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Role of Hydroxyl Containing Residues in the Intracellular Region of Rat Bradykinin B_2 Receptor in Signal Transduction, Receptor Internalization, and Resensitization

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Abstract In past reports we illustrated the importance of Y131, Y322, and T137 within the intracellular (IC) face of the rat bradykinin B2 receptor (rBKB2R) for signal transduction and receptor maintenance (Prado et al. [1997] J. Biol. Chem. 272:14638–14642; Prado et al. [1998] J. Biol. Chem. 273:33548–33555). In this report, we mutate the remaining hydroxyl possessing residues located within the rBKB2R IC region. Exchange of S139A (IC2) or T239V (IC3) did not affect BK activated phosphatidylinositol (PI) turnover or receptor internalization. Chimeric exchange of the last 34 amino acids of BKB2R C-terminus with the corresponding 34 amino acids of the rat angiotensin II AT1a receptor (rAT1aR), both containing an S/T cluster, resulted in a mutant with normal endocytosis and BK activated PI turnover. A more selective chimera of these S/T clusters, with an exchange of BKB2R (333–351) with a rAT1aR fragment (326–342), resulted in a receptor with a retarded internalization but a normal BK activated PI turnover. Subsequent mutation of rBKB2R T344V showed little change in receptor uptake but a pronounced loss of BK activated PI turnover. The mutation of S335A, S341A, S348A, and S350A resulted in very poor receptor internalization and loss of activated PI turnover. Closer examination of this serine cluster illustrated that the replacement of S348A led to poor internalization; whereas the retention of S348 and mutation of S341A resulted in a receptor with a much greater internalization than WT. These and other results suggest that the presence of S348 promotes internalization while the presence of S341 dampens it. Conversely, S341 and S350 proved important for receptor signaling. In sum, our results illustrate that the distal C-terminus including its S/T cluster is important for both rBKB2R internalization and signal transduction. Individual S/T residues within this cluster appear involved in either signal transmission or receptor uptake capacity. However, replacement of the entire distal tail region with the corresponding rAT1aR sequence, also containing an S/T cluster, enables the BKB2R/AT1aR chimera to act in a very similar manner to wild type rBKB2R. J. Cell. Biochem. 83: 435–447, 2001.

Key words: chimera; signal transduction; receptor internalization; receptor resensitization; C-terminus; bradykinin receptors; angiotensin II 1a receptors

Bradykinin (BK) is a powerful vasoactive agent, part of the kinin family of small peptide regulatory effectors. BK binds to the rat bradykinin B2 receptor (rBKB2R) which participates in a number of signaling events making the effector a commonly used activator. The rBKB2R signal is G-protein transduced leading to the activation of phospholipase C, phospholipase A_2, phospholipase D, and Ca^{2+} translocation [Liao and Homcy, 1993; Kennedy et al., 1996; Ricupero et al., 1997; Prado et al., 1998;Yang et al., 1999]. rBKB2R has also been implicated in triggering the action of mitogen activated protein kinase and endothelial nitric oxide synthase [Blaukat et al., 1999; Marrero

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et al., 1999]. Generally, BK functions as a vasorelaxant [Berthiaume et al., 1997].

Angiotensin II type 1a receptor (AT1aR) functions as a vasoconstrictor and is also G-protein linked [Smith et al., 1998]. Interestingly the two receptors share 40% homology within their intracellular (IC) face and are approximately of the same length. The length of the C-terminal IC tail of rBKB2R is 57 amino acids while that of rat (rAT1aR) is 63 amino acids. Both receptors contain S/T clusters within the distal tail. rBKB2R contains four serines at position 335, 341, 348, and 350 and two threonines at position 344 and 347. rAT1aR has 13 serine/threonine (S/T) residues in the distal half of the C-tail [Smith et al., 1998]. However, the region implicated in rAT1a receptor internalization contains nine S/T residues and is located within the position 326–342 [Smith et al., 1998].

The initiation and termination of effector triggered receptor function (receptor maintenance) is an essential step in regulating cellular signaling. Receptor maintenance occurs in the presence of agonist desensitization. Although the molecular mechanisms of this process are poorly understood, three major components appear involved. One is the uncoupling of the G-protein from the receptor, "receptor desensitization." The other is removal of the receptor from the plasma membrane termed "receptor internalization" and return of the inactive receptor to the cell surface, "receptor resensitization." The S/T regions of both the BKB2R and AT1aR have been proposed to play a role in receptor uptake and resensitization [Prado et al., 1997; Fathy et al., 1999]. The importance of the C-terminus in internalization is evident [Prado et al., 1997, 1998; Pizard et al., 1999]. However, clearly the C-terminus is not alone in promoting these receptor actions. For example, the two tyrosines located in the IC 2nd loop (IC2) and the proximal C-tail, and the threonine in the IC2 have also been implicated in rBKB2R signal transduction and receptor internalization and resensitization [Prado et al., 1997, 1998; Fathy et al., 1999]. Internalization, however, does not appear to be directly linked to desensitization, at least not in the rBKB2R [Prado et al., 1998].

In this study we set out to delineate the actions of the remaining individual hydroxyl containing residues in the IC region of the rBKB2R. We first examine the importance of S139 in the IC2 and T239 in the IC3. We then build chimeras between rBKB2R and rAT1aR within the distal C-terminus. We examine the effect of a large sequence exchange, 34 amino acids, within the distal tail and a smaller exchange involving 17 amino acids containing the S/T cluster of rBKB2R and the first S/T cluster at position 326–342 of the rAT1aR. We then construct a series of modifications of the S/T residues in the rBKB2R distal tail and explore the contribution of the individual hydroxyl containing residues to signal transduction, receptor desensitization, resensitization, and internalization.

MATERIALS AND METHODS

Materials

[^{3}H] BK (78 Ci /mmol), *myo*-[1,2-^{3}H] Inositol (45–80 Ci/mmol) and [^{3}H] arachidonate (60–100 Ci/mmol) were obtained from NEN Life Science Products. Restriction endonucleases were purchased from New England Biolabs. Oligonucleotides were synthesized by Life Technologies, Inc. All other reagents were from Sigma unless stated otherwise.

Site-Directed Mutagenesis

The Hind III site located in the multiple cloning site of pBluescript plasmid containing the rBKB2R was silenced by blunting the site followed by ligation. The next step was to introduce a silent mutation in the C-terminal segment of the rBKB2R at position 333 and 351 to contain restriction sites, Nsi 1 and Hind III, respectively. This clone allowed us to make any combination of mutations within the region 333–351 by constructing complementary oligonucleotides containing the desired mutant with Nsi 1 and Hind III at the 5′ and 3′ ends, respectively. The complementary oligonucleotides when annealed form a cassette that is ligated to the corresponding restriction site at position 333–351. The nucleotide sequence of the mutant construct is confirmed by sequencing and then subcloned into the Xho 1 and Xba 1 site of the expression vector, pCMIN [Zhou et al., 2000].

Oligonucleotides Used to Construct the Mutants

S139A sense strand 5′ CCG GTA CCT GGC ACT AGT GAA GAC CAT GGT GAT GGG CCG GAT GCG CGG GGT ACG CTG GGC 3′ antisense strand 5′ TTA GCC CAG CGT ACC

CCG CGC ATC CGG CCC ATC ACC ATG GTC TTC ACT AGT GCC AGG TA 3′.

T239V sense strand 5′ TTA AGG AAC AAC GAG ATG AAG AAG TTC AAG GAG GTC CAG GTG GAG AAG AAG GCC ACA GTA 3′ antisense strand 5′ CTA GTA CTG TGG CCT TCT TCT CCA CCT GGA CCT CCT TGA ACT TCT TCA TCT CGT TGT TCC 3′.

BKAT34 sense strand 5′ CCT AAA TGC ATG TCA CAC GCA GGC TTG TCA ACA AAA ATG AGC ACT CTT TCC TAC CGC CCT TCA GAT AAC ATG AGT 3′ antisense strand 5′ GTT TCG AAGCTTTCACTCCACTTCAAAAAAAGATGC-AGACTT TTTGGCAGATGAACTCATGTTAT-CTGAAGGGCGGTA.

BKAT17 sense strand 5′ CCTAAATGCATGT-CACACGCAGGCTTGTCAACAAAAAT GAGC-ACTCTTTCCTACCGCCCTTCAGATAAGCTT-CAGGAT 3′ antisense strand 5′ ATC CTG AAG CTT ATC TGA AGG GCG GTA GGA AAG AGT GCT CAT TTT TGT TGA CAA GCC TGC GTG TGA CAT GCA TTT AGG 3′.

SASS sense strand 5′ TGG GAG AGT CCG TCC AGA TGG AGA ACG CCA TGG GGA CTC TGA GGA CCT CTA TCT CGG TCG ACC GGC AGA TCC ACA 3′ antisense strand, 5′ TGT GGA TCT GCC GGT CGA CCG AGA TAG AGG TCC TCA GAG TCC CCA TGG CGT TCT CCA TCT GGA CGG ACT CTC CCA 3′.

ASSS sense strand 5′ TGG GAG AGG CCG TCC AGA TGG AGA ACT CCA TGG GGA CTC TGA GGA CCT CTA TCT CGG TCG ACC GGC AGA TCC ACA 3′ antisense strand 5′ TGT GGA TCT GCC GGT CGA CCG AGA TAG AGG TCC TCA GAG TCC CCA TGG AGT TCT CCA TCT GGA CGG CCT CTC CCA 3′.

AASS sense strand 5′ TGG GAG AGG CCG TCC AGA TGG AGA ACG CCA TGG GGA CTC TGA GGA CCT CTA TCT CGG TCG ACC GGC AGA TCC ACA 3′ antisense strand 5′ TGT GGA TCT GCC GGT CGA CCG AGA TAG AGG TCC TCA GAG TCC CCA TGG CGT TCT CCA TCT GGA CGG CCT CTC CCA 3′.

AADA sense strand 5′ TGG GAG AGG CCG TCC AGA TGG AGA ACG CCA TGG GGA CTC TGA GGA CCG ACA TCG CTG TCG ACC GGC AGA TCC ACA 3′ antisense strand 5′ TGT GGA TCT GCC GGT CGA CAG CGA TGT CGG TCC TCA GAG TCC CCA TGG CGT TCT CCA TCT GGA CGG CCT CTC CCA 3′.

A4V2 sense strand 5′ TGG GAG AGG CCG TCC AGA TGG AGA ACG CCA TGG GGG TTC TGA GGG TCG CTA TCG CGG TGG ACC GGC AGA TCC ACA 3′ antisense strand 5′AGC TTG TGG ATC TGC CGG TCC ACC GCG ATA GCG ACC CTC AGA ACC CCC ATG GCG TTC TCC ATC TGG ACG GCC TCT CCC ATG CA 3′.

AAAA sense strand 5′ TGG GAG AGG CCG TCC AGA TGG AGA ACG CCA TGG GGA CTC TGA GGA CCG CTA TCG CGG TCG ACC GGC AGA TCC ACA antisense strand 5′ AGC TTG TGG ATC TGC CGG TCG ACC GCG ATA GCG GTC CTC AGA GTC CCC ATG GCG TTC TCC ATC TGG ACG GCC TCT CCC ATG CA 3′.

T344V sense strand 5′ TGG GAG AGT CCG TCC AGA TGG AGA ACT CCA TGG GGA TTC TGA GGG TCT CTA TCT CGG TCG ACC GGC AGA TCC ACA 3′ antisense strand 5′AGC TTG TGG ATC GCC GGT CGA CCG AGA TAG AGA CCC TCA GAA CCC CCA TGG AGT TCT CCA TCT GGA CGG ACT CTC CCA TGC A 3′.

T347V sense strand 5′ TGG GAG AGT CCG TCC AGA TGG AGA ACT CCA TGG GGG TTC TGA GGA TCT CTA TCT CGG TCG ACC GGC AGA TCC ACA 3′ antisense strand 5′AGC TTG TGG ATC GCC GGT CGA CCG AGA TAG AGA CCC TCA GAA CCC CCA TGG AGT TCT CCA TCT GGA CGG ACT CTC CCA TGC A 3′.

TVTV sense strand 5′ TGG GAG AGT CCG TCC AGA TGG AGA ACT CCA TGG GGG TTC TGA GGG TCT CTA TCT CGG TCG ACC GGC AGA TCC ACA 3′ antisense strand 5′AGC TTG TGG ATC GCC GGT CGA CCG AGA TAG AGA CCC TCA GAA CCC CCA TGG AGT TCT CCA TCT GGA CGG ACT CTC CCA TGC A 3′.

Cell Culture and Transfection

Cell cultures and transfections in Rat-1 cells were performed as described [Prado et al., 1997, 1998]. Rat-1 cells were seeded at 100,000 cells/well in a 6-well plate in Dulbecco's modified Eagle's medium (DMEM), containing 5% fetal bovine serum supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin. Cells were grown at 37°C in a humidified atmosphere with 5% CO_2. For transfection, two solutions were prepared. One solution contained 2 μg of DNA in 20 μl of Opti-MEM medium (Life Technologies, Inc.) and the other solution contained 6 μl of Lipofectamine (Life Technologies, Inc.) and 14 μl of Opti-MEM medium. Both solutions were combined and incubated at room temperature for 15 min. The DNA–liposome complex was added to 1 ml of Opti-MEM medium and added to each well. Sixteen hours later, the transfection medium was replaced with complete medium. Next day, the transfected Rat-1 cells were detached with trypsin and seeded in a 60 cm^2

dish containing complete medium and 500 μg/ml of Geneticin (G418, Life Technologies, Inc.). The neomycin-resistant cell culture was then expanded and tested for the amount of specific binding to [^{3}H] BK.

Immunofluorescence Assays

Cells were grown for 24 h on microscopy glass slides as described [Prado et al., 1996]. Cells were fixed with 3% formaldehyde/2% sucrose in phosphate-buffered saline (PBS) and permeabilized with 0.5% Triton X-100, 0.3 M sucrose, 0.003 M $MgCl_2$, 0.05M NaCl, in 0.01 M HEPES pH 7.6. Cells were incubated with anti-B2 Bradykinin-R mAb (Transduction Laboratories, Lexington, KY) diluted 1:200 in 1% bovine serum albumin (BSA)/PBS. Bound antibodies were detected by incubation with fluorescein isothiocyanate (FITC)-conjugated secondary anti-mouse IgG antibody (Sigma Catalog no. 6257) at a dilution of 1:50 in 1% BSA/PBS. Cells were then treated with slow fade-light reagent (Molecular Probes) to minimize photobleaching. To assess receptor internalization, cells were incubated with 10 nM BK for 30 min at 37°C and then fixed and permeabilized as above. Cells were viewed under a Nikon TE 200 Inverted fluorescence microscope with a 100× oil immersion objective lens.

Receptor Binding and Internalization

Receptor binding studies and internalization of the rBKB2R in intact Rat-1 cells were carried out as described previously [Prado et al., 1997, 1998]. Briefly, to determine binding, 80–100% confluent cell monolayers in 24-well plates (Costar, Cambridge, MA) were incubated in binding buffer containing various concentrations of [^{3}H] BK in the absence (total binding) or presence of 10^{-7} M BK (nonspecific binding) for 2 h at 4°C. Cells were washed three times with ice-cold buffer and then solubilized with 0.2% sodium dodecyl sulfate. Radioactivity was determined in a β counter. The affinity and the number of binding sites were then determined. To determine receptor internalization, cells were incubated with 100 nM BK at different time points at 37°C. Cells were washed with ice-cold buffer and acid-stripped with 0.2 M acetic acid, pH 3.0, containing 0.5 M NaCl. The number of binding sites remaining at the cell surface was then determined by performing a binding assay as described above.

Desensitization and Resensitization

Stably transfected Rat-1 cells were detached and washed once in DMEM containing 5% fetal bovine serum. Cells were then washed a second time in physiological buffer solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 0.9 mM $CaCl_2$, 15 mM HEPES, 0.1% bovine serum albumin). Cells were resuspended at 1×10^7 cells/ml and incubated with FURA-2AM 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 1×10^7 cells/ml. Ca^{2+} mobilization experiments were performed using a Perkin Elmer LS-3B fluorescense spectrophotometer. IC calcium increase in the presence or absence of BK was measured as described [Prado et al., 1998]. Data were analyzed using the FURA program.

To examine desensitization, Ca^{2+} flux was determined upon exposure of cells to 10 nM BK followed by two washes and spins and subsequent reexposure to 10 nM BK. Resensitization was examined by exposing cells to a desensitizing dose of 10 nM BK for 2 min at 37°C. The cells were then washed with physiological buffer, resuspended at 1×10^7 cells/ml and incubated at 37°C at 5% CO_2 for 15 min to allow for recovery. Calcium flux was then measured upon exposure of the cells to 10 nM BK.

Molecular Modeling

The molecular model of the rBKB2R was built as described previously [Prado et al., 1998]. In short, the experimental data from rhodopsin [Schertler et al., 1993; Baldwin et al., 1997] was used to arrange the transmembrane helices. The loops and termini were then added, incorporating the secondary structural elements identified by BLAST-based [Altschul et al., 1990] homology analysis [Prado et al., 1998]. The secondary structure includes a well defined, amphipathic α-helix for residues 310–329 and a less well-defined helix for residues 335–350.

The model of the receptor was refined using molecular dynamics (MD) + simulations using the GROMACS program [van Aalten et al., 1995]. A novel water/decane/water simulation cell was used to mimic the hydrophilic/hydrophobic, biphasic nature of the membrane in a computationally simple model [Pellegrini et al.,

1998; Prado et al., 1998]. The transmembrane helices were introduced into a 40 Å layer of decane (518 molecules) and the extracellular and IC regions soaked with ~40 Å layers of water (12,906 molecules).

RESULTS

Diagram of Mutations

Figure 1 shows the location of OH containing residues within the IC face of rBKB2R. This includes S139 within IC2, T239 within the IC3, and the S/T cluster in the distal C-terminus. Figure 1 also illustrates the mutations carried out on S139, T239, and the carboxylic tail of BKB2R. As illustrated, S139 was replaced with alanine, T239 was replaced with valine. The last 34 amino acids of BKB2R were replaced with the last 34 amino acids of the rAT1aR (BK/AT34). The cluster of S/T at position 326–342 within the rATa1R C-tail region replaced the S/T cluster of the rBKB2R at position 333–351 (BK/AT17). Also the series of serines (position 335, 341, 348, and 350) and threonines (position 344 and 347) were replaced by alanine or valine, respectively, as illustrated. Aspartate was used to generate a negative charge at S348. All the described mutants bound BK with kinetics that approximate WT. The Bmax ranged from $3-7 \times 10^4$ receptors/cell.

Second and Third IC Loops

We formerly illustrated that Y131 and T137 located in the IC2 were critical for BKB2R internalization and signal capacity [Prado et al., 1998]. Figure 2a illustrates the effect of mutating S139, the remaining OH possessing residue in IC2 and T239, the only OH possessing residue in IC3, on BK stimulated PI production. Exchanging S139 for alanine or T239 for valine had little effect on signal capacity of the receptor. The receptor activity remained 86 and 74% of WT, respectively. Figure 2b illustrates receptor internalization. Again, the mutation of either S139A or T239V had little effect on endocytosis of rBKB2R. Both are only slightly lower in comparison to WT.

BK/AT1a Receptor C-Tail Chimeras

The consequence of exchange of the last 34 amino acids of BKB2R with the corresponding rAT1aR sequence (BK/AT34) and replacement

I. Second Intracellular Loop

```
                131    137 139
rBKB2R   DRYLALVKTMSMGRMRGVRWAK
 S139A   ----------A-----------
```

II. Third Intracellular Loop

```
                        239
rBKB2R   QVLRNNEMKKFKEVQTEKK
 T239V   ---------------V---
```

III. C-terminus of the BKB2R

```
                                                   348
                    322               335   341 344 347 350
BKAT34   -------------------------SHSSLSTKMSTLSYRPSDNMSSSAKKPASCFEVE
BKAT17   -------------------------SHSSLSTKMSTLSYRPS
rBKB2R   VGKRFRKKSREVYQAICRKGGCMGESVQMENSMGTLRTSISVDRQIHKLQDWAGNKQ
AAAA     -------------------------A-----A------A-A----------------
A4V2     -------------------------A-----A--V--VA-A----------------
T344V    ----------------------------------V----------------------
T347V    -------------------------------------V-------------------
TVTV     ----------------------------------V--V-------------------
AASS     -------------------------A-----A-------------------------
ASSS     -------------------------A-------------------------------
SASS     -------------------------------A-------------------------
SSSA     ----------------------------------------A----------------
SSAS     --------------------------------------A------------------
AADA     -------------------------A-----A------D-A----------------
```

Fig. 1. Amino acid sequence alignment of the wild type and mutant rBKB2R and the overall structure of the rBKB2R/rAT1aR chimeras. The complete sequence of the IC2, IC3, and the C-tail of the rBKB2R is shown using the single letter amino acid code. A dash under a given mutant receptor indicates identical residues while residues replaced are identified with the appropriate single letter code with the position number(s) given above. Receptor chimeras, BKAT17 and BKAT34, were generated by exchanging specific regions of the C-tail of rAT1aR and the rBKB2R as indicated by the underlined sequence.

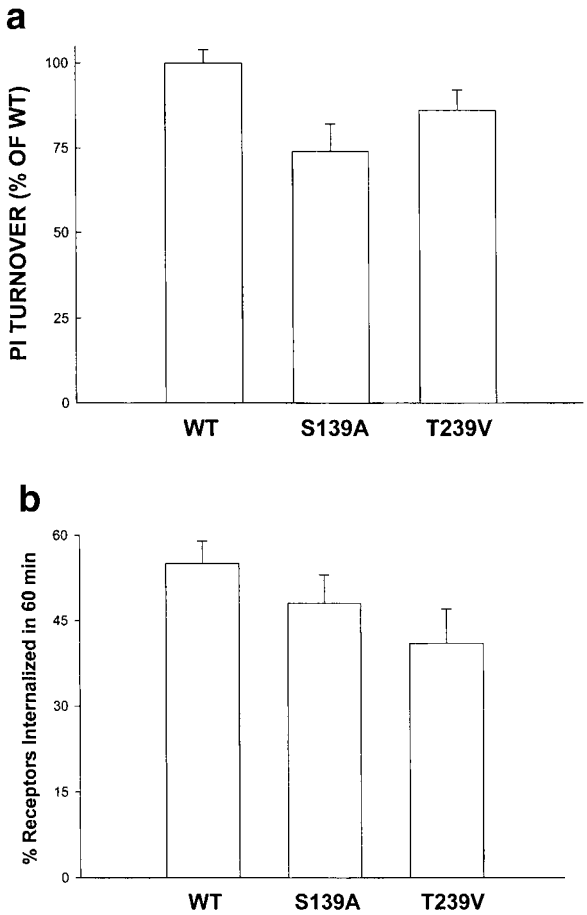

Fig. 2. Second and third intracellular loop mutant rBKB2 receptors. **a**: BK-induced PI turnover by Rat-1 cells expressing WT, S139A, and T239V mutant rBKB2 receptors. PI turnover was measured in myo-[^{3}H]inositol-labeled cells as described in the Methods section. Data represent triplicate wells from three experiments. **b**: Internalization profile of Rat-1 cells expressing WT, S139A, T239V mutants. Rat-1 cells transfected with given receptor were incubated with 100 nM BK for 60 min, acid-stripped, as described in the Methods section. Presence of the remaining surface receptors was determined by [^{3}H] BK binding as described in Methods section.

of the rBKB2R region (333–351) with the rAT1aR region 326–342 (BK/AT17) with respect to receptor uptake and resensitization, and signal capacity are illustrated in Figure 3. As shown in Figure 3a, 73% of the BK/AT34 mutant was internalized 60 min after exposure to 10 nM BK. This proved to be approximately the same as WT, which internalized 62% at 60 min. The BK/AT17 mutant was internalized at a considerably slower rate than either WT or BK/AT34, 37% internalizing during the same time span. As illustrated in Figure 3b, the capacity of the mutant receptors to stimulate PI turnover following activation with 10 nM BK for the BK/AT34 and BK/AT17 chimeras proved approximately the same as for WT. However, receptor resensitization of these two mutants, as determined by Ca^{2+} translocation following exposure to 10 nM BK, differed radically (Fig. 3c). Whereas BK/AT34 resensitized normally, BK/AT17 which internalized poorly also did not resensitize. Both mutants desensitized normally as WT.

BKB2R C-Tail Ser/Thr Mutations

We then mutated all 4 serines within the distal tail of BKB2R (position 335, 341, 348, and 350) to alanine (AAAA) followed by the two threonines (position 344 and 347) to valine (A4V2). Both the A4V2 and AAAA mutants displayed very poor internalization, no greater than 20% after 1 h (Fig. 4a). Next, the two threonines alone were mutated to the corresponding valine (TVTV). This mutant was internalized to a very similar degree as WT suggesting that these two residues were not a major factor in receptor uptake. On the other hand, AASS (Ser335 and 341 mutated to alanine) and SASS (Ser341 mutated to alanine) internalized very rapidly, to ~90% within 1 h following receptor activation with 10 nM BK. This suggests an involvement of S341 in the modulation of receptor uptake. When S348 was exchanged for alanine (SSAS), receptor internalization became poor, at approximately the same level as A4V2 and AAAA at less than 25% within 1 h after exposure to 10 nM BK. Conversion of alanine 348 of the AAAA mutant to aspartate (AADA) did not improve receptor internalization. The SSSA mutant (S350 to alanine) and ASSS mutant (S335 to alanine) internalized normally.

The capacity of the mutants to activate PI turnover differed markedly from their potential for internalization. As illustrated in Figure 4b, unlike the negligible role of the threonines in receptor uptake, their role in the activation of PI turnover proved important, with TVTV showing very poor signal transduction. Further scrutiny of threonine 344 and 347 attributed T344 (T344V) the critical role in BKB2R activation of PI turnover. In addition to T344, certain serines within the cluster also proved important for signal transduction. Both A4V2 and AAAA mutants proved very poor signal transducers (Fig. 4b). Interestingly, single mutations of serine to alanine at position 341 and 350 (SASS and SSSA) resulted in a receptor with a very

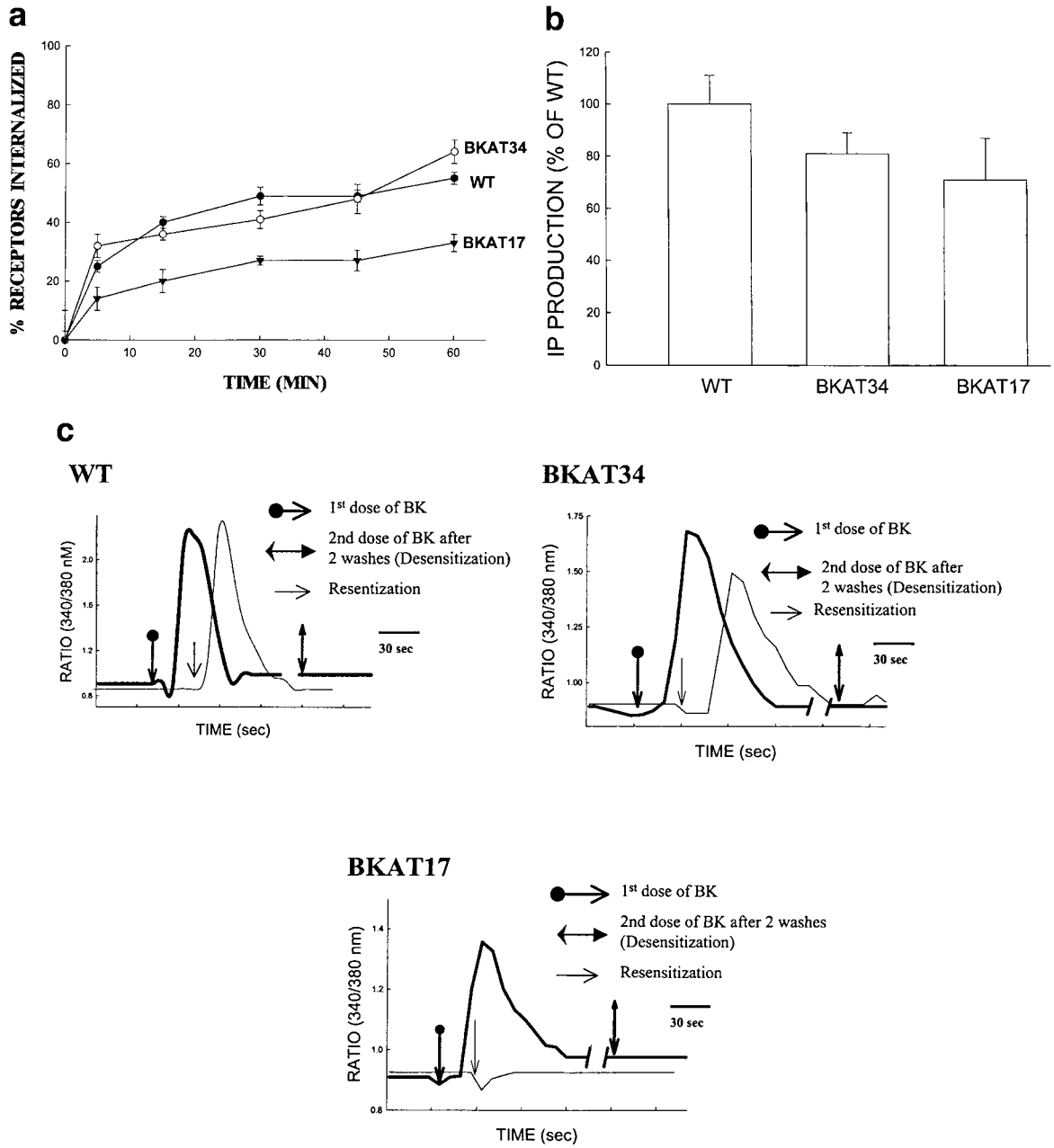

Fig. 3. BK/AT1a receptor chimeras. **a**: Internalization profile of Rat-1 cells expressing WT (●), BKAT34 (○), BKAT17 (▼). Cells containing these receptors were incubated with 100 nM BK for the times indicated. After acid stripping and three washes with ice-cold buffer, [^{3}H] BK binding to Rat-1 cells was measured. Results are mean of duplicate wells from three experiments. **b**: BK-induced PI turnover of Rat-1 cells expressing WT, BKAT34, and BKAT17 receptor chimeras. PI turnover was measured in myo-[^{3}H]inositol-labeled cells as described in Methods section. Data represent triplicate wells from three experiments. **c**: Desensitization and resensitization of BK/AT receptor chimeras. Dark tracing, Rat-1 cells expressing WT, BKAT34, and BKAT17 receptors loaded with Fura-2 were stimulated with a first dose of 10 nM BK (●→) followed by two washes (as indicated by gap) and a second dose of 10 nM BK (↔) to test for receptor desensitization. Light tracing represents Rat-1 cells expressing WT, BKAT34, and BKAT17 receptors which were stimulated with a second dose of 10 nM BK (→) after 15 min of recovery at 37°C from the first dose of 10 nM BK. Data are representative of three independent measurements.

poor receptor signaling capacity. On the other hand, the conversion of S335 to alanine (ASSS) or S348 to alanine (SSAS) proved irrelevant for receptor activated PI turnover.

We formerly showed a pattern of receptor resensitization as directly related to its uptake in BKB2R, with mutants displaying poor uptake not resensitizing while those displaying

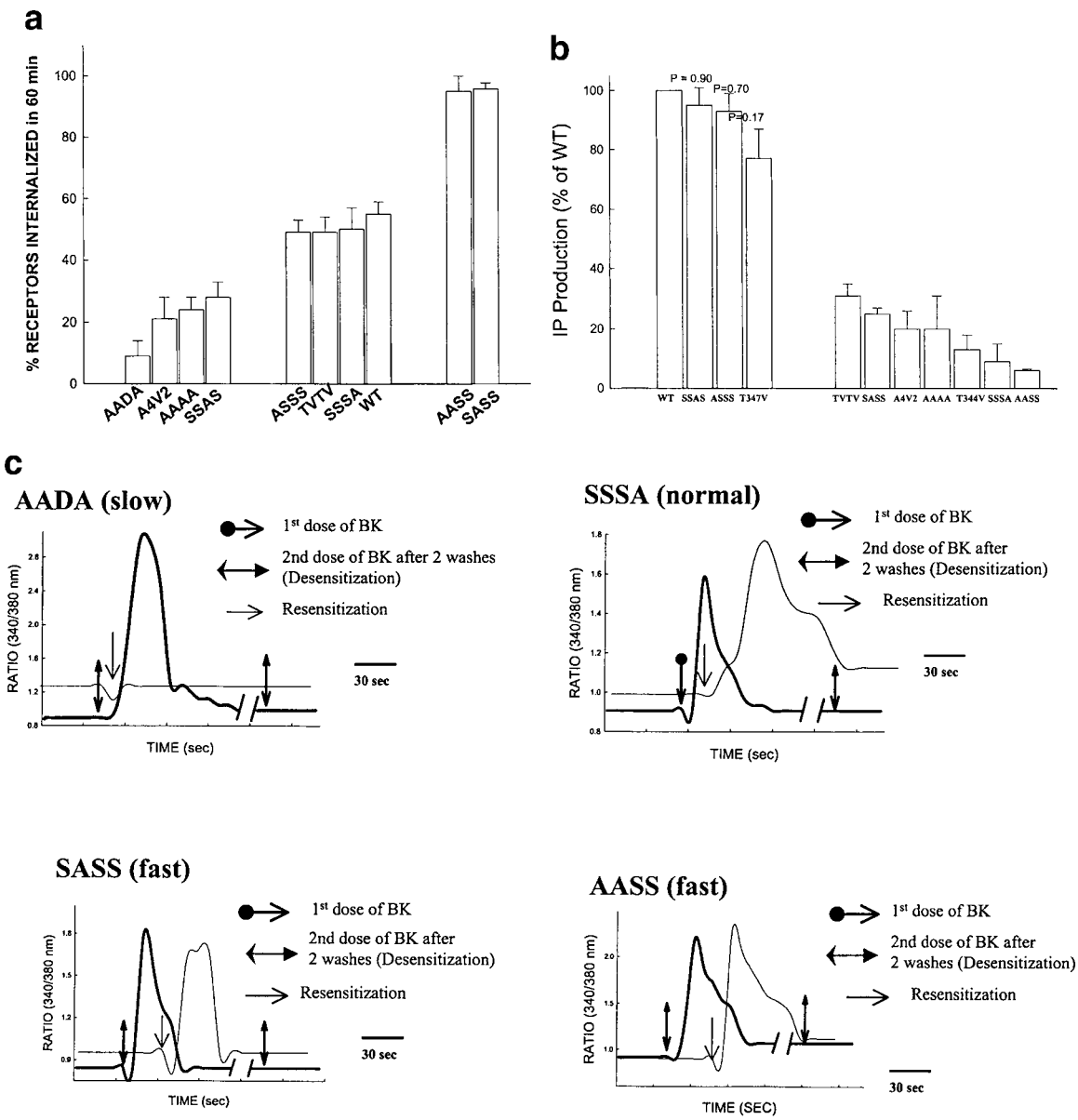

Fig. 4. C-terminal mutant rBKB2 receptors. **a**: Internalization of S/T mutants within the C-tail. The mutants are categorized into three groups: slow internalizing receptors (AADA, A4V2, AAAA, SSAS), normal internalizing receptors (ASSS, TVTV, SSSA, and WT), and fast internalizing receptors (AASS and SASS). To determine internalization, cells were incubated with 100 nM BK for 60 min and acid-stripped. The remaining receptors located at the cell surface were determined by [^{3}H] BK binding. **b**: BK-induced PI turnover of Rat-1 cells expressing WT, SSAS, ASSS, T347V, TVTV, SASS, A4V2, AAAA, T344V, SSSA, AASS receptor chimeras. Data represent triplicate wells from three experiments. **c**: Desensitization and resensitization of representative C-terminal mutant rBKB2 receptors expressed in Rat-1 cells. Dark tracing, Rat-1 cells expressing AADA (non-internalizing receptor), SSSA (normally internalizing receptor as WT), AASS and SASS (fast internalizing receptors) loaded with Fura-2 were stimulated with a first dose of 10 nM BK (●→) followed by two washes (indicated by gap) and a second dose of 10 nM BK (↔) to test for receptor desensitization. Light tracing indicates same cell samples as above which were stimulated with a second dose of 10 nM BK (→) after 15 min of recovery at 37°C from the first dose of 10 nM BK. Data are representative of three independent measurements.

normal or rapid uptake resensitizing normally [Prado et al., 1998]. This pattern was seen again with the chimera BKAT17 shown above and is also exemplified with AADA, the mutant with very poor internalizing capacity (Fig. 4a) which did not resensitize (Fig. 4c). SSSA, the normally internalized mutant (Fig. 4a) and SASS and AASS, the rapidly internalized mutants

(Fig. 4a) resensitized normally (Fig. 4c). Importantly, all mutants tested, no matter what their rate of internalization, desensitized completely as exemplified with the mutants shown in Figure 4c.

Immunofluorescence of rBKB2R

Receptor internalization was visualized using immunofluorescence (Fig. 5). WT and the mutant AADA, which displayed very poor uptake with the labeled procedure above, were used as examples. Panel a illustrates WT receptor not exposed to BK. In this case, a strong fluorescence at the plasma membrane is visible when FITC-fixed cells are incubated with the anti-B2 Bradykinin-R mAb. Panel b shows redistribution of fluorescence into distinct pockets within the cell following exposure of WT to 10 nM BK. Panel c shows an AADA transfected cell. As with WT, the AADA mutant exhibits a distinct, bright fluorescence on the cell surface before exposure to BK. After exposure to 10 nM BK the AADA mutant receptor remains on the cell surface with little internalization taking place, as illustrated in Panel d. Panel e, a non transfected Rat-1 cell, shows no distinct fluorescence.

Molecular Modeling

To clarify the apparent residue interaction within the S/T cluster in the distal C-tail region of the BKB2R, molecular modeling was performed on the C-terminus of BKB2R. Based on homology analysis, the C-terminus contains two α-helices (residues 310–329 and residues 335–350). As described previously [Prado et al., 1998], we explored different arrangements of the proximal helix to the seven helical bundle. During the simulations we observed a tendency for the helices to adopt a quasi-antiparallel arrangement (Fig. 6). The 310–329 helix was found to lie on the membrane surface on the outside (membrane face) of TM6. The five residues between the helices form a loop. The second helix, residues 335–350, lies on the outside of the helical bundle, near TM5 and TM4 pointing to interaction with IC2 and IC3, with S350 in close proximity to T137. Within the

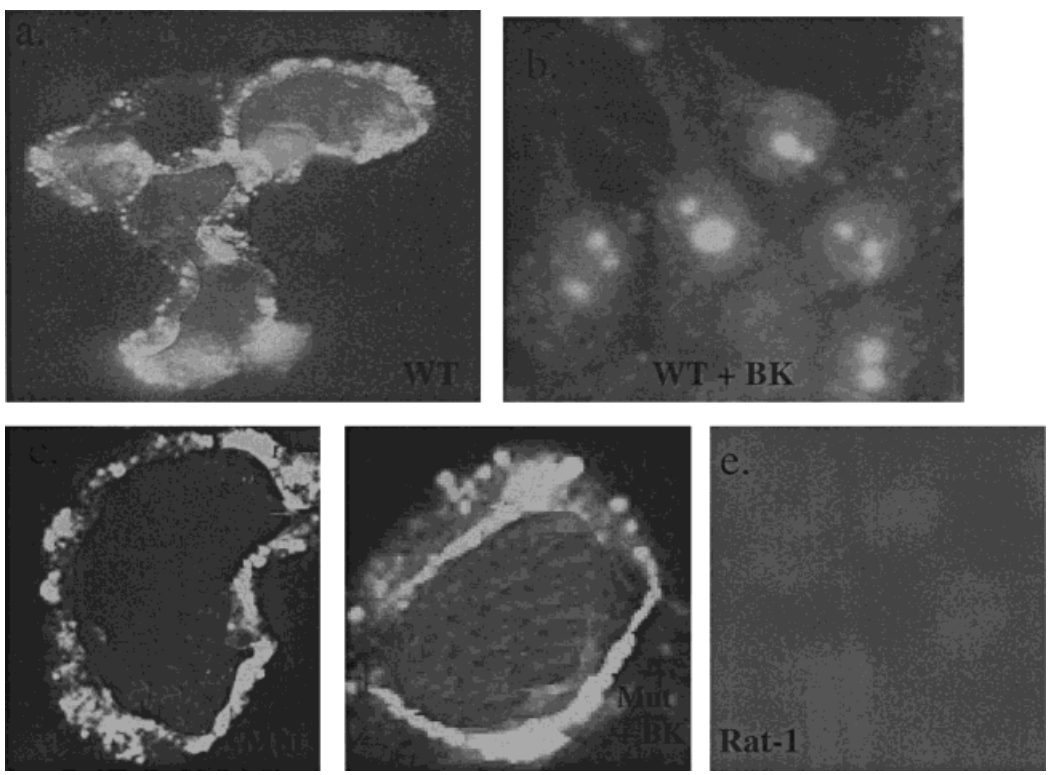

Fig. 5. Subcellular distribution of wild type and mutant rBKB2 receptor stably expressed in Rat-1 cells. Cells expressing wild type or AADA mutant rBKB2 receptors were incubated without (control) (panel a and c) or with 10 nM BK (panel b and d) for 30 min, fixed and permeabilized. The rBKB2 receptors were visualized with receptor-specific antibodies. Panel e shows no visible fluorescence in non-transfected Rat-1 cells.

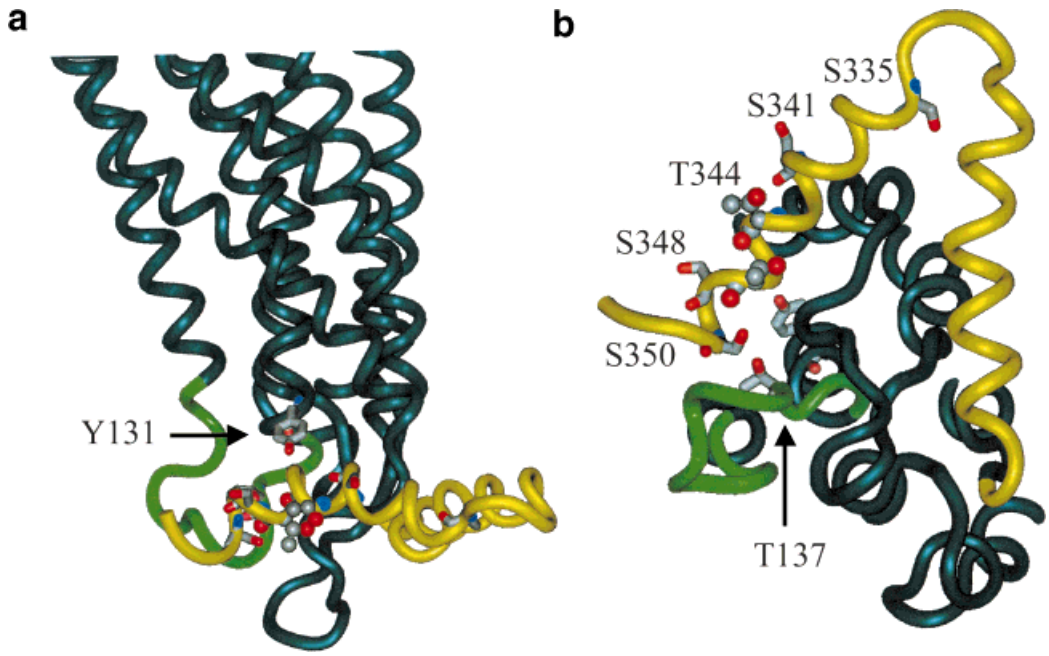

Fig. 6. The rBKB2 receptor conformation generated by MD simulations in a decane/water two-phase simulation cell. The MD simulations of the rBKB2R are shown in side (**a**) and bottom (**b**) views. The transmembrane helices are shown as blue cylindrical alpha helices while the IC2 and IC3 loops are shown as blue ribbons projecting loosely in the intracellular side. The C-terminus is shown as cyan colored ribbons with proximal and distal alpha helices.

helix, T344 and S341 are close to each other (separated by one helical turn) while S335 is distantly removed from any of the other hydroxyl containing residues within the C-terminus. Importantly, there is a large distance between S348 and T137, since S348 is on the opposite face of the C-terminal helix, projecting away from the IC2.

DISCUSSION

In a previous publication we noted that the replacement of T137, located in the proximal IC2, with proline or aspartate led to dramatic changes in rBKB2R function [Prado et al., 1998]. Exchange for proline resulted in a receptor which continued to signal but was not endocytosed. Exchange for aspartate resulted in a receptor which was endocytosed but displayed very poor signaling capacity. The replacement of Y131, also in the IC2, with alanine (Y131A) or serine (Y131S) led to poor BK activated signal capacity while Y131S resulted in a high rate of uptake and Y131A in a slowed rate of uptake. These results illustrated that the IC2 was involved in signal transmission as well as receptor uptake. Further results illustrated that simultaneous mutation of Y131 and Y322, located in the proximal C-terminus, to alanine or serine led to a selective normalization of the rBKB2R signaling capacity. Thus specific interaction between the IC2 and the C-terminus is apparently taking place in the rBKB2R. Indeed, receptor modeling showed that in the ligand-activated rBKB2R the C-terminus is located in the vicinity of the IC2 thus enabling Y131/Y322 interaction. These results point to the importance of motif interaction in G-protein coupled receptor function. The mutations of T137 also pointed to the importance of OH possessing residues within rBKB2R IC region in receptor internalization. Phosphorylation of Y131 and Y322 proved unnecessary for receptor self maintenance since replacement of both residues with either phenylalanine or serine resulted in normal uptake and resensitization [Prado et al., 1998]. However, the results obtained with the exchange of T137 with aspartate suggested that creating a negative charge at position 137 facilitates receptor uptake but negates receptor signal capacity [Prado et al., 1998].

In the present communication we proceeded to examine the role of the remaining OH containing residues within the IC face in rBKB2R function and maintenance. This consisted of

S139 in the IC2, T239 in the IC3 and the S/T cluster of four serines and two threonines located in the distal portion of the C-terminus. Nothing is known of the role of S139 or T239 in either rBKB2R function or maintenance. However, the S/T cluster in the distal tail has been implicated in receptor internalization [Pizard et al., 1999; Soskic et al., 1999]. In fact, a cluster of serine residues in the distal C-terminus exists in a number of G-protein coupled receptors (GPCR), including interleukin-8 [Prado et al., 1996], serotonin [Hartig et al., 1993], bombesin [Battey et al., 1991], and angiotensin [Balmforth et al., 1995]. Amino acid alignment of a number of G-protein linked receptors showed that the AT1aR family has the closest amino acid homology to BKB2R. In the AT1aR the cluster of serines and threonines within its distal tail at position 326–342 has been described as crucial for its endocytosis [Smith et al., 1998].

The replacement of either S139 with alanine or T239 with valine, neutral amino acids, had little effect on rBKB2R signal capacity or uptake function. This illustrated that the presence of an OH group in these positions is unimportant for BKB2R function or maintenance. To learn more about the overall function of the last 34 residues containing the C-terminal S/T cluster in the rBKB2R, we replaced the entire 34 amino acid sequence of the rBKB2R with the last 34 residues of AT1aR. We also replaced the 17 amino acids containing the S/T cluster within the BKB2R tail with the corresponding 17 amino acid region containing an S/T cluster from AT1aR reported to be critical in uptake. Both of the BK2R/AT1aR chimeras showed good signal capacity as illustrated with BK activated PI turnover. However, the BKAT17 mutant internalized poorly while BKAT34 internalized normally. Thus the S/T cluster within the C-tail of the AT1aR at position 326–342 is able to substitute for the single BKB2R S/T cluster with regard to signal transmission but not internalization. For internalization, additional non-hydroxyl containing motifs within the distal BKB2R tail appear necessary. However, when these motifs are replaced with the entire rAT1aR sequence containing the last 34 amino acids then internalization becomes normal. Thus, the distal tail section of rBKB2R appears interchangeable with the corresponding distal tail of the rAT1aR. It remains to be determined which motifs outside of the S/T cluster participate in determining receptor uptake.

Although replacement of the rBKB2R region containing the S/T cluster with the rAT1aR region 326–342 or the entire 34 distal residues results in a normally signaling receptor, the presence of one of the threonines and certain serines within the native BKB2R distal C-terminus is crucial for PI turnover. This was observed when the distal tail of rBKB2R was retained and mutations within its S/T cluster were carried out. In fact, all mutants generated with the exception of the exchange of S348 for alanine (SSAS), S335 for alanine (ASSS) or T347 for valine (T347V) resulted in defective signaling capacity. TVTV, T344V, SSSA, AASS, SASA, and SASS were all very poor signal transducers. The sum of these results suggests that the presence of both S341 and S350 is crucial to signal transmission.

Our results further suggest that certain serines participate in internalization. Fluorescence microscopy visualized the internalization of the receptor of BK-stimulated WT cells into IC compartments, probably into clathrin coated pits [Pizard et al., 1999] or to caveolae [Haasemann et al., 1998; Hukovic et al., 1998]. This visualization corroborated our internalization results obtained with the ^{3}H-BK technique. The AADA mutant, which was determined to undergo only minimal internalization by the labeled BK binding procedure, was correspondingly not internalized with the fluorescent microscopy procedure. The sum of these results points to the presence of S348 as crucial for normal BKB2R uptake. Replacement of any of the other three serines (335, 341, or 350) resulted in either no change in internalization (SSSA) or a substantially increased receptor uptake (AASS, SASS). Interestingly, replacement of S341 with alanine either alone (SASS) or in combination with S335 (AASS) resulted in a very rapidly internalized receptor with endocytosis at ~90% within 60 min of exposure to ligand suggesting that S341 acts to limit uptake. Thus cooperative residue effects may prove important for appropriate receptor uptake. This is exemplified by the apparent roles of S341 as a modulator and S348 as a promoter of uptake.

Receptor desensitization is an intrinsic property of the GPCRs. The OH possessing residues in the C-terminal domain have been linked to this process [Pals-Rylaarsdam et al., 1995;

Hukovic et al., 1998; Spurney, 1998]. However, with regard to desensitization of the BKB2R, our results show that neither exchange with the C-tail of the AT1aR nor alteration of any of the S/T residues within the C-tail or, in fact, within the IC face of the receptor itself to neutral, non-phosphorylatable, amino acids affected desensitization. All the mutants tested, whether they internalized, resensitized or not, desensitized in the same manner as WT. Thus desensitization of the BKB2R does not display dependence on any single phosphorylatable residue located in the IC of the BKB2R. In fact, within the span of experiments conducted here and in past reports [Prado et al., 1997, 1998] we mutated all the OH containing residues within the IC face of BKB2R to neutral amino acids such as valine or alanine for the T/S residues and phenylalanine or alanine for the tyrosine residues. These mutations were often fairly extensive with regard to the number of residues mutated at one time, including the conversion of all six S/T residues located within the distal tail of BKB2R. None of these exchanges resulted in a mutant lacking the ability to desensitize. This points to either, other non OH containing motifs within the IC face of the receptor, or transmembrane OH containing residues as ultimately being responsible for this agonist-induced process in this receptor.

On the other hand, experiments dealing with rBKB2R resensitization have correlated directly with receptor uptake. For example, we showed previously that T137 when converted to proline did not internalize and did not resensitize [Prado et al., 1998]. Also the Y131A/Y322A mutant which internalized more slowly than the other Y131, Y322 mutants and WT also resensitized poorly. Here we confirm these previous results. The mutants AADA and BKAT17 do not resensitize and also internalize very poorly. Both, the rapidly internalizing mutants SASS and AASS and the normally internalizing mutants BKAT34 and SSSA resensitized equally to WT. The phosphorylation of specific S/T residues within the IC face of the BKB2R with regard to these two processes awaits further determination.

At this time, it is not clear exactly how the actions and interactions of these serines and threonines within the BKB2R IC take place. In our receptor model we concluded that in the activated receptor the C-terminal tail of WT adopts two α-helices, consisting of residues 310–329 and residues 335–350. The serines/threonines within the distal tail would all be located on this latter helix. As illustrated in Figure 6, within this model S350 is located in proximity of T137 (located within the 2nd IC loop) a residue which was formerly shown to be critical for BKB2R signaling [Prado et al., 1998]. Interestingly S350 is also involved in receptor signaling. The proximity of S350/T137 could thus be construed as important in BKB2R signaling action. Additionally, S341 and T344 are also in proximity as illustrated in Figure 6. Again, both of these residues are important in BKB2R signal transmission. Endocytosis of the BKB2R is apparently accompanied by phosphorylation of the S/T cluster [Pizard et al., 1999]. Introduction of a large, negatively charged phosphate group at one of the S/T sites of the C-terminus, would be expected to disrupt the α-helix. In such a case, S348/T137 could move in close proximity of each other, in contrast to the non-phosphorylated state in which these residues face opposite directions within the helix. Although currently only a working model, the data presented here, combining targeted site-directed mutagenesis and molecular modeling, is providing insight into the structural differences of the receptor during signaling and endocytosis.

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