Lack of Evidence for AT$_1$R/B2R Heterodimerization in COS-7, HEK293, and NIH3T3 Cells

**HOW COMMON IS THE AT$_1$/R/B2R HETERODIMER?**

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It has been suggested previously (AbdAlla, S., Lother, H., and Quitterer, U. (2000) *Nature* 407, 94–98) that the angiotensin II type 1 receptor (AT$_1$/R) and the bradykinin B2 receptor (B2R) form constitutive heterodimers. Furthermore they demonstrate that AT$_1$/R signaling significantly increases in the presence of the B2R. These findings suggest that heterodimerization and potentiation of AT$_1$/R signaling is a universal phenomenon that occurs as a natural consequence of simultaneous expression of the two receptors. Hence this potential interaction is of great pharmacological and biological interest that adds an additional layer of complexity to the understanding of the cross-talk between the renin-angiotensin and kallikrein-kinin systems. Given the remarkable significance of this finding, scientists from four independent research groups have set out to reproduce and further examine the potential AT$_1$/R/B2R interaction. We have investigated functional potentiation by the B2R of AT$_1$/R signaling in three different cell lines using multiple assays including phosphoinositide hydrolysis, ERK activation, β-arrestin recruitment, and receptor selection and amplification technology, and we have examined dimerization using bioluminescence resonance energy transfer and regulated secretion/aggregation technology. However, although both the AT$_1$/R, Rs and B2Rs were functional in our systems and the systems were fine tuned to detect small changes in receptor function, we failed to detect any functional modulation by or physical interaction between the two receptor proteins. In contrast to the previous observations, our data collectively suggest that AT$_1$/R/B2R heterodimerization does not occur as a natural consequence of their simultaneous expression in the same cell nor does the B2R influence the AT$_1$/R signaling.

The angiotensin II type 1 (AT$_1$/R) receptor belongs to the superfamily of the seven-transmembrane (7TM) or G protein-coupled receptors. The AT$_1$/R is a key regulator of blood pressure and body fluid homeostasis, and its importance in renal and cardiovascular pathophysiology is underscored by the widespread use of receptor blockers and inhibitors of the angiotensin-converting enzyme in clinical practice (1, 2). There is mounting evidence that the AT$_1$/R may form both homo- and heterodimers and that dimerization could be important for the receptor function (3–14). For example, it has been shown that the AT$_1$/R decreases G$_s$ coupling when the receptor interacts with either the MAS or the angiotensin II type 2 (AT$_2$/R) receptors (6, 10–12). It has also been shown that the AT$_1$/R can form complexes with the β$_2$-adrenergic receptors and that it is possible to achieve dual receptor inhibition of AT$_1$/R and β$_2$-adrenergic receptor signaling using only a single receptor antagonist (9). The AT$_1$/R also co-immunoprecipitates with the Dopamine D1 and D3 receptor in tissue specimen obtained from Wistar-Kyoto and spontaneously hypertensive rats (13, 15). Finally AbdAlla et al. (5, 7, 16) have shown in a number of studies that the AT$_1$/R forms heterodimers with the B2R. From these studies, they derive three important pharmacological conclusions. First, the AT$_1$/R and the B2R form heterodimers in all the cellu-

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5 The abbreviations used are: AT$_1$/R, angiotensin II type 1; AT$_2$/R, angiotensin II type 2; Ang II, angiotensin II; AT$_1$/R, angiotensin II type 1 receptor; AT$_2$/R, angiotensin II type 2 receptor; B2R, bradykinin B2 receptor; BRET, bioluminescence resonance energy transfer; GFP, green fluorescent protein; RSAT, receptor selection and amplification technology; 7TM, seven-transmembrane; PI, phosphoinositide; DMEM, Dulbecco’s modified Eagle’s medium; ERK, extracellular signal-regulated kinase; P-ERK, phosphorylated ERK; Rluc, Renilla luciferase; GABA$_{w}$, γ-aminobutyric acid, type B; wt, wild type; ER, endoplasmic reticulum; IP, inositol phosphate.
lar systems examined including HEK293 cells (16), primary cells such as vascular smooth muscle cells (16), neurons (5), and platelets (7). These data indicate that the system is “universal” and that AT1, R/B2R heterodimerization occurs as a natural consequence of simultaneous AT1, R and B2R expression within the same cell (5, 7, 16). Second, when compared with the individually expressed receptors, the AT1, R/B2R heterodimer signals with higher potency and efficacy upon Ang II stimulation, whereas bradykinin signaling remains largely unaffected (16). Third, the B2R must be competent to engage its G protein signaling pathway to produce the functional AT1, R potentiation (16). From a medical perspective, the AT1, R/B2R interaction would be of outstanding value because the authors show that it can be linked to experimental hypertension (5), vascular smooth muscle cell contraction (16), and preeclampsia (7). The disease relevance of the dimer implies that development of AT1, R/B2R heterodimer-specific antagonists could be beneficial for the treatment of cardiovascular diseases. The interaction is also of great biological interest because it could add an additional layer of complexity to the understanding of the cross-talk between the renin-angiotensin and kallikrein-kinin systems (17, 18). Both systems are intricately connected. (i) The AT1, R mediates vasoconstriction, whereas the B2R is a vasodepressor (17, 18). (ii) Angiotensin-converting enzyme is responsible for both production of circulating Ang II and at the same time degradation of bradykinin (19). (iii) AT1, R activation leads to up-regulation of the B2R (17, 18). It has also been shown that many biological effects of AT1, R blockers such as losartan are in fact mediated by the release of bradykinin and, accordingly, can be blocked by the B2R inhibitor HOE130 (20–22). Finally heterodimerization between the AT2 receptor and B2R has also been proposed to enhance NO release (23), and it has been implicated in left ventricular remodeling after myocardial infarction in mice (24).

The concept of 7TM receptor heterodimerization is of emerging importance, and it will be crucial to address general questions with respect to experimental pitfalls, specificity, generality, and biological significance of a given heterodimeric interaction. Scientists from four independent groups set out to study the putative AT1, R/B2R heterodimerization in detail. Surprisingly although both the AT1, Rs and the B2Rs were functional in our studies, we were not able to establish any functional potentiation or physical interaction between the two receptors. Despite careful experimental fine tuning, our data do not support the concept of an AT1, R/B2R heterodimer occurring as a natural consequence of simultaneous AT1, R and B2R expression in the same cell.

**MATERIALS AND METHODS**

**Recombinant DNA Plasmids**

The rAT1aR and the hAT1, R have been described previously (3, 25). Using PCR-based methods, the rB2R was cloned from rat heart cDNA, and the hB2R was cloned from human smooth muscle cDNA. Both receptors were fully sequence-verified and subcloned into the pCDNA3.1 (Invitrogen) and pSI (Promega) expression vectors, respectively. The wild type rat B2R sequence is identical to that of GenBank™ accession number M59967 (26), and the human B2R is identical to that described in Hess et al. (27). The enhanced GFP-tagged bovine β-arrestin 2 and AT1, R-Rluc were reported previously (3, 25).

**Phosphoinositide (PI) Hydrolysis Assay**

To perform this assay, we seeded 2.5 × 10⁶ COS-7 cells in a p10 dish and transfected the cells with the amounts of receptor plasmid described under “Results” using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s protocol. After 1 day, the cells were seeded into 48-well plates coated with 0.25% poly-1-lysine (100,000 cells/well) and incubated in isoflurane-free Dulbecco’s modified Eagle’s medium (DMEM) supplemented with myo-[2-3H]inositol (1 μCi/ml) (Amersham Biosciences). Twenty hours after transfection, the cells were assayed as described previously (3, 28).

**ERK Phosphorylation Assay**

This assay was performed as described previously (3, 29). Briefly 2.5 million COS-7 cells were seeded into a p10 dish and grown in 10% fetal calf serum, DMEM overnight. After 24 h, the cells were transfected using Lipofectamine 2000 according to the manufacturer’s protocol. The day after transfection they were seeded onto 6-well plates. Next 45 h after transfection, the cells were serum-starved for 3 h, incubated with agonist for 12 min at 37 °C, and then lysed. SDS-PAGE and immunoblotting were performed as described previously (30, 31), and the bands were visualized using the enhanced chemiluminescence system (BD Biosciences). To assess the relative efficacies of agonists, a densitometric gel quantification of the phosphorylated ERK (P-ERK) and total ERK band intensities was performed, and the ratios of P-ERK to total ERK were normalized with reference to the receptor maximum Ang II response (100%).

**β-Arrestin Recruitment Assay**

This assay was performed as described previously (3, 32) except for minor changes. Briefly 2.5 million COS-7 cells were seeded into a p10 dish and grown in 10% fetal calf serum, DMEM overnight. After 24 h, the cells were transfected using Lipofectamine 2000 according to the manufacturer’s protocol. We used 20 μg of GFP-β-arrestin 2 and 1 μg of rAT1, R-Rluc or B2R-Luc DNA with either 3 μg of empty vector, B2R, or AT1, R as described in the text. The cells were isolated and submitted to either basal or agonist treatment for 20 min at room temperature before measuring the BRET² ratios as described in the text and previously (3).

**Receptor Selection and Amplification Technology (RSAT)**

RSAT was performed as described previously (25, 33, 34). Briefly NIH3T3 cells at 70–80% confluence were transfected with cDNA as follows (0–25 ng of receptor as described in the figures and 20 ng of β-galactosidase reporter/well of a 96-well plate) using the PolyFect Reagent (Qiagen, Valencia, CA) as described in the manufacturer’s protocol. One day after transfection, ligands were added in DMEM supplemented with 100 units/ml penicillin, 100 g/ml streptomycin, and 2% Cyto-SF3. After 6 days, the medium was removed by aspiration, cells were lysed, O-nitrophenyl-β-D-galactopyranoside was added, and the resulting absorbance was measured spectrophotometri-
cally. All concentration-response curves were performed in duplicate.

**Dimerization Assays**

**BRET2 Assay**—Except for minor differences, the BRET2 assay was performed as described previously (3, 28). Briefly 0.5 million COS-7 cells/well were seeded into a 6-well plate and grown in 10% fetal calf serum, DMEM overnight. After 24 h the cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol (varying amounts of DNA were used to assure equal expression levels between the different combinations). After 2 days, the cells were washed twice with phosphate-buffered saline to remove the indicator dye before detachment in phosphate-buffered saline. The cells were then split into two portions. The first portion was used to examine the GFP2 levels by fluorescent measurements as described previously (3, 28), and next the Rluc expression was determined by measuring the Coelenterazine h-induced luminescence as described previously (3, 28). The second portion of the cells was submitted to DeepBlueC excitation, and the luminescence at the dual bands (515/30 and 410/80 nm) was measured on a Fusion reader (PerkinElmer Life Sciences). The BRET2 ratio was determined according to the principle described in (32). The BRET2 ratio equals \( \frac{(\text{emission 515}/30)}{(\text{emission 410}/80) \times \text{Cf}} \) where Cf denotes the cross-Rluc luminescence cross-talk ratio into the 515/30 filter defined as \( \frac{(\text{emission 515}/30)}{(\text{emission 410}/80)} \) when Rluc expressed alone is excited.

**Regulated Secretion/Aggregation Technology**—This assay was developed by Rivera et al. (35) and is available from ARIAD Pharmaceuticals. The assay was performed as described previously (3). Briefly 2.5 \( \times 10^6 \) COS-7 cells were seeded in a p10 dish and transfected with 10 \( \mu \)g of Mt-AT1R (either wild type or mutant) cDNA alone or together with 2 \( \mu \)g of wild type B2R DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. After 24 h, the cells were split into 48-well plates coated with 0.25% poly-L-lysine (100,000 cells/well) and stimulated with or without AP21998 at 2 \( \mu M \) for 2 h prior to performing the PI hydrolysis assays as described above.

**RESULTS**

The B2R Does Not Enhance AT1R-mediated G Protein Activation in Various Cellular Models—AbdAlla et al. (5, 7, 16) have shown that simultaneous expression of functional B2Rs dramatically increases the ability of AT1Rs to activate Goq signaling. These studies were performed very carefully in a variety of systems including HEK293 cells (16), primary cells such as vascular smooth muscle cells (16), neurons (5), and platelets (7). Given the natural interest in this interaction and its significant (patho)physiological relevance, we set out to examine this interaction in more detail. To be able to detect enhanced signaling, it is pivotal that the assay is within a dynamic range that allows for detection of further increases in signaling. We therefore transfected increasing amounts of AT1R cDNA into COS-7 cells and measured the Ang II-induced PI hydrolysis (Fig. 1A). We observed that an increasing amount of DNA would result in increased signaling up to the level of 10 \( \mu g \) of DNA/10-cm culture dish. Next we analyzed the effect of B2R expression on AT1R signaling. To do so, we compared the Ang II-induced signaling in cells transfected with either 2 \( \mu g \) of AT1R alone or with 0.5, 2, or 5 \( \mu g \) of B2R as indicated in Fig. 1B. Surprisingly we did not observe any changes in the AT1R efficacy or potency even though our system is indeed able to pick up changes as shown in Fig. 1A. AT1R expression did not
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FIGURE 2. The B2R does not enhance AT<sub>1</sub>-, R-mediated ERK activation or β-arrestin recruitment in our cellular models. Western blot analysis of AT<sub>1</sub>-mediated ERK phosphorylation (A and B) and BRET<sup>2</sup> analysis of AT<sub>1</sub>-mediated β-arrestin recruitment (C, D, and E) are shown. A, to identify the dynamic range of receptor levels increasing amounts of AT<sub>1</sub>-R-wt cDNA were transfected into COS-7 cells, and cells were stimulated as described under “Materials and Methods.” P-ERK and total ERK (T-ERK) are shown for unstimulated cells or cells stimulated with 100 nM Ang II. Gel images are representatives from four different experiments. B, to test the effect of B2R expression on AT<sub>1</sub>-mediated ERK activation, AT<sub>1</sub>-R (2 μg) was co-transfected with either empty vector (2 μg) or B2R (2 μg) receptor DNA in COS-7 cells, and the cells were treated as described under “Materials and Methods.” Phosphorylated and total ERK proteins are shown for unstimulated cells or cells stimulated with 100 nM Ang II by representative gel images from four experiments. To assess the relative efficacies of activation, densitometric gel quantification of the P-ERK and total ERK band intensities was performed, and the density of the P-ERK bands was first normalized for expression levels using the intensity of the total ERK band as a reference. The P-ERK response was then normalized with reference to the wild type receptor maximum response values. Data are depicted as -fold response and represent the average (±S.D.) from four independent experiments. * indicates significant difference (p < 0.05) as determined by Student’s t test, and NS indicates no significance. C, to test whether the B2R expression affects AT<sub>1</sub>-mediated β-arrestin 2 recruitment, we co-expressed 1 μg of AT<sub>1</sub>-R-Rluc with 20 μg of GFP<sup>2</sup>-tagged β-arrestin 2 either alone or together with increasing amounts of untagged B2Rs. The cells were treated with either vehicle or increasing amounts of Ang II. The BRET<sup>2</sup> responses represent the average (±95% confidence interval) from four independent experiments. D, to test the functionality of the B2R in the β-arrestin recruitment assay 1 μg of B2R-Rluc was co-transfected with 20 μg of GFP<sup>2</sup>-tagged β-arrestin 2 and stimulated with increasing concentrations of bradykinin. The BRET<sup>2</sup> responses represent the average (±95% confidence interval) from four independent experiments. E, to see whether the B2R wild type could induce a “functional rescue” of the β-arrestin 2 recruitment by the AT<sub>1</sub>-R-K199A-Rluc, we co-expressed AT<sub>1</sub>-R-K199A-Rluc and GFP<sup>2</sup>-tagged β-arrestin 2 either alone or together with untagged AT<sub>1</sub>-wt or B2R-wt and analyzed β-arrestin 2 recruitment as aforementioned. These expression combinations were treated with vehicle or with a saturating Ang II dose (10<sup>-5</sup> M) as described under “Materials and Methods.” Data are depicted as -fold response and represent the average (±S.D.) from three independent experiments performed in duplicate. * indicates significant difference (p < 0.05) as determined by Student’s t test, and NS indicates no significance.

change by co-transfection of the B2R and was consistently in the range of 20–22 fmol of receptor/100,000 cells as determined by radioligand binding as described previously (25). The bradykinin receptor expression and function increased as a result of increasing amounts of B2R cDNA transfection. The expression levels of the B2R per 100,000 cells were 7 ± 3 fmol (for 0.5 μg of B2R), 23 ± 4 fmol (for 2 μg of B2R), and 50 ± 6 fmol (for 5 μg of B2R). Functional activity of the B2R was verified in the PI hydrolysis assay (Fig. 1C). In addition, we have tried several combinations of cDNAs in COS-7 cells, and we also have performed analysis in HEK-293 cells, but we have not observed any gain of function of the AT<sub>1</sub>,R as a result of B2R co-transfection (data not shown).

The B2R Does Not Enhance AT<sub>1</sub>,R-mediated β-Arrestin 2 Recruitment or ERK Activation in Different Cellular Models—To test whether potentiation by the B2R of AT<sub>1</sub>,R signaling could be detected with other functional assays, Ang II-mediated activation of the ERK cascade and β-arrestin 2 recruitment has been investigated. To analyze the Ang II-induced ERK activation, we first confirmed that the system was dynamic as described for the inositol 1,4,5-trisphosphate assay (Fig. 2A) and then examined the effects on Ang II-mediated AT<sub>1</sub>,R stimulation as a consequence of B2R co-transfection, but we did not see any changes in this assay either (Fig. 2B). To analyze the β-arrestin 2 recruitment, COS-7 cells were transfected with AT<sub>1</sub>,R-Rluc and β-arrestin 2-GFP either alone or in combination with the B2R, and the Ang II-induced BRET<sup>2</sup> signal was measured. As depicted in Fig. 2C, we failed to detect any changes in the AT<sub>1</sub>,R-mediated β-arrestin 2 recruitment. Failure to detect enhancement by B2R of AT<sub>1</sub>,R-mediated β-arrestin 2 recruitment is not due to lack of activity of the B2R in this functional assay because the B2R-Rluc is capable of inducing a strong β-arrestin 2 translocation response (Fig. 2D).

We have shown previously that it is possible to “transactivate” β-arrestin 2 recruitment to an AT<sub>1</sub>,R mutant (K199A) that does not bind Ang II by co-expression of the AT<sub>1</sub>,R-K199A-Rluc, β-arrestin 2-GFP, and an untagged version of the wild type AT<sub>1</sub>,R (3). To analyze whether we could perform a similar transacti-
vation using the B2R, we co-transfected the AT1R-K199A-Rluc, β-arrestin 2-GFP, and the B2R and measured the BRET2 value as a consequence of bradykinin stimulation. However, we did not observe any significant BRET2 increase (Fig. 2E).

The B2R Does Not Enhance AT1R-mediated Cellular Proliferation in NIH3T3 Cells—We also tested whether the B2R potentiated the Ang II-induced AT1R-mediated cellular proliferation. To this end, we transiently expressed the hAT1R in NIH3T3 cells together with a β-galactosidase reporter gene and determined its response to Ang II in the cellular proliferation assay RSAT (25, 36, 37). As depicted in Fig. 3A, we observed an increased AT1R expression resulted in an increased response. We also confirmed that the B2R was functionally expressed as stimulation with bradykinin resulted in a robust B2R-mediated response (Fig. 3C). We then co-transfected the AT1R and the B2R using a fixed amount of 5 ng of AT1R cDNA/well together with either 1, 2.5, 5, or 10 ng of B2R cDNA/well and stimulated with Ang II. As depicted in Fig. 3B, no functional enhancement of AT1R signaling by B2R co-transfection was observed in this assay either. To test whether RSAT was able to detect dynamic changes as a result of heterodimerization, we tested the signaling capacity of other previously reported heterodimers. Indeed we do find that RSAT is able to detect changes as a result of “heterodimerization.” First we compared signaling of AT1R expressed alone or when they were co-expressed with the AT2R. Here we observe a dramatic fall of both Ang II potency and efficacy upon AT2R co-expression (Fig. 3D). This finding is in good agreement with previous reports showing that AT2R expression can down-regulate AT1R-mediated signaling (38). We also tested whether RSAT was able to detect increased signaling as a result of co-expression or heterodimerization. To do so we expressed the GABAB1 and GABAB2 receptors either alone or in combination. Here we observed robust signaling when the two receptor subunits where co-expressed but did not observe any signaling when the receptor subunits were expressed individually (Fig. 3E). This is in good agreement with previous reports demonstrating that both subunits of the heterodimer are needed for functional expression (39).

The B2R Does Not Interfere with AT1R Homodimerization and Does Not Travel with the AT1R from the Endoplasmic Reticulum (ER) to the Cell Surface—To further analyze the proposed interaction between the AT1R and B2R, we utilized two different experimental approaches that allow for detection of
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FIGURE 4. AT$_1$R/B2R does not seem to dimerize in COS-7 cells. A, AT$_1$R-Rluc was expressed either alone (bar 1) or together with AT$_1$R-GFP$^2$ (bars 2–4) in COS-7 cells. To test whether the B2R could inhibit the robust BRET$^2$ signal generated from AT$_1$R-Rluc/AT$_1$R-GFP$^2$, the BRET$^2$ signal was compared from cells expressing either AT$_1$R-Rluc/AT$_1$R-GFP$^2$ alone (bar 2) or AT$_1$R-Rluc/AT$_1$R-GFP$^2$ together with either untagged wild type B2R (bar 3) or AT$_1$R (bar 4). Equal levels of receptor expression were determined by measurements of luminescence and fluorescence intensities, and the BRET$^2$ ratios were calculated as described under "Materials and Methods." The BRET$^2$ values observed for each receptor combination were compared with those of the AT$_1$R-Rluc and AT$_1$R-GFP$^2$ co-expression using a one-tailed paired Student’s $t$ test (*, $p < 0.05$). The data represent the average values ($\pm$ S.D.) from at least three individual experiments performed in duplicate.

B, the Fm4-AT1R was expressed either alone (Fig. 4A) or together with either untagged wild type B2R (Fig. 4B) or AT$_1$R (Fig. 4C). Equal levels of receptor expression were determined by measurements of luminescence and fluorescence intensities, and the BRET$^2$ ratios were calculated as described under "Materials and Methods." The BRET$^2$ values observed for each receptor combination were compared with those of the Fm4-AT1R-Rluc (Fig. 4A) expressed in COS-7 cells was determined. Data from four individual experiments performed in triplicate represent the normalized average values ($\pm$ S.D.) with reference to the basal condition in the absence of AP21998. Data were compared using a Student’s $t$ test; * indicates significant difference ($p < 0.05$), and NS indicates no significance. B, the Fm4-AT1R was expressed alone, and the induction of IP hydrolysis in response to Ang II (bars 3 and 4) and bradykinin (bars 5 and 6) when compared with basal is indicated. C, the B2R-wt was co-expressed with the Fm4-AT1R in COS-7 cells, and the effect of a 2-h incubation with AP21998 (2 $\mu$M) on the Ang II- or bradykinin (10$^{-6}$ M)-induced IP accumulation of the Fm4-AT1R (or the Fm4-AT1R-EEN(A)) expressed in COS-7 cells was determined. Data from four individual experiments performed in triplicate represent the normalized average values ($\pm$ S.D.) with reference to the basal condition in the absence of AP21998. Data were compared using a Student’s $t$ test; * indicates significant difference ($p < 0.05$), and NS indicates no significance. D, the mutant Fm4-AT1R-EEN(A), which does not activate G proteins, was expressed, and it was confirmed that it does not respond to Ang II stimulation either with or without AP21998 incubation. E, we then co-expressed the Fm4-AT1R-Rluc (or the Fm4-AT1R-EEN(A)) expressed in COS-7 cells was determined. Data from four individual experiments performed in triplicate represent the normalized average values ($\pm$ S.D.) with reference to the basal condition in the absence of AP21998. Data were compared using a Student’s $t$ test (*, $p < 0.05$), and NS indicates no significance. Thus, our data confirm that AT$_1$Rs are capable of forming homodimers but again fail to prove AT$_1$R/B2R heterodimerization.

We have shown previously that AT$_1$R dimerization occurs as early as in the ER; this is in good agreement with the majority of studies analyzing 7TM receptor heterodimerization (3, 40, 41). To test for potential AT$_1$R/B2R heterodimerization during receptor biosynthesis in the ER, the regulated secretion/aggregation technology (RPD) developed by Rivera et al. (35) was used. In principle, the protein of interest is N-terminally fused to a protein (dubbed Fm) that accumulates as aggregates in the ER and Golgi. However, incubation with a synthetic small molecule drug (AP21998) alleviates the aggregation and allows the fusion proteins to escape the ER and Golgi in an AP21998-gated fashion and travel to the cell surface (35). Using this assay, we have convincingly shown that we can use AP21998 to...
regulate the expression of an N-terminally fused, signaling-deficient, AT,
T R mutated in the DRY motif (D125R126Y127-
(M134) → E125R126A127(A134)) with four consecutive Fm,
fusion domains (dubbed Fm4-AT1R-EEA(A)) when this mutant was
expressed in COS-7 cells. To suggest that the AT,
R s interact as early as in the ER, AP21998 could be used to regulate the surface
expression of the AT1R-wt when it was co-expressed with the
Fm4-AT1R-EEA(A) (3).

An analogous approach was applied to study the potential
AT1R/B2R dimerization. Initially regulation of signaling of an
Fm-tagged AT1R-wt (dubbed Fm4-AT1R, R) by AP21998 addition
was established, and next it was confirmed that this receptor did not signal in response to bradykinin stimulation (Fig. 4B) by
measuring PI hydrolysis. COS-7 cells transiently transfected to
express the Fm4-AT1R and challenged with Ang II displayed a significant increase in IP production from 1.41 ± 0.09- 2.81 ± 0.5-fold when preincubated with AP21998 (Fig. 4B; p < 0.05).
Conversely treatment of COS-7 cells with AP21998 or bradykinin did not affect basal IP levels (Fig. 4B).

When COS-7 cells were transfected to co-express the Fm4-
AT1R together with the B2R-wt (Fig. 4C) a similar regulation of
Ang II-induced signaling as a consequence of AP21998 incubation was observed. On the other hand, preincubation with
AP21998 did not change the bradykinin-induced signaling (Fig. 4C).
In the presence or absence of AP21998, bradykinin
induced inositol hydrolysis 1.71 ± 0.32- 1.74 ± 0.31-fold, respectively, when compared with basal (Fig. 4C). These data
confirm (i) functional expression of the B2R in this cellular system and (ii) inability of the AT1R to retain the B2R in the ER,
indicating that the two receptors do not traffic together. We
also co-expressed the wild type AT1R and B2R with the previ-
ously reported Fm4-AT1R-EEA(A) mutant that does not activate G protein signaling. As depicted in Fig. 4D this receptor is not capable of inducing PI hydrolysis in response to Ang II stimulation. When we co-expressed the B2R or the AT1R-wt we observed that we were able to regulate AT1R signaling as depicted in Fig. 4F and described previously (3). When the
AT1R-wt was co-expressed with the Fm4-AT1R-EEA(A) mutant the Ang II-mediated PI hydrolysis response was 1.39 ± 0.11-
fold without AP21998 preincubation and 1.75 ± 0.11-fold in the presence of AP21998 when compared with basal (p < 0.05).
However, although we could regulate signaling of the AT1R wild type, we were still not able to detect any changes in brady-
kinin signaling. In the presence or absence of AP21998, the
bradykinin-induced PI hydrolysis was 1.82 ± 0.38- and 1.80 ± 0.38-fold, respectively, when compared with basal (Fig. 4E).
Additionally we performed experiments addressing whether
different amounts of B2R expression might lead to regulation;
however, we did not detect any signs of B2R regulation in any of the experiments performed herein (data not shown).

DISCUSSION

AbdAlla et al. (5, 7, 16) have shown that simultaneous
expression of functional B2Rs dramatically increases the ability of AT1R s to activate Goq signaling in three studies. This effect has been shown in a variety of cellular systems including HEK293 cells (16), primary cells such as vascular smooth muscle
cells (16), neurons (5), and platelets (7). When summarized,
their studies indicate that the system is universal and that
AT1R/B2R heterodimerization and the functional potentiation
occur as a natural consequence of simultaneous receptor
expression within the same cell (5, 7, 16). In this study, we have
tried to reproduce their findings; however, in contrast to their
reports, we did not observe indications of either physical inter-
action between the two receptors or functional consequences
of B2R expression on the AT1R-mediated responses.

The B2R Does Not Enhance AT1R-mediated Signaling in Any
Of Our Systems—First we set out to look for functional potentiation. To do so, we have analyzed a number of readouts such
as PI hydrolysis (Fig. 1), β-arrestin recruitment (Fig. 2), ERK
activation (Fig. 2), and cell proliferation (Fig. 3) and expressed
AT1R and B2R in multiple combinations and in three different
cellular systems (COS-7, HEK293, and NIH3T3 cells), but we
have not observed any gain of function on Ang II-induced AT1R
signaling as a result of B2R expression. This is very surprising
because our systems should be able to detect gain-of-function
signaling events at least to the extent reported by AbdAlla et al.
(5, 7, 16). Indeed we have measured the effects of the presence
of the B2R in the PI hydrolysis, ERK activation, and cellular
proliferation assays within the dynamic range of our system,
meaning that AT1R function correlates well with AT1R expression (Figs. 1–3). Furthermore we were able to detect functional
consequences as a result of co-expression of previously
reported heterodimers such as the AT1R/AT2R and GABAA111
and GABA22 receptors. In addition, we used several different
expression levels of the B2R receptor, and the clones used in our
study were functional in all systems tested (Figs. 1C, 2D, 3C, and
4, C and E). The latter observation is particularly important
because AbdAlla et al. (16) report that functional G protein
coupling was both necessary and sufficient for the observed
B2R-mediated AT1R signaling potentiation.

Lack of Evidence for AT1R/B2R Dimerization in this Study—
AbdAlla et al. (5, 7, 16) report that the AT1R and B2R form
deredimers and that this dimerization occurs in all the sys-
tems they have examined. This conclusion is based on observa-
tions using two classical interaction studies. First, they have
observed that each of the two receptors can be co-enriched by
immunoaffinity purification of the other receptor labeled by
radioactive ligands (5, 7, 16). Second, they have observed that
the two receptors co-internalize in response to ligand stimula-
tion (16). We have also tried to detect heterodimerization using
three different methods that have been used successfully to
study AT1R homodimerization and heterodimerization of wild
type and mutant AT1R (3), but these methods did not yield any
indication for AT1R/B2R heterodimerization. First, utilizing
the BRET2 assay, we were able to see that RLuc- and GFP-
tagged AT1Rs form homodimers and that this dimer formation is
a consequence of specific protein/protein interaction because
overexpression of wild type AT1R was sufficient to reduce the
BRET2 signal significantly (Fig. 4A). However, we were not able
to reduce the AT1R dimer formation by B2R overexpression,
clearly indicating that AT1R and B2R do not form dimers in this
system (Fig. 4A). Second, when testing whether the B2R could
be retained in the ER by artificially retaining the AT1Rs in the
ER using the RPD technology, none of the experimental strategies
allowed us to detect any intracellular retention of the B2R
The AT₁R/B2R Heterodimer Is Not a Universal Phenomenon

as a result of AT₁R retention (Fig. 4, B and C, and data not shown). This also strongly suggests that the two receptors do not dimerize because we were able to retain the AT₁R using this system (Fig. 4F). Furthermore the B2R was functionally expressed during the experiment (Fig. 4, C and E, and Ref. 3). This system has also been used successfully by Kaykas et al. (42) to show oligomerization of mutant and wild type Frizzled 4 receptors. Third, we used BRET² analysis to record association between AT₁R-Rluc and GFP²-β-arrestin 2 by stimulation of untagged wild type B2Rs, but this was also unsuccessful (Fig. 2, C–E). This experiment again suggests that these receptors do not dimerize because we can successfully rescue β-arrestin recruitment of a binding-deficient AT₁R mutant (AT₁R-K199A-Rluc) when co-expressed together with an untagged AT₁R wild type presumably because they form a functional homodimer (Fig. 2E and Ref. 3). This kind of “trans-β-arrestin recruitment” has also been observed for other heterodimers underscoring that the technique is suitable for dimer detection (43).

How Do We Reconcile the Data?—When looking at the data presented in the elaborate work by AbdAlla et al. (5, 7, 16) and the data we present in this study, the contrast is striking, and the data are hard to reconcile. They observed heterodimers and functional consequences in all their systems, but we cannot detect any signs of either physical or functional interaction between the two receptors. Our data do not exclude the possibility that the AT₁R/B2R heterodimer might indeed exist in some biological systems as suggested by AbdAlla et al. (5, 7, 16), but at least they strongly support the notion that this heterodimer does not occur in any of our cellular systems at any protein ratio explored herein. Thus, our data support the conclusion that the AT₁R/B2R heterodimer is probably not a universal phenomenon and that it requires more that simultaneous expression of the two receptors. This conclusion covers both the biochemical interaction between the two receptors and the pharmacological potentiation.

What Explains the Lack of Reconciliation?—Although we have shown that our system can detect AT₁R gain-of-function events, there are a few likely explanations that can reconcile the data.

The most obvious explanation would be that the cellular systems we have used lack a particular protein that mediates the interaction and that the cellular system or their growth conditions (CO₂ levels, serum batch growth media, etc.) could influence the results. Although such proteins have not yet been identified for any 7TM heterodimer to the best of our knowledge, it is often observed that different proteins modulate 7TM receptor function (44). However, we have investigated this interaction in three different cell lines that have been successfully used to study gain-of-function events in many cases in many laboratories (3, 40, 41). Accordingly the requirement of a particular protein, which is not present in our systems, would mean that this particular AT₁R/B2R interaction requires a very stringent environment of cofactors and therefore that it is not likely that the heterodimer occurs in most systems. It is also possible that the B2R or the AT₁R clones we have used are not completely identical, although we are using the same species of receptors as reported by AbdAlla et al. (5, 7, 16). However, this explanation would also render the interaction very vulnerable. AbdAlla et al. (5, 7, 16) have reported both functional and physical interaction between both human and rat AT₁R and rat B2R. We have used the human orthologs in our studies on cellular proliferation (Fig. 3) and in the PI hydrolysis assay performed in HEK293 cells (data not shown), whereas the PI hydrolysis in COS-7 cells (Fig. 1), the ERK and β-arrestin recruitment (Fig. 2), and the physical interaction data presented are performed on the rat AT₁R and B2R (Fig. 4). Finally it is possible that the lack of functional potentiation as a result of the B2R co-transfection is because the B2R is already expressed endogenously in the systems. This would mean that the functional potentiation “has already occurred” and that introducing more receptor would not result in additional potentiation. This explanation is not very likely because we do not observe any response to bradykinin before we transfect B2R cDNA into the cells, meaning that the endogenous B2R levels must be very low if present at all.

Conclusion and Future Challenges—The data presented by AbdAlla et al. (5, 7, 16) are certainly very interesting, and as mentioned in the Introduction, the renin-angiotensin and kallikrein-kinin systems interact on many levels of biology, and a heterodimer would add a new layer of complexity to this cross-talk (17, 18). Furthermore this interaction could also be interesting from a medical perspective because it has been linked to experimental hypertension and preeclampsia (5, 7). When summarized, their studies indicate that the system is universal and that AT₁R/B2R heterodimerization and the functional potentiation occur as a natural consequence of simultaneous AT₁R and B2R expression within the same cell (5, 7, 16). On the other hand, we have examined this interaction in several cellular systems that should be able to detect gain of function or physical interaction between the two receptors, and we do not find any signs of either interaction or potentiation. Accordingly our data suggest that the AT₁R/B2R heterodimer does not occur as a natural consequence of their simultaneous expression in the same cell but perhaps demands yet unidentified specific requirements. The AT₁R has been implicated in both homo- and heterodimerization, and at least five possible heterodimers have been reported (3–14). Because these heterodimeric interactions may have fundamental impact on AT₁R signaling their existence opens a novel pharmacological venue with possibilities for developing heterodimer-specific antagonists with higher efficiency and fewer adverse effects in the treatment of cardiovascular disease. Hopefully future studies will address the biological and pharmacological consequences of AT₁R dimerization and determine how many receptors actually form dimers with the AT₁R and how many of these dimers result in a gain-of-function or loss-of-function phenotype.

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