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The Tyrosine within the NPX_nY Motif of the Human Angiotensin II Type 1 Receptor Is Involved in Mediating Signal Transduction but Is Not Essential for Internalization

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SUMMARY

The NPX_nY motif is involved in the internalization process of several types of receptors, including lipoprotein receptors and G protein-coupled receptors. We replaced Tyr^{302} with either phenylalanine or alanine in the NPLFY site of the human angiotensin II receptor type 1 and determined the pharmacological properties of the resulting mutant receptors. Competitive binding experiments revealed that COS-7 cells transfected with either the wild-type or mutant receptors expressed approximately the same amount of high affinity binding sites (B_{max} ~70,000 sites/cell and K_d ~2 nM). Photoaffinity labeling of both native and mutant receptors revealed apparent molecular masses of 110 kDa. Incubation of transfected cells with 0.2 nM [^{125}I]Ang II at 37° revealed an efficient internalization of the wild-type receptor and the mutant receptors, although the mutant receptors were internalized at a slower rate. Interestingly, however, the transmembrane signaling was severely impaired in transfected cells expressing mutant receptors. No significant production of inositol-1,4,5-trisphosphate was observed when these cells were challenged for 3 min with a concentration of angiotensin II as high as 1 μM. This is in contrast to the dose-dependent stimulation of inositol-1,4,5-trisphosphate production in cells expressing the wild-type receptor. Thus, our results show that the Tyr^{302} in the NPX_nY motif of the human angiotensin II receptor type 1 is not essential for agonist binding properties or for internalization of the receptor but plays an important role in transmembrane signaling.

The octapeptide hormone Ang II exerts its numerous physiological effects on cardiovascular, endocrine, and neuronal systems by interacting with specific receptors (1). Two pharmacologically distinct types of receptors, belonging to the seven-transmembrane domain receptor family (2, 3), have been identified for this vasoactive peptide: AT_1 and AT_2 (4). The primary effector mechanism activated by the AT_1 is the hydrolysis of polyphosphoinositides by PLC, thus generating the second messengers $Ins(1,4,5)P_3$ and diacylglycerol (5). Diacylglycerol directly activates protein kinase C, whereas $Ins(1,4,5)P_3$ activates calmodulin kinase through the release of Ca^{2+} from intracellular stores. Ang II-induced PLC activation is mediated by a G protein designated G_q (6, 7). The precise functions and intracellular signaling pathways of the AT_2 have not been clearly defined.

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Endocytosis of cell surface receptors is a ubiquitous process that serves multiple functions. Constitutively recycling receptors, such as those for transferrin and low density lipoprotein, are shuttles that deliver their ligands to the cell interior and then return to the cell surface (8). Relatively little is known concerning the molecular basis of endocytosis of seven-transmembrane G protein-coupled receptors. Clathrin-coated vesicles are involved in the internalization of some types of receptors (9) but not of other types (10, 11). For many receptors, efficient endocytosis requires cytoplasmic determinants that serve as positive signals for triggering the internalization process. Determinants, such as serine and threonine residues in the third intracellular loop and carboxyl terminus of the β_2-adrenergic receptor, are involved in receptor internalization (12). A recent study of the AT_1 identified specific serine and threonine residues in the carboxyl terminus that are critical for agonist-induced internalization (13).

It has been shown that for single-transmembrane receptors, such as those for low density lipoprotein, transferrin, insulin, and epidermal growth factor, a tyrosine residue that

ABBREVIATIONS: Ang II, angiotensin II; AT_1, angiotensin II receptor type 1; AT_2, angiotensin II receptor type 2; $Ins(1,4,5)P_3$, inositol-1,4,5-trisphosphate; PLC, phospholipase C; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; hAT_1Y302F, human angiotensin II receptor type 1 Y302F; hAT_1Y302A, human angiotensin II receptor type 1 Y302A.

is present in the highly conserved NPXY motif found in the cytoplasmic portion of these receptors is required for receptor internalization. (8). Such a sequence is believed to be directly involved in the association of the receptor with the HA-2 adaptor protein of the membrane clathrin lattice, a process that leads to efficient endocytosis via clathrin-coated pits (14). A similar motif, NPX_nY, which is found at the boundary of the seventh transmembrane domain and the cytoplasmic tail, is highly conserved in G protein-coupled receptors. A recent study has shown that replacement of the tyrosine in this motif of the β_2-adrenergic receptor blocked receptor sequestration and resensitization (15). In the present study, Tyr^{302} of the NPLFY site in the human AT_1 was replaced by phenylalanine or alanine to evaluate the contribution of that amino acid in the internalization process (Fig. 1). Our results indicate that Tyr^{302} is not essential for the internalization of the human AT_1. Interestingly, however, this mutation appears to play a determining role in the activation of PLC.

Experimental Procedures

Materials. The cDNA clone of the human AT_1 subcloned in the mammalian expression vector pRc/CMV (Invitrogen, San Diego, CA) was kindly provided by Dr. S. Meloche (Université de Montréal). Lipofectamine and culture media were obtained from GIBCO Life Technologies (Gaithersburg, MD). BSA, Bacitracin, and GTPγS were purchased from Sigma Chemical Co. (St. Louis, MO). Pertussis toxin was obtained from Calbiochem (La Jolla, CA). Oligonucleotides were synthesized by General Synthesis and Diagnostics (Toronto, Ontario, Canada). *myo*-[^{3}H]Inositol (83 Ci/mmol) and [*tyrosyl*-3,5-^{3}H]Ang II (60 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). L-158,809 was a generous gift from Merck (Rahway, NJ). AG 1-X8 resin was obtained from Bio-Rad (Richmond, CA). [^{125}I]Ang II (1000 Ci/mmol) was prepared with iodogen as described by Fraker and Speck (16) and purified by high performance liquid chromatography on a C-18 column, and the specific radioactivity was determined by self-displacement in our binding system. Briefly, a saturation curve with increasing concentrations of [^{125}I]Ang II and a dose-displacement curve with a fixed concentration of [^{125}I]Ang II inhibited by increasing concentrations of unlabeled homologous peptide were performed simultaneously with the use of a bovine adrenal cortex membrane preparation. The specific radioactivity was determined through evaluation of the amount of [^{125}I]Ang II necessary to obtain an occupation ratio in the saturation curve corresponding to the occupation ratio obtained with a known amount of unlabeled peptide in the dose-displacement curve.

Site-directed mutagenesis. pRc/CMV containing the human AT_1 cDNA was digested with *Hin*dIII and *Xba*I endonucleases and cloned into M13 mp19 also digested with *Hin*dIII and *Xba*I. The codon changes in the human AT_1 cDNA were made by site-directed mutagenesis using an *in vitro* mutagenesis kit (Sculptor, Amersham) based on the phosphorothioate technique (17). Two oligonucleotides were constructed to introduce mutations in the tyrosine of the NPX_nY motif. The mutagenic primers are listed below (altered nucleotides are underlined): Tyr^{302}→phenylalanine (hAT_1Y302F): 5-CCAGAAAGCCAAAAAAAAAGAGGATT-3 and Tyr^{302}→alanine (hAT_1Y302A) 5-CCAGAAAGCCAGCAAAAAGAGGATT-3. After confirmation of site-directed mutation in each construct by DNA sequencing, the hAT_1Y302F and the hAT_1Y302A were excised from the mp19RF form by digestion with *Hin*dIII and *Xba*I and subcloned into the multiple cloning site of pRc/CMV that had been digested by these same restriction enzymes.

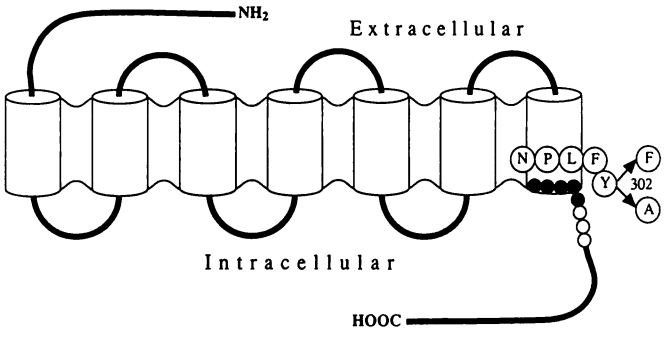

Fig. 1. Schematic representation of human AT_1. ●, NPX_nY motif found in many seven-transmembrane G protein-coupled receptors. Mutation of the human AT_1 was made by replacing Tyr^{302} with phenylalanine (hAT_1Y302F) or alanine (hAT_1Y302A).

Transfection of COS-7 cells. COS-7 cells were grown in DMEM containing 2 mM L-glutamine and 10% heat-inactivated fetal calf serum. Cells were seeded into six-well plates 2 days before transfection. After 2 days, the cells were washed once with serum-free DMEM and transfected with 1.5 μg of plasmid DNA and 10 μl of Lipofectamine in 1 ml of serum-free DMEM. The cells were incubated for 5 hr at 37°, and the media were replaced with a complete DMEM medium containing 100 IU/ml penicillin and 100 μg/ml streptomycin. All experiments were performed 48 hr after the initiation of transfection.

Binding experiments. Transfected COS-7 cells grown in six-well plates (3.5×10^5 cells/well) were washed twice with PBS and incubated for 30 min on ice in PBS containing 1 mM EDTA. Cells were then scraped gently, pooled, centrifuged at $200 \times g$ for 10 min at 4°, and resuspended in binding buffer (25 mM Tris·HCl, pH 7.4, 5 mM $MgCl_2$, 100 mM NaCl, 0.1% BSA). COS-7 cells (1.5×10^5–3.0×10^5, 35–70 μg of protein) were incubated in the binding buffer containing 0.2 nM of [^{125}I]Ang II and selected concentrations of Ang II or selected concentrations of [*tyrosyl*-3,5-^{3}H]Ang II in a final incubation volume of 0.5 ml. Incubations were performed at room temperature for 45 min. Nonspecific binding was measured in the presence of 1 μM unlabeled Ang II. Bound radioactivity was separated from free ligand by filtration through GF/C filters presoaked in binding buffer. The receptor-bound radioactivity was analyzed by γ counting or scintillation counting.

To determine the effect of GTPγS, crude membranes were prepared. At 48 hr after transfection, cells were washed in ice-cold binding buffer without BSA and underwent three cycles of freezing and thawing. Membranes were centrifuged at $16,000 \times g$ for 25 min. Binding assays were performed in 0.5 ml of binding buffer containing 0.1 mg/ml Bacitracin, 50–100 μg of membrane proteins, 0.2 nM [^{125}I]Ang II, and different concentrations of GTPγS. Incubations were performed at room temperature for 45 min and were terminated by vacuum filtration through presoaked glass-fiber filters and three rapid washes with 2 ml of cold medium. Bound radioactivity was measured by γ spectrometry.

Experimental data from displacement and saturation curves were analyzed by the curve-fitting program LIGAND with weighted nonlinear least-squares analysis to determine values for each parameter that minimizes the weighted sum of squares (18). The extra sum of squares test (F statistic) was used to compare the aptness of fit to models of one and two classes of receptors.

Photoaffinity labeling. Transfected COS-7 cells were photolabeled as described previously (19). Briefly, cells were incubated with 10 μCi of [^{125}I]Ang II/N_3 in 1 ml of medium containing 25 mM Tris·HCl, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, and 2 mg/ml BSA with or without L-158,809 (1 μM). After 45 min at room temperature, the cells were washed three times and irradiated for 30 min at 0° under filtered UV light (365 nm). The labeled cells were solubilized in modified RIPA buffer (50 mM Tris·HCl, pH 7.4, 1% Nonidet P-40, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride), and cell lysates were centrifuged at 15,000 rpm for 5 min to remove insoluble material. Cell extracts solubilized in modified RIPA buffer were incubated for 60 min at 37° in denaturing buffer containing 60 mM Tris·HCl, pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS, 125 mM dithiothreitol, and 3%

(w/v) bromophenol blue. Denatured proteins (25–50 μg) were subjected to electrophoresis in a 7.5% polyacrylamide gel and were run at 95 V for 90 min. The gel was stained with 0.05% (w/v) Coomassie Brilliant blue and dried before autoradiography for 2 weeks on Kodak X-Omat AR film.

Internalization assay. Internalization assay was done as described by Hunyady *et al.* (13). Briefly, transfected COS-7 cells in six-well culture plates were placed on ice, washed twice with PBS containing 0.1% dextrose, and kept on ice in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered Medium 199 (62.5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 0.01% Bacitracin, 0.1% BSA) until the internalization assay. Cells were incubated with [^{125}I]Ang II (0.2 nM) at 37° for different periods of time. Incubation was rapidly stopped by washing of the cells twice with ice-cold PBS. Cells were then incubated for 10 min in 2 ml of an ice-cold acid solution (150 mM NaCl, 50 mM acetic acid, pH 2). The supernatant containing the acid-released radioactivity was collected for analysis. Cells were then solubilized with 0.5 N NaOH, and their acid-resistant radioactive ligand content was also evaluated. Internalization values were expressed as percent calculated from the ratio of acid-resistant binding to total (acid-resistant plus acid-released) binding. Nonspecific binding was measured in the presence of 1 μM unlabeled Ang II.

Inositol phosphate hydrolysis. Labeling of transfected COS-7 cells was done according to the method of Balla *et al.* (20) with some modifications. Briefly, the culture medium was replaced with inositol-free DMEM containing 15 μCi/ml *myo*-[^{3}H]inositol 24 hr after transfection. In some experiments, pertussis toxin (100 ng/ml) was added simultaneously. After a 16–20-hr labeling period, cells were washed twice with PBS containing 0.1% dextrose and incubated in Medium 199 at 37° for 3 min with increasing concentrations of Ang II. Incubations were stopped by the addition of perchloric acid (5% v/v). Cells were scraped and centrifuged at 15,000 × *g* for 5 min. Inositol phosphates were then extracted from the supernatant with an equal volume of a mixture of 1,1,2-trichlorotrifluroethane and tri-*n*-octylamine (1:1). The samples were vigorously mixed and centrifuged at 15,000 × *g* for 1 min. The upper phase containing the inositol phosphates was applied to an AG 1-X8 resin column. [^{3}H]Inositol-labeled compounds were sequentially eluted by the addition of an ammonium formate/formic acid mixture of increasing ionic strength, as described by Berridge *et al.* (21).

Results

Ang II binding. The pRc/CMV vectors containing the cDNAs encoding the wild-type or the two mutant receptors (hAT$_1$Y302F and hAT$_1$Y302A) were transfected in COS-7 cells. Binding analysis was performed with [^{125}I]Ang II and increasing concentrations of unlabeled Ang II. Fig. 2A shows that increasing concentrations of unlabeled Ang II effectively inhibited [^{125}I]Ang II binding to wild-type and mutant receptors. The K_d values (and Hill coefficients) for wild-type AT$_1$, hAT$_1$Y302F, and hAT$_1$Y302A were 1.8 ± 0.4 nM (n_H = 0.81 ± 0.09; eight experiments), 3.4 ± 1.2 nM (n_H = 0.70 ± 0.04; three experiments), and 2.8 ± 0.4 nM (n_H = 0.87 ± 0.01; three experiments) with binding capacities (B_{max}) of 63,000 ± 23,000, 62,000 ± 31,000, and 66,000 ± 31,000 receptors/cell, respectively. Although the apparent affinities of the mutant receptors appear to be lower than that of the wild-type, K_d values were not statistically different (analysis of variance). The data analyzed by the LIGAND program were statistically relevant only when a one-site fit model was used. Fig. 2B shows a representative binding isotherm for [*tyrosyl*-3,5-^{3}H]Ang II that yielded K_d values of 1.1, 2.0, and 2.1 nM for wild-type AT$_1$, hAT$_1$Y302F, and hAT$_1$Y302A, respectively, and B_{max} values of 47,000, 40,000, and 44,000 receptors/cell

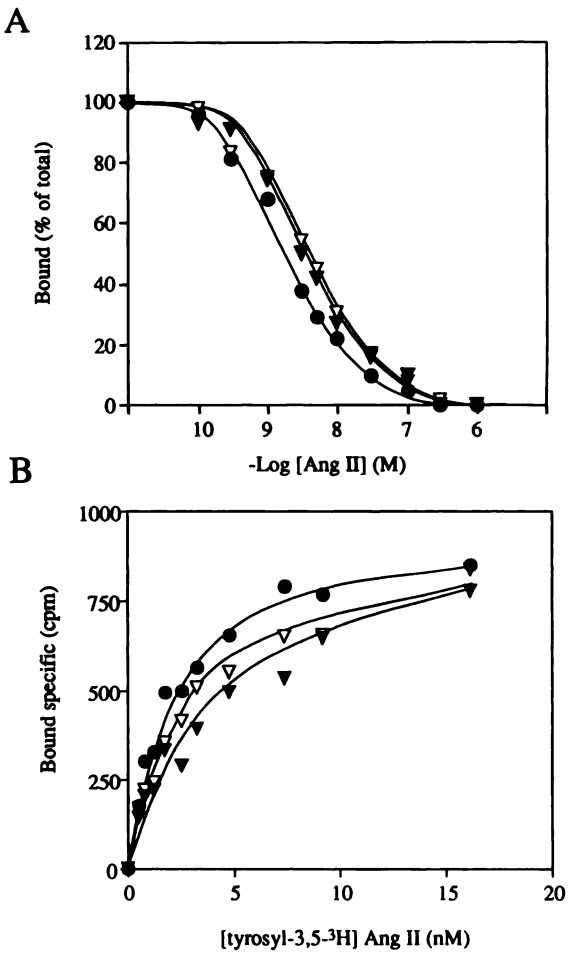

Fig. 2. A, Binding of [^{125}I]Ang II to transfected COS-7 cells. Cells expressing human AT$_1$ (●), hAT$_1$Y302F (▽), and hAT$_1$Y302A (▼) were incubated for 45 min at room temperature in a medium containing 0.2 nM [^{125}I]Ang II and increasing concentrations of Ang II. Data are expressed as values relative to total binding observed in the absence of unlabeled ligand (9214, 4156, and 3347 cpm) and were corrected for nonspecific binding (1004, 802, and 724 cpm) for the cells expressing human AT$_1$, hAT$_1$Y302F, and hAT$_1$Y302A, respectively. Each point represents the mean of triplicate determinations. These results are representative of at least three independent experiments. B, Binding isotherms of [*tyrosyl*-3,5-^{3}H]Ang II to transfected COS-7 cells. Cells expressing human AT$_1$ (●), hAT$_1$Y302F (▽), and hAT$_1$Y302A (▼) were incubated for 45 min at room temperature with increasing concentrations of [*tyrosyl*-3,5-^{3}H]Ang II as described in Experimental Procedures. Binding data were analyzed with a nonlinear least-squares curve-fitting procedure (LIGAND). Each point represents the mean of triplicate determinations. These results are representative of two independent experiments.

for wild-type AT$_1$, hAT$_1$Y302F, and hAT$_1$Y302A, respectively. K_d and B_{max} values are similar to those observed with bovine adrenal glomerulosa cells, which constitutively express high levels (50,000–100,000 sites/cell) of high affinity (0.5–1.5 nM) AT$_1$s (22).

Fig. 3 shows that GTPγS inhibited [^{125}I]Ang II binding to wild-type and mutant receptors in a dose-dependent fashion with IC$_{50}$ values of 11, 11, and 9 nM for wild-type AT$_1$, hAT$_1$Y302F, and hAT$_1$Y302A, respectively. Interestingly, the reduction in [^{125}I]Ang II binding on GTPγS treatment was much less marked in the mutated than in the wild-type receptor. This is indicative of an impaired coupling between mutant receptors and G proteins.

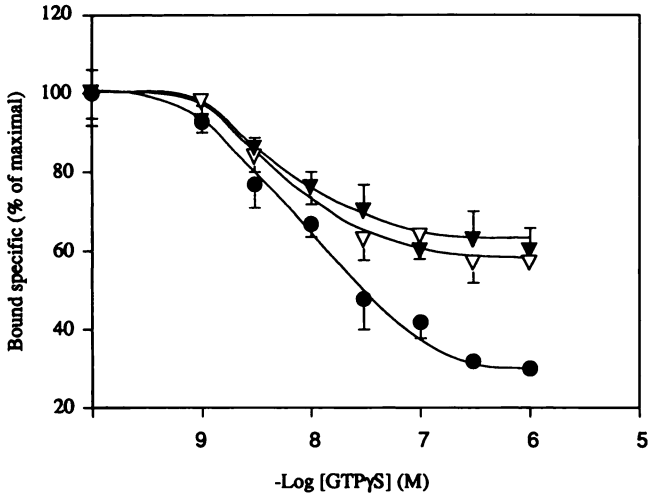

Fig. 3. Effect of GTPγS on [^{125}I]Ang II binding. Membranes of COS-7 cells transfected with wild-type human AT_1 (●), hAT_1Y302F (▽), and hAT_1Y302A (▼) were incubated in the presence of 0.2 nM of [^{125}I]Ang II and increasing concentrations of GTPγS. Nonspecific binding was evaluated in the presence of 1 μM Ang II. Results are presented as the mean ± standard deviation of triplicate determinations of two independent experiments.

Photoaffinity labeling of expressed Ang II receptors. Photolabeled receptors were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). The autoradiogram shows that the wild-type and the mutant receptors migrated as broad bands of similar apparent molecular masses. The broadness of the bands is consistent with a glycosylated protein, as previously reported (23, 24). All three receptors exhibited similar molecular masses of 110 kDa (Fig. 4; *lanes 1, 3, and 5*). The labeling of these receptors was specific because it was inhibited in the presence of 1 μM of the AT_1 antagonist L-158,809 (Fig. 4). No specific labeling was observed when nontransfected cells were photolabeled (Fig. 4). According to previously described procedures (26), we performed endoglycosidase treatment of COS cell-expressed photolabeled receptors; the results indicated that the wild-type and the mutant receptors could be deglycosylated, yielding apparent molecular masses of 34 kDa (data not shown).

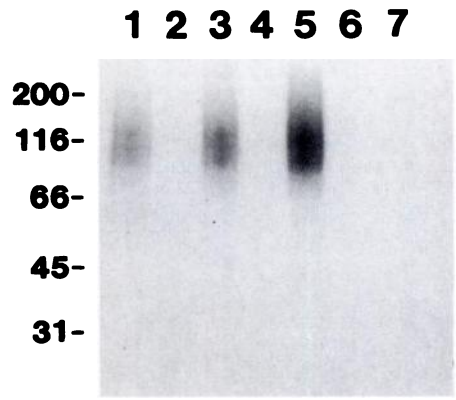

Fig. 4. Determination of molecular weight of human AT_1 and the mutant receptors expressed in COS-7 cells. Transfected COS-7 cells expressing wild-type AT_1 (*lanes 1 and 2*), hAT_1Y302F (*lanes 3 and 4*), and hAT_1Y302A (*lanes 5 and 6*) were incubated with [^{125}I]Ang II/N_3 (10 μCi) in the absence (*lanes 1, 3, and 5*) or the presence (*lanes 2, 4, and 6*) of L-158,809 (1 μM) and were subjected to UV irradiation. Nontransfected COS-7 cells were also incubated with 10 μCi of [^{125}I]Ang II/N_3 (*lane 7*). Irradiated material was resolved by 7.5% SDS-polyacrylamide gel electrophoresis followed by autoradiography as indicated in Experimental Procedures. Protein standards of indicated molecular masses (in kDa) were run in parallel.

Internalization of [^{125}I]Ang II. To evaluate the contribution of Tyr^{302} in receptor internalization, we assessed the kinetics of [^{125}I]Ang II internalization. Fig. 5 shows the time course of internalization of [^{125}I]Ang II in COS-7 cells expressing human AT_1, hAT_1Y302F, and hAT_1Y302A. During incubation at 37°, the acid-resistant binding of [^{125}I]Ang II to the native receptor increased rapidly (within 20 min) to a high value representing ~63% of total specific binding. This rapid uptake of radioactive material is consistent with rapid internalization of the hormone/receptor complex. No internalization of [^{125}I]Ang II was observed in nontransfected cells. Mutant receptors displayed internalization patterns similar to that of the wild-type receptor but with slower kinetics. The slower rate of receptor internalization was clearly demonstrated after 5 min of incubation when the acid-resistant binding to cells expressing the mutant receptors represented only 24% (hAT_1Y302F) and 30% (hAT_1Y302A) of total specific binding, whereas this value was ~50% with cells expressing the wild-type receptor. After 10 and 20 min of incubation at 37° with [^{125}I]Ang II, the apparent rates of receptor internalization were still different, but after 30 min of incubation, the acid-resistant binding reached a similar level (~63%) with all three types of receptor (data not shown). These results clearly indicate that Tyr^{302} is not essential for AT_1 internalization.

PLC activation. The functional properties of the transiently expressed wild-type and mutant receptors were eval-

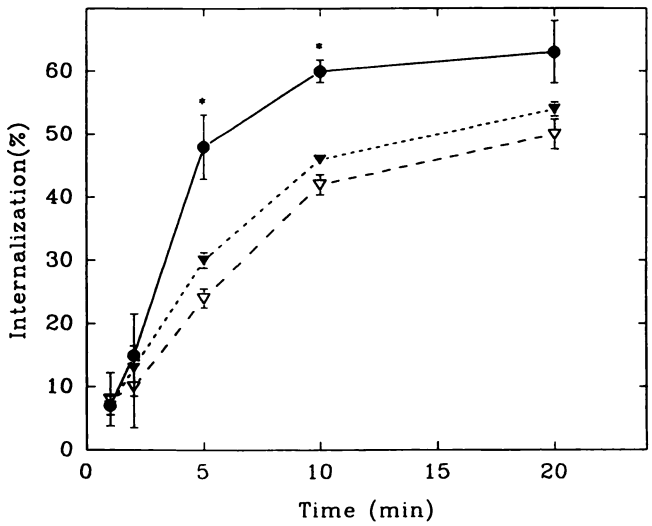

Fig. 5. [^{125}I]Ang II internalization in transfected COS-7 cells. Cells expressing wild-type AT_1 (●), hAT_1Y302F (▽) and hAT_1Y302A (▼) were incubated with [^{125}I]Ang II (0.2 nM) at 37° for different periods of time. Internalization was stopped by washing the cells with an acid solution at 0° for 10 min. Acid-resistant binding was evaluated as indicated in Experimental Procedures. Data are expressed as percent of total binding for each time point and represent mean ± standard deviation of triplicate values. Internalization was calculated from the ratio of acid-resistant binding to total binding (acid-resistant plus acid-released). Nonspecific binding was measured in the presence of 1 μM unlabeled Ang II. These results are representative of three independent experiments. Statistical analysis were made by a one-way analysis of variance combined with Bonferroni's test for multiple comparisons. *, $p \leq 0.05$ was considered statistically significant compared with wild-type. No significant difference was observed between mutants.

uated by measurement of Ang II-induced Ins(1,4,5)P_3 production. When COS-7 cells expressing the human AT_1 were incubated for 3 min in the presence of increasing concentrations of Ang II, their Ins(1,4,5)P_3 content increased progressively in a dose-dependent manner (Fig. 6). The threshold concentration was ~1 nM, and the maximal response was obtained with a concentration of 30 nM. The half-maximally effective concentration (EC_{50}) for Ins(1,4,5)P_3 production was ~10 nM. This Ang II-induced Ins(1,4,5)P_3 production was insensitive to a 16-hr pretreatment of cells with 100 ng/ml of pertussis toxin, ruling out the involvement of $G_{i/o}$ in this process. Under the same conditions, COS-7 cells expressing the mutant AT_1s did not show any significant increase in Ins(1,4,5)P_3 production. As expected, no increase in Ins(1,4,5)P_3 production was detected when nontransfected COS-7 cells or COS-7 cells expressing the plasmid alone were challenged with 100 nM Ang II (data not shown). These striking differences between the wild-type and the mutant receptors in their capacity to activate PLC are not related to different levels of expression because all three receptors were always expressed at similar levels (Fig. 2).

Discussion

We recently demonstrated a functional desensitization of the AT_1 (22), but the molecular determinants of the receptor that lead to G protein uncoupling and/or to its internalization are not fully understood. In the present study, we analyzed the role of the Tyr^{302} residue in the NPLFY motif located at the carboxyl terminus of the seventh transmembrane domain of the human AT_1. As shown in Fig. 1, we chose to substitute the Tyr^{302} for an alanine, which has no functional group beyond the β-carbon that could change the main conformation of the protein or impose severe electrostatic or steric effects (25). We also reasoned that the phenylalanine substitution would conserve the aromatic properties of the benzyl group and that it would enable us to evaluate the contribution of the hydroxyl group involved in receptor internalization, binding, and signal transduction. We systematically evaluated the level of expression of the wild-type and mutant receptors in transiently transfected COS-7 cells. Competitive binding and saturation experiments showed that all three receptors were expressed at similar levels in COS-7 cells and exhibited a high affinity for Ang II.

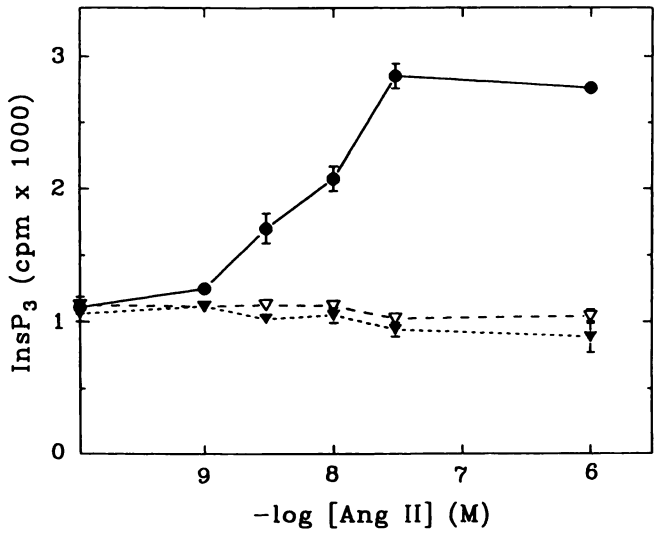

Fig. 6. Ins(1,4,5)P_3 production by transfected COS-7 cells. At 24 hr after the initial transfection, cells expressing human AT_1 (●), hAT_1Y302F (▽), and hAT_1Y302A (▼) were prelabeled for 18 hr with 15 μCi/ml *myo*-[^{3}H]inositol. Cells were then incubated for 3 min at 37° with increasing concentrations of Ang II in 1 ml of Medium 199. The incubations were stopped with perchloric acid, and the inositol phosphates were measured as described in Experimental Procedures. Data are expressed as mean ± standard deviation of triplicate values and are representative of three independent experiments.

The wild-type and mutant receptors expressed in COS-7 cells showed apparent molecular masses of 110 kDa. The broadness of the bands suggests that these receptors are glycosylated. These results differ from those previously reported where a single band of 60 kDa was observed when AT_1s of bovine adrenal gland were covalently labeled with [^{125}I]Ang II/N_3 (19, 23, 24). This difference in the molecular masses could be attributed to different degrees of glycosylation. This phenomenon has been previously observed by Carson *et al.*, who showed that in different tissues and species variations could be observed in the molecular weight of the AT_1 due to different levels of glycosylation (24). Preliminary experiments have revealed that the 34-kDa deglycosylated form of the COS-7 cell-expressed receptors is identical in molecular mass to that reported by Carson *et al.* (24). A similar observation was also made for the AT_2, whose apparent molecular mass varies, depending on the level of glycosylation, from 60 to 130 kDa, depending on the tissue or the species studied (26).

A recent study on the β_2-adrenergic receptor demonstrated that mutation of the tyrosine residue in the consensus NPX_nY sequence resulted in a complete loss of agonist-induced sequestration of the receptor (15). The authors proposed that the NPX_nY sequence may represent a general sequestration signal for seven-transmembrane G protein-coupled receptors. Our results do not concur with this hypothesis because the substitution of Tyr^{302} in the human AT_1 did not prevent receptor internalization. Similar results were also obtained for the gastrin-releasing peptide receptor, another member of the seven-transmembrane G protein-coupled receptor family, where the replacement of the tyrosine residue in the NPX_nY motif did not impair internalization (27). Moreover, the NPX_nY motif is also found in the AT_2, which does not exhibit ligand-induced internalization (13, 28). Alternatively, other seven-transmembrane G protein-coupled receptors lacking the NPX_nY motif have been described to undergo efficient ligand-mediated endocytosis. This is the case for the G protein-coupled receptor of parathyroid hormone and parathyroid hormone-related protein (29). It is thus clear that the Tyr^{302} of the NPX_nY motif is not essential for receptor internalization. However, we cannot completely exclude the implication of this motif in the ligand-induced endocytosis of the human AT_1 because the rate of internalization of the two mutant receptors was slower than that of the wild-type receptor. Clearly, there are other signals in the AT_1 involved in this process, as shown by Hunyady *et al.* (13). This group identified nonconserved serine/threonine residues in the cytoplasmic tail that appear to play an important role in receptor endocytosis. These residues and others found in the third intracellular loop of many seven-transmembrane domain receptors, such as the β-adrenergic (30), muscarinic (31), gastrin-releasing peptide (32), thyrotropin-

releasing peptide hormone (33), and yeast mating factor (34), have also been implicated in this process.

The gastrin-releasing peptide receptor, like the AT_1, is coupled to a G_q heterotrimeric protein complex. When these receptors are stimulated by an agonist, they activate a PLCβ1 that hydrolyzes phosphoinositides to produce Ins(1,4,5)P_3 and diacylglycerol. The substitution of the tyrosine residue of the NPX_nY motif for an alanine in the gastrin-releasing peptide receptor did not impair the agonist-induced PLC activation (27). We demonstrate that the homologous tyrosine residue plays an important role in Ang II-induced PLC activation. Modification of Tyr^{302} residue in human AT_1s impedes their ability to stimulate PLC, apparently due to a coupling defect between receptors and G proteins. This impaired ability to activate PLC is analogous to the impaired Ang II-induced Ins(1,4,5)P_3 production by the rat AT_{1a}Y292F mutant receptor (35). In the Y292F mutant, it was proposed that a direct hydrogen bond between Tyr^{292} and Asp^{74} is necessary for PLC activation. The main difference between tyrosine and phenylalanine residues is that the phenolic hydroxyl group that can act mainly as a hydrogen donor and less as an acceptor due to its weakly acidic nature. Tyrosine-to-phenylalanine mutants thus would lack only the hydrogen bridge capacity and maintain the aromatic/aromatic interactions. In general, weak stabilizing interactions do not result in total loss of binding capacity or efficiency. In a similar mutation on the closely related somatostatin receptor (type 5), substitution of Phe^{258} by tyrosine increased by 20-fold its affinity for somatostatin-14, presumably through an additional hydrogen bound with the ligand (36). The marked effect observed in our study with regard to PLC activation argues against the loss of a weak interaction but rather suggests the loss of a more critical function of the Tyr^{302}. It could be interesting to replace this tyrosine residue by serine or threonine to evaluate the implication of hydroxyl-containing amino acids.

The mechanism by which Ca^{2+}-mobilizing agonists induce internalization is still unclear. It has been suggested that G proteins, acting directly or via their effect on phosphoinositide metabolism, may regulate receptor endocytosis (37, 38). Our results show that although the PLC activation by hAT_1Y302F and hAT_1Y302A mutant receptors was clearly altered, receptor internalization was still detectable. It appears that internalization and PLC activation implicate distinct mechanisms. This is also supported by other studies showing that internalization-deficient mutant receptors were still able to stimulate their respective G proteins and induce signal transduction (15, 39).

In summary, the present findings suggest that the activation of PLC via a G protein is not an essential requirement for internalization of the human AT_1. These data also indicate that the Tyr^{302} of the AT_1 receptor is important for signal transduction and that the NPX_nY motif is not the unique determinant for internalization of seven-transmembrane G protein-coupled receptors.

While this manuscript was in revision, an article was published on the role of the NPLFY motif of the rat AT_{1a} (40). The authors report that the Tyr^{302} to alanine mutation does not affect the K_d values and is not involved in internalization. However, the report shows that the mutant receptor's ability to interact with G proteins and to stimulate inositol phosphate production was reduced. The slight discrepancy with our observations of a markedly impaired activation of phospholipase C may be explained by the prolonged stimulation with the ligand (20 min) in the presence of lithium, conditions that may permit slow accumulation of inositol phosphates.

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