Involvement of the Amino Terminus of the B₂ Receptor in Agonist-induced Receptor Dimerization*

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The mechanisms and the functional importance of G-protein-coupled receptor dimerization are poorly understood. We therefore analyzed dimerization of the bradykinin B₂ receptor. The binding of the agonist bradykinin to the B₂ receptor endogenously expressed on PC-12 cells led to the formation of receptor dimers, whereas the B₂ antagonist HOE140 did not induce dimerization, suggesting that B₂ receptor dimerization was linked to receptor activation. Addition of a peptide corresponding to the amino terminus of the receptor reduced the amounts of detected B₂ receptor dimers, whereas peptides derived from the extracellular loops had no effect. To further analyze the role of the amino terminus of the receptor in receptor dimerization, we created two different rat B₂ receptor variants with truncated amino termini, B₂65 and B₂53, starting at amino acids 53 and 65. In contrast to the wild-type B₂ receptor and to B₂65, bradykinin did not induce dimerization of the B₂65 receptor. Both receptor variants were similar to the wild-type B₂ receptor with respect to agonist binding and signal generation. However, B₂65 was not phosphorylated, did not desensitize, and was not down-regulated upon bradykinin stimulation. Likewise, antibodies directed to the amino terminus of the receptor partially reduced internalization of [³H]bradykinin on PC-12 cells. These findings suggest that the amino terminus of the B₂ receptor is necessary for triggering agonist-induced B₂ receptor dimerization, and receptor dimers are involved in receptor-mediated signal attenuation.

The mechanism and the function of agonist-induced receptor dimerization of G-protein-coupled receptors is still a matter of debate. For a variety of G-protein-coupled receptors, receptor dimers have been detected recently, e.g. for the β₂-adrenergic receptor (1), the dopamine D₂ receptor (2), the metabotropic glutamate receptor 5 (3), or the calcium sensing receptor (4). G-protein-coupled receptors seem to dimerize via two different mechanisms. Whereas dimerization of the dopamine D₂ and the β₂-adrenergic receptor occurs via transmembrane regions, the metabotropic glutamate receptor and the calcium sensing receptor dimerize via their large amino terminus and, to a lesser extent, via their transmembrane regions (1–4). G-protein-coupled receptors are classified into three main families according to the structure and length of their amino terminus and the localization of the agonist binding site (5). The metabotropic glutamate receptor and the calcium sensing receptor are members of family 3 receptors. In family 3 receptors, the amino terminus is not only involved in dimerization but also essential for agonist binding. In contrast, adrenaline and dopamine, which are agonists on family 1 receptors, bind within the seven transmembrane helices (5). The transmembrane regions are also involved in dimerization of these receptors (1, 2). Thus, the mechanism of receptor dimerization seems to be related to agonist binding. To further investigate this hypothesis, we analyzed dimerization of the bradykinin B₂ receptor. The B₂ receptor belongs also to family 1. However, receptors for catecholamines or dopamine belong to the 1a subfamily, whereas receptors for peptides like bradykinin are classified into the subfamily 1b (5). In contrast to family 1a receptors, 1b-type receptors are characterized by an agonist binding site within the amino terminus of the receptor and the extracellular loops (5). In accordance with its classification as a family 1b receptor, a binding site(s) for the agonist bradykinin was identified within the extracellular loop regions, e.g. the connecting loops between membrane domains IV and V and domains VI and VII (6–9). Because the agonist binding site of 1b receptors differs from that of 1a family receptors, we asked whether the sites necessary for receptor dimerization were also different between members of these two subfamilies. Here, we report that binding of bradykinin to the B₂ receptor endogenously expressed on PC12 cells or transiently expressed in HEK293 cells led to the formation of receptor dimers/oligomers, which were irreversibly captured by covalent cross-linking. The amino terminus of the receptor seemed to be involved in receptor dimerization because a B₂ receptor variant lacking the entire amino terminus, B₂53, was impaired in agonist-induced receptor dimerization.

EXPERIMENTAL PROCEDURES

Materials—[2,3-prolyl-3,4-³H]Bradykinin (specific activity, 98 Ci/mmOL), [Nα-2-³H]NOSitol (specific activity, 17 Ci/mmOL), Na₁₂⁵I (specific activity, 17.4 Ci/mg), and the chemiluminescence detection kit were from Amersham Pharmacia Biotech; bradykinin, desArg⁹-Lys⁵-bradykinin, and HOE140 (D-Arg⁰-Hyp³-Thi⁵-D-Tic⁷-Oic⁸-bradykinin) were from Bachem; fura-2/AM was from Calbiochem; and m-maleimido-5-5-dihydroxy sucinimidyl ester (MBS), 1,5-difluoro-2,4-dinitrobenzene (DFDNB), and iodogen (I₃,4,6-tetrachloro-3b-6b-diphenyl- glycoluril) were from Pierce.

Cell Culture and Cell Transfection—HEK293 and PC-12 cells (ATCC) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum and kept in a humidified 7.5% CO₂/92.5% air atmosphere at 37 °C. Absence of mycoplasma infection was routinely controlled. HEK293 cells were transfected by CaCl₂ phosphate precipitation with 20 μg of DNA/10⁶ cells (10). For B₂ receptor

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1 The abbreviations used are: MBS, m-maleimido-5-dihydroxy sucinimidyl ester; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; GRK, G-protein-coupled receptor kinase; PAGE, polyacrylamide gel electrophoresis.
B₂ Receptor Dimerization

Desensitization and down-regulation, the B₂ receptor was expressed at low levels (0.05–0.1 pmol/mg of protein) to avoid saturation of the downstream effector components of the cells. The amount of B₂-cDNA used for these experiments was 0.1–0.2 μg of DNA/10⁶ cells, and the total amount of transfected DNA was held constant by plasmid pCDNA3. Cross-linking was terminated by addition of 100 mM Tris, pH 8.0, and 10 mM dithiothreitol. Membranes were collected by centrifugation and solubilized in SDS sample loading buffer for 15 min at 25 °C instead of boiling to avoid unspecific receptor aggregation. Solubilized proteins were separated by SDS-PAGE, including 6 μl urea. Cross-linked bradykinin-B₂ receptor complexes were detected by anti-bradykinin antibodies (7). And indeed, bradykinin binding to the B₂ receptor lead to the formation of receptor dimers and higher order complexes purified by affinity chromatography (7). Similarly, HOE140-B₂ receptor complexes were detected by anti-HOE140 antibodies (6). To control B₂ receptor specificity, a 500-fold molar excess of HOE140 was used as a control for bradykinin cross-linking and a 500-fold molar excess of bradykinin as a control for HOE140 cross-linking. Bound antibodies were visualized with peroxidase-labeled secondary antibodies with enhanced chemiluminescence detection.

Effect of Peptides Corresponding to the Extracellular Domains of the Rat B₂ Receptor on B₂ Receptor Dimer Formation—PC-12 membranes containing 0.5–1 pmol of B₂ receptor/mg of protein were incubated in phosphate-buffered saline including protease inhibitors (6) and incubated without or with 10 μM of a peptide corresponding to the sequence of the connecting loop between membrane domains II and III (ED2), IV and V (ED3), and VI and V (ED4) (6) and to the amino terminus of the B₂ receptor (ED1) at 37 °C. At the time points indicated, the membranes were solubilized in SDS-sample buffer, and proteins were separated by 10% SDS-PAGE including 6 μl urea. [125I]Tybradykinin-labeled B₂ receptors were visualized by autoradiography.

Effect of Site-directed Antibodies on the Internalization of [3H]Bradykinin—PC-12 cells on six-well plates were incubated for 2 h at 4 °C in the absence (control) or presence of 100 nM affinity-purified antibodies to the amino terminus of the B₂ receptor (α-ED1) or to the connecting loop between membrane domains II and III (α-ED2), IV and V (α-ED3), and VI and VII (α-ED4) in incubation buffer (138 mM NaCl, 5 mM KC1, 1 mM MgCl₂, 1.6 mM CaCl₂, 1 g/liter glucose, 20 mM Na⁺-HEPES, pH 7.3). The generation of the antibodies has been performed essentially as described previously (6). Then, 5 nM [3H]bradykinin was added and incubated for an additional 1 h at 4 °C. To induce internalization, cells were shifted to 37 °C. At the time points indicated, cells were washed three times with 0.2 μM glycine, 0.5 mM NaCl, pH 3.0, followed by three washing steps with incubation buffer. Then, cells were dissolved by 1 M NaOH, and internalized [3H]bradykinin was determined in a β-counter.

RESULTS

Detection of Agonist-induced B₂ Receptor Dimerization on PC-12 Cells—To determine a potential functional role of receptor dimerization, we attempted to detect B₂ receptor dimers in a system with endogenously expressed B₂ receptors, and we chose PC-12 cells (11). To detect bradykinin-stimulated receptor dimerization, we stimulated cell membranes by bradykinin at 25 °C for 30 min and irreversibly captured receptor dimers by addition of the cross-linker MBS (0.2 mM). Cross-linked bradykinin-B₂ receptor complexes were detected by anti-bradykinin antibodies (7). And indeed, bradykinin binding to the B₂ receptor lead to the formation of receptor dimers and higher oligomerization states as shown in Fig. 1A, lane 1. The B₂ antagonist HOE140 did not significantly stimulate the formation of receptor aggregates when HOE140 was cross-linked to the B₂ receptor under similar conditions as described above and detected by anti-HOE140 antibodies (Fig. 1A, lane 3). To control B₂ receptor specificity, a 500-fold molar excess of HOE140 was used as a control for bradykinin cross-linking (Fig. 1A, lane 2) and a 500-fold molar excess of bradykinin as a control for HOE140 cross-linking (Fig. 1A, lane 4). The presence of a 500-fold molar excess of HOE140 or bradykinin abol-
cross-linker MBS (cf. Fig. 1A). This finding may be due to different length and different specificity of the applied cross-linkers. The specificity of the cross-linking was confirmed by the finding that a 500-fold molar excess of unlabeled bradykinin suppressed the labeling of the B2 receptor by \[^{125}\text{I} \text{Ty}r\]bradykinin (Fig. 1B, lane 5). Together, these findings suggest that peptides corresponding to the extracellular loops of the B2 receptor did not affect the capturing of receptor dimers by DFDNB.

In contrast, a peptide derived from the amino terminus of the B2 receptor (ED1) significantly reduced the ratio between labeled B2 receptor monomers versus dimers (Fig. 1C, lane 2) compared with the control without peptide (Fig. 1B, lane 4, and Fig. 1C, lane 1) or compared with the controls with peptides directed to the extracellular loops (Fig. 1B, lanes 1–3). The presence of a 500-fold molar excess of the B2 antagonist HOE140 abolished the labeling by \[^{125}\text{I} \text{Ty}r\]bradykinin, demonstrating B2 receptor specificity of the signal(s) (Fig. 1C, lane 3). Together, these findings may suggest that the amino terminus of the B2 receptor was involved in triggering B2 receptor dimerization induced by bradykinin.

Expression of Amino-terminally Truncated B2 Receptor Variants in HEK293 Cells—To further analyze the role of the amino terminus of the B2 receptor in receptor dimerization, we created two rat B2 receptor variants with truncated amino termini, B2\(^{65}\), starting with amino acid 53 after the conserved cysteine 52 (17), and B2\(^{66}\), lacking the entire amino terminus. The indicated positions of the amino acids are derived from the rat B2 receptor sequence with extended amino terminus (17). The wild-type B2 receptor and the two B2 receptor variants were expressed in HEK293 cells. Expression of the B2 receptor protein was detected in Western blot by anti-HOE140 antibodies after cross-linking of HOE140 to the B2 receptor. The antibodies detected a major protein of 55 ± 5 kDa in wild-type receptor-expressing cells (Fig. 2, A, lane 1, and B, lane 1), and proteins of 45 ± 6 and 43 ± 4 kDa were detected in cells expressing B2\(^{53}\) (Fig. 2B, lane 2) and B2\(^{65}\) (Fig. 2B, lane 3), respectively. No significant amounts of receptor dimers were detected (Fig. 2, A, lane 1, and B, lanes 1–3). The labeling of the B2 receptors by HOE140 was suppressed when the cross-linking was performed in the presence of a 500-fold molar excess of bradykinin, as demonstrated for the wild-type B2 receptor (Fig. 2A, lane 2). These findings demonstrate that binding of HOE140 to the different B2 receptors expressed in HEK293...
cells did not induce the formation of receptor dimers, although HOE140 was efficiently cross-linked to these receptors, revealing similar expression levels.

**Agonist-induced Receptor Dimerization of B₂ Receptors Expressed in HEK293 Cells**—Next we asked whether bradykinin stimulated receptor dimerization of the wild-type B₂ receptor and the truncated B₂ receptor variants expressed on HEK293 cells. Similarly as on PC-12 cells, B₂ receptor dimers were irreversibly captured by MBS and detected by anti-bradykinin antibodies in Western blot. Bradykinin induced the formation of receptor dimers and higher oligomerization states on membranes of HEK293 cells expressing wild-type B₂ receptors (Fig. 2C, lane 2). Although no oligomeric states larger than dimers were found with B₂<sup>53</sup>, the ratio between B₂ receptor monomers versus dimers was similar for the wild-type B₂ receptor and for B₂<sup>53</sup>

In contrast, there were no significant amounts of receptor dimers detectable following stimulation of the B₂<sup>65</sup> variant by bradykinin (Fig. 2C, lane 3). Bradykinin was efficiently cross-linked to the monomeric form of B₂<sup>65</sup> (Fig. 2C, lane 3), confirming that the truncation of further 12 amino acids in B₂<sup>65</sup> compared with B₂<sup>53</sup> did not reduce cross-linking efficiency (Fig. 2C, lane 3 versus lane 2). A 500-fold molar excess of HOE140 suppressed the specific labeling of the wild-type B₂ receptor, of B₂<sup>53</sup> and of B₂<sup>65</sup> by bradykinin, confirming B₂ receptor specificity (not shown). Together, these findings suggest that 12 amino acids upstream of membrane domain I were sufficient to support agonist-induced dimerization/oligomerization of the B₂ receptor.

**Functional Characterization of the B₂ Receptor Variants**—Next, we analyzed the interrelationship between B₂ receptor dimerization and receptor function, and we determined the KD values for the binding of [³H]bradykinin. The binding affinity of bradykinin for the wild-type B₂ receptor, for B₂<sup>53</sup> and of B₂<sup>65</sup> by bradykinin, confirming B₂ receptor specificity (not shown). Together, these findings suggest that 12 amino acids upstream of membrane domain I were sufficient to support agonist-induced dimerization/oligomerization of the B₂ receptor.

Next, we determined the bradykinin-induced rise in inositol phosphate levels of HEK293 cells expressing the different B₂
receptor variants. The concentration of bradykinin necessary to produce the half-maximum increase in inositol phosphate levels (EC_{50} value) was 34 ± 7 × 10^{-9}, 22 ± 8 × 10^{-9}, and 38 ± 6 × 10^{-9} M for the wild-type B_2 receptor, for B_2^{53}, and for B_2^{65}, respectively (cf. Fig. 3C). Thus, the amino terminus of the B_2 receptor is not essential for B_2 receptor activation.

**Interrelationship between Agonist-induced Receptor Dimerization and Desensitization—**B_2 receptor phosphorylation is the initial step in B_2 receptor desensitization (12, 13). Therefore, phosphorylation of the B_2 receptor variants by GRK2 was analyzed. GRK2 phosphorylated the B_2^{53} variant upon agonist stimulation (Fig. 3A, lane 5) similarly to the wild-type B_2 receptor (Fig. 3A, lane 2). No significant increase in B_2 receptor phosphorylation was detected after stimulation by a B_1-specific agonist, desArg^{10}-kallidin (Fig. 3A, lanes 3 and 6) compared with the unstimulated control (Fig. 3A, lanes 1 and 4). In contrast, GRK2 did not significantly phosphorylate the B_2^{65} variant upon bradykinin stimulation (Fig. 3A, lane 8) compared with the unstimulated control (Fig. 3A, lane 7). This experiment strongly suggests that agonist-induced phosphorylation of the B_2^{65} receptor by GRK2 was impaired.

Receptor phosphorylation is assumed to precede receptor desensitization (18, 19). Therefore, we next asked whether desensitization of the B_2^{65} variant was also impaired. The desensitization of the bradykinin signal was analyzed for the bradykinin-stimulated rise in [Ca^{2+}]_{i}, on Jurkat-2-loaded HEK293 cells expressing the different B_2 receptor variants. The bradykinin-induced rise in [Ca^{2+}]_{i}, was similar in wild-type B_2 receptor-expressing cells, in cells expressing B_2^{53}, and in cells expressing B_2^{65} (Fig. 3B, panels 1–3). To initiate receptor desensitization, the cells were preincubated for 5 min with 1 μM bradykinin. After removal of bradykinin, the cells were given a second pulse of bradykinin. A second stimulation by bradykinin did not significantly increase [Ca^{2+}]_{i}, in wild-type B_2 receptor-expressing cells (Fig. 3B, panel 4) or in cells expressing the B_2^{53} receptor (Fig. 3B, panel 5), indicating that the receptors were efficiently desensitized after prestimulation by 1 μM bradykinin. In contrast, the B_2^{65} receptor was not desensitized after the initial bradykinin stimulation, as revealed by a rise in [Ca^{2+}]_{i}, following a second pulse of bradykinin (Fig. 3B, panel 6).

**Down-regulation of the Different B_2 Receptor Variants—**The B_2^{65} receptor poorly dimerized, was not significantly phosphorylated, and did not desensitize upon bradykinin stimulation. Therefore, we asked whether the B_2^{65} receptor could be down regulated. To this end, the B_2 receptor-expressing cells were prestimulated by 10 μM bradykinin for 6 h. After washing, the bradykinin-stimulated increase in inositol phosphate levels was determined compared with the unstimulated control. After long-term bradykinin stimulation, the B_2 receptor-mediated signal was down-regulated on wild-type B_2 receptor-expressing cells and on B_2^{53} expressing cells. The signal was reduced by 28 ± 3 and 25 ± 5%, respectively (Fig. 3C, panels 1 and 2). In contrast, the B_2 receptor-mediated signal was not down regulated on B_2^{65}-expressing cells (Fig. 3C, panel 3). Thus, a B_2 receptor variant lacking the entire amino terminus had lost its capability to undergo agonist-induced dimerization, phosphorylation, desensitization, and down-regulation.

**Effect of Site-directed Antibodies on the Internalization of [^{3}H]bradykinin on PC-12 Cells—**Finally, we asked whether the amino terminus of the B_2 receptor was also involved in the signal attenuation process of a B_2 receptor expressed in a native system. To this end, we initially tested the effect of the peptide ED1 derived from the amino terminus of the B_2 receptor, which has been shown to reduce the ratio between detected B_2 receptor monomers versus dimers (cf. Fig. 1C). However, this peptide (10 μM) had no significant effect on B_2 receptor phosphorylation and internalization under the conditions applied (not shown). Therefore, we next analyzed the effect of site-directed antibodies to the extracellular domains of the B_2 receptor. The generation, immunoselection, and characterization of these antibodies was performed as described previously (6). The site-directed antibodies used for this study did not affect the binding of [^{3}H]bradykinin or the activation of the B_2 receptor (6). The antibodies to the amino terminus of the B_2 receptor (α-ED1) caused a significant reduction of internalized [^{3}H]bradykinin (Fig. 4, A and B) by 24 ± 4%, whereas site-directed antibodies to the extracellular loops (α-ED2, α-ED3_C, and α-ED4_A) had no effect on internalization of [^{3}H]bradykinin (Fig. 4A). Results obtained with the site-directed antibodies in phosphorylation studies were not consistent, probably due to the moderate effect of the antibodies (not shown). Nevertheless, our data indicate that the amino terminus of the B_2 receptor is also involved in B_2 receptor-mediated signal attenuation processes on PC-12 cells.

**DISCUSSION**

Although the detailed mechanism of receptor dimerization is far from being understood, different regions of G-protein-coupled receptors have been identified to be involved in receptor dimerization. Dimerization of the dopamine D_2 and the β_2-adrenergic receptor occur via transmembrane regions (1, 2). In contrast, the metabotropic glutamate receptor and the calcium sensing receptor dimerize via their large extracellular amino terminus and to a lesser extent via their transmembrane regions (3, 4). Although the B_2 receptor, as a family 1b receptor, lacks a large extracellular domain that is characteristic of
family 3 receptors, agonist-induced B₂ receptor dimerization also needs the amino terminus of the receptor because (i) a peptide corresponding to the amino terminus of the B₂ receptor partially suppressed bradykinin-induced receptor dimerization on PC-12 cells, and (ii) a B₂ receptor variant lacking the entire amino terminus, B₂₆₅, was impaired in agonist-induced receptor dimerization. The amino terminus, which was necessary for agonist-stimulated B₂ receptor dimerization, was not essential for agonist binding. Such a finding is in contrast to previous studies with the β₂-adrenergic, the dopamine, the calcium sensing, or the metabotropic glutamate receptor where the region(s) involved in receptor dimerization were overlapping with the agonist binding sites (1–4). This potential mechanistic difference of B₂ receptor dimerization may be related to the predicted topology of the B₂ receptor, which is different from rhodopsin with five transmembrane domains instead of seven and two membrane reentrant segments, membrane domains I and II (20). Thus, membrane domain I, which follows the amino terminus, is not membrane-spanning.

In addition to the mechanism, the functional importance of receptor dimerization is not clear. Receptor dimerization of the β₂-adrenergic receptor seems to be related to receptor activation because the amount or receptor dimers increased upon agonist stimulation, and a peptide derived from transmembrane domain VI partially suppressed receptor dimerization and receptor activation (1). Several other lines of evidence suggest, however, that receptor dimerization does not seem to be essential for receptor activation. The stoichiometry of the interaction between the receptor, G-protein, and effector molecule is assumed to be 1:1:1 (21), and a single receptor with a single Gα protein fused to its carboxyl terminus is functionally active (22). Therefore, the potential role of receptor dimerization may lie apart from receptor activation. And indeed, a B₂ receptor variant (B₂₆₅) with impaired agonist-induced receptor dimerization was “normal” with respect to signal generation. In contrast, this B₂ receptor variant was defective in agonist-induced receptor phosphorylation, desensitization, and down-regulation. Likewise, affinity-purified antibodies to the region that is necessary for receptor dimerization, the amino terminus of the B₂ receptor, partially reduced internalization of [³H]bradykinin on PC-12 cells. Thus, agonist-induced dimerization of the B₂ receptor seems to follow receptor-induced G-protein activation and to precede receptor phosphorylation, desensitization, and down-regulation.

Twelve amino acids upstream of membrane domain I may be sufficient to support agonist-induced B₂ receptor dimerization because B₂₆₅ was still capable of dimerizing after bradykinin stimulation, whereas B₂₆₅, lacking the entire amino terminus, did not significantly dimerize after bradykinin stimulation. Bradykinin-induced B₂ receptor dimerization did not depend on receptor overexpression because bradykinin-induced B₂ receptor dimerization was also detected on PC-12 cells with endogenously expressed B₂ receptors. Thus, agonist-induced B₂ receptor dimerization via the amino terminus of the receptor is a characteristic of B₂ receptor signaling.

In addition to agonist-induced dimerization, G-protein-coupled receptors tend to dimerize under native conditions via hydrophobic interactions (7). Receptor clustering via hydrophobic interactions within the (trans)membrane domains may be supported by high receptor density. Future work will have to determine whether, in addition to agonist-induced receptor dimerization, which has been shown to regulate signal attenuation processes, receptor clustering under basal conditions represents an additional mechanism of regulating B₂ receptor activity.

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