Evidence that pinacidil may promote the opening of ATP-sensitive K⁺ channels yet inhibit the opening of Ca²⁺-activated K⁺ channels in K⁺-contracted canine mesenteric artery

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- 1 The effects of cromakalim and pinacidil on contraction and ⁸⁶Rb efflux were investigated in strips of canine mesenteric artery.
- 2 Cromakalim and pinacidil relaxed arterial strips precontracted with 20.9 mM K⁺ with pD₂ values of 6.56 and 5.88, respectively.
- 3 High (above 10 μM) concentrations of pinacidil, but not cromakalim, relaxed arterial strips bathed by a medium containing 65.9 mM K⁺, and inhibited Ca²⁺-induced contractions in strips bathed by a medium containing 80 mM K⁺. These findings suggested that pinacidil may act as an inhibitor of Ca²⁺ influx.
- 4 In arterial strips preloaded with ⁸⁶Rb, cromakalim and pinacidil increased the basal ⁸⁶Rb efflux.
- 5 When the effects of cromakalim and pinacidil on ⁸⁶Rb efflux were determined in arterial strips contracted with 65.9 mM K⁺, both drugs increased ⁸⁶Rb efflux. The increase in ⁸⁶Rb efflux induced by pinacidil was much smaller than that induced by cromakalim. Under the same conditions, nifedipine decreased ⁸⁶Rb efflux.
- 6 After the addition of nifedipine to arterial strips contracted with 65.9 mM K⁺, pinacidil produced a greater increase in ⁸⁶Rb efflux than in the absence of nifedipine, whereas the effects of cromakalim were the same for the two conditions. Therefore, the effects of pinacidil on ⁸⁶Rb efflux may be the resultant of two opposing effects: an increased ⁸⁶Rb efflux due to the opening of ATP-sensitive K⁺ channels, and a decreased efflux due to the closing of Ca²⁺-activated K⁺ channels.
- 7 In causing relaxation, cromakalim was competitively antagonized by glibenclamide with a pA₂ value of 7.16. However, glibenclamide antagonism of pinacidil was not of the simple competitive type, suggesting that inhibition of Ca²⁺ influx may contribute to the relaxant action of pinacidil.
- 8 It may be concluded that although the ability of pinacidil to increase ⁸⁶Rb efflux via ATP-sensitive K⁺ channel opening was similar to that of cromakalim, the inhibition of Ca²⁺ influx by pinacidil may reduce the opening of Ca²⁺-activated K⁺ channels in K⁺-contracted arterial strips.

Introduction

Pinacidil is a new antihypertensive agent, which may relax vascular smooth muscle (Arrigoni-Martelli & Finucane, 1985; Cohen, 1986; Cohen & Colbert, 1986). The mechanism of action has not yet been fully established, but it has recently been suggested that vascular relaxant responses to pinacidil are associated with the opening of smooth muscle K⁺ channels (Bray *et al.*, 1987; Cook *et al.*, 1988a; Weston *et al.*, 1988; Videbaek *et al.*, 1988a). This mechanism of action was first proposed for the vasodilator effects of cromakalim on the basis that cromakalim enhanced ⁸⁶Rb (or ⁴²K) efflux from preloaded vascular smooth muscle preparations and hyperpolarized the cell membrane (Hamilton *et al.*, 1986; Weir & Weston, 1986; Quast, 1987; Cook *et al.*, 1988b; Quast & Baumlin, 1988). In vascular tissues, cromakalim inhibited contractions elicited by noradrenaline and low (below 30 mM) concentrations of K⁺ but was ineffective against high concentrations of K⁺ (Hamilton *et al.*, 1986; Weir & Weston, 1986). Recent pharmacological studies have demonstrated that glibenclamide, a blocker of adenosine 5'-triphosphate (ATP)-sensitive K⁺ channels, competitively antagonizes the vascular relaxant responses to cromakalim (Winquist *et al.*, 1989; Wilson, 1989; Caverio *et al.*, 1989; Buckingham *et al.*, 1989; Quast & Cook, 1989) and inhibits the cromakalim-induced increase in ⁸⁶Rb efflux from vascular tissues (Quast & Cook, 1989), thus suggesting that ATP-sensitive K⁺ channels are involved in the vasodilator effects of cromakalim. More recently, Standen *et al.* (1989) have demonstrated the presence

of ATP-sensitive K⁺ channels in arterial smooth muscle cells at the single channel level. They have also demonstrated that these channels are opened by cromakalim and inhibited by glibenclamide. These pharmacological and electrophysiological studies provide evidence that cromakalim produces vascular relaxation by opening ATP-sensitive K⁺ channels in vascular smooth muscle cells.

Fewer electrophysiological and ion efflux studies using pinacidil have been performed than for cromakalim. However, in rat portal vein, spontaneous electrical activity was abolished by pinacidil and, in this tissue and in rat mesenteric resistance vessels, a pinacidil-induced increase in ⁸⁶Rb (or ⁴²K) efflux and a marked hyperpolarization was observed (Bray *et al.*, 1987; Cook *et al.*, 1988a; Videbaek *et al.*, 1988a,b). It has been demonstrated that pinacidil, at high (above 10 μM) concentrations, inhibits the arterial contractions produced by high (above 60 mM) concentrations of K⁺ (Kaergaard Nielsen & Arrigoni-Martelli, 1981; Mikkelsen & Lederballe Pedersen, 1982; Cook *et al.*, 1988a; Videbaek *et al.*, 1988b; Hermsmeyer, 1988). Inasmuch as the response of arterial smooth muscle to high concentrations of K⁺ is known to depend on transmembrane Ca²⁺ influx (Bolton, 1979), the inhibition by pinacidil of contractions induced by high concentrations of K⁺ may reflect an action of this drug in inhibiting Ca²⁺ influx. Furthermore, this action of pinacidil will have a great influence on the Ca²⁺-activated K⁺ channels as has been demonstrated for other Ca²⁺ influx inhibitors (Casteels & Droogmans, 1985; Aaronson & Jones, 1985). In contrast with these observations, it was demonstrated that high concentrations (30 and 100 μM) of pinacidil had little or no effect on 80 mM K⁺-induced contractions in rat aorta (Bray *et al.*, 1987;

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Weston *et al.*, 1988). The purpose of the present study was to determine whether pinacidil interacts with both ATP-sensitive and Ca^{2+} -activated K^+ channels in strips of canine mesenteric artery. In the event of pinacidil affecting both types of K^+ channel, it was of further interest to determine the extent of any antagonism by glibenclamide of the responses to pinacidil.

Methods

Preparation of arterial strips for recording mechanical activity

Mongrel dogs of either sex weighing 7–12 kg were anaesthetized with sodium pentobarbitone (30 mg kg^{-1} , i.v.) and exsanguinated. Mesenteric artery, with an *in situ* outside diameter 0.6–0.8 mm, was excised and placed in Krebs solution of the following composition (mM): NaCl 115.0, KCl 4.7, CaCl_2 2.5, MgCl_2 1.2, NaHCO_3 25.0, KH_2PO_4 1.2 and dextrose 10.0. Helical strips (0.8 mm in width and 7 mm in length) of mesenteric artery were prepared as described previously (Asano *et al.*, 1987; 1988). The endothelium of the strip was removed by gently rubbing the endothelial surface with cotton pellets.

Strips were mounted vertically between hooks in water-jacketed baths containing 20 ml Krebs solution. The Krebs solution was maintained at 37°C and was gassed with a mixture of 95% O_2 and 5% CO_2 . The upper end of the strip was connected to a force-displacement transducer (TB-612T, Nihon Kohden Kogyo Co., Tokyo, Japan) for isometric tension recordings. Strips were stretched passively to optical length by imposing a resting tension of 0.8 g. The optimal resting tension was determined by a length-passive tension study (Asano *et al.*, 1987). After application of the resting tension, the strips were equilibrated for 90 min in Krebs solution with replacement of solution every 20 min. This resting tension was maintained throughout the experiments. All the experiments were conducted on phenoxybenzamine-treated strips to eliminate possible α -adrenoceptor responses to nor-adrenaline released by the K^+ -depolarization. To this end, strips were treated with $2 \mu\text{M}$ phenoxybenzamine during the first 60 min of the 90 min equilibration period (Asano *et al.*, 1988).

Relaxant responses to cromakalim and pinacidil

After equilibration, contractile responses of the strips to Krebs solution containing 65.9 mM K^+ (K^+ substitution for Na^+) were repeated two or three times until the responses were reproducible. To determine the relaxant responses to cromakalim, pinacidil and nifedipine, strips were contracted by the addition of 15 mM K^+ to Krebs solution (total K^+ concentration; 20.9 mM) before challenge with these drugs. In some experiments, the relaxant responses were determined in high K^+ (35.9 and 65.9 mM K^+ substitution for Na^+)-contracted strips. Cumulative concentration-response curves for the relaxant responses to these drugs were constructed. At the end of experiments, 0.1 mM papaverine was added to identify the position of the maximum relaxation. Relaxant responses to cromakalim, pinacidil and nifedipine are expressed as % of the papaverine-induced maximum relaxation.

Effects of glibenclamide on the relaxant responses to cromakalim or pinacidil were determined in strips exposed to Krebs solution containing 20.9 mM K^+ . Five strips from the same animal were prepared and subjected to different treatments; test strips were treated with glibenclamide (0.03 , 0.1 , 0.3 or $1 \mu\text{M}$), while control strips were treated with the vehicle for glibenclamide (ethanol). Glibenclamide was added 20 min before the K^+ -induced contraction. A single concentration-response curve for the K^+ channel opening drug was determined on each strip. Each individual concentration-response curve was plotted to obtain EC_{50} values for cromakalim or

pinacidil. The concentration-ratio, i.e., EC_{50} for cromakalim or pinacidil in the presence of glibenclamide divided by the EC_{50} in the control was obtained at varying concentrations of glibenclamide. Then the data were subjected to a Schild plot analysis according to the method of Arunlakshana & Schild (1959) and a pA_2 value for glibenclamide and a slope of the line were determined from the regression analysis.

Effects of cromakalim and pinacidil on Ca^{2+} -induced contraction in K^+ -depolarized strips

Concentration-response curves for Ca^{2+} in K^+ -depolarized strips were constructed as described previously (Asano *et al.*, 1987). In these experiments, strips were washed several times over 90 min with (nominally) Ca^{2+} -free, K^+ -depolarizing solutions (80 mM K^+ substitution for Na^+) and a cumulative concentration-response curve for Ca^{2+} was then constructed. Four strips from the same animal were prepared; one strip was treated with $10 \mu\text{M}$ cromakalim, one with $30 \mu\text{M}$ pinacidil, one with 10 nM nifedipine, while the remaining strip was used as a control.

Drug effects on ^{86}Rb efflux

Arterial strips were mounted vertically on stainless-steel rods and allowed to equilibrate in the Krebs solution for 90 min. The strips were then incubated for an additional 3 h in Krebs solution to which $14\text{--}20 \mu\text{Ci ml}^{-1}$ ^{86}Rb had been added. Each strip was then dipped three times (a total of 15 s) into non-radioactive Krebs solution to remove excess radioactivity, and transferred to a temperature-controlled superfusion chamber similar to that described by Su & Bevan (1970). The upper end of the strip was connected to the force-displacement transducer as in the tension experiments. A resting tension of 2.4 g was applied because larger strips were used. The strips were superfused at a rate of 1 ml min^{-1} with Krebs solution at 37°C . Strips were superfused for the next 30 min before application of the test drugs. The superfusate was sampled by use of a collection period of 2 min and counted for radioactivity in an Aloka autowell gammer counter. The radioactivity remaining in the strip at the end of an efflux sequence was determined by dissolving the strip in 0.5 ml of 1 N nitric acid and the volume of the sample was adjusted to 2.0 ml . The rate constant of ^{86}Rb efflux was then calculated as the radioactivity released from the strip per min at time t divided by the radioactivity remaining in the strip at that time (Imaizumi & Watanabe, 1981; Bolton & Clapp, 1984; Quast, 1987).

Drug effects on ^{86}Rb efflux were generally calculated as the peak value of efflux rate constant obtained in the presence of the drug divided by the basal value of the efflux rate constant averaged over 8–10 min before drug application. However, effects of cromakalim and pinacidil faded during application (see Figure 4) and the area under the curve (AUC) of the efflux rate constant *vs.* time plot was chosen as a better measure of the drug effect. AUCs were calculated from an increase in the efflux rate constant during the 10 min application time (Quast, 1987). In experiments involving either 20.9 or 65.9 mM K^+ , the K^+ -rich medium itself increased ^{86}Rb efflux. In the efflux experiments, the ^{86}Rb efflux rate constant averaged over 8–10 min before drug application was used as a baseline for the drug effect in strips exposed to Krebs solution containing 5.9 and 20.9 mM K^+ . When 65.9 mM K^+ was used, the efflux rate constant rose rapidly and then began to decline (Figure 6). Accordingly, the rate constant just before drug application was used as a baseline in strips exposed to Krebs solution containing 65.9 mM K^+ .

Statistical analysis

When assessing the EC_{50} value, responses to drugs were calculated as % of the maximum response obtained with each drug. The EC_{50} value was obtained from a plot of % response

vs. log concentration of the drug and expressed as a negative log (pD₂ value).

Unless specified, results shown in the text, table and figures are expressed as the mean value $\pm$ s.e.mean (n = number of preparations). Statistical analysis of the data was performed using Student's t test for paired or unpaired data, or by completely randomized design, one-way analysis of variance followed by Newman-Keuls test for a significant F ratio ($P < 0.05$), depending on which test was statistically appropriate. Two groups of data were considered to be significantly different when $P < 0.05$.

Drugs and chemicals

Drugs used were cromakalim (Beecham Pharmaceuticals Research Division), pinacidil (Lilly Research Laboratories), nifedipine (Bayer Yakuhin Ltd.), phenoxybenzamine hydrochloride (Nakarai Chemicals), glibenclamide (Sigma Chemical Co.) and papaverine hydrochloride (Wako Pure Chemical Industries). ⁸⁶RbCl (specific activity initially 1.5–2.9 mCi mg⁻¹) was purchased from Amersham International.

Cromakalim and pinacidil were dissolved in 60% ethanol and 0.01 N HCl, respectively to make a stock solution of 10 mM with further dilution in distilled water before use. Stock solutions of nifedipine (1 mM) and phenoxybenzamine (1 mM) were prepared in 50% ethanol with further dilution in distilled water before use. Glibenclamide was dissolved in 50% ethanol to make a stock solution of 1 mM with further dilution in the same solvent before use. Aqueous stock solutions were prepared for other drugs. Concentrations of drugs are expressed as final molar concentrations in the tissue bath.

Results

Relaxant responses to cromakalim, pinacidil and nifedipine

Relaxant responses to cromakalim and pinacidil were compared in arterial strips exposed to Krebs solution containing 20.9 mM K⁺ (Figure 1). The magnitude of the K⁺-induced contraction was $48.0 \pm 3.6\%$ ($n = 32$) of the maximum contraction induced by 65.9 mM K⁺. The addition of cromakalim or pinacidil in concentrations ranging from 30 nM to 30 μ M caused a concentration-dependent relaxation in these strips (Figure 1). The maximum relaxant response to 10 μ M cromakalim ($59.6 \pm 3.8\%$ of the papaverine-induced maximum relaxation) was significantly ($P < 0.001$) smaller than the response to 30 μ M pinacidil ($91.3 \pm 2.9\%$) (Figures 1 and 2). The pD₂ value for cromakalim (6.56 ± 0.05) was significantly ($P < 0.001$) greater than that for pinacidil (5.88 ± 0.09).

The relaxant responses to cromakalim and pinacidil were also determined in arterial strips exposed to Krebs solution containing 35.9 or 65.9 mM K⁺ (Figure 2). In the presence of these high concentrations of K⁺, relaxant responses to cromakalim and pinacidil were decreased. When the strips were contracted with 65.9 mM K⁺, cromakalim did not cause relaxation (Figure 2a). However, under the same conditions, pinacidil at relatively high (above 10 μ M) concentrations produced a relaxation (Figure 2b).

Nifedipine, a Ca²⁺ influx inhibitor, also caused a concentration-dependent relaxation in strips exposed to Krebs solution containing either 20.9 or 65.9 mM K⁺. The effect of nifedipine seemed independent of the K⁺ concentration (Figure 2c).

Effects on Ca²⁺-induced contractions

Ca²⁺-induced contractions in K⁺-depolarized strips were not altered by 10 μ M cromakalim (Figure 3). On the other hand, 30 μ M pinacidil produced a rightward displacement of the

Figure 1 Canine mesenteric artery: suppression of K⁺-induced contraction by K⁺ channel opening drugs. Note, in (a) and (b), the initial contractile response to K⁺ (65.9 mM). Following washout, contraction was subsequently induced by Krebs solution containing 20.9 mM K⁺. Note that cromakalim (a) and pinacidil (b) each produced concentration-dependent suppression of the contraction. The concentrations of cromakalim and pinacidil are expressed as μ M. Note also the final identification of the position of full relaxation by use of papaverine 0.1 mM (Pap).

concentration-response curve for Ca²⁺ (Figure 3). Nifedipine also antagonized Ca²⁺ (Figure 3).

⁸⁶Rb efflux studies

Exposure of arterial strips to 10 μ M cromakalim increased the ⁸⁶Rb efflux rate constant from the basal efflux of $2.32 \pm 0.22 \times 10^{-3}$ per min (measured between the 20th and 30th min of efflux) to a peak value of $3.66 \pm 0.12 \times 10^{-3}$ per min (Figure 4). The AUC of the increase in the efflux rate constant during the 10 min application time was $5.6 \pm 1.4 \times 10^{-3}$. Pinacidil (30 μ M) also increased ⁸⁶Rb efflux (Figure 4). On the other hand, nifedipine (100 nM) did not change ⁸⁶Rb efflux (Figure 4). The data presented in Table 1 indicate the effects of cromakalim and pinacidil on ⁸⁶Rb efflux as measured in the presence of various concentrations of K⁺.

Figure 2 Canine mesenteric artery precontracted by exposure to Krebs solution containing 20.9 (○), 35.9 (Δ) or 65.9 (▲) mM K⁺: log concentration-response curves for the relaxant actions of cromakalim (a), pinacidil (b) and nifedipine (c). Relaxation induced by 0.1 mM papaverine (Pap) was taken as 100%. Data points are means of values from the number of strips indicated beside each curve. Vertical lines represent s.e.mean.

Figure 3 Effects of cromakalim, pinacidil and nifedipine on Ca^{2+} -induced contractions in K^{+} -depolarized strips of canine mesenteric artery. Cromakalim ($10\text{ }\mu\text{M}$) ($\bullet$), pinacidil ($30\text{ }\mu\text{M}$) ($\blacktriangle$) or nifedipine (10 nM) ($\square$) was added 20 min before the concentration-response curve for Ca^{2+} was constructed. The control concentration-response curve ($\circ$) was constructed in the absence of any drugs. Contractile responses to Ca^{2+} are expressed as % of the contraction induced by 65.9 mM K^{+} which had been evoked in Ca^{2+} -containing Krebs solution. Each point is the mean, and vertical lines represent the s.e.mean of 5 determinations.

At a K^{+} concentration of 5.9 mM , pinacidil (10 and $30\text{ }\mu\text{M}$) caused a concentration-dependent increase in ^{86}Rb efflux. In contrast, cromakalim (3 – $30\text{ }\mu\text{M}$) did not exhibit a clear relationship between concentration and effect.

In order to correlate possible drug-induced alterations in ^{86}Rb efflux with mechanical effects, the effects of cromakalim

Figure 4 Effects of cromakalim, pinacidil and nifedipine on basal ^{86}Rb efflux from resting strips of canine mesenteric artery. Ordinate scale: ^{86}Rb efflux rate constant expressed as 10^{-3} per min. Abscissa scale: time (min) after start of the efflux period. Strips preloaded with ^{86}Rb were exposed to $10\text{ }\mu\text{M}$ cromakalim ($\circ$) ($n = 6$), $30\text{ }\mu\text{M}$ pinacidil ($\bullet$) ($n = 6$) or 100 nM nifedipine (Δ) ($n = 6$) at the 30th min of the efflux period as indicated by the bar. Data points are means and s.e.means (representative values) are shown by vertical lines. The basal ^{86}Rb efflux rate constant obtained in the absence any drugs was constant between the 18th min ($2.67 \pm 0.23 \times 10^{-3}$ per min, $n = 6$) and 68th min ($2.46 \pm 0.18 \times 10^{-3}$ per min, $n = 6$) of the efflux period.

Table 1 Effects of cromakalim and pinacidil on ^{86}Rb efflux from resting and K^{+} -contracted strips of canine mesenteric artery preloaded with ^{86}Rb

Condition ^a	Concentration of drug μM	Drug effect on ^{86}Rb efflux rate constant (AUC) ^b	
		Cromakalim	Pinacidil
$\text{K}^{+} 5.9\text{ mM}$	3	6.2 ± 1.5 (5)	ND
	10	5.6 ± 1.4 (6)	$1.5 \pm 0.5^{*}$ (4)
	30	6.1 ± 1.8 (4)	6.3 ± 1.4 (6)
$\text{K}^{+} 20.9\text{ mM}$	3	5.0 ± 2.5 (4)	ND
	10	9.4 ± 3.0 (6)	$2.1 \pm 0.1^{*}$ (4)
	30	8.9 ± 0.9 (4)	8.8 ± 2.8 (5)
$\text{K}^{+} 65.9\text{ mM}$	10	53.8 ± 7.6 (5)	ND
	30	ND	$18.2 \pm 4.3^{\dagger}$ (5)
$\text{K}^{+} 65.9\text{ mM} + \text{Nif}$	10	53.0 ± 7.3 (5)	ND
	30	ND	32.8 ± 5.8 (4)

^a Drug effects on ^{86}Rb efflux were determined in strips exposed to Krebs solution containing 5.9 (resting), 20.9 , 65.9 mM K^{+} , and strips exposed to 65.9 mM K^{+} plus 100 nM nifedipine (Nif).

^b The drug effects are expressed as AUC of the increase in ^{86}Rb efflux rate constant during the 10 min application time. For details, see Methods.

Figures in parentheses indicate the number of preparations used. Data are expressed as mean $\pm$ s.e.mean. ND, not determined.

^{*} Significantly different from the same concentration of cromakalim ($P < 0.05$).

[†] Significantly different from $10\text{ }\mu\text{M}$ cromakalim ($P < 0.05$).

and pinacidil on ^{86}Rb efflux were compared in strips exposed to Krebs solution containing 20.9 mM K^{+} (Figure 5). The ^{86}Rb efflux was increased by strip exposure to 20.9 mM K^{+} , but the addition of $10\text{ }\mu\text{M}$ cromakalim or $30\text{ }\mu\text{M}$ pinacidil caused a further increase in ^{86}Rb efflux (Figure 5). It should be noted that the concentrations of cromakalim and pinacidil

Figure 5 Effects of cromakalim, pinacidil and nifedipine on ^{86}Rb efflux from strips of canine mesenteric artery exposed to Krebs solution containing 20.9 mM K^{+} . Strips preloaded with ^{86}Rb were exposed to 20.9 mM K^{+} at the 30th min of the efflux period and then exposed to $10\text{ }\mu\text{M}$ cromakalim ($\circ$) ($n = 6$), $30\text{ }\mu\text{M}$ pinacidil ($\bullet$) ($n = 5$) or 100 nM nifedipine (Δ) ($n = 5$) at the 50th min. The ^{86}Rb efflux rate constant in the presence of 20.9 mM K^{+} was constant between the 32nd min and 70th min of the efflux period.

required to cause a significant increase in ⁸⁶Rb efflux in the presence of 20.9 mM K⁺ exceeded their respective EC₅₀ values as regards inducing relaxation (Table 1 and Figure 2). The effect of 10 μ M cromakalim in increasing ⁸⁶Rb efflux was significantly greater than that of 10 μ M pinacidil but was comparable to the effect of 30 μ M pinacidil (Figure 5 and Table 1). In contrast to cromakalim and pinacidil, nifedipine (100 nM) decreased ⁸⁶Rb efflux from strips exposed to Krebs solution containing 20.9 mM K⁺ (Figure 5).

Further comparison of the effects of cromakalim and pinacidil on ⁸⁶Rb efflux was made in strips exposed to Krebs solution containing 65.9 mM K⁺ (Figure 6). Exposure of strips to the K⁺-rich medium greatly increased ⁸⁶Rb efflux. Cromakalim and pinacidil further increased ⁸⁶Rb efflux. However, the effect of 30 μ M pinacidil was significantly smaller than that of 10 μ M cromakalim (Table 1). Nifedipine again decreased ⁸⁶Rb efflux (Figure 6). Compared with their effects in the presence of 5.9 mM K⁺, the effects of cromakalim and pinacidil on ⁸⁶Rb efflux in the presence of 65.9 mM K⁺ were augmented (Table 1). This augmentation was significantly greater in the case of cromakalim (9.6 fold) than for pinacidil (2.9 fold). To determine whether the difference between the effects of cromakalim and pinacidil was due to the relaxant effect of pinacidil, similar experiments were performed in the presence of nifedipine. In these experiments, 100 nM nifedipine was applied before the addition of cromakalim or pinacidil to inactivate the Ca²⁺-activated K⁺ channels via the inhibition of Ca²⁺ influx (Figure 6). In the presence of nifedipine, cromakalim and pinacidil again increased ⁸⁶Rb efflux. The effects of cromakalim were similar in the absence and presence of nifedipine (Table 1). On the other hand, the effect of pinacidil was significantly increased in the presence of nifedipine compared with that observed in the absence of nifedipine (Table 1).

Glibenclamide antagonism of cromakalim and pinacidil in strips exposed to Krebs solution containing 20.9 mM K⁺

Glibenclamide in concentrations ranging from 0.03 to 1 μ M produced a rightward displacement of the concentration-

Figure 6 Effects of cromakalim, pinacidil and nifedipine on ⁸⁶Rb efflux from strips of canine mesenteric artery exposed to Krebs solution containing 65.9 mM K⁺. Strips preloaded with ⁸⁶Rb were exposed to 65.9 mM K⁺ at the 30th min of the efflux period and then exposed to 10 μ M cromakalim (○) (*n* = 5), 30 μ M pinacidil (●) (*n* = 5) or 100 nM nifedipine (△) (*n* = 9) at the 40th min. In the nifedipine (Nif) experiment, the strips were exposed to 10 μ M cromakalim (□) (*n* = 5) or 30 μ M pinacidil (■) (*n* = 4) at the 60th min. The ⁸⁶Rb efflux rate constant in the presence of 65.9 mM K⁺ was constant between the 40th min and 50th min of the efflux period. The ⁸⁶Rb efflux rate constant in the presence of 65.9 mM K⁺ plus 100 nM nifedipine was constant between the 48th min and 70th min of the efflux period.

Figure 7 Effects of glibenclamide on the log concentration-response curves for cromakalim (a) and pinacidil (b) in strips of canine mesenteric artery exposed to Krebs solution containing 20.9 mM K⁺. Glibenclamide in a concentration of 0.03 (●), 0.1 (△), 0.3 (▲) or 1 (□) μ M was added 20 min before the K⁺-induced contraction. Control concentration-response curves (○) were constructed in the presence of 0.05% ethanol, the vehicle for glibenclamide. Relaxation induced by 0.1 mM papaverine (Pap) was taken as 100%. Each point is the mean, and vertical lines represent the s.e.mean of 6 determinations.

response curve for cromakalim (Figure 7a) or pinacidil (Figure 7b). At the concentrations used, glibenclamide did not modify the resting tone or K⁺-induced contractions. The Schild plot for antagonism by glibenclamide of cromakalim gave a regression line with a slope of 1.00 ± 0.15 (mean $\pm$ 95% CL) and a pA₂ value of 7.16 ± 0.07 (Figure 8a). In the case of glibenclamide antagonism of pinacidil, a slope of 0.85 ± 0.21 (mean $\pm$ 95% CL, not significantly different from unity) and a pA₂ value of 7.12 ± 0.08 were obtained (Figure 8b). However, the effects of glibenclamide on the concentration-response curves for pinacidil did not comprise a parallel rightward displacement (Figure 7b). The extent of rightward displacement of the concentration-response curve in the presence of 0.3 μ M glibenclamide calculated at the EC₅₀ (0.59 ± 0.07 log units) was significantly (*P* < 0.05) different from that calculated at the EC₇₅ (0.30 ± 0.04 log units). This difference was also noted in the effect of 1 μ M glibenclamide (1.04 ± 0.12 log units vs. 0.63 ± 0.09 log units) (Figure 7b). When these values at the EC₇₅ were used the Schild plot for glibenclamide antagonism of pinacidil gave a regression line with a slope of 0.79 ± 0.25 (mean $\pm$ 95% CL, not significantly different from unity) and a pA₂ value of 6.60 ± 0.10 (Figure 8b). If the Schild plot was constructed using the values at the EC₂₅, a typical competitive antagonism by glibenclamide of pinacidil was observed, since a regression line with a slope of 1.06 ± 0.18

Figure 8 Schild plots for glibenclamide antagonism of cromakalim (a) or pinacidil (b) in strips of canine mesenteric artery exposed to Krebs solution containing 20.9 mM K⁺. The plots were obtained by use of the concentration-ratios calculated from EC₅₀ (○), EC₇₅ (●) and EC₂₅ (△) values of the concentration-response curves in Figure 7. The lines indicate the regression lines of best fit through 4 (a) or 3 (b) data points from 6 determinations each. Vertical lines represent the s.e.mean of calculated values of log (concentration-ratio - 1) at points corresponding to the glibenclamide concentrations used.

(mean $\pm$ 95% CL) and a pA_2 value of 7.15 ± 0.08 was obtained (Figure 8b). When the values at the EC_{75} for cromakalim were used for the Schild plot, the points on the Schild plot were in good agreement with the former points (Figure 8a).

Discussion

The experiments described in the present study evaluated the effects of two K^+ channel openers, cromakalim and pinacidil, on tension and ^{86}Rb efflux in strips of canine mesenteric artery. Arterial relaxations induced by both cromakalim and pinacidil were associated with an increase in ^{86}Rb efflux. In addition to this K^+ channel opening activity, pinacidil at high (above $10 \mu M$) concentrations behaved like an inhibitor of Ca^{2+} influx. Cromakalim, however, showed no such action even at high concentrations. Evidence was also obtained that an action of pinacidil in inhibiting Ca^{2+} influx might affect the function of Ca^{2+} -activated K^+ channels and the antagonism by glibenclamide.

Both cromakalim and pinacidil produced arterial relaxation against the contraction induced by a low (20.9 mM) concentration of K^+ with an approximately five fold difference in their EC_{50} values. The apparent mechanism of action of cromakalim is to stimulate K^+ efflux, which in turn causes hyperpolarization and subsequent relaxation of arterial smooth muscle (Hamilton *et al.*, 1986; Weir & Weston, 1986; Quast, 1987; Cook *et al.*, 1988b; Quast & Baumlin, 1988). Under the conditions where the arterial strips were contracted with low concentrations of K^+ , the equilibrium potential for K^+ (E_K) would be more negative than the actual membrane potential. On the other hand, cromakalim did not produce the relaxation in strips contracted by 65.9 mM K^+ , presumably because the high K^+ reduced the E_K to a value of membrane potential less negative than that required to close voltage-dependent Ca^{2+} channels. Under these conditions, the cromakalim-induced increase in K^+ efflux would not hyperpolarize arterial smooth muscle sufficiently to inhibit transmembrane Ca^{2+} influx. Such a simple experiment easily allows Ca^{2+} influx inhibitors to be distinguished from vasodilators with K^+ channel opening activity, as demonstrated by Weston and his colleagues (Hamilton *et al.*, 1986; Bray *et al.*, 1987; Weston *et al.*, 1988; Hamilton & Weston, 1989). The present study has demonstrated that pinacidil inhibits Ca^{2+} influx as well as opening K^+ channels. This conclusion is based on the following two observations: (1) in contrast to cromakalim, pinacidil at high concentrations produced relaxation in strips contracted by 65.9 mM K^+ and (2) pinacidil inhibited the Ca^{2+} -induced contractions in strips depolarized by 80 mM K^+ , whereas cromakalim did not. The maximum relaxant response to pinacidil was greater than that to cromakalim. Perhaps the difference is due to the inhibitory action of pinacidil on Ca^{2+} influx. Similar effects of pinacidil on contractions induced by high concentrations of K^+ have been demonstrated in rabbit aorta (Kaergaard Nielsen & Arrigoni-Martelli, 1981; Cook *et al.*, 1988a), rat aorta (Mikkelsen & Lederballe Pedersen, 1982), rat mesenteric and femoral arteries (Videbaek *et al.*, 1988b), rat tail artery (Hermsmeyer, 1988) and human crural vein (Mikkelsen & Lederballe Pedersen, 1982).

^{86}Rb has been used as a marker of K^+ conductance in smooth muscles (Imaizumi & Watanabe, 1981; Bolton & Clapp, 1984; Hamilton *et al.*, 1986; Quast & Baumlin, 1988). In the resting strips, both cromakalim and pinacidil increased the basal ^{86}Rb efflux. The increase in ^{86}Rb efflux induced by $30 \mu M$ pinacidil was comparable to that induced by $10 \mu M$ cromakalim. When the effects of these concentrations of cromakalim and pinacidil were determined in strips contracted by 20.9 mM K^+ , the pinacidil-induced increase in ^{86}Rb efflux was also comparable to the cromakalim-induced increase. Compared with the effect of each drug on the basal ^{86}Rb efflux from the resting strips, the effects of cromakalim and pinacidil were not increased. However, the effects of two drugs on ^{86}Rb

efflux from strips contracted by 65.9 mM K^+ were significantly augmented.

The pinacidil-induced increase in ^{86}Rb efflux from strips contracted by 65.9 mM K^+ was only 34% of that of cromakalim (Table 1). This reduction in the effects of pinacidil on the K^+ -contracted strips may be the net balance of two opposing effects; an increased ^{86}Rb efflux due to the opening of ATP-sensitive K^+ channels, and a decreased efflux through other K^+ channels due to the relaxation of the K^+ -induced contractions. Arterial smooth muscle contraction induced by K^+ is associated with an increased ^{86}Rb efflux, presumably due to an opening of Ca^{2+} -activated K^+ channels (Bolton & Clapp, 1984; Casteels & Droogmans, 1985; Aaronson & Jones, 1985; see also this paper, Figures 5 and 6). On the other hand, the inhibition by drugs of transmembrane Ca^{2+} influx is associated with the closing of Ca^{2+} -activated K^+ channels (Casteels & Droogmans, 1985; Aaronson & Jones, 1985; see also this paper, Figures 5 and 6). In fact, pinacidil produced relaxation in strips contracted by 65.9 mM K^+ , whereas cromakalim did not. Our proposal that pinacidil has two opposing effects on K^+ channel activity is supported by the following observations: (1) the relaxant response to nifedipine was associated with the decreased ^{86}Rb efflux in strips contracted by K^+ and (2) after the inactivation of Ca^{2+} -activated K^+ channels by the addition of nifedipine, pinacidil produced a greater increase in ^{86}Rb efflux than in the absence of nifedipine, whereas the effects of cromakalim were the same for the two conditions. Under conditions where the Ca^{2+} -activated K^+ channels are inactivated, ^{86}Rb efflux due to opening of ATP-sensitive K^+ channel by $30 \mu M$ pinacidil was comparable to that by $10 \mu M$ cromakalim. These observations suggest that, to determine the function of ATP-sensitive K^+ channels, it is necessary to minimize the influence of the pinacidil-induced relaxation on other K^+ channels. To this end, the elevation of K^+ to demonstrate the simultaneous measurement of the effects of K^+ channel opening drugs on tension and ^{86}Rb efflux may not be the most appropriate method of comparing their abilities to open ATP-sensitive K^+ channels.

Glibenclamide is a potent blocker of ATP-sensitive K^+ channels in pancreatic β -cells (Zunkler *et al.*, 1988) and insulin-secreting cells (Schmid-Antomarchi *et al.*, 1987). These channels have also been identified in cardiac cells (Noma, 1983; Escande *et al.*, 1988; Sanguinetti *et al.*, 1988) and in arterial smooth muscle cells (Standen *et al.*, 1989). As described in the Introduction, a large number of pharmacological studies have revealed a competitive antagonism by glibenclamide of cromakalim in vascular tissues (Winqvist *et al.*, 1989; Wilson, 1989; Caverio *et al.*, 1989; Buckingham *et al.*, 1989; Quast & Cook, 1989). In the present study, glibenclamide was also demonstrated to antagonize the relaxant responses to cromakalim and pinacidil. Basically, the glibenclamide antagonism of pinacidil was not significantly different from that of cromakalim. The Schild plots for glibenclamide antagonism of cromakalim and pinacidil showed a competitive antagonism with a slope of unity and a pA_2 value of 7.16 (for cromakalim) and 7.12 (for pinacidil), respectively. These pA_2 values for glibenclamide were in good agreement with the pA_2 values for this substance reported in other studies (Wilson, 1989; Caverio *et al.*, 1989; Quast & Cook, 1989).

However, looking more closely at the effects of glibenclamide on the concentration-response curve for pinacidil, we noted that the extent of the rightward displacement of the concentration-response curve calculated at EC_{75} values was different from that calculated at EC_{50} or EC_{25} values. The Schild plot constructed from EC_{75} values for pinacidil, gave a regression line with a pA_2 value of 6.60, thus suggesting that this antagonism is not of the simple competitive type. Because pinacidil inhibits Ca^{2+} influx at high concentrations, and higher concentrations of pinacidil were necessary to obtain EC_{75} values in the presence of glibenclamide, the Ca^{2+} influx inhibitory action of pinacidil assumed progressively greater importance. Thus, it may be concluded that the ability of pinacidil to inhibit Ca^{2+} influx also contributed to the relax-

ant responses to the higher concentrations of this drug in addition to its K⁺ channel opening activity.

In conclusion, the present study compared the ability of cromakalim and pinacidil to produce relaxation and to increase ⁸⁶Rb efflux via the opening of ATP-sensitive K⁺ channels in strips of canine mesenteric artery. The ability of pinacidil to increase ⁸⁶Rb efflux via the opening of ATP-sensitive K⁺ channels was similar to that of cromakalim. Because of the ability of pinacidil at high concentrations to

inhibit Ca²⁺ influx, this similarity did not extend to the effects of these two drugs on the net ⁸⁶Rb efflux or to antagonism by glibenclamide.

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