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

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ABSTRACT

Although mutant receptors are highly useful to dissect the signal transduction pathways of receptors, they are difficult to study in physiological target tissues, due to the presence of endogenous receptors. To study AT_1 angiotensin receptors in their physiological environment, we constructed a mutant receptor, which differs only from the AT_{1A} receptor in its reduced affinity for candesartan, a biphenylimidazole antagonist. We have determined that the conserved S109Y substitution of the rat AT_{1A} receptor eliminates its candesartan binding, without exerting any major effect on its angiotensin II and peptide angiotensin receptor antagonist binding, internalization kinetics, β-arrestin binding, and potency or efficacy of the inositol phosphate response. To demonstrate the usefulness of this mutant receptor in signal transduction studies, we combined it with substitution of the highly conserved DRY sequence with AAY, which abolishes G protein activation. In rat C9 hepatocytes the S109Y receptor caused ERK activation with the same mechanism as the endogenous AT_1 receptor. After combination with the DRY/AAY mutation G protein-independent ERK activation was detected demonstrating that this approach can be used to study the angiotensin II-stimulated signaling pathways in cells endogenously expressing AT_1 receptors.

1. Introduction

Most of the physiological and pathophysiological actions of the octapeptide hormone angiotensin II (Ang II) are mediated by the type I angiotensin receptor (AT_1-R). This typical seven transmembrane domain, G protein-coupled receptor (GPCR) activates a wide variety of signaling pathways, including the G_q-mediated, phospholipase C-dependent phosphoinositide

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Abbreviations: Ang II, angiotensin II; AT_1-R, type I angiotensin receptor; AT_{1A}-R, type 1A angiotensin receptor; AT_{1B}-R, type 1B angiotensin receptor; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; TM, transmembrane domain; DMEM, Dulbecco's modified Eagle's medium; BRET, bioluminescence resonance energy transfer; eYFP, enhanced yellow fluorescent protein; eGFP, enhanced green fluorescent protein; SI-Ang II, [Sar^1,Ile^8]Ang II; Rhod-Ang II, rhodamine-conjugated Ang II; Rluc, Renilla luciferase; EGF-R, epidermal growth factor receptor; PKC, protein kinase C; PI3K, phosphoinositide 3-kinase

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hydrolysis and subsequent Ca^{2+} signaling, and G protein-independent signaling mechanisms. Ang II also induces activation of different MAP kinases via several mechanisms including transactivation of various growth factor receptors [1,2], and the more recently described β-arrestin-mediated ERK activation pathway [3]. It has been demonstrated that the latter pathway is G protein-independent, similar to the interaction of the receptor with different AT_1-R-associated proteins [2,4]. Since these different mechanisms are often cell specific, the physiological relevance of these mechanisms needs to be established in physiological target tissues, which express AT_1-Rs.

Creation of receptor mutants is a powerful tool for studying the different signal transduction pathways [5], but the use of this technique is limited in cells endogenously expressing the studied receptor, as it is extremely difficult to differentiate between the responses of wild-type and expressed receptors. As the significance of performing such studies in these kinds of cells, which are physiological targets of Ang II, would be high, our aim was to develop angiotensin receptors, which can be studied in physiological target tissues. We have used a pharmacological approach, by constructing a mutant receptor, which differs only from the wild-type receptor in its reduced affinity for biphenylimidazole antagonists. A series of mutations in the hemagglutinin epitope-tagged rat Ang II receptor type 1A (AT_{1A}-R) was created, and their ligand binding and signal transduction properties were determined. We have identified a single amino acid mutation in transmembrane domain (TM) III, which seems to interfere only with the binding of candesartan to the receptor, but leaves the other ligand binding and signaling properties intact. To demonstrate the usefulness of this mutant receptor in signal transduction studies, we combined this mutation with substitution of the highly conserved DRY sequence with AAY sequence, which we have shown to abolish G protein activation [6], however, it can activate G protein-independent, β-arrestin-mediated ERK activation [4]. This construct was used to study G protein-independent ERK activation in rat C9 cells, which endogenously express the AT_1-R.

2. Materials and methods

2.1. Materials

The cDNA of the rat vascular smooth muscle AT_{1A}-R was provided by Dr. K.E. Bernstein (Emory University, Atlanta, GA), and a *Hin*dIII/*Not*I fragment of this receptor was subcloned into pcDNA™ 3.1 vector and influenza hemagglutinin epitope was inserted as previously described [7,8]. The presence of the epitope tag had no effect on ligand binding or inositol phosphate signaling and internalization properties of the receptor, as described earlier [8]. β-Arrestin2-eGFP was kindly provided by Dr. Marc G. Caron (Duke University, Durham, NC). β-Arrestin2-Rluc and AT_{1A}R-eYFP was generated as previously described [9]. ^{125}I-Ang II and ^{125}I-[Sar^1,Ile^8]Ang II (SI-Ang II) was provided by Dr. R.C. Speth (Univ. Mississippi, University, MS), [^{3}H]inositol was from Amersham Pharmacia Biotech (Piscataway, NJ). Rhodamine-conjugated Ang II (Rhod-Ang II) was obtained from NEN Life Science Products (Boston, MA). Candesartan was a gift from AstraZeneca (Mölndal, Sweden). Anti-phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 antibodies were from Cell Signaling Technology® Inc. (Beverly, MA). DMEM, Opti-MEM® I, fetal bovine serum, Lipofectamine™, Lipofectamine™ 2000, Optifect™, Fura-2/AM and coelenterazine h were from Invitrogen™ Life Technologies (Carlsbad, CA). Clone 9 rat liver cells were obtained from ATCC (Manassas, VA). Unless otherwise stated, all other chemicals and reagents were from Sigma.

2.2. Site-directed mutagenesis

Mutations in the rat AT_{1A}-R were performed with the QuikChange® Site-Directed Mutagenesis Kit (Stratagene®, La Jolla, CA) according to manufacturer's suggestions, and verified using automated sequencing.

2.3. Transfections and cell culture

C9 rat liver epithelial cells were grown in 5% CO_2 in $NaHCO_3$-buffered F-12K nutrient mixture (Kaighn's modification) supplemented with 10% fetal calf serum (FCS), 100 μg/ml streptomycin, 100 IU/ml penicillin. For all studies, C9 cells between passages 3 and 10 were used because these cells exhibit maximum expression of their endogenous AT_1 receptors. COS-7 cells were grown in 5% CO_2 in DMEM containing glucose, glutamine, sodium bicarbonate, and supplemented with 10% FCS, 100 μg/ml streptomycin, 100 IU/ml penicillin. COS-7 cells were plated on 24 well plates. Seventy-two hours later the cells were transfected overnight with 0.5 μg receptor cDNA using Lipofectamine™ in Opti-MEM® I. Ligand binding, internalization and inositol phosphate measurements were performed 48 h after transfection. C9 cells were plated on 6 and 24 well plates 24 h before transfection in antibiotic-free F-12K medium. After transfection with Optifect™ in Opti-MEM® I for 6 h, cells were allowed to recover overnight in complete medium.

2.4. Binding assay and receptor endocytosis

Cell surface angiotensin receptor binding was measured in cold displacement studies using ^{125}I-SI-Ang II. The radioligand (2.5 kBq/ml, ~0.03 nM) and various concentrations of the non-labeled ligand were added in 0.5 ml of HEPES-buffered DMEM, and the cells were incubated overnight at 4 °C. Following incubation, cells were washed twice with PBS, and radioactivities were measured by γ-spectrometry after solubilization with 0.5 M NaOH/0.05% SDS. For data analysis non-linear regression (curve fit) with one site competition or homologous competitive binding was performed using GraphPad Prism 4.03 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

To determine the internalization kinetics of the AT_1-R mutants, ^{125}I-Ang II (2.5 kBq/ml, ~0.03 nM) was added in 0.25 ml of HEPES-buffered DMEM, and the cells were incubated at 37 °C for the indicated times. Incubations were stopped by placing the cells on ice and rapidly washing them twice with ice-cold PBS. Acid-released and acid-resistant radioactivities were separated and measured by γ-spectrometry as described previously [10]. The percentage of internalized ligand at each

time point was calculated from the ratio of the acid-resistant specific binding to the total (acid-resistant + acid-released) specific binding.

2.5. Inositol phosphate measurements

The culture medium was replaced 24 h after transfection with 0.5 ml inositol-free DMEM containing 10–20 μCi/ml [^{3}H]inositol, 2.5% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin as described earlier [11]. Twenty-four hours later the cells were washed twice, incubated in inositol-free DMEM containing 25 mM HEPES (pH 7.4) and 10 mM LiCl for 30 min at 37 °C, and stimulated with Ang II for 20 min. Inositol phosphates were extracted with the addition of 10 mM formic acid as described earlier [12]. The samples were applied to Bio-Rad AG1 X 8 columns (Bio-Rad Laboratories Inc., Hercules, CA), and washed three times with 3 ml water and twice with 3 ml 0.2 M ammonium-formate in 0.1 M formic acid to remove inositol and inositol monophosphates. After these washing steps, the combined $InsP_2$ (inositol bisphosphate) + $InsP_3$ (inositol trisphosphate) fractions were eluted with two 3-ml aliquots of 1 M ammonium-formate in 0.1 M formic acid, and radioactivities were determined by liquid scintillation counting.

2.6. Bioluminescence Resonance Energy Transfer (BRET) measurements

BRET was measured between β-arrestin2-Rluc and eYFP-tagged receptors [13]. For the BRET measurements, COS-7 cells were plated and transfected with Lipofectamine™ 2000 at the same time on white 96 well plates in Opti-MEM® I. After 48 h, medium was changed to HEPES-buffered F-12, and coelenterazine h was added for a final concentration of 5 μM. Sequential measurements at 485 and 530 nm were taken with a Mithras LB 940 Multilabel Reader (Berthold Technologies, Bad Wildbad, Germany). Normalized ratios were calculated by subtracting the 530 nm/485 nm ratio of the luciferase-tagged molecule co-expressed with an untagged receptor from the ratio obtained with the YFP-tagged partner.

2.7. Western blots

Twenty-four hours after transfection, C9 cells were serum-starved for 6 h, then preincubated or not with 100 nM candesartan and/or 1 μM AG1478 for 30 min and stimulated for various times with 10 nM of Ang II. Cells were scraped into SDS sample buffer containing protease and phosphatase inhibitors, briefly sonicated, boiled, and separated on SDS-polyacrilamide gels. The proteins were transferred to PVDF membranes and incubated with the appropriate primary and secondary antibodies. The antibodies were visualized by ECL, using SuperSignal® West Pico or Dura reagents (Pierce Biotechnology Inc., Rockford, IL). The films were scanned and quantified by densitometry.

2.8. Confocal microscopy

For confocal microscopy, C9 cells were grown on glass coverslips and transiently transfected using Optifect™ reagent following the manufacturer's recommendations. Images were detected with a Zeiss LSM 510 confocal laser-scanning microscope 48 h later. GFP and rhodamine were excited with argon (488 nm) and helium/neon (543 nm) lasers, respectively, and emitted fluorescence was detected in multitrack mode with 500–530 nm bandpass and 560 nm longpass filters [9].

2.9. Cytoplasmic [Ca^{2+}] measurements

C9 cells were grown on 10 cm dishes and transiently transfected using Optifect™ reagent following the manufacturer's recommendations. The cells were detached by trypsinization, allowed to recover for 1 h in HEPES-buffered F12K medium, and loaded with Fura-2/AM (2 μM, 45 min). The calcium measurement in populations of C9 cells (10^6 cells/ml) was performed in a fluorescence spectrophotometer (DeltaScan, PTI, Lawrenceville, NJ), in the absence or presence of candesartan as indicated.

2.10. Statistical analysis

All data are presented as means ± S.E.M. Differences between groups were analyzed by ANOVA combined with Holm–Sidak test or Kruskal–Wallis ANOVA on ranks combined with Dunn's test using the software SigmaStat for Windows 3.5 (Systat Software Inc., Richmond, CA). The value of P less than 0.05 was considered significant.

3. Results

3.1. Creation and characterization of the ligand binding of mutant AT_{1A}-Rs

Mammalian AT_1-Rs show high affinity for the biphenylimidazole antagonists, such as losartan or candesartan, whereas the avian and amphibian angiotensin receptors have similar functional properties, but do not bind these ligands [14]. Previous studies have shown several amino acids responsible for losartan binding, located in transmembrane domains III, IV, V, VI and VII of the rat Ang II receptor type 1B (AT_{1B}-R), with TM III being the most important [15–17]. Based on these data, we have created a series of mutant AT_{1A}-Rs, by introducing substitutions of the amino acids in TM III, which eliminate losartan binding of the amphibian angiotensin receptor (Table 1). To determine the binding of different ligands to the receptors, the constructs were expressed in COS-7 cells and cold displacement experiments were performed, in which a constant amount of ^{125}I-SI-Ang II was displaced with different concentrations of unlabelled Ang II, SI-Ang II and candesartan. The first mutant we tested was the V108I/S109T double mutant, which was described earlier by Ji et al. [16] to cause a large reduction in losartan affinity for the AT_{1B}-R without interfering with the binding of SI-Ang II to the receptor. As can be seen in Fig. 1A and Table 1, this mutation, as expected, greatly impaired the binding of candesartan to the AT_{1A}-R without affecting the SI-Ang II binding, but it increased the affinity of the receptor for the physiological agonist, Ang II. Since increased agonist affinity is characteristic of constitutively active GPCRs [18–20], and our

Table 1 – IC_{50} values (nM) determined by the displacement of ^{125}I-[Sar^1,Ile^8]Ang II for wild-type and mutant AT_{1A}-Rs expressed in COS-7 cells

	Ang II	Candesartan	SI-Ang II
HA-AT_{1A}	13.7 ± 0.5	0.7 ± 0.1	1.0 ± 0.1
V108I/S109T	2.6 ± 0.5*	>1000*	1.1 ± 0.2
V108A/S109T	12.1 ± 1.4	900 ± 106*	1.0 ± 0.2
V108I	8.5 ± 0.9	4 ± 0.6	1.1 ± 0.1
S109T	4.0 ± 0.6*	64 ± 12	1.1 ± 0.2
S109A	8.6 ± 0.9	0.8 ± 0.1	0.9 ± 0.1
S109C	8.7 ± 1.3	0.8 ± 0.1	1.1 ± 0.4
S109V	6.7 ± 0.7	7.4 ± 1.3	0.8 ± 0.1
S109N	10.5 ± 0.9	154 ± 4*	1.1 ± 0.2
S109Y	19.7 ± 0.8	>1000*	1.0 ± 0.2

Transfected cells were incubated with ^{125}I-SI-Ang II and different concentrations of unlabelled Ang II, candesartan and SI-Ang II as described in Section 2. Data are expressed as means ± S.E.M. from 3 to 9 independent experiments, each performed in duplicate. Statistically significant differences are marked with asterisk (*).

aim was to create a receptor, which has similar functional properties to the native receptor, except for non-peptide antagonist binding, we tested additional mutant receptors. In the next series of experiments the V108I and S109T single- (Fig. 1B), and the V108A/S109T double-mutant (Fig. 1A) receptors were analyzed. The Ang II binding affinity of the V108A/S109T mutant receptor was virtually identical to that of the AT_{1A}-R, whereas its binding affinity for candesartan was highly decreased. Studies with point mutant receptors showed that the S109T mutation interfered more with candesartan binding than the V108I mutation, however, it also significantly elevated the affinity for Ang II suggesting that this mutation was responsible for the increased Ang II affinity of the V108I/S109T double mutant receptor (Table 1). The values given in Table 1 are IC_{50} values, however, K_i values calculated using the equation described by Cheng and Prusoff [21], are less then 10% different, due to the low concentration of the radioligand used.

3.2. *Signaling properties of mutant AT_1-Rs in COS-7 cells*

To test the signal transduction properties the created receptor mutants were expressed in COS-7 cells and Ang II-induced inositol phosphate responses of these cells were measured. The COS-7 cells used did not contain endogenous AT_1-Rs and subsequently no inositol phosphate response was observed in untransfected cells. The higher affinity of the V108I/S109T mutant for Ang II translated into an increased potency to activate inositol phosphate signaling, as can be seen from the shift of the dose-response curve to the left, the EC_{50} changed statistically significantly from 0.65 ± 0.08 nM (AT_{1A}-R) to 0.39 ± 0.04 nM (V108I/S109T) and the maximal response was also increased (Fig. 2A). Although the V108A/S109T mutant carried the S109T mutation, which was responsible for the increased Ang II affinity of the V108I/S109T double mutant, it showed minimally reduced Ang II affinity and significantly reduced maximal inositol phosphate response. The EC_{50} of this mutant for the inositol phosphate response was 1.11 ± 0.43 nM. Because both double mutant receptors showed moderately altered signaling responses, additional studies were performed with point

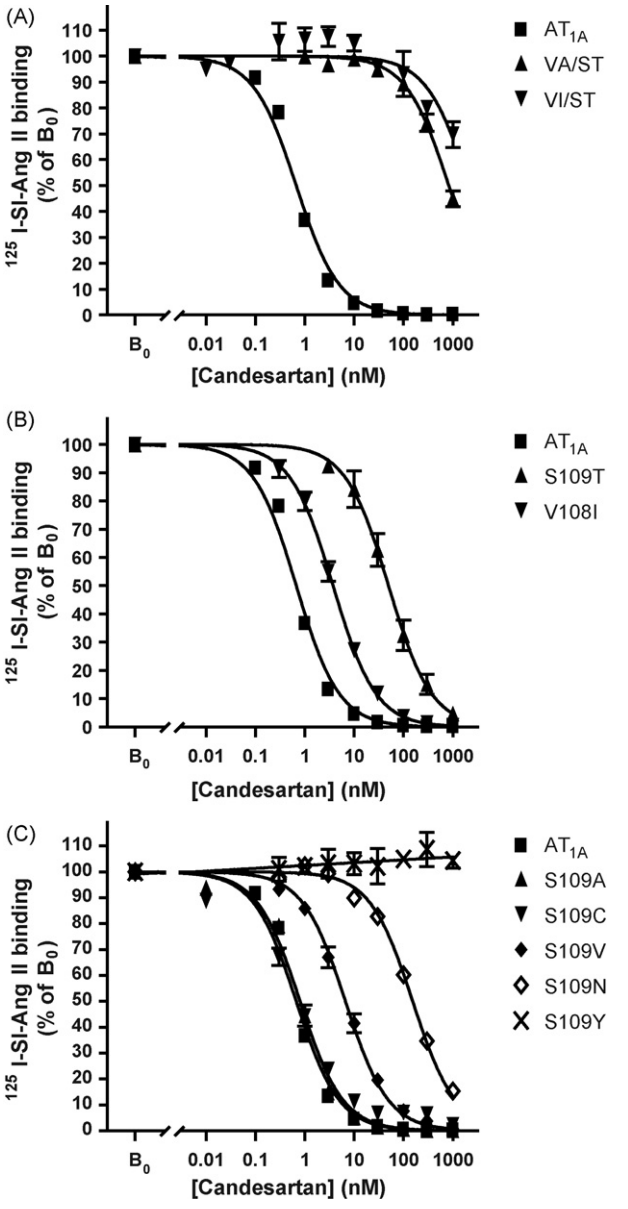

Fig. 1 – Competition binding curves for AT_{1A}-Rs and mutant AT_{1A}-R expressed in COS-7 cells. Transfected cells were incubated with ^{125}I-SI-Ang II and increasing concentrations of candesartan as described in Section 2. Bound ligand is expressed in percents of the total specific binding obtained without candesartan (B_0). Data are expressed as means ± S.E.M. from 3 to 9 independent experiments, each performed in duplicate. (A) Candesartan binding curves of AT_{1A}-R (■), V108A/S109T (▲), and V108I/S109T (▼) mutant AT_{1A}-Rs. (B) Candesartan binding curves of AT_{1A}-R (■), S109T (▲), and V108I (▼) mutant AT_{1A}-Rs. (C) Candesartan binding curves of AT_{1A}-R (■), S109A (▲), S109C (▼) S109V (◆), S109N (◇), and S109Y (×) mutant AT_{1A}-Rs.

mutant AT_1-Rs. Since the Ser-109 seemed more important in the binding of candesartan than the Val-108, we constructed a series of substitutions for this residue. Ser-109 was substituted with non-polar (S109V and S109A, the latter

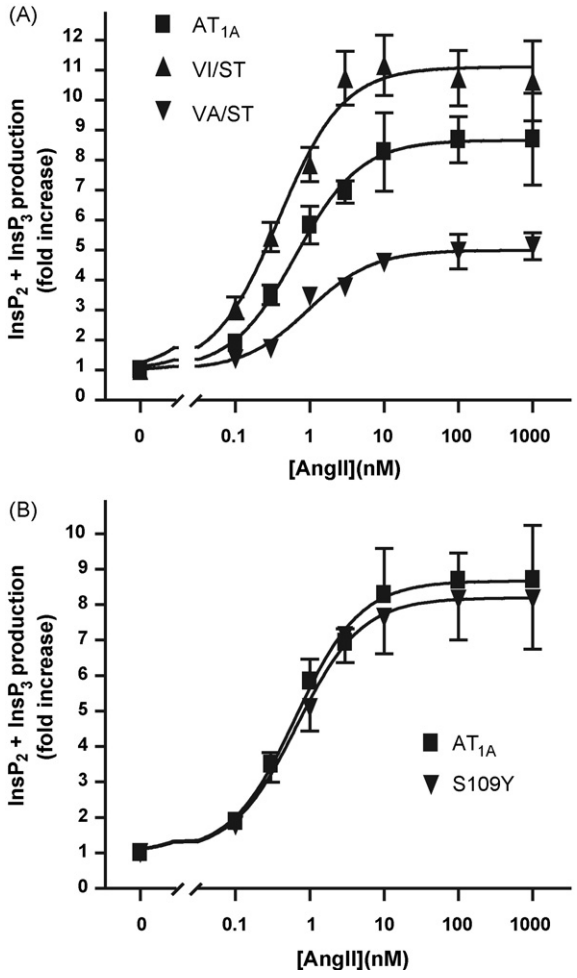

Fig. 2 – Inositol phosphate responses of AT_{1A}-Rs and mutant AT_{1A}-R. Transfected COS-7 cells were prelabeled with [^{3}H]inositol. The cells were pretreated with LiCl and incubated in the presence of various concentrations of Ang II as described in Section 2. Data are shown as fold increase over the unstimulated cells. All data are shown as means ± S.E.M. from three independent experiments, each performed in duplicate. (A) Inositol phosphate response of AT_{1A}-R (■), V108I/S109T (▲), and V108A/S109T (▼) mutant AT_{1A}-Rs. (B) Inositol phosphate response of AT_{1A}-R (■) and S109Y (▼) mutant AT_{1A}-Rs.

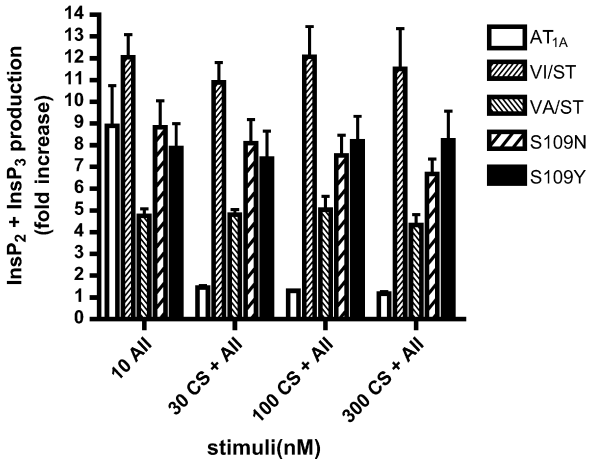

Fig. 3 – Inositol phosphate responses of AT_{1A}-Rs and mutant AT_{1A}-R in the presence of candesartan. Transfected COS-7 cells were prelabeled with [^{3}H]inositol. The cells were pretreated with LiCl and candesartan for 30 min and incubated in the presence of 10 nM Ang II (AII) as described in Section 2. Data are shown as fold increase over the unstimulated cells. Candesartan (CS) (300 nM) on its own did not cause any significant change in the inositol phosphate response. All data are shown as means ± S.E.M. from three independent experiments, each performed in duplicate.

mutant was previously published [16]) and polar (S109N, S109C and S109Y) residues. Fig. 1C and Table 1 shows the binding characteristics of these constructs. The S109A, S109V and S109C mutants did not differ considerably form the AT_{1A}-R in their candesartan affinities, the S109N mutant showed a marked decrease in binding this non-peptide antagonist, and S109Y mutants displayed a remarkable resistance to the compound, showing no displacement of labeled SI-Ang II even at micromolar concentration of candesartan. Binding of the peptide antagonist/partial agonist SI-Ang II was not affected in any of the mutant receptors. When tested for inositol phosphate generation, the S109Y (Fig. 2B) and S109N (data not shown) mutants did not differ significantly from the AT_{1A}-R in their maximal response and EC_{50} values, the latter being 0.67 ± 0.1 nM for the S109Y and 0.75 ± 0.06 nM for the S109N mutant receptor.

The effect of candesartan on the inositol phosphate response of the AT_{1A}-R, and the V108I/S109T, V108A/S109T, S109N and S109Y mutant receptors is shown in Fig. 3. While the response of the AT_{1A}-R to 10 nM of Ang II is completely abolished after preincubation with 30 nM of candesartan, only the S109N mutant receptor showed reduced inositol phosphate responses, whereas no major impairment of the signaling of V108I/S109T and V108A/S109T mutant receptors were detected, and the S109Y mutant completely retained its signaling ability even in the presence of 300 nM candesartan.

Even single amino acid mutations can alter the whole conformation of a GPCR, creating functionally inactive or, conversely, constitutively active receptor mutants, such as the N111G mutant [18,22,23]. A very sensitive test for the detection of constitutive activity in mutant receptors is their sensitivity for stimulation with peptide antagonists, such as SI-Ang II, which act in several test system as weak partial agonists of the wild-type receptor, but can act as full agonists of constitutively active receptors. We tested this possibility by incubating the mutants as seen in Fig. 3 with 1000 nM of SI-Ang II, which was used to reveal constitutive active properties of the D125A receptor mutant [6]. However, SI-Ang II caused no detectable activation of the S109Y mutant receptor (data not shown), which, in agreement with the normal agonist affinity of the receptor, shows that S109Y is not constitutively active.

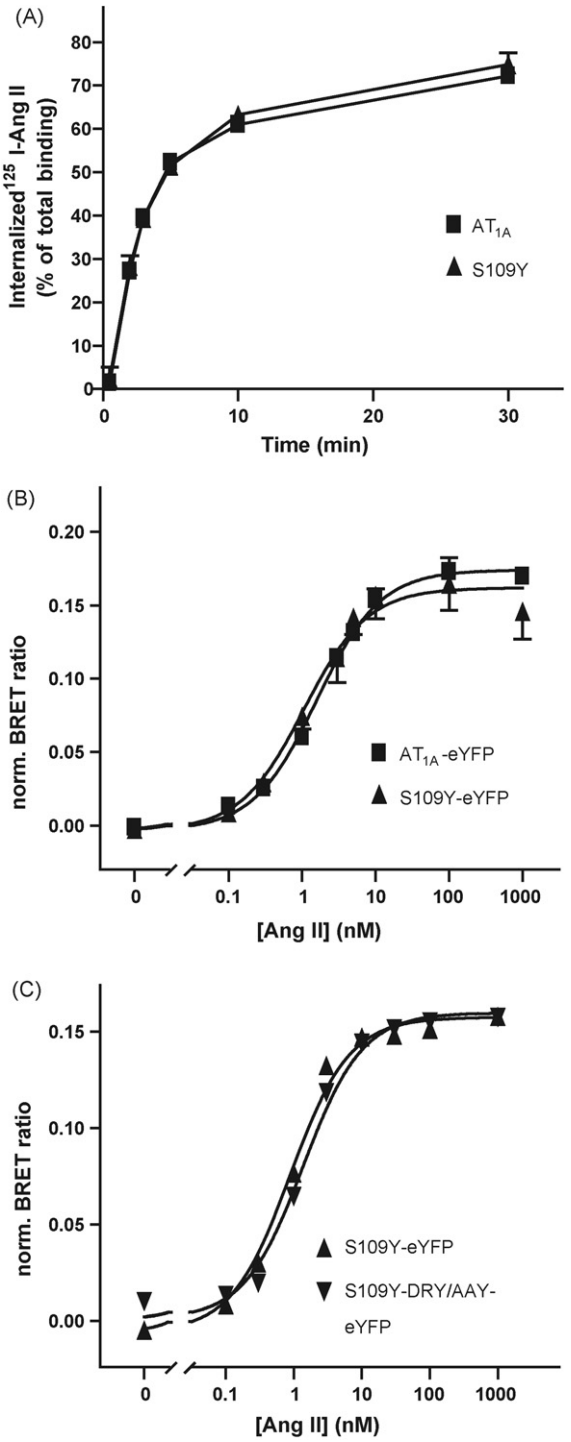

Fig. 4 – Internalization kinetics and BRET dose–response curves for AT_{1A}-R and mutant AT_{1A}-Rs. (A) Internalization kinetics of AT_{1A}-R (■) and S109Y (▲) mutant AT_{1A}-Rs were determined as described in Section 2. Internalized radioactivity was expressed as percent of the total specific binding at each time point. Values are shown as means ± S.E.M. from three independent experiments, each performed in duplicate. (B) BRET analysis of β-arrestin2 binding to AT_{1A}R-eYFP (■) and S109Y (▲) mutant AT_{1A}R-eYFP. Normalized BRET ratios were determined in COS-7 cells coexpressing β-arrestin2-Rluc and AT_{1A}R-eYFP or S109Y-AT_{1A}R-eYFP following stimulation with different concentrations of Ang II as described in Section 2. Values are shown as means ± S.E.M. from three independent experiments, each performed in triplicate. (C) BRET analysis of β-arrestin2 binding to S109Y (▲) and S109Y-DRY/AAY (▼) mutant AT_{1A}-Rs. Normalized BRET ratios were determined in COS-7 cells coexpressing β-arrestin2-Rluc and S109Y-AT_{1A}R-eYFP or S109Y-DRY/AAY-AT_{1A}R-eYFP following stimulation with different concentrations of Ang II. Values shown are means ± S.E.M. of the triplicates of a representative experiment.

3.3. *β-Arrestin2 binding of the S109Y mutant AT_1-R in COS-7 cells*

Based on these data, we focused our attention to the S109Y mutant, and performed further experiments to characterize it. Following agonist binding, activated AT_1-Rs are phosphorylated by G protein-coupled receptor kinases, which enable cytoplasmic proteins called β-arrestins to bind to the receptor. This process desensitizes the receptor, uncoupling it from the G protein, and directs it to clathrin-coated pits, from which it can internalize into endocytic vesicles [24–26]. Internalized receptors are separated from their ligand, dephosphorylated, resensitized and largely recycled to the plasma membrane [27,28]. In addition to their function in desensitizing the receptor, β-arrestins also act as scaffold proteins to organize signaling complexes on the surface on endosomes [29,30]. To test these properties of the S109Y mutant receptor, we followed the internalization using uptake of ^{125}I-Ang II into the cells. As shown in Fig. 4A, the internalization kinetics of the mutant receptor is indistinguishable from that of the AT_{1A}-R. As the internalization of the AT_1-R can also occur also via β-arrestin-independent mechanisms [31], we used BRET, a more direct approach to detect interactions between the receptor and β-arrestins [13]. Fig. 4B shows that the BRET dose–response curves between β-arrestin2-hRluc and AT_{1A}-eYFP or S109Y-AT_{1A}-eYFP are virtually identical, with EC_{50} values 1.6 ± 0.2 nM for AT_{1A}-eYFP and 1.1 ± 0.2 nM for S109Y-AT_{1A}-eYFP ($n = 3$). In another set of experiments the β-arrestin2 binding of the S109Y-AT_{1A}-eYFP receptor was compared with that of the S109Y-DRY/AAY-eYFP receptor. BRET dose–response curves of the Ang II-induced interactions of these receptors with β-arrestin did not show significant differences (Fig. 4C), which is consistent with earlier findings [4,6]. These data demonstrate that the ability of the mutant receptors to interact with β-arrestin2 is similar to that of the wild-type receptor.

3.4. *Expression of the S109Y mutant AT_1-R in C9 cells*

In the next step we expressed the mutant receptor in C9 cells, a rat hepatocyte cell line, which expresses endogenous AT_1-Rs and respond to Ang II stimulation with Ca^{2+} signal generation [32]. In these cells, Ang II activates the ERK1/2 MAPK through transactivation of the epidermal growth factor receptor (EGF-R), a process extensively characterized [33–35]. In control cells, transfected with GFP, Ang II caused rapid phosphorylation of ERK1/2, with a peak at 5 min, and a smaller increase was detected at 30 min. Candesartan and AG1478, an inhibitor of

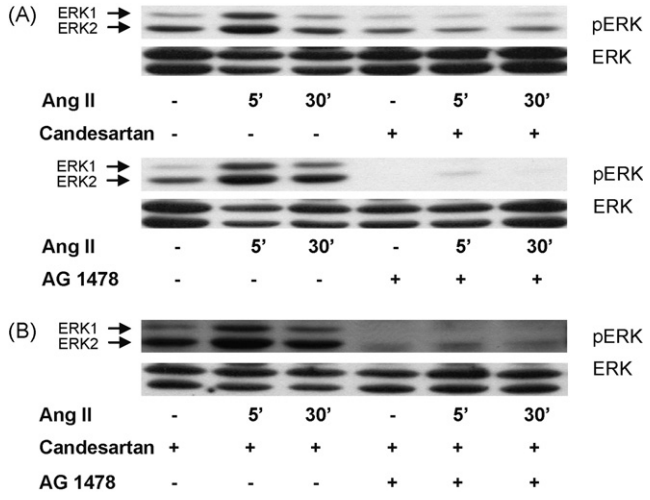

Fig. 5 – ERK1/2 activation transfected C9 cells. (A) Ang II activates ERK1/2 through an AT_1-R and EGF-R dependent way in C9 cells. In C9 cells transfected with GFP as a control Ang II (10 nM) caused a rapid phosphorylation of ERK1/2 with a peak at 5 min and decreasing at 30 min, which could be prevented by preincubation with either candesartan (100 nM) or AG1478 (1 μM). (B) The S109Y mutant AT_{1A}-R expressed in C9 cells activates ERK1/2 in the same EGF-R dependent manner as the endogenous receptor. In C9 cells transfected with the S109Y mutant AT_{1A}-R Ang II (10 nM) activated ERK1/2 in the presence of the AT_1-R blocker candesartan (100 nM). Preincubation with AG1478 (1 μM) prevented this activation. These pictures are representative of at least 10 independent experiments.

EGF-R, completely eliminated the ERK activation mediated by endogenous receptors (Fig. 5A). When transfected with the S109Y mutant receptor, ERK activation occurred even in the presence of candesartan with similar kinetics to the wild-type receptor and it could be blocked by AG1478, which suggests that it activated the same mechanism (Fig. 5B).

AT_1-R mutants incapable of activating G proteins have been shown to activate ERK1/2 through a β-arrestin2-dependent pathway [36,4], but these experiments were performed in cells with no endogenous AT_1-R. To test for the existence of G protein-independent ERK1/2 activation in C9 cells, the D125A/R126A mutation, which disrupts the highly conserved DRY motif in TM III proximal to the 2nd intracellular loop required for G protein activation, was combined with the S109Y mutation. These constructs were expressed in C9 cells. The surface expression levels of the S109Y and S109Y-DRY/AAY receptors in C9 cells (measured in the presence of 300 nM candesartan to block endogenous receptors) were 287 ± 26 fmol/mg protein and 306 ± 29 fmol/mg protein, whereas the expression of the endogenous receptors was 409 ± 35 fmol/mg protein in the same set of experiments ($n = 3$).

To demonstrate that the S109Y-DRY/AAY mutant receptor does not activate G proteins in C9 cells, we measured intracellular calcium levels in response to Ang II in the presence or absence of candesartan in C9 cells. Our data shows that while in non-transfected cells the response to Ang II (Fig. 6A) is abolished in the presence of candesartan (Fig. 6B), cells transfected with the S109Y mutant receptor retain their ability to elicit a Ca^{2+}-signal (Fig. 6C), whereas the cells expressing the S109Y-DRY/AAY mutant receptor does not activate this signaling pathway (Fig. 6D). The smaller Ca^{2+} signal of the expressed receptor (Fig. 6C) versus the endogenous receptor (Fig. 6A) is due to the incomplete transfection of the cells. Visualization of GFP-tagged β-arrestin2 in transfected C9 cells demonstrated that this S109Y-DRY/AAY mutant AT_1-R was able to mediate translocation of β-arrestin2 to the S109Y-DRY/AAY mutant receptor, similar to the S109Y mutant receptor (Fig. 7B). In candesartan-treated cells, the addition of Rhod-Ang II caused the previously cytoplasmic β-arrestin2-eGFP (Fig. 7A) to accumulate on the plasma membrane, and following that both molecules internalized into and colocalized in endocytic vesicles. The S109Y-DRY/AAY mutant receptor was also able to induce ERK1/2 phosphorylation (Fig. 8A), with different kinetic properties from the wild-type receptor. The ERK activation elicited by this mutant was lesser in magnitude, and showed a steady elevation even at 30 min (Fig. 8B), consistent with the earlier observations on the kinetics of the G protein-independent ERK activation [36].

4. Discussion

Structure–function studies in eukaryotic expression systems contributed greatly to our current understanding of the signal transduction pathways of angiotensin receptors and other GPCRs. However, signaling mechanisms of GPCRs, such as activation of MAP kinases, are highly cell specific. For example in many tissues, including vascular smooth muscle cells, stimulation by Ang II leads to the transactivation of growth factor receptors such as EGF, PDGF and IGF receptors [1,37,2] and the subsequent activation of MAP kinases; whereas in other cell types (e.g. HEK-293 or H295R cells), protein kinase C (PKC) activation directly leads to the activation of ERK [2,35]. While these mechanisms both require intact coupling of the receptor to G proteins, a more recently described pathway shows that just as the internalization of the receptor is independent of G protein activation [11,38], β-arrestins can mediate ERK activation independently from G protein coupling [30], and in some cell types, this ERK activation also involves EGF receptor transactivation [38,39]. However, the physiologically relevant signaling mechanisms in the target tissues of hormones and other mediators are difficult to analyze using expression of mutant receptors, due to the presence of endogenous receptors. This is a particularly interesting problem in case of the AT_1-R, since its signaling involves G protein-dependent and -independent pathways, including signal transduction pathways characteristic of GPCRs, growth factor and cytokine receptors [2].

To study the mechanism of Ang II-induced MAP kinase activation in cells expressing native angiotensin receptors, we have developed a mutant AT_1-R, which is deficient in candesartan binding, whereas completely normal in its other pharmacological properties and signal transduction characteristics. Positions 108 and 109 were selected to eliminate

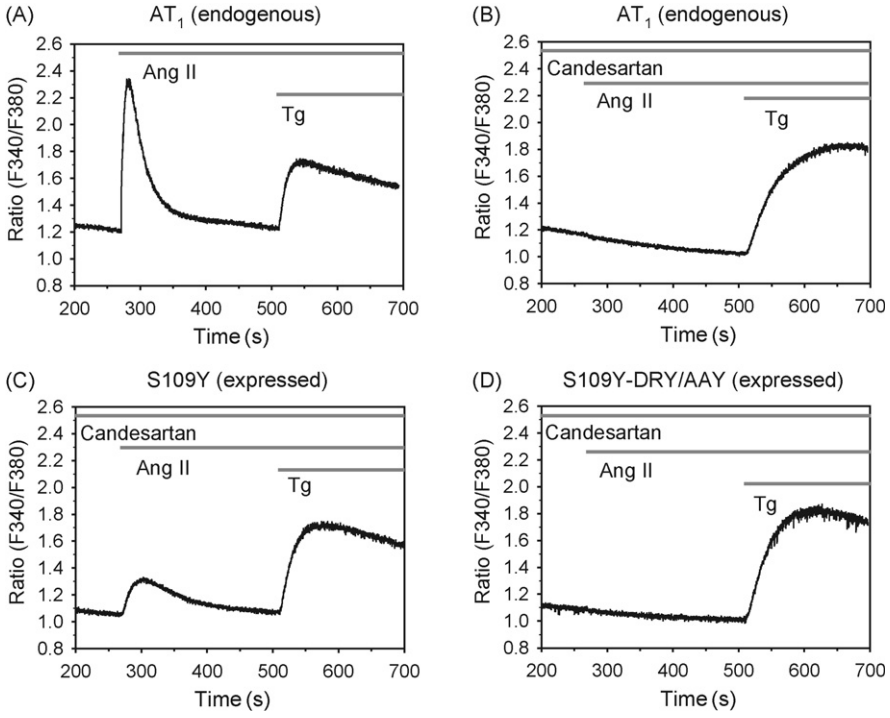

Fig. 6 – Ca^{2+}-signaling in transfected C9 cells. (A–D) C9 cells were detached and loaded with Fura2 as described in Section 2. Candesartan (1 μM), Angiotensin II (100 nM) and Thapsigargin (200 nM) were added as indicated on the graphs. Values are shown as means from two independent experiments. (A) Ang II-induced intracellular Ca^{2+}-signal in non-transfected C9 cells. (B) Ang II-induced intracellular Ca^{2+}-signal in non-transfected C9 cells in the presence of candesartan. (C) Ang II-induced intracellular Ca^{2+}-signal in C9 cells transfected with S109Y mutant AT_{1A}-R in the presence of candesartan. (D) Ang II-induced intracellular Ca^{2+}-signal in C9 cells transfected with S109Y-DRY/AAY mutant AT_{1A}-R in the presence of candesartan.

candesartan binding, based on previous studies, which showed that these residues strongly contribute to the lack of biphenylimidazole antagonist binding in amphibian angiotensin II receptors, and substitution of the amphibian residues strongly impairs non-peptide antagonist binding without affecting the binding of a peptide antagonist [15,16]. In the present study substitution of Ser-109 with threonine was combined with two different non-polar substitutions of Val-108. Although, as expected, these mutants showed impaired candesartan binding, the signaling characteristics of these mutant receptors showed minor differences compared to those of the AT_{1A}-R. Since the S109T mutant receptor displayed more impaired candesartan binding than the V108I mutant receptor, mutant receptors with substitutions of Ser-109 with various polar and non-polar residues were created. Interestingly, substitutions of Ser-109 with non-polar residues did not have a major effect on candesartan binding, suggesting that the polar group of Ser-109 is not involved directly in the binding of candesartan. However, substitution of Ser-109 with asparagine or tyrosine affected the candesartan binding more than the S109T substitution. Since the S109Y mutation eliminated candesartan binding, which caused no detectable displacement of labeled SI-Ang II in concentrations up to 1 μM, even though this antagonist has the highest affinity for the AT_1-R from the currently available non-peptide antagonists [40], the signaling properties of this mutant receptor were characterized.

A recent paper describes a new proposal for the binding orientation of non-peptide antagonists, such as candesartan [41]. According to this model, Val-108, which is adjacent to Ser-109, along with other residues, is involved in the formation of a lipophilic pocket between TM III, TM VI and TM VII in which the biphenyl ring of the ligand resides. Although the exact mechanism of the interference of the S109Y substitution with candesartan binding is not known, it is likely, that the substitution of the Ser-109 with tyrosine prevents the binding of candesartan by obstructing this lipophilic pocket, and/or a secondary lipophilic pocket, in which Ser-109 is directly involved [41]. Our data demonstrated that although the S109Y mutant is completely impaired in candesartan binding, the signaling properties of this mutant AT_1-R are indistinguishable from those of the AT_{1A}-R. Therefore, the S109Y mutant receptor was used to characterize the mechanisms involved in Ang II-induced MAP kinase activation in C9 cells, which express endogenous angiotensin receptors.

Previous studies have demonstrated that the major mechanism of Ang II-induced ERK activation in C9 cells is independent of the internalization of the AT_1-R receptor [42], and is mediated by transactivation of EGF receptors. This transactivation process involves PKC δ, Src, and the proline-rich tyrosine kinase PyK-2, but is independent of intracellular Ca^{2+} levels [34], and leads to the activation of matrix metalloproteinases, which in turn cause the shedding of

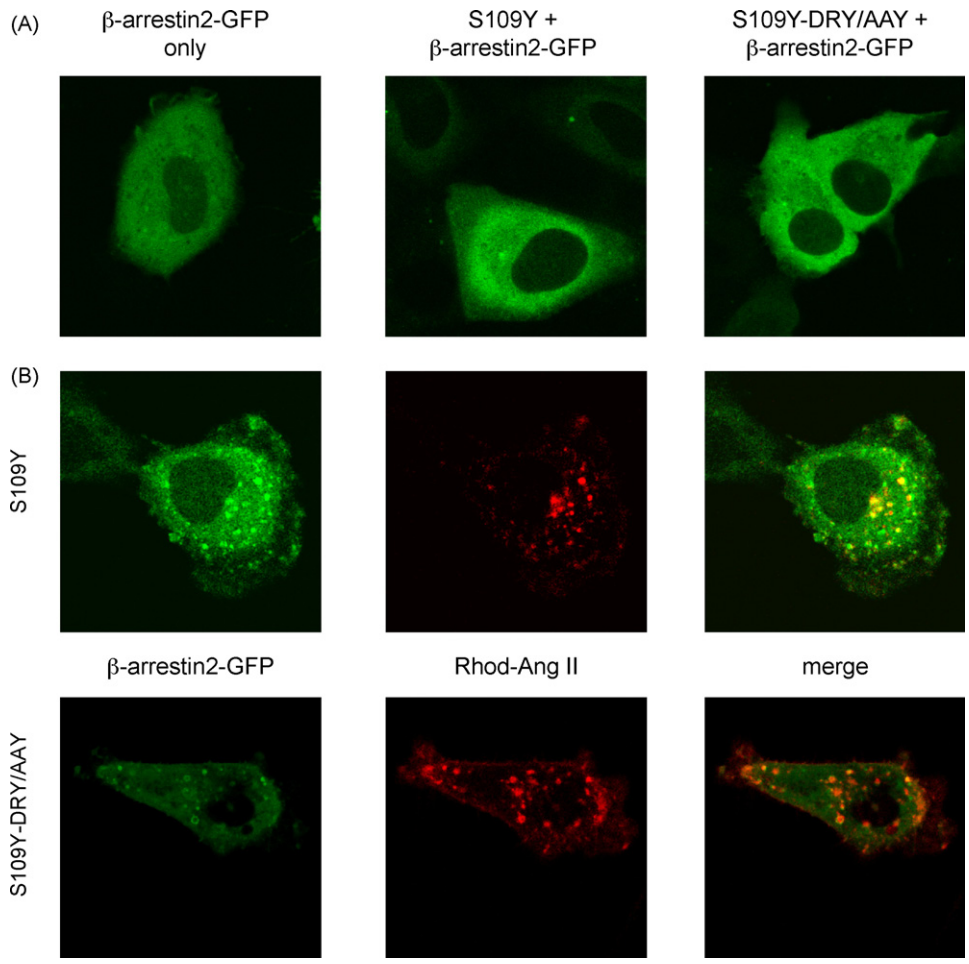

Fig. 7 – β-Arrestin2 translocation in transfected C9 cells in response to Ang II. (A) Localization of eGFP-labeled β-arrestin2 (β-arrestin2-eGFP) without Ang II-stimulation. C9 cells were transfected with β-arrestin2-eGFP alone (left), β-arrestin2-eGFP and S109Y (middle), or β-arrestin2-eGFP and S109Y-DRY/AAY mutant AT_{1A}-R (right). Without Ang II-stimulation, the β-arrestin2-eGFP localizes homogenously to the cytoplasm. (B) Localization of β-arrestin2-eGFP during stimulation of S109Y and S109Y-DRY/AAY mutant AT_{1A}-Rs. S109Y (top) and S109Y-DRY/AAY (bottom) mutant AT_{1A}-R were coexpressed with β-arrestin2-eGFP in C9 cells. The cells were preincubated with 300 nM candesartan and stimulated with 30 nM Rhod-Ang II for 20 min. The localization of β-arrestin2-eGFP (left panels) and Rhod-Ang II (middle panels) is shown. The superimposed image is shown on the right panels. Colocalized structures appear yellow. Colocalization was apparent within a minute of stimulation. The images shown are representative of four independent experiments.

heparin-binding EGF, and subsequent activation of the EGF-receptor [35]. AT_1-R activation by Ang II and EGF-induced activation of the EGF-R in C9 cells can also induce phosphorylation of the AT_1-R, which can be prevented by inhibiting the EGF-R with AG1478 or reduced by inhibition of PKC and phosphoinositide 3-kinase (PI3K) [33,43]. However, ERK1/2 activation by Ang II was found to be independent of PI3K activation in C9 cells [44].

Our data show that candesartan, in a concentration that completely blocks the endogenous receptors, has no detectable effect on the signal generation via the S109Y mutant receptors. The main mechanism of Ang II-induced MAP kinase activation in C9 cells, expressing S109Y mutant receptors, was mediated by EGF receptor transactivation. These data suggest that the signaling mechanism of the S109Y mutant receptor and that of the wild-type receptor are similar. To evaluate the presence of AT_1-R-mediated, G protein-independent signaling in C9 cells, the S109Y mutation was combined with the DRY/AAY mutation, which eliminates G protein coupling, without a major interference with receptor internalization, β-arrestin binding and G protein-independent signaling of the receptor [4,6], and this receptor was expressed in C9 cells. Using this approach, G protein-independent ERK activation was detected in C9 cells, which is the first demonstration of such mechanism in target cells of angiotensin II, which express endogenous angiotensin receptors. The G protein-independent mechanism was a minor component of ERK activation after 5 min stimulation with Ang II, but became a more significant component of ERK activation after 30 min stimulation, which is consistent with previous reports demonstrating that the kinetics of the G protein-independent, β-arrestin-mediated ERK activation pathway is slower than that of the G protein-mediated ERK activation [36].

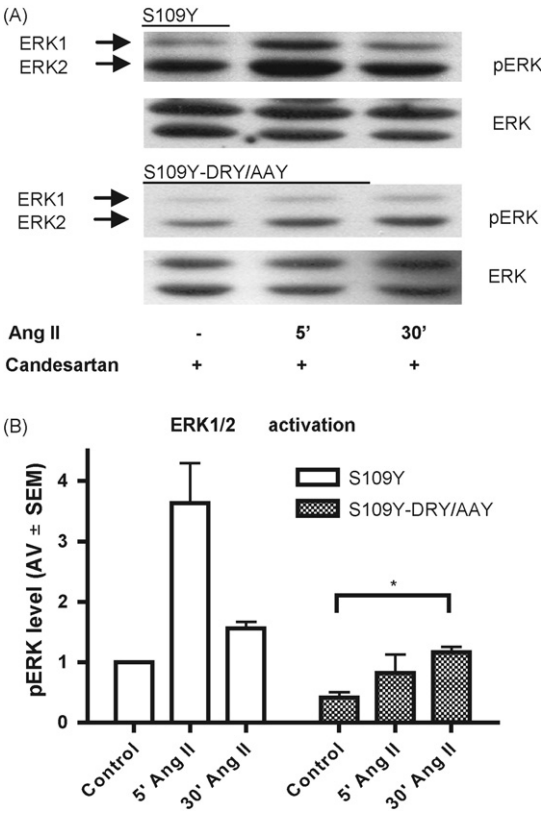

Fig. 8 – ERK1/2 activation in C9 cells transfected with S109Y and S109Y-DRY/AAY mutant AT_{1A}-R. (A) The G protein-independent ERK1/2 activation occurs with different kinetics in C9 cells. In C9 cells transfected with the S109Y-DRY/AAY mutant AT_{1A}-R Ang II (10 nM) caused ERK1/2 activation in the presence of candesartan (100 nM). The kinetic of this activation was different from the one observed with the endogenous and the S109Y mutant receptor, with a sustained activation even at 30 min. The images shown are representative of three independent experiments. (B) Quantification of the ERK1/2 activation of the S109Y and the S109Y-DRY/AAY mutant AT_{1A}-R. Films were scanned and quantified. Data are expressed as fold increase over the unstimulated cells expressing the S109Y mutant receptor. Values are shown as means ± S.E.M. from three independent experiments.

In summary, we have identified a single amino acid substitution mutant (S109Y) in the rat AT_{1A}-R, which selectively eliminates the binding of non-peptide 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. We combined this mutant with a previously published mutation to demonstrate the presence of Ang II-induced, G protein-independent ERK1/2 activation in cells expressing endogenous AT_1-R. These data are consistent with the relevance of G protein-independent signaling pathways to the mechanism of Ang II action, but additional studies are required to elucidate the exact physiological roles of these pathways in different target tissues of the hormone.

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