Pilocarpine-induced relaxation of rat tail artery by a non-cholinergic mechanism and in the absence of an intact endothelium

M.A. Tonta, ¹Helena C. Parkington, M. Tare & H.A. Coleman

Department of Physiology, Monash University, Clayton, Victoria 3168, Australia

1 The partial muscarinic agonist, pilocarpine, evoked concentration-dependent relaxation with an EC_{50} of 2.4×10^{-3} M in isolated segments of rat tail artery that were constricted with phenylephrine (10^{-8} to 2×10^{-7} M). Acetylcholine also evoked concentration-dependent relaxation but was more potent than pilocarpine (EC_{50} , 6.5×10^{-7} M).

2 The concentration-relaxation curves for pilocarpine were not affected by the muscarinic antagonists, atropine (10^{-9} M) or pirenzepine (5×10^{-7} M), while the concentration-relaxation curves for acetylcholine-evoked relaxation of the same tissues were shifted some 10 fold to the right by these concentrations of atropine and pirenzepine.

3 Acetylcholine failed to evoke relaxation following removal of the endothelium. The smooth muscle of the rat tail artery was some 10 fold more sensitive to the relaxing action of pilocarpine following denudation of the endothelium.

4 The effects of pilocarpine and acetylcholine on membrane potential were studied in tissues that were depolarized to -39 ± 1 mV with phenylephrine (5×10^{-8} to 2×10^{-7} M). In intact tissues, pilocarpine caused hyperpolarization, an effect that persisted in the presence of muscarinic antagonists. Acetylcholine also evoked hyperpolarization.

5 Following removal of the endothelium, pilocarpine (10^{-5} to 10^{-3} M) evoked hyperpolarization in 6 of 15 preparations and a decrease in the frequency of action potentials in the remainder. Both of these responses were associated with relaxation.

6 The effects of pilocarpine were compared with other agents that evoke endothelium-independent relaxation. The concentration-relaxation curves in response to pilocarpine and nitroprusside were shifted to the right by ferricyanide (10^{-5} M) and methylene blue (10^{-5} M). Glibenclamide (10^{-6} M) was without effect on the hyperpolarization and relaxation evoked by pilocarpine (10^{-4} to 10^{-3} M).

7 Thus, pilocarpine evokes relaxation of rat tail artery independently of the cholinergic system and it is suggested that this is achieved by decreasing the frequency of action potentials in the smooth muscle.

Keywords: Pilocarpine; endothelium; artery; relaxation; hyperpolarization; acetylcholine; nitroprusside; isoprenaline; cromakalim; smooth muscle

Introduction

Muscarinic agonists, such as acetylcholine, act on the endothelial cells of intact arteries to stimulate the release of factors that relax the underlying smooth muscle (Furchgott & Zawadski, 1980; Bolton *et al.*, 1984; Komori & Suzuki, 1987; Chen *et al.*, 1988; Feletou & Vanhoutte, 1988). More than one factor may be released from the endothelium. It is well established that endothelium-derived nitric oxide (NO) (Ignarro *et al.*, 1987; Palmer *et al.*, 1987; Furchgott, 1988) and prostacyclin (Moncada *et al.*, 1977; Lee *et al.*, 1990) relax arterial smooth muscle, and the hyperpolarization evoked by endothelium-derived hyperpolarizing factor (EDHF) (Chen *et al.*, 1988) would be expected to facilitate relaxation. The same type of muscarinic receptor is not necessarily involved in the release of the different endothelium-derived relaxing factors. Thus, activation of M_1 receptors is thought to be involved in the release of EDHF (Komori & Suzuki, 1987; Chen *et al.*, 1988), while M_2 receptors are coupled to the release of NO (Komori & Suzuki, 1987) and prostacyclin (Jaiswal & Malik, 1990).

Stimulation of the muscarinic receptors that are present on vascular smooth muscle cells most often results in contraction (Furchgott & Zawadski, 1980) and this is the predominant response to muscarinic agents in arteries denuded of their endothelium. However, Bolton and colleagues (1984)

have described carbachol-induced relaxation of mesenteric arteries but not aortae, in the absence of endothelium. The type of muscarinic receptor involved was not identified.

The partial muscarinic agonist, pilocarpine, has been used to increase blood flux in the finger in human subjects (Lindblad, L-E., Westerman, R.A. & Wajnbloom, D., personal communication). The drug was applied iontophoretically through the skin during measurement of blood flux with a laser doppler device. The site of action of the pilocarpine was unclear. It is likely that the laser doppler measured blood flux in the superficial blood vessels supplying the skin. Such vessels are likely to be influenced by temperature. In this study we used the tail artery from rats, a vessel that is involved in thermoregulation, to compare the effects of pilocarpine and acetylcholine and to investigate whether or not pilocarpine-induced vasorelaxation depended upon the presence of an intact endothelium.

Methods

Sprague-Dawley rats of either sex were decapitated and ring segments, 1–2 mm in length, of the ventral tail artery were isolated. Each segment was mounted on a myograph which allowed simultaneous recording of membrane potential and tension development (Parkington *et al.*, 1993). The tissues were continuously superfused with physiological saline solu-

¹ Author for correspondence.

tion containing (mM): NaCl 120, KCl 5, CaCl₂ 2.5, KH₂PO₄ 1, MgSO₄ 1.2, NaHCO₃ 25 and glucose 11; at 29°C and gassed with 95% O₂ and 5% CO₂. The endothelium was removed as required (see Results) with a roughened wire. The success of the procedure was verified by the failure of acetylcholine to evoke relaxation of a contraction induced by phenylephrine. The tissues were stretched in increments until the transmural pressure was equivalent to 70–80 mmHg (the mean blood pressure in rats). Intracellular impalements were made from the adventitial surface using microelectrodes filled with 1 M KCl and having resistances of 80–120 MΩ.

For most experiments, acetylcholine, pilocarpine, nitroprusside, cromakalim and isoprenaline were injected for 10 s directly into the perfusion line. With this method the concentration of drug reached steady state at the tissue within 3–5 s (Parkington *et al.*, 1993). In some experiments, pilocarpine was added to the superfusate and was applied to the tissue for 1 min. Atropine, pirenzepine, N^ω-nitro-L-arginine methyl ester (L-NAME), ferricyanide, methylene blue and glibenclamide were added to the superfusate as described in Results.

Glibenclamide and cromakalim were kind gifts from Hoechst and Beecham, respectively. All other drugs were purchased from Sigma Chemicals (U.S.A.).

Active tension was raised in most preparations by adding phenylephrine to the superfusate. The maximal contractile capacity was determined for each tissue at the beginning of the day, using exposure to 10⁻⁵ M phenylephrine for 2 min. The phenylephrine was then washed out and the tissue was allowed to recover for 30 min. For all subsequent procedures, the concentration of phenylephrine in the superfusate was chosen to achieve a contraction that was approximately 30% of maximal. For some experiments (see Results) the thromboxane-mimetic, U46619 (15S)-hydroxy-11α,9α-(epoxymeth-

ano) prosta-5Z,13E-dienoic acid), was used instead of phenylephrine.

Concentration-relaxation curves were obtained by expressing the maximum amplitude of the relaxation as a % of the contraction evoked by phenylephrine. A sigmoid curve was fitted to these data using the least squares method, with the aid of the computer software package InPlot (GraphPad Inc.). The concentration of relaxant which was effective in producing a response that was 50% of maximal (EC₅₀) was determined. The statistic quoted with each mean is the standard error based on the number of animals used. A *t* test was used to test for significant differences between the values of EC₅₀. A significance level of *P* < 0.05 was used throughout.

Results

Effect of pilocarpine and acetylcholine on the contraction evoked by phenylephrine

Phenylephrine, 10⁻⁸ to 2 × 10⁻⁷ M, constricted ring segments of rat tail artery to approximately 30% of maximal. Application of pilocarpine for 10 s caused concentration-dependent relaxation of this contraction (Figure 1). The relaxations evoked by 1 min exposure to lower concentrations of pilocarpine were similar in amplitude to those in response to 10 s application, but were appropriately longer in duration (Figure 2). At concentrations of approximately 3 × 10⁻³ M and above, the amplitude of the relaxation was larger when pilocarpine was applied for 60 s compared with 10 s (Figure 2).

The EC₅₀ for the relaxation in response to 10 s application of pilocarpine was 2.4 × 10⁻³ M (pD₂, 2.62 ± 0.03, *n* = 7). This response was not affected by 10⁻⁹ M of the general

Figure 1 Pilocarpine (Pilo) and acetylcholine (ACh) evoked concentration-dependent relaxation of intact segments of rat tail artery that were constricted with phenylephrine (5 × 10⁻⁸ M). The EC₅₀ for the relaxation evoked in 7 preparations of rat tail artery by pilocarpine (a) was 2.4 × 10⁻³ M (pD₂ 2.62 ± 0.03) and was unaffected by 10⁻⁹ M atropine (EC₅₀, 2.7 × 10⁻³ M; pD₂, 2.57 ± 0.04). The EC₅₀ for acetylcholine-evoked relaxation (b) was 6.5 × 10⁻⁷ M in control (pD₂, 6.19 ± 0.06) and was increased to 4.5 × 10⁻⁶ M by 10⁻⁹ M atropine (pD₂, 5.36 ± 0.08). In both (a) and (b): (●) control; (○) atropine 10⁻⁹ M.

muscarinic antagonist, atropine (EC_{50} , 2.7×10^{-3} M; pD_{20} 2.57 ± 0.04 , $n = 7$) (Figure 1). Pirenzepine, another muscarinic antagonist also failed to block the relaxation induced by pilocarpine. In this series of experiments the EC_{50} for pilocarpine was 4.0×10^{-3} M (pD_2 , 2.40 ± 0.03 , $n = 5$) in control solution and was 4.9×10^{-3} M and 5.4×10^{-3} M in pirenzepine 10^{-7} M and 5×10^{-7} M, respectively (pD_2 , 2.31 ± 0.03 and 2.27 ± 0.05 , $n = 5$) (Figure 3). Acetylcholine also

relaxed segments of rat tail artery. The EC_{50} for this response was 6.5×10^{-7} M (pD_2 , 6.19 ± 0.06 , $n = 7$) in control solution and this was increased to 4.5×10^{-6} M (pD_2 , 5.36 ± 0.08 , $n = 7$) by atropine (10^{-9} M) (Figure 1). In another series of experiments the EC_{50} for acetylcholine-evoked relaxation was increased from 5.2×10^{-7} M (pD_2 , 6.28 ± 0.06 , $n = 5$) in control to 2.3×10^{-6} M (pD_2 , 5.64 ± 0.16 , $n = 5$) in 10^{-7} M pirenzepine and to 6.5×10^{-6} M (pD_2 , 5.18 ± 0.13 , $n = 5$) in 5×10^{-7} M pirenzepine (Figure 3).

Role of the endothelium

The apparent lack of effectiveness of muscarinic antagonists in blocking pilocarpine-evoked relaxation was puzzling, and prompted us to question the role of the endothelium in the response. Accordingly, the effects of pilocarpine and acetylcholine on the contraction evoked by phenylephrine were examined in segments of tail artery from 6 rats before and after removal of the endothelium. Acetylcholine failed to relax denuded segments, even at increased concentrations (Figure 4a). In contrast, pilocarpine continued to evoke relaxation following removal of the endothelium (Figure 4a). The EC_{50} of 1.5×10^{-4} M (pD_2 , 3.82 ± 0.06) for pilocarpine-induced relaxation in 12 denuded preparations (those used as the controls in testing the effects of ferricyanide and methylene blue, see below) was some 10 fold shifted to the left compared with the EC_{50} of 2.4×10^{-3} M (pD_2 , 2.62 ± 0.03) in 7 intact tissues (those used in the studies of the effects of atropine, see above) (Figure 4b).

In view of the resistance of pilocarpine-induced relaxation to both muscarinic antagonists and the integrity of the endothelium, the possibility was considered that the response to pilocarpine might be due to an antagonism of the constriction evoked by phenylephrine. Five tissues, denuded of endothelium, were superfused with 3×10^{-9} or 10^{-8} M of the thromboxane-mimetic, U46619, which produced similar levels of constriction to phenylephrine. Pilocarpine caused relaxation of the constriction evoked by U46619 that was indistinguishable from its effects when phenylephrine was used to constrict (not shown).

Figure 2 (a) An example of the effects of 10^{-3} M pilocarpine, applied for 10 and 60 s, on the contraction evoked by 10^{-7} M phenylephrine. (b) The amplitudes of the relaxation evoked in 3 preparations by concentrations of 10^{-3} M pilocarpine or below were not different whether they were applied at close range for 10 s (●) or included in the superfusate for 60 s (○), although the responses to the latter mode of exposure were of longer duration. The responses evoked by larger concentrations of pilocarpine were larger in amplitude when applied for 60 s than for 10 s.

Figure 3 (a) Pilocarpine (Pilo)-induced relaxation in 5 preparations of rat tail artery (pD_2 , 2.40 ± 0.03) was unaffected by pirenzepine (pD_2 , 2.31 ± 0.03 and 2.27 ± 0.05 in 10^{-7} (○) and 5×10^{-7} M (X) pirenzepine, respectively); (●) control. (b) The relaxation evoked by acetylcholine (ACh) (EC_{50} , 5.2×10^{-7} M; pD_2 , 6.28 ± 0.06) was progressively reduced by pirenzepine at 10^{-7} M (○) (EC_{50} , 2.3×10^{-6} M; pD_2 , 5.64 ± 0.16) and 5×10^{-7} M (X) (EC_{50} , 6.5×10^{-6} M, pD_2 , 5.18 ± 0.13); (●) control.

Figure 4 (a) Shows that both pilocarpine (Pilo) and acetylcholine (ACh) caused relaxation of rat tail artery with endothelium intact and constricted with phenylephrine (10^{-8} M). (b) Acetylcholine failed to relax the same preparation following removal of the endothelium, while pilocarpine continued to be effective in evoking relaxation. (c) In tissues from which the endothelium had been removed (the 12 tissues used to test the effects of ferricyanide (see Figure 8) and methylene blue (Figure 9)), the sensitivity to pilocarpine was significantly increased (EC_{50} , 1.5×10^{-4} M; pD_2 , 3.82 ± 0.06) compared with the relaxation evoked in intact tissues (EC_{50} , 2.40×10^{-3} M; pD_2 , 2.62 ± 0.03) the 7 tissues used when testing atropine, (see Figure 1): (●) with endothelium; (○) without endothelium.

It is now well established that nitric oxide (NO) is an important vasorelaxant produced by the vascular endothelium and the production of NO by vascular smooth muscle cells has also been described more recently (Schini & Vanhoutte, 1991). N^G -nitro-L-arginine methyl ester (L-NAME) competes with L-arginine and reduces the biosynthesis of NO. Segments of intact tail artery from 7 rats were treated with L-NAME (2×10^{-5} M) for 20 min before and during application of pilocarpine. The relaxation evoked by application of 3×10^{-3} to 3×10^{-4} M pilocarpine was resistant to L-NAME. For example, the relaxation evoked by 3×10^{-3} M pilocarpine was $53 \pm 4\%$ in control and $53 \pm 5\%$ in L-NAME.

Vascular endothelium may also release the potent vasodilator prostaglandin, prostacyclin. In order to eliminate production of prostacyclin by either the endothelium or the smooth muscle cells, tissues from 4 rats were incubated with indomethacin (10^{-6} M) 20 min prior to and during application of pilocarpine. The relaxation in response to 3×10^{-3} M pilocarpine in control was $52 \pm 3\%$ and was $53 \pm 4\%$ in indomethacin.

Effects of pilocarpine on membrane potential

In order to gain further insights into the possible mechanisms whereby pilocarpine caused relaxation, membrane potential and tension were measured simultaneously in segments of rat tail artery. The membrane potential recorded from the smooth muscle cells of intact preparations at rest was -68 ± 1 mV ($n = 15$). Apart from spontaneous excitatory junction potentials, which were not associated with tension development, there were no fluctuations in membrane potential, and hence membrane potential was stable. Neither pilocarpine (10^{-4} to 10^{-2} M) nor acetylcholine (10^{-8} to 10^{-6} M) had any effect on membrane potential in 4 of these tissues at rest. The tension developed in preparations of rat tail artery resulting from stretching on the myograph was purely passive, and hence neither pilocarpine nor acetylcholine had an effect on tension at rest.

Inclusion of phenylephrine (5×10^{-8} M to 2×10^{-7} M) in the superfusing solution depolarized the membrane to -39 ± 1 mV ($n = 11$). The depolarization was not stable, and shallow oscillations occurred that consisted of peaks and troughs that were associated with phasic oscillations in tension (Figure 5). Application of pilocarpine to intact segments

Figure 5 (a) Phenylephrine (5×10^{-8} M) depolarized intact segments of rat tail artery to approximately -39 mV. Pilocarpine (Pilo) and acetylcholine (ACh) both evoked repolarization and relaxation. (b) In segments without endothelium the oscillations in membrane potential evoked by phenylephrine were usually topped with slow action potentials of varying amplitudes. Pilocarpine evoked repolarization, reduced the frequency of action potentials and relaxed the tissue following removal of the endothelium. Acetylcholine failed to affect membrane potential or tension in segments denuded of endothelium.

for 10 s resulted in the disappearance of the oscillations in the membrane potential, hyperpolarization of up to 5 mV negative of the level of the troughs, and relaxation (Figure 5). In 3 tissues, hyperpolarization that was 3.7 ± 0.8 mV in control was 3.9 ± 0.6 mV in the presence of atropine. Acetylcholine similarly abolished oscillations and evoked hyperpolarization and relaxation in these tissues (Figure 5).

Removal of the endothelium

Following removal of the endothelium the resting potential was -66 ± 2 mV ($n = 15$). In the presence of phenylephrine, the oscillations in membrane potential became more pronounced with troughs at -43 ± 1 mV and peaks at -40 ± 1 mV ($n = 15$). Slow, stunted action potentials of varying amplitudes usually occurred on the peaks of the oscillations. A phasic increase in tension was associated with each group of action potentials. Relaxation accompanied the brief cessations in action potential activity (Figures 5 and 6).

Pilocarpine abolished the action potentials, evoked hyperpolarization negative to the level of the troughs and produced relaxation (Figure 5). In contrast, acetylcholine never

caused relaxation following removal of the endothelium and action potential activity was uninterrupted (Figure 5) or increased in frequency when the concentration of acetylcholine was increased to 10^{-3} M.

The effects of pilocarpine on membrane potential were difficult to quantify. The reasons can be appreciated from Figure 6, which shows the responses to increasing concentrations of pilocarpine in two endothelium-denuded preparations. At concentrations of less than approximately 10^{-5} M, pilocarpine was without effect on either membrane potential or tension in either of the tissues. At 2.5×10^{-5} M pilocarpine, the frequency of action potentials was decreased and this was associated with relaxation. Hyperpolarization was not observed in either tissue. In the responses illustrated in Figure 6a, higher concentrations of pilocarpine caused hyperpolarization, up to a maximum of 7 mV negative to the level of the troughs, and relaxation. This type of response was observed in 6 out of the 15 tissues studied.

Relaxation also occurred in another 5 of the 15 tissues, although hyperpolarization negative to the level of the troughs was absent (Figure 6b). In these tissues the decrease in tension was associated with a decrease in the frequency of

Figure 6 Examples of the effects of various concentrations of pilocarpine on membrane potential and tension in 2 preparations of rat tail artery denuded of endothelium and depolarized and constricted with phenylephrine (a, 3×10^{-8} M; b, 2.5×10^{-8} M). Pilocarpine (10^{-6} M) had no effect (a and b). At 2.5×10^{-5} M pilocarpine, the frequency of spike action potentials was decreased (a and b). In (a) 1.5×10^{-4} M pilocarpine repolarized the membrane, while this concentration reduced the frequency of action potentials in (b). At 8×10^{-4} M, pilocarpine evoked a larger repolarization in (a) and decreased the frequency of action potentials further in (b).

Figure 7 (a) In a segment of rat tail artery denuded of endothelium and depolarized and constricted with phenylephrine (3×10^{-8} M), pilocarpine (Pilo) and sodium nitroprusside (SNP) evoked repolarization negative to the troughs in the oscillations, cessation of action potentials and relaxation. (b) These responses to both agonists were markedly reduced by potassium ferricyanide.

spikes. In the remaining 4 of the 15 tissues an intermediate few mV of hyperpolarization occurred but the principal response was a decrease in the frequency of action potentials.

Comparison with other vasorelaxants

Since pilocarpine appeared capable of relaxing the rat tail artery in the absence of the endothelium, its effects on membrane potential and tension were compared with other agents that cause direct relaxation of vascular smooth muscle. Thus, in all subsequent experiments the segments of artery were denuded of endothelium, prior to stretching, to ensure that exogenously applied agents did not evoke the release of endogenous vasorelaxants that could contribute to the responses. The denuded preparations were then rested for 1 h before being stretched and continuously superfused with phenylephrine. Phenylephrine evoked oscillations in membrane potential and the peaks of the depolarizations were topped with small, slow action potentials and increased tension as described above. Every tissue was tested with acetylcholine to ensure that no functional endothelium remained.

Nitroprusside

Nitroprusside is thought to release NO and increase guanosine 3':5'-cyclic monophosphate (cyclic GMP) in vascular smooth muscle cells which causes relaxation that is independent of the integrity of the endothelium (Ignarro & Kadowitz, 1985; Waldman & Murad, 1987). In 8 preparations of rat tail artery, denuded of endothelium, pilocarpine

and nitroprusside both caused cessation of the action potentials, hyperpolarization negative to the level of the troughs and relaxation (Figure 7). NO is oxidised by ferricyanide (Murad *et al.*, 1978). The hyperpolarization and relaxations induced by pilocarpine (10^{-4} to 10^{-3} M) and nitroprusside (3×10^{-8} to 10^{-7} M) were markedly attenuated by incubation for 10 min in potassium ferricyanide (10^{-5} M) (Figure 7). In 4 preparations the hyperpolarization evoked by 2.5×10^{-4} M pilocarpine was 4.6 ± 0.5 mV and this was reduced to 1.9 ± 0.4 mV by ferricyanide. In 8 preparations, the EC_{50} for the concentration-relaxation curve for pilocarpine was increased from 1.5×10^{-4} M (pD_2 , 3.82 ± 0.06) in control to 2.3×10^{-3} M (pD_2 , 2.64 ± 0.03) in 10^{-5} M ferricyanide, while the response to nitroprusside was shifted from 3.3×10^{-8} M (pD_2 , 7.48 ± 0.02) to 1×10^{-6} M (pD_2 , 6.00 ± 0.06) by ferricyanide (Figure 8).

Methylene blue

Soluble guanylate cyclase is thought to be blocked by methylene blue (Ignarro & Kadowitz, 1985; Waldman & Murad, 1987) and its effects on the relaxation evoked by pilocarpine and nitroprusside were studied in 4 preparations. Following 30 min incubation in methylene blue (10^{-5} M), the EC_{50} for the relaxation evoked by nitroprusside was significantly increased from 3.3×10^{-8} M (pD_2 , 7.48 ± 0.02) to 1.9×10^{-7} M (pD_2 , 6.73 ± 0.07) (Figure 9). The effect of methylene blue on the relaxation evoked by pilocarpine was small but significant, the EC_{50} was 1.3×10^{-4} M (pD_2 , 3.89 ± 0.06) in control and 2.9×10^{-4} M (pD_2 , 3.54 ± 0.11) in methylene blue (Figure 9).

Figure 8 In 8 preparations of rat tail artery, potassium ferricyanide (10^{-5} M) (O, $\square$) caused a significant shift in the EC_{50} for the relaxation evoked by pilocarpine ($\bullet$, $\circ$) (pD_2 , from 3.82 ± 0.06 in control to 2.64 ± 0.03), and nitroprusside ($\blacksquare$, $\square$) (pD_2 , from 7.48 ± 0.02 in control to 6.00 ± 0.06).

Figure 9 In 4 preparations of rat tail artery methylene blue (10^{-5} M) (O, $\square$) caused a small but significant shift in the pD_2 for the relaxation evoked by pilocarpine ($\bullet$, $\circ$) from 3.89 ± 0.06 to 3.54 ± 0.11 ($P < 0.03$). The pD_2 for nitroprusside ($\blacksquare$, $\square$) was shifted from 7.48 ± 0.02 in control to 6.73 ± 0.07 by methylene blue.

Figure 10 (a) In a segment of rat tail artery denuded of endothelium and depolarized and constricted with phenylephrine (7×10^{-8} M), pilocarpine (Pilo) and cromakalim (Crom) evoked repolarization negative to the troughs of the oscillations, cessation of action potentials and relaxation. (b) These responses to cromakalim were abolished by glibenclamide while the responses to pilocarpine persisted.

Isoprenaline

The β -adrenoceptor agonist, isoprenaline, stimulates cyclic AMP production in vascular smooth muscle and evokes relaxation in the absence of the endothelium. The rat tail artery is well supplied by adrenergic nerves containing noradrenaline. It was considered that the hyperpolarization and relaxation evoked by pilocarpine might result from the release of this noradrenaline. In 4 preparations of rat tail artery, denuded of endothelium, the hyperpolarization and relaxation in response to pilocarpine (10^{-4} to 10^{-3} M) was not affected by the β -adrenoceptor antagonist, propranolol (5×10^{-7} M). Furthermore, the hyperpolarization and relaxation evoked by isoprenaline (10^{-7} to 10^{-6} M) were not affected by ferricyanide (10^{-5} M) (not shown) while the responses to pilocarpine were reduced (see above).

Cromakalim

Cromakalim causes endothelium-independent hyperpolarization of vascular smooth muscle as a result of opening a class of potassium channels (Nakao *et al.*, 1988). This effect is accompanied by relaxation, presumably as a result of a reduction in calcium influx, since hyperpolarization would be expected to decrease the probability of opening of voltage-dependent calcium channels (Hirst & Edwards, 1989; Nelson *et al.*, 1990). In denuded segments of artery from 4 rats, both pilocarpine (10^{-4} to 10^{-3} M) and cromakalim (3×10^{-7} and 10^{-6} M) caused hyperpolarization, cessation of action potentials and relaxation (Figure 10). The responses to cromakalim were prolonged in duration. The 6.3 ± 0.3 mV hyperpolarization evoked by 10^{-6} M cromakalim was reduced to 0 mV by glibenclamide, while the 4.1 ± 0.4 mV hyperpolarization evoked by 2.5×10^{-4} M pilocarpine was unchanged at 4.0 ± 0.5 mV in glibenclamide.

Discussion

The results presented in this study demonstrate that, unlike acetylcholine, the ability of pilocarpine to hyperpolarize the depolarization and relax the constriction evoked by phenylephrine in the smooth muscle of rat tail artery does not depend on the presence of an intact endothelium and does not appear to result from interaction with muscarinic receptors. The ability of pilocarpine to hyperpolarize and relax the depolarization and constriction in response to α -adrenoceptors supports the observations of Bolton *et al.* (1984) in which they described relaxation in response to carbachol in guinea-pig mesenteric artery denuded of endothelium. In that study, the hyperpolarization evoked by carbachol in intact arteries was converted to depolarization following removal of the endothelium when the membrane was depolarized to around -60 mV. Further depolarization of denuded tissues, to -50 mV or more positive (see their Figure 11), restored the ability of carbachol to evoke hyperpolarization. In our study, in which phenylephrine depolarized the membrane to -45 to -35 mV, pilocarpine always evoked hyperpolarization or a decrease in the frequency of action potentials in preparations denuded of endothelium.

When compared with other agents that cause endothelium-independent hyperpolarization and relaxation, the responses to pilocarpine resemble most closely those induced by nitroprusside. Nitroprusside is believed to release NO within vascular smooth muscle (Ignarro & Kadowitz, 1985; Waldman & Murad, 1987). It has recently been demonstrated that arterial smooth muscle cells are capable of synthesizing NO (Schini & Vanhoutte, 1991). The relaxation evoked by pilocarpine was unaffected by agents that block biosynthesis of NO. Thus, it is unlikely that pilocarpine might be inducing

NO production by the smooth muscle. Alternatively, pilocarpine might be releasing NO. The principal endogenous substrate for the biosynthesis of NO is a guanidino nitrogen of L-arginine (Palmer *et al.*, 1988), and production of NO from the imidazole ring of pilocarpine would require a completely different mechanism. If such a mechanism exists, it might be expected that both histidine and histamine could act as sources of NO. We are unaware of any evidence supporting such an expectation.

The available evidence suggests that the NO liberated by nitroprusside stimulates soluble guanylate cyclase within vascular smooth muscle cells to bring about relaxation (Ignarro & Kadowitz, 1985; Waldman & Murad, 1987). Access of pilocarpine to directly stimulate a cytoplasmic cyclase, hence bypassing NO, seems unlikely. However, the cytoplasmic domain of the membrane-bound receptor for atrial-natriuretic peptide is thought to be a guanylate cyclase. Stimulation of this receptor from the extracellular medium leads to an increase in cyclic GMP and relaxation of vascular smooth muscle (Winkvist & Hintze, 1990).

Calcium influx through voltage-operated calcium channels is highly regulated within the range of membrane potential (-50 to -30 mV) evoked by phenylephrine in rat tail artery in this study (Nelson *et al.*, 1990). It is becoming increasingly clear that hyperpolarization of vascular smooth muscles is amongst the important mechanisms of vasodilatation (Standen *et al.*, 1989; Brayden, 1991). Pilocarpine evoked hyperpolarization in only 6 of the 15 preparations examined in this study. In the remaining 9 a decrease in the frequency of action potentials or a brief cessation of action potentials was observed. We have shown previously that the extent of the hyperpolarization induced by acetylcholine and exogenous NO in preparations of uterine artery was dependent upon the extent of the depolarization evoked by phenylephrine. Thus, when the membrane of the uterine artery was depolarized to around -30 mV, that is, more depolarized than the range in which action potentials occurred, acetylcholine and NO evoked limited hyperpolarization. In contrast, these agents evoked concentration-dependent hyperpolarization when the membrane was less depolarized and were very effective in the range in which action potentials were prominent (Tare *et al.*, 1990). In the present study, low concentrations of phenylephrine (10^{-8} – 2×10^{-7} M) rapidly depolarized intact segments of rat tail artery beyond the range of action potentials, while in denuded tissues it was difficult to drive the membrane potential beyond the range in which action potentials occurred. The tissues readily relaxed in the interval between bursts of action potentials evoked by phenylephrine. Pilocarpine appeared to exaggerate this situation by increasing the interval between action potentials or between bursts of action potentials. This is likely to explain the 10 fold increase in the sensitivity of denuded preparations of rat tail artery to the relaxing actions of pilocarpine. An effect of pilocarpine on membrane conductance is likely, although the magnitude or direction of such a change could not be tested in the myograph used in this study.

In conclusion, pilocarpine is capable of evoking relaxation of the smooth muscle of rat tail artery by a mechanism that is independent of muscarinic receptors and of the integrity of the endothelium. This is in stark contrast with the relaxation induced by acetylcholine. Of the agents tested that also induce relaxation that is independent of the endothelium, the actions of pilocarpine resembled those of nitroprusside most closely. The principal mechanism of this action of pilocarpine appears to involve a decrease in the frequency of firing of action potentials and presumably decreased calcium influx.

This study was supported by the National Heart Foundation of Australia. The authors thank Ms D. Clare for preparing the figures.

References

- BOLTON, T.B., LANG, R.J. & TAKEWAKI, T. (1984). Mechanisms of action of noradrenaline and carbachol on smooth muscle of guinea-pig anterior mesenteric artery. *J. Physiol.*, **351**, 549–572.
- BRAYDEN, J.E. (1991). Hyperpolarisation and relaxation of resistance arteries in response to adenosine diphosphate. *Circ. Res.*, **69**, 1415–1420.
- CHEN, G., SUZUKI, H. & WESTON, A.H. (1988). Acetylcholine releases endothelium-derived hyperpolarising factor and EDRF from rat blood vessels. *Br. J. Pharmacol.*, **95**, 1165–1174.
- FELETOU, M. & VANHOUTTE, P.M. (1988). Endothelium-dependent hyperpolarization of canine coronary smooth muscle. *Br. J. Pharmacol.*, **93**, 515–524.
- FURCHGOTT, R.F. (1988). Studies on relaxation of rabbit aorta by sodium nitrite: the basis for the proposal that the acid-activatable inhibitory factor from bovine retractor penis is inorganic nitrite and the endothelium-derived relaxing factor is nitric oxide. In *Vasodilatation: Vascular Smooth Muscle, Peptides, Autonomic Nerves and Endothelium*. ed. Vanhoutte, P.M. pp. 401–414. New York: Raven Press.
- FURCHGOTT, R.F. & ZAWADSKI, J.V. (1980). The obligatory role of endothelial cells in relaxation of arterial smooth muscle by acetylcholine. *Nature*, **288**, 373–376.
- HIRST, G.D.S. & EDWARDS, F.R. (1989). Sympathetic neuroeffector transmission in arteries and arterioles. *Physiol. Rev.*, **69**, 546–604.
- IGNARRO, L.J., BUGA, G.M., WOOD, K.S., BYRNS, R.E. & CHAUDHURI, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proc. Natl. Acad. Sci. U.S.A.*, **84**, 9265–9269.
- IGNARRO, L.J. & KADOWITZ, P.J. (1985). The pharmacological and physiological role of cyclic GMP in vascular smooth muscle relaxation. *Annu. Rev. Pharmacol. Toxicol.*, **25**, 171–191.
- JAISWAL, N. & MALIK, K.U. (1990). Prostacyclin synthesis elicited by cholinergic agonists is linked to activation of M₂ alpha and M₂ beta muscarinic receptors in the rabbit aorta. *Prostaglandins*, **39**, 267–280.
- KOMORI, K. & SUZUKI, H. (1987). Heterogeneous distribution of muscarinic receptors in the rabbit saphenous artery. *Br. J. Pharmacol.*, **92**, 657–664.
- LEE, L., BRUNER, C.A. & WEBB, R.C. (1990). Prostanoids contribute to endothelium-dependent coronary vasodilatation in guinea-pigs. *Blood Vessels*, **27**, 341–351.
- MONCADA, S., HERMAN, A.G., HIGGS, E.A. & VANE, J.R. (1977). Differential formation of prostacyclin (PGX or PGI₂) by layers of the arterial wall. An explanation for the anti-thrombotic properties of vascular endothelium. *Thromb. Res.*, **11**, 323–344.
- MURAD, F., MITTAL, C.K., ARNOLD, W.P., KATSUKI, S. & KIMURA, H. (1978). Guanylate cyclase: activation by azide, nitro compounds, nitric oxide, and hydroxyl radical and inhibition by hemoglobin and myoglobin. *Adv. Cyclic Nucl. Res.*, **9**, 145–149.
- NAKAO, K., OKABE, K., KITAMURA, K., KURIYAMA, H. & WESTON, A.H. (1988). Characteristics of cromakalim-induced relaxations in the smooth muscle cells of guinea-pig mesenteric artery and vein. *Br. J. Pharmacol.*, **95**, 795–804.
- NELSON, M.T., PATLAK, J.B., WORLEY, J.F. & STANDEN, N.B. (1990). Calcium channels, potassium channels and voltage dependence of arterial smooth muscle tone. *Am. J. Physiol.*, **259**, C3–C18.
- PALMER, R.M.J., FERRIGE, A.G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature*, **327**, 524–526.
- PALMER, R.M.J., REES, D.D., ASHTON, D.S. & MONCADA, S. (1988). L-Arginine is the physiological precursor for the formation of nitric oxide in endothelium-dependent relaxation. *Biochem. Biophys. Res. Commun.*, **153**, 1251–1256.
- PARKINGTON, H.C., TARE, M., TONTA, M.A. & COLEMAN, H.A. (1993). Stretch reveals three components in the hyperpolarisation of guinea-pig coronary artery in response to acetylcholine. *J. Physiol.*, **465**, 459–476.
- SCHINI, V.B. & VANHOUTTE, P.M. (1991). L-Arginine evokes both endothelium-dependent and -independent relaxations in L-arginine-depleted aortas of the rat. *Circ. Res.*, **68**, 209–216.
- STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarising vasodilators activate ATP-sensitive K⁺ channels in arterial smooth muscle. *Science*, **245**, 177–180.
- TARE, M., PARKINGTON, H.C., COLEMAN, H.A., NEILD, T.O. & DUSTING, G.J. (1990). Hyperpolarisation and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. *Nature*, **345**, 69–71.
- WALDMAN, S.A. & MURAD, F. (1987). Cyclic GMP synthesis and function. *Pharmacol. Rev.*, **39**, 163–196.
- WINQUIST, R.J. & HINTZE, T.H. (1990). Mechanisms of arterial naturetic factor-induced vasodilatation. *Pharmacol. Ther.*, **48**, 417–426.

(Received July 6, 1993
Revised December 9, 1993
Accepted March 4, 1994)