In the search for the structural elements participating in signal transduction, internalization, and resensitization of the bradykinin B2 receptor, we identified two critical motifs, one in the second intracellular loop (IC2), the other in the proximal C terminus. We previously described the contribution of tyrosines within each of the two motifs (Tyr131 and Tyr322) to signal transduction and receptor internalization (Prado, G. N., Taylor, L., and Polgar, P. (1997) J. Biol. Chem. 272, 14638–14642). Here, we investigate the effect of exchanging both tyrosine residues simultaneously for alanine, phenylalanine, or serine, termed YAYA (Y131A/Y322A), YFYF (Y131F/Y322F), and YSYS (Y131S/Y322S) receptors, respectively. All of these mutants bound bradykinin (BK) normally, with a Kd of approximately 1.1 nM. However, although phosphoinositide (PI) turnover in response to BK by Y131A and Y131S proved negligible, the YAYA mutant returned BK-activated PI turnover to wild type (WT). In contrast, PI turnover with YSYS remained unresponsive to BK. Importantly, the pattern of BK-activated arachidonate release differed markedly in the mutant receptors. For example, whereas Y131S ablated BK-activated arachidonic acid release, conversion of this mutant to YSYS returned the BK-activated receptor function to a level above that of WT. However, YAYA showed only a partial recovery from the poor BK response of Y131A. These and additional results suggest that Tyr131 and Tyr322 interact cooperatively in conjunction with at least two separate signaling pathways. Given these results, a molecular model of the receptor was generated with the IC2 and the proximal as well as distal C terminus (1).* This work was supported in part by National Institute of Health Grants HL25776 from NHLBI, AG00115 from NIA, and GM54082 (to D.F.M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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The bradykinin B2 receptor (BKB2R)1 cDNA has been successfully transfected into such cell types as hamster lung fibroblasts, Rat-1 fibroblasts and CHO cells with the expected binding properties (1–3). The BKB2R is a typical G protein-coupled receptor (GPCR) that has been reported to be associated with Gq (4–7), Gi, and G12 (8–10). Binding of a GPCR to more than one Go subunit is not unprecedented. For example, the angiotensin II type 1 (AT1) receptor couples to both Gi and Gq (11). Whereas Gq has been linked to phospholipase C activation (6, 7), the release of arachidonate, via phospholipase A2 activation, has been linked to Gi and Gq (12–14).

The cellular response of GPCRs to agonists is regulated under a tightly controlled process of desensitization and resensitization (15, 16). Desensitization prevents cells from uncontrolled stimulation, and resensitization allows cells to recover and maintain responsiveness (17). Both processes appear to be regulated at the receptor level. Recent studies by Haasemann et al. (18) showed that stimulation of BKB2R results in redistribution of the receptor and its internalization in caveolae (18). On/off regulatory elements have been identified in other receptors (19–22). However, to date, no clear consensus motifs have been identified to determine either internalization or resensitization. The DRYXII/VXXP motif located in the second intracellular loop (IC2) of the human muscarinic cholinergic receptor has been proposed as important for receptor internalization (23). Another important motif is the tyrosine-containing motif located in the proximal BKB2R C terminus. The motifs in this region have been implicated in receptor internalization in other GPCRs, such as neurokinin 1 receptor (17), parathyroid hormone receptor (24), and AT1 receptor (20). Studies have also shown that clathrin-associated protein complexes interact with tyrosine-based motifs (25).

Mutations exchanging single as well as multiple amino acids have been applied to resolve the actions of GPCRs. The importance of specific residues within the IC face of GPCRs was demonstrated recently by a number of investigators, using receptors such as BKB2 receptors (1), AT1 receptors (26), interleukin-8 receptors (27), and muscarinic acetylcholine receptors (28). Our previous results showed that Tyr131 and Tyr322 are crucial for signal transduction (1). Truncation of the distal C terminus of BKB2R exhibited normal BK-activated ARA release, PI turnover, and [Ca2+]i flux. Furthermore, our previous results showed that at least some of the structural elements involved in the internalization process are found in the IC2 and the proximal as well distal C terminus (1).

1 The abbreviations used are: BKB2R, bradykinin B2 receptor; BKB2, bradykinin B2; GPCR, G protein-coupled receptor; AT1, angiotensin II type 1; MD, molecular dynamics; WT, wild type; ARA, arachidonic acid; PI, phosphoinositide; DMEM, Dulbecco’s modified Eagle’s medium; IC, intracellular loop; TM, transmembrane.
Experimental Procedures

Materials—[1H]BK (78 Ci/mmol) was obtained from Amersham Pharmacia Biotech, myo-[1,2-3H]inositol (45–80 Ci/mmol) was obtained from NEN Life Science Products. Analytical grade Dowex-X8 (AG-1-X8, 100–200 mesh) was obtained from Bio-Rad. Restriction endonucleases were purchased from New England Biolabs. QuikChange mutagenesis kit was obtained from Stratagene Corp. (La Jolla, CA). Oligonucleotides were either synthesized from an in-house Applied Biosystems DNA synthesizer or purchased from Life Technologies, Inc. All other reagents were from Sigma unless stated otherwise.

Site-directed Mutagenesis—The QuikChange Site-Directed Mutagenesis (Stratagene) was used to generate mutant BKB2 receptors. Briefly, two primers complementary to each other were designed to contain the desired mutation. The mutation-containing DNA was synthesized using Ffu DNA polymerase. The parental strand, which is dam-methylated, was removed by digestion with Dpn I. The mutant strand was transformed into XL1-Blue supercompetent cells and then DNA isolated by the miniprep method. The synthesized mutant DNA was sequenced using an in-house facility using an automatic DNA sequencer (Applied Biosystem Inc, model 370A). Using the BKB2R as template, T137P, T137D, and T322F were constructed using oligonucleotides T137P (sense strand, 5'-ctgtgtagcgcctgctgg-3'; antisense strand, 5'-cgatgcacgacctccagag-3'); T137D (sense strand, 5'-ctggaagcgcgctggcctg-3'; antisense strand, 5'-catgagcactgctggcctg-3'); T322F (sense strand, 5'-agaggtttcccaggcaata-3'; antisense strand, 5'-catggacatggacttcaccag-3'); and Y322F (sense strand, 5'-cagcgccagggatcggtcgat-3'; antisense strand, 5'-cagcgccagggatcggtcgat-3'). These mutations were then incorporated into the molecular model (36).

Cell Culture and Transfection—Rat-1 cells were cultured on 100-mm plastic dishes at an initial concentration of 5 × 10^5 cells/ml and incubated with Fura-2/AM for 30 min (2 μM final concentration). After 30 min, the cell suspension was diluted 10× with physiological buffer solution and incubated for another 30 min. Cells were pelleted and resuspended at 1 × 10^5 cells/ml. Calcium mobilization was performed using a Perkin Elmer/LKB 11200 calcium imaging spectrophotometer. Intracellular calcium increase in the presence or absence of BK was measured as described (5). Data were analyzed using the FURA program.

To examine desensitization, cells were allowed to equilibrate at 37 °C for 5 min and then stimulated sequentially with 10 nM BK at 2-min intervals, without an intervening wash. Resensitization was examined by exposing cells to a desensitizing dose of 100 nM BK for 1 min at 37 °C. The cells were then washed with physiological buffer and resuspended at 1 × 10^5 cells/ml. Calcium mobilization assays as described above were carried out by stimulating cells with a second dose of 10 nM BK at various times after the first desensitizing dose to 10 nM BK.

Molecular Modeling—The molecular model of the BKB2R was built up using the topological arrangement of the transmembrane helices of rhodopsin (32, 33) following standard procedures using the program WHAT IF (34). The loops connecting the transmembrane domains were then added to complete the model. In an attempt to develop the conformational preferences of IC2 and the C terminus of the receptor, the corresponding sequences were submitted to a BLAST search (35). The homologous regions of each of these protein structures were then analyzed for conformational features. The secondary structural features were then incorporated into the molecular model (36).

To refine the molecular model, molecular dynamics (MD) simulations and energy minimizations were carried out with the GROMACS program (37). The membrane environment was mimicked by a 40-Å layer of decane molecules, with approximately 40-Å layers of water above and below (38). The simulation cell was 132 × 81 × 71 Å and consisted of 12,906 waters and 518 decanes. The seven helical bundle of the receptor was placed in the decane layer with the extra- and intracellular regions within the water phases. Twenty different MD simulations, with alternative starting structures, were carried out for 200 ps on an SGI Origin 2000 computer.
RESULTS

Mutation of the Intracellular Tyrosines of the BKB2 Receptor—Previous experiments pointed to Tyr131 within the DRY motif, which is located in IC2, as important for both PI turnover and ARA release. These experiments also pointed to a Tyr131/Tyr322 interaction. The location of Tyr131 and Tyr322 in the BKB2 receptor is shown in Fig. 1. To investigate this interaction further, we generated critical double mutants at the Tyr131 and Tyr322 positions. Both tyrosine residues were converted to a small hydrophobic (alanine), a large hydrophobic (phenylalanine), and a small hydrophilic (serine) hydroxyl-bearing residue. All the mutants generated showed little change in binding affinity compared with the WT receptor, as illustrated in Table I. Levels of expression obtained in Rat-1 cells stably transfected with the double mutants are also shown in Table I.

PI Turnover of Tyr131 and Tyr322 Mutants—The ability of the single mutants at Tyr131 and Tyr322 and double mutants YSYS, YAYA, and YFYF to signal PI turnover is illustrated in Fig. 2. Results show that in Rat-1 cells expressing YAYA, the double mutant containing the small hydrophobic alanine, the BK-activated receptor signals a PI turnover equal to that of WT. This is in stark contrast to the actions observed with the Y131A mutant (Fig. 2). However, the action of YFYF, which contains the large hydrophobic phenylalanine, unlike the single mutant Y131F, was markedly reduced by 90% compared with WT. PI turnover by YSYS, containing the small hydrophilic serine, was reduced by 68% as compared with WT. This marked reduction in response is approximately the same as that of Y131S and Y322S (Fig. 2).

ARA Release of Tyr131 and Tyr322 Mutants—To analyze another signaling pathway, the release of ARA in response to BK was measured in stably transfected Rat-1 cells. The ability of the double mutant receptors to stimulate ARA release proved considerably different from that observed with PI turnover (Fig. 3). In this case, Rat-1 cells expressing YAYA, the double mutant containing the small hydrophobic alanine, the BK-activated receptor signals a PI turnover equal to that of WT. This is in stark contrast to the actions observed with the Y131A mutant (Fig. 2). However, the action of YFYF, which contains the large hydrophobic phenylalanine, unlike the single mutant Y131F, was markedly reduced by 90% compared with WT. PI turnover by YSYS, containing the small hydrophilic serine, was reduced by 68% as compared with WT. This marked reduction in response is approximately the same as that of Y131S and Y322S (Fig. 2).

Role of Thr137 in Signal Transduction and Internalization—The IC2 of BKB2R contains a threonine at position 137, as shown in Fig. 1. This residue is the exact counterpart of proline in the DRYXX/VXXP motif, an internalization motif found in other GPCRs (18). The proline residue is conserved in a number of GPCR families, including the AT1 and BKB1 receptors. We tested the effect of changing threonine to proline at this position, T137P. The mutant receptor functioned normally, as did WT, with respect to both ARA release and PI turnover, as illustrated in Fig. 4A and B, respectively. However, as illustrated in Fig. 5, the function of T137P changes markedly with...
regard to internalization. T137P is internalized only minimally, at 5 and 15% after 30 min and 45 min, respectively.

We investigated the role of the putative phosphorylation site, threonine 137 in IC2. To mimic the effect of phosphorylation, we introduced an aspartate residue, a negatively charged amino acid. The exchange of aspartate or glutamate for serine or threonine allows for the assessment of a single phosphorylated residue on the structure and function of a protein (39, 40). Our results show that substitution of threonine 137 with aspartate (T137D) caused a drastic reduction in both PI turnover and ARA release compared with both WT and the T137P mutant receptor (Fig. 4, A and B). The T137D mutant receptor also exhibited a very different internalization profile in comparison to T137P. At 5 min, 38 and 46% of the receptors are internalized in T137D and WT receptors, respectively. This is in contrast to a negligible amount of T137P internalized. At 30 min, 63, 47, and 5% of the receptors were internalized for WT, T137D, and T137P, respectively (Fig. 5). These results suggest that the aspartate substitution at position 137 introduced a conformational change similar to that of a phosphorylated threonine, allowing for receptor internalization.

Internalization of WT and Tyrosine Mutant Receptors—The Tyr131/Tyr322 mutants were next compared with the WT receptor for their capacity to internalize following BK receptor activation. At 15 min, only YSYS internalized to approximately the same level as WT, at about 42% uptake. The YFYF and YAYA mutants displayed slow receptor uptake, at 19 and 30% uptake, respectively (Fig. 6). Interestingly, YAYA acted like WT with respect to PI turnover, whereas it displayed the slowest receptor uptake. On the other hand, YSYS, which showed poor linkage to PI turnover, displayed a very similar rate of uptake to WT. The action by these two mutant receptors, YAYA and YSYS, indicate that receptor signaling does not correlate with ligand-mediated internalization of BK.

Receptor Desensitization and Resensitization—Bradykinin-induced receptor desensitization was examined in cells stably transfected with WT and with mutants that show a slow rate of receptor uptake, namely T137P, YAYA, and YFYF. With BK stimulated ARA release, we observed differential desensitization. As illustrated in Fig. 7, WT and YFYF desensitized at 100%, YAYA desensitized to 80%, and T137P desensitized to only 50%. Interestingly, T137P displayed the slowest rate of internalization, whereas internalization of YFYF at 30 min equaled that of WT (Figs. 5 and 6). With regard to BK activated Ca\(^{2+}\) flux, all four receptors desensitized completely within seconds of exposure to BK. This was evidenced by total inability of BK to induce a second burst of Ca\(^{2+}\) flux after an initial exposure to BK (Fig. 8A).
Receptor resensitization was evaluated in WT, T137P, YAYA, and YFYF, as shown in Fig. 8B. Cells were treated with a saturating concentration of BK for 2 min. Excess BK was removed by washing. Five minutes after washing, there was no observed [Ca\textsuperscript{2+}]\textsubscript{i} response to a subsequent dose of saturating BK in WT or the mutant receptors (data not shown). However, complete resensitization to BK occurred within 15 min for WT (Fig. 8B). We then determined whether the different mutant receptors resensitized at 15 min. The resensitization process proved related to the rate of receptor internalization. Although the initial [Ca\textsuperscript{2+}]\textsubscript{i} response was low for YFYF, it recovered normally, as did WT, within 15 min, reaching its original [Ca\textsuperscript{2+}]\textsubscript{i}. On the other hand, YAYA showed only partial recovery after 15 min. No recovery was observed for T137P at 15 min and even up to 60 min (data not shown).

**Molecular Modeling**—The results from the BLAST search of the C-terminal domain indicated a high probability of an α-helix in the region just after transmembrane 7 (TM7), encompassing residues 310–329, and a second, less well-defined helix, encompassing residues 335–350. The first helix is amphipathic in nature, with a well-defined hydrophobic and hydrophilic face. Given the amphipathicity of the helix, we assumed that it is lying on the surface of the membrane, with the hydrophilic amino acids projecting into the aqueous phase. During the MD simulations, this helix always adopted such an orientation with respect to the water/decane interface, even when starting completely in the water or decane phase. The results for IC2 indicate the presence of a β-turn centered around Thr\textsuperscript{137} (i + 1 position of the turn). Both of these conformational features were incorporated into the molecular model of the receptor. Alternative starting structures were generated by moving the α-helical domain of residues 310–329 to different positions with respect to the helical bundle and IC2 (i.e. projecting toward TM1-TM2, on the other side adjacent to TM6-TM5, or directly underneath the helical bundle). During the simulations, a weak harmonic force with a target distance of 12 Å was applied to residues Tyr\textsuperscript{311} and Tyr\textsuperscript{322}. In this manner, the conformations of the cytoplasmic domains can adjust to adapt to this constraint. The results from the simulation with the helix near TM6-TM5 are shown in Fig. 9.
**DISCUSSION**

We have generated a number of mutants within the IC2 and proximal C terminus of the BKB2R. All constructs retained the binding kinetics of the WT receptor. However, the mutations showed marked and varied effects on BKB2R signal transduction, receptor internalization, and resensitization. These results suggest that no one motif within the IC face of the BKB2R is solely responsible for a given receptor function. Instead, cooperative interactions appear to be taking place among the various IC domains.

For example, replacement of Tyr\(^{322}\), located at the proximal C terminus, with the small, uncharged alanine had little effect on either BK-stimulated ARA release or PI turnover. However, replacement with the small, hydroxyl-containing serine reduced both events markedly. Replacement of Tyr\(^{131}\) with serine also markedly reduced both events. However, when both tyrosine residues, Tyr\(^{131}\) and Tyr\(^{322}\), were mutated to serine (YSYS), the consequent reduction of a bulky residue only impaired the activation of phospholipase C. The double mutation did not affect receptor-stimulated ARA release. In contrast, double mutation of Tyr\(^{131}\) and Tyr\(^{322}\) residues to alanine (YAYA) impaired ARA release but not PI turnover. In this case, a hydrophobic residue at both locations promotes phospholipase C action but hinders the release of ARA. However, a double substitution with phenylalanine, a bulky hydrophobic residue, hinders both signaling pathways. These results demonstrate that discrete sequences within the DBY motif of IC2 and the tyrosine-based motif in the proximal C terminus interact with each other and cause differential effects on BK-activated signal pathways. A possible explanation for this differential signaling is that Tyr\(^{322}\) of the BKB2R is phosphorylated and acts like the YIPP motif, similarly found in the proximal C tail of the AT1 receptor (41). However, our results and results reported by Blaukat et al. (42) indicate that this tyrosine residue is not phosphorylated. A more solid explanation is that the two IC regions are cooperatively involved in coupling to more than a single subset of G protein (Gq, Gi, or Gi-like proteins). Interaction between the IC loops and the proximal C tail has been proposed in other GPCRs (43). In the \(\beta_{2}\)-adrenergic receptor, the C-terminal portion of IC3 and the proximal C tail are important for Gs activation (44). In the BKB2R, a small hydrophobic residue such as alanine at Tyr\(^{131}\) and Tyr\(^{322}\) favors interaction with a G protein responsible for PI turnover (Gq). In all, residue size, hydrophobicity, and charge at these positions play important roles in signal transduction. Another example to illustrate the effect of charge is the substitution of the hydrophilic threonine residue at location 137 with either proline or aspartate. Exchange of threonine for proline had no effect on either ARA release or PI turnover. However, the T137D mutant receptor, which displays a negative charge, showed impaired PI turnover and ARA release. Our previous studies demonstrated that point mutations at Tyr\(^{131}\) to serine, alanine, or phenylalanine also affected the rate of receptor internalization as compared with that of WT (1). Y131A displayed the slowest rate of uptake, whereas Y131S displayed a rate considerably faster than WT. Our present results further demonstrate that the double mutant YAYA also displayed a very slow rate of uptake, notably slower than WT or the other
double mutants. These results confirm the importance of the DRY region in receptor internalization. Apparently, mutant receptors that display either the slowest or the most rapid rates of receptor internalization still show a degree of PI turnover similar to that of WT receptor. This suggests that signal transduction in relation to the Tyr\(^{131}\)/Tyr\(^{322}\) interaction is not involved directly with receptor uptake. A similar lack of correlation between internalization and signal transduction was observed in muscarinic (18), angiotensin/AT1 (45), and \(\beta_2\)-adrenergic receptors (46).

To further understand the role of IC2 in receptor uptake, we investigated the DRYXXIVXX\(\phi\) motif as proposed in muscarinic cholinergic receptor (23). We aligned this motif with 184 GPCRs belonging to the peptide subfamily. We found that proline is conserved in 71% of the receptors. Conversion of threonine in the BKB2R to proline resulted in a complete loss of receptor uptake. However, substitution of proline at this site did not affect signal transduction. Substitution with a negatively charged aspartate to generate T137D reversed these effects. T137D did not stimulate ARA release or PI turnover but returned receptor uptake to that of WT. This result points to the importance of a negative charge at this site and suggests that phosphorylation at Thr\(^{137}\) participates in termination of receptor function and initiation of receptor uptake. Blaukat et al. (42) demonstrated threonine/serine phosphorylation to be taking place in conjunction with BKB2R internalization and resensitization.

Our results further suggest that internalization and resensitization are linked, whereas desensitization is a more complicated process related to specific metabolic events regulated by BK. When resensitization was tested by measuring \([Ca^{2+}]_i\), T137P, which was only minimally taken up even after 60 min, showed no resensitization. On the other hand, YIFYF, with a moderate rate of internalization, resensitized similarly to WT. YAYA, which was also taken up poorly, but not to the extreme of T137P, resensitized somewhere between YIFYF and T137P. It remains to be resolved which receptor motif(s) determine internalization, which determine desensitization, and what correlation exists between these two actions.

Our present and previous results, in sum, clearly indicate cooperativity or interaction between the C-terminal tail and IC2 of the receptor. We postulate the presence of \(\alpha\)-helices (for the C terminus) and a \(\beta\)-turn (residues 137–138 of IC2) based on homology analysis with structurally defined proteins, following a procedure recently reported for the \(\alpha\)-opioid receptor (36). However, the relative orientation of the loops and termini, containing the \(\alpha\)-helices or \(\beta\)-turns, needs to be established. The aim of our modeling effort is to investigate the conformations with these two regions (IC2 and C terminus) in close proximity. Given the constraints (i.e. presence of the \(\alpha\)-helices and \(\beta\)-turn), we looked for conformations that are energetically feasible. There are three possible orientations that would place the C-terminal helix (residues 310–329) in close proximity to Tyr\(^{131}\); it could lie 1) on the membrane surface near TM6-TM5, 2) on the membrane surface near TM1-TM2, or 3) across the cytoplasmic ends of the helix bundle. All attempts at placing the C-terminal helix toward TM1-TM2 failed. The topological orientation of the helical bundle does not allow for Tyr\(^{131}\) and Tyr\(^{322}\) to come close without disrupting the C-terminal helix or the TM bundle. In contrast, during the simulations starting with the other two orientations, low energy conformations were adapted early and maintained for the remainder of the 200 ps of MD simulation. Of these two, we prefer the first, in which the C-terminal helix lies on the membrane surface, mimicked by a layer of decane molecules in the simulation, near TM6-TM5. In the resulting structure, the hydrophobic face of the amphipathic C-terminal helix projects into the membrane surface. This conformation also places Cys\(^{326}\), postulated to be a site of palmitoylation, adjacent to the membrane surface. One role of the palmitoylation of this residue would be to securely anchor this helix to the membrane surface. Based on this, we exclude the low energy conformations that place the \(\alpha\)-helix of the C terminus across the helix bundle, even though from an energetic perspective based on the MD simulations, it is feasible. In Fig. 9, two views of the resulting conformation from the MD simulation, placing Tyr\(^{131}\) and Tyr\(^{322}\) 8.1 Å distal from each other, are shown. The resulting conformation places IC3 juxtaposed to IC2 and the C terminus. This is important given the well documented role of IC3 in coupling to the G proteins.

Concerning Thr\(^{137}\) and the \(\beta\)-turn, the resulting conformations have this turn well exposed, projecting down into the solvent, readily available for phosphorylation. Mutations at this position indicate that following receptor activation, leading to signal transduction, phosphorylation at this position results in termination of the activated receptor structure. This termination is then followed by internalization and potential resensitization of the receptor. A model of the T137P mutation suggests that it stabilizes the \(\beta\)-turn structure, while removing the possibility of phosphorylation.

In summary, our results using BKB2R mutant receptors and consequent molecular modeling involving the distal IC2 and proximal C terminus point to important interaction between these two regions, not only with regard to regulation of signal transduction but also in conjunction with receptor internalization and resensitization. Apparently, the motifs encompassing Tyr\(^{131}\) and Tyr\(^{322}\) perhaps in conjunction with elements within the IC3, dictate BKB2R-G protein interaction.

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