MAS1 Receptor Trafficking Involves ERK1/2 Activation Through a β-Arrestin2–Dependent Pathway

Flavia M. Cerniello, Oscar A. Carretero, Nadia A. Longo Carbajosa, Bruno D. Cerrato, Robson A. Santos, Hernán E. Grecco, Mariela M. Gironacci

Abstract—The MAS1 receptor (R) exerts protective effects in the brain, heart, vessels, and kidney. R trafficking plays a critical function in signal termination and propagation and in R resensitization. We examined MAS1R internalization and trafficking on agonist stimulation and the role of β-arrestin2 in the activation of ERK1/2 (extracellular signal-regulated kinase 1/2) and Akt after MAS1R stimulation. Human embryonic kidney 293T cells were transfected with the coding sequence for MAS1R-YFP (MAS1R fused to yellow fluorescent protein). MAS1R internalization was evaluated by measuring the MAS1R present in the plasma membrane after agonist stimulation using a ligand-binding assay. MAS1R trafficking was evaluated by its colocalization with trafficking markers. MAS1R internalization was blocked in the presence of shRNAcaveolin-1 and with dominant negatives for Eps15 (a protein involved in endocytosed Rs by clathrin-coated pits) and for dynamin. After stimulation, MAS1R colocalized with Rab11—a slow recycling vesicle marker—and not with Rab4—a fast recycling vesicle marker—or LysoTracker—a lysosome marker. Cells transfected with MAS1R showed an increase in Akt and ERK1/2 activation on angiotensin-(1–7) stimulation, which was blocked when the clathrin-coated pits pathway was blocked. Suppression of β-arrestin2 by shRNA reduced the angiotensin-(1–7)–induced ERK1/2 activation, whereas Akt activation was not modified. We conclude that on agonist stimulation, MAS1R is internalized through clathrin-coated pits and caveolae in a dynamin-dependent manner and is then slowly recycled back to the plasma membrane. MAS1R induced Akt and ERK1/2 activation from early endosomes, and the activation of ERK1/2 was mediated by β-arrestin2. Thus, MAS1R activity and density may be tightly controlled by the cell. **(*Hypertension*. 2017;70:982-989. DOI: 10.1161/HYPERTENSIONAHA.117.09789.)** • **Online Data Supplement**

Key Words: angiotensin-(1–7) ■ arrestin ■ endocytosis ■ endosomes ■ MAS1 receptor ■ trafficking

The MAS1 receptor is a GPCR (G protein-coupled receptor) that exerts protective effects in the brain, heart, vessels, and kidney.[1–3] Furthermore, MAS1R stimulation inhibits cells growth and induces anti-inflammatory and antifibrotic effects.[1,4]Angiotensin-(1–7) (Ang-(1–7)) is the endogenous ligand for MAS1R.[5] Thus, the axis formed by Ang-(1–7) and MAS1R represents a counter-regulatory pathway within the renin–angiotensin system, opposing the pressor and proliferative arm represented by Ang II and the Ang II receptor, type 1 (AT_1R).

Although most published reports propose a detrimental effect with MAS1R deficiency, other reports show the adverse effects of Ang-(1–7) and the beneficial effects of MAS1R deficiency. For instance, a genetic deletion of MAS1R abolishes salt-induced hypertension in mice,[6] and MAS1R renal deficiency diminishes renal damage in models of renal insufficiency.[7] The genetic backgrounds may explain these apparently divergent conclusions. Thus, C57Bl/6 MAS1R-deficient mice remain normotensive, whereas FVB/N MAS1R-deficient animals exhibit elevated blood pressure.[8]

To ensure that extracellular stimuli are translated into intracellular signals of the appropriate magnitude and duration, most signaling cascades are tightly regulated. One of the mechanisms by which GPCR signaling is regulated is through internalization, where receptors are physically removed from the cell surface by endocytosis through different pathways, such as clathrin-coated pits (CCPs), caveolae, or endocytic pathways in which neither specific coat proteins nor a particular pinching machinery have been identified.[9,10] Once internalized, the R may be recycled back to the plasma membrane through tubules that emanate from the uncoated areas of the endosome by fast or slow kinetics or may be targeted to lysosomes for complete degradation.[11,12] Several lines of evidence show that Rs may also be directed to the nucleus, where they activate different signals from those activated from the plasma

Received May 30, 2017; first decision June 21, 2017; revision accepted August 11, 2017.

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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.117.09789/-/DC1.

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membrane.[13,14] Thus, R trafficking may regulate the ultimate response elicited by the R.

Another player in GPCR endocytosis is β-arrestin. β-arrestins act not only as regulators of desensitization but also as multifunctional adaptor proteins that have the ability to signal through multiple mediators, such as MAPKs (mitogen-activated protein kinases), nuclear factor-κB, and phosphoinositide 3-kinase, regulating GPCR signaling and trafficking.[9,15,16] For instance, β-arrestins scaffold various components of the ERK (extracellular signal-regulated kinase) cascade, bringing them into proximity to promote ERK activation.[17] Signaling that is mediated by β-arrestins has distinct biochemical and functional consequences from those mediated by G proteins, and several biased ligands and receptors have been identified that preferentially signal through either G protein- or β-arrestin–mediated pathways.[16]

Regarding MAS1R, we previously showed, through immunofluorescence, that MAS1R colocalized with the early endosome marker and CAV-1 (caveolin-1) after Ang-(1–7) stimulation, suggesting that both internalization pathways (CCP and caveolae) are involved in MAS1R internalization.[17] However, no knowledge about MAS1R trafficking has been reported. We hypothesize that MAS1R interacts with β-arrestins on agonist stimulation and is then internalized into early endosomes through clathrin and caveolar pathways in a dynamin-dependent manner. Once internalized, MAS1R may signal from the early endosome through a β-arrestin–dependent pathway and then may be recycled back to the plasma membrane. Thus, our aim was to investigate MAS1R trafficking on Ang-(1–7) stimulation and to determine whether it activates other signaling cascades independent of G protein activation once it is internalized.

Materials and Methods

All the experiments were performed with the chimera MAS1R-YFP (MAS1R fused to yellow fluorescent protein), but to make the reading easier, the notation MAS1R was used instead. We have previously shown that Ang-(1–7) elicited the same affinity for both the chimera MAS1R-YFP and MAS1R.[17]

Materials

Fetal bovine serum, penicillin–streptomycin, Lipofectamine 2000, goat antimouse antibody coupled to Alexa 594, and Dulbecco-modified Eagle medium were purchased from Invitrogen (Carlsbad, CA). Bovine seroalbumin, paraformaldehyde, phosphate buffered saline, and the protease inhibitors cocktail were from Sigma Chemical, Co (St. Louis, MO). Mouse anti Rab4, Rab11, or anti-CAV-1 antibodies were purchased from BD Biosciences. LysoTracker Red was from Molecular Probes. Ang-(1–7) was from Bachem. The plasmid containing MAS1R-YFP cDNA was obtained as previously described.[17,18] shRNA β-arrestin2 was from SantaCruz. All other chemicals were analytic grade reagents of the highest purity available.

Ligand Concentrations

Concentrations of 1 μmol/L of Ang-(1–7) were used in the present study because we have previously shown that 1 μmol/L of Ang-(1–7) induces R internalization.[17,18]

Detailed methods are provided in the online-only Data Supplement.

Statistical Analysis

All the average results are presented as the mean±SEM. Kruskal–Wallis test followed by Dunn multiple comparison test and a 1-way ANOVA computation combined with the Bonferroni test were used to analyze the data. A probability level of 0.05 was considered significant.

Results

MAS1R Is Internalized Through the Clathrin and Caveolar Pathways in a Dynamin-Dependent Manner

We previously showed, via immunofluorescence, that MAS1R colocalized with AP50—a key protein in the CCP pathway—and CAV-1 on agonist stimulation,[17] suggesting that MAS1R is internalized through both pathways. In the present study, we further studied the MAS1R internalization pathway but used a more specific approach. Here, we measured the presence of MAS1R in the plasma membrane after agonist stimulation by a ligand-binding assay in cells where either one or both endocytic pathways were blocked. HEK293T cells were cotransfected with the MAS1R-YFP coding sequence plus the dominant-negative Eps15Δ95/295 (Eps15) or shRNA CAV-1, and after 48 hours, the cells were stimulated with 1 μmol/L Ang-(1–7) for 15 minutes. Eps15 is constitutively associated with the plasma membrane adaptor complex AP-2, which participates in the recruitment of endocytosed receptors in CCP.[19] Thus, in the presence of the dominant negative for Eps15, the CCP pathway is reduced. As shown in Figure 1, MAS1R internalization decreased when the CCP or caveolar endocytosis pathways were reduced by the dominant negative for Eps15 or shRNA CAV-1, respectively. Figure S1 shows that the shRNA targeting CAV-1 effectively silenced the expression of CAV-1. When

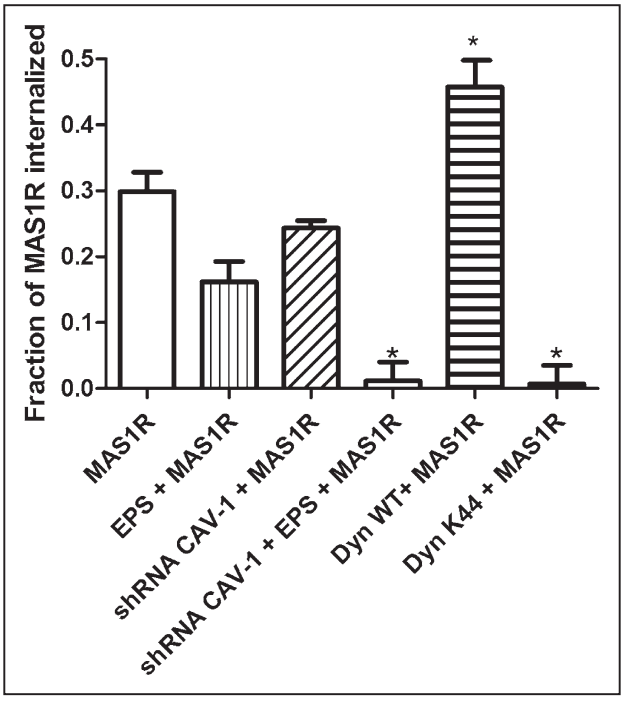

Figure 1. MAS1R is internalized via clathrin-coated pits and caveolae in a dynamin-dependent manner. HEK 293T cells transiently transfected with MAS1R alone or in combination with the dominant negatives for Eps15 (EPS) or shRNA caveolin-1 (shRNA CAV-1), shRNA CAV-1 plus EPS or wild-type dynamin (Dyn WT), or the dominant-negative dynamin K44A (Dyn K44), were incubated with 1 μmol/L angiotensin-(1–7) (Ang-(1–7)) for 15 min. Then, MAS1R internalization was determined by specific [^{125}I]Ang-(1–7) binding to intact cells, as described in the Methods section. The data are presented as the mean±SEM of triplicates of 4 independent experiments. *P<0.05 compared with MAS1R alone.

both pathways were blocked at the same time, MAS1R was not internalized on agonist stimulation (Figure 1), demonstrating that MAS1R was internalized by both CCP and caveolae.

GTPase dynamin is necessary for the scission of vesicles during clathrin-mediated and several types of clathrin-independent endocytosis.[8,9,20,21] To investigate the role of dynamin in MAS1R internalization, wild-type dynamin or the dominant-negative K44A dynamin were coexpressed with MAS1R in HEK 293T cells, and R internalization, on agonist stimulation, was investigated. The expression of wild-type dynamin induced an increase in MAS1R internalization after 1 µmol/L Ang-(1–7) stimulation for 15 minutes. In contrast, the expression of the dominant negative for dynamin (K44A dynamin) completely blocked MAS1R internalization in response to Ang-(1–7) stimulation (Figure 1). These data demonstrated that MAS1R endocytosis was dynamin-dependent.

Altogether, these data demonstrated that MAS1R was internalized through CCP and caveolar pathways in a dynamin-dependent manner.

Internalized MAS1R Is Recycled Back to the Plasma Membrane

Once internalized, the R may be targeted to lysosomes for complete degradation or be recycled back to the plasma membrane.[10,12,21] To investigate MAS1R trafficking once it is internalized, HEK293T cells transfected with MAS1R-YFP were stimulated with Ang-(1–7) for 15 minutes. We previously showed that after 15 minutes of agonist stimulation, ≈50% of the Rs were internalized.[17] After washing the medium, the cells were incubated in fresh media for different lengths of time, and the percentage of MAS1R present in the plasma membrane was evaluated using a ligand-binding assay. This fraction of Rs corresponded to the Rs recycled back to the plasma membrane. After 30 minutes, MAS1R was recycled back to the plasma membrane (Figure 2A). The same result was observed in the presence of 10 µmol/L cycloheximide (data not shown)—a protein synthesis inhibitor—indicating that the Rs quantified in the plasma membrane after agonist stimulation did not result from de novo synthesis.

Trafficking in the endosomal compartment is controlled by small GTP-binding proteins of the Rab family.[11,12,21,22] Rab4 regulates the rapid recycling of vesicles containing GPCRs to the cell surface, whereas Rab11 regulates slow vesicular recycling.[21,22] We investigated MAS1R recycling to the cell surface once internalized by measuring the colocalization of the R with Rab 4 or Rab 11 in MAS1R-YFP–transfected cells stimulated in the absence or presence of 1 µmol/L Ang-(1–7) for 15 and 30 minutes. Figure 2B shows that MAS1R-YFP colocalized with Rab11 but not with Rab4 after 30 minutes of stimulation with Ang-(1–7), suggesting that the R is recycled back to the plasma membrane by slow recycling vesicles. A similar result was obtained when the cells were incubated for 15 minutes with the agonist (data not shown).

MAS1R Is Not Targeted to Lysosomes on Agonist Stimulation

To investigate whether MAS1R was targeted to the lysosomes, MAS1R expression was evaluated using western blotting in MAS1R-YFP–transfected cells in the absence or presence of 1 µmol/L Ang-(1–7) treatment for 30 and 60 minutes. No changes in MAS1R expression were observed (Figure 3A), suggesting that MAS1R is not being degraded once internalized. To further confirm this result, the colocalization of MAS1R-YFP with LysoTracker—a lysosome marker—was evaluated. MAS1R-YFP did not colocalize with the lysosome marker in the MAS1R-YFP–transfected cells incubated with Ang-(1–7) for 30 minutes (Figure 3B), demonstrating that the R was not targeted to lysosomes for degradation after agonist stimulation. Similar results were obtained when the cells were incubated with the agonist for 60 minutes (data not shown).

MAS1R Is Not Present in the Nucleus After Agonist Stimulation

Some GPCRs are present in the nucleus[13,14] or are translocated to this cellular compartment after agonist stimulation, which was previously demonstrated for the AT_1R.[23,24] We investigated whether MAS1R was present in the nucleus and whether it was translocated to this compartment after Ang-(1–7) treatment. MAS1R was not present in the nucleus of the MAS1R-transfected cells. After 30 minutes of Ang-(1–7) stimulation, there was no MAS1R expression in the nucleus, demonstrating that MAS1R is not translocated to this compartment (Figure 3C). Similar results were obtained when the cells were incubated with the agonist for 45 and 60 minutes (data not shown).

MAS1R Induces ERK Activation From Early Endosomes Through a β-Arrestin2-Dependent Pathway

In addition to functioning as adaptor proteins to facilitate the endocytosis of GPCRs, β-arrestins scaffold a wide variety of signaling complexes bringing them into proximity to promote their activation.[15,16,21] We investigated whether MAS1R, once internalized, induced Akt and ERK1/2 activation and β-arrestin2 involvement in MAS1R-mediated Akt and ERK1/2 activation. HEK293T cells transfected with MAS1R and stimulated with Ang-(1–7) for 10 minutes showed an increase in Akt phosphorylation (Figure 4A). The blockade of MAS1R endocytosis with the dominant negative for dynamin or for Eps15 decreased Ang-(1–7)–induced Akt phosphorylation (Figure 4B). Conversely, the blockade of CAV-1 or β-arrestin2 did not modify the response of Ang-(1–7) on Akt activation (Figure 4B). Altogether, these results suggest that the MAS1R-mediated Akt activation occurs once the R is internalized through CCP via a β-arrestin2–independent pathway.

We then examined the effect of the RNAi-mediated suppression of β-arrestin2 expression on ERK1/2 activation after the stimulation of transiently expressed MAS1R in HEK293T cells. MAS1R-transfected cells were stimulated with 1 µmol/L Ang-(1–7) for 10 minutes, and ERK1/2 phosphorylation was evaluated. Ang-(1–7) induced an increase in ERK1/2 phosphorylation (Figure 5A). The stimulatory effect of Ang-(1–7) on ERK1/2 activation was diminished when MAS1R internalization was blocked by the dominant negatives for dynamin or for Eps15 (Figure 5B), whereas it was not modified when CAV-1 expression was suppressed (Figure 5B). The suppression of β-arrestin2 expression reduces receptor-mediated ERK1/2 activation by 75% (Figure 5C), suggesting that

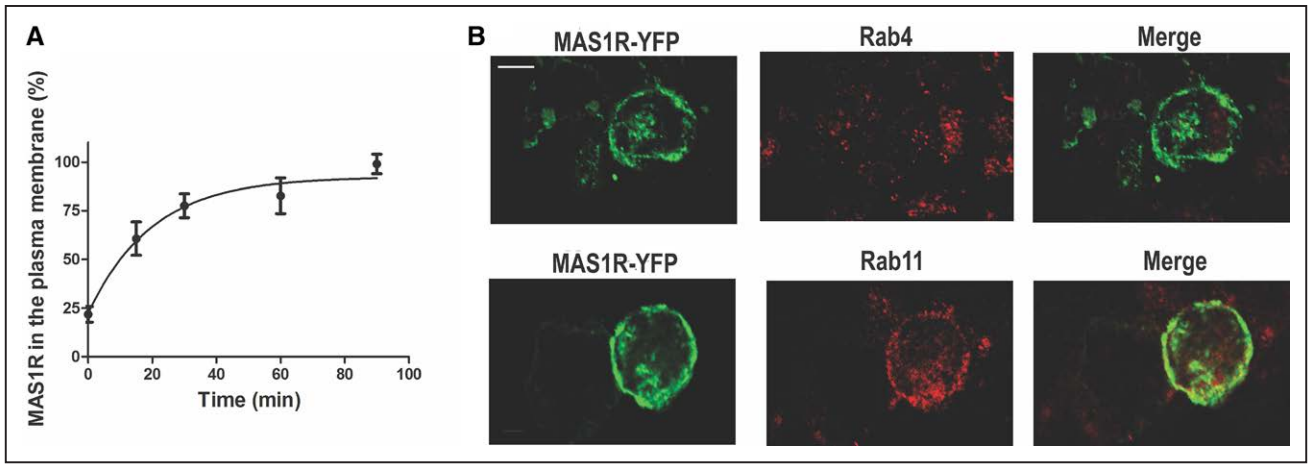

Figure 2. MAS1R is recycled back to the plasma membrane through slow recycling vesicles after agonist stimulation. **A**, HEK 293T cells, transiently transfected with MAS1R, were incubated with 1 μmol/L angiotensin-(1–7) (Ang-(1–7)) for 15 min. The medium was removed, and the cells were incubated, at different times, with fresh medium. The percentage of MAS1R recycled back to the plasma membrane was determined using a ligand-binding assay to intact cells, as described in the Methods section. The data are presented as the mean±SE of triplicates of 4 independent experiments. **B**, The colocalization of MAS1R-YFP (yellow fluorescent protein; green) and fast recycling vesicles (Rab4) or slow recycling vesicles (Rab11) markers (red) in MAS1R-YFP transfected HEK293T cells stimulated with Ang-(1–7) for 30 min. Three independent experiments were analyzed (text). Scale bar=10 μm.

MAS1R induces ERK1/2 activation through a β-arrestin2–dependent pathway once internalized through CCP.

Figure S1 showed that an siRNA targeting β-arrestin2 effectively silenced the expression of β-arrestin2.

Discussion

R trafficking is critical for the regulation of the temporal and spatial aspects of GPCR signaling. Through the shRNA-mediated knockdown of the CAV-1 protein or a dominant negative

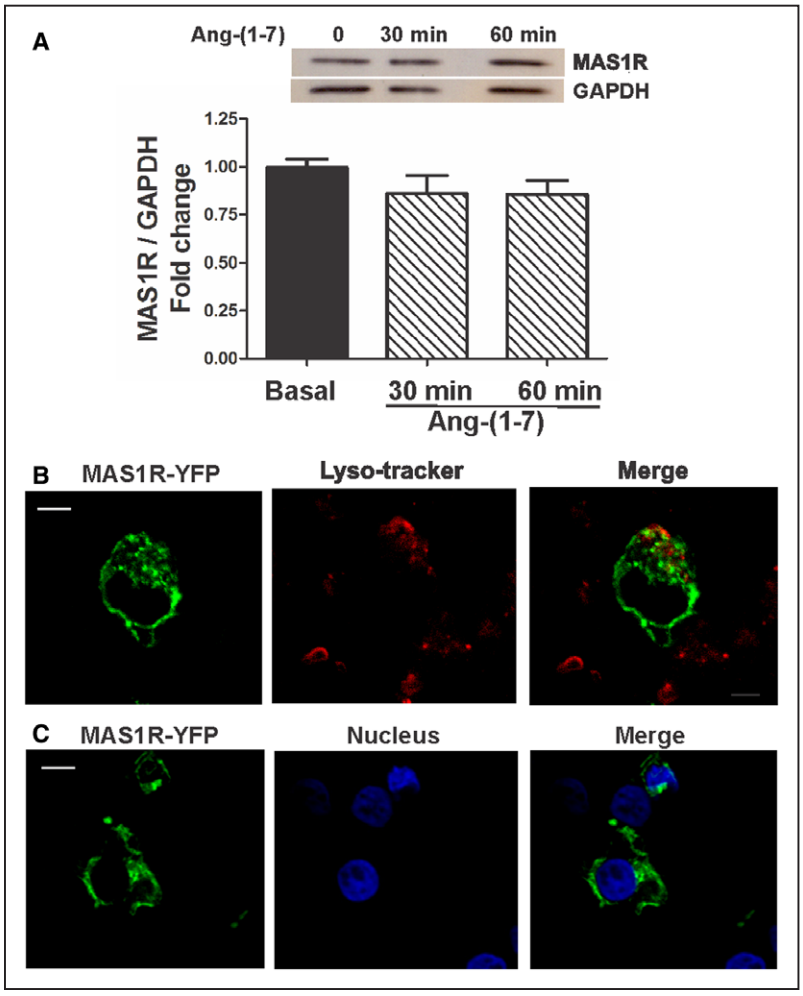

Figure 3. MAS1R is not targeted to the lysosomes or the nucleus after agonist stimulation. **A**, MAS1R expression in MAS1R-transfected HEK293T cells stimulated in the absence (basal) or presence of angiotensin-(1–7) (Ang-(1–7)) treatment for 30 and 60 min. The results are expressed as the fold change of the response detected in the basal conditions. Each bar represents the mean±SEM of 3 independent preparations. **B**, Colocalization of MAS1R-YFP (yellow fluorescent protein; green) and the lysosome marker LysoTracker (red) or (**C**) the nucleus (blue) in MAS1R-YFP transfected HEK293T cells stimulated with Ang-(1–7) for 30 min. Three independent experiments were analyzed (text). Scale bar=10 μm.

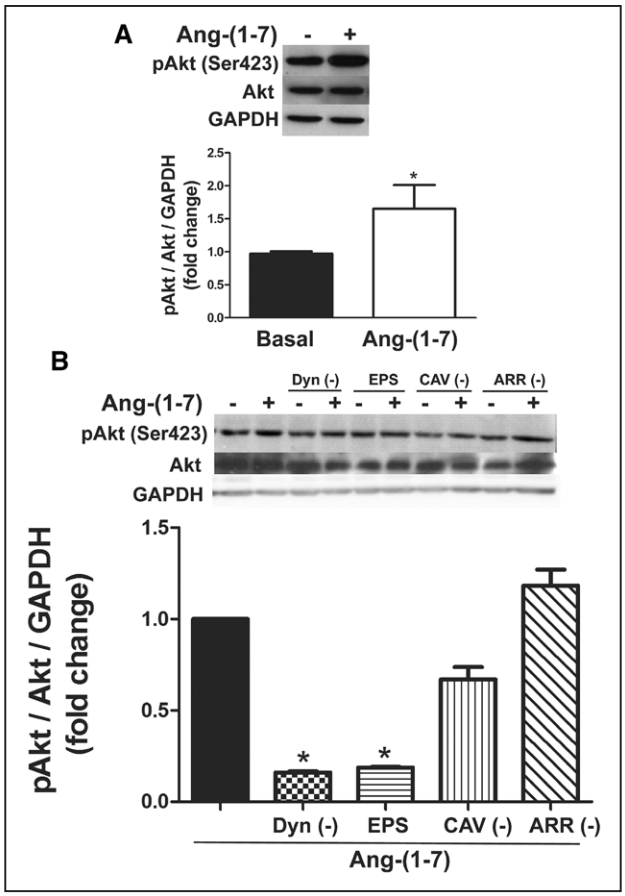

Figure 4. β-Arrestin2 is not involved in MAS1R-dependent Akt activation. **A**, Akt phosphorylation in the MAS1R-transfected cells incubated in the absence (basal) or presence of angiotensin-(1–7) (Ang-(1–7)) treatment for 10 min. A representative western blot of total and phosphorylated Akt after 10 min of agonist treatment. The results are expressed as the fold change of the response detected in the basal conditions. Each bar represents the mean±SEM of 4 independent preparations. **P*<0.05 vs basal. **B**, Akt phosphorylation in the cells transfected with MAS1R or MAS1R+dominant negative for dynamin (Dyn–) or MAS1R+dominant negative for Eps15 (EPS–) or MAS1R+siRNA β-arrestin2 (ARR–) or MAS1R+shRNA caveolin-1 (CAV–), which were incubated in the absence or presence of Ang-(1–7) for 10 min. A representative western blot of total and phosphorylated Akt after 10 min of agonist treatment. The results are expressed as the fold change of the response elicited by Ang-(1–7) in the MAS1R-transfected cells. Each bar represents the mean±SEM of 4 independent preparations. **P*<0.05 vs MAS1R-transfected cells treated with Ang-(1–7).

for a protein involved in the CCP pathway (Eps15) or a dominant negative for dynamin, we demonstrated that MAS1R was endocytosed through both the CCP and caveolae pathways in a dynamin-dependent manner. Once inside the cell, MAS1R was recycled back to the plasma membrane by slow recycling vesicles. Based on the present results, a proposed model depicting the intracellular trafficking of MAS1R is shown in Figure 6. In certain cases, the overexpression of proteins affects their function and results in promiscuous associations. In the present study, only MAS1R was overexpressed, and endogenous proteins, such as CAV-1 and β-arrestin2, were silenced; therefore, we disregard a promiscuous association between MAS1R and those proteins. In addition, ongoing experiments from our laboratory showed that MAS1R, endogenously expressed in hypothalamic neurons from rats, undergoes the same trafficking described in this work. Conversely, a study by Galandrin et al[25] showed that Ang-(1–7) acts as an AT_1R-biased agonist to selectively promote β-arrestin activation, which was performed with AT_1R and β-arrestin2 overexpression and that the association may result from overexpression.

Regarding other Rs of the renin–angiotensin system, the internalization of the AT_1R depends on the concentration of the ligand, that is, at high concentrations, the R is internalized through a dynamin and arrestin-independent manner,[26] but at physiological hormone concentrations, endocytosis of the AT_1R occurs predominantly via an arrestin and dynamin-dependent mechanism.[27] In contrast, the AT_2R is not internalized after agonist stimulation.[27] Thus, the mechanism by which GPCRs are internalized is specific for each R.

MAS1R internalization via CCP was coupled to Akt and ERK1/2 activation (present results). Why MAS1R endocytosis also occurred through caveolae is still without an explanation based on the present results. The integration of different endocytic routes is crucial for determining the net signaling output. In the case of the EGFR (epidermal growth factor R) and the transforming growth factor-β R, clathrin-mediated endocytosis couples the R with recycling, and sustainment of the signaling and nonclathrin-mediated endocytosis couples the Rs with degradation and signaling attenuation.[11] We disregard that MAS1R endocytosis, through caveolae, may be coupled to degradation because we showed that MAS1R expression was not modified and it was not targeted to the lysosomes after 60 minutes of agonist stimulation (Figure 3). Some GPCRs are often directed for lysosomal degradation via the conserved endosomal-sorting complex required for the transport machinery, but others are directed to the resensitization pathway,[28] as occurs with the MAS1R (the current results). Other GPCRs, such as the metabotropic glutamate receptors 1 and 5, the human follitropin receptor, different opioid receptors, acetylcholine receptors, or glycine receptors, undergo basal and agonist-induced proteasomal degradation.[28,29] For instance, the AT_1R is directed to lysosomes, but it is also recycled back to the plasma membrane by both slow and rapid recycling vesicles, and when it is heterodimerized with the dopamine receptor D_5R, it targets to proteasomes for degradation, with the half-life of the AT_1R ≈150 minutes.[30] Thus, R trafficking is specific for each R. Certainly, at some stage, a fraction of the MAS1Rs may be directed to lysosomes or proteasomes for degradation, but at the time interval assayed in the present study (≤60 minutes), the MAS1R was mostly recycled back to plasma membrane and not a significant amount of the MAS1R was detected in the lysosomes.

Trafficking of Rs from early endosomes involves the small GTP-binding proteins Rab4 and Rab11.[11,21,22] AT_1R uses both Rab4 and Rab11 for recycling.[25–27] Unlike AT_1R, MAS1R was recycled back only by slow recycling vesicles. After 30 minutes of being internalized, MAS1R was recycled to the plasma membrane (Figure 2), allowing, in this case, a stimulatory long-lasting effect on signaling.

Evidence from isolated nuclei indicates the presence of GPCRs on the nuclear membrane that can activate similar

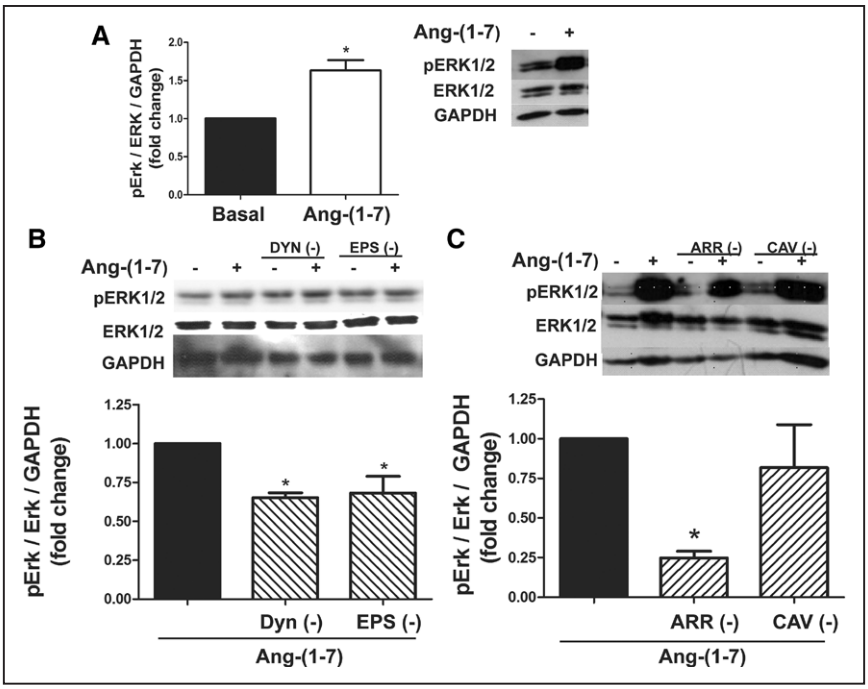

Figure 5. β-Arrestin2 is involved in MAS1R-dependent ERK1/2 (extracellular signal-regulated kinase 1/2) activation. **A**, ERK1/2 phosphorylation in MAS1R-transfected cells incubated in the absence (basal) or presence of angiotensin-(1–7) (Ang-(1–7)) for10 min. A representative western blot of total and phosphorylated ERK1/2 after 10 min of agonist treatment. The results are expressed as the fold change of the response detected in the basal conditions. Each bar represents the mean±SEM of 4 independent preparations. *P<0.05 vs basal. **B**, ERK1/2 phosphorylation in the cells transfected with MAS1R or MAS1R+dominant negative for dynamin (Dyn–) or MAS1R+dominant negative for Eps15 (EPS–), which were incubated in the absence or presence of Ang-(1–7) for 10 min. A representative western blot of total and phosphorylated ERK1/2 after 10 min of agonist treatment. The results are expressed as the fold change of the response elicited by Ang-(1–7) in the MAS1R-transfected cells. Each bar represents the mean±SEM of 4 independent preparations. **C**, ERK1/2 phosphorylation in the cells transfected with MAS1R or MAS1R+siRNA β-arrestin2 (ARR–) or MAS1R+shRNA caveolin-1 (CAV-1), which were incubated in the absence or presence of Ang-(1–7) for 10 min. A representative western blot of total and phosphorylated ERK1/2 after 10 min of agonist treatment. The results are expressed as the fold change of the response elicited by Ang-(1–7) in the MAS1R-transfected cells. Each bar represents the mean±SEM of 4 independent preparations. *P<0.05 vs MAS1R-transfected cells treated with Ang-(1–7).

G protein-dependent signaling pathways in the nucleus as at the cell surface.[13,14] For instance, the rat neuronal AT_1R is targeted to the nucleus when it is stimulated by Ang II, and this sequestration may have implications on the Ang II–induced expression of the tyrosine hydroxylase gene.[25] Ang II rapidly induces AT_1R internalization, nuclear translocation, and nuclear de novo synthesis of this receptor in vascular smooth muscle cells, modulating, in this way, sustained free cytosolic calcium and nuclear calcium.[24] Regarding MAS1R, isolated nuclei from the renal cortex of sheep express MAS1Rs that are functionally linked to nitric oxide formation.[31] These authors did not report MAS1R translocation to the nucleus on agonist stimulation. In our present study, nuclear MAS1R expression was not observed in HEK 293T cells, and there was not even translocation to the nucleus after Ang-(1–7) stimulation. We do not have an explanation for this discrepancy.

Endosomes are key signaling stations that sustain signals that originate from the plasma membrane and generate unique signals that are prohibited at the plasma membrane, thus, contributing to signal diversification and specificity.[11] Our present work showed that MAS1R-mediated Akt activation occurred once the R was internalized through CCP in a β-arrestin2–independent manner. Similar findings were reported for the epidermal growth factor R.[11] In endosomes that contain internalized epidermal growth factor R, 2 related Rab5 effectors are recruited, which lead to Akt activation.[11] On the contrary, we showed that ERK1/2 activation was also triggered when MAS1R was internalized on agonist stimulation into early endosomes via CCP but through a β-arrestin2–dependent manner. It is worth noting that, in other cell types, a decrease in ERK1/2 activity is reported on MAS1R stimulation.[32,33] This is consistent with the notion that ERK1/2 (and the MAPK pathway) transduces a variety of extracellular signals, resulting in different biological responses.

In the present study, Ang-(1–7) induced MAS1R-mediated signaling through β-arrestin2. It was previously shown that Ang-(1–7) acts as an AT_1R-biased agonist to selectively promote β-arrestin activation while blocking the detrimental Ang II/AT_1R/Gq axis.[25]

Perspectives

In conclusion, we demonstrated that, on Ang-(1–7) stimulation, MAS1R is internalized through CCP and caveolae in a dynamin-dependent pathway. Once internalized, MAS1R induces Akt activation in a β-arrestin2–independent mechanism. Conversely, β-arrestin2 is involved in MAS1R-induced ERK activation. Subsequently, MAS1R is recycled back to the plasma membrane through slow recycling vesicles (Figure 6). The elucidation of the mechanisms of MAS1R endocytosis and trafficking is essential not only for a better understanding

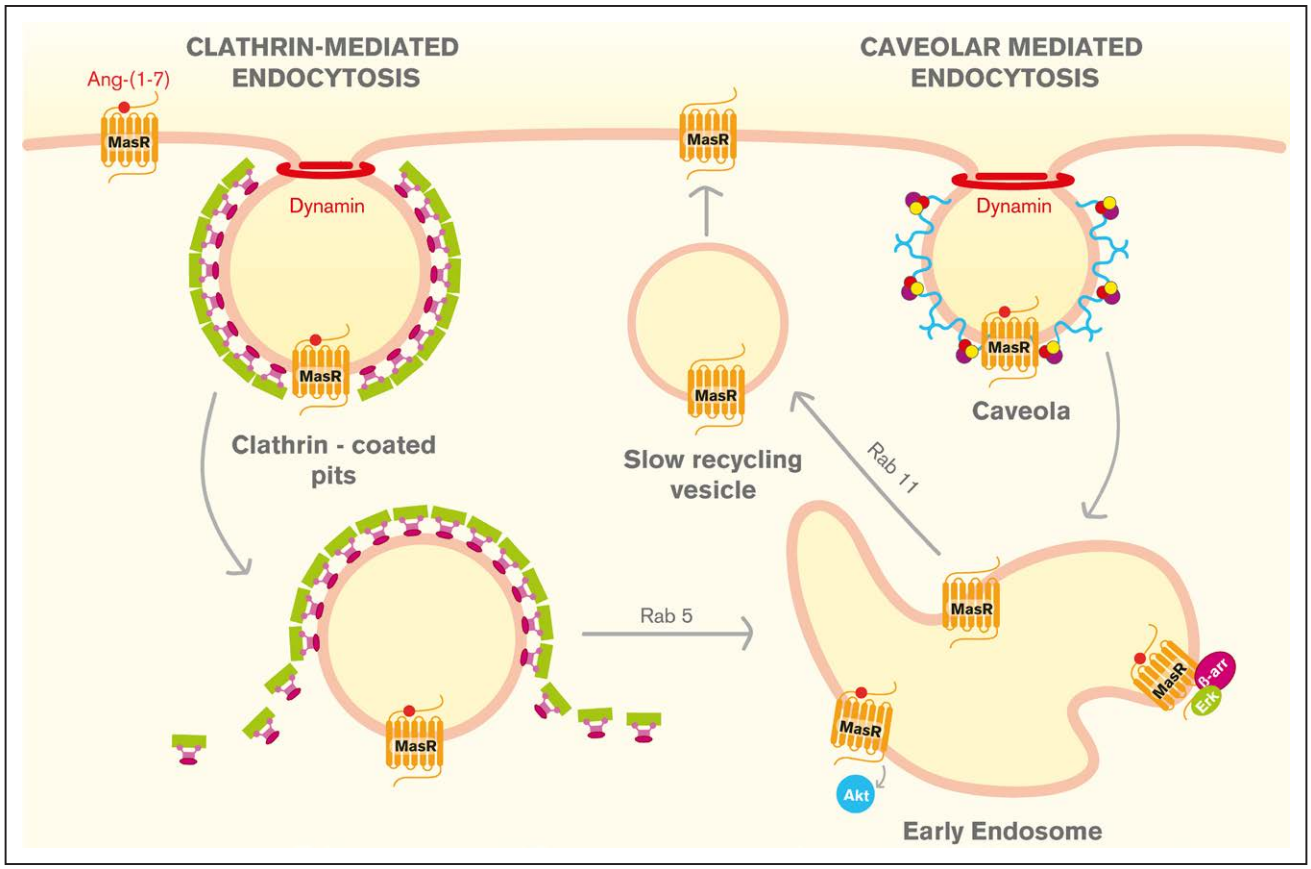

Figure 6. Schematic representation of MAS1R trafficking on agonist stimulation. After ligand binding, MAS1R (MasR) undergoes internalization by either clathrin-coated pits or caveolae through a dynamin-dependent mechanism. Endocytic vesicles fuse with early endosomes and stimulate Akt and ERK1/2 (extracellular signal-regulated kinase 1/2) phosphorylation. MAS1R-mediated ERK1/2 activation is mediated by β-arrestin2. Finally, the R is recycled back to the plasma membrane through a slow recycling route that depends on the action of Rab11.

the R biology but it also translates into a better understanding of GPCR signaling in human health and disease. This development will further our understanding of MAS1R pharmacology and function and open new opportunities for the development of strategies to therapeutically manipulate GPCR functions in diseases associated with altered GPCR signaling, such as hypertension and congestive heart failure.

Sources of Funding

This work was supported by the Universidad de Buenos Aires (20020160100134BA), the Agencia Nacional de Promoción Científica y Tecnológica (2013–2170), the Consejo Nacional de Investigaciones Científicas y Técnicas (11220110101097), and the National Institutes of Health Grant (HL028982).

Disclosures

None.

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Novelty and Significance

What Is New?

- R trafficking is critical for the regulation of the temporal and spatial aspects of R signaling.
- We provide evidence, for the first time, about MAS1R trafficking and the signaling pathway activated during this process.

What Is Relevant?

- On agonist stimulation, MAS1R induces Akt and ERK (extracellular signal-regulated kinase) activation once it is internalized into early endosomes, and the ERK activation is mediated by β-arrestin2.
- Subsequently, MAS1R is slowly recycled back to the plasma membrane.

Summary

R trafficking plays an important role in signal termination, propagation and resensitization. We showed that, on agonist stimulation, MAS1R is internalized through clathrin-coated pits and caveolae in a dynamin-dependent manner. MAS1R induced Akt and ERK1/2 activation from early endosomes, and only the activation of ERK1/2 was mediated by β-arrestin2. Subsequently, the receptor was slowly recycled back to the plasma membrane. In this way, MAS1 R activity and density may be tightly controlled by the cell.

Hypertension
Journal of the American Heart Association

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Hypertension. 2017;70:982-989; originally published online September 5, 2017;
doi: 10.1161/HYPERTENSIONAHA.117.09789
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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

ONLINE SUPPLEMENT

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METHODS

All the experiments were carried out with the chimera MAS1R-YFP, but to make the reading easier, the notation MAS1R was used instead. We have previously shown that Ang-(1-7) elicited the same affinity for both the chimera MAS1R-YFP and MAS1R.[1]

Materials

Fetal bovine serum, penicilin-streptomycin, Lipofectamine 2000, goat anti-mouse antibody coupled to Alexa 594 and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Carlsbad, CA, USA). Bovine seroalbumin, paraformaldehyde, phosphate buffered saline (PBS) and the protease inhibitors cocktail were from Sigma Chemical Co. (St. Louis, MO, USA). Mouse anti Rab4, Rab11 or anti-CAV-1 antibodies were purchased from BD Biosciences. LysoTracker Red was from Molecular Probes. Ang-(1-7) was from Bachem. The plasmid containing MAS1R-YFP cDNA was obtained as previously described.[1] shRNA ß-arrestin2 was from SantaCruz. All other chemicals were analytical grade reagents of the highest purity available.

Ligand concentrations

Concentrations of 1 µmol/L of Ang-(1-7) was employed in the present study because we have previously shown that 1 µmol/L of Ang-(1-7) induces R internalization.[1]

Cell culture and transfection

Human embryonic kidney (HEK) 293T cells were grown in DMEM high glucose supplemented with 10% heat-inactivated fetal bovine serum and penicillin-streptomycin at 37 °C in a humidified atmosphere at 95% air and 5% CO_2. The cells were transiently

transfected using Lipofectamine 2000 according to the manufacturer's instructions and were used 48 h post-transfection.
The following plasmids were employed: pEGFP-N1-Dyn2-WT (wild-type dynamin); pEGFP-N1-Dyn2-K44A (dominant negative for dynamin) and pEGFP-C2-Eps15Δ95/295 (dominant negative for Eps15), which were kindly provided by Dr. Pablo Ortiz (Henry Ford Hospital, Detroit, USA); pEYFP-N1-MAS1R-YFP (obtained in our lab as previously described[1]) and the plasmid gene silencer targeted to CAV-1 (shRNA CAV-1) obtained from ViraQuest.

[^{125}I]-Ang-(1-7) labeling
Ang-(1-7) was labeled in our laboratory by the lactoperoxidase method as previously described.[2] 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 [^{125}I] 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 subjected to competition radioligand binding assay as previously described.[1,3] Briefly, cells were incubated at 4ºC for 60 min with incubation buffer containing 2 nmol/L of [^{125}I]Ang-(1-7) [labelled as described above] in the absence or presence of increasing concentrations of unlabelled Ang-(1-7) (Bachem). 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). Nonspecific binding was determined in the presence of 10 µmol/L unlabeled Ang-(1-7). Specific binding was calculated by the subtraction of nonspecific binding from total binding.

Receptor internalization assay
MAS1R internalization was determined by measuring MAS1R present in the plasma membrane after 1 µmol/L Ang-(1-7) stimulation during 15 min at 37 ºC in cells transfected with the plasmid pEYFP-N1-MAS1R-YFP or pEYFP-N1-MAS1R-YFP + pEGFP-N1-Dyn2-WT or pEYFP-N1-MAS1R-YFP + pEGFP-N1-Dyn2-K44A or pEYFP-N1-MAS1R-YFP + pEGFP-C2-Eps or pEYFP-N1-MAS1R-YFP +shRNA CAV-1 or the empty plasmid. 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 [^{125}I]Ang-(1-7) radioligand binding assays as described above and previously.[3] The fraction of internalized R was expressed as the decrease in specific [^{125}I]Ang-(1-7) compared with the total binding obtained in untreated cells.

MAS1R recycled back to the plasma membrane
Cells transfected with the plasmid pEYFP-N1-MAS1R-YFP or the empty plasmid were incubated in DMEM (pH 7.4) in the absence or presence of 1 µmol/L Ang-(1-7) during 30 min at 37 ºC to allow MAS1R internalization. We have previously shown that at that time around 70% of MAS1Rs were internalized.[1] Cells were washed with PBS to remove unbound Ang-(1-7) following by an ice-cold acid buffer (0.2 mol/L acetic acid pH 3.5, 150

mmol/L NaCl) wash to remove the cell surface receptor-bound agonist. Cells were then incubated with fresh growth medium during different times to allow R recycling to the plasma membrane. Then, R present in the plasma membrane was measured by [^{125}I]Ang-(1-7) binding assay as described above and previously.[3] These experiments were carried out in the presence and absence of 10 µmol/L cycloheximide, a protein synthesis inhibitor.

MAS1R-YFP trafficking

Cells transfected with the plasmid pEYFP-N1-MAS1R-YFP or the empty plasmid were incubated in the presence and absence of 1 µmol/L Ang-(1-7) during different times. After two washes with PBS, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in PBS and incubated in blocking solution (PBS/0.2% Triton X-100/3% BSA) for 30 min at room temperature. MAS1R trafficking was evaluated by measuring the colocalization between MAS1R-YFP and endocytosis markers signals by immunofluorescence as previously described.[3] The following antibodies were employed: anti-Rab4 or anti Rab11 mouse monoclonal antibodies (diluted 1:150 in blocking solution). 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) for 2h at room temperature.

To evaluate MAS1R targeting to lysosome, colocalization between MAS1R-YFP and the lysosome marker LysoTracker Red was evaluated. Cells transfected with the plasmid coding for MAS1R-YFP were incubated in a probe-containing growth medium during 45 min. The medium was replaced by fresh medium and cells were incubated in the presence and absence of 1 µmol/L Ang-(1-7) during 30 and 60 min. After two washes with PBS cells were fixed with 4% paraformaldehyde.

Samples were mounted and imaged using an Olympus Fluoview FV1000 spectral laser scanning confocal microscope with a 60x oil immersion lens using dual excitation (473 nm for YFP and 559 nm for Alexa 594 and LysoTracker Red). Due to the spectral properties of the scan head, fluorescence emission was collected between 520 and 550 nm for YFP and 600-660 nm for Alexa 594 and LysoTracker Red. Images were obtained using sequential scanning for each channel to eliminate the cross-talk of chromophores. Quantitative colocalization was estimated by Pearson´s correlation coefficient and overlap coefficient according to Manders, which were calculated using Image-Pro Plus software (MediaCybernetics Inc.). Negative controls consisted of mocked transfected cells treated with blocking solution in the absence of the primary antibody.

ERK and Akt phosphorylation assay

Transfected cells with the plasmid pEYFP-N1-MAS1R-YFP or pEYFP-N1-MAS1R-YFP + pEGFP-N1-Dyn2-K44A or pEYFP-N1-MAS1R-YFP + pEGFP-C2-Eps or pEYFP-N1-MAS1R-YFP + shRNA CAV-1 or pEYFP-N1-MAS1R-YFP + siRNA ß-arrestin2 or the empty plasmid were incubated in the absence or presence of 1 µmol/L Ang-(1-7) during 10 min at 37 ^{o}C. 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 µg/ml aprotinin and 2 µg/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 meassure 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.

RESULTS

Cells were transfected with shRNA targeting CAV-1 or siRNA targeting β-arrestin2 and CAV-1 or β-arrestin2 expression were measured by Western-blot. Figures S1 showed that shRNA targeting CAV-1 or siRNA targeting β-arrestin2 effectively silenced expression of CAV-1 or β-arrestin2, respectively.

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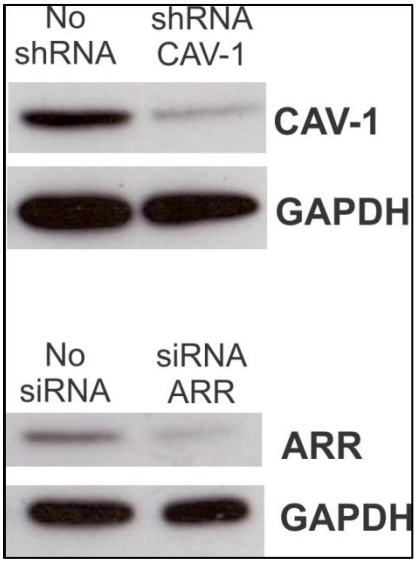

Figure S1. Expression of CAV-1 and β-arrestin2 (ARR) in cells transfected with shRNA CAV-1 or siRNA β-arrestin2