Kinins are potent proinflammatory peptides that are produced extracellularly and are rapidly degraded by extracellular peptidases and by intracellular peptidases accessed by kinins via receptor-mediated endocytosis. In this study, we developed model cell systems expressing the kinin B2 receptor (B2R) and the metalloendopeptidase thimet oligopeptidase (EC 3.4.24.15; EP24.15) either individually or together to address 1) the cellular and functional relationship between these proteins and 2) the participation of EP24.15 in the metabolism of bradykinin (BK) after BK internalization via B2R. B2R was localized almost exclusively in the plasma membrane, whereas EP24.15 was localized both intracellularly and on the cell surface and secreted in the media. Intracellular EP24.15 was present throughout the cell, both cytosolic and particulate, with less nuclear localization and no colocalization with either the endoplasmic reticulum marker calnexin or the Golgi marker GM130. No direct colocalization of B2R and EP24.15 was observed using immunofluorescence microscopy. However, the two proteins coimmunoprecipitated specifically, and EP24.15 attenuated maximal B2R responsiveness without influencing the potency of BK to stimulate phosphoinositide hydrolysis and intracellular Ca\textsuperscript{2+} mobilization. Cell surface-bound BK remained intact in cells overexpressing EP24.15 but was degraded intracellularly in an EP24.15-dependent manner upon B2R-mediated endocytosis. These results show that EP24.15 acts to negatively regulate B2R responsiveness, and it serves as an intracellular peptidase in the degradation of BK specifically internalized via this receptor.
iological roles of several of these peptidases as kininases have not yet been fully explored.

The metalloendopeptidase thimet oligopeptidase (EC 3.4.24.15; EP24.15) is a 78-kDa neuropeptide-metabolizing peptidase that is ubiquitously expressed primarily in the brain, gonads, and the pituitary gland (Orlowski et al., 1989; Shrimpton et al., 2002). In addition to kinins, this enzyme acts on many other substrates such as dynorphin A₁₋₈, amyloid precursor protein, gonadotropin-releasing hormone, and angiotensins I and II and has been implicated in several events including reproduction, cardiovascular homeostasis, and nociception (Shrimpton et al., 2002).

EP24.15 is a soluble enzyme that lacks any hydrophobic clusters of amino acids or a signal peptide and resides primarily in the cytosol (Shrimpton et al., 2002), but it may also be extracellularly associated with the plasma membrane (Crack et al., 1999; Jeske et al., 2004) and secreted into the media, both constitutively (Ferro et al., 1999; Oliveira et al., 2005) and after stimulation by, e.g., calcium (Ferro et al., 1999; Carreño et al., 2005). EP24.15 is implicated primarily in the extracellular metabolism of neuropeptides but also intracellularly in the antigen presentation of peptides generated by the proteasome (Portaro et al., 1999; Silva et al., 1999; Kim et al., 2003, Ferro et al., 2004).

EP24.15 acts on BK at the Ser⁵-Phe⁶ bond to produce BK(1–5) and BK(6–9). BK(1–5) has been detected extracellularly after incubation of BK with intact tracheal epithelial cells (Dendorfer et al., 1997), primary guinea pig alveolar and peritoneum macrophages (Vietinghoff and Paegelow, 2000), and primary rabbit vascular smooth muscle cells (Bengtson et al., 2006). Furthermore, EP24.15 inhibitors amplify BK-stimulated tracheal (Da Silva et al., 1992) and uterine contractions (Schriefer and Molineaux, 1993), cerebral microvascular permeability (Norman et al., 2003), and hypotension (Smith et al., 2000). Because current EP24.15 inhibitors do not effectively discriminate between EP24.15 and the highly related metalloendopeptidase neurolysin (EC 3.4.24.16; EP24.16), which produces the degradative pathways after B₂R-mediated endocytosis in the system in which B₂R and EP24.15 were expressed independently and together. Our results show that EP24.15 partially associates and negatively regulates B₂R responsiveness. Furthermore, BK rapidly accesses intracellular EP24.15 upon B₂R-mediated endocytosis, indicating that this enzyme is a candidate peptidase in the intracellular degradation of BK.

Materials and Methods

Cell Culture, Transfections, and Constructs. HEK293 cells (American Type Culture Collection, Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 10% CO₂ at 37°C. Transient transfections were done with the calcium phosphate precipitate method. Clonal stable cell lines were generated by choosing single colonies and propagating them in the presence of selection marker-containing media.

The human B₂R and B₁R cDNAs were subcloned into a pcDNA3.1 vector containing a hygromycin or zeocin selection marker. An artificial signal sequence and the FLAG (FLAG-B₁ or FLAG-B₂) or HA (HA-B₁ or HA-B₂) tag were added in series as described previously (Whistler et al., 2002; Enquist et al., 2007). The human EP24.15 cDNA in a pcMXL5 vector (Origene) was subcloned into the pcDNA3.1 vector with a FLAG sequence at the N terminus using the following protocol. The FLAG-B₁ pcDNA3.1 vector was first digested with BamH1 and HindIII to remove the signal and FLAG sequences from this vector. An oligonucleotide consisting of the Met-FLAG sequence flanked by the HindIII and BamH1 restriction sites (5’-agatgagactaaagaggtatgagcggc-3’) was then ligated into the digested B₂R pcDNA3.1 vector. The resultant construct was then digested with BamH1 and HindIII to remove the B₂R sequence. The EP24.15 pcMXL5 vector was restriction digested with BamH1 and Xho, and the EP24.15 sequence was ligated into the Met-FLAG-containing pcDNA3.1 vector to make a FLAG-tagged EP24.15 (FLAG-EP). To make HA-tagged EP24.15 (HA-EP), the FLAG-EP pcDNA3.1 vector was digested with Nhel and BamH1 and ligated with an oligonucleotide consisting of the Met-HA sequence flanked by the Nhel and BamH1 restriction sites (5’-agatgagactaaagaggtatgagcggc-3’). The FLAG-tagged CXCR4 receptor (FLAG-CXCR4) in the pcDNA3.1 vector was made by inserting FLAG sequence at the receptor N terminus immediately after the initial methionine using a polymerase chain reaction-ligation-polymerase chain reaction protocol as described previously (Fathy et al., 1998).

Immunoprecipitation and Immunoblotting. Cell lysates were subjected to immunoprecipitation (IP) and/or immunoblotting (IB) essentially as described previously (Kang and Leeb-Lundberg, 2002). In short, cells or membrane fractions were solubilized in lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM sodium phosphate, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and a complete protease inhibitor cocktail (Roche, Indianapolis, IN)] for 30 min at 4°C. The lysate was centrifuged at 13,000 g for 15 min at 4°C. For IP, the lysate was incubated overnight at 4°C with polyclonal antibodies raised against EP24.15 (1:2000) followed by incubation with protein A-Sepharose beads (Sigma-Aldrich, St. Louis, MO) for an additional 2 h at 4°C. Alternatively, the lysate was incubated overnight at 4°C with anti-FLAG-M2 affinity resin (Sigma-Aldrich). The beads were then washed extensively and sequentially in the extraction buffer and in 10 mM Tris-HCl, pH 7.4. Proteins were denatured in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer (6% β-mercaptoethanol) for 30 min at 37°C, fractionated by SDS-PAGE, transferred to a nitrocellulose membrane, and the membrane was blocked for at least 1 h in Tris-buffered saline and 10% nonfat milk. The proteins were stained by incubating with anti-HA.11 antibody (1:1000; Biosite Inc., San Diego,
CA), monoclonal anti-EP24.15 antibody (clone IVD6, 1:1000; Millipore, Billerica, MA), or polyclonal EP24.15 antibody (1:1000) for 1 h. Immunoreactive bands were visualized with a chemiluminescence immunodetection kit using peroxidase-labeled sheep anti-mouse or anti-rabbit antibody according to the procedure described by the supplier (PerkinElmer Life and Analytical Sciences, Waltham, MA).

**Fluorescence Microscopy.** Cells stably expressing HA-EP and FLAG-B2, individually or together, were grown on glass coverslips to 50% confluence and then treated in one of two ways. For cell surface labeling, cells were incubated in media containing 3.5 μg/ml primary mouse anti-FLAG M1 or mouse anti-HA11 monoclonal antibody, together or individually, for 30 min at 37°C to label surface receptors and proteases. Cells were then fixed using 3.7% formaldehyde in phosphate-buffered saline (PBS) and treated with blotto (3% dry milk, 0.1% Triton X-100, 1 mM CaCl₂, and 50 mM Tris-HCl, pH 7.4). For whole-cell labeling, cells were fixed using 3.7% formaldehyde in PBS and permeabilized using blotto. Then, the cells were incubated in blotto containing 3.5 μg/ml primary mouse anti-FLAG M1 or mouse anti-HA11 monoclonal antibody, 16.2 μg/ml rabbit anti-calnexin antibody (Sigma-Aldrich), or 0.5 μg/ml mouse anti-GM130 (BD Biosciences, San Jose, CA) for 1 h at room temperature. In all experiments, cells were then washed with PBS and visualized by incubation with Alexa Fluor 488- or Alexa Fluor 568-labeled anti-mouse IgG2b, anti-mouse IgG1, or anti-rabbit IgG antibody, individually or in combination. Images were collected using a Nikon Eclipse confocal microscope.

**BK Internalization.** Cells stably expressing FLAG-B2, alone or in combination with HA-EP, were first incubated in DMEM-0.1% bovine serum albumin (BSA) for 15 min at 37°C. This buffer was then replaced with an equal volume of fresh DMEM-0.1% BSA, and the cells were incubated with 1 to 3 nM [3H]BK (PerkinElmer Life and Analytical Sciences) for 30 min at 37°C. The cells were then rinsed twice with ice-cold PBS including 0.3% BSA. Surface-bound [3H]BK was stripped by incubating the cells with 50 mM glycine, pH 3.0, for 6 min at 4°C. This acid wash and the washed cells were collected for further fractionation and analysis of BK metabolism.

**Cell Fractionation.** Cells were scrapped off in ice-cold lysis buffer (1 mM Tris-HCl, pH 7.4, 2 mM EDTA) containing 50 μM phenylmethylsulfonyl fluoride. All subsequent steps were performed at 4°C. The cells were homogenized in an Ultra-Turrax homogenizer (7 × 10 s at 24,000 rpm; IKA Werke, Staufen, Germany). The homogenates were then transferred to Eppendorf tubes and centrifuged at 13,000g for 12 min at 4°C to obtain a high-density fraction (HDF) pellet, whereas the supernatant was centrifuged at 100,000g for 80 min to yield a low-density microsomal fraction (LDMF) pellet. For high-performance liquid chromatography (HPLC) analysis of BK metabolism, the pellets were extracted in 1 ml of 25% trichloroacetic acid. These fractions were also used for immunoblotting of EP24.15 and B-2R (see Fig. 2). In the case of B-2R, the cells had been treated with 1 μM BK for 30 min at 37°C.

**Analysis of BK Metabolism.** The HDF, LDMF, and acid wash samples were supplemented with 5 μmol of BK and applied on a C18 SepPak cartridge (Waters, Milford, MA) that had previously been sequentially washed with H₂O, 0.2% H₃PO₄, 50% CH₃CN/0.2% H₃PO₄, and 0.2% H₃PO₄. The SepPak flow-through volume was recycled over the column once (only HDF and LDMF). The cartridge was washed with 2 ml of 0.2% H₃PO₄ and then eluted with 2 ml of 50% CH₃CN/0.2% H₃PO₄. The eluates were lyophilized in a Speed-Vac lyophilizer (Savant, Farmingdale, NY), and the dry samples were resuspended in 250 μl of 12% CH₃CN, 0.2% H₃PO₄. The samples were then chromatographed by reverse-phase HPLC on a C18 column (5 μm; 250 × 4.6 mm) with a linear gradient system, i.e., 100% buffer A (0.1% trifluoroacetic acid) to 100% buffer B (0.1% trifluoroacetic acid in acetonitrile), run for 45 to 55 min with a flow rate of 1 ml/min. Fractions were collected each minute, and the UV absorbance was monitored at 214 nm. Data were analyzed with the software package TotalChrom version 6.2.0.0.1 with LC instrument control (PerkinElmer Life and Analytical Sciences). Fractions (1 ml) were then counted for radioactivity in an LS6000 liquid scintillation counter.

**Biotinylation.** Confluent cells were washed twice with ice-cold PBS and then incubated with 0.3 mg/ml disulfide-cleavable sulfo-NHS-SS-biotin (Pierce, Rockford, IL) in PBS for 30 min at 4°C with gentle agitation. The cells were then washed twice with Tris-buffered saline to quench the biotinylation reaction. Cells were then extracted in 0.1% Triton X-100, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 25 mM KCl, and 1 mM iodoacetamide containing a complete protease inhibitor cocktail, and cell debris was removed by centrifugation at 10,000g for 10 min at 4°C. FLAG-EP was immunoprecipitated in the extraction buffer by incubating with anti-FLAG M2 affinity resin as described above. Proteins were denatured in SDS-PAGE sample buffer without reducing agent followed by SDS-PAGE as described above. Biotinylated proteins were visualized by incubating with the Vectastain avidin-biotinylated enzyme complex immunoperoxidase reagent (Vector Laboratories, Burlingame, CA) followed by development with Western Blot Chemiluminescence Reagent Plus according to the procedure described by the supplier (PerkinElmer Life and Analytical Sciences).

**Cell Media Analysis.** Cells were grown to confluency in six-well dishes and incubated with serum-free DMEM overnight at 37°C. Media samples were taken from each well, mixed with SDS-PAGE sample buffer including 6% β-mercaptoethanol, and denatured for 30 min at 37°C. As positive controls, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 mM NaF, 10 mM sodium phosphate, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and a complete protease inhibitor cocktail) for 30 min at 4°C. The lysate was centrifuged at 13,000g for 15 min at 4°C, and the supernatant was mixed with SDS-PAGE sample buffer including 6% β-mercaptoethanol and denatured for 30 min at 37°C. The proteins were then fractionated and immunoblotted as described above.

**Phosphoinositide Hydrolysis.** Cells were seeded in six-well dishes and first labeled with 1 μCi/ml myo-[3H]inositol (PerkinElmer Life and Analytical Sciences) in DMEM/10% fetal bovine serum for 20 to 24 h at 37°C, washed four times in DMEM, and then further incubated in DMEM for 1 h at 37°C. This was followed by incubation in the absence and presence of various concentrations of BK in DMEM supplemented with 50 mM LiCl for 30 min at 37°C. Cells were then lysed with 0.1 M formic acid for 20 min at 4°C, transferred to Eppendorf tubes, and then centrifuged at 16,100g for 5 min at 4°C. The supernatants were added to anion exchange columns, which were washed twice with a low-salt solution (60 mM ammonium formate and 5 mM sodium borate). Inositol phosphates were then eluted with a high-salt solution (1 M ammonium formate and 0.1 M formic acid) and counted for radioactivity in a Beckman LS6000 liquid scintillation counter.

**Intracellular Ca²⁺ Mobilization.** Cells were seeded in 96-well dishes and then labeled for 60 min with Ca²⁺-fluorophore (Molecular Devices, Sunnyvale, CA). The cells were then incubated in the absence and presence of various concentrations of BK as shown in Fig. 5B. Intracellular Ca²⁺ release was measured immediately after agonist application in a Flex apparatus (Molecular Devices) for 2 min.

**Membrane Preparation and Radioligand Binding.** Membrane preparation and radioligand binding were performed essentially as described previously (Leeb et al., 1997). Cells were resuspended in a buffer containing 25 mM TES, pH 6.8, 0.5 mM EDTA, 0.2 mM MgCl₂, and 1 mM phenanthroline and homogenized using an Ultra-Turrax homogenizer at 20,500 rpm for 10 s. Membranes were isolated by centrifugation at 45,000g for 30 min at 4°C. The pellets were then resuspended in the above buffer supplemented with 0.1% BSA and 0.014% bacitracin (binding buffer). Protein concentration was determined with a Bradford assay. Membranes were diluted 600 times in binding buffer. Binding assays were performed in a total volume of 0.5 ml with varying concentrations of [3H]BK, with or without 1 μM BK. After incubation for 60 min at room temperature, assays were terminated by dilution with 4 ml of ice-cold PBS, 0.3%
BSA, and rapid vacuum filtration on Whatman GF/C filters previously soaked in 1% polyethyleneimine. The trapped membranes were then washed with an additional 2 × 4 ml of ice-cold PBS, 0.3% BSA. The filters were then counted for radioactivity in an LS6000 liquid scintillation counter.

**Statistics.** Data are presented as the means ± S.E.M. Student’s two-tailed t test for unpaired data were used to evaluate statistical significance. P values less than 0.05 were regarded as statistically significant. Data analysis was performed in the Prism program (GraphPad Software Inc., San Diego, CA).

**Results**

**Cellular Expression and Localization of EP24.15 and B₂R.** HEK293 cells express EP24.15 endogenously as determined by both IB and IP using monoclonal and polyclonal EP24.15-specific antibodies (Fig. 1, A and B). Because immunofluorescence microscopy with these antibodies did not detect any endogenous EP24.15, presumably due to low enzyme expression, we constructed cells stably expressing EP24.15 tagged in the N terminus with either the HA epitope (HA-EP) or the M2 FLAG epitope (FLAG-EP). Stable cells specifically expressed HA-EP and FLAG-EP with molecular masses apparently identical to that of endogenously expressed EP24.15 (Fig. 1, C and D). HA-EP was used primarily for protein IB and immunofluorescence microscopy, whereas FLAG-EP was used primarily for IP.

HA-EP was detected both cell-associated (Fig. 2A, lanes 2 and 3) and secreted (Fig. 2A, lanes 5 and 6), consistent with previous reports by other investigators (Ferro et al., 1999; Oliveira et al., 2005). Some cell-associated specific HA-EP species of lower molecular masses (60–70 kDa) were also detected (Fig. 2A, lanes 2 and 3), which were not present in the media (lanes 5 and 6) and may be degradation products. Confocal immunofluorescence microscopy of fixed and permeabilized cells showed that the cell-associated HA-EP was localized throughout the cell with slightly less staining in the nucleus (Fig. 3A, a and d) and without any colocalization with either the endoplasmic reticulum (ER) marker calnexin (Fig. 3A, a–c) or the Golgi marker GM130 (Fig. 3A, d–f). Immunolabeling of live nonpermeabilized cells did not reveal any specific HA-EP staining on the cell surface (data not shown). However, biotinylation of cells stably expressing FLAG-EP with a cell-impermeable birin analog revealed that this enzyme is localized also on the extracellular cell surface (Fig. 4A). A major biotinylated band was identified at approximately 75 kDa, but additional ones at approximately 65 kDa and >150 kDa were also identified. Each of the three biotinylated species corresponded to FLAG-EP-specific forms as determined by IB with both FLAG antibodies (Fig. 4B) and monoclonal EP24.15 antibodies (Fig. 4C). Two additional FLAG-EP-specific bands at approximately 45 kDa (Fig. 4B) corresponded to biotinylated bands that could only be observed after prolonged exposure of the streptavidin-horseradish peroxidase blots. However, IB with monoclonal EP24.15 antibodies did not identify these two bands (Fig. 4C). Specific membrane-associated HA-EP was also identified by IB in both a crude high-density particulate fraction (Fig. 2A, lane 9) and a low-density microsomal fraction of the cells (Fig. 2A, lane 10).

B₂R tagged in the N terminus with the M1 FLAG epitope (FLAG-B₂) was specifically expressed in HEK293 cells as a heterogeneous species centered at 65 to 70 kDa (Fig. 2B, lanes 2 and 3), to which BK bound with high affinity (Table 1). Figure 3A, g and j, shows that this species was localized...
Fig. 3. The relative cellular distribution of EP24.15 and B2R. A, HEK293 cells stably expressing HA-EP + FLAG-B2 were fixed and permeabilized before incubation with primary mouse anti-HA antibody (a), primary rabbit anti-EP24.15 antibody (d), primary mouse anti-FLAG M1 antibody (g and j), primary rabbit anti-calnexin antibody (b and h), and primary mouse anti-GM130 antibody (e and k). Secondary mouse anti-rabbit or rabbit anti-mouse Alexa Fluor 488- or Alexa Fluor 568-labeled antibodies were then added. Individual and merged images were collected using a Nikon Eclipse confocal microscope, 60× objective, 50-μm zoom. The images in a and b, d and e, g and h, and j and k were merged in c, f, i, and l, respectively. B, HEK293 cells stably expressing HA-EP + FLAG-B2 together were preincubated with primary mouse anti-FLAG M1 antibody for 30 min at 37°C followed by further incubation in the absence (−BK) or presence of 1 μM receptor-specific agonist (+BK) for an additional 30 min at 37°C as indicated. Cells were then fixed and permeabilized before incubation with primary mouse anti-HA antibody and secondary Alexa Fluor 488-labeled or Alexa Fluor 568-labeled anti-mouse antibody. Images were collected using a Nikon Eclipse confocal microscope, 60× objective, 50-μm zoom.

Fig. 4. Cell surface localization of EP24.15. A, HEK293 cells (Control) and cells stably expressing FLAG-EP or FLAG-EP + HA-B2 were labeled at 4°C with a biotin reagent as described under Materials and Methods. Cell lysates were then IP with anti-FLAG antibody and IB with streptavidin-horseradish peroxidase. B, cell lysates in A were IB with anti-FLAG antibody. C, cell lysates in A were IB with monoclonal anti-EP24.15. Molecular mass (Mr) standards (left-side arrows) and EP24.15 species (right-side arrows) are indicated, and the results are representative of experiments performed at least three times.

α-adrenergic receptor staining in immunofluorescence images (Fig. 3B). Celluar fractionation of BK-stimulated cells revealed the presence of a specific heterogeneous FLAG-B2 species centered at 65 to 70 kDa as well as some lower molecular mass receptor species in both HDF (Fig. 2B, lane 6) and LDMF (Fig. 2B, lane 7).

EP24.15 and B2R Association. Coexpression of FLAG-B2 with HA-EP did not significantly alter the affinity (Kd) of BK or the number of B2 receptor binding sites (Bmax) expressed either totally (Table 1) or on the cell surface (data not shown), as determined by specific radioligand binding. On the other hand, coexpression with HA-EP yielded a significant decrease (−29.3%, p < 0.001) in the Emax value for FLAG-B2-mediated BK stimulation of phosphoinositide (PI) hydrolysis without a change in the BK pEC50 value (8.31 ± 0.12), compared with cells stably expressing FLAG-B2 alone (8.47 ± 0.19) (Fig. 5A; Table 1). When measuring BK-stimulated intracellular Ca2+ mobilization in cells coexpressing FLAG B2 and HA-EP, we also observed a significant decrease in the Emax value (−20.7%, p < 0.05) without a change in the pEC50 value (8.91 ± 0.40), compared with cells only expressing FLAG-B2 (8.93 ± 0.33) (Fig. 5B). On the other hand, no significant change was observed in the time course of BK-stimulated Cu2+ mobilization with and without HA-EP. Considering that the levels of both total cellular and cell surface FLAG-B2 were not significantly different in these cells, these results show that EP24.15 lowers the maximal responsiveness of B2R without influencing the potency by which BK stimulates the receptor.
Table 1

<table>
<thead>
<tr>
<th>Receptor</th>
<th>$K_D$</th>
<th>$B_{max}$</th>
<th>pEC$_{50}$</th>
<th>$E_{max}$</th>
</tr>
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<tbody>
<tr>
<td>FLAG-B2</td>
<td>1.39 ± 0.08$^a$</td>
<td>2.13 ± 0.41$^a$</td>
<td>8.47 ± 0.19$^a$</td>
<td>14,845 ± 830$^a$</td>
</tr>
<tr>
<td>FLAG-B2/HA-EP</td>
<td>1.42 ± 0.24</td>
<td>1.58 ± 0.18</td>
<td>8.31 ± 0.12</td>
<td>10,497 ± 830$^a$</td>
</tr>
</tbody>
</table>

$^a$ Values were obtained by nonlinear regression analysis (Prism) of equilibrium ligand binding isotherms on particulate preparations using [3H]BK as radioligand.

$^b$ Values were obtained by nonlinear regression analysis (Prism) of dose-response curves on PI hydrolysis in intact cells as shown in Fig. 7.

$^{***}$, $P < 0.001$ for FLAG-B2/HA-EP vs. FLAG-B2.

Discussion

In this study, we developed a model cell systems that expressed B$_2$R and the metalloendopeptidase EP24.15 either individually or in combination to address 1) the cellular and functional relationship between these proteins and 2) the participation of EP24.15 in the metabolism of BK after BK internalization via B$_2$R. Our results show that EP24.15 negatively regulates B$_2$R by 1) associating with B$_2$R and attenuating receptor responsiveness and 2) acting as an intracellular peptidase to degrade BK internalized via B$_2$R.

The low abundance of the endogenously expressed EP24.15 in HEK293 cells prevented us from monitoring the distribution of this form in these cells. On the other hand, epitope-tagged EP24.15 was clearly visible, and it was found to be widely distributed in the cells with cytosolic, particulate, and secreted forms of the enzyme, which is consistent with previous observations of endogenous EP24.15 in other cells with higher endogenous enzyme expression (Crack et al., 1999; Ferro et al., 1999; Jeske et al., 2004; Oliveira et al., 2005).

The particulate form of EP24.15 was localized both on the extracellular face of the plasma membrane and in endocytic vesicles, whereas no colocalization with either the ER marker calnexin or the Golgi marker GM130 was observed. B$_2$R was almost exclusively expressed in the plasma membrane with a
small fraction of presumably maturing receptors that were also detected in the Golgi.

An association of B2R and EP24.15 was clearly indicated by their highly specific coIP, which seems to be physiologically relevant because it occurred with both endogenous and recombinant epitope-tagged EP24.15. Overexpression of EP24.15 also led to an increase in higher molecular mass B2R species detected on IB, providing additional evidence for an association between these proteins. In addition, overexpression of EP24.15 yielded a decrease in B2R re-

Fig. 6. Association of EP24.15 and B2R. A, cell lysates of HEK293 cells and cells stably expressing FLAG-B1 and FLAG-B2 and transiently expressing FLAG-CXCR4 were IP with anti-FLAG antibody and then IB with anti-FLAG antibody or polyclonal EP24.15 antibody. B, cell lysates of HEK293 cells and cells stably expressing HA-B2 were IP with polyclonal anti-EP24.15 antibody and then IB with anti-HA antibody. C, cell lysates of HEK293 cells and cells stably expressing FLAG-EP or FLAG-EP + HA-B2 were IP with anti-FLAG antibody and then IB with anti-HA antibody. Molecular mass (M_r) standards (left-side arrows) are indicated, and the results are representative of experiments performed at least three times.

Fig. 7. EP24.15-dependent degradation of internalized BK. HEK293 cells stably expressing FLAG-B2 or FLAG-B2 + HA-EP were incubated with [3H]BK at 37°C for 30 min and then washed with low pH buffer at 4°C and fractionated. The radioactivity in the low pH buffer (A and B), LDMF (C and D), and HDF (E and F) was then extracted and subjected to reverse-phase HPLC on a C18 column as described under Materials and Methods. The results are presented as percentage of total (% of Total), where total represents the radioactivity applied to the C18 column and varied between $2 \times 10^5$ and $20 \times 10^5$ dpm. Each experiment included standard BK and BK(1–5). The result is representative of three experiments.
sponsiveness both on PI hydrolysis and intracellular Ca\textsuperscript{2+} mobilization. These results suggest that the interaction between B\textsubscript{2R} and EP24.15 causes an inhibition of B\textsubscript{2R} function. However, until such a complex has been isolated, the possibility remains that EP24.15 interferes with B\textsubscript{2R}-mediated BK signaling downstream of the receptor. The fact that the lowered maximal B\textsubscript{2R} response was not caused by decreased BK availability, e.g., by increased degradation of free unbound BK, was shown by the unaltered BK potency on B\textsubscript{2R}-mediated stimulation of PI hydrolysis and intracellular Ca\textsuperscript{2+} mobilization.

Cell surface-associated EP24.15 was not detected by immunofluorescence microscopy but was clearly indicated by biotinylation, which suggests either that the cell surface fraction of EP24.15 is relatively small or that optimal antigen access requires detergent treatment. B\textsubscript{2R} was localized almost exclusively in the plasma membrane, suggesting that that is where the receptor interacts with EP24.15. Attempts to coIP B\textsubscript{2R} with biotinylated EP24.15 also failed, which again may be due to the limited size of the cell surface EP24.15 pool in these cells.

The plasma membrane form of EP24.15 has been reported to be localized in flotillin-containing membrane lipid rafts in the A7r5 mouse pituitary cell line and the G71-7 hypothalamic neuronal cell line as determined by immunofluorescence microscopy and cell fractionation, and it was proposed that it is from here that the constitutively secreted form is released (Jeske et al., 2003). Furthermore, this enzyme also cofractionated with B\textsubscript{2R} in cultured trigeminal neurons (Jeske et al., 2006), which is consistent with previous reports of the presence of both B\textsubscript{2R} and receptor-bound BK in lipid rafts in some cells (de Weerd and Leeb-Lundberg, 1997; Haasemann et al., 1998; Lamb et al., 2002). Pretreatment with methyl-\beta-cyclodextrin to chelate cholesterol and disrupt lipid rafts led to the loss of EP24.15 from the plasma membrane (Jeske et al., 2006) and release into the media (Jeske et al., 2003), whereas B\textsubscript{2R} remained associated with the membrane (Jeske et al., 2006). In HEK293 cells, B\textsubscript{2R} and EP24.15 remained associated despite pretreatment of cells with methyl-\beta-cyclodextrin (data not shown), suggesting that their association does not depend on lipid rafts in these cells. One study identified a direct interaction between these two proteins involving the B\textsubscript{2R} C-terminal tail by yeast-two-hybrid screening and subsequently by coIP (Shivakumar et al., 2005). An apparent interaction occurred both in the plasma membrane and in endosomal compartments. Nevertheless, several questions remain about the molecular details and cellular localization of the B\textsubscript{2R}-EP24.15 interaction.

Peptide ligands for GPCR generally form very high affinity complexes with their respective receptors, causing them to dissociate extremely slowly from the receptors. Indeed, BK binding to the high-affinity, GTP-sensitive B\textsubscript{2R} in myometrial plasma membranes involves the sequential formation of at least three binding states with decreasing dissociation rates, with the most slowly dissociating state having an equilibrium dissociation constant (K\textsubscript{D}) of 8 pM and a dissociation rate constant (k\textsubscript{-1}) of 4.4 \times 10\textsuperscript{-5} s\textsuperscript{-1} (Leeb-Lundberg and Mathis, 1990). In primary myometrial cells, such binding is indicated by the significant amount of slowly reversible acid-labile, i.e., cell surface, BK binding to B\textsubscript{2R} that forms at 37°C (Tropea et al., 1992). After washing of HEK293 cells stably expressing B\textsubscript{2R} and EP24.15, which removes extracellular but not plasma membrane-bound EP24.15, acid-labile BK binding to B\textsubscript{2R} remains as intact BK. This result suggests that cell surface-bound EP24.15 does not access cell surface receptor-bound BK. On the other hand, the limited amount of BK that dissociates from the receptor before receptor-mediated internalization may still be susceptible to degradation.

Due to this slowly reversible BK binding, significant ligand dissociation is likely to be preceded by receptor-mediated BK internalization, which is indicated by the acid-resistant BK binding that forms at 37°C (Munoz and Leeb-Lundberg, 1992; Tropea et al., 1992). Thus, receptor-bound BK may not access peptidases until after internalization. Rapid intracellular degradation of internalized BK may be absolutely necessary for efficient receptor regulation because B\textsubscript{2R} rapidly recycles and resensitizes in most cells (Leeb-Lundberg et al., 2005). Indeed, in DDT1 MF-2 smooth muscle cells, BK rapidly enters two intracellular degradative pathways after B\textsubscript{2R}-mediated endocytosis, which both in part yield BK(1–5) (Munoz and Leeb-Lundberg, 1992).

Expression of EP24.15 in HEK293 cells clearly resulted in degradation of internalized BK. In LDMF, introduction of EP24.15 led to a very specific cleavage of BK to BK(1–5). Because the cleavage of internalized BK would require the presence of EP24.15 on the luminal side of endocytic vesicles, this activity is probably not derived from cytosolic EP24.15 but rather from EP24.15 originally located on the extracellular side of the plasma membrane and subsequently internalized into endosomal compartments. However, to determine precisely where in the BK-receptor endocytic process that EP24.15-catalyzed BK cleavage occurs, further investigation is required. In HDF, which is considerably more heterogeneous than LDMF and likely to contain many intracellular structures, introduction of EP24.15 yielded a heterogeneous mixture of smaller BK products that only partially overlapped with the BK(1–5) standard. Thus, EP24.15 also seems to trigger and participate in cascades of peptidase actions in some compartments. Nevertheless, these results show that EP24.15 is capable of accessing internalized BK and contribute to the degradation of this peptide agonist.

In all, our results show that EP24.15 negatively regulates B\textsubscript{2R} responsiveness and acts as an intracellular peptidase to degrade BK specifically internalized via B\textsubscript{2R}. Receptor-peptidase interactions may be of general importance because peptide agonists dissociate slowly and receptor-mediated peptide endocytosis is a common process. Furthermore, localized degradation of agonist may be critical for subsequent postendocytic receptor sorting. It is noteworthy that evidence has recently been provided that B\textsubscript{2R} is capable of directly interacting also with some other peptidases that process BK such as ACE, and that this may be important for some actions of ACE inhibitors (Chen et al., 2006). Finally, our approach circumvents the use of cell-impermeable pharmacological inhibitors to discriminate between peptidases, particularly between highly homologous ones such as EP24.15 and EP24.16.

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References


