G protein-independent signaling of the AT_{1A}-angiotensin receptor in C9 cells

Ph.D. thesis

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1. Introduction

The octapeptide hormone angiotensin II (Ang II) is the main effector molecule of the renin-angiotensin system. Ang II plays a fundamental role in several physiological and pathophysiological processes, including the regulation of blood pressure, the maintenance of the sodium and fluid homeostasis, the development of hypertension, and subsequent smooth muscle and cardiomyocyte remodeling. Most of the known effects of Ang II are mediated by the AT_1 angiotensin receptor (AT_1R), a 7 transmembrane domain, G protein-coupled receptor.

The AT_1R activates a number of signaling pathways after agonist binding, the most well known being the different heterotrimeric G proteins, mainly $G_{q/11}$, but also $G_{12/13}$ in some cell types, and $G_{i/o}$ in rodents. $G_{q/11}$ activates phospholipase C, which leads to inositol-phosphate and Ca^{2+}-signal generation, and activation of protein-kinase C, and this is the main signal transduction pathway in most of the physiological target tissues of Ang II, such as in adrenal, cardiac muscle, renal and smooth muscle cells. Besides the fast, G protein-mediated effects, Ang II is regulating processes like cell growth, division, migration, or gene expression. These effects are usually mediated by the

Sirokmány G, ***Szidonya L****, Káldi K, Gáborik Z, Ligeti E, Geiszt M.*

Sec14 homology domain targets p50RhoGAP to endosomes and provides a link between Rab and Rho GTPases

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IF: 5,854

Turu G, Simon A, Gyombolai P, ***Szidonya L****, Bagdy G, Lenkei Z, Hunyady L.*

The role of diacylglycerol lipase in constitutive and angiotensin AT_1 receptor-stimulated cannabinoid CB1 receptor activity

J Biol Chem 2007;282:7753-7757.

IF: 5,854

transactivation of different cytoplasmic tyrosine-kinases or growth factor receptors. In the C9 rat hepatocyte cell line the main pathway of AT_1R-mediated ERK1/2 mitogen-activated protein-kinase (MAPK) activation is through the transactivation of epidermal growth factor receptors (EGFR).

After agonist binding, the receptor is phosphorylated by specific kinases, followed by the binding of arrestin molecules from the cytoplasm to the phosphorylated receptors, which uncouples the receptor from the G proteins, and targets it to the internalization machinery. Depending on the strength of the interaction between the receptors and β-arrestins, the receptors are classified into two groups, and the AT_1R is a class B receptor, meaning that it maintains a strong and sustained interaction with arrestin molecules.

In the last few years, a number of observations suggested that besides the classical, G protein-dependent signaling pathways, AT_1R activates G protein-independent mechanisms as well. The best characterized of these mechanisms is the signaling through arrestin proteins. To study this phenomenon, several experimental approaches have been developed, one of which are the different mutant AT_1Rs, selectively impaired in their ability to activate G proteins, but able to be phosphorylated, to bind arrestin and to internalize.

One such mutant is the DRY/AAY mutant AT_1R, which is unable to activate G proteins, but induces the translocation of β-arresztin2 to the plasma membrane, and co-internalizes with it into endocytic vesicles. During the desensitization and internalization of the AT_1R, β-arresztin2 organizes a multi-protein signaling complex, including cRaf-1, MEK1, and ERK2 proteins, the members of the MAPK cascade leading to activation of ERK1/2. The overexpression of arrestin diminishes the inositol-phosphate response evoked by Ang II, which is in line with the role of arrestins in desensitization, but at the same time, it leads to increased ERK-activation. The ERK activated through this mechanism colocalizes with AT_1R and arrestin on the surface of endosomes, which prevents it from entering the nucleus, and so it does not activate gene transcription.

It is difficult to study mutant receptors in cells expressing endogenous AT_1Rs (such as C9 cells), since the addition of Ang II stimulates both the wild-type and the expressed receptors. The highly cell-specific nature of signaling pathways means that the most interesting would be to study exactly these kinds of cells. To find a solution to this problem, we tried to develop a mutant receptor that would not bind the non-peptide antagonists of the AT_1R. This mutant

5. List of publications

Related to the thesis

Turu G, ***Szidonya L****, Gáborik Z, Buday L, Spät A, Clark AJL, Hunyady L.*

Differential β-arrestin binding of AT_1 and AT_2 angiotensin receptors

FEBS Lett 2006;580:41-45.

IF: 3,415

Szidonya L*, Süpeki K, Karip E, Turu G, Várnai P, Clark AJL, Hunyady L.*

AT_1 receptor blocker-insensitive mutant AT_{1A} angiotensin receptors reveal the presence of G protein-independent signaling in C9 cells

Biochem Pharmacol 2007;73:1582-1592.

IF: 3,617

Other publications

Gáborik Z, Szaszák M, ***Szidonya L****, Balla B, Paku S, Catt KJ, Clark AJL, Hunyady L.*

β-arrestin- and dynamin-dependent endocytosis of the AT_1 angiotensin receptor

Mol Pharmacol 2001;59:239-247.

IF: 5,297

study of these mutants in cells expressing endogenous AT_1Rs. Another possible use for this mutant is the study of the dimerization of the receptor, as it would allow us to differentiate between two kinds of AT_1Rs expressed in the same cells, or enable the selective stimulation of one of the receptors.

receptor, after expressed in cells with endogenous AT_1Rs, would enable us to block the wild-type receptors with the antagonist, and study the expressed receptors in a cell, which is normally the target of Ang II.

Previous studies demonstrated that in the case of the AT_1R, different amino-acid residues are responsible for the binding of peptide and non-peptide ligands. One of the key facts leading to this finding was that the amphibian AT_1R, unlike its mammal counterpart, does not bind the biphenylimidazole antagonists of the receptor. Comparing the differences between the receptors in the two species, the III. transmembrane helix turned out to be important in the binding of non-peptide ligands, so we focused our attention to this region during our experiments.

2. Objectives

The main aims of our experiments were the following:

1. Setting up a BRET assay to detect and monitor the kinetics of the molecular interaction between the AT_1R and β-arresztin2

2. Development of a candesartan-resistant AT_1R mutant receptor to study the signal transduction pathways of cells expressing endogenous AT_1Rs

3. Detection of G protein-independent signaling in C9 cells expressing endogenous AT_1Rs

belongs to the class B receptors with respect to β-arrestin binding.

Using this method, we confirmed that the DRY/AAY mutant AT_1R, incapable of G protein-activation, is able to bind β-arrestin2 with the same affinity as the wild-type receptor.

Expressing the S109Y-$AT_{1A}R$ in the C9 rat hepatocyte cell line we studied the Ang II-induced activation of the ERK1/2 MAPK, and determined that this mutant activates ERK with the same mechanism as the endogenous AT_1R.

Using the S109Y-DRY/AAY mutant $AT_{1A}R$, we found that Ang II can cause G protein-independent ERK-activation in C9 cells, which was not previously studied with mutant receptors in cells expressing endogenous AT_1Rs.

In summary we have identified a single amino acid substitution mutant (S109Y) in the rat AT_{1A}-R, which selectively eliminates the binding of nonpeptide antagonists to the receptor, without having any detectable effect on the binding of peptide ligands, as well as signal transduction, internalization and β-arrestin binding properties of the receptor. This mutant receptor provides a new opportunity to study the role of G protein-independent signaling of the AT_1R in physiological processes. The S109Y mutant receptor can also be combined with other mutations, which would allow the

4. Results and conclusions

In my Ph.D. studies I focused on the signal transduction of the AT_{1A} angiotensin receptor, which we studied also in cells expressing endogenous AT_1Rs. For these experiments we needed a mutant AT_{1A}R resistant to the blockers of this receptor.

During our experiments, we managed to construct a single amino acid mutant AT_{1A}R (S109Y-AT_{1A}R), which is practically insensitive to candesartan, a non-peptide antagonist of the receptor. This mutation did not exert any major effect on the binding of the physiological agonist Ang II, nor the peptide antagonist SI-Ang II to the receptor.

We characterized the inositol-phosphate response, internalization kinetic, and β-arresztin2-binding of the S109Y-AT_{1A}R, and found that these were similar to that of the wild-type AT_{1A}R. We determined that the S109Y mutation does not cause constitutive activity in the AT_{1A}R.

The recruitment of β-arrestin2 to the receptor was followed by bioluminescence resonance energy transfer, and we found that Ang II induces stable interaction between the two molecules, which corresponds to the fact that AT_1R

3. Methods

Plazmid constructions: The receptor mutants were created by site-directed mutagenesis in the HA epitope-tagged HA-$AT_{1A}R$ cDNA derived from rat vascular smooth muscle. We constructed a yellow fluorescent protein-tagged (eYFP) receptor and a Renilla luciferase-tagged (Rluc) β-arresztin2 construct as well.

Cell culture and transfection: To study the wild-type and mutant receptors, we expressed them in different cell cultures. We used the simian renal COS-7, the human embryonic renal HEK-293, and the rat hepatocyte epithelial C9 cell lines. The cells were transfected by Lipofectamine, Lipofectamine 2000, and Optifect reagents.

Ligand binding studies: The ligand binding parameters of the expressed wild-type and mutant AT_1Rs were determined in cold competition experiments, using radioactively labeled ^{125}I-SI-Ang II, with 6 hours or overnight incubations at 4 ˚C.

Study of the inositol-phosphate response: To follow the classical, G protein-dependent signaling of the expressed mutant receptors, we measured the Ang II-induced inositol-phosphate accumulation. We incubated the transfected COS-7 with tritium-labeled inositol, and after its incorporation into

inositol lipids, we stimulated with Ang II. After the extraction of inositol-bisphosphates and –trisphosphates, the radioactivity was measured by liquid scintillation counting.

Receptor internalization studies: To determine the internalization kinetics of AT_1R mutants, the transfected COS-7 cells were incubated in the presence of radioactive ^{125}I-Ang II at 37 ˚C for different times, and after the extracellularly bound ligand were separated with an acidic wash step, the quantity of intracellular and extracellular ligand were counted.

Bioluminescence resonance energy transfer (BRET) measurements: We followed the BRET between a β-arresztin2-Rluc and eYFP-labeled AT_1Rs expressed in COS-7 and HEK-293 cells. After the addition of coelenterazine h and Ang II we measured the intensity of light emitted at 485 and 530 nm. The presence of molecular interaction is revealed by the increase of the ratio of light emitted at 530 and 485 nm.

Western blot: Transfected C9 cells were serum-starved for 6 hours, preincubated with the appropriate inhibitors (AG 1478, candesartan) for 30 minutes, and stimulated with Ang II for different times. The proteins were resolved on SDS polyacrilamide gels and transferred to PVDF membranes, followed by incubation with primary antibodies against total

and phosphorylated p44/42 MAPK, and HRP-conjugated secondary antibodies, and detected by ECL.

Confocal laser microscopy: C9 cells were grown on coverslips and transfected with β-arrestin2-eGFP and mutant $AT_{1A}Rs$. After preincubation with candesartan, the cells were stimulated using Rhod-Ang II. Images were taken using a Zeiss LSM 510 confocal laser microscope, excited by argon and helium/neon lasers. Emission was detected using a 500–530 nm band pass filter for GFP, and a 560 nm long pass filter for rhodamine.

Cytoplasmic $[Ca^{2+}]$-measurements: C9 cells were transfected with the appropriate mutant AT_1Rs, and 24 hours later, the cells were detached, and loaded with Fura-2/AM. The Ca^{2+}-measurements were done in cell suspensions, using excitation at 340 and 380 nm, and emission detected at 505 nm, in the absence or presence of candesartan.

Statistical analysis: The data are presented as average ± standard error of the means. The differences between groups were analyzed using ANOVA.