

Human Bradykinin B₂ Receptor Is Activated by Kallikrein and Other Serine Proteases

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ABSTRACT

Bradykinin (BK) and kallidin (Lys-BK), liberated from kininogens by kallikreins, are ligands of the BK B₂ receptor. We investigated whether kallikreins, besides releasing peptide agonist, could also activate the receptor directly. We studied the effect of porcine and human recombinant tissue kallikrein and plasma kallikrein on [Ca²⁺]_i mobilization and [³H]arachidonic acid release from cultured cells stably transfected to express human BK B₂ receptor (CHO/B₂, MDCK/B₂, HEK/B₂), and endothelial cells were used as control cells. As with BK, the actions of kallikrein were blocked by the B₂ antagonist, HOE 140. Kallikrein was inactive on cells lacking B₂ receptor. Kallikrein and BK desensitized the receptor homologically but there was no cross-desensitization. Furthermore, 50 nM human cathepsin G and 50 nM trypsin also activated the receptor; this also was blocked by HOE 140. Experiments ex-

cluded a putative kinin release by proteases. [³H]AA release by BK was reduced by 40% by added kininase I (carboxypeptidase M); however, receptor activation by tissue kallikrein, trypsin, or cathepsin G was not affected. Prokallikrein and inhibited kallikrein were inactive, suggesting cleavage of a peptide bond in the receptor. Kallikreins were active on mutated B₂ receptor missing the 19 N-terminal amino acids, suggesting a type of activation different from that of thrombin receptor. Paradoxically, tissue kallikreins decreased the [³H]BK binding to the receptor with a low K_D (3 nM) and inhibited it 78%. Thus, kallikreins and some other proteases activate human BK B₂ receptor directly, independent of BK release. The BK B₂ receptor may belong to a new group of serine protease-activated receptors.

Kallikrein was discovered in 1925 as a hypotensive agent in urine (Frey and Kraut, 1926), and, in the 1930s, the drop in blood pressure it causes was attributed to the release of an agent from a plasma protein (Werle et al., 1937). Since then, a great variety of kallikrein activities have been ascribed to the split products released from plasma protein substrates, either the independently discovered bradykinin (BK) (Rocha e Silva, 1963) or kallidin (Lys-BK) (Erdős, 1979). The former is liberated by plasma kallikrein, the latter by most tissue kallikreins, with the exception of the rat kallikrein (Bhoola et al., 1992). Rat kallikrein, however, has a direct effect: it contracts the rat uterus without releasing a kinin (Chao et al., 1981).

Tissue and plasma kallikreins are the products of different genes (Ashley and MacDonald, 1985; Bhoola et al., 1992), whereas the synthesis of their substrates, high- and low-molecular-weight kininogen, is directed by one gene (Bhoola et al., 1992). The nonapeptide BK and the decapeptide Lys-BK act on the B₂ receptor (Drouin et al., 1979; Bhoola et al., 1992), which is expressed constitutively and ubiquitously. It is normally present in endothelial cells, smooth muscles, and certain neurons, where its activation causes pronounced hypotension,

bronchoconstriction, pain, and inflammation (Erdős, 1979). The B₂ receptor belongs to the seven-transmembrane domain G protein-coupled receptor superfamily (Hess et al., 1992). BK activates the B₂ receptor by coupling either Gα_q protein (Gutowski et al., 1991; Liao and Homcy, 1993), which in turn activates phospholipase C, or the Gα_i protein (Liao and Homcy, 1993), acting through phospholipase A₂. The consequences are the release of NO and arachidonic acid (AA), the main indirect cardiovascular actions of BK attributed to the release of prostaglandins and/or NO (Bhoola et al., 1992).

Angiotensin I converting enzyme (ACE) has dual functions; it releases angiotensin II and inactivates bradykinin (BK) (Yang et al., 1971). Inhibitors of ACE have been used successfully in a variety of clinical conditions related to heart or kidney functions (HOPE Investigators, 2000). The beneficial effects that follow administration of ACE inhibitors have frequently been attributed to enhancing the actions of BK on B₂ receptor (Bhoola et al., 1992; Linz et al., 1995). Kinins decreased the duration of postischemic reperfusion arrhythmias and improved cardiac functions (heart rate, cardiac output, and oxygenation of the heart) and metabolic param-

ABBREVIATIONS: BK, bradykinin; Lys-BK, kallidin; AA, arachidonic acid; ACE, angiotensin I converting enzyme; CHO, Chinese hamster ovary; HEK, human embryonic kidney; MDCK, Madin-Darby canine kidney; BPAE, bovine pulmonary artery cells; PCR, polymerase chain reaction; D-Val-Leu-Arg-AMC, D-valyl-leucyl-arginyl-amino-methyl-coumarin; DFP, diisopropyl fluorophosphate; AM, acetoxymethyl ester; PAR, protease activated receptor.

eters (ATP, creatine phosphate, and glycogen levels) (Linz et al., 1995). ACE inhibitors also potentiate the actions of BK on the B₂ receptor indirectly and independently of blocking peptide breakdown, through interaction of ACE with B₂ receptor (Minshall et al., 1997; Erdös et al., 1999; Marcic et al., 1999). The B₂ receptor antagonist HOE 140 reverses the cardioprotective actions of kinins and some of those of ACE inhibitors (Linz et al., 1995; Marcic et al., 1999).

Here we report that the BK B₂ receptors are also directly activated by proteases. To demonstrate the protease activation of the receptor, we have examined the action of tissue and plasma kallikreins, trypsin, and also cathepsin G on [Ca²⁺]_i mobilization and [³H]AA release from Chinese hamster ovary (CHO), Madin-Darby canine kidney (MDCK), and human embryonic kidney (HEK) 293 cells stably transfected with the human B₂ receptor cDNA or bovine pulmonary arterial endothelial cells (BPAE). Consequently, kallikreins may play a direct role in the modulation of cellular responses and in the regulation of blood pressure via B₂ receptor activation.

Experimental Procedures

Materials

CHO, HEK 293, and MDCK cells were purchased from American Type Culture Collection (Rockville, MD). BPAE cells were obtained from Clonetics (Walkersville, MD). The cDNA encoding the human BK B₂ receptor was donated by Dr. K. Jarnigan (Syntex Co., Palo Alto, CA). Using polymerase chain reaction (PCR), the cDNA of human prokallikrein was isolated from the human kidney cDNA library obtained from Dr. Graeme Bell of the University of Chicago. Purified human urinary prokallikrein was prepared from human urine in our laboratory. Mammalian expression vector pCDNA3 was from Invitrogen (San Diego, CA); lipofectin and geneticin (G418) were from Life Technologies (Gaithersburg, MD). [5,6,8,9,11,12,14,15-³H(N)]-AA (100 Ci/mmol) and [³H]BK were purchased from American Radiolabeled Chemicals (St. Louis, MO). The fura-2/acetoxymethyl ester (AM) was obtained from Molecular Probes (Eugene, OR) and D-valyl-leucyl-arginyl-amino-methyl-coumarin (D-Val-Leu-Arg-AMC) from Enzyme Systems Products (Livermore, CA). Cathepsin G from human leukocytes was from Athens Inc. (Athens, GA) and human plasma kallikrein from Enzyme Research Lab (South Bend, IN). BK, porcine pancreatic kallikrein, thermolysin, trypsin, culture media, penicillin, and other peptides and chemicals were purchased from Sigma Chemical (St. Louis, MO). HOE 140 was a gift from Dr. B. A. Schölkens (Hoechst Co., Frankfurt, Germany). Carboxypeptidase M (Erdös and Skidgel, 1997) was obtained in this laboratory as recombinant enzyme (F. Tan and R. Skidgel, unpublished observations).

Cell Culture and Transfection

Construction of Human Prokallikrein Expression Vector. The human kidney cDNA library used was constructed in the bacteriophage λgt10 vector. The complete human prokallikrein cDNA was isolated from this library by a PCR method using two synthetic primers carrying the complementary sequence of exon 1 or exon 5. The human tissue prokallikrein was then cloned into the *EcoRI* site of pCDNA3. The direction of the insert was determined by restriction enzyme digestion and sequencing.

Stable Transfection of the Human Tissue Prokallikrein. MDCK cells were grown in Ham's F-12 culture medium supplemented with L-glutamine, penicillin-streptomycin, HEPES buffer, and 10% fetal bovine serum. One day before transfection, cells were seeded into 60-mm dishes at 30 to 40% confluence. Cells were then transfected with the human prokallikrein cDNA inserted into pCDNA3 vector using lipofectin. After 2 h of incubation at 37°C, cells

were thoroughly washed with Ham's F-12 medium and 2 ml of the same medium containing geneticin (G418; 800 μg/ml) was added to start the selection of stably transfected cells. Different clones were selected and propagated using cloning rings.

Activation of Human Recombinant Prokallikrein. MDCK cells previously transfected with human prokallikrein cDNA, as described above, were washed and incubated with serum-free Dulbecco's modified Eagle's medium. Prokallikrein was released from these cells into the medium over a period of 24 h. The prokallikrein-containing medium or purified urinary human prokallikrein was activated by thermolysin (1 μM) in 0.4 M Tris buffer containing 20 mM CaCl₂ at 37°C, pH 8.0, for 30 min. Activation by thermolysin was stopped with the inhibitor phosphoramidon (10 μM) (Noda et al., 1985; Takada et al., 1985).

Kallikrein Assay. Kallikrein activity was determined fluorometrically (Takada et al., 1985; Abe et al., 1995) with D-Val-Leu-Arg-AMC substrate. Fifty microliters of kallikrein solution was mixed with 445 μl of 0.1 M Tris-HCl, pH 8.0. The reaction was initiated with 5 μl of AMC substrate (final concentration, 0.1 mM). The amount of fluorogenic coumarin derivative liberated was measured in a spectrofluorometer with excitation at 380 nm and emission at 460 nm and the increase in fluorescence was monitored with a recorder. Active kallikrein concentration in the medium was determined using a standard dose-response curve obtained with commercial tissue kallikrein.

Inhibition of Kallikrein. Kallikrein (1 μM) was incubated at 37°C with 1 mM diisopropyl fluorophosphate (DFP) for 1 h. The samples were then exhaustively dialyzed at 4°C to remove the DFP. The inactivation of kallikrein was tested with the substrate, D-Val-Leu-Arg-AMC, by spectrofluorometry (Takada et al., 1985; Abe et al., 1995). In other experiments, 1 μM kallikrein was incubated at 37°C with 100 μM aprotinin for 1 h before being applied to the cells.

Stable Transfection of B₂ Receptor. The human B₂ receptor cDNA was inserted into pCDNA3 vector. CHO, HEK 293, or MDCK cells were stably transfected with the B₂ receptor and cloned as described above for prokallikrein (CHO/B₂, HEK/B₂, or MDCK/B₂).

Endothelial Cells. BPAE cells, which constitutively express BK B₂ receptors, were grown to confluence as described (Marcic et al., 1999).

Construction of 1–19 Deletion of Human B₂ Receptor Expression Vector. The 1–19 deletion of B₂ receptor cDNA (N-del-B₂) was obtained by combining a PCR-amplified 300–741 fragment with (742–1770) *SacI-XbaI* fragment of the cDNA of B₂ receptor. In the PCR reaction, the sequence of the upstream primer is 5'-CATGC-CATGGAATGCCCCCAAGTGGAG-3', which contains a translation initiation codon in the reading frame. The downstream primer used was 5'-CATGGGTGAGCTCAGGAGCA-3'. The 441-base-pair PCR fragment was cloned into PCR-E vector from Promega (Madison, WI) and sequenced. The 441-base-pair fragment with the correct sequence was recovered by digestion with *NotI* and *SacI* and ligated with 1 kb *SacI-XbaI* fragment of B₂ receptor into *NotI-XbaI* site of pCDNA3 expression vector. The sequence change is as follows: Wild-type, NH₂-MLNVTLQGPTLNGTFAQSKCPQVE... 1–19 Del, NH₂-MECPQVE...

Radioligand Binding. To select the clone with the highest expression of B₂ receptors and to determine the effect of kallikrein on BK binding to the receptor, [³H]BK saturation binding was performed (Minshall et al., 1997). Briefly, in routine assay, CHO cell monolayers in 6-well trays were washed with serum-free Ham's F-12 medium, placed on ice, and [³H]BK was added to 2.5 nM. Nonspecific binding was determined in the presence of 10 μM unlabeled BK. After an incubation for 1 h at 4°C, cell monolayers were washed with serum-free medium to eliminate the excess ligand, solubilized in 0.5 ml of a solution containing 0.1 M NaOH, 0.1 M NaHCO₃, and 1% SDS, and transferred to vials to be counted. Specific binding was calculated as the difference between total binding and nonspecific binding. Receptor numbers on CHO, HEK 293, and MDCK cell surface were determined to be 10⁶, 1.1 × 10⁶, and 1.9 × 10⁵ per cell.

[Ca²⁺]_i Mobilization. [Ca²⁺]_i was measured using a microspectrofluorometer (PTI Deltascan, Princeton, NJ), or with an Attofluor RatioVision digital fluorescence microscopy system, and the Ca²⁺-sensitive fluorescent dye, fura-2/AM. BPAE or transfected CHO cells were grown on glass coverslips and then incubated with 2 to 5 μM fura-2/AM for 1 h, washed with buffer, preincubated 15 min, subsequently placed in a Sykes-Moore chamber (Bellco, Vineland, NJ) filled with 200 μl of the same medium and mounted on an inverted microscope coupled to a microspectrofluorometer. The excitation wavelengths were 340 and 380 nm, and the emission 510 nm. The signals are represented in figures as a ratio of bound/free Ca²⁺, F₃₄₀/F₃₈₀ (Marcic et al., 1999).

[³H]AA Release. Transfected cells were grown to confluence in a 6-well dish. The medium was replaced with 1 ml of Ham's F-12 medium containing 0.5 μCi/ml of [³H]AA and cells were loaded for 16 h at 37°C. After washings with the incubation medium (Ham's F-12 medium complemented with 0.1% bovine serum albumin) the cells were incubated for 30 min at 37°C with either medium alone, 1 to 50 nM BK, or enzymes with or without 1 μM B₂ receptor blocker, HOE 140. Medium was removed and its [³H]AA content was determined by scintillation counting. The amount of released [³H]AA (ordinate) is shown after subtracting the background spontaneous release into medium (Minshall et al., 1997).

Statistical Analysis. Means and S.E. were calculated for the experiments and statistical significance of differences between means was tested by one-way analysis of variance (Excel; Microsoft, Redmond, WA).

Results

The effects of BK and kallikreins were tested on human B₂ receptor expressed in transfected CHO, HEK 293, MDCK, and endothelial cells.

Activation of Human Recombinant Prokallikrein. MDCK cells were transfected with cDNA of human prokallikrein, synthesized, and released into culture medium that contained no active kallikrein (C. Hecquet and E. Erdös, unpublished observations) similar to rat kallikrein (Abe et al., 1995). Human prokallikrein (Lottspeich et al., 1979) was fully activated by 100 nM thermolysin (Takada et al., 1985). The concentration of the enzyme secreted into the medium by 5 × 10⁶ cells in 24 h was 15 nM as determined by fluorometric assay. The activity of the enzyme was completely blocked by 100 μM aprotinin, an inhibitor of serine proteases.

[³H]AA Release. AA liberation from the cells expressing B₂ receptor was measured as a marker of phospholipase A₂ activation and release of prostaglandins. The amounts of BK and of the enzymes employed were determined in pilot studies. The log molar concentrations of the agents were plotted against [³H]AA release. The concentrations, which caused between 50 and 100% activation, were selected for the routine assays. Taking the amount of [³H]AA spontaneously liberated into the medium in 30 min as the baseline: with CHO/B₂ cells, 50 nM BK enhanced it 6-fold, (*n* = 9) and this increase was blocked by 1 μM B₂ antagonist HOE 140. Fifty nanomolar porcine pancreatic kallikrein, 4 nM human recombinant tissue kallikrein, or 50 nM trypsin also augmented the release of [³H]AA (by 4.3 ± 0.6, *n* = 10; 5.8 ± 0.4, *n* = 4; and 5 ± 0.5, *n* = 3; respectively). All effects were blocked by 1 μM HOE 140 (Fig. 1A). Both human and porcine tissue kallikrein were ineffective on CHO cells not transfected to express the B₂ receptor; the release of AA from these cells was insignificant (1.02 ± 0.4, 1.15 ± 0.4, *n* = 3 each; Fig. 1B).

Cathepsin G. Cathepsin G was used as a control serine protease because it is a chymotrypsin-type enzyme, (Molino et al., 1994; Salvesen, 1998). As such, it should not release kinins from kininogens and it does not inactivate BK (Skidgel et al., 1991). Fifty nanomolar human cathepsin G also enhanced the amount of [³H]AA released into the medium 2.5-fold (±0.3, *n* = 3) in 30 min compared with the baseline. The effect was abolished by 1 μM HOE 140. Similar to the other proteases tested, cathepsin G did not increase [³H]AA release from CHO cells lacking the B₂ receptor (Fig. 1C).

Human Plasma Kallikrein. Fifty nanomolar human plasma kallikrein also elevated the release of [³H]AA from CHO/B₂ cells by 4.63 ± 0.5 (Fig. 1D, *n* = 4) in 30 min compared with the baseline, and this liberation of AA was abolished by HOE 140. As with the other serine proteases, in control experiments, plasma kallikrein did not enhance [³H]AA release from CHO cells lacking the B₂ receptor (Fig. 1D, *n* = 3). These experiments indicate that kallikreins and other serine proteases act directly on the B₂ receptor.

[Ca²⁺]_i Mobilization and Desensitization. [Ca²⁺]_i mobilization stimulated by agonist was determined as a marker of the phospholipase C activation via B₂ receptor upstream and NO release downstream. Both BK (10 nM) (Fig. 2A, *n* = 12) and pancreatic kallikrein (10 nM) (Fig. 2B, *n* = 10) induced a similar transient increase of the [Ca²⁺]_i level in transfected MDCK cells. Kallikrein activated the receptor in less than 10 s. Each agonist desensitized the receptor homologically, because a repeated dose of the agonist did not cause a second response. However, BK did not desensitize the receptor to kallikrein (Fig. 2C, *n* = 6) and kallikrein did not desensitize the receptor to BK (Fig. 2D, *n* = 4). Consequently, there was no cross-desensitization of the B₂ receptor by porcine pancreatic kallikrein and BK in CHO cells, although both actions were blocked by 100 nM HOE 140 (Fig. 2E, *n* = 5). This is taken as an additional strong indication that kallikrein has a direct effect on the receptor. Porcine pancreatic kallikrein did not elevate [Ca²⁺]_i level in MDCK cells lacking the B₂ receptor (Fig. 2F, *n* = 5), where ionomycin was still active.

As shown in Fig. 3, A and B, where the effect of kallikrein was simultaneously recorded in 18 HEK/B₂ cells, kallikrein did not desensitize the B₂ receptor to BK. Similar results were observed in 66 BPAE cells, which express B₂ receptor constitutively (Fig. 3, C and D).

Cathepsin G Mobilizes [Ca²⁺]_i in HEK/B₂ Cells. Cathepsin G also activated the B₂ receptor to raise [Ca²⁺]_i level (Fig. 4). BK (10 nM) or cathepsin G (10 nM) increased the [Ca²⁺]_i level transiently and desensitized the receptor homologically to subsequent administration of BK or cathepsin G (results not shown). However, there was no cross desensitization of the B₂ receptor by cathepsin G or by BK (Fig. 4, A and B, *n* = 4). Cathepsin G was inactive in cells pretreated with 100 nM HOE 140 (Fig. 4C) and in HEK 293 cells lacking the B₂ receptor expression (Fig. 4D).

Effect of Kininase. To further confirm the activation of B₂ receptor by kallikreins and to eliminate a putative kinin release by kallikreins, activation of the B₂ receptor was done in the presence of added recombinant carboxypeptidase M. Carboxypeptidase M is a kininase I-type enzyme that converts BK to desArg⁹ BK (Erdös and Skidgel, 1997). DesArg⁹ BK acts on the B₁ but not on the B₂ receptor (Bhoola et al., 1992). The effects of 50 nM CPM on [³H]AA release from the

transfected CHO cells induced by 50 nM BK, porcine pancreatic kallikrein, and the other serine proteases, cathepsin G and trypsin, in 30 min have been compared. The release of [³H]AA was reduced by 40% by added carboxypeptidase M, but the activation of the B₂ receptor by kallikrein, trypsin, or cathepsin G in the presence of carboxypeptidase M was not significantly modified, as measured by [³H]AA release (data not shown).

Effect of Prokallikrein and Inhibited Kallikrein. To demonstrate that B₂ receptor is activated only by active proteases, we tested human tissue prokallikrein and inhibited kallikrein on the receptor. The active site of human kallikrein and, as a control, that of porcine kallikrein (results not shown) were blocked by 100 μM aprotinin or DFP. Recombinant kallikrein, inhibited by DFP or aprotinin, prokallikrein, and, as a control, thermolysin-activated human kallikrein, were tested on [Ca²⁺]_i mobilization and [³H]AA release from MDCK or CHO cells expressing the B₂ receptor

(results not shown). Activated prokallikrein (50 nM) was active as described above but 50 nM prokallikrein, DFP-treated kallikrein, or aprotinin-treated kallikrein did not elevate [Ca²⁺]_i level (*n* = 5) or increase [³H]AA release (*n* = 3), suggesting that catalytic activity is necessary for the activation of the B₂ BK receptor.

N-Terminal Deleted B₂ Receptor. The amino-terminal, extracellular portion of the human BK B₂ receptor consists of 26 residues with a lysine in the 19th position, (Lys¹⁹) (Hess et al., 1992). Because of the specificity of tissue kallikrein to cleave peptide bonds at basic amino acid residues (Bhoola et al., 1992), it may hydrolyze the extracellular portion of the receptor either at the C-terminal end of Lys¹⁹ or even before Lys¹⁹, which is similar to cleaving the low-molecular-weight kininogen at a Met-Lys bond to release Lys-BK. To investigate whether kallikrein would hydrolyze the receptor similarly to thrombin cleaving the extracellular peptide of its receptor, a protease-activated receptor (PAR-1) (Dery et al.,

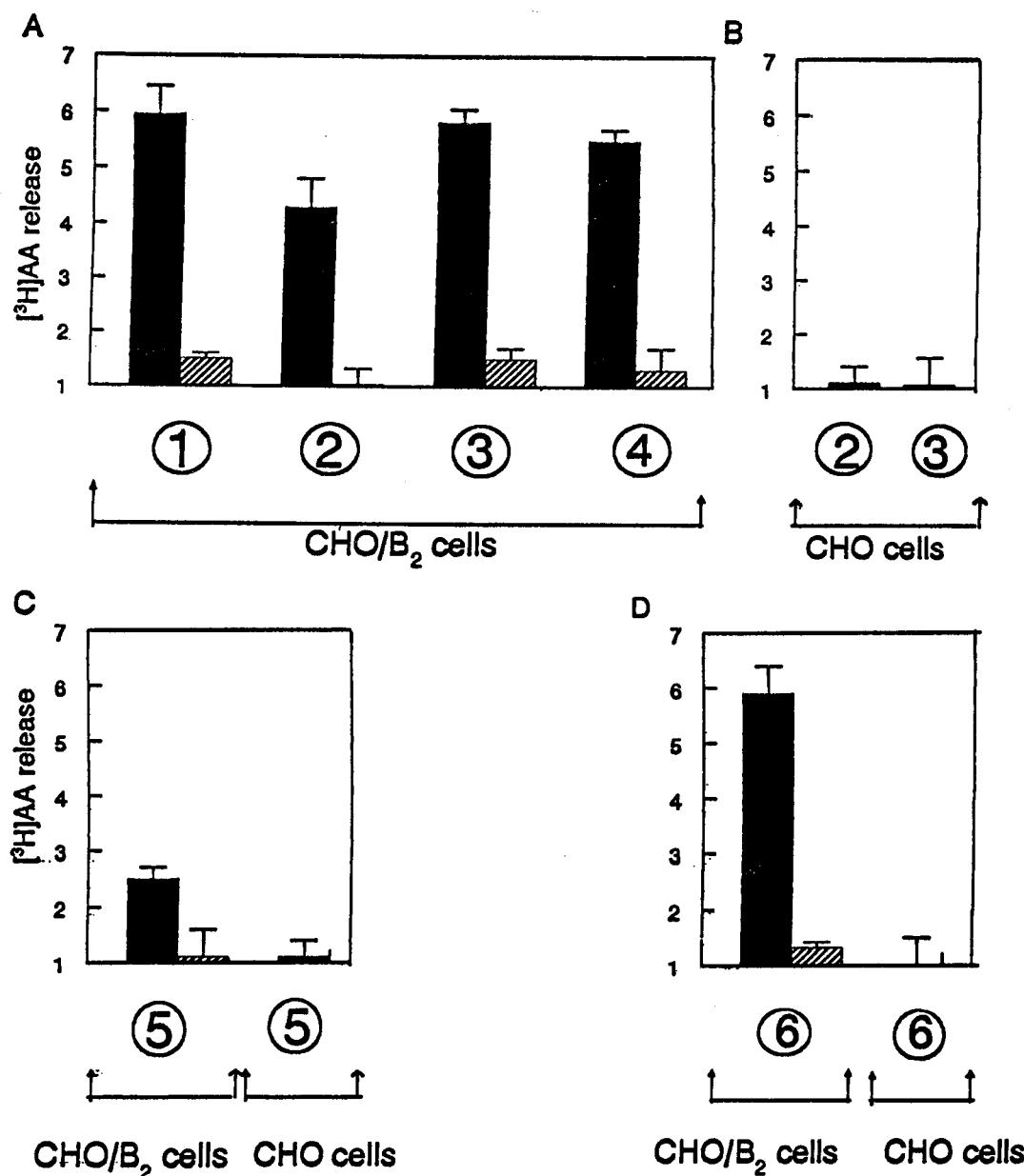


Fig. 1. Release of AA via activation of B₂ receptor by serine proteases. HOE 140 inhibited the activity of proteases. Proteases ineffective on CHO cells not expressing receptor. Ordinate, arachidonic acid release. Solid columns, agonist alone. Cross-hatched, in presence of HOE 140 1 μM. A, 1, BK, 50 nM (*n* = 10); 2, porcine pancreatic kallikrein, 50 nM (*n* = 10); 3, human recombinant tissue kallikrein, 4 nM (*n* = 4); 4, trypsin 50 nM (*n* = 4) ± S.E.M. B, tissue kallikrein (2 and 3 as in A), 50 nM inactive in cells lacking B₂ receptor (*n* = 3). C, activation of B₂ receptor by cathepsin G (5), 50 nM, was also blocked by HOE 140; the enzyme was inactive in nontransfected cells (*n* = 3). D, human plasma kallikrein, 50 nM, activated CHO/B₂ cells; this was blocked by HOE 140 (6) and was inactive in non-transfected cells.

1998), a human B_2 receptor mutant lacking the 19 amino acids of the N-terminal sequence of the receptor (Nt-del B_2), was constructed. The mutant lacked basic amino acids at the remaining N-terminal extracellular portion.

Figure 5 compares the level of [3 H]AA released by porcine pancreatic kallikrein or BK from cells transfected with wild-type human B_2 receptor or with the mutated human receptor. Transfected CHO cells expressing an equivalent number of mutated receptors (10^6 receptors per cell) on the cell surface as the wild-type CHO- B_2 cells were selected by using radioligand binding assay (see *Experimental Procedures*) for the experiments. The amount of [3 H]AA released induced within 30 min by either 50 nM BK or 50 nM porcine pancreatic kallikrein in the cells transfected with wild-type B_2 receptor, CHO/ B_2 (5.9 ± 0.5 and 5 ± 0.5 over baseline, $n = 3$), were similar to those of the mutated B_2 transfected cells (CHO/Nt-del- B_2) (5.5 ± 0.7 and 5.4 ± 1 , $n = 3$).

[3 H]BK Binding Displacement by Kallikrein. Besides activating the BK receptor, kallikrein (somewhat paradoxically) also competes with BK for a site on B_2 receptor. To determine whether kallikrein affects the binding of BK to the receptor, [3 H]BK was used in displacement binding studies (Fig. 6). Compared with the maximum binding, which is

taken as 100%, both purified human urinary kallikrein and porcine pancreatic kallikrein modulated the binding of the BK to the receptor with low EC_{50} values of 2.2 ± 0.5 and 10 ± 0.6 nM, respectively; the maximum inhibition was $77 \pm 3\%$ ($n = 3$) and $78 \pm 3\%$ ($n = 3$). Accordingly, kallikrein binds to the B_2 to decrease the binding of BK. Interestingly, human kallikrein was more potent than porcine kallikrein in displacing BK from the human receptor, suggesting species specificity. Trypsin and cathepsin G did not significantly modify the binding of BK to the receptor (Fig. 6). Trypsin detaches cells at a concentration over 10^6 M, which explains the sudden drop in BK binding.

Figure 7 shows the [3 H]BK binding displacement by the active and by the inhibited form of kallikreins. Purified human urinary kallikrein was inhibited by either 1 mM DFP or 100 μ M aprotinin completely. Figure 7 inset shows inhibition of the hydrolysis of D-Val-Leu-Arg-AMC by human tissue kallikrein.

Compared with the maximum binding, DFP-inhibited human urinary kallikrein also was still active, to modulate the binding of the BK to the receptor with a K_D value of 2.0 ± 0.5 nM. The maximum inhibition of BK binding was $80 \pm 3\%$ ($n = 3$).

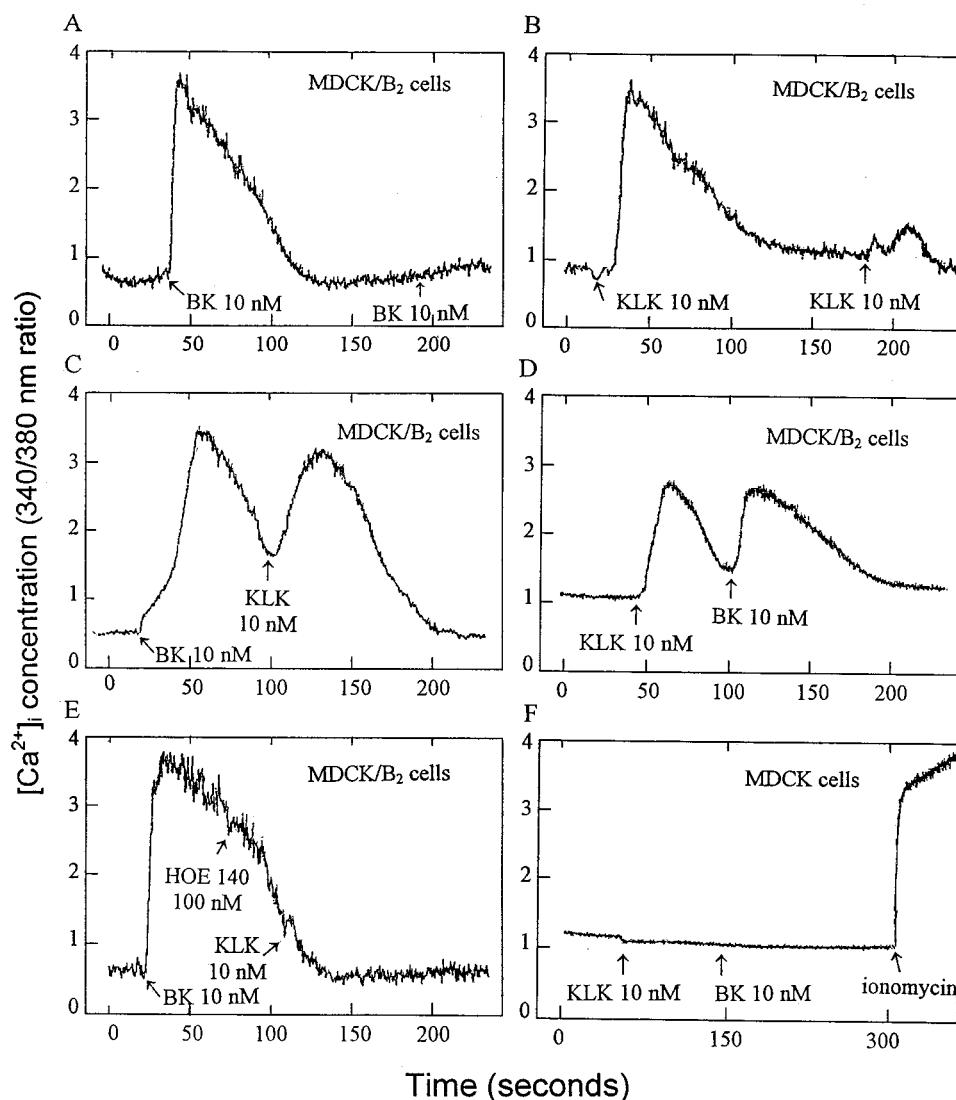


Fig. 2. Mobilization of $[Ca^{2+}]_i$ level in B_2 receptor transfected cells by BK and kallikrein (KLK). Homologous desensitization of the receptor by 10 nM BK (A) and 10 nM kallikrein (B) but no cross-desensitization of the receptor (C, D). Abscissa, time in seconds. Ordinate, relative $[Ca^{2+}]_i$ level. (BK, $n = 12$; pancreatic kallikrein, $n = 10$). HOE 140 blocks kallikrein activity (E), both agents inactive in absence of B_2 receptor. Ionomycin is still active in MDCK cells lacking B_2 (F).

In contrast, aprotinin-treated kallikrein did not affect the binding of BK to the receptor, probably because of the steric interference caused by the fact that the aprotinin kallikrein complex has a much higher molecular weight than kallikrein phosphorylated at a serine residue by DFP. In additional control studies, aprotinin (100 μ M) did not affect the binding of BK to the B₂ receptor (data not shown). Prokallikrein was much less active in displacing [³H]BK from the receptor ($34 \pm 6\%$ at 100 nM). Even this low degree of inhibition might have been caused by the presence of a trace amount of active kallikrein in the prokallikrein solution, as shown on the D-Val-Leu-Arg-AMC hydrolysis curve (Fig. 7, inset). The failure of the proenzyme to inhibit binding can be attributed to the lack of conformational change induced by activation of prokallikrein (Takada et al., 1985).

Discussion

We report here that the human BK B₂ receptor, besides being activated by peptide ligands, is also activated by proteases, including plasma and tissue kallikrein, trypsin, and cathepsin G. Recombinant human tissue kallikrein was most potent, and inactive kallikreins (inhibited or prokallikrein)

had no effects. Kinins release prostaglandins and NO; the liberation of these agents are among the important functions of the peptides (Bhoola et al., 1992). Increase in [Ca^{2+}]_i level or AA release was measured as markers of their biosynthesis (Marcic et al., 1999). Kallikreins and the other serine proteases tested induced a transient [Ca^{2+}]_i mobilization and AA release, which were blocked by a specific B₂ receptor antagonist, HOE 140. The enzymes were ineffective on cells lacking B₂ receptors.

Human endothelial cells express the BK B₂ receptor, among others (Marcic et al., 1999), as well as PARs of thrombin and trypsin (Molino et al., 1997). In the presence of zinc ions, high-molecular-weight kininogen binds to the endothelial cells and may serve as substrate for a kallikrein to liberate BK (Colman et al., 1997). Therefore, our main concern was to show that kallikreins and the enzymes tested did not act by releasing kinins from a kininogen hypothetically bound to the membrane of transfected cells. We believe that results obtained show that this is not the case.

First, although both BK and kallikreins elevate [Ca^{2+}]_i level and desensitize the receptor homologously, there was no cross-desensitization of the B₂ receptor by kallikrein and BK

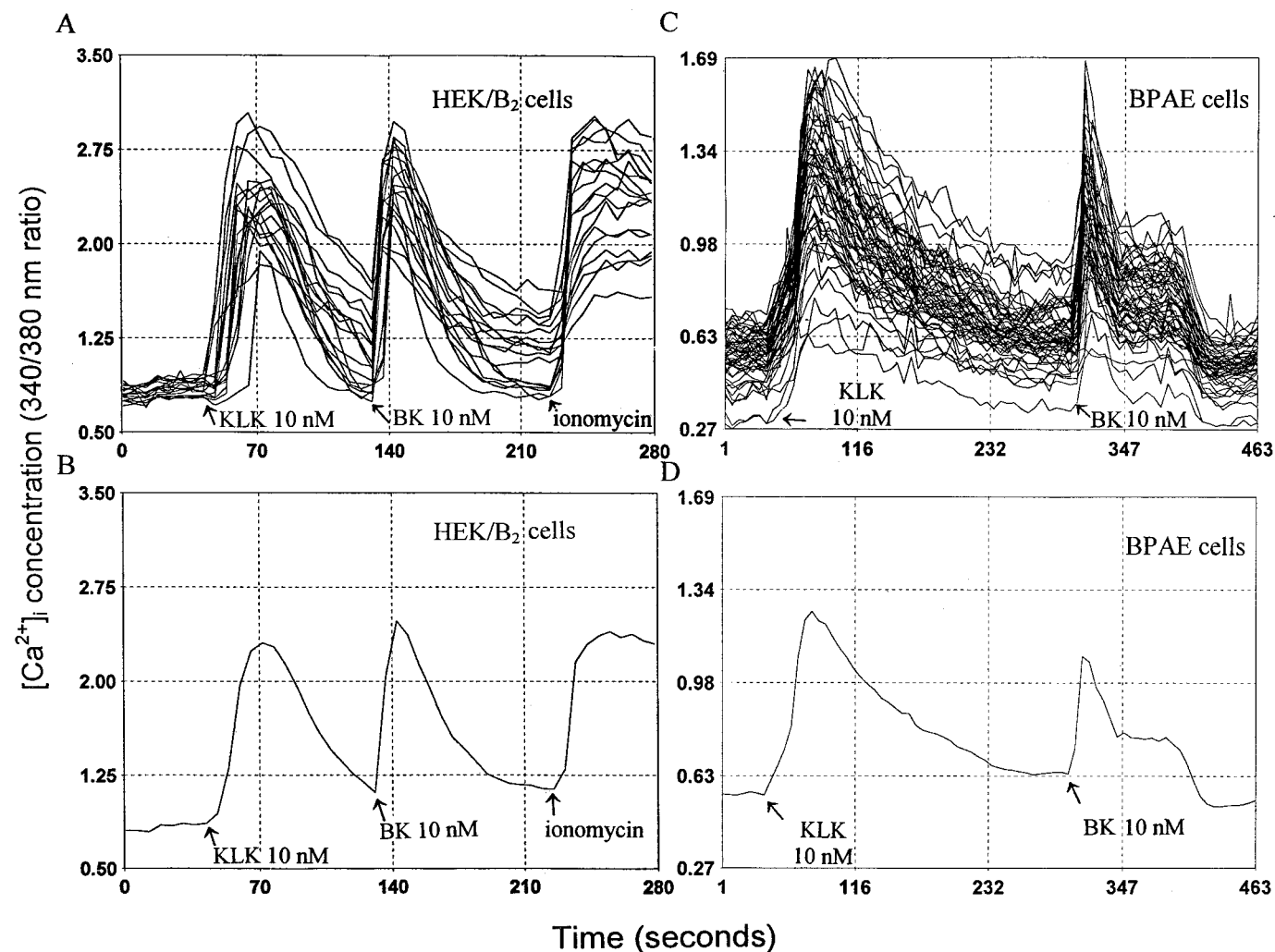


Fig. 3. [Ca^{2+}]_i mobilization induced by 10 nM BK and 10 nM porcine pancreatic kallikrein (A, B) or 10 nM human recombinant kallikrein (C, D). A, measurement in 18 single HEK/B₂ cells. B, mean values for A. C, measurement in 66 single BPAE cells. D, mean values for C. Note: there is no cross-desensitization between kallikrein and BK. Abscissa, time in seconds. Ordinate, relative [Ca^{2+}]_i level ($n = 3$).

in CHO, MDCK, or endothelial cells. Administration of BK desensitized the receptor to a second dose of BK (Fig. 2) but not to kallikrein and vice versa. If the response to kallikrein were caused by the kinins released from kininogen precursor, the first dose of kallikrein would desensitize the B₂ receptor to the following dose of BK. These experiments are also taken as an indication that BK and kallikreins both activate the B₂ receptor, but not necessarily at the same site.

Second, carboxypeptidase M, which cleaves Arg⁹ of BK (Erdös and Skidgel, 1997), did not affect activation by proteases, although it lowered the BK effect on B₂ by 40%.

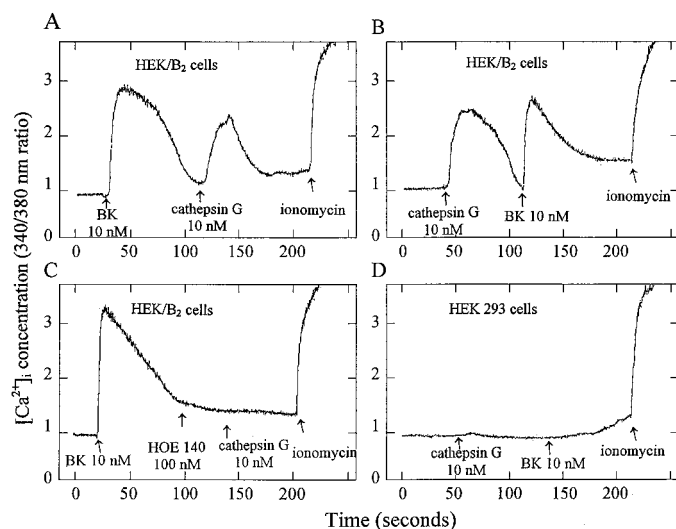


Fig. 4. Effect of cathepsin G on $[Ca^{2+}]_i$ level. Cathepsin G (Cat G) activates B₂ receptor in HEK B₂ cells (A, B) but no cross-desensitization with BK (10 nM). HOE 140 (100 nM) blocks cathepsin G activity (C). Cathepsin G and BK are inactive on HEK 293 cells lacking B₂ receptor but ionomycin still raises $[Ca^{2+}]_i$ level (D). Abscissa, time in seconds. Ordinate, relative $[Ca^{2+}]_i$ level ($n = 3$).

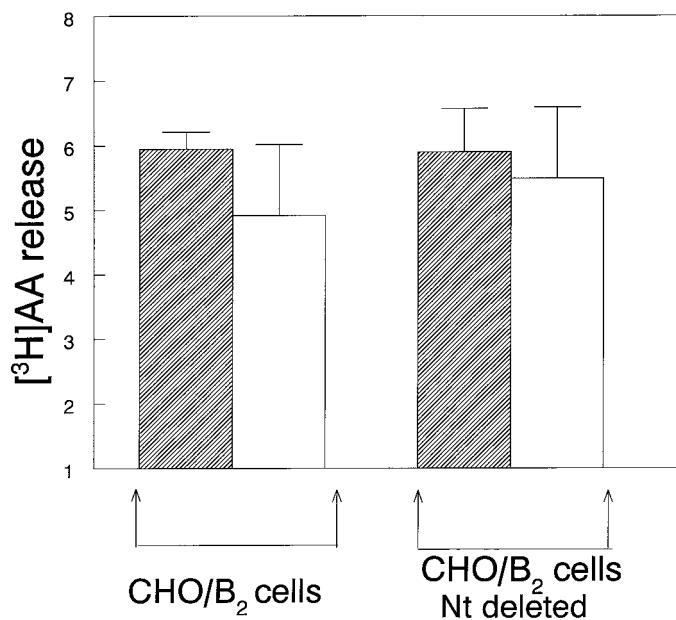


Fig. 5. Stimulation of B₂ receptor truncated at N terminus by BK and pancreatic kallikrein to release arachidonic acid. The extracellular N-terminal 19 residues of the B₂ receptor including Lys¹⁹ were deleted (Nt). BK (crosshatched bars, 50 nM) and kallikrein (open bars, 50 nM) were equally active on wild-type and Nt B₂ receptors ($n = 3$). Ordinate, \pm S.E.M. as in Fig. 1.

Third, cathepsin G, mainly a chymotrypsin-type of enzyme, should not release kinins from kininogens but can be a potentially important activator of the B₂ receptor (Molino et al., 1994). Cathepsin G is released by activated neutrophils at sites of injury or inflammation, reaching an estimated concentration of 0.5 μ M (Hung et al., 1992), and it is also present on plasma membrane (Skidgel et al., 1991). Human cathepsin G activated the B₂ receptor in the transfected cells, enhanced AA release, and elevated $[Ca^{2+}]_i$.

Fourth, trypsin releases kinin more from denatured proteins than from native proteins (Diniz and Carvalho, 1963; Habermann, 1970), but trypsin also activated the receptor. The $[Ca^{2+}]_i$ level of all proteases increased immediately (<12 s) when added to the CHO/B₂ cells.

Fifth, the experiments were also repeated using zinc-free medium and cells were washed with Ca₂-EDTA to sequester zinc that may be on cell membrane to anchor kininogen. These experiments (data not shown) yielded the same results.

The experiments make it evident that the phenomena described are not caused by the release of kinins by hydrolysis of putative cell surface kininogen, especially because the studies were done on thoroughly washed cells in serum-free medium. The cells were grown on glass coverslips to study $[Ca^{2+}]_i$ mobilization, and AA release was performed in the presence of bovine serum albumin to exclude even a trace of plasma kininogens that might be bound to plastic culture plates. Besides, the results obtained with three different types of cells transfected to express B₂ receptor and with endothelial cells were quite similar.

Thermolysin, a metalloprotease, hydrolyzes peptide bonds at the amino side of hydrophobic amino acids and activates human prokallikrein by cleaving the Arg⁰-↓-Ile⁺¹ bond (Takada et al., 1985). Recombinant human tissue prokallikrein, released into culture medium from transfected MDCK cells, is fully activated by thermolysin, which releases a heptapeptide (Lottspeich et al., 1979; Noda et al., 1985; Takada et al., 1985). The newly revealed N-terminal Ile forms an internal bridge with an Asp residue to induce a change in conformation, exposing the active site (Takada et al., 1985). Rat prokallikrein has a Val¹ instead of Ile¹ and is activated by thermolysin only about half as much as human prokallikrein (C. Hecquet and E. Erdös, unpublished observations; Abe et al., 1995).

Kallikrein activates the B₂ receptor, very likely by cleaving a peptide bond(s), given that prokallikrein and inhibited kallikrein were inactive. Kallikrein hydrolyzes peptides at the carboxylic side of arginine and sometimes lysine, preferentially at Phe-(or Leu)-Arg-Xaa bonds (Bhoola et al., 1992). The extracellular part of the B₂ receptor has several potential cleavage sites for kallikrein. Lys¹⁹ is in the N-terminal end; others are in extracellular loops (Hess et al., 1992). We explored the possibility that kallikrein cleaves the receptor in a manner similar to the activation of PAR by thrombin (Dery et al., 1998). PAR-1, -3, and -4 thrombin receptors are G protein-coupled receptors activated by cleaving at the N-terminal end to form a tethered ligand (Bohm et al., 1998; Dery et al., 1998). This also applies to the PAR-2, tryptase, and trypsin receptors (Dery et al., 1998). Thrombin receptor has an N-terminal exodomain of 94 residues, activated at the Arg⁴¹-↓-Ser⁴² bond (Bohm et al., 1998; Dery et al., 1998). The N terminus of the human BK B₂ receptor has an estimated 26 amino acids; kallikrein may cleave at Lys¹⁹ to

activate the receptor. To explore that idea, kallikrein was tested on a B₂ receptor mutant with a 19-residue N-terminal exodomain peptide deleted. Tissue kallikrein activates the B₂ receptor differently than thrombin does the PARs because it was equally active with the mutated receptor and wild-type receptor. Other possible sites for cleavage and activation by kallikrein are located in the extracellular loops (Hess et al., 1992). Cathepsin G, a chymotrypsin-type enzyme, may activate by cleaving at a different residue (Molino et al., 1994),

although it hydrolyzes some proteins at arginine (Bohm et al., 1998). In all of the above experiments, HOE 140 blocked the activation of the receptor, and the proteases were inactive on cells lacking the B₂ receptor.

Kallikrein was also tested on cells other than transfected CHO, HEK 293, or MDCK cells. Kallikrein activated the B₂ receptors in endothelial cells that constitutively express them, as shown by elevation of [Ca²⁺]_i level (Fig. 3, C and D). Again the effects were not caused by a potential BK release,

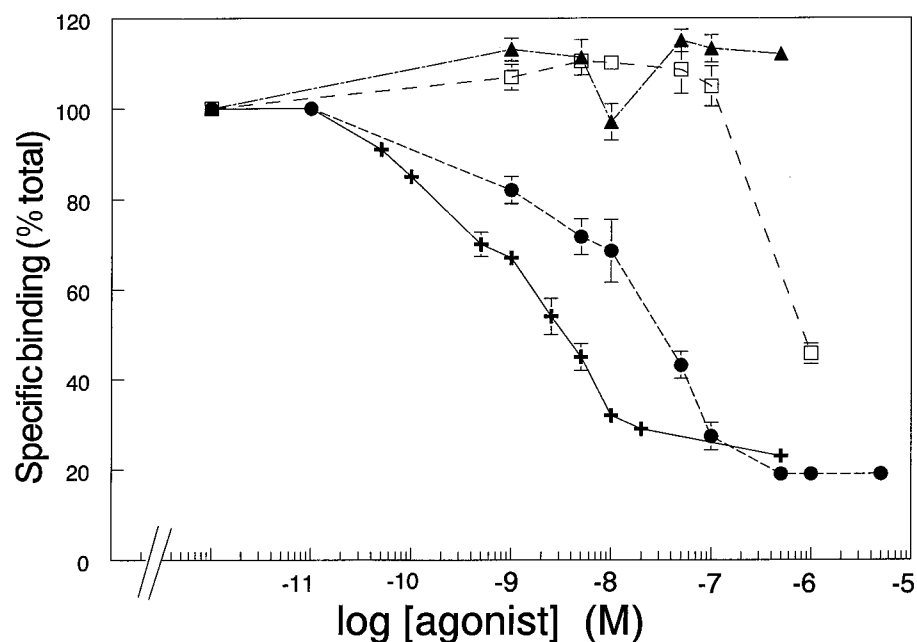


Fig. 6. Binding displacement of [³H]BK by serine proteases on CHO/B₂ cells. B₂ receptor transfected CHO cells were incubated with [³H]BK in the presence or absence of increasing concentrations of human tissue kallikrein (+), porcine pancreatic kallikrein (●), cathepsin G (▲), or trypsin (□). Specific binding was calculated as the difference between total binding and nonspecific binding and expressed as percentage of total binding (ordinate). Abscissa, concentration of agonist proteases (*n* = 3) ± S.E.M.

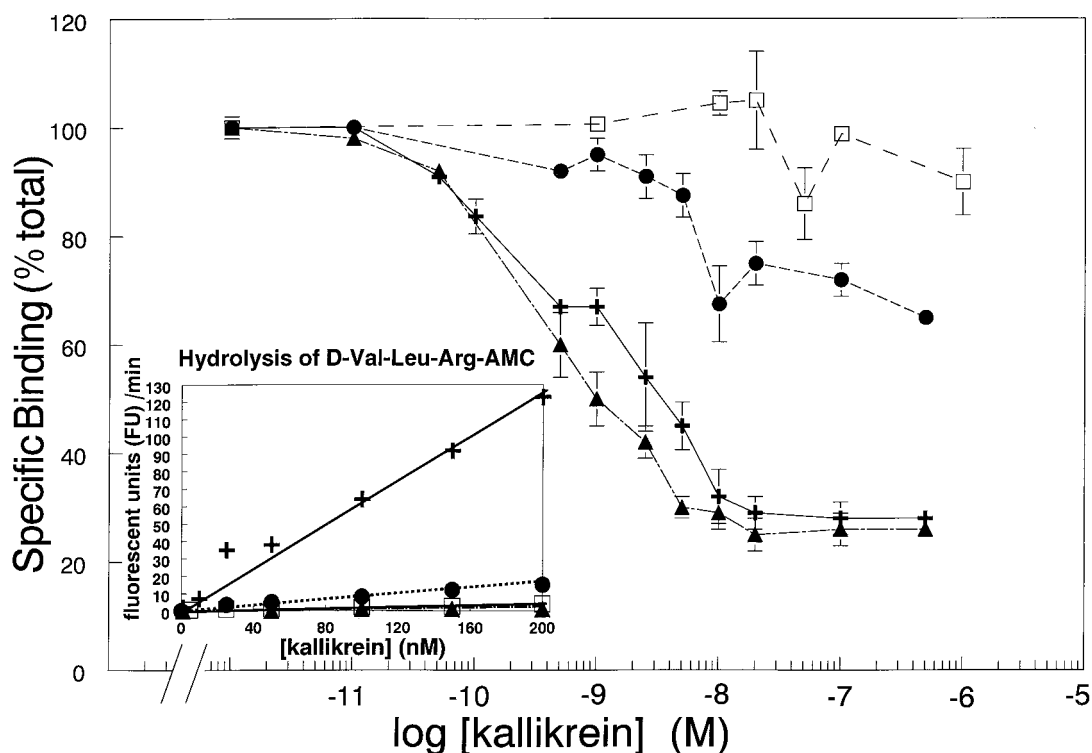


Fig. 7. Binding displacement of [³H]BK by kallikrein on CHO/B₂ cells. B₂ receptor transfected CHO cells were incubated with a constant concentration of [³H]BK in the presence or absence of increasing concentrations of 1) thermolysin-activated human tissue kallikrein (+); 2) human tissue prokallikrein (●); 3) DFP-treated human kallikrein (▲); or 4) aprotinin-treated human kallikrein (□). Ordinate and abscissa as in Fig. 5. Inset, hydrolysis of substrate by the kallikrein preparation (*n* = 3) ± S.E.M.

because there was no cross-desensitization between kallikrein and BK, although both agents homologously desensitized the receptor in endothelial cells as well.

The physiological and pathological relevance of kallikrein activity has been the subject of many studies. For example, the level of human urinary kallikrein has been associated with blood pressure control: hypertensive patients excrete less kallikrein in urine (Margolius, 1995). Renal kallikrein is located along the luminal membrane of the connecting tubular cells, where it is released (Scicli et al., 1976), and on the basolateral side of distal tubular cells (Yamada and Erdös, 1982). Kallikrein releases kinins into the lumen of the distal nephron, and kinin receptors are present in the collecting duct (Schanstra et al., 1999). Kallikrein can enter the circulation from the basolateral side of the nephron; this bipolar secretion was also shown in MDCK cells (Abe et al., 1995); possibly, however, tissue kallikrein may act more on B₂ receptors at the luminal side.

A direct activation of the B₂ receptor by a plasma or tissue kallikrein can be important in a variety of conditions at the multiple sites of actions of kinins (Erdös, 1979). These range from algogenic effects to reconditioning the heart and reducing peripheral resistance (Bhoola et al., 1992; Linz et al., 1995).

Tissue kallikrein possesses a dual activity. It may enhance apparent BK actions by activating its receptor directly, but, at low concentration, it can decrease BK binding to the receptor by competing with it (Figs. 6 and 7). Tissue kallikrein affected BK binding to receptor, even when inhibited by DFP; thus, this competitive binding was independent of protease activity. Kallikrein inhibited by a higher molecular weight inhibitor, aprotinin, did not compete with BK for a binding site, suggesting that kallikrein coupled to protein inhibitors such as kallistatin (Chao et al., 1997) will neither cleave nor bind to the B₂ receptor. Aprotinin in separate experiments did not affect the binding of peptide ligand to the receptor (not shown). Prokallikrein, which has a different conformation than active kallikrein, was much less competitive. In general, the other protease activators of the B₂ receptor did not compete with BK for attachment to the receptor. Two ligands can bind simultaneously to a receptor to form a ternary complex (Hulme, 1996). Kallikrein significantly decreases the BK binding to the receptors by 80%, but 20% of the receptors still bind BK. Kallikrein, being of much higher molecular weight, probably binds to the receptor also at other sites different from the BK and may decrease its affinity for kinin-type agonists.

In summary, kallikreins and some other proteases activate the BK B₂ receptor directly, independently of kinin release. Paradoxically, tissue kallikrein decreases [³H]BK binding to the receptor. Kallikreins may play a direct role, for example, in the regulation of blood pressure or cardiac function by activating the B₂ BK receptor, which may belong to a newly established group of PARs.

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