Spontaneous Formation of a Proteolytic B1 and B2 Bradykinin Receptor Complex with Enhanced Signaling Capacity*

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B1 bradykinin receptor (B1R) induction is critical in the adaptation of the kinin-mediated inflammatory response from a B2 bradykinin receptor (B2R) subtype to a B1R subtype that occurs during chronic insult. Here, we show that B1R spontaneously forms a proteolytic plasma membrane complex with B2R along with increased receptor signaling capacity. Co-expression of hemagglutinin-tagged B2R with FLAG-tagged B1R in HEK293 cells resulted in degradation of B2R as determined by the diminution of the intact 65-kDa B2R species and the appearance of proteolytic B2R products at 30–40 kDa and by the reduction in B2R bradykinin binding sites. On the other hand, the 55-kDa B1R remained intact. Receptor co-expression also led to an increase in constitutive and agonist-stimulated receptor signaling. Selective immunoprecipitation with epitope-specific antibodies revealed a spontaneously formed heterologous receptor complex, which was composed of the intact 35-kDa B1R and the B2R degradation products. Cellular fractionation, cell surface biontination, and immunolabeling and confocal microscopy showed that B2R-B1R complexes were present on the cell surface. This is the first evidence that a heterologous G protein-coupled receptor complex in the plasma membrane is linked to proteolytic degradation of a participating receptor, and this mechanism may contribute to the adaptation of the kinin response from a B2 type to a B1 type during chronic insult.

Kinins are potent agonists who participate in the inflammatory and pain responses to insults by acting through two receptor subtypes named B1 and B2 (1). During sustained insult, the kinin-mediated response adapts from a B2 type in the acute phase to a B1 type in the chronic phase (2). This adaptation is explained in part by an induction of B1R from essentially a null level, which is triggered by pro-inflammatory cytokines (3) and important in the chronic response (4, 5). B1R induction may be necessary to prolong the kinin response because it has been observed that the cellular B2 response desensitizes rapidly, whereas the B1 response is sustained (6). Indeed, B1R is constitutively active, i.e. active in the absence of agonist (7).

It is now well established that G protein-coupled receptors are capable of hetero-oligomerizing in the cell (8), but in only a few cases has a direct causal relationship been established between a hetero-oligomer and a functional outcome. The best described hetero-oligomeric G protein-coupled receptor system is the GABAB receptor, in which the type 1 receptor is responsible for recognizing GABA, the type 2 receptor for agonist-dependent receptor G protein coupling, and expression of both receptors is required for proper trafficking to the plasma membrane (9–11). Other heterologous G protein-coupled receptor combinations have revealed receptor trans-activation and changes in pharmacological profiles, agonist efficacy, and receptor internalization.

B1R and B2R subtypes are co-expressed on many cell structures on which B1R induction may be readily observed including primary sensory neurons, which are critical for the pain response (12–16). Furthermore, cellular cross-talk between B1R and B2R occurs via bradykinin (BK) stimulation of B2R, which leads to an autocrine up-regulation of B1R through an interleukin-1β-mediated mechanism (16). Also, in some cells that co-express the receptors, such as PC3 prostate cancer cells, the pharmacological profile of kinin signaling through each receptor subtype suggests the co-engagement of the other subtype in the response (15).

On this basis, we hypothesized that in cells that co-express B1R and B2R, these receptors directly interact to enhance signaling. In this report we investigated the effect of heterologous co-expression of B1R and B2R. Our results show that B1R promotes the formation of a plasma membrane complex with B2R, which involves proteolytic degradation of B2R along with increased constitutive and agonist-stimulated kinin receptor signaling. This event may be part of a cellular adaptation of kinin signaling in the inflammatory and pain response from a B2 type to a B1 type that occurs during sustained insult.

**EXPERIMENTAL PROCEDURES**

*Cell Culture, Mutation, and Transfection—*HEK293 cells were grown, and mutations and transfections were done as described previously (7). The FLAG and HA epitopes were inserted at the receptor N terminus immediately following the initial methionine.

*Plasma Membrane Fractionation—*Enrichment of plasma membranes followed the procedure by Smart et al. (17), with a few modifications as described previously by us (18).

*Functional Receptor Assays—*Receptor activities were assayed by monitoring PI hydrolysis in cells transfected with a series of receptor cDNA amounts and labeled with 1 μCi/ml [3H]inositol as described previously (7). B1R and B2R densities were determined using saturating concentrations (2–3 nM) of [3H]BK and [3H]BK (PerkinElmer Life Sciences), respectively. The slope factors of the increases in basal cel-
lular PI hydrolysis and agonist-stimulated PI hydrolysis as a function of the level of receptor expression were used as parameters of constitutive and agonist-dependent receptor activity.

Cell Surface Biotinylation—Cells were biotinylated with 4 ml of 0.3 mg/ml non-permeable polyethylene oxide-maleimide activated biotin (Pierce) for 30 min at 4 °C, washed two times with ice-cold phosphate-buffered saline, and quenched with 1 mM dithiothreitol/phosphate-buffered saline for 10 min at 4 °C. After washing three times with ice-cold

![Fig. 1](image1) Identification of B1R and B2R and their level of N-glycosylation. A. cells transfected with pcDNA3 vector (-; lane 1) and FB1 (lanes 2–4) were immunoprecipitated (IP) with anti-B1R-(317–353) antibody (lanes 1 and 2) and anti-FLAG antibody (lanes 3 and 4) and immunoblotted (IB) with anti-FLAG antibody. The anti-FLAG immunoprecipitates were treated without (lane 3) and with (lane 4) PNGase F for 2 h at 37 °C before immunoblotting. B. cells transfected with pcDNA3 vector (-; lane 1) and HB2 (lanes 2–4) were immunoprecipitated (IP) with anti-B2R-(310–364) antibody and immunoblotted (IB) with anti-HA antibody. The immunoprecipitates were treated without (lane 3) and with (lane 4) 2 units of PNGase F for 37 °C before immunoblotting.

![Fig. 2](image2) B1R-dependent proteolytic degradation of B2R. A. cells transfected with pcDNA3 (-; lane 1), HB2 (lane 2), HB2 + WT B1R (B1) (lane 3), HB2 + WT 5HT2A (2A) (lane 4), HB2 + WT CXCR4 (CXCR) (lane 5), WT 5HT2A + WT B1R (lane 6), and WT CXCR4 + WT B1R (lane 7) were immunoblotted with anti-HA antibody. pcDNA3 was used to equalize the amount of DNA transfected. B. cells transfected with pcDNA3 and HB2 without and with increasing amounts of WT B1R were immunoblotted with HA antibody. C. cells transfected with pcDNA3 (-; lane 1), HB2 (lanes 2 and 3), and FB1 (lane 5), either individually or together (lane 4). A plasma membrane-enriched cellular fraction was immunoblotted with anti-HA antibody or anti-FLAG antibody. D. cells were transfected with HB2 and FB1 in the absence and presence of increasing amounts of WT B1R and B2R, respectively, and cell surface receptor binding was then analyzed. Data are presented as mean ± S.E. of at least three experiments. E. cells transfected with pcDNA3 vector (-; lane 1) and HB2 without (lane 2) and with WT B1R (lanes 3) were immunoprecipitated (IP) with anti-B2R-(310–364) antibody and immunoblotted (IB) with anti-HA antibody. In A–C and E, molecular mass standards (in kDa) (left side arrows) and major receptor species (right side arrows) are indicated, and the results are representative of experiments performed three times.
phosphate-buffered saline, cells were immunoprecipitated and electroblotted as described below. Biotinylated receptors were detected with streptavidin conjugated to horseradish peroxidase (Vector Laboratories).

Immuno precipitation and Immunoblotting—Cells were subjected to immunoprecipitation and/or immunoblotting essentially as described previously (7). For immunoprecipitation, the lystate was incubated with anti-FLAG M2 antibody (1:300; Stratagene), anti-HA antibody (1:300; Covance), or polyclonal antibodies raised against the B1R and B2R C-terminal tail residues 317–353 (anti-B1R-317–353 and 310–364 (anti-B2R-310–364), respectively (18), followed by incubation with protein A- Sepharose beads with or without precoupled rabbit anti-mouse IgG. For immunoblotting, samples were subjected to SDS-PAGE under reducing conditions (6% β-mercaptoethanol) on 12% gels, electrotransferred onto nitrocellulose membranes, and then stained with anti-FLAG M2 antibody (1:1000) or anti-HA antibody (1:1000). Immunoreactive bands were visualized with a chemiluminescence immunodetection kit using peroxidase-labeled sheep anti-mouse antibody according to the procedure described by the supplier (PerkinElmer Life Sciences).

Immunoelectron Microscopy—The FLAG and HA antibodies were labeled with colloidal gold of different sizes (5 and 11 nm, respectively), as described previously (19). Cells co-expressing FLAG-tagged B1R (FB1) and HA-tagged B2 receptor (HB2) were incubated with the gold-labeled antibodies for 30 min at 4 °C. The cells were fixed for 1 h at room temperature and then fixed overnight at 4 °C in 2.5% glutaraldehyde in 0.15 M sodium cacodylate, pH 7.4 (cacodylate buffer). The fixed samples were washed with cacodylate buffer and postfixed for 1 h at room temperature in 1% osmium tetroxide in cacodylate buffer. The samples were then dehydrated in a graded series of ethanol and embedded in Epon 812 using acetone as intermediate solvent. Specimens were sectioned with a diamond knife into 50-nm-thick, ultrathin sections on an LKB ultramicrotome. The sections were then stained with uranyl acetate and lead citrate. Specimens were observed in a Jeol JEM 1230 electron microscope operated at 80 kV accelerating voltage. Images were recorded with a Gatan Multiscan 791 charge-coupled device camera.

Data Analysis—Where indicated, data are presented as the mean ± S.E. and were compared using Student’s t test.

RESULTS

Identification of Human B1R and B2R and Their Relative Level of Glycosylation—To study the consequence of B1R and B2R co-expression, the respective receptors were structurally modified with the FLAG and HA epitopes at their N termini and then expressed in HEK293 cells. The 35-kDa FB1 (Fig. 1A, lane 2) and 65-kDa hemagglutinin-tagged B2R (HB2) (Fig. 1A, lane 2) were then monitored with epitope-specific antibodies and antibodies against human B1R C-terminal residues 317–353 (anti-B1R-317–353) and human B2R C-terminal tail residues 310–364 (anti-B2R-310–364). The different relative molecular mass of intact B2R and B1R was due to differential N-linked glycosylation. As shown in Fig. 1B, lanes 3 and 4, treatment of anti-B2R-310–364 immunoprecipitates with 2 units of PNGase F for 2 h at 37 °C promoted a decrease in the relative mass of HB2 from 65 kDa to ~40 kDa. On the other hand, treatment of anti-FLAG immunoprecipitates did not change the relative mass of FB1 (Fig. 1A, lanes 3 and 4).

B1R-promoted Proteolytic Processing of B2R—Co-expression of HB2 and WT B1R resulted in the diminution of the intact 65-kDa HB2 species and the appearance of heterogeneous products at 30–40 kDa (Fig. 1A, lane 3). The B1R-promoted effect on B2R was also observed in a highly enriched plasma membrane fraction of the cell (Fig. 1C, top panel, lane 4). The effect of B1R on B2R was dose-dependent (Fig. 2B, lanes 2–6) and was paralleled by the loss of cell surface binding sites for the selective B2R agonist (**H)BK (Fig. 2D) (69 ± 8% of control binding at 2 μg of B1R DNA). On the other hand, B2R did not alter either the integrity of the 35-kDa FB1 (Fig. 2C, bottom panel, lanes 4 and 5) or the cell surface binding sites for the selective B1R agonist (**H)DK (Fig. 2D). Neither the WT serotonin 5HT2A receptor nor the WT chemokine receptor CXCR4 had any apparent effect on B2R integrity (Fig. 2A, lanes 4 and 5) or (**H)BK binding (100 ± 2% of control binding at 2 μg of CXCR4 DNA). Furthermore, B1R had no apparent effect on the integrity of FLAG-tagged CXCR4 (data not shown). To directly address whether B2R was proteolytically degraded, we compared HB2 expressed alone and together with B1R, immunoprecipitated with anti-B2R-310–364 antibody and then immunoblotted with anti-HA antibody. Proteolytic cleavage would be indicated if, after introduction of B1R, the HA antibody, which recognizes the B2 receptor N-terminal tail, reacted less with the anti-B2R-310–364 immunoprecipitate, which recognizes the B2 receptor C-terminal tail. Fig. 2E shows an almost complete loss of anti-HA reactivity after co-expression of HB2 with B1R. These results provide direct evidence that the decrease in B2R molecular mass is caused by proteolytic B2R cleavage.

Spontaneous Formation of Proteolytic Hetero-oligomeric B1R and B2R Complexes—Relatively stable detergent-resistant homooligomeric B1R structures were readily apparent on gels (Fig. 1A, lane 2, and Fig. 1C, bottom panel, lanes 4 and 5). To determine whether the B1R-promoted degradation of B2R involved receptor hetero-oligomerization, attempts were made to co-immunoprecipitate the two receptors. Immunoprecipitation of HB2 with HA antibodies yielded the co-immunoprecipitation of FB1.
B1R and B2R complexes by cell surface biotinylation. If cells transfected with pcDNA3 (lane 1), FB1 (lane 2), or FB1 with HB2 (lane 3) were biotinylated, immunoprecipitated (IP) with HA antibody or FLAG antibody, and stained with streptavidin conjugated to horseradish peroxidase. In lane 4, biotinylated cells transfected with FB1 and HB2 individually were mixed and immunoprecipitated with HA antibody. Molecular mass (in kDa) standards (left side arrows) and receptor species (right side arrows) are indicated, and the results are representative of experiments performed three times.

We have shown previously that B1R is constitutively active and B2R interaction is also indicated at the functional level. B1R complexes were also investigated using immunoelectron microscopy. To do so, FLAG antibodies were labeled with 5-nm gold-labeled antibodies for 30 min at 4 °C. The cells were then processed as described under “Experimental Procedures.” The bar at the bottom indicates 0.2 µm. The arrows indicate some 5- and 11-nm gold heterodimer complexes. Inset, magnified area containing complexes.

Kinetics of hetero-oligomeric B1R and B2R complex formation. A, cells transfected with FB1 and HB2 in ratios of 2:1 (lane 1), 2:2 (lane 2), 4:1 (lane 3), and 4:2 (lane 4) (µg DNA/µg DNA) were immunoprecipitated (IP) with HA antibody and immunoblotted (IB) with FLAG antibody. B, cells transfected with FB1 without (lane 1) and with HB2 (lanes 2–5) were incubated in the absence (lanes 1 and 2) and presence of DK (lane 3), BK (lane 4), and kallidin (KD) (lane 5) for 30 min at 37°C, and immunoprecipitated (IP) with FLAG antibody or HA antibody and immunoblotted (IB) with FLAG antibody. Molecular mass (in kDa) standards (left side arrows) and receptor species (right side arrows) are indicated, and the results are representative of experiments performed three times.

Increased Signaling of the Hetero-oligomeric B1R and B2R Complexes—Next, we analyzed whether the spontaneous B1R and B2R interaction is also indicated at the functional level. We have shown previously that B1R is constitutively active.
because it dose-dependently increases PI hydrolysis in the absence of agonist (Fig. 7B, Mock), whereas B2R is essentially inactive on its own (Fig. 7A, Mock) (7). Interestingly, in the presence of a constant amount of B1R, which by itself partially increased PI hydrolysis, B2R dose-dependently increased basal PI hydrolysis with a slope that was at least 14-fold higher than that in the absence of B1R (Fig. 7A). An increase (2-fold) in constitutive signaling also occurred when B2R was held constant and B1R was varied (Fig. 7B). Neither mock-transfection with pcDNA3 nor co-transfection with CXCR4 caused a change in B2R activity (data not shown). Fig. 7C shows that B1R and B2R co-expression also increased the ability of B1R to be stimulated by 1 μM desArg9BK. This response remained B1R-specific because desArg9[Leu8]kallidin, a highly selective B1 antagonist, effectively inhibited the response, whereas the selective B2 inverse agonist NPC17731 did not inhibit the response (data not shown). Furthermore, NPC17731 had no effect on the increase in basal PI hydrolysis caused by co-expression, excluding the conversion of B2R to a more active conformational state as the source of this increase (20, 21). These results show that the spontaneous interaction of B1R and B2R is functionally significant and translates into an increased coupling efficacy of the receptor to Gq and subsequent phospholipase Cβ activity.

**DISCUSSION**

Our observation is, to our knowledge, the first to implicate proteolysis of a G protein-coupled receptor in its participation in an oligomeric plasma membrane receptor complex. Such complexes are novel species that may have broad significance in receptor signaling and regulation, and the complexes and the involved protease(s) could represent new targets for therapeutic intervention.

Multiple approaches were used to show spontaneously formed functional hetero-oligomeric B1R and B2R complexes. These include receptor co-immunoprecipitation using epitope-specific antibodies against both the N-terminal and C-terminal domains of the receptors, direct identification of complexes by immunoelectron microscopy using antibodies labeled with differently sized gold particles, and constitutive receptor activity. The immunoelectron microscopy indicated the presence of receptor dimers.

Regulated proteolytic degradation of plasma membrane receptors through endocytic targeting to lysosomes is well characterized (22). Recently, increased attention has been drawn to plasma membrane proteolysis in receptor function (23–25). It is most likely that the degraded B2R products are formed after B1R and B2R complex formation rather than before it. Indeed, when in a complex with B1R, B2R may become susceptible to an intracellular or membrane-bound protease that may or may not have been recruited by B1R or to which B2R is recruited by B1R. Furthermore, hetero-oligomeric B2R may be more prone to degradation than homo-oligomeric B2R, which has been shown to exist (26). Whether or not an endocytic mechanism is involved in the proteolysis is not clear, but this possibility cannot be excluded because the complex may recycle from an intracellular degradative compartment back to the plasma membrane. Alternatively, proteolysis may occur while the receptors are in the endoplasmic reticulum or en route to the surface, if complexes are capable of forming at these points.

The presence of a spontaneously formed proteolytic receptor complex in the plasma membrane that correlates with increased constitutive and agonist-stimulated receptor signaling efficacy argues strongly that the complex is functionally significant. The lack of effect of the B2R inverse agonist NPC17731 suggests that the increase involves an allosteric enhancement by the B2R fragment of the inherent signaling of B1R. Indeed, this effect may be related to that which B2R elicits by hetero-oligomerizing with the angiotensin II AT1 receptor (27).

The possibility that B2R and B1R are typical protease-activated receptors seems unlikely because kinins are their natural receptor ligands. It has been reported that B2R is activated in cell culture by some extracellular proteases, which could be released from neutrophils and bacteria during insult, but without any direct evidence for receptor proteolysis (28). Starving the cells in the absence of serum for 24 h had no effect on B2R degradation, suggesting a cell-associated plasma membrane-bound or intracellular protease rather than a serum-derived extracellular protease as the cause of the degradation.

Induction of B1R expression by pro-inflammatory cytokines such as interleukin-1β is an important mechanism underlying the adaptation of the kinin-mediated inflammatory pain response from a B2 type to a B1 type during chronic insult.
Considering that these receptor subtypes are co-expressed on numerous cellular structures including primary sensory neurons, which are critical for the pain response, a proteolytic B1R-B2R complex could be involved in assuring the fading of the B2-type response and in augmenting the kinin signal. Indeed, a proteolytic plasma membrane mechanism to remove B2R may be necessary because this receptor (a) recycles relatively rapidly after agonist-promoted internalization (29, 30) and (b) down-regulates to a very limited extent (if at all), even after prolonged agonist exposure (days) (30, 31).

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