Role of Cytoplasmic Tail of the Type 1A Angiotensin II Receptor in Agonist- and Phorbol Ester–Induced Desensitization

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Abstract—To investigate mechanisms underlying the agonist-induced desensitization of the type 1A angiotensin II receptor (AT₁A-R), we have stably expressed in Chinese hamster ovary (CHO) cells the wild-type receptor and truncated mutants lacking varying lengths of the cytoplasmic tail. Assay of inositol 1,4,5-trisphosphate (IP₃) formation in response to agonist demonstrated that the truncated mutants T318, T328, and T348 lacking the last 42, 32, or 12 amino acid residues, respectively, couple with G_q protein with an efficiency similar to that of full-length receptors, whereas coupling of G_q protein was abolished in the T310 truncated mutant devoid of the carboxyl-terminal 50 amino acids. Exposure of CHO/AT₁A-R cells expressing the wild-type AT₁A-R to angiotensin II resulted in rapid and dose-dependent homologous desensitization of receptor-mediated IP₃ formation, which was independent of the receptor internalization. Mastoparan, an activator of G protein–coupled receptor kinase (GRK), induced desensitization of the AT₁A-R. The agonist-induced desensitization of the receptor was largely prevented by heparin, a potent inhibitor of GRK, whereas it was only partially attenuated by a protein kinase C (PKC)-specific inhibitor. The homologous or heterologous desensitization of the receptor was greatly impaired in the truncated mutants T318 and T328, lacking the Ser/Thr-rich (13 or 12 Ser/Thr residues) cytoplasmic tail of the AT₁A-R. Deletion of the last two Ser residues, including one PKC consensus site in the receptor tail, prevented only phorbol 12-myristate 13-acetate–induced desensitization by 30%. Moreover, we found an agonist-induced translocation of a heparin-sensitive kinase activity. The angiotensin II–stimulated heparin-sensitive kinase could phosphorylate a thioredoxin fusion protein containing the entire AT₁A-R cytoplasmic tail (N²⁹⁵ to E³⁵⁹), which lacks consensus phosphorylation sites for GRK1, GRK2, and GRK3. The heparin-sensitive kinase may not be GRK2, GRK3, or GRK6 expressed in CHO/AT₁A-R cells, since angiotensin II did not induce translocation of these receptor kinases. Potential Ser/Thr phosphorylation sites located between S⁻³²⁸ and S⁻³⁴⁷ in the cytoplasmic tail of AT₁A-R seem to play a critical role in the heterologous and homologous desensitization of the receptor. A heparin-sensitive kinase other than GRK2, GRK3, or GRK6 may be involved in the agonist-induced homologous desensitization of the AT₁A-R. (Circ Res. 1998;82:523-531.)

Key Words: angiotensin II ■ desensitization ■ AT₁A receptor ■ phorbol ester

The type 1 Ang II receptors, AT₁A-R and AT₁B-R, undergo rapid homologous downregulation on exposure to Ang II.¹,²,³ Multiple mechanisms, such as desensitization and concurrent internalization, seem to be involved in this response to maintain cellular homeostasis. The mechanism of desensitization is complex; however, receptor phosphorylation seems to be an initial and major step. Extensive mutagenesis studies on the β₂-adrenergic receptor revealed that phosphorylation of two Ser residues located on the third cytoplasmic loop and in the proximal portion of the cytoplasmic tail by protein kinase A is involved in the heterologous desensitization of the receptor, whereas agonist-induced homologous desensitization requires phosphorylation of many Ser/Thr residues in the distal portion of the receptor cytoplasmic tail by β-ARK. Phosphorylation of the cytoplasmic tail by β-ARK induces arrestin-like protein binding to the receptor, which has been proposed to block fully the physical coupling of receptor to G protein and adenylyl cyclase.⁴ This indicates multiple mechanisms for desensitization by phosphorylation. In the G₁₉/₁₁-coupled AT₁A-R, the desensitization mechanisms are not well understood. Although the receptor phosphorylation is associated with desensitization, whether they are causally related is not clear.⁵ Conflicting views exist as to the sites of phosphorylation. Thomas et al⁶ proposed that the second cytoplasmic loop may be the site of phosphorylation responsible for AT₁A-R desensitization, whereas Balmforth et al⁵ reported observations indicating involvement of a long cytoplasmic tail in the receptor desensitization, without identifying specific domains.

The desensitization of the AT₁ receptor was shown to occur independent of the receptor internalization in rat cardiomyocytes.⁷ We were able to show that the desensitization of AT₁B-R can occur even if receptor internalization is inhibited.
and concluded that the two phenomena are distinct receptor functions even though they occur concurrently.7 Although AT1a-R and AT1b-R share a high degree of structural homology, they have some considerable differences in the cytoplasmic tail region, particularly in positions of Ser/Thr residues. Du et al1 have recently reported that AT1a-R and AT1b-R are regulated in opposite directions on low salt feeding. Since AT1a-R is the dominant subtype of the AT1 receptor in most of tissues, except for the adrenal and pituitary glands, determination of the mechanism of its desensitization has long been overdue.

Since we have been able to obtain cells expressing AT1a-R at a high level, which will enable us to quantify the down-regulation of the receptor and its functions, we have performed studies directed to the delineation of the receptor domains involved in the desensitization using various mutants of AT1a-R, and a thioredoxin fusion protein containing the entire carboxyl-terminal region of the receptor. To gain insight into the type of kinases involved in this process, we have also applied reagents that stimulate or inhibit PKC and GRKs. We have obtained evidence that the proximal portion (K310 to L317) of the receptor cytoplasmic tail is involved in the AT1a-R coupling to Gq and that the potential Ser/Thr phosphorylation sites located between S328 and S347 in the cytoplasmic tail play a critical role in the agonist- and phorbol ester (PMA)-induced desensitization. Finally, the type and role of protein kinases involved in agonist-induced desensitization of the AT1a-R were examined.

**Materials and Methods**

**Materials**

Cell culture media, fetal calf serum, genetin (G418 sulfate), and protein tyrosine kinase inhibitors were obtained from Life Technologies, Inc.; [32P]ATP, (y-32P)ATP, and [3H]IP, radioreceptor assay kits were purchased from Dupont NEN; CHO cells (CHO-K1) were obtained from American Type Culture Collection; Ang II and [Sar, Ile8]Ang II were obtained from Peninsula Laboratories; the entire coding region of rat kidney AT1A-R cDNA (an open reading frame encoding 359 amino acid residues) was subcloned into the BsuXI site of the pRc/CMV expression vector. Truncated mutants lacking carboxyl-terminal segments of varying lengths were prepared by inserting a stop codon. Sites of truncation are shown in Fig 1. The mutated DNA was confirmed by digestosynucleotide sequencing using appropriate primers, as described by Sanger et al,1 and subcloned into the expression vector pRc/CMV. CHO-K1 cells were grown in Ham's F-12 medium supplemented with 10% fetal calf serum in a humidified incubator with 5% CO2. At 50% confluence, CHO cells were transfected with WT or truncated AT1a-R plasmid DNA using a calcium phosphate precipitation/glycercol shock procedure previously described.2 After selection in G418 (500 μg/mL), resistant colonies were isolated, amplified, and screened for receptor expression by radioligand binding assay. Cells were propagated in flasks with Ham's F-12 medium supplemented with 10% fetal calf serum under a selection pressure of 150 μg/mL G418 in an atmosphere of 95% air and 5% CO2 at 37°C.

**Radioligand Binding Assay**

Monooiodinated [125I]-[Sar, Ile8]Ang II was prepared by the lactoperoxidase method and purified by reversed-phase HPLC as described previously12 except that a 0% to 80% acetonitrile gradient was applied for 60 minutes with a flow rate of 1.0 mL/min. Binding studies were performed with cells at or near confluence essentially as described previously.2 The assay buffer consisted of 50 mmol/L Tris-HCl, pH 7.4, 100 mmol/L NaCl, 5 mmol/L MgCl2, 0.25% BSA, and 0.5 mg/mL bacitracin. For determination of receptor affinity and density, competition binding studies were performed in the presence of 50 pmol/L [125I]-[Sar, Ile8]Ang II and increasing concentrations of unlabelled ligands (10-11 to 10-6 mol/L). For measurement of the surface receptor binding capacity, the cells were pretreated as indicated and then incubated in the presence of 100 pmol/L Ang II for the indicated period at 37°C. Cells were then washed with ice-cold 50 mmol/L glycine and 150 mmol/L NaCl, pH 3.0, for 5 minutes at 4°C, followed by two saline washes. The binding studies were performed at near-saturating conditions with 2 nmol/L [125I]-[Sar, Ile8]Ang II for 3 hours at 4°C.

**Measurement of IP3 Production**

Cells were subcultured into 12-well dishes and were exposed to Ang II at or near confluence; the treatment was stopped by the addition of 1/5 vol of 100% ice-cold trichloroacetic acid to the plates. The cells were then harvested by scraping and transferred to polypropylene tubes. The cell extract was vortexed thoroughly and then centrifuged for 10 minutes at 6000g at 4°C. The supernatant was removed and warmed to room temperature for 15 minutes. Levels of IP3 in each supernatant were determined by use of a radioimmunooassay kit from Dupont NEN following the manufacturer's instructions.

**Permeabilization of CHO Cells**

CHO cells bearing AT1a-R at subconfluence in 12-well plates were incubated with prewarmed calcium-free PBS and then washed twice with KG buffer (mmol/L: KCl 120, NaCl 30, MgCl2, 2.5, K2HPO4, 1, PIPES 10, EGTA 2, and CaCl2 0.5, pH 7.2). Cells were then incubated for 30 seconds with 0.0075% digitonin at room temperature in KG-A buffer (KG buffer supplemented with 5 mmol/L glucose and 2 mmol/L ATP), followed by three washes with KG buffer. Under this condition, >95% of the cells were permeabilized as assessed by staining with trypan blue. After permeabilization, the cells were loaded with mastoparan or low molecular weight heparin (average molecular weight, 3000) by incubation with 100 μmol/L mastoparan or 10 μmol/L heparin for 15 minutes in KG-A buffer at 37°C, respectively.
Desensitization of Receptors

Studies were performed essentially as described previously. In brief, the mastoparan- or heparin-loaded cells, or cells pretreated with chemicals as indicated, were incubated with or without 100 nmol/L Ang II in serum-free medium for indicated periods at 37°C. The reaction was stopped by washing the cells with 150 mmol/L NaCl/50 mmol/L glycine (pH 3.0) for 5 minutes at 4°C and with prewarmed PBS twice. For measurement of the IP3 level in response to Ang II, the cells were again stimulated with Ang II (100 nmol/L), and 15 seconds later the IP3 mass was assayed as described above.

Preparation of the Bacterial Thioredoxin-AT1A Fusion Protein

A region of the rat AT1A-R cDNA encoding the entire cytoplasmic tail (N295 to E359) was amplified by polymerase chain reaction and inserted into pBluescript KS(+) TA vector, and DNA was sequenced. Then, the BamHI-SalI fragment was cut and inserted into the same sites of the thiorefusion expression plasmid pTrxFus vector in the frame. Induction of pTrxFus-AT1A–transformed *Escherichia coli* with tryptophan (100 μg/mL) resulted in the production of a fusion protein of ~19 kD (11.6 kD of thioredoxin plus 7.5 kD for N295 to E359). The fusion protein was purified with activated ThioBond resin (Invitrogen Corp) according to manufacturer’s protocols.

Preparation of Cell Fractions and Western Blotting

Serum-starved cells were washed twice with PBS and stimulated with 100 nmol/L Ang II for the indicated periods. Then, the cells were washed with ice-cold PBS and scraped into 0.5 mL per dish of lysis buffer. The supernatant, representing the cytosolic fraction, was centrifuged at 100 000g for 20 minutes and resuspended in lysis buffer. The supernatant, representing the cytosolic fraction, was centrifuged at 100 000g for 20 minutes, and was then homogenized three times (30 seconds each) in a Polytron homogenizer (Brinkmann). Unbroken cells and cell nuclei were sedimented at 800g for 20 minutes and resuspended in lysis buffer. The supernatant, representing the cytosolic fraction, was centrifuged at 100 000g for 20 minutes, and the pellet was resuspended in lysis buffer. The supernatant, representing the cytosolic fraction, was centrifuged at 100 000g for 20 minutes, 60 minutes. Preparations were kept at ~80°C and used for Western blot or kinase assay. Samples containing equal amounts of protein (50 to 100 μg) were separated by 7.5% SDS-PAGE gels and transferred to PVDF membranes. Membranes were probed with 0.3 to 0.5 μg/mL GRK polyclonal antibodies, and immunoreactive bands were visualized with the enhanced chemiluminescence detection system (New England Biolabs). For Western blotting of PLCs, CHO/AT1A-R cells or VSMCs were lysed on ice in lysis buffer containing 1% Triton X-100. The detergent-lysed cell extracts were separated by 7.5% SDS-PAGE gels and subjected to Western blotting with PLC polyclonal antibodies as described above.

In Vitro Phosphorylation of Thioredoxin-AT1A Fusion Protein

For measuring the PKC-dependent or Src kinase–dependent phosphorylation of thioredoxin-AT1A fusion protein, purified thioredoxin-AT1A fusion protein (5 μg) was incubated with PKC (mixture of α, β, and γ, 50 ng per tube) or Src kinase (19 U per tube) in kinase buffer (mmol/L: Tris-HCl 20, MgCl2 10, EGTA 1, and dithiothreitol 2, pH 7.4) containing 1 μmol/L [γ-32P]ATP for 30 minutes or 60 minutes at 30°C (final volume, 100 μL). For phosphorylation of the fusion protein by membrane preparations from CHO/AT1A cells, an aliquot of cell membrane fractions (90 μg) that had been precleared by activated ThioBond resin, was incubated with purified thioredoxin-AT1A fusion protein (5 μg) in the above kinase buffer containing 50 μmol/L [γ-32P]ATP for 30 minutes at 30°C. The reaction was terminated by the addition of 1 mL ice-cold TE buffer (mmol/L: Tris-HCl 10 and EDTA 10, pH 7.4). Thiobond resin was added, collected by centrifugation (13 000g for 10 seconds), and washed four times with 1 mL of TE buffer. Bound protein was dissociated by boiling in 1× SDS–PAGE sample buffer. Proteins were resolved by 15% SDS-PAGE gels. Gels were then dried and analyzed by autoradiography.

**Results**

Desensitization of the AT1A-R–Mediated IP3 Formation

To investigate mechanisms underlying the agonist-induced desensitization of AT1A-R, we expressed cloned rat WT AT1A-R in...
CHO cells that lack endogenous AT₁ receptors. The binding of 125I-[Sar,1Ile8]Ang II, a peptidic Ang II receptor antagonist, to the cells bearing WT AT₁A-R was dose-dependently inhibited by Ang II, [Sar,1Ile8]Ang II, and an AT₁A-R–selective antagonist, losartan. In contrast, PD124319, a selective AT₂ receptor antagonist, had no effect on the binding of 125I-[Sar,1Ile8]Ang II (data not shown). The AT₁ receptor activates PLC through Gq/11.11 Previous studies of the AT₁A-R have shown that Ang II–induced formation of IP₃ was inhibited 75% by the tyrosine kinase inhibitor genistein and was associated with the activation of PLC₂ in VSMCs lacking PLC₁. Western blot analysis demonstrated that PLC₂ (150 kD), PLC₃ (150 kD), and PLC₆ (145 kD) were clearly detected in CHO cells expressing high levels of WT AT₁A-R (CHO/AT₁A-R cells), whereas only PLC₃ and PLC₆ were detected in VSMCs (Fig 2). To determine whether Ang II–induced formation of IP₃ was sensitive to the tyrosine kinase inhibitors or calmodulin inhibitor W-7, we measured IP₃ response to Ang II in CHO/AT₁A-R cells with three different tyrosine inhibitors (genistein, herbimycin-A, and lavendustin A) or W-7. The results (not presented) showed that neither compound affected the IP₃ response to Ang II in intact cells. This observation suggests that the IP₃ response to Ang II in CHO/AT₁A-R cells is probably mediated by PLC₂ through Gq/11.11
To ascertain whether agonist-induced desensitization of AT₁A-R expressed in CHO cells occurs, we determined the effect of a pretreatment with Ang II on the subsequent maximal Ang II–stimulated IP₃ formation. The pretreatment with Ang II resulted in a subsequent attenuation of IP₃ response to Ang II. By varying the time of preexposure to the agonist from 1 minute to 30 minutes, we found that Ang II rapidly induced receptor desensitization as early as 1 minute (P<.05), with complete desensitization observed by pretreatment for 15 minutes (Fig 3A). Attenuation of IP₃ production was maximal when cells were pretreated with 10 to 100 nmol/L Ang II, and half-maximal effects were observed after pretreatment with ≈3 nmol/L Ang II (Fig 3B).

Involvement of GRKs in Agonist-Induced Homologous Desensitization of AT₁A-R
We and others²,¹³,¹⁴ have shown that PKC is involved in the heterologous desensitization of the AT₁ receptor. Treatment of CHO/AT₁A-R cells with PMA (100 nmol/L) for 10 minutes completely abolished receptor-mediated production of IP₃ with no effect on the basal level of IP₃ (Fig 4). This effect of PMA was completely antagonized by pretreating the cells with 10 μmol/L of a newly tested in vitro and in vivo nontoxic PKC-specific inhibitor, GF109203X.¹⁵ Consistent with the effects of the PKC inhibitor, downregulation of PKC by overnight incubation with PMA (1 μmol/L)¹⁶ completely blocked the desensitization of the AT₁A-R by PMA (data not shown). Like AT₁B-R, desensitization of AT₁A-R by a low Ang II concentration (1 nmol/L) was abolished by GF109203X.

Figure 2. Immunoblots showing the expression of PLCs. Equal amounts of cell lysates (100 μg) were separated by 7.5% SDS-PAGE and transferred to PVDF membranes. The blocked membranes then were probed with polyclonal antibodies (Ab) to PLC₁ through PLC₆, and PLC₂. The position of PLC is indicated by the arrows.

Figure 3. Time course and dose response of the Ang II–induced desensitization of AT₁A-R. CHO/AT₁A-R cells were incubated with 100 nmol/L Ang II for the indicated periods (A) or with different concentrations of the agonist for 15 minutes (B). After acid washing, cells were restimulated with 100 nmol/L Ang II, and the IP₃ mass generated in 15 seconds was measured by radioreceptor binding assay as described in “Experimental Procedures.” Results are expressed as percentage of levels in cells not restimulated with Ang II. Data are mean±SE of three separate experiments. A 100% response represents the complete desensitization of the receptor.

Figure 4. PKC-dependent desensitization of AT₁A-R. CHO/AT₁A-R cells were pretreated with 10 μmol/L GF109203X (GF) for 30 minutes to inhibit PKC and then incubated for an additional 10 minutes with 100 nmol/L PMA or 15 minutes with 1 or 100 nmol/L Ang II. Cells were acid-washed and restimulated with 100 nmol/L Ang II for 15 seconds, and IP₃ mass was determined by radioreceptor binding assay. Results are expressed as percentage of levels in cells not restimulated with Ang II. Data are mean±SE of four experiments. A 100% response represents the complete desensitization of the receptor.
whereas the desensitizing effect at a high agonist concentration (100 nmol/L) was only partially prevented by GF109203X (Fig 4). The partial attenuation was seen even at a high dose of this compound (20 μmol/L) or with PKC depletion by overnight treatment with 1 μmol/L PMA (data not shown). These results suggest that a PKC-independent pathway, probably involving GRKs, contributes to the homologous desensitization of the AT1A-R in transfected CHO cells.

We then studied the role of GRKs in agonist-induced homologous desensitization of AT1A-R. Mastoparan, a wasp venom peptide, has been known to mimic the agonist-bound (active form) GPCRs and activate GRKs.17,18 Incubation of permeabilized CHO/AT1A-R cells with 100 μmol/L mastoparan for 10 minutes completely abolished the receptor-mediated IP3 response to Ang II (Fig 5). On the other hand, pretreatment of the permeabilized cells with 10 μmol/L heparin, a potent GRK inhibitor,19,20 for 20 minutes largely prevented the Ang II–induced desensitization of the AT1A-R–mediated IP3 response. The pretreatment with heparin decreased the basal level of IP3 only slightly (Fig 5).

These results indicate that PKC plays a major role in the heterologous desensitization of AT1A-R and that a heparin-sensitive GRK(s) may be involved in the agonist-induced homologous desensitization of the receptor in transfected CHO cells.

### Impaired Heterologous or Homologous Desensitization of Truncated Mutants of AT1A-R

To address the possible role of the cytoplasmic tail in the regulation of AT1A-R, we constructed truncated mutants of AT1A-R and stably expressed them in CHO cells. As shown in Fig 1, T310 and T318 truncation removes all Ser/Thr residues, including three potential PKC phosphorylation sites in the cytoplasmic tail of the AT1A-R; T328 truncation removes all but the Ser236; and T348 truncation removes a potentially farnesylated cysteine and two Ser residues in the tail, including one PKC phosphorylation site, S146AK. Clonal, stably transfected cells expressing T310, T318, or T348 AT1A-R were prepared and matched with CHO/AT1A-R cells expressing WT AT1A-R at a high level. Cells expressing the T328 receptor were compared with cells bearing low levels of WT AT1A-R (Table). As shown in the Table, Scatchard analysis revealed that the dissociation constants (Kd) for 125I-[Sar1,Ile8]Ang II among the WT AT1A-R and its mutants were similar, indicating that all the mutants possessed similar ligand binding affinity. Assay of IP3 formation in response to Ang II demonstrated that truncated mutants (T318, T312, and T348) of AT1A-R couple with effector PLC with an efficiency similar to that of full-length receptors. By contrast, the IP3 formation on Ang II stimulation was barely detected in cells expressing high levels of truncated mutant T310, indicating the region between K310 and L317 in the cytoplasmic tail of the AT1A-R that is involved in the receptor coupling to Gq protein (Table).

As shown in Fig 6, pretreatment of cells expressing WT AT1A-R or mutant T348 with 100 nmol/L Ang II abolished the IP3 response of the cells to a subsequent maximal stimulation with Ang II. By contrast, the Ang II–induced attenuation of the IP3 response was markedly impaired in both cell clones expressing T318 or T328 AT1A-R, indicating that potential

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**Figure 5.** GRK-dependent desensitization of AT1A-R. CHO/AT1A-R cells were permeabilized by 0.0075% digitonin for 30 seconds and then treated with 100 μmol/L mastoparan or with 10 μmol/L heparin for 15 minutes in KG-A buffer before incubation with 100 nmol/L Ang II for 15 minutes. Cells were acid-washed and then restimulated with 100 nmol/L Ang II for 15 seconds, and IP3 mass was determined by radioreceptor binding assay. Changes of IP3 mass are expressed as percentage of that seen in control cells not restimulated with Ang II. Data are mean±SE of four independent experiments.
Ser/Thr phosphorylation sites located between S328 and S347 in the cytoplasmic tail are important for the agonist-induced desensitization. In addition to the impaired agonist-induced desensitization, the PMA-dependent heterologous desensitization of the receptor was also dramatically impaired in the truncated mutants T318 and T328 (Fig 6). Furthermore, deletion of the last two Ser residues (mutant T348), including one PKC phosphorylation site in the receptor tail, prevented PMA-induced desensitization of the AT1A-R by ~30% (Fig 6).

Phosphorylation of Thioredoxin-AT1A Fusion Protein by PKC
To determine whether the cytoplasmic tail of AT1A-R could be phosphorylated by PKC, we generated a thioredoxin fusion protein containing the entire carboxyl tail of the AT1A-R (Fig 7A). The purified thioredoxin-AT1A fusion protein was then used in an in vitro PKC phosphorylation assay. It was not a surprise that the thioredoxin-AT1A fusion protein was highly phosphorylated by PKC (mixture of α, β, and γ) (Fig 7B), since there are three potential PKC phosphorylation sites in the carboxyl tail of the AT1A-R. However, the thioredoxin-AT1A fusion protein was not phosphorylated by Src kinase, even though a Src kinase substrate peptide (KVEKIGEGEGTVGVKK) was highly phosphorylated (205 ± 17 235 cpm, 8-fold incorporation over background). There was no phosphorylation of the thioredoxin protein by PKC or Src kinase (Fig 7B).

Ang II Did Not Induce Translocation of GRK2, GRK3, or GRK6
Agonist-induced translocation of GRK1, GRK2, or GRK3 is considered the first step involved in the homologous desensitization of rhodopsin or β-adrenergic receptors, respectively. To determine whether Ang II could induce translocation of GRK from cytosol to the plasma membranes, CHO/AT1A-R cells were pretreated with 1 μmol/L PMA overnight to deplete PKC and then exposed to 100 nmol/L Ang II for 15 minutes. Plasma membranes were prepared, and the kinase activity associated with the membranes was determined using thioredoxin-AT1A fusion protein as a substrate. As shown in Fig 7C, the phosphorylation of the thioredoxin-AT1A fusion protein was increased dramatically by membranes derived from Ang II–treated CHO/AT1A-R cells depleted of PKC. This indicated that the cytoplasmic tail of AT1A-R could be phosphorylated by a PKC-independent mechanism. Moreover, the Ang II–induced kinase activity in the membrane fraction was completely inhibited by the GRK inhibitor heparin (100 nmol/L) (Fig 7C) but not by the PKC-specific inhibitor GF109203X or the PKA inhibitor H-89 (data not shown). To determine which individual GRK is selectively activated (by translocation) on Ang II stimulation, CHO/AT1A-R cells or

Figure 6. Impaired homologous and heterologous desensitization of truncated mutants of AT1A-R. Cells expressing WT or truncated mutants of AT1A-R were pretreated with 100 nmol/L Ang II for 15 minutes or 100 nmol/L PMA for 10 minutes. Cells were acid-washed and restimulated with 100 nmol/L Ang II for 15 seconds. IP3 mass was determined by radioreceptor binding assay. Results are expressed as percent of levels in cells not restimulated with Ang II. Data are mean ± SE of three independent experiments. A 100% response represents the complete desensitization of the receptor.

Figure 7. Phosphorylation of thioredoxin-AT1A fusion protein by PKC and a heparin-sensitive kinase from the membrane of Ang II–treated CHO/AT1A-R cells. A, Expression and purification of the thioredoxin-AT1A fusion protein. Shown is a Coomassie blue stain of the purified fusion protein indicating the relative position on a 12% SDS-PAGE gel. Indicated on the left are the positions of molecular weight standards. β-ME indicates β-mercaptoethanol. B, Phosphorylation of the fusion protein by PKC. Thioredox control protein (Thio.) or thioredoxin-AT1A fusion protein (Thio.-AT1A) was incubated with PKC (mixture of α, β, and γ) or Src kinase for indicated times at 30°C. C, CHO/AT1A-R cells pretreated with 1 μmol/L PMA overnight to deplete PKC and then exposed to 100 nmol/L Ang II for 15 minutes as indicated. The membranes were prepared and incubated with thioredoxin (Thio.) or thioredoxin-AT1A fusion protein (Thio.-AT1A) in the absence or presence of 100 nmol/L heparin for 30 minutes at 30°C. The reaction was stopped, and ThioBond resin (Invitrogen Corp) was added and collected. Bound fusion protein was dissociated and resolved by 15% SDS-PAGE. The position of the thioredoxin-AT1A fusion protein is indicated by the arrow.
VSMCs were stimulated with Ang II for different time periods, and the cytosol and plasma membranes were prepared. Western blot experiments showed that GRK2 was almost equally distributed in cytosol or plasma membranes of the CHO/AT1A-R cells, whereas GRK3 was mainly present in cytosol (Fig 8A). Ang II treatment did not induce translocation of GRK2 or GRK3 in CHO/AT1A-R cells, since the protein level of GRK2 or GRK3 in the cytosolic and membrane fractions was not changed by Ang II stimulation (Fig 8A). Similar results with GRK2 and GRK3 were obtained before and after Ang II treatment in VSMCs (data not shown). In addition, acute PMA treatment had no effect on the translocation of GRK2 or GRK3 in either CHO/AT1A-R cells (Fig 8A) or VSMCs (data not shown). However, translocation of GRK2 was observed by agonist UK14,304 treatment in MDCK cells stably expressing α2-adrenergic receptors23 (Fig 8B). GRK5 was undetectable in CHO/AT1A-R cells but was detected in VSMCs (Fig 8C). GRK6 was detected only in the plasma membranes of both CHO/AT1A-R cells (Fig 8D) and VSMCs (not shown). The localization of GRK6 was not changed on Ang II stimulation of CHO/AT1A-R cells (Fig 8A) or VSMCs (data not shown). On the other hand, PKC was rapidly activated by Ang II in CHO/AT1A-R cells as well as in VSMCs (not shown), as reported previously.24,25

**Discussion**

In the present study, we have used four truncated mutants of AT1A-R (T310, T318, T328, and T348) lacking the potential carboxyl-terminal phosphorylation sites but retaining the normal binding of Ang II intact. We showed that the proximal portion (K310 to L317) of the receptor cytoplasmic tail is involved in the AT1A-R coupling to Gq, whereas the middle portion (S328 to S347) containing the potential Ser/Thr residues (eight Ser and two Thr residues) phosphorylation sites plays an important role in the agonist- and PMA-induced desensitization of the AT1A-R. Furthermore, the thioredoxin-AT1A fusion protein containing the entire cytoplasmic tail of the AT1A-R can be heavily phosphorylated by PKC and by a membrane-associated heparin-sensitive kinase from Ang II-treated CHO/AT1A-R cells depleted of PKC.

It has been noted that Ang II action is accompanied by a rapid and profound desensitization known as tachyphylaxis. However, the mechanisms by which Ang II receptors desensitize are still not clear. It seems that receptor sequestration or internalization does not play a major role in causing the homologous desensitization of the AT1A-R expressed in CHO cells, since the complete desensitization induced by 15 minutes of incubation with 100 nmol/L Ang II still occurred even when the receptor internalization was almost completely blocked by concanavalin A2 or phenylarsine oxide treatment (loss of membrane receptors: 12 ± 2% with concanavalin A + Ang II or 8 ± 0.6% with phenylarsine oxide + Ang II versus 50 ± 4% with Ang II alone). Similar findings have been reported for the AT1 receptors in cardiomyocytes,1 AT1B-R expressed in CHO cells,2 AT1A-R expressed in 293 cells,3 and other PLC-linked GPCRs.26,27 Thus, rapid desensitization of the AT1A-R-mediated IP3 formation may well be a consequence of the agonist-induced biochemical modification of the receptor, such as receptor phosphorylation.

The cytoplasmic tail of the AT1A-R contains multiple Ser/Thr residues (13 of the last 59 amino acids), and there are three consensus PKC phosphorylation sites (S311TK, S338YR, and S346AK).28,29 Removal of all Ser/Thr residues in the tail (truncated mutant T318) or removal of all Ser/Thr residues but the S326 (mutant T328) dramatically impaired agonist- and PMA-induced desensitization of the receptor. Deletion of the last two Ser residues, including one PKC consensus site, prevented only the PMA-induced heterologous desensitization by ~30%, suggesting that two other PKC consensus sites (S311TK and S338YR) may chiefly contribute to the PKC-
dependent desensitization of the AT₁A-R. Since the immuno-
precipitable antibody against the AT₁ receptor is not available,
we cannot measure the receptor phosphorylation on agonist or
PMA stimulation. We made and prepared a purified thio-
redoxin-AT₁A fusion protein that contains the entire cytoplasmic
tail of AT₁A-R. The thioredoxin-AT₁A fusion protein was
highly phosphorylated by PKC and by a membrane-associated
heparin-sensitive kinase from Ang II–treated CHO/AT₁A-R
cells depleted of PKC, but not by Src kinase in vitro despite the
presence of four tyrosine residues in the receptor cytoplasmic
tail. Taken together, these results indicate that the phos-
phorylation of the receptor cytoplasmic tail is involved in the
homologous and heterologous desensitization of the AT₁A-R
and that the potential Ser/Thr phosphorylation sites important
for the AT₁A-R desensitization are located between S 328 and
and S 347 (containing eight Ser and two Thr residues) of the receptor
cytoplasmic tail. Studies are now in progress to identify the
major phosphorylation site(s) by the agonist or PMA
treatment.

The PKC-specific inhibitor GF109203X completely sup-
pressed the desensitization of AT₁A-R by Ang II at a low
concentration (1 nmol/L). This suggests that PKC plays a
major role in heterologous desensitization at a near-physi-
ological agonist concentration. The desensitizing effect at a higher
agonist concentration (100 nmol/L) is only partially prevented
by the inhibition of PKC. Previous studies have shown that the
PKC inhibitor staurosporine suppressed agonist-induced Ang
II receptor desensitization in Xenopus oocytes.14 Pfeilschifter
and coworkers40–42 have obtained evidence implicating PKC in
the Ang II–induced desensitization in glomerular mesangial
cells. We have demonstrated that PKC is only partially
involved in agonist-induced desensitization of AT₁B-R in
transfected CHO cells.5 Similar findings have also been re-
ported for several other PLC-linked GPCRs.23,24 In contrast,
PKC depletions or treatment with the selective PKC inhibitor
RO31-7519 did not affect the rapid agonist-induced desensi-
tization of the Ang II receptor in neonatal cardiac myocytes.1
Recently, Oppermann et al30 reported that PKC inhibition
with staurosporine reduced agonist-induced phosphorylation
of AT₁A-R by 40% to 50% in transfected 293 cells but did not
affect the agonist-induced desensitization of the receptor.
In aggregate, these studies and ours suggest that the agonist-
induced desensitization of AT₁A-R may only be partially
regulated by PKC in a cell type–specific manner.

A wasp venom peptide, mastoparan, has been shown to mimic
the agonist-bound (active form) GPCRs and activate GRKs.17,18
Challenging the permeabilized cells expressing WT AT₁A-R, with
mastoparan completely abolished the receptor-mediated IP3 re-
sponse to Ang II. Pretreatment of the permeabilized cells with the
GRK inhibitor heparin largely prevented the agonist-induced
desensitization of AT₁A-R. Furthermore, treatment of the cells
depleted of PKC with Ang II induced the translocation of a
heparin-sensitive kinase activity, which could phosphorylate the
thioredoxin-AT₁A fusion protein containing the entire cytoplas-
mic tail of AT₁A-R. These data indicate that the heparin-sensitive
kinase may be mainly involved in the agonist-induced homolo-
gous desensitization of AT₁A-R. Six members of the GRK family
have been cloned to date and, on the basis of structural
similarities, have been divided into three subfamilies: (1) rhodop-
sin kinase (GRK1), (2) the β-adrenergic receptor kinase subfamily
(GRK2 and GRK3), and (3) the GRK4 subfamily (GRK4,
GRK5, and GRK6). GRKs also seem to have different patterns of
expression in tissues. GRK1 and GRK4, for instance, are primar-
ily expressed in retinal cells and testis, respectively, suggesting very
specific functions. Whereas, other GRKs are expressed in many
tissues, suggesting their broad roles in regulating the functions of
GPCRs. Overexpression (>20-fold) of either GRK2, GRK3, or
GRK5 was shown to enhance agonist-induced AT₁A-R phos-
phorylation equally well, illustrating a role for GRKs (GRK2,
GRK3, and GRK5) in the agonist-induced phosphorylation and
desensitization of the AT₁A-R. Since active GPCRs (agonist-
bound form), in the presence of charged phospholipids, can
directly associate with and activate GRKs,36,57 it is possible that
overexpression (20-fold) of either GRK2, GRK3, or GRK5
could result in nonspecific direct interaction between Ang II–
bound AT₁A-R with the overexpressed GRK, leading to the
enhanced agonist-induced phosphorylation of AT₁A-R, since experi-
ments did not show a significant difference between indi-
vidual GRKs in their ability to enhance receptor phosphorylation
in response to Ang II.1 As described above, we have demonstrated
that the cytoplasmic tail of AT₁A-R plays a critical role in the
agonist-induced homologous desensitization of the receptor.
The cytoplasmic tail of AT₁A-R does not contain the consensus
phosphorylation sites for GRK1 (the presence of acidic residue on
the C-terminal side of a Ser/Thr), GRK2, and GRK3 (the
presence of acidic residue localized to the N-terminal side of a
Ser/Thr).32,33 Stimulation of CHO/AT₁A-R cells or VSMCs with
Ang II did not induce translocation of GRK2 and GRK3. GRK5
was not detected in CHO/AT₁A-R cells but was detected only in
the membrane fraction of quiescent VSMCs. GRK6 was detected
only in the plasma membranes of both CHO/AT₁A-R and VSMCs.
The localization of GRK6 was not affected by Ang II stimulation
of either CHO/AT₁A-R cells or VSMCs. Moreover, we
immunoprecipitated GRK6 and measured its catalytic activity
using a soluble GRK substrate, casein, according to method of
Pitcher et al.50 The activity of GRK6 was not affected by Ang II
stimulation in CHO/AT₁A-R cells (not shown). Therefore, it
seems that GRK2, GRK3, or GRK6 might not be involved in the
agonist-induced homologous desensitization of the AT₁A-R,
at least in CHO/AT₁A-R cells and VSMCs. Recently, a novel
heparin-sensitive 40-kD receptor kinase, designated as MEK, was
purified from porcine cerebellum. It was identified in the CHO
cells expressing the m3-muscarinic receptor, and the activity of
MEK in the membrane fraction was increased after the agonist
stimulation.40

In summary, the data presented have demonstrated that
potential Ser/Thr phosphorylation sites located between S 328
and S 347 in the cytoplasmic tail of AT₁A-R play a critical role in
the heterologous and homologous desensitization of the re-
ceptor. Although a recent study reported that the cytoplasmic
tail of AT₁A-R is not involved in desensitization, the prelimi-
nary observation needs to be reconsidered, since no acid-
washing step was taken to remove the receptor-bound Ang II
before a second challenge of the cells with agonist during the
measurement of Ca²⁺ mobilization. However, the precise role
played by the cytoplasmic tail of the AT₁A-R in the complex
series of biochemical mechanisms underlying receptor regula-
tion needs to be explored further. The present results with the
T310 truncated mutant indicate that the proximal portion (K310 to L317) of the AT1A-R cytoplasmic tail is directly involved in receptor coupling to Gq. Recently, T. Sano (unpublished data, 1997) detailed experiments showing that a specific sequence, Y152-L158-L161, in the proximal portion of the rat AT1A-R cytoplasmic tail is essential for coupling and activation of Gq. Clearly, more work is needed to identify the Ser/Thr residues between S286 and S347 involved in the homologous and heterologous desensitization of AT1A-R.

Acknowledgments

This study was supported in part by National Institutes of Health grants HL-58205, HL-35323, andDK-20593. The authors thank J.R. Keefer for the MDCK cells expressing α2A-adrenergic receptor and Dr E.J. Landon for reading this manuscript.

References


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Circ Res. 1998;82:523-531
doi: 10.1161/01.RES.82.5.523

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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