Collagenase-2 and -3 Mediate Epidermal Growth Factor Receptor Transactivation by Bradykinin B₂ Receptor in Kidney Cells

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ABSTRACT

We have previously shown that stimulation of extracellular signal-regulated protein kinase (ERK) by bradykinin (BK) in murine inner medullary collecting duct (mIMCD)-3 cells is mediated by epidermal growth factor receptor (EGFR) transactivation. The mechanism of EGFR transactivation seemed to be novel, because it does not require phospholipase C, Ca²⁺, and calmodulin, protein kinase C, Go₁, subunits, or EGFR-B₂ receptor heterodimerization. In this study, we demonstrated the involvement of matrix metalloproteinases (MMPs) in B₂ receptor-induced EGFR transactivation using their broad-spectrum inhibitors batimastat and N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (Galardin); GM-6001 inactive form, N-[(2R)-2-(carboxymethyl)-4-methylpentanoyl]-L-tryptophan methylamide (Galardin); GM-6001 inactive form, N-t-butoxycarbonyl-L-leucyl-L-tryptophan methylamide; AB, antibody; HSPG, heparan sulfate proteoglycan; PD98059, 2-amino-3-methoxyflavone; AG-1478, 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG-1478) and thus dependent on EGFR tyrosine kinase activity. Inhibition of both collagenases resulted in ~65% decrease of BK-induced ERK activation, supporting roles for both enzymes. Stimulation of mIMCD-3 cells with 10 nM BK increased the activity of collagenases in concentrated culture media within 10 min. Moreover, recombinant MMP-13 and MMP-8, when applied to mIMCD-3 cells for 10 min without BK, stimulated tyrosine phosphorylation of EGFR and caused ~250% increase over basal ERK phosphorylation comparable with BK-induced ERK activation. Collagenases-induced ERK activation was inhibited by 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG-1478) and thus dependent on EGFR tyrosine kinase activity. This study demonstrates a novel role for collagenase-2 and -3 in signaling of the G₄-coupled BK B₂ receptor in mIMCD-3 cells.

The bradykinin (BK) B₂ receptor is a prototypical G protein-coupled receptor (GPCR) that has been shown to activate extracellular signal-regulated protein kinase (ERK)1/2 in vascular smooth muscle cells (Velarde et al., 1999), endothelial cells (Bernier et al., 2000), PC-12 cells (Dikie et al., 1996), and various tumor cell lines (Drube and Liebmann, 2000; Graness et al., 2000). Little is presently known about the role of the vasoactive nonapeptide BK in ERK1/2 signaling in kidney cells. Activation of ERK1/2 by BK in kidney cells has been demonstrated for cultured mesangial cells, although its mechanism remains unclear (Jaffa et al., 1997;...
El-Dah et al., 1998). As for renal tubular cells, it has been shown that BK activates ERK1/2 in rabbit cortical collecting duct cells via a protein kinase C-dependent mechanism (Lal et al., 1998).

In our previous work, we provided evidence that BK stimulates early mitogenic signals associated with activation of ERK1/2 in renal epithelia cell line derived from the inner medullary collecting duct of mice (mIMCD-3 cell line) (Mukhin et al., 2003). We showed that BK stimulated proliferation of mIMCD-3 cells that was dependent on activation of epidermal growth factor receptor (EGFR) tyrosine kinase and ERK1/2. Our results suggested that the bradykinin B2 receptor stimulates Ras-dependent activation of ERK1/2 in mIMCD-3 cells via transactivation of the EGFR.

The mechanisms underlying GPCR-induced EGFR transactivation have not been clearly defined. One transactivation pathway recently proposed for the β2-adrenergic receptor includes the isoproterenol-induced formation of a β2-adrenergic receptor-EGFR complex (Maudsley et al., 2000). Multiple G protein-coupled receptors transactivate the EGFR through the intermediary actions of the calcium-dependent nonreceptor tyrosine kinase PYK-2 and of Src (a nonreceptor tyrosine kinase) kinase in various native and transfected cell types (Grosset al., 2000; Keely et al., 2000). The thymoxane A2 receptor transactivates the EGFR through Gαq, phospholipase C, and protein kinase C-mediated phosphorylation of the thymoxane A2 receptor, which results in coupling to Gq and Src-dependent activation of the EGFR (Gao et al., 2001). The angiotensin II AT1A receptor transactivates the EGFR through activation of matrix metalloproteinases and release of tethered heparin-bound EGF (HB-EGF) in mesangial, vascular smooth muscle cells, and C9 hepatocytes (Eguchi et al., 2001; Uchiyama-Tanaka et al., 2001; Shah et al., 2004). The M1 muscarinic and thrombin receptors use a similar pathway in Rat-1 cells and in rat smooth muscle cells, respectively (Prenzel et al., 1999; Kalnes et al., 2000). In contrast, carbachol-induced transactivation of the EGFR in colonic epithelial cells involves the matrix metalloproteinase (MMP)-dependent release of transforming growth factor (TGF)-α (McCole et al., 2002). The gelatinases MMP-2 and MMP-9 have been implicated in transactivation of the EGFR by the Gα13-coupled gonadotropin-releasing hormone receptor in gonadotropic cells (Roelle et al., 2003).

Bradykinin B2 receptor-induced activation of the EGFR has been described in COS-7 cells transiently transfected with bradykinin B2 receptors (Adomeit et al., 1999). In contrast, BK-induced activation of ERK1/2 in A431 epidermoid carcinoma cells is independent of the EGFR (Graness et al., 2000).

In our previous work, we suggested that the mechanism of B2 receptor-induced transactivation of EGFR in mIMCD-3 cells is different from the mechanisms described in the literature, for it does not involve complex formation between the B2 receptor and EGFR, and it does not require pertussis toxin-sensitive G proteins, Ca2+, calmodulin, phospholipase C, or protein kinase C activity (Mukhin et al., 2003). In the current study, we test the hypothesis that matrix metalloproteinases are involved in this signaling pathway.

MMPs, a large family of zinc-dependent, matrix-degrading enzymes, are thought to play a central role in degradation of extracellular matrix (ECM) (Nagase and Woessner, 1999). In the kidney, the accumulation of ECM molecules can lead to interstitial fibrosis and glomerulosclerosis (He et al., 1995; Norman and Lewis, 1996). MMPs are also involved in normal kidney development and potentially play roles in diabetic nephropathy and in inflammatory glomerulonephritis (Lenz et al., 2000). Despite the emerging roles of MMPs in the kidney, little is presently known about possible interactions between BK receptors and MMPs. The effect of BK on the expression of MMP-3, MMP-20, and membrane type-1-MMP has been described for cultured granulosa cells (Kimura et al., 2001). In vivo studies on the B2 receptor knockout mice suggest that the B2 receptor might play a protective role in renal tubulointerstitial fibrosis (Schanstra et al., 2002), but a possible connection between the B2 receptor and MMPs in kidney cells remains unclear. Herein, we demonstrate novel roles for collagenase-2 and -3 (MMP-8 and MMP-13, respectively) in Gq-coupled B2 receptor signaling in mIMCD-3 cells.

Materials and Methods

Materials. Cell culture supplies were obtained from Invitrogen (Grand Island, NY), or Corning Life Sciences (Cambridge, MA). Bradykinin and anti-β-actin antibody were from Sigma-Aldrich (St. Louis, MO). Phospho-ERK and phospho-MEK kits were obtained from Cell Signaling Technology Inc. (Beverly, MA). The Ras activation kit, EGFR polyclonal antibody, and anti-phospho-EGFR (Tyr1173) monoclonal antibody were from Upstate Biotechnology (Lake Placid, NY). MMP inhibitors and neutralizing antibody against TGF-α were from Calbiochem (San Diego, CA). Purified MMP-8 from human neutrophils, recombinant MMP-13, MMP-13 neutralizing antibodies, and type 1 collagenase activity assay kit were from Chemicon International (Temecula, CA). MMP-8 and MMP-13 antibodies, MMP-8 siRNA, MMP-13 siRNA, and control siRNA were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Neutralizing antibody against HB-EGF was from R&D Systems (Minneapolis, MN). The Mouse MMP MultiGene-12 RT-PCR Profiling kit was from SuperArray Bioscience Corporation (Frederick, MD).

Cell Culture. mIMCD-3 cells were obtained from American Type Culture Collection (Manassas, VA). mIMCD-3 cells were grown in equal mixtures of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Transfections of mIMCD-3 Cells: MMP-8 and MMP-13 Silencing. Transfections of mIMCD-3 cells were achieved by nucleofection with an Amaz maximize Biosystems instrument (Giesen, Germany) to transfer DNA or RNA directly into the nucleus of the cell. Cells (1 × 106) were resuspended in 100 µl of Nucleofector Solution R (Amaz maximize Biosystems), and nucleofected with either 100 nM MMP-8 siRNA or MMP-13 siRNA or control siRNA, or with combination of MMP-8 and MMP-13 siRNAs (Santa Cruz Biotechnology, Inc.) using manufacturer’s protocol T-16. Forty-eight hours postnucleofection, cells were stimulated with BK or vehicle, lysed, and analyzed for MMP-8 expression by Western blotting with an anti-MMP-8 goat polyclonal antibody, for MMP-13 expression by Western blotting with an anti-MMP-13 rabbit polyclonal antibody, and for ERK activation. Blots were reprobed with a mouse monoclonal anti-β-actin antibody to control for protein loading and for silencing specificity.

ERK Assay. ERK phosphorylation was assessed by immunoblot using phosphorylation state-specific antibodies as described previously (Mukhin et al., 2003) in mIMCD-3 cells treated for various times with varying concentrations of BK, EGF, MMPs, or vehicle.

Ras Assay. Ras activation was assessed by a nonradioactive Ras assay kit (Upstate Biotechnology) as described previously (Mukhin et al., 2003). Quiescent mIMCD-3 cell monolayers were pretreated with inhibitors or vehicle for 30 min, stimulated with 10 nM BK or
vehicle for 5 min, and lysed in a 1 ml/100-mm dish of Mg\textsuperscript{2+} lysis buffer (150 mM NaCl, 25 mM HEPES, pH 7.5, 1 mM EDTA, 10 mM MgCl\textsubscript{2}, 1% Igepal CA-630, 25 mM sodium fluoride, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 10 μg/ml each of aprotinin and leupeptin, and 10% glycerol). Cell lysates were precleared by incubating with glutathione-agarose for 10 min at 4°C. Precleared lysates (1 μg/μl total cell protein) were incubated with 10 μg of Raf-1 Ras binding domain (glutathione S-transferase fusion protein, corresponding to the Ras binding domain of Raf-1) and bound to glutathione-agarose for 30 min at 4°C. The agarose beads were collected by centrifugation, washed three times with Mg\textsuperscript{2+} lysis buffer, resuspended in 2× Laemmli sample buffer, boiled for 5 min, and subjected to SDS-polyacrylamide gel electrophoresis and subsequent immunoblot analysis with monoclonal anti-Ras IgG.

**EGF Receptor Phosphorylation Assay.** The phosphorylation state of EGFR was assessed by immunoprecipitation/Western blotting studies as described previously (Mukhin et al., 2003). Quiescent mIMCD-3 cells, grown in 100-mm dishes, were pretreated with vehicle or inhibitors for 30 min. Cells were subsequently treated with 10 nM BK, 1 ng/ml EGF, or vehicle for 5 min and lysed in radiimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium fluoride, 1 mM Na\textsubscript{3}VO\textsubscript{4}, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin). Cell lysates were precleared by incubating with protein A-agarose bead slurries, with incubation for 2 h more at 4°C. The immunocomplexes were captured by the addition of protein A-agarose bead slurries for 30 min at 4°C. Precleared lysates (1 μg/μl total cell protein) were incubated with 4 μg of anti-EGFR polyclonal IgG (Upstate Biotechnology) overnight at 4°C. The immunocomplexes were captured by incubating with glutathione-agarose for 10 min at 4°C. Preincubated glutathione-agarose was washed four times with Hanks’ balanced salt solution on an autosampler device, followed by boiling for 5 min. The affinity-purified immunocomplexes were transferred to a biotin-binding 96-well plate and detected with streptavidin-enzyme complex. The addition of enzyme substrate resulted in a colored product, which was detected by its absorbance at 492 nm.

**Results**

**MMPs Are Involved in Bradykinin-Induced ERK1/2 Activation and EGFR Transactivation in mIMCD-3 Cells.** In the first set of experiments, we tested the involvement of MMPs in B\textsubscript{2} receptor-induced EGFR transactivation and ERK1/2 phosphorylation using synthetic hydroxamate-based broad-spectrum MMP inhibitors batimastat and GM-6001. Pretreatment of mIMCD-3 cells for 30 min with 5 μM batimastat or 5 μM GM-6001 completely blocked BK-induced activation of ERK without affecting EGF-induced ERK activation (Fig. 2). The negative control for GM-6001 was without effect. Columns in Fig. 2 are presented as mean ± S.E.M. and were analyzed for repeated measures by Student’s t test for unpaired two-tailed analysis. Differences were considered significant at P < 0.05.

**BK-Induced Activation of Ras in mIMCD-3 Cells Is MMPs-Dependent.** Because we demonstrated previously that BK-induced ERK activation is Ras-dependent (Mukhin et al., 2003), we studied the effects of MMP inhibitors on BK-induced activation of Ras. Quiescent mIMCD-3 cells monolayers were pretreated with MMP inhibitors or vehicle for 30 min and stimulated with 10 nM BK or 1 ng/ml EGF for 5 min. GM-6001 abolished BK-induced, but not EGF-induced, phosphorylation of EGFR (n = 4), whereas a negative control for GM-6001 was without effect. Pretreatment with batimastat produced the same effect on BK-induced phosphorylation of EGFR (not shown). The inset in Fig. 1B shows a representative immunoblot.
MMP Inhibitors Do Not Affect Ca\textsuperscript{2+} Signaling in mIMCD3 Cells. We have shown previously that BK increases intracellular Ca\textsuperscript{2+} release in mIMCD-3 cells (Mukhin et al., 2001). In the next set of experiments, we tested whether MMP inhibitors are able to influence this effect. We used a FLIPR (Molecular Devices) fluorometric imaging plate reader system to measure intracellular Ca\textsuperscript{2+} in mIMCD-3 cells. GM-6001 (5 μM), 5 μM GM-1489 (another broad-spectrum MMP inhibitor), and 10 μM MMP inhibitor III (selective for MMP-1, -2, -3, -7, and -13) had no effect on BK-induced intracellular Ca\textsuperscript{2+} release as measured by Fig. 2.

Fig. 1. Metalloproteinases are involved in bradykinin-induced ERK1/2 activation and transactivation of EGFR in mIMCD-3 Cells. mIMCD-3 cells were pretreated with vehicle or broad-spectrum inhibitors of MMPs: 5 μM batimastat, 5 μM GM-6001, or inactive form of GM-6001 (5 μM). Cells were then stimulated with 10 nM BK or with 1 ng/ml EGF for 5 min. A, ERK1/2 phosphorylation was measured as described under Materials and Methods. Experiments were performed at least four times in duplicates. Data are presented as mean ± S.E.M. (n = 4; *, P < 0.01 versus vehicle-treated cells; †, P < 0.01 versus control). B, in the inset, a representative immunoblot of phospho-EGFR is shown. EGFR phosphorylation was measured as described under Materials and Methods. Experiments were performed at least four times. Data are presented as mean ± S.E.M. (n = 4; ‡, P < 0.05 versus vehicle-treated cells; †, P < 0.05 versus control).

Fig. 2. Bradykinin-induced activation of Ras in mIMCD-3 cells is metalloproteinase-dependent. mIMCD-3 cells were pretreated with vehicle or broad-spectrum inhibitors of MMPs: 5 μM batimastat or 5 μM GM-6001; or inactive form of GM-6001 (5 μM). Cells were then stimulated with 10 nM BK or with 1 ng/ml EGF for 5 min. Ras activity was measured using Ras activation assay kit as described under Materials and Methods. Experiments were performed at least three times in duplicates. Data are presented as mean ± S.E.M. *, P < 0.01 versus vehicle-treated cells; †, P < 0.01 versus control.

Fig. 3. Inhibitors of MMPs do not affect Ca\textsuperscript{2+} signal in mIMCD-3 cells. mIMCD-3 cells were pretreated with vehicle (rows 2 and 3), 5 μM GM-1489 (rows 4 and 5), 5 μM inactive form of GM-6001 (rows 6 and 7), 5 μM GM-6001 (rows 8 and 9), or 10 μM MMP inhibitor III. Cells were then stimulated with 10 nM BK (B and C), 1 μM ATP (D and E), 1 μM Ca\textsuperscript{2+} ionophore A23187 (F and G), or vehicle (A and H). Intracellular Ca\textsuperscript{2+} release was measured in FLIPR experiments as described under Materials and Methods.
FLIPR (Fig. 3), suggesting that MMP inhibitors selectively affect BK-induced EGFR phosphorylation. This FLIPR experiment also shows that the effects of inhibitors on ERK and EGFR are not due to toxic effects of the inhibitors.

**Characterization of Metalloproteinase Types Present in mIMCD-3 Cells.** Because to date ~28 different MMPs have been identified (Egeblad and Werb, 2002), we needed to determine which isoforms are present in mIMCD-3 cells to plan more specific experiments. We extracted RNA from the cells using the TRIzol reagent and used a mouse MMP MultiGene RT-PCR Profiling kit. Thus, we established that mIMCD-3 cells express mRNAs for the following MMPs: MMP-2, MMP-3, MMP-8, MMP-9, MMP-11, MMP-13, MMP-14, MMP-15, MMP-17, MMP-19, MMP-20, and MMP-23 as well as for all four tissue inhibitors of metalloproteinases (Table 1).

**Inhibitor Data Suggest the Involvement of Collagenase-2 and -3 in the BK-Induced EGFR Transactivation and ERK1/2 Phosphorylation.** In an attempt to identify the MMPs that mediate BK-induced EGFR transactivation, we used a number of commercially available broad-spectrum as well as more selective MMP inhibitors. Those studies showed that selective inhibitors for MMP-8 and MMP-13 were able to block BK-induced EGFR phosphorylation and ERK activation. Inhibitors for MMP-1, -2, -3, -7, or -9 were without effect (Table 2).

**Neutralizing Antibody against MMP-13 Decreases BK-Induced ERK Activation.** To support the involvement of MMP-13 in BK-induced ERK activation, we preincubated mIMCD-3 cells with 0.1 mg/ml neutralizing MMP-13 antibody for 2 h before stimulation with 10 nM BK for 5 min. Control samples were preincubated with 0.1 mg/ml normal goat IgG. Figure 4A shows that neutralizing MMP-13 antibody reduced BK-induced ERK phosphorylation by about ~50%, supporting a role for MMP-13 in this process.

**Transfection of mIMCD-3 Cells with MMP-8 siRNA Decreases BK-Induced ERK Activation.** To support the involvement of MMP-8 in BK-induced ERK activation, we used RNA-mediated interference to knock down the expression of MMP-8. mIMCD-3 cells were nucleofected with either 100 nM MMP-8 siRNA or with control siRNA. Forty-eight hours postnucleofection, cells were stimulated with vehicle or 10 nM BK for 5 min, lysed, and analyzed for ERK phosphorylation. mIMCD-3 cells transfected with MMP-8 siRNA demonstrated ~50% less BK-induced ERK activation than cells transfected with control siRNA (Fig. 4B). Figure 4C demonstrates effective silencing of MMP-8 expression in mIMCD-3 cells transfected with MMP-8 siRNA. Immunoblot with antibody against β-actin shows the equal amount of β-actin in the lysates from cells transfected with MMP-8 siRNA and with control siRNA, supporting silencing specificity and serving as a control for protein loading.

**Combined Inhibition of MMP-8 and MMP-13 Further Decreases BK-Induced ERK Activation.** Because inhibition of MMP-8 by transfection with MMP-8 siRNA and inhibition of MMP-13 neutralizing antibody resulted only in partial (~50%) decrease in BK-induced ERK phosphorylation (Fig. 4), in the next set of experiments we used the combination of both treatments to block the activity of both collagenses and to clarify the role of two specific MMPs in BK-induced ERK activation. The results are presented in Fig. 5. Cells transfected with MMP-8 siRNA were further preincu-

**Table 1**

mIMCD-3 cells express mRNA for multiple MMPs

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Expected Size</th>
<th>Signal Intensity</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>MMP-1</td>
<td>368</td>
</tr>
<tr>
<td>2</td>
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<td>508</td>
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<td>11</td>
<td>MMP-14</td>
<td>439</td>
</tr>
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</table>

**Table 2**

MMPs inhibitors data

<table>
<thead>
<tr>
<th>Inhibitors of Matrix Metalloproteinases</th>
<th>Known Effects on MMPs</th>
<th>Inhibits ERK1/2 Activation</th>
<th>Inhibits EGFR Transactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Batimastat</td>
<td>Broad spectrum, blocks MMP-1, -2, -3, -8, and -9</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>GM-6001</td>
<td>Broad spectrum, blocks MMP-1, -2, -3, -8, and -9</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Inactive form of GM-6001</td>
<td>Negative control for GM-6001</td>
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<td>No</td>
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<tr>
<td>MMP-2 inhibitor II</td>
<td>Inhibits MMP-1, -2, -3, -7, and -9</td>
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<tr>
<td>MMPs inhibitor III</td>
<td>Inhibits MMP-1, -2, -3, -7, and -13</td>
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<td>N.D.</td>
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<td>N.D.</td>
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<td>No</td>
</tr>
<tr>
<td>MMP-8/MMP-13 inhibitor I</td>
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<tr>
<td>MMP-8 inhibitor I</td>
<td>Specific MMP-8 inhibitor</td>
<td>Yes</td>
<td>Yes</td>
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N.D., not determined.
mMCD-3 cells transfected with MMP-8 siRNA. mIMCD-3 cells were nucleofected with either MMP-13 siRNA alone or with both MMP-8 and MMP-13 siRNAs. Forty-eight hours postnucleofection, cells were stimulated with vehicle or 10 nM BK for 5 min, lysed, and analyzed for ERK phosphorylation. mIMCD-3 cells transfected with MMP-13 siRNA (MMP-13 siRNA) demonstrated ~50% less BK-induced ERK activation than cells transfected with control siRNA (Fig. 5A).

Cells transfected with both siRNAs (MMP-8 and MMP-13 siRNA) demonstrated ~68% decrease in BK-induced ERK activation. Figure 5B demonstrates effective silencing of MMP-13 expression in mIMCD-3 cells transfected with MMP-13 siRNA. Immunoblot with antibody against β-actin shows the equal amount of β-actin in the lysates from cells transfected with MMP-13 siRNA and with control siRNA, supporting silencing specificity and serving as a control for protein loading. The residual (~30–35%) BK-induced activation of ERK in mIMCD-3 cells that were treated to block both MMP-8 and MMP-13 is due most likely to the incomplete blockade of collagenases, although we cannot exclude possible involvement of other enzymes in this process.

### BK Treatment Stimulates the Activity of Collagenases in mIMCD-3 Cells

The next set of experiments was aimed to determine whether BK stimulates the activity of MMPs in mIMCD-3 cells. To answer this question, we measured collagenase activity in concentrated conditioned media from mIMCD-3 cells that were treated with 10 nM BK for different periods. The activities of type 1 collagenases (MMP-1, -8, and -13) were assessed by assaying biotinylated collagen as a substrate for active collagenases. Experiments were performed at least four times in triplicate. The results presented on Fig. 6A demonstrated that BK treatment stimulated collagenase activity in mIMCD-3 cells within 10 min. We also assessed MMP gelatinase activity, but we were able to detect only basal activity of gelatinases, and not BK-dependent activity in the same samples (data not shown). This suggests that the most likely enzymes involved in BK-induced ERK activation are collagenase-2 and -3, rather than gelatinases MMP-2 or MMP-9.

We also used this collagenase activity assay to confirm that silencing of MMP-8 and MMP-13 expression by nucleofection of mIMCD-3 cells with siRNAs indeed inhibited the collagenases activity under our experimental conditions (Fig. 6B).

Cells were transfected with control siRNA, MMP-8 siRNA, MMP-13 siRNA, or with combination of both MMP-8 and MMP-13 siRNAs. Forty-eight hours postnucleofection, cells were stimulated with vehicle or 10 nM BK for 5 min, and collagenase activity in concentrated conditioned media was measured as described under Materials and Methods. Figure 6B demonstrates that silencing of MMP-8 and MMP-13 expression actually inhibits BK-induced activation of collagenases in mIMCD-3 cells. However due to the nature of this
assay (nonlinear calibration curves) and to highly uneven errors we were not able to show that this inhibition was statistically significant. The major pitfall of this assay is the lack of specificity, because it cannot distinguish between the different collagenases; therefore, more specific assays are needed for the quantitative determinations of active and pro-MMP-8 and MMP-13. It is also essential that the determined MMP activity may reflect a balance of active MMP and its inhibitors (tissue inhibitor of metalloproteinases) that are also present in mIMCD-3 cells (Table 1).

**BK-Induced ERK Phosphorylation Does Not Require the Release of HB-EGF or TGF-α.** Because most of the published mechanisms of MMP-dependent EGFR activation by GPCRs involve extracellular release of EGF-like growth factors such as HB-EGF and/or TGF-α (Frenzel et al., 1999; Kalmes et al., 2000; Uchiyama-Tanaka et al., 2001; McCole et al., 2002; Shah et al., 2004), we next decided to test whether EGF-like ligands are involved as extracellular soluble factors in BK-induced transactivation of the EGFR in mIMCD-3 cells. In the first set of experiments, we used a glycosaminoglycan heparin, which is structurally similar to the heparan sulfate proteoglycan (HSPG) that is present on the cell surface and serves as coreceptor for many growth factors. Heparin acts as a competitor for HSPG and can adsorb the HB-EGF as well as other HSPG-tethered molecules (e.g., amphiregulin or betacellulin). mIMCD-3 cells were pretreated with 0.1 mg/ml heparin for 30 min before stimulation with 10 nM BK or with 1 ng/ml EGF for 5 min. We also pretreated cells with 0.1 mg/ml neutralizing antibody against HB-EGF for 1 h and measured phosphorylation of ERK after 5 min of stimulation with BK or EGF. We have previously used this antibody to successfully inhibit HB-EGF- and serotonin-induced ERK activation in renal mesangial cells (M. Gooz, submitted for publication; data not shown). In another set of experiments, we pretreated mIMCD-3 cells with 50 μg/ml neutralizing antibody against TGF-α, another potential EGFR ligand, before stimulation with 10 nM BK or 10 ng/ml TGF-α. TGF-α neutralizing antibody blocked TGF-α-induced ERK activation, supporting that this antibody is functional under our experimental conditions. We found that BK-induced MMP-dependent phosphorylation of EGFR does not require extracellular release of TGF-α and/or HB-EGF, in that neutralizing TGF-α and HB-EGF antibodies (as well as heparin) failed to prevent ERK activation by BK. Results are shown in Fig. 7, A and B.  

**Metalloproteinases Stimulate ERK1/2 Activation and EGFR Phosphorylation in the Absence of BK.** To further assess the possible involvement of MMPs of the collagenase class in ERK signaling in mIMCD-3 cells, we studied the ability of exogenous activated collagenases to stimulate ERK phosphorylation. Activated MMP-8 from human neutrophils as well as recombinant MMP-13, were able to stimulate ERK activity when applied to mIMCD-3 cells in the absence of BK. The results are presented in Fig. 8. MMP-8- and MMP-13-induced ERK activation was not due to release of HB-EGF, because heparin did not block it (Fig. 8). MMP-induced ERK phosphorylation did not require the B2 receptor because pretreatment with 1 μM HOE-140 (Icatibant; B2 receptor antagonist) did not block ERK phosphorylation induced by collagenases. The same pretreatment abolished BK-induced ERK phosphorylation. MMP-8-induced as well as BK-induced ERK phosphorylation was MEK-dependent because pretreatment with 10 μM PD98059 (specific inhibitor of the ERK kinase MEK-1) abolished ERK phosphorylation. Finally, MMP-8- and MMP-13-induced ERK activation seemed to be dependent on EGFR kinase activity, because a selective inhibitor of EGFR tyrosine kinase, AG-1478 (100 nM for 30 min), effectively blocked the phosphorylation. A role for
EGFR is further supported by experiments showing that activated MMP-8 and MMP-13 also stimulated EGFR phosphorylation in mIMCD-3 cells (Fig. 9).

**Discussion**

The current work describes a novel mechanism of EGFR transactivation by the Gq-coupled bradykinin B2 receptor that involves activation of collagenase-2 and -3. In addition, we demonstrate for the first time that 1) collagenases (rather than gelatinases) are activated by the B2 receptor in a cultured murine cell model of the IMCD, 2) collagenases are involved in a cross-talk between the B2 receptor and EGFR, and 3) EGFR activation does not require the release of HB-EGF or TGF-α.

Several mechanisms of EGFR transactivation by Gq-coupled receptors have been demonstrated. A protein kinase C-dependent mechanism of EGFR transactivation has been shown for the receptor of gonadotropin-releasing hormone in pituitary T3-1 gonadotropes and in transfected COS-7 cells (Grosse et al., 2000). Metalloproteinases-dependent EGFR activation by HB-EGF was described for angiotensin II AT1 receptors in vascular smooth muscle cells (Eguchi et al., 2001), glomerular mesangial cells (Uchiyama-Tanaka et al., 2001), C9 hepatocytes (Shah et al., 2004), and for muscarinic M1 and thrombin receptors in other cell types (Prenzel et al., 1999; Kalmes et al., 2000). Carbachol-stimulated transactivation of the EGFR by M3 receptors in T84 cells is mediated by metalloproteinase-dependent extracellular release of TGF-α and intracellular Src activation (McCole et al., 2002).

Bradykinin has been shown to induce tyrosine phosphorylation of the EGFR in COS-7 cells where protein kinase C activation and EGFR transactivation independently activate ERK (Adomeit et al., 1999). Previously, we identified a novel mechanism of BK-induced transactivation of EGFR in mIMCD-3 cells that does not involve phospholipase C, elevations of Ca²⁺, calmodulin, protein kinase C, Gsα subunits, or B2 receptor heterodimerization with EGFR (Mukhin et al., 2003). In the current study, we looked further into this mechanism testing the hypothesis that B2 receptor-induced trans-
The B2 receptor plays a protective role in renal tubulointerstitial fibrosis, probably by increasing the activity of a plasminogen activator/MMP-2 cascade (Schanstra et al., 2002), but it does not mediate the antifibrotic effect of angiotensin-converting enzyme inhibitors (Schanstra et al., 2003). Thus, a possible connection between the B2 receptor and MMPs in kidney cells remains unclear.

Our data support the involvement of MMPs in a BK-induced signaling pathway that leads to the activation of ERK1/2 in mIMCD-3 cells. Using broad-spectrum inhibitors of MMPs, batimastat, and GM-6001, we demonstrated that MMPs are involved in BK-induced ERK activation and EGFR phosphorylation (Fig. 1, A and B), and in Ras activation (Fig. 2). To plan more specific experiments, we first established that mIMCD-3 cells express mRNAs for the MMP-2, MMP-3, MMP-8, MMP-9, MMP-11, MMP-13, MMP-14, MMP-15, MMP-17, MMP-19, MMP-20, and MMP-23 (Table 1). Next, we used a panel of commercially available chemical inhibitors for different MMPs present in mIMCD-3 cells that allowed us to propose involvement of MMP-8 (collagenase-2) and/or MMP-13 (collagenase-3) in B2 receptor-induced EGFR transactivation (Table 2). Because we questioned the specificity of MMP inhibitors, the next series of experiments were aimed to inhibit MMP-8 and MMP-13 with other methods to support the inhibitor data. Figure 4A shows that neutralizing MMP-13 antibody reduced BK-induced ERK phosphorylation by about 50%, supporting a role for MMP-13 in this process. The same level of decrease in BK-induced ERK phosphorylation was achieved by transfection of mIMCD-3 cells with MMP-8 siRNA (Fig. 4B) or with MMP-13 siRNA (Fig. 5). The simultaneous blockade of MMP-8 and MMP-13 resulted in a further decrease in BK-induced ERK phosphorylation, supporting roles for both collagenases. Having established that collagenases are involved in BK-induced ERK stimulation in mIMCD-3 cells, we hypothesized that BK stimulates the activity of collagenases. Figure 6A demonstrates that BK treatment stimulates collagenase activity in concentrated conditioned media from mIMCD-3 cells.

To further assess possible involvement of MMPs in ERK signaling in mIMCD-3 cells we studied the ability of exogenous activated collagenases to stimulate ERK phosphorylation. Interestingly, exogenous activated collagenase-2 and -3 induced ERK activation (Fig. 8) and EGFR phosphorylation (Fig. 9). MMP-8 and MMP-13-induced ERK activation was MEK-dependent, did not require B2 receptor, was not due to release of HB-EGF, and required EGFR kinase activity (Fig. 8).

Thus, our data support specific roles for MMP-8 and MMP-13 in BK-induced antibody against MMP-13, 3) siRNA stud-
ies, 4) stimulation of collagenase activity by BK, and 5) phosphorylation of EGFR and ERK by exogenously applied activated MMP-8 and MMP-13.

Although there are several studies describing the role of MMPs in mediating EGFR transactivation induced by GPCRs, most of them have reported MMP-dependent EGFR ligand shedding. The EGFR can be activated by an expanding group of growth factors: EGF, HB-EGF, TGF-α, epiregulin, amphiregulin, epigen, neuregulins, and betacellulin (Harris et al., 2003). Among these, HB-EGF has been most implicated in mediating GPCR-induced EGFR transactivation (Prenzel et al., 1999; Kalmes et al., 2000; Eguchi et al., 2001; Gschwind et al., 2001; Uchiyama-Tanaka et al., 2001; Shah et al., 2004; Mifune et al., 2005). Metalloproteinase-dependent TGF-α release also has been described as a mechanism for GPCR-induced EGFR transactivation (McCole et al., 2002; Zhao et al., 2004).

To test the mechanism of MMP-dependent ERK activation in mIMCD-3 cells, we tried to block BK-induced activation of ERK1/2 by heparin or neutralizing HB-EGF antibody (Fig. 7A). Neither treatment changed the ability of BK to activate ERK1/2, suggesting that B2 receptor does not use HB-EGF-like growth factor to activate EGFR. To test the possibility that MMP-dependent extracellular release of TGF-α is involved in BK-induced signaling, we used neutralizing antibody against TGF-α (Fig. 7B). Treatment with neutralizing TGF-α antibody did not change the ability of BK to activate ERK1/2, but it blocked TGF-α-induced ERK1/2 activation, suggesting that B2 receptor does not use TGF-α.

Thus, we demonstrated that B2 receptor-induced ERK phosphorylation in mIMCD-3 cells does not require the release of HB-EGF or TGF-α. Inability of heparin to inhibit BK-induced signaling also does not suggest the importance of amphiregulin and betacellulin, other heparin-binding members of EGF family. How exactly collagenases mediate BK-induced transactivation of EGFR is not clear at the moment (Fig. 10). One possibility is that MMP-8 and/or MMP-13 upon stimulation with BK cause shedding of other nonheparin-sensitive EGFR ligands (e.g., epiregulin). Another possibility would be that in this case collagenases activate EGFR without releasing EGFR ligand, probably by proteolytic activation of EGFR molecule. Regulation of EGFR activity during apoptosis by proteolytic cleavage in a caspase-dependent manner has been described in A431 cells, although in this case protease inactivated EGFR (Bae et al., 2001). MMP-mediated ectodomain shedding has been shown for ErbB2 and ErbB4 receptors, the members of the EGF family (Codony-Servat et al., 1999; Junttila et al., 2000). Although the shedding of ErbB4 molecule is activated by protein kinase C stimulation and most likely is mediated by tumor necrosis factor-α-converting enzyme (Junttila et al., 2000), the cleavage of ErbB2 does not require protein kinase C activation and involves metalloprotease different from tumor necrosis factor-α-converting enzyme, which can be inhibited by tissue inhibitor of metalloproteinases-1 (Codony-Servat et al., 1999). Finally, BK-induced stimulation of collagenases may cause the recruitment of other mediators of EGFR activation such as nonreceptor tyrosine kinases Src that has been shown to mediate EGFR p90RAS release in response to GPCR ligands in head and neck cancer cells (Zhang et al., 2004).

In conclusion, this study demonstrates a novel mechanism of EGFR transactivation by the Gα2 coupled bradykinin B2 receptor that involves activation of collagenase-2 and -3 but does not require the release of HB-EGF or TGF-α. Although the exact mechanism of BK-induced MMP-dependent transactivation of EGFR is still to be defined, these findings reveal a novel functional link between collagenases and EGFR, suggesting that besides their matrix-degrading abilities, MMP-8 and MMP-13 are essential for BK-induced proliferation of kidney cells.

References


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