Br. J. Pharmacol. (1988), **95**, 103–108

Pinacidil opens K^+-selective channels causing hyperpolarization and relaxation of noradrenaline contractions in rat mesenteric resistance vessels

Lars M. Videbæk, Christian Aalkjær & Michael J. Mulvany

Biophysics Institute, Aarhus University, Aarhus C, Denmark

1 The effects of pinacidil on noradrenaline-induced tone, smooth muscle membrane potential and ^{42}K- and ^{86}Rb-efflux from isolated mesenteric resistance vessels (internal diameter 200 μm) of the rat have been studied.

2 Pinacidil (0.3–10 μM) produced concentration-dependent suppression of noradrenaline-induced tone.

3 Pinacidil (0.3–10 μM) caused concentration-dependent hyperpolarization of the smooth muscle.

4 In rat resistance vessels loaded with ^{42}K, pinacidil (1–10 μM) significantly increased the ^{42}K-efflux rate constant.

5 With the use of ^{86}Rb as a marker for K^+, 1 μM pinacidil did not affect the ^{86}Rb-efflux rate constant, while 10 μM pinacidil transiently increased the ^{86}Rb rate constant.

6 The results indicate that the relaxant action of pinacidil in these vessels is due to the opening of K^+-channels and consequent hyperpolarization. The K^+-channels opened are selective for ^{42}K over ^{86}Rb.

Introduction

Pinacidil ((±)-N-cyano-4-pyridyl-*N*-1,2,2-trimethylpropyl-guanidine monohydrate) is a new antihypertensive vasodilator, probably acting directly on vascular smooth muscle (Arrigoni-Martelli *et al.*, 1980). The cellular mechanism of action has not yet been fully determined, but it has been suggested that pinacidil belongs to a new group of smooth muscle relaxants, the K^+-channel openers (Bray *et al.*, 1987; Southerton *et al.*, 1987). The relaxant properties of these agents are thought to be due to opening of K^+-channels in the smooth muscle cell membrane, thus increasing the K^+-permeability and thereby hyperpolarizing the cell membrane. When the smooth muscle cell membrane is insurmountably depolarized by a high potassium solution, the relaxant effect is abolished or markedly diminished. The hyperpolarization seen with these agents is therefore thought to be the cause of the relaxation. The relaxant effect of pinacidil is also accompanied by a hyperpolarization and, when ^{86}Rb is used as a marker for K^+, efflux experiments suggest an increase in K^+-permeability (Bray *et al.*, 1987; Southerton *et al.*, 1987). There appears, however, to be a discrepancy between the concentrations of pinacidil that cause mechanical relaxation and the higher concentrations needed for an increase in ^{86}Rb-permeability (Cook *et al.*, 1988). This discrepancy could be because the increase in ^{86}Rb-permeability is not directly related to the mechanical and electrical events seen. Alternatively, it could be due to ^{86}Rb not being a good substitute for K^+ in the K^+-channels opened by pinacidil. It was then the purpose of this study to examine these possibilities by using ^{42}K for the efflux experiments.

Methods

Preparation

We used rat resistance vessels (internal diameter ca. 200 μm) isolated from the mesenteric bed of male, 12–15 weeks old normotensive Wistar-Kyoto rats, killed with CO_2 on the day of the experiment.

Solutions

Vessels were dissected, mounted and held relaxed in a physiological salt solution (PSS) of the following composition (mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18,

$MgSO_4$ 1.17, $NaHCO_3$ 25, $CaCl_2$ 2.5, ethylenediaminetetracetic acid (EDTA) 0.026, glucose 5.5. For the loading of ^{42}K, PSS containing ^{42}K (Risø Research Station, Roskilde, Denmark) sp. act. ca. 12 Ci mol^{-1} was used. The solution used for loading in ^{86}Rb-efflux experiments was PSS containing 0.1 mM ^{86}Rb (Risø Research Station), sp. act. ca. 4.0 Ci mol^{-1} ($K^+ + {}^{86}Rb$). For loading, vials containing 50 μl of the loading solution were kept in a container at 37°C through which 5% CO_2 in O_2 saturated with water was circulated. All other solutions were held at 37°C and bubbled with 5% CO_2 in O_2 to give pH 7.4.

Mechanical experiments

Segments of mesenteric resistance vessels (about 2 mm long) were dissected free from surrounding tissue and mounted as a ring preparation on a myograph allowing isometric wall tension measurements (Mulvany & Halpern, 1977). The vessels were threaded on to two stainless-steel wires (diameter 40 μm), which were fastened to a force transducer and a micrometer, respectively and placed in a 10 ml organ bath. After a rest period (30–60 min) the vessels were set to a normalized internal circumference (L_1), estimated to be 0.9 times the internal circumference the vessels would have had *in situ* when relaxed and under a transmural pressure of 100 mmHg, which is where force development has been found to be maximal (Mulvany & Halpern, 1977). The normalized internal diameter (l_1) was taken as L_1/π.

The effect of pinacidil (0.1–10 μM) was examined by inducing tone in the vessels with noradrenaline (5 μM) and when the maximal effect of noradrenaline had been obtained (after 5 min) pinacidil was added cumulatively every 5 min. The relaxations obtained were compared with control measurements made previously on the same vessel, where the same procedure was used, but with the time-matched addition of vehicle. At the end of each 5 min period, immediately before addition of a new concentration of pinacidil, the active wall tension was measured. Active wall tension is wall tension in excess of the wall tension in PSS. Wall tension is measured as wall force divided by the wall length (= twice segment length) (Mulvany & Halpern, 1977). For each vessel an IC_{50} was calculated as the molar concentration of pinacidil which reduced the active wall tension to 50% of active wall tension in the time-matched control of the same vessel.

Membrane potential measurements

In these experiments mesenteric resistance vessel segments were dissected, mounted on a myograph and normalized as in mechanical experiments. Membrane potential was measured by using glass microelectrodes (resistance ca. 100 MΩ when filled with 3 M KCl) as described previously (Mulvany *et al.*, 1982). PSS was continuously circulated through the organ bath (volume 4 ml) at 7 ml min^{-1} by use of a peristaltic pump. A total of 23 cells were impaled, and the effect of pinacidil (0.3–10 μM) on membrane potential in resting vessels was examined, either by adding pinacidil directly into the organ bath (7 measurements) or by changing the circulating PSS with PSS containing pinacidil (22 measurements). No one concentration of pinacidil was examined more than once on an impaled cell, and the maximal hyperpolarization was measured.

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

Segments (3 mm long) of mesenteric resistance vessels (internal diameter ca. 200 μm) were dissected as for mechanical experiments and each threaded on a 40 μm stainless-steel wire, which was then fastened to a stainless-steel handle used for transferring the vessel segments through the different solutions. The vessel segments were then preincubated in PSS for at least 60 min before being loaded with either ^{86}Rb or ^{42}K for 60 min. The isotopes were allowed to efflux from the tissues during 2 min collection periods (except for the first min) in vials containing 1 ml of PSS. After efflux for 7 min, pinacidil was added to the efflux medium for the next 6 min. Finally, the vessel segments were placed in vials containing 1 ml of distilled water. The vials were filled with 4 ml of Quickszint 212 scintillation liquid (Zinsser Analytic) and counted for radioactivity in a liquid scintillation counter (Mark III, model 6880, Searle Analytic Inc.). The results for ^{42}K-efflux were corrected for isotopic decay. Efflux rate constants, determined as fractional loss of isotope per min, were calculated for each 2 min efflux period (Aalkjær & Mulvany, 1983).

Drugs

Stock solutions of pinacidil and noradrenaline were made fresh every day. Pinacidil (Leo Pharmaceutical Products) was dissolved in a small volume of acetic acid and diluted with PSS. The final concentration of acetic acid did not exceed 0.001%. (−)-Noradrenaline hydrochloride (Sigma Chemicals) was dissolved in distilled water.

Statistics

In Results, values are given as mean ± s.e.mean or for the efflux studies as geometric mean with the

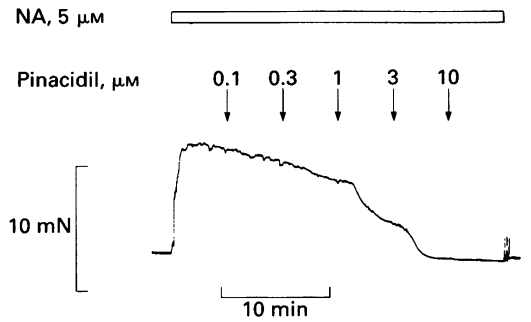

Figure 1 Record showing the relaxing effect of pinacidil on noradrenaline-induced tone in a rat resistance vessel (internal diameter 178 μm, segment length 1.86 mm).

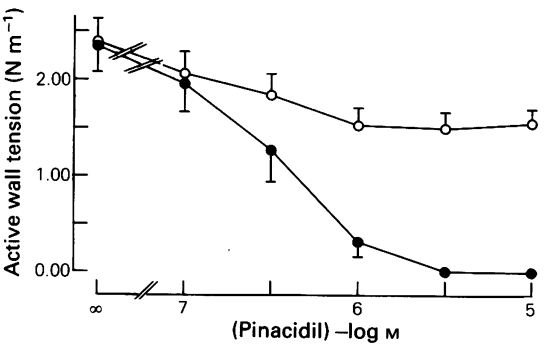

Figure 2 Effect of pinacidil (●) on active wall tension in noradrenaline precontracted rat resistance vessels (internal diameter 216 ± 11 μm, $n = 7$), and vehicle-treated controls of the same vessels (○). Points show means with s.e.mean indicated by vertical bars. Abscissa scale gives negative log of pinacidil concentration.

limits of s.e.mean indicated in parentheses; n = number of vessels or cells. Differences between observed means were tested by the two-tailed t-test. $P < 0.05$ was considered significant.

Results

Mechanical experiments

The mechanical effect of pinacidil was evaluated using a protocol illustrated by the record shown (Figure 1). After inducing a tone in a mesenteric resistance vessel with noradrenaline, pinacidil was added cumulatively. Pinacidil caused a concentration-dependent relaxation of the vessel. Figure 2 shows average results from 7 vessels, where $-\log IC_{50}$ was 6.35 ± 0.16 (IC_{50} = 0.45 μM).

Membrane potential measurements

The resting membrane potential of smooth muscle cells from 11 rat mesenteric resistance vessels (internal diameter 199 ± 7 μm) was −57.1 mV (Table 1). Figure 3 shows the abrupt hyperpolarizing effect of pinacidil, which was maintained during exposure to pinacidil and reversed when pinacidil was removed from the superfusate. The hyperpolarization (13–16 mV) was of approximately the same size with 1 μM, 3 μM and 10 μM pinacidil (Table 1). With 0.3 μM pinacidil, the cell membrane hyperpolarized by about 6 mV.

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

All concentrations of pinacidil (1–10 μM) produced a significant ($P < 0.001$) increase in ^{42}K-efflux rate constant (Figure 4), an increase that, except for 10 μM pinacidil, was maintained at the same level during the 6 min of efflux in pinacidil. Pinacidal 1 μM caused a 45% increase and pinacidil 10 μM caused a 90%

Table 1 Effect of pinacidil on resting membrane potential in rat mesenteric resistance vessel

Concentration of pinacidil (μM)	*Membrane potential* (mV)	*Hyperpolarization* (mV)
0	−57.1 ± 1.2 (23)	
0.3	−61.0 ± 2.7 (5)	6.0 ± 0.8 (5)
1.0	−72.2 ± 3.0 (7)	13.3 ± 2.1 (7)
3.0	−73.3 ± 1.9 (8)	16.8 ± 0.6 (8)
10.0	−75.9 ± 2.3 (9)	16.4 ± 0.9 (9)

Each value is mean ± s.e.mean (number of measurements).

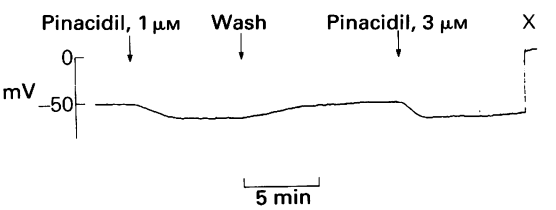

Figure 3 Record showing hyperpolarizing effect of pinacidil on resting membrane potential in a rat resistance vessel (internal diameter 212 μm, resting membrane potential −52 mV). Arrows indicate circulation of pinacidil in given concentrations. At Wash, recirculation of PSS is begun. Microelectrode is withdrawn at X.

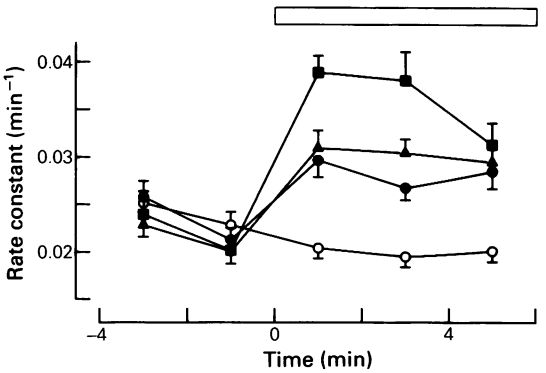

Figure 4 Effect of pinacidil (bar) on ^{42}K-efflux from rat resistance vessels. From time 0 the efflux medium contained pinacidil, 1 μM (●, $n = 27$), 3 μM (▲, $n = 27$) and 10 μM (■, $n = 19$) compared to basal loss of ^{42}K (○, $n = 49$). Points show geometric means with limits of s.e.mean shown by vertical bars.

increase in ^{42}K-efflux rate constant. In contrast, pinacidil at 1 μM, a concentration causing over 80% relaxation of a noradrenaline-induced tone, did not affect the ^{86}Rb-efflux rate constant in the examined mesenteric resistance vessels (Figure 5). Pinacidil 10 μM caused a significant ($P < 0.02$) increase (72%) in the ^{86}Rb-efflux rate constant, an increase that was not maintained.

The basal ^{42}K-efflux rate constant after 13 min of efflux in PSS was 0.0201 min^{-1} (0.0189–0.0213), while the corresponding basal ^{86}Rb rate constant

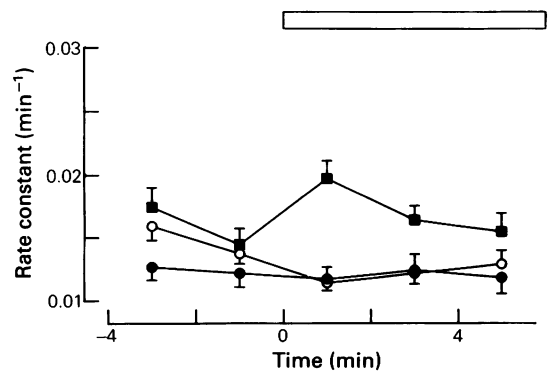

Figure 5 Effect of pinacidil (bar) on ^{86}Rb-efflux from rat resistance vessels. At time 0 the efflux medium contained pinacidil, 1 μM (●, $n = 16$), 10 μM (■, $n = 27$) compared to basal loss of ^{86}Rb (○, $n = 35$). Points show geometric means with limits of s.e.mean shown by vertical bars.

was 0.0129 min^{-1} (0.0119–0.0140). Thus the basal ^{86}Rb/^{42}K rate constant ratio was 0.64.

Discussion

The concentrations of pinacidil necessary for relaxation of the rat (200 μm) mesenteric vessels were similar to the concentrations found previously to be effective *in vitro* (Kauffman *et al.*, 1986; Bray *et al.*, 1987; Southerton *et al.*, 1987) and also in accordance with the plasma concentrations found to have an antihypertensive effect *in vivo* (Carlsen *et al.*, 1981; Eilertsen *et al.*, 1982). Since the vessels we have studied are small enough to contribute to the peripheral resistance (Bohlen, 1986), the results support previous indications that the antihypertensive effect of pinacidil could be due to a direct action on the vascular smooth muscle.

The concentration-dependent hyperpolarizing effect of pinacidil (0.3–1 μM) observed in the present study is consistent with the electrical events first reported in rat portal vein (Bray *et al.*, 1987) and rat aorta (Southerton *et al.*, 1987). Those reports also showed that pinacidil increased the ^{86}Rb-efflux rate constant in the same tissues, ^{86}Rb being used as a marker for K^+. These findings, in addition to the markedly diminished relaxation of K^+-depolarized vessels, led to the suggestion that pinacidil was a K^+-channel opening drug, holding the membrane potential close to the K^+ equilibrium potential, thereby resisting the depolarizing actions of agonists (Bray *et al.*, 1987; Southerton *et al.*, 1987). A difficulty with this hypothesis was, however, that in low concentrations of pinacidil where a marked mechanical effect was seen, it was not possible to detect an increase in ^{86}Rb-efflux rate (Cook *et al.*, 1988).

The present study appears to explain the cause of this discrepancy. As indicated above, pinacidil (1–10 μM) caused a significant increase in ^{42}K-efflux rate constant indicating an increase in K^+-permeability, while no increase in ^{86}Rb-efflux rate constant was seen with 1 μM pinacidil, and 10 μM produced an increase that was not maintained. Therefore it seems that the channels concerned, particularly with 1 μM pinacidil, are highly selective for ^{42}K over ^{86}Rb, thus explaining why we did not see the same effect of pinacidil on ^{86}Rb-efflux. Not only the pinacidil-stimulated but also the basal efflux was underestimated using ^{86}Rb as a tracer for K^+ (^{86}Rb/^{42}K = 0.64). These findings are in accordance with a recent study in rat aorta (Smith *et al.*, 1986) where basal ^{86}Rb-efflux was only 80% of the basal ^{42}K-efflux, a study in which the K^+- and noradrenaline-induced increases in K^+-efflux were also underestimated by ^{86}Rb. This selectivity for K^+ over Rb^+ is a property shared by many of the large

number of well-defined K^+-channels, although the degree of selectivity differs between classes of channels (Latorre & Miller, 1983). Even though the nature of the K^+-channels opened by pinacidil is still unknown (Bray *et al.*, 1987), the relatively high selectivity for ^{42}K over ^{86}Rb displayed by the pinacidil-sensitive K^+-channels, especially with low concentrations of pinacidil, could give a pointer towards the K^+-channels most likely to be involved. Pharmacological experiments with compounds reported to block K^+-channels, will probably also add further knowledge to the channel-opening properties of pinacidil, but since selective blockers for a single class of K^+-channels are very rare, investigations into the precise nature of the channels involved will probably have to make use of patch clamp techniques.

Our findings strongly suggest that the source of the hyperpolarization and the accompanying mechanical relaxation of rat mesenteric resistance vessels is an increase of K^+-permeability. How hyperpolarization and relaxation are related is still, however, unknown. Possible mechanisms are that either the receptor-operated Ca^{2+}-channels are potential sensitive (Mulvany *et al.*, 1982) or that hyperpolarization closes voltage-operated Ca^{2+}-channels.

A parallel to the above mentioned results are findings with another compound, cromakalim (Beecham), which although chemically unrelated to pinacidil, also has the qualities of a K^+-channel opener (Hamilton *et al.*, 1986; Weir & Weston, 1986a,b). In rat portal vein, cromakalim causes relaxation, hyperpolarization and increase in ^{86}Rb-efflux, all in the same concentration-range (Hamilton *et al.*, 1986; Weir & Weston, 1986b). However, in rat aorta, cromakalim did not produce an increase in ^{86}Rb-efflux comparable to that observed in portal vein (Weir & Weston, 1986b). Furthermore, in smooth muscle of the guinea-pig bladder, cromakalim has been found to increase ^{43}K-efflux but not ^{86}Rb-efflux (Foster & Brading, 1987), the K^+-channels opened being selective for ^{43}K over ^{86}Rb. The available evidence thus suggests that both pinacidil and cromakalim may have similar modes of action.

In conclusion, the results indicate that pinacidil increases K^+-permeability by opening K^+-channels that are selective for ^{42}K over ^{86}Rb in the smooth muscle cell membrane of rat mesenteric resistance vessels. This causes a hyperpolarization of the cell membrane and thus a mechanical relaxation. Pinacidil therefore appears to belong to the group of K^+-channel openers, but the nature of the K^+-channels opened by pinacidil is a subject for further investigation, as is the causal relation between hyperpolarization and mechanical relaxation.

This work has been supported by Leo Pharmaceutical Products, Ballerup, Denmark, to whom we express our thanks. We also thank Kirsten Olesen, Tina Frederiksen and Jørgen Andresen for expert technical assistance. L.M.V. is a Danish Medical Research Council scholar.

References

AALKJÆR, C. & MULVANY, M.J. (1983). Sodium metabolism in rat resistance vessels. *J. Physiol.*, **343**, 105–116.

ARRIGONI-MARTELLI, E., KAERGAARD NIELSEN, C., BANG OLSEN, U. & PETERSEN, H.J. (1980). N″-cyano-N-4-pyridyl-N′-1,2,2-trimethylpropylguanidine, monohydrate (P 1134): a new, potent vasodilator. *Experientia*, **36**, 445–447.

BOHLEN, H.G. (1986). Localization of vascular resistance changes during hypertension. *Hypertension*, **8**, 181–183.

BRAY, K.M., NEWGREEN, D.T., SMALL, R.C., SOUTHERTON, J.S., TAYLOR, S.G., WEIR, S.W. & WESTON, A.H. (1987). Evidence that the mechanism of the inhibitory action of pinacidil in rat and guinea-pig smooth muscle differs from that of glyceryl trinitrate. *Br. J. Pharmacol.*, **91**, 421–429.

CARLSEN, J.E., KARDEL, T.K., HILDEN, T., TANGØ, M. & TRAP-JENSEN, J. (1981). Immediate central and peripheral haemodynamic effects of a new vasodilating agent Pinacidil (P1134) in hypertensive man. *Clin. Physiol.*, **1**, 375–384.

COOK, N.S., QUAST, U., HOF, R.P., BAUMLIN, Y. & PALLY, C. (1988). Similarities in the mechanism of action of two new vasodilator drugs: pinacidil and BRL 34915. *J. Cardiovasc. Pharmacol.*, **11**, 90–99.

EILERTSEN, E., HART, J.W., MAGNUSSEN, M.P., SØRENSEN, H. & ARRIGONI-MARTELLI, E. (1982). Pharmacokinetics and distribution of the new antihypertensive agent pinacidil in rat, dog and man. *Xenobiotica*, **12**, 177–185.

FOSTER, C.D. & BRADING, A.F. (1987). The effect of potassium channel antagonists on the BRL 34915 activated potassium channel in guinea-pig bladder. *Br. J. Pharmacol.*, **92**, 751P.

HAMILTON, T.C., WEIR, S.W. & WESTON, A.H. (1986). Comparison of the effects of BRL 34915 and verapamil on electrical and mechanical activity in rat portal vein. *Br. J. Pharmacol.*, **88**, 103–111.

KAUFFMAN, R.F., SCHENCK, K.W., CONERY, B.G. & COHEN, M.L. (1986). Effects of pinacidil on serotonin-induced contractions and cyclic nucleotide levels in isolated rat aortae: comparison with nitroglycerin, minoxidil, and hydralazine. *J. Cardiovasc. Pharmacol.*, **8**, 1195–1200.

LATORRE, R. & MILLER, C. (1983). Conduction and selectivity in potassium channels. *J. Membrane Biol.*, **71**, 11–30.

MULVANY, M.J. & HALPERN, W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ. Res.*, **41**, 19–26.

MULVANY, M.J., NILSSON, H. & FLATMAN, J.A. (1982). Role of membrane potential in the response of rat small mesenteric arteries to exogenous noradrenaline stimulation. *J. Physiol.*, **332**, 363–373.

SMITH, J.M., SANCHEZ, A.A., JONES, A.W. (1986). Comparison of rubidium-86 and potassium-42 fluxes in rat aorta. *Blood Vessels* **23**, 297–309.

SOUTHERTON, J.S., TAYLOR, S.G., WEIR, S.W. & WESTON, A.H. (1987). An investigation into the mechanism of action of pinacidil in rat blood vessels. *Br. J. Pharmacol.*, **90**, 126P.

WEIR, S.W. & WESTON, A.H. (1986a). Effect of apamin on responses to BRL 34915, nicorandil and other relaxants in the guinea-pig taenia caeci. *Br. J. Pharmacol.*, **88**, 113–120.

WEIR, S.W. & WESTON, A.H. (1986b). The effects of BRL 34915 and nicorandil on electrical and mechanical activity and on ^{86}Rb efflux in rat blood vessels. *Br. J. Pharmacol.*, **88**, 121–128.

(Received December 10, 1987
Revised April 14, 1988
Accepted April 27, 1988)