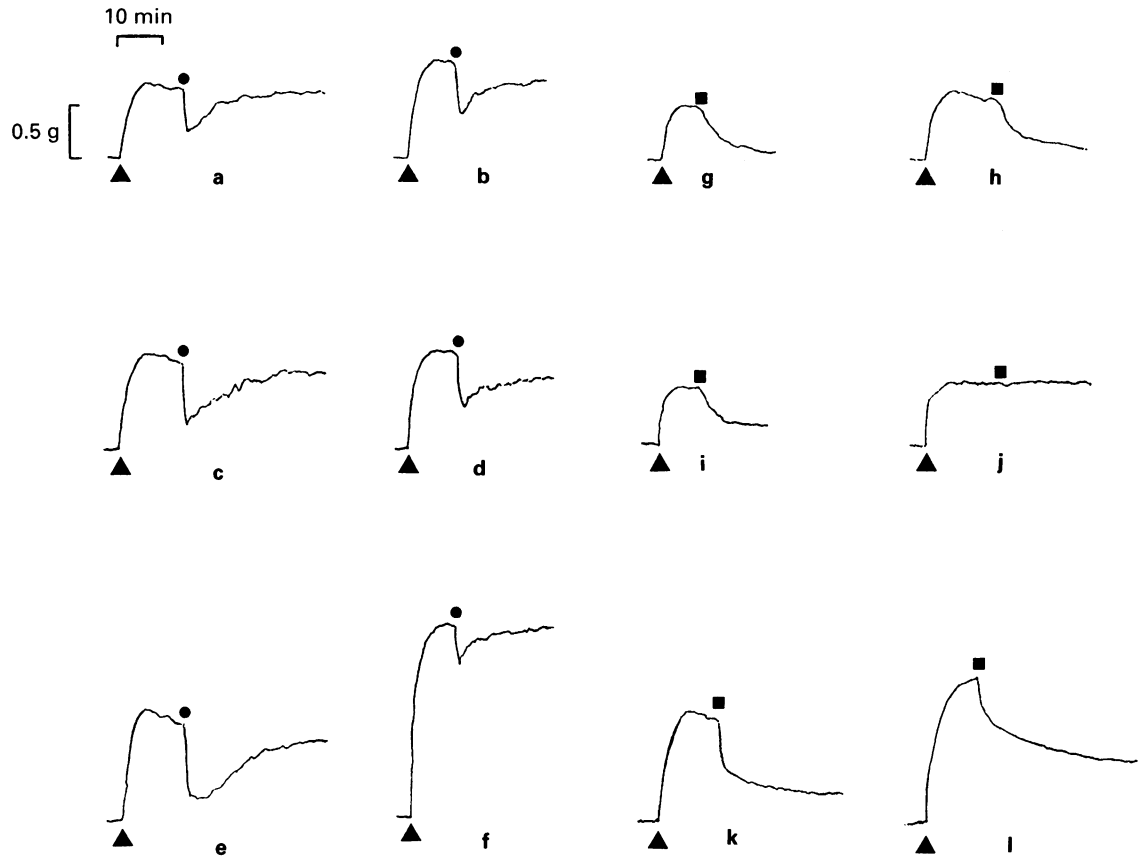

Figure 2 Representative traces showing the effects of glibenclamide (GBC) and L^G-monomethyl L-arginine (L-NMMA) on acetylcholine (ACh, ●, left panel) and cromakalim (CRK)-induced relaxations (■, right panel) in rat aorta. Left panel: (a,c,e) control responses. Tissues were precontracted with phenylephrine (PE, 1 μM) (▲), and then exposed to ACh (0.3 μM, ●). Following washout, vehicle, dimethylsulphoxide (DMSO), GBC (10 μM) or L-NMMA (250 μM) was added for 40 min. Re-exposure to PE (▲) and ACh (●) in presence of (b) DMSO; (d) GBC; (f) L-NMMA. Right panel: (g,i,k) control responses. Tissues were precontracted with PE (1 μM) (▲), and then exposed to CRK (10 μM, ■). Re-exposure to PE (▲) and CRK (■) in presence of (h) vehicle (DMSO); (j) GBC (10 μM); (l) L-NMMA (250 μM).

for 20 min produced a small but significant increase in the basal $^{86}Rb^+$ efflux rate coefficient (Figure 5b). In the continuing presence of TEA (10 mM), the response to acetylcholine (measured as AUC post exposure to TEA/AUC pre-exposure) was reduced to 33% of control values (no TEA) (Table 2). TEA (10 mM) completely inhibited cromakalim (1 μM)-induced efflux (Table 2).

Ba^{2+}: Although a small reduction in acetylcholine-induced $^{86}Rb^+$ efflux was observed in the presence of Ba^{2+} (100 μM) this was not significant (Figure 5c, Table 2). In contrast, the flux-inducing effects of cromakalim were almost completely inhibited by Ba^{2+} (100 μM, Table 2).

Ouabain: With an identical protocol, exposure to ouabain (300 μM) produced a slowly developing but progressively increasing baseline. In the continuing presence of ouabain, both acetylcholine- and cromakalim-induced effluxes were significantly inhibited (Table 2) compared to control responses where ouabain was not included in the perfusing solution (acetylcholine response, Figure 5d).

Studies in pig coronary arteries

The basal rates of efflux of $^{86}Rb^+$ and $^{42}K^+$ (measured between 30–35 min into efflux, $n = 6$ preparations) from pig coronary artery were 0.328 ± 0.003 10^{-2} min^{-1} and 0.751 ± 0.02 10^{-2} min^{-1}, respectively. In the absence of agonist these rates were well maintained, in contrast to the basal efflux in rat aorta which tended to fall slightly throughout the experiment (see Figure 1).

Effects of substance P on tracer efflux In pig coronary artery rings, substance P (30 nM) induced a small, concentration-dependent, transient increase in the efflux of $^{86}Rb^+$ and $^{42}K^+$ (Figure 6a). In a manner similar to that of acetylcholine in rat

Table 1 Vasorelaxant effects of acetylcholine (ACh), substance P and cromakalim (CRK) and their inhibition by glibenclamide (GBC) and L^G-monomethyl L-arginine (L-NMMA)

	Rat aorta[a]		*Pig coronary*[a]	
	ACh	*CRK*	*Substance P*	*CRK*
Substance	0.3 μM/10 μM	0.3 μM/10 μM	30 nM	0.3 μM/10 μM
(Control)	87 ± 7/98 ± 9	90 ± 6/84 ± 6	102 ± 5	98 ± 5/103 ± 4
GBC (1 μM)	96 ± 6/99 ± 7	29 ± 8*/30 ± 3*	97 ± 6	10 ± 7*/85 ± 6*
GBC (10 μM)	97 ± 7/97 ± 5	20 ± 8*/35 ± 4*	99 ± 8	4 ± 1*/52 ± 6*
L-NMMA (250 μM)	34 ± 4*/51 ± 6*	83 ± 14/74 ± 5	98 ± 12	111 ± 14/106 ± 3

[a] Tissues were precontracted with phenylephrine 1 μM (aorta) or prostaglandin $F_{2\alpha}$ 10 μM (pig coronary artery). Results in presence of inhibitor or vehicle are expressed as % of initial relaxant response in absence of inhibitor (see Methods). Values are mean ± s.e.mean of 4–7 observations; * denotes $P < 0.05$ compared to control value.

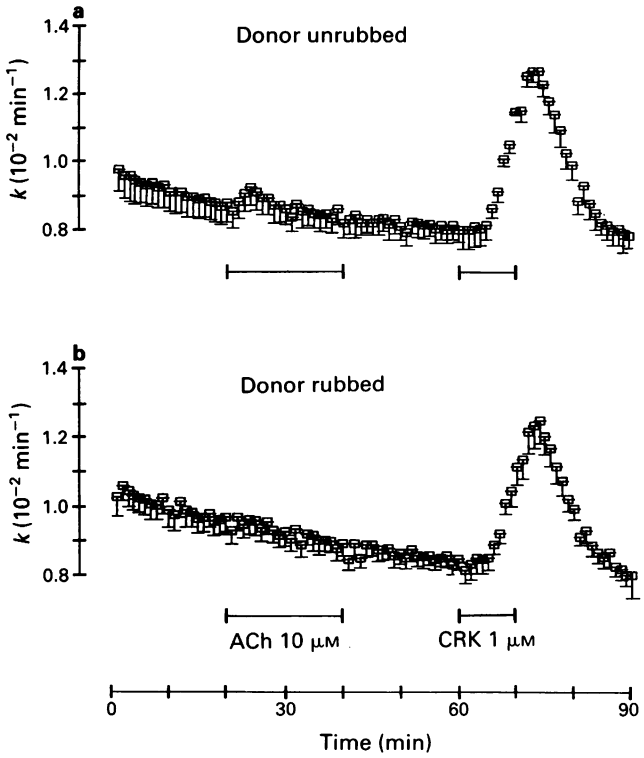

Figure 3 Donor/acceptor experiments in rat aorta, showing the efflux of $^{86}Rb^+$ induced by acetylcholine (ACh, 10 μM) and cromakalim (CRK, 1 μM), from rubbed (endothelium-denuded) rings of rat aorta (a) in the presence of an unrubbed, unlabelled donor tissue; (b) in the presence of a rubbed, unlabelled donor tissue. ACh and CRK were perfused at the times indicated by the horizontal bars. Values are mean for 6 experiments; vertical bars show s.e.mean.

aorta (see Figure 1), this effect of substance P was completely abolished by removal of the endothelium, whereas in the same tissue segments (i.e. rubbed tissues) the response to cromakalim was unaffected (results not shown).

Following washout of substance P, a second increase in efflux of both $^{86}Rb^+$ and $^{42}K^+$ could be produced, although this was reduced by approximately 20% when compared to the initial peak (Figure 6a, Table 3). Preincubation with glibenclamide (10 μM) for 20 min had no effect on the basal efflux of $^{86}Rb^+$ or $^{42}K^+$ and in the continuing presence of glibenclamide, efflux induced by substance P was unaffected (Figure 6b). In contrast, the effects of cromakalim were inhibited by glibenclamide (0.5–10 μM) in a concentration-dependent manner (Figure 7 and Table 3).

In a separate series of experiments, tissues were preincubated with L-NMMA (250 μM) during the $^{86}Rb^+$ loading period and for a further 30 min before exposure to a single concentration of substance P (30 nM). L-NMMA was without effect on either the basal or substance P-induced increase in $^{86}Rb^+$ efflux in this tissue (Table 3).

Tissue bath studies Exposure of coronary artery rings to $PGF_{2\alpha}$ (10 μM), usually produced a slowly developing contraction which reached a maintained level after approximately 30–60 min. In some preparations the contraction developed more rapidly (15 min). However, no differences were observed in the inhibitory effects of substance P, following either type of contraction (Figure 8).

Substance P (30 nM) produced a maximal relaxation of pig coronary artery rings which had been precontracted with $PGF_{2\alpha}$ (10 μM) (Figure 8). Like the inhibitory effects of acetylcholine in rat aorta, this relaxation was transient in nature and inhibited completely by endothelial removal (data not shown). These mechanical changes were completely unaffected by preincubation of the tissues for 40 min with glibenclamide

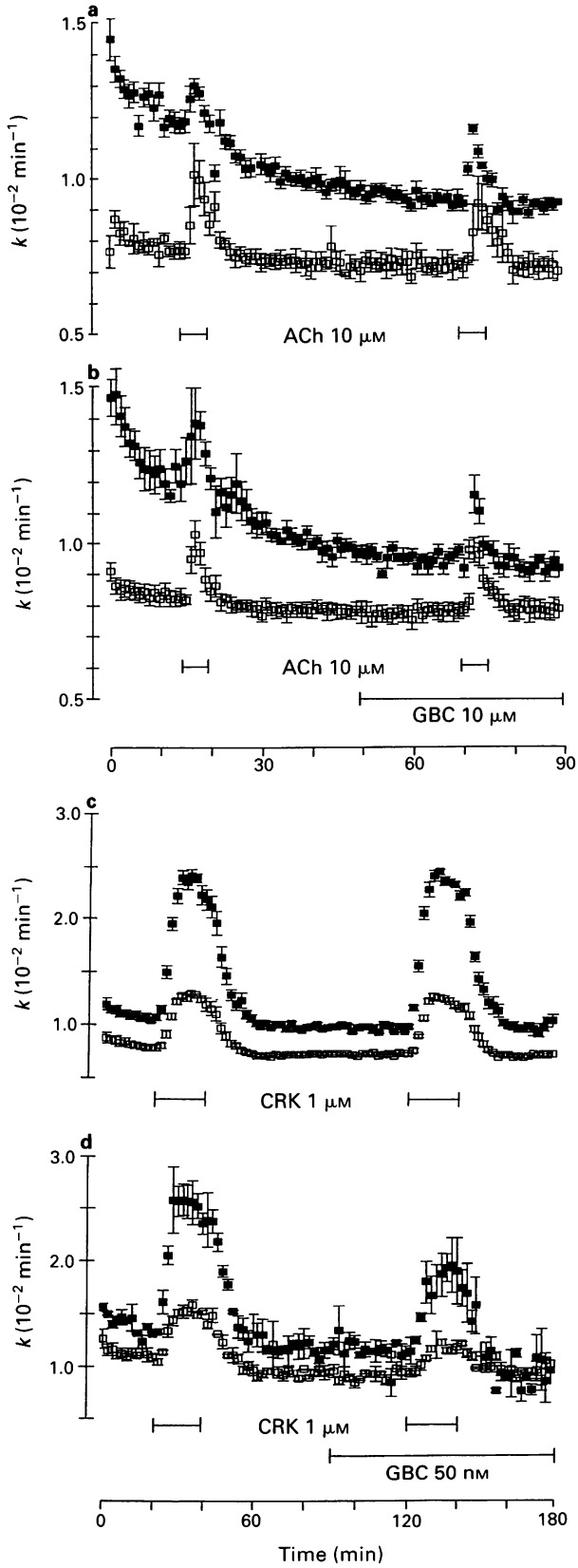

Figure 4 Effects of glibenclamide (GBC) on acetylcholine (ACh) and cromakalim (CRK)-induced efflux of $^{42}K^+$ (■) and $^{86}Rb^+$ (□) from rat aorta. (a) Control: ACh (10 μM) was perfused for 5 min at the times indicated. (b) Effect of GBC on ACh-induced tracer efflux. GBC (10 μM) was added 20 min prior to the second exposure to ACh as indicated. (c) Control: double pulse protocol for CRK (1 μM, perfused for 20 min). (d) Effect of GBC (0.05 μM, added 30 min prior to the second exposure to CRK) on CRK-induced tracer efflux. (a and b) Effluxes of $^{42}K^+$ and $^{86}Rb^+$ were determined in separate experiments; (c and d) double labelling experiments. Values are mean with s.e.mean shown by vertical bars, $n = 6$.

Table 2 Comparison of $^{86}Rb^+$ effluxes induced by acetylcholine (10 μM) or cromakalim (1 μM) in rat aorta[a]

Substance[b]	*Acetylcholine* AUC_2/AUC_1 (%)[c]	*Cromakalim* AUC_2/AUC_1 (%)[c]
(Control)	0.87 ± 0.06 = 100%	1.09 ± 0.07 = 100%
GBC 0.05 μM	/	48 ± 10*
0.5 μM	/	0 ± 0*
10 μM	104 ± 6	0 ± 0*
L-NMMA 250 μM	63 ± 14*	82 ± 9
D-NMMA 250 μM	96 ± 8	/
TEA 10 mM	33 ± 10*	0 ± 0*
Ba^{2+} 100 μM	83 ± 10	20 ± 3*
Ouabain 300 μM	54 ± 6*	73 ± 5*

[a] A double pulse protocol was used, except for L^G-monomethyl L-arginine (L-NMMA) and D-NMMA which were present during the $^{86}Rb^+$ loading period (120 min) and a 10 min wash (see Figure 5 and Methods).

[b] The applied agents were without effect on the basal rate of $^{86}Rb^+$ efflux ($k = 0.65 \pm 0.03\ 10^{-2}\,min^{-1}$) except tetraethylammonium (TEA), Ba^{2+} and ouabain which increased k by 0.07 ± 0.02, 0.08 ± 0.06 and 0.13 ± 0.03 $10^{-2}\,min^{-1}$ after 20 min perfusion time (n = 6–10, mean ± s.e.mean).

[c] Data are expressed as AUC_2/AUC_1 (mean ± s.e.mean, n = 6–10) where AUC_1 was 0.016 ± 0.002 (n = 10) for 20 min stimulation with acetylcholine (10 μM) and 0.164 ± 0.0005 (n = 9) for cromakalim (1 μM) (AUC in dimensionless units, see Quast & Baumlin, 1988). AUC_2/AUC_1 was then normalized to 100%. Statistically significant differences, compared to control values are indicated by * $P < 0.05$. GBC = glibenclamide.

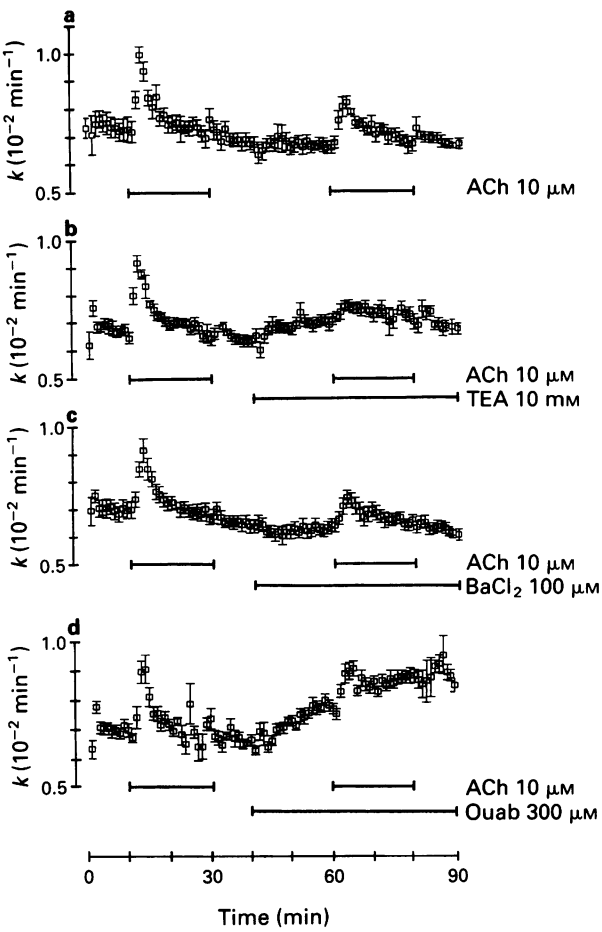

Figure 5 Acetylcholine (ACh)-induced $^{86}Rb^+$ efflux in the presence of tetraethylammonium (TEA), Ba^{2+} and ouabain (Ouab). (a) Control responses to ACh (10 μM), using a double pulse protocol. After first exposure to ACh, tissues were superfused with PSS containing (a) vehicle, (b) TEA, 10 mM, (c) Ba^{2+}, 100 μM or (d) ouabain, 300 μM for 20 min, prior to re-exposure to ACh. Values are mean of 6–8 observations; vertical bars show s.e.mean.

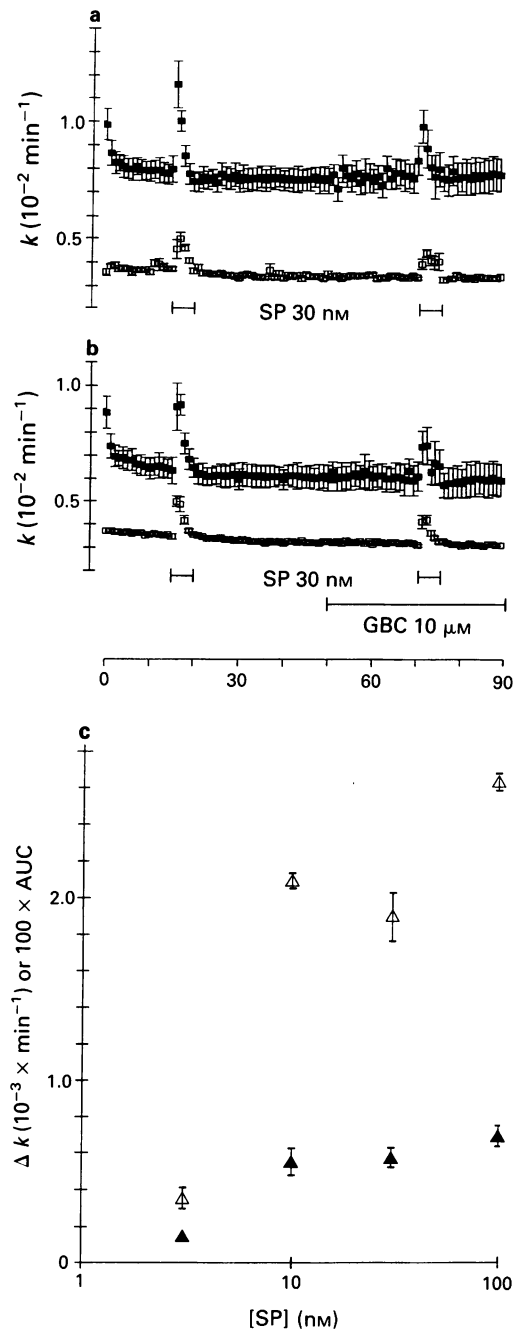

Figure 6 Substance P (SP)-induced efflux of $^{42}K^+$ (■) and $^{86}Rb^+$ (□) from pig coronary artery rings and effect of glibenclamide (GBC). (a) Effect of substance P (30 nM) perfused for 5 min at the times indicated (control). (b) Effect of GBC (10 μM), added 20 min prior to the second exposure to substance P. (c) Plot of Δk (△) and AUC (▲) vs concentration of substance P. Effluxes of $^{42}K^+$ and $^{86}Rb^+$ were determined in separate experiments. Values are mean of 6–8 observations; s.e.mean shown by vertical bars.

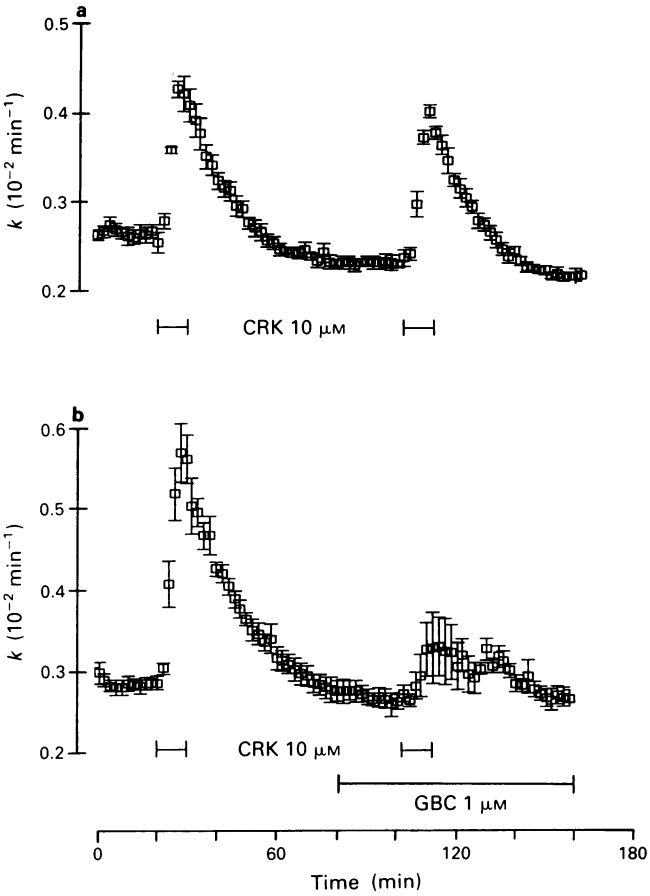

Figure 7 Effect of glibenclamide (GBC) on cromakalim (CRK)-induced $^{86}Rb^+$ efflux from pig coronary artery rings. (a) Control responses to CRK (10 μM) using a double pulse protocol. Tissues were exposed to CRK for 10 min at the times indicated. (b) Effect of GBC (1 μM), added 20 min prior to and during second exposure to CRK. Values are mean of 8 observations; s.e.mean shown by vertical bars.

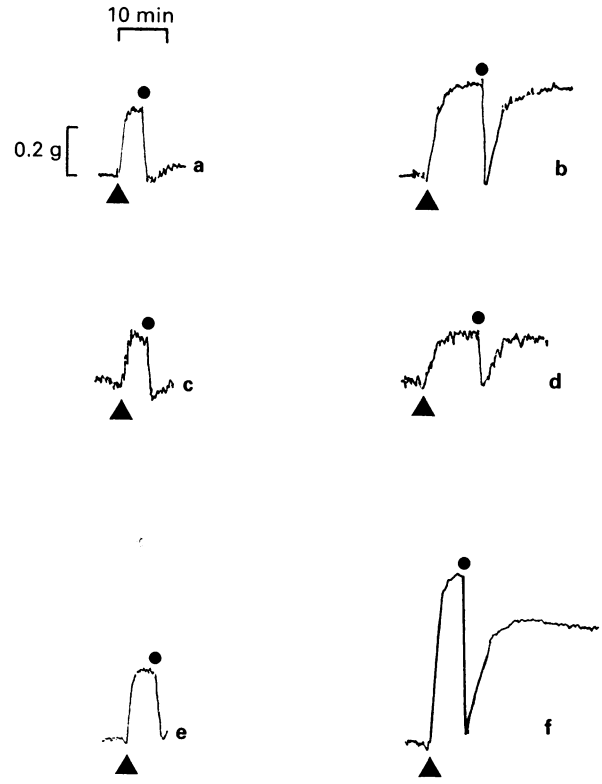

Figure 8 Representative traces showing the effects of glibenclamide (GBC) and L^G-monomethyl L-arginine (L-NMMA) on substance P-induced relaxations in pig coronary artery. (a,c,e) Control responses. Tissues were precontracted with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10 μM) (▲), and then exposed to substance P (30 μM, ●). Following washout, vehicle (DMSO), GBC (10 μM) or L-NMMA (250 μM) was added for 40 min. Re-exposure to $PGF_{2\alpha}$ and substance P in presence of (b) DMSO, (d) GBC, (f) L-NMMA.

(10 μM). Although the contractile response to $PGF_{2\alpha}$ was potentiated by preincubation with L-NMMA (see Figure 8f), the inhibitory effects of substance P were unaffected by this pretreatment (Table 1).

Cromakalim (1 and 10 μM) produced a slowly developing inhibition of tone in coronary artery rings, which could be inhibited by pretreatment with glibenclamide (1 and 10 μM). The inhibitory effects of cromakalim were not significantly inhibited by pretreatment with L-NMMA (Table 1).

Table 3 Effects of glibenclamide (GBC) and L^G-monomethyl L-arginine (L-NMMA) on the $^{86}Rb^+$ efflux induced by substance P (30 nM) and cromakalim (1 and 10 μM) in porcine coronary artery

Substance	*Substance P* (30 nM) AUC_2/AUC_1 (%)	*Cromakalim* (1 μM/10 μM) AUC_2/AUC_1 (%)
(Control)	0.82 ± 0.02 = 100%	1.0 ± 6 = 100% (1 μM) 1.0 ± 6 = 100% (10 μM)
GBC 0.1 μM	/	27 ± 1*/55 ± 1*
0.3 μM	/	8 ± 4*/28 ± 3*
3 μM	92 ± 14	/
10 μM	113 ± 17	/
L-NMMA 250 μM	111 ± 13	/

Substance P: AUC_1 = 0.0056 ± 0.0004 (absolute units, n = 14, mean ± s.e.mean)
Cromakalim: AUC_1 (1 μM) = 0.034 ± 0.002 (absolute units, n = 8, mean ± s.e.mean) (10 μM) = 0.062 ± 0.005 (absolute units, n = 8, mean ± s.e.mean)
* $P < 0.05$, compared to control.

Neither substance P nor cromakalim had any effects on the basal tension of coronary artery rings (data not shown).

Discussion

The present study has shown that several major differences exist between the pharmacological profiles of acetylcholine- and substance P-induced $^{86}Rb^+/^{42}K^+$ efflux in comparison to cromakalim-induced effects. These include: (1) the complete lack of sensitivity of acetylcholine and substance P to inhibition by glibenclamide; (2) the endothelium-dependence of both the relaxant responses and tracer efflux induced by acetylcholine and substance P; (3) the relative lack of sensitivity of acetylcholine-induced efflux to inhibition by Ba^{2+} and TEA; (4) the rapid inactivation kinetics of the $^{86}Rb^+/^{42}K^+$ efflux induced by endothelium-dependent vasodilators and (5) differences in the magnitude of efflux induced by acetylcholine and substance P as compared to cromakalim, although each of these substances were equally effective as relaxants.

Origin of acetylcholine-induced efflux

In rat aortic rings, acetylcholine produced a small transient increase in $^{86}Rb^+$ and $^{42}K^+$ efflux. Taylor *et al.* (1988) had previously shown that acetylcholine transiently hyperpolarized this tissue and elicited a concomitant increase in $^{86}Rb^+$ efflux. To investigate whether the small increases in $^{86}Rb^+$ efflux observed here with acetylcholine stem from the endothelial cells or the smooth muscle cells, donor/acceptor experiments were performed. The rationale for these experiments was derived from the observation that in endothelium-denuded tissues no tracer efflux in response to acetylcholine

was observed. Therefore, it was possible that acetylcholine was acting either directly to increase the permeability of the endothelium for K^+ or indirectly, by the release of factors from the endothelium, which lead to an increase in $^{86}Rb^+$ efflux from either the endothelium or the underlying smooth muscle. In endothelium-denuded preparations which were loaded with $^{86}Rb^+$, efflux of this tracer was only observed in the presence of an endothelium-intact donor (unlabelled) tissue. Thus, the efflux must have been a result of a factor(s) released from the endothelium which acts on the smooth muscle.

In these donor/acceptor experiments the observed efflux was considerably smaller than that obtained from endothelium-intact tissues. This may reflect the small amounts or the short half-life of EDRF/EDHF released by the endothelium, the effects of which are normally facilitated by the close association with the underlying smooth muscle. Alternatively, a component of the efflux induced by acetylcholine from tissues with intact endothelium, may be derived from the endothelium itself. It has recently been shown that activation of cultured endothelial cells by endothelium-dependent vasodilators leads to hyperpolarization due to the opening of Ca^{2+}-dependent K^+-channels (Lueckhoff & Busse, 1990), and this may therefore, contribute to the increased efflux observed after exposure to acetylcholine.

Nature of acetylcholine-induced efflux

Some information on the nature of the endothelium-derived hyperpolarizing factor in rat aorta was obtained with the EDRF synthesis inhibitor, L-NMMA at concentrations that inhibit endothelium-mediated vasorelaxation maximally (250 μM, Palmer *et al.*, 1988). Acetylcholine (10 μM)-induced $^{86}Rb^+$ efflux was inhibited by L-NMMA (but not D-NMMA) by 37%, suggesting that these effects were specific for the L-enantiomer. In contrast, cromakalim-induced effects were not significantly inhibited by L-NMMA.

The observation that part of the response was resistant to the actions of L-NMMA, suggested that at least two factors may be released from the endothelium. It also suggests that in the presence of acetylcholine at least part of the observed efflux is induced by EDRF. The present result is consistent with those of Tare *et al.* (1990) who showed that EDRF (NO) could hyperpolarize guinea-pig uterine artery. However, in other vessels there is evidence against a role of EDRF (NO) in acetylcholine-induced hyperpolarization, for example in canine mesenteric arteries (Komori *et al.*, 1988), and in rabbit femoral (Huang *et al.*, 1988) and cerebral arteries (Brayden, 1990). These differences may relate to the vessel studied and this matter clearly requires further clarification. In the present study, L-NMMA inhibited the relaxant and efflux responses to acetylcholine (10 μM) by 50% and 37%, respectively. Thus, at this acetylcholine concentration, EDRF and EDHF appear to make approximately equal contributions to the observed responses. At the lower concentration of acetylcholine (1 μM) it seems likely that of the two factors released, EDRF plays a greater role in mediating relaxation (EDRF release has previously been estimated to produce 60–80% of the acetylcholine-induced relaxation; Chen & Suzuki, 1989).

Substance P produces an endothelium-dependent hyperpolarization of pig coronary artery (Bény *et al.*, 1986), and also hyperpolarizes pig coronary artery endothelial cells in primary culture (Brunet & Bény, 1989). In support of these results, substance P increased the efflux of $^{86}Rb^+$ from pig coronary artery. However, in contrast to acetylcholine, the effects of substance P on tracer efflux (and agonist-induced tone) were not antagonized by L-NMMA. This suggests that substance P acts to release factors from the endothelium which hyperpolarize (EDHF) and/or relax (EDRF) the coronary artery but which are synthesized via pathways different from those leading to EDRF (= nitric oxide, NO) synthesis in other vessels, and are therefore probably different from NO.

The nature of the $^{86}Rb^+$ efflux induced by acetylcholine was also investigated with K^+-channel blocking agents and ouabain, an inhibitor of Na^+/K^+ ATPase. Tetraethylammonium, a K^+-channel blocker with broad specificity, inhibited the acetylcholine-stimulated efflux suggesting that at least a part of this response was a result of K^+ ($^{86}Rb^+$) flux through ion channels. The results obtained with ouabain are, on the other hand, more difficult to interpret. Activation of Na^+/K^+ ATPase leads to membrane hyperpolarization (see Fleming, 1980, for review) and has been implicated in endothelium-dependent hyperpolarization of smooth muscle (De Mey & Vanhoutte, 1980; Rapoport *et al.*, 1985; Feletou & Vanhoutte, 1988). Conversely, inhibition of the pump leads to cell depolarization (Fleming, 1980) and increases in intracellular Ca^{2+} via Na^+/Ca^{2+} exchange (see Brading & Lategan, 1985, for discussion). Accordingly, exposure to ouabain alone produced a slowly-developing increase in the baseline efflux of $^{86}Rb^+$. However, although activation of the pump by acetylcholine may provide an explanation as to the relatively small amount of induced efflux, it does not reconcile the fact that in the presence of ouabain, acetylcholine-induced flux was reduced, when theoretically, due to an increase in the electrochemical driving force for $^{86}Rb^+$ to leave the cell, an increase in $^{86}Rb^+$ efflux would be predicted. As similar qualitative results were obtained with cromakalim, i.e. the cromakalim-induced efflux was also inhibited by ouabain, these observations may reflect that in the presence of ouabain there is a decreased release of the hyperpolarizing factor(s) (in the case of acetylcholine) due to depolarization of the endothelium or may reflect the voltage- and Ca^{2+}-dependence of the Rb^+-permeable channels opened by these hyperpolarizing factors and by cromakalim. It has been suggested that Ca^{2+}-influx or extracellular Ca^{2+} may be involved in the closure of K^+-channels opened by cromakalim, in accordance with this suggestion (Coldwell & Howlett, 1988).

Pharmacology of acetylcholine-induced effects

The most interesting observation made was that the sulphonylurea glibenclamide, a potent and selective blocker of K_{ATP} in pancreatic β-cells and related cell lines (Trube *et al.*, 1986; Schmidt-Antomarchi *et al.*, 1987; Sturgess *et al.*, 1988) was completely without effect on both the relaxant and efflux-inducing properties of both acetylcholine and substance P in rat aorta and pig coronary, respectively. In rabbit middle cerebral artery, however, glibenclamide has previously been shown to inhibit the hyperpolarization induced by acetylcholine suggesting that in this vessel this effect is mediated by an opening of K_{ATP} (Standen *et al.*, 1989; Brayden, 1990). This difference may relate to the inherent physiological properties of the vessels studied since we have used large, tonic vessels, in contrast to Standen *et al.* (1989) and Brayden (1990), who used small (resistance) vessels.

In contrast to the lack of effect of glibenclamide (10 μM) on the relaxant and tracer efflux stimulating effects of acetylcholine and substance P in rat aorta and pig coronary artery, respectively, the effects of cromakalim in these vessels were antagonized by concentrations of glibenclamide up to 200 times lower. This is in agreement with others, who suggest that the effects of cromakalim may be exerted at an ATP-dependent K^+-channel in smooth muscle (see e.g. Quast & Cook, 1989b; Standen *et al.*, 1989).

In addition to the differential effects of glibenclamide, on the cromakalim- and acetylcholine-induced $^{86}Rb^+/^{42}K^+$ efflux in the rat aorta, differences were obtained with non-selective K^+-channel blockers. TEA, at 10 mM, fully inhibited the $^{86}Rb^+$-efflux induced by cromakalim whereas responses to acetylcholine were only partially inhibited (by 65%), thus demonstrating that the ion channels involved in these two responses differ in their sensitivity to TEA. Similarly Ba^{2+} (100 μM) was unable to reduce appreciably acetylcholine-induced $^{86}Rb^+$ efflux, whereas cromakalim-induced efflux was much more sensitive to inhibition. Ba^{2+}, at these concentra-

tions, is an effective and relatively specific inhibitor of K_{ATP} in skeletal (Quayle *et al.*, 1988) and vascular smooth muscle (Standen *et al.*, 1989). Thus, the relative insensitivity of the acetylcholine-induced $^{86}Rb^+$ efflux in rat aorta to blockade by Ba^{2+} provides additional support for the contention that the channels involved in this response differ from K_{ATP} channels, and from the channels opened by cromakalim in smooth muscle.

To show that the lack of effect of glibenclamide on endothelium-dependent vasodilators was not restricted to the actions of acetylcholine in the rat aorta, similar experiments were carried out with substance P in the pig coronary artery. Like acetylcholine in aorta, substance P produced a concentration- and endothelium-dependent increase in $^{42}K^+$ and $^{86}Rb^+$ efflux and relaxation of $PGF_{2\alpha}$-induced contractions. These responses were completely unaffected by glibenclamide suggesting that the relaxant response and $^{42}K^+$ and $^{86}Rb^+$-efflux produced by substance P were not associated with the activation of glibenclamide-sensitive K^+-channels.

In conclusion, in the present experiments clear differences in the profiles of action of cromakalim and acetylcholine in rat aorta, were observed. While both agents opened K^+-channels permeable to both $^{86}Rb^+$ and $^{42}K^+$, only cromakalim opened K^+-channels which were inhibited by glibenclamide. A similar pattern emerged in pig coronary artery where substance P increased the efflux of $^{86}Rb^+/^{42}K^+$ an effect which was also unaffected by glibenclamide. In both tissues, the effects of cromakalim were inhibited by glibenclamide in a concentration-dependent manner. These results together with the differential sensitivity of the stimulated $^{86}Rb^+$ effluxes in rat aorta to TEA and Ba^{2+} strongly suggest that the K^+-channel opened by endothelium-dependent vasodilators such as acetylcholine and substance P is distinct from that opened by cromakalim, at least in the two large vessels studied here.

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(Received August 24, 1990
Revised October 18, 1990
Accepted October 19, 1990)