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ACE inhibitor potentiation of bradykinin-induced venoconstriction

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1 Angiotensin-converting enzyme (ACE) inhibitors exert their cardiovascular effects not only by preventing the formation of angiotensin II (AII), but also by promoting the accumulation of bradykinin in or at the vessel wall. In addition, certain ACE inhibitors have been shown to augment the vasodilator response to bradykinin, presumably by an interaction at the level of the B_2 receptor. We have investigated whether this is a specific effect of the ACE inhibitor class of compounds in isolated endothelium-denuded segments of the rabbit jugular vein where bradykinin elicits a constrictor response which is exclusively mediated by activation of the B_2 receptor.

2 Moexiprilat and ramiprilat ($\leqslant$ 3 nM) enhanced the constrictor response to bradykinin three to four fold. Captopril and enalaprilat were less active by approximately one and quinaprilat by two orders of magnitude. Moexiprilat and ramiprilat, on the other hand, had no effect on the constrictor response to AII or the dilator response to acetylcholine.

3 The bradykinin-potentiating effect of the ACE inhibitors was not mimicked by inhibitors of amino-, carboxy-, metallo- or serine peptidases or the synthetic ACE substrate, hippuryl-L-histidyl-L-leucine, at a concentration which almost abolished the residual ACE activity in the vessel wall. In contrast, angiotensin-(1–7) (10 μM), an angiotensin I metabolite, significantly enhanced the constrictor response to bradykinin.

4 Ramiprilat did not alter the binding of [^{3}H]-bradykinin to a membrane fraction prepared from endothelium-denuded rabbit jugular veins or to cultured fibroblasts, and there was no ACE inhibitor-sensitive, bradykinin-induced cleavage of the B_2 receptor in cultured endothelial cells.

5 These findings demonstrate that ACE inhibitors selectively potentiate the B_2 receptor-mediated vascular effects of bradykinin. Their relative efficacy appears to be independent of their ACE-inhibiting properties and might be related to differences in molecule structure. Moreover, the potentiation of the biological activity of bradykinin by this class of compounds does not seem to be mediated by a shift in affinity of the B_2 receptor or a prevention of its desensitization, but may involve an increase in the intrinsic activity of unoccupied B_2 receptor molecules.

Keywords: ACE inhibitors; acetylcholine; angiotensin II; angiotensin-(1–7); bradykinin; B_2 receptor; jugular vein, rabbit; vasoconstriction

Introduction

Angiotensin-converting enzyme (ACE) inhibitors are widely used in the treatment of hypertension and heart failure. Their beneficial effects on blood pressure and left ventricular hypertrophy in patients with an activated renin-angiotensin system is explained by an inhibition of the biosynthesis of angiotensin II (AII), one of the most powerful endogenous vasoconstrictor and growth-promoting substances. However, several studies during the last decade, have demonstrated that this mechanism cannot solely account for the clinical effects of these drugs, especially in patients with normal plasma renin levels. ACE also catalyzes the degradation of the potent endothelium-dependent vasodilator peptide, bradykinin, to biologically inactive fragments, and therefore it has been proposed that an accumulation of endogenous bradykinin in the vicinity of the vessel wall also plays an important role in the cardiovascular effects of this class of compounds (Linz *et al*., 1995). By protecting endogenously synthesized bradykinin from proteolysis, ACE inhibitors indeed seem to be capable of enhancing the bradykinin-induced synthesis of the potent vasodilator and growth-inhibitory autacoids, nitric oxide (NO), prostacyclin (PGI_2) and endothelium-derived hyperpolarizing factor (EDHF), from both cultured and native endothelial cells (Wiemer *et al*., 1991; Mombouli *et al*., 1992; Hecker *et al*., 1993).

However, the same ACE inhibitors are also capable of relaxing preconstricted coronary and femoral artery segments either directly (van Wijngaarden *et al*., 1991; Hecker *et al*., 1993; Moroi *et al*., 1994) or following exposure to threshold concentrations of bradykinin (Auch-Schwelk *et al*., 1993; 1995; Desta *et al*., 1994). This effect is presumably brought about by an ACE-independent potentiation of the B_2 receptor-mediated dilator response to endogenous or exogenous bradykinin (Hecker *et al*., 1994b). Considering that probably only very small amounts of bradykinin are released from the endothelium under physiological conditions (Linz *et al*., 1995; Derad *et al*., 1996), it is likely that a potentiation of the biological efficacy of bradykinin rather than its local accumulation contributes to the cardiovascular effects of ACE inhibitors (Busse & Fleming, 1996).

We have recently suggested that the bradykinin-potentiating effect of ACE inhibitors may be due to an interaction of these drugs with the B_2 receptor itself, inhibition of receptor internalization or potentiation of a post-receptor signal transduction mechanism (Hecker *et al*., 1994b). We have now investigated the bradykinin-potentiating effect of the ACE inhibitor class of compounds by comparing the activity of five different ACE inhibitors in the rabbit jugular vein, a vascular model in which bradykinin elicits a B_2 receptor-mediated constriction. In addition, we have investigated the specificity of this effect of the ACE inhibitors, i.e. whether it is mimicked by

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other peptidase inhibitors and whether it is specific for bradykinin or extends to other vasoactive substances. Moreover, we have attempted to characterize further the underlying mechanism of action of these compounds.

Methods

Organ bath and superfusion experiments

Male New Zealand White rabbits (1.8–2.0 kg body weight) were anaesthetized with 60 mg kg^{-1}, i.v., pentobarbitone sodium (Nembutal, Sanofi, München, Germany) and exsangiunated by cutting through both the aorta and vena cava. The left and right jugular vein were dissected, cleansed of adventitial adipose and connective tissue, and cut into rings of 3–4 mm width which were mounted between K30 force transducers (Hugo Sachs Elektronik, March, Germany) and a rigid support for measurement of isometric force. The rings were incubated in 10 ml organ baths (Schuler-Organbad, Hugo Sachs Elektronik) containing warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs-Henseleit solution, pH 7.4 (composition in mM: Na^+ 144.0, K^+ 5.9, Cl^- 126.9, Ca^{2+} 1.6, Mg^{2+} 1.2, $H_2PO_4^-$ 1.2, SO_4^{2-} 1.2, HCO_3^- 25.0 and D-glucose 11.1) to which the cyclo-oxygenase inhibitor, diclofenac (1 μM), was added. In another series of experiments, four ring segments were simultaneously superfused at 3.0 ml min^{-1} with the same salt solution. Passive tension was adjusted over a 30 min equilibration period to 0.5 g. In the majority of experiments, the endothelium was mechanically removed, and its absence confirmed by the lack of a relaxant response to acetylcholine (1 μM). For this purpose, the segments were pre-constricted to 1.5 g with U46619 (10–30 nM).

Thereafter, a cumulative concentration-response curve was established for AI, AII, acetylcholine (following pre-constriction with U46619) or bradykinin by adding increasing doses of the agonists to the organ bath or injecting them into the superfusate. After washout, a second concentration-response curve was established in the presence or absence of the various test compounds. At the end of each experiment, the maximum change in tension (organ bath) or the area under the curve (AUC) of the constrictor/dilator response (superfusion) was determined planimetrically.

Plasma membrane preparation

Endothelium-denuded jugular veins (from approximately 30 animals) were first pulverised in liquid nitrogen with the aid of a mortar and a pestle. The resulting powder was further homogenized with a glass-glass potter in ice-cold 50 mM Tris-HCl buffer (pH 7.4) containing 1.15% (w/v) KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM glucose, 0.1 mM DL-dithiothreitol (DTT), 2 mg l^{-1} leupeptin, 2 mg l^{-1} pepstatin A, 10 mg l^{-1} trypsin inhibitor and 44 mg l^{-1} phenylmethylsulphonyl fluoride (PMSF) in a total volume of 20 ml. Subcellular fractions were prepared by differential centrifugation at $1{,}000 \times g$ for 10 min, at $10{,}000 \times g$ for 20 min and at $100{,}000 \times g$ for 60 min. The $100{,}000 \times g$-sediment, which in addition to the endoplasmic reticulum also contains the plasma membrane fraction (Hecker *et al*., 1994a), was resuspended in 1 ml of 50 mM Tris-HCl buffer (pH 7.4) containing 10% (v/v) glycerol, 0.1 mM EDTA, 0.1 mM DTT, 2 mg l^{-1} leupeptin, 2 mg l^{-1} pepstatin A, 10 mg l^{-1} trypsin inhibitor and 44 mg l^{-1} PMSF, and stored at −70°C. Protein concentrations were measured by using the Bio-Rad protein assay (Bio-Rad, München, Germany) and bovine serum albumin as a standard.

Binding experiments with [^{3}H]-bradykinin

Binding experiments, in the absence and presence of ramiprilat (0.3 μM), were performed with 50 μg of the membrane fraction in 20 mM PIPES, pH 6.8 containing 1 mM hippuryl-L-histidyl-L-leucine (to suppress any residual ACE activity), 2 nM [^{3}H]-bradykinin (spec. act. 39.2 TBq $mmol^{-1}$), and non-labelled bradykinin (0–10 μM) in a total volume of 0.3 ml at 4°C. Incubations were initiated by the addition of the membrane fraction and continued for 90 min with gentle agitation. Bound and unbound ligand were separated by filtration of the samples through polyethylenimine (0.3%, 30 min) pretreated GF52 fibreglass filters (Schleicher & Schüll, Dassel, Germany). The filters were washed three times with 2-propanol (4 ml, 14%) and bound radioactivity was determined by β-scintillation counting. Binding experiments with human intact foreskin fibroblasts (HF-15 cells), cultured in 24-well multiwell plates as previously described (Roscher *et al*., 1990), were performed in HEPES-buffered RPMI 1640 medium containing 2 mM bacitracin, 2 nM [^{3}H]-bradykinin (spec. act. 39.2 TBq $mmol^{-1}$) and non-labelled bradykinin (0–100 μM) in a total volume of 0.2 ml for 90 min at 4°C. The unbound ligand was removed by washing the adherent cells four times with 1 ml ice-cold HEPES-buffered RMPI medium. The cells were dissolved in 1 M NaOH followed by β-scintillation counting to determine the cell-bound radioactivity.

Analysis of B_2 receptor integrity

Porcine aortic endothelial cells were isolated and cultured as described by Lückhoff *et al*. (1987). They were passaged once by using trypsin-EGTA (0.05/0.02%, w/v) and labelled with [^{32}P]-H_3PO_4 (3.7 MBq ml^{-1} in a total volume of 3 ml) for 10 h in HEPES-buffered, serum-free DMEM (Applichem, Darmstadt, Germany). Thereafter, fresh medium was added and the cells were stimulated with bradykinin (1 μM) for 10 min at 37°C, in the absence or presence of ramiprilat (0.3 μM). The cells were then washed three times with 50 mM Tris (pH 7.5)/150 mM NaCl and then lysed by using a mixture of detergents in the presence of various protease and phosphatase inhibitors. Details of this procedure as well as that of the immunoprecipitation of the cell lysates with a polyclonal anti-B_2 receptor antibody (AS 346) are described elsewhere (Blaukat *et al*., 1996). The precipitated proteins were analysed by sodium dodecylsulphate-polyacrylamide (10%) gel electrophoresis (SDS-PAGE) in the presence of 5 M urea followed by autoradiography.

Materials

Angiotensin I (AI), AII, angiotensin-(1–7) bradykinin, des-Arg^9-bradykinin and hippuryl-L-histidyl-L-leucine were obtained from Bachem (Heidelberg, Germany); bacitracin from Fluka (Buchs, Switzerland); acetylcholine, 3,4-dichloroisocoumarin (3,4-DCI), DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA) and phosphoramidon from Sigma (Deisenhofen, Germany); and diclofenac (Voltaren) from Ciba-Geigy (Wehr, Germany). [^{3}H]-bradykinin was from Amersham (Braunschweig, Germany) and [^{32}P]-H_3PO_4 from ICN (Meckenheim, Germany); 9,11-dideoxy-11α,9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619) was kindly supplied by Upjohn (Kalamazoo, MI, U.S.A.); captopril and moexiprilat from Schwarz Pharma (Monheim, Germany); enalaprilat from Merck, Sharp & Dohme (München, Germany); quinaprilat from Gödecke (Freiburg, Germany); apstatin, ramiprilat and Hoe 140 (Icatibant) from Hoechst-Marion-Roussel (Frankfurt, Germany); and D-Arg[Hyp^3]-bradykinin by Prof. D. Regoli (University of Sherbrooke, Canada).

Statistical analysis

Unless indicated otherwise, all data in the figures and text are expressed as means ± s.e. mean of n observations. Statistical evaluation was performed by one-way analysis of variance followed by a Bonferroni t-test for multiple comparisons with a P value <0.05 considered statistically significant.

Results

Specificity of the bradykinin-potentiating effect of the ACE inhibitors

Bradykinin elicited a prominent constriction of the endothelium-denuded venous segments (Figures 1a, 2 and 3) which was completely abrogated in the presence of the B_2 receptor antagonist, Hoe 140 (0.1 μM). The concentration-dependent constrictor response to bradykinin was not modified by the presence of an intact endothelium and not mimicked by the B_1 receptor agonist, des-Arg^9-bradykinin (not shown). Moreover, no tachyphylaxis was observed upon repeated administration of bradykinin to the same segment (Figure 4a). When the segments were exposed to bradykinin in the presence of ramiprilat (0.3 μM), there was a marked shift of the concentration-response curve of bradykinin to the left (EC_{50} decreased from 741 pM to 54 pM) with the most striking effect at subthreshold concentrations of the peptide, i.e. $\leqslant$30 pM (Figure 1a). At this concentration (0.3 μM), ramiprilat strongly inhibited the constrictor response to AI (Figure 1b), indicative of an effective inhibition of the residual ACE activity in the endothelium-denuded segments. In contrast to its bradykinin-potentiating effect, ramiprilat neither affected the AII-induced contraction in endothelium-denuded segments (Figure 1c) nor the acetylcholine-induced relaxation in U46619-preconstricted, endothelium-intact segments (Figure 1d). Comparable results were obtained with 0.1 μM moexiprilat. In another series of experiments, ramiprilat also potentiated the constrictor effect of the ACE-resistant bradykinin analogue, D-Arg[Hyp^3]bradykinin (Hecker *et al*., 1994b), to a similar extent as with bradykinin (not shown).

The synthetic ACE substrate, hippuryl-L-histidyl-L-leucine, at a concentration (2 mM) that caused a similar inhibition of the constrictor response to AI as 0.3 μM ramiprilat (Figure 1b) and which has previously been shown to prevent the degradation of bradykinin by rabbit lung ACE as effectively as 1 μM ramiprilat (Hecker *et al*., 1994b), had no effect on the concentration-response curve of bradykinin (Figure 1a). In another series of experiments, ramiprilat (0.3 μM), on the other hand, retained its bradykinin-potentiating effect even in the presence of the ACE substrate (Figure 2a).

The aminopeptidase P inhibitor, apstatin (10 μM, Figure 3a) (Kitamura *et al*., 1995), the serine protease inhibitor, 3,4-DCI (10 μM, Figure 3b) (Harper *et al*., 1985), the carboxypeptidase inhibitor, MGTA (10 μM, Figure 3c), and the metalloprotease inhibitor, phosphoramidon (10 μM, Figure 3d), failed to affect

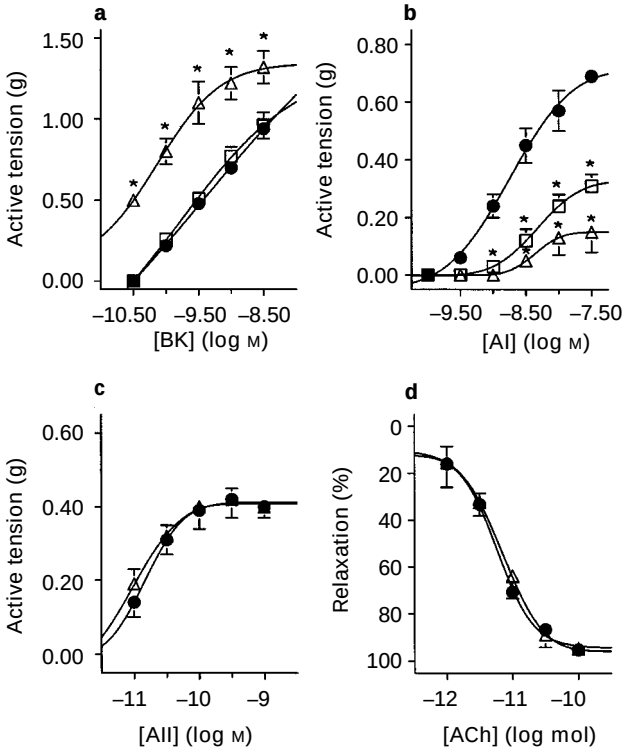

Figure 1 (a,b) Effects of ramiprilat (△, 0.3 μM), hippuryl-L-histidyl-L-leucine (□, 2 mM) and vehicle (●) on the constrictor response to (a) bradykinin (BK) or (b) AI in endothelium-denuded segments of the rabbit jugular vein ($n=5$; $^*P<0.05$ vs control). (c, d) Effects of ramiprilat (△, 0.1 μM) and vehicle (●) on the constrictor response to AII (c) in endothelium-denuded ($n=5$) and on the relaxant response to acetylcholine (ACh, d) in endothelium-intact, U46619-preconstricted ($n=10$) segments of the rabbit jugular vein. Experiments (a)–(c) were performed in organ baths, experiment (d) with superfused segments. Vertical lines show s.e.mean.

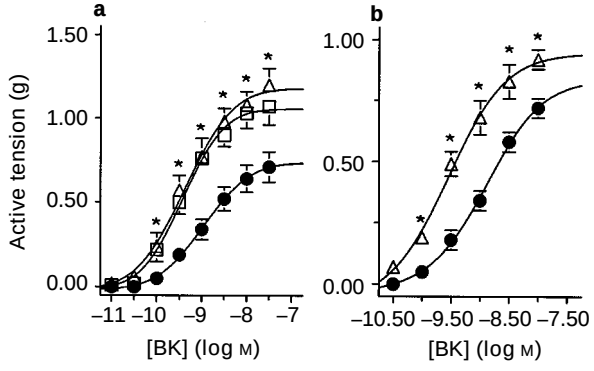

Figure 2 Effects of (a) ramiprilat (△, 0.3 μM), ramiprilat plus 2 mM hippuryl-L-histidyl-L-leucine (□) and vehicle (●) ($n=7$; $^*P<0.05$ vs control), and (b) of angiotensin-(1–7) (△, 10 μM) and vehicle (●) ($n=5$; $^*P<0.05$ vs control) on the constrictor response to bradykinin (BK) in endothelium-denuded segments of the rabbit jugular vein suspended in organ baths. Vertical lines show s.e.mean.

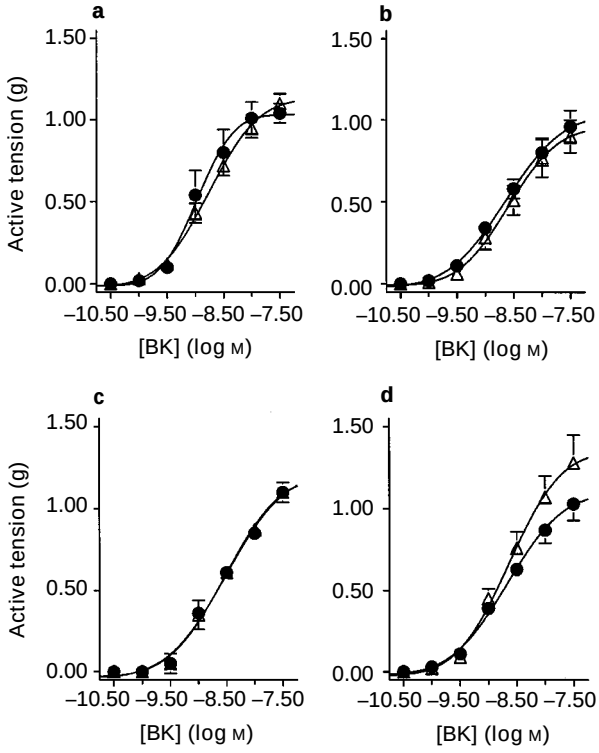

Figure 3 Effects of (a) apstatin (△, 10 μM), (b) 3,4-DCI (△, 10 μM), (c) MGTA (△, 10 μM), (d) phosphoramidon (△, 10 μM) and vehicle (●) on the constrictor response to bradykinin (BK) in endothelium-denuded segments of the rabbit jugular vein suspended in organ baths ($n=4$). Vertical lines show s.e.mean.

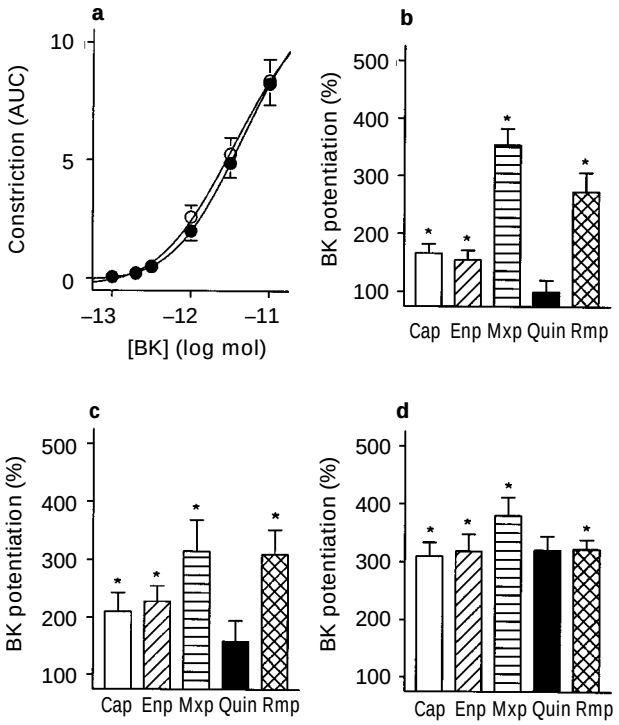

Figure 4 (a) Lack of tachyphylaxis upon repeated exposure to bradykinin (BK). The figure shows the first (●) and the second (○) control dose-response curve in endothelium-denuded superfused segments of the rabbit jugular vein. (b–d) Effects of captopril (Cap), enalaprilat (Enp), moexiprilat (Mxp), quinaprilat (Quin) and ramiprilat (Rmp) on the constrictor response to 1 pmol bradykinin at a concentration of (b) 3 nM, (c) 30 nM and (d) 300 nM ($n=4-6$; $^*P<0.05$ vs control).

the constrictor response to bradykinin. In contrast, the AI metabolite (Welches *et al*., 1993), angiotensin-(1–7) (10 μM), which had no effect on the tone of the endothelium-denuded segments itself (not shown), elicited a significant shift of the concentration-response curve of bradykinin to the left (EC_{50} decreased from 1.51 nM to 0.28 nM, Figure 2b).

Comparison of the bradykinin-potentiating activity of different ACE inhibitors

To compare the relative efficacy of the various ACE inhibitors without any influence of tachyphylaxis, endothelium-denuded ring segments were superfused with Krebs-Henseleit solution to which increasing doses of bradykinin were added at 10 min intervals. The area under the curve of the resulting constrictor responses was compared to that obtained in the presence of 3, 30 or 300 nM captopril, enalaprilat, moexiprilat, quinaprilat or ramiprilat. There was indeed no tachyphylaxis (Figure 4a) and all five ACE inhibitors similarly potentiated the constrictor response to bradykinin at 300 nM (Figure 4d). Although full dose-response curves were established in the presence of the ACE inhibitors, for reasons of clarity only their effects on the constrictor response to 1 pmol bradykinin is shown, which represents the ED_{20} value (Figure 4a). Lowering the ACE inhibitor concentration by one (Figure 4c) or two (Figure 4b) orders of magnitude revealed significant differences with respect to their relative potency. While ramiprilat and moexiprilat retained their bradykinin-potentiating effect at 3 nM, the captopril- and enalaprilat-induced potentiation at 3 nM was approximately 30% of that observed in response to 300 nM. Quinaprilat failed to affect the constrictor response to bradykinin at this concentration and had only a weak potentiating effect (<30%) at 30 nM. Thus, there appeared to be distinct differences between the five ACE inhibitors with respect to their ability to potentiate the bradykinin-induced contraction.

Effects of ramiprilat on the affinity of the B_2 receptor

The prominent ramiprilat-induced leftward shift in the concentration-response curve of bradykinin (Figure 1a) indicated that the ACE inhibitor may increase the affinity of the B_2 receptor. Therefore, microsomes, which also contain part of the plasma membrane, were prepared from endothelium-denuded rabbit jugular veins, and the binding of [^{3}H]-bradykinin to this membrane fraction was examined in the absence and presence of ramiprilat (0.3 μM). To avoid any interference with the binding assay due to degradation of the ligand by the residual ACE activity potentially present in this membrane fraction, [^{3}H]-bradykinin binding was analysed in the presence of 1 mM hippuryl-L-histidyl-L-leucine. Microsomal B_2 receptor density was estimated to be 100 fmol mg^{-1}. [^{3}H]-bradykinin binding revealed a single binding site of intermediate affinity with a K_d value of approximately 0.8 nM that closely matched the EC_{50} value for the bradykinin-induced constriction (0.7 nM). However, co-incubation with ramiprilat did not affect the affinity of the receptor (Figure 5a).

To verify the lack of effect of ramiprilat on the affinity of the B_2 receptor, we performed an additional series of experiments with cultured HF-15 fibroblasts. These cells also revealed a single binding site for [^{3}H]-bradykinin with a density of approximately 250 fmol mg^{-1} and a K_d value of 10.9 nM (Figure 5b). Co-incubation with ramiprilat (0.3 μM) also did not affect [^{3}H]-bradykinin binding to the HF-15 cells (Figure 5b).

Effect of ramiprilat on the integrity of the B_2 receptor

Since ramiprilat did not reveal any effect on the affinity of the B_2 receptor and because the recycling of the B_2 receptor after binding of the ligand has been shown to be too slow to account for the bradykinin-potentiating effect of the ACE inhibitors (Munoz & Leeb-Lundberg, 1992), we examined the possibility that the B_2 receptor is proteolytically cleaved upon exposure to bradykinin. Such a proteolytic cleavage had previously been reported for another G protein-coupled peptide receptor, the V_2 receptor (Kojro & Fahrenholz, 1995) and we reasoned, therefore, that by preventing this proteolysis, the ACE inhibitors might be capable of augmenting the biological efficacy of bradykinin. The effect of ramiprilat on the integrity of the B_2 receptor was studied in cultured endothelial cells where the ACE inhibitor potentiates the bradykinin-mediated release of NO and PGI_2 (Wiemer *et al*., 1991).

Immunoprecipitation of the B_2 receptor followed by SDS-PAGE analysis revealed seven phosphorylated proteins, one of which (approximately 69 kDa) corresponded to the molecular mass of the intact B_2 receptor (Abd Alla *et al*., 1993). This

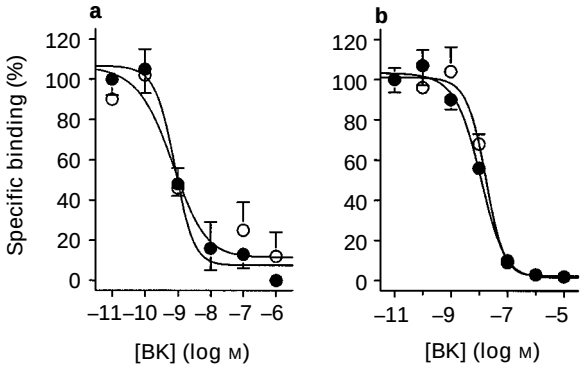

Figure 5 Binding of [^{3}H]-bradykinin to (a) rabbit jugular vein microsomes or (b) cultured HF-15 fibroblasts in the absence (●) and presence (○) of 0.3 μM ramiprilat (triple determination with the same batch of microsomes or cells). Vertical lines show s.e.mean.

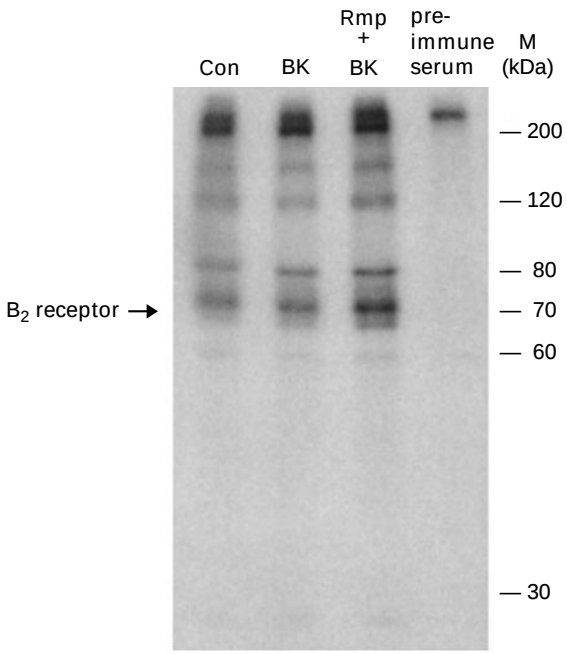

Figure 6 Typical SDS-PAGE/autoradiography analysis of B_2 receptor integrity in cultured endothelial cells in the absence and presence of bradykinin (BK, 1 μM) and/or ramiprilat (Rmp, 0.3 μM). As a control, the lack of precipitation of the B_2 receptor protein (indicated by the arrow) with pre-immune serum is also shown.

pattern did not differ between control and bradykinin-stimulated cells and apart from slightly enhancing the intensity of the 80 kDa band and that of the B_2 receptor (an effect which is likely to be due to difference in the effectiveness of the immunoprecipitation), the ACE inhibitor also did not modify this pattern (Figure 6).

Discussion

The present findings demonstrate that five different ACE inhibitors are capable of potentiating the B_2 receptor-mediated constrictor effect of bradykinin in the rabbit jugular vein. This effect of the ACE inhibitors appears to be specific for bradykinin, as the constrictor response to AII was not affected by moexiprilat or ramiprilat. Moreover, we have previously shown that ramiprilat does not affect the constrictor response to U46619 in this vascular preparation (Hecker *et al*., 1994b). Although we observed that the ACE inhibitors did not potentiate the endothelium-dependent relaxant response to acetylcholine in the rabbit jugular vein, enalprilat has been shown to augment the increase in forearm blood flow in healthy volunteers elicited by intra-arterial infusion of acetylcholine (Nakamura *et al*., 1992). In addition, captopril and enalapril have been shown to enhance the vasodilator response to both acetylcholine and bradykinin in conscious dogs (Zanzinger *et al*., 1994). The potentiating effect of the ACE inhibitors on the acetylcholine-induced vasodilation in the latter study was abolished in the presence of the B_2 receptor antagonist Hoe 140. It would appear, therefore, that the dilator response to acetylcholine *in vivo* involves the secondary release of endogenous bradykinin, hence explaining the apparent lack of specificity of the ACE inhibitors.

The ACE inhibitor-induced potentiation of the constrictor response to bradykinin seems to be independent of ACE or kininase II inhibition for the following reasons. The synthetic ACE substrate hippuryl-L-histidyl-L-leucine which suppressed the ACE-dependent constrictor response to AI (this study) and blocks the bradykinin-degrading activity of rabbit lung microsomes (Hecker *et al*., 1994b) as effectively as ramiprilat, had no effect on the constrictor response to bradykinin. Moreover, ramiprilat potentiated the constrictor response to bradykinin even in the presence of the substrate. The ACE inhibitor also markedly enhanced the constrictor effect of the ACE-resistant B_2 receptor agonist, D-Arg[Hyp^3]bradykinin. Similar findings with this bradykinin analogue have also been obtained previously in bovine and human isolated coronary arteries (Auch-Schwelk *et al*., 1995) and in our earlier study in the coronary microcirculation of the rabbit (Hecker *et al*., 1994b). Short retro-inverted peptide analogues of AI and bradykinin also have been shown to potentiate the bradykinin response in the guinea-pig ileum independently of their ACE-inhibitory activity (Carmona & Juliano, 1996), and differences between the ACE-inhibitory and bradykinin-potentiating activity of different ACE inhibitors have been observed in guinea-pig airways (Murata *et al*., 1995), guinea-pig ileum and rat uterus (Rodrigues *et al*., 1992). These findings, together with the fast kinetics of the constrictor response to bradykinin in the rabbit jugular vein and the rather slow degradation of bradykinin by ACE in the endothelium-intact bovine coronary artery (Bossaller *et al*., 1992), all suggest that inhibition of bradykinin degradation by ACE cannot account for the bradykinin-potentiating effect of the ACE inhibitors.

Several proteases apart from ACE have been proposed to be responsible for the degradation of bradykinin *in vivo* such as carboxypeptidase N, neutral endopeptidase 24.11 and aminopeptidase P (Welches *et al*., 1993; Kitamura *et al*., 1995). Since ACE inhibitors are not absolutely specific for ACE, the potential degradation of bradykinin in the rabbit jugular vein by proteases other than ACE was probed by examining the effects of inhibitors of these proteases on the constrictor response to bradykinin. However, neither MGTA, phosphoramidon, apstatin nor 3,4-DCI affected the constrictor response to bradykinin. It is thus unlikely that the ACE inhibitor-induced potentiation of the constrictor response to bradykinin was mediated by the blockade of another bradykinin-degrading protease.

There were distinct differences in the relative efficacy of the various ACE inhibitors. Most notable was that moexiprilat and ramiprilat markedly enhanced the constrictor response to bradykinin at a concentration of 3 nM, while quinaprilat was virtually inactive even at 30 nM. With the exception of captopril (IC_{50} 23–35 nM), the IC_{50} values of the other ACE inhibitors for the inhibition of ACE activity are virtually identical (1–5 nM) (Unger & Gohlke, 1994). This finding not only reinforces the notion of a bradykinin-potentiating effect of the ACE inhibitors which is unrelated to their ACE-inhibitory activity, but also suggests that these compounds bind with different affinities to a binding site other than ACE.

A marked rise in the plasma level of angiotensin-(1–7), a bioactive fragment of AI (Welches *et al*., 1993), has been observed in rats following ACE blockade (Campbell *et al*., 1991). Although in the present study angiotensin-(1–7) alone had no effect on the tone of the isolated jugular vein, it significantly augmented the constrictor response to bradykinin in a manner similar to the effect of the ACE inhibitors. Angiotensin-(1–7) has also been shown to potentiate the hypotension induced by bradykinin in conscious rats (Paula *et al*., 1995). AI, on the other hand, failed to mimic the effects of ramiprilat in the rabbit isolated heart (Hecker *et al*., 1994a). It is tempting to speculate, therefore, that angiotensin-(1–7) and perhaps other AI fragments (Carmona & Juliano, 1996) share a common mechanism of action with the ACE inhibitors.

With respect to the mechanism responsible for the bradykinin-potentiating activity of the ACE inhibitors, we considered the possibility that this class of compounds enhances the affinity of the B_2 receptor for bradykinin. The reason for this assumption was the parallel leftward shift in the concentration-response curve of bradykinin induced by the ACE inhibitors and data showing that the B_2 receptor can exist both in a high (for picomolar concentrations of bradykinin) and in a low to intermediate state of affinity (for nanomolar concentrations of bradykinin) (Hall, 1992). However, ramiprilat did not affect either the binding of [^{3}H]-bradykinin in a mixed membrane

fraction prepared from endothelium-denuded segments of the rabbit jugular vein or [^{3}H]-bradykinin binding to cultured HF-15 fibroblasts. It is thus unlikely that ACE inhibitors potentiate the biological activity of bradykinin by increasing the affinity of the B_2 receptor for the peptide.

In addition, we considered the possibility that ACE inhibitors may interfere with the internalization and degradation of the B_2 receptor. Since ACE and the B_2 receptor are apparently colocalized in the plasma membrane (Trifilieff *et al*., 1994), we speculated that – should bradykinin elicit a proteolytic cleavage of its receptor in a similar manner as the vasopressin-induced cleavage of the V_2 receptor (Kojro & Fahrenholz, 1995) – ACE inhibitors perhaps potentiate the effects of bradykinin by preventing the agonist-induced cleavage of the B_2 receptor. As a model, we chose cultured endothelial cells where ACE inhibitors such as enalaprilat, moexiprilat or ramiprilat enhance the intracellular concentration of free Ca^{2+} and, as a consequence, the formation of NO and PGI_2 (Hecker *et al*., 1992). However, immunoprecipitation of the ^{32}P-labelled B_2 receptor followed by SDS-PAGE analysis did not reveal any bradykinin-induced fragmentation of the receptor protein after an incubation period of 10 min. Although this finding does not preclude the possibility that ACE inhibitors interfere with the potential degradation of the B_2 receptor, this process is, like the recycling of the B_2 receptor, following agonist-induced internalization (Munoz & Leeb-Lundberg, 1992) presumably too slow to account for the bradykinin-potentiating effect of the ACE inhibitors.

In summary, our data demonstrate that ACE inhibitors selectively potentiate the B_2 receptor-mediated contractile response to bradykinin in the rabbit jugular vein. Their relative efficacy seems to depend on structural differences, but is unrelated to ACE inhibition *per se*. The bradykinin-potentiating effect of this class of compounds does not appear to be accounted for by a shift in the affinity of the B_2 receptor or the prevention of its degradation. When considering that the B_2 receptor exhibits spontaneous activity even in the absence of bradykinin (Leeb-Lundberg *et al*., 1994) and that the receptor protein and ACE may be colocalized in the plasma membrane, it is tempting to speculate that the binding of an ACE inhibitor to ACE induces a change in conformation of the enzyme, which is transduced to the receptor protein and results in an increased activity of the B_2 receptor. This notion is supported by the finding that the administration of ACE inhibitors to isolated segments of the rabbit jugular vein, which had not been exposed to bradykinin before, results in a small but significant contractile response that is abolished by the B_2 receptor antagonist Hoe 140 (Hecker *et al*., 1994b). Moreover, this ACE inhibitor-mediated increase in the activity of unoccupied B_2 receptors could add to the cellular response triggered by a small number of bradykinin-occupied receptor molecules, hence turning a threshold constriction elicited by low concentrations of bradykinin into a distinct contractile response.

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