Cyclic GMP release and vasodilatation induced by EDRF and atrial natriuretic factor in the isolated perfused kidney of the rat

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- 1 Guanosine 3':5'-cyclic monophosphate (cyclic GMP) release and vascular tone was measured in the isolated kidney of the rat perfused at constant flow with Krebs-Henseleit solution. The effects of 3 vasodilators, acetylcholine (ACh), atrial natriuretic factor (ANF) and sodium nitroprusside (SNP) on the renal release of cyclic GMP and vascular tone were examined. The ability of the endothelial-derived relaxing factor (EDRF) inhibitors, haemoglobin and gossypol, to modify vasodilatation and vasodilator-induced changes in cyclic GMP releases from the kidney was also investigated.
- 2 Renal cyclic GMP release was elevated 8 fold by ANF (0.01 μM), 5 fold by SNP (1 μM) and 3 fold by ACh (0.3 μM).
- 3 For ACh, both the increase in renal cyclic GMP release and the vasodilatation were reduced by the EDRF inhibitors, haemoglobin (1 μM) and gossypol (15 μM). For SNP, neither the increase in renal cyclic GMP release nor vasodilatation were inhibited by gossypol (15 μM).
- 4 For ANF, neither the increase in cyclic GMP release from the kidney nor its vasodilator activity were affected by haemoglobin (1 μM).
- 5 EDRF inhibitors reduced the basal release of cyclic GMP from $0.32 \pm 0.06 \text{ pmol min}^{-1}$ to $0.18 \pm 0.03 \text{ pmol min}^{-1}$, gossypol being more effective than haemoglobin.
- 6 The results are consistent with the ability of ACh to induce EDRF-mediated vasodilatation in the isolated perfused kidney of the rat. Basal EDRF release appears to contribute approximately 50% to the basal release of cyclic GMP from this preparation. The renal vasodilator action of ANF however, is independent of EDRF, although the renal vascular endothelium cannot be discounted as a site at which ANF stimulates cyclic GMP production.

Introduction

The arterial vasodilator activity of endothelial-derived relaxing factor (EDRF) is mediated by activation of the soluble (cytosolic) form of guanylate cyclase elevating guanosine 3':5'-cyclic monophosphate (cyclic GMP) (Forstermann *et al.*, 1986). Atrial natriuretic factor (ANF) which also elevates cyclic GMP is thought to activate the particulate (membrane bound) form of guanylate cyclase through an endothelium-independent mechanism (Winqvist *et al.*, 1984). The vascular endothelium may, however, contain functional receptors for ANF since endothelial removal reduced the ability of ANF to stimulate cyclic GMP from rabbit aorta (Linz *et al.*, 1986) and in cultured bovine endothelial cells ANF-induced elevation of cyclic GMP content was 10 fold greater than in vascular smooth muscle (Schenk *et al.*, 1985).

In marked contrast to ANF, little is known about the renal properties of EDRF and its role in the control of renal vascular resistance. In other vascular beds including, the rabbit isolated ear (Griffith *et al.*, 1988) and the rat mesentery (Byfield *et al.*, 1986, Randall & Hiley, 1988), angiographic techniques or the use of detergents have indicated a role for EDRF in the control of arteriolar resistance. In the rat isolated perfused kidney, we have previously been unable to produce selective inhibition of acetylcholine (ACh)-induced vasodilatation with deoxycholate (Burton *et al.*, 1987), although some degree of selectivity was achieved with the detergent CHAPS. Bhardwaj & Moore (1988) employing a similar rat kidney preparation, however, demonstrated that the potentiation of renal vasoconstriction produced by CHAPS could not be reproduced by EDRF inhibitors, questioning the selectivity of detergents for the endothelium in the rat isolated kidney. While EDRF

release in the kidney, unlike the heart (Amecua *et al.*, 1988), has yet to be demonstrated, initial experiments with EDRF inhibitors (Burton *et al.*, 1988; Bhardwaj & Moore, 1988) are consistent with the presence of EDRF-mediated renal vasodilatation.

The present study was performed to establish (a) whether measurements of renal cyclic GMP synthesis were consistent with the presence of EDRF-mediated vasodilatation in the kidney and (b) whether EDRF may mediate either the increase in renal cyclic GMP synthesis or the renal vasodilatation induced by ANF. Experiments were performed in the isolated perfused kidney of the rat to examine the effect of the EDRF inhibitors haemoglobin (Edwards *et al.*, 1986) and gossypol (Alheid *et al.*, 1987) on vasodilator-induced changes in renal cyclic GMP release. The vasodilators studied were ACh, ANF and sodium nitroprusside (SNP).

Methods

Isolated perfused kidney of the rat

Male Wistar rats (200–300 g) were anaesthetized with sodium pentobarbitone (60 mg kg⁻¹, i.p.), the jugular vein cannulated and heparin (200 units kg⁻¹) administered by i.v. bolus injection. Following a flank incision the left renal artery was cannulated via an incision made in the aorta, the left adrenal artery being tied when necessary. The kidney was removed to a thermostatically controlled perfusion cabinet (37°C) and perfused with a Krebs-Henseleit solution of composition (mm): Na⁺ 143, K⁺ 5.9, Ca²⁺ 2.6, Mg²⁺ 1.2, Cl⁻ 128, H₂PO₄⁻ 2.2, HCO₃⁻ 24.9, SO₄²⁻ 1.2 and glucose 10, containing theophylline (1 mm) and gassed with 95% O₂ and 5% CO₂. Renal

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Figure 1 Effects of (a) methoxamine (3 μM) alone ($n = 6$) and together with either (b) acetylcholine (ACh, 0.3 μM, $n = 6$), or (c) sodium nitroprusside (SNP 1 μM, $n = 6$) on the time course of renal cyclic GMP release from the isolated perfused kidney of the rat following vasoconstriction with methoxamine (3 μM). Representative traces of renal perfusion pressure obtained from single kidneys are shown by the continuous line.

perfusion pressure was recorded electronically and the combined renal venous and tubular effluent collect for cyclic GMP assay.

Cyclic GMP assay

Following collection of renal effluent, the samples were immediately boiled (2 min) to inhibit phosphodiesterase activity and stored at -20°C . Samples were extracted and concentrated before assay as follows: 4 ml of each sample was acidified with 1 M HCl and subjected to ion exchange chromatography using a Dowex cation exchange resin. The column sizes were $9 \times 0.6\text{ cm}$ and the resin was regenerated after each sample with 1 M HCl. Columns were eluted with distilled water and control experiments using renal effluent 'seeded' with known concentrations of cyclic GMP showed that fractions eluted between 7–14 ml diluted parallel to a standard curve of cyclic GMP in distilled water. Control experiments using [^3H]-cyclic GMP demonstrated that $47.7 \pm 4.1\%$ ($n = 4$) of the ^3H added could be recovered in the fraction assayed for cyclic GMP.

All samples were freeze-dried and re-constituted in 250 μl distilled water and a 200 μl aliquot acetylated by the addition of 2 μl acetic anhydride and 4 μl triethylamine. The derivatised nucleotide was immunoassayed using guanosine 3'-cyclic phosphoric acid 2'-O-succinyl 3-[^{125}I]-iodotyrosine methyl ester, 5nCi per tube. Calibration binding curves were constructed by use of standard concentrations of cyclic GMP (7.8 fmol–2 pmol per tube) dissolved in Krebs-Henseleit solution which had previously been subjected to ion exchange chromatography.

Experimental protocol

Each kidney received only a single dose of each vasodilator which was infused for a 6 min period during renal vasoconstriction with the α_1 -adrenoceptor agonist methoxamine (3 μM). In initial time course experiments, cyclic GMP was assayed in renal effluent collected at 1 min intervals during vasodilator infusion. The time of maximum cyclic GMP release was 0–3 min during infusion with ACh (0.3 μM) but, 4–6 min during infusion with SNP (1 μM) or ANF (0.01 μM). Values for cyclic GMP quoted in the text for ACh were therefore obtained in 0–3 min, while those for SNP or ANF were obtained in 4–6 min. Doses of vasodilators were chosen to produce approximately 80% of the maximum vasodilator response obtained to each agent in the absence of theophylline. The use of the EDRF inhibitors, gossypol and haemoglobin, required different protocols. Kidneys were pretreated with a 10 min infusion of gossypol (15 μM) 30 min before dilator stimulation. In contrast, haemoglobin (1 μM) was infused 1 min before and throughout the period of vasodilator administration.

Materials

Dowex 50 (H^+ form 4% cross-linked 200–400 dry mesh) (Sigma); guanosine 3'-cyclic phosphoric acid 2'-O-succinyl 3-[^{125}I]-iodotyrosine methyl ester (New England Nuclear); atrial natriuretic factor (rat 8–33 amino acids) (Pennisula); acetylcholine chloride, sodium nitroprusside, gossypol (all Sigma). Cyclic GMP antisera were obtained from Professor B. Brown, Department of Human Metabolism and Clinical Biochemistry, Sheffield. Methoxamine hydrochloride was a gift from the Wellcome Foundation.

Haemoglobin was prepared by a modification of the method of Edwards *et al.* (1986). Rat blood was spun at 3000 *g* for 20 min the supernatant discarded and the red cell concentrate then washed 3 times with 10 ml Krebs-Henseleit solution: 2 ml of the remaining red cell concentrate was then lysed with 2 ml water and spun to remove the cellular debris. The supernatant was chromatogrammed on a Sephadex G75 column ($2.5 \times 25\text{ cm}$) and was eluted with Krebs-Henseleit solution at 30 ml h^{-1} . The haemoglobin fraction was assayed by the cyanmethaemoglobin method (Drabkin & Austin, 1935).

Statistics

Results were evaluated by either paired or unpaired Student's *t* test where appropriate.

A preliminary account of some of the results presented in this paper was communicated to the British Pharmacological Society Meeting in London, December 1988.

Results

Renal vasoconstriction

Methoxamine (3 μM) increased the renal perfusion pressure from 61.7 ± 4.8 to $153.3 \pm 9.9\text{ mmHg}$ ($P < 0.001$, $n = 6$). The renal release of cyclic GMP was not significantly different ($n = 6$) in the absence ($0.26 \pm 0.07\text{ pmol min}^{-1}$) or in the presence ($0.20 \pm 0.08\text{ pmol min}^{-1}$) of methoxamine-induced vasoconstriction.

Renal vasodilators

The vasoconstrictor response to methoxamine was inhibited $77.8 \pm 9.5\%$ ($n = 6$) by ACh (0.3 μM), $57.2 \pm 7.1\%$ ($n = 5$) by ANF (0.01 μM) and $46.8 \pm 12.7\%$ ($n = 5$) by SNP (1 μM) and these vasodilator responses were sustained over at least a 6 min period during which time renal effluent was collected for cyclic GMP assay. All three vasodilators increased the renal release of cyclic GMP but there were differences in both the

extent and the duration of the increases obtained. Release was increased from 0.33 ± 0.04 to $0.91 \pm 0.23 \text{ pmol min}^{-1}$ ($P < 0.05$, $n = 6$) by ACh ($0.3 \mu\text{M}$, 0–3 min), however this increase declined during the 4–6 min period of the infusion despite the dilator response being sustained (Figure 1). Both vasodilator response and the increase in cyclic GMP release produced by SNP were slower in onset than those obtained with ACh. The increase in renal cyclic GMP release induced by SNP ($1 \mu\text{M}$, 4–6 min) from 0.23 ± 0.07 to $1.34 \pm 0.47 \text{ pmol min}^{-1}$ ($P < 0.05$, $n = 5$) did not reach a maximum until the 4–6 min period of its infusion (Figure 1). ANF ($0.01 \mu\text{M}$) produced an immediate and sustained effect on cyclic GMP release which had increased from 0.63 ± 0.36 to $5.80 \pm 1.96 \text{ pmol min}^{-1}$ ($P < 0.05$, $n = 5$) during the 4–6 min period of the ANF infusion.

EDRF inhibitors

The influence of haemoglobin on both vascular tone and renal cyclic GMP release measured in the presence of ACh and ANF is demonstrated in Figure 2 using non-paired statistics. In the presence of haemoglobin ($1 \mu\text{M}$), the vasodilator response to ANF remained unchanged and was accompanied by a significant increase in cyclic GMP release from 0.16 ± 0.06 to $8.44 \pm 1.60 \text{ pmol min}^{-1}$ ($P < 0.001$, $n = 5$). However, haemoglobin ($1 \mu\text{M}$) markedly reduced dilator responses to ACh which were unaccompanied by any increase in renal cyclic GMP release from 0.34 ± 0.16 to $0.23 \pm 0.06 \text{ pmol min}^{-1}$ ($n = 5$).

The effect of gossypol ($15 \mu\text{M}$) on vascular tone and cyclic GMP release produced in the presence of ACh and SNP is demonstrated in Figure 3 using non-paired statistics. Follow-

Figure 2 Effect of haemoglobin ($1 \mu\text{M}$) on renal vasodilatation and renal cyclic GMP release in the presence of either (a) acetylcholine ($0.3 \mu\text{M}$) or (b) atrial natriuretic factor ($0.01 \mu\text{M}$). Paired histograms denote vasodilatation or cyclic GMP release measured in the absence (open) or presence (hatched) of haemoglobin. Vertical bars represent the s.e.mean. * $P < 0.001$ (non-paired).

Figure 3 Effect of gossypol ($15 \mu\text{M}$) on renal vasodilatation and renal cyclic GMP release in the presence of either (a) acetylcholine ($0.3 \mu\text{M}$) or (b) sodium nitroprusside ($1 \mu\text{M}$). Paired histograms denote vasodilatation or cyclic GMP release measured either without (open) or with (hatched) gossypol pretreatment. Vertical bars represent s.e.mean. * $P < 0.001$ (non-paired).

ing treatment with gossypol ($15 \mu\text{M}$), the vasodilator response to SNP was not inhibited and was associated with a significant increase ($P < 0.001$) in renal cyclic GMP release from 0.16 ± 0.03 to $1.09 \pm 0.24 \text{ pmol min}^{-1}$. However, following gossypol, there was a marked reduction in the vasodilator response to ACh which was not associated with any increase in cyclic GMP release, from 0.09 ± 0.01 to $0.11 \pm 0.02 \text{ pmol min}^{-1}$ ($n = 6$).

Basal cyclic GMP release

The basal release of cyclic GMP from the kidney was $0.33 \pm 0.06 \text{ pmol min}^{-1}$ ($n = 33$). The basal release was significantly lower ($P < 0.05$) in kidneys receiving an EDRF inhibitor at $0.18 \pm 0.03 \text{ pmol min}^{-1}$ ($n = 26$). In kidneys which later received either ACh or SNP, the basal release of cyclic GMP ($0.27 \pm 0.05 \text{ pmol min}^{-1}$, $n = 11$) was significantly lower ($P < 0.01$) when pretreated with gossypol ($15 \mu\text{M}$, $0.12 \pm 0.02 \text{ pmol min}^{-1}$, $n = 11$). In kidneys which later received either ACh or ANF, although the mean basal release of cyclic GMP ($0.46 \pm 0.16 \text{ pmol min}^{-1}$, $n = 11$) was lower in the presence haemoglobin ($1 \mu\text{M}$) ($0.30 \pm 0.07 \text{ pmol min}^{-1}$, $n = 10$) the difference was not significant at the 5% level.

Discussion

This paper contains the first report of measurements of cyclic GMP release from the isolated kidney and required the presence of theophylline in the perfusate to bring the basal release rate to within the limit of detection of the assay system. Cyclic GMP synthesis is normally assessed from the

cyclic GMP content of tissue homogenates rather than whole organ release. Evidence that renal cyclic GMP release may also reflect the level of cyclic GMP synthesis in the rat isolated kidney was provided by the increase in renal cyclic GMP release produced by ANF and SNP, standard stimulators of the particulate (Winqvist *et al.*, 1984) and soluble (Forstermann *et al.*, 1986) forms of guanylate cyclase respectively. The use of the isolated perfused kidney allowed the vascular reactivity of the kidney to be assessed in parallel with renal cyclic GMP synthesis.

In arterial smooth muscle ACh activates soluble guanylate cyclase indirectly by stimulating the synthesis of EDRF (Forstermann *et al.*, 1986). Renal vasodilatation induced by ACh in the isolated kidney was accompanied by a 3 fold increase in cyclic GMP release although, the vascular reactivity of the isolated kidney was partially depressed by the presence of the phosphodiesterase inhibitor, theophylline. The increase in cyclic GMP release was maximal after 2 min but declined towards basal levels after 6 min despite a sustained vasodilator response. A similar time course has been demonstrated for the effects of ACh on the tissue content of cyclic GMP and generated tension in arterial rings (Gruetter & Lemke, 1986). The ability of ACh to increase renal cyclic GMP release was abolished by the structurally dissimilar EDRF inhibitors, haemoglobin and gossypol, which also inhibited ACh-induced renal vasodilatation. Such data are consistent with the hypothesis that EDRF-mediated vasodilatation can be stimulated in the isolated perfused kidney by acetylcholine. It is also consistent with the chemical identity of EDRF being nitric oxide (Palmer *et al.*, 1987) since nitric oxide is known to activate soluble guanylate cyclase in the kidney (Arnold *et al.*, 1977).

Both the increase in cyclic GMP release produced by ANF in the isolated perfused kidney and its renal vasodilator activity remained unaffected by treatment with haemoglobin, which acts as an EDRF inhibitor by binding nitric oxide (Gibson & Roughton, 1957). EDRF synthesis would not therefore appear to be involved in either the renal vasodilator activity of ANF or its ability to stimulate cyclic GMP synthesis. While the renal vasodilator response to ANF at the dose employed was smaller than that produced by ACh, the increase in cyclic GMP release was considerably larger. In isolated arteries, the ability of ANF to stimulate cyclic GMP is markedly enhanced by the presence of the endothelium (Linz *et al.*, 1986) and in cultured endothelial cells cyclic GMP elevation by ANF was some 10 fold greater than that in vascular smooth muscle (Schenk *et al.*, 1985). The renal vascular endothelium therefore could also represent a site for ANF to stimulate cyclic GMP synthesis in the isolated perfused kidney. However, since ANF stimulates a different form of guanylate cyclase from EDRF or the nitrovasodilators, such a difference could equally be explained by a greater synthetic capacity or a wider distribution of the particulate form of the enzyme. It has been proposed that ANF stimulates cyclic GMP in a variety of renal cell types (Takeda *et al.*, 1986). At the present time the importance of cyclic GMP to endothelial cell function and, the importance of the endothelium to the renal properties of ANF remain uncertain.

In isolated arterial rings, endothelial removal reduces the tissue content of cyclic GMP (Rapoport & Murad, 1983), indicating the presence of basal EDRF synthesis. Evidence for the presence of basal EDRF release from the isolated perfused kidney was provided by the results obtained with the EDRF inhibitors. Combination of the data obtained for both gossypol and haemoglobin showed a significant reduction in basal cyclic GMP release. Gossypol, in particular, reduced the

basal outflow of cyclic GMP from the kidney by 60% to $0.1 \text{ pmol min}^{-1}$ indicating that the kidney may continuously synthesize and/or release EDRF. The precise mechanism of action of gossypol as an EDRF inhibitor is unknown. While gossypol may, like other antioxidants, act by generating superoxide ions to break down nitric oxide (Moncada *et al.*, 1986), it potentiates rather than inhibits the dilator response to nitroprusside, thought to be a nitric oxide generating agent. Due to the irreversible nature of its effect and prolonged exposure time, gossypol was considered to be a better agent than haemoglobin to detect a change in basal cyclic GMP release.

However, the contribution which the basal EDRF release may make to the basal perfusion pressure in the rat isolated kidney is less certain, since although EDRF inhibitors produce a small increase in the basal perfusion pressure, ACh-induced vasodilatation cannot be detected in the absence of generated tone. Methoxamine was employed to elevate the renal vascular tone on the assumption that the endothelial α -adrenoceptor was of the α_2 -subtype. Methoxamine therefore, being an α_1 -selective agonist, would be unlikely to stimulate cyclic GMP release through the endothelium. Although as expected, methoxamine did not increase cyclic GMP release from the isolated perfused kidney, an interaction between the vascular effects of methoxamine and EDRF is still possible in the kidney since it has been proposed that the basal EDRF release inhibits the contractile activity of any spasmogen (Bullock *et al.*, 1986). In the present isolated perfused kidney preparation vasoconstriction was not potentiated by the EDRF inhibitors, in agreement with the experiments of Bhardwaj & Moore (1988). One conclusion could be that the basal release of EDRF is subthreshold for vasodilatation in this preparation. However, the possibility that renal EDRF synthesis may have been inhibited by the perfusion conditions employed cannot be ignored.

The validity of using measurements of cyclic GMP synthesis to support the presence of EDRF-mediated vasodilatation relies on the assumption that the properties of EDRF are mediated by this nucleotide. Although evidence from arterial tissues is strong (Murad, 1986), it must be remembered that agonists such as ACh can elevate cyclic GMP in tissues where EDRF has not been proposed as a mediator of its action (Waldman & Murad, 1987) and may also dilate smooth muscle by hyperpolarizing the cell membrane (Taylor *et al.*, 1988). The possibility that EDRF may possess other renal properties besides vasodilatation must also be considered. In a recirculating model of the isolated perfused kidney of the rat, the EDRF inhibitor gossypol reduced the glomerular filtration rate at doses that had no effect on perfusate flow (Pathak *et al.*, 1984). In addition, effluent from the rabbit aorta has been demonstrated to produce endothelium-dependent inhibition of renin release from dog kidney slices (Vidal *et al.*, 1988) although, recent evidence suggests that cyclic GMP may stimulate rather than inhibit renin release (Abu-Kishak *et al.*, 1989).

In conclusion, (a) the presence of EDRF-mediated vasodilatation in the isolated perfused kidney of the rat is supported by the pattern of change in both cyclic GMP release and vascular tone produced by vasodilators in the presence of EDRF inhibitors, (b) neither the increase in cyclic GMP release nor the vasodilator activity of ANF would appear to be dependent on EDRF synthesis, (c) renal EDRF synthesis may contribute approximately 50% to the basal release of cyclic GMP by this preparation.

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