Replacement of the Transmembrane Anchor in Angiotensin I-converting Enzyme (ACE) with a Glycosylphosphatidylinositol Tail Affects Activation of the B$_2$ Bradykinin Receptor by ACE Inhibitors*

Received for publication, December 1, 1999, and in revised form, January 14, 2000 Published, JBC Papers in Press, March 15, 2000, DOI 10.1074/jbc.M909490199

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To investigate further the relationship of angiotensin I-converting enzyme (ACE) inhibitors to activation of the B$_2$ bradykinin (BK) receptor, we transfected Chinese hamster ovary cells to stably express the human receptor and either wild-type ACE (WT-ACE), an ACE construct with most of the cytosolic portion deleted (Cyt-del-ACE), or ACE with a glycosylphosphatidylinositol (GPI) anchor replacing the transmembrane and cytosolic domains (GPI-ACE). BK or its ACE-resistant analogue were the agonists. All activities (arachidonic acid release and calcium mobilization) were blocked by the B$_2$ antagonist HOE 140. B$_2$ was desensitized by repeated administration of BK but resensitized to agonist by ACE inhibitors in the cells expressing both B$_2$ and either WT-ACE or Cyt-del-ACE. In GPI-ACE expressing cells, the B$_2$ receptor was still activated by the agonists, but ACE inhibitors did not resensitize. Pretreatment with filipin returned the sensitivity to inhibitors. In immunocytochemistry, GPI-ACE showed patchy, uneven distribution on the plasma membrane that was restored by filipin. Thus, ACE inhibitors were inactive as long as GPI-ACE was sequestered in cholesterol-rich membrane domains. WT-ACE and B$_2$ receptor in Chinese hamster ovary cells co-immunoprecipitated with antibody to receptor, suggesting an interaction on the cell membrane. ACE inhibitors augment BK effects on receptors indirectly only when enzyme and receptor molecules are sterically close, possibly forming a heterodimer.

Renin was discovered over a century ago (1), and kallikrein was discovered about 25 years later (2). Many of the cascading events initiated by these proteases are integrated by angiotensin I to angiotensin II activation and which are caused by blocking the enzymatic breakdown of bradykinin (BK) or kallidin. The very extensive clinical applications of ACE inhibitors, not only in treating hypertension but also in treating cardiac conditions, (e.g. congestive heart failure or after myocardial infarction), and in diabetic nephropathies (9–11), have kept attention focused on this issue. In laboratory experiments and in some clinical studies (12, 13), many effects of ACE inhibitors were abolished by the BK B$_2$ receptor blocker HOE 140. Although it was assumed that these effects were due to inhibiting the inactivation of BK, early bioassays already indicated that substances that did not prolong the half-life of BK still potentiated its actions on the isolated guinea pig ileum (14). Experiments on isolated guinea pig atria demonstrated that ACE inhibitors can resensitize the heart tissue desensitized by a B$_2$ receptor agonist (15). More precisely formulated, an unresolved issue was whether the potentiation of BK effects by ACE inhibitors (12, 16, 17) was caused only by blocking its enzymatic hydrolysis. Investigations using cultured cells transfected with cDNA of the human B$_2$ receptor provided evidence that ACE inhibitors do not act on the B$_2$ receptor directly but enhance BK effects and resensitize the receptor to BK only if ACE is also present on the plasma membranes of cells (18, 19). The enhancement of the activity of the peptide and the reactivation of the receptor (thus reversing tachyphylaxis) was the same when, instead of BK, its ACE-resistant peptide analogues were used. Results similar to those in transfected cells were obtained with endothelial cells that constitutively express the enzyme and the receptor (18, 19). The resensitization of the desensitized receptor by ACE inhibitors occurs with the agonist still present in the medium and without adding more peptide, making it difficult to attribute these results to only blocking the breakdown of BK. This effect is also observed with some endogenous peptides that act as inhibitors or slow substrates of ACE (19). Thus, ACE inhibitors and some endogenous peptides augment BK effects on the receptor by inducing cross-talk between ACE and the B$_2$ receptor (19).

We reported elsewhere the effects on B$_2$ receptor activation of a recombinant, mutant ACE that combined part of the N-terminal end with the C-domain of ACE but deleted most of the

\[ \text{PI-PLC, phosphatidylinositol-specific phospholipase C; WT-ACE, wild-type ACE; Z, benzoyloxy carbonyl;} \]

Subsequently, it became obvious that inhibitors of ACE affect the metabolism of both peptides (6). The successful clinical applications of ACE inhibitors have gone far beyond controlling elevated blood pressure (7, 8), but questions remain regarding which of the beneficial effects are due to inhibiting angiotensin II activation and which are caused by blocking the enzymatic breakdown of bradykinin (BK) or kallidin. The very extensive

* This study was supported in part by NHLBI, National Institutes of Health Grants HL36473 and HL58118 and NIDDK, National Institutes of Health Grant DK41431. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡ These abbreviations used are: ACE, angiotensin I-converting enzyme; AA, arachidonic acid; **HAA, [5,6,8,9,11,12,14,15-**H]**N]**AA; BK, bradykinin; Chaps, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; CHO, Chinese hamster ovary; CHO/AB cell, CHO cell expressing both human B$_2$ receptor and wild-type ACE; Cyt-del-ACE, ACE with partially deleted cytosolic domain; GPI, glycosylphosphatidylinositol; HBSS, Hank's balanced salt solution; HEK293, human embryonic kidney 293; Hip-His-Leu, benzoyl-Gly (hippuryl)-His-Leu; PL-

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GPI Anchoring of ACE Affects Activation of the B₂ Receptor

N-domain. We continued these studies as described below to explore the importance of the cytosolic and transmembrane domains of ACE in the activation of the B₂ receptor by ACE inhibitors and, as a consequence, its steric relation to the receptor. Here, we report the results with two ACE constructs: one containing the transmembrane domain with most of its cytosolic portion deleted, and a chimeric ACE construct that results in its expression on plasma membranes with a glycosylphosphatidylinositol (GPI) anchor. These experiments indicate that the cytosolic and transmembrane domains are not required for activation of the B₂ receptor by ACE inhibitors, but ACE has to be in the immediate vicinity of the receptor for the activation to occur, possibly due to heterodimer formation.

**EXPERIMENTAL PROCEDURES**

**Materials—**BK, benzoyl-Gly (hippuryl)-His-Leu (Hip-His-Leu), filipin, tissue culture medium, buffers and reagents were from Sigma. [3H]AA, 100 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). The original ACE wild-type cDNA was kindly donated by Prof. P. Corvol of Collège de France (Paris, France). B₂ receptor cDNA was provided by Dr. K. Jarnigan (Syntex, Palo Alto, CA). Chinese hamster ovary (CHO) cells and human embryonic kidney 293 (HEK293) cells were from the American Type Culture Collection (Manassas, VA). Mammalian expression vectors pcDNA3 and pCEP4 were from Invitrogen (Carlsbad, CA). Anti-B₂ receptor antibodies were kindly provided by Prof. W. Müller-Esterl of Gutenberg University (Mainz, Germany). Geneticin (G-418) was from Life Technologies, Inc. Hygromycin B was purchased from Calbiochem (San Diego, CA). Phosphatidylinositol-specific phospholipase C from Bacterium thuringiensis was from Oxford Glycosystems (Oxford, United Kingdom).

**Cell Culture—**CHO cells were grown in 100-mm-diameter Petri dishes in Ham’s F-12 culture medium supplemented with 1-glutamine, antibiotic, Hepes buffer, and 10% fetal bovine serum. Cells were routinely subcultured using trypsin-EDTA to mobilize them. For transfec-tion, CHO cells were plated at a density of 1 x 10⁶ cells per 60-mm dish, 1 day prior to transfection.

**Construction of ACE with Cytosolic Domain Deleted (Cyt-del-ACE)—** ACE cDNA fragment 3743–3886 was obtained by polymerase chain reaction with two primers, ACED1 (5’-GACAGGGCGCGCTGCAG) and ACED2 (5’-GCTCTAGAGGCTGGCTGGGCTGGA). After digestion with NotI and XbaI, the fragment encoding base pairs 3742–3886 of the ACE sequence, followed by a codon for Phe and a stop codon in the 3’ end was obtained by using carboxypeptidase M cDNA as template and two primers, MGPI-1 (5’-AGACGGCCGGCCGCTACG) and MGPI-2 (5’-GCTCTAGAGGCTGGCTGGGCTGGA), in polymerase chain reaction amplification. The amplified product encodes residues 403–426 of carboxypeptidase M, containing the complete GPI anchor signal, the putative anchor attachment site (Ser-proline, Ser-lysine), and the 3 residues N-terminal to this site. It fits into the correct reading frame of human ACE through the connecting NotI site. An ACE/GPI-pcDNA3 construct was obtained by ligating the ~100-base pair polymerase chain reaction fragment digested with NotI and XbaI with a gel-purified 9-kilobase pair fragment of ACE-pcDNA3 digested with NotI and XbaI. The ACE/GPI-pcDNA3 construct was sequenced to prove that it contains the coding region for the extracellular domain of ACE (base pairs 1–3748, encoding residues 1–1242, counting the signal peptide, yielding a mature sequence of 1213 residues from ACE) fol-

lowed by the coding sequence of the GPI anchor signal of carboxypeptidase M in the same reading frame. The C-terminal sequence of ACE/GPI is as follows: LPLDGPGRDHS*AATKPSLFLVSLIIHFPK (underlining indicates the carboxypeptidase M sequence; * indicates the GPI anchor attachment site). The plasmid DNA of ACE/GPI-pcDNA3 was purified through a phenol-chloroform extraction and transiently transfected HEK293 cells with Superfect reagent (Qiagen) according to the manufacturer’s protocol.

To establish cell lines with stable expression, CHO cells were transfected with human mutant or wild-type ACE cDNA in a pcDNA3 expression vector (carrying the neomycin resistance gene) using the Superfect method with serum-free DMEM/F-12 medium without antibiotics as described (19). Clones were isolated by cloning rings and grown to confluence (19).

**Screening of Clones for ACE Activity—**Individual clones were evaluated both for cell-associated and released ACE activity. Cells were incubated in fresh serum-free medium; after 24 h, medium was collected, and the cells were washed and lysed in 3 ml of 8 m NaCl in phosphate-buffered saline, pH 7.4, or 3 ml of 13 m Chaps in 50 m NaCl, pH 7.4, with 100 m NaCl. Both the supernatants and the lysates were centrifuged for 15 min at 900 x g and their aliquots were assayed for ACE activity in 50 m Tris-maleate, pH 7.4, with 150 m NaCl, with Hip-His-Leu or Z-Phe-His-Leu (1 m) as described (22, 23). The reaction was terminated by addition of 0.28 m NaOH and the assay mixture then incubated with 100 ml of 20 m o-phthalaldehyde– hydroxylamine in methanol for 10 min at room temperature. A fluorescent derivative of the His-Leu product. This reaction was stopped by the addition of 200 ml of 3 m HCl. Fluorescence of the samples was determined at wavelengths of 365 nm excitation and 500 nm emission.

**Transfection with Human B₂ Receptor cDNA—**Clones with highest levels of ACE expression were transfected with a human B₂ BK receptor cDNA cloned in pcDNA3 together with pCEP4 DNA at a ratio of 10:1, using the Superfect transfection method (19). Following transfection, cells were selected in Ham’s F-12 medium containing 0.5 mg/ml hygromycin B (pCEP4 contains the hygromycin B resistance gene). After selection, different clones were harvested and propagated using cloning rings (19). Cells expressing both human B₂ receptor and wild-type ACE were designated CHO/AB cells.

**Radionuclide Binding on Selected Clones—**Clones with the highest expression of B₂ receptors were selected by [3H]BK saturation binding on whole cell monolayers. Equilibrium binding of 0.05–20 nM [3H]BK with or without 10 μM unlabeled BK was done in Ham’s F-12 cell culture medium for 1 h at 37°C (18). Bound radioactivity was separated from excess [3H]BK by washing. The cells were then solubilized in 0.5 m NaOH (1 ml per 106 cells) for 15 min. The NaOH-lysates were centrifuged at 9000 x g and their aliquots were desalted and transferred to 20-ml liquid scintillation vials to be counted. Clones with high expression of B₂ receptors on the cell surface were chosen and used further.

**Enzymatic Release of the Membrane Anchor of ACE—**HEK293 cells transfected with ACE/GPI-pcDNA3 or CHO cells stably transfected with full-length somatic ACE were harvested by scraping into ice-cold phosphate-buffered saline and were washed by centrifugation at 4°C. For studies of the release of ACE, cells were resuspended in 10 m Hepes, pH 7.4, containing 150 m NaCl, 0.25 m sucrose, and protease inhibitors (1 m phenylmethylsulfonyl fluoride, 10 μM trans-epoxysuccinyl-l-tycylamido-(4-undanide)butane, 100 μM leupeptin, 1 μM pepstatin A, and 1 μg/ml aprotinin) with or without 300 m phosphatidylinositol-specific phospholipase C (PI-PLC) or with 0.1% trypsin treated with t-lysylamide-2-phénylénoléthyl chlorométhyl ketone (Sigma) in the same buffer without protease inhibitors. Cells were incubated for 2 h at 37°C and then centrifuged at 14,000 x g for 30 min. The supernatant was saved, and the pellet was resuspended in the same buffer containing 0.5% Chaps detergent by mixing for 15 min at room temperature. ACE activity was measured in both the pellet and supernatant.

To determine whether enzymatic release of ACE removed the hydrophobic anchor, Triton X-114 partitioning was used (24). In these experiments, cells were lysed by sonication, and cell homogenates were centrifuged at 10,000 x g for 10 min and then at 100,000 x g for 60 min to obtain a final membrane pellet. To solubilize membrane-bound ACE, the membrane fraction was mixed with 0.1% Chaps detergent overnight at 4°C and then centrifuged at 50,000 x g for 1 h to pellet insoluble material. To release the hydrophobic membrane anchor, solubilized membrane fractions were treated at 37°C for 2 h with phosphate-buffered saline alone (control) or 30 mlnitrosonitrile/PLC in the presence of the same protease inhibitors as above or with 0.01% trypsin without protease inhibitors. Triton X-114 partitioning was carried out by diluting samples 10-fold in phosphate-buffered saline followed by

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mixing with a 1% final concentration of Triton X-114 at 4 °C for 1 h. Samples were warmed to 37 °C and centrifuged for 1 min in a micro-
centrifuge to separate the phases. The detergent pellet and supernatant were separated for determination of enzyme activity.

Measurement of Changes in [Ca^{2+}o], and [H]AA—Free cytosolic calcium ([Ca^{2+}i]), was measured using a microspectrofluorometer (PTI Deltascan, Princeton, NJ) or Attofluor Ratiometric with fura-2/AM re-
agent. Cells were grown to confluence on glass coverslips and then incubated with 2–5 μM fura-2/AM for 1 h at 37 °C (19). Cells were washed with buffer, incubated for an additional 15 min, and then mounted in a Sykes-Moore chamber (Belco, Vineland, NJ) at room temperature. Cellular fluorescence at 510 nm was measured following excitation at wavelengths of 340 or 380 nm. Changes in [Ca^{2+}i], were detected by the ratio of intensities at 340 and 380 nm, and the amount of free calcium in the cytosol was calculated. In some experiments, cytosolic calcium concentrations were recorded in a single cell or in 100 cells simultaneously using an Attofluor Ratiometric microspectroflu-
orometer (25). [H]AA release upon receptor stimulation was measured as before (18), following incorporation of [H]AA into membrane phospholipids.

Desensitization and Resensitization of the B2 Receptor—After desen-
sitization of the receptor by initial exposure of cells to a kinin (15, 18), the restoration of sensitivity to the agonist (resensitization) was mea-
sured either by [H]AA release or by mobilization of [Ca^{2+}i]. For exam-
ple, monolayers of transfected CHO cells loaded with [H]AA were stimulated with 1 μM BK or its ACE-resistant analogue for 30 min. Then, without removal of the agonist, cells were exposed to either 5 nM enalaprilat or 5 nM ramiprilat without adding more kinin to show resensitization of the receptor, or as a control, to demonstrate desensitiza-
tion, a second dose of kinin was added instead of the ACE inhibitor.

The amount of [H]AA released was determined by taking AA re-
leased during the first 30 min as baseline and normalizing to the amount released during the reactivation by adding buffer alone for 5 min.

[Ca^{2+}i], mobilization was measured in cells first exposed to 10–100 nM BK. After the initial [Ca^{2+}i], response, without removal of BK from the medium, CHO cells were exposed either to BK again to confirm desensitization or to enalaprilat or another agent to resensitize the receptors.

Filipin Treatment—CHO cells expressing GPI-ACE and B2 receptor or B2 receptor alone were treated in monolayers with Ham’s F-12 medium (10% fetal bovine serum) containing 10 nM filipin for 30 min at 37 °C.

Immunocytochemistry—Approximately 1 x 10^6 CHO cells expressing B2 receptor and GPI-ACE were grown to confluence on coverslips and then incubated for 2 h in phenol red-free and serum-free medium. The cells were treated with 10 nM filipin or vehicle for 30 min, fixed 20 min in Hanks’ balanced salt solution containing 20 mM Hepes (HBSS), 2% paraformaldehyde, and rinsed with 100 mM glycine (3 x 5 min each) to quench free aldehydes groups. The cells were then washed three times in HBSS and preincubated for 30 min in HBSS, 0.2% BSA, 5% goat serum, 0.1% Triton X-100, 0.01% NaN3, Rabbit polyclonal antiserum to human ACE (19:1:200) was applied overnight at 4 °C. Coverslips were washed three times with HBSS, 0.2% BSA, 0.01% NaN3, preincubated for 30 min in HBSS, 0.2% BSA, 5% goat serum, 0.1% Triton X-100, 0.01% NaN3, and finally exposed to 4 μg/ml goat anti-rabbit secondary anti-
body (Alexa 568 conjugate) for 75 °C (19). Cells were

Desensitization and Resensitization of the BK B2 Recep-
tor—To show that the agonist BK uniformly desensitizes the B2 receptor in transfected cells, we stimulated B2 receptors in CHO/AB cells with 100 nM BK to elevate [Ca^{2+}i], level and desensitizes human B2 receptor to second dose of BK in CHO cells expressing B2 receptor and WT-ACE. Ramiprilat (RAM) (1 μM) resensitizes the receptor to BK present in the medium. A, simultaneous measurements in 100 CHO/AB cells. B, tracing showing calculated mean value from A.

**RESULTS**

**Desensitization and Resensitization of the BK B2 Recep-
tor**—To show that the agonist BK uniformly desensitizes the B2 receptor in transfected cells, we stimulated B2 receptors in CHO/AB cells with 100 nM BK to elevate [Ca^{2+}i], and monitored it simultaneously in 100 cells. Fig. 1A illustrates the individual cellular responses to the first application of BK and that the second dose of the peptide was inactive. Fig. 1B shows the computer calculated mean values from 100 cells. The sensitiv-
ity to BK present in the medium was restored by adding the ACE inhibitor enalaprilat (1 μM) (Fig. 1B). Thus, the agonist BK indeed desensitized the receptor, and an ACE inhibitor resen-
sitized it. In these and in subsequent experiments, 1 μM HOE 140 (12) was always used as a control (not shown), and it blocked all the primary effects of BK and the resensitization of the receptor to BK as reported (18, 19).

**Effect of Changes in ACE Structure**—To investigate the im-

**FIG. 1.** BK desensitizes the B2 receptor, ACE inhibitor resen-
sitizes it. BK (100 nM) raises the [Ca^{2+}i], level and desensitizes human B2 receptor to second dose of BK in CHO cells expressing B2 receptor and WT-ACE. Ramiprilat (RAM) (1 μM) resensitizes the receptor to BK present in the medium. A, simultaneous measurements in 100 CHO/AB cells. B, tracing showing calculated mean value from A.
portance of the cytoplasmic and transmembrane domains of the ACE molecule (27–29) in augmenting BK effects on B2 receptors, we created two different ACE constructs (Fig. 2). The first is Cyt-del-ACE; the second one (GPI-ACE) is a chimeric ACE in which the sequence encoding the transmembrane and cytoplasmic domains was deleted from the cDNA and replaced with a sequence from carboxypeptidase M encoding the C-terminal 24 residues, comprising the signal for attachment of a GPI-membrane anchor (20). These mutants were stably transfected and expressed in CHO cells together with human BK B2 receptor.

**Cyt-del-ACE**—The mutant was obtained to test whether or not the potentiation of B2 receptor by ACE inhibitors, and thus the cross-talk between the enzyme and the receptor, would be mediated by an intracellular mechanism involving the intracellular portion of ACE. For this purpose, a human ACE mutant, Cyt-del-ACE, was used. This construct lacks most of the intracellular domain of WT-ACE (27, 28); 18 carboxyl-terminal amino acids were deleted, and an extra Phe was added to the C terminus as a consequence of the construction method (Fig. 2). The truncation removes three out of five serine residues, which may serve as potential phosphorylation sites within the cytoplasmic domain (30). The extracellular and transmembrane domains of Cyt-del-ACE were unchanged.

Seven clones were tested for stable expression of B2 receptor and ACE in CHO cells. The selected clone expressed 82,000 B2 receptors per cell and 13,000 ACE molecules per cell.

The effect of enalaprilat on B2 receptor number was tested on Cyt-del-ACE-expressing cells at two concentrations of the inhibitor. Enalaprilat (5 nM) increased B2 receptor number 2.8-fold (n = 3; p < 0.05); the difference between the effects of these two concentrations of enalaprilat was not significant.

We also investigated whether enalaprilat can desensitize the B2 receptor through interaction with Cyt-del-ACE (Fig. 3). The B2 receptor was desensitized by the initial application of 1 μM BK, as shown by lack of a response to an additional dose of 1 μM BK (Fig. 3). Enalaprilat (5 nM) desensitized B2 receptor-mediated [3H]AA release, resulting in a 6.5 ± 0.8-fold (n = 3; p < 0.005) increase over buffer alone, and 1 μM enalaprilat potentiated the response 8.4 ± 1.2-fold (n = 3; p < 0.005).

Enalaprilat (1 μM) also desensitized the B2 receptor to BK present in the medium, when a rise in [Ca2+]i of 2.7 ± 0.6-fold was recorded (n = 6; p < 0.005; Fig. 4). In the absence of BK, enalaprilat was inactive (not shown).

These results, which agree with the data obtained with full-sized WT-ACE (18, 19), suggest that ACE inhibitors can potentiate BK effects on B2 receptors acting through Cyt-del-ACE, which lacks most of the intracellular domain. This is taken as an indication that for an interaction between ACE and the B2 receptor, either the transmembrane anchor of ACE, the extracellular portion, or both, but not most of the intracellular portion, are important.

**GPI Anchored ACE**—To investigate the role of the transmembrane anchor, we employed a chimeric ACE cDNA encoding the extracellular domain of ACE (residues 1–1213 of the mature protein) followed by the C-terminal 24 residues of carboxypeptidase M (20, 21) (Fig. 2). Thus, the final 64 residues of ACE containing the transmembrane and cytoplasmic domains were replaced with the 24-residue GPI anchor signal sequence of the carboxypeptidase. When this construct was expressed in transiently transfected HEK293 cells, high ACE activity was present in membrane-bound form, as determined after subcellular fractionation and immunofluorescent staining (not shown).

To prove that GPI-ACE was indeed anchored by the GPI tail, HEK293 cells transfected with WT-ACE or GPI-ACE were treated either with buffer alone as control or with 30 milli-units/ml PI-PLC or 0.01% trypsin. As shown in Table I, both PI-PLC and trypsin released the GPI-ACE from the cells into the supernatant, whereas buffer alone did not. In contrast, WT-ACE was not released by PI-PLC or buffer but was cleaved from the membrane with 0.01% trypsin (Table I). To prove that cleavage by PI-PLC or trypsin removed the hydrophobic membrane anchor from GPI-ACE, membrane fractions from transfected HEK293 cells that were first solubilized with Chaps detergent were treated with either buffer alone, 30 milli-units/ml PI-PLC, or 0.01% trypsin and then subjected to Triton X-114 partitioning (24). Membrane fractions from CHO cells stably transfected with WT-ACE were used as controls. In these experiments, only 15% of the GPI-ACE partitioned into the detergent-soluble fraction.
the aqueous phase in the buffer control, indicating that the majority of the enzyme contained the hydrophobic GPI anchor, whereas after PI-PLC treatment, 76% and after trypsin treatment, 60% of the enzyme partitioned into the aqueous phase (mean values from two separate experiments). In contrast, 68% of WT-ACE treated with trypsin partitioned into the aqueous phase, but only 26 or 15% was found in the aqueous phase after PI-PLC or buffer treatment. Taken together, the above data show that the C-terminal hydrophobic region of carboxypeptidase M functions as a signal for addition of the GPI tail to anchor the extracellular domain of ACE to the plasma membrane, which is normally anchored by a transmembrane peptide.

CHO cells were stably transfected with the GPI-ACE construct and selected by Geneticin (G-418) resistance. Ten clones were tested for ACE activity using 1 mM Z-Phe-His-Leu as the substrate, and a clone with the highest expression was chosen (21–29 nmol/min/mg protein; 50,000 ACE molecules/cell). These cells were subsequently co-transfected with a cDNA encoding the B2 receptor and a pCEP4 vector and selected using hygromycin B resistance. Six clones were tested by [3H]AA release (18) and the clone with the highest expression was chosen; 95,000 B2 receptor molecules/cell. The level of ACE expression was not changed by the second transfection (50,000 molecules/cell).

In the GPI-ACE and B2 transfected CHO cells, an ACE- and carboxypeptidase-resistant analogue, [Phe⁸(CH₂NH)Arg⁹]BK (10 nM) (31), activated the B2 receptor as shown by AA release and increase in [Ca²⁺]i level. This peptide stimulated the release of [³H]AA 2.9 ± 0.3-fold over basal values (n = 3) and enhanced [Ca²⁺]i level 3.4 ± 0.5-fold (n = 8). These results are similar to those obtained with CHO/AB cells expressing WT-ACE (18, 19). However, in GPI-ACE-containing cells, 1 μM ramiprilat failed to resensitize the B2 receptors desensitized by the agonist (see below). This is in contrast to results routinely obtained with WT-ACE (18, 19) or as reported above with Cyt-del-ACE. Thus, BK analogue in the medium in the presence of an ACE inhibitor did not release [³H]AA or elevate [Ca²⁺]i after the receptor was desensitized by the first dose of the agonist (Figs. 5 and 6).

These experiments indicate that GPI-ACE is unable to interact with the BK B2 receptor, which could be explained by two possible mechanisms. The first one is that the transmembrane domain of ACE directly interacts with the BK B2 receptor.
Alternatively, the steric relationship of GPI-ACE to B₂ might be altered. GPI-anchored proteins are known to partition into sphingolipid and cholesterol-rich microdomains on the plasma membrane (32–37). GPI-ACE may be sequestered in these rafts or similar lipid structures and therefore unable to interact with the BK B₂ receptor. In addition, GPI anchors confer higher lateral mobility compared with transmembrane domains (34).

To distinguish between these possibilities, we attempted to restore the steric relationship between the B₂ receptor and GPI-ACE by employing a cholesterol-depleting agent, filipin (38), which is known to disperse GPI-anchored proteins from the cholesterol rich domains in the plasma membrane.

The CHO cells expressing GPI-ACE and B₂ receptor were pretreated in a monolayer with 10 nM filipin for 30 min at 37 °C. Viability of the cells was preserved after this treatment, and they responded to stimulation by 10 nM BK analogue with a typical $[\text{Ca}^2+]$ mobilization response (Fig. 6). In control experiments without added ramiprilat, cells did not respond to a second dose of BK ($n = 4$; not shown).

Fig. 6B, a recording from a single CHO cell stimulated with an ACE-resistant BK analogue, shows that filipin restored the ability of ramiprilat to resensitize the receptor. In eight additional experiments done with approximately 100 individual cells each, filipin restored the activity of ramiprilat (1 μM) to resensitize the receptor in 84 ± 5% of the cells to the BK analogue (10 nM) present in the medium. The mean $[\text{Ca}^{2+}]$ response to the BK analogue after filipin and resensitization by ramiprilat was 120 ± 8% of the original response. As in all other experiments, the ACE inhibitor did not enhance the $[\text{Ca}^{2+}]$ level in the absence of an agonist (not shown).

We also tested whether ramiprilat resensitized the desensitized B₂ receptor to release $[^{3}\text{H}]$AA. In four experiments, in CHO cells expressing GPI-ACE and B₂ receptors, 1 μM ramiprilat failed to resensitize the receptor to stimulate the release of $[^{3}\text{H}]$AA after desensitization by 1 μM BK analogue (Fig. 5). However, if the cells were pretreated with 10 nM filipin for 30 min at 37 °C, 1 μM ramiprilat resensitized $[^{3}\text{H}]$AA release by 1 μM BK analogue present in the medium, increasing it over basal release by 5.7 ± 0.8-fold ($n = 4$; Fig. 5).

**Immunocytochemistry**—The localization of ACE on the plasma membrane of CHO cells stably expressing either WT-ACE or GPI-ACE was studied by immunostaining and confocal microscopy. WT-ACE was distributed uniformly on the plasma membrane of CHO cells (Fig. 7A). In contrast, in the cells expressing GPI-ACE, immunostaining with antisera to human ACE showed a patchy distribution and aggregated clusters on the plasma membrane (Fig. 7C). When cells expressing WT-ACE were treated with 10 nM filipin for 30 min prior to fixation and immunostaining, filipin did not alter the membrane-associated staining pattern of WT-ACE (Fig. 7B). However, pretreatment with filipin dispersed the patchy immunostaining pattern of GPI-ACE on the cells (Fig. 7D) to reveal a more uniform distribution of GPI-ACE. These data are consistent with the hypothesis that filipin, by binding to cholesterol, disrupts GPI-anchored protein clusters accumulated on the membrane in areas rich in cholesterol (32–37).

**Co-Immunoprecipitation of B₂ Receptors and ACE**—The above data are in agreement with a physical interaction between ACE and the B₂ receptor requiring the extracellular domain of ACE. To further test the steric relationship between B₂ receptor and ACE and their possible interaction, we immunoprecipitated the two proteins with anti-B₂ receptor antibodies. CHO/AB cells in monolayers were exposed to buffer alone, enalaprilat (1 μM), BK (1 μM), or BK and enalaprilat for 30 min. (Fig. 8). Cells were then solubilized with detergent, the lysates immunoprecipitated with anti-B₂ receptor antibodies. ACE was present in all precipitates as detected by Western blotting with rabbit polyclonal anti-ACE antibodies. ACE was present in all precipitates as detected with the antibodies, showing that the enzyme and receptor formed a complex (Fig. 8). When immunoprecipitation was performed with normal rabbit serum or with antiserum to the

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\text{TABLE I}
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**Release of ACE from HEK293 cells transfected with the wild type (WT-ACE) or chimeric enzyme containing the C-terminal tail of carboxypeptidase M (GPI-ACE)**

Transfected cells were scraped, washed, and then incubated either with buffer, with PI-PLC and protease inhibitors, or with trypsin, for 2 h at 37 °C. Cells were centrifuged at 14,000 × g for 30 min, and ACE activity in the supernatant and pellets was measured with Z-Phe-His-Leu. Results shown are mean values from two or three separate transfections. Total ACE activity among the different transfections did not differ by more than 2-fold and ranged from 40–80-fold above the background activity measured in nontransfected cells.

| Transfected cells | Treatment | Activity |
|-------------------|-----------|----------|
|                   |           | % of total |
|                   |           | Supematant | Pellet |
| WT-ACE            | Buffer control | 20 | 80 |
|                   | 0.01% Trypsin | 78 | 22 |
|                   | 30 milliunits/ml PI-PLC | 28 | 72 |
| GPI-ACE           | Buffer control | 18 | 82 |
|                   | 0.01% Trypsin | 66 | 34 |
|                   | 30 milliunits/ml PI-PLC | 67 | 33 |

**Fig. 5.** ACE inhibitor is inactive in cells expressing GPI-ACE and B₂ receptor; filipin restores activity. CHO cells expressing GPI-ACE and B₂ receptors loaded with $[^{3}\text{H}]$AA were pretreated and desensitized with 1 μM ACE-resistant BK analogue (BKan) for 30 min. Following this, addition of buffer alone, 1 μM BK analogue, or 1 μM ramiprilat (RAM) did not increase $[^{3}\text{H}]$AA release over baseline. In cells pretreated with filipin (10 nM; 30 min), 1 μM BK analogue had no effect, but 1 μM ramiprilat resensitized the receptor to it to release $[^{3}\text{H}]$AA. ($n = 4$; *, $p < 0.005$, compared with buffer alone).
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**FIG. 6.** Filipin restores resensitization of the B₂ receptor to BK by ACE inhibitor to elevate [Ca²⁺]i in GPI-ACE-expressing cells. A, tracing showing in a single CHO cell, expressing GPI-ACE and B₂ receptor, ACE-resistant BK analogue (10 nM) (BKcan) elevates [Ca²⁺]i, but ramiprilat (RAM) does not resensitize the receptors. B, tracing from a single CHO cell expressing GPI-ACE and B₂ receptor that was pretreated with filipin (10 nM; 30 min). This treatment restores the ACE inhibitor effect and ramiprilat resensitizes B₂ receptor to BK analogue in the medium.

**FIG. 7.** Effect of filipin on WT-ACE and GPI-ACE localization on plasma membrane of CHO cells. CHO cells stably expressing WT-ACE (A and B) or GPI-ACE (C and D) were treated with vehicle (A and C) or 10 nM filipin (B and D) for 30 min, fixed, and labeled with anti-ACE antiserum. Note the uniform membrane distribution of WT-ACE as compared with the punctate, patchy membrane distribution of GPI-ACE, and note the dispersion of GPI-ACE by filipin treatment. (The experiment was repeated three times with similar results.)

B₂ receptor using CHO cells expressing only B₂ or only ACE (18, 19), no immunoreactivity to anti-ACE antibodies was detected (not shown). In control experiments, Western blotting of cell lysates without immunoprecipitation revealed a dark band of the expected molecular weight in cells expressing WT-ACE, whereas cells expressing only the B₂ receptor were negative (Fig. 8). Thus, WT-ACE and B₂ receptor, when transfected into CHO cells, form a complex, very likely a heterodimer, owing to the very close steric relationship. This gross complex formation was sustained even in the presence of agonist and ACE inhibitor.

**DISCUSSION**

Human ACE is a single chain protein that can be roughly divided into three parts: 1) a large extracellular portion containing two homologous active sites on the N and C domains; 2) a hydrophobic domain near the C terminus that functions as the transmembrane anchor; and 3) a short cytoplasmic domain (27, 28) (Fig. 2). Our previous studies have shown that ACE inhibitors potentiate BK and reactivate the B₂ receptor to kinin. This may mean that the transmembrane domain of ACE and the receptor have to be in close proximity, possibly forming a heterodimer mediated through the extracellular domains of ACE and the receptor. Two different, active ACE inhibitors, enalaprilat and ramiprilat, gave the same results, showing that their effect was group-specific (i.e. their ability to bind ACE) and not related to any unique aspect of their structures (12).

Receptors can form dimers via interactions between their cytosolic domains (40). To determine whether the cytosolic domain of ACE was important in our studies, we created Cyto-ACE, where the major part of the cytosolic domain was deleted, including three out of five of the possible serine phosphorylation sites (30). This deletion had no effect on the potentiation of BK and resensitization of its receptor by an ACE inhibitor.

To establish whether the transmembrane domain was critical for this interaction, we expressed a chimeric ACE cDNA containing the two extracellular active site domains and the C-terminal GPI anchor signal sequence from carboxypeptidase M (20, 21, 24) to replace the cytoplasmic and transmembrane domains of ACE. This resulted in a chimeric ACE that was still membrane-associated via a GPI-anchor but completely lacked transmembrane and cytoplasmic domains. In these cells, BK and an ACE-resistant BK analogue (31) activated the B₂ receptor as before, but in contrast to cells expressing WT-ACE and the B₂ receptor, ACE inhibitor did not resensitize the receptor to kinin. This may mean that the transmembrane domain of ACE is crucial for its interaction with the B₂ receptor, implying that the cross-talk takes place within the membrane. A more likely explanation is based on the following. It has been established that GPI-anchored proteins are concentrated in cholesterol- and sphingolipid-rich microdomains, also termed lipid rafts, on the cell membrane that can exclude many transmembrane proteins (32–37). GPI-ACE would be sequestered in such lipid rafts. Furthermore, GPI-anchors in general enhance the lateral mobility of proteins on cell membrane (34). This should also apply to GPI-ACE, if it is compared with WT-ACE anchored by transmembrane and cytosolic portions to the cell membrane (27–29). Consequently, the distance of GPI-ACE from the B₂ receptor would increase on the cell surface and deny an enzyme receptor interaction. When the cells expressing both GPI-ACE and B₂ receptor were depleted of cholesterol by filipin, which resulted in the dispersion of GPI-
anchored proteins, it led to the full restoration of the response to ACE inhibitors. This indicates that the lack of reactivation with GPI-ACE is due to sequestration in lipid rafts and not necessarily to the increased lateral movement. The absence of resensitization of the B2 receptor in cells expressing GPI-ACE and restoration of the response by filipin also indicate that close physical proximity is required for cross-talk between B2 receptors and ACE.

The conclusions reached after using filipin in a large number of cells are supported by immunocytochemistry. As shown before by immunohistochemistry (41, 42), when antibody to ACE was used, WT-ACE was uniformly distributed on the plasma membrane of cells (Fig. 7). In contrast, GPI-ACE had a patchy distribution, indicative of aggregated clusters of the enzyme. After employing filipin, GPI-ACE was dispersed on the membrane of the cells and distributed more uniformly, and the aggregate was dissolved. Thus, filipin changed the distribution of GPI-ACE on the membrane to resemble more that of WT-ACE.

G-protein linked receptors, including the B2 BK receptor, can form homodimers and even heterodimers with other receptors or different proteins (43–45). The dimer formation has been associated with receptor desensitization and endocytosis (46). The results of the present studies, showing co-immunoprecipitation of ACE and the BK receptor, indicate an interaction between these two proteins on the plasma membrane. This suggests how ACE inhibitors may affect BK receptor activity via ACE. ACE inhibitors, by stabilizing the ACE-B2 receptor heterodimer, could inhibit B2 receptor homodimer formation. However, we found no apparent difference in co-immunoprecipitation in the presence or absence of an ACE inhibitor. Thus, the reactivation by ACE inhibitor may reflect a conformational change in ACE transduced to the B2 receptor in a preformed heterodimeric complex, which results in abolishing desensitization and resensitizing the receptor to the agonist. Resensitization of the receptor by ACE inhibitors can enhance the activity of the agonist up to 8-fold (Refs. 18 and 19 and see under “Results”).

It was reported that ACE inhibitors reduce the sequestration of the B2 receptor into caveolae, a location that was also found to be enriched in ACE (47). This implied that the inhibition of receptor sequestration by an ACE inhibitor would be responsible for its ability to resensitize the cellular receptor to a BK-stimulated increased [Ca2+]i level (47). These studies might have offered an interpretation for receptor reactivation. For example, ACE inhibitors may interfere with the caveolar sequestration of the ACE and B2 receptor heterodimer complex. However, other findings argue against this interpretation. First, although ACE has been reported to be in caveolae, caveolin-rich fractions can be contaminated with other membrane compartments, depending on the method of isolation. In fact, in the studies reporting a procedure for the isolation of highly purified caveolar fractions, ACE was found to be associated only with plasma membrane fractions and excluded from caveolar fractions, resulting in its use as a marker for membrane fractions that do not contain caveolae (49, 50). Furthermore, the resensitization of the receptor to BK by ACE inhibitors is immediate, happening in seconds (18, 19). This rapid response does not correlate with the physical sequestration of the receptor, which can be on the order of 5–15 min (47). All of this is taken as an indication that the effect of ACE inhibitors on caveolar sequestration is unlikely to be responsible for the reactivation of the response to BK. That could be a secondary phenomenon that occurs after the sequence of events that mediate the initial desensitization and resensitization.

Our previous experiments (19) indicate that the pathway mediating the action of resensitized B2 receptors differs from the pathway involved in the immediate response to BK. Consequently, the receptor resensitized to the same peptide can initiate signaling through alternate pathways.2 Judging from experiments based on AA release and [Ca2+]i elevation, both Gβγ and Gq-linked B2 receptors (48, 51) are involved in signaling in the reactivation process. Similarly to WT-ACE (18, 19), in cells expressing the receptor and Cyt-del-ACE, the release of AA by BK was stimulated at a much lower concentration of ACE inhibitor (5 nM) than the elevation of [Ca2+]i (0.1–1 μM). In addition, in the present experiments as well, ACE inhibitors increased the BK receptor sites only when used at the higher concentration (18).

The release of prostaglandins and NO mediate the important cardiovascular effects of BK and kallidin (16, 17), and this can be a consequence of the clinical application of ACE inhibitors (7–11). Prostaglandin release is attributed to activation of phospholipase A2 by Gq-coupled B2 receptors, followed by AA liberation, whereas phospholipase C is activated by a Goq complex (51). The latter reaction leads to elevated [Ca2+]i level and subsequently to enhanced NO synthesis and release. ACE inhibitors are more potent in augmenting BK effects and resensitizing B2 receptors if they are coupled to Goq than if they are coupled to Giq (18, 19). At the end of the transduction chain, this can lead to enhanced release of prostaglandins over NO liberation in the presence of ACE inhibitors in some cells and tissues (52).

In conclusion, ACE and the B2 receptor expressed on cell membranes are stERICALLY closely associated, probably forming a heterodimer, and ACE inhibitors and other agents with af-
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finity for the active center likely alter the heterodimer interaction to promote a conformation of the B₂ receptor that can more efficiently induce signal transduction.

Acknowledgments—We are grateful to Dr. Francois Alhenc-Gelas, INSERM U367 (Paris, France) for very useful discussion and suggestions, Sara Bahnmaier for manuscript preparation, and H. Z. Wang for technical assistance.

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