Angiotensin-Converting Enzyme Inhibitor Ramiprilat Interferes With the Sequestration of the B₂ Kinin Receptor Within the Plasma Membrane of Native Endothelial Cells

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Background—ACE (kininase II) inhibitors have been shown to exert their beneficial cardiovascular effects via the inhibition of both angiotensin II formation and bradykinin breakdown. Because recent evidence suggests that ACE inhibitors may also interfere with B₂ kinin receptor signaling and thus enhance the vascular response to bradykinin, we examined whether the distribution of B₂ kinin receptors within the plasma membrane of native endothelial cells is affected by an ACE inhibitor.

Methods and Results—Localization of the B₂ kinin receptor in membranes prepared from native porcine aortic endothelial cells was evaluated by means of specific [³H]bradykinin binding and immunoprecipitation of the B₂ receptor from isolated membranes. Effects of bradykinin and ramiprilat on intracellular signaling were determined by monitoring the activation of the extracellularly regulated kinases Erk1 and Erk2 as well as [Ca²⁺], increases in fura 2–loaded endothelial cells. Stimulation of native endothelial cells with bradykinin 100 nmol/L resulted in the time-dependent sequestration of the B₂ receptor to caveolin-rich (CR) membranes, which was maximal after 5 minutes. Pretreatment with ramiprilat 100 nmol/L for 15 minutes significantly attenuated the recovery of B₂ kinin receptors in CR membranes while increasing that from membranes lacking caveolin. This effect was not due to the inhibition of bradykinin degradation, because no effect was seen in the presence of an inhibitory concentration of the synthetic ACE substrate hippuryl-L-histidyl-L-leucine. Ramiprilat also decreased [³H]bradykinin binding to CR membranes when applied either before or after bradykinin stimulation. Moreover, ramiprilat resulted in reactivation of the B₂ receptor in bradykinin-stimulated cells and induced a second peak in [Ca²⁺], and reactivation of Erk1/2.

Conclusions—The ACE inhibitor ramiprilat interferes with the targeting of the B₂ kinin receptor to CR membrane domains in native endothelial cells. Therefore, effects other than the inhibition of kininase II may account for the effects of ramiprilat and other ACE inhibitors on the vascular system. (Circulation. 1999;99:2034-2040.)

Key Words: angiotensin n enzymes n bradykinin n cells

The crucial role of the vascular endothelium in modulating vascular tone and blood flow to tissues is attributed to the production of vasoactive endothelium-derived autacoids such as nitric oxide, prostacyclin, and endothelin.1 Attenuation of endothelium-dependent vasodilator responsiveness and a reduction in the production of bioactive nitric oxide has been reported in a number of clinical conditions, including hypertension, diabetes, and coronary artery disease,2 and can be reversed by inhibitors of ACE.3,4 This class of compounds also promotes the regression of the changes in arterial media-to-lumen ratio and ventricular hypertrophy associated with hypertension,5 reverses medial thickening in spontaneously hypertensive rats,6 and accelerates endothelial regrowth after arterial injury.7

Although ACE inhibitors are widely accepted vascular protective agents, it is only relatively recently that their beneficial effects have been considered to be due not only to inhibition of angiotensin II formation but also to an accumulation of bradykinin.8 However, because no consistent potentiating effects of ACE inhibitors on circulating kinin levels were evident, it was proposed that these substances instead modulate the concentration of kinins within the vicinity of the endothelium.9,10 While endothelial cells possess the biochemical machinery necessary to generate vasoactive kinins from an endogenous source,11–13 there is increasing experimental evidence to suggest that ACE inhibitors may also exert a direct effect on the endothelial B₂ kinin receptor.14,15 To elucidate the effects of ACE inhibitors on the B₂ kinin receptor, we studied the effects of bradykinin and ramiprilat on the distribution of the B₂ receptor within the plasma membrane of native porcine aortic endothelial cells.
Methods

Materials
Ramiprilat and icatibant were obtained from Hoechst Marion Roussel. Bradykinin and histidine-tartaric acid were purchased from Bachem. [3H]bradykinin (98 Ci/mmol) and [125I]NaI were purchased from Du Pont NEN. All other substances were obtained from Sigma Chemical Co. A B2 receptor–specific antipeptide antiserum was raised against a CR36 peptide and used for immunoprecipitation. The monoclonal antibody against ACE (C78 MAb) was kindly provided by Prof P. Büning (Hoechst Marion Roussel). The antibody against the activated extracellularly regulated kinases (clone 12D4) was from Biomol.

Incubation and Cell Fractionation of Native Porcine Aortic Endothelial Cells
Freshly isolated porcine aortas were slit longitudinally, mounted in an open chamber, and washed twice with a HEPES-modified Tyrode’s solution (mmol/L: NaCl 132, KCl 4, CaCl2 1, MgCl2 0.5, glucose 5, and HEPES 9.5; pH 7.4), and the exposed endothelial layer was incubated in the presence and absence of agonists as indicated in the Results. Thereafter, the incubation was stopped by replacement of the incubation medium with ice-cold HEPES-Tyrode’s solution, and the endothelial cells were harvested by scraping. Endothelial cells from 10 aortas were collected by centrifugation and lysed at 4°C in a glass/Teflon homogenizer in 1 mL of a buffer (mmol/L: sucrose 250, EDTA 1, and Tricine 20; pH 7.6) containing (μg/mL) PMSF 44, leupeptin 2, pepstatin A 2, and trypsin inhibitor 10. From this lysate, a crude supernatant fraction was prepared by centrifugation at 1000g for 10 minutes. The resulting pellet was resuspended in buffer (1 mL), and the homogenization and centrifugation steps were repeated. Both supernatants were combined, layered on top of 30% Percoll, and centrifuged at 84 000g for 18 minutes. The purified plasma membrane fraction was collected, and caveolin-rich (CR) membrane domains were prepared from this fraction as previously described. The caveolin-poor (CP) plasma membrane was contained within the lower fraction (1 mL) after the first gradient centrifugation.

Radioiodination of Proteins and Immunoprecipitation
Proteins in CR membranes were adjusted to 100 μL and incubated with 1 mCi carrier-free [125I]NaI over a solid oxidative phase of Iodo-gen for 15 minutes. Separation of unreacted iodine was achieved by acetone precipitation of labeled proteins and washing of precipitates in 70% (vol/vol) ethanol. Thereafter, membrane proteins were solubilized in 100 μL 1% (wt/vol) SDS. After dilution with 900 μL Tris (50 mmol/L, pH 7.5) containing Nonidet P-40 (1%, wt/vol), deoxycholate (0.5%, wt/vol), and NaCl (150 mmol/L), samples were precleared with Pansorbin (Calbiochem) and immunoprecipitated with specific B2 receptor antiserum as described. Immune complexes were subjected to SDS-PAGE under nonreducing conditions in the presence of urea 5 mol/L. The gels were stained with Coomassie Brilliant Blue and dried for autoradiography. Figured is the ratio of fluorescent intensity of total specific binding compared with control.

Statistical Analysis
Data are expressed as mean±SEM of n experiments. Statistical evaluation was performed by use of ANOVA for repeated measures, followed by a Bonferroni test. Values of P<0.05 were considered to be statistically significant.

Results
Effect of Bradykinin Stimulation on the Distribution of the B2 Kinin Receptor Within the Plasma Membrane
Localization of the B2 receptor in native porcine endothelial cells was evaluated by determining the specific binding of [3H]bradykinin to the CR and the CP plasma membranes. Under resting conditions, bradykinin binding was 660±85 fmol/mg protein (n=14) in CR and 190±25 fmol/mg protein (n=14) in CP membranes (note that CR represents ≤10% of total cellular protein). Nonspecific binding was 30% to 40% of total binding. Stimulation of endothelial cells with bradykinin 100 nmol/L resulted in a significant time-dependent increase in [3H]bradykinin binding to CR membranes that peaked 5 minutes after application of the agonist. Thereafter, [3H]bradykinin binding decreased but was still elevated over control levels after 15 minutes (Figure 1). Prolonged incubation of endothelial cells with bradykinin 100 nmol/L resulted in a significant time-dependent increase in [3H]bradykinin binding to CR membranes that peaked 5 minutes after application of the agonist. Thereafter, [3H]bradykinin binding decreased but was still elevated over control levels after 15 minutes (Figure 1). Prolonged incubation of endothelial cells with bradykinin 100 nmol/L resulted in a significant time-dependent increase in [3H]bradykinin binding to CR membranes that peaked 5 minutes after application of the agonist. Thereafter, [3H]bradykinin binding decreased but was still elevated over control levels after 15 minutes (Figure 1).

To determine whether the bradykinin-stimulated increase in [3H]bradykinin binding to CR membranes was due to an increased affinity of the receptor or its physical translocation, membrane proteins were radioactively labeled, and the B2 receptor was immunoprecipitated. Stimulation of endothelial cells with bradykinin led to a time-dependent increase in recovery of the B2 receptor from CR membranes (Figure
2A). The time course of this translocation was similar to the bradykinin-induced enhancement of \([3H]\)bradykinin binding. This effect was confirmed by Western blotting (Figure 2B).

**Effect of Ramiprilat on \([3H]\)Bradykinin Binding to CR and CP Membranes**

Pretreatment of endothelial cells with ramiprilat significantly decreased \([3H]\)bradykinin binding to CR and enhanced binding to CP membranes. Moreover, the ACE inhibitor partially prevented the increase in binding to CR membranes prepared from bradykinin-stimulated cells. In the same experiment, no significant effect of ramiprilat could be detected in CP membranes (Figure 3). This effect of ramiprilat is unlikely to reflect interference with the binding of bradykinin to its receptor, because we have previously shown the ineffectiveness of ramiprilat in displacing \([3H]\)bradykinin from the B2 receptor.\(^{15}\) In addition, a ramiprilat-induced decrease in B2 receptor protein in CR membranes was confirmed by Western blotting (data not shown).

To further examine the effect of ramiprilat on B2 receptor translocation, endothelial cells were pretreated with bradykinin 100 nmol/L for 20 minutes. At this time, no significant change in \([3H]\)bradykinin binding to CR membrane domains was observed compared with control (data not shown; refer to Figure 1). However, the presence of ramiprilat during the last 5 minutes of the bradykinin stimulation significantly decreased \([3H]\)bradykinin binding to CR (56±9% compared with bradykinin alone, \(n=4, P<0.01\)) and increased binding to CP membranes (113±3% compared with bradykinin alone, \(n=4, P=NS\)). Pretreatment of cells with the selective B2 receptor antagonist icatibant 100 nmol/L for 15 minutes also significantly decreased \([3H]\)bradykinin binding to CR membranes and increased binding to CP membranes (Figure 4). The synthetic ACE substrate hippuryl-L-histidyl-L-leucine, at a concentration (1 mmol/L) that blocks the degradation of bradykinin by ACE, failed to alter \([3H]\)bradykinin binding under both basal and bradykinin-stimulated conditions (data not shown).

**Effect of Ramiprilat on Bradykinin-Stimulated Ca\(^{2+}\) Signaling in Endothelial Cells**

Superfusion of fura 2–loaded cultured endothelial cells with bradykinin 100 nmol/L resulted in a biphasic elevation in \([Ca^{2+}]_i\). The subsequent addition of higher concentrations of bradykinin (0.1 to 1 \(\mu\)mol/L) to these cells failed to induce a further change in \([Ca^{2+}]_i\), (Figure 5A). This is consistent with previous reports that prolonged exposure of endothelial cells to high concentrations of bradykinin results in receptor desensitization.\(^{21}\) This desensitization is homologous for
bradykinin because the $\text{Ca}^{2+}$ responses to such agents as ATP 100 nmol/L, thrombin 3 nmol/L, and histamine 1 $\mu$mol/L remained unaffected (data not shown). Pretreatment of endothelial cells with ramiprilat 100 nmol/L for 15 minutes significantly enhanced the $\text{Ca}^{2+}$ response to bradykinin (data not shown), as previously described, 9 but failed to affect the peak $\text{Ca}^{2+}$ response to either histamine or thrombin. 22 The peak histamine 1 $\mu$mol/L– and thrombin 3 nmol/L–induced increases in $[\text{Ca}^{2+}]$ were 586±52 and 463±27 nmol/L in the absence versus 547±36 and 487±31 nmol/L in the presence of ramiprilat, respectively ($n=4$).

Application of ramiprilat 100 nmol/L to endothelial cells treated with bradykinin 100 nmol/L for 15 minutes consistently resulted in a second peak increase in $[\text{Ca}^{2+}]$, that ranged between 20% and 40% of the initial bradykinin-induced increase (Figure 5B). The simultaneous application of ramiprilat and bradykinin 15 minutes after an initial bradykinin stimulation resulted in a secondary peak increase in $[\text{Ca}^{2+}]$ that was identical to that observed after the application of ramiprilat alone. This effect can be attributed solely to the ACE inhibitor, because a 10-fold greater concentration of bradykinin failed to induce a secondary peak (see Figure 5A). The secondary increase in $[\text{Ca}^{2+}]$, was still observed when ramiprilat was applied in a $\text{Ca}^{2+}$-free buffer but was not observed in response to the coadministration of ramiprilat and icatibant (data not shown).

**Effect of Ramiprilat on Basal and Bradykinin-Stimulated Activation of the Extracellularly Regulated Kinases Erk1 and Erk2.**

Bradykinin 100 nmol/L elicited the time-dependent activation of Erk1/2 in native endothelial cells, which peaked at 5 minutes and had returned to near baseline values after 20 minutes (Figure 6). Pretreatment of cells with ramiprilat 100 nmol/L for 15 minutes tended to increase Erk1/2 activation, although this effect did not reach statistical significance. Ramiprilat, however, enhanced the bradykinin-induced activation of both kinases as assessed 5 minutes after application of the agonist. In a desensitization protocol similar to that described above, ramiprilat induced a secondary activation of Erk1/2 that was not observed in response to higher concentrations of bradykinin (compare lanes 3 and 5 in Figure 6).

**Localization of ACE in the Plasma Membrane of Native Endothelial Cells**

ACE and the caveolar marker protein caveolin-1 were detected in the lysate, plasma membrane, and CR membrane domains from native endothelial cells (Figure 7). ACE, but not caveolin-1, was also detected in the CP membranes. Neither bradykinin nor ramiprilat, either alone or in combination, influenced the distribution of ACE within the plasma membrane (data not shown).

**Discussion**

In the present study, we have demonstrated that incubation of native porcine aortic endothelial cells with ramiprilat decreased $[^{3}H]$bradykinin binding to CR membranes while increasing that in plasma membranes lacking the caveolar marker protein. In addition to this effect on unstimulated...
cells, the ACE inhibitor was able to inhibit and/or partially reverse the bradykinin-induced translocation of the B₂ receptor to CR membranes and to reactivate signaling events initiated by the B₂ receptor.

The endothelial B₂ kinin receptor is a G protein–coupled receptor that is rapidly desensitized and internalized in response to high agonist concentrations. Although many members of this receptor family bind to β-arrestin and are internalized via clathrin-coated pits, the B₂ kinin receptor appears to be sequestered to caveolae before internalization. Indeed, the translocation of the B₂ kinin receptor and its associated G proteins (Gᵢₒ and Gₑₐ) to caveolae after application of the agonist has been described in a smooth muscle cell line (DDT₁ MF-2 cells). Similarly, the B₂ receptor was shown by immunoelectron microscopy to be randomly distributed on the plasma membrane of a carcinoma cell line under resting conditions and was sequestered to vesicles containing caveolin-1 after the application of bradykinin.

Although caveolae isolated by density centrifugation are reported to be contaminated with other membrane fractions, the results of the present study are in agreement with the above-mentioned studies, because we also observed a bradykinin-induced translocation of the B₂ receptor to CR membranes. The bradykinin-stimulated increase in [³H]bradykinin binding to CR membranes was time-dependent and transient. That these effects can be attributed to the targeting of the B₂ receptor to a specific membrane compartment rather than a change in the receptor binding affinity was demonstrated by immunoprecipitation of radioactively labeled B₂ receptors and Western blotting. The limited amount of protein obtained from native cells prohibited Scatchard analysis and competition binding studies.

Our findings that the B₂ receptor can be recovered from both CR and CP membranes and that ramiprilat decreased [³H]bradykinin binding in the CR fraction, without displacing bradykinin from its receptor, suggest that the ACE inhibitor attenuates a basal flux or cycling of the kinin receptor through caveolae. This effect, however, must be attributed to an effect distinct from enzyme inhibition, because the synthetic ACE substrate hippuryl-L-histidyl-L-leucine, at a concentration that blocks the degradation of bradykinin by ACE, failed to alter [³H]bradykinin binding under either basal or bradykinin-stimulated conditions. Icatibant also induced a redistribution of the B₂ receptor within the plasma membrane and enhanced bradykinin binding to CP while attenuating that to CR membranes. Because this selective receptor inverse agonist stabilizes the B₂ receptor in a G protein–uncoupled and thus inactive form, our observations support the concept that a certain proportion of B₂ receptors are inherently active in the absence of agonist occupancy and are continuously sequestered to caveolae.

ACE inhibitors potentiate many of the vascular responses to bradykinin, an effect originally attributed to the accumulation of kinins generated locally within the vascular wall. There are, however, situations in which ACE inhibitors amplify the response to bradykinin, although accumulation of the peptide cannot be assumed to occur, eg, in the isolated perfused heart and superfused cultured endothelial cells. Because the effects of ACE inhibitors in such models were sensitive to icatibant, we proposed that ACE inhibitors are able to interact with and modulate the activity of the B₂ receptor. Indeed, ACE inhibitors enhance the bradykinin-induced contraction of the endothelium-denuded rabbit jugular vein and increase the tone of this preparation, which lacks measurable ACE activity.

Prolonged treatment of native and cultured endothelial cells with a high concentration of bradykinin results in the desensitization of the B₂ receptor and failure of the subsequent application of even higher concentrations of the agonist to elicit a biological response. Under such experimental conditions, the ACE inhibitor was able to inhibit and/or partially reverse the bradykinin-induced translocation of the B₂ receptor to CR membranes and to reactivate signaling events initiated by the B₂ receptor.
conditions, ACE inhibitors appear to be able to reactivate the B₂ receptor–mediated signaling cascade, resulting in an immediate secondary increase in [Ca²⁺], and activation of Erk1/2. These effects were observed only when bradykinin was used as agonist and not in cells pretreated with either histamine or thrombin, suggesting that ACE inhibitors selectively potentiate bradykinin-induced signaling pathways. A similar reactivation of B₂ signaling by enalaprilat was recently reported in CHO cells transfected with both ACE and the B₂ receptor. Although a number of pharmacological studies imply that ACE inhibitors directly activate the B₂ receptor, there is at present no biochemical evidence to suggest that this is the case. Indeed, no effect of enalaprilat could be observed in CHO cells transfected with the B₂ receptor but lacking ACE, and we have been unable to detect specific binding of [³H]ramiprilat to the B₂ receptor isolated from fibroblasts (W.M.-E., unpublished observations). It therefore seems likely that the effects of ACE inhibitors on bradykinin-activated signaling are mediated by a crosstalk between ACE and the B₂ receptor. This crosstalk, however, must occur independently of ACE activity, because ramiprilat potentiated bradykinin-induced responses even in the presence of ACE-saturating concentrations of hippuryl-L-histidyl-L-leucine. Negative cooperativity has been proposed to participate in the desensitization of the B₂ receptor such that after their interaction with bradykinin, B₂ receptors may interact with one another, resulting in an acceleration of the dissociation of the bound ligand and an apparent decrease in the receptor affinity. Because a protein/protein interaction or clustering of B₂ receptors may be involved in this phenomenon and may precede translocation to CR membrane domains, this is a possible point in the desensitization pathway at which ACE inhibitors could intervene. Ramiprilat may also interfere with bradykinin-induced signaling processes at other points and affect, for example, mechanisms determining receptor phosphorylation or act in the opposite manner to icatibant and mediate secondary increase in [Ca²⁺].

In summary, our data represent the first evidence that ACE inhibitors elicit icatibant-sensitive responses in vascular cells.

Acknowledgments

This study was supported by the Deutsche Forschungsgemeinschaft (SFB 553, B3, and B5) and the Commission of the European Communities (BMH4-CT96-0979). The authors are indebted to Professor Peter Bünning for kindly providing the ACE antibody and to Dr Amparo Riesco and Isabel Winter for expert technical assistance.

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_Circulation_. 1999;99:2034-2040
doi: 10.1161/01.CIR.99.15.2034

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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