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The action of diazoxide and minoxidil sulphate on rat blood vessels: a comparison with cromakalim

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1 The actions of diazoxide and minoxidil sulphate have been compared with those of cromakalim in rat aorta and portal vein.

2 Diazoxide and minoxidil sulphate hyperpolarized the rat portal vein in a similar manner to cromakalim.

3 Cromakalim, diazoxide and minoxidil sulphate increased ^{42}K and ^{86}Rb efflux from rat portal vein, although minoxidil sulphate had only a small effect on ^{86}Rb efflux.

4 Cromakalim, diazoxide and minoxidil sulphate increased ^{42}K efflux from rat aorta but only cromakalim and diazoxide increased ^{86}Rb efflux from this tissue.

5 Glibenclamide inhibited the relaxant actions of cromakalim, diazoxide and minoxidil sulphate on rat aorta and the increase in ^{42}K efflux produced by these agents in this tissue.

6 Diazoxide relaxed an 80 mM KCl-induced contraction of rat aorta, whilst cromakalim and minoxidil sulphate were without effect.

7 Cromakalim, diazoxide and minoxidil sulphate had no effect on cyclic AMP or cyclic GMP concentrations in rat aorta.

8 It is concluded that diazoxide and minoxidil sulphate like cromakalim exhibit K^+ channel opening properties in vascular smooth muscle. Diazoxide exerts an additional inhibitory action not related to the production of cyclic AMP or cyclic GMP. The action of minoxidil sulphate may be primarily located at a K^+ channel which is relatively impermeable to ^{86}Rb.

Introduction

Diazoxide is a sulphonamide derivative with antihypertensive properties (Rubin *et al.*, 1962). In addition to its ability to lower blood pressure, diazoxide produces hyperglycaemia, an action associated with decreased insulin secretion from the pancreas (Loubatières *et al.*, 1986). Subsequently, Henquin & Meissner (1982) showed that diazoxide hyperpolarizes mouse pancreatic β cells and increases ^{86}Rb efflux from rat perfused pancreatic islets. These effects are now known to be associated with the opening of a K^+ channel which is modulated by changes in intracellular ATP concentrations (Trube *et al.*, 1986). Minoxidil sulphate is the active metabolite of the antihypertensive drug minoxidil (Johnson *et al.*, 1982). This agent relaxes rabbit isolated mesenteric artery, an action which is inhibited by the K^+ channel blocker tetraethylammonium and is associated with an increase in ^{42}K efflux. From these data, it has been concluded that the inhibitory effects of minoxidil sulphate are produced by the opening of membrane K^+ (Meisheri *et al.*, 1988).

The aim of the present study was to investigate further the mechanism of action of diazoxide and minoxidil sulphate on rat isolated aorta and portal vein. The actions of these agents are compared with those of the benzopyran derivative cromakalim (BRL34915: Ashwood *et al.*, 1986). This potent antihypertensive agent (Buckingham *et al.*, 1986; Buckingham, 1988) opens K^+ channels in several blood vessels and other smooth muscle-containing tissues (Hamilton & Weston, 1989). The consequences of the resulting hyperpolarization are believed to underlie the antihypertensive action of this compound (Hamilton & Weston, 1989). By use of a variety of techniques it was hoped to determine whether the opening of K^+ channels is involved in the relaxant actions of minoxidil sulphate and diazoxide and to detect any similarities or differences between the three antihypertensive agents. Preliminary observations have been presented (Bray *et al.*, 1988).

Methods

Experiments were carried out on portal veins and segments of thoracic aorta isolated from male Sprague-Dawley rats (300–400 g) which were killed by stunning and bleeding. All animals were supplied by the University of Manchester Animal Unit.

Tissue bath experiments

Aorta Each segment of aorta was cut into four rings, approximately 0.5 cm long. Each ring was opened along its longitudinal axis to form a flat sheet and a thread attached via a small bent pin to each longitudinally-cut edge. The endothelium was removed by rubbing the intimal surface with a cotton bud moistened with physiological salt solution (PSS). Strips were mounted for isometric tension recording under a resting tension of 1 g in a tissue bath containing PSS at 37°C, pH 7.4.

After a 75 min equilibration period in PSS, the spasmolytic effects of cromakalim, diazoxide or minoxidil sulphate were evaluated by exposing tissues to KCl (either 20 or 80 mM). When the maximum mechanical effects of these spasmogens had been obtained, the ability of cromakalim, diazoxide or minoxidil sulphate to relax the maintained KCl-induced contraction was investigated by use of a cumulative protocol.

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Where appropriate, tissues were pre-incubated with glibenclamide for 45 min and the relaxant effects of cromakalim, diazoxide or minoxidil sulphate were examined in the continuing presence of glibenclamide. The effect of glibenclamide on the relaxant actions of nifedipine and sodium nitroprusside was also investigated with a similar protocol.

Portal vein Whole portal veins, each approximately 2 cm in length, were mounted for isometric recording under a resting tension of 0.5 g. Mechanical activity was quantified by use of integrators. Veins were equilibrated for 1 h and then exposed to increasing concentrations of cromakalim, diazoxide or minoxidil sulphate, by use of cumulative protocol, until spontaneous mechanical contractions had been abolished.

Determination of cyclic AMP and cyclic GMP levels

These experiments were carried out with segments of rat aorta. The anatomical position of each strip was noted (upper, upper middle, lower middle, lower) before it was assigned, by a balanced design, to the appropriate treatment group. Each tissue was impaled on a needle and equilibrated for 90 min in 80 ml PSS at 37°C, pH 7.4 gassed with 100% O_2. During this time, the PSS was changed once. At the end of this period KCl (20 mM) was added to mimic tissue bath conditions. Cromakalim, diazoxide or minoxidil sulphate in the appropriate concentration was then added and after varying times, the tissues were plunged into liquid nitrogen. Once frozen, the preparations were transferred to individual test tubes containing 1 ml 10% trichloroacetic acid on ice and allowed to thaw. After homogenisation in a Potter glass/glass homogeniser, the homogenate was centrifuged at 3000 *g* for 15 min at 4°C and the supernatant removed for cyclic nucleotide analysis. The remaining precipitate was dissolved in 1 ml 1 M NaOH and a portion was used for protein determination with Pierce Protein Assay Reagent. Bovine serum albumin in 1 M NaOH was used to construct a standard curve.

The supernatant remaining after centrifugation was extracted 4 times with 3 volumes water-saturated ether, the ether phase being discarded each time. Residual ether was removed with nitrogen gas. Samples of the extract were acetylated and assayed for guanosine 3':5'-cyclic monophosphate (cyclic GMP) or adenosine 3':5'-cyclic monophosphate (cyclic AMP) with the appropriate assay kit (NEN).

Measurement of membrane potential

Simultaneous measurement of the electrical and mechanical activity of rat portal vein was carried out as described by Hamilton *et al.* (1986). When recordings had been stable for several minutes tissues were exposed to cromakalim, diazoxide or minoxidil sulphate which were added to the calibrated reservoirs which supplied PSS to the recording chamber at a rate of approximately 2 ml min^{-1}. Microelectrodes of resistance 50–80 MΩ, filled with 3 M KCl were employed and estimates of the magnitude of the resting membrane potential were made by measuring the oscilloscope deflection which occurred following deliberate electrode withdrawal.

^{86}Rb and ^{42}K efflux

Whole portal veins were prepared for use in these experiments. Tissues were attached to a perspex gassing manifold with syringe needles and, after a 10 min equilibration period in PSS at 37°C, were loaded with ^{86}Rb or ^{42}K by incubation with $^{86}RbCl$ (5 μCi ml^{-1} for 90 min) or $^{42}K_2CO_3$ (1.57 μCi ml^{-1} for 180 min), respectively. The efflux of these isotopes from the tissues was assessed by transferring them into 3 ml aliquots of PSS at 2 min intervals. After 8 such 2 min periods, tissues were exposed to PSS alone (controls) or to PSS containing cromakalim, diazoxide or minoxidil sulphate in varying concentrations for the next five collection periods. For the last 4 collection intervals the tubes contained PSS alone. The tissues were blotted and their ^{42}K and ^{86}Rb content was determined, together with that in the collecting tubes, in a manner similar to that described by Smith *et al.* (1986) by gamma counting or β counting with the Cerenkov Method. Endothelium-free segments of rat aorta were prepared and mounted on the gassing manifold as for portal veins. In these experiments ^{42}K and ^{86}Rb efflux were measured simultaneously (dual isotope experiments) using 4 min collection periods with a 20 min exposure to either cromakalim, diazoxide or minoxidil sulphate. The loading solution contained $^{42}K_2CO_3$ (1.57 μCi ml^{-1}) and $^{86}RbCl$ (5 μCi ml^{-1}) and the tissues were incubated in this solution for 3 h. The total radioactivity in the collection tubes was counted in a gamma counter. In some of these experiments, the PSS contained glibenclamide 1 μM throughout the efflux period. The tubes were recounted after 14 half-lives of ^{42}K had elapsed (7 days) and the counts were corrected for ^{86}Rb decay. The values obtained were then subtracted from the counts measured initially to yield the counts due to ^{42}K.

The efflux data were expressed in terms of the rate coefficient (fractional loss of ^{86}Rb and ^{42}K from the tissue standardised for a 1 min period) expressed as a percentage.

Drugs and solutions/statistical analysis

The following substances were used: (±)-cromakalim (BRL34915, Beecham); diazoxide (Glaxo); forskolin (Sigma); glibenclamide (Hoechst); $^{42}K_2CO_3$ (University of Manchester Reactor Facilities, Risley); minoxidil sulphate (see below); nifedipine (Bayer); $^{86}RbCl$ (Amersham); sodium nitroprusside (Sigma). Minoxidil sulphate was prepared as follows: a mixture of 2,4-diamino-6-(1-piperidinyl)pyrimidine-3-oxide (1 g), chlorosulphonic acid (1.11 g, 0.0096 mol) and diisopropylethylamine (2.47 g, 0.191 mol) in chloroform (25 ml) was stirred overnight. The mixture was concentrated and the residue stirred with aqueous sodium bicarbonate, filtered and washed with ether to yield off-white crystals (1.3 g). The crystals were stirred at room temperature with ethanol, filtered and dried to give 1 g minoxidil sulphate as off-white crystals containing 1 mol of ethanol. These were stored at −70°C and a stock solution was prepared freshly each day. The physiological salt solution (PSS) had the following composition (mM): NaCl 129.7, KCl 5.9, $CaCl_2$ 2.54, $MgCl_2$ 1.19, MOPS 10 and glucose 11.1. The solution was gassed with 100% O_2 and adjusted to pH 7.4 at 37°C with NaOH. When KCl was used as a spasmogen, the stated concentration excludes the KCl (5.9 mM) already present in the PSS. When tissues were loaded with ^{42}K, KCl was excluded from the PSS and replaced with $^{42}K_2CO_3$ to give a final concentration of 5.9 mM.

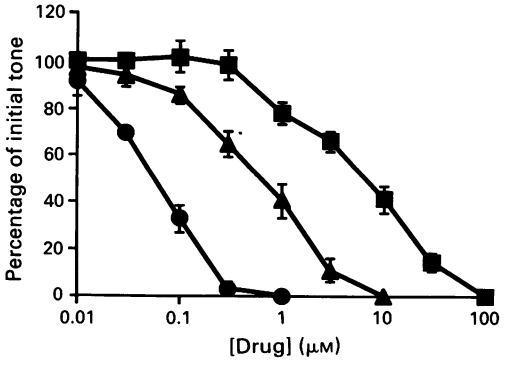

Figure 1 Effect of cumulatively increasing concentrations of cromakalim (●), diazoxide (■) and minoxidil sulphate (▲) on spontaneous mechanical activity of rat portal vein. Points show mean values ($n = 6$) and the vertical bars represent s.e.mean. Ordinate scale: % control integrated mechanical activity in the 2 min period before drug exposure.

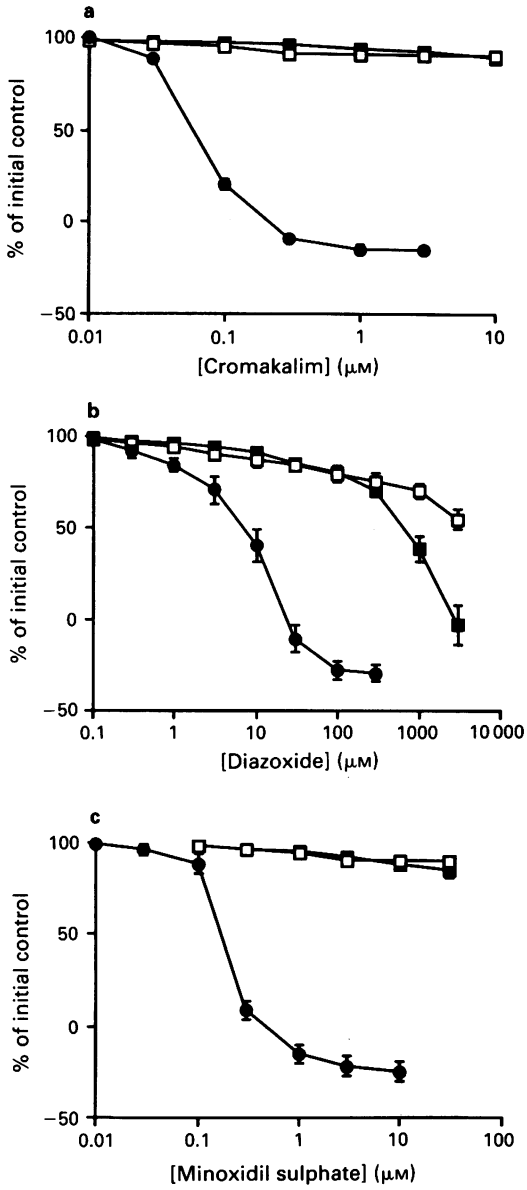

Figure 2 Relaxant effects of cromakalim, diazoxide and minoxidil sulphate against rat aorta precontracted with 20 mM or 80 mM KCl. (a) Cromakalim against 20 mM KCl (●) and 80 mM KCl (■), compared with solvent control (□). (b) Diazoxide against 20 mM KCl (●) and 80 mM KCl (■), compared with solvent control (□). (c) Minoxidil sulphate against 20 mM KCl (●) and 80 mM KCl (■), compared with solvent control (□). The points show mean values ($n = 5$ or 6) and the vertical bars represent s.e.mean. Ordinate scale: percentage of control maximum response to 20 mM or 80 mM KCl as appropriate.

Table 1 Effects of diazoxide, minoxidil sulphate and cromakalim on membrane potential of rat portal vein smooth muscle recorded with intracellular microelectrodes

	Hyperpolarization (mV)
Cromakalim	
1 μM	11.0 ± 1.9 (8) $P \leq 0.01$
10 μM	27.8 ± 2.4 (8) $P \leq 0.01$
Diazoxide	
0.1 mM	2.3 ± 1.3 (4) $P > 0.05$
1 mM	16.0 ± 2.4 (5) $P \leq 0.01$
Minoxidil sulphate	
10 μM	No change (4)
100 μM	6.6 ± 1.0 (5) $P \leq 0.01$

Each value is the mean maximum hyperpolarization ± s.e.mean with the number of observations in parentheses.

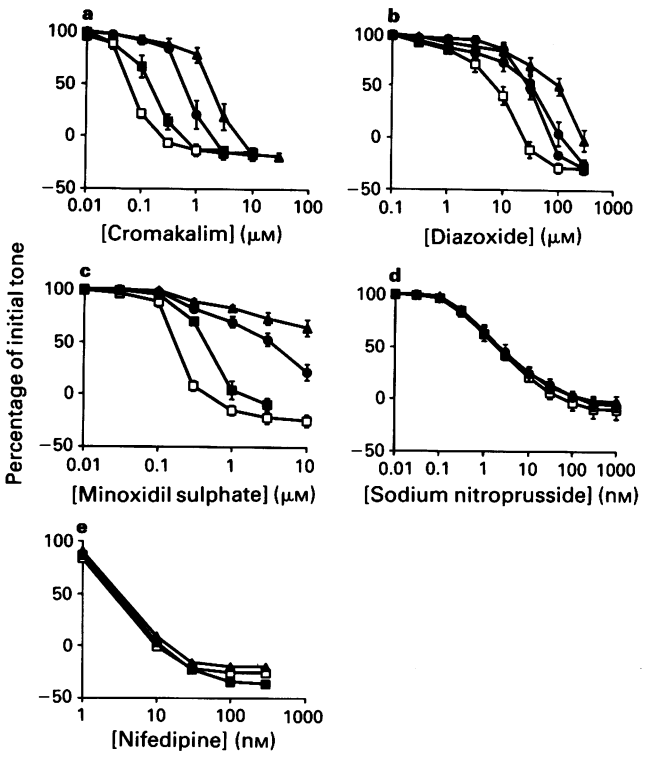

Figure 3 Effect of glibenclamide on the relaxant action of cromakalim, diazoxide, minoxidil sulphate, sodium nitroprusside and nifedipine in rat aorta, precontracted with 20 mM KCl. The effects of glibenclamide 100 nM (■), 300 nM (●) and 1 μM (▲), compared with solvent control (□) on (a) cromakalim, (b) diazoxide, (c) minoxidil sulphate, (d) sodium nitroprusside and (e) nifedipine are shown. The points show mean values ($n = 6$) and the vertical bars represent s.e.mean. Ordinate scale: percentage of control maximum response to 20 mM KCl.

The significance of differences between two means was assessed by a two-tailed Student's t test or analysis of variance, as appropriate.

Results

Tissue bath studies

Rat portal vein Cromakalim (0.01–1 μM), diazoxide (0.5–100 μM) and minoxidil sulphate (0.5–10 μM) each produced complete inhibition of spontaneous activity in the rat portal vein (Figure 1). IC_{50} values for this action were 84 nM, 7.1 μM and 630 nM respectively. Examination of the experimental traces showed that cromakalim, diazoxide and minoxidil sulphate had an identical action on the spontaneous activity of the portal vein. In each case, as the drug concentration was increased, the amplitude of individual tension waves was markedly reduced before any reduction in their frequency was observed.

Rat aorta Cromakalim (0.3–1 μM), diazoxide (0.3–100 μM) and minoxidil sulphate (0.1–3 μM) produced a concentration-dependent relaxation of a 20 mM KCl-induced contraction. The IC_{50} values for this effect were 69 nM, 11 μM and 205 nM, respectively. The time course of the relaxant action of minoxidil sulphate was much slower than that observed with cromakalim or diazoxide.

Diazoxide (0.3–3 mM) produced a concentration-dependent relaxation of an 80 mM KCl-induced contraction, while cromakalim (0.01–10 μM) and minoxidil sulphate (0.1–30 μM) were each without effect (Figure 2).

Glibenclamide (0.1–1 μM) inhibited the ability of cromakalim, diazoxide and minoxidil sulphate to relax a 20 mM KCl-

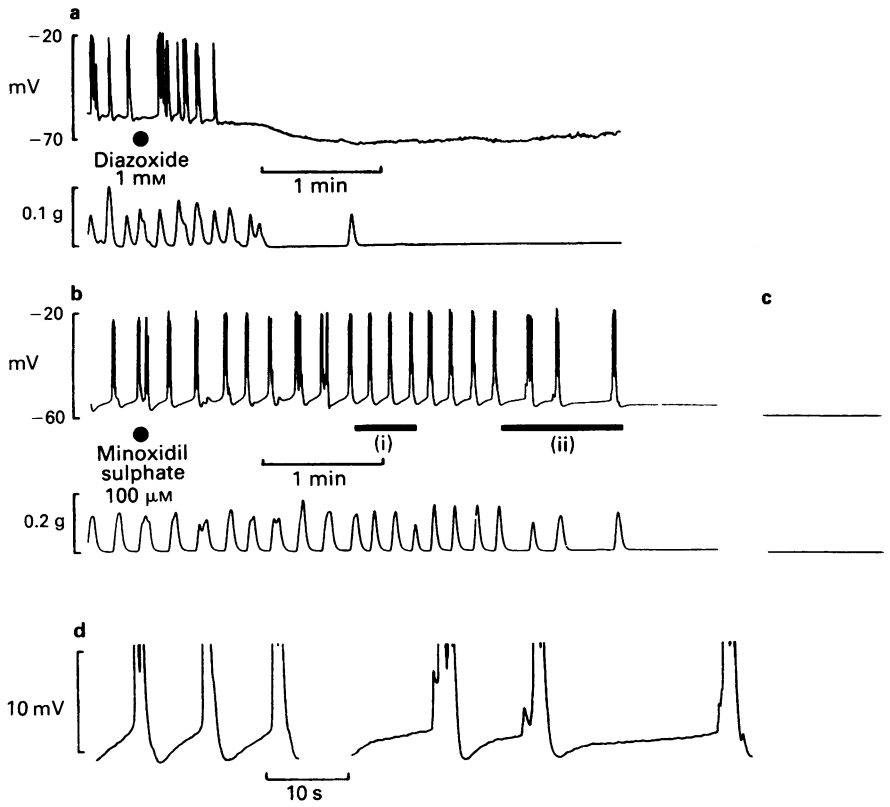

Figure 4 The effects of (a) diazoxide, 1 mM and (b, c) minoxidil sulphate 100 μM on membrane potential (upper trace) and mechanical activity (lower trace), recorded simultaneously in rat portal vein. The filled circles show the points of drug addition. (c) A later, continuous segment of the recording shown in (b) made 10 min after initial exposure to minoxidil sulphate. In (d) segments of the membrane potential trace (continuous lines (i) and (ii)) shown in (b) are presented at greater amplification to illustrate the effects of minoxidil sulphate on pacemaker activity.

induced contraction (Figure 3). At the concentrations of glibenclamide tested, cromakalim and diazoxide appeared to be inhibited in a competitive manner. Schild analysis of the inhibitory action of glibenclamide on cromakalim and diazoxide yielded slopes of 1.21 ± 0.14 and 0.92 ± 0.21 and pA_2 values of 7.2 in both cases. In contrast, minoxidil sulphate was inhibited by glibenclamide in an apparently non-competitive manner. Glibenclamide (0.3–1 μM) had no inhibitory action against nifedipine or sodium nitroprusside (SNP)-induced relaxation of a 20 mM KCl-induced contraction.

Intracellular microelectrode studies

The effects of cromakalim, diazoxide and minoxidil sulphate on membrane potential of rat portal vein are summarized in Table 1. The resting membrane potential of rat portal vein was −56.0 ± 1.4 mV ($n = 16$). Diazoxide (0.1 and 1 mM) produced complete inhibition of spontaneous activity and hyperpolarized the membrane by 2.3 ± 1.3 mV ($n = 4$) and 16.0 ± 2.4 mV ($n = 5$). Diazoxide abolished the spontaneous electrical spiking which is characteristic of electrical activity in rat portal vein within 3 min and produced maximum hyperpolarisation in 5–6 min. Minoxidil sulphate (10 μM) abolished spontaneous electrical spiking without detectable hyperpolarization. The larger (100 μM) concentration of minoxidil sulphate used abolished spontaneous electrical spiking and hyperpolarized the membrane potential by 6.6 ± 1 mV ($n = 5$). At this concentration, minoxidil sulphate took up to 5 min to abolish spontaneous spiking and 10–12 min to produce maximum hyperpolarization. Representative tracings of the effects of diazoxide and minoxidil sulphate on membrane potential and mechanical activity are shown in Figure 4. Cromakalim (1 and 10 mM) abolished spontaneous electrical spiking within 1–2 min and hyperpolarized the membrane potential by 11.0 ± 1.9 mV ($n = 8$) and 27.8 ± 2.4 mV ($n = 8$) respectively within 8 min. Both the electrical and mechanical effects of cromakalim and diazoxide were reversible on washout whereas these effects of minoxidil sulphate were very difficult to reverse.

The effects of minoxidil sulphate (100 μM) on a smooth muscle pacemaker cell in rat portal vein were observed in a single experiment (Figure 4). Minoxidil sulphate slowed the rate of rise of the pacemaker potential, reducing the frequency of the electrical multispike complexes and associated mechanical events. Similar effects were also produced by cromakalim ($n = 3$) and by diazoxide ($n = 1$) in the few experiments in which pacemaker potentials were observed. This action is probably responsible for the decrease in the frequency of spontaneous activity observed with minoxidil sulphate and other K channel opening agents in rat portal vein.

Effects on ^{42}K and ^{86}Rb efflux

Portal vein Cromakalim (1 and 10 μM), diazoxide (0.1 and 1 mM) and minoxidil sulphate (10 and 100 μM) each produced significant increases in ^{42}K efflux (Figure 5). For equivalent relaxant concentrations, the order of potency for this effect was cromakalim > diazoxide > minoxidil sulphate. Cromakalim (1 and 10 μM) and diazoxide (0.1 and 1 mM) each produced large increases in ^{86}Rb efflux, whereas minoxidil sulphate (10 and 100 μM) produced only a small but significant increase (Figure 6). The order of potency for increasing ^{86}Rb efflux was also cromakalim > diazoxide > minoxidil sulphate for equivalent relaxant concentrations. Comparison of the changes in ^{42}K efflux and ^{86}Rb efflux showed that cromakalim, diazoxide and minoxidil sulphate produced larger increases in ^{42}K efflux relative to ^{86}Rb efflux at a given drug concentration.

Rat aorta The effects of cromakalim (1 and 10 μM), diazoxide (0.1 and 1 mM) and minoxidil sulphate (3 and 30 μM) on ^{42}K

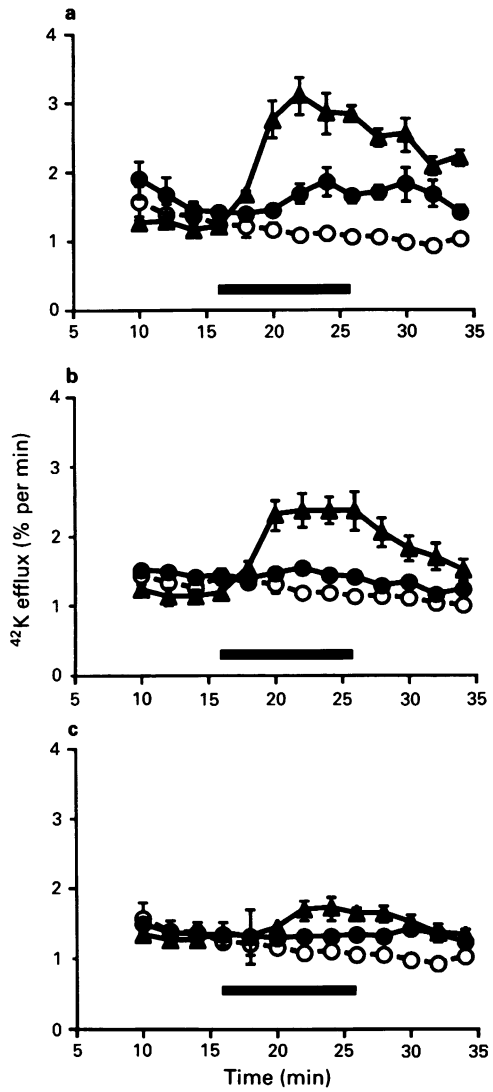

Figure 5 Effects of cromakalim, diazoxide and minoxidil sulphate on the efflux of ^{42}K from rat portal vein. (a) Effect of cromakalim 1 μM (●) and 10 μM (▲). (b) Effect of diazoxide 100 μM (●) and 1 mM (▲). (c) Effect of minoxidil sulphate 10 μM (●) and 100 μM (▲). In each case the basal ^{42}K efflux is represented by the broken line (○). The points signify mean values (n = 5) and the vertical lines represent s.e.mean. The horizontal bar indicates the period of drug or solvent exposure.

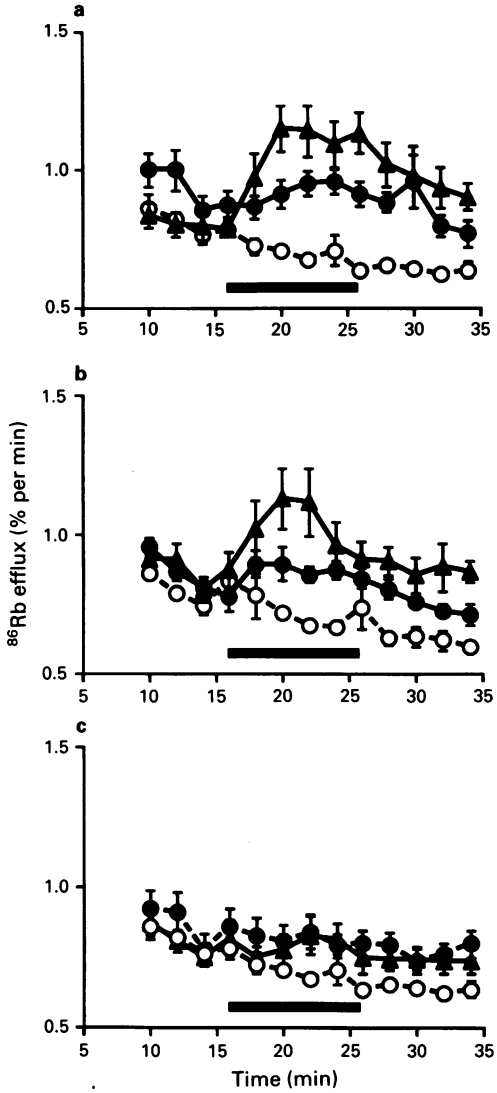

Figure 6 Effects of cromakalim, diazoxide and minoxidil sulphate on the efflux of ^{86}Rb from rat portal vein. (a) Effect of cromakalim 1 μM (●) and 10 μM (▲). (b) Effect of diazoxide 100 μM (●) and 1 mM (▲). (c) Effect of minoxidil sulphate 10 μM (●) and 100 μM (▲). In each case the basal ^{86}Rb efflux is represented by the broken line (○). The points signify mean values (n = 5) and the vertical lines represent s.e.mean. The horizontal bar indicates the period of drug or solvent exposure.

and ^{86}Rb efflux were investigated. Cromakalim (1 and 10 μM), diazoxide (0.1 and 1 mM) and minoxidil sulphate (3 and 30 μM) each increased ^{42}K efflux from rat aorta (Figure 7). The order of potency for this action was cromakalim > diazoxide > minoxidil sulphate. These effects were antagonised by glibenclamide (1 μM) which itself had no effect on basal ^{42}K efflux (Figure 8). Cromakalim (1 and 10 μM) and diazoxide (1 mM) each produced a significant increase in ^{86}Rb

Table 2 Effects of various relaxants on cyclic AMP and cyclic GMP concentrations in rat aorta

	Cyclic AMP (pmol mg^{-1} protein)	*Cyclic GMP* (pmol mg^{-1} protein)
Control	7.95 ± 1	0.058 ± 0.02
Cromakalim		
1 μM	5.3 ± 0.49	0.039 ± 0.01
10 μM	6.2 ± 0.46	0.049 ± 0.005
Diazoxide		
100 μM	8.8 ± 0.9	0.069 ± 0.018
1 mM	7.0 ± 1.4	0.13 ± 0.05
Minoxidil sulphate		
3 μM	10.5 ± 1.6	0.037 ± 0.016
30 μM	9.6 ± 2.6	0.039 ± 0.001
Sodium nitroprusside		
1 μM	Not determined	42.7 ± 6.1 $P < 0.01$
Forskolin		
1 μM	15.6 ± 2.2 $P < 0.05$	Not determined

Each value is the mean derived from 5 observations ± s.e.mean.

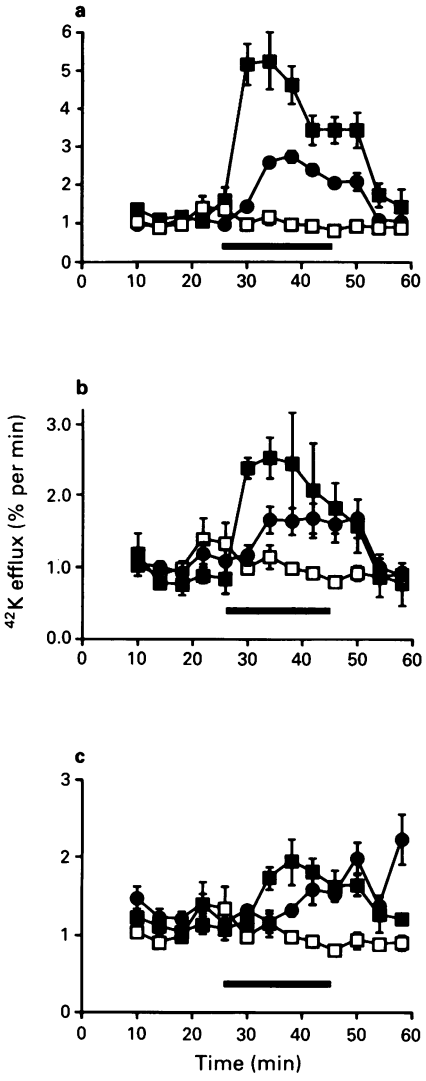

Figure 7 Effects of cromakalim, diazoxide and minoxidil sulphate on ^{42}K efflux from rat aorta. (a) Effect of cromakalim 1 μM (●) and 10 μM (■). (b) Effect of diazoxide 100 μM (●) and 1 mM (■). (c) Effect of minoxidil sulphate 3 μM (●) and 30 μM (■). In each case the basal ^{42}K efflux is represented by (□). The points signify mean values (n = 5) and the vertical lines represent the s.e.mean. The horizontal bar indicates the period of drug or solvent exposure.

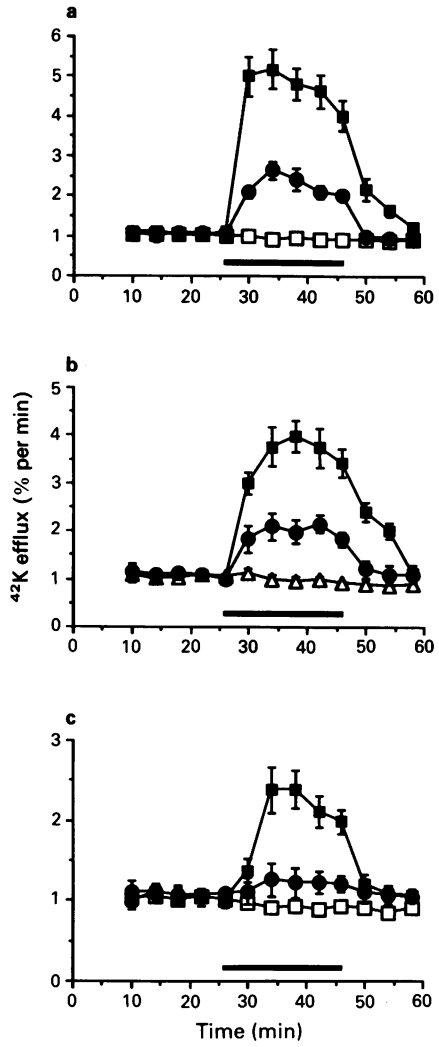

Figure 8 Effect of (a) cromakalim 10 μM, (b) diazoxide 1 mM and (c) minoxidil sulphate 30 μM on ^{42}K efflux from rat aorta in normal PSS (■) and in PSS containing glibenclamide 1 μM (●). In each case, basal ^{42}K efflux is represented by the open symbols. The points signify mean values (n = 5) and the vertical lines represent s.e.mean. The horizontal bar indicates the period of drug or solvent exposure.

efflux whereas minoxidil sulphate had no significant effect at either concentration tested (Figure 9). Glibenclamide 1 μM also antagonized the effects of cromakalim, 10 μM and diazoxide, 1 mM on ^{86}Rb exchange (data not shown). Cromakalim and diazoxide produced larger changes in ^{42}K efflux relative to ^{86}Rb efflux at both concentrations tested.

Cyclic nucleotide studies

The effects of IC_{100} and 10 fold IC_{100} concentrations of cromakalim, diazoxide and minoxidil sulphate on cyclic AMP and cyclic GMP levels in 20 mM KCl-contracted segments of rat aorta were examined. Cromakalim (1 and 10 μM), diazoxide (0.1 and 1 mM) and minoxidil sulphate (1 and 10 μM) had no significant effect on intracellular cyclic AMP concentration. Forskolin (1 μM) produced a significant increase in cyclic AMP concentration, indicating the viability of the assay technique. Cromakalim (1 and 10 μM) and minoxidil sulphate (3 and 10 μM) had no effect on intracellular cyclic GMP concentration. Diazoxide produced a small, but non-significant increase in cyclic GMP levels which was negligible compared with that obtained with a maximum relaxant concentration of sodium nitroprusside (1 μM). These effects are summarized in Table 2.

Discussion

Diazoxide and minoxidil sulphate each produced complete inhibition of spontaneous activity in the rat portal vein. The pattern of loss of activity was similar to that produced by cromakalim, used as a standard, and to that previously described for pinacidil (Southerton *et al.*, 1988). The order of potency for this action was cromakalim > minoxidil sulphate > diazoxide, with an approximate potency ratio of 1:10:100 when IC_{50} values were compared.

Diazoxide abolished spontaneous electrical activity and produced a concentration-dependent hyperpolarization of rat portal vein. Minoxidil sulphate exerted similar inhibitory effects on spontaneous electrical discharges, but the degree of hyperpolarization was less than that generated by diazoxide. No previous data on the effects of minoxidil sulphate on membrane potential in whole portal vein have been published, although hyperpolarization of isolated single cells from rabbit portal vein was detected by Leblanc *et al.* (1989). The results with diazoxide are consistent with those of a previous study by Rhodes & Sutter (1971) on rabbit anterior mesenteric vein. Using the sucrose gap method these workers showed that diazoxide had a hyperpolarizing action. Cromakalim also abolished spontaneous spike generation and produced a concentration-dependent hyperpolarization of the portal vein, confirming the results of Hamilton *et al.* (1986).

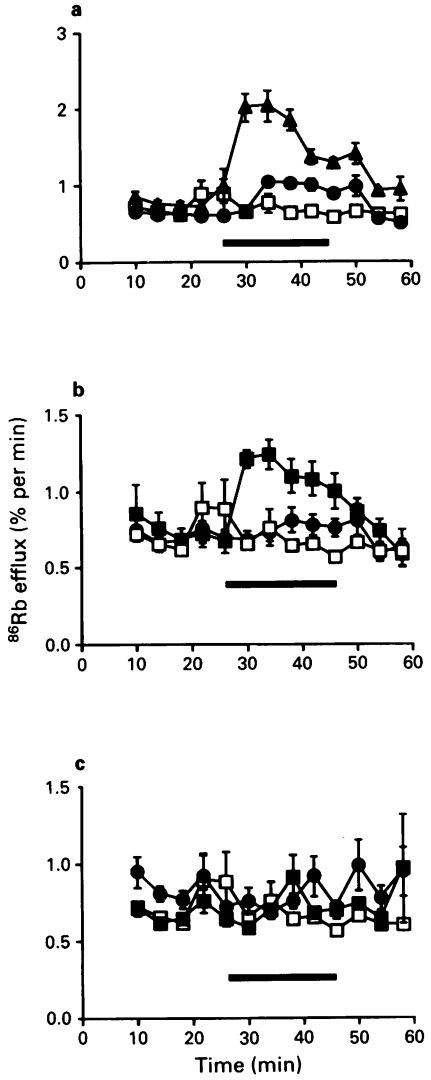

Figure 9 Effects of cromakalim, diazoxide and minoxidil sulphate on ^{86}Rb efflux from rat aorta. (a) Effect of cromakalim 1 μM (●) and 10 μM (▲). (b) Effect of diazoxide 100 μM (●) and 1 mM (■). (c) Effect of minoxidil sulphate 1 μM (●) and 10 μM (■). In each the basal ^{86}Rb efflux is represented by (□). The points show mean values (n = 5) and the vertical lines represent the s.e.mean. The horizontal bar indicates the period of drug or solvent exposure.

In rat portal vein, cromakalim produced the largest hyperpolarization followed by diazoxide, while minoxidil sulphate was the least effective when equivalent relaxant concentrations were compared. Furthermore, at concentrations which abolished spontaneous mechanical activity diazoxide produced only a 2 mV hyperpolarization and minoxidil sulphate had no effect on membrane potential. This observation suggests that diazoxide and minoxidil sulphate may have relaxant actions in rat portal vein in addition to K^+ channel opening. Alternatively, these agents may selectively inhibit pacemaker cells in the rat portal vein. Such a mechanism was originally suggested for cromakalim in this tissue by Hamilton *et al.* (1986) and has been proposed by Quast & Baumlin (1988) to explain discrepancies between the concentrations of cromakalim required to inhibit spontaneous activity and to stimulate ^{42}K efflux in guinea-pig portal vein. In the present study, diazoxide, minoxidil sulphate and cromakalim suppressed pacemaker potentials in rat portal vein before any marked hyperpolarization was observed. Thus these agents may open K^+ channels in pacemaker cells at concentrations lower than those required to hyperpolarize the other smooth muscle cells in the rat portal vein.

Diazoxide, minoxidil sulphate and cromakalim increased ^{42}K and ^{86}Rb efflux from rat portal vein. These results together with observed changes in membrane potential confirm that both diazoxide (Cook *et al.*, 1988) and minoxidil sulphate (Meisheri *et al.*, 1988) exert K^+ channel opening actions in vascular smooth muscle. In each case, the increase in ^{42}K efflux observed was greater than the increase in ^{86}Rb efflux. Quast & Baumlin (1988) showed that cromakalim stimulated quantitatively greater increases in ^{42}K efflux compared to ^{86}Rb efflux in rat aorta and guinea-pig portal vein. Such data support the general conclusion that ^{86}Rb can be used as a ^{42}K substitute, although absolute changes in K^+ efflux will be underestimated due to differences between the physical properties of the two ions (Smith *et al.*, 1986). Similar to the results obtained in rat portal vein, cromakalim and diazoxide produce larger changes in ^{42}K efflux relative to ^{86}Rb efflux from rat aorta. However, in the present study, minoxidil sulphate in contrast to diazoxide and cromakalim had no effect on ^{86}Rb efflux from rat aorta, even at a concentration ten times greater than that required for complete relaxation of a 20 mM KCl-induced contraction. Minoxidil sulphate did, however, increase ^{42}K efflux from rat aorta. Thus in rat aorta minoxidil sulphate may open a K^+ channel which is relatively impermeable to ^{86}Rb. Recently Foster *et al.* (1989) showed that cromakalim stimulated ^{43}K efflux, but not ^{86}Rb efflux, from guinea-pig urinary bladder indicating that a similar situation may exist in this tissue for cromakalim. These observations collectively suggest that ^{86}Rb may not be a totally adequate substitute for ^{42}K in evaluating putative K^+ channel openers.

In rat aortic strips, diazoxide, minoxidil sulphate and cromakalim were each able to relax fully a 20 mM KCl-induced contraction. The order of potency for this effect was the same as for inhibition of spontaneous activity in rat portal vein (cromakalim > minoxidil sulphate > diazoxide) with an approximate potency ratio of 1:3:160. Minoxidil sulphate, like cromakalim was without effect on aortic strips contracted with 80 mM KCl, while diazoxide was capable of producing maximal relaxation of these contractions. The ability of an agonist to relax contractions induced by low, but not high concentrations of extracellular K^+ is an indication of a K^+ channel opening action (Hamilton & Weston, 1989). Effects of other smooth muscle relaxants are inhibited in high K^+ solutions (isoprenaline: Allen *et al.*, 1985; sodium nitroprusside: Ito *et al.*, 1985). These observations are consistent with a relatively selective K^+ channel opening action of minoxidil sulphate and cromakalim, but suggest that diazoxide must exert additional effects. In rabbit pulmonary artery, Thorens & Haeusler (1979) showed that concentrations of diazoxide (300 μM–3 mM) which in the present study relaxed an 80 mM KCl-induced contraction of rat aorta, may exert a calcium entry-blocking action. Thus diazoxide may possess dual K^+ channel opening and calcium entry blocking activity.

The apparently selective K^+-channel opening action of both minoxidil sulphate and cromakalim contrasts with their effects in isolated single cells. In rabbit and rat portal veins under voltage clamp conditions, both minoxidil sulphate (Leblanc *et al.*, 1989) and cromakalim (Okabe *et al.*, 1990) inhibited calcium currents at concentrations which enhanced K^+-currents in these cells. The evidence for such an action on calcium currents in whole tissues is relatively weak and the contribution of this effect to the pharmacological profile of these agents is the subject of further study.

Glibenclamide, the hypoglycaemic sulphonylurea, which blocks ATP-regulated K^+ channels in pancreatic β-cells (Schmid-Antomarchi *et al.*, 1987; Sturgess *et al.*, 1988) inhibits the relaxant effects of cromakalim on rabbit aorta and mesenteric artery (Quast & Cook, 1988; Wilson *et al.*, 1988) and of diazoxide, minoxidil sulphate and cromakalim in rat portal vein (Winquist *et al.*, 1989). In the present study, the relaxation of 20 mM KCl-induced contractions produced by diazoxide, minoxidil sulphate and cromakalim in rat aortic strips was inhibited by glibenclamide with the order of potency; minoxidil sulphate > cromakalim > diazoxide. Schild analysis showed that the effect of glibenclamide on cromakalim and

diazoxide-induced relaxation was apparently competitive, whereas the relaxant action of minoxidil sulphate appeared to be non-competitively inhibited. Glibenclamide also antagonized the increase in ^{42}K efflux produced by cromakalim, diazoxide and minoxidil sulphate in rat aorta. In contrast, glibenclamide had no inhibitory effect on relaxations produced by sodium nitroprusside or by nifedipine, which were used as standards to produce relaxation by activation of guanylate cyclase or by blockade of L-type Ca^{2+}-channels respectively. It would appear therefore that glibenclamide is a relatively selective inhibitor of K^+ channel openers in vascular smooth muscle.

In pancreatic β-cells glibenclamide selectively inhibits the opening of ATP-regulated K^+ channels which can be opened by diazoxide (Trube *et al.*, 1986; Sturgess *et al.*, 1988; Zünkler *et al.*, 1988). The observation in the present study that glibenclamide inhibits the relaxant actions of diazoxide, minoxidil sulphate and cromakalim, coupled with the finding that all three agents open K^+ channels, suggests that the action of these K^+ channel openers may be exerted at a K^+ channel similar to the ATP-regulated K^+ channel in pancreatic β-cells. The *in vivo* hypotensive action of cromakalim in rats is also antagonized by glibenclamide (Buckingham *et al.*, 1989; Cavero *et al.*, 1989), while the hypotensive actions of diazoxide are known to be inhibited by the hypoglycaemic sulphonylurea, tolbutamide (Wales *et al.*, 1967), further supporting this view. High concentrations of cromakalim activate ATP-regulated K^+ channels in guinea-pig ventricular myocytes (Escande *et al.*, 1988; Osterrieder, 1988), an action which is also inhibited by glibenclamide (Escande *et al.*, 1988).

There are conflicting results about the ability of cromakalim to open the pancreatic ATP-regulated K^+ channel. Ashford *et al.* (1988) showed that cromakalim has no opening action against ATP-dependent K^+ channels in a rat pancreatic tumour cell line (CRI-GI). However, Dunne *et al.* (1989, 1990) have recently shown that high concentrations of cromakalim (100–400 μM) do open the ATP-dependent K channel in the RINm5F cell line, although to a much lesser extent than diazoxide. Cromakalim does not produce the marked increase in plasma glucose associated with antihypertensive doses of diazoxide *in vivo* (Cook *et al.*, 1988). Furthermore, the substance galanin which is located in nerves which innervate the pancreas (Dunning *et al.*, 1986) and which activates the pancreatic ATP-dependent K^+ channel (De Weille *et al.*, 1988) does not relax rat aorta or portal vein (Longmore *et al.*, 1989) but has contractile effects in these tissues.

These observations suggest that the ATP-regulated K^+ channel in pancreatic cells is not identical to that opened by the K^+ channel openers in vascular smooth muscle. However, the K^+ channel opened by these agents in smooth muscle may be similar to the ATP-regulated K^+ channel found in cardiac myocytes. Until recently, there was no direct evidence of an ATP-regulated K^+ channel in vascular smooth muscle, although many different voltage- and Ca^{2+}-gated channels have been described (for a review see Cook, 1988). However, evidence of an ATP-regulated K^+ channel in rat mesenteric artery and for an action of cromakalim at this site has recently been obtained (Standen *et al.*, 1989).

Several previous patch clamp studies have demonstrated that cromakalim interacts with a variety of smooth muscle K^+-channel types. Trieschmann *et al.* (1988) demonstrated that diazoxide and cromakalim increased the opening probability of the large conductance Ca^{2+}-dependent K^+ channel in isolated patches from human mesenteric arterial smooth muscle cells. Gelband *et al.* (1988) also showed that cromakalim increased the opening probability of the large conductance Ca^{2+}-dependent K^+ channel from rabbit aorta when isolated into planar lipid bilayers, by decreasing the mean closed time of the channel. In contrast, however, Beech & Bolton (1987) found no effect of cromakalim on the large conductance Ca^{2+}-dependent K^+ channel in isolated smooth muscle cells of rat portal vein by use of the whole cell voltage clamp method. Thus the exact nature of the K^+ channel opened by cromakalim and other K^+ channel openers remains to be resolved.

In the present study, no significant effect of diazoxide, minoxidil sulphate or cromakalim on intracellular cyclic AMP or cyclic GMP concentrations was detected. This suggests that the actions of these substances are independent of changes in these cyclic nucleotides. Previous studies have shown that the relaxant action of cromakalim is independent of changes in cyclic AMP and cyclic GMP in the bovine retractor penis (Gillespie & Sheng, 1988), rabbit mesenteric artery (Coldwell & Howlett, 1988) and rat aorta (Newgreen *et al.*, 1988a,b). The K^+ channel opening drug pinacidil also has no effect on either cyclic AMP or cyclic GMP levels in the rat aorta (Kauffman *et al.*, 1986; Southerton *et al.*, 1988). Conversely, the compound nicorandil has K^+ channel opening properties in rat portal vein (Weir & Weston, 1986) and it increases cyclic GMP concentration in bovine coronary artery (Holzmann, 1983) and rat aorta (Newgreen *et al.*, 1988b). However, these actions are believed to be independent via two separate pathways (Newgreen *et al.*, 1988a,b).

The results of the present study suggest that diazoxide and minoxidil sulphate have K^+ channel opening actions on vascular smooth muscle. However, diazoxide exerts an additional action which is not mediated by changes in cyclic nucleotide concentration. The ion flux experiments in rat aorta suggest that these agents may act on two different K^+ channels, one of which is permeable to Rb and the other relatively impermeable to this ion. The ability of minoxidil sulphate to exert a selective action on a Rb-impermeable channel suggests that the blood pressure lowering effect of minoxidil sulphate (and of cromakalim and diazoxide) may be causally related to this action. Such a conclusion is supported by the results obtained with the K^+ channel opener pinacidil in small rat blood vessels (Videbaek *et al.*, 1988) and is the subject of further investigation.

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