A comparison of vasodilator activity of agents activating cyclic nucleotides with those inhibiting their metabolism in rabbit isolated ear artery

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1 The effects of forskolin, a direct activator of adenylate cyclase and sodium nitroprusside, a direct activator of guanylate cyclase, were studied on rabbit isolated ear arteries preconstricted with 80 mM potassium.

2 Bolus injection of these two compounds resulted in vasodilatation. They had similar potencies in this tissue but forskolin had a significantly longer duration of action than sodium nitroprusside.

3 In the same tissue, perfusion with isobutylmethylxanthine (IBMX), a non-selective phosphodiesterase (PDE) inhibitor, or zaprinast, selective for the PDE primarily responsible for the metabolism of guanosine 3':5'-cyclic monophosphate (cyclic GMP), resulted in vasodilatation. However, SK&F 94120 selective for cyclic AMP-PDE (PDE III), primarily responsible for the metabolism of adenosine 3':5'-cyclic monophosphate (cyclic AMP), resulted in vasodilatation only at very high concentrations. The rank order of potency for the compounds was IBMX > zaprinast > SK&F 94120.

4 The effects of these three PDE inhibitors were studied on the vasoconstriction produced by perivascular sympathetic nerve stimulation in the absence of raised potassium. IBMX and zaprinast, caused a reduction in the response at 50 Hz stimulation frequency and a shift in the frequency-response curve to the right. SK&F 94120 did not displace the frequency-response curve but did reduce the response at 50 Hz. The same order of potency for the inhibition of the vasoconstrictor responses to perivascular sympathetic nerve stimulation was found as for vasodilatation i.e. IBMX > zaprinast > SK&F 94120.

5 These results indicate that in the same tissue direct activation of adenylate and guanylate cyclase results in vasodilatation. Non-specific PDE and cyclic GMP-PDE inhibition also resulted in vasodilatation and inhibition of vasoconstrictor responses to sympathetic nerve stimulation. However a selective cyclic AMP-PDE (PDE III) inhibitor did not result in vasodilatation, except at very high concentrations, or inhibit sympathetic vasoconstrictor responses except to reduce the response at 50 Hz stimulation. These findings provide further support for the ability of PDE inhibitors to be tissue selective.

Introduction

Changes in intracellular cyclic nucleotide concentrations are known to mediate the effects of many drugs and hormones on vascular smooth muscle. The relaxation of smooth muscle by β -adrenoceptor agonists is associated with a rise in intracellular adenosine 3':5'-cyclic monophosphate (cyclic AMP) concentration (Andersson, 1973; Poch & Holzmann, 1980). On the other hand, the relaxation in response to nitrates is brought about by a rise in intracellular guanosine 3':5'-cyclic monophosphate (cyclic GMP) concentration (Itoh *et al.*, 1985). Thus both cyclic

nucleotides are implicated in the relaxation of vascular smooth muscle and studies on the time course of these events have revealed that the rise in intracellular cyclic nucleotide concentrations precedes the mechanical events (Kukovetz *et al.*, 1979).

Compounds which inhibit phosphodiesterase (PDE), responsible for the metabolism of cyclic nucleotides, also result in vasodilatation (Kramer & Wells, 1979). Recently, different PDE isoenzymes have been identified and characterized. Selective inhibitors for these isoenzymes have been identified (Weishaar *et al.*, 1986; Reeves *et al.*, 1987) and measurement of their effects on vascular smooth muscle

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may yield further information for the role of cyclic nucleotides in mediating smooth muscle relaxation.

We have investigated the vasodilator effects of PDE inhibitors with differing isoenzyme selectivity; (a) isobutylmethylxanthine (IBMX) which is non-selective, (b) zaprinast which primarily inhibits the metabolism of cyclic GMP and (c) SK&F 94120 (5-(4-acetamidophenyl)pyrazin-2[1H]-one) which inhibits the metabolism of cyclic AMP (Reeves *et al.*, 1987), and compared their effects with forskolin and sodium nitroprusside which activate adenylate and guanylate cyclase respectively (Seamon & Daly, 1981; Itoh *et al.*, 1985). The tissue used was the rabbit isolated ear artery perfused at constant flow.

Methods

New Zealand white rabbits of either sex (2–4 kg) were pretreated with heparin (Pularin, Evans Medical) $1,250 \text{ iu kg}^{-1}$ i.v. immediately prior to anaesthesia with a mixture of sodium pentobarbitone (Sagatal, May & Baker) 17 mg kg^{-1} and sodium-5-ethyl-5-1-methyl-propyl-2-thiobarbiturate (Inactin, Byk Gulden) 45 mg kg^{-1} i.v. and killed by exsanguination. The central ear arteries were cannulated and a 3–4 cm length dissected free from the ear and placed in a silicone envelope to prevent drying out.

The arteries were perfused at 2 ml min^{-1} (Watson-Marlow 502s pump) at 32°C . The vascular perfusion pressure, recorded by a pressure transducer (Bell & Howell type 4-422), was measured from a side-arm cannula close to the perfusion cannula. The output was amplified (Lectromed 3552 conditioning amplifier) and recorded (Lectromed Multitrace 4 type 5041). Changes in perfusion pressure were used as an index of changes in the resistance of the artery; a fall in perfusion pressure indicated vasodilatation, a rise, vasoconstriction. The perfusion pump produced a pulsatile pressure, this was not electrically means since the time course of the R-C circuits would have biased the measurement of the duration of action. All responses were measured from the minimum of the pulse pressure and are the peak responses. For the comparison of the potencies of the drugs, doses required to produce a given dilatation or constriction were calculated by straight line interpolation from bracketing doses. The data were analysed by Student's paired *t* test to compare the effects of bolus injection of forskolin and sodium nitroprusside, and the effects of the different PDE inhibitors on perivascular sympathetic nerve stimulation. Student's *t* test was used for the comparison of the effects of perfusion with different PDE inhibitors. The data were analysed by use of a statistical package (RSE, BBN Software Products) on a VAX 785 computer (Digital Equipment Corporation). Dif-

ferences were considered to be significant when $P < 0.05$.

Precontracted arteries

The arteries were precontracted by perfusion with 80 mM KCl in modified Krebs-Henseleit solution of the following composition (mM): NaCl 43.9, KCl 78.8, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2, CaCl_2 2.5, NaHCO_3 25.0, KH_2PO_4 1.2, glucose 5.0, Na pyruvate 2.0, bubbled with 95% O_2 and 5% CO_2 to give a pH of 7.3–7.4. Bolus injection of compounds into the perfusate were made by injecting 10 or $30 \mu\text{l}$ volume with a Hamilton syringe through a short length of thick walled rubber tubing just proximal to the artery. Injection of $30 \mu\text{l}$ of the vehicles used to dissolve forskolin and sodium nitroprusside had no effect on vascular tone. For continuous infusion of compounds, the appropriate molar concentrations were dissolved in the Krebs-Henseleit solution which perfused the arteries.

Perivascular sympathetic nerve stimulation

The arteries were prepared as described above, and perfused with Krebs-Henseleit solution of composition (mM): NaCl 118.0, KCl 4.7, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 1.2, CaCl_2 2.5, NaHCO_3 25.0, KH_2PO_4 1.2, glucose 5.0, Na pyruvate 2.0, bubbled with 95% O_2 and 5% CO_2 to give a pH of 7.3–7.4. Stimulation of the perivascular sympathetic nerves was achieved by placing the proximal end of the artery over a bipolar silver stimulating electrode using stimulus parameters of 1 to 50 Hz, 5 ms duration, and 20 V which was supra-maximal, from a Grass S11 stimulator (Grass Instruments, Quincy, Mass., U.S.A.). The control frequency-response curve was obtained, followed by perfusion with Krebs-Henseleit solution containing the inhibitor for 20 min before obtaining the second frequency-response curve, this was then followed by a period of 1 h of perfusion with Krebs-Henseleit solution to washout the inhibitor, after which the third curve was obtained. Three successive frequency-response curves, without compound present, were shown to be highly reproducible over the time taken to complete the protocol.

Drugs

The following compounds were used and prepared as follows: forskolin (Calbiochem, Cambridge, U.K.) dissolved in absolute ethanol to make a 10^{-2} M stock solution. Sodium nitroprusside (Sigma Chemical Co., St. Louis, MO., U.S.A.) dissolved in distilled water to make a 10^{-2} M stock solution. All stock solutions were diluted with Krebs-Henseleit solution. SK&F 94120 (Smith Kline and French Labs Ltd, Welwyn Garden City), zaprinast (May & Baker, Dagenham)

and IBMX (Aldrich Chemical Co. Gillingham) were dissolved in 0.5 ml 1 M NaOH diluted with one litre of Krebs-Henseleit solution to make a 10^{-4} M solution (pH 7.3–7.4).

Results

Vasodilator effects of forskolin and sodium nitroprusside in potassium constricted arteries

In eight arteries injection of either compound produced a dose-dependent dilatation of the precontracted arteries. Threshold doses were 3×10^{-10} mol, for forskolin and nitroprusside. The doses required to produce a 25 mmHg fall in perfusion pressure were not significantly different, 8.7 ± 0.4 , $12.9 \pm 1.0 \times 10^{-10}$ mol for forskolin and nitroprusside respectively. Solubility of forskolin limited the highest concentration of forskolin that could be injected to 3×10^{-8} mol which resulted in a dilatation of 89.7 ± 8.5 mmHg, sodium nitroprusside at this same dose resulted in a dilatation of 90.1 ± 19.1 mmHg. For sodium nitroprusside this was also the highest dose that could be given which allowed recovery of the perfusion pressure to the control values. These results are illustrated in Figure 1.

In these eight arteries the duration of responses to forskolin was much greater than that to nitroprusside. For forskolin, 1×10^{-9} mol, the time to 50% recovery ($t_{50\%}$) of the peak dilatation, 6.1 ± 1.7 min, was significantly greater than that for nitroprusside, 2.9 ± 0.4 min, at 1×10^{-9} mol (Figure 2).

Figure 1 Vasodilatation in rabbit isolated ear arteries precontracted with K^+ (80 mM) following bolus injection of forskolin (●) or sodium nitroprusside (■) each tested on the same 8 arteries. Points are mean with s.e.mean shown by vertical bars.

Figure 2 Rabbit isolated ear arteries: an example of the vasodilator responses to bolus injection of sodium nitroprusside 10^{-9} mol, and forskolin 10^{-9} mol.

Vasodilator effects of continuous infusion of different phosphodiesterase isoenzyme inhibitors

Each inhibitor was tested on a separate group of eight arteries. Perfusion with the non-specific PDE inhibitor IBMX and the PDE I selective inhibitor zaprinast resulted in concentration-dependent vasodilatation. The concentrations required to produce a 50 mmHg fall in perfusion pressure of the arteries were $<10^{-6}$ M for IBMX and $2.18 \pm 0.01 \times 10^{-6}$ M for zaprinast. Perfusion with the selective PDE III inhibitor SK&F 94120 resulted in a minimal dilatation, statistically significant only at 3×10^{-4} M. These data are shown in Figure 3.

Effects of different phosphodiesterase inhibitors on the vasoconstrictor responses to sympathetic perivascular nerve stimulation

Supramaximal electrical stimulation resulted in a frequency-dependent increase in perfusion pressure

Figure 3 Vasodilatation in rabbit isolated ear arteries precontracted with K^+ (80 mM)-containing Krebs-Henseleit solution following addition of isobutylmethylxanthine (●), zaprinast (■), or SK&F 94120 (▲). Points are mean with s.e.mean shown by vertical bars; 8 separate arteries for perfusion with each drug.

of the arteries over the range 1 to 50 Hz. At 100 Hz responses did not exceed those at 50 Hz and usually were reduced. In eight arteries three repeated frequency-response curves were reproducible; the mean frequencies required to produce a 50 mmHg rise in perfusion pressure (EF_{50}) were: 5.8 ± 1.1 Hz for curve 1 control; 4.4 ± 0.8 Hz for curve 2, carried out 20 min after curve 1 and equivalent to the curve in the presence of drug; 4.7 ± 0.7 Hz for curve 3, carried out 1 h after curve 2 and equivalent to the curve performed after 1 h washout of the drug. These EF_{50} values were not significantly different. There was no significant difference in the responses at 50 Hz for the three curves: they were 143 ± 30 , 155 ± 31 , 123 ± 20 mmHg, curves 1 to 3 respectively. These data are shown in Figure 4a, for clarity only curves 1 and 2 are shown.

In eight arteries the inclusion of IBMX, 1×10^{-4} M, in the perfusion fluid reduced vasoconstrictor responses to sympathetic nerve stimulation. The EF_{50} significantly increased from 4.1 ± 0.5 to 27.3 ± 7.1 Hz and the response at 50 Hz was significantly reduced from 168 ± 23 to 67 ± 19 mmHg (Figure 4b). Following washout for 1 h the EF_{50} returned towards control values, 11.7 ± 6.5 Hz, as did the response at 50 Hz, 128 ± 27 mmHg; neither response was significantly different from control curve values.

Zaprinast, 1×10^{-6} M in eight arteries, significantly increased the EF_{50} from 3.7 ± 0.5 to 8.3 ± 1.7 Hz, and decreased the response at 50 Hz from 191 ± 17 to 114 ± 18 mmHg. Following washout for 1 h the EF_{50} only partially returned to control values, 7.1 ± 1.1 Hz, as did the response at 50 Hz, 139 ± 29 mmHg; these responses were significantly different from control curve values; Figure 4c (only two curves shown, the control and that in the presence of the drug).

In contrast SK&F 94120, 1×10^{-4} M in eight arteries, did not significantly alter the EF_{50} value, 5.4 ± 0.5 to 9.3 ± 2.1 Hz, but the response at 50 Hz was significantly reduced, 182 ± 31 to 146 ± 29 mmHg. Following washout for 1 h the EF_{50} was not significantly different from the control value 8.1 ± 1.3 Hz; however, the maximum response was slightly reduced further to 130 ± 27 mmHg, still significantly different from control (Figure 4d, only 2 curves, control and in the presence of drug shown).

Discussion

These experiments have demonstrated similar vasodilator potencies for compounds which, in the same tissue, activate guanylate or adenylate cyclase directly i.e. sodium nitroprusside and forskolin respectively. However, there was a significant difference in the duration of action of the compounds. In

Figure 4 Vasoconstriction during sympathetic nerve stimulation in rabbit ear arteries. Frequency-response curves are shown before (●) and during (■) perfusion of the arteries with (a) Krebs-Henseleit solution without drug, (b) Krebs-Henseleit solution containing isobutylmethylxanthine, 10^{-4} M, (c) Krebs-Henseleit solution containing zaprinast, 1×10^{-6} M or (d) Krebs-Henseleit solution containing SK&F 94120, 1×10^{-4} M. All points are mean with s.e.mean shown by vertical bars, for clarity, two of three curves performed in the same 8 arteries are shown. The third curve was performed after 1 h washout of the drug (not shown). A different set of arteries was used to test each drug and to test the reproducibility of the 3 curves in the absence of drug.

the same tissue, PDE inhibitors with differing isoenzyme selectivity have been shown to result in vasodilatation in potassium precontracted tissues and also to inhibit the responses to perivascular sympathetic nerve stimulation, in each case with a similar order of potency, IBMX > zaprinast > SK&F 94120.

The weak vasodilator activity of SK&F 94120 was unexpected, since Gristwood *et al.* (1986) showed that it was a vasodilator *in vivo*. In that paper, SK&F 94120 ($0.1 \text{ mg kg}^{-1} \text{ min}^{-1}$) resulted in a significant decrease in total peripheral vascular resist-

ance, resulting from reductions in vascular resistance of the heart, kidneys, duodenum, and stomach. No *in vitro* vasodilator data were described in the paper, yet SK&F 94120 was shown to have inotropic effects *in vitro* in guinea-pigs, cats, marmosets, and dogs.

Penetration of SK&F 94120 into vascular cells is needed to achieve inhibition of the tissue enzyme. Its IC_{50} for the low K_m cyclic AMP-PDE (PDE III) is $11 \mu M$, with no inhibition of cyclic GMP-PDE (PDE I), PDE II or the high K_m cyclic AMP-PDE (PDE IV) (Leigh *et al.*, 1987). SK&F 94120 has been shown to act as a positive inotrope over the same concentration range as it inhibits PDE III (Gristwood *et al.*, 1986) suggesting that myocyte penetration is no barrier to activity on the tissue enzyme. Thus the weak vasodilator activity in the rabbit ear artery may imply little or no turnover of cyclic AMP which is metabolised by PDE III.

IBMX is non-selective with IC_{50} values of 11.5, 4.2, $13.0 \mu M$ for PDE I, III and IV respectively, and zaprinast is selective for PDE I, IC_{50} values 10, 400, $300 \mu M$ for PDE I, III and IV (Leigh *et al.*, 1987). The greater potency of IBMX relative to zaprinast may reflect its ability to inhibit a wider range of PDE enzymes or, if PDE I is the primary basis for activity, reflect greater cell penetration.

Previous investigations of PDE inhibitors have shown that PDE inhibition results in relaxation of precontracted vascular smooth muscle (Ljung *et al.*, 1975; Kramer & Wells, 1979; Kukovetz *et al.*, 1981; Schoeffter *et al.*, 1987). However, a correlation between the relaxant effects of the PDE inhibitors and increases in intracellular cyclic nucleotide concentrations has not been established. Vegesna & Diamond (1986) used a series of xanthine derivatives and papaverine with phosphodiesterase inhibitory activity in a rabbit aortic ring preparation. They showed dissociation between vasodilator activity and phosphodiesterase inhibition i.e. some compounds were more potent in causing relaxation than would be expected from their low potency for the inhibition of the hydrolysis of cyclic nucleotides, whilst others were more potent for the inhibition of cyclic nucleotide metabolism, than for relaxation of smooth muscle. They also reported that some agents which were of equivalent potency for the relaxation of smooth muscle, caused differential increases in cyclic nucleotide concentrations. Fujioka (1984), using basilar arteries of dog and guinea-pig, also failed to find a causal relationship between cyclic AMP and the effects of papaverine, which was thought to act by inhibition of voltage-dependent influx of calcium and inhibition of receptor activated release of intracellularly stored calcium.

The use of more selective inhibitors of the different PDE isoenzymes by Schoeffter *et al.* (1987) in rat thoracic aorta, demonstrated a correlation between

intracellular cyclic AMP concentrations and relaxation. However they concluded that an increase in intracellular cyclic GMP by inhibition of the cyclic GMP-PDE with zaprinast did not by itself lead to relaxation of the tissue. They reported a 3.3 fold increase in cyclic GMP with zaprinast, $50 \mu M$, yet they measured a 10, 20, 60% relaxation at 5, 50, $500 \mu M$. Our findings are at odds with those of Schoeffter *et al.* (1987), because we have shown a greater potency of action for zaprinast for the relaxation of vascular smooth muscle with 50% reduction in perfusion pressure at $55 \mu M$ compared to $500 \mu M$ needed in their study.

The arteries used in our investigation were perfused with pulsatile pressure at constant flow. Recent studies have shown that this may cause the release of EDRF (Holtz *et al.*, 1984; Rubanyi *et al.*, 1986) which is known to increase cyclic GMP concentrations and so result in vasodilatation (Holzmann, 1982; Rapoport & Murad, 1983). Griffith, (1985) showed that responses to EDRF could be potentiated by zaprinast thus confirming the role of cyclic GMP in mediating the response of EDRF. Griffith *et al.* (1987) provided evidence for a possible physiological role for the endogenous release of EDRF. They showed that EDRF may control the perfusion of an arterial vascular bed, the rabbit ear artery, during high rates of perfusion by maintaining a constant pressure gradient across each vessel, particularly in the first order branching vessels from the main artery. The endothelium is intact in our preparations, despite being perfused with high potassium Krebs-Henseleit solution (Griffith *et al.*, 1984), since in four arteries perfused under identical conditions and for the same time required to perform the dose-response curves to perfusion with PDE inhibitors, carbachol, 1×10^{-8} mol injected as a bolus, resulted in a peak dilatation of 31 ± 6 from 97 ± 16 mmHg (unpublished observations). The effectiveness of IBMX and zaprinast in causing vasodilatation in our preparations, compared to the findings of Schoeffter *et al.* (1987), may therefore be due to an increased basal turnover of cyclic GMP caused by EDRF release, since the actions of PDE inhibitors must be in part dependent upon the turnover rate of cyclic nucleotides. The lack of effect of SK&F 94120 in our preparations could therefore be accounted for by a corresponding lack or very slow rate of turnover of cyclic AMP, since it would appear from the equipotency of forskolin and sodium nitroprusside that both cyclic nucleotides result in vasodilatation.

Tissue selectivity of cyclic AMP-PDEs may however account for the lack of effect of SK&F 94120. Schoeffter *et al.* (1987) used rolipram and Ro 20-1724 as cyclic AMP-PDE inhibitors whereas we have used SK&F 94120. Reeves *et al.* (1987) showed that SK&F 94120 is selective in its inhibition of

PDE III which is a subtype of the cyclic AMP-PDE and that rolipram and Ro 20-1724 are inhibitors of PDE IV, a clearly distinct subtype of the cyclic AMP-PDE with a high K_m for cyclic AMP. The activities of these cyclic AMP-PDE subtypes has previously been shown to have differential functional significance in another tissue (Gristwood & Owen, 1986) where PDE III inhibition by SK&F 94120 causes an increase in cyclic AMP of the heart associated with an increase in the contractility, whereas rolipram also caused an increase in the cyclic AMP but did not affect the contractility of the heart. With this precedent in heart muscle, the apparently different effects of cyclic AMP-PDE inhibition on vascular smooth muscle could be accounted for by the inhibition of these different subtypes of cyclic AMP-PDE.

There is a further significant difference between the two studies, that of the precontracting agent used. Thus the differences in the vasodilator effects of cyclic AMP-PDE inhibition could be the result of using potassium to precontract the arteries in our experiments, and the use of 5-hydroxytryptamine by Schoeffter *et al.* (1987). Work to study possible differences in potency of PDE inhibitors caused by differing precontracting agents is currently under way. Preliminary results obtained by Morrison & Pollock (1988) indicate that sodium nitroprusside relaxations of rat aortic rings were significantly smaller with potassium as a precontracting agent than with nor-adrenaline. Therefore reduced vasodilator potencies of drugs tested against potassium precontraction may explain the minimal effect of the cyclic

AMP-PDE inhibitor SK&F 94120 in our preparations.

We have shown for the first time that PDE inhibition can attenuate the vasoconstrictor responses to perivascular sympathetic nerve stimulation. The frequency-response relationship was shifted to the right and the maxima of the responses were reduced by IBMX and zaprinast. However SK&F 94120 only weakly inhibited the response at 50 Hz. Thus the same order of potency was found for the inhibition of neural vasoconstrictor responses, as for dilatation of the tissue when precontracted with potassium. It would therefore seem that there is some common underlying mechanistic reason for the differences in potency of cyclic nucleotide PDE inhibitors for the relaxation of vascular smooth muscle, perhaps either basal turnover rates of cyclic nucleotides or tissue selectivity of the inhibitors.

In order to resolve these differences in potency of the PDE inhibitors and the differences in duration of action of forskolin, and sodium nitroprusside the correlation of time course and concentrations of intracellular cyclic nucleotides and resulting vasodilator effects should be investigated. These studies are in progress.

It remains for the functional significance of this inhibition to be determined *in vivo*. It could be expected that non-specific PDE inhibition and inhibition of cyclic GMP-PDE would result in vasodilatation. *In vivo* there might be an elevated turnover of cyclic AMP compared to that *in vitro* which may account for the activity of SK&F 94120 as a vasodilator *in vivo* (Gristwood *et al.*, 1986).

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