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Global Chimeric Exchanges Within the Intracellular Face of the Bradykinin B2 Receptor With Corresponding Angiotensin II Type Ia Receptor Regions: Generation of Fully Functional Hybrids Showing Characteristic Signaling of the AT1a Receptor

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Abstract The intracellular (IC) face of the G-protein coupled receptors (GPCR), bradykinin (BK) B2 and angiotensin (AT) 1a, is similar in sequence homology and in size. Both receptors are known to link to Gαi and Gαq but differ markedly in a number of physiologic actions, particularly with respect to their hemodynamic action. We made single as well as multiple, global replacements within the IC of BKB2R with the corresponding regions of the AT1aR. When stably transfected into Rat-1 cells, these hybrid receptors all bound BK with high affinity. Single replacement of the intracellular loop 2 (IC2) or the distal 34 residues of the C-terminus (dCt) with the corresponding regions of AT1aR resulted in chimera, which turned over phosphotidylinositol (PI) and released arachidonic acid (ARA) as WT BKB2R. In contrast, incorporation of the AT1aR IC3 in a single replacement abolished signal transduction. However, the simultaneous exchange of IC2 and IC3 of BKB2R with AT1aR resulted in a receptor responding to BK with PI turnover and ARA release approximately 4-fold greater than WT BKB2R. Likewise, the simultaneous replacement of IC2 and dCt resulted in a 2.8- and 1.6-fold increase in PI turnover and ARA release, respectively. In contrast, the dual replacement of IC3 and dCt could not overcome the deleterious effects of the IC3 replacement, resulting in very low PI activation and ARA release. Replacement of all three IC domains (IC2, IC3, and dCt) resulted in PI closer to that of AT1aR than BKB2R. The uptake of the receptor chimeras was similar to that of WT BKB2R with the exception of the IC3/dCt dual mutant, which exhibited very poor internalization (18% at 60′). When transfected into Rat-1 cells, the AT1aR markedly increased the expression of connective tissue growth factor (CTGF) mRNA, while BK slightly decreased it. The dual IC2/dCt and triple IC2/IC3/dCt hybrids both upregulated CTGF mRNA in response to BK. These results show that the IC face of the BKB2R can be exchanged with that of AT1aR, producing hybrid receptors, which take on the functional characteristics of AT1aR. The characterization of the chimera with stepwise replacement of the IC domains should allow for assignment of specific roles to the individual loops and C-terminus in the signaling and internalization of the BKB2R and facilitate the generation of a receptor with BKB2R binding and AT1aR function. J. Cell. Biochem. 85: 809–819, 2002.

Key words: signal transduction; chimera; intracellular loops; C-terminus; bradykinin; bradykinin B2 receptor; angiotensin type 1a receptor; AT1a; connective tissue growth factor; CTGF; angiotensin; angiotensin II; hybrid receptors; mutant receptors; mutations

The binding of bradykinin (BK) to its B2 receptor (BKB2R) results in a wide spectrum of biological effects including vasodilatation, smooth muscle relaxation, pain, asthma, inflammation, and coughing during treatment with angiotensin-converting enzyme (ACE) inhibitors [Fox et al., 1996]. Airway hyper responsiveness is also linked to the action of BKB2R [Folkerts et al., 2000]. Experiments with transgenic mice substantiate the role for

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BKB2R in the regulation of vascular tone [Wang et al., 1997]. It is constitutively expressed with a typical G-protein coupled receptor (GPCR) character. It couples to Gαi2 and Gαi3? often characterized by the action of phospholipase A_2 (PLA2) and the release of arachidonic acid (ARA) and Gαq/11 characterized by phospholipase C (PLC) activity and the turnover of phosphatidylinositol (PI) [Ricupero et al., 1997; Yang et al., 1999]. The mobilization of Ca^{2+} is another characteristic of this receptor in response to its ligand, BK [Ricupero et al., 1997; Yang et al., 1999]. BKB2R has been cloned from rat, mouse, canine, and human [McEachern et al., 1991; Hess et al., 1992, 1994, 2001]. Angiotensin II (Ang II) type 1 (AT1a) receptor has also been cloned from the rat [Murphy et al., 1991]. It is also a GPCR and, as BKB2R, has been reported to couple to Gαq/11 and Gαi [Wang et al., 1995; Shibata et al., 1996]. The AT1aR shares some homology with the BKB2R within the IC face [Prado et al., 2001]. It is also of very similar length to the BKB2R within the IC face [Prado et al., 2001]. However, the physiologic roles of this receptor such as its hemodynamic actions are often opposed to those of the BKB2R [Armin et al., 2001].

Large chimeric exchanges within receptor subtypes have been accomplished. For example, chimeric exchanges of some IC loops between angiotensin II type 1 (AT1R) and type 2 (AT2R) have been reported [Wang et al., 1995]. AT1R couples to a Gαq signaling pathway, whereas the AT2R does not. While substitution of the IC2 and the C-tail between the two receptors did not affect AT1R function, the exchange of the IC3 resulted in the loss of function in AT1R [Wang et al., 1995]. Chimeric exchange between related receptors has also been accomplished. For example, while the BKB2R undergoes rapid and pronounced internalization, the bradykinin B1 receptor (BKB1R), which responds to $desArg^{10}$-kallidin, displays slow and limited internalization [Zhou et al., 2000]. Swapping of the C-terminus of the BKB2R with that of the BKB1R led to a decrease in receptor internalization [Faussner et al., 1998]. Chimeric exchange between divergent (heterologous) GPCRs has also been accomplished. This includes exchanges between M1-muscarinic receptors (M1AChR) and β-adrenergic receptors (β-AdRs) [Wong et al., 1990]. When the IC3 of the β2-adrenergic receptor (β2-AdR) was substituted into the corresponding regions of the M1AchR, a 2–4-fold stimulation of adenyl cyclase (AC) was observed [Wong et al., 1990]. It has also been shown that a proline-rich region of the IC3 determines the different Gs coupling and sequestration of β1-AdR vs. β2-AdR [Green and Liggett, 1994]. Also, we showed previously that the replacement of the last 34 amino acids of the C-terminus of BKB2R with those from the C-terminus of AT1aR resulted in a receptor, which functioned, internalized and resensitized as BKB2R WT [Prado et al., 2001].

In this communication, we continue to focus on the intracellular face of the BKB2R and the ability of large intracellular segments of the AT1R to replace those of BKB2R. We report that, indeed, specific global substitutions of the BKB2R IC motifs with the corresponding AT1aR regions result in hybrid receptors able to continue to function as WT BKB2R. However, certain hybrids take on functional characteristics of the AT1aR such as increase in connective tissue growth factor (CTGF) mRNA expression. CTGF is a protein, which is involved in collagen synthesis and is known to be upregulated by TGF-β [Frazier et al., 1996] and downregulated by cAMP [Duncan et al., 1999]. Its expresion has been suggested to be also upregulated by angiotensin II [Finckenberg et al., 2001].

MATERIALS AND METHODS

Materials

[^{3}H] BK (78 Ci/mmol), myo-[1,2-^{3}H] inositol (45–80 Ci/mmol), [^{3}H] arachidonate (60–100 Ci/mmol), and ^{32}P-dCTP were obtained from NEN Life Science Products (Boston, MA). TGF-β was purchased from R&D Systems (Minneapolis, MN). 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 Life Technologies Inc. (Rockville, MD). QuikChange mutagenesis kit was obtained from Strategene Corp (LaJolla, CA). RNAwiz™ RNA isolation reagent was purchased from Ambion Corp (Austin, TX). All other reagents were from Sigma (St. Louis, MO) unless stated otherwise. The BKB2R cDNA was produced in our laboratory. The cDNA for the AT1aR was obtained from Dr. K. Sandberg.

Site-Directed Silent Mutagenesis of the Intracellular Regions of the rBKB2R

To facilitate the investigation of the structure-function relationships of intracellular

regions of the BKB2R, we introduced five silent mutations to create restriction sites to easily swap the 2nd intracellular (i2) or 3rd intracellular loops (i3) or the distal C-tail (Ct) of the BKB2R with corresponding regions of the AT1aR. The silent mutations created preserved the amino acid sequence of the coding region and in turn created five unique restriction sites, which are the following: AgeI (position 130) and BlpI (position 149) were introduced for second loop substitution; AlfII (position 226) and SpeI (position 245) for third loop substitution, and NsiI (position 333) for distal C tail swapping. QuickChange™ Site-Directed Mutagenesis Kit (Stratagene) was used to generate these silent mutations. The oligonucleotides used were the following:

AgeI: sense strand 5′ CTT GTG AGT ATC GAC CGG TAC CTG GCG CTG G 3′; anti-sense strand 5′ CCA GCG CCA GGT ACC GGT CGA TAC TCA CAA G 3′;
BlpI: sense strand 5′ GGG GTA CGC TGG GCT AAG CTG TAC AGC CTG G 3′; anti-sense strand 5′ CCA GGC TGT ACA GCT TAG CCC AGC GTA CCC C 3′;
AlfII: sense strand 5′ GAA TCA TGG AGG TCT TAA GGA ACA ACG AGA TG 3′; anti-sense strand 5′ CAT CTC GTT GTT CCT TAA GAC CTG CAT GAT TC 3′;
SpeI: sense strand 5′ GAA GGC CAC TGT ACT AGT GCT GGC TGT CCT G 3′; anti-sense strand 5′ CAG GAC AGC CAG CAC TAG TAC AGT GGC CTT C 3′;
NsiI: sense strand 5′ CCG GAA GGG AGG ATG CAT GGG AGA G 3′; anti-sense strand 5′ CTC TCC CAT GCA TCC TCC CTT CCG G 3′;

The synthesized BKB2R mutations were confirmed by DNA sequencing. This clone now allowed us to make any combination of mutations within the regions 130–149 (IC2), 226–245 (IC3), and 333–351 (Ct) by synthesizing complementary oligonucleotides containing counterpart regions of other GPCRs such as AT1aR.

Generation of Chimera Mutants

To construct chimera mutants, the pBluescript-rBKB2R cassette was digested with two unique enzymes corresponding to either second or third loop or C-tail. The corresponding sense and anti-sense oligonucleotides of the AT1AR loop or C-tail of interest were then annealed at equal molar ratio and ligated into the digested cassette vector. The mutants were confirmed by DNA sequencing. The XhoI–XbaI fragment containing the chimera receptor mutant was subcloned to the bicistronic mammalian expression vector, pCMIN [Zhou et al., 2000]. The oligonucleotides used to construct the chimeras were the following:

Ai2 sense strand CCG GTA CCT GGC ACT AGT GCA CCC AAT GAA GTC TCG CCT CCG CCG CAC GAT GCT GGT AGC3′; anti-sense strand 5′ TTA GCT ACC AGC ATC GTG CGG CGG AGG CGA GAC TTC ATT GGG TGC ACT AGT GCC AGG TA 3′;
Ai3 sense strand 5′ TTA AAG AAG GCA TAT GAA ATT CAA AAG AAC AAA CCA AGA AAC GAT GAC ATC TTT AGG3′; antisense strand 5′ CTAGCCTAAA GATGTCATCG TTTCTTGGTT TGTTCTTTTG AATTTCATAT GCCTTCT 3′.
DCt 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 AAG CTT TCA CTC CAC TTC AAA AAA AGA TGC AGA CTT TTT GGC AGA TGA ACT CAT GTT ATC TGA AGG GCG GTA 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 six-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. For transfection, the Lipofectamine method (Life Technologies, Inc.) was used as described [Prado et al., 1997, 1998].The neomycin-resistant mass cell culture was then allowed to expand for several weeks and then tested for the amount of specific binding to [^{3}H] BK or [^{125}I]Ang II.

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.

IP Formation

Untransfected and stably transfected Rat-1 cells were incubated with 1 μCi/ml myo-[^{3}H] inositol in 1 ml of growth medium for 16–24 h and the levels of inositol phosphates were determined 1 day later as described [Prado et al., 1997].

Release of Arachidonate

Rat-1 cells were prelabeled with [^{3}H] arachidonate (0.2 μCi/well) for 16 h as described previously [Prado et al., 1997]. Briefly, cells were washed with Dulbecco's medium 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 scintillation β-counter after addition of 2 ml of Ecolite scintillation fluid.

Northern Blot Analyses

All incubations for Northern blots were done in duplicate. Total RNA was extracted from cells by RNAwiz™ RNA Isolation Reagent (Ambion Corp.). RNA was fractionated by electrophoresis on a 1% agarose and 8% formaldehyde denaturing gel. The fractionated RNA was transferred overnight to nylon membranes from NEN Life Science Products (Boston, MA) and fixed by ultraviolet crosslinking. Approximately 25 ng of cDNA probes for CTGF were labeled with ^{32}P using Ready-to-Go DNA Labeling beads (dCTP) from Pharmacia Biotech (Piscataway, NJ). The RNA blots were probed with given ^{32}P-labeled cDNA at 65°C in a Rapid-hybridization buffer from Amersham Life Science (Amersham, UK) and washed with 2 × SSC buffer at 28°C for low stringency, then with 0.2 × SSC buffer at 50°C for high stringency. Radioactivity associated with each band was visualized by autoradiography with an intensifying screen at −80°C. Autoradiograms were scanned and the intensity of the bands quantitated using Sigma/Scan Image software (Jandel Scientific, San Rafael, CA). To correct for loading errors photographs of the ethidium bromide stained 18s rRNAs were scanned and quantitated using the same program.

Molecular Modeling

The BKB2 receptor and chimera containing IC domains of the AT1a receptor were modeled following previously published procedures [Prado et al., 1997, 1998, 2001]. Briefly, the receptor chimera was assembled by replacement of the appropriate residues, including deletions and additions. The chimera was subsequently soaked in a water/decane/water simulation cell and extensive simulations (energy minimization, molecular dynamics) carried out.

RESULTS

Expression of Chimeric BKB2 Receptors

The intracellular regions of many G-protein coupled receptors (GPCRs) have been implicated in the functional specificity of signal transduction. To assess whether intracellular domains, i.e., the intracellular loops and C-terminus of BKB2 and AT1a receptors were interchangeable, seven hybrid receptors were constructed in which the IC2, IC3, or the distal 34 residues of the terminal C-tail of BKB2R (Ct) were substituted in various combinations with the equivalent domains of the AT1aR. The nomenclature and schematic diagrams of the receptor chimeras are shown in Figure 1. The single hybrids are Ai2, Ai3, and ACt, where the second, third, and distal C-tail regions of BKB2R were exchanged with the corresponding regions of the AT1aR, respectively. The double exchanges were the IC2 and IC3, the IC2 and Ct, the IC3 and Ct, termed as Ai23, Ai2Ct, Ai3Ct, respectively. The triple exchange chimera, Ai23Ct, contains exchanges at IC2, IC3, and Ct. All hybrid receptor genes were expressed in Rat-1 cells by stable transfection and retained high affinity binding for [^{3}H] BK.

Signal Transduction Following Single Intracellular Exchange

We first determined the functional activity of the single regional exchange hybrids. WT

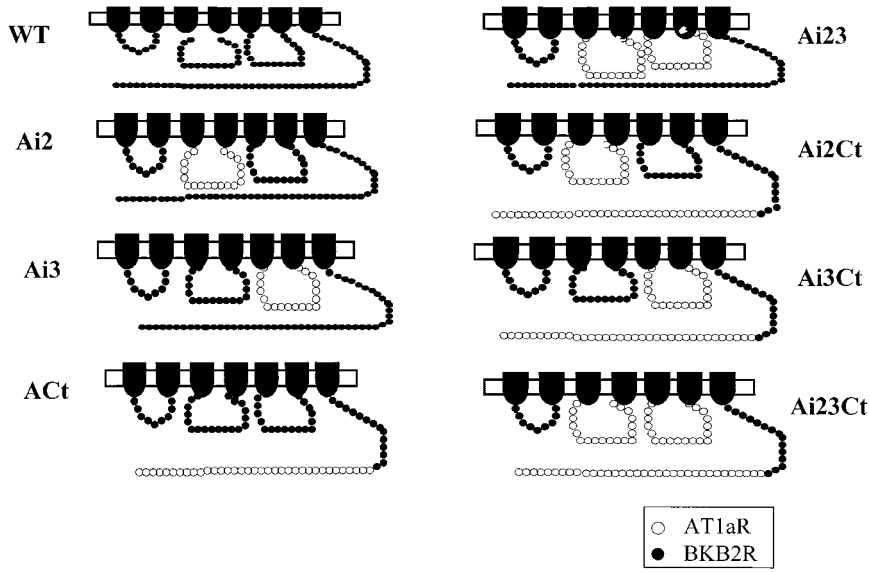

Fig. 1. Schematic representation of the BKB2R/AT1aR chimeric mutants. A schematic diagram of the seven transmembrane and intracellular regions of each receptor chimera constructed is shown. Intracellular regions are shown in either dark or light circles representing BKB2R or AT1aR respectively. Chimeras were constructed by exchanging the IC2, IC3, or the distal C-terminus 34 amino acids (Ct) of BKB2R with AT1aR. The abbreviations used to identify different receptor chimeras are shown on either sides of the diagram.

BKB2R and AT1aR were stably transfected into Rat-1 cells and PI turnover (related to Gαq activity), and ARA release (associated with Gαi activity) were tested in response to 100 nM BK or 100 nM Ang II. As illustrated in Figure 2a, BKB2R displayed a considerably higher inositol phosphate (IP) formation than AT1aR. Both the Ai2 and ACt activated PI turnover was very similar to that of WT BKB2R. The IC3 exchange, Ai3, resulted in a receptor unable to activate PI turnover in response to BK. The release of ARA followed a very similar pattern, as shown in Figure 2b. The WT AT1aR caused a somewhat lower ARA release than WT BKB2R. Both Ai2 and ACt responded to BK with an ARA release approximately the same as WT BKB2R. The Ai3 chimera displayed very poor signaling properties.

Signal Transduction Following Multiple Intracellular Exchange

The signaling properties of the multiple exchange chimera between BKB2R and AT1aR is illustrated in Figure 3. None of these mutant receptors showed constitutive activity with respect to either ARA release or PI turnover. All controls were approximately the same. Each response was determined as agonist response minus basal activity. The Ai23 chimera results in a receptor whose signaling response is increased 4-fold relative to WT. This large increase is seen for both ARA release and IP production. The Ai2Ct response to BK is also increased with respect to PI turnover (3-fold higher relative to WT), while it displays approximate WT signaling in ARA release. The dual chimera incorporating AT1aR 3rd loop and distal portion of the C-terminus tail, Ai3Ct, resulted in very poor signaling, similar to the single mutant Ai3 (Fig. 2).

Receptor Uptake of the BKB2R/AT1aR Chimera

Endocytosis of the receptor hybrids between the IC domains of BKB2R and AT1aR is shown in Figure 4. The IC2 replacement receptor (Ai2) and distal tail receptor (ACt) demonstrated rapid uptake at 75 and 70%, respectively, by 60 min. Exchange of the IC3 alone resulted in uptake only somewhat lower than WT BKB2R, Figure 4a. The dual mutants, Ai2Ct and Ai23, were taken up slightly more rapidly than WT BKB2R, at $\sim$60% as compared to 48% for WT (Fig. 4b). On the other hand, Ai3Ct displayed very poor uptake at 18%. The triple mutant, with IC2, IC3, and distal tail exchange (Ai23Ct) endocytosed to a very similar degree as WT BKB2R.

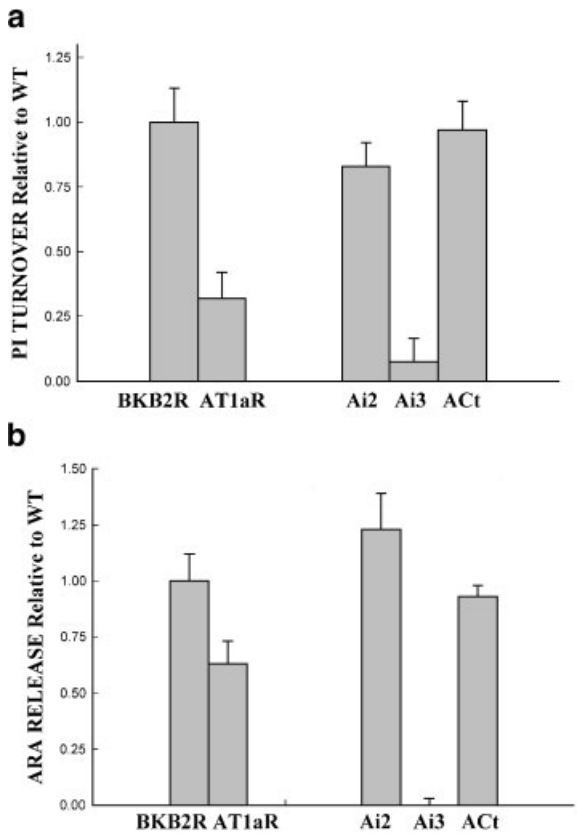

Fig. 2. BKB2R mutants with a single intracellular exchange. **a**: Bradykinin-induced inositol phosphate production in Rat-1 cells expressing chimeric mutants, wild type AT1a, and BKB2 receptors. PI turnover was measured in myo-[^{3}H]inositol-labeled cells expressing WT and the indicated chimeric receptors. Results are expressed as hormone stimulated IP production minus basal IP normalized to that of WT. Data represent duplicate wells from three experiments. **b**: Bradykinin-induced ARA release in Rat-1 cells expressing chimeric mutants, wild type AT1a, and BKB2 receptors. ARA release was measured in [^{3}H] arachidonate labeled cells expressing WT and chimeric receptors. Results are expressed as hormone stimulated ARA release minus basal ARA normalized to that of WT. Data represent triplicate wells from three experiments.

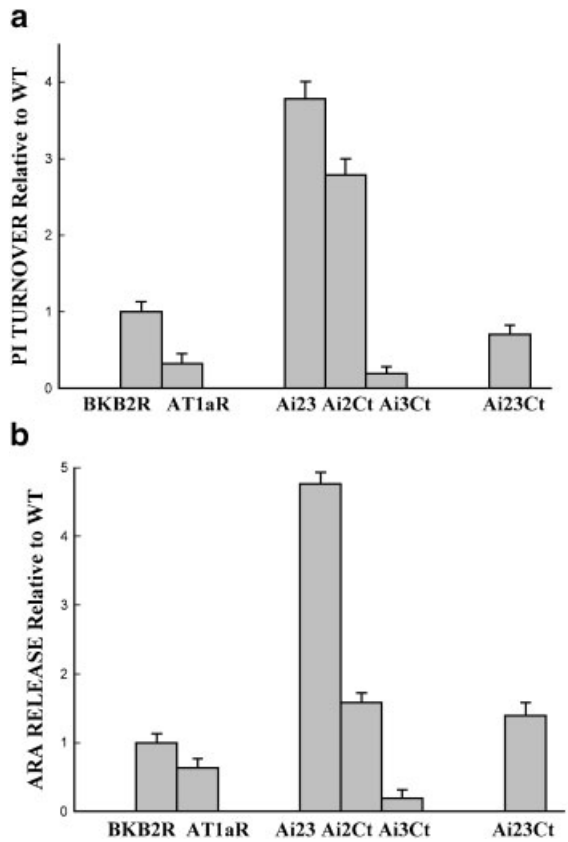

Fig. 3. BKB2R mutants with double or triple intracellular exchanges. **a**: Bradykinin-induced inositol phosphate production in Rat-1 cells expressing chimeric and wild type BKB2 receptors. Results are expressed as bradykinin stimulated IP production minus basal IP normalized to that of WT as described under Materials and Methods. **b**: Bradykinin-induced ARA release in Rat-1 cells expressing chimeric and wild type BKB2 receptors. Results are expressed as bradykinin stimulated ARA release minus basal ARA normalized to that of WT. Data represent triplicate wells from three experiments as described under Materials and Methods.

Increase of CTGF mRNA Expression in Response to BK by AT1aR/BKB2R Hybrid Receptor Transfected Rat-1 Cells

CTGF has been shown to be an important agent for the action of TGF-β on connective tissue production. Here, we show that CTGF expression is also upregulated by Ang II. As illustrated in Figure 5a, CTGF mRNA content in Rat-1 cells in response to 100 nM BK in BKB2R WT transfected cells falls slightly below that of the control (BK untreated cells). In the same phenotype Ang II had no effect on CTGF mRNA level, while treatment with BK in the presence of 12 μM forskolin resulted in considerably less CTGF mRNA than control. TGF-β at 5 ng/ml tripled CTGF mRNA content. When Rat-1 cells were transfected with AT1aR cDNA, Figure 5b, CTGF mRNA in response to 100 nM Ang II increased markedly, approaching that of the TGF-β treated cells. In the presence of 12 μM forskolin, a non specific activator of adenyl cyclase, the effect of Ang II was abrogated. Figure 5c illustrates the effect of BK on Ai23 transfected cells. Here, we show a sizable increase in the CTGF mRNA in response to 100 nM BK as IC2 and IC3 of the BKB2R were replaced by the corresponding AT1aR regions. A very similar increase was observed with regard to BK treatment of Ai23Ct (Fig. 5d) transfected

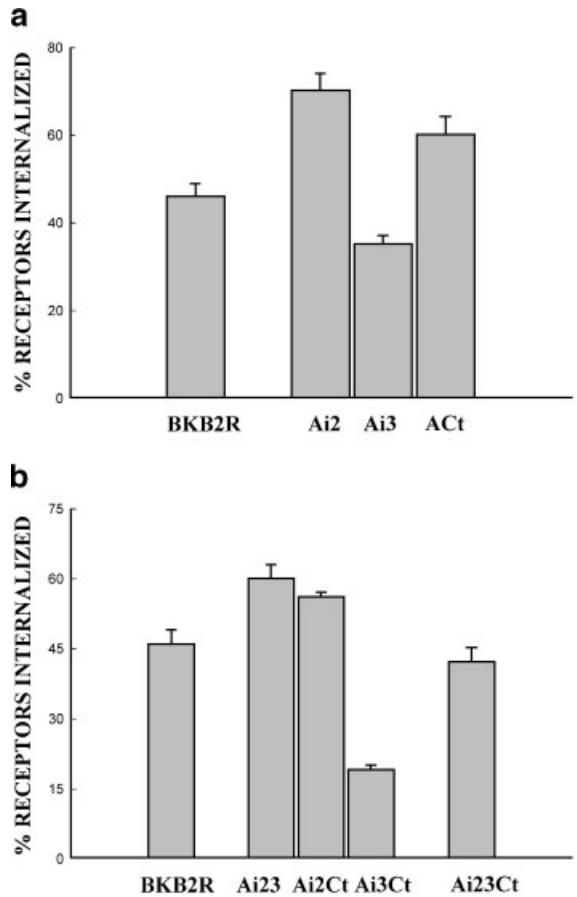

Fig. 4. Internalization of BK/AT1a receptor chimeras. **a**: Internalization profile of WT BKB2 receptors and chimeric mutants with a single IC region of the BKB2 receptor replaced with an AT1a receptor. Cells were incubated with 100 nM BK for 60 min. After acid stripping and three washes with ice-cold 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. **b**: Internalization profile of WT BKB2 receptors and chimeric mutants with 2 or 3 IC regions of the BKB2 receptor replaced with the AT1a receptor. Results represent the percentage of receptors internalized in 60 min as compared to receptors internalized in 0 min.

cells with CTGF mRNA increased approximately 3-fold above control.

Molecular Modeling

Molecular models of the receptor chimeras examined here were assembled, and simulations were carried out using our membrane-mimetic simulation cell. The results from the assembly of the Ai3 receptor hybrid are illustrated in Figure 6. The global topological arrangement of the IC face is illustrated in Figure 6a. The structural features of IC2 of BKB2R were determined experimentally [Piserchio et al., 2001], while the presence of the two α-helices in the C-terminus is based on homology modeling as previously detailed [Prado et al., 1998]. The exchange of BKB2R IC3 with that of AT1aR results in unfavorable interactions between the IC3 of AT1aR (shown in yellow) and the IC2 of BKB2R (shown in light green). The amino acids which appear involved are K237, R239 of IC3 and K136 of IC2, displayed both as a cytoplasmic view (Fig. 6b) and side view (Fig. 6c). During the simulations these two regions are displaced from each other, alleviating the unfavorable interaction of the positively charged residues.

DISCUSSION

The aim of this study was to determine whether global exchange of IC regions between unrelated receptors with similar sequence lengths and similar G-protein interactions can result in functional receptor hybrids. Using BKB2R as the recipient and the AT1aR as the donor, regions within the BKB2R IC were systematically replaced with the corresponding AT1aR sequences. The primary reason for using the AT1aR as the donor was that the length of its overall IC domain and regional length are very similar to BKB2R. Additionally, AT1aR is known to link to Gαi and Gαq with the ability to increase IP formation and release arachidonate from lipid stores similarly to BKB2R. However, AT1aR performs physiologic roles often contrary to those of BKB2R and the generation of functional hybrids could prove useful for gene therapy. Initially, the IC2, IC3, and the distal C-tail of the BKB2R were replaced as single mutations with the corresponding regions of the AT1aR. Following this approach, two or three of these intracellular regions were exchanged simultaneously converting the BKB2R to a hybrid whose IC face was predominantly AT1aR.

We demonstrated previously that in the WT BKB2R the distal end of the C-terminus (the C-terminal 34 residues) is crucial for signal transduction as well as receptor endocytosis and resensitization [Prado et al., 2001]. Although the S/T complex within this region was important for these functions, other motifs, as yet undetermined, within this region also appeared contributive [Prado et al., 1998, 2001]. At the same time, we illustrated that this entire region could be interchanged with the corresponding region of AT1aR without altering the BKB2R receptor signal capacity, uptake and

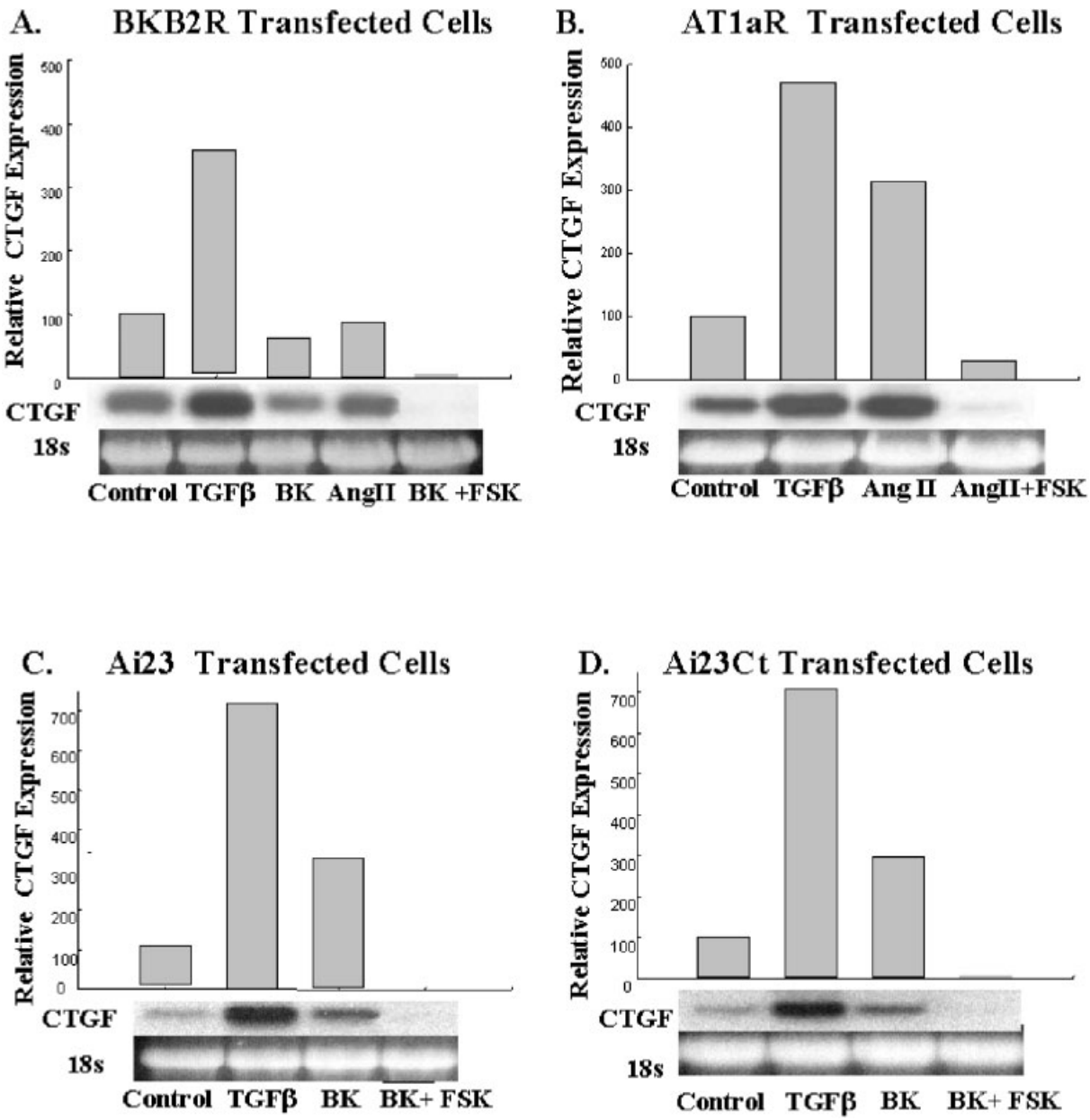

Fig. 5. Effect of TGF-β, AngII, BK and BK plus forskolin on CTGF mRNA levels in cells expressing BKB2, AT1a, Ai23, or Ai23Ct receptors. Stably transfected cells expressing (**a**) BKB2R, (**b**) AT1aR, (**c**) Ai23, and (**d**) Ai23Ct were grown to confluence and treated with TGF-β (5 ng/ml) or BK (100 nM) or AngII (100 nM) with or with out forskolin (12 μM) for 2 h. Total RNA from one well of a six-well plate was run per lane and probed with CTGF cDNA as described in Materials and Methods. The northern blot analysis is a representative of two separate experiments. Ethidium bromide staining showing the 18S rRNA represents loading control.

resensitization [Prado et al., 2001]. Here, we show that in addition to the exchangeability of the distal tail, the exchange of the IC2 with the AT1aR also results in a functional receptor mutant, which has the ability to generate IP and arachidonate approximately equal to WT BKB2R and continues to endocytose. However, the IC3 of BKB2R, although about same size as AT1aR, was not successfully interchangeable. The IC3 mutant (Ai3) displayed very poor signal capacity. This suggests that the IC3 of AT1aR is not compatible with the other IC domains of BKB2R with respect to coupling and activation of Gαi and Gαq. The results from the multiple domain chimera provide the possible sources of this lack of compatibility.

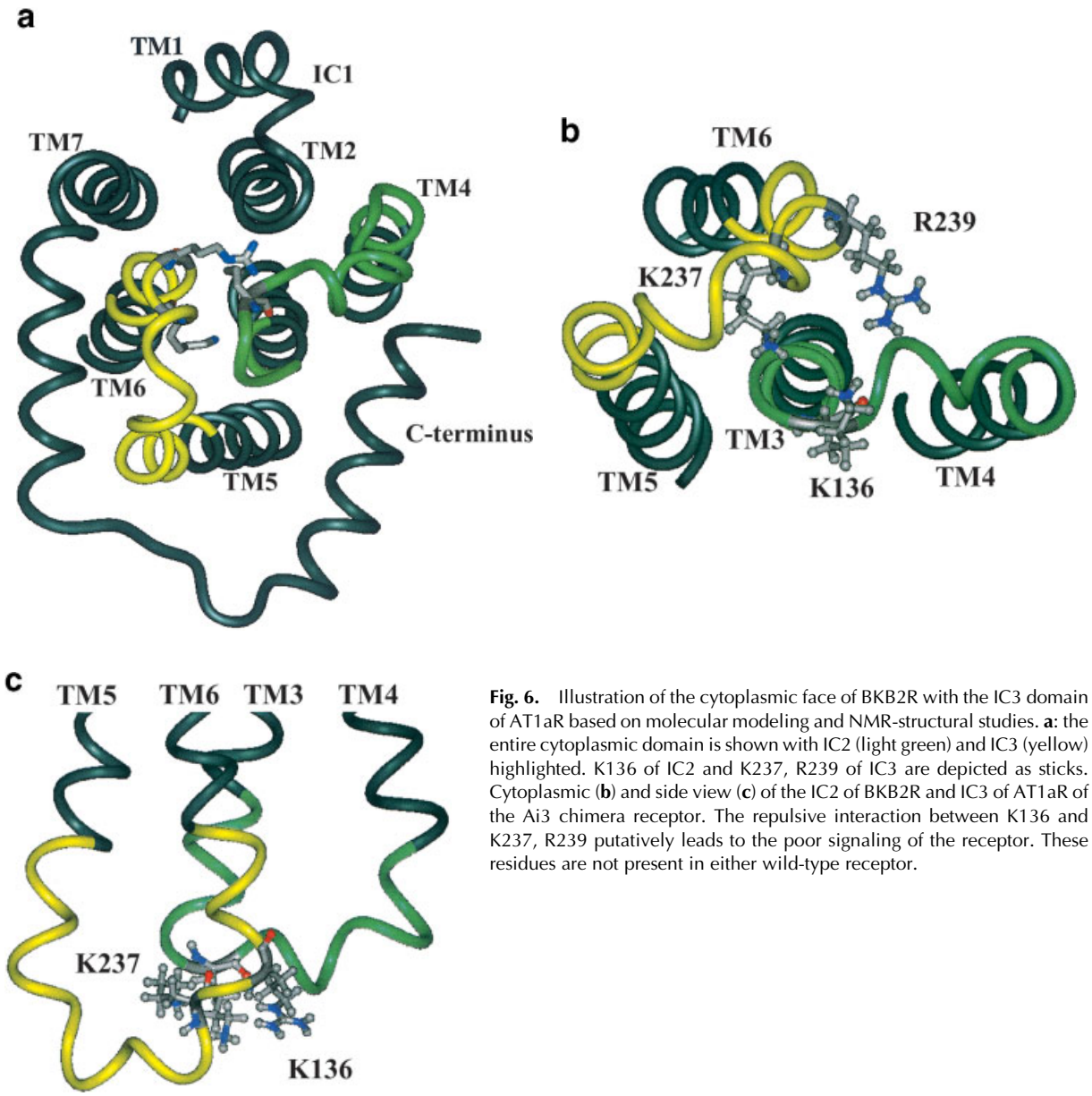

Fig. 6. Illustration of the cytoplasmic face of BKB2R with the IC3 domain of AT1aR based on molecular modeling and NMR-structural studies. **a**: the entire cytoplasmic domain is shown with IC2 (light green) and IC3 (yellow) highlighted. K136 of IC2 and K237, R239 of IC3 are depicted as sticks. Cytoplasmic (**b**) and side view (**c**) of the IC2 of BKB2R and IC3 of AT1aR of the Ai3 chimera receptor. The repulsive interaction between K136 and K237, R239 putatively leads to the poor signaling of the receptor. These residues are not present in either wild-type receptor.

Combining the switching of both IC2 and IC3 of BKB2R with the corresponding regions of AT1aR (Ai23) results in a superactive receptor, both in ARA release and PI production. This increased signal capacity is not accompanied by increased basal activity, illustrating that these mutant receptors are manifesting true increases in BK responsive signaling action. These observations suggest that the two regions, IC2 and IC3, are functionally related and that unfavorable (with respect to signaling) interactions between the IC2 of BKB2R and the IC3 of AT1aR are responsible for the lack of signaling of Ai3. Supporting this hypothesis is the lack of recovery of signaling in the dual mutant, Ai3Ct (i.e., the unfavorable interaction with the IC3 of AT1aR is not within the C-terminus of BKB2R). Importantly, the exchange of all of the IC domains of BKB2R for those of AT1aR restores signaling.

To further probe for possible interactions between the IC2 of BKB2R and IC3 of AT1aR, extensive molecular modeling was carried out. Based on the molecular models, we find that K237 and R239 within the IC3 of the AT1aR are in close proximity to K136 of IC2 of BKB2R in

the Ai3 chimera (Fig. 6). The consequent repulsion between the IC2 and IC3 could account for the lack of function. Importantly, this interaction is not present in WT BKB2R (BKB2R contains a valine and threonine at the corresponding positions to K237 and R239). In the dual chimera receptor containing both IC2 and IC3 of AT1aR, the K136 is replaced with a histidine, not uncharged under the experimental conditions used or most physiological environments. Future experiments such as a mutation of K237 and R239 within the AT1aR third loop to V237 and T239 will focus on the specific interactions between these two loops which lead to a functioning receptor.

The dual mutant, containing the IC2 and distal C-terminus (Ai2Ct) of AT1aR also exhibits an improved signaling capacity over either single mutant or WT BKB2R with respect to both ARA release and IP generation. We previously illustrated that the C-terminus of the BKB2R, both distal and proximal is interactive with the proximal segment of the IC2 [Prado et al., 1998, 2001]. For example, we have shown a functional correlation between Y131 (of IC2) and Y322 (of the proximal C-terminus) of BKB2R [Prado et al., 1997]. Studies targeting the cluster of hydroxyl containing amino acids of the distal C-terminus suggest a correlated role of IC2 and the C-terminus in internalization of the receptor [Prado et al., 1998, 2001]. It is therefore interesting to note that the uptake of Ai3 is only slightly below that of WT BKB2R at 38% within 1 h compared to 48% for WT. Also, the ACt mutant displays active uptake. However, the double exchanges IC3/Ct (Ai2Ct) hybrid endocytoses very poorly with an uptake at 18% in 60 min. To return uptake to normal a triple exchange hybrid, Ai23Ct, had to be constructed. This hybrid exhibits uptake very similar to WT BKB2R. At this time, it is not clear which form of uptake the Ai23Ct emulates. Both BKB2R and AT1aR are known to endocytose and resensitize [Prado et al., 1998, 2001; Anborgh et al., 2000]. However, AT1aR has been reported to endocytose via clathrin vesicles, while BKB2R is taken up into caveolae [de Weerd and Leeb-Lundberg, 1997; Anborgh et al., 2000]. It remains to be determined by which route the triple exchange hybrid is endocytosed.

As illustrated in Figure 5, cells transfected with the AT1aR cDNA respond to Ang II with increased CTGF mRNA. Correspondingly, BKB2R cDNA transfected cells respond to BK with a slight lowering of CTGF mRNA and with no response to Ang II. The Ai23Ct and Ai23 hybrids respond to BK with an increased CTGF mRNA approaching that obtained with the AT1aR transfected cells. These results show that, despite both AT1aR and BKB2R linking to Gαi and Gαq, their action can be differentiated in vitro. As with TGF-β, the effect by Ang II on the expression of CTGF is shut down by cAMP. This action by cAMP to limit the expression of genes associated with connective tissue production in fibroblastic cell types has been described previously [Choung et al., 1998].

In summary, our results illustrate that global exchanges between the BKB2R and AT1aR, heterologous GPCRs, sharing functional activities and of similar IC length, can lead to hybrid receptors, which continue to signal and are able to take on the function of the donor receptor (AT1aR) while still responding to the recipient's ligand, BK.

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