Carboxypeptidase M and Kinin B1 Receptors Interact to Facilitate Efficient B1 Signaling from B2 Agonists*

Received for publication, December 3, 2007, and in revised form, January 9, 2008 Published, JBC Papers in Press, January 10, 2008, DOI 10.1074/jbc.M709837200

Xianming Zhang†, Fulong Tan†‡, Yongkang Zhang§, and Randal A. Skidgel†‡§
From the Departments of †Pharmacology and §Anesthesiology, University of Illinois at Chicago College of Medicine, Chicago, Illinois 60612

Kinin B1 receptor (B1R) expression is induced by injury or inflammatory mediators, and its signaling produces both beneficial and deleterious effects. Kinins cleaved from kininogen are agonists of the B2R and must be processed by a carboxypeptidase to generate B1R agonists des-Arg²-bradykinin or des-Arg¹⁰-kallidin. Carboxypeptidase M (CPM) is a membrane protein potentially well suited for this function. Here we show that CPM expression is required to generate a B1R-dependent increase in [Ca²⁺]i in cells stimulated with B2R agonists kallidin or bradykinin. CPM and the B1R interact on the cell membrane, as shown by co-immunoprecipitation, cross-linking, and fluorescence resonance energy transfer analysis. CPM and B1R are also co-localized in lipid raft/caveolin-enriched membrane fractions, as determined by gradient centrifugation. Treatment of cells co-expressing CPM and B1R with methyl-β-cyclodextrin to disrupt lipid rafts reduced the B1R-dependent increase in [Ca²⁺]i in response to B2R agonists, whereas cholesterol treatment enhanced the response. A monoclonal antibody to the C-terminal β-sheet domain of CPM reduced the B1R response to B2R agonists without inhibiting CPM. Cells expressing a novel fusion protein containing CPM at the N terminus of the B1R also increased [Ca²⁺]i when stimulated with B2R agonists, but the response was not reduced by methyl-β-cyclodextrin or CPM antibody. A B1R- and CPM-dependent calcium signal in response to B2R agonist bradykinin was also found in endothelial cells that express both proteins. Thus, a close relationship of B1Rs and CPM on the membrane is required for efficiently generating B1R signals, which play important roles in inflammation.

Carboxypeptidase M (CPM)² was discovered as a glycosylphosphatidylinositol (GPI)-anchored membrane protein with B-type carboxypeptidase activity (1–3) and is a member of the “regulatory” or carboxypeptidase N/E subfamily of metallo-carboxypeptidases (4–8) based on its cDNA sequence (9), genomic structure (10), and x-ray crystal structure (11). The overall structure of CPM is composed of a 295-residue N-terminal catalytic domain, followed by an 86-residue conical β-sandwich (transshreyretin-like domain) and a unique 25-residue extension to which the GPI anchor is post-translationally attached (11).

CPM cleaves only C-terminal Arg or Lys residues, and some of its endogenous substrates include bradykinin, anaphylatoxins C3a, C4a, and C5a, Arg- or Lys-enkephalins, epidermal growth factor, and hemoglobin (2, 7, 12, 13). CPM preferentially cleaves C-terminal Arg as exemplified by the kinetics with Arg⁶-Met⁵-enkephalin ($K_m = 46 \mu M$, $k_{cat} = 394 \text{ min}^{-1}$) versus Lys⁶-Met⁵-enkephalin ($K_m = 375 \mu M$, $k_{cat} = 663 \text{ min}^{-1}$) (2). Bradykinin exhibits the lowest $K_m$ (16 μM) of any CPM substrate tested (2), a concentration that is still much higher than the typical physiological concentration of this peptide in the nanomolar range. However, peptidases in vivo typically work at substrate concentrations far below the $K_m$ as exemplified by angiotensin I-converting enzyme (ACE), which has the same relatively high $K_m$ (16 μM) with angiotensin I (14), a major physiological substrate. The development of ACE inhibitors as effective agents for treating hypertension and cardiovascular diseases was based in large part on the critical role of this enzyme in converting angiotensin I to II in vivo (15).

The kinin peptides bradykinin and kallidin (Lys-bradykinin) are generated by the proteolytic action of plasma or tissue kallikrein on high or low molecular weight kinogen (16, 17). Removal of the C-terminal Arg from bradykinin or kallidin inactivates these peptides as agonists of the constitutively expressed B2 receptor (B2R) (16, 17). However, this conversion is a required processing step to generate des-Arg⁹-bradykinin or des-Arg¹⁰-kallidin (16, 18), metabolites that have a variety of biological activities mediated by specific activation of a different B1 receptor (B1R) whose expression is induced by inflammatory mediators (19, 20). Thus, CPM acts as a cell surface processing enzyme to generate des-Arg⁹-bradykinin or des-Arg¹⁰-kallidin, and without this catalytic conversion, B1R signaling could not occur. This is of potential importance in inflammatory or pathological responses. For example, B1R activation stimulates extracellular signal-regulated kinase phosphorylation, prostaglandin production (20), and inducible nitric-oxide synthase-mediated high output NO production (21–23), which inhibits protein kinase Cε activity in endothelial cells (24).
In cytokine-treated human lung microvascular endothelial cells, we showed that nanomolar concentrations of B2 agonists kallidin or bradykinin generated a robust output of NO that could be inhibited by about 50% with either a B1R antagonist or carboxypeptidase inhibitor (22). These results show that, at concentrations far below the \( K_{\text{D}} \), CPM can convert sufficient kinin peptide to B1R agonist to give a physiological response. One possible explanation of these findings is that the B1R and CPM are closely associated and that the orientation of CPM on the cell membrane allows efficient delivery of B1R agonist to the receptor. The x-ray crystal structure of CPM suggested a possible mechanism for favorable orientation of CPM on the membrane involving the unique C-terminal extension and positively charged residues in the C-terminal \( \beta \) sandwich domain that may interact with the phospholipid head groups (11).

The present study was undertaken to explore the possible relationship between the B1R and agonist-generating enzyme CPM on the cell membrane. We show that CPM expression is required for generating B1R signals in response to B2 agonists. Furthermore, CPM and B1Rs are colocalized on the membrane and interact, as shown by immunoprecipitation, cross-linking, and fluorescence resonance energy transfer (FRET) analysis. These data indicate that the close relationship of B1Rs and CPM on the membrane allows the efficient generation of B1R signals that play important roles in inflammatory processes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Low glucose Dulbecco’s modified Eagle’s medium was obtained from Invitrogen. Fetal bovine serum was from Atlanta Biologicals. \( \text{dl-2-mercaptomethyl-3-guanidino-ethylthiopropanoic acid (MGTA)} \) was from Calbiochem. Cholesterol, methyl-\( \beta \)-cyclodextrin (M-\( \beta \)-CD), protein A, HOE 140, des-Arg\(^9\) HOE 140, bradykinin, des-Arg\(^9\)-bradykinin, des-Arg\(^{10}\)-kallidin, polylysine, and suberic acid bis(3-sulfo-N-hydroxysuccinimide ester) (BS\(^3\)) were from Sigma. Kallidin was from Bachem. Fura-2/AM and anti-GFP polyclonal IgG were from Molecular Probes. Anti-V5 monoclonal antibody was from Invitrogen. Anti-CPM monoclonal antibody was from Novocastra. Anti-Ga\(_q/11\) and anti-B1R polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-caveolin-1 monoclonal antibody was from BD Biosciences. Goat antimouse and anti-rabbit IgG-conjugated horseradish peroxidase were from Pierce. 5-Dimethylaminonaphthalene-1-sulfonyl-1-alamyl-1-arginine (dansyl-Ala-Arg) was synthesized and purified as described previously (25). Common chemicals were from Fisher.

**Cells**—Human embryonic kidney (HEK293) cells were from the American Type Culture Collection, and bovine pulmonary artery endothelial cells were from Genlantis. Cells were maintained in Dulbecco’s modified Eagle’s medium containing 100 units/ml penicillin, 100 mg/ml streptomycin, and 10% (HEK293 cells) or 15% (BPAEC) fetal bovine serum.

**Generation of Receptor and CPM Constructs**—The cDNA for human kinin B1R was a kind gift from Dr. Fredrik Leeb-Lundberg (University of Lund, Sweden). The cDNA for human CPM was cloned in this laboratory as described (9, 26). Wild type (WT) B1R and CPM cDNAs were cloned into pcDNA3 or pcDNA6 (Invitrogen) for expression in mammalian cells.

C-terminally tagged B1Rs were generated by amplifying B1R cDNA using PCR and then cloned into pEGFP-N1 (Clontech) for B1R-GFP or into pcDNA6-HisV5C (Invitrogen) for B1R-V5 expression, both at the NotI/BamHI restriction sites. B1R was also cloned into pIRES (Clontech) at the NotI/XhoI sites, together with enhanced green fluorescent protein at the SalI/NotI sites, to achieve the coexpression of B1R and GFP separately but at the same time in the same cells.

Because of the lack of signal peptides on CFP or YFP, N-terminal tagging of the B1R using standard fluorescent protein vectors led to low or no membrane expression. To efficiently express N-terminal CFP- or YFP-tagged B1R or CPM, the signal peptide coding sequence of human ACE was inserted into the pECFP-C1 or pEYFP-C1 vectors (Clontech) upstream of and in the same reading frame as ECFP or EYFP (at the Nhel/A gel sites) to create pECFP-sig-C1 and pEYFP-sig-C1. The B1R cDNA was then cloned into pECFP-sig-C1 or pEYFP-sig-C1 downstream of the fluorescent protein at the XhoI/BamHI sites. For CPM, PCR-amplified cDNA lacking the native CPM signal peptide was cloned into pECFP-sig-C1 or pEYFP-sig-C1 at the XhoI/BamHI sites.

To generate a fusion protein with CPM attached to the extracellular N terminus of the B1R (CPM-B1R), CPM cDNA (nucleotides 1–1390) was inserted in frame to the 5′ end of the B1R coding sequence and cloned into pcDNA3 or pcDNA6 at the BglII/XhoI sites.

All of the PCR fragments used were amplified using high fidelity TaqDNA polymerase. Constructs were verified by DNA sequencing performed by the DNA Services Facility of the Research Resources Center, University of Illinois at Chicago.

**Transfection and Establishment of Stable Cell Lines**—HEK293 cells, at 70–80% confluence in 6-well plates, were transfected with SuperFect (Invitrogen) reagent containing 5 \( \mu \)g of DNA according to the manufacturer’s instructions. After 48 h of transfection, cells were transferred to selective medium containing 418 (500 \( \mu \)g/ml) or blasticidin (5 \( \mu \)g/ml) according to the resistance gene contained in the vector. The cells were cultured for 15–30 days in selective medium and then diluted to the resistance gene contained in the vector. The cells were cultured for 15–30 days in selective medium and then diluted for single clone selection. For B1R selection, the increase in [Ca\(^{2+}\)] was determined using fura-2/AM (21). HEK293 cells stably expressing either B1R, CPM, or both were grown on polylysine-coated glass coverslips to 80% confluence and then loaded with 2 \( \mu \)M fura-2/AM for 60 min at 37°C. Cells were washed and then stimulated with various concentrations of B1R or B2R agonists as indicated, and the fluorescence emission at 510 nm was monitored after excitation at 340 and 380 nm using a PTI Deltascan microspectrofluorometer. The area under the curve was integrated using Origin 7.0 software (OriginLab Corp.).

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3 F. Tan, R. A. Skidgel, unpublished results.
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**Determination of CPM Activity**—CPM activity was measured using dansyl-Ala-Arg substrate as described (25, 27). Briefly, live cells in 12- or 24-well plates were incubated in 200 µl of PBS containing 200 µM dansyl-Ala-Arg for 30–60 min at 37 °C. The medium was transferred to 10 × 75-mm glass tubes, and the reaction was stopped with 150 µl of 1.0 M citrate, pH 3.0, followed by extraction with 1.0 ml of chloroform. The fluorescence in the chloroform layer was measured at 495 nm (340 nm excitation) in a spectrofluorometer. The activity of CPM is expressed as fluorescent units/min/10^6 cells.

**Immunoprecipitation**—The cells were lysed in 200 µl of 50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 0.1% SDS (radioimmune precipitation buffer) containing 1% protease inhibitor mixture (Sigma) and then sonicated for 30 s on ice. The supernatants were collected by centrifugation at 14,000 × g for 10 min at 4 °C and diluted 10-fold with Tris buffer (10 mM Tris, 150 mM NaCl, pH 7.4). Antibody was added to the diluted samples, and after overnight incubation at 4 °C, protein A-coupled agarose beads (15 µl) were added and then further incubated for at least 8 h at 4 °C. After washing with Tris buffer three times, the beads were suspended in SDS-PAGE loading buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.1 M dithiothreitol, 0.01% bromphenol blue) and boiled for 5 min. After centrifugation at 14,000 × g for 5 min, supernatants were analyzed by Western blotting.

**Density Gradient Centrifugation**—Detergent-free preparation of lipid rafts was done using OptiPrep density gradient centrifugation as described (28) with slight modifications. Cells were scraped into PBS, and then centrifuged at 1000 × g for 5 min at 4 °C. (In some experiments, cells were incubated with 50 nM [3H]des-Arg^10^-kallidin for 90 min on ice and then washed three times with PBS before lysing). The cell pellets were lysed with 1 ml of base buffer (20 mM Tris-HCl, pH 7.8, 250 mM sucrose) containing 1 mM CaCl_2, 1 mM MgCl_2, and 1% protease inhibitor mixture by passage through a 22-gauge needle 20 times and then centrifuged at 1000 × g for 10 min at 4 °C. After centrifugation, the postnuclear supernatant was removed, the pellets were lysed and centrifuged again, and the two postnuclear supernatants were combined. An equal volume (2 ml) of base buffer containing 50% OptiPrep was mixed with the postnuclear supernatant and then layered on top with successive 1.6-ml fractions of base buffer containing 20%, 15%, 10%, 5%, and 0% OptiPrep. Gradients were centrifuged for 3 h at 52,000 × g in an SW-41 rotor, and then 0.7-ml fractions were collected (17 total). The fractions were assayed for CPM activity or bound [3H]des-Arg^10^-kallidin. Aliquots of gradient fractions were also mixed with 10× concentrated radioimmune precipitation buffer in a 9:1 ratio, sonicated for 15 s, and then used for Western blotting.

**Detergent Resistance Assay**—HEK293 cells stably expressing CPM and B1R (without or with [3H]des-Arg^10^-kallidin preincubation as above) were incubated in the absence or presence of 10 mM M-β-CD for 30 min at 37 °C. Cells were scraped and transferred into 1.5-ml tubes, collected by centrifugation, and incubated with PBS containing various concentrations of Nonidet P-40 for 30 min on ice. Supernatants were collected after centrifugation at 14,000 × g for 10 min at 4 °C and analyzed for CPM by activity assay and Western blotting or for B1R by counting [3H]des-Arg^10^-kallidin and Western blotting. The radioactivity and CPM activity were expressed as the ratio of that found in the supernatant from cells treated with M-β-CD to that of nontreated cells.

**Cross-linking of B1R and CPM**—Cross-linking was carried out on whole cells using BS^3 similar to procedures described previously (29). Briefly, cells were scraped into PBS and centrifuged for 10 min at 1000 × g. The cell pellets were resuspended in PBS containing 2 mM BS^3 and rotated for 2 h at 4 °C. After washing with PBS, cells were lysed in radioimmune precipitation buffer, sonicated for 30 s, and centrifuged at 14,000 × g for 10 min. The supernatant was used for immunoprecipitation as described above.

**Western Blotting**—Cells were lysed in radioimmune precipitation buffer with sonication for 30 s on ice. After centrifugation at 14,000 × g for 10 min, the supernatant was collected and boiled with 2× concentrated loading buffer for 5 min. The protein samples were separated on an 8% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The blots were blocked with 5% nonfat dry milk in PBS with 0.5% Tween 20 (PBST) for 2 h at room temperature. The membranes were washed with PBST and incubated with primary antibodies overnight at 4 °C. Anti-rabbit or anti-mouse (Pierce) peroxidase-conjugated secondary antibodies were added to the membranes at a dilution of 1:3000, and incubation was continued for 1.5 h at room temperature. The bands were visualized by chemiluminescence (Pierce).

**Confocal Microscopy and Measurement of FRET**—Fluorescence imaging and FRET analysis were performed using an LSM 510 confocal microscope according to the protocol as previously described by Liu et al. (30). Cells were grown on polylysine-coated glass coverslips and fixed with 3% paraformaldehyde. For fluorescence imaging, CFP and YFP excitation wavelengths were 458 and 514 nm, and emission fluorescence was measured at 485 ± 30 and 545 ± 30 nm, respectively. For FRET, selective photobleaching of YFP was performed by repeatedly scanning a region of the specimen with the 514 nm wavelength set at the maximum intensity to photobleach at least 85% of the original acceptor fluorescence. For determination of FRET efficiency in the selected bleach area, the average pixel intensity (I) of the CFP signal was determined from the unmixed pre- and postbleach images using Zeiss software. Relative FRET efficiency was calculated as (1 − (CFP<sub>prebleach</sub>/CFP<sub>postbleach</sub>)) × 100%.

**Statistical Analysis**—Data are expressed as means ± S.E. Statistical analysis was performed using Student’s t test. Values of p < 0.05 were considered significant.

**RESULTS**

**B2R Agonists Only Induce a B1R-dependent Calcium Signal in Cells Co-expressing B1R and CPM**—HEK293 cells stably expressing the WT B1R or WT CPM or both WT B1R and WT CPM were established. In cells stably expressing both WT B1R and WT CPM, B1R agonists des-Arg^9^-bradykinin and des-Arg^10^-kallidin stimulated a dose-dependent increase in intracellular calcium concentration ([Ca^{2+}]) (Fig. 1, A and B). Interestingly, the B2R agonists bradykinin and kallidin also caused a dose-dependent increase in [Ca^{2+}], (Fig. 1, A and B), but the
Effect of Receptor Antagonists and CPM Activity on B1R Signaling Stimulated by B2R Agonist—In HEK293 cells stably expressing WT CPM and B1R-V5, both des-Arg<sup>10</sup>-kallidin (B1R agonist) and kallidin (B2R agonist) stimulated an increase in [Ca<sup>2+</sup>]<i>i</i>, and neither response was blocked by specific B2R antagonist HOE 140 (Fig. 2, A and B). In contrast, the specific B1R antagonist des-Arg-HOE 140 blocked the response to both B1R and B2R agonists (Fig. 2, A and B). The CPM inhibitor MGTA blocked the increase in [Ca<sup>2+</sup>]<i>i</i>, stimulated by B2R agonist kallidin but did not affect activation of the B1R directly by des-Arg<sup>10</sup>-kallidin (Fig. 2, A and B). Similar results were obtained in cells coexpressing WT CPM and either WT B1R, B1R-GFP, or WT B1R and GFP (data not shown).

To further explore the role of CPM activity in this response, we selected two clones that had similar responses to B1R agonist des-Arg<sup>10</sup>-kallidin (Fig. 2D) and similar expression of B1R-GFP (Fig. 2F) but had different activities and expression of WT CPM (Fig. 2, E and F). The clone with the highest CPM activity and expression gave the greatest increase in [Ca<sup>2+</sup>]<i>i</i>, in response to B2R agonist, whereas the clone with lower activity gave a diminished response (Fig. 2C). In contrast, the increase in [Ca<sup>2+</sup>]<i>i</i>, induced by B1R agonist des-Arg<sup>10</sup>-kallidin did not depend on CPM activity (Fig. 2D). Interestingly, B1R-GFP migrated on SDS-PAGE primarily as an oligomer, consistent with a previous report showing that homo-oligomerization of B1Rs is required for cell surface expression (31). To confirm that the high molecular weight form of the B1R was due to homo-oligomerization, a stable cell line co-expressing B1R-GFP and B1R-V5 was established. Immunoprecipitation with anti-GFP or anti-V5 antibody co-immunoprecipitated B1R-V5 or B1R-GFP respectively, as shown by Western blotting (Fig. 2G).

Colocalization and Heterodimerization of CPM and B1Rs—Based on the kinetics determined for bradykinin hydrolysis by purified CPM in vitro (<i>K<sub>m</sub></i> = 16 μM; <i>k<sub>cat</sub></i> = 147 min<sup>-1</sup>) (2), it is unlikely that CPM would generate sufficient concentrations of des-Arg-kinin from 10 or 100 nM B2R agonist in the bulk extracellular fluid to give a response within 10–20 s, as shown in Fig. 1. However, it is possible that the des-Arg kinin generated on the cell surface by CPM is preferentially targeted to a B1R in close proximity or in a microdomain such that the concentration achieved is much higher than an equivalent amount of agonist distributed free in solution. This would require a close
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![Image](image-url)

**FIGURE 2. Effect of receptor antagonists and CPM activity on B1R signaling stimulated with B2R agonist.** A, in cells stably expressing WT CPM and B1R-V5, B1R antagonist des-Arg HOE 140 (1 μM) and CPM inhibitor MGTA (20 μM) blocked the increase in [Ca<sup>2+</sup>], caused by kallidin (KD; 1 μM), but B2R antagonist HOE 140 (1 μM) did not. B, in cells stably expressing WT CPM and B1R-V5, only B1R antagonist des-Arg HOE 140 inhibited the increased [Ca<sup>2+</sup>], mediated by des-Arg<sup>10</sup>-kallidin (DAKD; 1 μM). In A and B, the cells were stimulated only with antagonists/inhibitors (left three bars) or preincubated with antagonists for 60 s followed by stimulation with kallidin or des-Arg<sup>10</sup> kallidin. The [Ca<sup>2+</sup>], was recorded and quantified as in Fig. 1. Data are expressed as mean ± S.E. (n = 3). *, p < 0.05 versus kallidin (A) or des-Arg<sup>10</sup> kallidin (B). C, correlation of CPM expression and activity with the increase in [Ca<sup>2+</sup>], mediated by B2R agonist (1 μM kallidin) in two different stable clones (designated by number) coexpressing WT CPM and B1R-GFP. D, the increase in [Ca<sup>2+</sup>], induced by 1 μM des-Arg<sup>10</sup> kallidin in these clones. E, CPM activity in these clones measured with dansyl-Ala-Arg. F, the expression of WT CPM and B1R-GFP in cell lysates of these clones as determined by Western blotting with anti-CPM and anti-GFP antibody, respectively. In C–E, the data are expressed as mean ± S.E. (n = 3). In F, the results are representative of three separate experiments. G, coimmunoprecipitation of B1R-V5 with B1R-GFP. Cells stably expressing only B1R-GFP or both B1R-GFP and B1R-V5 were lysed. Immunoprecipitation (IP) was performed as described using anti-GFP or anti-V5 antibody followed by immunoblotting (IB) using anti-V5 or anti-GFP antibody, respectively. AU, arbitrary units.

There are discrepant reports about the localization of B1Rs in lipid rafts/caveolae. One study showed that B1Rs moved into caveolae after agonist stimulation, whereas another provided evidence for the partial localization of B1Rs in lipid rafts whose distribution did not change after agonist stimulation (33, 34). In HEK293 cells stably expressing WT CPM and WT B1R, CPM protein (Fig. 4A) and activity (Fig. 4B) was distributed in caveolin-1 enriched lipid raft fractions on an OptiPrep density gradient of membranes prepared by a detergent-free procedure. G<sub>αq</sub>, a protein confirmed to exist in lipid rafts (35), was also found in fractions containing CPM (Fig. 4A). The B1R distributed into two peaks: one in the same fractions containing CPM, caveolin-1, and G<sub>αq</sub> and one in higher density fractions that probably represents B1Rs in nonlipid raft membranes (Fig. 4B).

Lipid rafts are resistant to detergent solubilization at low temperatures and have also been called detergent-insoluble

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**association between the B1R and CPM.** To investigate this possibility, coimmunoprecipitation studies were carried out in HEK293 cells stably co-expressing B1R and GFP and WT CPM. As shown in Fig. 3A, coimmunoprecipitation of B1R resulted in coimmunoprecipitated CPM from two different clones coexpressing CPM and B1Rs, but not from cells expressing only WT CPM. The interaction between CPM and B1R was further verified by chemical cross-linking as shown in Fig. 3B. Only in cells coexpressing CPM and B1R/GFP was there a high molecular weight cross-linked band (at 100 kDa) that was immunoprecipitated by B1R antibody and also contained CPM (Fig. 3B). Furthermore, YFP-B1R and CFP-CPM were colocalized in cells stably expressing both (data not shown). Moreover, FRET analysis using acceptor photobleaching showed that CFP-B1R fluorescence was increased after YFP-CPM photobleaching (Fig. 3C) with a calculated FRET efficiency of 7.8 ± 3.1% (n = 4).

Colocalization of B1Rs and CPM in Membrane Microdomains—CPM is a cell surface glycoprotein attached to the plasma membrane by a GPI anchor (3, 26) that confers localization to lipid raft membrane microdomains (28, 32).
glycolipid-enriched fractions (36). M-β-CD disrupts lipid rafts/caveolae by sequestration of cholesterol found in high concentrations in these domains (37). To determine whether M-β-CD increases detergent solubility of CPM and B1Rs, HEK293 cells stably expressing B1R-V5 and WT CPM were preincubated with 10 mM M-β-CD for 30 min and then solubilized with 0.5% or 1.0% Nonidet P-40 for 30 min on ice. As shown in Fig. 5, CPM activity and protein as well as B1R protein and [3H]des-Arg10-kallidin binding solubilized by Nonidet P-40 were increased significantly when cells were pretreated with M-β-CD compared with PBS alone. However, M-β-CD treatment alone did not solubilize either CPM or B1Rs (Fig. 5).

To evaluate the importance of the colocalization of B1R and CPM in lipid rafts, we measured the increase in [Ca^{2+}⁻], mediated by B2R and B1R ligands in HEK293 cells expressing B1R/GFP and WT CPM after treatment with 10 mM M-β-CD. As shown in Fig. 6A, M-β-CD decreased the calcium response induced by B1R agonist des-Arg10-kallidin by about 40–50%, indicating that B1R signaling is affected. However, the response to B2R agonist kallidin, which requires conversion by CPM, was reduced to a much greater extent (about 75%) by M-β-CD (Fig. 6A).

In control experiments, concentrations of M-β-CD 10 mM or lower did not significantly affect the activity and or membrane expression of CPM (Fig. 6, C and D). The effect was probably due to the cholesterol-sequestering ability of M-β-CD, which disrupts lipid rafts and the association of CPM and B1R. This is supported by the fact that cholesterol-saturated M-β-CD had less effect on the kallidin and des-Arg10-kallidin-mediated increase in [Ca^{2+}⁻], than M-β-CD alone (Fig. 6B). Moreover, when the cells were cultured in cholesterol-saturated medium for 24 h, the increase in [Ca^{2+}⁻], induced by kallidin was significantly increased, whereas that stimulated by des-Arg10-kallidin was not (Fig. 6E). Direct evidence for disruption of B1R and CPM interactions was obtained by a significant reduction in the coimmunoprecipitation of WT B1R and WT CPM by M-β-CD treatment (Fig. 6, F and G).

CPM consists of two major domains, as revealed by x-ray crystallography: a 300-residue N-terminal carboxypeptidase subdomain containing all of the catalytic residues, followed by an 80-residue C-terminal β-sandwich transhyretin-like subdomain, a unique feature shared by other members of the carboxypeptidase N/E “regulatory” metallo-carboxypeptidase subfamily (11, 38). Although the function of the C-terminal domain is still unclear, it has been hypothesized to be a potential binding site (e.g. between the 50-kDa catalytic subunit and 83-kDa regulatory subunit of carboxypeptidase N) (39) or a membrane interaction site for CPM (11). Epitope mapping of a highly specific monoclonal antibody to CPM (Novocastra) revealed that it binds to residues 326–335 (VFDQNGNPLP), between residues 326–335 (VFDQNGNPLP), and residues 9 and 10 in the C-terminal domain (11). To determine whether this antibody might disrupt the ability of CPM to deliver agonist to the B1R, we preincubated cells stably expressing WT B1R and WT CPM with 500 ng/ml CPM antibody or IgG control for 30 min at 37 °C prior to the addition of either B1R or B2R agonists. Indeed, CPM antibody preincubation significantly reduced the increase in [Ca^{2+}⁻], induced by B2R agonist bradykinin (Fig. 7A) but did not affect the increase in [Ca^{2+}⁻], induced by B1R agonist des-Arg10-kallidin (Fig. 7A).
mediated by B1R agonist des-Arg\textsuperscript{10}-kallidin (Fig. 7B). The antibody preincubation did not inhibit cell surface CPM activity but instead resulted in a small, but significant, increase in CPM activity in these cells (Fig. 7C).

![Image](49x400 to 299x676)

**FIGURE 4.** The distribution of CPM and B1R in lipid raft/caveolin-enriched fractions. The lysates from cells stably expressing B1R-V5 and WT CPM were fractionated using OptiPrep density gradient centrifugation as described. Cells were incubated with 5 nM \[^{[3H]}\text{des-Arg}^{10}\text{-kallidin}\] for 90 min on ice for labeling B1R before lysing. The numbers represent fractions taken starting at the top (lower density) and going to the bottom (higher density) of the centrifuge tube. A, CPM, G\textsubscript{\alpha q}, and caveolin-1 had the same distribution as determined by Western blotting of each fraction. B, distribution of B1R and CPM as determined by \[^{[3H]}\text{des-Arg}^{10}\text{-kallidin}\] bound and CPM activity with dansyl-Ala-Arg in each fraction (expressed as a percentage of the total). The data are representative of three experiments.

![Image](54x145 to 402x287)

**FIGURE 5.** M-\(\beta\)-CD decreases the resistance of B1R and CPM to detergent solubilization. Cells stably expressing WT CPM and WT B1R (A and B) or B1R-V5 (C) were incubated without or with 5 nM \[^{[3H]}\text{des-Arg}^{10}\text{-kallidin}\] (DAKD) for 90 min on ice. Cells were then treated with 10 mM M-\(\beta\)-CD or PBS for 30 min, followed by incubation with buffer containing various concentrations of Nonidet P-40 for 30 min on ice. Supernatants were collected by centrifugation, and the activity of CPM (A) or \[^{[3H]}\text{DAKD}\]-bound B1R (B) was measured. The fold increase relative to non-Nonidet P-40 cells is shown. The data are expressed as mean ± S.E. of three experiments. *, \(p < 0.05\) versus PBS. C, determination of WT CPM and B1R-V5 in the supernatant after Nonidet P-40 solubilization with or without M-\(\beta\)-CD pretreatment by Western blotting using anti-CPM and anti-V5 antibody, respectively. The last two lanes show that pretreatment of cells with M-\(\beta\)-CD alone did not solubilize B1R or CPM. The data are representative of three experiments.

**B2R Agonists Induce a Calcium Signal in Cells Expressing a CPM-B1R Fusion Protein**—To further investigate the importance of colocalization of B1Rs and CPM in B2R agonist-mediated increase in \([\text{Ca}^{2+}]_i\), we generated a B1R with CPM fused to its N terminus. This construct forces a covalent colocalization of the two proteins that should not be disruptable by M-\(\beta\)-CD or CPM antibody. As shown in Fig. 8A, CPM-B1R was a functional B1R as des-Arg\textsuperscript{10}-kallidin dose-dependently stimulated an increase in \([\text{Ca}^{2+}]_i\). The B2R agonist kallidin also caused a dose-dependent increase in \([\text{Ca}^{2+}]_i\). Unexpectedly, the CPM activity in CPM-B1R stable cells was only slightly higher than the basal activity in nontransfected HEK293 cells and much lower than that in cells coexpressing B1Rs and CPM (Fig. 8B). CPM-B1R also formed oligomers as noticed with the other B1R constructs (Fig. 8C). However, M-\(\beta\)-CD no longer caused a greater decrease in the calcium response mediated by B2R agonist bradykinin compared with B1R agonist des-Arg\textsuperscript{10}-kallidin (Fig. 8D). Moreover, anti-CPM antibody did not affect the increase in \([\text{Ca}^{2+}]_i\), caused by either B1R or B2R agonist (Fig. 8E).

**B2R Agonist Stimulates a B1 Response via Endogenous CPM in Bovine Pulmonary Artery Endothelial Cells (BPAEC)**—To determine whether this pathway was operative in nontransfected cells, we used BPAEC that constitutively express both B1Rs and B2Rs, as shown by the increase in \([\text{Ca}^{2+}]_i\), caused by both des-Arg\textsuperscript{9}-bradykinin and bradykinin (Fig. 9A). The B2R antagonist HOE 140 reduced the magnitude of the increase in \([\text{Ca}^{2+}]_i\), caused by bradykinin (Fig. 9A), and the remaining response was slower in onset (data not shown), similar to the B2 agonist response found in HEK293 cells transfected with CPM and B1Rs (Fig. 1A). The remaining response was dependent on CPM conversion of bradykinin to des-Arg\textsuperscript{9}-bradykinin, since either B1R antagonist des-Arg\textsuperscript{10}-Leu\textsuperscript{9}-kallidin or CPM inhibitor MGTA inhibited it (Fig. 9A). CPM activity was detected in the BPAEC and could be inhibited by MGTA (Fig. 9B). CPM was expressed in BPAEC, as determined by Western analysis, and expression of CPM was not affected by MGTA (Fig. 9C).

**DISCUSSION**

Many cellular processes involve the assembly of protein complexes that carry out reactions in a more efficient and targeted way than would occur freely in solution (40). The best described examples are intracellular processes regulated by protein machines, such as the spliceosome, proteasome, or transcriptional and translational machinery (40). However, there is emerging evidence that analogous protein assemblies may occur on the cell surface, possibly in lipid microdomains (41–43). Although interactions between peptide receptors and related peptidases have not been extensively studied, recent evidence indicates that ACE and B2 kinin
receptor do interact on the cell surface (44, 45). Because ACE readily inactivates bradykinin, it was previously thought that ACE inhibitors enhance B2R signaling solely by blocking degradation of the B2 agonist (46). However, several reports provide evidence that ACE inhibitors potentiate/resensitize B2R signaling apart from their ability to block bradykinin degradation (e.g., with ACE-resistant B2 agonists) (47). Subsequent studies showed that ACE and B2Rs form heterodimers on the cell surface, which leads to an increase in B2R signaling efficiency (44, 45). This interaction facilitates signaling and enhances the biological effects of B2R agonists. ACE inhibitors enhance B2R signaling solely by blocking degradation of the B2 agonist, which results in increased B2R signaling efficiency. This interaction is important in understanding the clinical effects of ACE inhibitors in various cardiovascular diseases.
CPM and B1 Receptor Interaction Facilitates Signaling

**FIGURE 8.** B1R and B2R agonists stimulate an increase in [Ca\textsuperscript{2+}]\textsuperscript{i}, in cells stably expressing a CPM-B1R fusion protein. A, cells stably expressing the CPM-B1R fusion protein were stimulated with indicated concentrations of kallidin (KD) or des-Arg\textsuperscript{10}-kallidin (DAKD), and the calcium signal was recorded and quantified. Data are expressed as mean ± S.E. (n = 3). B, CPM activity in cells stably expressing the CPM-B1R fusion protein. The CPM activity in naive HEK293 cells and cells stably expressing CPM-B1R or WT CPM and WT B1R was measured. The data are expressed as mean ± S.E. (n = 4). * p < 0.05 versus naive HEK293 cells; # p < 0.05 versus CPM-B1R. C, the expression of the CPM-B1R protein in stably expressing cells was detected by Western blotting using anti-CPM antibody. The data are representative of three experiments. D, M-β-CD has the same effect on the increase in [Ca\textsuperscript{2+}]\textsuperscript{i}, mediated by B1R and B2R agonists in cells stably expressing CPM-B1R. The increase in [Ca\textsuperscript{2+}]\textsuperscript{i}, stimulated by B2R agonist bradykinin (BK; 1 μM) or B1R agonist DAKD (1 μM) was recorded in cells without or with pretreatment by 10 mM M-β-CD for 30 min. The area under the curve is shown as mean ± S.E. (n = 3). E, anti-CPM monoclonal antibody has no effect on the [Ca\textsuperscript{2+}]\textsuperscript{i}, induced by B2R or B1R agonist in cells stably expressing CPM-B1R. Cells were incubated with 500 ng/ml antibody for 30 min at 37 °C and then activated with bradykinin (1 μM) or DAKD (1 μM), and the [Ca\textsuperscript{2+}]\textsuperscript{i}, was measured and quantified. The data shown are the mean ± S.E. of at least three experiments. AU, arbitrary units.

**FIGURE 9.** B2R agonist stimulates a B1R- and CPM-dependent increase in [Ca\textsuperscript{2+}]\textsuperscript{i}, in BPAEC. A, BPAEC were incubated with B2R antagonist HOE 140 (10 μM) alone or in combination with B1R antagonist des-Arg\textsuperscript{10}-Leu\textsuperscript{10}-kallidin (DAKD; 10 μM) or CPM inhibitor MGTA (20 μM) for 10 min. After incubation, the calcium signal stimulated by B2R agonist bradykinin (BK; 1 μM) or B1R agonist des-Arg\textsuperscript{10}-bradykinin (DBAK; 1 μM) was measured and quantified. The data are expressed as mean ± S.E. (n = 3). # p < 0.05 versus bradykinin; *, p < 0.05 versus HOE 140 plus bradykinin. B and C, the activity (B) and expression (C) of CPM in BPAEC. The activity of CPM in BPAEC was measured after treatment with or without MGTA (20 μM). The data shown are mean ± S.E. (n = 6). * p < 0.05 versus control. After measuring activity, cells were lysed, and CPM protein in the lysate was detected by Western blotting using anti-CPM antibody. The data are representative of three experiments. AU, arbitrary units; FU, fluorescent units.

Cell membrane and are co-localized in the same membrane microdomain (44, 45), indicating that conformational changes in ACE upon inhibitor binding are transmitted to the B2R, which results in allosteric modification of its signaling properties.

Given the essential role of carboxypeptidase cleavage in processing the native kinins released from kininogen precursors into B1R agonists, membrane-bound CPM seems ideally suited to carry out this function. For efficient signaling to occur, CPM and the receptor would probably need to be localized in close proximity, possibly interacting in a membrane microdomain. Indeed, in the present study, we show that B1R signaling in cells exposed to B2R agonists is dependent on the co-expression and activity of CPM. Furthermore, CPM and B1Rs are co-localized in lipid raft domains and interact on the cell surface as evidenced by FRET analysis, cross-linking, and coimmunoprecipitation studies. Disruption of this interaction with M-β-CD or monoclonal antibody to CPM reduces B1R signaling in response to B2R agonists. Furthermore, a CPM-B1R fusion protein also signals in the presence of B2R agonists. Taken together, these data indicate a close functional relationship between B1Rs and CPM on the cell surface that enhances B1 signaling in the presence of B2R agonists.

Although there exist other mammalian B-type CPs that could potentially be involved in generating B1 agonists, their localization and other properties make them unlikely candidates to play this role in vivo. For example, carboxypeptidase E has an acidic pH optimum and is localized in secretory granules, whereas carboxypeptidase Z is localized in the extracellular matrix (48, 49). Although not well characterized enzymatically, some members of the newly described Nna-1-like carboxypeptidases are proposed to have broad substrate specificity based on modeling and initial expression studies (50). They have been named the cytosolic carboxypeptidase subfamily because of their intracellular localization (51), which would preclude their ability to cleave extracellular peptide substrates. Two plasma carboxypeptidases, carboxypeptidase N and carboxypeptidase U (or TAFI), are known to cleave the C-terminal Arg of bradykinin (52–54), but carboxypeptidase U is normally present in an inactive proform and only becomes activated by thrombin/thrombomodulin during coagulation (55), whereas carboxypeptidase N prefers cleaving C-terminal Lys and has relatively poor kinetic constants with bradykinin (1). In addition, dilution of the peptide products into the large blood volume before reaching the B1R would compromise the efficiency of this process. The carboxypeptidase functions that relate to cell surface phenomena, such as generating a
receptor ligand, are better served by a membrane-bound enzyme in close proximity to the receptor. Carboxypeptidase D is a membrane-bound B-type carboxypeptidase, and although it does cleave bradykinin and can be detected on the plasma membrane, it is primarily localized in the trans-Golgi network and has an acidic pH optimum of 6.2 with only about 20–25% activity remaining at pH 7.4 (56–58). Thus, carboxypeptidase D is unlikely to play a major role in generating B1 agonists.

CPM is GPI-anchored on the plasma membrane and has a neutral pH optimum (1–3, 59). In addition, CPM has structural features that may orient it on the membrane to make it well suited to cleave substrates close to the cell surface. The structure of the last half of the C-terminal tail of CPM (starting with Pro391) is fixed due to the presence of 5 Pro residues, a disulfide bond between Cys224 and Cys393, and a helical turn composed of Tyr399–Leu402 (11). In mature GPI-anchored CPM, the four C-terminal amino acids (Pro403–Ser406) and glycan moieties of the GPI anchor would presumably form a partially flexible 20-Å “tether” that could restrict the movement of CPM with respect to the membrane. This could allow seven positively charged side chains on the C-terminal transthyretin-like domain (Lys379, Arg230, Arg196, His210, Arg215, Lys384, and Lys358) to interact with negatively charged phospholipid head groups of the membrane so that its active site groove would point along the membrane (11). The need for proper orientation on the membrane is supported by the finding that a GPI-anchored form of the 50-kDa subunit of carboxypeptidase N (which does not have these features on its transthyretin-like domain) is expressed on the membrane but is not enzymatically active on live cells.

The B1R is not found constitutively in most cells, but its expression is up-regulated in response to injury or inflammatory mediators (17, 20). Although CPM is expressed constitutively in many cell types (7, 59), its expression can be increased about 2–3-fold in cytokine-treated human endothelial cells (22) or in aortas from pigs treated with lipopolysaccharide (60). Thus, the potential for conversion of kinins to des-Arg-kinins and B1R signaling is enhanced in inflammatory or pathological responses. Whether B1R signaling has overall beneficial or deleterious effects in inflammation or sepsis is difficult to predict and depends on the model system being used. B1R antagonists have been proposed as potentially useful drugs in treating inflammation, sepsis, and pain (61), supported largely by studies in various animal models. For example, B1R knock-out protects mice from lipopolysaccharide-induced hypotension, reduces pain in response to thermal or chemical stimuli as well as neuropathic pain, and reduces intestinal ischemia/reperfusion inflammation and lethality (62). However, B1R signaling also has numerous beneficial effects, such as promoting angiogenesis and neovascularization during wound healing (62–64), protecting kidneys from renal fibrosis and attenuating cardiac remodeling in stroke-prone spontaneously hypertensive rats (65, 66), and reducing lethality in a porcine model of endotoxic shock (67). B1R stimulation also results in high output NO production in human endothelial cells (21, 22) via activation of extracellular signal-regulated kinase and acute stimulation of inducible nitric-oxide synthase activity via phosphorylation of Ser245 (23). One outcome of high output NO production in endothelial cells is inhibition of protein kinase Cε activity (24). This could have beneficial effects in the heart, since overexpression of protein kinase Cε resulted in dilated cardiomyopathy and disruption of myofilibrillar proteins and their interactions (68). ACE inhibitors also act as direct agonists of B1Rs to stimulate NO production (21, 69, 70), and some of their beneficial therapeutic effects could arise from stimulation of B1R signaling. For example, ACE inhibitor treatment improves endothelial function in septic patients (71) or in rabbits with lipopolysaccharide-induced endotoxic shock by an NO-dependent mechanism (72). ACE inhibitors also promote angiogenesis in an NO-dependent manner that results from B1R activation (73).

In conclusion, we have shown that CPM expression is required to generate a B1R-dependent increase in [Ca2+]i in cells stimulated with B2R agonists kallidin or bradykinin. Furthermore, CPM and the B1R are found in the same membrane microdomains, and their heterodimerization on the cell membrane is important for efficient B1R signaling in response to B2R agonists. The C-terminal β-sheet domain of CPM is probably involved in this interaction, since a monoclonal antibody to this domain reduced the B1R response to B2R agonists without inhibiting CPM activity. In bovine or human endothelial cells, B2R agonists stimulate a calcium signal (this study) or nitric oxide production (22) that is B1R- and CPM-dependent. A close relationship of B1Rs and CPM on the membrane is thus required for efficiently generating B1R signals, which play important roles in inflammatory processes (61, 62).

Acknowledgment—We thank Kai Zhang for excellent technical assistance.

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