

Heteromerization Between the Bradykinin B₂ Receptor and the Angiotensin-(1–7) Mas Receptor

Functional Consequences

Bruno D. Cerrato, Oscar A. Carretero, Brana Janic, Hernán E. Grecco, Mariela M. Gironacci

Abstract—Bradykinin B₂ receptor (B₂R) and angiotensin-(1–7) Mas receptor (MasR)–mediated effects are physiologically interconnected. The molecular basis for such cross talk is unknown. It is hypothesized that the cross talk occurs at the receptor level. We investigated B₂R–MasR heteromerization and the functional consequences of such interaction. B₂R fused to the cyan fluorescent protein and MasR fused to the yellow fluorescent protein were transiently coexpressed in human embryonic kidney293T cells. Fluorescence resonance energy transfer analysis showed that B₂R and MasR formed a constitutive heteromer, which was not modified by their agonists. B₂R or MasR antagonists decreased fluorescence resonance energy transfer efficiency, suggesting that the antagonist promoted heteromer dissociation. B₂R–MasR heteromerization induced an 8-fold increase in the MasR ligand-binding affinity. On agonist stimulation, the heteromer was internalized into early endosomes with a slower sequestration rate from the plasma membrane, compared with single receptors. B₂R–MasR heteromerization induced a greater increase in arachidonic acid release and extracellular signal-regulated kinase phosphorylation after angiotensin-(1–7) stimulation, and this effect was blocked by the B₂R antagonist. Concerning serine/threonine kinase Akt activity, a significant bradykinin-promoted activation was detected in B₂R–MasR but not in B₂R-expressing cells. Angiotensin-(1–7) and bradykinin elicited antiproliferative effects only in cells expressing B₂R–MasR heteromers, but not in cells expressing each receptor alone. Proximity ligation assay confirmed B₂R–MasR interaction in human glomerular endothelial cells supporting the interaction between both receptors in vivo. Our findings provide an explanation for the cross talk between bradykinin B₂R and angiotensin-(1–7) MasR–mediated effects. B₂R–MasR heteromerization induces functional changes in the receptor that may lead to long-lasting protective properties. (*Hypertension*. 2016;68:00-00. DOI: 10.1161/HYPERTENSIONAHA.116.07874.)

• **Online Data Supplement**

Key Words: B₂ receptor ■ heteromerization ■ internalization ■ Mas receptor

The renin–angiotensin system and the kallikrein–kinin system contribute to fluid homeostasis and blood pressure regulation. The renin–angiotensin system is composed of 2 arms with opposing functions. The pressor arm, represented by the angiotensin type 1 receptor (AT₁R), angiotensin-converting enzyme (ACE), and angiotensin II, is responsible for the vasoconstrictive, proliferative, fibrotic, and hypertensive effects of the renin–angiotensin system. In contrast, the second arm exerts depressor and cardiovascular protective effects through angiotensin-(1–7) (Ang-(1–7)), the Ang-(1–7)–specific receptor Mas that transduces the main physiological actions of Ang-(1–7), and ACE2 that catalyzes the generation of Ang-(1–7) from angiotensin II.^{1–3}

Bradykinin, generated by the kallikrein–kinin system, exerts cardioprotective, vasodilatory, and depressor properties through B₂ receptor (B₂R) stimulation.^{4,5} Several studies showed the existence of a cross talk between the renin–angiotensin system and kallikrein–kinin system.^{6,7} For instance, kallikrein not only catalyzes the generation of kinin from kininogen, but also acts as a prorenin-activating enzyme leading to an increase in angiotensin II.⁶ In addition, ACE not only generates the vasopressor angiotensin II but also responsible for the proteolytic degradation of bradykinin.^{6,7}

Not only was a cross talk between both systems reported at the enzymatic level, but also between the components of these systems. For example, Ang-(1–7) exerts kinin-like effects and

Received May 16, 2016; first decision June 10, 2016; revision accepted July 26, 2016.

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The online-only Data Supplement is available with this article at <http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.116.07874/-DC1>.

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DOI: 10.1161/HYPERTENSIONAHA.116.07874

potentiates the effects of bradykinin.^{6,8–11} In fact, bradykinin was devoid of the effect on blood vessels when the specific Ang-(1–7) receptor, the Mas receptor (MasR), was blocked or genetically knocked down.¹²

On the other hand, the Ang-(1–7) effects such as the neuroinhibitory action on noradrenergic neurotransmission,¹³ the stimulatory effect on nitric oxide generation,¹⁴ or the facilitation of the baroreflex control of the heart rate¹⁵ completely disappeared when the specific bradykinin receptor subtype B₂R was blocked. However, in not all circumstances, the effects of these peptides depend on both B₂R and MasR.¹⁶ Despite that, the function and the molecular basis for such cross talk are still unknown.

It could be hypothesized that the cross talk between both pathways occurs at the receptor level. Indeed, both B₂R and MasR belong to the G-protein-coupled receptor (GPCR) family. GPCRs exist as homo- or hetero-oligomers. Such structural organization has been found to be essential for receptor function. GPCR oligomerization affects important receptor functions like biosynthesis, plasma membrane diffusion or velocity, ligand-binding, pharmacology, signaling, and trafficking properties.^{17–19} Taking into account that bradykinin actions are blocked by an MasR antagonist or when MasR is knocked down, or that Ang-(1–7) response disappears when B₂R is blocked, we hypothesized that B₂R and MasR may interact directly through a heteromer formation. Our aim was to investigate the heteromerization between B₂R and MasR and to determine its functional consequences.

Materials and Methods

Cell Culture and Transfection

Human embryonic kidney293T cells obtained from the American Type Culture Collection were grown in high-glucose Dulbecco Modified Eagle Medium, 10 mg/mL sodium pyruvate, 2 mmol/L L-glutamine, 10% (vol/vol) FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere at 95% air and 5% CO₂. Cells were transiently transfected with cDNA (3 µg/9.5 cm² tissue culture well) encoding for MasR fused to enhanced yellow fluorescence protein (MasR–yellow fluorescence protein [YFP]) and B₂R fused to enhanced cyan fluorescence protein (B₂R–cyan fluorescence protein [CFP]) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and were used 48 hour post transfection. MasR–YFP cDNA was obtained as previously described.²⁰ B₂R–CFP cDNA was a gift from Dr Faussner (Ludwig-Maximilians-University Munich, Munich, Germany). This construct was subcloned into BamHI–XhoI sites of pcDNA3.1 plasmid. The cloned genes were confirmed by restriction analysis and sequencing. Physical and biological containment of both recombinant plasmids conform to the National Institutes of Health guidelines.

Human glomerular endothelial cells were kindly gifted by Dr María Marta Amaral. Human glomerular endothelial cells were isolated as previously described²¹ from kidneys removed from different pediatric patients undergoing nephrectomies performed at Hospital Nacional “Alejandro Posadas,” Buenos Aires, Argentina (written informed consent was obtained from the next of kin, caretakers, or guardians on behalf of the minor/children participants involved). The Ethics Committee of the University of Buenos Aires approved the use of human renal tissues for research purposes. Detailed methods are provided in the [online-only Data Supplement](#).

Data Analysis

The results are presented as mean±SEM. One-way ANOVA computation combined with the Bonferroni test was used to analyze data with

unequal variance between each group. A probability level of 0.05 was considered significant.

Results

B₂R Interacts With MasR

B₂R–MasR heteromerization was investigated by fluorescence resonance energy transfer (FRET),²² so we generated fusion proteins of the B₂R and MasR sequences tagged at their carboxyl terminus to the enhanced cyan fluorescence protein and, respectively (Data Supplement). Human embryonic kidney293T cells were cotransfected with B₂R–CFP and MasR–YFP, and interaction between B₂R and MasR was evaluated by FRET measured by acceptor photobleaching.²² Figure 1A shows a representative FRET experiment. Fluorescence of YFP and CFP was observed in the cell membrane and cytoplasm of human embryonic kidney293T cells coexpressing B₂R–CFP and MasR–YFP, at prephotobleaching. Acceptor photobleaching resulted in a decrease in YFP fluorescence and in a positive FRET (Figure 1B). To quantify FRET, we calculated the apparent FRET efficiency, which for tight interactions is proportional to the fraction of the donor in complex with the acceptor. For cells transiently coexpressing B₂R–CFP and MasR–YFP with similar donor enhanced cyan fluorescence protein to acceptor enhanced yellow fluorescence protein ratio, a mean apparent FRET efficiency of 0.31±0.02 was measured (Figure 1C) demonstrating that both B₂R and MasR constitutively interact by a heteromer formation.

To further corroborate FRET specificity between B₂R and MasR, FRET was evaluated in cells transiently coexpressing B₂R–CFP and MasR–YFP with the native B₂R (without an enhanced cyan fluorescence protein moiety) or with the native MasR (without an enhanced yellow fluorescence protein moiety). If an interaction between B₂R and MasR exists, a decrease in FRET apparent efficiency would be expected because of the formation of heteromers between B₂R–CFP and untagged Mas or MasR–YFP and untagged B₂R. As it is shown in Figure 1C, the presence of the untagged receptors resulted in a significant reduction in FRET apparent efficiency, demonstrating that untagged receptors are competing with tagged receptors for heteromer formation, thus reinforcing the interaction between both B₂R and MasR. Altogether, these results show that FRET arises from the interaction between the receptor moieties of the fusion proteins, and hence demonstrate the existence of constitutive B₂R–MasR heteromers.

The influence of ligands on FRET efficiency was investigated by incubating the cells cotransfected with B₂R–CFP plus MasR–YFP with 1 µmol/L bradykinin or Ang-(1–7), for 15 minutes at 37°C, before FRET analysis. The formation of B₂R–MasR heteromer was not altered by either of the agonists because FRET was not modified when the cells were stimulated with bradykinin or Ang-(1–7) (Figure 1D), demonstrating that the oligomerization state of B₂R–MasR complexes is not modulated by the agonist. On the contrary, the addition of B₂R or MasR antagonists decreased FRET efficiency to ≈50% of the initial value, indicating that the antagonist promoted the dissociation of the heteromer (Figure 1D).

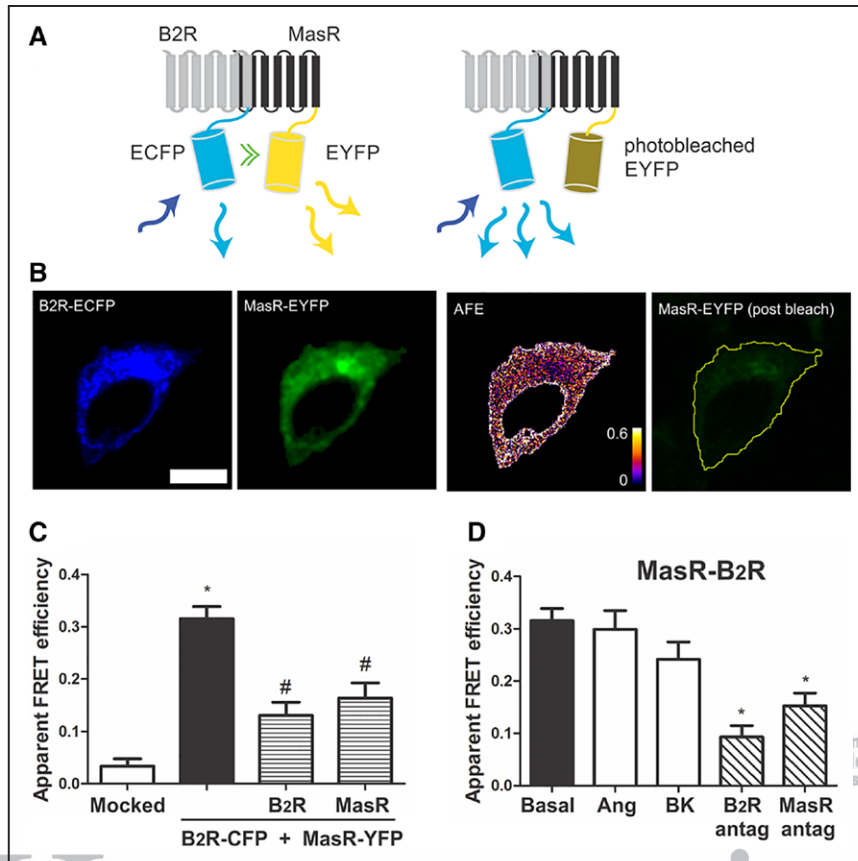


Figure 1. B_2 receptor (B_2R) interacts with Mas receptor (MasR). **A**, Schematic representation of fluorescence resonance energy transfer (FRET). B_2R fused to ECFP (FRET donor) and MasR fused to EYFP (FRET acceptor) are coexpressed in the same cell. On excitation of the donor molecule (blue arrow) not only the donor emits the energy (light blue arrow) but also part of the energy is transferred to the acceptor (green double arrow) which also emits (yellow arrows; **left**). Photobleaching of the acceptor molecule abolishes energy transfer, which is evidenced by an increase in the donor emission (light blue arrows; **right**). **B**, Representative images of the FRET measurements (scale bar, 10 μ m): B_2R -cyan fluorescence protein (CFP) (FRET donor) and MasR-cyan fluorescence protein (YFP) (FRET acceptor) before acceptor photobleaching, apparent FRET efficiency (AFE) and resulting acceptor image after photobleaching. The AFE values can be between 0 and 1 and are proportional to the fraction of interacting B_2R molecules. **C**, Apparent FRET efficiency of cells transfected with the empty vector (mocked) or the vectors containing the DNA coding for B_2R -CFP+MasR-YFP (black bar), B_2R -CFP+MasR-YFP+untagged B_2R (patterned bar) or B_2R -CFP+MasR-YFP+untagged MasR (patterned bar). * P <0.05 vs mocked; # P <0.05 vs B_2R -CFP+MasR-YFP. **D**, Influence of ligands on B_2R -MasR heteromer formation. Before FRET measurements, human embryonic kidney 293T cells transiently coexpressing B_2R -CFP+MasR-YFP were incubated without (basal) or with 1 μ mol/L bradykinin (BK) or Ang-(1-7) or the B_2 receptor antagonist Hoe or the Mas receptor antagonist A779 15 min at 37°C. * P <0.05 vs basal. Values are expressed as the mean \pm SEM from 4 independent experiments. Twenty-five cells were analyzed per experiment.

All the subsequent experiments were performed with the chimeras B_2R -CFP and MasR-YFP, but to make the reading easier, the notations B_2R and MasR were used instead.

MasR Displays Higher Affinity to Its Ligand When Expressed in Combination With B_2R

To determine whether B_2R -MasR interaction leads to changes in receptor pharmacology, ligand-binding properties of each receptor were examined. Competition radioligand-binding experiments were performed in B_2R -MasR cotransfected cells and cells transfected with either B_2R or MasR alone (Figure 2). From these curves, the ligand-binding affinity constant was determined. Binding affinity of bradykinin for the specific B_2R remained unchanged in B_2R - and B_2R -MasR-expressing cells ($K_D=3.56\pm0.55\times10^{-10}$ mol/L in B_2R -transfected cells and $5.8\pm0.68\times10^{-10}$ mol/L in B_2R -MasR-transfected cells; Figure 2A). Conversely, an 8-fold increase in the binding affinity of Ang-(1-7) for its

MasR was observed in cells expressing both B_2R and MasR ($K_D=1.15\pm0.28\times10^{-9}$ mol/L in MasR-transfected cells and $1.39\pm0.55\times10^{-10}$ mol/L in B_2R -MasR-transfected cells, P <0.05; Figure 2B). These results suggested that heteromerization modified the pharmacological properties of MasR but not of B_2R .

B_2R -MasR Heteromer Internalizes Into Early Endosomes on Agonist Stimulation

To determine whether heteromerization affects the trafficking properties of these receptors, agonist-induced redistribution of receptors from the cell surface (ie, internalization) was examined. To investigate whether B_2R -MasR heteromer was targeted to early endosome, B_2R -CFP- and MasR-YFP-cotransfected cells were stimulated with 1 μ mol/L Ang-(1-7) during 15 minutes, and then colocalization with the early endosome marker Rab5 was evaluated. After Ang-(1-7) treatment, B_2R and

MasR internalization was observed as small bright spots within the cell (Figure S2A and S2B in the [online-only Data Supplement](#)), in a distribution pattern that is characteristic of receptors located in intracellular endocytic vesicles. Quantitative colocalization analysis revealed Pearson correlation coefficient >0.6 indicating that both B_2R and MasR were colocalized. Thus, B_2R and MasR cointernalized on ligand stimulation. We also evaluated whether the heteromer is endocytosed to early endosome on ligand stimulation. The analysis revealed that a fraction of the oligomer B_2R -MasR colocalized with the early endosome marker Rab5 (Figure S2C and S2D; Pearson's correlation coefficient: 0.82 ± 0.1 , $P < 0.01$ by 1-tail Wilcoxon matched pairs signed ranks test). Similar results were observed after bradykinin stimulation. Altogether, these results showed that the oligomer B_2R -MasR was targeted mostly to early endosomes after agonist stimulation.

Ang-(1-7) Induces B_2R Internalization When B_2R -MasR Heteromer Is Formed

To further confirm the existence of B_2R -MasR heteromer and its internalization, we investigated whether selective stimulation of one of the receptor promotes cointernalization of both receptors. To induce receptor internalization, cells were stimulated with the selective B_2R agonist bradykinin (1 $\mu\text{mol/L}$) or the selective MasR agonist Ang-(1-7)

(1 $\mu\text{mol/L}$) for 30 minutes. The amount of B_2R or MasR present in the plasma membrane after agonist stimulation was evaluated by radioligand-binding assays. B_2R -transfected cells stimulated with bradykinin or MasR-transfected cells stimulated with Ang-(1-7) showed a significant decrease in the amount of receptor present in the plasma membrane (Figure 3A and 3B, respectively), indicating that the receptor was internalized on agonist stimulation. When B_2R -MasR-transfected cells were stimulated with bradykinin, there was a decrease of $91 \pm 4\%$ in B_2R and $53 \pm 3\%$ in MasR in the plasma membrane (Figure 3C and 3D). Conversely, when B_2R -MasR-transfected cells were stimulated with Ang-(1-7), there was a decrease of $58 \pm 4\%$ in B_2R and $82 \pm 6\%$ in MasR present in the plasma membrane (Figure 3C and 3D).

These results clearly demonstrate that in cells coexpressing both receptors, the selective stimulation of one of the GPCRs promotes cointernalization of both receptors.

B_2R -MasR Heteromerization Results in a Decreased Rate of Ang-(1-7)-Mediated MasR Sequestration

Because B_2R -MasR interaction may lead to changes in the rate of receptor sequestration from the plasma membrane after agonist stimulation, we evaluated the percentage of internalized MasR after Ang-(1-7) treatment. Cells were stimulated with Ang-(1-7) (1 $\mu\text{mol/L}$) during different times for the time intervals ranging from 5 to 40 minutes, and the amount of MasR present in the plasma membrane was determined by radioligand-binding assay. Receptor sequestration was then defined as the decrease in specific [^{125}I]Ang-(1-7) binding compared with the total binding obtained in untreated cells.

Cells transiently coexpressing B_2R and MasR revealed a slower sequestration of MasR on stimulation with Ang-(1-7), compared with cells transiently expressing only MasR (Figure 3E). The rate of Ang-(1-7)-induced MasR internalization was significantly decreased by the coexpression of B_2R ($P < 0.05$), as the estimated $t_{1/2}$ was 10.6 ± 1.2 and 16.9 ± 0.8 minutes in MasR- and in B_2R - and MasR-expressing cells, respectively.

B_2R -MasR Heteromerization and Downstream Signaling

We determined whether heteromerization leads to alterations in functional coupling. Arachidonic acid (AA) release in response to bradykinin or Ang-(1-7) was examined in cells expressing individual or both receptors. Cells expressing both B_2R and MasR showed an increase in AA release under basal condition which was ≈ 2 -fold higher than that observed in cells expressing one of the receptors (Figure 4A). Bradykinin or Ang-(1-7) treatment resulted in a significant increase in AA release in cells transiently expressing B_2R or MasR alone or together, being the effect elicited by Ang-(1-7) greater in cells expressing both receptors (Figure 4A).

The increase in AA release induced by Ang-(1-7) (1 $\mu\text{mol/L}$) in cells coexpressing both B_2R and MasR was blocked by the B_2R antagonist Hoe 140 (10 $\mu\text{mol/L}$). Conversely, the increase in AA release induced by bradykinin in cells

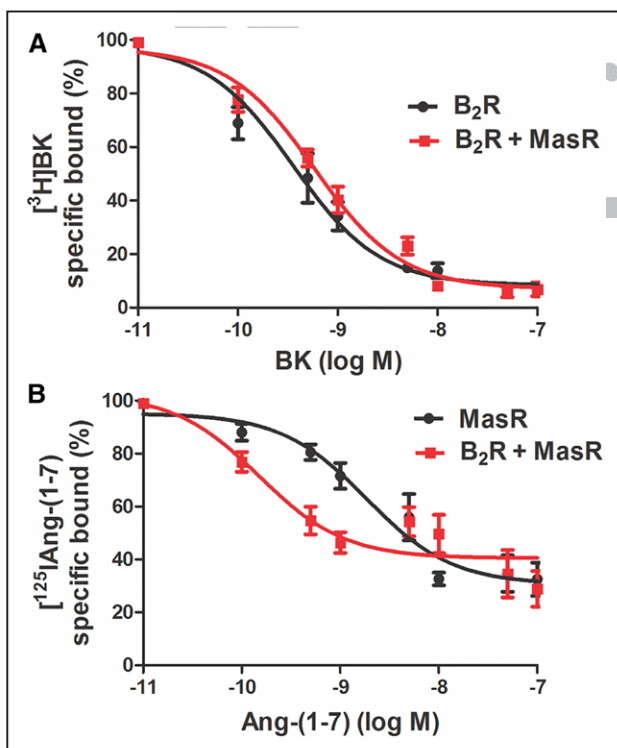


Figure 2. Mas receptor (MasR) displays higher affinity to its ligand when expressed in combination with B_2R receptor (B_2R). **A**, Competition binding assay with [^3H] bradykinin in the presence of increasing concentrations of bradykinin in cells expressing B_2R (black line) or B_2R -MasR (red line). **B**, Competition binding assay with [^{125}I]Ang-(1-7) in the presence of increasing concentrations of Ang-(1-7) in cells expressing MasR (black line) or B_2R -MasR (red line). Data represent means \pm SE from 4 independent experiments performed in triplicate.

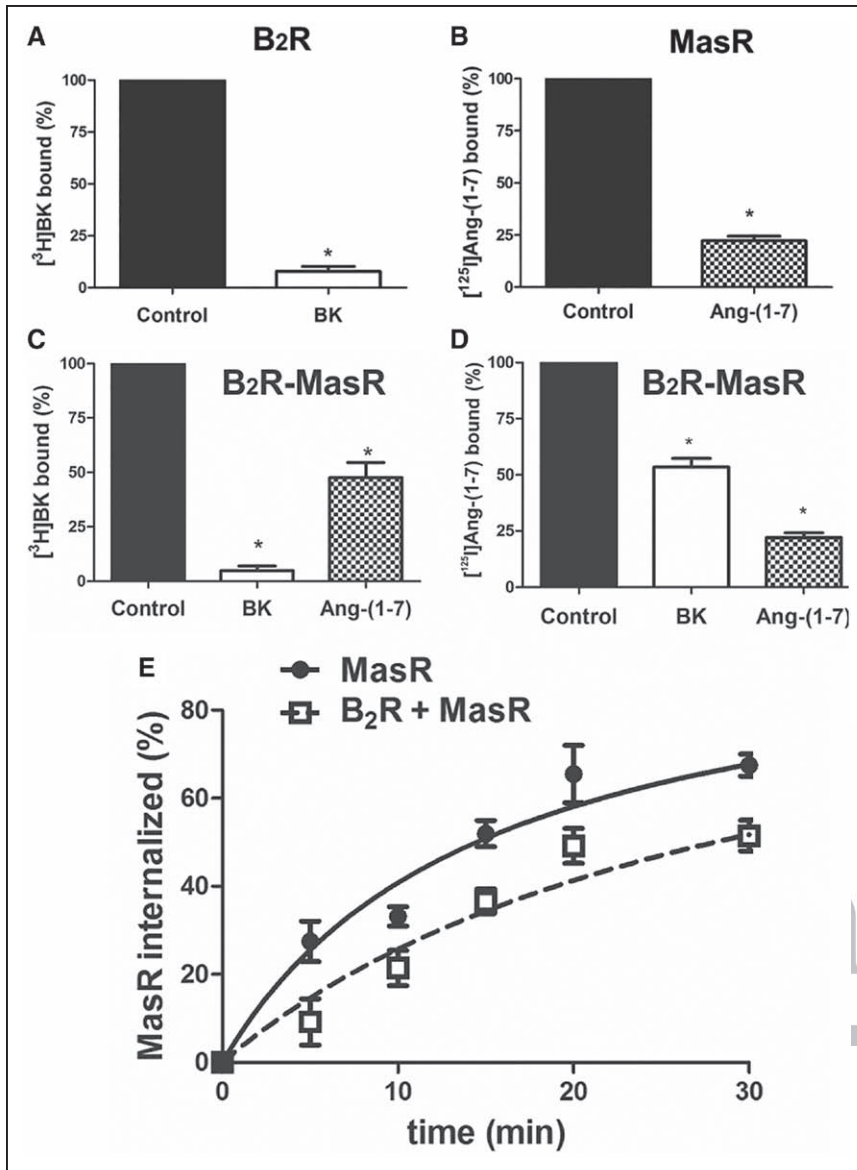


Figure 3. Agonist-mediated internalization of receptors. Cells expressing (A) B_2 receptor (B_2R), (B) Mas receptor (MasR) or (C and D) B_2R -MasR were incubated in the absence (control, black bar) or presence of (1 μ mol/L) bradykinin (white bar) or (1 μ mol/L) Ang-(1-7) (patterned bar) for 15 min, and the percentage of B_2R (A and C) or MasR (B and D) present in the membrane was analyzed by ligand binding assay as described under Methods section of this article. * $P < 0.05$ vs control. E, Effect of B_2R coexpression on the time course of MasR sequestration. Cells expressing MasR (solid line) or B_2R -MasR (dotted line) were incubated with Ang-(1-7) (1 μ mol/L) for the indicated time intervals and receptor sequestration determined as described under Methods. Values are mean+SEM of 4 different experiments each performed in triplicate.

coexpressing both B_2R and MasR was blocked by the MasR antagonist A779 (10 μ mol/L; Figure 4B). These results support B_2R -MasR heteromerization.

We also investigated whether B_2R -MasR heteromerization affects extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase activation. Bradykinin (1 μ mol/L) had no effect on ERK phosphorylation in cells transiently expressing B_2R alone, whereas Ang-(1-7) (1 μ mol/L) induced a significant increase in ERK phosphorylation in cells transiently expressing MasR alone (Figure 4C). Cells transiently coexpressing both B_2R and MasR showed no change in ERK phosphorylation under basal condition compared with that observed in cells expressing one of the receptors (Figure S3A), indicating that the B_2R -MasR heteromer does not modify constitutive ERK activation. In cells expressing both B_2R and MasR, ERK phosphorylation was not modified by bradykinin treatment, but it was increased in response to Ang-(1-7), and

this effect was greater compared with cells expressing only MasR. The Ang-(1-7)-stimulated ERK phosphorylation was prevented by pretreatment with the B_2R antagonist (Figure 4D).

The effect of B_2R -Mas heteromerization on serine/threonine kinase Akt phosphorylation was also evaluated. Bradykinin or Ang-(1-7) had no effect on Akt phosphorylation in cells transiently expressing B_2R or MasR alone, respectively (Figure 4E). Cells transiently coexpressing both B_2R and MasR showed no change in Akt phosphorylation under basal condition compared with that observed in cells expressing one of the receptors (Figure S3B). Conversely, bradykinin induced an increase in Akt phosphorylation in cells expressing both B_2R and MasR, and this effect was partially prevented by pretreatment with the MasR antagonist (Figure 4F).

Altogether, these data support the presence of B_2R -MasR heteromerization and the consequences on downstream signaling.

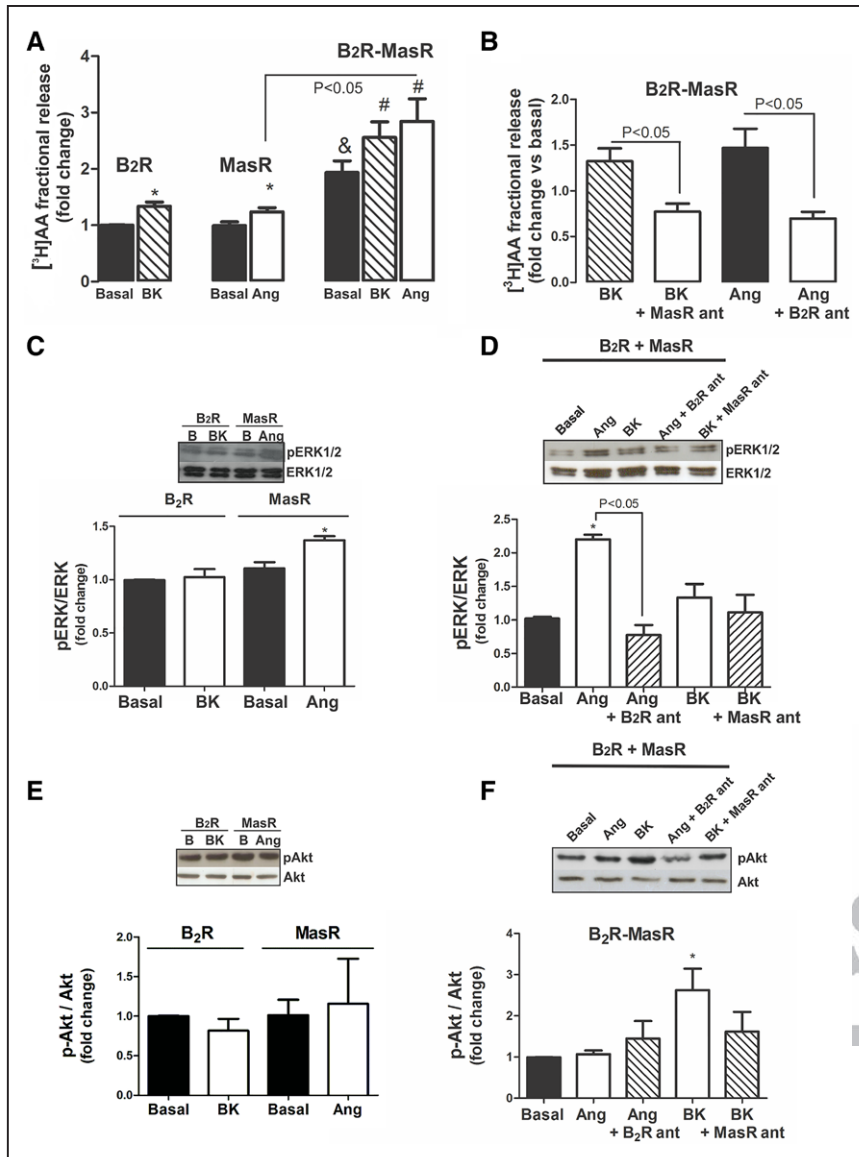


Figure 4. B₂R–MasR heteromerization and downstream signaling. **A**, [³H]JAA release from cells expressing B₂ receptor (B₂R), Mas receptor (MasR) or B₂R–MasR, incubated in the absence (basal, black bar) or presence of bradykinin (patterned bar) or bradykinin+MasR antagonist (white bar) or Ang-(1–7) (white bar). Results are presented as fold change of the response detected in untreated B₂R-transfected cells. **P*<0.05 vs untreated B₂R- or MasR-transfected cells; and *P*<0.05 vs cells transfected with only 1 receptor; #*P*<0.05 vs untreated B₂R–MasR-transfected cells. **B**, [³H]JAA release from cells expressing B₂R–MasR, incubated with bradykinin (patterned bar) or bradykinin+MasR antagonist (white bar) or Ang-(1–7) (black bar) or Ang-(1–7)+B₂R antagonist (white bar). Results are presented as fold change of the response detected in untreated B₂R–MasR-transfected cells. Values are mean±SEM of 4 independent experiments performed in triplicate. **C–F**, Effect of B₂R–MasR heteromerization on extracellular signal-regulated kinase (ERK) 1/2 and Akt phosphorylation. ERK 1/2 (**C**) and Akt (**E**) phosphorylation in human embryonic kidney293T (HEK293T) cells expressing B₂R or MasR incubated in the absence (basal) or presence of bradykinin or Ang-(1–7), respectively. ERK 1/2 (**D**) and Akt (**F**) phosphorylation in HEK293T cells expressing B₂R–MasR incubated in the absence (basal) or presence of Ang-(1–7) or Ang-(1–7)+B₂R antagonist or bradykinin or bradykinin+MasR antagonist for 15 min. **P*<0.05 vs basal. Results are expressed as fold change of the response detected in basal conditions. Each bar represents the mean±SEM of 4 independent preparations.

BK or Ang-(1–7) Induces Antiproliferative Effect Only When B₂R–MasR Heteromer is Formed

We tested whether B₂R–MasR heteromerization may lead to changes in the biological response, eg, cellular proliferation. Cells expressing individual or both receptors did not show differences in cellular growth (Figure 5A). Bradykinin or Ang-(1–7) (100 nmol/L or 1 μmol/L) were without effect on cellular proliferation in cells expressing only B₂R or MasR, respectively (Figure S4 and Figure 5B, respectively). However, in cells coexpressing both receptors, 1 μmol/L bradykinin or Ang-(1–7) induced a decrease in cellular proliferation (43±5% reduction in bradykinin-treated cells and 36±6% reduction in Ang-(1–7)-treated cells; Figure 5B). There was a tendency to reduce cellular proliferation after incubation with 100 nmol/L bradykinin or Ang-(1–7) (Figure S4) not statistically significant. This reduction was lower than that observed with higher concentrations of the ligands. Thus, bradykinin or Ang-(1–7)

induced an antiproliferative response when B₂R heteromerized with MasR.

B₂R–MasR Heteromer Formation in Physiological Conditions

To establish that our results are not specific to transfected human embryonic kidney293T cells and that B₂R–MasR oligomerization also occurs in cells that natively express both receptors, we evaluated B₂R–MasR interaction in mesenteric vascular bed from rats. On immunoprecipitation of the B₂R from mesenteric vascular bed lysate, we observed coprecipitation of the MasR (Figure 6A). Conversely, on immunoprecipitation of the MasR from mesenteric vascular bed lysate, we observed coprecipitation of the B₂R with 2 bands detected in this situation (Figure 6B). The lower band may correspond to the immature B₂R, as previously suggested.²³

To further confirm B₂R–MasR interaction, we performed proximity ligation assay (PLA). Human glomerular

endothelial cells were incubated in the presence or absence of B_2 R and MasR antibodies and PLA was performed following the manufacturer's instructions. Figure 6C showed a positive PLA signal observed in cells incubated with the antibodies (left). Conversely, a PLA signal was absent in cells not incubated with the primary antibodies (right). Taken together, the data show that B_2 R and MasR form heteromers in physiological conditions, supporting the interaction between both receptors in vivo.

Discussion

The present study demonstrates that B_2 R and MasR form constitutive and agonist-independent functional heteromers. The results of coimmunoprecipitation experiments in rat mesenteric vascular bed and of positive PLA in human glomerular endothelial cells provide direct evidence that these receptors also form heteromers in vivo (present results).

Previous studies have demonstrated the heteromerization of MasR with AT_1 R and that this heteromerization inhibited the actions of angiotensin II.²⁴ In this situation, MasR acts as a physiological antagonist of AT_1 R. B_2 R has been described to form constitutive heteromers with AT_1 R. AT_1 R- B_2 R

heteromerization was shown to enhance AT_1 R-stimulated signaling under pathophysiological conditions such as experimental and human pregnancy hypertension.^{25,26} However, the universality of the formation of AT_1 R- B_2 R heteromers has been questioned.⁶ B_2 R also forms heteromers with B_1 R in cotransfected HEK293 cells and in natively expressing endothelial cells, resulting in significant internalization and desensitization of the B_1 R response in cells pretreated with the B_2 R agonist.²⁷ Concerning B_2 R and MasR, to our knowledge,

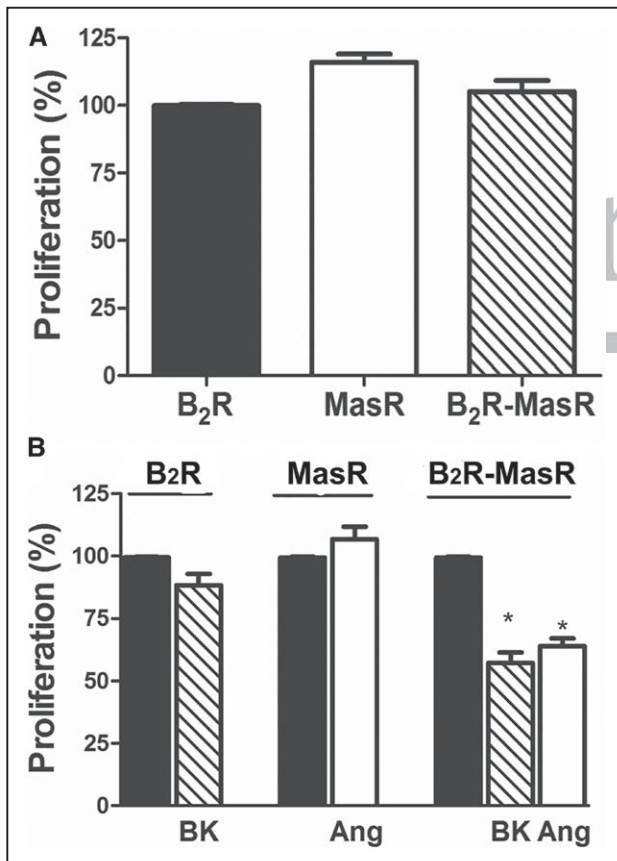


Figure 5. B_2 R-MasR heteromerization and cellular proliferation. Cellular proliferation was evaluated in human embryonic kidney 293T cells expressing B_2 receptor (B_2 R), Mas receptor (MasR), or B_2 R-MasR incubated in the absence (**A** and black bar in **B**) or presence of bradykinin or Ang-(1-7) as described in Methods. Results are expressed as the percentage change of proliferation detected in untreated B_2 R-transfected cells (**A**) or in basal conditions (black bars in **B**). * $P < 0.05$ vs its corresponding basal. Each bar represents the mean \pm SEM of quintuplicate of 4 independent experiments.

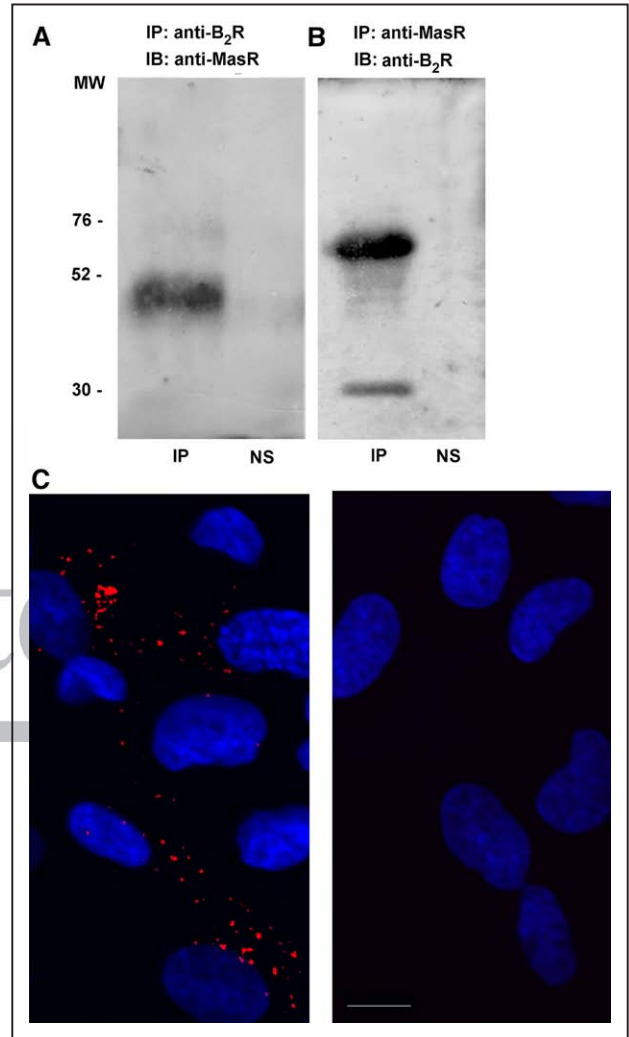


Figure 6. Mas and B_2 receptors interact in physiological conditions. Immunoprecipitation of lysates from Wistar rat mesenteric vascular beds were performed using (**A**) anti- B_2 receptor (B_2 R) or (**B**) anti-Mas receptor (MasR) antibodies. Immunoprecipitates were analyzed by SDS-PAGE and immunoblotted using the indicated antibodies. These blots are representative of 4 different experiments with similar qualitative results. NS indicates nonspecific, which refers to omission of the primary antibody in the coimmunoprecipitation assay (**C**) B_2 R and MasR interaction measured by proximity ligation assay (PLA) in human tubulointerstitial and glomerular endothelial cells. Fixed cells were permeabilized with 0.2% Triton X-100 for 1 hour, and then blocked in blocking buffer for 1 h. B_2 R (antimouse) and MasR (antirabbit) antibodies were diluted in blocking solution, and incubated with the cells overnight at 4°C (**A**). PLA was conducted using Duolink In Situ Orange Starter Kit Mouse/Rabbit (Sigma, St. Louis, MO) following manufacturer's instructions. Cells presented in image **B** were subjected to the same protocol but without the addition of the primary antibodies. Scale bar, 15 μ m.

this is the first evidence showing B₂R–MasR heteromerization, which is constitutive and independent of the presence of physiological ligands.

B₂R–MasR heteromerization may explain the potentiation in bradykinin or Ang-(1–7) responses when both ligands are incubated together.^{6,8–11,28} We could not disregard that bradykinin–Ang-(1–7) interaction may result not only from B₂R–MasR heteromerization but also from ACE inactivation by Ang-(1–7). It has been shown that Ang-(1–7) at micromolar levels inhibits ACE, thus enhancing bradykinin vasodilatory effects.^{11,29,30} In contrast, the addition of B₂R or MasR antagonists decreased FRET efficiency to ≈50% of the initial value, suggesting that the antagonist promoted the dissociation of the heteromer and that the formation of B₂R–MasR heteromer is essential for agonists to display many of its biological responses. For instance, bradykinin or Ang-(1–7) induced an antiproliferative response when B₂R–MasR heteromer is formed and not when each receptor (B₂R or MasR) is expressed alone (present results) showing the importance of this interaction for some of the effects elicited by bradykinin or Ang-(1–7). B₂R–MasR heteromerization may also explain the lack of effect of Ang-(1–7) when B₂R is blocked^{13–15} or the absence of the bradykinin-induced vasodilatory response when MasR is knocked down.¹² In agreement, our present study shows that the Ang-(1–7)-induced increase in AA release or in ERK phosphorylation was prevented by the B₂R antagonist or that the bradykinin-stimulated Akt phosphorylation was prevented by the MasR antagonist.

Heteromerization may result in changes in affinity for a specific ligand, receptor trafficking, maturation, and signaling.^{17–19} Several reports demonstrated changes in pharmacological properties of the receptors because of heteromerization. Our present study showed that the coexpression of B₂R leads to an increase in the affinity of Ang-(1–7) for its receptor Mas, although the vice versa situation was not observed. This result may explain the potentiation in the vasodilatory or blood pressure-lowering responses when bradykinin and Ang-(1–7) are present together.^{6,8–11,28}

A receptor that is capable of producing a biological response in the absence of a bound ligand is said to display constitutive activity. Our study showed that constitutive ERK or Akt activation was not modified by B₂R–MasR heteromerization. Conversely, AA release was increased 2-fold in cells expressing both B₂R and MasR. We may speculate that in this last situation not only receptor heteromerization but also a cross talk between both receptors may occur, and this may explain the increase in AA release.

B₂R–MasR heteromerization also influences downstream signaling. Our present study showed that B₂R–MasR heteromerization induced a greater response concerning AA release and ERK phosphorylation on Ang-(1–7) stimulation. Furthermore, bradykinin-stimulated Akt phosphorylation only when both B₂R and MasR were coexpressed but not when the B₂R was expressed alone, suggesting that this was a biochemical fingerprint associated to the heteromer because it was abolished when heteromerization was disrupted by the cross-antagonist. For instance, β₁-adrenergic receptor (AR)-β₂-AR heteromerization induced a reduced ability of the

β₂-AR to stimulate ERK-phosphorylation³¹ or the AT₁R–D₂R heteromerization affected calcium mobilization but not cAMP signaling coupled to AT₁R.³² In both examples as it happens in B₂R–MasR heteromerization (present results) 1 receptor modifies downstream signaling of the partner receptor, giving a biochemical fingerprint, which is characteristic of receptor heteromerization.³³

Akt phosphorylation is one of the downstream signaling mediating Ang-(1–7) actions in cultured cells and tissues.^{1,2} It was surprising to find that Ang-(1–7) stimulation did not elicit Akt activation (present results). Despite that, constitutive Akt activation was present in MasR-transfected cells, suggesting that MasR activation is coupled to Akt activation despite the fact that Ang-(1–7) was absent. We do not have an explanation for the absence of effect of Ang-(1–7) on Akt phosphorylation. Conversely, it does not imply that B₂R–MasR interaction does not take place in nontransfected cells because in the present work we provide evidence that this interaction occurs in vascular bed from rats and in renal cells from humans.

Receptor endocytosis serves a variety of purposes, including receptor downregulation, recycling, and relocalization of the cell signaling. B₂R follows an internalization pathway involving redistribution to caveolae, through an arrestin- and dynamin-independent pathway.³⁴ However, other evidence showed that B₂R was targeted to early endosome after agonist stimulation.³⁵ MasR is internalized on ligand stimulation into early endosomes via a clathrin-dependent pathway.²⁰ Our study showed that B₂R–MasR heteromer was internalized mostly into early endosomes and that stimulation of only one of the receptors was sufficient to promote cointernalization of the entire dimer, reinforcing the concept not only of B₂R–MasR heteromer formation but also of cointernalization on agonist stimulation.

Heteromerization of GPCRs can affect the internalization properties of the individual receptors.¹⁷ Our present data demonstrate that B₂R–MasR heteromerization results in a delayed sequestration of the MasR when stimulated with its ligand Ang-(1–7). Similar observations were made for other GPCRs.^{36–38} Rapid desensitization and receptor internalization and trafficking tightly control the temporal and spatial regulation of GPCR signaling and hence the biological response. Both B₂R and MasR have been associated with cardioprotective, vasodilatory, and hypotensive responses.^{1–5} In addition, the present study showed that Ang-(1–7) induced antiproliferative responses when B₂R–MasR heteromer was formed but not when MasR is expressed alone. The delayed sequestration of MasR may cause this receptor to be present for a longer time in the plasma membrane, thus allowing a long-lasting protective response.

Conclusions

In conclusion, we have demonstrated that B₂R and MasR can form heteromers that are internalized on agonist stimulation. This heteromer revealed a delayed sequestration of the MasR on stimulation with Ang-(1–7). Furthermore, B₂R coexpression induced an increase in the affinity ligand binding properties of MasR. Altogether, these changes in receptor functional characteristics may lead to long-lasting protective

biological properties. B₂R–MasR heteromerization leads to altered functional properties in the putative heteromer and suggest that heteromerization may represent a regulatory cross talk process arising through the creation of a receptor form with distinct functional properties. This is the first time that B₂R–MasR heteromerization has been experimentally demonstrated. The existence of B₂R–MasR heteromer may help in comprehending the mechanisms underlying bradykinin and Ang-(1–7) cross talk. Putting such evidence out to the community is an important, necessary step toward further investigating the functional significance of such interaction in animal models. Furthermore, it would open new strategies for drug development acting through one of these receptors. Compounds targeting B₂R–MasR heteromer could provide an alternative pharmacological approach for cardiovascular diseases treatment.

Acknowledgments

We acknowledge the generous gift of human glomerular endothelial cells from Dr María Marta Amaral.

Sources of Funding

This work was supported by Universidad de Buenos Aires (20020120100194), Agencia Nacional de Promoción Científica y Tecnológica [2013–2170], and Consejo Nacional de Investigaciones Científicas y Técnicas [11220110101097], and by National Institutes of Health Grant [HL028982] and Henry Ford Health System institutional funds.

Disclosures

None.

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Novelty and Significance

What Is New?

- Both B₂ receptor (B₂R) and Mas receptor (MasR) are associated with cardioprotective, vasodilatory, and hypotensive responses. We provide evidence for the first time that B₂R form heteromeric complexes with MasR on the cell surface.

What Is Relevant?

- B₂R–MasR heteromerization increases ligand affinity and induces a delayed sequestration of the complex from the cell membrane, which may allow long-lasting protective biological responses.

Summary

B₂R form heteromeric complexes with MasR on the cell surface. B₂R–MasR heteromerization increases ligand affinity and induces a delayed sequestration of the complex from the cell membrane. Treatment with MasR or B₂R antagonists decreases B₂R–MasR interaction while the agonists display antiproliferative effects or Akt activation only when B₂R–MasR heteromer is formed. The existence of B₂R–MasR heteromer may help comprehending the mechanisms underlying bradykinin and Ang-(1–7) cross talk and developing drugs targeting these 2 systems involved in cardiovascular regulation. Compounds targeting B₂R–MasR heteromer could provide an alternative pharmacological approach for cardiovascular diseases treatment.

Hypertension

Heteromerization Between the Bradykinin B₂ Receptor and the Angiotensin-(1–7) Mas Receptor: Functional Consequences

Bruno D. Cerrato, Oscar A. Carretero, Brana Janic, Hernán E. Grecco and Mariela M. Gironacci

Hypertension. published online August 22, 2016;

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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SUPPLEMENTAL DATA

Heteromerization Between the Bradykinin B₂ Receptor and the Angiotensin-(1-7) Mas Receptor: Functional Consequences

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METHODS

Cell culture and Transfection

HEK293T cells obtained from the American Type Culture Collection were grown in high-glucose Dulbecco's Modified's Eagle Medium, 10 mg/mL sodium pyruvate, 2 mM L-glutamine, 10% (vol/vol) FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37° C in a humidified atmosphere at 95% air and 5% CO₂. Cells were transiently transfected with cDNA (3 µg per 9.5-cm² tissue culture well) encoding for MasR fused to EYFP (MasR-YFP) and B₂R fused to ECFP (B₂R-CFP) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and were used 48 h post-transfection. MasR-YFP cDNA was obtained as previously described.¹ B₂R-CFP cDNA was a gift from Dr. Faussner (Ludwig-Maximilians-University Munich, Munich, Germany). This construct was subcloned into BamHI-XhoI sites of pcDNA3.1 plasmid. The cloned genes were confirmed by restriction analysis and sequencing. Physical and biological containment of both recombinant plasmids conform to the National Institutes of Health guidelines.

Human glomerular endothelial cells (HGEC) were kindly gifted by Dr. María Marta Amaral. HGEC were isolated as previously described² from kidneys removed from different pediatric patients undergoing nephrectomies performed at Hospital Nacional "Alejandro Posadas", Buenos Aires, Argentina (written informed consent was obtained from the next of kin, caretakers, or guardians on the behalf of the minors/children participants involved). The principles of the Declaration of Helsinki and Title 45, U.S. Code of Federal Regulations, Part 46, Protection of Human Subjects, Revised November 13, 2001, effective December 13, 2001, were followed. The Ethics Committee of the University of Buenos Aires approved the use of human renal tissues for research purposes.

Ligand concentrations

Concentrations of 1 $\mu\text{mol/L}$ of BK or Ang-(1-7) were employed in the present study. Due to the short half-life of BK and Ang-(1-7) (less than 1 min), high concentrations of these peptides was employed just to assure that they reach the receptors in amount enough to measure a response. Many researchers have employed those concentrations used in our study.³⁻¹² Furthermore, we have previously used high concentrations of Ang-(1-7 to induce R desensitization followed by R internalization.¹ In order to not change ligand concentration, we performed the rest of the experiments with the same concentration as the first ones, that is 1 $\mu\text{mol/L}$.

Animals

Male Wistar rats weighting 300–350 g were used (n=5) were used for coimmunoprecipitation study. Animals were handled in accordance with the National Institutes of Health's animal care guidelines. The animal research protocol was approved by the Institutional Animals Care and Use Committee of the School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina.

FRET quantification by Acceptor Photobleaching

HEK293T cells cotransfected with B₂R-CFP and MasR-YFP cDNA were fixed and analyzed for the presence of FRET using APB.¹³ Microscopy images were acquired by a confocal scanning microscope (Spectral FV1000 Olympus) using a 60X UPLSAPO oil immersion objective lens with a numerical aperture of 1.35. The samples were excited for ECFP and EYFP with the 457 nm and 488 nm lines of an argon laser, respectively, in sequential mode. The samples were illuminated through the 457/488 dichroic filter and the emission was collected in the 465–495 nm band for ECFP and 500–530 nm band for EYFP. The intensity of the donor molecule was imaged before (*D*) and after (*Dapb*) photobleaching of the acceptor molecule. To control for unwanted photodestruction of the donor during imaging, two images were acquired in each case. The background intensity in each image was measured from the mean intensity of a region without cells, and subtracted from the images for all subsequent calculations. Cells exhibiting large decrease in the donor intensity (> 5%) due to imaging bleaching, as measured from two consecutive images, were discarded. For the remaining cells, FRET efficiency was calculated in each pixel as $1 - D/Dapb$. The number of cells analyzed per experiment was 25. Results were expressed as apparent FRET efficiency (AFE), where $AFE = 1 - (\text{donor intensity before photobleaching}) / (\text{donor intensity after photobleaching})$.

[¹²⁵I]-Ang-(1-7) labeling

Ang-(1-7) was labeled in our laboratory by the lactoperoxidase method as previously described.¹⁴ Five μg of Ang-(1-7) was dissolved in 20 μl of 0.05 mol/L phosphate buffer (pH 7.5) and iodinated with 0.5 mCi of [¹²⁵I] Na (Perkin Elmer) in the presence of 5 μg of lactoperoxidase (Sigma-Aldrich) and 5 μg of hydrogen peroxide (Sigma-Aldrich). Hydrogen peroxide addition was repeated twice at 5 min interval. Finally, the monoiodinated fraction was purified by high performance liquid chromatography. The specific activity was 2000 Ci/mmol.

Radioligand Binding

Cells were transfected with the constructs that encodes for B₂R-CFP, MasR-YFP or B₂R-CFP plus MasR-YFP and subjected to competition radioligand binding assay as previously described.¹ Briefly, cells were incubated at 4°C for 60 min with incubation buffer containing 2 nmol/L of [¹²⁵I]Ang-(1-7) [labelled as described previously³] or [³H]BK (Perkin Elmer), in the absence or presence of increasing concentrations of unlabelled Ang-(1-7) (Bachem) or BK (Bachem), respectively. Incubation was stopped by rinsing the cells three times with ice-cold PBS. Cells were solubilized by incubation with 0.1 mol/L NaOH for 60 min and radioactivity was measured using a gamma counter (Wallac Wizard 1470 Automatic gamma counter, Perkin-Elmer) or a β counter (Beckman LS-5000 TD). Nonspecific binding was determined in the presence of 10 μmol/L unlabeled Ang-(1-7) or BK. Specific binding was calculated by the subtraction of nonspecific binding from total binding. K_D was calculated using Graphpad Prism (Graphpad Software Inc.).

Receptor internalization and sequestration

Cells transfected with the constructs that encodes for B₂R-CFP, MasR-YFP or B₂R-CFP plus MasR-YFP were incubated in the absence or presence of Ang-(1-7) (1 μmol/L) or BK (1 μmol/L). Cells were then placed on ice, washed 3 times with ice-cold PBS and the amount of receptor present in the plasma membrane was determined by [¹²⁵I]Ang-(1-7) or [³H]BK radioligand binding assays as described above. Receptor sequestration was defined as the decrease in specific [¹²⁵I]Ang-(1-7) or [³H]BK binding, compared with the total binding obtained in untreated cells. The amount of internalized receptors as a function of time was fitted using the following equation: $y = A (1 - e^{-k t_{1/2}})$ where A is the maximum number of internalized receptors and k is the rate coefficient. This equation was used to estimate the half-time of internalization ($t_{1/2}$) as $t_{1/2} = \ln 2/k$.

Arachidonic acid (AA) release

Cells were transfected with the constructs encoding for B₂R-CFP, MasR-YFP or B₂R-CFP plus MasR-YFP. Negative controls consisted of mocked transfected cells. AA release was measured as previously described.¹ Briefly, 24 hours post-transfection cells were labeled with [³H]AA (0.2 μCi/well) (Perkin Elmer) for 16 h. Cells were then washed with Dulbecco's Modified's Eagle Medium containing 2% bovine seroalbumin (Sigma-Aldrich) and incubated in the absence or presence of Ang-(1-7) (1 μmol/L) or BK (500 nmol/L) for 30 min at 37° C. Radioactivity in the supernatant was measured. For total cellular radioactivity, cells in each well were solubilized with 1 mol/L NaOH and counted. [³H]AA released into the medium was expressed as percent of the total cellular radioactivity and referred to as fractional release.

B₂R-MasR hetero-oligomer internalization

B₂R-MasR hetero-oligomer internalization upon agonist stimulation was evaluated by analyzing colocalization between MasR-YFP and B₂R-CFP and the early endosome marker Rab5 by immunocytochemistry, as previously described.¹ Forty eight hours after the transfection, cells were incubated in the absence and presence of 1 μmol/L Ang-(1-7) or 1 μmol/L BK for 15 min at 37°C. After two washes with PBS, cells were fixed with 4% paraformaldehyde (Sigma-Aldrich), permeabilized with 0.2% Triton X-100 (Sigma-Aldrich) in PBS and incubated in blocking solution (PBS/0.2% Triton X-100/3% BSA) for 30 min at room temperature. Cells were then incubated with anti Rab5 mouse monoclonal antibody (diluted to 1:150 in blocking solution) (BD Biosciences) overnight at 4°C. The

samples were rinsed twice in PBS/0.2% Triton X-100, and exposed to the secondary antibody (goat anti-mouse antibody coupled to Alexa 594, dilution 1:600 in blocking solution) (Molecular Probes) for 2h at room temperature. Samples were mounted and imaged with confocal scanning microscope (Spectral FV1000 Olympus) using a 60X UPLSAPO oil immersion objective lens with a numerical aperture of 1.35. The sample was excited for ECFP and EYFP with the 457 nm and 488 nm lines of an argon laser, respectively, in sequential mode. The sample was illuminated through a 457/488 dichroic filter and the emission was collected in the 465–495 nm band for ECFP and 500–530 nm band for EYFP. For the colocalization experiments with Rab5, images were acquired with a third channel using a 543 nm laser with a 405/488/543/635 dichroic mirror. Images were obtained using sequential scanning for each channel to eliminate the cross-talk of chromophores. After establishing the threshold for the background pixels, we masked individual cells and early endosomes using CellProfiler (<http://www.cellprofiler.org/>). For each cell, we created a ‘non-endosome’ mask by subtracting the endosome mask obtained from the red channel from the cell mask. We then calculated the Pearson correlation coefficient between pixels in the CFP and YFP images among pixels belonging to both regions in each cell using CellProfiler. We used a 1-tail Wilcoxon Matched Pairs Signed Ranks Test to determine if the correlation inside the early endosomes was significantly larger than outside.

ERK and Akt phosphorylation assay

After treatments, cells were homogenized in ice cold buffer (pH 7.4) containing 24 mmol/L Hepes, 1 mmol/L EDTA, 2 mmol/L tetrasodium pyrophosphate, 70 mmol/L sodium fluoride, 1 mmol/L β -glycerophosphate, 1% Triton X-100, 1 mmol/L PMSF, 10 μ g/ml aprotinin and 2 μ g/ml leupeptin. Equal amount of proteins were subjected to 10% SDS-PAGE and transferred electrophoretically to polyvinylidene difluoride membrane. Nonspecific binding sites on the membrane were blocked by incubation with 5% milk in Tris-buffered saline solution containing 0.1% Tween 20. Membranes were subsequently probed with rabbit anti- ERK1/2-phosphoThr202/Tyr204 (pERK1/2) (1/2000) (Cell Signaling) or rabbit anti-Akt-phospho Ser473 (1/1000) (Cell Signaling) to measure ERK or Akt phosphorylation, respectively, followed by incubation with goat anti-rabbit IgGs coupled to horseradish peroxidase (Amersham Biosciences). Total protein content (non-phosphorylated) was evaluated by reblotting membranes with anti-ERK 1/2 (1/2000) or anti-Akt (1/1000) antibodies (Cell Signaling). Immunoreactive bands were visualized by chemiluminescence detection (Pierce ECL Plus Western Blotting Substrate, Thermo Scientific) and quantified by densitometry. Protein loading in gels was evaluated by reblotting membranes with anti-GAPDH antibody (Cell Signaling). pERK1/2 and pAkt were normalized to ERK1/2 and Akt, content in the same sample, respectively.

Co-immunoprecipitation

Mesenteric vascular bed from adult male Wistar rat incubated with 2 mmol/L dithiobis succinimidyl propionate during 30 min at 37° C was homogenized in lysis buffer (20 mmol/L Hepes, 0.3 mol/L sucrose, 2 mmol/L EDTA containing 0.025% triton-X100 and a protease inhibitor cocktail, pH =7.4) and then centrifuged at 14000xg for 15 min. Protein content in the supernatant was adjusted to 1 mg/mL, and 500 μ L was immunoprecipitated with an anti-B₂ (Santa Cruz) or anti-Mas (Santa Cruz) receptor antibody (2 μ g/mL). Immunocomplexes were isolated by incubation with 10% vol/vol protein A-Sepharose

(Sigma-Aldrich) for 2 h and eluted with 30 μ l of non-reducing sample buffer. The eluate was subjected to Western blot, 10 % SDS/PAGE and transferred electrophoretically to polyvinylidene difluoride membrane, which were subsequently probed with rabbit anti-MasR (1/750) or rabbit anti-B₂R (1/750) antibodies, followed by incubation with goat anti-rabbit IgGs coupled to horseradish peroxidase (Amersham Biosciences). Immunoreactive bands were visualized by chemiluminescence detection (Pierce ECL Plus Western Blotting Substrate, Thermo Scientific) and quantified by densitometry.

As a control, immunoprecipitation in HEK293T cells lacking one of the receptors was performed. Cells were transfected with B₂R or B₂R + MasR and immunoprecipitation was performed with an anti-MasR antibody as described above.

Cellular proliferation assay

Cells were placed in 96-well microplates at a density of $2 \cdot 10^4$ cells/well and transfected with the constructs encoding for B₂R-CFP, MasR-YFP or B₂R-CFP plus MasR-YFP. Negative controls consisted of mocked transfected cells. Cellular proliferation was measured by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) method as previously described.¹⁵ Briefly, 5 hours post-transfection cells were incubated in the absence or presence of Ang-(1-7) or BK (1 μ mol/L) at 37°C and 48 hours later, cell proliferation was determined using the CellTiter 96-AQueous Non-radioactive Cell Proliferation Assay (Promega Corporation) according to the manufacturer's instructions. Briefly, after incubation a tetrazolium compound (MTS) and an electron-coupling reagent (phenazine methosulfate) were added to the medium. The plates were incubated at 37°C under a 5% CO₂ atmosphere for 3 h. Absorbance at 492 nm corresponding to the conversion of MTS into a soluble formazan product (indicative of a viable cell) was measured using a microtiter plate reader (Biotrak II; Amersham Biosciences).

RESULTS

B₂R-CFP is Properly Expressed and Fully Active

To investigate heteromerization between B₂R and MasR by FRET, we generated fusion proteins of the B₂R and MasR sequences tagged at their carboxyl terminus to the ECFP and EYFP, respectively. MasR fused to EYFP (MasR-YFP) was characterized previously.¹ Since the fusion of ECFP to the C-terminal of the receptor may alter its correct folding and hence its functionality, we first characterized the C-terminally tagged B₂R-CFP.

HEK293T cells transfected with the DNA coding for the chimera showed that B₂R-CFP was found predominantly in the plasma membrane (Figure S1A). However, intracellular localization of the fusion protein was also detectable, especially in cells showing higher levels of receptor expression. In some cells, the B₂R-CFP was present around the nucleus, presumably in the endoplasmic reticulum, and it probably represented newly synthesized molecules passing through the secretory pathway.

The functional integrity of the B₂R-CFP tagged receptor was investigated by BK binding to the receptor. As shown in Figure 1B, increasing concentrations of unlabeled BK displaced the binding of [³H]BK to the receptor expressed in transfected cells (K_i : $8.9 \pm 0.1 \times 10^{-9}$ mol/L). These results demonstrated that B₂R-CFP attained a correct folding and that it binds its physiological ligand. Cells transfected with the empty vector showed BK binding which was one third less than the binding observed in B₂R-CFP transfected cells (mocked

transfected cells: 15000 cpm of specific binding vs B₂R-CFP transfected cells: 45000 cpm specific binding).

After being stimulated by its ligand, B₂ receptor is endocytosed. We evaluated the functionality of B₂R-CFP by measuring B₂R-CFP internalization upon ligand exposure. Figure S1C shows that B₂R-CFP receptor is endocytosed after ligand stimulation.

Receptor functionality and coupling were evaluated by measuring B₂ receptor signaling. To determine the functionality of B₂R-CFP, HEK293T cells transfected with the B₂R-CFP construct and labeled with [³H]AA were exposed to BK during different times. As shown in Figure S1D, 500 nmol/L BK caused an increase in [³H]AA release in B₂R-CFP transfected cells after 30 min stimulation. Cells transfected with the empty vector (mock) showed no change in [³H]AA release upon BK stimulation (Figure S1C).

Collectively, these data show that B₂R-CFP is properly expressed and fully active.

Regarding the fusion protein MasR-YFP, we have previously shown that the YFP fused to the C terminus of MasR does not alter its functional properties.¹

Coimmunoprecipitation control

As a control, immunoprecipitation in HEK293T cells lacking one of the receptors was performed. As it is shown in Figure S4A, immunoprecipitation performed with an anti-MasR antibody in cells expressing only B₂R did not cause B₂R immunoprecipitation. Conversely, in cells coexpressing both B₂R and MasR, immunoprecipitation performed with an anti-MasR antibody caused B₂R coimmunoprecipitation. These results confirm those observed in rat mesenteric vascular bed.

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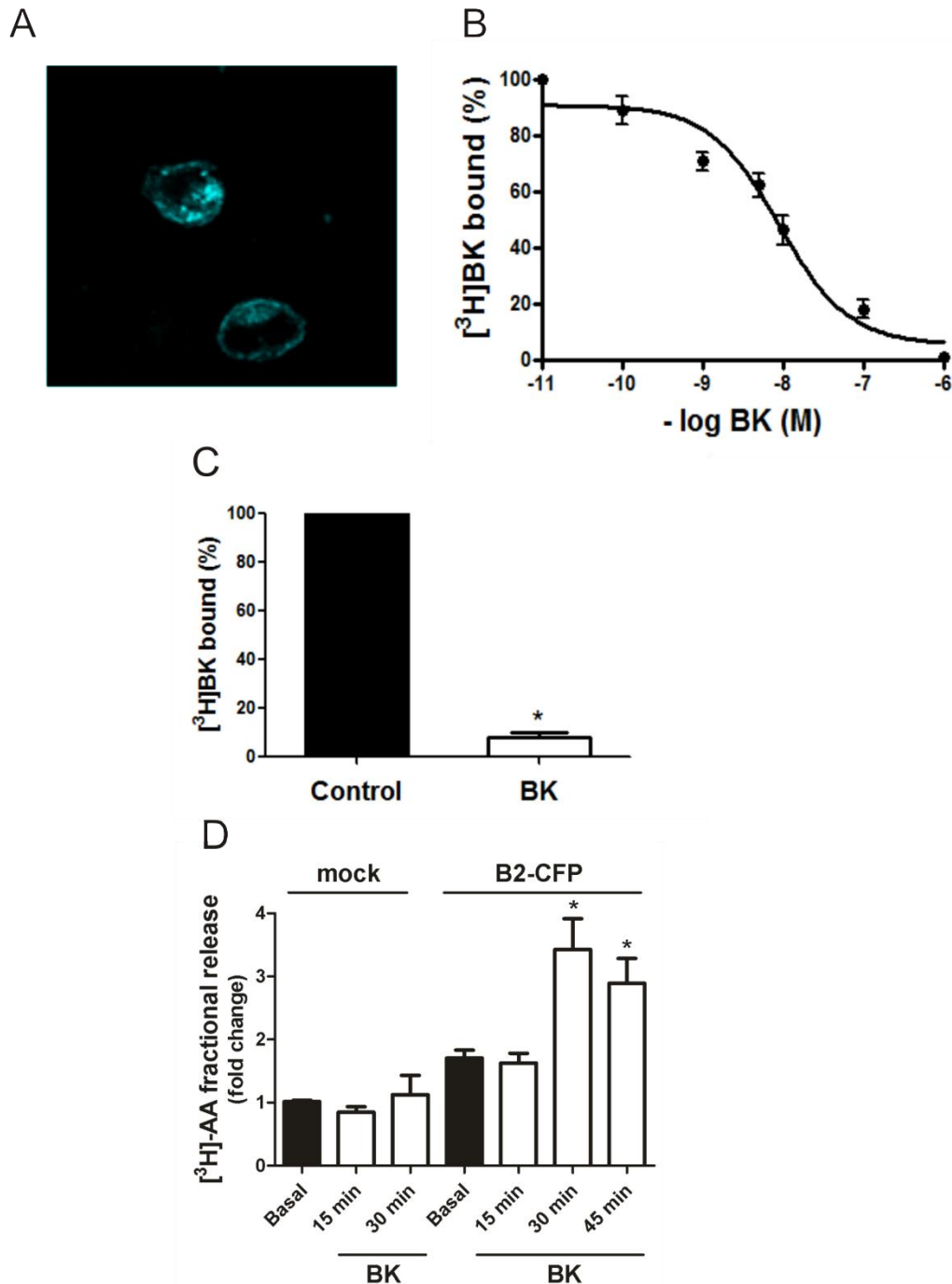


Figure S1. (A) Expression and cellular localization of the B₂R-CFP fusion protein in transfected HEK 293T cells. (B) Specific binding of [³H]BK to B₂R-CFP transfected cells in the presence of increasing concentrations of BK. Values are mean_±SE. (C) Internalization of B₂R-CFP. B₂R-CFP transfected cells were incubated in the absence (control, black bar) or presence of BK (white bar) for 15 min, and the percentage of B₂R present in the membrane analyzed by ligand binding assay as described under Methods. Values are mean_±SEM of at least three different experiments each performed in triplicate. * P<0.05 compared to control. (D) [³H]AA release from mocked transfected cells or cells

transfected with the DNA coding for B₂R-CFP in the absence (basal) and presence of 500 nmol/L BK during different times. Results are presented as fold change over basal in mocked transfected cells, taking them as 100%. Values are mean \pm SEM. * $P < 0.05$ compared with basal.

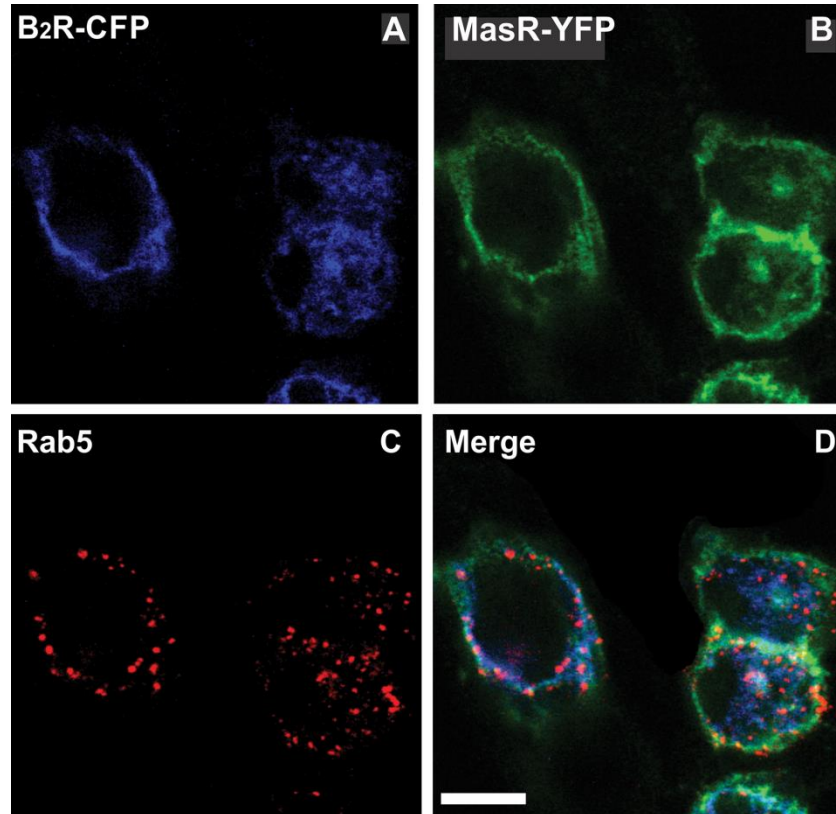


Figure S2: B₂R-MasR heterodimer is internalized into early endosomes upon Ang-(1-7) stimulation. Panels show images of (A) B₂R-CFP, (B) MasR-YFP, (C) the early endosome marker Rab5 and (D) a merged image of A, B and C. Scale bar: 10 μ m. Images are representative of four independent experiments (20 cells analyzed in each experiment).

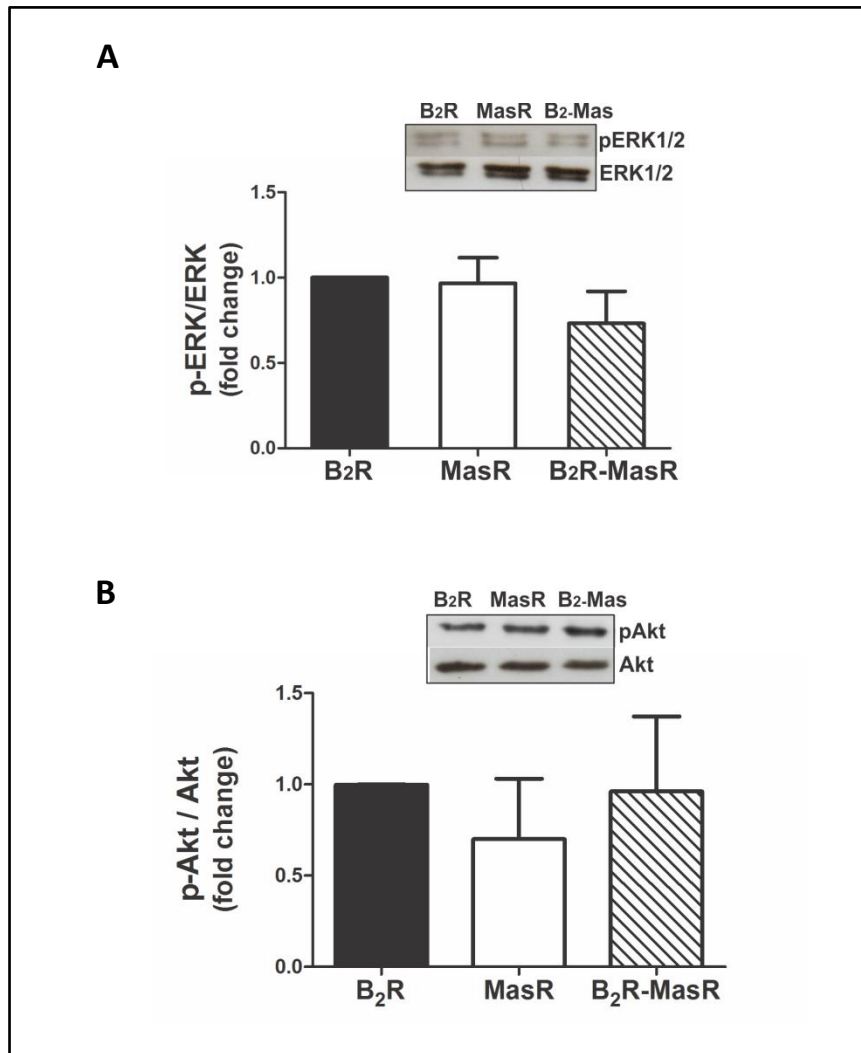


Figure S3: (A) ERK 1/2 or Akt (B) phosphorylation under basal conditions in HEK293T cells expressing B₂R or MasR or B₂R-MasR. Results are expressed as fold change of the response detected in basal conditions. Each bar represents the mean \pm SEM of 4 independent preparations.

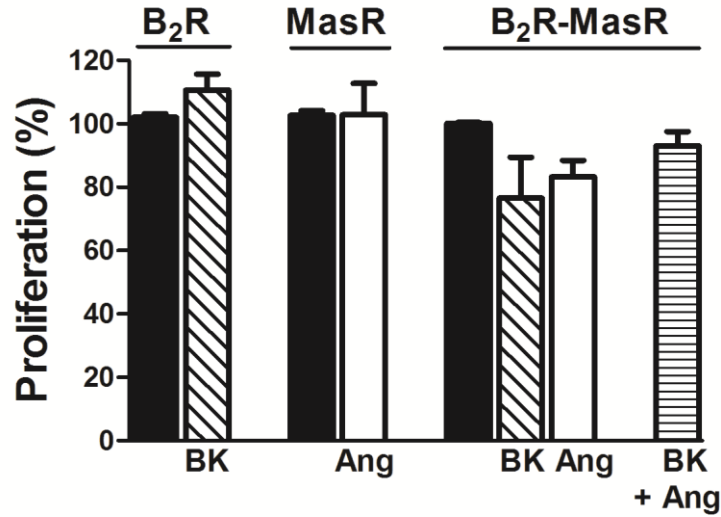


Figure S4. Cellular proliferation was evaluated in HEK293T cells expressing B₂R, MasR or B₂R-MasR incubated in the absence (black bar) or presence of BK or Ang-(1-7) (Ang) or BK + Ang. Results are expressed as the percentage change of proliferation detected in basal conditions (black bars). Each bar represents the mean \pm SEM of quintuplicate of 4 independent experiments.

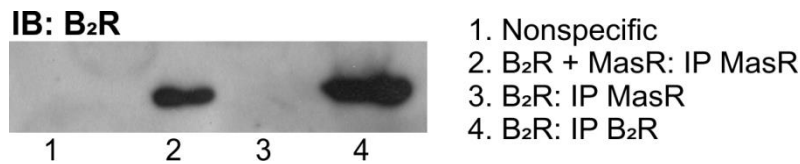


Figure S5. Immunoprecipitation of lysates from HEK293T cells transfected with B₂R and immunoprecipitated with an anti-MasR (well 3) or anti-B₂R (well 4) antibody or with B₂R + MasR and immunoprecipitated with anti-MasR antibody (well 2). Immunoprecipitates (IP) were analyzed by SDS-PAGE and immunoblotted (IB) using the indicated antibody. These blots are representative of three different experiments with similar qualitative results.