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Activation of ERK, JNK, Akt, and G-Protein Coupled Signaling by Hybrid Angiotensin II AT1/Bradykinin B2 Receptors Expressed in HEK-293 Cells

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Abstract Bradykinin (BK) and angiotensin II (AngII) often have opposite roles in cardiovascular diseases. Our aim here was to construct hybrid receptors which bind AngII but signal as BK. Various sequences of the intracellular face of the AngII type I receptor, AT1R, were replaced with corresponding sequences from the bradykinin B2 receptor (BKB2R). The hybrids demonstrated a number of signaling characteristics of the BKB2R. For example, the hybrids demonstrated BK as opposed to AngII like phosphorylation of Akt and JNK. The hybrids containing the BKB2R intracellular loop 2 (IC2) displayed minimal G-protein, Gαi/Gαq, linked signaling. Computer based molecular models suggested that Ser-Met-Gly from the IC2 of the BKB2R is detrimental for the Gαi/Gαq coupled functions of this hybrid. The return of Lys-Ser-Arg of the AT1R to this hybrid led to almost full recovery of Gαi and Gαq activation. The design and production of AT1/BKB2 hybrid receptors is a potential approach in the treatment of hypertension related diseases where the presence of AngII, its AT1 receptor and the consequent signal transduction has proven detrimental. J. Cell. Biochem. 101: 192–204, 2007.

Key words: angiotensin II type I receptor; bradykinin B2 receptor; chimeric receptor; GPCR; signal transduction; MAPK; Akt; molecular modeling

The regulation of vascular tone is a complex process controlled by multiple effectors. Accumulating evidence suggests that angiotensin II (AngII) mediates vasoconstriction, endothelial dysfunction and vascular remodeling in cardiovascular diseases, such as systemic and pulmonary hypertension, atherosclerosis, and heart failure [Hunyady and Catt, 2005]. Limiting the presence of AngII and/or the action of its AT1 receptor (AT1R) has proven to be important in the treatment of these diseases [Conlin, 2005; Ribeiro, 2006]. Evidence is accumulating that bradykinin (BK) through its B2 receptor (BKB2R) antagonizes the actions of AT1R. BK induces various actions, such as vasodilatation, anticoagulation and hypotension [Leeb-Lundberg et al., 2005]. In animal models genetic dysfunction of the kallikrein–kinin system ultimately results in hypertension [Brochu et al., 2002].

At this time, the signal cascades which differentiate the actions of these two receptors are not well understood. In fact, BKB2R and

Abbreviations used: ARA, arachidonic acid; AngII, angiotensin II; AT1R, angiotensin II type I Receptor; BK, bradykinin; BKB2R: bradykinin type B2 receptor; ERK, extracellular regulated kinase; GPCR, G-protein coupled receptor; IP, inositol phosphate; JNK, c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; WT, wild-type.

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AT1R share many signaling paths. They both couple to Gαi and Gαq/11 [Prado et al., 2002; Leeb-Lundberg et al., 2005; Hunyady and Catt, 2006]. Both receptors also activate the MAPKs, including ERK1/2, JNK, and p38 [Liu et al., 2006]. However some differences in their signaling have been reported. For example RhoA has been reported to be activated by AngII but inactivated by BK in human lung fibroblasts [Huang et al., 2006]. This small G-protein is proving important in cardiovascular regulatory processes [Rolfe et al., 2005]. Another important cardiovascular regulator, Akt, may be a target for therapeutic intervention in AngII caused hypertensive responses [Vecchione et al., 2005]. Although often beneficial as a pro-growth, anti-apoptotic agent [Hemmings, 1997; Cantley, 2002], the activation of the PI3K/Akt complex is often linked to a variety of diseases including cardiac hypertrophy, heart failure, preconditioning and hypertension [Oudit et al., 2004; Benkirane et al., 2006]. For example, blood pressure which rises in AngII-infused rats is attenuated by rosiglitazone, an activator of PTEN which inactivates the PI3k/Akt pathway [Benkirane et al., 2006].

With the eventual goal of generating AT1 hybrid receptors which respond to AngII but generate signals which counter some of the negative actions of the AT1R, we have constructed global chimeric exchanges between the BKB2 and AT1 receptors. Previously we reported on chimeric receptors in which the intracellular domains of BKB2R were replaced with those from the AT1 [Yu et al., 2002]. We found that these hybrid receptors continued to bind BK and signal. In this communication we focus on the intracellular face of the AT1R and the ability of global intracellular segments of the BKB2R to replace those of the AT1R. The second and third intracellular loops (IC2, IC3) and the carboxyl-terminus of the AT1R were replaced, both individually and simultaneously, with the corresponding domains of the BKB2 receptor. Many of these AT1 hybrid receptors maintained AngII binding, but acquired the signaling properties of the WT BKB2 receptor. Furthermore, molecular models suggested that maintaining certain endogenous charge interactions within and among the ICs is crucial for functional hybrid formation. Experimental results supported this prediction.

MATERIALS AND METHODS

Materials

[^{3}H] BK (78 Ci/mmol), [^{3}H] AngII (52.5 Ci/mmol), myo-[1,2-^{3}H] inositol (60 Ci/mmol), and [^{3}H] arachidonate (65.9 Ci/mmol) were obtained from Perkin Elmer Life Sciences (Boston, MA). Analytical grade AG-1-X8 resin (100–200 mesh) was obtained from Bio-Rad (Hercules, CA). Antibodies for detection of phospho-ERK, ERK, phospho-JNK, JNK, phospho-Akt, and Akt were purchased from Cell Signaling Technologies (Beverly, MA). Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). Oligonucleotides, G418, and Lipofectamine 2000 were obtained from Invitrogen (Carlsbad, CA). QuikChange® mutagenesis kit was obtained from Stratagene (La Jolla, CA). Compass DNA Purification Kit was from American Bioanalytical (Natick, MA). Plasmid Midi Kit was purchased from Qiagen (Valencia, CA). Protease inhibitor cocktail was from Roche Diagnostics (Indianapolis, IN). BCA Protein Assay Kit was from Pierce (Rockford, IL). ECL Western Blotting Detection Reagents were from Amersham Biosciences (Piscataway, NJ). The rat BKB2R cDNA was cloned in our laboratory. The rat AT1aR cDNA was obtained from Dr. K. Sandberg (Georgetown University).

Site-Directed Mutagenesis of the Intracellular Regions of the AT1R

To facilitate the construction of the chimeric mutants between AT1R and BKB2R, we introduced five silent mutations creating unique restriction sites in order to swap the IC2, IC3 or the C-tail of the AT1R with the corresponding regions of the BKB2R. The silent mutations created preserved the amino acid sequence of the coding region. The restriction sites created were: AgeI (at position 126) and BstEII (at position 147) for second loop substitution; SpeI (position 214) and EcoRV (position 237) for third loop substitution; and MfeI (position 295) for 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′-CGT GTC TCA GCA TCG ACC GGT ACC TGG CCA TC-3′; anti-sense strand 5′-GAT GGC CAG GTA CCG GTC GAT GCT GAG ACA CG-3′; *BstEII:* sense strand 5′-GAT GCT GGT GGC CAA GGT CAC CTG CAT CAT CAT CTG-

3′; anti-sense strand 5′-CAG ATG ATG ATG CAG GTG ACC TTG GCC ACC AGC ATC-3′; *SpeI:* sense strand: 5′- CCC TTT CCT TAT CAT TCT CAC TAG TTA TAC CC TTA TTT GGA AAG CTC-3′; antisense strand: 5′-GAG CTT TCC AAA TAA GGG TAT AAC TAG TGA GAA TGAT AAG GAA AGG G-3′; *EcoRV:* sense strand: 5′- GAA CAA ACC AAG AAA CGA TGA TAT CTT TAG GAT AAT TAT GGC G -3′; antisense strand: 5′- CGC CAT AAT TAT CCT AAA GAT ATC ATC GTT TCT TGG TTT GTT C -3′; *MfeI* sense strand: 5′- CTG CAT AGC GTA TTT TAA CAA TTG CCT GAA CCC TCT GTT C -3′; antisense strand: 5′- GAA CAG AGG GTT CAG GCA ATT GTT AAA ATA CGC TAT GCA G -3′.

The AT1R cassette was confirmed by DNA sequencing. This cassette receptor now allowed us to make any combination of mutations within the regions 126–147, 214–237, and 295–359 by synthesizing complementary oligonucleotides containing counterpart regions of other GPCRs such as BKB2R.

Generation of Hybrid Receptors

To construct the AB(2) mutant, the pcDNA3.1-AT1R cassette was digested with AgeI and BstEII. The corresponding sense and anti-sense oligonucleotides for the IC2 region of the BKB2 receptor (5′-CCG GTA CCT GGC CAT CGT CAA GAC CAT GTC CAT GGG CCG GAT GCG CGG GG-3′ and 5′-GTG ACC TTG GCC ACC AGT ACC CCG CGC ATC CGG CCC ATG GAC ATG GTC TT-3′) were then annealed at equal molar ratio and ligated into the AgeI/BstEII digested vector cassette. The same procedure was performed to construct the mutant AB(KSR). The oligonucleotides used were the following: sense strand: 5′-CCG GTA CCT GGC CAT CGT CAA GAC CAT GAA GTC TCG CCG GAT GCG CGG GGT ACT GGT GGC CAA G-3′ and antisense strand: 5′-GTG CCT TGG CCA CCA GTA CCC CGC GCA TCC GGC GAG ACT TCA TGG TCT TGA CGA TGG CCA GGT A-3′.

To construct the entire C-terminus AT1R/BKB2R hybrid, PCR was performed to amplify the C-terminus of BKB2R using the rat BKB2R cDNA as a template. The 5′ PCR primer used was 5′-GAA CAA TTG CCT GAA CCC TCT GTT CTA CGT GAT TGT GGG CAA GCG CTT C-3′ and the 3′ primer used was 5′-CCG CTC GAG TCA CTG CTT GTT CCC CGC CCA ATC-3′. The PCR product was digested with MfeI and BamHI. The EcoRI-XhoI fragment of the AT1R was ligated into the EcoRI-XhoI sites in the pGEM vector. The PCR product was then ligated into the pGEM-AT1R fragment cassette digested with MfeI and BamHI. The fragment in pGEM vector was then ligated into the pcDNA3.1-AT1R vector digested with EcoRI and XhoI thus reconstituting the full-length receptor cDNA with the C-terminus of the AT1R replaced by residues derived from the corresponding region of rat BKB2 receptor.

After obtaining the single loop mutants AB(2), AB(3), and AB(T), combinational loop mutants AB(2, 3), AB(2, T), AB(3, T), and AB(2, 3, T) were constructed by exchanging the EcoRI-XhoI fragments containing the 3rd loop and tail with rest of the receptor. The XhoI-XbaI fragments containing the chimeric receptors from pcDNA3.1 were then subcloned into the bicistronic mammalian expression vector, pCMIN(+) for stable transfections [Zhou et al., 2000].

All the mutants described above were sequenced by an in-house facility. 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 HEK-293 cells were performed as described by Yu et al. [2005]. HEK-293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified CO_2 (5%) incubator. HEK-293 cells were transiently or stably transfected in 12-well plates using Lipofectamine 2000 according to the protocol of the manufacturer (Invitrogen). Stable transfectants were selected in the presence of 0.5 mg/ml G418. The G418 resistant cell culture was then expanded and tested for specific binding to [^{3}H]Ang II.

Ligand Binding

Receptor binding studies of the BKB2R, AT1R, and mutant receptors in intact HEK293 cells were carried out as described by Yu et al. [2005]. Briefly, confluent cell monolayers in 24-well plates were incubated in binding buffer (50 mmol/L Tris, 120 mmol/L NaCl, 4 mmol/L KCl, 10 μg/ml bacitracin, 10 mmol/L glucose, 0.1% BSA, 1 mmol/L $CaCl_2$, 5 mmol/L $MgCl_2$, 10 mmol/L HEPES pH 7.35) containing various

concentrations of [^{3}H]-AngII ranging from 0.04 to 10 nM in the absence (total binding) or presence of 100 nM unlabeled AngII (non-specific binding) for 2 h at 4°C. Cells were washed three times with ice-cold buffer and then solubilized with 0.2% SDS. Radioactivity was determined in a PACKARD β counter after addition of 2 ml of Ecolite scintillation fluid. Equilibrium binding data (K_d and B_{max}) were analyzed by best fit to a single site model using the SigmaPlot® 8 program (SPSS Inc.).

Phosphoinositide Turnover

HEK293 cells stably transfected with AT1 and BKB2 receptors were incubated with 1 μCi/ml myo-[^{3}H]inositol in 1 ml of growth medium and the levels of inositol phosphates (IPs) determined 24 h later as described by Prado et al. [1997]. Briefly, 10 min prior to ligand stimulation, cells were exposed to DMEM containing 20 mM $LiCl_2$ and 20 mM HEPES, pH 7.4. Cells were then exposed to 100 nM BK or AngII 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 and the formic acid soluble material isolated by centrifugation and neutralized by the addition of 10 ml 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 2M ammonium formate, pH 5.0, as described. Radioactivity was determined in a Packard liquid scintillation counter.

Arachidonic Acid Release

HEK-293 cells stably transfected with AT1 and BKB2 receptors were labeled with [^{3}H]arachidonate (0.2 Ci/well) for 16 h as described by Prado et al. [1997]. Briefly, cells were washed and incubated with 500 μl of DMEM containing 2 mg/ml bovine serum albumin and incubated with 100 nM BK or AngII for 20 min at 37°C. Medium was removed and centrifuged at 800*g*. The control group was treated identically except for the addition of BK or AngII. Radioactivity in the supernatant was determined in a scintillation counter after addition of 2 ml of Ecolite scintillation fluid.

Calcium Mobilization

Mobilization of Ca^{2+} was determined as reported previously with some modifications [Prado et al., 1998]. Wild-type or mutant receptor transfected HEK-293 cells were trypsinized and washed two times in physiological buffer solution (140 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 10 mmol/L glucose, 0.9 mmol/L $CaCl_2$, 15 mmol/L HEPES, 0.1% BSA). The HEK-293 cells were resuspended at 1.5×10^7 cells/ml and incubated with Fura-2/AM for 30 min (2 μmol/L final concentration). After 30 min, the cell suspension was diluted five times with physiological buffer solution and incubated for another 15 min. Cells were pelleted and resuspended at 1×10^6 cells/ml. Ca^{2+} mobilization experiments were performed using a Hitachi F-2500 Fluorescence Spectrophotometer.

Molecular Models of the AT1 and BKB2 Receptors

The generation of the molecular models has been previously described [Yu et al., 2005]. The receptor hybrids were created using the MSI Insight II program (Accelrys®) and then soaked in a two-phase simulation cell of water/decane. The receptors were fully energy minimized, followed by extensive molecular dynamics simulations employing the GNU GROMACS simulation program (http://www.gromacs.org).

Western Blot Analysis

HEK-293 cells stably transfected with WT and mutant receptors were incubated with 100 nM AngII or BK for 5 min. The cells were then washed twice with ice-cold PBS. Cell lysates were prepared by addition of ice-cold RIPA buffer, 150 mM NaCl, 1.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 (Sigma, St. Louis, MO) and 1× complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) and sedimented at 12,000 rpm in a microcentrifuge at 4°C for 20 min. The proteins were fractionated on 10% SDS–PAGE gels and Western blots were carried out using antibodies against phosphorylated or unphosphorylated ERK1/2, JNK or Akt. Proteins were detected by chemiluminescence and the film scanned with an Epson Perfection 3170 scanner using Epson Scan (version 1.22A) software. The image was then analyzed using Sigma Scan10 (Jandel Scientific, San Rafael, CA) to determine the intensity of each band.

Statistical Analysis and Data Analysis

The K_d, B_{max} of BK and AngII binding were estimated using the SigmaPlot® 8 Program Pharmacology Module (SPSS Inc.). Statistical evaluation of the data was carried out using the student *t*-test. Probability values less than 0.05 were considered significant for PI turnover and arachidonic acid release.

RESULTS

Generation of Hybrid Receptors With the AT1R as Recipient and BKB2R as Donor

Sequences of the IC2, IC3, and C-terminus tail of the WT AT1R and BKB2R are shown in Table I. Although the sequences differ markedly, the number of residues in each intracellular domain is similar. In Figure 1 the global exchanges of each receptor hybrid are illustrated in schematic form. For single exchanges, the IC2, IC3 or C-tail of the *A*T1R (recipient) were replaced with the corresponding region from the *B*KB2R (donor) and referred to as AB(2), AB(3), and AB(T), respectively. The hybrid receptors with multiple domain exchanges are named similarly, AB(2,3), AB(2,T), AB(3,T) and AB(2,3,T). The binding parameters (K_d and B_{max}) of the WT and hybrid receptors are shown in Table II. The B_{max} of all the single exchange receptors was similar to that of WT AT1R at 1 pmole receptor/mg protein. The B_{max} of the double replacement receptors, AB(2,T) and AB(3,T), was also within the WT AT1R range. However, the AB(2,3) hybrid displayed a considerably lower B_{max}, at approximately 10% of WT AT1R. The additional replacement of the C-terminus, AB(2,3,T), returned the B_{max} to 25% of WT AT1R. Interestingly, the K_d for all the receptors including the double and triple exchanges was very similar, approximately 5 nM. However, because of the considerably lower B_{max} of the two multi-exchange hybrids containing IC2 and IC3 exchanges, these two receptors were not included in the subsequent experiments.

G-protein Coupled Signal Transductions

G-protein coupled signaling by the hybrids was determined by measuring phosphatidylinositol (PI) turnover (related to Gαq and PLC activity) and arachidonic acid (ARA) release (associated with Gαi and PLA_2 activity). As illustrated in Figure 2a, IP production by HEK-293 cells expressing the AT1R and BKB2R in response to their respective ligands was very similar. The inhibitor of PLCβ, U73312, at 10μM which was determined to cause maximal inhibition (results not shown) reduced PI turnover by WT AT1R in response to AngII by approximately 77%. The chimeric receptors AB(3) and AB(T) and AB(3,T) activated PI turnover to a similar extent as WT AT1R. The hybrids containing IC2 [AB(2) and AB(2,T)] showed negligible PI turnover. As shown in Figure 2b, the release of ARA followed a very similar pattern. The AB(3), AB(T) and AB(3,T) expressing cells responded to AngII with an ARA release approximately the same as WT AT1R. The AB(2), AB(2,T) hybrids displayed very poor ARA release. None of these receptor hybrids showed constitutive activity with respect to either ARA release or PI turnover.

Calcium Mobilization

Ca^{2+} mobilization in response to AngII by the cells expressing the chimeric constructs is shown in Figure 3. Untransfected wild-type

TABLE I. Amino Acid Sequences of the Intracellular Regions of the Wild Type AT1 and BKB2 Receptors

IC2:
AT1: 131 VHPMKSRLRRT
B2R: 165 VKTMSMGRMRG
IC3:
AT1: 218 IWKALKKAYEIQKNKPRNDD
B2R: 252 IMQVLRNNEMKKFKEVQTEK
C-tail:
AT1: 303 GFLGKKFKKYFLQLLKYIPPKAKSHSSLSTKMSTLSYRPSDNMSSSAKKPASCFEVE
B2R: 338 VIVGKRFRKKSREVYQAICRKGGCMGESVQMENSMGTLRTSISVDRQIHKLQDWAGNKQ

The sequences of AT1R and BKB2R were retrieved from SwissProt: Rat Angiotensin II type 1 Receptor (AG2R_RAT)—P25095; Rat Bradykinin B2 Receptor (BRB2_RAT)-P25023. The putative intracellular loops of BKB2R and AT1R were determined by aligning the amino acid sequences of these receptors with the bovine rhodopsin sequence using the ClustalW program. The transmembrane helical regions of bovine rhodopsin have been identified based on its crystal structure [Palczewski et al., 2000]. The numbers at the beginning and end of each sequence indicate the number of the amino acid in the sequence.

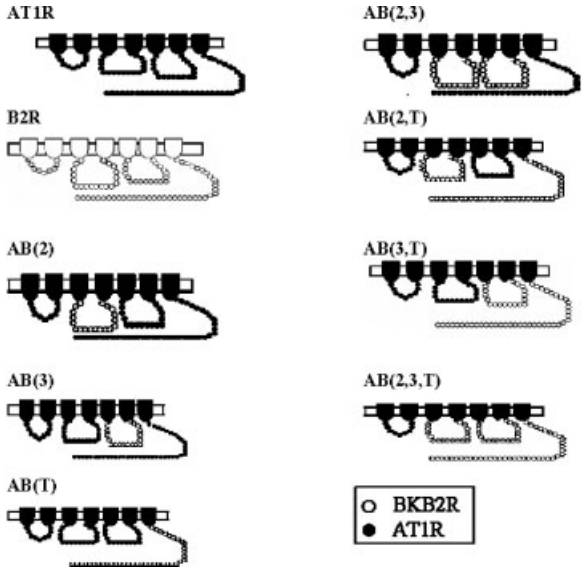

Fig. 1. Schematic representation of the hybrid receptors. Hybrid receptors were created using the AT1R as the recipient and using the BKB2R as the donor. Amino acids from BKB2R are shown as empty circles and those from AT1R are shown as solid circles.

HEK-293 cells showed no response to AngII. The maximum calcium response of the cells expressing WT AT1R was approximately two-fold the maximum calcium response of cells expressing the WT BKB2R. Calcium mobilization by the cells expressing AB(3), AB(T), and AB(3,T) was similar to that of cells expressing the WT BKB2R. The AB(2) cells, which displayed minimal PI turnover, translocated Ca^{2+} considerably less than WT BKB2R. However, the poorest Ca^{2+} mobilization was displayed by

TABLE II. Binding Parameters of WT and Hybrid Receptors Expressed in HEK-293 Cells

Receptor	B_{max} (pmole/mg protein)	K_d (nM)
BKB2R	1.12 ± 0.15	5.2 ± 1.5
AT1R	1.23 ± 0.17	4.9 ± 1.5
AB(2)	1.20 ± 0.11	4.3 ± 1.2
AB(3)	0.94 ± 0.11	3.9 ± 1.4
AB(T)	1.10 ± 0.10	4.2 ± 0.9
AB(2,3)	0.14 ± 0.01	2.0 ± 0.8
AB(2, T)	1.06 ± 0.08	5.8 ± 1.0
AB(3, T)	0.96 ± 0.08	4.5 ± 1.3
AB(2, 3, T)	0.31 ± 0.01	2.6 ± 0.9

Ligand binding experiments were carried out using [^{3}H] AngII or [^{3}H]BK on HEK-293 cells expressing wild type or hybrid receptors. Ligand concentrations ranged from 0.04 to 10 nM. The K_d and B_{max} of ligand binding were calculated using the SigmaPlot® 8 Program Pharmacology Module (SPSS Inc.). B_{max} is presented as pmole receptor per mg protein. Data are representative of at least two separate experiments.

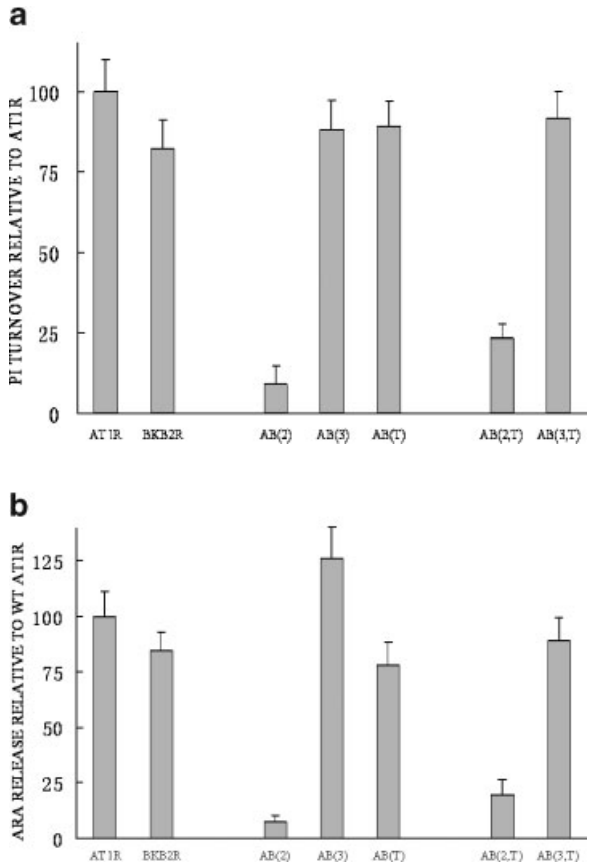

Fig. 2. **a**: Inositol phosphate production in HEK-293 cells expressing WT and hybrid receptors. PI turnover was measured in myo-[^{3}H]inositol-labeled cells as described in Materials and Methods. Results are presented as ligand stimulated IP production minus basal IP normalized to that of wild type receptors. Data represent the average of triplicate samples ± SD from a representative experiment of at least three experiments. **b**: ARA release in HEK-293 cells expressing WT and hybrid receptors. ARA release was measured in arachidonate labeled cells as described in Materials and Methods. Results are presented as effecter stimulated ARA release minus basal ARA, normalized to that of wild-type receptors. Data represent the average of triplicate samples ± SD from a representative experiment of at least three separate experiments.

the AB(2,T) cells which also exhibited minimal PI turnover in response to AngII.

ERK Activation

The activation of ERK by AngII and BK through their respective WT receptors was very similar as illustrated in Figure 4a. Western analysis showed approximately the same activation of ERK by either WT AT1R or BKB2R in response to AngII or BK, after 5 min of ligand exposure. All the single and multiple loop replacement cells demonstrated an increase in

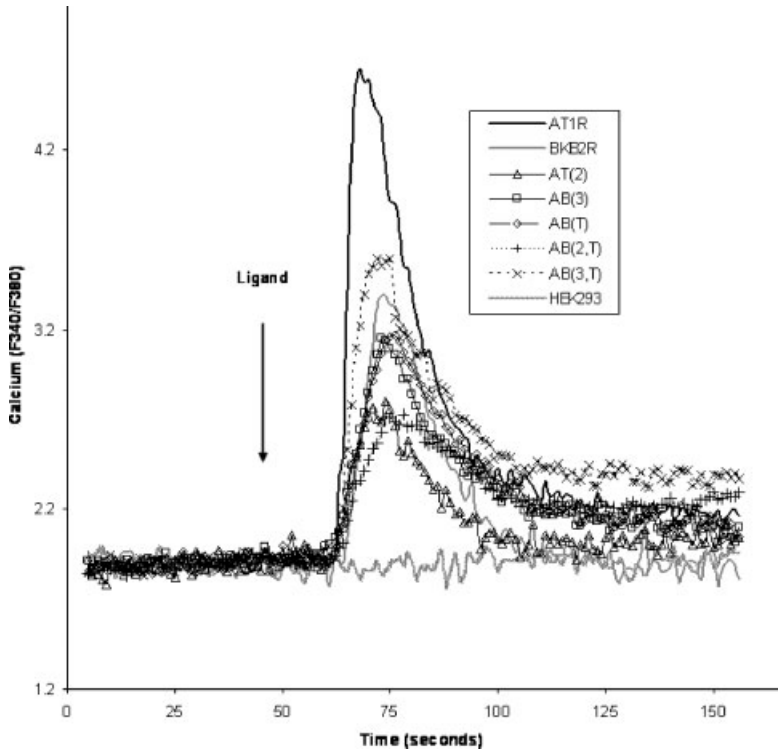

Fig. 3. Calcium mobilization. Calcium mobilization was measured in HEK-293 cells as described in Materials and Methods. Results from untransfected HEK cells and HEK cells transfected with WT AT1R or BKB2R are drawn as thick lines. Single loop hybrids, AB(2), AB(3), AB(T) are drawn with triangle, square, or diamond, respectively; multiple loop exchange mutants, AB(2,T) and AB(3,T) are drawn with cross and star, respectively.

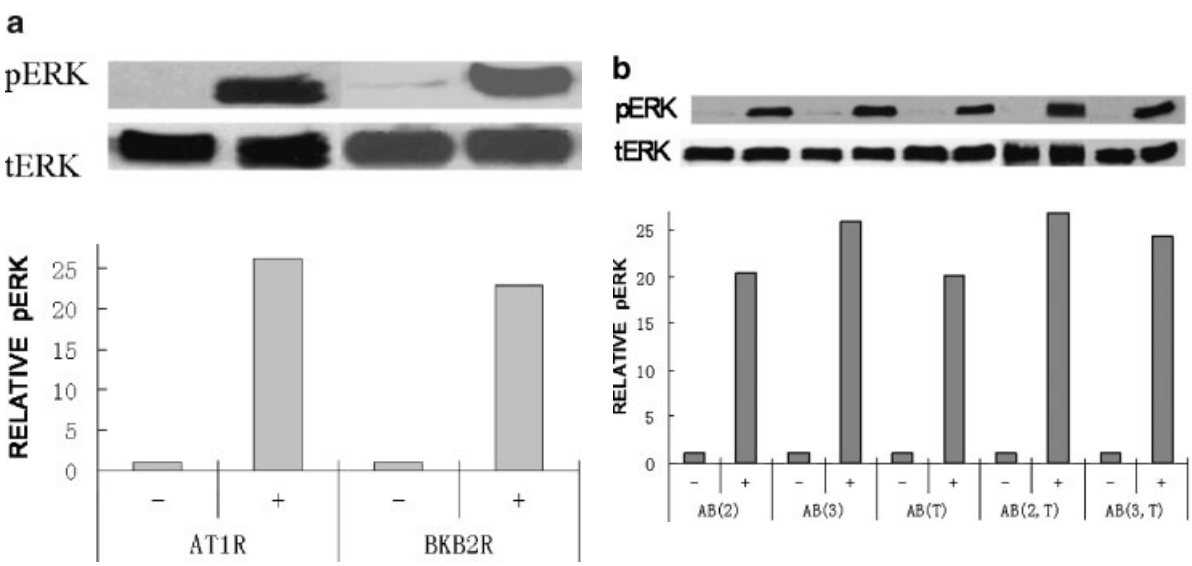

Fig. 4. ERK activation in (**a**) WT receptors and (**b**) hybrid receptors. Western blot analysis of cell lysates of HEK-293 cells expressing either AT1R or BKB2R. Cells were incubated with or without 100 nM AngII or BK for 5 min. The upper bands are pERK and the lower bands are total ERK. The results are representative of at least three experiments. The quantitation of the western is illustrated as a graph which shows fold change in spot intensity of the bands with basal level as 1.

pERK in response to AngII to approximately the same extent as cells expressing either WT AT1R or BKB2R (Fig. 4b).

JNK Activation

HEK-293 cells transfected with WT AT1R showed a modest increase in phosphorylated JNK (pJNK) above basal in response to 100 nM AngII. In contrast, BKB2R transfected cells, in response to 100 nM BK, displayed a considerably higher level of pJNK (Fig. 5a). The strength of the JNK activation signal in the single loop replacement mutant cells varied according to which loop was replaced. The AB(2) replacement showed a similar increase of pJNK as that seen in the WT AT1R. The AB(3) and AB(T) replacements demonstrated JNK activation comparable to that observed in WT BKB2R. The multiple loop substitutions behaved in similar fashion to WT BKB2R, activating JNK markedly more than WT AT1R (Fig. 5b).

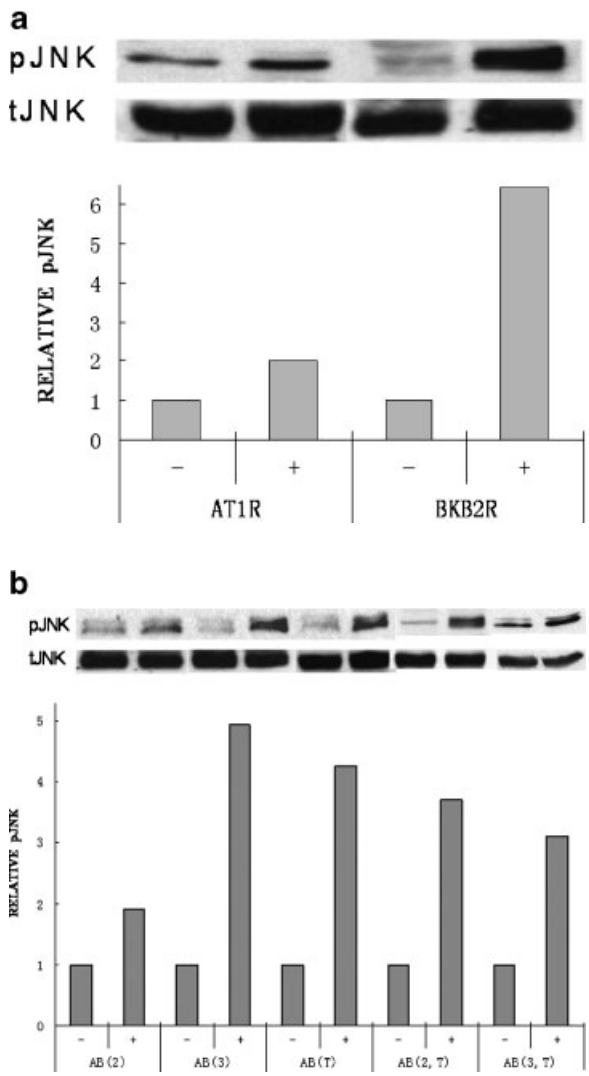

Fig. 5. JNK activation in (**a**) WT receptors and (**b**) hybrid receptors. The experiments were performed as described in Figure 4 except that specific antibodies against JNK and pJNK were used.

Akt Activation

AngII caused a sizable phosphorylation of Akt through the WT AT1 receptor. This is illustrated in Figure 6a. The response of Akt to BK is the opposite. Exposure of BKB2R transfected HEK-293 cells to BK resulted in an approximate 60% loss of phosphorylated Akt (pAkt) compared to the basal level. As shown in Figure 6b, unlike in the WT AT1 expressing cells, the single exchange AB(3) and double exchange AB(3,T) cells demonstrated a pronounced (50–60%) decrease in pAkt in response to AngII. A more modest loss of pAkt (approximately 30%) in response to AngII was observed in the AB(2), AB(T), and AB(2,T) hybrid expressing cells.

Molecular Modeling

The molecular models of the receptors were assembled and the simulations were carried out using a membrane-mimetic simulation cell. The model of the WT AT1R proved stable, with no major conformational changes observed during the molecular dynamics simulation. As shown in Figure 7a, focusing on the interactions of the IC2 and IC3 loops, the intracellular ends of transmembrane region 3 (TM3) and transmembrane region 4 (TM4) are in close proximity to each other and are stabilized by a charge–charge interaction between R137 and D125. Residues 136–139 (SRLR) of IC2 form a perfect helical turn. In addition, a *coulombic* interaction between the end of the third (D236) and second (K135) intracellular loops further defines the conformation of IC2. Finally, a specific kink around phenylalanine at position 133 (F-133) places the histidine (H132) ring at the tip of the IC2 loop, directly facing toward the intracellular phase, fully solvated with water in the simulation cell. In contrast, using the G-protein coupling deficient mutant, AB(2), the results from the modeling (Fig. 7b), illustrate a clear disruption of the *coulombic* K125-R137 interaction within the IC2 and also a loss

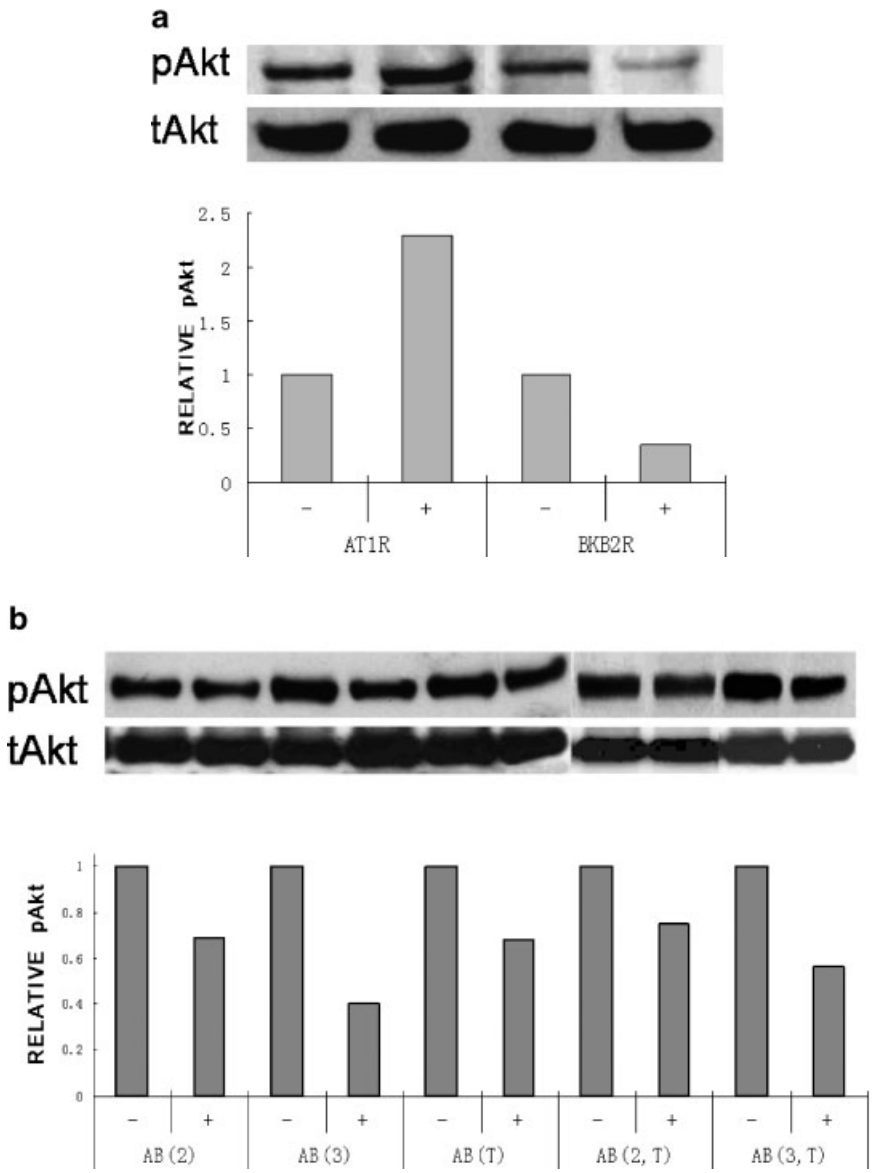

Fig. 6. Akt activation in (**a**) WT receptors and (**b**) hybrid receptors. The experiments were performed as described in the legend for Figure 4 except that specific antibodies against Akt and pAkt were used.

of interaction between K135 (IC2) and D236 (IC3).

Retention of K135 and R137 in the AB(2) Hybrid to Retain WT AT1 Charge Interactions

In an attempt to rescue the loss of Gi/Gq coupling by the AB(2) hybrid, its SMG motif at 135–137 obtained from the WT BKB2R was exchanged for the corresponding KSR from the WT AT1R [mutant named AB(KSR)]. Gαi/Gαq related signaling of the AB(KSR) is compared to WT AT1R and the AB(2) receptor signaling in Figure 8. AngII stimulated PI turnover by the AB(KSR) expressing cells was essentially at the same level as that seen in WT AT1R expressing cells. PI turnover by AB(2) was negligible, approximately 7% of WT AT1. ARA release showed a very similar pattern. These results illustrate the importance of charge interactions in AT1R coupling to G-protein.

DISCUSSION

Many studies, particularly those involving angiotensin converting enzyme (ACE) inhibitors, have now demonstrated that BK, through its BKB2 receptor, ameliorates a number of the

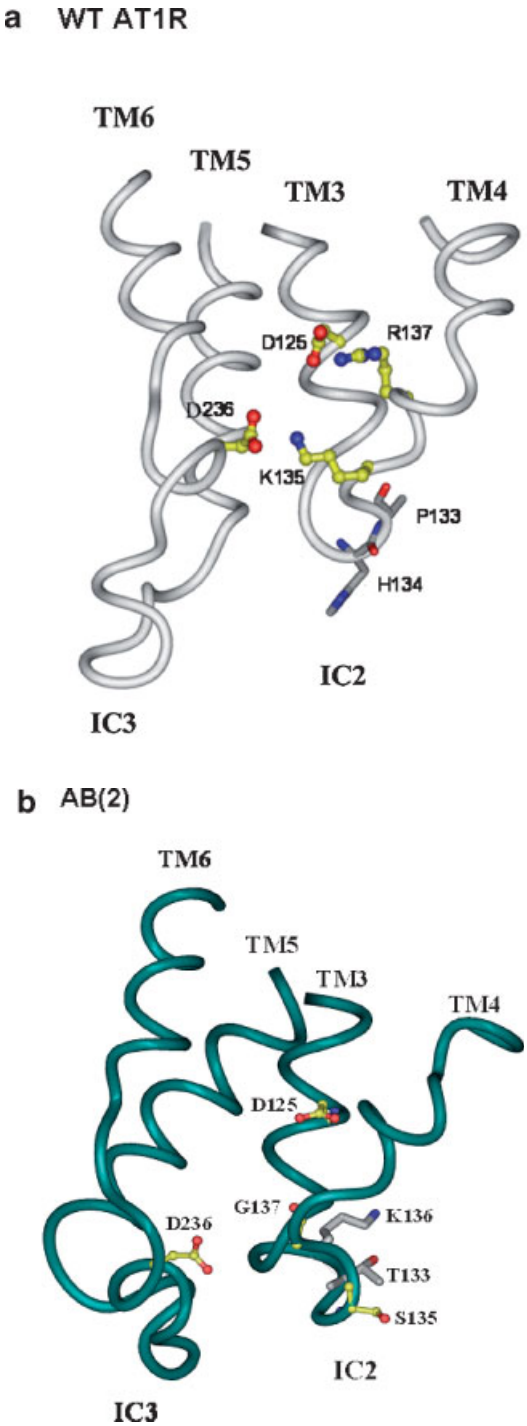

Fig. 7. Molecular model of IC2 and IC3 from (**a**) WT AT1R and (**b**) from the hybrid AB(2). The models depict a number of important interactions between the second and third intracellular loops of the AT1R receptor. TM represents a transmembrane domain, and IC2, IC3 represent the second and third intracellular loops, respectively. Individual amino acids discussed in the text are highlighted.

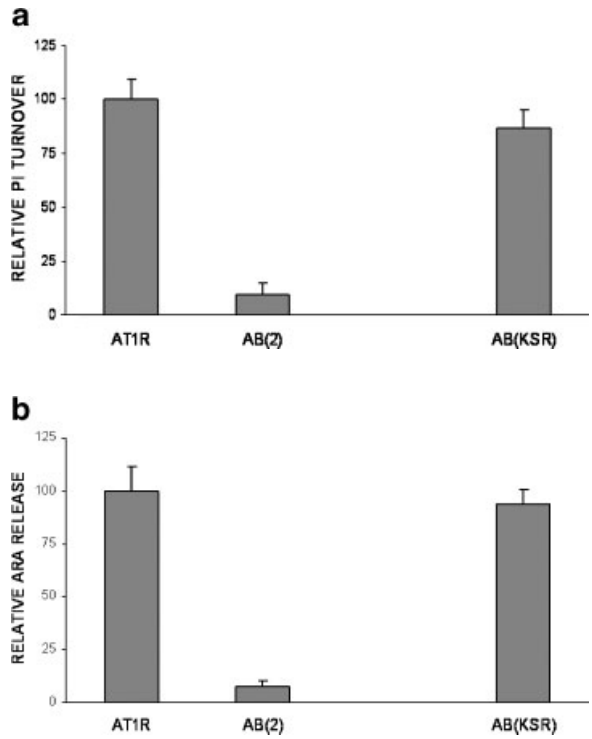

Fig. 8. **a**: Inositol phosphate production and (**b**) ARA release in HEK-293 cells expressing the AB(KSR) hybrid receptor. The PI turnover and ARA release experiments were performed as described in Figure 2.

deleterious effects of the AT1R [Linz et al., 1995; Landmesser and Drexler, 2006]. The aim of the present study was to generate AT1/BKB2 hybrid receptors which respond to AngII but signal as BK. Extensive sequences of the AT1R, including the entire IC2, IC3 and the C-terminus were replaced with those from the BKB2R, individually and in combination. The exchanges involving the IC2 AB(2), and the IC2 and C-terminus, AB(2,T) mutants bound AngII as WT AT1R in terms of both K_d and B_{max} (Table II). However, these mutants exhibited marginal PI turnover and ARA release. Both parameters have been associated with G-protein coupling as reviewed by Prado et al. [2002] and Touyz and Berry [2002]. Molecular modeling suggested that the Lys^{135}-Ser^{136}-Arg^{137} (KSR) sequence in the IC2 of the AT1R is an important conformational motif. As illustrated in Figure 7a, a number of strong *coulombic* interactions involved the K135 and R137 of this region. With the introduction of the IC2 from BKB2R both of these residues were replaced with residues lacking a charge (K135 with S; R137 with G). Thus, we hypothesized

that interaction of the receptor with G-protein would be altered. Verification was obtained by incorporation of the KSR motif from the WT AT1 IC2 into the AB(2) mutant. The resulting mutant AB(KSR), contained the IC2 of BKB2R with the exception of SMG which was replaced by KSR. This mutant bound AngII as WT AT1R and regained G-protein coupling capacity. Previous reports by others showed that mutations in the highly conserved $D^{125}R^{126}Y^{127}$ sequence within the proximal end of the IC2 of the AT1R results in the loss of Gαq coupling [Seta et al., 2002; Gaborik et al., 2003]. However, in the present case, the IC2 of both the BKB2 and AT1 receptors contains the DRY motif. Therefore it is the KSR sequence within the IC2 of the AT1R which is the motif responsible for the AT1 specific coupling mechanism. All other single replacements, not involving the IC2, as well as the double replacement AB(3,T) displayed PI turnover and ARA release comparable to WT BKB2R and AT1. Thus the IC2 of the AT1R clearly plays an important role in Gαi/Gαq coupling. Molecular modeling of other mutants agreed (results not shown), illustrating that introduction of the IC3 or C-terminus from the BKB2R did not alter the conformation of the endogenous IC2 loop. The helical turn for residues 136–139 remained, and the R137 and D125 salt bridge remained unchanged. Because of this lack of conformational change, the AB(3), AB(T), and AB(3,T) mutants remain completely functional with respect to G- protein interactions.

The pattern of Ca^{2+} mobilization by the various hybrid receptors is another indication that the IC2 of the AT1 plays an important role in G- protein related signaling. Replacement of the IC2 within the AT1R resulted in a highly muted Ca^{2+} spike. The AB(2,T) hybrid also showed very poor Ca^{2+} mobilization. Otherwise all other single and double replacement mutants tested emulated Ca^{2+} mobilization by BKB2R as opposed to AT1R, which showed a much higher Ca^{2+} translocation than the WT BKB2R. Ca^{2+} mobilization has been associated with the generation of IP_3 via the activation of PLCβ [Capponi, 1996; Griendling et al., 1997; Dinh et al., 2001]. Thus the Ca^{2+} mobilization results further point to a lack of G-protein coupling in the presence of the AT1 mutants containing the IC2 from the BKB2R. However, since Ca^{2+} entry into the cytosol in the IC2 single exchange mutant was reduced but not abolished, clearly the generation of IP_3 is not the only mechanism for AngII effected Ca^{2+} mobilization. Other mechanisms are taking place which do not require Gαq coupling.

While the binding parameters of the hybrids, AB(2,T) and AB(3,T), are much like those of the WT AT1R, the hybrids containing both BKB2 IC2 and IC3 show muted expression, particularly the AB(2,3) receptor. Interestingly, the expression of the AB(2,3,T) receptor improved markedly with a B_{max} at approximately 25% of WT. The reason for the reduction and partial recovery of the B_{max} of these receptors is not clear at this time. Further modeling and point mutations within these regions are necessary to obtain a better understanding.

Previously we have found that both the AT1 and BKB2 receptors contain certain sequences within their cytosolic faces which are irreplaceable with respect to binding of the ligand or interaction with Gαi and Gαq. For example, in BKB2R the IC1 has to contain N63 to continue to bind [Yu et al., 2005]. In the proximal tail BKB2R has to contain KSRE to continue to signal with Gαi and Gαq [Yu et al., 2004]. These results point to the importance of hydrophobic moieties, *coloumbic* forces and the action of charged residues in critical segments of the inner face of both receptors. Apparently charge–charge interactions, both attractive and repulsive, are necessary for the functional receptor conformation to be maintained [Yu et al., 2002, 2004, 2005]. There are also requirements for hydrophobic residues in critical locations in the inner face of the receptor.

Little has been done so far with either the AT1 or BKB2 receptors to identify the motifs which are involved in kinase signaling. One mutagenesis study on the AT1R showed that deletion of A and L from the AT1 221, 222, positions, the proximal end of the IC3, reduced ERK activation by more than 85% while not affecting the activation of JNK [Haendeler et al., 2000]. It is interesting to note that the corresponding motif in the BKB2R is the almost identical, AL in AT1R and VL in BKB2R. And in both receptors this hydrophobic sequence is followed by a positively charged residue, K and R, respectively. Participation of this motif would explain the continued ERK phosphorylation by all the AT1 hybrids used in this study since the motif is retained in each hybrid. Also several groups have shown that when the IC2 was mutated at various positions, the AT1 receptor

lost Gαi/Gαq coupled signaling but still activated ERK [Seta et al., 2002; Gaborik et al., 2003]. These reports agree with the findings here that the activation of ERK takes place in the absence or at most at a minimal Gi/Gq activation. In fact, we showed previously that in the Rat-1 cells, the AT1R activates JNK and p38 MAPK in addition to ERK in the presence of only a marginal Gαi and Gαq coupling [Liu et al., 2006]. A similar result was obtained with the human IMR-90 lung fibroblasts [Huang et al., 2006]. The studies here confirm these results and further illustrate that these actions occur in totally unrelated cell types, HEK-293, Rat-1, and IMR-90. The other interesting aspect of these results is that the activation of each MAPK, ERK, and JNK, appears independent of the other. Our results further show that whereas both WT AT1 and BKB2 receptors activate JNK equally in Rat-1 cells [Liu et al., 2006], in HEK-293 cells the BKB2R is clearly a more potent activator of JNK than the AT1R.

Akt/PKB signaling plays an important role in cardiovascular diseases [Oudit et al., 2004]. The activity of Akt/PKB is regulated by PI3K/PTEN via the modulation of PIP_3 levels. In general, AngII increases the phosphorylation of Akt [Takahashi et al., 1999; Haendeler et al., 2000; Dugourd et al., 2003], whereas the effect of BK is more variable and cell type specific. We are finding that in HEK-293 cells the WT AT1 and BKB2 receptors effect Akt in an antagonistic manner. Whereas AngII, through the AT1R, activates Akt, BK through the BKB2R, inactivates it markedly to around 40% of basal. Igarashi et al. [2001] found similar effect of BK in bovine aortic endothelial cells. Results with our hybrid receptors show that the exchange of any of the three regions (IC2, IC3, or C-terminus), whether singly or in combination, resulted in the reduction of pAkt although to different extents. As with the MAPKs, the single AB(3) exchange demonstrated the greatest reduction of pAkt suggesting that the key to this action originates in the IC3 of the BKB2R. Further experiments will be needed to determine whether the mechanism of this inactivation involves the recruitment of a phosphatase such as PTEN by the BKB2R.

Construction of functional hybrids among the GPCRs is an area with important implications with regard to the understanding of the motifs which determine receptor signaling and the generation of receptors with selected signaling potential. Construction of hybrid receptors expressing selective signaling may prove of particular importance in the control diseases where the receptor is not under homeostatic control. Clearly, the negative action of the AngII AT1 receptor in cardiovascular diseases such as hypertension is a good example of such a deleterious receptor. The functional fine-tuned AT1R/BKB2R hybrids may lead to the treatment of these diseases at molecular level.

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