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K317, R319, and E320 Within the Proximal C-Terminus of the Bradykinin B2 Receptor Form a Motif Important for Phospholipase C and Phospholipase A_2 but not Connective Tissue Growth Factor Related Signaling

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Abstract We showed previously that large domain exchanges between the bradykinin B2 (BKB2) and angiotensin II type 1a (AT1a) receptors can result in functional hybrids. However, when we proceeded to exchange the entire bradykinin B2 receptor (BKB2R) C-terminal tail with the AT1aR C-terminus, the hybrid, while continuing to bind BK and be endocytosed as wild type (WT) BKB2R, lost much of its ability to activate phosphatidylinositol (PI) turnover or the release of arachidonic acid (ARA). In this study, we constructed chimeric receptors within the proximal C-terminus between the BKB2R and AT1aR or bradykinin B1 receptor (BKB1R). The mutant and WT receptor cDNAs were stably transfected into Rat-1 cells. Also, point mutations were generated to evaluate the role of the individual residues within this region. These chimeric studies revealed that the proximal portion of the BKB2R C-tail is crucial for G protein-linked BKB2R functions. This region could not be swapped with the AT1aR to obtain a BK activated PI turnover or ARA release. Further studies demonstrated that the distal portion (325–330) of this region is exchangeable; however, the middle portion (317–324) is not. Small motif exchanges within this section identified the KSR and EVY motifs as crucial for $G_{\alpha q}$, $G_{\alpha i}$ related signaling of the BKB2R. Point mutations then showed that the charged amino acids K317, R319, and E320 are the residues critical for linking to PI turnover and ARA release. However, these proximal chimeras showed normal receptor uptake. Interestingly, while apparently not activating G protein-linked signaling, the proximal tail AT1aR exchange mutant and the entire C-terminus exchange hybrid continued to cause a substantial bradykinin effected increase in connective tissue growth factor (CTGF) mRNA level, as WT BKB2R. J. Cell. Biochem. 92: 547–559, 2004.

Key words: bradykinin B2 and B1 receptors; angiotensin II AT1 receptor; G protein coupled receptors; chimera; mutagenesis; carboxyl-terminal tail; signal transduction; molecular modeling; connective tissue growth factor; hybrid receptors

The bradykinin B2 receptor (BKB2R) is known to participate in a number of physiologic functions related to inflammation, pain, asthma, and blood vessel dilation [Kaplan et al., 1998; Liebmann, 2001; Prado et al., 2002]. The biological function, cloning, and the various intracellular (IC) motifs participating in signaling and self maintenance of this receptor have been recently reviewed [Prado et al., 2002]. BKB2R is a G protein coupled receptor (GPCR) and has been widely used in many cascading reactions. It couples to $G_{i2\alpha}$ and G_{i3} and $G_{\alpha q/11}$, characterized by phospholipase C (PLC) and phospholipase A_2 (PLA_2) activition and the turnover of phosphatidylinositol (PI), the release of arachidonic acid (ARA), and the mobilization of Ca^{2+} [Marceau and Bachvarov, 1998; Prado et al.,

Abbreviations used: BK, bradykinin; BKB2R, bradykinin B2 receptor; BKB1R, bradykinin B1 receptor; AT1aR, angiotensin II type 1a receptor; GPCR, G protein coupled receptor; CTGF, connective tissue growth factor; ARA, arachidonic acid; PI, phosphatidylinositol; IC, intracellular; TM, transmembrane; WT, wild type; MD, molecular dynamics.

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2002]. BK also elicits signaling responses that are typically associated with the involvement of growth factors, protein kinases, and protein tyrosine phosphatases [Liebmann, 2001].

The angiotensin type 1 receptor (AT1R) is relatively close to BKB2R in sequence length and homology within the IC face. The rat and mouse AT1 receptors exist as two distinct subtypes, termed angiotensin II type 1a (AT1a) and AT1b [de Gasparo et al., 2000]. The AT1a is the predominant type [Kitami et al., 1992]. The AT1R also couples to $G_{\alpha q/11}$ and $G_{\alpha i}$ [de Gasparo et al., 2000; Olivares-Reyes et al., 2001; Hunyady et al., 2002]. However, the physiologic roles of this receptor such as the regulation of vascular tension and effects on cardiac hypertrophy are generally opposed to those of the BKB2R [Audoly et al., 2000; de Gasparo et al., 2000; Touyz and Schiffrin, 2000].

Connective tissue growth factor (CTGF) is a member of a family of cysteine rich secreted proteins. CTGF has been reported to stimulate fibroblast proliferation and extracellular matrix production [Leask et al., 2002]. The AT1R increases CTGF mRNA levels [Iwanciw et al., 2003; Ruperez et al., 2003a,b]. CTGF has been reported to induce collagen synthesis [Duncan et al., 1999]. CTGF has also been reported to be involved in wound healing [Pawar et al., 1995]. Bradykinin is known to participate in wound healing [Frimm Cde et al., 1996; Plendl et al., 2000; Spillmann et al., 2002]; however, its receptor, the BKB2R, has not been well investigated as to its role in CTGF production. Bradykinin has formerly been shown to increase collagen production in human lung fibroblasts when its action on prostaglandin production was blocked [Goldstein and Polgar, 1982].

Formation of hybrids among GPCRs can be used to identify functional motifs and address the molecular interplay between multiple protein domains and can provide a rational targeting of residues for fine point mutagenesis studies [Prado et al., 2002].

We demonstrated previously that the simultaneous exchanges of the IC loops 2, 3 and the distal tail or exchange of the distal tail alone of the BKB2R with AT1aR results in functional hybrids which bind BK, continue to promote ARA release and PI turnover and be endocytosed [Yu et al., 2002a,b]. In this communication, we demonstrate that certain motifs within the proximal region of the BKB2R C-terminus are not exchangeable with the AT1aR in term of G protein-linked signaling. The specific motifs as well as individual residues within the proximal tail region which are not exchangeable and are crucial for BKB2R G protein coupled signaling function are identified. We also show that the hybrids containing the whole tail or the proximal tail AT1aR exchanges, although unable to signal through the classic PLC and PLA_2 paths, nevertheless continue to increase CTGF mRNA levels in response to BK.

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. Analytical grade Dowex-X8 (AG-1-X8, 100–200 mesh) was obtained from Bio-Rad (Hercules, CA). Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Oligonucleotides were synthesized by Invitrogen, Inc. (Carlsbad, CA). QuikChange® mutagenesis kit was obtained from Stratagene (LaJolla, CA). Rat-1 cells were obtained from Dr. Robert Weinberg (Whitehead Institute, MIT). All other reagents were from Sigma (St. Louis, MO) unless stated otherwise.

Site-Directed Mutagenesis

The QuickChange® Site-Directed Mutagenesis method (Stratagene®) was used to mutate single amino acids of BKB2R. Briefly, two primers complementary to each other were designed to contain the desired mutation. The mutation-containing DNA was synthesized using *Pfu* 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 mini-prep method. Using the pBlueScript-rBKB2R as a template, K317Y, S318A, R319L, E320Q, and ELY were constructed using oligonucleotides synthesized by Invitrogen, Inc.

Construction of Chimeras

To facilitate swapping of the proximal C-terminus of BKB2R with different sequences, a silent *Nsi*I restriction site was created at position 333 by site-directed mutagenesis [Prado et al., 2001]. *Afe*I is a convenient natural restriction site located at position 311. To construct proximal C-terminus chimeric mutants,

the pBluescript-rBKB2R cassette was digested with *Afe*I and *Nsi*I and then ligated with double-stranded synthetic oligonucleotides encoding the corresponding region with desired mutations. To construct the entire C-terminus BKB2R/AT1aR chimera, PCR was performed to amplify the C-terminus of AT1aR using the rat AT1aR receptor cDNA as a template. The PCR product was digested with *Afe*I and *Bam*HI. The fragment was then ligated into the BKB2R/pBluescriptII SK(+) vector digested with the same enzymes thus reconstituting the full-length receptor cDNA with the C-terminus portion of the BKB2R replaced by amino acid sequences derived from the corresponding region of rat AT1a receptor. The *Xho*I–*Xba*I fragment containing the chimera receptor mutant was subcloned into the bicistronic mammalian expression vector, pCMIN(+) [Zhou et al., 2000] for transfection.

All the chimeric and point-mutated constructs described above were sequenced by an in-house facility using an automatic DNA sequencer (Applied Biosystem, Inc., Foster City, CA, model 370A). Pure plasmid DNA for transfection into mammalian cells was isolated with the Qiagen® Plasmid Midi kit.

Cell Culture and Transfection

Cell culture and transfections in Rat-1 cells which lack endogenous BKB2R were performed as previously described [Prado et al., 1998]. Rat-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM), containing 5% fetal bovine serum supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified CO_2 (5%) incubator. Transfections were performed using the calcium phosphate method (ProFection® Mammalian Transfection Systems, Promega, Inc., Madison, WI) according to the protocol of the manufacturer. Stable transfectants were selected in the presence of 0.5 mg/ml G418 (Invitrogen, Inc.) added to the culture medium 48 h after the transfection. The neomycin-resistant cell culture was then expanded and tested for the amount of specific binding to [^{3}H]BK. All the mutants described here bound BK with kinetics that approximate wild type (WT). The B_{max} ranged from 25 to 83×10^3 receptors/cell (Table I). We previously showed that bradykinin-stimulated IP production and ARA release were not receptor number dependent in cells with receptors/cell between 25,000 and 140,000 [Ricupero et al., 1997].

TABLE I. Binding Parameters of [^{3}H]BK in Transfected Rat-1 Cells

Receptor	B_{max} (receptors/cells $\times 10^3$)	K_d (nM)
BKB2R (WT)	53.9 ± 3.2	1.9
wcAT	33.4 ± 2.0	2.5
dAT	27.5 ± 1.4	1.8
pAT	25.7 ± 1.2	1.9
dpAT	75.5 ± 4.5	2.1
mpAT	27.8 ± 1.2	1.6
YFL	26.5 ± 3.9	2.6
QLL	59.9 ± 3.6	2.9
pB1	82.3 ± 4.9	3.1
mpB1	27.7 ± 1.4	2.7
K317Y	51.6 ± 6.5	1.8
S318A	72.5 ± 5.0	1.7
R319L	55.2 ± 4.9	1.9
E320Q	70.4 ± 10.7	2.3
ELY	28.0 ± 1.7	2.1
Y322A	73.9 ± 4.4	1.4

The binding assay was carried out as described under "Materials and Methods." Data are representative of three separate experiments.

Ligand Binding and Receptor Internalization

Ligand binding and internalization of the WT and mutated BKB2R in intact Rat-1 cells were carried out as described previously [Prado et al., 1998]. Briefly, to determine binding, 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 100 nM 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 liquid scintillation counter (Pharmacia-LKB 1217, Gaithersburg, MD). The number of specific BK binding sites (B_{max}) and dissociation constants (K_d) were calculated using the Scatchard analysis. To determine receptor internalization, cells were incubated with 100 nM BK for 1 h at 37°C. Cells were then 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.

Phosphoinositide Turnover

Rat-1 cells were incubated with 1 μCi/ml myo-[^{3}H]inositol in 1 ml of growth medium for 16–24 h. Ten minutes prior to ligand stimulation, cells were incubated to DMEM containing 20 mM $LiCl_2$ and 20 mM HEPES, pH 7.4. Cells were then exposed to 100 nM bradykinin for

30 min at 37°C, and the incubations were terminated by removal of the media and addition of 0.5 ml of 10 mM ice-cold formic acid. Cells were scraped off and the formic acid soluble material isolated by centrifugation and neutralized by adding 10 ml of 5 mM sodium tetraborate. Total [^{3}H]IPs were extracted using a Dowex AG 1-X8 formate resin in an anion exchange column and eluted with 2 M ammonium formate, pH 5.0, as described [Prado et al., 1997]. Following the addition of 4 ml of Ecolite® scintillation fluid (ICN Biomedical, Inc., Aurora, OH), samples were counted for radioactivity in a liquid scintillation counter (Pharmacia-LKB 1217).

Release of Arachidonate

Rat-1 cells were pre-labeled with [^{3}H]arachidonate (0.2 μCi/well) for 16 h as described previously [Prado et al., 1997]. Briefly, cells were washed and incubated with 500 μl of DMEM containing 2 mg/ml bovine serum albumin (radioimmunoassay grade, Sigma). Cells were then incubated with 100 nM bradykinin for 20 min at 37°C. Medium was removed and centrifuged at 800*g*. Radioactivity was determined in a Pharmacia Biotech, Inc. scintillation counter after addition of 2 ml of Ecolite® scintillation fluid (ICN Biomedical, Inc.).

CTGF mRNA Determination by Real-Time PCR

Confluent Rat-1 cells expressing WT or mutant BKB2R were incubated without or with 5 ng/ml TGFβ1 or 100 nM BK at 37°C for 2 h. Total RNA was isolated from the cells using an RNeasy kit (Qiagen, Valencia, CA) and cDNA was made using SuperScript II reverse transcriptase (Invitrogen, Inc.). The expression of CTGF was measured by real-time PCR following standard procedures in an ABI 7700 system (Applied Biosystem, Inc.). The CTGF primers used in real-time PCR were: 5′-TGTGTGATGAGCCCAAGGA-3′ and 5′-TCAGGGCCAAATGTGTCTTC-3′.

Molecular Modeling

The molecular model of the BKB2R was built as described previously [Prado et al., 1997, 1998, 2001]. Briefly, the experimental data from the recent X-ray structure of rhodopsin were used to generate the topological arrangement of the transmembrane (TM) helices, with the loops and termini added to complete the model. The structural features of IC2 were determined experimentally [Piserchio et al., 2002], while the presence of the α-helices in the C-terminus is based on homology modeling as previously described [Prado et al., 1998].

The model of the receptor was refined using molecular dynamics (MD) simulations and energy minimizations using the GROMACS program, version 3.1 [Berendsen et al., 1995; Lindahl et al., 2001]. A series of mutated receptors were made computationally, representing each of the chimeras previously described. Each receptor was soaked in a three-phase solvent box composed of water and decane used to mimic the hydrophilic/hydrophobic, biphasic nature of the membrane in a computationally simple model. The TM helices were introduced into a layer of decane (~835 molecules) to simulate the lipid phase of the solvent box and the extracellular and IC regions soaked with water (~21,380 molecules). The charges of the ionizable side-chain groups were introduced according to the experimental pH, no counter-ions were considered. The entire system was minimized using deepest descent. In order to equilibrate the solvent and optimize the solvent–protein interactions, an initial MD simulation of 10 ps was implemented constraining all heavy atoms of the peptide to their initial positions with a force constant of 1,000 kJ mol^{-1} nm^{-1}. Subsequently, molecular dynamic simulations were carried out for 300 ps at 300 K, with a time step of 2 fs and periodic boundary conditions.

Statistical Analysis

Statistical evaluation of the data was carried out using the Student's *t*-test. Probability values less than 0.05 were considered significant for PI turnover and ARA release, 0.045 for receptor internalization.

RESULTS

Chimeric Studies Between BKB2R and AT1aR of the Proximal C-Terminus

In a previous communication, we showed that the distal part (S335–Q366) of the C-terminus (dCt) of the BKB2R can be exchanged with the corresponding rat AT1aR region and result in a functional mutant receptor [Prado et al., 2001]. We now proceeded to determine whether the entire C-terminus can be successfully exchanged between these two receptors. This exchange construct is shown in Figure 1a and termed wcAT. Smaller regions of the C-terminus of

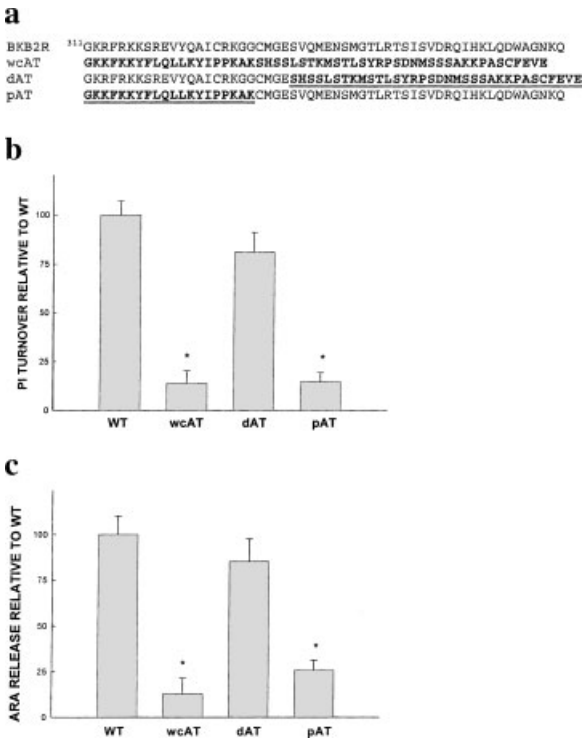

Fig. 1. Chimeric changes of the whole, distal, and proximal region of bradykinin B2 receptor (BKB2R) C-tail with the corresponding region of angiotensin II type 1a receptor (AT1aR). **a**: Amino acid sequences in C-tail region of wild type (WT) BKB2R and chimeric mutants. wcAT, **w**hole **C**-tail of BKB2R substituted with that of AT1aR; dAT, pAT, **d**istal or **p**roximal part of BKB2R C-tail substituted with the corresponding part of AT1aR respectively. The complete sequence of the C-tail is shown using the single letter amino acid code. Bold letter indicates the mutated residue. **b**: BK-stimulated inositol phosphate production in Rat-1 cells expressing WT BKB2R and chimeric receptors. Phosphatidylinositol (PI) turnover was measured in myo-[^{3}H]inositol-labeled cells as described under ''Materials and Methods.'' Results are presented as ligand stimulated IP production minus basal IP normalized to that of WT. Data represent the average of triplicate wells ±SE from a representative experiment of at least three experiments. **c**: BK-stimulated arachidonic acid (ARA) release in Rat-1 cells expressing WT BKB2R and chimeric receptors. ARA release was measured in [^{3}H]arachidonate labeled cells as described under ''Materials and Methods.'' Results are presented as hormone stimulated ARA release minus basal ARA normalized to that of WT. Data represent the average of triplicate wells ±SE from a representative experiment of at least three experiments. *Was used to represent significant difference ($P < 0.05$) from the WT BKB2R.

BKB2R were also exchanged with the AT1aR including the distal BKB2R (335–366) and proximal BKB2R (311–330) regions, denoted as dAT and pAT, respectively (Fig. 1a). The mutant constructs were stably transfected into Rat-1 cells.

PI turnover and ARA release were then determined in response to bradykinin and compared to the action of WT BKB2R. As illustrated in Figure 1b, BK responsive IP production by the wcAT and pAT mutant transfectants was reduced to below 15% of WT BKB2R. On the other hand, dAT remained at approximately the same level as WT BKB2R. With regard to ARA release by these mutant transfectants, the dAT mutant showed the same activity as WT BKB2R while the wcAT and pAT were below 25% of WT BKB2R (Fig. 1c). These results suggest that motifs within the proximal C-tail are important for the functional integrity of both $G_{\alpha q}$ and $G_{\alpha i}$ associated actions of the BKB2R.

To further examine the effect of the proximal tail in signal transduction of the BKB2R, additional domain swaps of this region were generated. As illustrated in Figure 2a, the most proximal part of the C-terminus (311–316) of BKB2R and AT1aR posses an almost identical sequence. Therefore, the segment 317–330 located immediately distal to this region became the area of focus. In the mutant termed mpAT, the middle region of proximal tail, KSREVYQA was replaced by the corresponding residues from AT1aR (YFLQLLKY). In the dpAT mutant, the distal part of proximal C-tail (BKB2R, ICRKGG, and AT1aR, IPPKAK) was exchanged. Figure 2b shows the IP producing activity of these two mutants. In this case, dpAT showed activity essentially equal to that of WT while mpAT showed very poor activity. A similar result was obtained with regard to ARA release, Figure 2c. The dpAT remained the same as WT BKB2R while the mpAT mutant action was reduced to below 25% of WT BKB2R.

Chimeric Exchanges Between BKB1R and BKB2R of the Proximal C-Terminus

To investigate whether the incompatibility of the proximal C-terminus is unique to the BKB2R/AT1aR exchanges, chimeras were constructed where the entire or the middle portion of the proximal BKB2R C-terminus were exchanged with corresponding areas of the BKB1R. This is depicted in Figure 3a. pB1 represents the entire proximal C-terminus exchange while mpB1 represents the middle of the proximal tail exchange. The capacity to turn over PI in response to BK by either of these mutants was significantly higher when compared to pAT. Nevertheless both mutants exhibited a reduced PI turnover capacity, approximately 50% of WT BKB2R, as shown in Figure 3b. With regard to ARA release, both

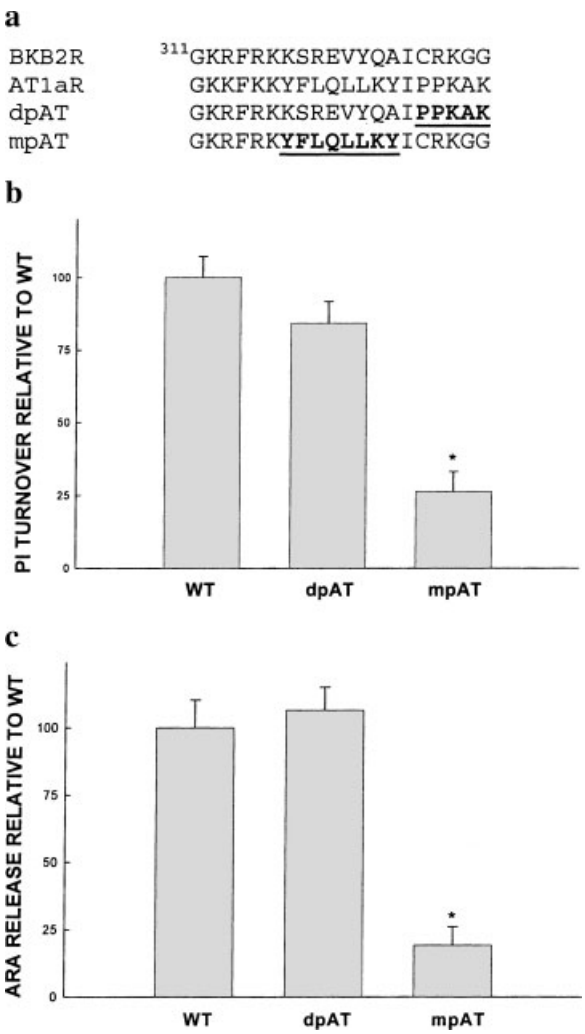

Fig. 2. Chimeric studies on proximal C-tail region between BKB2R and AT1aR. **a**: Amino acid sequences in the proximal C-tail region of mutants dpAT and mpAT. dpAT, **d**istal region of **p**roximal C-tail of BKB2R substituted with that of AT1aR; mpAT, **m**iddle section of **p**roximal BKB2R C-tail substituted with that of AT1aR. For clarity, the amino acid sequences of this region of WT BKB2R and WT AT1aR are also shown. Bold letter indicates the mutated residue. **b**: BK-stimulated inositol phosphate production in Rat-1 cells expressing WT BKB2R and chimeric receptors. Same experimental procedures as described in Figure 1b. **c**: BK-stimulated ARA release in Rat-1 cells expressing WT BKB2R and chimeric receptors. Same experimental procedures as described in Figure 1c. *Was used to represent significant difference ($P < 0.05$) from the WT BKB2R.

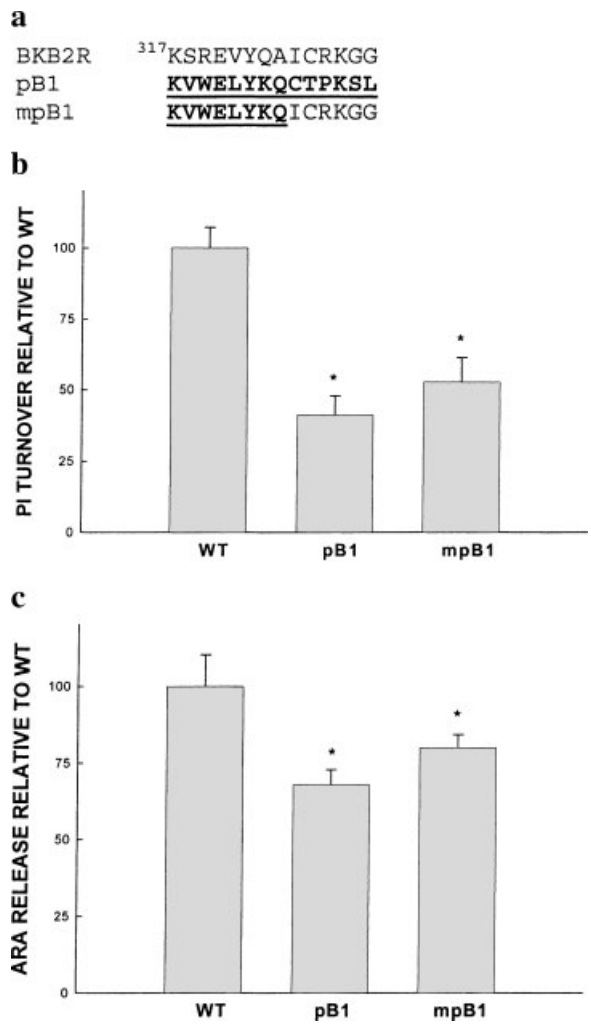

Fig. 3. Chimeric studies on proximal C-tail region between BKB2R and bradykinin B1 receptor (BKB1R). **a**: Amino acid sequences in the proximal C-tail region of the chimeric mutants. pB1, **p**roximal C-tail of BKB2R substituted with that of hBK**B1**R; mpB1, **m**iddle region of **p**roximal BKB2R C-tail substituted with that of hBK**B1**R. For clarity, the amino acid sequence of this region in WT BKB2R is also shown. **b**: BK-stimulated inositol phosphate production in Rat-1 cells expressing WT BKB2R and chimeric receptors. Same experimental procedures as described in Figure 1b. **c**: BK-stimulated ARA release in Rat-1 cells expressing WT BKB2R and chimeric receptors. Same experimental procedures as described in Figure 1c. *Was used to represent significant difference ($P < 0.05$) from the WT BKB2R.

mutants show a somewhat reduced activity relative to WT BKB2R (Fig. 3c).

Mutagenic Studies on the Middle Region of BKB2R Proximal C-Terminus

The results above in sum point to the importance of the KSREVY sequence in the middle region of BKB2R proximal C-terminus. To develop a more detailed picture of the importance of the individual residues within this motif, smaller sequence replacements as well as single point mutations were carried out. As illustrated in Figure 4a, the EVY motif of BKB2R was replaced with the corresponding AT1aR, QLL (QLL mutant). Also, an identical exchange of the BKB2R EVY motif was made with the

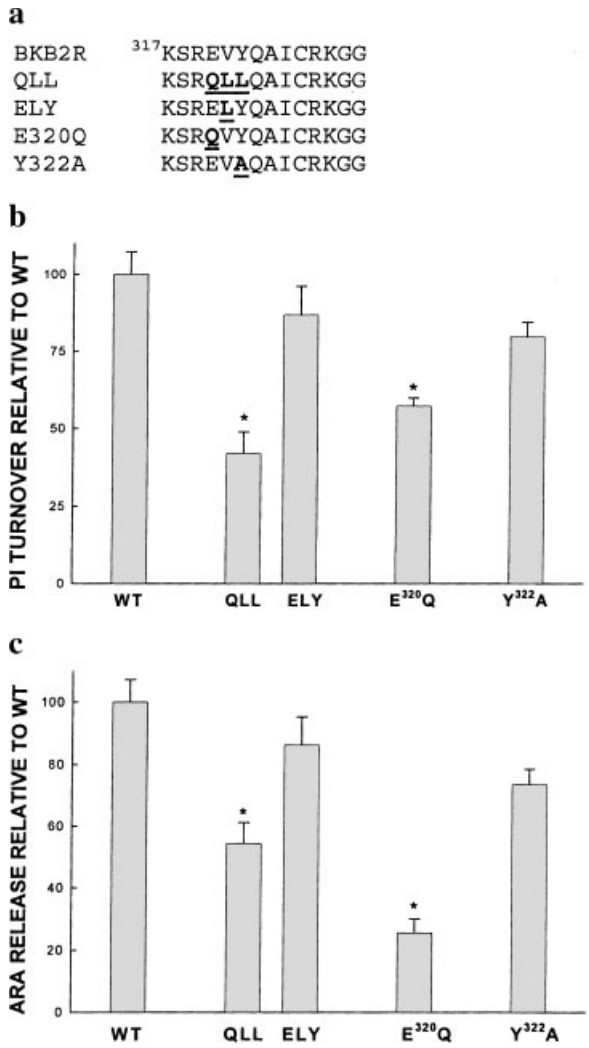

Fig. 4. Chimeric and point mutation studies on EVY motif. **a**: Diagram of chimeric mutant constructs, QLL and ELY. EVY motif in BKB2R changed to corresponding QLL of AT1aR or ELY of hBKB1R, respectively. The sequences of point mutations E320Q, Y322A are also illustrated. **b**: BK-stimulated inositol phosphate production in Rat-1 cells expressing WT BKB2R and mutated receptors. Same experimental procedures as described in Figure 1b. **c**: BK-stimulated ARA release in Rat-1 cells expressing WT BKB2R and mutated receptors. Same experimental procedures as described in Figure 1c. *Was used to represent significant difference ($P < 0.05$) from the WT BKB2R.

BKB1R, ELY sequence (ELY mutant). This was followed by point mutations, E320 to Q and Y322 to A (Fig. 4a). With regard to the EVY motif replacements, when this motif was replaced by QLL (an AT1aR replacement), the chimera function to turn over IP was reduced to approximately 50% of WT BKB2R. Changing EVY to the corresponding motif ELY (found in BKB1R) led to a mutant with a WT response (Fig. 4b). To evaluate the function of the negatively charged E320 in this motif, it was mutated to glutamine. The mutant (E320Q) demonstrated significantly lowered PI generating capacity compared to the WT BKB2R. The effect of mutating Y322 to A proved of marginal importance [Prado et al., 1997]. This mutant signaled in a manner similar to WT. Thus, the E320 is of importance for normal PI turnover by BKB2R. A very similar pattern is seen with respect to ARA release. However, in this case, the E320Q mutant exhibited the poorest signal capacity.

Chimeric studies on KSR motif were then performed. KSR was first replaced with the YFL from AT1aR (YFL mutant, Fig. 5a). When replacing KSR with YFL, the chimeric receptor lost almost all signaling function (Fig. 5b,c). As shown before, the mutant pB1 showed decreased signaling activity compared with WT (reshown in Fig. 5 for comparison). However, when the KVW motif in pB1 was changed back to KSR, termed KSR mutant (Fig. 5a), the signaling capacity (PI turnover and ARA release) of this mutant was recovered to the level of WT BKB2R (Fig. 5b,c). This "gain of function" confirms the importance of KSR sequence in BKB2R signal transduction.

Figure 6a illustrates the point mutations made within the KSR motif. The mutations consisted of K317 to Y, S318 to A, and conversion of R319 to L. The PI turnover and ARA release of these point mutations are illustrated in Figure 6b,c, respectively. Both changing K317 to Y and R319 to L compromised receptor function. The alanine replacement for S318 actually increased the receptor signal capacity to slightly higher for PI turnover and to considerably higher for ARA release.

Molecular Modeling

Employing the previously established model of BKB2R, extensive molecular modeling and MD simulations were carried out to examine the role of the residues in the mid-region of the proximal C-terminus BKB2R. This region of BKB2R, residues 312–326 (KRFRKKSREVYQAIC), is homologous to rhodopsin (residues 311–323, KQFRN–CMVTTLCC), with a two-site gap in rhodopsin corresponding to the KS of the BKB2R. In rhodopsin, Figure 7a, this region forms an α-helix, helix 8, that is perpendicular to the seven TM helices. As illustrated in Figure 7b the model of BKB2R likewise contains a helix, which although not perfectly

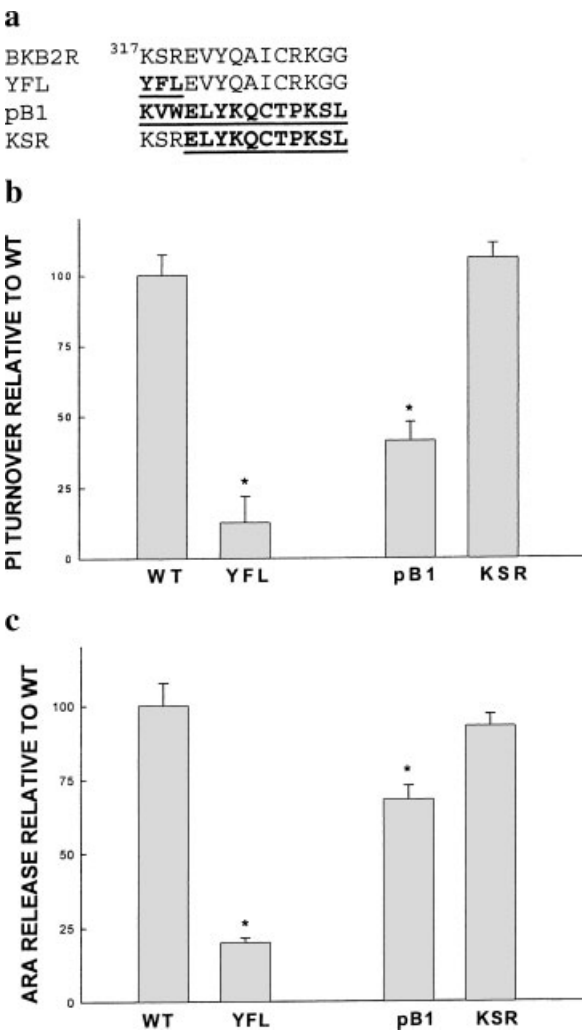

Fig. 5. Chimeric studies on KSR motif. **a**: Diagram of chimeric mutant constructs, YFL and KSR. YFL: KSR motif in BKB2R was changed to corresponding YFL of AT1aR. KSR: Change the **KVW** motif in the mutant pB1 (showed in Figure 3a) back to KSR. Bold letters indicate the chimeric exchanged residues. **b**: BK-stimulated inositol phosphate production in Rat-1 cells expressing WT BKB2R and mutated receptors. Same experimental procedures as described in Figure 1b. **c**: BK-stimulated ARA release in Rat-1 cells expressing WT BKB2R and mutated receptors. Same experimental procedures as described in Figure 1c. *Was used to represent significant difference ($P < 0.05$) from the WT BKB2R.

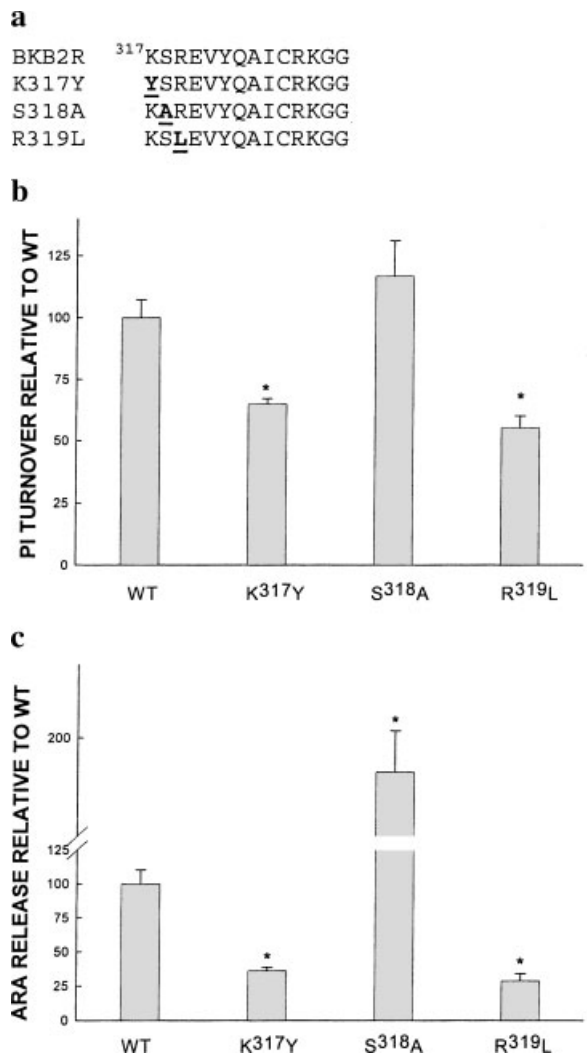

Fig. 6. Point mutations on KSR motif. **a**: Diagram of mutants with point mutations in KSR motif. K317Y, S318A, and R319L present the corresponding point mutations on K317, S318, and R319. Bold letters indicate the mutated residues. **b**: BK-stimulated inositol phosphate production in Rat-1 cells expressing WT BKB2R and mutated receptors. Same experimental procedures as described in Figure 1b. **c**: BK-stimulated ARA release in Rat-1 cells expressing WT BKB2R and mutated receptors. Same experimental procedures as described in Figure 1c. *Was used to represent significant difference ($P < 0.05$) from the WT BKB2R.

amphipathic has a hydrophobic face, F314, S318, V321, Y322 (not labeled in figure) and hydrophilic face, K317, R319, E320 (labeled in figure) which based on the MD simulations project towards the membrane and cytosol, respectively. K317, R319, and E320, projecting away from the membrane, are accessible for interaction with other proteins (e.g., G proteins, kinases). Based on MD simulations of mutant receptors these residues, (K317, R319, and E320) are particularly important for the stability of this helix, with mutation of these sites invariably altering the helical character of the region particularly with respect to maintaining the α-helix lying upon the cytosolic membrane surface. The Coulombic interaction between K317 and E320 provides additional stability to the α-helix. Replacing these residues with those from the AT1aR (i.e., K317Y, E320Q) removes this favorable salt-bridge.

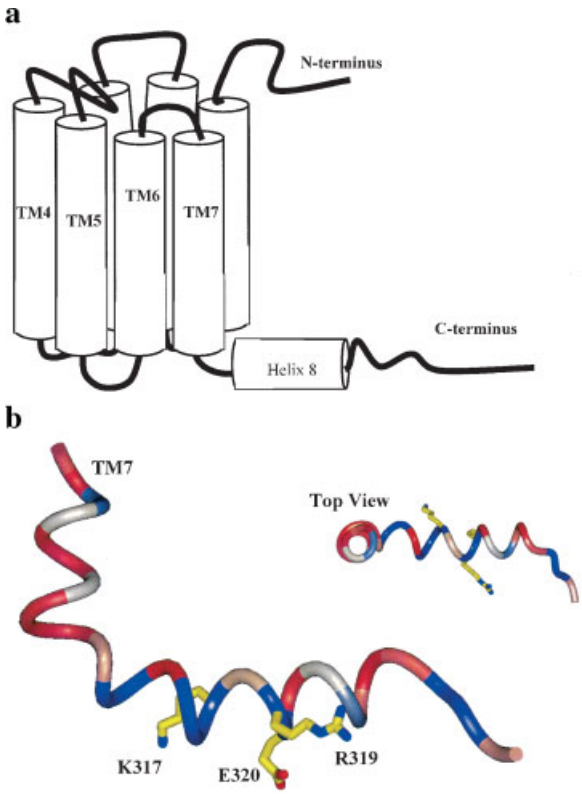

Fig. 7. Structural features of the proximal C-terminus of BKB2R. **a**: Schematic illustration of the topological arrangement of the proximal C-terminal α-helix (helix 8) of BKB2R. **b**: The BKB2R residues C302–G329 is illustrated as a ribbon, colored according to the hydrophobicity of the amino acids (red, hydrophobic; blue, charged/hydrophilic). This region includes the cytoplasmic end of transmembrane (TM)7 and the proximal C-terminus of BKB2R. The side chains of K317, E319, and R320 are depicted. Inset: The structure has been rotated 90°, looking directly onto TM7 from the extracellular portion.

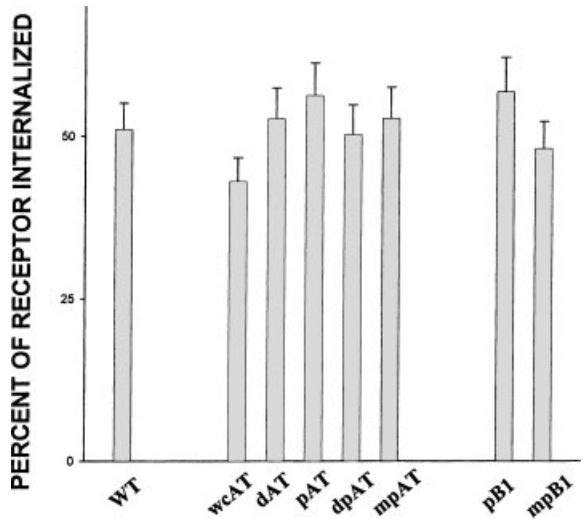

Fig. 8. Receptor internalization of global C-terminus exchanges of the BKB2R with AT1aR and BKB1R. Using 0.045 *P* value as the standard, none of these large receptor exchanges affected endocytosis compared to WT BKB2R. Cells were incubated with 100 nM BK for 60 min. After acid stripping and three washes with ice-cold buffer, [^{3}H]BK binding to Rat-1 cells was measured as described under ''Materials and Methods.'' Results represent the percentage of receptors internalized in 60 min as compared to receptors internalized in 0 min. Values are the mean ± SE from two to three experiments in triplicate.

Receptor Internalization

Since the proximal tail of the BKB2R appeared important for signal transduction, its role in the endocytosis of the receptor was determined. For this purpose the effect of the global C-terminus exchanges with AT1aR and BKB1R receptor uptake was examined. The chimeras wcAT, pAT, dpAT, and mpAT involved exchanges with AT1aR and the chimeras pB1 and mpB1 involved exchanges with BKB1R (all mutants constructs illustrated in the figures above). As shown in Figure 8, none of these large receptor exchanges affected endocytosis compared to WT BKB2R.

CTGF mRNA

We then proceeded to determine whether the proximal tail (pAT) or entire tail (wcAT) mutants, which activate either IP formation or ARA release in response to BK only minimally, were able to transduce another form of signal in response to BK. Using real-time PCR procedure we determined that BK caused an approximate three fold increase in CTGF mRNA levels in Rat-1 cells stably transfected with WT BKB2R. Interestingly both mutants, pAT and wcAT, which did not show an increase in either PI turnover or ARA release in response to BK, increased CTGF mRNA levels in response to BK (Fig. 9). The pAT mutant increased approximately two fold above control and the wcAT mutant increased approximately six fold above control.

DISCUSSION

We showed previously that the distal portion of the BKB2R C-terminus (S335-Q366) is fully exchangeable with that of the AT1aR [Prado et al., 2001]. To extend the BKB2R/AT1aR chimera construct, the entire C-tail of the two receptors was exchanged. Surprisingly, switching the whole C-tail of BKB2R with that of the AT1aR resulted in a receptor which both binds BK and is fully endocytosed, but is nonfunctional in terms of PLC and PLA2 related

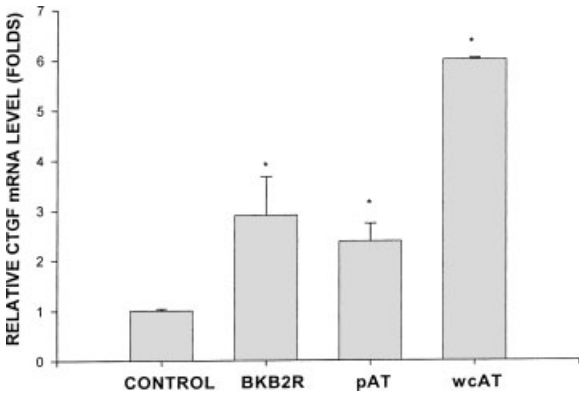

Fig. 9. Relative connective tissue growth factor (CTGF) mRNA levels following BK stimulation. Stably transfected Rat-1 cells were incubated with bradykinin (100 nM) at 37°C for 6 h. Total RNA (1 μg) isolated from 6-well plate cultures was reverse transcribed and the resulting cDNA (0.2 μl) was subjected to real-time PCR as described in ''Materials and Methods.'' Control, Rat-1 cells; BKB2R, Rat-1 cells stably transfected with WT BKB2R; pAT, Rat-1 cells transfected with the pAT mutant; wcAT, Rat-1 cells transfected with the wcAT mutant. *Was used to represent significant difference ($P < 0.05$) from the control.

signaling. Even exchanging the IC2 and IC3 simultaneously with the whole tail did not rescue the dampened signaling (data not shown). The compatibility of the distal portion of C-tail between BKB2R and AT1aR [Prado et al., 2001] suggested that the proximal part of the C-tail is the reason for the failure of the whole C-tail BK/AT chimera. The goal of this article was to illuminate the role of the proximal C-tail in BKB2R signaling function and self maintenance.

There is a very high homology between the BKB2R and AT1aR within the very proximal portion of the C-tail (G311 to K316 in the BKB2R). This makes this region an unlikely cause for the unexchangeability of the C-termini. Thus, we focused on the middle (317KSREVYQA324) and distal (325ICRKGG330) portions of the proximal C-terminus. Our results showed that it is the BKB2R sequence of the middle part that is crucial for successful signaling of this receptor. Certain residues within this region proved to be not exchangeable between these two receptors. On the other hand, the chimera with the substitution of the five amino acid (CRKGG) making up the distal part of the proximal BKB2R C-terminus with the corresponding PPKAK of AT1AR functioned just as WT BKB2R (Fig. 2).

To examine more closely the role of the motifs within the middle section of the BKB2R proximal C-terminus, the amino acids of this BKB2R region were exchanged with those from the AT1aR and (human) BKB1R. The AT1aR sequence is very different from that of the BKB2R while the BKB1R shows some homology to BKB2R (both receptor subtypes possess K317, E320, and Y322). Therefore, it was of interest to determine to what degree this region of BKB2R could be exchanged with the corresponding region of BKB1R. Two chimeras pB1 (entire proximal C-tail) and mpB1 (middle section of proximal C-tail) were constructed (Fig. 3). Both of these mutants displayed significantly better signaling than the corresponding AT1aR chimeras. But neither mutant signaled as well as WT BKB2R. Since the last two amino acids (QA) of this region vary greatly in BKB2R from different species, the first six amino acids, KSREVY, became the region of focus. This motif is conserved in BKB2Rs from a number of species. This region was further divided into two motifs: EVY and KSR.

The existence of the EV/LY motif in BKB2R/BKB1R, and its absence in AT1aR suggested this motif is important for receptor function. The chimeric studies confirmed this deduction. An ELY exchange for EVY was insignificant, whereas the QLL from the AT1aR could not be successfully exchanged to get a fully functional receptor. To further study the role of individual amino acids within this motif, the negatively charged E320 was mutated to non-charged glutamine, which exists in the AT1aR. This point mutant significantly lowered signaling capacity compared to WT BKB2R. This pointed to the importance of the negatively charged E320 in BKB2R signaling.

As illustrated in Figure 3b, exchanging KSREVYQA for the KVWELYKQ of BKB1R resulted in only a partially functional receptor. The PI turnover is only about 50% of that of WT BKB2R. Since changing V321 in BKB2R to L from the BKB1R resulted a fully functional receptor, the KSR motif, which is KVW in BKB1R and YFL in AT1aR, seems to be crucial for BKB2R signaling function. In fact, Figure 5 illustrates that by only changing the KVW motif of pB1 back to KSR, the new mutant (KSR) showed normal signaling function as WT BKB2R. On the other hand, replacement of KSR motif with YFL from AT1aR resulted in a non-functional receptor. These results substantiate that the KSR motif is important in BKB2R function. The lack of the KSR motif is the reason why the proximal tail exchanges between the

BKB2R and BKB1R generated only a partially functional receptor. Point mutations in this region further showed that the positively charged K317 and R319 are crucial for normal receptor function. When these are changed to non-charged amino acids (K317Y and R319L as shown in Fig. 6), the resulting mutants displayed significantly lower PI turnover and ARA release compared to WT BKB2R. Exchange of alanine for S318 did not alter PI turnover of the mutant receptor. It is interesting that the ARA release of this mutant increased to almost two fold of WT BKB2R. This suggests that S318 plays a role in the differential regulation of PI turnover and ARA release.

As illustrated in Figure 7, the proximal C-terminus of BKB2R including KSREVY adopts an α-helix, analogous to helix 8 in rhodopsin observed at a 90° angle to the TM helices of the 7-helical bundle [Palczewski et al., 2000]. Helix 8 has been implicated as a site of interaction between the C-terminus and the βγ-subunit of transducin [Phillips and Cerione, 1992] and hypothesized to be mobile enough to interact with the C-termini of α- and γ-subunits [Ernst et al., 2000]. Though in rhodopsin this region is anchored to the membrane by palmitoyl ester cysteine residues, the region in BKB2R has no such anchors, perhaps conferring even greater flexibility for interaction. However, this helical region of BKB2R shows a distinct amphipathic nature. The non-charged part of the helix, consisting of residues S318, V321, and Y322, during the MD simulations demonstrated interaction with the membrane, maintaining the proper positioning of the helix with respect to the 7-helical bundle. Indeed, MD simulations in which these residues are replaced with charged residues resulted in reduced lifetimes of the helix association with the membrane. The MD simulations also indicated that the K317, R319, and E320 are likewise important for maintaining the α-helix, lying upon the cytosolic membrane surface. The Coulombic interaction between K317 and E320 provides additional stability to the α-helix. Replacing these residues with those from the AT1aR (i.e., K317Y, E320Q) removes this favorable salt-bridge.

The role of the distal region of BKB2R C-tail in receptor internalization has been studied extensively [Blaukat et al., 1996; Prado et al., 1997, 2001; Faussner et al., 1998; Pizard et al., 1999; Bachvarov et al., 2001; Yu et al., 2002b]. However, there is little information on the role of the proximal tail in receptor uptake. Using 0.045 P values as the cutoff point, the present results showed that the proximal tail chimeric mutants tested internalized at about the same rate as WT BKB2R. Chimeric exchanging the whole, middle, or distal part of BKB2R proximal tail to the corresponding part of AT1aR without changing the behavior of receptor uptake suggests that if the proximal section of the tail is involved in the regulation of uptake, as was suggested previously [Prado et al., 2001], then global exchanges of this region are able to replace the role of the original BKB2R residues. This point is summed up by the wcAT mutant in which the entire tail of BKB2R is replaced with the entire tail of AT1aR. The endocytosis of this mutant is not statistically different from WT BKB2R while signal transduction is impaired. This also suggests receptor signaling and uptake are not dictated by the same amino acid motifs at least in the proximal tail region. Interestingly, the BKB1R which contains an abbreviated distal tail, has been reported not to be internalized [Zhou et al., 2000]. With regard to mutants created herein, both BKB1R exchanges (pB1 and mpB1) retained the BKB2R distal portion of the tail and internalized normally, which suggests distal C-tail is sufficient for BKB2R uptake.

It is interesting that despite a lack of ability to transduce signals via the PLC or PLA_2 paths, the mutants containing the entire C-terminus AT1aR sequence or the proximal AT1aR (303–322) did respond to BK stimulation with an increase in CTGF mRNA level. Further work is necessary, at this time, to pinpoint the path and receptor motif(s) responsible for this induction. Nevertheless, this result is illustrates that another signal mechanism is taking place here in addition to the classical BKB2R associated $G_{\alpha q}$ or $G_{\alpha i}$ transduced pathways.

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