A noninternalized nondesensitized truncated AT1A receptor transduces an amplified ANG II signal

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Conchon, Sophie, Nicolas Peltier, Pierre Corvol, and Eric Clauser. A noninternalized nondesensitized truncated AT1A receptor transduces an amplified ANG II signal. Am. J. Physiol. 274 (Endocrinol. Metab. 37): E336–E345, 1998.—The structural determinants of the rat angiotensin (ANG) II AT1A receptor involved in receptor internalization, desensitization, and activation are investigated by producing six mutants that had progressively larger deletions of the cytoplasmic tail (−13, −19, −24, −31, −46, and −56 residues, respectively). After stable transfection of the cDNAs into Chinese hamster ovary cells, all mutants, except the most truncated, exhibit normal [3H]-ANG II affinities (dissociation constant (Kd) = 0.19–0.70 nM) compared with the wild-type (WT) receptor (Kd = 0.62 nM) and are able to activate a Gq/11 protein and a phospholipase C as measured by the ANG II-induced inositol phosphate (IP) turnover in the different clones. However, one of these mutants, Δ329 (deletion of 31 residues), exhibits a peculiar phenotype. This mutant shows a reduced ligand-induced internalization as measured by the acid-washing procedure (only 32% of receptors are internalized vs. 83% for WT). Moreover, the Δ329 mutant is less desensitized by a pretreatment with either ANG II (15% desensitization of ANG II-stimulated IP turnover vs. 60% for WT receptor) or the phorbol ester phorbol 12-myristate 13-acetate (no desensitization vs. 29% for WT receptor). These functional modifications of the Δ329 mutant are associated with the transduction of an amplified signal as demonstrated on both IP turnover and an integrated physiological effect of ANG II. Taken together, these data indicate that the sequence 329SLSTKMS335 of the rat AT1A receptor is involved in both receptor internalization and desensitization. This is the first demonstration that a desensitization- and internalization-defective AT1A receptor mutant is also hyperreactive and mediates augmented cellular responses.

angiotensin II receptor; mutagenesis; endocytosis; Chinese hamster ovary cells

ANGIOTENSIN II (ANG II) is a vasoactive peptide that acts on its target tissues through the interaction with cell surface receptors. These receptors, members of the seven transmembrane domain receptor family, have been divided into several types (AT1 and AT2) and subtypes (AT1A and AT1B) based on their pharmacological properties, amino acid sequence, and/or tissue distribution (for review, see Ref. 7). AT1 receptors are G protein-coupled receptors, which are responsible for most of the ANG II physiological actions. The signal transduction via these receptors follows a classical pathway, which involves Gq/11 protein(s) and phospholipase(s) Cβ, which produce second messengers and result in Ca2+ mobilization and protein kinase (PK) C activation (7). More recently, other signaling pathways, such as the JAK-STAT pathway or the Shc-p21ras pathway, were demonstrated to be activated by the AT1 receptor, but the exact physiological significance of this observation is not known.

In parallel to the activation of this signal transduction pathway, the AT1 receptor, as for many other receptors of the same family, undergoes internalization of the ligand-receptor complexes (1) and phosphorylation (25). These two modifications are generally considered to participate in the process of receptor desensitization, defined as the attenuation of the signal due to serial agonist applications.

On one hand, the morphological aspects of the internalization-sequestration process, which involves specific clustering of the receptors in clathrin-coated pits (32), are well known, whereas the molecular mechanisms and the functional consequences of this internalization are still a matter of debate. For the AT1 receptors, this process is induced by peptide but not by nonpeptidic ligands and is independent of G protein coupling (9). The AT1 sequence involved in the internalization process is not the consensus sequence [NPX(1–2)Y] located at the junction of the seventh transmembrane domain and the COOH-terminal tail (17), as described for the β2-adrenergic receptors (3) or the low-density lipoprotein (LDL) and tyrosine kinase receptors (27), but more probably a sequence in the COOH-terminal tail. Indeed, several authors (16, 33) have shown that deletions or mutations in different segments of the proximal part of the COOH-terminal tail impaired the AT1A receptor internalization.

On the other hand, receptor phosphorylation on serine and threonine residues is a major event, which follows receptor activation and largely participates to receptor desensitization. G protein-coupled receptors can be phosphorylated by two different types of kinases: 1) second-messenger-activated kinases, such as PKA or PKC, which produce a negative feedback and a nonspecific mechanism of desensitization; and 2) specific kinases that form the growing family of G protein-coupled receptor kinases (GRK) (26). One example of such an homologous desensitization is the phosphorylation by GRK2 of the β2-adrenergic receptor, which subsequently displays a high affinity for a cytosolic protein, β-arrestin. This interaction between the β-arrestin and the phosphorylated receptor suppresses G protein interaction and uncouples the receptor. The sites and pattern of phosphorylation vary from one receptor to another but are located mainly in the COOH-terminal tail or the third intracellular loop; this also varies for the same receptor from one cellular model to another (29).

Despite a better understanding of the molecular mechanisms involved in receptor internalization, phosphorylation, and desensitization, the precise relation-
ships between these three phenomena are still a matter of debate and vary from one receptor to another.

To establish precisely the sequences of the COOH-terminal tail of the AT1A receptor involved in activation, internalization, and desensitization, six mutants of this receptor with progressive deletions of the COOH-terminal were characterized. We studied the signaling, physiological actions, internalization, and desensitization of these mutants. This enabled the identification of a deletion mutant that was neither internalized nor desensitized. Interestingly, this mutant receptor transduces an amplified signal through the membrane.

MATERIALS AND METHODS

Construction of truncated AT1A mutants. The expression plasmid used for the construction of the truncated mutants was pEAT1A/C and has been described previously (8). This plasmid consists of a synthetic AT1A cDNA containing multiple unique endonuclease restriction sites inserted into the Hind III and Xba I sites of the eukaryotic expression vector pECE (11). The mutants Δ347, Δ341, Δ336, and Δ329 were generated by double digestions of pEAT1A/C with Sma I (site located after the stop codon of AT1A cDNA) and Sac I, Stu I, Sal I, and Xho I, respectively. The cohesive ends generated by each of these enzymes was blunt-ended with either T4 DNA polymerase or DNA polymerase I (Klenow fragment), and the linear construction was subsequently religated using T4 DNA ligase. The presence of stop codons in each of the three linear constructions was subsequently recircularized using T4 DNA ligase. The cohesive ends generated by each of these enzymes was blunt-ended with either T4 DNA polymerase or DNA polymerase I (Klenow fragment), and the linear construction was subsequently religated using T4 DNA ligase. The presence of stop codons in each of the three linear constructions was subsequently recircularized using T4 DNA ligase.

For the other two constructions (Δ314 and Δ304), the restriction site used for the deletion was not unique. Therefore, the EcoR I-Xba I fragment of the synthetic AT1A cDNA, coding for the COOH-terminal half of the receptor, was subcloned into the plasmid pMT21 (11). This intermediate construct was called pMT21C. Δ314 was obtained by a double digestion of pMT21C with Sma I and Pst I followed by a T4 DNA polymerase treatment and ligation. For Δ304, the Nsi I site, located in a region corresponding to the middle of the seventh transmembrane domain of the receptor, and the Sma I site were used. The missing part of this transmembrane domain was reconstructed with a linker corresponding to nucleotides 868–905, which was inserted between the Nsi I and Sma I sites. In both cases, the EcoR I-Xba I insert was then excised and inserted into the EcoR I and Xba I sites of a plasmid, pENZ, consisting of pECE with the Hind III-EcoR I fragment of the synthetic AT1A cDNA. All these constructions were confirmed by sequencing (Sequenase version 2.0, USB).

Cell culture stable transfection. Chinese hamster ovary (CHO) K1 cells were cotransfected with 2 µg of pSVNeo (Pharmacia) and 10 µg of the expression plasmids by the calcium phosphate coprecipitation method (31). Transfected cells were selected by their resistance to neomycin (GIBCO-BRL). The cell populations giving high levels of binding were subcloned by limiting dilution to obtain pure cell lines with high expression levels of the mutant receptors. The CHO AT1A/V3 cell line was obtained by transfection of the already characterized CHO AT1A cell line with the expression plasmid pECE containing the vasopressin V2 receptor cDNA. Because these cells were already neomycin resistant, the selection was achieved by cotransfection with a plasmid that confers resistance to hygromycin.

Cells were then maintained at 37°C in a 5% CO2 atmosphere in Ham’s F-12 medium supplemented with 10% fetal calf serum plus 0.5 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Boehringer Mannheim).

Binding studies. [3H]ANG II and ANG II (Sigma) were labeled by the chloramine-T method and the monodiodinated product was purified by high-performance liquid chromatography.

Cells were subcultured into 24-well culture trays and incubated for 45 min at 22°C with 0.5 nM [3H]ANG II or [125I]-ANG II in the presence of increasing amounts of nonlabeled [3H]ANG II or ANG II, respectively, in 50 mM trishydroxymethylaminomethane HCl, 6.3 mM MgCl2, 125 mM NaCl, 1 mM EDTA, and 1 mg/ml bovine serum albumin (BSA, pH 7.6). Nonspecific binding was determined in the presence of 1 µM [3H]ANG II or ANG II. Each experiment was carried out in duplicate. Binding data were analyzed with a nonlinear least squares curve-fitting procedure (Ebdal-Ligand, Elsevier-Biosoft, Cambridge, UK).

Analysis of the guanosine 5’-O-(3-thiotriophosphate) (GTP·S) action on ANG II binding was performed on cell membranes as previously described (15). Binding of 0.4 nM of 125I-ANG II to 5–10 µg of membrane proteins was performed in the absence or presence of GTP·S (10^-9 to 10^-5 M) at 25°C for 2 h. Bound tracer was separated from free tracer and the radioactivity was measured by γ-spectrometry.

Internalization assay. Internalization of wild-type and mutant AT1A was measured as a percentage of 125I-[3H]ANG II resistant to acid wash, as described previously (9). Transfected cells were incubated with 0.4 µM 125I-[3H]ANG II in binding buffer with or without 1 µM [3H]ANG II for 180 min at 4°C, washed twice, and placed in binding buffer alone at 37°C for various times. Finally, cells were placed at 4°C, and for half of the replicate wells, total and nonspecific binding was measured after cell lysis with 1 M NaOH. In the other wells, surface-bound 125I-[3H]ANG II (total and nonspecific) was determined after incubating the cells in 50 mM glycine and 125 mM NaCl (pH 3) for 5 min. Internalized radioactivity (total and nonspecific) was determined after lysis of the cells in 1 M NaOH.

Inositol phosphate production. ANG II stimulation of inositol phosphate (IP) production was performed as described previously (35). Cells were subcultured in 12-well culture trays, labeled with 2 µCi/ml myo-[3H]inositol for 24 h, preincubated for 10 min with 10 mM LiCl, and then incubated with increasing concentrations of ANG II for 30 min at 37°C in the presence of 10 mM LiCl. After purification on a Dowex 1 X-8 anion exchange resin (Bio-Rad), the total IP fraction was measured. For desensitization experiments, labeled cells were preincubated for 15 min with 100 nM ANG II or arginine vasopressin (AVP) and rinsed three times, and after 15 min without agonist, they were incubated for a further 15 min with 100 nM ANG II or AVP. When used, phorbol 12-myristate 13-acetate (PMA) was added at the indicated concentration during the preincubation (30 min) and incubation (15 min) periods.

ANG II inhibition of insulin-induced [14C]glucose incorporation into glycogen. Confluent cells grown in 12-well plates were incubated for 1 h with the indicated concentrations of ANG II in phosphate-buffered saline (PBS), 0.1% BSA, 0.7 mM CaCl2, 0.5 mM MgCl2, and 100 nM insulin. Cells were then exposed for 3 h to [14C]glucose (5 mM, 2 µCi). After three washes with ice-cold PBS, the cells were lysed with 30% KOH and transferred to glass tubes. Total glycogen was precipitated with ethanol as described previously (20), and the amount of radioactivity incorporated was determined using a scintillation counter.
Statistics. Results are expressed as means ± SE. Statistical significance was assessed by analysis of variance.

RESULTS

The COOH-terminal cytoplasmic tail of AT\textsubscript{1}A receptors consists of \(\sim 60\) amino acids. To test the role of this region in the biological functions (binding, activation, internalization, and desensitization) of the AT\textsubscript{1}A receptor, six mutants were constructed in which progressively larger portions of the cytoplasmic tail were removed. Thus, as shown in Fig. 1, receptor mutants \(\Delta 347, \Delta 341, \Delta 336, \Delta 329, \Delta 314,\) and \(\Delta 304\) lack the last 13, 19, 24, 31, 46, and 56 residues of the cytoplasmic tail, respectively.

CHO cells were stably transfected with the cDNAs encoding these truncated receptors and were tested for their \(\text{\textsuperscript{125}I}-\text{Sar}^1\)ANG II binding activity. No \(\text{\textsuperscript{125}I}-\text{[Sar}^1\text{]ANG II binding could be detected in cells trans-
The binding characteristics of $^{125}$I-[Sar$^1$]ANG II to CHO pure cell lines expressing the full-length receptor and the five other truncated mutants are shown in Table 1. All these receptors display comparable affinities for this peptidic agonist. Moreover, the wild-type $AT_{1A}$ receptor (dissociation constant ($K_d$) = 0.645 ± 0.027 nM) and the $\Delta 329$ mutant ($K_d$ = 0.343 ± 0.051 nM) present similar affinities for the natural peptide ANG II.

The internalization of the mutant receptors from the cell surface after exposure to $^{125}$I-[Sar$^1$]ANG II was measured using the acid-wash method. As shown in Fig. 2, treatment of cells expressing the wild-type $AT_{1A}$ with this agonist resulted in a rapid loss of the receptors from the cell surface, with a maximal internalization of 83% of the total specific binding within 20 min at 37°C. The time necessary to internalize 50% of the ligand-receptor complexes ($t_{1/2}$) was 4.22 min. Time courses of internalization of $\Delta 347$, $\Delta 341$, and $\Delta 336$ were comparable to the time course of the full-length receptor, with an acid-resistant $^{125}$I-[Sar$^1$]ANG II fraction reaching a maximum of 76, 86, and 83%, respectively, after 20 min at 37°C and similar $t_{1/2}$ (4.05, 3.63, and 4.19 min, respectively). The truncation of $>31$ COOH-terminal residues of $AT_{1A}$ resulted in a drastic decrease of its ligand-mediated internalization (32% for $\Delta 329$ and 18% for $\Delta 314$). $\Delta 329$-reduced internalization had a time-course pattern comparable to $AT_{1A}$, since the maximal effect occurred after 20 min at 37°C, and the $t_{1/2}$ was 5.18 min, whereas the $\Delta 314$ internalization pattern appeared to be different: the maximum was reached only after 90 min at 37°C. For this mutant, the $t_{1/2}$ was ~14 min. These results are summarized in Fig. 1.

The ability of cells expressing the full-length receptor and the truncated mutants to stimulate IP production in response to increasing concentrations of ANG II was analyzed. LiCl was added to allow an accumulation of proteins in the lysosomal compartment of the cell. The amount of IP production was measured in CHO cell lines expressing $AT_{1A}$ with this agonist for various time periods to allow internalization. Noninternalized tracer was removed by acid washing, and internalized tracer levels were determined after NaOH treatment. Results are expressed as percentage of total specific binding and represent means ± SE of 3 independent experiments performed in duplicate.

The respective maximal binding capacities ($B_{max}$) of each cell line and represent means ± SE of 3 independent experiments performed in duplicate.

Fig. 2. Time course of [Sar$^1$]ANG II-induced receptor internalization. Chinese hamster ovary (CHO) cells expressing $AT_{1A}$ (●), $\Delta 347$ (○), $\Delta 341$ (●), $\Delta 336$ (●), $\Delta 329$ (●), and $\Delta 314$ (●) were prelabeled with $^{125}$I-[Sar$^1$]ANG II at 4°C for 3 h. Cells were then washed and incubated at 37°C for various time periods to allow internalization. Noninternalized tracer was removed by acid washing, and internalized tracer levels were determined after NaOH treatment. Results are expressed as percentage of total specific binding and represent means ± SE of 3 independent experiments performed in duplicate.

Fig. 3. Angiotensin II (ANG II)–induced stimulation of IP production. Chinese hamster ovary (CHO) cells expressing $AT_{1A}$ (●), $\Delta 347$ (○), $\Delta 341$ (●), $\Delta 336$ (●), $\Delta 329$ (●), and $\Delta 314$ (●) were prelabeled with $^{125}$I-[Sar$^1$]ANG II at 4°C for 3 h. Cells were then washed and incubated at 37°C for various time periods to allow internalization. Noninternalized tracer was removed by acid washing, and internalized tracer levels were determined after NaOH treatment. Results are expressed as percentage of total specific binding and represent means ± SE of 3 independent experiments performed in duplicate.

Table 1. Binding parameters of $^{125}$I-[Sar$^1$]ANG II in transfected CHO cells

<table>
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<tr>
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<th>$K_d$, nM</th>
<th>$B_{max}$, 10$^6$ sites/cell</th>
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<tr>
<td>$AT_{1A}$</td>
<td>0.617 ± 0.179</td>
<td>1.54 ± 0.13</td>
</tr>
<tr>
<td>$\Delta 347$</td>
<td>0.700 ± 0.118</td>
<td>1.30 ± 0.15</td>
</tr>
<tr>
<td>$\Delta 341$</td>
<td>0.552 ± 0.104</td>
<td>1.47 ± 0.18</td>
</tr>
<tr>
<td>$\Delta 336$</td>
<td>0.186 ± 0.013</td>
<td>0.34 ± 0.11</td>
</tr>
<tr>
<td>$\Delta 329$</td>
<td>0.201 ± 0.019</td>
<td>1.26 ± 0.34</td>
</tr>
<tr>
<td>$\Delta 314$</td>
<td>0.198 ± 0.070</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>$\Delta 304$</td>
<td>ND</td>
<td>ND</td>
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Values are means ± SE obtained from 3 separate experiments for which each point was performed in duplicate. Dissociation constants ($K_d$) and maximal binding capacities ($B_{max}$) represent affinity and number of sites. ND, not detectable.
of GTPγS on the binding of the native agonist ANG II to membranes from transfected cells. As shown in Fig. 4, GTPγS displayed a slightly better effect on the Δ329 mutant compared with the wild-type receptor, but this difference was not significant. However, GTPγS remained almost without effect on the binding of ANG II to the Δ314 mutant, suggesting a major alteration of the interaction between this mutant and G proteins.

This hyperreactivity of the Δ329 mutant was also investigated on an integrated biochemical effect of ANG II in CHO cells. The inhibitory action of ANG II on insulin-dependent incorporation of glucose into glycogen was analyzed for wild-type and Δ329 receptors. This physiological effect of ANG II was first described in hepatocytes (19) and could be reproduced in CHO cells. As shown in Fig. 5, in the CHO Δ329 cells, ANG II induced a 73% inhibition of the glucose incorporation triggered by 100 nM insulin, with an EC₅₀ of 1.40 nM. In CHO Δ329 cells, the inhibitory effect of ANG II reached a maximum of 91%, and the EC₅₀ of 0.15 nM was statistically different from the wild type (P < 0.05).

For some G protein-coupled receptors, internalization and the desensitization-resensitization processes have been shown to be closely related. We therefore studied whether the enhanced response observed with Δ329 reflected a modification of the desensitization pattern of this mutant. The stimulation of IP turnover in response to serial ANG II applications was measured for the wild-type and truncated receptors. Because our interest was to measure not a time-dependent IP accumulation but the effect of two consecutive agonist applications, the experiment was performed without LiCl. Therefore the following results cannot be compared with those described above. In addition, the time course of this IP production measured by the present assay is longer than the duration of the desensitization process. This explains why the second application of ANG II was done before the return of the IP values to the basal level.

Under these conditions, basal cellular IP content was 318 ± 25 counts/min (cpm) (see Fig. 6A, bar 1). After a single treatment with ANG II, IP production reached 675 ± 31 cpm (bar 2). However, two sequential treatments with ANG II gave rise to a significantly lower level of IP production (bar 4; 592 ± 29 cpm; P < 0.01) compared with treatment with a single application of ANG II. Moreover, because the pretreatment with ANG II raised the basal level of IP production somehow (to 448 ± 39 cpm, bar 3), the actual stimulation induced by the second application of ANG II was that much smaller at 144 cpm compared with 357 cpm after a single treatment with ANG II. This would suggest a 60% desensitization of the IP response to ANG II via the AT₁ receptor (see also Fig. 7).

For the Δ329 mutant, the basal IP cellular content was comparable to that of the CHO AT₁ receptor (305 ± 37 cpm). The IP production reached 1,275 ± 126 cpm after a single ANG II stimulation and 1,499 ± 120 cpm after two sequential treatments with ANG II. Fifteen minutes after the ANG II pretreatment, the IP level remained above the basal initial value (673 ± 55 cpm). Taking this into account, a second ANG II application induced an IP production of 826 cpm. Thus the IP production after two successive ANG II treatments was only 15% lower than after a single agonist exposure (823 cpm vs. 970 cpm; see also Fig. 7). Therefore the IP...
response induced by ANG II via the Δ329 mutant appeared only slightly desensitized.

The same procedure was applied to the Δ347 and Δ341 mutants, which behave as the wild-type receptor (data not shown). Unfortunately, the Δ336 and Δ314 mutants could not be tested because the low density of sites did not allow any measurable IP production in the absence of LiCl.

In the mutant Δ329, which had a modified internalization pattern and was less desensitized with exposure to ANG II, the three potential PKC phosphorylation sites present in the wild-type receptor were deleted. Thus we investigated whether a short-term application of the phorbol ester PMA (30 min), by activating PKC, would differentially modify the amplitude of the ANG II-induced IP production via wild-type and mutated receptors (Fig. 6B). In CHO AT1A cells, PMA produced a dose-dependent inhibition of the ANG II-induced IP production, which reached, for 10^{-6} M PMA, 29% of the stimulation observed without PMA. Because, under the same conditions, PMA had no effect on the ANG II-induced IP production in CHO cells expressing the Δ329 mutant, the implication of PKC-mediated phosphorylation in the desensitization process of the AT1A receptor could be postulated.

To study homologous vs. heterologous desensitization, a CHO cell line stably expressing both AT1A and vasopressin V3 receptors (10), which are coupled to the same signaling pathways, was produced. When these cells were submitted to ANG II stimulation, with or without an ANG II pretreatment, the responses were similar to those described for CHO AT1A cells (data not shown). To verify that the procedure of serial ANG II applications described above measures the desensitization of the receptor and not of an element further downstream in this signaling pathway, the effect of an ANG II pretreatment on the AVP-evoked IP production in these cells was analyzed (Fig. 7). When cells were preincubated with 100 nM ANG II before the 100 nM AVP application, the amplitude of the response reached 114% of the maximal stimulation obtained without pretreatment. This experiment demonstrated that an ANG II pretreatment did not promote the desensitization of any other element of this signaling pathway. Therefore the results described above represented exclusively the desensitization of the receptor.

However, the amplitudes of the ANG II-induced IP production with or without an AVP pretreatment were comparable. Therefore the activation of phospholipase C and subsequently of PKC via the V3 receptor was not able to induce a desensitization of the AT1A receptor and vice versa.

DISCUSSION

In the present study, the functional consequences of successive deletions of the COOH-terminal tail on signaling, internalization, and desensitization of the rat AT1A receptor were investigated. The first result of this study is that these successive deletions up to amino acid 314 do not drastically modify the expression at the cell surface or the binding affinity of the receptor for agonist ligands (Table 1). This is in accordance with the report of Ohyama et al. (22), which demonstrated that the deletion of the COOH-terminal sequence upstream of amino acid 309 does not drastically alter the expression and pharmacological properties of the AT1A receptor. However, the Δ304 mutant of the present study does not present any detectable binding for ANG II, which indicates that the sequence between residues 304 and 309 is involved in the binding site of ANG II or more probably plays a role in the folding, processing, and/or transport process at the cell surface of the receptor.
The most interesting mutant characterized in this study is the Δ329 mutant, which displays reduction of both internalization and desensitization associated with the transduction of an amplified signal. Several classes of functional alterations of G protein-coupled receptors resulting from natural or site-directed mutations have been described. These include loss-of-function mutations as well as gain-of-function mutations, such as those resulting in the constitutive activation of the receptor and its corresponding signaling pathways (21). However, the Δ329 mutant of the AT1A receptor belongs to a new class of functional mutants, presenting an amplification of the physiological response, probably due to an alteration of densensitization and/or internalization. Approaching phenotypes have been described for the β2-adrenergic receptors (14), and it would be interesting to investigate whether mutations of other receptors could determine such a "phenotype" and whether natural mutations of receptors could correspond to this phenotype in vivo.

In our series of mutants, the Δ329 is the largest deletion mutant, which presents a major impairment of its internalization. Indeed, the deletion mutants Δ347, Δ341, and Δ336 exhibit a ligand-induced internalization similar to the wild-type receptor, whereas Δ314 and Δ329 mutants are poorly internalized despite their ability to transduce the signal. Several sequences of the receptors have been identified as important for internalization of membrane-bound receptors. An example is the NPX(1–2)Y sequence, which is essential for the internalization of the LDL, tyrosine kinase (27), and β2-adrenergic receptors (3). However, this sequence corresponding to the sequence 300NPLFY304 of the AT1A receptor is not involved in the internalization of this receptor (17). In the present paper, a 329SLSTKMS335 sequence seems to be essential for AT1A receptor internalization, and an additional 15-amino acid deletion further reduces this internalization (17.9 vs. 31.8%; Figs. 1 and 2). This result is in agreement with a previous report, indicating that two regions of the AT1A receptor COOH-terminal tail are important for internalization (33). This result should also be compared with that reported by Hunyady et al. (16), who identify a 327TKMSTLS338 sequence as essential for internalization. This sequence only partially overlaps the sequence identified in this paper. This apparent discrepancy could be explained by the present strategy of tail deletions, which uses a synthetic AT1A cDNA with multiple unique restriction sites leading to the addition of two or three amino acids at the COOH-terminal of each deletion mutant. Therefore the Δ336 mutant presents a COOH-terminal sequence (TKMSRV), which may represent a fairly well-conserved substitution of the sequence described by Hunyady et al. The internalization sequence described in this paper could be classified in those serine- and threonine-rich sequences located within the COOH-terminal tail or the third intracellular loop and identified as important for internalization of the G protein-coupled receptors. The precise role of this AT1A receptor sequence is not known. Given the fact that this sequence is serine and threonine-rich, it has been proposed that this sequence is phosphorylated after receptor activation (16). Indeed, there are two PKC phosphorylation sites (Ser331 and Ser338) in or close to this sequence and several other serines or threonines. This could suggest a link between AT1A receptor phosphorylation and internalization. However, it is unlikely for this receptor, since several reports including ours show that ANG II peptidic antagonists are able to internalize the receptor without activating its G protein coupling and signal transduction (9, 15); in addition, an AT1A receptor mutant, which binds ANG II normally but is not coupled to G protein, is still internalized (9, 15). This relationship between phosphorylation and internalization has been investigated extensively for other G protein-coupled receptors. For some receptors, such as the gastrin-releasing peptide receptor, internalization...
is at least partially dependent on PKC phosphorylation of the receptor (5). Phosphorylation of the β-adrenergic receptor was not considered as necessary for receptor internalization; however, recent data suggest that GRK2 and β-arrestin are implicated in β-adrenergic receptor internalization (12). Similarly, the involvement of GRK2 in muscarinic M₂ receptor internalization is also controversial (24, 36). These data suggest that the relationship between receptor phosphorylation and internalization considerably varies from one receptor to another, and it is difficult today to delineate a common paradigm to define these relationships among this family of receptors.

Another explanation for the role of this internalization sequence would be that it interacts with a specific intracellular protein implicated in the internalization process. This protein could belong to, for example, the adaptin family, which is one of the main components of the plasma membrane coat pits, a major structural and morphological element of internalization (30).

In addition to its impaired internalization, the Δ329 mutant exhibits a reduced ANG II-induced desensitization. Because the deleted sequence contains several serines and threonines, one possible explanation is that this altered desensitization is due to a modification of the AT₁A receptor phosphorylation pattern. This hypothesis is indirectly supported by the observation that PMA, via PKC activation, is able to desensitize the wild-type AT₁A receptor but not the Δ329 mutant. This absence of PMA-induced desensitization of the Δ329 mutant is similar to the observations of Balmforth et al. (2) for a Δ319 mutant. Such a role of PKC in the AT₁A receptor desensitization was previously reported using PKC inhibitors (4). However, the use of pharmacological concentrations of PKC inhibitors or activators in these studies and their limited effect on desensitization both suggest that this mechanism is only subsidiary in the physiological state. Indeed, further experiments using a cell line expressing both the ANG II AT₁A and the vasopressin V₁ receptors, which are both coupled to phospholipase C and PKC (6), do not indicate that the desensitization of the AT₁A receptor proceeds from a heterologous mechanism involving PKC but instead from a homologous or another mechanism.

Therefore the reduction of homologous desensitization of the Δ329 mutant receptor could be due to either a reduction of ligand-induced internalization or an impairment of specific phosphorylation by an unidentified GRK. The possibility that the desensitization defect of the Δ329 mutant could be the consequence of its impaired internalization is unlikely. Indeed, in CHO AT₁A and CHO Δ329 cells, concanavalin A drastically reduces receptor internalization but has no effect on ANG II-induced IP production (data not shown). Moreover, there is accumulating evidence from the literature that the inhibition of ANG II receptor internalization in different cell types using KCl, sucrose, or other inhibitors either has no effect or leads to a reduction of the signal transduction (18, 28). Therefore the most likely hypothesis is that the loss of homologous desensitization of the Δ329 mutant is due to the deletion of a sequence phosphorylated by a specific GRK or to an interaction with a specific protein involved in this desensitization process. Taken together, these data support the hypothesis that desensitization of the AT₁A receptor is closely related to receptor phosphorylation either by a nonspecific PKC-associated mechanism or more probably by a GRK. A recent paper (23) analyzing the phosphorylation mechanisms of the AT₁A receptor confirms these hypotheses, showing that a tagged recombinant AT₁A receptor expressed in HEK 293 cells is desensitized and that this desensitization correlates temporally with receptor phosphorylation by both PKC and GRK.

Finally, probably as the result of its impaired desensitization, the Δ329 mutant is unique in its ability to transduce an amplified signal compared with the other mutants and the wild-type receptor. The Δ329 mutant amplifies stimulation of the IP turnover induced by ANG II compared with the wild-type receptor. Despite similar values of basal IP turnover and a similar number of binding sites in both cell lines, the EC₅₀ is eightfold lower (0.09 nM for Δ329 mutant vs. 0.75 nM for wild-type receptor), and the maximal stimulation of IP turnover is twofold higher for the Δ329 mutant. This result is confirmed by another effect of ANG II in these cell lines: the ANG II-mediated inhibition of insulin-induced stimulation of glycogen synthesis is amplified in the CHO cell line expressing the Δ329 mutant compared with that expressing the wild-type receptor. These data should be compared with those of the literature. On one hand, the Δ319 mutant expressed in HEK 293 cells (2) induces a similar maximal stimulation of the IP turnover compared with the wild-type receptor, despite a 2.5-fold reduction of the number of sites, a fact not pointed out by the authors. If the correlation between maximal IP stimulation and Bmax is taken into account, the above result can be interpreted as a signal amplification for the Δ319 mutant. On the other hand, the Δ314 mutant presented in our study does not transduce an amplified signal (IP production similar to wild-type receptor) and presents a major alteration of G protein interaction as demonstrated by the action of GTPγS on the ANG II binding (Fig. 4). In addition, a closely related mutant induces a normal Ca²⁺ mobilization in response to ANG II (34). Taken together, these results suggest that the deletion of the amino acid sequence 329–336 is responsible for the transduction of an amplified signal, whereas further deletion of the amino acid sequence 314–319 reduces the signal transduction ability of the AT₁A receptor. Therefore the most probable explanation of the signal amplification is the impairment of receptor desensitization via phosphorylation. In addition, the reduction of the coupling efficiency by further deletion is likely due to an alteration of the G protein coupling.

In conclusion, the phenotype described for Δ329 could reflect major impairments of two mechanisms dependent on ANG II binding: receptor internalization and homologous desensitization, which could result from receptor phosphorylation or other mechanisms. In the future, it will be very interesting to analyze in...
detail the amino acids of this sequence involved in the desensitization process by performing single or multiple amino acid mutations or deletions. The parallel characterization of the receptor phosphorylation will be possible using functional epitope-tagged receptors. Finally, the analysis of the phenotype produced by the expression of this internalization- and desensitization-defective AT1A mutant in transgenic animals could be of physiological and pathological significance.

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