Tissue Kallikrein Actions at the Rabbit Natural or Recombinant Kinin B₂ Receptors

Steeve Houle, Giuseppe Molinaro, Albert Adam, François Marceau

Abstract—We have examined whether exogenous human tissue kallikrein exerts pharmacological actions via the bradykinin B₂ receptor; specifically, whether the protease can bind to, cleave, internalize, and/or activate a fusion protein composed of the rabbit B₂ receptor conjugated to the green fluorescent protein (B₂R-GFP). The enzyme partially digested the fusion protein at 1 μmol/L, but not 100 nmol/L, and promoted B₂R-GFP endocytosis in HEK 293 cells (≥50 nmol/L). Trypsin and endopeptidase Lys-C, but not plasma kallikrein, also cleaved B₂R-GFP. Phospholipase A₂ was activated by 50 nmol/L tissue kallikrein in HEK 293 cells expressing B₂R-GFP, and this was mediated by the receptor, as shown by the effect of a B₂ receptor antagonist and by the lack of response in untransfected cells. However, 500 nmol/L kallikrein elicited a strong receptor-independent activation of phospholipase A₂. Tissue kallikrein competed for [³H]bradykinin binding to B₂R-GFP only at 1 μmol/L. A simulation involving kallikrein treatment of HEK 293 cells, pretreated or not with human plasma, evidenced the formation of immunoreactive bradykinin. The enzyme (50 nmol/L) contracted the rabbit isolated jugular vein via its endogenous B₂ receptors, but the effect was tachyphylactic, and there was no cross-desensitization with bradykinin effects. Aprotinin prevented all pharmacological responses to tissue kallikrein, indicating that the enzyme activity is required for its effect. The local generation of kinins is a plausible mechanism for the pharmacological effects of lower concentrations of tissue kallikrein (50 to 100 nmol/L); higher levels (0.5 to 1 μmol/L) can not only initiate the degradation of rabbit B₂ receptors but also exert nonreceptor-mediated effects. (Hypertension. 2003;41:611-617.)

Key Words: receptors, bradykinin ■ kallikrein ■ rabbits ■ fluorescence

K allikreins are a heterogeneous group of serine proteases capable of releasing bradykinin (BK)-related peptides (kinins) from kininogens.¹ The classic human tissue kallikrein KLK1 (hK1), notably present in renal tissue and released into urine, has been used as a pharmacological agent in animals, lately under the form of DNA expression vectors used in gene therapy strategies or transgenic animals.² The mechanism of the observed effects (hypotension, cardioprotection, angiogenesis, etc) frequently involved the BK B₁ receptor (selective for a class of kinin metabolites) was also involved in the effect of human tissue kallikrein.³

The mode of action of kallikrein at the cell level is not entirely clear. The proteinase-activated receptors (PARs) are not believed to possess other ligands than proteases, but tissue kallikrein does not activate PAR-1 or PAR-2.⁴ The human B₂R has been proposed to be a specific binding site for human or porcine tissue kallikrein and trypsin, with pharmacological activation of these receptors on binding.⁵ Moreover, extracellular proteases can initiate the destruction of G-protein–coupled receptors (GPCRs), as shown by the effects of chymotrypsin on the receptor for anaphylatoxin C5a⁶ or of trypsin on the BK B₁R.⁷

We have previously shown the feasibility of using a construct composed of the rabbit B₂R fused to the green fluorescent protein (GFP) to identify treatments that lead to B₂R degradation.⁸ The B₂R-GFP fusion protein retains the pharmacological profile, the functional properties, and the nanomolar affinity for [³H]BK, virtually indistinguishable from the wild-type B₂R.⁹ These investigations failed to support the concept that BK-induced endocytosis is a mechanism for B₂R downregulation, as the receptors were restricted to the recycling endosome compartment, did not enter lysosomes, and were eventually completely recycled to the cell surface. However, B₂R-GFP exposure to extracellular trypsin rapidly (10 minutes) produced a small immunoreactive protein similar to GFP, suggesting that limited proteolysis of B₂R was followed by rapid degradation by mechanisms endogenous to the cell.⁸

We have examined whether human tissue kallikrein exerts pharmacological actions via the B₂R; specifically, whether the protease can bind to, cleave, internalize, and/or activate...
the B,R-GFP. The physiological consequences of kallikrein interaction with the rabbit wild-type B,R have been verified by using the contractility of the rabbit jugular vein.

Methods

Drugs

Purified human plasma kallikrein and tissue (urinary) kallikrein were purchased from Calbiochem. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of tissue kallikrein, followed by silver nitrate staining, revealed a single protein band. Sequencing-grade proteases were from Sigma (bovine trypsin) or Roche (endoprotease Lys-C from *Lysobacter enzymogenes*). BK was purchased from Bachem. LF 16.0687, a competitive antagonist of BK at the rabbit B,R, was a gift from Laboratoires Fournier (Dax, France). When aprotinin (Sigma) was used as an inhibitor of the pharmacological actions of tissue kallikrein (50 to 500 nmol/L), its final concentration at the cell level was 10 μmol/L, but the enzyme and aprotinin had been preincubated together (50-fold more-concentrated stock) for 1 hour at 37°C before application (the kallikrein stock was also preincubated at 37°C in control experiments).

Cells

The derivation of an HEK 293 cell line stably expressing B,R-GFP and its properties are described elsewhere. Nontransfected HEK 293 cells were used in control experiments. Confocal microscopy was applied to the cells as described. An arachidonic acid release assay was performed as described elsewhere to evaluate the activation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) after stimulation with tissue kallikrein in untransfected HEK 293 cells or cells stably expressing B,R-GFP (24-well plates).

Protease Digestion of Surface Molecules in Intact Cells

The protease or BK treatments were applied to confluent 25-cm<sup>2</sup> flasks of HEK 293 cells stably expressing B,R-GFP or transfected cells. The culture medium was removed from the cell flasks, which were rinsed and filled with 500 mL serum-free α-minimal essential medium containing 71 μmol/L cycloheximide to avoid interference from newly synthesized receptors. The cell flasks were further incubated for 30 minutes at 37°C before extraction. The protease or BK treatments were applied for the last 10 minutes of the incubation period.

Immunoblots

Relative to other anti-GFP antibodies previously used by us for immunoblots, the monoclonal antibodies to GFP (Clontech; clone JL-8, used at dilution 1/1000) exhibited an exceptionally low background in total cell extracts of untransfected HEK 293 cells. For the analysis of B,R-GFP in total cell extracts, immunoblots were generally performed as described previously. Immunoblots of low- and high-molecular-weight kininogens were also conducted on HEK 293 cells exposed or not to citrated human plasma; the procedure outlined above was applied by using characterized polyclonal antibodies raised against human high-molecular-weight kininogen (dilution 1/25 000) and the appropriate secondary antibodies.

Binding Assay

The plasma membranes from HEK 293 cells stably expressing B,R-GFP were recovered after cell fractionation by using a sucrose/tricine buffer system as described elsewhere; the pellet of the third centrifugation was used as the source of material (150 000 × g, 3 hours). The binding of 2 nmol/L [H]BK (Perkin Elmer Life Sciences; 90 Ci/mmol) to these membranes (10 μg/tube, suspended in 500 μL phosphate-buffered saline [PBS], pH 7.4, supplemented with 0.02% Na<sub>2</sub>SO<sub>4</sub>, 0.1% bovine serum albumin, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 μmol/L captopril) was established in the presence or absence of a cold competitor (tissue kallikrein, BK, or the antigenist LF 16.0687). All tubes were incubated on ice for 90 minutes, and then the [H]BK-membrane complexes were adsorbed to glass fiber filters (GF/C, Whatman, presoaked in polyethyleneimine, 0.5% in PBS, 2 hours) by using a 24-channel cell harvester (Brandell Corp). The filters were washed 5 times with cold PBS and were subsequently analyzed for bound radioactivity by scintillation counting.

Enzyme Immunoassay of Kinins in the Culture Medium of HEK 293 Cells

To test whether tissue kallikrein could release BK-like peptides from B,R-GFP–expressing or untransfected HEK 293 cells, confluent 75-cm<sup>2</sup> flasks were rinsed with Earle’s balanced salt solution containing 1 μmol/L orthophenanthroline, filled with 5 mL undiluted human citrated plasma for 30 minutes, and then rinsed and incubated in Earle’s solution (5 mL) for 30 minutes. Then, tissue kallikrein (50 nmol/L) was added to some of the flasks. After a further 10 minutes’ incubation at 37°C, 3 mL of the medium was removed and transferred in 15 mL of ice-cold ethanol. A variation of the protocol aimed at measuring very low amounts of kinins released by kallikrein in cells not treated with plasma in larger flasks (175-cm<sup>2</sup>, 10 mL Earle’s solution filling, of which 6 were transferred in 30 mL ethanol). The suspensions were incubated on ice for 1 hour, centrifuged to remove the precipitated proteins, and stored at −80°C until assayed. For that purpose, the ethanol extract was evaporated to dryness in a Speed vac system and then processed precisely as described for the separate measurements of immunoreactiveBK and des-Arg<sup>9</sup>-BK. The results are expressed as the estimated kinin concentration in the initial medium.

Contractility Assay

A local ethics committee approved the procedures based on rabbits. Rabbit jugular vein contractile responses to tissue kallikrein, BK (mediated by B,Rs), or histamine (mediated by H<sub>1</sub> receptors) were measured as reported, except for captopril in the Krebs’ buffer, which was omitted in the present experiments.

Results

Assay for B,R Cleavage Based on Detection of the B,R-GFP Immunoreactive C-Terminal Fragments

When untransfected HEK 293 cell extracts were immunoblotted with the anti-GFP monoclonal antibodies, essentially no background was observed (Figure 1, lane 2). Transfection with a GFP-coding vector produced the expected strong immunoreactive band at ~27 kDa (Figure 1, lane 1). As previously reported with different anti-GFP antibodies, B,R-GFP–specific immunoreactivity was expressed as a wide band of 101 to 105 kDa; faint additional bands suggested that spontaneous degradation of the fusion protein occurs in the cells, with a protein resembling GFP as 1 of the main immunoreactive metabolites (Figure 1, lane 3). Short trypsin digestion (10 minutes, 1 μmol/L) of untransfected cells did not reveal immunoreactive bands (data not shown) but produced ~31- and 27-kDa C-terminal immunoreactive fragments in cells expressing B,R-GFP (Figure 1, lanes 5 or 10). Treatment of transfected cells with an alternate serine protease, endoproteinase Lys-C (10 minutes, 0.3 μmol/L), produced somewhat different results, with the reinforcement of an ~75-kDa band that existed in transfected cells not exposed to enzymes, and the appearance of lower-molecular-weight bands (Figure 1, lane 6). Plasma kallikrein (10 nmol/L to 1 μmol/L, 10 minutes) failed to digest B,R-GFP (Figure 1, lanes 7, 11, and 12), whereas tissue kallikrein (1 μmol/L, 10 minutes) produced a third pattern of digestion, with a 28-kDa major product (Figure 1, lane 8). However, tissue kallikrein...
failed to digest B$_2$R-GFP at lower concentrations (10 or 100 nmol/L, Figure 1, lanes 13 and 14). All observed digestions were partial. B$_2$R-GFP fragmentation does not occur when cells are stimulated with BK (100 nmol/L, 10 minutes, Figure 1, lane 4), suggesting that receptor degradation is not secondary to B$_2$R signaling. The B$_2$R antagonist LF 16.0687 (10 μmol/L) did not inhibit the digestion of B$_2$R-GFP by trypsin, endoproteinase Lys-C, or tissue kallikrein (data not shown).

**Subcellular Redistribution of Fluorescence**

Ten-minute treatments with tissue kallikrein promoted the internalization of B$_2$R-GFP membrane-associated fluorescence in cells stably expressing the fusion protein (Figure 2). The effect of kallikrein was concentration dependent (50 and 500 nmol/L tested). The internalized fluorescence was either finely granular or concentrated into ill-defined structures, similar to what is observed after BK treatment (Figure 2). The effects of kallikrein on fluorescence distribution were at least partially inhibited by the protease inhibitor aprotinin (final concentration, 10 μmol/L) or the receptor antagonist LF 16.0687 (the latter drug being active only against the lower concentration level of the enzyme).

**Functional Response to Tissue Kallikrein in Cells**

As the action of tissue kallikrein on B$_2$R has been proposed, we have investigated the functional effect of the enzyme on B$_2$R-GFP by using a PLA$_2$ assay (Figure 3). Both BK (1 nmol/L) and tissue kallikrein (50 nmol/L) significantly increased arachidonate release from cells expressing B$_2$R-GFP (Figure 3A) but not from untransfected cells (Figure 3B). Further insight into the role of the receptor in kallikrein action is provided by combining treatments with the competitive nonpeptide B$_2$R antagonist LF 16.0687: a concentration (1 μmol/L) that is sufficient to significantly reduce the effect of BK (1 nmol/L) also significantly decreased the tissue kallikrein effect (50 nmol/L; Figure 3A). This enzyme level is in a concentration range that does not promote detectable digestion of the fusion protein as assessed by immunoblotting (Figure 1). The 500 nmol/L concentration is closer to the level where digestion is observed (Figure 1). At this concentration, tissue kallikrein profusely released arachidonate from cells expressing B$_2$R-GFP or untransfected cells (Figures 3A and 3B). LF 16.0687 did not prevent the effect of the 500 nmol/L enzyme concentration in the first type of cells. Aprotinin inhibited arachidonate release induced by tissue

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**Figure 1.** Immunoblot of HEK 293 cell extracts based on monoclonal anti-GFP antibodies: effect of protease or BK treatments. HEK 293 cells stably expressing B$_2$R-GFP were extracted after 30-minute incubation in serum-free medium containing 71 μmol/L cycloheximide (control condition, lane 3). Other cells were additionally treated for the last 10 minutes of incubation with BK (100 nmol/L, lane 4), trypsin (100 nmol/L, lane 9; 1 μmol/L, lanes 5 or 10), endoproteinase Lys-C (0.3 μmol/L, lane 6), plasma kallikrein (10 nmol/L, 100 nmol/L, or 1 μmol/L; lanes 11, 12, and 7, respectively), or tissue kallikrein (10 nmol/L, 100 nmol/L, or 1 μmol/L; lanes 13, 14, and 8, respectively). Extracts from untransfected HEK 293 cells (lane 2) and HEK 293 cells transiently transfected with GFP (lane 1) were also immunoblotted as controls. The results are representative of at least 2 experiments for each reaction.

**Figure 2.** Subcellular localization of the B$_2$R-GFP fusion protein in stably transfected HEK 293 cells maintained in serum-free culture medium and treated for the last 10 minutes of incubation with tissue kallikrein (50 or 500 nmol/L) after a 30-minute pretreatment with saline (control), aprotinin (10 μmol/L), or LF 16.0687 (1 μmol/L). All cells were also pretreated with cycloheximide (71 μmol/L, to avoid synthesis of B$_2$R-GFP) (magnification approximately ×1800).
kallikrein (50 nmol/L) in cells expressing B2R-GFP (Figure 3C), indicating that the enzyme activity is required for its action. Aprotinin did not inhibit BK-induced PLA2 activation.

**Competition of [3H]BK Binding to B2R-GFP**

As tissue kallikrein apparently stimulates the B2R (Figure 3A) and as the enzyme is reported to bind with high affinity to this receptor,5 we tested whether it could displace the specific binding of [3H]BK (2 nmol/L) to the membranes of HEK 293 cells expressing B2R-GFP (Figure 4). No competition was seen for the 1 to 100 nmol/L concentration range of the protease, but some competition was seen at 1 μmol/L. By contrast, BK and the antagonist LF16.0687 were effective competitors of the radioligand binding.

**Immunoreactive BK Release From HEK 293 Cells Exposed to Tissue Kallikrein**

An alternative explanation for the stimulation of B2R-GFP by tissue kallikrein is the formation of kinins in the vicinity of the receptors from kininogen produced or taken up by the cells. To test this possibility, immunoreactive BK and des-Arg9-BK (1 of the BK metabolites) were measured in the supernatant of cell flasks exposed or not to tissue kallikrein (50 nmol/L, Table). Values close to the detection limits were observed in flasks without cells but containing both the Earle’s solution and tissue kallikrein. Confluent HEK 293 cells (75-cm² flasks) expressing B2R-GFP first exposed to undiluted human plasma and then washed and treated with tissue kallikrein released large quantities of immunoreactive BK and des-Arg9-BK into the supernatant medium (Table), supporting the notion that kinin generation in the system is dependent on the presence of kininogen. Even higher concentrations were recorded in untransfected cells pretreated with plasma and then treated with kallikrein, suggesting that the receptor presence reduces the diffusion of the newly formed kinins into the supernatant. The conversion of immunoreactive BK into the metabolite des-Arg9-BK was ineffective. Further use of the immunoassays in 175-cm² flasks not
pretreated with human plasma (allowing sample concentration and a higher sensitivity) showed that HEK 293 cells release small quantities of immunoreactive BK under the effect of tissue kallikrein.

To validate the procedure wherein HEK 293 cells are exposed to human plasma to load them with kininogens, we extracted proteins from cells exposed or not to plasma and ran immunoblots for both the low- and high-molecular-weight kininogens (60 and 110 kDa, respectively, Figure 5). The procedure was highly effective, whether the cells expressed B2R-GFP or not. The lower-molecular-weight band was further identified as a kininogen by its reaction with anti-BK antibodies (same antibodies used as in the enzyme immunoassay applied to immunoblotting; data not shown). The assay was not sensitive enough to detect kininogen presence on cells not exposed to plasma.

**Immunoreactive Kinins Measured in the Medium Contained in Cell Flasks Pretreated or Not Pretreated With Citrated Human Plasma and With Tissue Kallikrein (50 nmol/L)**

<table>
<thead>
<tr>
<th>Type of Flask/Pretreatment</th>
<th>Cell Type in Flask</th>
<th>Tissue Kallikrein Treatment</th>
<th>n</th>
<th>Immunoreactive BK</th>
<th>Immunoreactive des-Arg^9^BK</th>
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</thead>
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<tr>
<td>75 cm^2</td>
<td>None, no plasma</td>
<td>Yes</td>
<td>14</td>
<td>4±1</td>
<td>8±2</td>
</tr>
<tr>
<td>Human plasma pretreatment in flasks with cells</td>
<td>HEK 293 cells expressing B2R-GFP</td>
<td>No</td>
<td>6</td>
<td>77±4*</td>
<td>16±2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>6</td>
<td>213±49†</td>
<td>69±8††</td>
</tr>
<tr>
<td></td>
<td>Untransfected HEK 293 cells</td>
<td>No</td>
<td>6</td>
<td>88±11*</td>
<td>7±2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>4</td>
<td>1647±84†††</td>
<td>155±10†††</td>
</tr>
<tr>
<td>175 cm^2</td>
<td>None</td>
<td>Yes</td>
<td>6</td>
<td>3.1±0.4</td>
<td>3.4±0.4</td>
</tr>
<tr>
<td>No pretreatment</td>
<td>Untransfected HEK 293 cells</td>
<td>No</td>
<td>12</td>
<td>4.5±1.0</td>
<td>5.2±0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yes</td>
<td>12</td>
<td>11.4±1.8†††</td>
<td>8.3±2.7</td>
</tr>
</tbody>
</table>

Values (pg/mL of cell supernatant) are mean±SEM of the number of determinations indicated by n. In 75 cm^2 flasks, values inferior to 9.6 or 19.2 pg/mL for BK or des-Arg^9^BK, respectively, are interpolated in a nonlinear part of the enzyme immunoassay calibration curves and are therefore less precise (these values are 4.8 and 9.6 pg/mL, respectively, in 175 cm^2 flasks). The sets of value for each size of flasks and for each type of immunoreactive kinin are heterogeneous (P<0.001) by Kruskall-Wallis test, except for immunoreactive des-Arg^9^BK in 175 cm^2 flasks. The Mann-Whitney test was applied to compare kinin concentrations from flasks with no cells (first line in each set of values) with concentrations in other cell flasks (P<0.001). To evaluate the effect of kallikrein, values from cells exposed to kallikrein treatment were compared with the corresponding group control without kallikrein treatment (preceding line; †P<0.05; ††P<0.01; †††P<0.001). Finally, the effect of the cell type on flasks pretreated with plasma and further treated with kallikrein was tested (fifth line compared with third line; ‡P<0.01).

**Effect of Tissue Kallikrein on Rabbit Jugular Vein Contractility**

These experiments were performed to verify that a submicromolar concentration of tissue kallikrein could stimulate wild-type rabbit B2R expressed at a physiological level. Tissue kallikrein (50 nmol/L) rapidly induced a sizeable contraction in the isolated rabbit jugular vein, but the tissue was completely desensitized when a second application of the protease was done at 30-minute interval (Figure 6A, top tracing). The tissues desensitized to kallikrein were not desensitized to BK (10 nmol/L, Figure 6A, top tracing); in fact, the agonist BK applied at 30-minute intervals did not desensitize the preparation to itself or to kallikrein (Figure 6A, bottom tracing). Pretreatment of naive tissues with the B2R antagonist LF 16.0687 (1 µmol/L) prevented the contractile response to tissue kallikrein (50 nmol/L, in tissues exposed for the first time to the protease) and, in fact, allowed a very small relaxant response to kallikrein in most pretreated tissues (Figure 6B). LF 16.0687 also reduced the response to BK (10 nmol/L) by 49*† 69% (Figure 6B).

**Figure 4.** Binding of 2 nmol/L [3H]BK to membranes from HEK 293 cells stably transfected with B2R-GFP as a function of treatment with tissue kallikrein, BK, or the antagonist LF 16.0687. Cold drugs were coincubated at the indicated concentrations with the radioligand at 0°C. Values are the mean±SEM of the number of determinations indicated by n. In 75 cm^2 flasks, values inferior to 9.6 or 19.2 pg/mL for BK or des-Arg^9^BK, respectively, are interpolated in a nonlinear part of the enzyme immunoassay calibration curves and are therefore less precise (these values are 4.8 and 9.6 pg/mL, respectively, in 175 cm^2 flasks). The sets of value for each size of flasks and for each type of immunoreactive kinin are heterogeneous (P<0.001) by Kruskall-Wallis test, except for immunoreactive des-Arg^9^BK in 175 cm^2 flasks. The Mann-Whitney test was applied to compare kinin concentrations from flasks with no cells (first line in each set of values) with concentrations in other cell flasks (P<0.001). To evaluate the effect of kallikrein, values from cells exposed to kallikrein treatment were compared with the corresponding group control without kallikrein treatment (preceding line; †P<0.05; ††P<0.01; †††P<0.001). Finally, the effect of the cell type on flasks pretreated with plasma and further treated with kallikrein was tested (fifth line compared with third line; ‡P<0.01).

**Figure 5.** Immunoblot of kininogens in extracts of HEK 293 cells based on antibodies raised against high-molecular-weight kininogen. Cells stably expressing B2R-GFP (tracks 3, 4) or untransfected cells (1, 2) were exposed to human citrated plasma and rinsed (2, 4) or left untreated (1, 3) before extraction. Both high- and low-molecular-weight kininogens are present in extracts from plasma-treated cells.
nmol/L) subsequently recorded but did not influence the contractile effect of histamine (100 μmol/L; Figure 6B). Similarly, aprotinin selectively prevented the contractile effect of tissue kallikrein (Figure 6C).

**Discussion**

Short treatments with sequencing-grade extracellular trypsin or endoproteinase Lys-C should result in the cleavage of the rabbit β2R sequence after Arg (trypsin) or Lys (both enzymes) residues. The number of these residues is small (4) in the extracellular domains of the receptor, and they are not well conserved in sequences from other mammalian species. None of the predicted primary C-terminal products (≥38.5 kDa) can be identified with certainty from the digested cells (Figure 1). Tissue kallikrein cleavage site(s) are less predictable from the amino acid sequence, but subtle differences in the digestion patterns suggest that the 3 tested enzymes

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**Figure 6.** Analysis of tissue kallikrein-induced contraction of the rabbit jugular vein. A, Tracings showing the contractile response to kallikrein and investigating desensitization and cross-desensitization with BK. Closed symbols refer to drug applications; open symbols, to the first of a series of tissue wash-outs with fresh Krebs' buffer. The tracings are representative of 2 experiments. B and C, Effect of continuous treatment of tissues with LF 16.0687 (1 μmol/L) or protease inhibition by aprotinin (final concentration, 10 μmol/L). C) on the responses to kallikrein, BK, and histamine. The kallikrein stimulation was always the first, and histamine the last. Values are the mean±SEM of 4 or 5 (B) or 4 (C) determinations. The effect of LF 16.0687 or aprotinin was assessed by the Mann-Whitney test for each contractile agent (*P<0.05, **P<0.01).

A submicromolar level of tissue kallikrein (50 nmol/L) stimulates the recombinant β2R-GFP (PLA2 assay, Figure 3A) or the wild-type β2R contained in the rabbit jugular vein (contractility, Figure 5) in a manner sensitive to both the selective antagonist LF 16.0687 and the protease inhibitor aprotinin. Furthermore, such a low concentration of kallikrein does not stimulate HEK 293 cells devoid of β2Rs (Figure 3B).

Hecquet et al suggested that tissue kallikrein exerts its effect on β2R after high-affinity binding and without the need for a catalytically active enzyme. At variance with them, we did not observe competition of [1H]BK binding to rabbit β2R-GFP by kallikrein at submicromolar concentrations. Such binding may occur at a site not conserved in the rabbit sequence. Limited proteolysis of the resting β2R may release a structural constraint of the molecule and mimic a conformation similar to that produced by agonist stimulation. However, receptor hydrolysis is not evident in cells expressing high levels of β2R-GFP and exposed to tissue kallikrein at 100 nmol/L in immunoblots (Figure 1). An entirely different interpretation of the pharmacological effect of kallikrein could be based on the cleavage of kininogen produced or taken up by cells with the generation of kinins. There is surface uptake of kininogen via more or less specific “receptors” in endothelial and other cell types. We have easily simulated kininogen cell uptake by exposing HEK 293 cells expressing or not β2R-GFP to human plasma (Figure 3B).
used in the immunoassays are not discriminative for N-terminal variants of the kinin sequence, so Lys-BK is included in the BK concentration, but not intact kininogen, as proteins are precipitated before the assay. Some receptor-mediated uptake of BK is likely, based on the difference of immunoreactive kinin concentrations measured in the culture media of cells that expressed or not recombinant B2-R-GFP (Table; this is why kinin release by kallikrein has been tested in untransfected HEK 293 cells when plasma pretreatment was not applied). Rabbit blood was in contact with the jugular vein before tissue isolation in experiments reported in Figure 6. An indirect mechanism of action of kallikrein through the local generation of kinins would explain the drastic desensitization of the jugular vein repeatedly exposed to kallikrein, as the local kininogen stores would be consumed to form BK. There is no cross-desensitization between kallikrein and BK (Figure 6), which suggests that receptor presence is not limiting, and therefore, that there is no important degradation of B2Rs exposed to 50 nmol/L of kallikrein.

In our estimation, the pharmacological activity of lower concentration levels (50 to 100 nmol/L) of tissue kallikrein on the rabbit B2R is highly tachyphylactic in isolated tissues, likely to be indirect, and dependent on the local formation of kinins. Local generation of kinins, which subsequently activate cell receptors, probably accounts for the effect of human kallikrein in various animal models. Transgenic mice with a liver-targeted expression of the enzyme and a hypotensive phenotype exhibited a maximal kallikrein serum level of 7 nmol/L. Only higher kallikrein levels (0.5 to 1 μmol/L) can cleave and degrade the B2Rs but also exert nonreceptor-mediated effects.

Perspectives

Results shown here and elsewhere based on the rabbit BK B2R suggest that this receptor does not efficiently function as a PAR when the pharmacological actions of kallikrein are considered. However, receptor degradation initiated by extracellular proteases (such as those secreted by activated neutrophils) is a possible mechanism for the downregulation of B2Rs observed in injured tissues.

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References


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