THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 21, pp. 18547–18561, May 27, 2011

Cross-talk between Carboxypeptidase M and the Kinin B1 Receptor Mediates a New Mode of G Protein-coupled Receptor Signaling*[S]

Received for publication, December 21, 2010, and in revised form, March 24, 2011 Published, JBC Papers in Press, March 31, 2011, DOI 10.1074/jbc.M110.214940

Xianming Zhang[1], **Fulong Tan, Viktor Brovkovych, Yongkang Zhang, and Randal A. Skidgel**[2]
From the Department of Pharmacology, University of Illinois College of Medicine, Chicago, Illinois 60612

G protein-coupled receptor (GPCR) signaling is affected by formation of GPCR homo- or heterodimers, but GPCR regulation by other cell surface proteins is not well understood. We reported that the kinin B1 receptor (B1R) heterodimerizes with membrane carboxypeptidase M (CPM), facilitating receptor signaling via CPM-mediated conversion of bradykinin or kallidin to des-Arg kinin B1R agonists. Here, we found that a catalytically inactive CPM mutant that still binds substrate (CPM-E264Q) also facilitates efficient B1R signaling by B2 receptor agonists bradykinin or kallidin. This response required co-expression of B1R and CPM-E264Q in the same cell, was disrupted by antibody that dissociates CPM from B1R, and was not found with a CPM-E264Q-B1R fusion protein. An additional mutation that reduced the affinity of CPM for C-terminal Arg and increased the affinity for C-terminal Lys inhibited the B1R response to bradykinin (with C-terminal Arg) but generated a response to Lys^9-bradykinin. CPM-E264Q-mediated activation of B1Rs by bradykinin resulted in increased intramolecular fluorescence resonance energy transfer (FRET) in a B1R FRET construct, similar to that generated directly by a B1R agonist. In cytokine-treated human lung microvascular endothelial cells, disruption of B1R-CPM heterodimers inhibited B1R-dependent NO production stimulated by bradykinin and blocked the increased endothelial permeability caused by treatment with bradykinin and pyrogallol (a superoxide generator). Thus, CPM and B1Rs on cell membranes form a critical complex that potentiates B1R signaling. Kinin peptide binding to CPM causes a conformational change in the B1R leading to intracellular signaling and reveals a new mode of GPCR activation by a cell surface peptidase.

G protein-coupled receptors (GPCRs)[3] compose the largest known human gene family and represent targets for ~30% or more of all prescription drugs (1). Unique properties of GPCRs make them especially suited to transmit extracellular signals into the cytosol. These include structural flexibility that allows the existence of multiple dynamic conformational states that can be altered or stabilized by ligand binding to their extracellular domains, leading to intracellular signal generation (2). Historically, drugs have been targeted to orthosteric binding sites of the natural GPCR ligands. However, it is becoming clear that ligand binding to allosteric sites on the receptor can trigger or alter receptor signaling, for example causing biased agonism or modulation of orthosteric agonist effects (3, 4). In fact, it has been suggested that the entire extracellular surface of a GPCR can be considered to contain potential ligand-binding sites for allosteric control of receptor functions (5).

GPCR signaling is regulated not only by small molecule binding to allosteric or orthosteric sites, but also by protein/protein interactions, most notably with intracellular proteins such as the canonical G proteins, GPCR kinases, and arrestins (6). On the membrane, receptor signaling can be influenced by formation of GPCR homo- and heterodimers (7–9). However, very few other membrane proteins (*e.g.* receptor activity-modifying proteins or RAMPs I–III) have been described to regulate GPCR signaling (6).

We recently found that the glycosylphosphatidylinositol (GPI)-anchored enzyme carboxypeptidase M (CPM) interacts with the kinin peptide B1 GPCR (B1R) in lipid raft membrane microdomains (10). This interaction plays an important functional role in kinin signaling. Bradykinin (BK) (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) or kallidin (KD) (Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) are the peptides initially released by kallikrein from the precursor kininogen and are specific agonists of the kinin B2 receptor (11–13). CPM on the membrane or carboxypeptidase N in the plasma specifically cleave the C-terminal Arg from BK or KD to generate the specific B1R agonists des-Arg^9-BK (DABK) or des-Arg^{10}-KD (DAKD) (11–13). The interaction of CPM and the B1R on cell membranes provides a mechanism for efficient delivery of enzymatically generated agonist in close proximity to the B1R, enhancing signaling. Indeed, we found that disruption of the CPM·B1R com-

* This work was supported, in whole or in part, by National Institutes of Health Grants DK41431 and HL60678.
[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4.
[1] Recipient of an American Heart Association postdoctoral fellowship.
[2] To whom correspondence should be addressed: Dept. of Pharmacology (m/c 868), University of Illinois College of Medicine, 835 S. Wolcott, Chicago, IL 60612. Tel.: 312-996-9179; Fax: 312-996-1648; E-mail: rskidgel@uic.edu.
[3] The abbreviations used are: GPCR, G protein-coupled receptor; CP, carboxypeptidase; iNOS, inducible NOS; B1R, kinin B1 receptor; B2R, bradykinin B2 receptor; CFP, cyan fluorescent protein; CPM, carboxypeptidase M; $[Ca^{2+}]_i$, intracellular calcium concentration; GPI, glycosylphosphatidylinositol; ACE, angiotensin I-converting enzyme; MGTA, DL-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid; BK, bradykinin; KD, kallidin; DABK, des-Arg^9-bradykinin; DAKD, des-Arg^{10}-kallidin; DALKD, des-Arg^{10}-Leu^9-kallidin; dansyl-Ala-Arg, 5-dimethyl-aminonaphthalene-1-sulfonyl-L-alanyl-L-arginine; TC, tetracysteine; FlAsH, fluorescein arsenical hairpin binder; CPD-DIII, carboxypeptidase D domain III; K^9-BK, Lys^9-bradykinin; K^{10}-KD, Lys^{10}-kallidin; HLMVEC, human lung microvascular endothelial cells; TER, transendothelial electrical resistance; CT peptide, CPM C-terminal domain peptide.

plex greatly reduced B1R signaling in response to administration of BK or KD (10).

Signaling via the B1R, whose expression is induced by injury or inflammation, can have both beneficial and deleterious effects (14–16). We found that B1R stimulation leads to $G\alpha_i$ and ERK-mediated acute activation of inducible nitric-oxide synthase and prolonged high output NO production in human lung microvascular endothelial cells (17–19). Endothelium-specific expression of B1Rs in transgenic rats increased hypotension and lethality in response to lipopolysaccharide (LPS) (20), whereas B1R knock-out protected mice from LPS-induced hypotension, reduced neuropathic pain, and pain in response to thermal or chemical stimuli (14). However, B1R activation is also beneficial, for example in protecting kidneys from ischemia/reperfusion injury (21), promoting vasodilation, angiogenesis and neovascularization during wound healing (14, 22, 23), and reducing renal fibrosis and cardiac remodeling (24, 25). B1R signaling also participates in the therapeutic effects of angiotensin-converting enzyme (ACE) inhibitors in diabetes (26).

Because CPM is extracellular, tethered to the membrane by a GPI anchor inserted into the outer leaflet of the bilayer, it can only interact with the extracellular loops of B1R. The x-ray crystal structure of CPM revealed the presence of charged residues and structural features in its C-terminal β-sandwich domain that could restrict its movement and orient it on the membrane in a favorable configuration for interaction with substrates or proteins on or near the cell surface (10, 27). Because of the potential for extracellular interactions with the B1R to cause or affect receptor signaling, we wondered if enhancement of B1R signaling by CPM goes beyond generation of des-Arg-kinin agonists. To explore this, we made a point mutation of the catalytic glutamic acid (E264Q), which we previously showed generates catalytically inactive CPM that retains its substrate binding ability (28), similar to results reported for the same mutation of the related family member carboxypeptidase E (29). We unexpectedly found that kinin peptides BK and KD that are specific B2R agonists efficiently stimulated B1R signaling in cells co-expressing B1Rs and CPM-E264Q, without conversion to B1R agonist kinins. This response required co-expression of B1Rs and CPM-E264Q in the same cells and was disrupted by agents that dissociated the enzyme from B1Rs. The B1R response to KD or BK mediated by CPM-E264Q resulted in increased intramolecular fluorescence resonance energy transfer (FRET) in a B1R-TC-CFP construct that was similar to the increased FRET stimulated by B1R agonist. In cytokine-treated primary human endothelial cells, disruption of the interaction of the B1R and CPM inhibited B1R-mediated NO production stimulated by BK and loss of endothelial barrier function. These data indicate that substrate binding to CPM complexed to the B1R can cause a conformational change in the receptor resulting in intracellular signaling and reveal a new mode of GPCR activation dependent on heterodimerization with a GPI-anchored enzyme.

EXPERIMENTAL PROCEDURES

Materials—Low glucose Dulbecco's modified Eagle's medium (DMEM) was obtained from Invitrogen. Fetal bovine serum was from Atlanta Biologicals. DL-2-Mercaptomethyl-3-guanidinoethylthiopropanoic acid (MGTA) was from Calbiochem. Protein A, HOE 140, des-Arg^9-HOE 140, bradykinin (BK), des-Arg^9-bradykinin (DABK), des-Arg^{10}-kallidin (DAKD), des-Arg^{10}-Leu^9-kallidin (DALKD), polylysine, furylacryloyl-Ala-Lys and DL-1,4-dithiothreitol (DTT) were from Sigma. Kallidin (KD) was from Bachem. Lys^9-bradykinin (K^9-BK), Lys^{10}-kallidin (K^{10}-KD), CPM C-terminal domain peptide (CT peptide; residues 299–312), and scrambled CT peptide were synthesized by Chi Scientific. Fura-2/AM was from Molecular Probes. Anti-CPM monoclonal antibody was from Novocastra. Anti-B1R polyclonal antibody was from Santa Cruz Biotechnology. Goat anti-mouse and anti-rabbit IgG-conjugated HRP were from Pierce. Polyclonal antiserum to CPD was raised in rabbits as described previously (30). 5-Dimethylaminonaphthalene-1-sulfonyl-L-alanyl-L-arginine (dansyl-Ala-Arg) was synthesized and purified as described previously (31). The TC-FlAsH^{TM} II in-cell tetracysteine tag detection kit was purchased from Invitrogen. Common chemicals were from Fisher.

Generation of Receptor and Carboxypeptidase Constructs—The cDNA for human kinin B1R was a kind gift from Dr. Fredrik Leeb-Lundberg of the University of Lund, Sweden. The cDNA for human CPM was cloned as described previously (10, 28, 32). WTB1R and CPM cDNAs were cloned into pcDNA3 or pcDNA6 vectors (Invitrogen) for expression in mammalian cells. B1R was also cloned into pIRES (Clontech) at the NheI/XhoI sites, together with enhanced GFP at the SalI/NotI sites. This results in the co-expression of B1R and GFP (separately) at the same time in the same cells to facilitate selecting clones.

The B1R-TC-CFP construct was generated as follows. Insertion of the tetracysteine (TC) CCPGCC coding sequence between Gly^{242} and Arg^{243} in third intracellular loop was achieved by two steps of PCR amplification. A sense primer (5′tgc tgt cct ggt tgt tgc cgc aag gat agc aag acc aca) coding for Arg^{243}–Thr^{249} with a CCPGCC coding sequence overhang at its 5′ end was used together with a B1 C-terminal primer to amplify a B1R-TC C-terminal fragment (encoding CCPGCC plus Arg^{243}–Asn^{353}). The B1R C-terminal primer (5′tgg atc cgg att ccg cca caa aag ttg gaa) was designed to eliminate the stop codon and fuse the C-terminal Asn codon into the reading frame of cyan fluorescent protein (CFP) through a BamHI cleavage site. In a similar way, the cDNA coding for the B1R N-terminal fragment (encoding residues 1–242) with a C-terminal overhang encoding CCPGCC was PCR-amplified with a B1R 5′ end primer (5′cta gct agc atg gca tca tcc tgg ccc) with the reverse primer (5′ gca aca acc agg aca gca gcc ccc gca cct tgt cct gct), which contained the reverse sequence for Ser^{235}–Gly^{242} plus CCPGCC at its 5′ end. Electrophoretically purified cDNAs encoding the modified B1R N- and C-terminal fragments were annealed and used as template for PCR amplification with B1R 5′ and 3′ end primers to obtain a full-length B1R containing the TC insert cDNA (B1R-TC). Subsequently, the B1R-TC cDNA was digested with NheI and BamHI and cloned into pECFP-N1 to generate B1R-TC-CFP.

The CPM mutants CPM-E264Q, CPM-S180N, and CPM-S180N/E264Q were produced by site-directed mutagenesis using procedures described previously (28). To generate a fusion protein with CPM and CPM-E264Q attached to the extracellular N terminus of the B1R (CPM-B1R or CPM-

E264Q-B1R), CPM or CPM-E264Q cDNA (nucleotides 1–1390) were inserted in-frame into the 5′ end of the B1R coding sequence and cloned into pcDNA3 or pcDNA6 at the BglII/XhoI sites.

To generate the CPD-DIII construct, the NheI-HindIII fragment (2686–4587 bp) of the human CPD cDNA (33), which contains the complete domain III, including the transmembrane domain and cytosolic tail, was blunt-ended with dNTPs and Klenow fragment. The 3′ end HindIII site (at bp 4587), which is well beyond the stop codon, was changed to an XhoI site by cloning into a Bluescript KS vector for the convenience of further cloning. This fragment was ligated to an EcoRI-NaeI fragment (1–304 bp) of CPD cDNA, encoding the translation initiation site, signal peptide, and N-terminal 60 residues of CPD and cloned into the EcoRI-XhoI site of pcDNA3.

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

Cell Culture—Human embryonic kidney (HEK293) cells were from the American Type Culture Collection. Cells were maintained in DMEM containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum. Primary human lung microvascular endothelial cells (HLMVEC) were from Lonza and cultured in T-25 or T-75 flasks coated with 0.1% gelatin in endothelial cell basal medium (EBM®-2, Lonza) supplemented with EGM®-2 SingleQuots® kit (Lonza) and 10% fetal bovine serum (Atlanta Biologicals). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2, and cells between passage 3 and 6 were used for assay.

Transfection and Establishment of Stable Cell Lines—HEK293 cells, at 70–80% confluence in 6-well plates, were transfected with SuperFect (Qiagen) reagent containing 5 μg of DNA per the manufacturer's instructions. After 48 h, cells were transferred to selective medium containing G418 (500 μg/ml) or blasticidin (5 μg/ml) depending on the resistance gene in the vector. The cells were cultured for 15–30 days in selective medium and then diluted for single clone selection. For B1R and B1R-TC-CFP selection, the increase in intracellular calcium ($[Ca^{2+}]_i$) stimulated by agonist DAKD was evaluated for each clone. For WTCPM selection, the enzyme activity was measured for each clone. Clones stably expressing CPM-E264Q, CPM-S180N, CPM-S180N/E264Q, or CPD-DIII were selected by Western blot analysis using the appropriate antibody to detect protein expression. Cells were then transfected with B1R cDNA, and the B1R-positive clones were selected to generate B1R and carboxypeptidase double stable clones.

Co-culture of Cells Expressing B1R and CPM—HEK cells stably expressing B1Rs were mixed with cells stably expressing WTCPM or CPM-E264Q at a 1:1 ratio. These cells were seeded on polylysine-coated glass coverslips for the $[Ca^{2+}]_i$ measurement or into 24-well plates for the determination of CPM activity and expression. After 24–36 h of culture, the calcium response to B1R or B2R agonist and CPM activity were determined as described above.

Measurement of Increased Intracellular Ca^{2+}—Increases in $[Ca^{2+}]_i$ were determined using fura-2/AM (10). Control HEK293 cells or stable clones expressing constructs described above were grown on polylysine-coated glass coverslips to 80% confluence and then loaded with 2 μM 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. Area under the curve was integrated using Origin 8.0 software (OriginLab Corp.). To investigate the effect of receptor antagonists or carboxypeptidase inhibitors on the calcium response, the cells were preincubated with antagonist or inhibitor for 60–90 s before addition of receptor agonist. To examine the effect of reducing reagent on $[Ca^{2+}]_i$, the cells were incubated with various concentrations of DTT for 10 min at 37 °C before treatment with B1R or B2R agonist.

Determination of Carboxypeptidase Activity—Carboxypeptidase cleavage of C-terminal Arg or Lys was measured using dansyl-Ala-Arg substrate or furylacryloyl-Ala-Lys as described previously (10, 31, 34, 35).

Measurement of BK Degradation—BK (10 μM) was incubated for 30 min with HEK cells (80% confluent) stably expressing B1Rs alone or co-expressing CPM, CPM-E264Q, or the CPM-E264Q-B1R fusion protein in 6-well culture plates. The supernatants were collected and acidified by addition of trifluoroacetic acid, and BK and DABK were quantitated by HPLC analysis using a C-18 reversed phase column and ultraviolet detector as described previously (13, 36).

Immunoprecipitation—Immunoprecipitation was carried out as described previously (10). Briefly, supernatants from lysates of cells stably expressing B1Rs and carboxypeptidases were diluted 10-fold with Tris buffer (50 mM Tris/HCl, 150 mM NaCl, pH 7.4), and antibody was added at the appropriate dilution. 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, boiled for 5 min, and centrifuged at 14,000 × *g* for 5 min, and supernatants were analyzed by Western blotting.

Western Blotting—Cells were lysed in 50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 0.1% SDS with sonication for 30 s on ice. After centrifugation at 14,000 × *g* for 10 min, the supernatant was collected and boiled with an equal volume of 2× concentrated SDS-PAGE loading buffer for 5 min. The protein samples were separated on an 8% SDS-polyacrylamide gel and transferred to a PVDF membrane. The blots were blocked with 5% nonfat dry milk in PBS with 0.1% Tween 20 for 2 h at room temperature. The membranes were washed with the same buffer 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) (10).

Change in B1R Intramolecular FRET Mediated by Kinin Peptides—Intramolecular FRET between the fluorescein arsenical hairpin binder (FlAsH)-labeled CCPGCC in the B1R third intracellular loop, and C-terminal CFP was determined as described previously (37) with minor modification. Briefly, the HEK cells stably expressing B1R-TC-CFP and/or CPM mutants

were stained with 2 μM FlAsH-EDT2 for 30 min at 37 °C. After incubation, the cells were washed with 250 μM 2,3-dimercaptopropanol in buffer three times (5 min each) to remove nonspecific FlAsH binding and then the cells were resuspended in Hanks' balanced salt solution buffer. Cells were excited at 430 nm (CFP excitation), and emission was recorded from 490 to 600 nm using an Aminco Bowman Series 2 spectrofluorometer. For detection of change in FRET, the emission at 530 nm (FlAsH emission) was recorded continuously after stimulation with the indicated peptides while exciting at 430 nm. The FRET change was expressed as Δemission at 530 nm.

BK Binding Assay—HEK cells stably expressing B1Rs and CPM-E264Q were incubated with 100 nM [^{3}H]BK for 90 min at room temperature. After washing with PBS (three times), the cells were further incubated with 1 μM DABK or 20 μM MGTA for 60 min. Cells were washed three times with PBS and lysed, and the binding of [^{3}H]BK was determined by measuring radioactivity in a scintillation counter. The data were normalized using the total binding from the control cells not treated with DABK or MGTA.

Measurement of NO Production—HLMVEC were treated with 5 ng/ml IL-1β and 100 units/ml IFN-γ for 16–24 h to induce B1R and iNOS expression (19, 38). Cells were pretreated for 30 min with 1 μM HOE140 (B2R antagonist) without or with 500 ng/ml CPM monoclonal antibody, 50 μM CT peptide, or scrambled CT peptide. Cells were then stimulated with 100 nM or 1 μM BK, and NO production was measured for 20 min in real time with a porphyrinic microsensor as described previously (38, 39). The current was proportional to the NO released, and a computer-based Gamry VP600 potentiostat was used to monitor NO concentration over time. Each electrode was calibrated with an NO standard. The concentration of NO achieved 20 min after addition of agonist was used to quantitate the results.

Determination of Transendothelial Electrical Resistance—Transendothelial electrical resistance (TER) reflects endothelial monolayer permeability and was measured as described previously (40, 41). Briefly, HLMVEC were grown to confluence on 10 μg/ml fibronectin-coated gold electrodes (ECIS cultureware 8W10E) and then treated with 5 ng/ml IL-1β and 100 units/ml IFN-γ for 16 h. Cells were then placed in fresh EBM-2 medium supplemented with 1% fetal bovine serum for 1 h, and electrodes were mounted in the Electric Cell Substrate Impedance Sensing Module 1600R (ECIS, Applied Biophysics), and base-line TER was allowed to stabilize. HLMVEC monolayers were pretreated with 10 μM HOE140 to block any B2R response. Cells were then stimulated with 1 μM BK alone or combined with 200 μM pyrogallol (superoxide generator) without or with 1 μM DALKD (B1R antagonist), 50 μM CT peptide, or 50 μM scrambled CT peptide, and TER was recorded.

Statistical Analysis—Data are expressed as mean ± S.E. For two group comparisons, Student's *t* test was used. Analysis of variance was used for more than two group comparisons, which was followed by Tukey's test to identify the difference between groups (using Prism software version 5.0). Values of $p < 0.05$ were considered significant.

RESULTS

BK or KD Stimulates Increased $[Ca^{2+}]_i$ in Cells Stably Expressing B1Rs and CPM-E264Q—Metallocarboxypeptidases contain an active site Glu critical for polarizing and increasing the nucleophilicity of the bound solvent (water) required for catalytic hydrolysis of the C-terminal peptide bond, but this residue is not involved in substrate binding (42). Indeed, mutation of this catalytic Glu in CPM (Glu^{264}) or the related enzyme CPE (Glu^{300}) resulted in catalytically inactive enzymes that retained their substrate-binding ability (28, 29). An HEK cell line stably expressing B1Rs and CPM-E264Q was established, and CPM activity was measured. In contrast to cells co-expressing B1Rs and WTCPM, there was no increase in CPM activity over basal in cells expressing B1Rs and CPM-E264Q (Fig. 1*A*), consistent with our previous finding that this mutant lacks enzymatic activity (28). Cells stably expressing only B1Rs did not produce a significant increase in $[Ca^{2+}]_i$ in response to 1 μM B2R agonist KD (Fig. 1*B*), but cells co-expressing B1Rs and WT CPM did (Fig. 1*C*), consistent with our previous findings (10). In both cases, cells responded to 1 μM B1R agonist DAKD as expected (Fig. 1, *B* and *C*). Surprisingly, cells stably expressing B1Rs and CPM-E264Q also produced a significant increase in $[Ca^{2+}]_i$ in response to 0.1 or 1 μM KD (Fig. 1*D*), although the response was less than that stimulated by equivalent concentrations of B1R agonist DAKD (Fig. 1*E*). BK, the other natural B2R agonist, also stimulated a similar increase in $[Ca^{2+}]_i$ in these cells (data not shown). The calcium response to 1 μM KD in cells stably expressing B1Rs and CPM-E264Q was significantly inhibited by MGTA (Fig. 1*F*). MGTA, an effective inhibitor of CPM (28) and related carboxypeptidases (43, 44), is an arginine derivative that binds to the active site via the side chain binding pocket and contains a free –SH group that complexes the active site zinc (44). The response was also significantly blocked by B1R antagonist des-Arg HOE140 but not by B2R antagonist HOE140 (Fig. 1*F*). MGTA and the receptor antagonists had no activity on their own (Fig. 1*F*). These data indicate that the calcium response to KD or BK is mediated through the B1R and that binding of these kinin peptide substrates to CPM is sufficient to stimulate a B1R response without conversion to B1R agonist.

Although CPM-E264Q lacks detectable activity with the fluorescent dipeptide substrate dansyl-Ala-Arg as reported previously (28) and shown above, we wanted to formally rule out the possibility that co-expression of CPM-E264Q with the B1R results in a heterodimer that cleaves KD or BK via a novel catalytic mechanism. We measured BK hydrolysis in live HEK cells stably expressing either WTCPM or CPM-E264Q with or without B1Rs. HEK cells stably expressing WTCPM or WTCPM + B1R converted BK to DABK, whereas nontransfected HEK cells or cells expressing CPM-E264Q or B1Rs + CPM-E264Q did not (Fig. 2). Thus, CPM-E264Q facilitates B1R signaling by binding BK or KD without converting them to B1R agonists.

To determine whether inhibition of CPM by a mechanism that would not compete with BK binding to the active center could mimic the finding with CPM-E264Q, we treated cells with dithiothreitol (DTT), which dose-dependently inhibited CPM activity in cells stably expressing WTCPM and B1R, with-

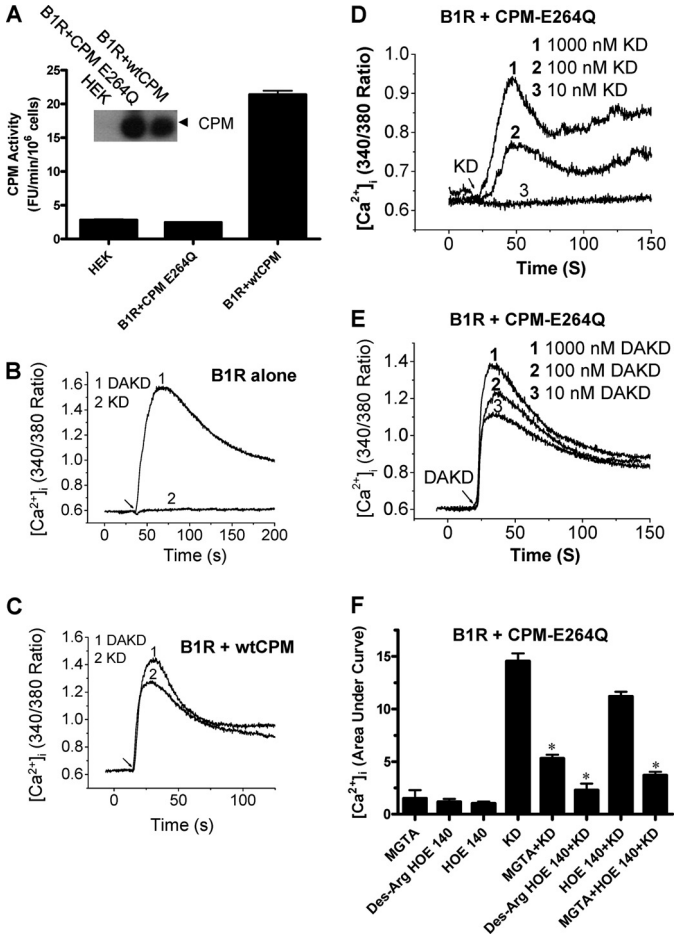

FIGURE 1. **KD stimulates an increase in $[Ca^{2+}]_i$ in cells expressing CPM-E264Q and B1R.** *A,* expression and activity of WTCPM and CPM-E264Q in stably transfected HEKcells co-expressing B1Rs. CPM activity was measured with the fluorescent substrate dansyl-Ala-Arg. The data are expressed as mean ± S.E. ($n = 3$). The *inset* shows the expression of WTCPM and CPM-E264Q as determined by Western blotting. Data are representative of three experiments. *B–E,* tracings showing the increase in $[Ca^{2+}]_i$ induced by B1R and B2R agonists in HEK cells stably expressing B1R (*B*), B1R and WTCPM (*C*), or B1R and CPM-E264Q (*D* and *E*). The concentrations of agonists were 1 μM unless otherwise indicated. The *traces* are representative of three independent experiments. *F,* CPM inhibitor and B1R antagonist block the increase in $[Ca^{2+}]_i$ mediated by KD. HEK cells stably expressing CPM-E264Q and B1R were pretreated for 60–90 s with vehicle or the following agents: CPM inhibitor MGTA (20 μM); B1R antagonist des-Arg^9-HOE 140 (10 μM); B2R antagonist HOE 140 (10 μM). Cells were then stimulated with 1 μM KD, and the increase in $[Ca^{2+}]_i$ was recorded and quantified by integrating the area under the curve with software Origin 8.0. The data are expressed as mean ± S.E. ($n = 3$). *, $p < 0.05$ *versus* KD alone. *FU,* fluorescence units.

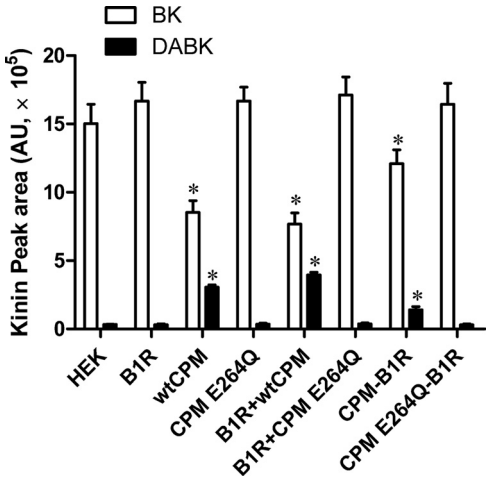

FIGURE 2. **CPM-E264Q expressed alone or with the B1R does not cleave BK to generate B1R agonist DABK.** HEK cells stably expressing the indicated proteins were incubated with 10 μM BK for 30 min. The remaining BK and product DABK were measured by HPLC as described under "Experimental Procedures." The data are expressed as mean ± S.E. of three experiments. *, $p < 0.05$, *versus* HEK or B1R alone. *CPM-B1R* indicates the B1R fusion protein with the C terminus of WTCPM attached to the N terminus of the B1R. *CPM-E264Q-B1R* indicates the B1R fusion protein with the C terminus of CPM-E264Q attached to the N terminus of the B1R. *AU*, arbitrary units.

out altering CPM protein levels (Fig. 3, *A* and *B*). Concentrations of DTT that inhibited CPM activity to basal levels found in nontransfected cells only partially decreased the calcium response induced by 1 μM KD (Fig. 3, *C* and *E*). DTT did not affect B1R responses to 1 μM DAKD (Fig. 3, *D* and *E*), indicating it did not affect B1R signaling directly. Interestingly, MGTA further inhibited the calcium response induced by 1 μM KD in cells stably expressing B1Rs and WTCPM after treatment with DTT (Fig. 3*F*). This is consistent with the interpretation that DTT treatment inhibited CPM enzymatic activity without denaturing it, still allowing it to bind kinin peptides and mediate the activation of the B1R. This is also supported by the finding that 10 mM DTT did not interfere with the increased $[Ca^{2+}]_i$ induced by 1 μM KD or B1R agonist DAKD in HEK cells stably expressing B1Rs and CPM-E264Q (Fig. 3*G*).

Effect of Carboxypeptidase D Domain III (CPD-DIII) and CPM Mutations on the BK-stimulated Calcium Response Mediated by the B1R—Human CPD is a membrane-anchored enzyme consisting of three carboxypeptidase domains, each with similarity to human CPM with sequence identities ranging from 27 to 45% (33). Domains 1 and 2 have enzymatic activity but domain 3 (containing the C-terminal transmembrane anchor) does not (45, 46). In the place of the catalytic Glu equivalent to Glu^{264} in CPM, CPD-DIII has Tyr^{1248}. As a negative control, we established cell lines stably expressing CPD-DIII and B1Rs which, as expected, did not exhibit any detectable carboxypeptidase activity over basal (Fig. 4*A*), although the protein was expressed (Fig. 4*B*). In these cells, BK (1 μM) did not stimulate increased $[Ca^{2+}]_i$ in contrast to the cells expressing B1Rs + CPM-E264Q (Fig. 4*C*). Cells co-expressing CPD-DIII + B1Rs did give an equivalent calcium response to direct application of the B1R agonist DAKD (Fig. 4*C*).

CPM specifically cleaves only C-terminal Arg or Lys from peptides, but it has a clear preference for Arg (13, 31), the C-terminal residue on BK or KD. The crystal structures of human CPM (27) and the active subunit of carboxypeptidase N (47) suggested that active site residue Ser^{180} in CPM might mediate its preference for C-terminal Arg and the corresponding Asn^{203} in carboxypeptidase N its specificity for C-terminal Lys. Indeed, we mutated Ser^{180} in CPM to Asn, expressed and purified the mutant protein, and found it decreased the k_{cat}/K_m for C-terminal Arg substrate by ~100-fold and increased C-terminal Lys hydrolysis by ~2-fold, largely due to changes in the K_m values.[4] To determine whether the specificity of binding affected the ability of CPM-E264Q to mediate B1R responses to BK, we established stable cell lines co-expressing the B1R with either

[4] F. Tan, P. A. Deddish, and R. A. Skidgel, unpublished data.

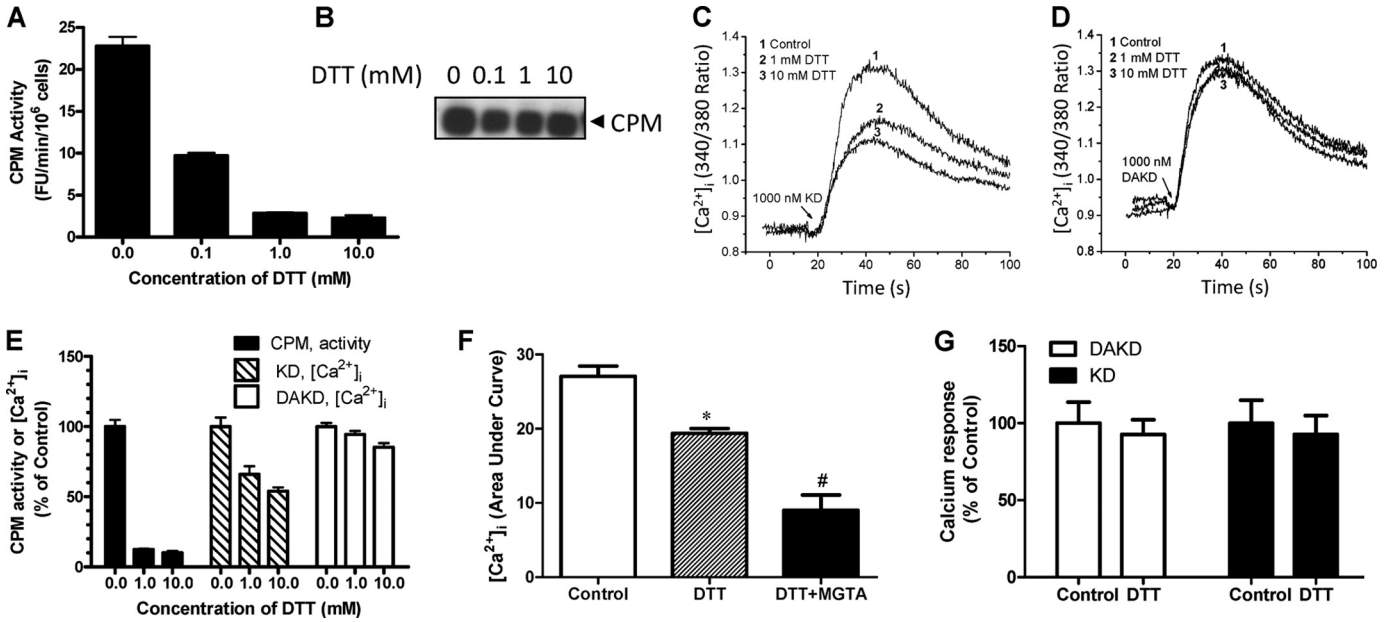

FIGURE 3. **KD stimulates an increase in $[Ca^{2+}]_i$ in cells expressing B1R and WTCPM whose activity is inhibited with reducing reagent.** *A,* HEK cells stably expressing WTCPM and B1R were incubated with the indicated concentration of DTT for 10 min at 37 °C. The activity of CPM in these cells was then measured as described under "Experimental Procedures." The data are expressed as mean ± S.E. ($n = 3$). *FU*, fluorescence units. *B,* cells from *A* were lysed, and CPM in the lysates was analyzed by Western blotting with anti-CPM monoclonal antibody. The data are representative of three independent experiments. *C* and *D*, HEK cells stably expressing WTCPM and B1R were pretreated with the indicated concentration of DTT for 10 min at 37 °C, followed by the addition of 1 μM KD (*C*) or B1R agonist DAKD (*D*), and the increase in $[Ca^{2+}]_i$ was recorded as described under "Experimental Procedures." At concentrations that completely inhibit CPM activity, DTT only partially decreases the increase in $[Ca^{2+}]_i$ mediated by KD (*C*) but does not inhibit the calcium response to B1R agonist DAKD (*D*). The *traces* are representative of three experiments. *E,* in cells stably co-expressing B1Rs and WTCPM, the effect of increasing concentrations of DTT on CPM activity and the increase in $[Ca^{2+}]_i$ stimulated by 1 μM KD or DAKD was quantified as in Fig. 1. Results are expressed as % of control cells not treated with DTT. The data are expressed as mean ± S.E. ($n = 3$). *F,* HEK cells stably expressing B1R and WTCPM were incubated with 10 mM DTT for 10 min at 37 °C and then treated with 20 μM MGTA for 60 s before addition of 1 μM KD to stimulate a calcium response, which was quantified as described under "Experimental Procedures." The data are expressed as mean ± S.E. ($n = 3$). *, $p < 0.05$ *versus* control; #, $p < 0.05$ *versus* DTT. *G,* DTT did not reduce the increase in $[Ca^{2+}]_i$ induced by B2R and B1R agonists in HEK cells stably expressing B1R and CPM-E264Q. The cells were treated with KD or DAKD and the calcium response measured and quantified as in *C*. The data are shown as mean ± S.E. ($n = 3$).

CPM-S180N or CPM-S180N/E264Q double mutant. Cells co-expressing B1R + CPM-S180N exhibited carboxypeptidase activity for furylacryloyl-Ala-Lys, which was equivalent to that exhibited by cells expressing B1R + WTCPM (Fig. 4*A*), even though CPM-S180N expression was significantly lower than that of WTCPM (Fig. 4*B*). Cells co-expressing B1R + CPM-S180N did not appreciably cleave dansyl-Ala-Arg, and cells co-expressing CPM-S180N/E264Q had no detectable carboxypeptidase activity with either substrate over background (Fig. 4*A*). BK (1 μM) stimulated an increase in $[Ca^{2+}]_i$ in the cells stably expressing CPM-E264Q and B1R (Fig. 4*C*) as above. However, in cells stably expressing B1Rs + CPM-S180N, 1 μM BK gave only a minor calcium response and had no effect on cells co-expressing B1Rs + CPM-S180N/E264Q (Fig. 4*C*). These data indicate that reduced binding affinity of CPM-S180N/E264Q for BK (with C-terminal Arg) eliminated its ability to generate a B1R signal. To determine whether a response with CPM-S180N/E264Q could be restored by changing the C-terminal residue of BK or KD, we generated bradykinin or kallidin analogs containing C-terminal Lys residues instead of Arg (*i.e.* K^9-BK and K^{10}-KD). Indeed, 1 μM K^9-BK or K^{10}-KD stimulated a significant increase in $[Ca^{2+}]_i$ in cells stably co-expressing B1Rs with CPM-S180N or CPM-S180N/E264Q compared with cells stably expressing the B1R alone (Fig. 4*D*). The greater response in cells co-expressing CPM-S180N is due to its ability to both bind K^9-BK or K^{10}-KD and cleave them to generate B1R agonist, whereas the CPM-S180N/E264Q mutant only binds but does not cleave K^9-BK or K^{10}-KD. In contrast, cells co-expressing B1Rs + CPM-E264Q or CPD-DIII did not generate an increase in $[Ca^{2+}]_i$ in response to K^9-BK or K^{10}-KD beyond the signal generated in cells expressing B1Rs alone (Fig. 4*D*). Taken together, these data indicate that kinin peptide binding to CPM-E264Q is required for generation of B1R-dependent signals.

Our previous studies showed that WTCPM and B1R form a complex on the cell membrane as supported by their co-localization in lipid raft domains, co-immunoprecipitation, and FRET between YFP-CPM and CFP-B1R (10). We carried out co-immunoprecipitation experiments to determine whether the CPM mutants also interact with the B1R. When B1Rs were stably co-expressed with CPM-E264Q, CPM-S180N, or CPM-S180N/E264Q, all CPM mutants co-immunoprecipitated with B1R as did WTCPM (Fig. 4, *E* and *F*); however, CPD-DIII did not co-immunoprecipitate (data not shown). Thus, the effects of the CPM active site mutations on B1R signaling were due to changes in CPM substrate binding and/or hydrolysis and not its binding to the B1R.

Effect of BK on B1R Responses in Co-cultured Cells Separately Expressing WTCPM, CPM-E264Q, or B1R—In contrast to CPM-E264Q, WTCPM converts BK to DABK, which can diffuse to activate nearby or more distant B1Rs. To determine whether B1R responses mediated by WTCPM or CPM-E264Q required expression of the two proteins in the same cell, HEK cells stably expressing only WTCPM or CPM-E264Q were

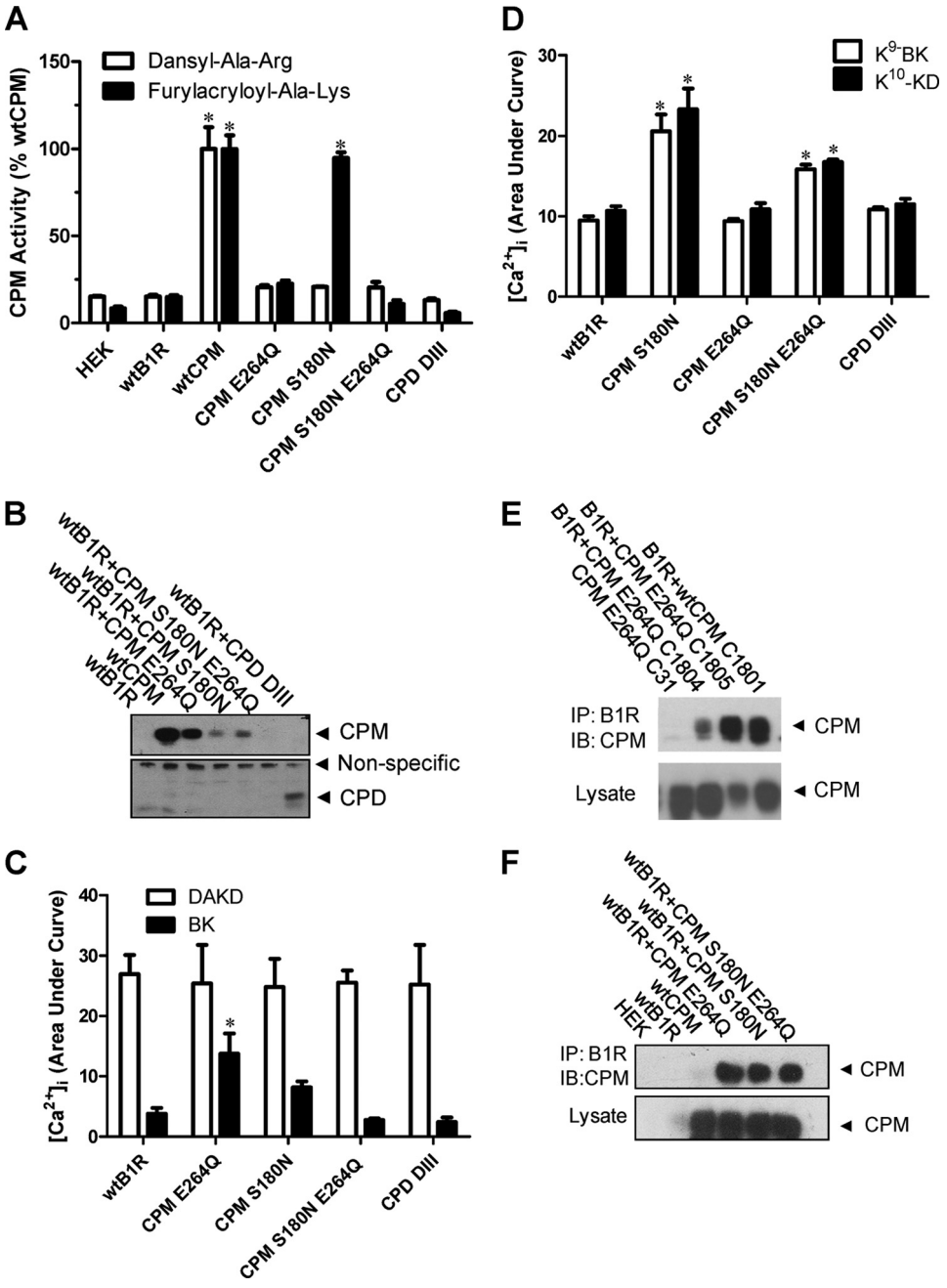

FIGURE 4. **Effect of CPM mutations on the ability of B2R agonists to induce an increase in $[Ca^{2+}]_i$ in cells co-expressing B1Rs.** *A,* activity of CPM, CPM mutants, and CPD DIII in cells stably co-expressing B1Rs were measured with dansyl-Ala-Arg or furylacryloyl-Ala-Lys as described under "Experimental Procedures." *B,* cells from *A* were lysed, and the expression of CPM, its mutants, and CPD-DIII was determined by Western blotting. *C,* HEK cells stably expressing B1R alone or co-expressing CPM-E264Q, CPM-S180N, CPM-S180N/E264Q or CPD-DIII were stimulated with B1R agonist DAKD (1 μM) or B2R agonist BK (1 μM), and the increase in $[Ca^{2+}]_i$ was recorded and quantified by integrating area under the curve. *D,* same cells as in *C* were stimulated with the C-terminal Lys derivatives, K^9-BK (1 μM) or K^{10}-KD (1 μM), and the increase in $[Ca^{2+}]_i$ was recorded and quantified as in *C*. *E,* different HEK cell clones (designated by number) stably expressing CPM-E264Q without or with the B1R were lysed and immunoprecipitated (*IP*) with antibody to the B1R followed by immunoblotting (*IB*) for CPM as described under "Experimental Procedures." *F,* HEK cells alone (HEK) or stably expressing B1R, WTCPM, or B1R plus CPM-E264Q, CPM-S180N, or CPM-S180N/E264Q were lysed and co-immunoprecipitation was performed as in *E*. The data are expressed as mean ± S.E. ($n = 3$) in *A, C,* and *D*; *, $p < 0.05$ *versus* B1R alone. The data in *B, E,* and *F* are representative of three experiments.

mixed with cells stably expressing only B1Rs in a 1:1 ratio. After 24–36 h in culture, CPM activity and the calcium response to BK or DABK were determined. As shown in Fig. 5, *A* and *D*, 1 μM DABK (B1R agonist) stimulated an increase in $[Ca^{2+}]_i$ in the cells stably expressing B1Rs alone or in the mixed cultures of these cells with cells stably expressing WTCPM or CPM-E264Q but not in HEK cells expressing only WTCPM or CPM-E264Q. As expected, because only half the cells were expressing B1Rs, the response in the mixed cultures was about half that detected in cells stably expressing B1Rs alone (Fig. 5, *A* and *D*). In contrast, whereas 1 μM BK did induce a significant increase in $[Ca^{2+}]_i$ in the mixed culture of cells expressing WTCPM and B1R (Fig. 5*A*), it did not increase $[Ca^{2+}]_i$ in mixed cells expressing CPM-E264Q and B1R (Fig. 5*D*). BK did not stimulate a

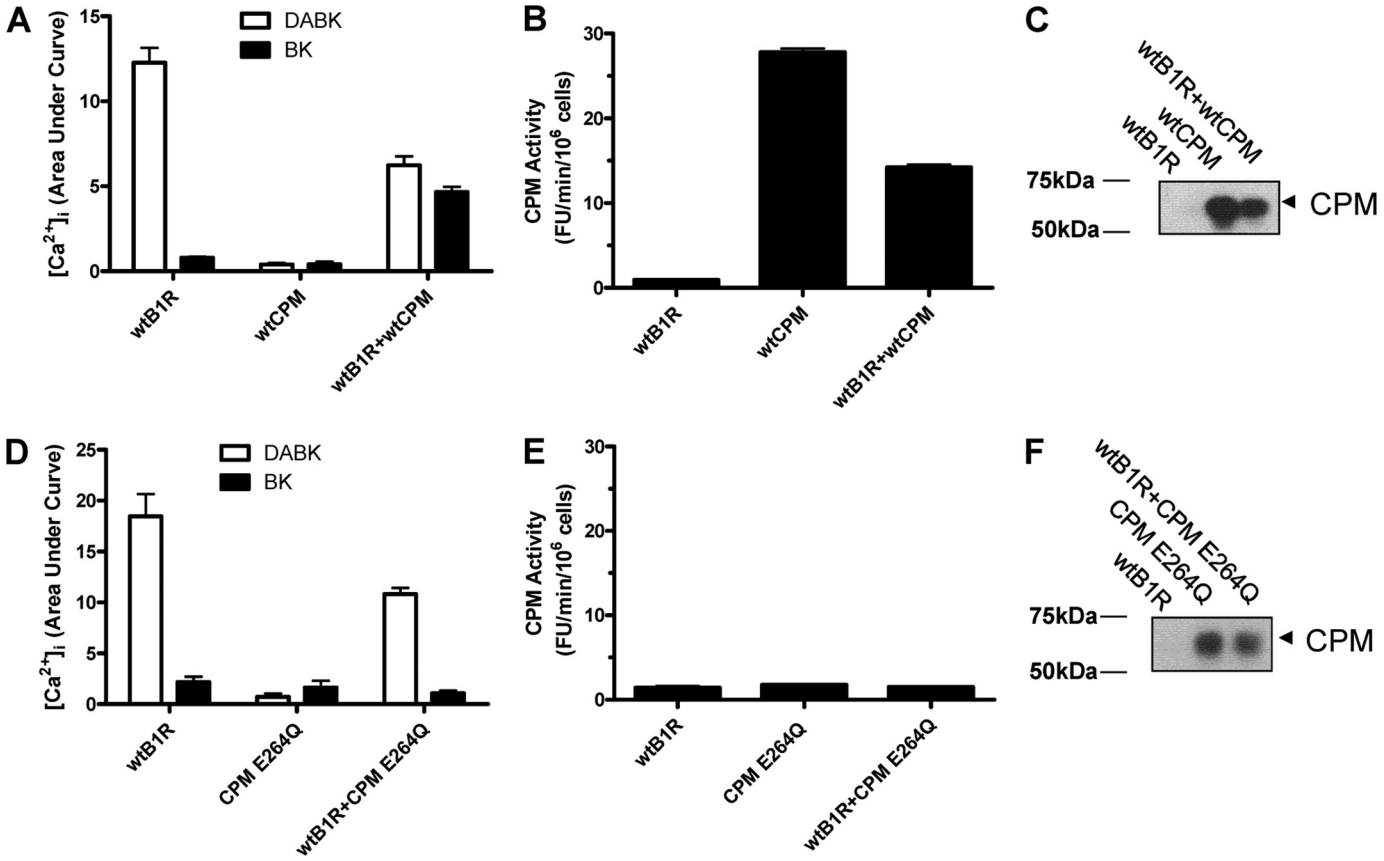

FIGURE 5. **Effect of BK on B1R responses in co-cultured cells separately expressing WTCPM, CPM-E264Q, or B1R.** HEK cells stably expressing either WTCPM, CPM-E264Q, or B1R were cultured separately or mixed at a 1:1 ratio (WTCPM/B1R or CPM-E264Q/B1R) and cultured for 24–36 h. *A,* calcium response to DABK (1 μM) or BK (1 μM) in HEK cells expressing only B1R or WTCPM alone or in a mixed co-culture of the same cells (*wtB1R+WTCPM*). The activity (*B*) and expression (*C*) of CPM were measured in HEK cells stably expressing only WTCPM, B1R, or in the mixed co-culture. *D,* calcium response to DABK (1 μM) or BK (1 μM) in HEK cells expressing only B1R or CPM-E264Q alone or in a mixed co-culture of the same cells (*wtB1R+CPM-E264Q*). The activity (*E*) and expression (*F*) of CPM were measured in HEK cells only stably expressing CPM-E264Q, B1R, or in the mixed co-culture. The data are expressed as mean ± S.E. ($n = 3$) in *A, B, D,* and *E*. The data are representative of three experiments in *C* and *F*.

calcium response in cells only stably expressing B1Rs (Fig. 5, *A* and *D*), and neither DABK nor BK increased $[Ca^{2+}]_i$ in cells only stably expressing WTCPM or CPM-E264Q (Fig. 5, *A* and *D*).

The activity and protein level of WTCPM in the mixed cells was about 50% of that in the cells stably expressing only WTCPM as expected (Fig. 5, *B* and *C*). Only low basal carboxypeptidase activity (equivalent to cells expressing B1Rs alone) was detected in cells expressing CPM-E264Q alone or mixed with cells expressing B1Rs (Fig. 5*E*), but Western blotting confirmed protein expression of CPM-E264Q (Fig. 5*F*). These data indicate that B1R agonist generated by WTCPM in one cell can diffuse to activate B1Rs in adjacent cells in the mixed culture. However, the B1R response generated by BK via CPM-E264Q requires co-expression with B1Rs in the same cells as the response was lost when the two proteins were expressed in different cells in the mixed culture.

Effect of a Monoclonal Antibody Targeting an Epitope on the C-terminal β-Sheet Domain of CPM—We previously found that a CPM monoclonal antibody specific for a 10-amino acid epitope (residues 302–311) on the CPM C-terminal domain does not block CPM activity, but inhibits B1R/CPM interaction and reduces CPM-dependent B1R signaling to BK or KD (10). In this study, we found that the anti-CPM monoclonal antibody also significantly decreased the calcium response to BK in cells stably expressing CPM-E264Q and B1R (Fig. 6*A*). In fact, the inhibition of the response was much greater in cells stably expressing CPM-E264Q and B1R (75%) than that (40%) in cells stably expressing WTCPM and B1R (Fig. 6*B*). The lesser effect seen with WTCPM is likely due to the ability of the active enzyme to generate B1R agonist, although delivery to the B1R is less efficient, whereas with CPM-E264Q, the response is completely dependent on its interaction with the B1R as no agonist is produced.

KD Does Not Increase $[Ca^{2+}]_i$ in Cells Stably Expressing a CPM-E264Q-B1R Fusion Protein—We previously showed that a CPM-B1R fusion protein, in which the C terminus of WTCPM is fused to the N terminus of the B1R, is a functional B1R receptor that also responds to B2R agonists BK or KD (10). We generated a similar B1R fusion protein containing covalently attached CPM-E264Q at the N terminus (CPM-E264Q-B1R). Cells expressing this fusion protein did not convert BK to B1R agonist DABK, whereas cells expressing the WTCPM-B1R fusion protein did (Fig. 2). In cells stably expressing CPM-E264Q-B1R, B1R agonist DAKD (1 μM) elicited a calcium response that was similar to that generated by cells expressing the WTCPM-B1R fusion protein (Fig. 7, *B* and *C*). However, KD (1 μM) produced almost no increase in $[Ca^{2+}]_i$ in the cells stably

expressing CPM-E264Q-B1R, but it did stimulate a significant increase in $[Ca^{2+}]_i$ in the cells stably expressing WTCPM-B1R (Fig. 7, *A* and *C*) as we reported before (10). Thus, tethering WTCPM to the N terminus of the B1R still allows efficient delivery of enzymatically generated agonist to the B1R. However, close proximity of CPM-E264Q afforded by covalent linkage to the B1R is not sufficient to generate a response to KD. These data, together with the inhibitory effect of the CPM monoclonal antibody, indicate that the CPM-E264Q-mediated response requires proper orientation/binding of CPM-E264Q and B1R on the membrane, which is not replicated by covalent linkage.

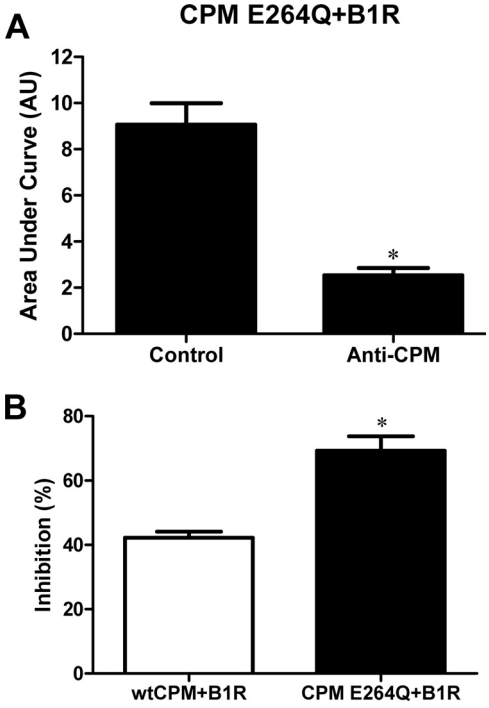

FIGURE 6. **A monoclonal antibody that disrupts the interaction of CPM with the B1R inhibits the calcium response mediated by BK.** HEK cells stably expressing B1Rs and either WTCPM or CPM-E264Q were incubated with or without an anti-CPM monoclonal antibody (500 ng/ml) for 30 min. The increase in $[Ca^{2+}]_i$ was recorded after stimulation with BK (1 μM) and quantified by integrating the area under the curve. *A,* CPM monoclonal antibody decreased the calcium response to BK in HEK cells stably expressing CPM-E264Q and B1Rs. *B,* relative inhibition of the calcium response to 1 μM BK by anti-CPM monoclonal antibody in cells stably expressing B1Rs and either WTCPM or CPM-E264Q. The data are expressed as mean ± S.E. ($n = 3$). *, $p < 0.05$ *versus* control without antibody treatment in *A*. *, $p < 0.05$ *versus* cells stably expressing WTCPM and B1R in (*B*).

Effect of MGTA and DABK on BK Binding to Cells Expressing the B1R and CPM-E264Q—One mechanism by which BK or KD might stimulate a B1R-dependent response in cells co-expressing CPM-E264Q is by cooperative binding of the kinin C terminus to CPM and the N terminus to B1R, resulting in receptor activation. To address this question, we measured the ability of a B1R agonist (which would not bind CPM) or a CPM inhibitor (which would not bind the B1R) to displace BK binding on cells co-expressing CPM-E264Q and B1Rs. As shown in Fig. 8, the CPM inhibitor MGTA almost completely displaced bound [^{3}H]BK, whereas the B1R agonist DABK had little effect. These results do not support the idea of simultaneous kinin binding to the active site of CPM and orthosteric binding site of the B1R but could be consistent with primary binding to CPM, which, through allosteric effects, activates the B1R.

Induction of a Conformational Change in the B1R by BK When Co-expressed with CPM—To determine whether binding of BK to CPM-E264Q would result in a conformational change in the B1R consistent with activation, we used an intramolecular FRET approach previously described to detect ligand-dependent conformational changes in GPCRs (37, 48). In this method, a tetracysteine motif is inserted into the third intracellular loop that binds the FRET acceptor, a small molecule called FlAsH, and a CFP FRET donor at the C terminus (37). The TC FlAsH-binding motif (CCPGCC) was inserted into the third intracellular loop of the B1R between Gly^{242} and Arg^{243}, and CFP was fused to the C terminus to generate B1R-TC-CFP (supplemental Fig. 1). This receptor was fully functional, as in HEK cells expressing B1R-TC-CFP, and B1R agonist DAKD stimulated a robust calcium response, but BK had no effect (supplemental Fig. 2*A*). B1R agonist also stimulated a substantial increase in ERK phosphorylation, and HEK cells expressing this construct specifically bound [^{3}H]DAKD at a level similar to that of the WTB1R (data not shown).

There was basal FRET between CFP and FlAsH in B1R-TC-CFP, as shown by confocal imaging using acceptor photobleaching and in a spectrofluorometer where a prominent emission peak at 530 nm (the FlAsH emission) was seen after excitation of CFP at 430 nm (supplemental Fig. 2, *B* and *C*). To determine whether FRET changed upon agonist stimulation,

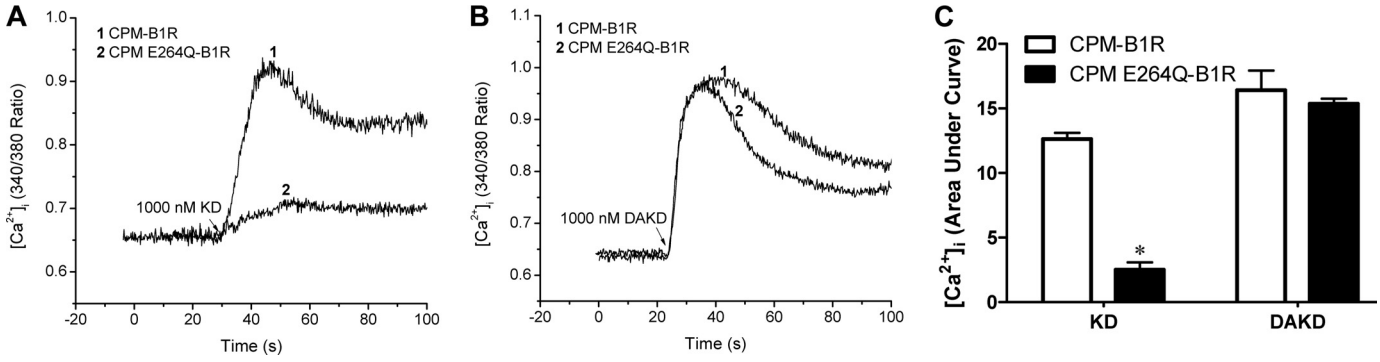

FIGURE 7. **Calcium response to kinin peptides in HEK cells stably expressing CPM-B1R or CPM-E264Q-B1R fusion proteins.** HEK cells stably expressing fusion proteins with the N terminus of the B1R fused to the C terminus of either WTCPM (CPM-*B1R*) or CPM-E264Q (*CPM E264Q-B1R*) were stimulated with 1 μM KD (*A*) or 1 μM DAKD (*B*), and the increase in $[Ca^{2+}]_i$ was measured. *C,* data were quantified by integrating area under the curve and are expressed as mean ± S.E. ($n = 3$). *, $p < 0.05$ *versus* CPM-B1R.

cells labeled with FlAsH reagent were excited at 430 nm, and the emission at 530 nm was continuously monitored. The B1R agonist DAKD caused a sharp rise in emission at 530 nm, indicating increased FRET, whereas the B1R antagonist DALKD or BK gave no response (Fig. 9, *A* and *B*). Pretreatment of cells with DALKD inhibited the increased FRET in response to DAKD (Fig. 8*B*). These data show that the FlAsH/CFP-labeled B1R is functional, and the fluorophores are close enough to exhibit FRET, and the FRET signal increases upon agonist stimulation, consistent with a conformational change that reduces the distance between the CFP and FlAsH fluorophores.

To determine the role of CPM in activating B1R-TC-CFP, we tested the calcium response in HEK cells stably expressing B1R-TC-CFP without or with CPM, inactive CPM-E264Q, or the inactive CPD-DIII (supplemental Fig. 2*D*). BK did not stimulate a calcium response in cells expressing only B1R-TC-CFP nor in cells co-expressing B1R-TC-CFP and CPD-DIII (supplemental Fig. 2*E*). However, BK did stimulate an increase in intracellular Ca^{2+} when B1R-TC-CFP was co-expressed with WTCPM or CPM-E264Q (supplemental Fig. 2*E*), similar to results with WTB1R (Fig. 1). All cells responded similarly to 1 μM B1R agonist DAKD (supplemental Fig. 2*F*), indicating equivalent B1R function.

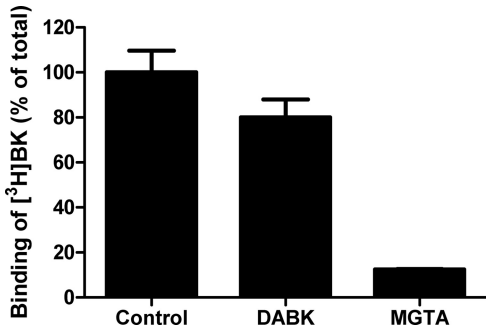

FIGURE 8. **Displacement of BK binding to cells stably co-expressing B1Rs and CPM-E264Q.** HEK cells stably expressing B1R and CPM-E264Q were incubated with [^{3}H]BK and then incubated with either DABK or MGTA, and the residual binding of [^{3}H]BK was determined as described under "Experimental Procedures." CPM inhibitor MGTA blocked the binding of BK, whereas the B1R agonist DABK had little effect. The data are expressed as mean ± S.E. ($n = 3$).

To determine whether BK can cause a conformational change in the B1R mediated by CPM, the change in intramolecular FRET of B1R-TC-CFP was examined. BK induced a significant increase in FRET in cells stably expressing B1R-TC-CFP and WTCPM or CPM-E264Q but not in cells expressing only B1R-TC-CFP or co-expressing CPD-DIII (Fig. 9*C*). The increase in FRET in cells co-expressing WTCPM and B1R-TC-CFP was greater than in cells co-expressing CPM-E264Q and B1R-TC-CFP, consistent with the differences in the calcium response (supplemental Fig. 2*E*). These data indicate that BK causes a CPM-mediated conformational change in the B1R that reduces the distance between FlAsH in the third intracellular loop and the C-terminal CFP, similar to that induced by B1R agonist.

Role of the CPM/B1R Interaction in Primary Endothelial Cells under Inflammatory Conditions—CPM is present in human endothelial cells, and its expression can be increased 2–3-fold by cytokine treatment that also up-regulates B1R expression (11, 16, 36, 38, 49, 50). We previously showed that in cytokine-pretreated HLMVEC, B1R stimulation leads to acute activation of iNOS and high output NO production via ERK-mediated phosphorylation of Ser^{745} in the iNOS reductase domain (17, 19). To investigate the role of the CPM/B1R interaction in this response, we designed a peptide ("CT peptide") corresponding to residues 299–312 in the CPM C-terminal domain (Ac-KGQVFDQNGNPLPN-NH_2) containing the 10-residue epitope (302–311) recognized by the monoclonal antibody to CPM that inhibits its interaction with the B1R (10). In control studies with HEK cells stably co-expressing CPM and B1Rs, 50 μM CT peptide did not directly inhibit CPM activity measured in live cells with dansyl-Ala-Arg substrate. CPM activity was 17.2 ± 1.0 *versus* 16.4 ± 0.4 fluorescence units/min/10^6 cells ($n = 6$) in control *versus* CT peptide-treated cells. However, preincubation of cells with the CT peptide significantly reduced co-immunoprecipitation of CPM with the B1R (supplemental Fig. 3). The CT peptide also inhibited the cal-

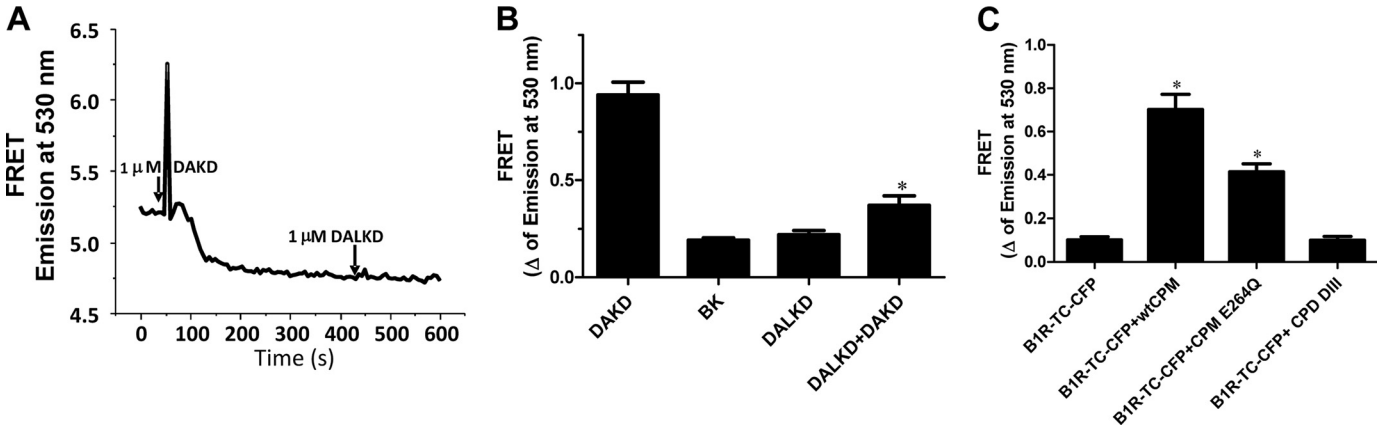

FIGURE 9. **Effect of kinin peptides on the intramolecular FRET of B1R-TC-CFP.** *A*, sample tracing of the increase in FRET stimulated by B1R agonist DAKD. HEK cells expressing B1R-TC-CFP and labeled with FlAsH reagent were excited at 430 nm, and emission at 530 nm was continuously monitored after addition of 1 μM DAKD agonist or DALKD antagonist. *B*, increase in FRET of B1R-TC-CFP induced by B1R agonist is inhibited by B1R antagonist DALKD. The data are expressed as mean ± S.E. ($n = 3$). *, $p < 0.05$ *versus* DAKD. *C*, change in intramolecular FRET of B1R-TC-CFP in response to BK. HEK cells stably expressing B1R-TC-CFP alone or with WTCPM, CPM-E264Q, or CPD DIII were labeled with FlAsH-EDT2 and washed as described under "Experimental Procedures." Cells were stimulated with 1 μM BK, and the FlAsH emission at 530 nm was recorded in real time while exciting CFP at 430 nm. The data are expressed as mean ± S.E. ($n = 4$). *, $p < 0.05$ *versus* B1R-TC-CFP.

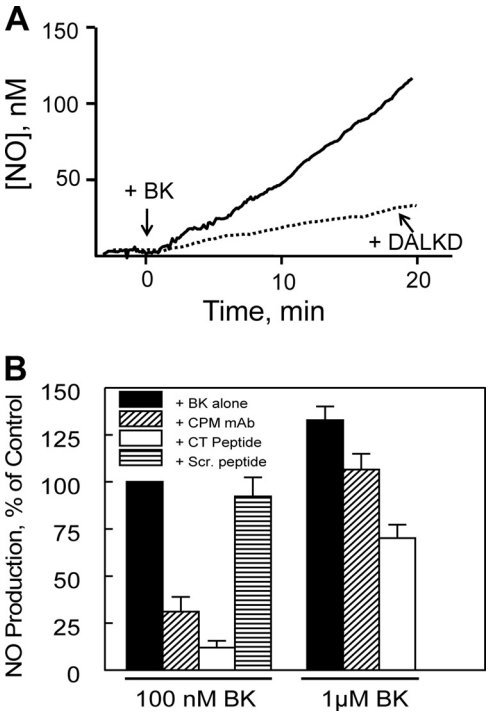

FIGURE 10. **Disruption of the CPM/B1R interaction inhibits B1R-mediated NO production in response to BK in human endothelial cells.** *A,* cytokine-treated HLMVEC were preincubated for 30 min with 1 μM HOE140 (B2R antagonist) without (*solid line*) or with (*dotted line*) 1 μM DALKD (B1R antagonist). At time 0, 100 nM BK was added, and NO production was measured in real time for 20 min with a porphyrinic microsensor. *B,* cells were pretreated with 1 μM HOE140 without or with 500 ng/ml CPM monoclonal antibody, 50 μM CT peptide, or scrambled CT peptide (*Scr. peptide*) for 30 min. Cells were stimulated with 100 nM or 1 μM BK, and NO production was measured for 20 min. Shown are mean values as % control (100 nM BK alone = 100%) ± S.E. (n = 3).

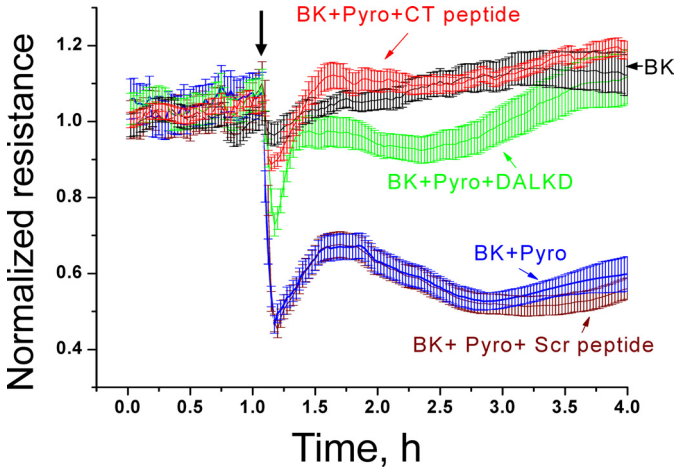

FIGURE 11. **B1R-dependent increase in endothelial permeability caused by BK combined with superoxide depends on CPM/B1R interaction.** HLMVEC, grown to confluence on gold electrodes coated with 10 μg/ml fibronectin, were cytokine-pretreated (10 ng/ml IL-1β + 100 units /ml IFN-γ, 16 h). Medium was changed; cells were allowed to stabilize, and all samples were pretreated with 10 μM HOE140 to block B2R responses. Cells were then treated with 1 μM BK (added at the *black arrow*) alone (*black line*) or combined with 200 μM pyrogallol (*Pyro*; superoxide generator) without (*blue line*) or with 1 μM B1R antagonist DALKD (*green line*), 50 μM CT peptide (*red line*), or 50 μM scrambled (*Scr*) peptide (*brown line*), and TER was measured. Results show mean values ± S.E. for n = 4.

cium response to 1 μM KD in cells co-expressing CPM and B1Rs, but not the response to 1 μM B1R agonist DAKD (supplemental Fig. 3).

We investigated the role of the CPM/B1R interaction in B1R-dependent activation of iNOS (17, 19) using cytokine-treated HLMVEC preincubated with B2R antagonist HOE140 to block B2R responses (Fig. 10). 100 nM BK stimulated a prolonged output of NO, measured in real time with a porphyrinic microsensor, that was B1R-dependent as it was blocked by antagonist DALKD (Fig. 10*A*). HLMVEC (in the presence of HOE140) were then preincubated with CPM monoclonal antibody, CT peptide, or a scrambled CT peptide with the same amino acids (Ac-GPQGDNVQPKFNLN-NH_2), and then NO production in response to 100 nM or 1 μM BK was measured. Both the CPM antibody and CT peptide, but not the scrambled peptide, substantially blocked B1R-mediated NO production in response to 100 nM BK (Fig. 10*B*), indicating the CPM/B1R interaction is critical in mediating this response. Interestingly, the CPM antibody and CT peptide were less effective in inhibiting the response to 1 μM BK (Fig. 10*B*), indicating that at a 10-fold higher substrate (BK) concentration, the amount of B1R agonist generated by CPM is sufficient to diffuse to and activate dissociated B1Rs to generate a partial response. These data are consistent with an important role for CPM in mediating and enhancing B1R signaling in primary human endothelial cells, especially at low *in vivo* concentrations of kinins.

Although iNOS-derived high output NO has been associated with loss of lung endothelial barrier function (51), NO itself is unlikely to be the proximal mediator as it is not highly reactive and is rapidly removed by reaction with hemoglobin (52). However, peroxynitrite ($ONOO^-$), formed by the rapid diffusion-limited reaction of NO with O_2^-, is a potent, diffusible oxidant that can mediate endothelial barrier disruption by either protein nitration or oxidation of sensitive thiols in signaling proteins (51, 52). We used TER (40, 41) to investigate the role of CPM/B1R interaction in endothelial barrier disruption. Stimulation of cytokine-pretreated HLMVEC with BK alone, in the presence of HOE140 to block B2Rs, produced a modest increase in TER (Fig. 11). However, BK combined with pyrogallol (which auto-oxidizes to produce O_2^-) caused a profound drop in resistance, consistent with the generation of $ONOO^-$ mediating an increase in endothelial permeability (Fig. 11). This drop in resistance was reversed by B1R antagonist DALKD, showing that the response was B1R-mediated (Fig. 11). Importantly, the CPM CT peptide almost completely reversed decrease in resistance caused by BK + pyrogallol, whereas the scrambled peptide had no effect (Fig. 11). These data emphasize the importance of the CPM/B1R interaction in mediating B1R-iNOS signaling and effects on barrier function in primary endothelial cells that express both proteins at native levels.

DISCUSSION

Protein complexes form functional units that operate more efficiently and can be more finely regulated than mixtures of independent proteins, with typical examples including the proteasome and transcriptional or translational machinery (53). Cell signaling also relies on coordinated protein interactions to transmit extracellular signals across the cell membrane and propagate them intracellularly (54). The role of GPCR interactions with non-GPCR extracellular membrane proteins has not

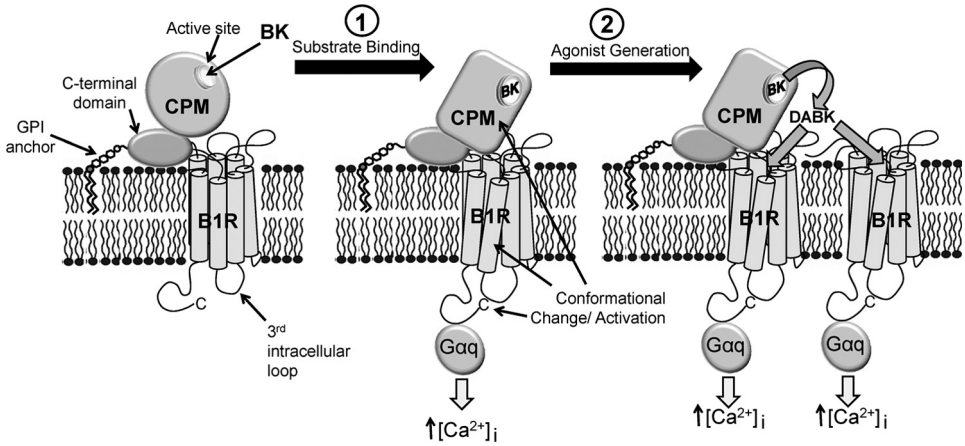

FIGURE 12. **Model of CPM/B1R interactions and signaling in response to BK.** A model of CPM and its potential membrane orientation and basal interaction with the B1R is shown in the *left panel*. Based on our results, BK (or KD) released from the kininogen precursor can stimulate B1R signaling in two ways via CPM. *1,* binding as a substrate causes a conformational change in CPM that is transmitted via protein/protein interaction to the B1R, resulting in G protein coupling and activation of calcium signaling. *2,* catalytic conversion of BK (or KD) to B1R agonist that can further activate the associated receptor or additional B1Rs. For the catalytically inactive CPM-E264Q mutant, only the first mechanism of activation is possible. For further details, see text.

been well studied, but cell surface peptidases represent interesting candidates to regulate signaling. Although enzymatic hydrolysis itself is a key mechanism by which peptide hormone activity is altered or terminated, peptidase functions might extend beyond peptide degradation to include direct protein/protein interactions with peptide-activated GPCRs. The extracellular loops of GPCRs contain potential ligand-binding sites for allosteric control of receptor functions (5); thus, a membrane peptidase whose active site domain is extracellular could regulate receptor function by interacting with such a site. For example, there is growing evidence for a functional complex between ACE and the B2R (55). ACE enzymatically inactivates BK, thus ACE inhibitors prolong BK half-life, indirectly enhancing B2R responses. However, ACE inhibitors potentiate the B2R effects of BK analogs that are not cleaved by ACE and can reactivate B2Rs that have been desensitized (55, 56). Because ACE and B2Rs also form a heterodimeric complex on the cell membrane (56, 57), it is likely that this potentiation of B2R signaling happens via a conformational change in ACE upon inhibitor binding that is transmitted to an extracellular binding site on the receptor (55).

We previously found that the interaction of CPM and B1R on the membrane plays an important role in B1R signaling when cells are presented with B2R agonists BK or KD as would occur *in vivo* (10). Because these kinins are the peptides released from the precursor kininogen, they require further processing to generate B1R agonists. The assembly of CPM and B1R into a functional protein complex can thus facilitate B1R signaling by generating B1R agonist in close proximity to the receptor and is concordant with the current views that B1R signaling requires generation of DABK and DAKD (12, 58). However, this study shows that the CPM·B1R protein complex provides an additional novel pathway for initiation of B1R-mediated kinin signaling that is dependent on substrate binding to CPM but independent of kinin cleavage (Fig. 12). This is supported by several lines of evidence. First, BK or KD efficiently stimulated B1R- and CPM-dependent increases in $[Ca^{2+}]_i$ in cells stably expressing B1R and CPM-E264Q, a mutant that is a catalytically inactive but retains its substrate binding ability (28). That Glu^{264} is not involved in substrate binding is consistent with the x-ray crystal structure of CPM and its catalytic role in abstracting a proton from the zinc-bound water and increasing its nucleophilicity to allow hydrolysis of the scissile peptide bond (27). Second, the competitive active site-directed inhibitor MGTA blocked the ability of BK or KD to increase $[Ca^{2+}]_i$ in cells stably expressing B1R and CPM-E264Q. Third, the ability of KD to stimulate increased $[Ca^{2+}]_i$ was still present after elimination of WTCPM catalytic activity by the reducing agent DTT. The mechanism by which reducing agents inhibit CPM enzymatic activity remains unclear, but it may involve reduction of disulfide bonds between Cys^{225}–Cys^{267} and/or Cys^{268}–Cys^{121}, which participate in formation of the wall of the circular pit forming the active site of CPM (27). This might alter the orientation of the catalytic residues such that hydrolysis cannot occur but still allow substrate binding. This is supported by the fact that the competitive inhibitor MGTA reduced the calcium response to KD after DTT treatment. Moreover, DTT treatment did not affect the calcium response to KD in cells expressing B1Rs and CPM-E264Q, showing that DTT inhibited catalytic activity without globally denaturing CPM. Fourth, the ability of catalytically inactive CPM-E264Q to generate an increase in $[Ca^{2+}]_i$ when co-expressed with B1Rs correlated with its substrate specificity as determined by mutating the specificity-determining residue Ser^{180}. Thus, CPM-E264Q, with a higher affinity for C-terminal Arg than Lys, was able to facilitate a B1R-dependent calcium response with native BK (containing C-terminal Arg), but not a BK analog containing C-terminal Lys (K^9-BK). Conversely, CPM-S180N/E264Q, with a higher affinity for C-terminal Lys, generated a B1R-dependent calcium response with K^9-BK but not native BK with C-terminal Arg. Finally, BK interaction with CPM-E264Q resulted in a B1R conformational change similar to that stimulated by B1R agonist directly as detected by an increase in intramolecular FRET in B1R-TC-CFP.

Because CPM-E264Q cannot generate a diffusible agonist, generation of a B1R signal by BK or KD via this catalytically inactive mutant likely requires not only close physical association with the B1R but also proper orientation on the membrane,

as supported by the present data. For example, CPM-E264Q co-immunoprecipitated with the B1R and the calcium response mediated by CPM-E264Q in response to BK required its co-expression with B1Rs in the same cells. The crystal structure of human CPM and molecular modeling suggests that the C-terminal residues (Pro^{403}–Ser^{406}) and glycan moieties of the GPI anchor form a partially flexible ~20-Å-long tether, restricting the mobility of CPM with respect to the membrane. This would allow electrostatic contacts between seven positively charged, surface-located residues in the C-terminal domain and negatively charged membrane phospholipid headgroups to orient CPM so its active site groove points along the cognate membrane (27). These interactions might contribute to the stability and proper conformation of the CPM·B1R complex in the membrane. This is consistent with the present data showing that a monoclonal antibody specific for an epitope in the C-terminal domain of CPM, which we previously showed disrupted the co-immunoprecipitation of WTCPM and the B1R (10), blocked the ability of CPM-E264Q to mediate the B1R-dependent increase in $[Ca^{2+}]_i$ in response to BK. In fact, the inhibition of the calcium response was greater with CPM-E264Q than with WTCPM because of the ability of the latter enzyme to generate B1R agonist that could still diffuse to a more distant B1R and cause a response, albeit less efficiently. Furthermore, the WTCPM-B1R fusion protein was still capable of generating a significant B1R response to KD, whereas the CPM-E264Q-B1R fusion was not. Thus, covalent fusion of the C terminus of CPM to the N terminus of the B1R likely does not allow for a proper orientation of CPM-E264Q on the membrane and/or interaction with the B1R for this noncatalytic effect to be manifested.

Taken together, the above data indicate that WTCPM promotes B1R signaling in two ways. First, it causes B1R activation upon binding of B2R agonists BK or KD. Second, it generates B1R agonist that can further activate the bound receptor or diffuse and activate adjacent receptors (Fig. 12). This is consistent with the relative responsiveness to BK of the various CPM/B1R systems we used to investigate this phenomenon. In mixed co-cultures of cells singly expressing either WTCPM or B1R, where receptor activation depended on diffusion of agonist generated by CPM, a significant increase in $[Ca^{2+}]_i$ was only achieved with 1 μM BK (Fig. 5 and supplemental Fig. 4). In cells co-expressing CPM-E264Q and B1Rs, where only interaction-dependent receptor activation was possible, both 1 μM and 100 nM (but not 10 nM) BK generated a significant calcium response (Fig. 1 and supplemental Fig. 4). With cells co-expressing WTCPM and B1Rs, where both CPM/B1R interaction and agonist generation occurred, a significant response was generated with as little as 10 nM BK (Fig. 1 and supplemental Fig. 4). This is also consistent with the results of experiments on natively expressed CPM and B1R in HLMVEC where disruption of the CPM·B1R complex with monoclonal antibody or CT peptide more effectively inhibited the B1R-mediated NO production in response to 100 nM BK than to 1 μM BK (Fig. 10).

There are two potential mechanisms by which CPM-E264Q/B1R interaction could cause B1R signaling in response to BK or KD, the natural B2R agonists. First, CPM might participate in the binding of KD or BK simultaneously with the B1R, resulting in activation of the receptor. Second, kinin peptide binding to CPM could alter its conformation which, through protein/protein interaction, elicits an active conformation of the B1R. Regarding the first possibility, the presence or absence of the C-terminal arginine is the key switch that determines the specificity of kinin peptides for the B2R or B1R because of interactions with Lys^{118} in transmembrane domain 3 of the B1R, corresponding to Ser^{111} in the B2R (16). Potentially, binding of the C-terminal Arg in the active site groove of CPM could shield the positive charge from Lys^{118}, allowing the kinin N terminus to bind the B1R, shown to be mediated by the extracellular negatively charged residues Glu^{273} and Asp^{291} flanking the third extracellular loop (16, 59). However, because the CPM active site interacts with three C-terminal residues of its substrates (27), it could prevent productive binding of remaining peptide to the receptor. This is because 9 of the 11 residues in the B1R identified to be important for agonist binding reside in transmembrane domains (59). Thus, although binding of the C-terminal of BK or KD to CPM might allow the N terminus to bind the extracellular B1R residues Glu^{273} and Asp^{291}, it seems unlikely that the rest of the peptide would be able to interact with B1R transmembrane residues required to elicit an appropriate conformational change of the receptor leading to the activated state. In addition, the displacement of BK binding to cells expressing CPM-E264Q and B1Rs by the CPM inhibitor, but not by B1R agonist, argues against this explanation. The displacement data, however, are consistent with the second possibility that binding to CPM allosterically activates the B1R by protein/protein interaction (Fig. 12). This is also consistent with our finding of increased intramolecular FRET of the B1R complexed with CPM-E264Q after stimulation with BK. Nevertheless, these data do not exclude the possibility that binding of the C-terminal of BK to CPM causes the N-terminal of BK to interact with the B1R at a site different from the orthosteric site to cause a response.

The competitive inhibitor MGTA binds to the active site of CPM, but in contrast to BK or KD binding, it does not elicit a response on its own, and it blocks the ability of KD or BK to generate a B1R signal. This could be due to a different mode of binding than substrate; MGTA is a simple arginine derivative whose –SH group complexes the active site zinc. The crystal structure of domain 2 of carboxypeptidase D (a related carboxypeptidase) complexed with guanidinoethylmercaptosuccinic acid (a similar arginine derivative) shows that it occupies the same position as the specificity pocket for the P_1' Arg of substrates (45). However, modeling of a bound substrate in the active site of CPM showed that active site residues interact with not only the P_1' but also the P_1 and P_2 residues of the substrate (27). Although compared with substrates, MGTA interacts with a more limited number of active site residues, its affinity for CPM (K_i = 2 nM) is much higher than that of substrate BK (K_m = 16 μM). The lack of effect of MGTA could be compatible with either of the above proposed mechanisms for B1R activation. In the case of conformational change of CPM upon substrate binding being transmitted to the B1R, much tighter binding and longer residence time of MGTA and more limited interaction with active site residues may reduce the amount of conformational movement below a threshold required for

CPM-mediated activation of the B1R. If shared binding between CPM and the B1R is required for this response, MGTA would only interact with the active site pocket of CPM and could not simultaneously interact with the B1R.

For the CPM/B1R interaction to be physiologically relevant, the two proteins would have to be co-expressed in the same cells *in vivo*. CPM is constitutively expressed in a wide variety of cell types, including renal, vascular, neural, pulmonary, and immune cells (11, 36, 60–62). Although the B1R is not typically expressed constitutively, injury or inflammatory mediators upregulate its expression in most cell types (16, 63). Importantly, endotoxin or cytokines, which effectively induce B1R expression (16, 38, 63), also increase CPM expression (38, 49, 50). Thus, although not verified for all cell types, it is likely that cells expressing B1Rs will also express CPM.

B1Rs play important roles in the endothelium, for example by promoting angiogenesis (22) and stimulating nitric oxide production (17–19, 64). Endothelial B1Rs can also have deleterious effects as shown by increased hypotension and lethality in response to lipopolysaccharide administration in transgenic rats with endothelium-specific overexpression of B1Rs (20). CPM is also expressed in endothelial cells (12, 38, 49), and we previously showed that B1R-dependent calcium signaling in response to BK could be blocked by a CPM inhibitor in bovine pulmonary artery endothelial cells (10). In this study, we found that disruption of the B1R-CPM heterodimer greatly reduced B1R-dependent NO production stimulated by BK and blocked the increase in endothelial permeability in response to BK and pyrogallol. Taken together, these data show that CPM and B1Rs form a critical complex on endothelial cells required for efficient generation of B1R signals in response to KD or BK that are released from kininogen during inflammatory processes.

In conclusion, we found that in addition to generating B1R agonists, CPM mediates BK or KD stimulation of B1R signaling by a novel mechanism that is independent of the enzymatic activity of CPM (Fig. 12). This effect of CPM critically depends on the integrity of its complex with the B1R and its relative orientation on the membrane. This unusual signaling function might be mediated by transmittal of a CPM conformational change (upon substrate binding) to the B1R or, alternatively, by co-participation with the B1R in binding the kinin peptide. In this way, CPM can potentiate B1R signaling at the low physiological concentration of B2R agonists (far below the K_m value for BK) generated under inflammatory conditions (13, 16, 38). This novel way of regulating GPCR signaling could be exploited to develop drugs to alter the CPM/B1R interaction and thereby regulate kinin signaling that plays important roles in inflammatory processes and cardiovascular function.

Acknowledgments—We thank Kai Zhang and Svitlana Brovkovych for excellent technical assistance.

REFERENCES

1. Jacoby, E., Bouhelal, R., Gerspacher, M., and Seuwen, K. (2006) *Chem. Med. Chem.* **1,** 761–782
2. Kobilka, B. K., and Deupi, X. (2007) *Trends Pharmacol. Sci.* **28,** 397–406
3. Kenakin, T. P. (2009) *Trends Pharmacol. Sci.* **30,** 460–469
4. Wang, L., Martin, B., Brenneman, R., Luttrell, L. M., and Maudsley, S. (2009) *J. Pharmacol. Exp. Ther.* **331,** 340–348
5. Kenakin, T. P. (2008) *Mol. Pharmacol.* **74,** 541–543
6. Ritter, S. L., and Hall, R. A. (2009) *Nat. Rev. Mol. Cell Biol.* **10,** 819–830
7. Milligan, G., and Smith, N. J. (2007) *Trends Pharmacol. Sci.* **28,** 615–620
8. Pin, J. P., Neubig, R., Bouvier, M., Devi, L., Filizola, M., Javitch, J. A., Lohse, M. J., Milligan, G., Palczewski, K., Parmentier, M., and Spedding, M. (2007) *Pharmacol. Rev.* **59,** 5–13
9. Rios, C. D., Jordan, B. A., Gomes, I., and Devi, L. A. (2001) *Pharmacol. Ther.* **92,** 71–87
10. Zhang, X., Tan, F., Zhang, Y., and Skidgel, R. A. (2008) *J. Biol. Chem.* **283,** 7994–8004
11. Skidgel, R. A., and Erdös, E. G. (1998) *Immunol. Rev.* **161,** 129–141
12. Skidgel, R. A., Stanisavljevic, S., and Erdös, E. G. (2006) *Biol. Chem.* **387,** 159–165
13. Skidgel, R. A., Davis, R. M., and Tan, F. (1989) *J. Biol. Chem.* **264,** 2236–2241
14. Pesquero, J. B., and Bader, M. (2006) *Biol. Chem.* **387,** 119–126
15. Campos, M. M., Leal, P. C., Yunes, R. A., and Calixto, J. B. (2006) *Trends Pharmacol. Sci.* **27,** 646–651
16. Leeb-Lundberg, L. M., Marceau, F., Müller-Esterl, W., Pettibone, D. J., and Zuraw, B. L. (2005) *Pharmacol. Rev.* **57,** 27–77
17. Brovkovych, V., Zhang, Y., Brovkovych, S., Minshall, R. D., and Skidgel, R. A. (2011) *J. Cell. Mol. Med.* **15,** 258–269
18. Kuhr, F., Lowry, J., Zhang, Y., Brovkovych, V., and Skidgel, R. A. (2010) *Neuropeptides* **44,** 145–154
19. Zhang, Y., Brovkovych, V., Brovkovych, S., Tan, F., Lee, B. S., Sharma, T., and Skidgel, R. A. (2007) *J. Biol. Chem.* **282,** 32453–32461
20. Merino, V. F., Todiras, M., Campos, L. A., Saul, V., Popova, E., Baltatu, O. C., Pesquero, J. B., and Bader, M. (2008) *J. Mol. Med.* **86,** 791–798
21. Kakoki, M., McGarrah, R. W., Kim, H. S., and Smithies, O. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104,** 7576–7581
22. Parenti, A., Morbidelli, L., Ledda, F., Granger, H. J., and Ziche, M. (2001) *FASEB J.* **15,** 1487–1489
23. Emanueli, C., Bonaria Salis, M., Stacca, T., Pintus, G., Kirchmair, R., Isner, J. M., Pinna, A., Gaspa, L., Regoli, D., Cayla, C., Pesquero, J. B., Bader, M., and Madeddu, P. (2002) *Circulation* **105,** 360–366
24. Hagiwara, M., Murakami, H., Ura, N., Agata, J., Yoshida, H., Higashiura, K., and Shimamoto, K. (2004) *Hypertens. Res.* **27,** 399–408
25. Moniwa, N., Agata, J., Hagiwara, M., Ura, N., and Shimamoto, K. (2006) *Biol. Chem.* **387,** 203–209
26. Couture, R., and Girolami, J. P. (2004) *Eur. J. Pharmacol.* **500,** 467–485
27. Reverter, D., Maskos, K., Tan, F., Skidgel, R. A., and Bode, W. (2004) *J. Mol. Biol.* **338,** 257–269
28. Tan, F., Balsitis, S., Black, J. K., Blöchl, A., Mao, J. F., Becker, R. P., Schacht, D., and Skidgel, R. A. (2003) *Biochem. J.* **370,** 567–578
29. Qian, Y., Varlamov, O., and Fricker, L. D. (1999) *J. Biol. Chem.* **274,** 11582–11586
30. McGwire, G. B., Tan, F., Michel, B., Rehli, M., and Skidgel, R. A. (1997) *Life Sci.* **60,** 715–724
31. Tan, F., Deddish, P. A., and Skidgel, R. A. (1995) *Methods Enzymol.* **248,** 663–675
32. Tan, F., Chan, S. J., Steiner, D. F., Schilling, J. W., and Skidgel, R. A. (1989) *J. Biol. Chem.* **264,** 13165–13170
33. Tan, F., Rehli, M., Krause, S. W., and Skidgel, R. A. (1997) *Biochem. J.* **327,** 81–87
34. Deddish, P. A., Skidgel, R. A., and Erdös, E. G. (1989) *Biochem. J.* **261,** 289–291
35. Skidgel, R. A. (1995) *Methods Enzymol.* **248,** 653–663
36. Skidgel, R. A., Johnson, A. R., and Erdös, E. G. (1984) *Biochem. Pharmacol.* **33,** 3471–3478
37. Hoffmann, C., Gaietta, G., Bünemann, M., Adams, S. R., Oberdorff-Maass, S., Behr, B., Vilardaga, J. P., Tsien, R. Y., Ellisman, M. H., and Lohse, M. J. (2005) *Nat. Methods* **2,** 171–176
38. Sangsree, S., Brovkovych, V., Minshall, R. D., and Skidgel, R. A. (2003) *Am. J. Physiol. Heart Circ. Physiol.* **284,** H1959–H1968
39. Brovkovych, V., Stolarczyk, E., Oman, J., Tomboulian, P., and Malinski, T. (1999) *J. Pharm. Biomed. Anal.* **19,** 135–143

40. Paria, B. C., Vogel, S. M., Ahmmed, G. U., Alamgir, S., Shroff, J., Malik, A. B., and Tiruppathi, C. (2004) *Am. J. Physiol. Lung Cell. Mol. Physiol.* **287,** L1303–L1313
41. Tiruppathi, C., Malik, A. B., Del Vecchio, P. J., Keese, C. R., and Giaever, I. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89,** 7919–7923
42. Gomis-Rüth, F. X. (2008) *Crit. Rev. Biochem. Mol. Biol.* **43,** 319–345
43. Fricker, L. D., Plummer, T. H., Jr., and Snyder, S. H. (1983) *Biochem. Biophys. Res. Commun.* **111,** 994–1000
44. Plummer, T. H., Jr., and Ryan, T. J. (1981) *Biochem. Biophys. Res. Commun.* **98,** 448–454
45. Aloy, P., Companys, V., Vendrell, J., Aviles, F. X., Fricker, L. D., Coll, M., and Gomis-Rüth, F. X. (2001) *J. Biol. Chem.* **276,** 16177–16184
46. Eng, F. J., Novikova, E. G., Kuroki, K., Ganem, D., and Fricker, L. D. (1998) *J. Biol. Chem.* **273,** 8382–8388
47. Keil, C., Maskos, K., Than, M., Hoopes, J. T., Huber, R., Tan, F., Deddish, P. A., Erdös, E. G., Skidgel, R. A., and Bode, W. (2007) *J. Mol. Biol.* **366,** 504–516
48. Nakanishi, J., Takarada, T., Yunoki, S., Kikuchi, Y., and Maeda, M. (2006) *Biochem. Biophys. Res. Commun.* **343,** 1191–1196
49. Hadkar, V., Sangsree, S., Vogel, S. M., Brovkovych, V., and Skidgel, R. A. (2004) *Am. J. Physiol. Lung Cell. Mol. Physiol.* **287,** L35–L45
50. Schremmer-Danninger, E., Offner, A., Siebeck, M., and Roscher, A. A. (1998) *Biochem. Biophys. Res. Commun.* **243,** 246–252
51. Sittipunt, C., Steinberg, K. P., Ruzinski, J. T., Myles, C., Zhu, S., Goodman, R. B., Hudson, L. D., Matalon, S., and Martin, T. R. (2001) *Am. J. Respir. Crit. Care Med.* **163,** 503–510
52. Pacher, P., Beckman, J. S., and Liaudet, L. (2007) *Physiol. Rev.* **87,** 315–424
53. Alberts, B. (1998) *Cell* **92,** 291–294
54. Scott, J. D., and Pawson, T. (2009) *Science* **326,** 1220–1224
55. Erdös, E. G., Tan, F., and Skidgel, R. A. (2010) *Hypertension* **55,** 214–220
56. Marcic, B., Deddish, P. A., Skidgel, R. A., Erdös, E. G., Minshall, R. D., and Tan, F. (2000) *J. Biol. Chem.* **275,** 16110–16118
57. Chen, Z., Deddish, P. A., Minshall, R. D., Becker, R. P., Erdös, E. G., and Tan, F. (2006) *FASEB J.* **20,** 2261–2270
58. Skidgel, R. A., and Erdös, E. G. (2007) *Int. Immunopharmacol.* **7,** 1888–1899
59. Ha, S. N., Hey, P. J., Ransom, R. W., Bock, M. G., Su, D. S., Murphy, K. L., Chang, R., Chen, T. B., Pettibone, D., and Hess, J. F. (2006) *Biochemistry* **45,** 14355–14361
60. Nagae, A., Abe, M., Becker, R. P., Deddish, P. A., Skidgel, R. A., and Erdös, E. G. (1993) *Am. J. Respir. Cell Mol. Biol.* **9,** 221–229
61. Nagae, A., Deddish, P. A., Becker, R. P., Anderson, C. H., Abe, M., Tan, F., Skidgel, R. A., and Erdös, E. G. (1992) *J. Neurochem.* **59,** 2201–2212
62. Deiteren, K., Hendriks, D., Scharpé, S., and Lambeir, A. M. (2009) *Clin. Chim. Acta* **399,** 24–39
63. Bhoola, K. D., Figueroa, C. D., and Worthy, K. (1992) *Pharmacol. Rev.* **44,** 1–80
64. Ignjatovic, T., Stanisavljevic, S., Brovkovych, V., Skidgel, R. A., and Erdös, E. G. (2004) *Mol. Pharmacol.* **66,** 1310–1316