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Agonist-Induced Phosphorylation of the Angiotensin AT_{1a} Receptor Is Localized to a Serine/Threonine-Rich Region of Its Cytoplasmic Tail

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ABSTRACT

The agonist-induced phosphorylation sites of the rat AT_{1a} angiotensin receptor were analyzed using epitope-tagged mutant receptors expressed in Cos-7 cells. Angiotensin II-stimulated receptor phosphorylation was unaffected by truncation of the cytoplasmic tail of the receptor at Ser342 (Δ342) but was abolished by truncation at Ser325 (Δ325). Truncation at Ser335 (Δ335), or double-point mutations of Ser335 and Thr336 to alanine (ST-AA), reduced receptor phosphorylation by ~50%, indicating that in addition to Ser335 and/or Thr336, amino acids within the Ser326-Thr332 segment are also phosphorylated. Agonist-induced phosphorylation of the ST-AA and Δ335 receptors was partially inhibited by staurosporine, suggesting that the single protein kinase C consensus site in the Ser326-Thr332 segment (Ser331) is phosphorylated. The impairment of receptor phosphorylation was broadly correlated with the attenuation of agonist-induced internalization rates (Δ325 < Δ335 < ST-AA < Δ342 < wild-type) and with the increasing rank order of magnitude of inositol phosphate production normalized to an equal number of receptors (Δ325 > Δ335 > ST-AA = Δ342 > wild-type). These results demonstrate that agonist-induced phosphorylation of the AT_{1a} receptor is confined to an 11-amino-acid serine/threonine-rich segment of its carboxyl-terminal cytoplasmic tail and implicate this region in the mechanisms of receptor internalization and desensitization.

The superfamily of GPCRs, which mediate the biological responses of cells to diverse extracellular stimuli such as light, odor, neurotransmitters, biogenic amines, and hormones, has been the subject of intensive study in recent years. The current paradigm of GPCR activation entails an agonist-induced change in receptor conformation that facilitates the exchange of GDP for GTP on the α subunits of cognate heterotrimeric G proteins (reviewed in Hamm, 1998). Activated G protein α subunits, together with liberated $\beta\gamma$ complexes, modulate the activities of several effector molecules, including enzymes such as adenylate cyclase (via G_i and G_s) and phospholipase C (via $G_{q/11}$). However, in many cases the responses of cells to agonists are limited by rapid quenching (or desensitization) of the signals generated by activated GPCRs (Hausdorff, 1990; Bohm, 1997). Activated GPCRs are also internalized (or sequestered) into cells and then may be targeted to lysosomes for proteolytic degradation (Hoxie *et al*., 1993) or resensitized and recycled back to the plasma membrane, where they become available for further ligand binding (Bohm, 1997).

The mechanism of desensitization is believed to result from the phosphorylation of activated GPCRs by GRKs and/or second

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ABBREVIATIONS: GPCR, G protein-coupled receptor; Ang II, angiotensin II; AT_{1a}-R, type 1a angiotensin receptor; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; GRK, G protein-coupled receptor kinase; HA, hemagglutinin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LB, lysis buffer; PAGE, polyacrylamide gel electrophoresis; PKC, protein kinase C; PNGase, peptide *N*-glycosidase; SDS, sodium dodecyl sulfate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

messenger-activated kinases (for reviews, see Inglese *et al*., 1993; Lefkowitz, 1993). Although GRK-mediated phosphorylation of GPCRs is sufficient for partial receptor desensitization, full desensitization requires the subsequent binding of β-arrestin proteins, which sterically hinder the coupling of receptors to G protein or proteins (Ferguson *et al*., 1996a, 1996b). Receptor-bound β-arrestins also seem to act as molecular adapters in the subsequent internalization of some GPCRs via clathrin-coated pits (Goodman *et al*., 1996). Resensitization of desensitized GPCRs results from the dephosphorylation of phosphorylated receptors by GPCR phosphatases (Pitcher *et al*., 1995) and the consequent dissociation of β-arrestin.

Although this paradigm of GPCR function is well established, it is based largely on studies of a limited number of receptors, in particular the G_s-coupled β-adrenergic receptor (Ferguson *et al*., 1995; Freedman *et al*., 1995; Fredericks *et al*., 1996; January *et al*., 1997). In contrast, relatively little is known about the nature and role of agonist-induced phosphorylation in the function of the $G_{q/11}$-coupled AT_1-R. This has been largely due to the inability to achieve adequate resolution of the phosphorylated AT_1-R from additional co-precipitating phosphoproteins in SDS-PAGE. However, the use of an improved technique that removes extraneous phosphoproteins before immunoprecipitation has facilitated the demonstration of agonist-induced phosphorylation of endogenous AT_1-Rs in bovine adrenal glomerulosa cells (Smith *et al*., 1998). Here, we applied this methodology to localize the phosphorylation sites of an epitope-tagged rat AT_{1a}-R transiently expressed in Cos-7 cells. By using a series of truncation mutants, we demonstrate that the major agonist-induced phosphorylation sites of the rat AT_{1a}-R are located in an 11-amino-acid serine/threonine-rich segment between Ser326 and Thr336 of the receptor carboxyl-terminal intracellular region.

Experimental Procedures

Materials. DMEM, P_i-free DMEM, inositol-free DMEM, FBS, and antibiotic solutions were from Biofluids (Rockville, MD). Angiotensin II was from Peninsula Laboratories (Belmont, CA). ^{125}I-[Sar^1,Ile^8]Ang II and ^{125}I-Ang II were from Covance Laboratories (Vienna, VA). *myo*-[2-^{3}H]Inositol was from Amersham (Arlington Heights, IL). $^{32}P_i$ was from Andotek (Tustin, CA). Protein A-Sepharose was from Oncogene Research Products (Cambridge, MA). PNGase F (E.C. 3.5.1.52) was from Boehringer-Mannheim (Indianapolis, IN). The HA.11 mouse monoclonal antibody was from BAbCo (Berkeley, CA). OptiMEM and LipofectAMINE were from Life Technologies (Gaithersburg, MD). Staurosporine and TPA were from Sigma Chemical (St. Louis, MO).

Mutagenesis of the rat AT_{1a} receptor cDNA. The influenza HA epitope (YPYDVPDYA) was inserted after the codons of the amino-terminal first two amino acids (MA) into the cDNA of the rat AT_{1a} receptor subcloned into pcDNAI/Amp (InVitrogen, San Diego, CA) as described previously (Smith *et al*., 1998). Using the *Eco*RI site within the coding region and the *Not*I site 3′ from the AT_{1a}-R sequence, previously described mutant (non-HA tagged) rat AT_{1a} receptor sequences (Hunyady *et al*., 1994) were subcloned into the HA-tagged rat AT_{1a} receptor.

Transient expression of HA-AT_{1a}-Rs. Cos-7 cells were seeded at 6×10^5 cells/10-cm dish or 3.7×10^4 cells/24-well culture plate in DMEM containing 10% (v/v) FBS, 100 μg/ml streptomycin, and 100 IU/ml penicillin (Cos-7 medium) and cultured for 3 days before transfection using 0.5 ml (24-well plate) or 5 ml (10-cm dish) of OptiMEM containing 10 μg/ml LipofectAMINE and the required DNA (1 μg/ml) for 6 hr at 37°. After changing to fresh Cos-7 medium, the cells were cultured for an additional 2 days before use. Binding of ^{125}I-[Sar^1,Ile^8]Ang II to intact cells was performed as described previously (Hunyady *et al*., 1996).

HA-AT_{1a}-R phosphorylation assay. Transfected Cos-7 cells in 10-cm dishes were metabolically labeled for 4 hr at 37° in P_i-free DMEM containing 0.1% (w/v) BSA and 100 μCi/ml $^{32}P_i$. After three washes in KRH [118 mM NaCl, 2.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM glucose, 0.1% (w/v) BSA, 20 mM HEPES, pH 7.4], cells were incubated in the same medium for 10 min in a 37° water bath. Vehicle or 100 nM Ang II was then added for an additional 5 min. After three washes with ice-cold PBS, cells were drained before scraping into LB (50 mM Tris, pH 8.0, 100 mM NaCl, 20 mM NaF, 10 mM Na pyrophosphate, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml pepstatin, 10 μg/ml benzamidine, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 1 μM okadaic acid) and probe-sonicated (Sonifier Cell Disruptor; Heat Systems Ultrasonics, Plainview, NY) for 2 × 20 sec. After removal of nuclei at 750 × *g*, membranes were pre-extracted by the addition of an equal volume of LB containing 2 M NaCl and 8 M urea followed by overnight tumbling at 4°. The membranes then were collected at 200,000 × *g* and solubilized in LB+ [LB supplemented with 1% (v/v) Nonidet P-40, 1% (w/v) Na deoxycholate and 0.1% (w/v) SDS] with Dounce homogenization. After clarification at 14,000 × *g*, solubilized membranes were incubated with 2% (v/v) protein A-Sepharose for 1 hr at 4°. The precleared supernatant was incubated overnight at 37° with 10 units/ml PNGase F before immunoprecipitation of deglycosylated HA-AT_{1a}-Rs by the addition of 1 μl of HA.11 antibody and 2% (v/v) protein A-Sepharose overnight at 4°. After washing of the Sepharose-bound immune complexes in LB+ lacking protease inhibitors, ^{32}P-labeled phospho-HA-AT_{1a}-Rs were eluted in Laemmli's sample buffer for 1 hr at 48° and resolved by SDS-PAGE on a 8–16% gradient resolving gel. Phospho-HA-AT_{1a}-Rs were then visualized and quantified in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

To quantify the relative phosphorylation of mutant HA-AT_{1a}-Rs, membrane lysates were normalized to an equal number of HA-AT_{1a}-Rs before immunoprecipitation. Cos-7 cells from replicate 10-cm dishes were detached by trypsinization 24 hr after transfection, reseeded into 24-well plates, cultured for an additional 24 hr, and subjected to radioligand binding competition assay using ^{125}I-[Sar^1,Ile^8]Ang II. B_{max} values were obtained from Scatchard analysis of the binding data using the LIGAND program.

AT_{1a}-R internalization assay. ^{125}I-Ang II was added in serum-free DMEM at 37° to transfected Cos-7 cells in 24-well plates for the indicated times. Incubations were stopped by rapid washing with ice-cold PBS, and acid-released and acid-resistant radioactivities were separated and measured by γ-spectrometry as described previously (Hunyady *et al*., 1994). The percent of internalized ligand at each time point was calculated from the ratio of the acid-resistant specific binding to the total (acid-released plus acid-resistant) specific binding.

Inositol phosphates measurements. Transfected Cos-7 cells in 24-well plates were labeled by overnight incubation in inositol-free DMEM containing 0.1% (w/v) BSA, 2.5% (v/v) FBS, antibiotics, and 20 μCi/ml *myo*-[2-^{3}H]inositol. After washing and preincubation with 10 mM LiCl for 30 min, 1 μM Ang II was added for an additional 30 min. Inositol phosphates were extracted as described (Hunyady *et al*., 1998) and applied to BioRad AG 1-X8 columns (Hercules, CA). After washing three times with water and twice with 0.2 M ammonium formate, the combined $InsP_2$/$InsP_3$ fractions were eluted with 1 M ammonium formate in 0.1 M formic acid, and radioactivity values were determined by liquid scintillation counting. At the expression levels used in this study, there was a linear relationship between cell surface receptor expression and the magnitude of agonist-stimulated inositol phosphate production (Hunyady *et al*., 1995).

Results

Binding parameters of mutant HA-AT_{1a}-Rs. The rat AT_{1a}-R contains as many as 19 potential serine/threonine phosphorylation sites, 13 of which (11 serine and two threonine) are located in the distal 34-amino-acid segment of its carboxyl-terminal intracellular tail (Fig. 1). Depending on the exact locations of their membrane boundaries, the intracellular loops contain up to three serine and five threonine residues. To localize the major agonist-induced phosphorylation sites to specific regions of the receptor and to explore the role of such phosphorylation in receptor signaling and internalization, a series of truncation mutants was created by introducing stop codons at Ser342 (Δ342), Ser335 (Δ335), and Lys325 (Δ325) of an influenza HA epitope-tagged rat AT_{1a} receptor (HA-AT_{1a}-R) (Fig. 1).

The binding parameters of these mutants, together with those of a double-point mutation to alanine at Ser335 and Thr336 (ST-AA), were determined by Scatchard analysis of ^{125}I-[Sar^1,Ile^8]Ang II binding to intact Cos-7 cells expressing each receptor (Table 1). Although the K_d values for each mutant receptor were not significantly different from that of the wild-type receptor, their expression levels (especially those of the truncation mutants) were appreciably lower than that of the wild-type receptor (Table 1). To assess the relative degree of phosphorylation of mutant receptors, B_{max} values obtained from Scatchard analysis of ^{125}I-[Sar^1,Ile^8]Ang II binding to intact replicate transfected Cos-7 cells were used to normalize ^{32}P-labeled solubilized membranes to an equal number of HA-AT_{1a}-Rs before immunoprecipitation.

Phosphorylation of mutant HA-AT_{1a}-Rs. The photoaffinity-labeled HA-AT_{1a}-R expressed in Cos-7 cells migrates as a diffuse smear of M_r 85,000–145,000 in SDS-PAGE (presumably due to heterogeneity arising from variable degrees of receptor glycosylation; Smith *et al*., 1998) but shifts to a discrete doublet with M_r ~40,000 after enzymatic deglycosylation. This finding is consistent with the predicted size (41 kDa) of the nonglycosylated AT_1 receptor (Murphy *et al*., 1991). Unlike the less diffuse migration pattern of the photoaffinity-labeled endogenous AT_1-R in bovine adrenal glomerulosa cells (which migrates as a broad band of M_r 60,000–65,000; Smith *et al*., 1998), the broad migration pattern of the HA-AT_{1a}-R expressed in Cos-7 cells, together with the presence of comigrating nonreceptor phosphoproteins, renders unsatisfactory the quantification of (glycosylated) phospho-HA-AT_{1a}-Rs. For this reason, the solubilized ^{32}P-labeled phospho-HA-AT_{1a}-Rs were subjected to enzymatic deglycosylation with PNGase F (Lemp *et al*., 1990) before immunoprecipitation and SDS-PAGE. The deglycosylated phospho-HA-AT_{1a}-R doublets were not only more discrete but also separated from the extraneous phosphoproteins and, accordingly, could be more accurately quantified.

Using this approach, no basal phosphorylation of the wild-type HA-AT_{1a}-R, or of any of the mutant receptors (data not shown) was detected in control cells. In contrast, treatment of the transfected cells for 5 min with 100 nM Ang II caused marked phosphorylation of the wild-type receptor (Fig. 2). However, the introduction of a stop codon at Lys325 (Δ325) upstream of the 13 serine/threonine residues of the receptor tail completely abolished receptor phosphorylation. These data indicate that none of the potential intracellular loop sites of the HA-AT_{1a}-R expressed in Cos-7 cells are phosphorylated and that the agonist-induced phosphorylation sites are located exclusively in the receptor intracellular tail downstream of Lys325. To further localize these phosphorylation sites, the carboxyl-terminal 18-amino-acid segment (which contains five serine residues) was removed from the HA-AT_{1a}-R by the introduction of a stop codon at Ser342 (Δ342). However, this mutant receptor displayed no significant difference in Ang II-induced phosphorylation compared with the wild-type HA-AT_{1a}-R, indicating that the major agonist-induced phosphorylation sites are located upstream of Ser342 in the serine/threonine-rich 13-amino-acid segment between Ser326 and Ser338.

To localize these phosphorylation sites, a stop codon was introduced at Ser335 (Δ335) and its phosphorylation status was compared with that of the ST-AA double-point mutant. Consistent with the incremental reductions in molecular size, sequential truncation of the carboxyl-terminal tail increased the electrophoretic mobility of the deglycosylated phospho-HA-AT_{1a}-Rs in SDS-PAGE with the rank order Δ335 > Δ342 > ST-AA = wild-type. Although Ang II caused a similar degree of phosphorylation of the Δ335 and ST-AA receptors, the magnitude of this phosphorylation was ~50% of that of the wild-type and Δ342 receptors. These data indicate that the HA-AT_{1a}-R is phosphorylated on multiple sites in the Ser326-to-Ser338 segment. Furthermore, the similar degrees of phosphorylation observed for both the Δ335 and ST-AA mutants, which is consistent with the deduced absence (discussed above) of major phosphorylation sites downstream of Pro341, indicate that Ser335 and/or Thr336 (but not Ser338) is a major site or sites for agonist-induced HA-AT_{1a}-R phosphorylation. However, the residual phosphorylation observed with the Δ335 and ST-AA mutants also indicates the existence of additional phosphorylation sites in the Ser326-to-Thr332 segment.

This segment contains a single residue (Ser331) that is situated within a consensus sequence for phosphorylation by

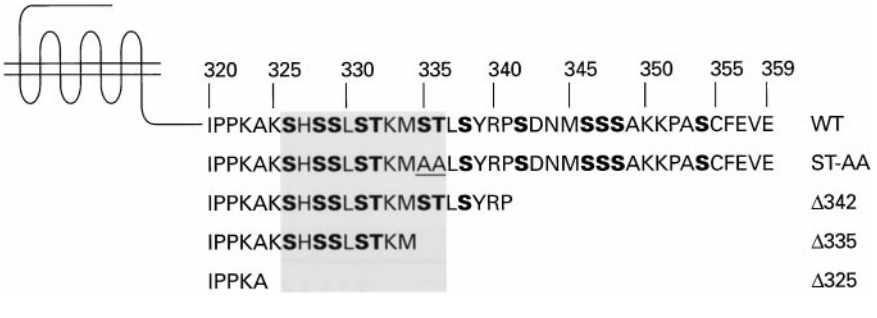

Fig. 1. Amino acid sequences of mutant HA-AT_{1a}-Rs. *Bold*, Potential serine and threonine phosphorylation sites; *underlined*, ST-AA mutation sites; *shaded box*, 11-amino-acid serine/threonine-rich region that contains the sites of agonist-induced phosphorylation.

PKC. Because PKC is activated by Ang II in target cells (Catt *et al.*, 1993), its role in agonist-induced phosphorylation of the ST-AA and Δ335 receptors was determined by pretreating cells with a concentration of staurosporine (500 nM) that is sufficient to inhibit PKC but has no effect on GRKs (Oppermann *et al.*, 1996). This reduced agonist-stimulated phosphorylation of the ST-AA and Δ335 receptors by about 50%, whereas direct activation of PKC (using the phorbol ester TPA) was sufficient to cause partial phosphorylation of each receptor (Fig. 3). These data suggest that Ang II stimulates phosphorylation of the ST-AA and Δ335 receptors on Ser331 via PKC and that a non-PKC (presumably GRK-dependent) pathway mediates the phosphorylation of an additional site or sites in the Ser326-to-Thr332 segment.

Internalization of mutant AT_{1a}-Rs. The effects of truncation of the tail of the AT_{1a}-R and hence sequential removal of its phosphorylation sites were assessed. Although truncation of the AT_{1a}-R at Ser342 (Δ342) caused little change in the receptor agonist-induced internalization rate, truncation at Lys325 (Δ325) almost completely abolished receptor internalization (Fig. 4). Truncation at Ser335 (Δ335) markedly reduced receptor internalization (although to a slightly lesser extent than that of Δ325), whereas the ST-AA mutant internalized at a rate that was intermediate between those of the Δ342 and Δ335 mutants. Hence, sequential removal of the receptor phosphorylation sites was correlated with incremental impairment of receptor internalization.

Inositol phosphate responses of mutant HA-AT_{1a}-Rs. Each of the mutant HA-AT_{1a}-Rs was able to couple to G_q because each receptor stimulated the production of inositol phosphates to a similar extent as the wild-type receptor when expressed in Cos-7 cells (Fig. 5a). However, when these data were normalized to equal receptor expression (derived from B_{max} values), it became apparent that the ability of the agonist-activated mutant receptors to stimulate inositol phosphate production was greater than that of the wild-type receptor (Fig. 5b). The rank order of magnitude with which the mutants stimulated inositol phosphates production (Δ325 > Δ335 > Δ342 > wild-type) correlated with the degree of truncation of the receptor tail.

TABLE 1

Binding parameters for mutant HA-AT_{1a}-Rs.

Intact Cos-7 cells expressing the indicated receptors were subjected to radioligand binding competition assays for 6 hr at 4° using ^{125}I-[Sar^1,Ile^8]-Ang II; K_d and B_{max} values were calculated using the LIGAND program. The B_{max} value for the wild-type receptor was 1.61 ± 0.87 pmol/mg of protein. The data represent mean values ± standard error from three independent experiments.

Receptor	K_d	B_{max}
	nM	*% WT*
WT	1.48 ± 0.34	100 ± 54
ST-AA	1.23 ± 0.38	73 ± 33
Δ342	1.16 ± 0.32	45 ± 24
Δ335	1.14 ± 0.25	47 ± 27
Δ325	1.28 ± 0.21	36 ± 22

WT, wild-type.

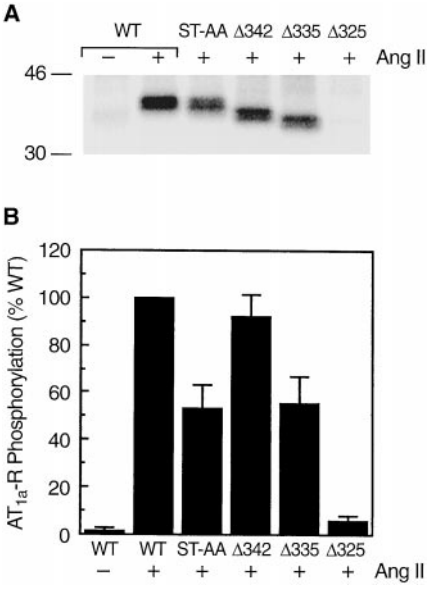

Fig. 2. Agonist-induced phosphorylation of mutant HA-AT_{1a}-Rs. A, Cos-7 cells expressing the indicated receptors were labeled with $^{32}P_i$ for 4 hr before the addition of vehicle or 100 nM Ang II. Membrane lysates normalized to an equal number of receptors were prepared as described in Experimental Procedures. After overnight incubation at 37° in the presence of 10 units/ml PNGase F, deglycosylated HA-AT_{1a}-Rs were precipitated by the anti-HA antibody and resolved by SDS-PAGE. Phosphorylated receptors then were visualized and quantified in a PhosphorImager. B, Quantification of mean ± standard error HA-AT_{1a}-R phosphorylation from four independent experiments.

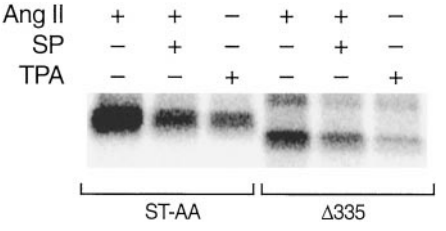

Fig. 3. The role of PKC in agonist-induced phosphorylation of mutant HA-AT_{1a}-Rs. Cos-7 cells expressing ST-AA or Δ335 receptors were labeled with $^{32}P_i$ for 4 hr and pretreated with vehicle or 500 nM staurosporine (*SP*) for 10 min before stimulation with 100 nM Ang II or 200 nM TPA for an additional 5 min as indicated. After overnight incubation at 37° in the presence of 10 units/ml PNGase F, deglycosylated HA-AT_{1a}-Rs (which were not normalized to an equal number of receptors) were precipitated by the anti-HA antibody and resolved by SDS-PAGE. Phosphorylated receptors then were visualized in a PhosphorImager.

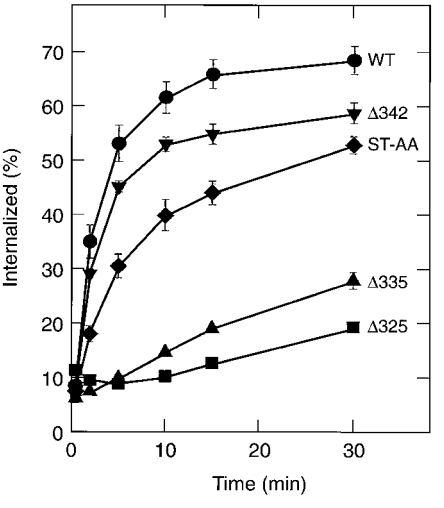

Fig. 4. Internalization kinetics of mutant AT_{1a}-Rs. Cos-7 cells expressing the indicated receptors were incubated with ^{125}I-Ang II at 37° for the indicated times. Acid-resistant and acid-sensitive binding (cpm) were determined as described in Experimental Procedures, and the internalized (acid-resistant) binding was expressed as percent of the total binding at each time point. The data represent mean ± standard error values from three to four independent experiments.

Discussion

Agonist-induced phosphorylation has been demonstrated for a variety of GPCRs including the β-adrenergic (Ferguson *et al*., 1995; Freedman *et al*., 1995; Fredericks *et al*., 1996; January *et al*., 1997), α-adrenergic (Eason *et al*., 1995), δ-opioid (Pei *et al*., 1995), endothelin (Freedman *et al*., 1997), adenosine (Palmer *et al*., 1995), vasopressin (Innamorati *et al*., 1997), and somatostatin (Hipkin *et al*., 1997) receptors. However, there have been relatively few unequivocal reports of AT_1-R phosphorylation. This has been due in large part to the inability to distinguish the immunoprecipitated phospho-AT_1-R from more abundant phosphoproteins that either genuinely or spuriously coprecipitate with the receptor (Smith *et al*., 1998). Despite these problems, unequivocal agonist-induced phosphorylation of a transiently expressed epitope-tagged AT_1-R (Oppermann *et al*., 1996), and of a stably expressed $(His)_6$-tagged AT_1-R (Balmforth *et al*., 1997) has been reported in human embryonic kidney 293 cells. We recently developed methodology that allowed us to demonstrate phosphorylation of the endogenous AT_1-R in primary cultures of bovine adrenal glomerulosa cells (Smith *et al*., 1998). Here, we successfully applied this technique to localize the phosphorylation sites of an HA epitope-tagged rat AT_{1a}-R expressed in Cos-7 cells. However, because the expressed receptor migrates as a diffuse smear of M_r 85,000–145,000 in SDS-PAGE and because this region also contains (despite the use of our improved methodology) some additional nonreceptor phosphoproteins, initial attempts to quantify HA-AT_{1a}-R phosphorylation were unsatisfactory.

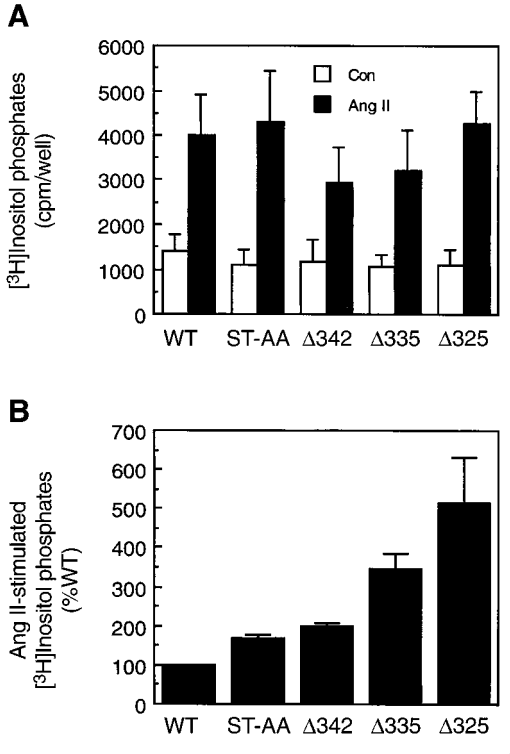

Fig. 5. Inositol phosphate responses of mutant HA-AT_{1a}-Rs. [^{3}H]inositol-labeled Cos-7 cells expressing the indicated receptors were preincubated with 10 mM LiCl for 30 min before the addition of vehicle or 1 μM Ang II for an additional 30 min. [^{3}H]Inositol phosphates were measured as described in Experimental Procedures. A, Mean ± standard error basal and Ang II-stimulated [^{3}H]inositol phosphates from three independent experiments. B, Ang II-stimulated data are normalized to an equal number of receptors. Receptor expression levels are shown in Table 1.

We therefore used the enzyme PNGase F (Lemp *et al*., 1990) to cleave N-linked carbohydrate moieties from solubilized ^{32}P-labeled Cos-7 cell membrane glycoproteins before immunoprecipitation. Interestingly, after this treatment, the deglycosylated phospho-HA-AT_{1a}-R ran as a doublet in SDS-PAGE, as did the deglycosylated photoaffinity-labeled receptor (data not shown). Furthermore, immunoblotting with the anti-HA antibody of PNGase F-treated membranes from unstimulated Cos-7 cells expressing the HA-AT_{1a}-R also revealed a doublet with M_r ~40,000 (data not shown). Because these cells were transfected with a single DNA species, it is unclear why the deglycosylated HA-AT_{1a}-R migrates as a doublet in SDS-PAGE. It is possible that the two bands result from a (nonglycosylation) post-translational processing event, such as lipidation, of a subset of receptors. However, this seems unlikely because the only potential attachment site of a lipid anchor to the HA-AT_{1a}-R (at Cys355) is absent from the truncated receptors, yet these also run as doublets in SDS-PAGE (Fig. 2).

The use of PNGase F revealed the phospho-HA-AT_{1a}-R as a discrete doublet with M_r ~40,000, which, being free from additional phosphoproteins, was more readily quantified. The data obtained from the various mutant receptors indicated that the major agonist-induced phosphorylation sites of the HA-AT_{1a}-R expressed in Cos-7 cells are located in an 11-amino-acid (Ser326-Th336) segment, which contains five serine and two threonine residues, in the receptor cytoplasmic tail. GRKs seem to phosphorylate the receptor at Ser335 and/or Thr336, as well as an additional site or sites in the Ser326-Thr332 segment, whereas PKC seems to phosphorylate at Ser331. Quantification of the phosphorylation status of additional multiple-point mutant HA-AT_{1a}-Rs should clarify whether these deductions are correct.

Previous studies have suggested that the consensus sequence for GRK-mediated phosphorylation of GPCRs consists of a diacidic motif (Fredericks *et al*., 1996). The cytoplasmic tail of the rat AT_{1a}-R contains only three acidic residues (at Asp343, Glu357 and Glu359) (Fig. 1). However, all three of these acidic residues are absent from the Δ342 truncation mutant receptor. Because agonist-induced phosphorylation of this receptor was not significantly different from that of the wild-type receptor, it seems that none of these acidic residues represent the consensus sequence for GRK-mediated phosphorylation of the HA-AT_{1a}-R expressed in Cos-7 cells. However, the HA-AT_{1a}-R does contain a diacidic motif (Asp236-Asp237) at the carboxyl-terminal end of its third intracellular loop (Murphy *et al*., 1991). Future experiments using additional mutant receptors should clarify whether this motif represents the diacidic consensus sequence for GRK-mediated HA-AT_{1a}-R phosphorylation. Should the Asp236-Asp237 motif prove to be a GRK consensus sequence, it would be unique for a GPCR in its not being adjacent to the GRK phosphorylation sites on the cytoplasmic tail but instead being situated on the third intracellular loop. Alternatively, should the Asp236-Asp237 motif prove not to be required for GRK phosphorylation, the GRK consensus sequence of the HA-AT_{1a}-R would instead be unique by virtue of its not being a diacidic motif.

Truncations (or mutation) of the cytoplasmic tail of the HA-AT_{1a}-R that caused removal of its phosphorylation sites were correlated with attenuation of the rate of agonist-induced receptor internalization. Thus, although the inter-

nalization rates of both the wild-type and Δ342 mutant receptors (which exhibited the same degree of phosphorylation) were similar, internalization of the Δ325 mutant (which did not phosphorylate) was virtually abolished. The internalization rates of the partially phosphorylated ST-AA and Δ335 mutants were intermediate between those of the wild-type and Δ325 receptors, although the Δ335 mutant internalized at a rate that was slower than the ST-AA mutant. The latter finding probably reflects the absence in the Δ335 (but not the ST-AA) mutant of the Leu337 residue of the Ser335-Thr336-Leu337 motif, which we previously identified as a major determinant of AT_{1a}-R internalization (Hunyady *et al*., 1994). However, the correlation between reduced phosphorylation and impaired internalization of the ST-AA mutant compared with the wild-type receptor indicates that phosphorylation on the Ser335 and/or Thr336 residues of the Ser335-Thr336-Leu337 motif plays a role in internalization. In addition, because the partially phosphorylated Δ335 mutant internalized slightly faster than the Δ325 mutant, we cannot rule out the possibility that phosphorylation at an additional site or sites in the Ser326-Thr332 segment also plays a role in the internalization process. However, the putative PKC-mediated phosphorylation of Ser331, indicated by the partial inhibitory effect of staurosporine on the agonist-induced phosphorylation of the Δ335 and ST-AA receptors, does not seem to play a role in receptor internalization because substitution of this residue for alanine had no effect on the internalization rate of the full-length AT_{1a}-R (Hunyady *et al*., 1994). Furthermore, because the Δ325 receptor is not phosphorylated, it is also likely that the previously described role in receptor internalization of a hydrophobic region in the amino-terminal cytoplasmic tail of the AT_1-R (Thomas *et al*., 1995a) operates independently of receptor phosphorylation.

It should be noted that receptor phosphorylation and the initial rates of receptor internalization were assessed during the early stages (5 min) of agonist stimulation, whereas inositol phosphate accumulation was measured 20 min after the addition of agonist. Care therefore should be taken in comparing changes in inositol phosphate production with those of receptor phosphorylation and internalization. However, when the inositol phosphate data were normalized to an equal number of receptors, increasing truncation of the cytoplasmic tail of the HA-AT_{1a}-R was correlated with an increased capacity of each receptor for intracellular signal generation. This could result from increased coupling of the mutant receptors to G_q and/or increasing attenuation of receptor desensitization. Because the available data indicate that residues distal to Lys325 in the cytoplasmic tail of the AT_1-R are not involved in coupling to G_q (Hunyady *et al*., 1994; Thomas *et al*., 1995b; Conchon *et al*., 1997; Sano *et al*., 1997; Gaborik *et al*., 1998), the latter possibility seems more likely.

However, although the nonphosphorylated Δ325 receptor elicited the largest signaling response and the fully phosphorylated wild-type receptor elicited the weakest signaling response, there were discrepancies between the degree of receptor phosphorylation and the magnitude of inositol phosphate production for the other mutant receptors. Thus, although phosphorylation of the wild-type and Δ342 receptors was similar, the Δ342 receptor elicited a larger signaling response than the wild-type receptor. Also, although phosphorylation of the ST-AA and Δ335 receptors was similar (but only ~50% of the wild-type and Δ342 receptors), the Δ335 receptor elicited a larger signaling response than the ST-AA receptor. These findings suggest that a sequence located in the segment downstream of Pro341 limits agonist-induced signaling at the HA-AT_{1a}-R. If the enhanced signaling observed for the various mutant HA-AT_{1a}-Rs results from impaired receptor desensitization, this putative sequence may be involved in stabilizing the binding of β-arrestin to the phosphorylated HA-AT_{1a}-R. However, because endocytosis of the AT_1-R has been shown to be β-arrestin independent (Zhang *et al*., 1996), the absence of such a motif from the truncation mutants would not affect the internalization rates of these receptors.

In conclusion, we demonstrated agonist-induced phosphorylation of an HA epitope-tagged rat AT_{1a}-R transiently expressed in Cos-7 cells. Measurement of the magnitudes of phosphorylation of a series of mutant HA-AT_{1a}-Rs have localized the receptor GRK and PKC phosphorylation sites to an 11-amino-acid serine/threonine-rich segment of its cytoplasmic tail. Phosphorylation of residues in this segment seems to be involved in agonist-induced internalization and desensitization of the HA-AT_{1a}-R. Although internalization of the AT_1-R has been shown to be β-arrestin independent (Zhang *et al*., 1996), our results imply that receptor phosphorylation is still required for this process. The development of a quantitative assay of HA-AT_{1a}-R phosphorylation should permit precise mapping of the receptor phosphorylation sites and identification of the specific consensus sequence or sequences for GRK-mediated phosphorylation. These advances should aid in the elucidation of mechanisms involved in the internalization and desensitization of the AT_1-R.

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