Agonist-induced Phosphorylation of the Angiotensin II (AT1A) Receptor Requires Generation of a Conformation That Is Distinct from the Inositol Phosphate-signaling State*

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G protein-coupled receptors are thought to isomerize between distinct inactive and active conformations, an idea supported by receptor mutations that induce constitutive (agonist-independent) activation. The agonist-promoted active state initiates signaling and, presumably, is then phosphorylated and internalized to terminate the signal. In this study, we examined the phosphorylation and internalization of wild type and constitutively active mutants (N111A and N111G) of the type 1 (AT1A) angiotensin II receptor. Cells expressing these receptors were stimulated with angiotensin II (Ang-II) and [Sar1,Ile4,Ile8]AngII, an analog that only activates signaling through the constitutive receptors. Wild type AT1A receptors displayed a basal level of phosphorylation, which was stimulated by AngII. Unexpectedly, the constitutively active AT1A receptors did not exhibit an increase in basal phosphorylation nor was phosphorylation enhanced by AngII stimulation. Phosphorylation of the constitutively active receptors was unaffected by pretreatment with the non-peptide AT1 receptor inverse agonist, EXP3174, and was not stimulated by the selective ligand, [Sar1,Ile4,Ile8]AngII. Paradoxically, [Sar1,Ile4,Ile8]AngII produced a robust (~ 85% of AngII) dose-dependent phosphorylation of the wild type AT1A receptor at sites in the carboxyl terminus similar to those phosphorylated by AngII. Moreover, internalization of both wild type and constitutive receptors was induced by AngII, but not [Sar1,Ile4,Ile8]AngII, providing a differentiation between the phosphorylated and internalized states. These data suggest that the AT1A receptor can attain a conformation for phosphorylation without going through the conformation required for inositol phosphate signaling and provide evidence for a transition of the receptor through multiple states, each associated with separate stages of receptor activation and regulation. Separate transition states may be a common paradigm for G protein-coupled receptors.

Seven transmembrane-spanning receptors that couple to heterotrimeric guanyl nucleotide-binding proteins (G proteins) are activated by an array of sensory and hormonal stimuli. Current theories (1–4) for G protein-coupled receptor (GPCR) activation predict that receptors spontaneously isomerize between an inactive (R) and active (R*) state and that, in the absence of agonist, structural constraints maintain an equilibrium between R and R* that favors the inactive R state. The active R* conformation is selected (or induced) by agonist binding and couples to and activates G proteins, which initiate signaling. This two-state model has been revised to include two active states, R* and R**, in the so-called three-state model (5, 6), which accommodates experimental evidence that one receptor can couple to different G protein effector pathways with distinct agonist potency profiles. In either model, the active states are then targeted for phosphorylation by specific GPCR kinases (GRKs), which only recognize the active conformation, and by second messenger-activated kinases (e.g., protein kinase C). Proteins termed arrestins bind to phosphorylated receptors and sterically hinder further association of the receptor with G protein and thereby terminate signaling (7). For some GPCRs, arrestins also act as adaptors to target the receptors for clathrin-mediated internalization (7) and to promote coupling to tyrosine kinase signaling pathways (8).

Experimental evidence for R/R*/R** has come from the observation that overexpression of many GPCRs leads to some degree of constitutive (or agonist-independent) activity, ostensibly by increasing the overall amount (not the proportion) of R*/R** available to interact with G proteins (1, 4). Moreover, constitutively active GPCRs can arise from naturally occurring and engineered mutations (9), presumably as a result of transforming the receptor to an active state. Generally, these constitutively active GPCRs have proven to be constitutively phosphorylated and desensitized (4), providing support for the model that the active state is also the conformation targeted for phosphorylation, internalization and desensitization.

Type 1 angiotensin receptors (AT1) are GPCRs that mediate the actions of angiotensin II (AngII), an octapeptide hormone (Asp1-Arg2-Val3-Tyr4-Ile5-His6-Pro7-Phe8) which regulates blood pressure and water and salt balance. Stimulation of AT1 receptors (two subtypes, AT1A and AT1B, exist in rodents) leads to Gq/11-mediated activation of phospholipase C-β1, which generates diacylglycerol and inositol (1,4,5)trisphosphate. No naturally occurring, constitutively active AT1 receptor mutants

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¶ The abbreviations used are: G protein, heterotrimeric guanyl nucleotide-binding protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; AngII, angiotensin II; AT1, AT1A, and AT1B, type 1 AngII receptor subtypes; CHO-K1, Chinese hamster ovary cells; HA, influenza hemagglutinin antigen; NH2-AT1A, NH2-terminally HA-tagged AT1A receptor; WT-1D4, N111A-1D4, N111G-1D4, wild type and mutated AT1A receptors with a carboxyl-terminal 1D4 epitope.
have been reported, but engineered mutation of Asn111 to glycine (N111G) or alanine (N111A), in the third transmembrane helix of the AT1A receptor, leads to partial activation (10–12). Thus, in the absence of AngII binding, the N111G mutant displays ~50%, and the N111A mutant ~20% (10), of the maximal agonist-induced production of inositol phosphates. In the wild type AT1A receptor, interaction of TyrR of AngII with Asn111 in the receptor appears to act as the trigger to convert R to R* and allow receptor activation. Small side-chain substitutions (glycine/alanine) of Asn111 in AT1A presumably release this conformational switch, allowing constitutive activity and removing the requirement of TyrR in AngII for maximal receptor activation.

In this study, we examined the phosphorylation and internalization of the N111A and N111G constitutively active mutants of the AT1A receptor. We anticipated that these mutants would display an elevated basal phosphorylation to parallel their enhanced G protein-phospholipase C-β1 coupling. Instead, we observed that the phosphorylation of the constitutively active AT1A receptors is not increased basally or following stimulation by AngII. The constitutively active receptors retained the ability to internalize in response to AngII. Rather surprisingly, the non-signaling AngII analog, [Sar1,Ile4,Ile8]AngII, induced robust phosphorylation of the wild type AT1A receptor, but this analog did not stimulate internalization of either wild type or constitutively active mutant AT1A receptors. Thus, we provide evidence for the existence of multiple AT1A receptor states capable of selectively mediating various aspects of receptor function.

**EXPERIMENTAL PROCEDURES**

**Materials**—The monoclonal antibody 1D4, produced by the Cell Culture Center (Endotronics Inc., Minneapolis), was a kind gift from Dr. Robert Graham (Vitor Chang Cardiac Research Institute, Sydney, Australia); the 12CA5 monoclonal antibody was purified from hybridoma culture media using an influenza hemagglutinin antigen (HA)-peptide affinity column; 125I-AngII was provided by Dr. Conrad Sernia (Department of Physiology and Pharmacology, University of Queensland, Brisbane, Australia) or purchased from NEN Life Science Products (Melbourne, Australia); [Sar1,Ile4,Ile8]AngII from the Biotechnology Center (Endotronics Inc., Minneapolis), was a kind gift from Dr. Robert Graham (Vitor Chang Cardiac Research Institute, Sydney, Australia); [Sar1,Ile4,Ile8]AngII from the DuPont Merck Pharmaceutical Co. (Wilmington, DE); AngII from Aus...
AT$_1$A Receptors—Receptor phosphorylation was determined in CHO-K1 cells expressing 1D4 epitope-tagged wild type (WT-1D4) or constitutively active mutants (N111A-1D4 and N111G-1D4) of the rat AT$_1$A receptor. Cells were transiently transfected into CHO-K1 cells and receptor phosphorylation was measured in response to maximal stimulation (100 nM AngII, 10 min, 37 °C), determined as described under “Experimental Procedures.” Top panel, a representative phosphoimage illustrating the AngII-stimulated phosphorylation of wild type and mutated AT$_1$A receptors. The positive (NHA-AT1A) and negative (NHA-TK325) controls for the phosphorylation assay displayed an appropriate robust and lack of phosphorylation, respectively, in response to AngII. Note the strong AngII-induced phosphorylation of WT-1D4, but the lack of AngII-stimulated phosphorylation for the N111A-1D4 and N111G-1D4 receptors. Middle panel, comparison of cell surface receptor expression for the various receptor constructs expressed as a percentage of the NHA-AT1A receptor. Receptor expression was determined for each phosphorylation experiment by radioligand binding assay to allow normalization of phosphoimager data. The data are means ± S.D. from six (1D4-tagged receptors) or four (NHA-AT1A) separate experiments. The data for the negative control (NHA-TK325) are the mean only of data from two experiments. Dynamic phosphorylation—Some antagonists are able to inhibit the constitutive activity of GPCRs and these ligands are referred to as inverse agonists (4). EXP3174 is a non-peptide antagonist of the AT$_1$ receptor and pretreatment with EXP3174 of cells expressing the constitutively active AT$_1$A mutants (N111A and N111G) reduces the basal inositol phosphate generation by 70–90% (10, 11). Thus, EXP3174 is an inverse agonist of the AT$_1$ receptor (see also Fig. 1B). We next examined whether conversion of the N111A and N111G receptors back to the basal (R) state by treatment with EXP3174 would allow a subsequent AngII-induced receptor phosphorylation event to occur. As shown in Fig. 3, EXP3174 treatment (100 nM, 16 h) did not reveal a cryptic AngII-induced phosphorylation of N111A-1D4 or N111G-1D4 receptors; these receptors remained poorly phosphorylated both basally and following AngII stimulation.

**Phosphorylation of AT$_1$A Receptors by [Sar$^1$,Ile$^4$,Ile$^8$]AngII—** Constitutively active AT$_1$A receptors may represent an intermediate transition state (R') between the basal (R) and fully active (R$^*$) forms of the receptor (10, 12). In the wild type receptor, transition to the R' state appears dependent upon the...
docking of Tyr8 and Phe8 side-chains of AngII onto the receptor protein. Full activation of the AT1A receptor (as measured by inositol phosphate production) then depends upon additional determinants within the AngII peptide. A [Sar1, Ile4, Ile8]AngII analogue (Sar = sarcosine, N-methylglycine) has an enhanced affinity ($K_D = 0.3 \text{ nM}$) for the AT1A receptor and is a full agonist (10). In contrast, a Tyr4 and Phe8 substituted analog (i.e. [Sar1, Ile4, Ile8]AngII) has a reduced affinity ($K_D = 300 \text{ nM}$) and is unable to activate inositol phosphate signaling through the wild type receptor, even at concentrations 300 times its $K_D$ (10).

Interestingly, the constitutive mutant (N111G) has an increased affinity for [Sar1, Ile4, Ile8]AngII ($K_D = 6 \text{ nM}$) compared with wild type receptor, and this analogue promotes maximal signaling of N111G. If the maximally signaling form (R*) of the receptor is the state targeted for phosphorylation (hereafter termed R*), then [Sar1, Ile4, Ile8]AngII, which drives the constitutive mutant receptor from R− into the fully active R* form, should be expected to promote phosphorylation of the N111G receptor. Conversely, the phosphorylation of the wild type receptor should not be stimulated by [Sar1, Ile4, Ile8]AngII. Remarkably, we observed that 30 μM [Sar1, Ile4, Ile8]AngII (~500 times the $K_D$ for N111G) was incapable of causing phosphorylation of the constitutively active AT1A receptors (Fig. 4). This result suggests that the R− and R* signaling forms can be differentiated from the phosphorylated form of the receptor, Rp. Paradoxically, 30 μM [Sar1, Ile4, Ile8]AngII (~100 times the $K_D$) caused a robust phosphorylation of the wild type AT1A receptor (~85% of that produced by 100 nM AngII, also ~100 times the $K_D$) (see Fig. 4). This observation also illustrates a distinction between the phosphorylated form, Rp, and the active signaling form, R*.

**Characterization of AT1A Receptor Phosphorylation by [Sar1, Ile4, Ile8]AngII**—To further analyze the phosphorylation generated by [Sar1, Ile4, Ile8]AngII, we treated cells expressing the WT-1D4 receptor with various doses of AngII and [Sar1, Ile4, Ile8]AngII and determined receptor phosphorylation. As shown in Fig. 5, maximal phosphorylation is achieved by 10 nM AngII (~10 times the $K_D$) with a half-maximal phosphorylation occurring at 1 nM, which is equivalent to the $K_D$. For [Sar1, Ile4, Ile8]AngII, maximal phosphorylation was achieved by 3 μM (~10 times the $K_D$) with a half-maximal phosphorylation occurring at 300 nM, which approximates the $K_D$. Thus, for both AngII and [Sar1, Ile4, Ile8]AngII, the degree of phosphorylation correlates well with the affinity of the ligands for the wild type receptor.

To determine whether AngII and [Sar1, Ile4, Ile8]AngII were phosphorylating similar sites on the wild type AT1A receptor, we next compared the AngII- and [Sar1, Ile4, Ile8]AngII-induced phosphorylation of wild type and mutant receptors. We (13) and others (14) have previously shown that most, if not all, AngII-induced phosphorylation occurs within the serine/threonine-rich AT1A carboxyl terminus (see also Fig. 2). The region in the middle of the carboxyl terminus between Thr332 and Ser338 (13), in particular Ser335Thr336 (14), is an important site for AngII-stimulated phosphorylation. We have also recently shown that triple mutation of three putative protein kinase C phosphorylation sites at Ser331, Ser335 and Ser348 to alanine in the carboxyl terminus causes significant decreases in both AngII- and phorbol ester-induced phosphorylation (15). Hence, we compared the phosphorylation produced by AngII and [Sar1, Ile4, Ile8]AngII on wild type (NHA-AT1A) and mutated (NHA-S335/T336A and NHA-S331/S335/S348A) receptors. As
The phosphorylation of NHA-AT1A and [Sar, Ile, Ile]AngII was determined in response to AngII (100 nM) and [Sar, Ile, Ile]AngII (30 μM) for 10 min at 37 °C. Top panel, representative phosphoimage. Middle panel, comparison of cell surface receptor expression for the double and triple alanine mutants expressed as a percentage of the wild type HA-tagged receptor (NHA-AT1A). Bottom panel, phosphoimaging data normalized for receptor expression (means ± S.D.) for three separate experiments.

**DISCUSSION**

In this study, the phosphorylation and internalization of wild type and mutant AT1A receptors following stimulation by AngII and an analog, [Sar, Ile, Ile]AngII, was examined. We observed that the constitutively active AT1A receptors (N111A and N111G) are poor substrates for phosphorylation and that this cannot be modulated by AngII or [Sar, Ile, Ile]AngII stimulation or by prior conversion of the constitutive receptors to the basal state by pretreatment with the inverse agonist, EXP3174. Unexpectedly, the wild type receptor was efficiently phosphorylated by the supposedly inactive analog, [Sar, Ile, Ile]AngII, indicating that the molecular switches required for phosphorylation are distinct from those necessary for G protein-mediated signaling. The fact that AngII, but not [Sar, Ile, Ile]AngII, stimulated the internalization of both wild type and constitutively active AT1A receptors suggests that the processes of internalization and phosphorylation can also be clearly differentiated. Thus, the AT1A receptor appears to exist in multiple conformational states, each relating to a distinct transitional stage of receptor activation (i.e. basal, intermediate, signaling, phosphorylation, and internalization). In Fig. 8, we provide a model, which summarizes the proposed
transition and interconversion of the AT$_{1A}$ receptor during the process of receptor activation and regulation.

Based on the frequently applied two-state model for receptor activation (3), constitutively active GPCRs are thought to attain a conformation that mimics the active (R*) state and should therefore be recognized and phosphorylated by GRKs. Indeed, a number of constitutively active GPCRs, such as the α$_{2A}$-adrenergic receptor (20), the β$_{2}$-adrenergic receptor (21), the 5-hydroxytryptamine (5-HT$_{2C}$) receptor (22), rhodopsin (23) and the bradykinin B2 receptor (24), have been reported to display enhanced agonist-independent phosphorylation. In support of the idea that the receptor state that propagates signals to G protein is also the one targeted for phosphorylation, January et al. (25) demonstrated that, for a series of β$_{2}$-adrenergic receptor agonists of varying coupling efficiencies, the degree of agonist strength on the wild type β$_{2}$-adrenergic receptor correlated well with the capacity to mediate phosphorylation, internalization, and desensitization. In contrast to these studies, we observed that the constitutively active AT$_{1A}$ receptors displayed a basal level of phosphorylation, which was similar to that of the unstimulated wild type receptor, and that this basal phosphorylation was not increased by stimulation with AngII or the substituted analog, [Sar$^1$,Ile$^4$,Ile$^8$]AngII. The fact that AngII and [Sar$^1$,Ile$^4$,Ile$^8$]AngII can cause maximal inositol phosphate signaling through the constitutively active receptors (10) argues against the assumption that the active, signaling state of GPCRs is the form recognized by GRKs and targeted for phosphorylation.

A distinction between signaling and phosphorylation is also sustained by our discovery that the AngII analog, [Sar$^1$,Ile$^4$,Ile$^8$]AngII, causes robust phosphorylation of the wild type AT$_{1A}$ receptor. Remarkably, this occurs despite an inability of this analog to promote G$_{q}$-mediated inositol phosphate signaling through the AT$_{1A}$ receptor (10). Maximal [Sar$^1$,Ile$^4$,Ile$^8$]AngII-mediated phosphorylation was about 85% of that produced by AngII, and relative to its affinity for the wild type receptor, was as efficacious as AngII at inducing phosphorylation. Moreover, [Sar$^1$,Ile$^4$,Ile$^8$]AngII seemingly mediates phosphorylation at the same sites as AngII in the receptor carboxyl terminus, based on a similar degree of inhibition of phosphorylation for wild type and carboxyl-terminally mutated receptors. Thus, to our knowledge, this represents the first example of an apparently "silent" analog, with respect to signaling, being able to generate almost complete "agonist-like" phosphorylation.

Given the robust nature of [Sar$^1$,Ile$^4$,Ile$^8$]AngII-induced phosphorylation as well as the apparent similarity of sites phosphorylated in the AT$_{1A}$ carboxyl terminus by AngII and [Sar$^1$,Ile$^4$,Ile$^8$]AngII, this strongly suggests that this analog is stabilizing a conformation in the AT$_{1A}$ receptor that is an excellent substrate for GRK-mediated phosphorylation.

Our observation that the optimal conformation for GRK-mediated phosphorylation is different from that which couples the receptor to G protein signaling, corroborates, and can be most directly compared, to a very recent study (26) of consti-
tively active α1β-adrenergic receptors. In their study, Mhatiouty-Kodja et al. (26) investigated the phosphorylation and internalization of α1β-adrenergic receptors made constitutively active through mutation at two separate sites in the receptor molecule: Asp142 in the DRY motif at the end of the third transmembrane-spanning domain and Ala293 in the carboxyl-terminal region of the third intracellular loop. Mutations at Ala293 (A293I and A293E) caused constitutive signaling (inositol phosphate production), which correlated well with an enhanced basal (agonist-independent) receptor phosphorylation. In direct contrast, mutations at Asp142 (D142A and D142T), while yielding greater levels of constitutive activation compared with the Ala293 mutants, showed no increase in basal phosphorylation as well as a lack of epinephrine-induced phosphorylation. This result, which is very similar to our data on the phosphorylation of the AT1A, Asn111 mutants, suggests that multiple conformational states exist for receptor activation, some of which serve as targets for phosphorylation. That both Asp142 in the α1β-adrenergic receptor and Asn111 of the AT1A receptor are located in the third transmembrane-spanning region, albeit at different levels of the helix, may be relevant to these similar observations and points to a shared mechanism of constitutive activity.

Phosphorylation of the AT1A receptor carboxyl terminus has been implicated in the mechanism of receptor internalization. Progressive truncation of the AT1A carboxyl terminus (13, 14), or specific mutation of serine and threonine residues in the central region of the receptor tail (Thr332→Ser338) to alanine (13), produces a concomitant decrease in both receptor phosphorylation and internalization. It was therefore somewhat surprising in the present study to observe that, despite the lack of AngII-mediated phosphorylation of N111A and N111G, the internalization kinetics for 125I-AngII of N111A was unaffected and only slightly reduced in the case of N111G. This would suggest that AngII-induced phosphorylation of the AT1A receptor is not mandatory for internalization. Moreover, given the strong phosphorylation of the wild type receptor by [Sar1, Ile4, Ile8]AngII, in the absence of appreciable internalization, it would appear that phosphorylation is incapable of driving the internalization process. Taken together, these observations support the concept of separate receptor states for receptor phosphorylation and for targeting receptors for endocytosis.

Similarly, clear distinctions can also be drawn between the signaling and internalizing forms of the AT1A receptor. Specifically, [Sar1, Ile4, Ile8]AngII can produce maximal inositol phosphate signaling through the constitutively active AT1A receptors (10), suggesting it can promote a conformation in the receptor that approximates the active R state. However, we observed no internalization of constitutive or wild type receptors in response to saturating concentrations (30 μM) of this analog. Additionally, we (27) and others (28) have previously reported that another AngII analog, [Sar1, Ile4, Ile8]AngII, which signals very poorly through the wild type receptor (10), is capable of causing a degree of AT1A receptor internalization that approximates that of AngII. This separation of signaling and internalization is corroborated by earlier reports, which showed that some G protein uncoupled AT1A receptors mutants (e.g. D74E, Y302A), with severely compromised signaling to AngII (29, 30), displayed almost wild type levels of receptor internalization (19, 28, 30). Furthermore, we have demonstrated that truncation of the AT1A carboxyl terminus produces a receptor mutant that couples well to G protein and signals in response to AngII stimulation, but exhibits vastly reduced internalization (16). The literature contains many such examples of GPCR mutants that are uncoupled from signaling but retain a capacity for robust internalization or fully signaling receptors that are compromised with respect to endocytosis. Hence, it appears likely that GPCRs in general are able to attain a conformation(s) that allows specific interaction with components of the internalization machinery while preventing productive coupling to heterotrimeric G proteins.

Important clues as to the role of individual amino acid residues in the octapeptide, AngII, in promoting various receptor states can be gleaned from a comparison of the receptor functions evoked by the AngII analogs, [Sar1, Ile4, Ile8]AngII and [Sar1, Ile4, Ile8]AngII. For example, both analogs poorly activate inositol phosphate production (10), indicating the crucial role for Tyr4 and Phe8 of AngII in specifying agonism, in particular Phe8 (for full discussion, see Ref. 31). Moreover, both analogs cause robust phosphorylation of the wild type receptor (Ref. 13 and this study), indicating that docking of Tyr4 and Phe8 of AngII onto their respective partners in the AT1A receptor (i.e. Asn111 in transmembrane helix 3 and His156 in transmembrane domain 6), is dispensable for promoting a conformation required for phosphorylation. These analogs do differ in that [Sar1, Ile4]AngII (27, 28), but not [Sar1, Ile4, Ile8]AngII (this study), causes robust internalization, identifying Tyr4 in AngII as a crucial determinant of AT1A receptor endocytosis. Despite the unequivocal importance of the aromatic-amide bond between Tyr4 in AngII and Asn111 in AT1A for receptor activation (31), it is interesting that the constitutively active Asn111 mutants (N111A and N111G) retain a capacity for AngII-induced internalization. Perhaps other points of contact made between Tyr4 and the receptor (e.g. Phe77, Val108, Leu112, and Tyr292), suggested by our molecular modeling studies, may be more important for dictating internalization. In regard to inducing phosphorylation, positions other than Tyr4 and Phe8 of AngII must be involved, and it will therefore be important to examine the capacity for phosphorylation of AngII analogs substituted at other positions.

In conclusion, data from the present study, as well as accumulating evidence from other GPCRs (32–37), strongly support the notion of multiple receptor conformational states and the transition of receptors through these states in the process of receptor activation and deactivation. Using constitutively active AT1A receptor mutants and novel analogs of AngII, which select different transitional phases of receptor activation, has provided a unique insight into the physical separation of these putative conformational states. Activation selective analogs, such as [Sar1, Ile4, Ile8]AngII, represent powerful tools to further dissect the molecular mechanisms that underlie the generation of GPCR signals and their subsequent termination by phosphorylation, internalization, and desensitization.

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