Angiotensin (1-7) Induces Mas Receptor Internalization

Mariela M. Gironacci, Hugo P. Adamo, Gerardo Corradi, Robson A. Santos, Pablo Ortiz, Oscar A. Carretero

Abstract—Angiotensin (Ang) (1-7) is the endogenous ligand for the G protein–coupled receptor Mas, a receptor associated with cardiac, renal, and cerebral protective responses. Physiological evidence suggests that Mas receptor (MasR) undergoes agonist-dependent desensitization, but the underlying molecular mechanism regulating receptor activity is unknown. We investigated the hypothesis that MasR desensitizes and internalizes on stimulation with Ang-(1-7). For this purpose, we generated a chimera between the MasR and the yellow fluorescent protein (YFP; MasR-YFP). MasR-YFP–transfected HEK 293T cells were incubated with Ang-(1-7), and the relative cellular distribution of MasR-YFP was observed by confocal microscopy. In resting cells, MasR-YFP was mostly localized to the cell membrane. Ang-(1-7) induced a redistribution of MasR-YFP to intracellular vesicles of various sizes after 5 minutes. Following the time course of [^{125}I]Ang-(1-7) endocytosis, we observed that half of MasR-YFP underwent endocytosis after 10 minutes, and this was blocked by a MasR antagonist. MasR-YFP colocalized with Rab5, the early endosome antigen 1, and the adaptor protein complex 2, indicating that the R is internalized through a clathrin-mediated pathway and targeted to early endosomes after Ang-(1-7) stimulation. A fraction of MasR-YFP also colocalized with caveolin 1, suggesting that at some point MasR-YFP traverses caveolin 1–positive compartments. In conclusion, MasR undergoes endocytosis on stimulation with Ang-(1-7), and this event may explain the desensitization of MasR responsiveness. In this way, MasR activity and density may be tightly controlled by the cell. **(*Hypertension*. 2011;58:176-181.)**

Key Words: receptor internalization ■ desensitization ■ angiotensin 1-7 ■ Mas receptor ■ trafficking

The renin-angiotensin (Ang) system consists of 2 distinct and counterregulatory axes. The classic Ang-converting enzyme/Ang II/Ang II type 1 receptor (AT_1R) axis is responsible for the vasoconstrictive, proliferative, hypertensive, and fibrotic actions of the renin-Ang system. Its hyperactivity is associated with hypertension and cardiovascular diseases, such as cardiac hypertrophy, heart failure, stroke, coronary artery disease, and end-stage renal disease. This axis is the primary target for the antihypertensive therapy.[1] The Ang-converting enzyme 2/Ang-(1-7)/Mas receptor (MasR) axis constitutes an alternative axis that represents an intrinsic mechanism to induce vasoprotective actions by counterregulating the Ang-converting enzyme/Ang II/AT_1R axis, thus inducing many beneficial effects in cardiovascular diseases. This vasoprotective axis of the renin-Ang system could be targeted for novel therapeutics strategies.[1,2]

Ang-(1-7) is the endogenous ligand for the G protein–coupled receptor (GPCR) Mas.[3] Prolonged stimulation of MasR with Ang-(1-7) or with high concentrations of the ligand caused an attenuation of receptor responsiveness,[4–6] suggesting receptor desensitization. Receptor desensitization represents an important physiological "feedback" mechanism that protects against both acute and chronic receptor overstimulation and is the consequence of a combination of different mechanisms.[7] These mechanisms include the uncoupling of the receptor from heterotrimeric G proteins in response to receptor phosphorylation, followed by the internalization of cell surface receptors to intracellular membranous compartments. Once internalized, the receptor may be recycled back to the cell surface in a resensitized state competent for signaling, or may be sorted to lysosomes or proteasome for degradation, a process important for signal termination.[7–9] Thus, receptor trafficking has critical function in signal termination and propagation, as well as receptor resensitization. The rates of GPCR internalization, recycling, and lysosomal sorting differ widely among receptors, suggesting that different mechanisms control trafficking of distinct receptors.[8] Thus, the spatial and temporal control of GPCRs determines the specificity of receptor-mediated signal transduction among the distinct downstream effectors and the ultimate cellular response.

The underlying molecular mechanism of MasR desensitization is unknown. In this study we investigated the early fate of MasR after agonist exposure. To better image receptor

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From the Departamento de Química Biológica (M.M.G., H.P.A., G.C.), Instituto de Química y Fisicoquímica Biológicas, Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Buenos Aires, Argentina; Hypertension and Vascular Research Division (P.O.C., O.A.C.), Henry Ford Hospital, Detroit, MI; Department of Physiology (R.A.S.), Federal University of Minas Gerais, Belo Horizonte, Brazil.

Correspondence to Mariela M. Gironacci, Instituto de Química y Fisicoquímica Biológicas, Consejo Nacional de Investigaciones Científicas y Técnicas, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 1113 Buenos Aires, Argentina. E-mail mariela@qb.ffyb.uba.ar

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trafficking, we generated a chimera between the MasR and the yellow fluorescent protein (YFP; MasR-YFP).

Methods

Materials

FBS, penicillin-streptomycin, Lipofectamine 2000, goat antimouse antibody coupled to Alexa 594, and DMEM were purchased from Invitrogen (Carlsbad, CA). BSA, paraformaldehyde, PBS, and the protease inhibitors mixture were from Sigma Chemical Co (St Louis, MO). Mouse antigreen fluorescent protein monoclonal antibody was from Clontech. Mouse anti-AP50, anti-Rab5, anti-caveolin 1 (Cav-1), or anti-early endosome antigen 1 (EEA1) antibodies were purchased from BD Biosciences. [^{3}H]arachidonic acid was from Perkin Elmer (Boston, MA). Ang-(1-7) and [D-Ala^7]-Ang-(1-7) were synthesized in our laboratory by the Merrifield solid-phase procedure, as described previously.[10] Peptide purity (>97%) was confirmed by matrix-assisted laser desorption mass spectrometry. All of the other chemicals were analytic grade reagents of the highest purity available.

DNA Construction

The YFP cDNA was attached to the carboxy-terminus end of the cDNA encoding the Mas receptor by the megaprimer method[11] into *Xho*I-*Apa*I sites of pEYFP-N1 plasmid (Clontech). The construct was verified by DNA sequencing.

Cell Culture and Transfection

Human embryonic kidney 293T cells were grown in DMEM high glucose supplemented with 10% heat-inactivated FBS and penicillin-streptomycin at 37°C in a humidified atmosphere at 95% air and 5% CO_2. Cells were transiently transfected using Lipofectamine 2000 according to instructions of the manufacturer and were used 36 hours posttransfection.

MasR-YFP Expression

The expression of MasR fused to YFP in transfected human embryonic kidney 293T cells was evaluated by Western blot as described previously.[10] MasR-YFP expression was evaluated with a mouse antigreen fluorescent protein monoclonal antibody (dilution 1/1000). MasR-YFP expression was also evaluated by laser scanning confocal microscopy (Olympus Fluoview FV1000, Tokyo, Japan).

[^{125}I]Ang-(1-7) Binding Studies

Thirty-six hours posttransfection, cells on 12-well plates were rinsed 2 times with DMEM and equilibrated on ice with incubation buffer (DMEM containing 0.2% BSA and a protease inhibitors mixture pH 7.4) for 30 minutes. Subsequently, the plates were incubated at 4°C for 60 minutes with incubation buffer containing 0.5 nmol/L of [^{125}I]Ang-(1-7) (labeled in our laboratory as described previously[12]). Incubation was stopped by rinsing the cells 3 times with ice-cold PBS. Cells were solubilized by incubation with 0.1 mol/L of NaOH for 60 minutes, and the radioactivity was measured. Nonspecific binding was determined in the presence of 10 μmol/L of unlabeled Ang-(1-7), which was no higher than 15%. Specific binding was calculated by the subtraction of nonspecific binding from total binding. Competition binding experiments were performed, and inhibition constant was calculated using GraphPad Prism (GraphPad Software, Inc, San Diego, CA).

Arachidonic Acid Release

Twenty-four hours posttransfection, cells were labeled with [^{3}H] arachidonic acid (AA; 0.2 μCi/well) for 16 hours, as described previously[3]. Then, cells were washed with DMEM containing 2% BSA and incubated with Ang-(1-7) during different times at 37 °C. Radioactivity in the supernatant was measured. For total cellular radioactivity, cells in each well were solubilized with 1 mol/L of NaOH and counted. [^{3}H]AA released into the medium was expressed as percentage of the total cellular radioactivity and referred to as fractional release.

Receptor Internalization Assay

It was measured according to the method described by Thomas et al.[13] Briefly, 36 hours posttansfection, cells on 12-well plates were rinsed 2 times with DMEM, preincubated with incubation buffer (DMEM containing 0.2% BSA and a protease inhibitors mixture pH 7.4) for 30 minutes, and then incubated with 0.5 nmol/L of [^{125}I]Ang-(1-7) (labeled in our laboratory as described previously[12]) during different times. After 2 washes with ice-cold PBS, surface-bound [^{125}I]Ang-(1-7) associated with noninternalized receptors was separated by treating the cells with an ice-cold acid solution (0.2 mol/L of acetic acid and 0.5 mol/L of NaCl; pH 5.5) for 5 minutes on ice, and radioactivity was determined (acid-sensitive fraction). Cells, which contain the internalized [^{125}I]Ang-(1-7), were lysed by incubation with 0.1 mol/L of NaOH for 60 minutes, and the radioactivity was measured (acid-insensitive fraction). The index of receptor internalization was determined as acid-insensitive counts per minute as a percentage of the total binding (acid sensitive + acid insensitive). Nonreceptor-mediated [^{125}I]Ang-(1-7) and surface binding were measured in the presence of 10 μmol/L of unlabeled Ang-(1-7) and subtracted from total binding to calculate the specific values. Curve fitting was performed with GraphPad Prism (GraphPad Software, Inc). In another set of experiments, transfected cells were incubated with the MasR antagonist [D-Ala^7]-Ang-(1-7) (100 nmol/L) during 15 minutes, and then internalized [^{125}I]Ang-(1-7) was determined as described.

MasR-YFP Trafficking

Trafficking pathway was evaluated by colocalization between MasR-YFP and endocytosis markers signals by immunocytochemistry. Briefly, 36 hours posttansfection, cells were incubated with 10 μmol/L of Ang-(1-7) (synthesized in our laboratory by the Merrifield method, as described previously[10]) for 15 minutes at 37°C. After 2 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 minutes at room temperature. Cells were then incubated with different primary antibodies (anti-AP50, anti-Rab5, anti-Cav-1, or anti-EEA1 mouse monoclonal antibodies, diluted 1:150 in blocking solution) overnight at 4°C. The samples were rinsed twice in PBS/0.2% Triton X-100 and exposed to the secondary antibody (goat antimouse antibody coupled to Alexa 594, dilution 1:600 in blocking solution) for 2 hours at room temperature.

Samples were mounted and imaged using an Olympus Fluoview FV1000 spectral laser scanning confocal microscope with a ×60 oil immersion lens using dual excitation (473 nm for YFP and 559 nm for Alexa 594). Because of the spectral properties of the scan head, fluorescence emission was collected between 520 and 550 nm for YFP and 600 to 660 nm for Alexa 594. Images were obtained using sequential scanning for each channel to eliminate the cross-talk of chromophores. Quantitative colocalization was estimated by Pearson correlation coefficient and Manders overlap coefficient,[14] 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. Some images (Figure 3) were acquired using a laser scanning confocal system (Visitech International, Sunderland, United Kingdom) mounted on a Nikon TE2000-eclipse microscope, ×100 1.3 NA lens, and visualized at 514 nm excitation, 550 LP emission. Identical laser, slit, and acquisition settings were used to obtain all of the images.

Data Analysis

All of the average 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 P level of 0.05 was considered significant.

Results

Characterization of MasR-YFP

To better image MasR trafficking, we generated the chimera C-terminally YFP tagged MasR. Because the fusion of YFP

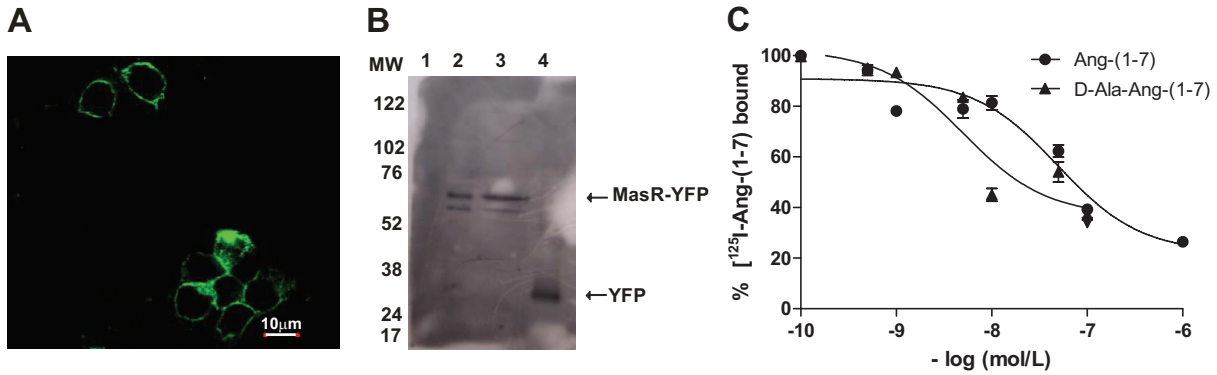

Figure 1. Characterization of MasR-YFP. **A**, Expression and cellular localization of the MasR-YFP fusion protein in transfected HEK 293T cells. **B**, Immunoblot of extracts of HEK 293T cells transfected with empty vector (lane 1), the DNA encoding MasR-YFP (lanes 2 and 3) or YFP (lane 4). **C**, Specific binding of [^{125}I]Ang-(1-7) to MasR-YFP transfected cells in the presence of Ang-(1-7) or the Mas R antagonist (D-Ala-Ang-(1-7). Values are mean±SEM.

to the C-terminal of the receptor may alter its correct folding and, hence, its functionality, we first characterized the C-terminally tagged MasR-YFP. Human embryonic kidney 293T cells transfected with the DNA coding for the chimera showed that MasR-YFP was found predominantly in the plasma membrane (Figure 1A). However, intracellular localization of the fusion protein was also detectable, especially in cells showing higher levels of receptor expression. In some cells, the MasR-YFP was present around the nucleus, presumably in the endoplasmic reticulum, and probably represents newly synthesized molecules passing through the secretory pathway, but intranuclear localization of the receptor was not observed.

MasR-YFP expression was also investigated by Western blot. Figure 1B shows that transfected cells expressed a protein with a molecular weight corresponding with the MasR-YFP chimera (68 kDa).

The functional integrity of the YFP-tagged receptor was investigated by Ang-(1-7) binding. As shown in Figure 1C, increasing concentrations of unlabeled Ang-(1-7), as well as the Mas receptor antagonist [D-Ala^7]-Ang-(1-7), displaced the binding of [^{125}I]Ang-(1-7) to transfected cells (inhibition constant: $4.92\pm0.12\times10^{-8}$ mol/L for Ang-[1-7] and $4.96\pm0.18\times10^{-9}$ mol/L for [D-Ala^7]-Ang-[1-7]). These results demonstrate that MasR-YFP attained a correct folding in the plasma membrane and binds its physiological ligand, as well as its antagonist. Cells transfected with the empty vector showed undetectable Ang-(1-7) binding (total binding: 480±68 versus nonspecific binding 395±87 counts per minute).

Receptor functionality and coupling were evaluated by measuring MasR signaling. MasR activation is coupled to AA release.[3,5] To determine the functionality of MasR-YFP, human embryonic kidney 293T cells transfected with the MasR-YFP construct and labeled with [^{3}H]AA were exposed to increasing concentration of Ang-(1-7). As shown in Figure 2, 100 nmol/L of Ang-(1-7) caused an increase in [^{3}H]AA release in MasR-YFP–transfected cells. A higher concentration (1 μmol/L) produced a smaller response, suggesting receptor desensitization. When cells were incubated with Ang-(1-7) for a prolonged period, a decrease in the Ang-(1-7)–induced [^{3}H]AA release was observed (Figure 2), suggesting receptor desensitization. Cells transfected with the empty vector (mock) showed no changes in [^{3}H]AA release on Ang-(1-7) stimulation (Figure 2).

Collectively, these data show that MasR-YFP is properly expressed and fully active. Furthermore, our results show that higher concentrations or longer times of stimulation with

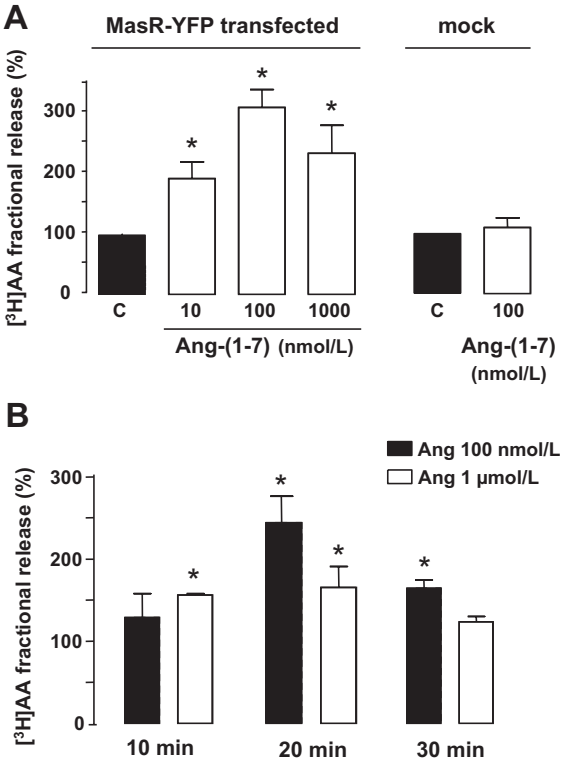

Figure 2. A, [^{3}H]AA release from mocked transfected cells or cells transfected with the DNA coding for MasR-YFP in the absence (C) and presence of Ang-(1-7) during 15 minutes. Results are presented as the percentage of the response detected in control, taking them as 100%. Values are mean±SEM *$P<0.05$ compared with control. **B**, [^{3}H]AA release from MasR-YFP transfected cells in the presence of 100 nmol/L or 1 μmol/L Ang-(1-7) (Ang) during 10, 20 and 30 minutes. Results are presented as the percentage of the response detected in control, taking them as 100%. Values are mean±SEM *$P<0.05$ compared with control.

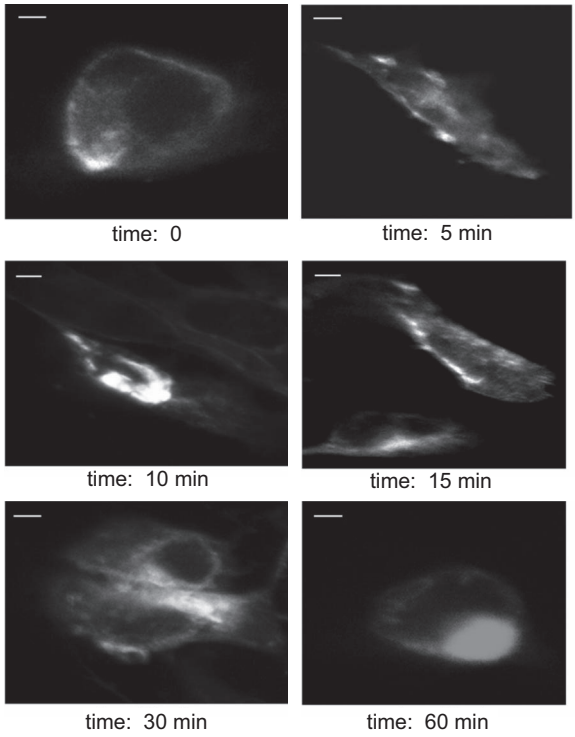

Figure 3. Agonist-induced subcellular distribution of MasR-YFP. Subcellular localization of the MasR-YFP fusion protein in HEK 293T transfected cells and stimulated with 1 μmol/L Ang-(1-7) for the defined time periods. Scale bar=10 μm.

Ang-(1-7) caused a decrease in its response (AA release), demonstrating receptor desensitization.

Ang-(1-7) Induces MasR-YFP Internalization

To investigate whether Ang-(1-7) induces Mas receptor internalization, MasR-YFP–transfected cells were incubated with 1 μmol/L of Ang-(1-7) during different times, and the relative cellular distribution of MasR-YFP was observed by laser scanning confocal microscopy. In resting cells, MasR-YFP was mostly localized on the cell membrane and in the endoplasmic reticulum. Treatment of cells with Ang-(1-7) induced redistribution in fluorescence after 5 minutes of stimulation, changing the localization of MasR-YFP to intracellular vesicles of various sizes (Figure 3), suggesting internalization of the receptor on agonist stimulation.

The capacity of MasR-YFP to internalize in response to Ang-(1-7) was determined by following the time course of [^{125}I]Ang-(1-7) endocytosis. Half of MasR-YFP underwent endocytosis after 10 minutes, and this internalization was partially blocked by the Mas receptor antagonist [D-Ala^7]-Ang-(1-7) (Figure 4).

MasR-YFP Trafficking

The Rab family of small GTPases is integral in determining the fate of a GPCR.[15] Different Rab GTPase family members selectively associate with specific intracellular structures, including both recycling and sorting endosomes, where they mediate multiple steps of vesicular membrane trafficking, including vesicle budding, docking, and fusion.[15] Rab5 plays a central role in endocytosis via clathrin-coated pits and

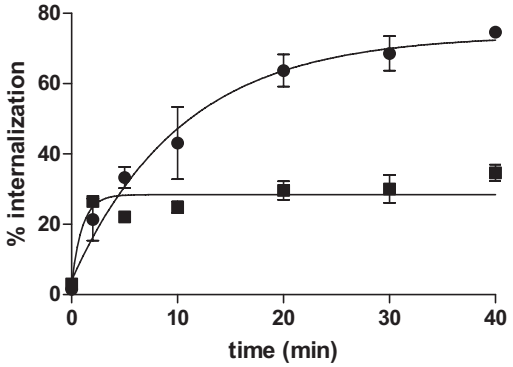

Figure 4. Agonist-induced internalization of MasR-YFP. MasR-YFP transfected cells were incubated with [^{125}I]Ang-(1-7) in the absence (circles) or presence of the Mas R antagonist [D-Ala^7]-Ang-(1-7) (squares) and internalized [^{125}I]Ang-(1-7) was evaluated as described in Methods. An index of internalization was calculated by expressing the internalized radioactivity (acid-resistant) as a percentage of the total binding (acid-resistant plus acid-susceptible). Data are means±SEM of 4 independent determinations.

subsequent fusion of vesicles with early endosomes.[15] To investigate whether MasR-YFP was targeted to early endosome, MasR-YFP–transfected cells were stimulated with Ang-(1-7) during 15 minutes, and then colocalization of MasR-YFP with Rab5 was investigated. A fraction of MasR-YFP colocalized with Rab5 (Figure 5A), showing that the receptor was internalized and targeted to early endosomes. In addition to Rab5, early endosomes contain Rab5 effectors and regulator proteins, including EEA1.[9] To provide further confirmation that MasR-YFP is targeted to early endosome, colocalization studies between MasR-YFP and the EEA1 marker protein after agonist stimulation were performed. Figure 5B shows that MasR-YFP also colocalized with EEA1, confirming that MasR-YFP is targeted to early endosomes after Ang-(1-7) stimulation.

Most GPCRs are endocyted through clathrin-mediated endocytosis.[8,9,16] However, some GPCRs preferentially localize to and/or internalize via specialized lipid raft/caveolae microdomains of the plasma membrane.[9,17,18] Caveolae are Cav-1–enriched smooth invaginations of the plasma membrane that form a subdomain of lipid rafts and represent a nonclathrin internalization pathway. To begin studying the mechanisms by which MasR is internalized, MasR-YFP–transfected cells were stimulated with Ang-(1-7), and then colocalization of MasR-YFP with markers of the clathrin or caveolin pathway were investigated. We evaluated whether MasR-YFP is endocyted through clathrin-coated pits by analyzing its colocalization with the adaptor protein complex 2 (AP-2, μ or AP50), which is an essential component of the clathrin-coated vesicle machinery.[9] On Ang-(1-7) stimulation, a fraction of MasR-YFP colocalized with AP50 (Figure 5C), which suggests that MasR-YFP is internalized via a clathrin-mediated pathway.

To further study whether the caveolae-dependent pathway was involved in the endocytosis of MasR-YFP, colocalization with Cav-1 was evaluated. After Ang-(1-7) stimulation, colocalization of MasR-YFP and the Cav-1–specific signal was observed (Figure 5D).

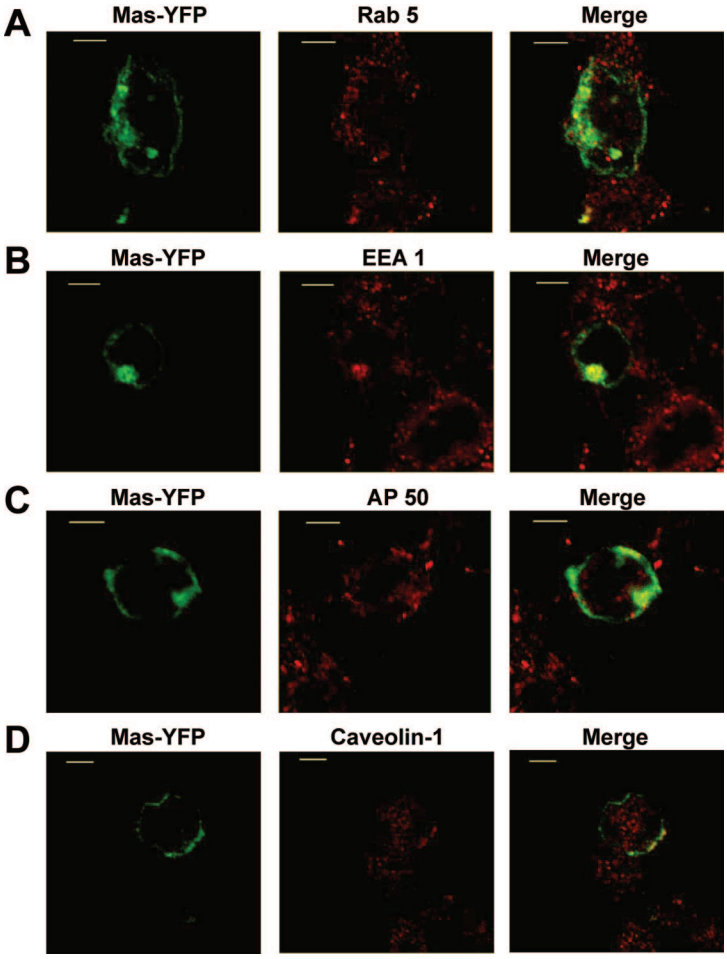

Figure 5. MasR-YFP trafficking. Colocalization of MasR-YFP (green) and early endosomes markers (Rab 5 and EEA1), clathrin-coated pits (AP50) or caveolin-1 (caveolae marker) (red) in MasR-YFP transfected HEK293T cells stimulated with Ang-(1-7) during 15 minutes. Three independent experiments were analyzed. See text for analysis. Scale bar=5 μm.

Altogether, these results indicate that MasR-YFP is internalized into early endosomes via a clathrin-dependent pathway. However, colocalization with Cav-1 suggests that, at some point in the receptor trafficking, MasR-YFP traverses Cav-1–positive compartments.

Discussion

The endocytic pathway tightly controls the activity of GPCR. Ligand-induced endocytosis can drive receptors into divergent lysosomal and recycling pathways, producing essentially opposite effects on the strength and duration of cellular signaling, allowing for the fine tuning of signal magnitude and duration.[9,19] Our study shows that Ang-(1-7) induces MasR internalization, a process that is involved in the feedback desensitization of GPCR responsiveness that protects against receptor overstimulation.[7] This event may explain the decrease in Ang-(1-7) response in releasing AA from tissue lipids and in stimulating 6-keto-prostaglandin F1α production in rabbit aortic smooth muscle cells,[5] phosphatidylcholine biosynthesis in the rat renal cortex,[4] and the attenuation in responsiveness in the Ang-(1-7)–induced proliferation of endothelial progenitor cells from sham or infarcted rodents.[6]

GPCR desensitization also acts to filter information from multiple receptor inputs into an integrated and meaningful biological signal. Present results show that MasR-YFPs are internalized together with Ang-(1-7). This event may be important for directing Ang-(1-7) to certain cellular locations for full expression of its biological response, as it happens with Ang II at the renal tissue.[20,21] Renal AT_1Rs are responsible for internalizing Ang II, and the presence of substantial Ang II in endosomes in both control and Ang II–infused hypertensive rats supports their internalization into a protected compartment that prevents degradation of some of the internalized Ang II.[20,21] In this way, the internalized Ang II activates various signaling pathways, contributing to fibrogenic proliferative responses while also migrating to the nucleus to exert transcriptional effects.[21]

The fact that a fraction of 20% of the binding of radiolabeled Ang-(1-7) was not completely displaced by Ang-(1-7) or its antagonist or that its endocytosis was not completely prevented by the MasR antagonist suggests that some of the Ang-(1-7) may be bound to an unknown receptor or be internalized by another mechanism. For instance, Gonzalez-Villalobos et al[22] have shown that the scavenger receptor megalin binds and internalizes Ang-(1-7).

GPCR endocytosis, in addition to playing a role in receptor desensitization, has been shown to have other important functions in regulating and even promoting GPCR signaling.[19,23] Recent studies indicate that GPCRs can continue signaling after internalization together with their agonists.[19,23] GPCR endocytosis appears to be required for efficient

mitogen-activated protein kinase signaling by certain GPCRs.[19] Growing evidence shows that caveolae are not only involved in endocytosis but also function as cell surface signal transduction domains by affecting both signaling selectivity and coupling efficacy,[17,18] as happens for the AT_1R.[24,25] After agonist binding, the AT_1R moves to caveolae, and this event is involved in the reactive oxygen species–dependent AT_1R signaling regulating vascular smooth muscle cells hypertrophy.[24,25] It is increasingly evident that endocytosis and signaling are not only connected but likely inextricably intertwined. Our present results show that MasR is internalized on ligand stimulation into early endosomes via a clathrin-dependent pathway; however, colocalization with Cav-1 suggests that at some point in the receptor trafficking MasR-YFP traverses Cav-1–positive compartments (present results). The relative contribution of both clathrin- and caveolae-dependent pathways in MasR desensitization deserves further future investigation.

Perspectives

Through desensitization and internalization, MasR activity and density may be tightly controlled by the cell. The broad data available reveal a degree of specificity and plasticity in the cellular regulation of GPCRs by endocytic membrane trafficking. We showed that MasR is internalized on ligand stimulation into early endosomes via a clathrin-dependent pathway; however, at some point in the receptor trafficking, MasR-YFP traverses Cav-1–positive compartments. To our knowledge this is the first report on MasR regulation, and it opens the way for a better understanding of MasR biochemistry. Within the cardiovascular system, regulation of GPCR endocytosis and trafficking is of fundamental importance both for physiological homeostasis and molecular response to physiological perturbation.[16,19] Many studies suggest that the endocytic trafficking of GPCR is highly controlled and has profound functional consequences in vivo.[16,19] Elucidating those mechanisms will grow our understanding of GPCR pharmacology and function and open new opportunities for the development of strategies to therapeutically manipulate GPCR function in diseases associated with altered GPCR signaling, such as hypertension and congestive heart failure.[26,27]

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Disclosures

None.

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