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L-NAME-resistant bradykinin-induced relaxation in porcine coronary arteries is NO-dependent: effect of ACE inhibition

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1 NO synthase (NOS) inhibitors partially block bradykinin (BK)-mediated vasorelaxation. Here we investigated whether this is due to incomplete NOS inhibition and/or NO release from storage sites. We also studied the mechanism behind ACE inhibitor-mediated BK potentiation.

2 Porcine coronary arteries (PCAs) were mounted in organ baths, preconstricted, and exposed to BK or the ACE-resistant BK analogue Hyp^3-$Tyr(Me)^8$-BK (HT-BK) with or without the NOS inhibitor L-NAME (100 μM), the NO scavenger hydroxocobalamin (200 μM), the Ca^{2+}-dependent K^+-channel blockers charybdotoxin + apamin (both 100 nM), or the ACE inhibitor quinaprilat (10 μM).

3 BK and HT-BK dose-dependently relaxed preconstricted vessels (pEC_{50} 8.0 ± 0.1 and 8.5 ± 0.2, respectively). pEC_{50}'s were $\approx$10 fold higher with quinaprilat, and $\approx$10 fold lower with L-NAME or charybdotoxin + apamin. Complete blockade was obtained with hydroxocobalamin or L-NAME + charybdotoxin + apamin.

4 Repeated exposure to 100 nM BK or HT-BK, to deplete NO storage sites, produced progressively smaller vasorelaxant responses. With L-NAME, the decrease in response occurred much more rapidly. L-Arginine (10 mM) reversed the effect of L-NAME.

5 Adding quinaprilat to the bath following repeated exposure (with or without L-NAME), at the time BK and HT-BK no longer induced relaxation, fully restored vasorelaxation, while quinaprilat alone had no effect. Quinaprilat also relaxed vessels that, due to pretreatment with hydroxocobalamin or L-NAME + charybdotoxin + apamin, previously had not responded to BK.

6 In conclusion, L-NAME-resistant BK-induced relaxation in PCAs depends on NO from storage sites, and is mediated *via* stimulation of guanylyl cyclase and/or Ca^{2+}-dependent K^+-channels. ACE inhibitors potentiate BK independent of their effect on BK metabolism.
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Keywords: ACE inhibitor; bradykinin; coronary artery; nitric oxide; NO synthase

Abbreviations: ACE, angiotensin-converting enzyme; B_2, bradykinin type 2 receptor; BK_{Ca}, large-conductance voltage and Ca^{2+}-activated K^+-channel; CRC, concentration-response curve; Hoe140, D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin; L-NAME, N^{ω}-nitro-L-arginine methyl ester HCl; 7-NI, 7-nitroindazole; NO, nitric oxide; NOS, NO synthase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PCA, porcine coronary artery; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; SK_{Ca}, small-conductance Ca^{2+}-activated K^+-channel; U46619, 9,11-dideoxy-11α,9α-epoxy-methano-prostaglandin $F_{2\alpha}$

Introduction

Bradykinin induces vasodilation *via* endothelial bradykinin type 2 (B_2) receptors. This effect can be blocked partly by inhibitors of nitric oxide synthase (NOS), suggesting a role for *de-novo* synthesis of nitric oxide (NO) from L-arginine by NOS (Palmer *et al*., 1988; Gardiner *et al*., 1990; Rees *et al*., 1990; Mombouli *et al*., 1992; Bjornstad-Ostensen *et al*., 1997). The relaxant effect of bradykinin that is not blocked by NOS inhibitors is generally attributed to so-called endothelium-derived hyperpolarizing factors, of which the exact identity has not yet been established. Several candidates have been proposed, including prostacyclin, potassium and cytochrome P-450 products of arachidonic acid (Mombouli & Vanhoutte, 1997; Edwards *et al*., 1998; Fisslthaler *et al*., 1999). However, NOS inhibitors, even at high concentrations, do not block NO release completely (Cohen *et al*., 1997). Moreover, *in-vivo* studies in the rat hindlimb (Davisson *et al*., 1996) and *in-vitro* studies in the isolated perfused rat heart (Danser *et al*., 1998) have shown that bradykinin also induces release of NO from stores of NO-containing factors, such as S-nitroso-thiols and dinitrosyl iron (II) thiol complexes (Ignarro, 1990; Myers *et al*., 1990; Verdernikov *et al*., 1992). Depletion of such stores occurred only after repeated exposure to bradykinin or after prolonged inhibition of NOS, treatments which themselves do not alter the response to NO (Davisson *et al*., 1996; Danser *et al*., 1998). Taken together therefore, bradykinin-induced relaxation in the presence of NOS inhibitors might also be due to NO generated by residual NOS activity or to NO released from storage sites.

Angiotensin-converting enzyme (ACE) inhibitors block bradykinin degradation. Accumulation of bradykinin is believed to contribute to the beneficial effects of ACE inhibitors in hypertension and heart failure, although elevated bradykinin levels were not always found during ACE inhibitor treatment (Miki *et al*., 1996; Campbell *et al*., 1999). Two recent studies propose that ACE inhibitors potentiate bradykinin beyond blocking its hydrolysis, by inhibiting desensitization of its receptor. These studies were performed in Chinese hamster ovary cells transfected with human B_2 receptors and human ACE (Minshall *et al*., 1997) and in porcine aortic endothelial cells that naturally express these proteins (Benzing *et al*., 1999). The mechanism underlying the ACE inhibitor-induced inhibition of B_2 receptor desensitization is currently unknown,

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but it may involve interference with the translocation of B_2 receptor to caveolin-rich membrane domains (Haasemann *et al*., 1998; Benzing *et al*., 1999; Marcic *et al*., 1999). Dendorfer *et al*. (2000) however, using the isolated perfused rat Langendorff heart, found no evidence for ACE inhibitor-induced B_2 receptor upregulation and suggested that the ACE inhibitor-induced potentiation of bradykinin was due to inhibition of bradykinin degradation in the vicinity of B_2 receptors (e.g., in caveolae).

It was the aim of the present study to investigate, in intact porcine coronary arteries (PCAs), (1) whether the NOS inhibitor-resistant bradykinin-induced vasorelaxation involves NO, and (2) whether the ACE inhibitor quinaprilat potentiates bradykinin *via* blockade of bradykinin metabolism or *via* other mechanisms. To address the second question, we used the ACE-resistant bradykinin analogue [Hyp^3-Tyr(Me)^8]-bradykinin (Rhaleb *et al*., 1990; Minshall *et al*., 1997).

Methods

Drugs

Bradykinin (acetate salt), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619), substance P (acetate salt), L-arginine HCl, N$^{\omega}$-nitro-L-arginine methyl ester HCl (L-NAME), aminoguanidine, 7-nitroindazole (7-NI), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), hydroxocobalamin (acetate salt), indomethacin, glibenclamide, charybdotoxin, apamin, sulphaphenazole and captopril were from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). [Hyp^3-Tyr(Me)^8]-bradykinin was from Calbiochem/Novabiochem AG, Läufelfingen, Switzerland. D-Arg[Hyp^3,Thi^5,D-Tic^7,Oic^8]-bradykinin (Hoe140) was a kind gift of Dr W. Linz, Hoechst, Frankfurt, Germany. Quinaprilat was a kind gift of Dr H. van Ingen, Parke-Davis, Hoofddorp, The Netherlands. 7-NI, indomethacin, glibenclamide and quinaprilat were dissolved in dimethylsulphoxide. Sulphaphenazole was dissolved in ethanol. Hydroxocobalamin was dissolved in methanol. All other chemicals were dissolved in saline.

Tissue collection

Porcine coronary arteries were obtained from 31 2–3 month-old pigs (Yorkshire × Landrace, weight 10–15 kg). The pigs had been used in *in-vivo* experiments studying the effects of α-adrenoceptor and serotonin receptor agonists and antagonists under pentobarbital (600 mg, i.v.) anaesthesia (de Vries *et al*., 1999; Willems *et al*., 1999). The Ethics Committee of the Erasmus University Rotterdam dealing with the use of animals for scientific experiments approved the protocol for this investigation. Hearts were explanted at the end of the experiment, and the coronary arteries were removed immediately and stored overnight in a cold, oxygenated Krebs bicarbonate solution of the following composition (mmol l^{-1}): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25 and glucose 8.3; pH 7.4. Vessels were then cut into segments of approximately 4 mm length, suspended on stainless steel hooks in 15 ml organ baths containing Krebs bicarbonate solution, aerated with 95% O_2/5% CO_2, and maintained at 37°C.

Organ bath studies

All vessel segments were allowed to equilibrate for at least 30 min and the organ bath fluid was refreshed every 15 min during this period. Changes in tissue contractile force were recorded with a Harvard isometric transducer (South Natick, MA, U.S.A.). The vessel segments, stretched to a stable force of about 15 mN, were exposed to 30 mmol l^{-1} K^+ twice. The functional integrity of the endothelium was verified by observing relaxation to 1 nM substance P after preconstriction with 1 μM $PGF_{2\alpha}$. Subsequently, the tissue was exposed to 100 mmol l^{-1} K^+ to determine the maximal contractile response to K^+. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 min. Thereafter, the following experiments were performed.

First, possible mediators of the relaxant effect of bradykinin with and without NOS inhibition were investigated. Vessels were pre-incubated for 30 min in the absence or presence of the non-selective NOS inhibitor L-NAME (100 μM), the inducible NOS inhibitor aminoguanidine (1 mM), the neuronal NOS inhibitor 7-NI (10 μM), the guanylyl cyclase inhibitor ODQ (10 μM), the NO scavenger hydroxocobalamin (200 μM), the cyclooxygenase inhibitor indomethacin (10 μM), the ATP-sensitive K^+-channel inhibitor glibenclamide (1 μM), the large-conductance voltage and Ca^{2+}-activated K^+-channel (BK_{Ca}) blocker charybdotoxin + the small-conductance Ca^{2+}-activated K^+-channel (SK_{Ca}) blocker apamin (both 100 nM), the cytochrome P-450 inhibitor sulphaphenazole (10 μM) or the B_2 receptor antagonist Hoe140 (1 μM). Vessels were then preconstricted with 10 μM $PGF_{2\alpha}$ or 1 μM U46619 and concentration-response curves (CRCs) to bradykinin (0.1 nM – 1 μM) were constructed.

Second, to test whether desensitization of the bradykinin-induced relaxation occurs more rapidly with NOS inhibition (due to more rapid depletion of NO storage sites), preconstricted vessel segments were exposed three times to a concentration of bradykinin (0.1 μM) that is capable of inducing maximal relaxation. Each subsequent exposure was started as soon as the effect of the previous exposure had disappeared, i.e., after approximately 15 min. To investigate whether L-arginine could reverse the effect of L-NAME, the repetitive exposure experiments were repeated in the presence of L-arginine (10 mM), using a concentration of bradykinin (10 nM) that induces submaximal relaxation. To rule out NOS inhibitor-related differences in B_2 receptor desensitization, we constructed two consecutive bradykinin CRCs in a preconstricted vessel segment in the presence or absence of L-NAME.

Third, the effect of ACE inhibition on bradykinin-induced relaxation was investigated. Vessel segments were preconstricted with 10 μM $PGF_{2\alpha}$ or 1 μM U46619 and CRCs were constructed to quinaprilat (1 nM – 10 μM) to verify the presence of endogenous bradykinin. Next, in the presence of the highest concentration of quinaprilat, CRCs were constructed to bradykinin and the ACE-resistant bradykinin analogue [Hyp^3-Tyr(Me)^8]-bradykinin (0.1 nM – 1 μM). In addition, quinaprilat (10 μM) or captopril (100 μM) were added to preconstricted vessels with desensitized B_2 receptors (i.e., vessels that had been exposed three times to a concentration of bradykinin (0.1 μM) that is capable of inducing maximal relaxation; see above). For comparison, quinaprilat (10 μM) was also added to preconstricted vessels that had been exposed three times to another ACE substrate, substance P, at a concentration (1 nM) that is capable of inducing maximal relaxation.

Statistical analysis

Data are given as mean ± s.e.mean and expressed as a percentage of the contraction in response to $PGF_{2\alpha}$ or U46619. CRCs were analysed using the logistic function

described by de Lean *et al*., (1978) to obtain pEC_{50} ($-^{10}\log EC_{50}$) values. The addition of L-NAME, ODQ, hydroxocobalamin, L-NAME + ODQ or L-NAME + hydroxocobalamin caused an increase in basal tone of 6 ± 1 mN ($n = 31$), 11 ± 2 mN ($n = 6$), 8 ± 1 mN ($n = 6$), 8 ± 1 mN ($n = 6$) and 9 ± 2 mN ($n = 6$), respectively. The $PGF_{2\alpha}$- and U46619-induced preconstrictions were corrected for this increase in baseline. Statistical analysis was by ANOVA, followed by *post hoc* evaluation (according to Tukey or Dunnett where appropriate). P values < 0.05 were considered significant.

Results

Precontractions

The $PGF_{2\alpha}$- and U46619-induced precontractions in control vessels did not differ and amounted to approximately 30% (13 ± 1 mN, $n = 31$) of the maximal contraction induced by 100 mmol l^{-1} K^+. Precontractions were not affected by aminoguanidine, 7-NI, indomethacin, glibenclamide, charybdotoxin + apamin, sulphaphenazole or Hoe140. In the vessel segments pretreated with L-NAME, ODQ, hydroxocobalamin, L-NAME + ODQ, or L-NAME + hydroxocobalamin, the precontractions (23 ± 1 mN, $n = 31$; 20 ± 2 mN, $n = 6$; 28 ± 2 mN, $n = 6$; 21 ± 3 mN, $n = 6$; and 24 ± 2 mN, $n = 6$, respectively) were approximately 2 fold higher than in control vessel segments ($P < 0.01$), which illustrates the importance of endogenous NO generation by endothelial NOS in this preparation.

Mediators of the relaxant effect of bradykinin

Bradykinin caused complete relaxation of preconstricted vessel segments in a concentration-dependent manner ($pEC_{50} = 8.03 \pm 0.05$, $n = 31$; Figure 1). The bradykinin CRC was not affected by aminoguanidine ($pEC_{50} = 8.21 \pm 0.11$, $n = 5$), 7-NI ($pEC_{50} = 7.98 \pm 0.22$, $n = 5$), indomethacin ($pEC_{50} = 7.58 \pm 0.22$, $n = 5$), glibenclamide ($pEC_{50} = 8.35 \pm 0.28$, $n = 5$) or sulphaphenazole ($pEC_{50} = 7.63 \pm 0.07$, $n = 6$). L-NAME ($pEC_{50} = 6.93 \pm 0.07$, $n = 25$; $P < 0.01$ vs control) and ODQ ($pEC_{50} = 7.19 \pm 0.30$, $n = 5$; $P < 0.05$ vs control) shifted the CRC of bradykinin to the right, while in the presence of hydroxocobalamin ($n = 6$) relaxation was only observed at the highest concentration of bradykinin (Figure 1). Charybdotoxin + apamin also shifted the CRC of bradykinin to the right ($pEC_{50} = 6.47 \pm 0.10$, $n = 6$; $P < 0.01$ vs control, Figure 1). Complete blockade of the response was obtained with Hoe140 (Figure 1). The addition of indomethacin ($pEC_{50} = 7.19 \pm 0.16$, $n = 4$) or sulphaphenazole ($pEC_{50} = 7.08 \pm 0.04$, $n = 6$) on top of L-NAME did not cause a further rightward shift of the bradykinin CRC as compared to L-NAME alone (data not shown), nor did the addition of ODQ on top of L-NAME ($pEC_{50} = 7.24 \pm 0.12$, $n = 5$; Figure 2). Charybdotoxin + apamin in combination with L-NAME completely blocked the response to all bradykinin concentrations tested (Figure 2), whereas in the presence of L-NAME + hydroxocobalamin ($n = 6$) relaxation was again observed at the highest concentration of bradykinin only (Figure 2).

Desensitization of the bradykinin-induced effect

The construction of a bradykinin CRC resulted in B_2 receptor desensitization, as evidenced by the approximate 10 fold rightward shift that was observed when constructing a second bradykinin CRC in the same vessel segment (Figure 3). A similar rightward shift was observed in the presence of L-NAME.

Repeated exposure of preconstricted vessel segments to 0.1 μM bradykinin produced progressively smaller relaxant responses (Figure 4, top panel). The response to the third bradykinin dose was less than 50% of the response to the first bradykinin dose (Figure 4, bottom panel). A similar pattern was observed in the presence of charybdotoxin + apamin,

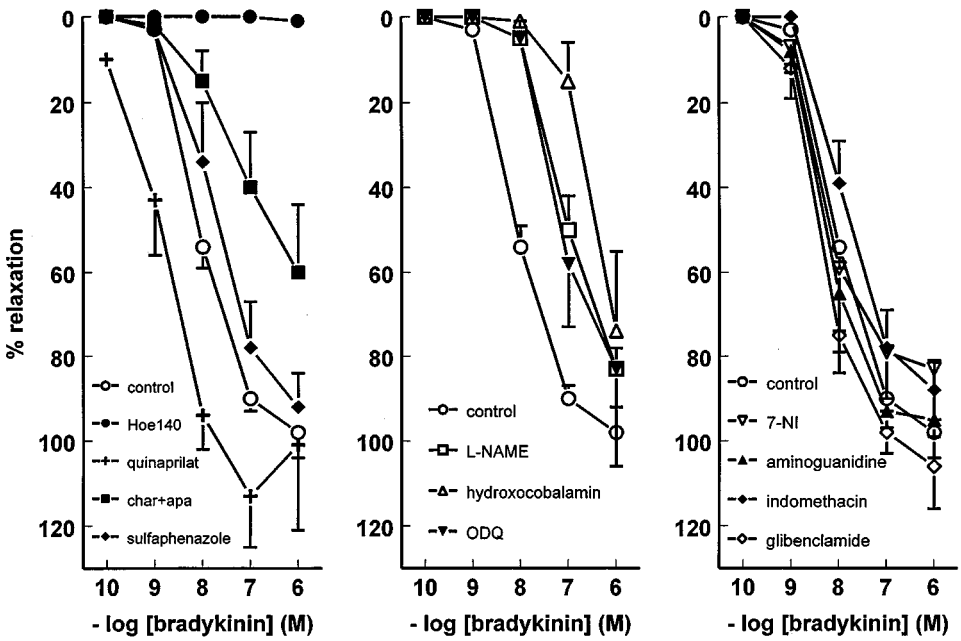

Figure 1 Relaxations of PCAs, preconstricted with 10 μM $PGF_{2\alpha}$ or 1 μM U46619, to bradykinin in the absence (control) or presence of 1 μM Hoe140, 10 μM quinaprilat, 100 nM charybdotoxin (char) + 100 nM apamin (apa), 10 μM sulphaphenazole, 100 μM L-NAME, 200 μM hydroxocobalamin, 10 μM ODQ, 10 μM 7-NI, 1 mM aminoguanidine, 10 μM indomethacin or 1 μM glibenclamide. For the sake of clarity, data have been divided across three panels, and the control curve is shown in each panel. Data (mean ± s.e.mean of five to 31 experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$ or U46619.

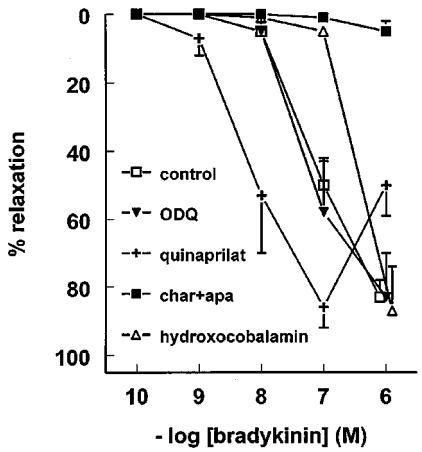

Figure 2 Relaxations of PCAs, following preconstriction with 10 μM $PGF_{2\alpha}$ or 1 μM U46619, to bradykinin in the presence of 100 μM L-NAME without (control) or with 10 μM ODQ, 10 μM quinaprilat, 100 nM charybdotoxin (char) + 100 nM apamin (apa) or 200 μM hydroxocobalamin. Data (mean ± s.e.mean of five to 25 experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$ or U46619.

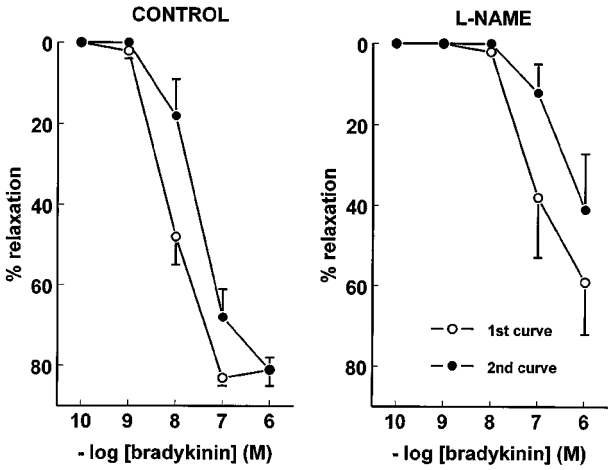

Figure 3 Two consecutive concentration-response curves, obtained in the same PCA vessel segment following preconstriction with 10 μM $PGF_{2\alpha}$, to bradykinin in the absence (left panel) or presence (right panel) of 100 μM L-NAME. Data (mean ± s.e.mean of five experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$.

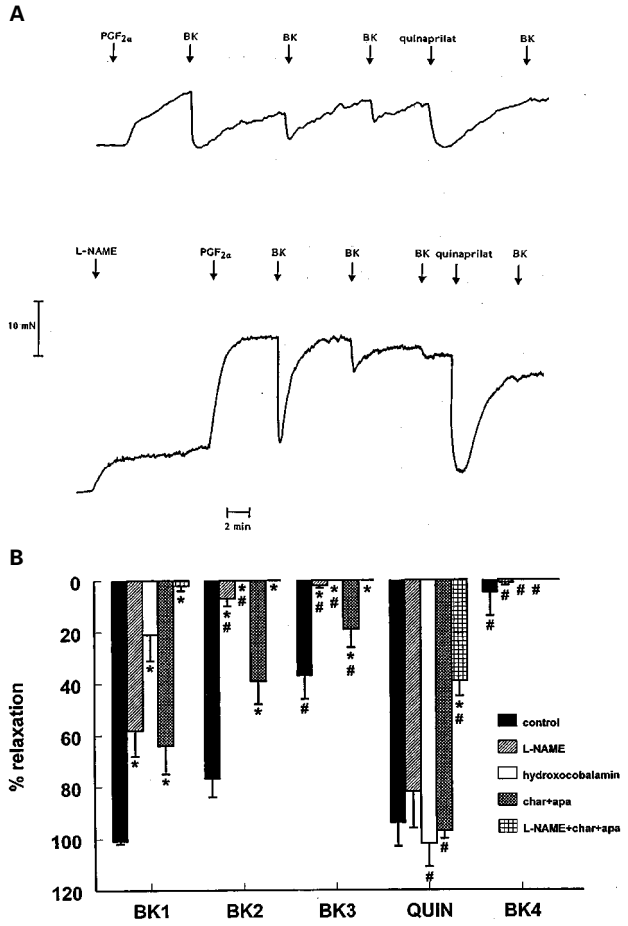

Figure 4 (A) Original tracings of an experiment in which a PCA in the absence (control; top) or presence (bottom) of 100 μM L-NAME was preconstricted with 10 μM $PGF_{2\alpha}$ and subsequently exposed to 0.1 μM bradykinin (BK, three times), 10 μM quinaprilat and 0.1 μM bradykinin. (B) Relaxations of PCAs, following preconstriction with 10 μM $PGF_{2\alpha}$ or 1 μM U46619, to three consecutive bradykinin doses (0.1 μM; BK1, BK2, BK3), 10 μM quinaprilat (QUIN) and a fourth bradykinin dose (0.1 μM; BK4) in the absence or presence of 100 μM L-NAME, 200 μM hydroxocobalamin, 100 nM charybdotoxin (char) + 100 nM apamin (apa), or 100 μM L-NAME + 100 nM charybdotoxin (char) + 100 nM apamin (apa). Data (mean ± s.e.mean of six to 24 experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$ or U46619. *$P<0.01$ vs control; #$P<0.05$ vs BK1.

although the relaxation to bradykinin in the presence of these drugs was always smaller than under control conditions (Figure 4, bottom panel). In the presence of L-NAME (Figure 4, top panel) or hydroxocobalamin (Figure 4, bottom panel), the relaxation observed in response to the first dose of bradykinin was significantly smaller than under control conditions, and relaxation was virtually absent in response to the second and third bradykinin dose. Results obtained with L-NAME + hydroxocobalamin were not different from those with hydroxocobalamin alone (data not shown). Charybdotoxin + apamin combined with L-NAME fully prevented all responses to bradykinin (Figure 4, bottom panel).

Exposing preconstricted vessels three times to a submaximal concentration of bradykinin (10 nM) revealed that, under control conditions, the relaxation in response to the third bradykinin dose was not significantly different from the response to the first bradykinin dose (Figure 5). In the presence of L-NAME, the first bradykinin dose induced a modest relaxation, and no further response was observed at the second and third exposure to bradykinin. L-Arginine reversed the inhibitory effect of L-NAME, and tended to enhance the effect of bradykinin in the absence of L-NAME (P = NS).

Effect of ACE inhibition on bradykinin-induced relaxation

Quinaprilat alone did not cause relaxation of preconstricted vessel segments (data not shown), thereby ruling out the presence of endogenous bradykinin. In the presence of the ACE inhibitor, the CRC to bradykinin was shifted to the left ($pEC_{50} = 8.89 \pm 0.20$, $n = 5$; $P<0.05$ vs control, see Figure 1). This was also the case in vessel segments that had been pre-incubated with L-NAME ($pEC_{50} = 8.03 \pm 0.23$, $n = 5$; $P<0.05$ vs L-NAME alone, see Figure 2). Quinaprilat caused a similar leftward shift of the CRC to the ACE-resistant analogue [Hyp^3-$Tyr(Me)^8$]-bradykinin (pEC_{50}'s resp. 8.50 ± 0.18 and 9.18 ± 0.06 without and with quinaprilat, $n = 4$; $P<0.05$, Figure 6). Hoe140 fully blocked the effects of [Hyp^3-$Tyr(Me)^8$]-bradykinin, confirming that this agonist induces relaxation *via* stimulation of B_2 receptors.

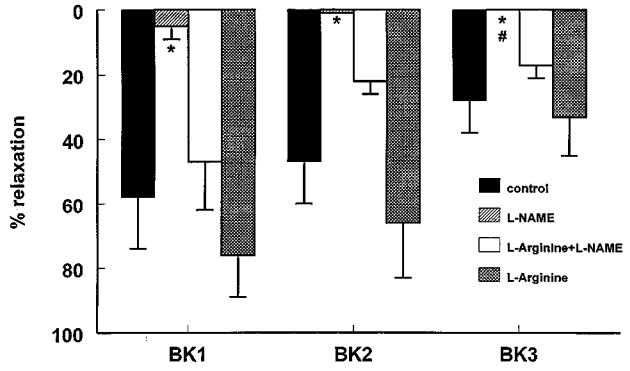

Figure 5 Relaxations of PCAs, following preconstriction with 10 μM $PGF_{2\alpha}$, to three consecutive bradykinin doses (10 nM; BK1, BK2, BK3) in the absence or presence of 100 μM L-NAME, 100 μM L-NAME + 10 mM L-arginine, or 10 mM L-arginine. Data (mean ± s.e.mean of five to seven experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$. $^*P<0.01$ vs control; $\#P<0.05$ vs BK1.

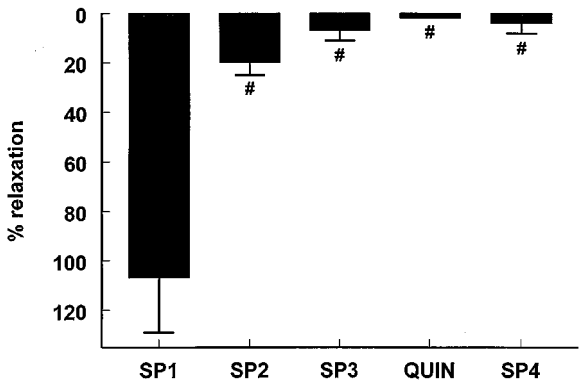

Figure 7 Relaxations of PCAs, following preconstriction with 10 μM $PGF_{2\alpha}$, to three consecutive doses of substance P (1 nM; SP1, SP2, SP3), 10 μM quinaprilat (QUIN) and a fourth dose of substance P (1 nM; SP4). Data (mean ± s.e.mean of five experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$. $\#P<0.01$ vs SP1.

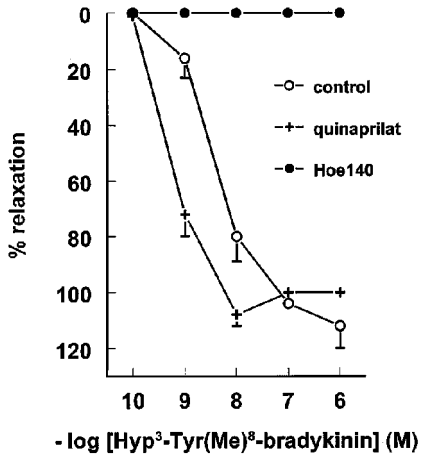

Figure 6 Relaxations of PCAs, preconstricted with 10 μM $PGF_{2\alpha}$, to [Hyp^3-$Tyr(Me)^8$]-bradykinin in the absence or presence of 1 μM Hoe140 or 10 μM quinaprilat. Data (mean ± s.e.mean of four experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$.

Quinaprilat added to vessel segments that had been exposed three times to 0.1 μM bradykinin, after the effect of the last dose of bradykinin had disappeared, caused complete relaxation, even in L-NAME- or hydroxocobalamin-pretreated vessel segments that previously had not responded to bradykinin (Figure 4). Quinaprilat also induced complete relaxation of vessel segments pretreated with charybdotoxin + apamin, whereas in vessel segments pretreated with L-NAME combined with charybdotoxin + apamin, which previously had not shown any response to bradykinin, the ACE inhibitor induced a modest relaxant response. Similar results were obtained with captopril ($n=5$, data not shown). A fourth bradykinin dose, added after the effect of quinaprilat or captopril had disappeared, induced no further effect.

Results obtained with bradykinin and quinaprilat in the presence of aminoguanidine ($n=5$), 7-NI ($n=5$), indomethacin ($n=4$), glibenclamide ($n=4$) and sulphaphenazole ($n=6$) were not different from those obtained in the absence of these inhibitors (data not shown). Moreover, results obtained with [Hyp^3-$Tyr(Me)^8$]-bradykinin and quinaprilat ($n=4$) exactly mimicked those with bradykinin and quinaprilat (data not shown). Hoe140 completely prevented the quinaprilat-induced potentiation (data not shown). The effect of quinaprilat was specific for bradykinin, since it was not observed in combination with substance *P* (Figure 7).

Discussion

NO is responsible for bradykinin-induced vasorelaxation in porcine coronary arteries

The results of the present study show that the B_2 receptor-mediated relaxant effects of bradykinin in PCAs, at doses up to 0.1 μM, depend on NO, either synthesized *de novo* by endothelial NOS or derived from NO storage sites. These data fully support the interaction between endothelial NOS and B_2 receptors that was recently described by Golser *et al.*, (2000). We found no evidence for a role of NO synthases other than endothelial NOS in PCAs, since neither aminoguanidine, a preferential inhibitor of inducible NOS (Joly *et al.*, 1994; Boulanger *et al.*, 1998), nor 7-NI, a preferential inhibitor of neuronal NOS (Moore *et al.*, 1993; Boulanger *et al.*, 1998) affected basal tone or the bradykinin CRC. Furthermore, neither prostaglandins, ATP-sensitive K^+-channels nor cytochrome P-450 products appeared to be involved in the bradykinin-induced vasodilation in PCAs. The latter contrasts with a recent observation in PCAs showing that bradykinin-induced relaxation in the presence of the NOS inhibitor N^{ω}nitro-L-arginine was due to the release of 11,12-epoxyeicosatrienoic acid, an arachidonic acid metabolite formed by cytochrome P-450 (Fisslthaler *et al.*, 1999). However, the EC_{50} of this effect was approximately 1 μM (Fisslthaler *et al.*, 1999), and we did not test bradykinin concentrations above 1 μM.

The 10 fold rightward shift of the bradykinin CRC induced by both the non-selective NOS inhibitor L-NAME and the guanylyl cyclase inhibitor ODQ contrasts with the much more complete blockade observed in the presence of the NO scavenger hydroxocobalamin. Hydroxocobalamin did not block the effect of 1 μM bradykinin. This is not due to the formation of relaxant prostaglandins or cytochrome P-450 products at this concentration of bradykinin, since indomethacin and sulphaphenazole did not affect the bradykinin CRC in the presence of L-NAME. The most likely explanation is that, at the concentration used in the present study (200 μM), hydroxocobalamin did not completely scavenge all NO (Li & Rand, 1999). The maximum solubility of hydroxocobalamin in

methanol (10 mg/ml) prevented us from reaching higher concentrations in the organ bath. Taken together therefore, NO release is responsible for the bradykinin-induced relaxation in PCAs, at least at bradykinin concentrations up to 0.1 μM, and NOS blockade as well as guanylyl cyclase blockade cannot prevent this relaxation completely.

The modest effect of ODQ might be due to the fact that this drug inhibits guanylyl cyclase reversibly (Garthwaite *et al*., 1995). Alternatively, NO-induced relaxation may occur independently of guanylyl cyclase. In support of the first possibility, we observed that ODQ fully inhibited the relaxant effects of low (<1 μM) but not of high ($\geqslant 1$ μM) concentrations of the NO donor S-nitroso-N-acetylpenicillamine (Danser *et al*., 1999, unpublished observations). In support of the second possibility, NO has been described to induce hyperpolarization directly *via* activation of Ca^{2+}-dependent K^+-channels (Bolotina *et al*., 1994), and bradykinin is known to activate these channels through stimulation of tyrosine kinase (Lee *et al*., 1993; Ogiwara *et al*., 1995). Indeed, in the present study the BK_{Ca} blocker charybdotoxin and the SK_{Ca} blocker apamin partially blocked the bradykinin-induced relaxation when given together, and fully blocked the bradykinin-induced effects when given in combination with L-NAME. Charybdotoxin plus apamin also blocked the bradykinin-induced hyperpolarization of PCA rings (Quignard *et al*., 1999). Thus, the most likely explanation for our findings is that the bradykinin-induced NO release causes vasodilation *via* stimulation of guanylyl cyclase and/or *via* direct activation of Ca^{2+}-dependent K^+-channels.

Release of NO from storage sites?

The limited effect of NOS blockade on NO release has been described before in rabbit carotid arteries (Cohen *et al*., 1997), and may involve either residual NOS activity or release of NO from storage sites (Davisson *et al*., 1996; Danser *et al*., 1998). To investigate the latter, we exposed coronary arteries repeatedly to the same concentration of bradykinin. Previous studies have shown that repetitive exposure to bradykinin or acetylcholine will cause depletion of NO storage sites, and that this will occur more rapidly in the presence of NOS inhibitors (Davisson *et al*., 1996; Colombari *et al*., 1998; Danser *et al*., 1998). In the present study, exposure to bradykinin, both with and without L-NAME, resulted in a relaxant effect which lasted approximately 10–15 min. The disappearance of the relaxation is suggestive for bradykinin B_2 receptor desensitization and/or bradykinin metabolism.

Desensitization was similar with and without L-NAME (Figure 3). Subsequent exposures to bradykinin initiated progressively smaller relaxant effects, and the decrease in response was much more rapid in the presence of L-NAME. L-Arginine reversed the rapid decrease in response to consecutive bradykinin doses in the presence of L-NAME. Taken together, these findings support the concept of bradykinin-coupling to NO storage sites. With NOS activity intact, the NO pools are continuously supplied with fresh NO and relaxation can be obtained multiple times, even when B_2 receptors are desensitized. During NOS inhibition the NO storage sites will become depleted, especially during exposure to drugs that cause release of NO from these sites. In an earlier study in isolated perfused rat hearts, we found that a 30 min exposure to L-NAME was sufficient to cause depletion of all existing NO pools (Danser *et al*., 1998). Depletion may also occur during exposure to high levels of superoxide anion (Arnal *et al*., 1996), although in PCAs superoxide anions scavengers did not affect the response to bradykinin (Pomposiello *et al*., 1999).

The nature and localization of NO pools is currently unknown. Although NO pools have been demonstrated in vascular smooth muscle cells (Venturini *et al*., 1993), the pools in the present study are most likely localized in endothelial cells, in view of the fact that we (Danser *et al*., 1999, unpublished results) and others (Mombouli *et al*., 1992) have found that bradykinin-induced relaxations are virtually absent following endothelium removal. The long half life of NO present at storage sites, evidenced by the fact that bradykinin was still capable of inducing relaxation after the vessels had been exposed to L-NAME for more than 30 min, is compatible with the idea that NO pools consist of stable NO-containing compounds, such as S-nitroso-thiols and dinitrosyl iron (II) thiol complexes (Ignarro, 1990; Myers *et al*., 1990; Verdernikov *et al*., 1992).

ACE inhibitors potentiate bradykinin independently of their effect on bradykinin metabolism

Finally, the ACE inhibitor quinaprilat, added at a time when the relaxant effect of bradykinin had disappeared, immediately restored the vasorelaxation, both with and without L-NAME. The effect of quinaprilat could be mimicked by captopril, and did not occur in combination with the B_2 receptor antagonist Hoe 140 or without prior exposure to bradykinin. The latter finding suggests that not all bradykinin has been metabolized at the time the ACE inhibitor is added to the organ bath. When added prior to bradykinin, quinaprilat shifted the bradykinin CRC approximately 10 fold to the left, both with and without L-NAME. It is unlikely that the potentiating effects of ACE inhibition are due simply to inhibition of bradykinin metabolism, as suggested by Dendorfer *et al*., (2000), because (1) the effect of quinaprilat was also observed in combination with the ACE-resistant bradykinin analogue [Hyp^3-$Tyr(Me)^8$]-bradykinin, (2) the effect of quinaprilat was not observed in relationship with the vasorelaxant ACE substrate substance P, and (3) quinaprilat even induced complete relaxation in vessel segments that had previously not responded to bradykinin, i.e., vessel segments that had been preincubated with hydroxocobalamin or charbdotoxin + apamin + L-NAME. Taken together therefore, our data support the concept of ACE inhibitor-induced bradykinin potentiation independently of the effect of these drugs on bradykinin metabolism. The mechanism underlying this phenomenon is currently unknown, but it may involve the ACE inhibitor-induced resensitization of desensitized B_2 receptors that has been described in isolated cells (Minshall *et al*., 1997; Benzing *et al*., 1999). Resensitized B_2 receptors may still cause relaxation *via* coupling to remaining NO pools, even after exposure to L-NAME and hydroxocobalamin, since we do not know whether these drugs, combined with repetitive exposure to bradykinin, have resulted in complete depletion of all existing NO pools. Alternatively, non-NO-related mechanisms may have come into play. These mechanisms do not involve prostaglandins, ATP-sensitive K^+-channels or cytochrome P-450 products, since indomethacin, glibenclamide and sulphaphenazole did not affect the quinaprilat-induced relaxation.

Conclusions and possible clinical implications

In conclusion, the L-NAME-resistant bradykinin-induced relaxation, at least at physiological bradykinin concentrations (i.e., concentrations up to 0.1 μM; Campbell *et al*., 1993), is NO-dependent, and is mediated *via* stimulation of guanylyl cyclase and/or Ca^{2+}-dependent K^+-channels. NO is either

synthesized *de novo* by endothelial NOS or released from storage sites. Depletion of such sites or a decrease in their number might be involved in the impaired endothelium-dependent vasodilatory response observed in subjects with hypertension or atherosclerosis (Hirooka *et al*., 1992; Zeiher *et al*., 1993). ACE inhibitors potentiate bradykinin-induced vasorelaxation independently of their effect on bradykinin metabolism. Such potentiation has also been observed *in vivo* in human subjects (Hornig *et al*., 1997; Kuga *et al*., 1997). Since elevated bradykinin levels have not been found consistently during ACE inhibitor treatment (Miki *et al*., 1996; Campbell *et al*., 1999), our findings might explain, at least in part, the beneficial effects of ACE inhibitors in hypertension and heart failure, as well as the ACE inhibitor-induced reversal of the impaired endothelium-dependent vasorelaxation in hypertensive patients (Hirooka *et al*., 1992).

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