Effects of bradykinin in the rat isolated perfused heart: role of kinin receptors and endothelium-derived relaxing factor

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1 The effects of bradykinin (BK) in the microcirculation of the isolated perfused heart of the rat were examined. The kinin receptors mediating the effects of BK were characterized and the role of endothelium-derived relaxation factor (EDRF) and prostacyclin investigated.

2 The dose-related vasodilator responses elicited by bolus doses of BK (0.001–10.0 nmol) were competitively blocked by the selective kinin B₂ receptor antagonist [D-Arg⁰,Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin (pA₂ = 6.8). Des-Arg⁹-bradykinin, a selective kinin B₁ receptor agonist had no vasodilator activity at doses of up to 10 nmol.

3 L-N^G-nitro arginine (100 µM; L-NOArg), an inhibitor of endothelium-dependent vasodilatation, reduced the duration but not the magnitude of the BK vasodilator response. This action of L-NOArg was not reversed by L-arginine (100 µM).

4 Superoxide dismutase (10 units ml⁻¹), haemoglobin (10 µM) and methylene blue (MB; 1 µM), all known to modify EDRF-mediated responses, failed to alter the vasodilator action of BK.

5 Gossypol (1–15 µM), a presumed inhibitor of EDRF biosynthesis, caused a marked drop in perfusion pressure followed by vasoconstriction. These changes in coronary tone were accompanied by an irreversible depression of cardiac contractility and heart rate. Over the same concentration range gossypol abolished the vasodilator action of BK (1.0 nmol), however it also blocked the endothelium-independent vasodilator response to sodium nitroprusside (30 nmol) and the vasoconstrictor effect of endothelin-1 (10 pmol) which suggests non-specific toxic actions of gossypol.

6 Bolus injections of BK (0.001–1.0 nmol) failed to elevate basal levels of prostacyclin (PGI₂) as shown by assaying for its stable metabolite 6-keto-PGF_{1α}. In addition, BK-induced vasodilatation was not blocked by flurbiprofen (2 µM) or BW755C (7.5 µM) which are inhibitors of the arachidonic acid pathway. When added with L-NOArg (100 µM), flurbiprofen (10 µM) did not potentiate the inhibitory action of L-NOArg on the BK response.

7 These results show that the vasodilator action of BK in the rat heart is dependent on the activation of the kinin B₂ receptors but independent of PGI₂ release. Although a conclusive role for EDRF could not be established, this study has questioned the suitability of several agents commonly used as inhibitors of EDRF-mediated responses.

Keywords: EDRF; nitric oxide; prostacyclin; bradykinin; kinin receptors; coronary microcirculation

Introduction

Bradykinin (BK) is a potent vasoactive agent which relaxes different vascular smooth muscle preparations via an endothelium-dependent mechanism (Altura & Chand, 1981; Cherry *et al.*, 1982). Endothelium-dependent relaxation involves the release of an endothelium-derived relaxing factor (EDRF) (Furchgott & Zawadzki, 1980) which has been identified as nitric oxide (NO) (Palmer *et al.*, 1987) with L-arginine as its physiological precursor (Palmer *et al.*, 1988a,b; Schmidt *et al.*, 1988). Although the release of this labile intermediate from many large vessels is well documented (Furchgott, 1984) little is known about its role in the microcirculation. Recent studies have indeed suggested that EDRF/NO mediates the vasodilator action of acetylcholine (ACh) and other drugs in resistance vessels (Cherry & Gillis, 1987; Stewart *et al.*, 1987; Bhardwaj & Moore, 1988; Amezcua *et al.*, 1988) and that BK releases NO from the perfused guinea-pig heart (Kelm & Schrader, 1988). However, other studies could not establish an exclusive role for NO (Aisaka *et al.*, 1989) nor could they rule out an additional direct vasodilator action of the drugs on the vascular smooth muscle (Stewart & Piper, 1988). It is therefore not clear whether the vasodilatation of resistance vessels

caused by compounds such as ACh and BK are mediated exclusively by the release of EDRF.

The present study was undertaken to establish the underlying mechanism(s) of action of BK on the resistance vessels in the isolated perfused heart of the rat. The role of EDRF in this model was investigated by use of superoxide dismutase (Gryglewski *et al.*, 1986a,b), methylene blue (Martin *et al.*, 1985), oxyhaemoglobin (Martin *et al.*, 1985, 1986), gossypol (Alheid *et al.*, 1987) and L-N^G-nitro arginine (Moore *et al.*, 1990) which are known to modify EDRF-mediated responses in several different vascular preparations. Characterization of the functional receptors mediating the actions of BK was carried out by use of [D-Arg⁰,Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin, a selective antagonist of the kinin B₂ receptors (Vavrek & Stewart, 1985; Regoli *et al.*, 1990) and des-Arg⁹-bradykinin, a selective B₁ receptor agonist (Regoli & Barabe, 1980).

In addition to EDRF, BK is also known to release prostacyclin (PGI₂), a potent vasodilator in the rat and guinea-pig perfused heart (Schrör *et al.*, 1978; Belo & Talesnik, 1982). In vascular endothelial cells the BK-induced release of PGI₂ is coupled to that of EDRF and is mediated by the activation of kinin B₂ receptors (de Nucci *et al.*, 1988; D'Orleans-Juste *et al.*, 1989). Thus the possibility of PGI₂ being released by BK in the rat heart has also been investigated. This was carried out by direct radioimmunoassay of its stable metabolite 6-keto-PGF_{1α} and by use of agents known to inhibit its synthesis. Some of our findings have been presented in a preliminary form (Baydoun & Woodward, 1989).

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Methods

Isolation and perfusion of rat heart

Hearts obtained from male Wistar rats (250–350 g) were perfused by a modified Langendorff technique (Broadley, 1979) at a constant flow rate of 10 ml min^{-1} at 37°C of Krebs-Henseleit solution containing 5.9 mM K^+ . The hearts were allowed to equilibrate for 20 min before raising coronary tone with Krebs-Henseleit buffer containing 3.2 mM K^+ . Changes in aortic perfusion pressure were used to monitor changes in coronary tone. The force of cardiac contraction was measured with a tension transducer attached to the apex of the heart and this was also used to trigger a heart rate meter. Vasodilator agents (in $10 \mu\text{l}$ aliquots) were administered to hearts as bolus injections into the perfusate, 3 cm proximal to the aortic cannula. All other compounds were added to the buffer reservoir and perfused through the hearts for a minimum period of 10 min before obtaining responses to the drugs. Commercial haemoglobin was prepared for experimental use according to the method of Martin *et al.* (1985).

Radioimmunoassay of prostacyclin

The release of PGI_2 was monitored by measurement of its stable metabolite, 6-keto- $\text{PGF}_{1\alpha}$, present in the cardiac effluent collected at 15 s intervals before, during and after the administration of bradykinin. The amount of 6-keto- $\text{PGF}_{1\alpha}$ in the effluent was determined by a standard radioimmunoassay technique using an antibody raised in rabbits (Salmon, 1978). Samples were assayed undiluted without prior extraction or purification (limit of detection was 0.05 ng ml^{-1} in the perfusate).

Materials

Bradykinin (BK), superoxide dismutase (SOD), haemoglobin (HB), gossypol, L- N^G -nitro arginine (L-NOArg) and Des-Arg⁹-bradykinin were obtained from Sigma (Poole, Dorset); [D-Arg⁰,Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin and endothelin-1 from Novabiochem (Nottingham), sodium nitroprusside (SNP) and methylene blue (MB) from British Drug House (Poole, Dorset); flurbiprofen from Boots (Nottingham) and BW755C (3-amino-1-[*m*-(trifluoromethyl)-phenyl]-2-pyrazoline) from Wellcome Laboratories (Beckenham, Kent).

Statistical analysis

Results are expressed as mean $\pm$ s.e.mean for n separate experiments. pA_2 was calculated according to Arunlakshana & Schild (1959).

Results

Effects of bradykinin and Des-Arg⁹-bradykinin

Bolus injections of BK (0.001–10.0 nmol) induced a dose-dependent vasodilatation in the perfused rat heart producing a maximum drop in coronary perfusion pressure of $42.8 \pm 4.5 \text{ mmHg}$ at 1 nmol ($n = 10$). Over the same dose range, BK had very little effect on the force of contraction but produced a dose-related increase in heart rate in four out of ten experiments. As shown in Figure 1, the initial response on coronary tone was a marked and rapid drop in perfusion pressure which was sustained for over 10 min at higher doses.

In contrast to BK, the selective B_1 receptor agonist, Des-Arg⁹-bradykinin (0.001–10 nmol), was completely inactive. This indicates that B_1 receptors are not present in the rat heart and suggests that the effects of BK in this model are mediated via the kinin B_2 receptors.

Figure 1 A representative trace of the effects of bradykinin (BK) on perfusion pressure (PP), developed tension (DT) and heart rate (HR, beats per min, b.p.m.) in the isolated perfused heart of the rat. This tracing is one of 10 similar experiments.

Effects of kinin receptor antagonists

The infusion of the selective kinin B_2 receptor antagonist [D-Arg⁰,Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin (100 nm and 200 nm) competitively antagonized the vasodilator action of BK (0.001–10.0 nmol) and caused a parallel rightward shift of the BK dose-response curve (Figure 2). A pA_2 value of 6.8 was calculated from these data. This value is comparable to that determined by Vavrek & Stewart (1985) in the rat uterus ($\text{pA}_2 = 6.4$), the guinea-pig ileum ($\text{pA}_2 = 6.3$) and in the dog carotid artery ($\text{pA}_2 = 6.4$).

In addition to reducing the magnitude of the responses, [D-Arg⁰,Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin also shortened the duration of the responses to BK. On its own [D-Arg⁰,Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin had a transient vasodilator action reducing the basal coronary tone by $7.2 \pm 0.3 \text{ mmHg}$ ($n = 5$) and $11.5 \pm 2.0 \text{ mmHg}$ ($n = 4$) at 100 nm and 200 nm respectively.

The role of endothelium-derived relaxing factor

Neither the infusion of SOD (10 units ml^{-1}) nor haemoglobin ($10 \mu\text{M}$) had any effect on the basal coronary tone or on the vasodilator action of BK. Methylene blue ($1 \mu\text{M}$) elevated basal

Figure 2 Vasodilator action of bradykinin (BK) in the perfused rat heart in the absence (●) and presence of [D-Arg⁰,Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin, 100 nm (□) ($n = 5$) and 200 nm (▲) ($n = 4$). The graph shows the mean with s.e.mean indicated by vertical bars.

Figure 3 Tracing depicting the effects of gossypol ($3 \mu\text{M}$) on perfusion pressure (PP), developed tension (DT) and heart rate (HR) in the isolated perfused heart of the rat. This trace is representative of three separate similar experiments.

coronary tone by $14.0 \pm 1.7 \text{ mmHg}$ ($n = 3$) but failed to modify the responses to BK ($0.01\text{--}1.0 \text{ nmol}$). At the same concentration ($1 \mu\text{M}$), MB did inhibit vascular relaxation induced by ACh in precontracted rat aortic strips (data not shown). Higher concentrations ($>10 \mu\text{M}$) of MB could not be used in our studies because of the marked depression in cardiac contractility and vasodilatation produced by the compound itself (Baydoun *et al.*, 1990).

Infusion of gossypol ($1\text{--}15 \mu\text{M}$) caused an initial drop in coronary tone followed by a sustained rise. This effect was accompanied by a depression of contractility and heart rate which was irreversible, even on washing for periods of up to 90 min (Figure 3). At these high concentrations, known to inhibit the actions of EDRF in other vascular preparations (Alheid *et al.*, 1987; Bhardwaj & Moore, 1988), gossypol abolished the vasodilator action of BK (1.0 nmol); however, it also inhibited the endothelium-independent dilator response to sodium nitroprusside (30 nmol) and the response to endothelin-1 (10 pmol) which is a potent vasoconstrictor peptide (Yanagisawa *et al.*, 1988; Baydoun *et al.*, 1989). Lower concentrations ($<0.5 \mu\text{M}$) of gossypol had no significant effect on the actions of BK ($0.01\text{--}1.0 \text{ nmol}$).

Effects of L-nitro arginine

In contrast to the agents used above, the infusion of L-NOArg through the heart elevated coronary tone and induced a positive inotropic action (Figure 4). At $100 \mu\text{M}$, L-NOArg increased basal perfusion pressure by $24.0 \pm 3.6 \text{ mmHg}$ ($n = 5$) and the developed tension increased from 8.0 ± 0.6 to $9.7 \pm 0.5 \text{ g}$ ($n = 5$).

Following L-NOArg ($100 \mu\text{M}$), bolus injections of BK ($0.01\text{--}1.0 \text{ nmol}$) produced responses which, although comparable in magnitude to the controls, were less well sustained. This decrease in duration which is expressed as half-recovery time ($t_{1/2}$) in Figure 5 was particularly pronounced at the higher doses of BK.

Infusion of L-arginine ($10\text{--}100 \mu\text{M}$) for 30 min failed to reverse the effects of L-NOArg on the duration of the BK response nor did it modify the vasodilator action of BK in the absence of L-NOArg. When administered as bolus injections, L-arginine ($1\text{--}30 \text{ nmol}$) produced a drop in basal tone ($6.2 \pm 0.5 \text{ mmHg}$; $n = 3$) the magnitude of which was independent of the dose applied.

Release of prostacyclin

Radioimmunoassay of cardiac effluent for PGI_2 release showed that levels of 6-keto- $\text{PGF}_{1\alpha}$ ($0.061 \pm 0.006 \text{ ng ml}^{-1}$; $n = 4$) detected following bolus injections of 0.1 nmol BK were not significantly different from basal levels

($0.063 \pm 0.004 \text{ ng ml}^{-1}$; $n = 7$). In addition to this, the infusion of either flurbiprofen ($2 \mu\text{M}$) or BW755C ($7.5 \mu\text{M}$) abolished the basal release of PGI_2 but did not inhibit the vasodilator action of BK. At the concentrations used BW755C however induced a marked drop in perfusion pressure, reducing the basal tone by $26.4 \pm 1.9 \text{ mmHg}$ ($n = 3$). A representative trace of the actions of BW755C is shown in Figure 6.

Figure 4 Effect of L- N^{G} -nitro arginine (L-NOArg) on bradykinin (BK)-induced vasodilatation in the rat isolated perfused heart. This tracing represents one of five separate experiments. PP denotes perfusion pressure, DT developed tension and HR heart rate.

Figure 5 Duration of the vasodilator response to bradykinin under control conditions (solid columns) and in the presence of $100 \mu\text{M}$ L- N^{G} -nitro arginine (open columns). Results represent the mean with s.e.mean shown by vertical bars ($n = 5$) of the time taken for the vasodilator response to return to 50% of its maximum.

Figure 6 Effect of BW755C ($7.5 \mu\text{M}$) on the vasodilator action of bradykinin (BK) in the rat isolated perfused heart. This tracing represents one of three separate experiments.

In view of the observation that EDRF and PGI_2 release appear to be coupled in aortic endothelial cells (de Nucci *et al.*, 1988; D'Orleans-Juste *et al.*, 1989) we examined the combined effect of L-NOArg (100 μM) and flurbiprofen (10 μM) on the vasodilator response to BK. This combination, like the action of L-NOArg alone, only reduced the duration of the BK-induced vasodilatation (data not shown). Flurbiprofen therefore did not potentiate the antagonistic action of L-NOArg.

Discussion

The data obtained from this study show that BK is a potent vasodilator of the resistance vessels in the rat isolated perfused heart, mediating its effects via the activation of the kinin B_2 receptors. The role of these receptors was confirmed by the potent competitive inhibition obtained with the kinin B_2 receptor antagonist [D-Arg⁰,Hyp³,Thi^{5,8},D-Phe⁷]-bradykinin and by the lack of a vasodilator action with the selective B_1 receptor agonist, Des-Arg⁹-bradykinin.

The responses elicited by BK were short-lived at lower doses but longer-lasting at the higher dose-range, indicating that the response consists of more than one component. This is supported by the finding that L-NOArg, a guanidino-substituted analogue of L-arginine (Moore *et al.*, 1990), reduced the duration but not the magnitude of the BK-induced vasodilatation. Since L-NOArg is known to inhibit the biosynthesis of NO (Ishii *et al.*, 1990; Moore *et al.*, 1990), it could imply that NO mediates the later phase of the vasodilator action of BK in the rat isolated heart. However, the inability of L-arginine to reverse the effects of L-NOArg raises the question as to whether L-NOArg is reducing the duration of the BK response by inhibiting the production of NO. It is possible that L-NOArg is having a direct inhibitory action on the later component of the BK response independent of an inhibition of NO synthesis.

The inability of MB (Martin *et al.*, 1985) and haemoglobin (Martin *et al.*, 1985; 1986) to inhibit, and SOD (Gryglewski *et al.*, 1986b) to potentiate the response to BK, adds support to the argument that BK does not produce vasodilatation in the rat heart by releasing NO. However, the lack of effect with haemoglobin and SOD does need to be treated with caution. These are both large molecular weight compounds which may not have access to the subendothelial site of action of NO. In addition if there is a high level of endogenous SOD and/or other superoxide free radical scavengers in the heart, these could mask the effects of exogenous SOD.

In contrast to SOD and haemoglobin, MB is a much smaller molecule which does have access to the extravascular space as indicated by its ability to depress cardiac contractility (Baydoun *et al.*, 1990). Although a low concentration (1 μM) of MB did increase coronary tone, possibly as a result of the inhibition of basal EDRF release, it had no effect on the BK-induced vasodilatation. Several studies have employed higher concentrations of MB (up to 100 μM) to block endothelium-mediated responses in tissues such as rabbit aortic rings

(Martin *et al.*, 1985; Warner *et al.*, 1989), and the perfused mesentery of the rat (Bhardwaj & Moore, 1988). However, in the rat heart, high concentrations of MB cause marked coronary dilatation and an irreversible depression of cardiac contractility (Baydoun *et al.*, 1990). As a result of this, these high concentrations of MB could not be used with any confidence in this study as they are probably producing non-specific detrimental effects.

In contrast to SOD, haemoglobin and MB, gossypol an inhibitor of the biosynthesis and/or release of EDRF (Alheid *et al.*, 1987), abolished the vasodilator action of BK. However, cardiac contractility and heart rate were irreversibly depressed and over the same concentration range gossypol also abolished the endothelium-independent dilator responses to sodium nitroprusside and the vasoconstrictor responses to endothelin-1. These findings suggest that in the rat heart the effects of gossypol are nonspecific and they do question the use and selectivity of the compound in inhibiting endothelium-mediated responses.

Finally, our study has shown clearly that metabolites of arachidonic acid, in particular PGI_2 , are not involved in mediating the vasodilator action of BK in the rat isolated heart. The action of BK was not associated with an increase in the level of 6-keto- $\text{PGF}_{1\alpha}$ in the perfusate, neither was it inhibited by flurbiprofen, or BW755C at concentrations which inhibited the basal release of PGI_2 . This finding is consistent with that of Stewart & Piper (1988) who have also concluded that PGI_2 does not mediate the vasodilator action of BK in the perfused guinea-pig heart. In bovine aortic endothelial cells, de Nucci *et al.* (1988) and D'Orleans-Juste *et al.* (1989) showed that BK-induced release of EDRF and PGI_2 synthesis were coupled, and that dual inhibition of EDRF and PGI_2 synthesis were required in order to block the actions of BK. From our data, the combination of flurbiprofen with L-NOArg had no greater inhibitory action on the effects of BK than L-NOArg alone. This shows that the rat coronary circulation behaves differently from bovine aortic endothelial cells in its response to BK.

In conclusion, this study has revealed that the vasodilator action of BK in the rat isolated perfused heart is mediated by the activation of the kinin B_2 receptors. The response appears to be biphasic with the sustained phase possibly involving the release of EDRF by virtue of its sensitivity to L-NOArg inhibition. However, the inability of L-arginine to reverse the inhibition and the lack of specific action by the other agents used, suggests that EDRF may not be the mediator. The suitability of several agents which are commonly used as tools for investigating endothelium-mediated responses is however questioned by some of our findings. It is also evident that extrapolation cannot always be made from studies using large conducting vessels and endothelial cells from these vessels to studies which involve the microvasculature.

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