Phosphorylation of the Type 1A Angiotensin II Receptor by G Protein-coupled Receptor Kinases and Protein Kinase C*

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The type 1A angiotensin II receptor (AT1A-R), which mediates cardiovascular effects of angiotensin II, has been shown to undergo rapid agonist-induced desensitization. We investigated the potential role of second messenger-activated kinases and G protein-coupled receptor kinases (GRKs) in the regulation of this receptor. In 293 cells transfected with the AT1A-R, a 3-min challenge with angiotensin II engendered a 46% decrease in subsequent angiotensin II-stimulated phosphoinositide hydrolysis in intact cells. This agonist-induced desensitization correlated temporally and dose-dependently with the phosphorylation of the receptor to a stoichiometry of 1 mol of phosphate/mol of receptor, as assessed by immunoprecipitation of receptors from cells metabolically labeled with 32P. Agonist-induced receptor phosphorylation was reduced by 40-50% by either overexpression of a dominant negative K220R mutant GRK2 or treatment of the cells with the protein kinase C (PKC) inhibitor staurosporine, in a virtually additive fashion. Cellular overexpression of GRK2K220R not only inhibited agonist-induced AT1A-R phosphorylation, but also prevented receptor desensitization, as assessed by angiotensin II-stimulated GTPase activity in membranes prepared from agonist-treated and control cells. In contrast, PKC inhibition by staurosporine did not affect homologous desensitization of the AT1A-R. Overexpression of GRKs 2, 3, or 5 significantly augmented the agonist-induced AT1A-R phosphorylation 1.5- to 1.7-fold (p < 0.001). These findings suggest a role for receptor phosphorylation by one or several GRKs in the rapid agonist-induced desensitization of the AT1A-R.

Agonist binding to a G protein-coupled receptor activates catalytic cascades of intracellular mediators which greatly amplify the response to an extracellular stimulus. In many instances, this same event triggers counter-regulatory pathways which attenuate receptor signaling (1). Several distinct, yet highly coordinated mechanisms act together to bring about receptor desensitization. Rapid agonist-specific or homologous desensitization is due to the functional uncoupling of the heterotrimeric G protein from the receptor. On a molecular level, phosphorylation of agonist-occupied receptors by G protein-coupled receptor kinases (GRKs)† or by second messenger-activated kinases have been shown to attenuate receptor interaction with G proteins (2). While these mechanisms underlying receptor desensitization have been studied most extensively for the rhodopsin and β-adrenergic receptor systems, they may also be operative in the regulation of several other G protein-coupled receptors.

This study focuses on the vascular type 1 angiotensin II receptor (AT1-R), which has long been known to undergo rapid desensitization (3). Most of the known effects of angiotensin II, the major effector molecule of the renin-angiotensin system, are mediated via this receptor (reviewed in Ref. 4). Two different, highly homologous subtypes (AT1A-R and AT1B-R) of this receptor were identified in rat and mouse by cloning experiments. Although functionally and pharmacologically indistinguishable from the AT1A-R, the AT1B-R is the predominant form in most tissues including the kidney, heart, liver, and aorta (5). Modulation of the AT1A-R gene in mice by gene targeting experiments has lent further support to the notion that angiotensin II exerts its hemodynamic effects mainly by the type 1A angiotensin II receptor (6). Two recent studies have provided evidence that rapid desensitization of the AT1-R involves G protein uncoupling and receptor phosphorylation. First, short-term desensitization of the AT1-R in bovine adrenal glomerulosa cells was found to correspond to a shift in receptor affinity for agonist from high (G protein-coupled) to low (uncoupled) (7). As GTPyS mimicked this effect, it was concluded that agonist-induced desensitization results from the uncoupling of AT1-R from its G protein. Second, the AT1-R in rat vascular smooth muscle cells was demonstrated to be phosphorylated in response to agonist stimulation (8). However, neither the kinases which phosphorylate the receptor nor the functional consequences of receptor phosphorylation were directly addressed.

The present study was designed to determine whether G protein-coupled receptor kinases and/or second messenger-activated kinases phosphorylate and desensitize the AT1A-R in intact cells. To this end, effects of kinase inhibitors on receptor phosphorylation and desensitization were studied. Finally, we asked whether individual GRKs can phosphorylate the AT1A-R. Three members of the G protein-coupled receptor kinase family, i.e. GRK2 (βARK1), GRK3 (βARK2), and GRK5, were chosen for this study on the basis of their widespread tissue expression (9).

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The abbreviations used are: GRK, G protein-coupled receptor kinase; 293 cells, human embryonic kidney cells; AT1-R, type 1 angiotensin II receptor; AT1A-R, type 1A angiotensin II receptor; βARK, β-adrenergic receptor kinase; G protein, guanine nucleotide-binding protein; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; GTPyS, guanosine 5′-3-O-(thio)triphosphate.
**EXPERIMENTAL PROCEDURES**

Materials—Tissue culture media and fetal bovine serum were from Life Technologies, Inc.; 293 cells were from the American Type Culture Collection; protein A-Sepharose CL-4B was from Pharmacia Biotech Inc.; okadaic acid and staurosporine were from Calbiochem; A23187, angiotensin II, dibutyryl-cAMP, forskolin, 3-isobutyl-1-methylxanthine, phorbol 12-myristate 13-acetate, and protease inhibitors were from Sigma; [35S]methionine/[35S]cysteine (Tran35S-label) was from NEN Research Products; myo-[2-3H]inositol and [Sar1,125I-Tyr4,Val5,D-(4-NH$_2$)]angiotensin II were from Du Pont NEN. I-AT1AR (10 $\mu$Ci/ml) was purchased from Amersham. Okadaic acid (1 $\mu$m) was added 10 min prior to cellular stimulation. Following the addition of different stimuli as indicated, cells were washed twice with ice-cold Dulbecco's PBS and then solubilized in SDS sample buffer.

Intact Cell Phosphorylation—Three days after transfection, 293 cells in 6-well dishes were washed once with phosphate-free Dulbecco's modified Eagle's medium and incubated at 37°C for 60 min in the same medium containing $^{32}$P (150 $\mu$Ci/ml). Okadaic acid (1 $\mu$m) was added 10 min prior to cellular stimulation. Following the addition of different stimuli as indicated, cells were washed twice with ice-cold Dulbecco's PBS and then solubilized in SDS sample buffer. Membranes were removed by centrifugation at 254 nm for 30 min. Membranes were washed once and solubilized in SDS sample buffer.

**RESULTS**

We first demonstrated that the AT$_{1}$A Receptor in transiently transfected 293 cells undergoes rapid agonist-induced desensitiza-
Pretreatment for 3 min with 100 nM angiotensin II reduced the subsequent maximal angiotensin II-stimulated phosphoinositide hydrolysis by 46% as compared to vehicle-pretreated control cells (Fig. 1, top panel). Attenuation of inositol phosphate generation was maximal when cells were pretreated with 10 to 30 nM angiotensin II and half-maximal effects were observed after pretreatment with approximately 2 nM angiotensin II (Fig. 1, Bottom Panel). By varying the time of pre-exposure to the agonist from 30 s to 12 min, we found that angiotensin II rapidly induced receptor desensitization with maximal effects observed by pretreatment for 1 min (data not shown).

Agonist-induced internalization of the AT1A-R is a well-established phenomenon (19). However, receptor internalization does not appear to account for the diminished inositol phosphate response in cells desensitized in these experiments. As determined by flow cytometry using a monoclonal antibody (12CA5) which is specific for the amino-terminal epitope-tag of our AT1A-R construct, incubation of AT1A-R-expressing cells with 100 nM angiotensin II for 3 min resulted in the loss of only 15 ± 2% of plasma membrane receptors. In the range of receptor expression used in these experiments, such a small decrease in receptor number, modeled by transfecting cells with varied amounts of plasmid, has no effect on angiotensin II-stimulated phosphoinositide hydrolysis (data not shown).

We then studied whether the AT1A-R is phosphorylated upon agonist stimulation. 293 Cells expressing the AT1A-R were equilibrated with 32Pi to label their ATP pool and were stimulated with increasing concentrations of agonist. In a dose-dependent fashion, angiotensin II induced within 5 min the phosphorylation of a 50–90-kDa band which was absent in untransfected 32Pi-labeled control cells (Fig. 2). This protein co-migrated with the photoaffinity-labeled AT1A-R from transfected 293 cell membranes. Phosphorylation of the AT1A-R was induced by as little as 0.1 nM angiotensin II and reached a maximum at 100 nM angiotensin II; the EC50 value was 4.5 nM. By labeling cells to equilibrium with 32P, and [35S]methionine, the amount of phosphate incorporated in the AT1A-R was determined to be 1.0 ± 0.4 (mean ± S.D.) mol of phosphate per mol of receptor in cells stimulated with 200 nM angiotensin II. In unstimulated cells, the AT1A-R contained 0.2 ± 0.02 mol of phosphate per mol of receptor.

To investigate which kinases effect AT1A-R phosphorylation, we challenged cells with both specific activators and inhibitors of the second messenger-dependent kinases, protein kinase C (PKC) or cAMP-dependent protein kinase (PKA). Whereas the phorbol ester PMA significantly increased AT1A-R phosphorylation above basal values, neither the PKA activators forskolin or dibutyryl cAMP, nor the calcium ionophore A23187, had an effect on receptor phosphorylation (Fig. 3). The amount of 32P

![Phosphorylation of the AT1A Receptor](http://www.jbc.org/Downloaded from)
incorporated into the receptor upon stimulation of PKC was only 40% of that achieved by angiotensin II stimulation. Furthermore, staurosporine, which at this concentration (500 nM) blocks both PKA and PKC activity, inhibited the agonist-induced AT1A-R phosphorylation by only 42%. These results imply that both PKC, the kinase activated by signaling pathways downstream of the AT1A-R, and one or several protein kinases which are not affected by staurosporine, participate in the agonist-induced phosphorylation of this receptor.

To test the hypothesis that agonist-induced AT1A-R phosphorylation is effected by GRKs endogenously expressed in 293 cells, cells were co-transfected with a GRK2 dominant negative mutant (GRK2K220R). This mutant GRK retains the ability to bind the agonist-occupied receptor, but is devoid of catalytic activity and therefore acts as a competitive inhibitor of GRK activity. Overexpression of this mutant at an excess over endogenous GRKs has been found to inhibit GRK-mediated receptor phosphorylation (13) and desensitization (20). As shown in Fig. 4, the overexpression of GRK2K220R reduced receptor phosphorylation consequent to angiotensin II stimulation for 5 min by 50%, as compared to control cells, but did not affect agonist-independent receptor phosphorylation through the PKC pathway. The combined inhibition of PKC by staurosporine and GRKs by GRK2K220R overexpression additively reduced agonist-induced AT1A-R phosphorylation, resulting in an inhibition of receptor phosphorylation by 85% as compared to control cells. These findings imply that agonist-induced AT1A-R phosphorylation is mediated by two different types of kinases: by one or more GRKs, which are directly activated by the agonist-occupied receptor, and by PKC, which is activated by second messengers generated consequent to receptor stimulation.

Since both GRK and PKC activity appear important in agonist-induced AT1A-R phosphorylation, we sought to characterize the kinetics of kinase activities. Previously, it had been demonstrated that GRK and CAMP-dependent protein kinase activity on the β2-adrenergic receptor proceed by distinct kinetics (21, 22). The time course of angiotensin II-induced receptor phosphorylation revealed the existence of a biphasic process, characterized by a rapid (t1/2 ~ 30 s) and a somewhat slower (t1/2 ~ 3 min) time course (Fig. 5). Inhibition of PKC by staurosporine eliminated any agonist-induced receptor phosphorylation occurring after 3 min, but did not affect the rapid phase of AT1A-R phosphorylation. Conversely, PKC-mediated

**Fig. 3.** Phosphorylation of the AT1A-R by angiotensin II and activators of second messenger-dependent kinases. 32P-labeled 293 cells expressing the AT1A-R were preincubated without or with 500 nm staurosporine for 20 min at 37 °C prior to cellular stimulation. Cells were stimulated for 5 min at 37 °C with 200 nm angiotensin II, 2 μM PMA, 10 μM calcium ionophore A23187, 50 μM forskolin plus 1 mM 3-isobutyl-1-methylxanthine, or 2 mM dibutyryl-cAMP. Epitope-tagged receptors were immunoprecipitated and analyzed by SDS-PAGE. a shows the autoradiogram from one representative experiment. b shows the extent of AT1A-R phosphorylation as determined by PhosphorImager analysis. Receptor phosphorylation is expressed as percent of that seen in unstimulated control cells. Data are mean ± S.E. of three experiments; **, p < 0.001; *, p < 0.05 compared with basal levels.

**Fig. 4.** Additive inhibition of agonist-induced AT1A-R phosphorylation by the GRK2K220R dominant negative mutant and staurosporine. 293 cells were co-transfected with the plasmids pcDNA I-AT1A-R and either pcDNA I (empty) or the pcDNA I-GRK2K220R construct (gARKK220R). 32P-labeled 293 cells were preincubated in the presence or absence of 500 nm staurosporine for 20 min, prior to cellular stimulation. Cells were exposed to medium alone, 200 nm angiotensin II, or 2 μM PMA for 5 min. Epitope-tagged receptors were immunoprecipitated and analyzed by SDS-PAGE. a shows the autoradiogram from one representative experiment. b shows the extent of AT1A-R phosphorylation as determined by PhosphorImager analysis. Receptor phosphorylation is expressed as a percentage of that seen in unstimulated control cells in the absence of staurosporine. Data are mean ± S.E. of three experiments; *, p < 0.05 compared with angiotensin II-stimulated control cells in the absence of GRK2K220R.
receptor phosphorylation stimulated by PMA proceeded with a half-time of approximately 3 min. These observations support the notion that rapid agonist-induced AT1A-R phosphorylation derives principally from GRK activity. Receptor phosphorylation seen with protracted (over 3 min) agonist exposure, however, may derive significantly from PKC.

If GRKs are involved in the agonist-induced AT1A-R phosphorylation, overexpression of these kinases in 293 cells might be expected to enhance the angiotensin II-mediated receptor phosphorylation. Receptor immunoprecipitation from labeled 293 cells which overexpressed either GRK2, GRK3, or GRK5 revealed agonist-induced AT1A-R phosphorylation which was 1.5–1.7-fold greater (p < 0.001) than that observed in control cells (Fig. 6). These experiments did not show significant differences between individual GRKs in their ability to enhance receptor phosphorylation in response to agonist. The increased basal phosphorylation of the AT1A-R seen in cells which overexpress GRK5, the kinase which is constitutively associated with the cellular membrane (12), was also observed in studies which investigated the role of GRKs in the phosphorylation of β1-adrenergic (13) or δ-opioid receptors (23). As shown by immunoblotting analysis, the transfection of 293 cells with pcDNA I constructs encoding GRK2, GRK3, or GRK5 resulted in cellular expression of these kinases at levels which were at least 20-fold higher than endogenous levels of GRK2 or GRK5.

To assess the effect of GRK inhibition on agonist-induced receptor desensitization, we exposed 293 cells expressing the AT1A-R with or without GRK2K220R to angiotensin II for 3 min, removed the agonist with an acid wash, and prepared membranes for the determination of angiotensin II-stimulated GTPase activity (Fig. 7). Exposure of control cells to medium containing angiotensin II decreased agonist-stimulated membrane GTPase activity by 85% (mean of 3 experiments). In cells overexpressing GRK2, pretreatment with this agonist decreased angiotensin II-stimulated membrane GTPase activity by 91% (n = 4). In contrast, exposure of cells overexpressing GRK2K220R to angiotensin II failed to diminish angiotensin II-stimulated membrane GTPase activity even at the highest concentration of agonist used (250 nM) (data not shown). These results were confirmed in five experiments performed in duplicate. Comparing these data with those obtained from control cells, a 1000-fold dilution of horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies (Jackson ImmunoResearch Laboratories) and diaminobenzidine as the peroxidase substrate with NiCl2 enhancement.

II-stimulated membrane GTPase activity at all. Thus, inhibition of GRK activity by GRK2K220R eliminates agonist-induced receptor desensitization assessed with maximal agonist stimulation.

Assessed in intact cells, however, GRK2K220R appears to behave differently. In phosphoinositide hydrolysis experiments (data not shown), we observed that GRK2K220R overexpression decreased even maximal angiotensin II-stimulated phosphoinositide hydrolysis in intact cells by over 90% just as wild type GRK2 did. Taken together with the GTPase findings, these data indicate that overexpressed GRKs may inhibit receptor signaling in intact cells not only by increasing receptor phosphorylation, but also by binding to the activated receptor and preventing receptor/G protein coupling. This latter function can also be served by GRK2K220R. The dampening effect of

FIG. 6. Augmentation of angiotensin II-induced AT1A-R phosphorylation by GRKs 2, 3, or 5. 293 cells were co-transfected with pcDNA I-AT1A-R and either pcDNA I (empty), pcDNA I-GRK2 (GRK2), pcDNA I-GRK3 (GRK3), or pcDNA I-GRK5 (GRK5). 32P-labeled cells were exposed to medium alone or 200 nM angiotensin II for 3 min. Receptors were immunoprecipitated and analyzed by SDS-PAGE. A shows the autoradiogram from one representative experiment. b summarizes the results of five experiments performed in duplicate. Receptor phosphorylation is expressed as a percentage of that seen in unstimulated control cells. Shown are mean ± S.E.; **, p < 0.001 compared with angiotensin II-stimulated control cells. c, immunoblot depicting GRK expression in 293 cells transiently transfected with pcDNA I-AT1A-R and either empty vector, pcDNA I-GRK2, pcDNA I-GRK3, or pcDNA I-GRK5. After electrophoretic separation (10% SDS-PAGE) of cellular lysates (10 μg of cellular protein per lane) and electrotransfer to nitrocellulose, proteins were visualized with anti-GRK2/3 mouse monoclonal antibody C5/1 (M. Oppermann, unpublished data; 10 μg/ml) or rabbit anti-GRK5 antisemur (12) at 1:1000. A 1000-fold dilution of horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies (Jackson ImmunoResearch Laboratories) and diaminobenzidine as the peroxidase substrate with NiCl2 enhancement.

FIG. 5. Time course of activation-induced AT1A-R phosphorylation. 32P-labeled 293 cells expressing AT1A-R were preincubated without (filled symbols) or with (open circles) 500 nm staurosporine (20 min/37°C) prior to cellular stimulation. Cells were challenged with 200 nM angiotensin II (circles) or 2 μM PMA (triangles) for the indicated time periods. Epitope-tagged receptors were immunoprecipitated and analyzed by SDS-PAGE. Receptor phosphorylation is expressed as a percentage of the radioactivity measured in samples of unstimulated cells in the absence of staurosporine. Data are mean values obtained from three separate experiments.
GRK2K220R on the process of signal generation is avoided by determining agonist-stimulated GTPase activity in washed membranes.

The role of PKC-mediated AT1A-R phosphorylation in receptor desensitization was examined in intact cells which were pretreated with staurosporine prior to a desensitizing stimulus with angiotensin II (data not shown). To maximize potential effects of PKC, cells were treated as in Fig. 1, except that the pretreatment with angiotensin II was 12 min instead of 3 min. Pretreatment with 150 nM staurosporine for 10 min prior to stimulation with angiotensin II was 12 min instead of 3 min. Pretreatment with 150 nM staurosporine prior to an agonist-desensitizing stimulus was completely prevented AT1A-R desensitization as assessed by angiotensin II-stimulated membrane GTPase activity. In contrast, while PKC was also found to phosphorylate the AT1A-R after stimulation with angiotensin II, the functional consequences of PKC-mediated receptor phosphorylation appear unimportant, at least when assessed at maximal levels of receptor stimulation. Recently, desensitization has been demonstrated with a truncated mutant of AT1A-R which lacks the carboxyl-terminal 45 amino acids, encompassing 13 serinethreonine residues (27). Although this result may seem difficult to reconcile with our own findings, the Ser and Thr residues important for AT1A-R desensitization have not yet been determined. Indeed, four serine and threonine residues in the first or second cytoplasmic loops, not affected by this deletion mutant, involve receptor regions critical for G protein coupling (28) and may represent functionally important phosphorylation sites.

The functional differentiation of PKC-mediated from GRK-mediated receptor phosphorylation seems to relate to their differing time courses of action. In this regard, our findings are supported by the kinetic analysis of angiotensin II-induced translocation of PKC in vascular smooth muscle strips (29). The spatial translocation of the cytosolic kinase to the membrane, which is a prerequisite for PKC activation, was found to peak at 5 to 10 min and then gradually decline to background values. These data are in accord with the PKC-mediated AT1A-R phosphorylation, which we determined to proceed with a t1/2 of about 3 min. The different time course of GRK- versus PKC-mediated receptor phosphorylation resembles the kinetics of GRK- and PKA-mediated phosphorylation and desensitization of the β2-adrenergic receptor (22). In this system, the GRK's rapidly induced receptor phosphorylation and desensitization with a t1/2 of about 15 s, whereas the second messenger-dependent enzyme PKA had a t1/2 of 2 to 3.5 min.

Whereas several phospholipase C-coupled receptors have
been shown to undergo negative feedback inhibition via PKC (30–32), conflicting data have been reported in the past with regard to the AT1-R. PKC depletion or treatment with the selective PKC inhibitor Ro 31-7519 was shown not to affect the rapid agonist-induced desensitization in neonatal cardiac myocytes (33) or in CHO cells expressing the human AT1-R (34). In our own experiments, PKC inhibition with staurosporine did not affect homologous desensitization of the AT1A-R. In contrast, Pfeilschifter and co-workers (35–38) have accumulated ample evidence implicating PKC in the angiotensin II-induced homologous desensitization in glomerular mesangial cells. In aggregate, these studies and ours suggest that the AT1A-R might be regulated by PKC in a cell type-specific manner.

Using staurosporine as a PKC inhibitor and a dominant negative GRK2K220R mutant to prevent endogenous GRKs from interacting with the receptor, each type of kinase was shown to account for 40–50% of the receptor phosphorylation induced by agonist within 5 min. The virtually additive effect of the two kinase inhibitors identifies GRKs and PKC as the main, if not the only, kinases which cause short-term agonist-induced phosphorylation of the AT1A-R. The minimal residual receptor phosphorylation in the presence of both staurosporine and the dominant negative GRK2K220R mutant is probably due to incomplete efficacy of these inhibitors. Indeed, even when present in 15-fold molar excess, GRK2K220R was shown to inhibit only 60% of GRK2 activity on the β2-adrenergic receptor in the presence of G protein βγ subunits (20).

Phosphorylation of the AT1A-R by PKA was reported in a previous study, which demonstrated phosphorylation of the receptor upon prolonged (15 min) stimulation with forskolin (8). This result is in contrast to our findings which, however, were obtained by incubation with forskolin for only 5 min. While we cannot definitely rule out a possible role for PKA in the regulation of the AT1A-R, this enzyme is not an effector kinase of the Gq-coupled AT1A-R and therefore would not be expected to be involved in agonist-induced receptor phosphorylation. In the same study, evidence was provided for both constitutive and agonist-induced tyrosine phosphorylation of the AT1A-R (8). Phosphoamino analysis of the receptor showed that stimulation with angiotensin II induces mainly serine phosphorylation, which supports our findings of GRK- and PKC-mediated receptor phosphorylation. Tyrosine phosphorylation was observed to a small extent and only after prolonged (≥20 min) stimulation with angiotensin II. In our own studies, the tyrosine kinase inhibitor genistein had no effect on receptor phosphorylation or functional consequences of angiotensin II stimulation (data not shown). Tyrosine phosphorylation therefore does not appear to contribute to the rapid agonist-induced desensitization of the AT1A-R.
