A agonist-induced receptor phosphorylation has been considered to play a pivotal role in transmembrane signal transduction systems by regulating receptor function and distribution. Previous studies have demonstrated that phosphorylation of G protein-coupled receptors, including α- and β-adrenergic receptors (ARs), rhodopsin receptors, and muscarinic cholinergic receptors (mChRs), induces receptor desensitization by reducing the affinity for binding agonist ligands and/or reducing the ability to interact with biochemical effectors. This modulation of function has been shown to be associated with phosphorylation of serine and/or threonine residues of the receptor proteins via cyclic AMP-dependent protein kinase (PKA) and agonist-specific receptor kinases (GRKs) such as β-Ar kinase (BARK) and rhodopsin kinase. 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 important in eliciting homologous desensitization at higher agonist concentrations. In all instances, phosphorylation of serine and/or threonine residues of the receptor proteins appears to attenuate receptor function.

In contrast, the tyrosine kinase receptors appear to be susceptible to positive modulation by phosphorylation. With these receptors, autophosphorylation of tyrosine residues may lead to enhanced tyrosine kinase activity of the receptors and increased receptor function. 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 receptors (including those for low-density lipoprotein and transferrin), a specific motif containing a tyrosine residue is proposed to promote receptor internalization. Recently, serine and/or threonine phosphorylation has been suggested to be involved in mChR internalization as well.

We have cloned the cDNA for the rat vascular type 1 angiotensin II receptor (AT₅R), a member of the G protein–coupled receptor superfamily. The cDNA for the AT₅R encodes a protein with several potential phosphorylation sites for serine/threonine and tyrosine kinases, raising the possibility that phosphorylation of the AT₁AR might play an important role in regulating receptor function. Furthermore, we have previously shown that phenylarsine oxide, a tyrosine phosphatase inhibitor, inhibits AT₁AR internalization in cultured rat aortic smooth muscle (RASM) cells, suggesting the possible involvement of tyrosine phosphorylation in the regulation of AT₁AR processing. With the development of antibodies to the AT₁AR, it has become possible to test these hypotheses directly.

We designed this study to determine whether angiotensin 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 AT₁AR is a phosphoprotein in unstimulated RASM cells and that phosphorylation of the AT₁AR is significantly increased on Ang II stimulation.
The AT₁R 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). [³²P]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, MgCl₂ 1, CaCl₂ 1.5, and glucose 10. Phosphate-buffered saline (PBS) solution contained (mmol/L) NaCl 100, Na₂HPO₄ 80, and NaH₂PO₄ 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 (GBHCO 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 µCi/mL of [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, Na₃VO₄ 1, MgCl₂ 1, CaCl₂ 1.5, and glucose 10. Phosphate-buffered saline (PBS) solution contained (mmol/L) NaCl 100, Na₂HPO₄ 80, and NaH₂PO₄ 20 (pH 7.4).  

Immunoblotting for Tyrosine Phosphorylation

Lysates of nonlabeled RASM cells were immunoprecipitated with anti-PY as described above. Phosphotyrosine-containing proteins were subjected to SDS-PAGE and transferred to Immobilon-P (Millipore Corp), and the membrane was subjected to autoradiography. For determination of the specific radioactive band that corresponded to the AT₁AR, 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 spectrometry.

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 AT₁R, 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 spectrometry.
FKB 2. Une graphs show time course of angiotensin II (Ang II)-induced phosphorylation of the Ang II type 1 receptor (AT1AR). A, Total phosphorylation of the AT1AR is expressed as percent control of "P content of AT1AR-specific bands. B, Tyrosine phosphorylation of the AT1AR is expressed as percent control of the signal intensity of AT1AR-specific bands of anti-phosphotyrosine antibody immunoprecipitates. Data are mean±SEM of three to five independent experiments. *P<.05, **P<.01 vs control.

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 phosphotyrosine residues. The ability of Ang II to induce phosphorylation of its receptor on both serine and tyrosine
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. Anti-AT identifies a 43-kD band when a nonglycosylated form of the AT1AR is synthesized in vitro using a rabbit reticulocyte lysate system. However, in several other rat tissues, immunoblotting with anti-AT identifies a 49-kD band. These differences in the apparent molecular weight of the AT1AR are most likely due to variations in glycosylation, because there are three potential glycosylation sites in the AT1AR molecules and because previous studies have pointed out differences in the carbohydrate content of the receptors depending on species and tissues.

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, 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, a result in agreement with our immunocyt 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. 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. 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 AT1AR 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. However, PKA is not normally activated on AT1AR stimulation in RASM cells, suggesting that this kinase is likely to be involved in heterologous rather than homologous receptor regulation. Indeed, in -ARs, serine phosphorylation mediated by PKA is postulated to play a role in heterologous desensitization of receptors. In particular, a recent study has shown that activation of -ARs leads to heterologous desensitization, internalization, and downregulation of mChRs via phosphorylation on serine. 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 -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. Specifically, GRKs and PKA control the efficacy of receptor coupling to G proteins by rapidly phosphorylating the receptor at serine/threonine residues. 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. 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. This motif, NPXY, occurs 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...
processing, including internalization, recycling, or re-sensitization of the receptors.

The present study suggests that Ang II induces phosphorylation 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 phosphorylation of the AT1AR and to determine the physiological consequences of AT1AR phosphorylation.

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**References**


