A
gonist-induced receptor phosphorylation has been considered to play a pivotal role in trans-
membrane signal transduction systems by reg-
ulating receptor function and distribution. Previous
studies have demonstrated that phosphorylation of G
protein–coupled receptors, including α- and β-adrener-
gic receptors (ARs), rhodopsin receptors, and musca-
rinic cholinergic receptors (mChRs), induces receptor
desensitization by reducing the affinity for binding
agonist ligands and/or reducing the ability to interact
with biochemical effectors.1,4 This modulation of func-
tion has been shown to be associated with phosphory-
lization of serine and/or threonine residues of the recep-
tor proteins via cyclic AMP–dependent protein kinase
(PKA) and agonist-specific receptor kinases (G pro-
tein–coupled receptor kinases [GRKs]) such as β-AR
kinase (BARK) and rhodopsin kinase.5,6 Studies on
agonist-induced phosphorylation of β-AR suggest that
PKA plays a major role in heterologous desensitization
at low agonist concentrations, whereas BARK is impor-
tant in eliciting homologous desensitization at higher
agonist concentrations.1,2,7 In all instances, phosphory-
lization of serine and/or threonine residues of the recep-
tor proteins appears to attenuate receptor function.8
In contrast, the tyrosine kinase receptors appear to
be susceptible to positive modulation by phosphoryla-
tion. With these receptors, autophosphorylation of ty-
rosine residues may lead to enhanced tyrosine kinase
activity of the receptors and increased receptor func-
tion.8 In receptors with intrinsic tyrosine kinases—such
as those for insulin, insulin-like growth factor-1, and
epidermal growth factor—and in so-called type 2 recep-
tors (including those for low-density lipoprotein and
transferrin), a specific motif containing a tyrosine resi-
due is proposed to promote receptor internalization.9
Recently, serine and/or threonine phosphorylation has
been suggested to be involved in mChR internalization
as well.10
We have cloned the cDNA for the rat vascular type 1
angiotensin II receptor (AT1αR), a member of the G
protein–coupled receptor superfamily.11 The cDNA for
the AT1αR encodes a protein with several potential
phosphorylation sites for serine/threonine and tyrosine
kinases, raising the possibility that phosphorylation of
the AT1αR might play an important role in regulating
receptor function. Furthermore, we have previously
shown that phenylarsine oxide, a tyrosine phosphatase
inhibitor, inhibits AT1αR internalization in cultured rat
aortic smooth muscle (RASM) cells,12 suggesting the
possible involvement of tyrosine phosphorylation in
the regulation of AT1αR processing. With the develop-
ment of antibodies to the AT1αR, it has become possible
to test these hypotheses directly.
We designed this study to determine whether angio-
tensin II (Ang II) induces phosphorylation of its own
receptors in intact RASM cells and if so to identify the
amino acids phosphorylated by Ang II. Our results
indicate that the AT1αR is a phosphoprotein in unstim-
ulated RASM cells and that phosphorylation of the
AT1αR is significantly increased on Ang II stimulation.
The \( \text{AT}_{1}\text{AR} \) is phosphorylated on both serine and tyrosine residues, suggesting a dual level of control.

### Methods

#### Materials

Ang II, forskolin, phorbol 12-myristate-13-acetate (PMA), and ionomycin were purchased from Sigma Chemical Co. Losartan was a gift from Dr R.D. Smith (Du Pont de Nemours Co). Orthophosphate was purchased from Du Pont NEN. Monoclonal anti-phosphotyrosine antibody (anti-PY) was from UBI, Inc. All other chemicals were of the highest grade commercially available. The composition of HEPES-buffered saline solution (BSS) was (mmol/L) HEPES 20 (pH 7.4), NaCl 130, KCl 5, MgCl\(_2\) 1, CaCl\(_2\) 1.5, and glucose 10. Phosphate-buffered saline (PBS) solution contained (mmol/L) NaCl 100, Na\(_2\)HPO\(_4\) 80, and NaH\(_2\)PO\(_4\) 20 (pH 7.4).

#### Culture of RASM Cells

Primary cultures of RASM cells were obtained by enzymatic dissociation of aortic medial tissue from male Sprague-Dawley rats, as described elsewhere. Cells were passaged in Dulbecco's modified Eagle's medium (Hazelton) containing 10% calf serum (GIBCO Laboratories), as previously described. For experiments, RASM cells from passages 5 through 15 were seeded on 100-mm dishes and fed every other day. On reaching confluence, RASM cells were incubated in serum-free medium for 48 hours before all experiments.

#### Preparation of Cell Lysate and Immunoprecipitation

RASM cells were washed three times with BSS (37°C). For phosphorylation analysis, cells were labeled with 400 \( \text{mCi}/\text{mL} \) of \( \text{[32P]Orthophosphate} \) in BSS at 37°C for 6 hours. Ang II, forskolin, PMA, ionomycin, and losartan were added to the BSS for the final period of the incubation as indicated. The reaction was terminated by aspirating the buffer and washing with ice-cold PBS on ice. The cells were then lysed in ice-cold lysis buffer containing (mmol/L) HEPES 50 (pH 7.5) and phenylmethylsulfonyl fluoride 1, as well as 1% Triton X-100, NaCl 50, NaF 50, sodium pyrophosphate 10, EDTA 5, Na\(_2\)VO\(_4\) 1, 10 \( \mu \text{g}/\text{mL} \) aprotinin, and 10 \( \mu \text{g}/\text{mL} \) leupeptin. Equal protein aliquots of the lysate (1 mg protein) were subjected to immunoprecipitation. Immunoprecipitation was performed according to the method of Molloy et al\(^\text{a}\) using 1 \( \mu \text{g}/\text{mL} \) of monoclonal anti-AT\(_{1}\text{AR} \) antibody against the peptide corresponding to amino acids 17 through 24 of the AT\(_{1}\text{AR} \) (anti-AT, prepared as described in detail previously in Reference 14) or 2 \( \mu \text{g}/\text{mL} \) of monoclonal anti-PY. In some experiments, antibody against peptide corresponding to amino acids 183 through 190 (anti-Ang2.183) or amino acids 230 through 237 (anti-Ang2.230)\(^\text{a}\) was used to identify the specificity of the immunoprecipitate. Immunoprecipitates were collected with protein A-Sepharose (Pharmacia) and subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

#### Phosphorylation Analysis

After SDS-PAGE, anti-AT immunoprecipitates were transferred to Immobilon-P (Millipore Corp), and the membrane was subjected to autoradiography. For determination of the specific radioactive band that corresponded to the AT\(_{1}\text{AR} \), the membrane was immunoblotted with anti-AT, and immunoreactive bands were visualized with a phosphatase detection system (Kirkegaard & Perry Laboratories). The immunoreactive bands were excised from the membrane, and the radioactivity of the bands was quantified by liquid scintillation spectroscopy.

#### Phosphoamino Acid Analysis

The radioactive bands corresponding to the AT\(_{1}\text{AR} \) were excised from the blotted membrane as described above and subjected to hydrolysis with 5.7\( \text{N} \) HCl at 110°C for 1 hour.\(^\text{a}\)

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**Figure 1.** Blots show angiotensin II (Ang II)–induced phosphorylation of the Ang II type 1 receptor (\( \text{AT}_{1}\)AR). A, Immunoblotting with anti-AT. Anti-AT immunoprecipitates of rat aortic smooth muscle (RASM) cells show a single specific band of 52 kD by immunoblotting with anti-AT (left). Incubation with 300 \( \mu \text{g}/\text{mL} \) of the immunizing peptide for anti-AT blocked the immunoreactive 52-kD band (right). B, Immunoblotting with other anti-AT\(_{1}\text{AR} \) antibodies. Immunoprecipitates with anti-Ang2.183 (lane 1) or anti-Ang2.230 (lane 2) show a similar band of 52 kD by immunoblotting with each antibody. Incubation with the immunizing peptide for each antibody blocked the immunoreactivity of the 52-kD bands (lanes 2 and 4). C, Ang II–induced phosphorylation of the AT\(_{1}\text{AR} \). Autoradiogram shows the 52-kD band of anti-AT immunoprecipitates of \( \text{[32P]Orthophosphate} \)–labeled RASM cells. – indicates control; +, 100 \( \mu \text{mol}/\text{L} \) Ang II for 20 minutes. D, Ang II–induced tyrosine phosphorylation of the AT\(_{1}\)AR. Immunoreactive band shows anti-AT (52 kD) of anti-phosphotyrosine antibody immunoprecipitates. – indicates control; +, 100 \( \mu \text{mol}/\text{L} \) Ang II for 20 minutes.

After lyophilization, the hydrolysate was spotted onto a thin-layer cellulose plate (JT Baker Inc) and subjected to one-dimensional electrophoresis at 600 V for 1.5 hours in acetic acid/pyridine/water (50:5:945, pH 3.5). Radiolabeled phosphoamino acids were identified by autoradiography and comparison with unlabeled standards revealed with ninhydrin.

#### Immunoblotting for Tyrosine Phosphorylation

Lysates of nonlabeled RASM cells were immunoprecipitated with anti-PY as described above. Phosphotyrosine-containing proteins were subjected to 10% SDS-PAGE and transferred to Immobilon-P. Immunoreactive bands for anti-AT were visualized as described above. The relative changes in the content of phosphotyrosine-containing AT\(_{1}\text{AR} \) were quantified by scanning the immunoreactive bands using laser densitometry.

#### Statistical Analysis

Data are expressed as mean±SEM. ANOVA followed by a multiple comparison test was used for comparisons on initial data before expression as percentage of control. A value of \( P<.05 \) was considered statistically significant.

#### Results

Immunoprecipitation of RASM cells with anti-AT identified a single band of molecular weight 52 kD on immunoblotting with anti-AT (Fig 1A). The identity of the 52-kD band as the AT\(_{1}\)AR was confirmed in several ways. First, anti-AT immunoreactive 52-kD bands were specifically blocked by incubation with an excess concentration of the immunizing peptide used in producing the anti-AT (Fig 1A). Second, similar bands were observed when immunoblotting was performed using two other anti-AT\(_{1}\text{AR} \) antibodies made independently against amino acids in the third extracellular domains (anti-Ang2.183) or against those in the third intracellular loop (anti-Ang2.230) of the AT\(_{1}\)AR. Furthermore,
incubation with the peptide used in producing each of these antibodies also blocked the immunoreactive 52-kD band (Fig 1B). These findings demonstrate that the anti-AT immunoreactive 52-kD band is specific for the AT1AR.

To determine whether the AT1AR is phosphorylated in intact cells, we incubated RASM cells with [32P]orthophosphate and isolated the AT1AR by immunoprecipitation with anti-AT. In the basal state, immunoprecipitates contained a labeled band of molecular weight 52 kD, corresponding to the AT1AR as indicated by immunoblotting with anti-AT (Fig 1C). The phosphorylation of the band was significantly increased by stimulation with 100 nmol/L Ang II. Immunoprecipitation with anti-AT also brought down labeled proteins of 61, 73, 89, and 110 kD. However, none of these bands reacted with anti-AT on immunoblotting. As shown in Fig 2A, Ang II induced a rapid (within 1 minute) and significant increase in AT1AR phosphorylation, with a peak at 20 minutes (226±28% of control, P<.01, n=5). The increase in phosphorylation was sustained for at least 4 hours. The Ang II–induced phosphorylation of the AT1AR was completely inhibited by pretreatment (15 minutes) with 1 μmol/L losartan, a specific AT1AR antagonist (losartan, 98±13%; losartan+Ang II, 108±17% of control; n=4), indicating that the receptor phosphorylation is mediated by an Ang II–AT1AR interaction.

We performed phosphoamino acid analysis to determine the characteristics of the basal and Ang II–induced phosphorylation of the AT1AR (Fig 3). In the basal state, the major phosphoamino acid was serine, with a small amount of phosphate found in tyrosine residues. After 20 minutes of Ang II, both serine and tyrosine phosphorylation were increased. Phosphothreonine was not evident in either state.

Subsequent analysis of Ang II–induced tyrosine phosphorylation of the AT1AR was performed using anti-PY immunoprecipitation, followed by immunoblotting with anti-PY. Phosphorytrosine-containing AT1AR was detected in the basal state but was not significantly increased until 10 minutes after stimulation of Ang II and peaked at 20 minutes (163±17%, P<.01, n=5) (Figs 1D and 2B). The tyrosine phosphorylation declined within 60 minutes to a new level, which remained slightly elevated over basal levels for at least 4 hours. Losartan completely inhibited the Ang II–dependent tyrosine phosphorylation of the AT1AR (losartan, 103±11%; losartan+Ang II, 106±12%; n=4). The AT1AR tyrosine phosphorylation was dose dependent, with a threshold of 0.1 nmol/L (122±18% of control, n=3), an EC50 of 4.0±2.0 nmol/L (n=3), and a maximal effect at 100 nmol/L Ang II (178±29%, n=3).

To determine whether the ubiquitous protein kinases PKA, protein kinase C (PKC), or Ca2+-dependent protein kinases were able to induce AT1AR phosphorylation, we used the PKA activator forskolin, the PKC activator PMA, and the Ca2+ ionophore ionomycin. As shown in Fig 4, forskolin (10 μmol/L for 15 minutes) increased the total phosphorylation of the AT1AR to 230±49% (P<.01, n=3), with no effect on tyrosine phosphorylation. Neither PMA (100 nmol/L for 20 minutes) nor ionomycin (2 μmol/L for 2 minutes) had any effect on total or tyrosine phosphorylation of the AT1AR.

Discussion

In the present study we demonstrate that the AT1AR is phosphorylated not only constitutively but also in an agonist-dependent manner in intact RASM cells. The AT1AR also appears to be phosphorylated by PKA but not PKC. Serine residues are the major phosphoamino acids in both the basal and Ang II–stimulated states, with a smaller but significant content of phosphothreonine residues. The ability of Ang II to induce phosphorylation of its receptor on both serine and tyrosine residues
has not yet been reported among members of the G protein–coupled receptor superfamily and suggests regulation by multiple kinases.

The 52-kD band was consistently detected as a single immunoreactive band on immunoprecipitation followed by immunoblotting, using any of three independent antibodies for the AT1AR against the different portions of the AT1AR molecules (anti-AT, anti-Ang2.183, and anti-Ang2.230). Furthermore, the peptide competition tests with each immunizing peptide showed the specific inhibition of the immunoreactive band for each antibody. These findings demonstrate that the immunoreactive 52-kD band is specific for the AT1AR. The band most likely represents a glycosylated form of the AT1AR. The calculated molecular weight of the deduced, nonglycosylated AT1AR is 41 kD.11 Anti-Ang2 identifies a 43-kD band when a nonglycosylated form of the AT1AR is synthesized in vitro using a rabbit reticuloocyte lysate system.14 However, in several other rat tissues, immunoblotting with anti-Ang2 identifies a 49-kD band.15 These differences in the apparent molecular weight of the AT1AR are most likely due to variations in glycosylation and effectors.56 Specifically, GRKs and PKA control the efficacy of receptor coupling to G proteins and effectors.5,6

Presently, the pathway by which Ang II leads to phosphorylation of its own receptor is not known. Since the AT1AR couples to both phospholipases C and D in RASM cells,13,18 the kinase involved in the agonist-dependent phosphorylation of the AT1AR might be either a Ca2+-dependent protein kinase or PKC. Neither ionomycin nor PMA, however, induced AT1AR phosphorylation. Alternatively, an agonist-specific receptor kinase, such as BARK, may mediate Ang II–induced AT1AR phosphorylation. In support of this possibility, no second messenger appears to be involved in the agonist-dependent phosphorylation by BARK or rhodopsin kinase,8 a result in agreement with our ionomycin and PMA experiments. Furthermore, the phosphorylation catalyzed by BARK and rhodopsin kinase has been proposed to occur in the serine/threonine-rich C-terminal tail of the respective receptor.25 The AT1AR has several serine/threonine-rich domains in the C-terminal tail, and serine residues appear to be the major site phosphorylated by Ang II. Thus, it seems likely that a member of the GRK family may be involved in agonist-dependent phosphorylation of the AT1AR. The kinase responsible for the basal phosphorylation of the AT1AR may be different, because most GRKs isolated to date require agonist stimulation for activation of the phosphorylation effect.

Recent evidence suggests that Ang II induces tyrosine phosphorylation of several proteins in RASM cells.14,19 Although these findings imply that Ang II activates tyrosine kinases and/or inhibits tyrosine phosphatases, the precise pathways involved are unknown. The delay in onset of AT1R tyrosine phosphorylation on Ang II stimulation as compared with that for total phosphorylation suggests that activation of a complex protein kinase cascade may be required for tyrosine phosphorylation of the AT1AR. The inability of PMA and ionomycin to increase AT1AR tyrosine phosphorylation suggests that this cascade does not include PKC or Ca2+-dependent protein kinases.

The observation that forskolin increased the total phosphorylation of AT1AR is not unexpected, because there is a potential consensus sequence for PKA-mediated phosphorylation in the C-terminal tail of the AT1AR.11 However, PKA is not normally activated on AT1AR stimulation in RASM cells,20 suggesting that this kinase is likely to be involved in heterologous rather than homologous receptor regulation. Indeed, in βARs, serine phosphorylation mediated by PKA is postulated to play a role in heterologous desensitization of receptors.5 In particular, a recent study has shown that activation of βARs leads to heterologous desensitization, internalization, and downregulation of mChRs via phosphorylation on serine.21 Thus, the PKA-mediated phosphorylation of the AT1AR may indicate that this receptor is also susceptible to heterologous regulation by PKA-coupled receptors, suggesting a possible interaction between vasoconstrictors such as Ang II and vasodilators such as β-adrenergic agonists. It is plausible that agonist-induced phosphorylation of the AT1AR may play a role in the regulation of receptor function. Rapid desensitization of G protein–coupled receptors has been shown to be caused by intrinsic changes in the receptors that decrease the effectiveness with which they can activate G proteins and effectors.5,6 Recently, GRKs and PKA control the efficacy of receptor coupling to G proteins by rapidly phosphorylating the receptor at serine/threonine residues.2,5,7 By analogy, it is likely that Ang II–induced phosphorylation of the AT1AR may function in homologous desensitization. Alternatively, receptor phosphorylation may be involved in the regulation of receptor processing. We have previously shown that Ang II–induced internalization of the AT1AR can be prevented by phenylarsine oxide, a tyrosine phosphatase inhibitor.12 In so-called type II receptors (acceptors) and receptor tyrosine kinases, a tyrosine-containing motif has been proposed to play a crucial role in internalization of the receptors.7 This motif, NPXY, does, in a slightly modified version (NPXXY), occur in the AT1AR. Taken together, these observations raise the possibility that phosphorylation of the AT1AR on tyrosine may be involved in the regulation of receptor phosphorylation catalyzed by BARK and rhodopsin kinase has been proposed to occur in the serine/threonine-rich C-terminal tail of the respective receptor.
processing, including internalization, recycling, or re-
sensitization of the receptors.

The present study suggests that Ang II induces phos-
phorylation of its own receptors through modulation of
not only serine/threonine but also tyrosine kinases.
Future studies will be designed to clarify the precise
mechanisms responsible for serine and tyrosine phos-
phorylation of the AT$_1$AR and to determine the physi-
ological consequences of AT$_1$AR phosphorylation.

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