Enhancement of Bradykinin and Resensitization of Its B2 Receptor

Branislav Marcic, Peter A. Deddish, Herbert L. Jackman, Ervin G. Erdös

Abstract—We studied the enhancement of the effects of bradykinin B2 receptor agonists by agents that react with active centers of angiotensin-converting enzyme (ACE) independent of enzymatic inactivation. The potentiation and the desensitization and resensitization of B2 receptor were assessed by measuring [3H]arachidonic acid release and [Ca2+]i mobilization in Chinese hamster ovary cells transfected to express human ACE and B2 receptor, or in endothelial cells with constitutively expressed ACE and receptor. Administration of bradykinin or its ACE-resistant analogue desensitized the receptor, but it was resensitized (arachidonic acid release or [Ca2+]i mobilization) by agents such as enalaprilat (1 μmol/L). Enalaprilat was inactive in the absence of ACE expression. La3+ (100 μmol/L) inhibited the apparent resensitization, probably by blocking the entry of extracellular calcium. Enalaprilat resensitized the receptor via ACE to release arachidonic acid by bradykinin at a lower concentration (5 nmol/L) than required to mobilize [Ca2+]i, (1 μmol/L). Monoclonal antibodies inhibiting the ACE N-domain active center and polyclonal antiseraum potentiated bradykinin. The snake venom peptide BPP5a and metabolites of angiotensin and bradykinin (angiotensin-[1–9], angiotensin-[1–7], bradykinin-[1–8]; 1 μmol/L) enhanced arachidonic acid release by bradykinin. Angiotensin-(1–9) and -(1–7) also resensitized the receptor. Enalaprilat potentiated the bradykinin effect in cells expressing a mutant ACE with a single N-domain active site. Agents that reacted with a single active site, on the N-domain or on the C-domain, potentiated bradykinin not by blocking its inactivation but by inducing crosstalk between ACE and the receptor. Enalaprilat enhanced signaling via ACE by Goi, in lower concentration than by Go-q-coupled receptor. (Hypertension. 1999;33:835-843.)

Key Words: angiotensin-converting enzyme inhibitors • kininase II • endothelial cells • G proteins • [Ca2+]i • arachidonic acid • angiotensin-(1–9)

Therapy with angiotensin I–converting enzyme (ACE) inhibitors initially was aimed at lowering elevated blood pressure.1 By now, however, it has gained much wider applications in combating heart and kidney diseases, such as congestive heart failure and diabetic nephropathy, involving millions of patients.2–7 Inhibitors of ACE affect both angiotensin II (Ang II) and bradykinin metabolism by blocking the production of the vasoconstrictor peptide and inactivating the vasodilator peptide,8 but these actions alone do not completely explain, for example, the beneficial effects of ACE inhibitors on the heart. These effects are not only due to lowering systemic blood pressure and peripheral vascular resistance. In laboratory experiments, many of the improvements in cardiac function brought about by ACE inhibitors are blocked by the bradykinin B2 receptor antagonist Hoe 140.9–14 We have observed, on the isolated atria15 and ileum16 of guinea pig, that ACE inhibitors potentiate the actions of bradykinin indirectly at the receptor level. Using cultured Chinese hamster ovary (CHO) cells cotransfected with the cDNA of human ACE and B2 receptor, we showed that ACE inhibitors augment the release of signal transduction products by bradykinin independent of inhibiting the degradation of bradykinin but have no direct effect on the B2 receptor.17 On the basis of accumulated evidence, it was suggested that the above effects and the resensitization of the receptor, desensitized by an agonist, are due to a crosstalk between ACE and the B2 receptor on the plasma membrane of the cells. We have also reported that angiotensin-(1–7), a substrate cleaved by the N-domain active site of ACE and an inhibitor of the C-domain active site in vitro, potentiates bradykinin at the receptor level in a manner similar to that of ACE inhibitors.18

The present report extends and reconfirms the previous observations, mainly in different cells, by using ACE inhibitors, inhibitory and noninhibitory monoclonal and polyclonal antibodies, a mutated ACE molecule, and endogenous peptide and snake venom peptide substrates of ACE,8,19 to show
that agents that react with at least 1 of the active centers of ACE with sufficient affinity enhance the effect of bradykinin on the cell membrane receptor. The receptor desensitized by an agonist was re-sensitized by the agents tested, as shown by the response of the receptor to the kinins still present in the cell medium. Besides arachidonic acid (AA) release, we measured mobilization of Ca\(^{2+}\) in the cells to assess another function, probably exerted through a different G protein coupled to the activated receptor. The experiments were done with cells that were cotransfected or serially transfected to overexpress ACE and B\(_2\) receptor. In addition to transfected cells, we also used cultured endothelial cells that constitutively expressed ACE and bradykinin receptors with basically similar results.

### Methods

#### Materials

CHO cells were purchased from American Type Culture Collection (Rockville, Md). The cDNA of human ACE, the cDNA encoding the human B\(_2\) receptor, and the neomycin-resistant gene (pHBA-pr-3pneo) were gifts from Prof P. Corvol (Collège de France, Paris), from Syntex Co (Palo Alto, Calif), and from Dr L.H. Kesed (University of Southern California, Los Angeles), respectively. Mammalian expression vectors pcDNA1 and pcDNA3 were from Invitrogen; lipofectin, monoclonal antibodies 3AS, 12HS, and 9B9 against the N-domain of human ACE were provided by Dr Sergei Danilov (University of Illinois, Chicago).[^21][^22] [H]bradykinin (107 to 114 Ci/mmol) was from NEN. [5,6,8,9,11,12,14,15-\(^{13}\)H]arachidonic acid ([H]AA; 100 Ci/mmol) was purchased from American Radiolabeled Chemicals. Bradykinin, Ang I, normal rabbit protein; this rate is comparable to that of native human deamidase.19 The major product, Ang-(1–9), was purified from platelets.22 The enzyme was treated with recombinant human deamidase, also known as lysosomal protective protein or cathepsin A.21,22 The enzyme hydrolyzed Ang I to Ang-(1–9) at a rate of 5 \(\mu\)mol/min per milligram protein; this rate is comparable to that of native human deamidase purified from platelets.22 The major product, Ang-(1–9), was purified for use in our assays by C18 reversed-phase high-performance liquid chromatography.

#### Production of [des-Leu\(^{10}\)]Ang I (Ang-[1–9])

Ang I was treated with recombinant human deamidase, also known as lysosomal protective protein or cathepsin A.21,22 The enzyme hydrolyzed Ang I to Ang-(1–9) at a rate of 5 \(\mu\)mol/min per milligram protein; this rate is comparable to that of native human deamidase purified from platelets.22 The major product, Ang-(1–9), was purified for use in our assays by C18 reversed-phase high-performance liquid chromatography.

#### Human ACE Construct

Plasmid pBACE (ACE-9–4024); provided by F. Alhenc-Gelas, INSERM, Unit 367, Paris, France) was digested with EcoRI restriction enzyme in order to obtain the full-length ACE cDNA. The fragment corresponding to ACE (4–43 kb) was excised from 1% agarose gel, purified with a DNA CleanUp kit (Promega), and ligated into the pECE expression vector at the EcoRI restriction site (provided by E. Clauser, INSERM Unit 36, Paris, France). This vector was used for transfecting CHO cells.17

#### Cell Culture

CHO cells were grown in 100-mm-diameter Petri dishes in Ham’s F-12 culture medium supplemented with L-glutamine, antibiotic-antimycotic, HEPES buffer, and 10% fetal bovine serum. Cells were subcultured routinely with trypsin-EDTA. CHO cells were plated at a density of 1 \(\times\) 10^6 cells per 100-mm dish 1 day before the transfection procedure.

### Screening and Selection of Clones

Up to 150 clones derived from each mixed population of transfected cells were analyzed for their release of ACE into the culture medium. The clone with the highest ACE activity in the medium, clone 3C11, was chosen (donated by Dr S. Danilov). This clone was evaluated for both cell-associated and released ACE activity, which yielded a concentration of about 1 \(\times\) 10^6 ACE molecules per cell (S. Danilov and D. Miletich, unpublished observations, 1998).

#### Transfection of Selected Clone With B\(_2\) Receptor cDNA

The selected 3C11 ACE clone was transfected with human B\(_2\) receptor cDNA inserted into the pCEP4 vector[^23] (a gift from Syntex Co, Palo Alto, Calif). After transfection, cells were subjected to selection using Ham’s F-12 medium containing 0.5 mg/mL hygromycin B (the pCEP4 vector contains hygromycin B resistance gene). After selection, 4 different clones were harvested and propagated using cloning rings.

#### Radioligand Binding on Selected Clones

To select the clone with the highest expression of B\(_2\) receptors, we performed [H]bradykinin saturation binding on whole-cell monolayers expressing ACE and B\(_2\) receptors.17 One clone with the highest expression of B\(_2\) receptors on the cell surface was chosen. This CHO-AB clone binds 232 to 279 fmol of [H]bradykinin per 10^6 cells, which corresponds to the expression of 2 \(\times\) 10^7 B\(_2\) receptors per cell. The ACE activity in CHO-AB cells ranged from 196 to 292 nmol of Hip-His-Leu hydrolyzed per minute per milligram protein.

#### Enzyme Assay

In general, enzymatic activity of ACE was assayed using Hip-His-Leu substrate.20 Briefly, enzyme samples were incubated at 37°C with 1 mmol/L final concentration of Hip-His-Leu in 50 mmol/L Tris-maleate (pH 7.4) plus 150 mmol/L NaCl. After incubation, the reaction was stopped by adding 0.28 mol/L NaOH, and released His-Leu was measured by adding 100 \(\mu\)L of 20 mg/mL o-phenaldialdehyde and incubating for 10 minutes at room temperature; this step was followed by adding 200 \(\mu\)L of 3 mol/L HCl. Fluorescence was measured at an excitation wavelength of 363 nm and an emission wavelength of 500 nm.

#### Inhibition Studies

The effect of Ang-(1–9) on the hydrolysis of Hip-His-Leu by human kidney ACE was determined by preincubating 2 mmol/L enzyme for 30 minutes at 4°C in a concentration range of 15 to 40 mmol/L Ang-(1–9) before addition of substrate. The IC\(_{50}\) was then calculated from the inhibition curve. The inhibition by 5-S,S-5-benzamido-4-oxo-6-phenylhexanoyl-L-proline (keto-ACE[^16]) was carried out similarly.

#### Measurement of Changes in Intracellular Free Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) and of [H]AA

Measurements of [Ca\(^{2+}\)], were made using a microspectrofluorometer (PTI Deltascan) and the Ca\(^{2+}\)-sensitive fluorescent dye fura 2-AM.23 Cells were grown to confluence on glass coverslips and then incubated with 2 to 5 mmol/L fura 2-AM for 1 hour at 37°C. Cells were washed with buffer, incubated for an additional 15 minutes, and then mounted in a Sykes-Moore chamber (Belco) at room temperature on an inverted microscope coupled to the microspectrofluorometer. Cellular fluorescence at 510 nm was measured following excitation at wavelengths of 340 and 380 nm. Changes in [Ca\(^{2+}\)]\(_i\) are given as the ratio of intensities at 340 and 380 nm.
Potentiation of Bradykinin and Desensitization and Resensitization of Receptor

Monolayers of CHO-AB cells, loaded with [3H]AA, were exposed to 1 μmol/L HT-BK given alone or together with enalaprilat or another agent for 30 or 60 minutes. [3H]AA released into the medium was determined, and the amount released relative to that released by HT-BK alone was calculated. In experiments using antibodies to human ACE, confluent monolayers of CHO-15AB cells were pretreated for 1 hour.

It was determined in control experiments that the potentiating agents were inactive in cells lacking ACE or in the absence of receptor agonist. The B2 receptor blocker Hoe 140 (0.1 to 1.0 μmol/L) was routinely used to show the direct involvement of the bradykinin receptor.

After desensitization by initial exposure of cells to kinin, sensitivity to bradykinin (resensitization) was measured either by [3H]AA release or by mobilization of [Ca2+]. For example, monolayers of [3H]AA-loaded CHO-AB cells were stimulated with 1 μmol/L HT-BK for 30 minutes. Then, without removal of HT-BK, cells were exposed to either 5 nmol/L enalaprilat, 1 μmol/L bradykinin, or other agent given as control for an additional 5 minutes; the amount of [3H]AA released was determined, taking AA released during the first 30 minutes as baseline and normalizing to the amount released during the additional 5 minutes by buffer alone.

[Ca2+]i mobilization was measured in cells first exposed to 100 nmol/L bradykinin. After the initial [Ca2+]i response, without removal of the bradykinin dose, CHO or endothelial cells were then exposed again either to bradykinin, to confirm desensitization, or to 1 μmol/L enalaprilat or to another agent. In control experiments, Hoe 140 (0.1 to 1.0 μmol/L) blocked the responses.

Statistics

Data in the figures and text are expressed as mean±SEM of n observations, n being 3 or more. [Ca2+]i, is represented as the percentage of mean fluorescence intensity increase relative to control levels. Statistical evaluation was performed by one-way ANOVA for matched values. Values of P<0.05 were considered statistically significant.

Results

Expression of B2 Receptor and ACE

To determine the importance of the ratio of receptor to enzyme, we investigated the potentiation of B2 receptor responses by ACE inhibitors in CHO cells transfected sequentially by human ACE cDNA first, and then with human B2 receptor cDNA using selective cloning (see Methods). These CHO-AB cells expressed much higher concentrations of both proteins than found previously: an estimated 2×105 B2 receptors per cell and about 1×106 ACE molecules per cell, established by assaying enzyme activity (S. Danilov and D. Miletich, personal communication). Besides bradykinin, its analogue HT-BK, which is more resistant to ACE than the parent compound, was used as agonist. The ratio of B2 receptors to ACE was 1:5 in CHO-AB cells, whereas in the previously used cotransfected CHO-15AB cells, it was 1:1.17

In the CHO-AB cells, interactions between B2 receptor and ACE as affected by inhibitors were studied by determining the B2 receptor number on the cell surface, measuring [3H]AA release, and assaying changes in [Ca2+]i mobilization induced by B2 receptor agonists.

Binding of [3H]Bradykinin in CHO-AB Cells

The effects of 2 concentrations of enalaprilat (1 μmol/L and 5 nmol/L) on active B2 receptor number on CHO-AB cells were tested as reported previously with the different CHO-AB cells. 15 AB cells. Enalaprilat (1 μmol/L) increased the number of bradykinin binding sites from 76±9 to 248±36 fmol/106 cells at 37°C (n=5) ([3H]bradykinin concentration, 0.1 nmol/L; P<0.005). Interestingly, 5 nmol/L enalaprilat, which inhibited 75% to 90% of ACE activity of these cells, did not increase the available bradykinin binding sites. These findings are similar to those obtained with CHO-15AB cells.17

[3H]AA Release

The effect of enalaprilat on [3H]AA release induced by HT-BK was tested in CHO-AB cells. When the amount of [3H]AA released by 1 μmol/L HT-BK was taken as baseline, 5 nmol/L enalaprilat enhanced release by 59±22% (P<0.05) and 1 μmol/L enalaprilat by substantially more (163±32%; n=3, P<0.005) (Figure 1). When CHO-15AB cells were compared with CHO-AB cells, the maximum amounts of [3H]AA released were 5±3 and 100±15 fmol/106 cells (n=4), respectively. These numbers strongly suggest that the B2 receptors expressed in these 2 cell systems (106/cell in CHO-15AB cells; 2×105 in CHO-AB cells) were actively connected to G proteins that participate in signal transduction in the cells.

We also used measurement of [3H]AA release to address another aspect of B2 receptor–ACE interaction: resensitiza-
Enalaprilat in a concentration of 5 nmol/L increased the Ca^{2+} level or 1 μmol/L EPT alone (Figure 2). Enalaprilat, in concentrations of either 5 nmol/L or 1 μmol/L, resensitized the receptor (n=3). When enalaprilat was added alone to the first desensitizing dose of bradykinin still present in the well, the ACE inhibitor restored the sensitivity of the receptor to bradykinin as measured by [3H]AA released in 5 minutes after addition of enalaprilat. Enalaprilat in a concentration of 5 nmol/L increased the release by 620±81% (P<0.005) compared with the addition of buffer alone or a second dose of bradykinin, whereas 1 μmol/L enalaprilat induced a somewhat higher resensitizing response—a 900±110% increase (P<0.005). The esterified prodrug form of enalaprilat, enalapril, was inactive. The B2 receptor blocker Hoe 140 (1 μmol/L) abolished the resensitization by enalaprilat (n=3, data not shown). These experiments also make it evident that resensitization of the B2 receptor by ACE inhibitors cannot be attributed to blocking peptide breakdown.

**Resensitization of B2 Receptor and Ca^{2+} Mobilization**

To explore resensitization of the B2 receptor further, we studied the increase in [Ca^{2+}], caused by bradykinin (Figure 3, n=7). Addition of bradykinin to CHO-AB cells increased [Ca^{2+}], very likely by initially mobilizing it from intracellular Ca^{2+} stores.28 The receptor was desensitized by the first dose of bradykinin (100 nmol/L) and did not respond to a second dose (data not shown, n=4). However, when enalaprilat (1 μmol/L) was added alone without any additional agonist 100 to 200 seconds after the first dose of bradykinin, the level of mobilized [Ca^{2+}], was elevated again 170±80% higher than by the original dose of bradykinin. Here again, the B2 receptor antagonist Hoe 140 (1 μmol/L) blocked this effect of enalaprilat (n=6, data not shown). In CHO cells, which expressed only B2 receptors but no ACE (CHO-3B), bradykinin mobilized Ca^{2+}, but added enalaprilat did not resensitize the receptor (Figure 4, n=6). Enalaprilat lacking the presence of the agonist bradykinin or HT-BK was inactive (data not shown).

**Endothelial Cells**

To further establish that ACE inhibitors enhance the effects of bradykinin beyond inhibiting its inactivation in cells that constitutively express both the enzyme and the receptor, we studied cultured bovine pulmonary artery endothelial cells, which have both ACE and B2 receptors. In these experiments, enalaprilat (1 μmol/L) restored the bradykinin response, thus resensitizing the receptor to the agonist. The second elevation of [Ca^{2+}], level induced by enalaprilat in these cells did not exceed the first one triggered by 100 nmol/L bradykinin (n=6, Figure 5).

We also investigated where, after resensitization, the raised level of [Ca^{2+}], was coming from. Applying 100 μmol/L La^{3+} to the bovine endothelial cells did not block the initial calcium mobilization by bradykinin but did inhibit resensitization by enalaprilat (n=3). This finding indicates that after resensitization of the receptor to bradykinin by enalaprilat, the second peak of [Ca^{2+}], (blocked by La^{3+}) represents influx from the extracellular fluid, whereas the primary effect of
bradykinin mobilizes calcium from intracellular stores (Figure 6).

**Role of the N-Domain of ACE in Potentiation of the B2 Receptor Response**

**Antibodies**

The role of the active center of ACE in the N-domain in the potentiation of bradykinin was investigated by using monoclonal antibodies directed against this domain. The potentiation was assessed by measuring [3H]AA released from CHO-15AB cells by HT-BK. As control, the amount of [3H]AA released by 1 μmol/L HT-BK was taken as 1, after subtracting the spontaneously released AA. Three different monoclonal antibodies, designated 3A5, 12H5, and 9B9,20 were used at a concentration of 5 μg/mL. All 3 of these antibodies react only with the N-domain of ACE. The 3A5 and 12H5 antibodies, inhibitory antibodies that complex the N-domain active center of ACE, augmented HT-BK–induced [3H]AA release by 80±6 and 108±17%, respectively (n=4, P<0.05; Figure 7). The antibody 9B9, which binds to the N-domain of ACE but does not inhibit it, did not potentiate (n=4). The control IgG was also inactive (n=4). At the dilution used (1:100 vol/vol), polyclonal rabbit antiserum to human somatic 2-domain ACE29 augmented HT-BK–induced [3H]AA release by 150±20% (P<0.005, n=4). As another control, enalaprilat (1 μmol/L) in these experiments enhanced [3H]AA release by 190±22% (P<0.005, n=4).

**Mutated ACE**

The role of the N-domain active site of ACE was further studied in CHO cells that expressed a mutated ACE containing only 1 active site in the N-domain. These CHO cells [CHO (E)] were transfected with D960 ACE cDNA in which the C-domain Glu960 was mutated to Asp (donated by F. Alhenc-Gelas and S. Danilov).30 This mutation inactivates the catalytic center in the C-domain; consequently, ACE inhibitors very likely react with the remaining N-domain active center. These cells were subsequently transfected with human B2 receptor, and resensitization of the receptor was tested by measuring [Ca2+]i levels, as described above (n=5, Figure 8). The B2 receptor was desensitized by stimulation with bradykinin (100 nmol/L) first. As before, enalaprilat (1 μmol/L), given 70 to 100 seconds after bradykinin, resensitized the B2 receptor and, in 5 experiments done in 5 different cell population samples, enhanced the initial response by 70±15%.

These data strongly suggest an active role for the N-domain of ACE in potentiation of B2 receptor responses, and specifically point out the importance of inhibitor binding to the N-domain active center.

**Figure 6.** La3+ (100 μmol/L) blocks apparent resensitization of B2 receptor by enalaprilat (EPT) in endothelial cells. Shown is the time course of the increase in [Ca2+] induced by 100 nmol/L bradykinin. La3+ blocks the effect of EPT (A and B) but not the first effect of the agonist bradykinin (B). Shown is 1 experiment representative of 3.

**Figure 7.** Effect of antibodies to ACE on [3H]AA release by HT-BK in CHO-15AB cells. Confluent monolayers of cells were pretreated with either buffer alone, 1 of 3 monoclonal antibodies to human ACE (3A5, 12H5, or 9B9; 5 μg/mL), nonimmune mouse IgG (5 μg/mL) polyclonal anti-ACE antiserum (Pcl; 1:100 vol/vol), or enalaprilat (EPT, 1 μmol/L) for 1 hour. Cells were then stimulated with 1 μmol/L HT-BK for 30 minutes. Amount of released [3H]AA is shown after background was subtracted. Data are mean±SEM (n=4). *P<0.05; **P<0.005.

**Figure 8.** Role of N-domain active center of ACE in resensitization of B2 receptor by enalaprilat (EPT) in CHO cells expressing B2 receptors and mutated ACE with an inactive C-domain active center [CHO(E)/B2]. Shown is the time course of increase in [Ca2+]i. The first rise in [Ca2+]i is induced by 100 nmol/L BK. EPT (1 μmol/L) resensitizes the receptor. Shown is 1 experiment representative of 5.
Role of the C-Domain

To investigate the role of the C-domain active center in B₂ receptor potentiation, we re-sensitized the B₂ receptor in CHO-AB cells with keto-ACE. Keto-ACE is a relatively specific inhibitor of the C-domain active site of ACE (IC₅₀ for bradykinin = 0.5 μmol/L). Resensitization was determined by measuring [Ca²⁺], in CHO-AB cells. The B₂ receptor was desensitized by stimulating it with the first dose of bradykinin (10 nmol/L). Subsequently, 1 μmol/L keto-ACE was added 120 to 200 seconds later to restore 85±13% of the initial response (n=4).

These results indicate that the selective interaction of an inhibitor with the C-domain of ACE is sufficient to re-sensitize the B₂ receptor.

Potentiation of B₂ Receptor by ACE Substrates

We also tested whether relatively slowly hydrolyzed ACE substrates can augment bradykinin effects. The snake venom peptide pGlu-Lys-Trp-Ala-Pro (BPP5a) is cleaved by ACE, but it is a potent inhibitor as well. Another substrate, Ang-(1–9), was produced by cleaving the His³-Leu⁹ bond of Ang I by human recombinant cathepsin A, lysosomal protective protein to release Ang-[1–9] (see Methods). It inhibited human somatic ACE with an IC₅₀ of 2±0.5 μmol/L (n=3). The peptide alone did not release [³H]AA, but when given together with HT-BK, it potentiated the release of [³H]AA from CHO-AB cells by 185±26% (n=3, P<0.05; Figure 9). BPP5a (1 μmol/L), administered simultaneously with 1 μmol/L HT-BK, potentiated its effect by 480±48% (n=3, P<0.005; Figure 9) and elevated [Ca²⁺]. Ang-(1–9) also re-sensitized the B₂ receptor desensitized by bradykinin (n=4); 1 μmol/L Ang-(1–9) given 100 seconds after the first dose of 100 nmol/L bradykinin re-sensitized the receptor and enhanced [Ca²⁺], level 2-fold (data not shown).

Two more substrates of ACE were tested to re-sensitize the B₂ receptor in CHO-AB cells that was desensitized first by bradykinin (10 to 100 nmol/L). Bradykinin-(1–8) (1 μmol/L) was added 50 to 100 seconds after the initial dose of the agonist, and the peak [Ca²⁺], released was 92±22% of the initial response (n=3). Another endogenous substrate, Ang-(1–7), was used in the same type of experiments. Ang-(1–7) (1 μmol/L) added to the cells 100 to 230 seconds after bradykinin induced a second [Ca²⁺], peak of 124±34% of the first peak (n=5). These peptides, just like other potentiators of bradykinin, were inactive in the absence of bradykinin. In the absence of bradykinin, Ang-(1–7) was equally ineffective in endothelial cells.

Discussion

These experiments were carried out to explore further the relationship of ACE to the B₂ receptor and the mode of activation of the receptor by agents that have an affinity to the active center, but their actions in these studies cannot be attributed to inhibition of the cleavage of bradykinin. In some early experiments done in the isolated surviving guinea pig ileum, the contractions induced by bradykinin could be potentiated by a variety of agents that were not necessarily enzyme inhibitors. Studies on blood vessels also indicated that the mode of action of ACE inhibitors goes beyond protecting bradykinin against enzymatic breakdown. It was proposed that ACE inhibitors potentiate bradykinin by increasing the affinity of B₂ receptor.

We observed, using isolated guinea pig ileum and left atria, that ACE inhibitors, when given before bradykinin is added to the organ bath, enhance the effects of bradykinin on the B₂ receptor, but they also re-sensitize the preparation if given after the peptide agonist that desensitized it. These experiments could not exclude a direct effect of ACE inhibitors on the B₂ receptor, so to decide this issue, we transfected cultured cells. In CHO cells cotransfected with ACE and B₂ receptor, the concentrations of the two proteins were similar to those found normally in endothelial cells, and the potentiation phenomenon was clearly demonstrated there. ACE inhibitors were inactive with CHO cells that expressed only the B₁ receptor.

Bradykinin analogues that are more resistant to ACE but are ligands of B₂ receptors also are potentiated by ACE inhibitors, just as bradykinin. The action of bradykinin is immediately enhanced even in tissues or cells in which its inactivation is slow; for example, ACE inhibitors enhanced inositol 1,4,5-triphosphate (IP₃) release in CHO-15AB cells in seconds.

Augmenting bradykinin activity on the receptor by ACE inhibitors includes increasing the number of receptor sites, preserving high-affinity receptors, re-sensitizing the desensitized receptors, and decreasing receptor internalization, which results in the increased release of signal transduction products.

All of these may contribute to the improved functioning of the damaged heart (e.g., after myocardial infarction, or by diminishing reperfusion injury and subendothelial tissue proliferation after vascular endothelial damage), as seen in clinical subjects and in laboratory experiments. ACE inhibitors also support renal function in kidney diseases, such as in diabetic nephropathy.

Here we investigated further how this crosstalk between ACE and B₂ receptors on the cell membrane can be induced. Two types of CHO cells were used; both expressed human ACE and B₂ receptor, although in different ratios. CHO-AB15 cells were cotransfected with the cDNA of ACE and the B₂ receptor and expressed them at a ratio of 1:1, whereas CHO-AB cells were transfected sequentially by selective cloning (see Methods). They overexpressed ACE and B₂...
receptor in a ratio of 5:1. It is very likely that all receptors were coupled to G proteins and participated in signal transduction. ACE inhibitors given after bradykinin administration make the receptor respond without addition of a second dose of the ligand; thus they abolish tachyphylaxis, as shown by AA release and elevated $[Ca^{2+}]$. The prodrug form of ACE inhibitor, enalapril, was inactive in these tests.

Besides using transfected cells, we also carried out experiments with bovine pulmonary artery endothelial cells, which reacted similarly to transfected cells. For example, here, Ang-(1–7), which is a substrate of the N-domain and an inhibitor of the C-domain of ACE, also potentiated bradykinin.18 (The experimental conditions used to establish potentiation, desensitization and resensitization of receptor, are given in detail in Methods). Ang-(1–7) also resensitized the receptor without having a direct effect on the B₂ receptor by itself. These and previous experiments18 offer an explanation of how this derivative of Ang II, at least in experiments in vitro, can have kininlike action,41,42 without acting on the receptor by itself.

Other ACE substrates, [des-Arg⁹]bradykinin (bradykinin-[1–8]) and Ang-(1–9), also reverse the desensitization of the B₂ receptor by a ligand; this was demonstrated by the increase in $[Ca^{2+}]$. These peptides are metabolites of the parent endogenous peptides bradykinin, Ang I, and Ang II. Bradykinin-(1–8) is released by plasma carboxypeptidase N or tissue plasma membrane carboxypeptidase M by the removal of the C-terminal arginine of bradykinin.43 This step converts it from being a B₁ ligand to an agonist of the B₂ receptor.28 In our experiments, it also affected the B₂ receptor indirectly by potentiating bradykinin through interaction with ACE.

Of these peptides, Ang-(1–9) is of particular interest. It is released by the hydrolysis of the His⁵-Leu¹⁰ bond in Ang I. Thus it cannot be converted to the vasoconstrictor Ang II by ACE. This cleavage was attributed initially to a carboxypeptidase A-type enzyme in platelets,44 and the resulting peptide inhibited ACE. Here we used a recombinant human enzyme, deamidase (cathepsin A, lysosomal protective protein)22 that cleaved Ang I avidly by releasing Leuⁱ⁰. (It can cleave Ang I further at the Phe⁴-His⁹ bond and thus release Ang II.) Possibly, the platelet carboxypeptidase A–type enzyme is identical with this protein.

To decide which domain and active center of ACE is involved in the potentiation process, we used mutated ACE and monoclonal antibodies. The N-domain of ACE is distal to the plasma membrane in the membrane-anchored enzyme,30 which is found frequently on microvilli.29 ACE is in contact with peptide substrates in fluids that pass through body conduits, for example, the renal proximal tubules. The C-domain is anchored to plasma membrane by the transmembrane and cytosolic portions of this single-chain protein.30,45 The mutated ACE expressed in CHO cells lacked an active C-domain; only the N-domain had an active center.30 This mutated ACE on the cell membrane acted similarly to the somatic 2-domain enzyme used in the other experiments; an ACE inhibitor potentiated bradykinin activity on the B₂ receptor in these cells as well. The evidence of the involvement of the C-domain active center alone is less convincing, but keto-ACE, at a concentration that inhibited the C-domain active center18 only, did resensitize the B₂ receptor.

We tested monoclonal39 and polyclonal39 antibodies to further probe the involvement of the active sites of ACE. Of the 3 monoclonal antibodies, 2 (3A5 and 12H5) that reacted with the active center on the N-domain distal to the cell membrane (Figure 7) enhanced $[^{3}H]AA$ release by bradykinin. The third antibody, the noninhibitory 9B9, had no effect. Polyclonal antiserum to human ACE augmented the action of bradykinin on the B₂ receptor. Thus, it appears that agents that react with either one of the active centers enhance the action of agonists on the B₂ receptor.

During potentiation of bradykinin and resensitization of the B₂ receptor, both $[^{3}H]AA$ release and $[Ca^{2+}]$ increase are augmented. AA, a precursor of prostaglandins, is liberated after bradykinin activates phospholipase A₂ by the Go₃-coupled receptor. Mobilization of Ca²⁺ by bradykinin is initiated by the Go₃ protein–linked receptors via phospholipase C.46–48 This results in the release of IP₃ and diacylglycerol, as well as stored Ca²⁺ from endoplasmic reticulum. The resensitization of the desensitized B₂ receptor enhanced $[^{3}H]AA$ release and raised $[Ca^{2+}]$. La⁺, which can competitively antagonize Ca²⁺,49 blocked the effect of the resensitization of the receptor by ACE inhibitor, as shown by the lack of an appearance of a second peak of $[Ca^{2+}]$. Since lanthanaum did not affect the first mobilization of calcium by bradykinin, it acted by blocking the entry of extracellular calcium from the medium50 (Figure 6). Thus, before resensitization of the receptor, bradykinin first mobilizes intracellular Ca²⁺ from internal stores and from the endoplasmic reticulum51; at the same time, there is an immediate desensitization of the receptor. Giving an ACE inhibitor, without any additional B₂ agonist, enhanced the entry of calcium from the extracellular fluid because of the bradykinin already present in the medium and acting on resensitized receptors.

The rapidity of resensitization and potentiation indicates that the first step may involve a conformational change, steric rearrangement in the receptor protein. Such a step may induce transformation of an inactive (R) receptor to the active form (R*)2 by enzyme receptor crosstalk. This is also suggested by previous experiments in which the high-affinity sites were preserved and the receptor number was increased by ACE inhibitor.17 However, that does not explain the activity of a low dose of inhibitor (5 nmol/L), which did not elevate bradykinin binding sites but still enhanced $[^{3}H]AA$ release.

Although the phosphorylation of the receptor, followed by endocytosis, is a well-accepted mode of desensitization of G protein–linked transmembrane receptors,25–27,53,54 it appears that desensitization (tachyphylaxis) of the B₂ receptor is immediate, possibly owing to a conformational change. Such an instantaneous desensitization of a receptor by an agonist is a common experience during bioassays on isolated organs. This suggested induced change in the receptor may affect the linking of G protein–coupled receptors to caveolin in the caveolar microdomains53 and subsequent or simultaneous phosphorylation.

Beyond these speculations, studies also indicate that ACE inhibitors activate the B₂ receptor coupled with the G protein Go₃ quantitatively differently than the receptor coupled with...
Goα, Enalaprilat reactivated the receptor to release [3H]AA at a lower concentration (5 nmol/L) than it upregulated the receptor or augmented Ca2+ mobilization (1 μmol/L; Figures 1 through 3). The first reaction is attributed to the receptor signaling through Goα-coupled receptor, and the last reaction to Goα proteins. These findings can be interpreted in various ways. For example, phospholipase A2, which has a crucial role in AA release, is possibly activated at a different, lower active receptor density than phospholipase C involved in Ca2+ mobilization through IP3.47,48 Thus, the potentiation of bradykinin on the B2 receptor can involve different processes, yielding the release of prostaglandins and nitric oxide28,56 as the augmented final signal transduction products. Nevertheless, because of these differences, bradykinin may cause a preferential liberation of prostaglandins compared with nitric oxide in some cells and tissues after administration of an ACE inhibitor. Such an effect is suggested by the experiments of Yu et al.,57 who found that the vasoactivity of bradykinin on rabbit renal microvessels is primarily due to prostaglandins. It certainly is a challenge to decipher more of the intricate steps involved in the potentiation of bradykinin effects on the B2 receptor.

Acknowledgments

These studies were supported in part by National Heart, Lung, and Blood Institute MERIT HL-36473 and HL-58118. We are grateful to Dr Sergei Danilov for donating the monoclonal antibodies and the assistance, and to Dr Richard D. Minshall for useful discussions. These studies were supported in part by National Heart, Lung, and Blood Institute MERIT HL-36473 and HL-58118. We are grateful to Dr Sergei Danilov for donating the monoclonal antibodies and the assistance, and to Dr Richard D. Minshall for useful discussions.

References


Enhancement of Bradykinin and Resensitization of Its B₂ Receptor
Branislav Marcic, Peter A. Deddish, Herbert L. Jackman and Ervin G. Erdös

_Hypertension_. 1999;33:835-843
doi: 10.1161/01.HYP.33.3.835

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/33/3/835

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Hypertension_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

**Reprints:** Information about reprints can be found online at:
http://www.lww.com/reprints

**Subscriptions:** Information about subscribing to _Hypertension_ is online at:
http://hyper.ahajournals.org/subscriptions/