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ACCELERATED COMMUNICATION

Homologous and Heterologous Phosphorylation of the AT_2 Angiotensin Receptor by Protein Kinase C

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ABSTRACT

The angiotensin AT_2 receptor is an atypical seven transmembrane domain receptor that is coupled to activation of tyrosine phosphatase and inhibition of MAP kinase, and does not undergo agonist-induced internalization. An investigation of the occurrence and nature of AT_2 receptor phosphorylation revealed that phorbol ester-induced activation of protein kinase C (PKC) in HA-AT_2 receptor-expressing COS-7 cells caused rapid and specific phosphorylation of a single residue (Ser^{354}) located in the cytoplasmic tail of the receptor. Agonist activation of AT_2 receptors by angiotensin II (Ang II) also caused rapid PKC-dependent phosphorylation of Ser^{354} that was prevented by the AT_2 antagonist, PD123177, and by inhibitors of PKC. In cells coexpressing AT_1 and AT_2 receptors, Ang II-induced phosphorylation of the AT_2 receptor was reduced by either PD123177 or the AT_1 receptor antagonist, DuP753, and was abolished by treatment with both antagonists or with PKC inhibitors. These findings indicate that the AT_2 receptor is rapidly phosphorylated via PKC during homologous activation by Ang II, and also undergoes heterologous PKC-dependent phosphorylation during activation of the AT_1 receptor. The latter process may regulate the counteracting effects of AT_2 receptors on growth responses to AT_1 receptor activation.

The superfamily of seven transmembrane domain G protein-coupled receptors (GPCRs) mediates the responses of cells to light, odorants, neurotransmitters, biogenic amines, and numerous hormones. The current view of GPCR function and regulation, which is based largely on studies of the β_2-adrenergic receptor (β_2-AR), invokes an agonist-dependent change in receptor conformation that allows receptor coupling to cognate G protein(s). This conformational change also promotes receptor phosphorylation by G protein-coupled receptor kinases (GRKs) and/or second messenger-activated kinases. The subsequent binding of β-arrestin proteins desensitizes the receptor by sterically inhibiting its coupling to G proteins, and also mediates its internalization via clathrin-coated pits (reviewed in Bohm et al., 1997; Krupnick and Benovic, 1998; Pitcher et al., 1998). Although this model of β_2-AR action has been extrapolated to other GPCRs, it does not apply to all of them, and some such receptors use modified or alternative mechanisms of desensitization and internalization, or none at all. For example, the gonadotropin-releasing hormone receptor functionally desensitizes and internalizes very slowly, and does not undergo agonist-induced phosphorylation (Neill et al., 1997). Also, the parathyroid hormone receptor internalizes independently of phosphorylation (Malecz et al., 1998), and endocytosis of the m1, m3, and m4 muscarinic receptors (Lee et al., 1998), and possibly the AT_1 angiotensin receptor (AT_1-R) (Zhang et al., 1996), appears to be independent of β-arrestins. In contrast to most other GPCRs, the AT_2 angiotensin receptor (AT_2-R) does not undergo internalization in the presence of its endogenous agonist ligand, Ang II (Hunyady et al., 1994; Hein et al., 1997).

Although the AT_1-R and AT_2-R exhibit high affinity for the octapeptide hormone, Ang II, and both are members of the

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ABBREVIATIONS: GPCR, G protein-coupled receptor; AT_1-R and AT_2-R, types 1 and 2 angiotensin II receptors, respectively; β_2-AR, β_2-adrenergic receptor; BIM, bisindolylmaleimide; DG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; GRK, G protein-coupled receptor kinase; HA, hemagglutinin; PKC, protein kinase C; PNGase F, peptide *N*-glycosidase F; SP, staurosporine; TFMS, trifluoromethanesulfonic acid; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; Ang II, angiotensin II; PAGE, polyacrylamide gel electrophoresis.

GPCR superfamily, they share only 32 to 34% amino acid sequence homology (Kambayashi et al., 1993; Mukoyama et al., 1993) and have completely different functions. All of the classical actions of Ang II in the regulation of salt/water balance and blood pressure control are mediated by the G_q-coupled AT_1-R. Due to its central role in cardiovascular regulation, many aspects of the structure and function of the AT_1-R have been analyzed and elucidated. Although less is known about the functions of the AT_2-R, recent studies have shown that its activation can counteract the mitogenic and hypertensive effects mediated via the AT_1-R. AT_2-R activation exerts antiproliferative and/or apoptotic effects in certain cells (Yamada et al., 1996), and exerts a hypotensive effect in AT_1-R-deficient mice (Oliverio et al., 1998). In addition, the wide distribution of the AT_2-R in fetal tissues, in contrast to its limited expression in adult tissues, suggests a role for this receptor in developmental processes (de Gasparo and Siragy, 1999).

In contrast to the well characterized signal transduction pathways that mediate Ang II actions through the AT_1-R, those that are activated by the AT_2-R are less well defined. For example, AT_2-R activation has been reported to either stimulate (Gohlke et al., 1998) or inhibit (Bottari et al., 1992) cyclic GMP production, and to activate phosphotyrosine phosphatases (such as SHP-1) (Bottari et al., 1992; Kambayashi et al., 1993; Nahmias et al., 1995; Tsuzuki et al., 1996; Bedecs et al., 1997) as well as serine/threonine phosphatase activity (Huang et al., 1996). In addition, there is conflicting evidence about the extent to which the AT_2-R can couple to G proteins (Bottari et al., 1991; Kambayashi et al., 1993; Kang et al., 1993; Mukoyama et al., 1993; Zhang and Pratt, 1996).

Although the AT_1 receptor and many other GPCRs have been found to undergo agonist-induced phosphorylation of their cytoplasmic domains, phosphorylation of the AT_2-R has not yet been investigated. In this study, we undertook an analysis of AT_2-R phosphorylation to seek insights into the activation and signaling mechanism(s) of this receptor. To this end, the phosphorylation of an epitope-tagged rat AT_2-R was investigated in transiently transfected COS-7 cells.

Experimental Procedures

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and antibiotic solutions were from Biofluids (Rockville, MD). Ang II, [Sar^1,Ile^8]Ang II, and CGP42112 were from Peninsula Laboratories (Belmont, CA). DuP753 and PD123177 were generous gifts from Dr. P. C. Wong (DuPont, Wilmington, DE). ^{125}I-[Sar^1,Ile^8]Ang II and ^{125}I-[Sar^1,(4-N_3)Phe^8]Ang II were from Covance Laboratories (Vienna, VA), and $^{32}P_i$ was from ICN (Costa Mesa, CA). Protein A Sepharose was from Oncogene Research Products (Cambridge, MA), and the HA.11 mouse monoclonal antibody was from BAbCo (Berkeley, CA). Peptide *N*-glycosidase F (PNGase F: E.C. 3.5.1.52) was from Boehringer Mannheim (Indianapolis, IN). Bisindolylmaleimide (BIM) and staurosporine (SP) were from Calbiochem (San Diego, CA). OptiMEM and LipofectAMINE were from Life Technologies, Inc. (Gaithersburg, MD). Trifluoromethanesulfonic acid (TFMS) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) were from Sigma (St. Louis, MO).

Epitope-Tagging and Mutagenesis of the Rat AT_2-R. A *Hin*dIII/*Nsi*I fragment of the rat AT_2 receptor was subcloned into the eukaryotic expression vector, pcDNAI/Amp (Invitrogen, San Diego, CA), as previously described (Hunyady et al., 1994). The influenza hemagglutinin (HA) epitope (YPYDVPDYA) was inserted after the N-terminal methionine residue using the Mutagene kit (Bio-Rad, Hercules, CA), and its sequence was verified using Sequenase II (Amersham, Arlington Heights, IL). The presence of the epitope tag had no effect on the ligand binding properties of the HA-AT_2-R (data not shown). Site-directed mutagenesis was achieved using the Quick Change kit (Stratagene, La Jolla, CA), and mutant sequences were verified by dideoxy sequencing using Thermosequenase (Amersham, Arlington Heights, IL).

Transient Expression of HA-AT_2-Rs. COS-7 cells were maintained in DMEM containing 10% (v/v) fetal bovine serum, 100 μg/ml streptomycin, and 100 IU/ml penicillin (COS-7 medium). Cells were seeded at 8×10^5 cells/10-cm dish in COS-7 medium and cultured for 3 days before transfection using 5 ml/dish OptiMEM containing 10 μg/ml LipofectAMINE and the required DNA (1 μg/ml) for 6 h at 37°C. After changing to fresh COS-7 medium, the cells were cultured for an additional 2 days before use. HA-AT_2-Rs were photoaffinity-labeled with ^{125}I-[Sar^1,(4-N_3)Phe^8]Ang II as described (Smith et al., 1998a). To quantify the relative phosphorylation of mutant HA-AT_2-Rs, membrane lysates were normalized to an equal number of HA-AT_2-Rs (on the basis of B_{max} values obtained from radioligand displacement assays using replicate transfected cells) before immunoprecipitation as described (Smith et al., 1998b).

HA-AT_2-R Phosphorylation Assay. Transfected Cos-7 cells in 10-cm dishes were metabolically labeled for 4 h at 37°C in P_i-free DMEM containing 0.1% (w/v) BSA and 100 μCi/ml $^{32}P_i$ as described previously (Smith et al., 1998b). In brief, after three washes in KRH (118 mM NaCl, 2.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 10 mM glucose, 20 mM HEPES, pH 7.4), cells were incubated in the same medium for 10 min in a 37°C water bath. Vehicle or 100 nM Ang II was then added for an additional 5 min. After three washes with ice-cold PBS, cells were drained before scraping into lysis buffer (LB−: 50 mM Tris, pH 8.0, 100 mM NaCl, 20 mM NaF, 10 mM sodium pyrophosphate, 5 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml pepstatin, 10 μg/ml benzamidine, 1 mM AEBSF [4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride], 1 μM okadaic acid) and probe-sonicated (Sonifier Cell Disruptor; Heat Systems Ultrasonics, Plainview, NY) for 2 × 20 s. After removal of nuclei at 750*g*, membranes were pre-extracted by the addition of an equal volume of LB− containing 2 M NaCl and 8 M urea, followed by overnight tumbling at 4°C. The membranes were then collected at 200,000*g* and solubilized in LB+ [LB− supplemented with 1% (v/v) NP 40, 1% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS] with Dounce homogenization. After clarification at 14,000*g*, solubilized membranes were incubated with 2% (v/v) protein A Sepharose for 1 h at 4°C. HA-AT_2-Rs were immunoprecipitated from solubilized membrane lysates using the HA.11 monoclonal antibody and 2% (v/v) protein A Sepharose as described (Smith et al., 1998b).

Chemical Deglycosylation of HA-AT_2-Rs. After washing of the Sepharose-bound immune complexes in LB+ lacking protease inhibitors, ^{32}P-labeled phospho-HA-AT_2-Rs were eluted into 50 μl of buffer containing 2% (v/w) SDS, 5% (v/v) β-mercaptoethanol, and 80 mM Tris (pH 6.8) for 1 h at 48°C. After the addition of ovalbumin as carrier, proteins were precipitated by the addition of ice-cold trichloroacetic acid, collected by centrifugation, washed twice in ice-cold acetone, and dried in a rotary evaporator. Samples were then subjected to chemical deglycosylation using TFMS as previously described (Horvath et al., 1989). After chemical deglycosylation, proteins were redissolved in sample buffer and incubated for 1 h at 48°C before SDS-polyacrylamide gel electrophoresis (PAGE). After drying (Gel-Dry; Novex, San Diego, CA), ^{32}P-labeled phospho-HA-AT_2-Rs were visualized in a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Results

Enzymatic Deglycosylation of the Phospho-HA-AT_2-R. Like the rat AT_{1a}-R (Smith et al., 1998a), the photoaffinity-labeled HA-tagged rat AT_2-R (HA-AT_2-R) expressed in COS-7 cells migrated as a diffuse high-molecular-

weight band in SDS-PAGE (Figs. 1 and 2), probably as a result of heterogeneous receptor glycosylation. Treatment of HA-AT_2-R-expressing COS-7 cells with TPA to activate PKC caused a concentration-dependent increase in the phosphorylation of proteins that comigrated as a broad high-molecular-weight band with the photoaffinity-labeled receptor (Fig. 1A). To determine whether these represent the phosphorylated HA-AT_2-R, the photoaffinity-labeled and putative phospho-HA-AT_2-Rs were treated with PNGase F (Lemp et al., 1990), which completely deglycosylates the HA-AT_{1a}-R (Smith et al., 1998b; Jayadev et al., 1999), before immunoprecipitation. However, overnight treatment with PNGase F (10 U/ml) deglycosylated only a minority of the AT_2-R receptor component, as indicated by its migration in SDS-PAGE to the predicted M_r of the 41-kDa nonglycosylated HA-AT_2-R polypeptide (Kambayashi et al., 1993; Mukoyama et al., 1993). Most of the receptors appeared as a diffuse band with migration intermediate between the fully glycosylated and completely deglycosylated forms (Fig. 1, B and C). Even after overnight incubation with a high concentration (50 U/ml) of PNGase F (not shown), or with 10 U/ml PNGase F for up to 72 h (Fig. 1C), there was no appreciable increase in the proportion of the completely deglycosylated receptor.

The increased migration of a minority of the PKC-stimulated phosphoproteins to the same position as the completely deglycosylated photoaffinity-labeled receptor (Fig. 1B) confirmed their identity as the fully deglycosylated phospho-HA-AT_2-R. Enzyme treatment also increased the mobility of additional phosphoproteins that comigrated with the partially deglycosylated photoaffinity-labeled receptor (Fig. 1B). However, although PNGase F fully or partially deglycosylated most of the photoaffinity-labeled receptor (Fig. 1, B and C), the majority of the proteins whose phosphorylation was stimulated by PKC (and which comigrated with the fully glycosylated photoaffinity-labeled receptor) showed no change in migration after the same enzyme treatment (Fig. 1B).

These results indicate that the minority of phosphoproteins whose migration in SDS-PAGE increased to coincide with those of the fully and partially deglycosylated photoaffinity-labeled receptors represent the phospho-HA-AT_2-R. Conversely, the majority of the PKC-stimulated phosphoproteins whose migration did not increase after PNGase F treatment are nonreceptor phosphoproteins that coprecipitate with the HA-AT_2-R. Because enzymatic deglycosylation of the HA-AT_2-R was incomplete and did not facilitate the investigation of HA-AT_2-R phosphorylation, the immunoprecipitated phospho-HA-AT_2-Rs were subjected to chemical deglycosylation with TFMS (Horvath et al., 1989).

Chemical Deglycosylation of the Phospho-HA-AT_2-R. In contrast to PNGase F, treatment of the immunoprecipitated photoaffinity-labeled HA-AT_2-R with TFMS for 60 to 90 min gave rise solely to the completely deglycosylated recep-

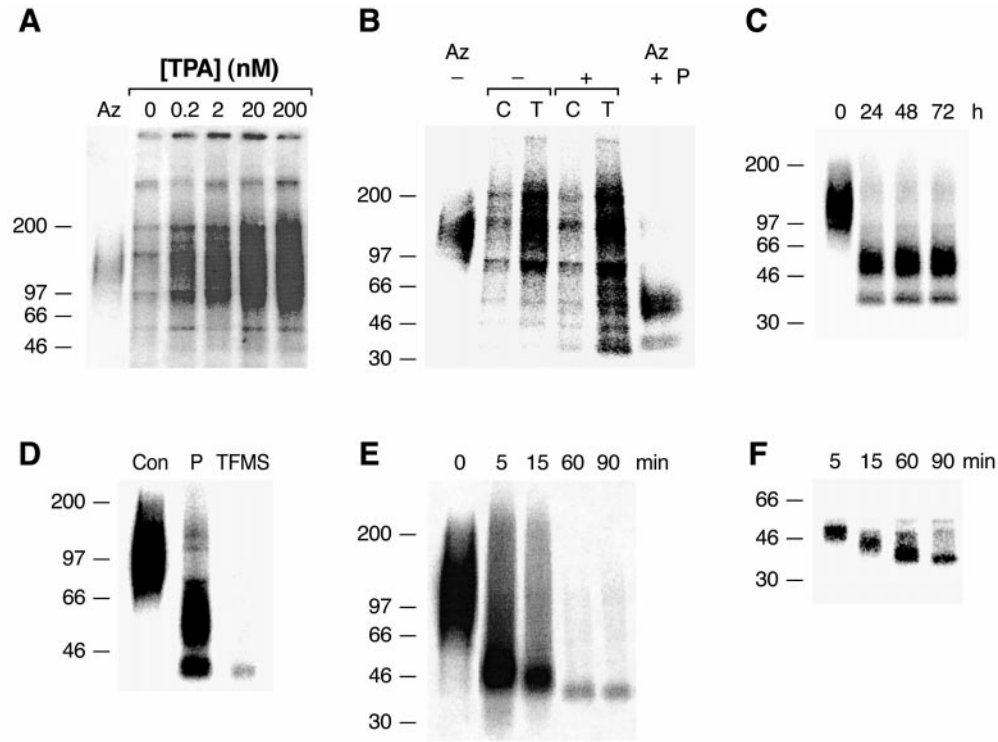

Fig. 1. Deglycosylation of the phospho-HA-AT_2-R. A, membrane lysates prepared from ^{32}P-labeled HA-AT_2-R-expressing COS-7 cells were exposed to the indicated concentrations of TPA for 5 min and subjected to immunoprecipitation with the anti-HA antibody. B, membrane lysates from ^{32}P-labeled cells exposed to vehicle (C) or 200 nM TPA (T) for 5 min were incubated overnight in the presence or absence of 10 U/ml PNGase F (P) as indicated before immunoprecipitation with the anti-HA antibody. Membrane lysates from photoaffinity-labeled cells (Az) were incubated without (−) or with (+) 10 U/ml PNGase F (P) before immunoprecipitation with the anti-HA antibody. C, membrane lysates from photoaffinity-labeled cells were incubated for the indicated times with 10 U/ml PNGase F before immunoprecipitation with the anti-HA antibody. D, membrane lysates prepared from photoaffinity-labeled cells were incubated overnight with 10 U/ml PNGase F (P), or 1 h with TFMS as indicated. E, membrane lysates prepared from photoaffinity-labeled cells were incubated for the indicated times with TFMS. F, membrane lysates prepared from ^{32}P-labeled cells exposed to 200 nM TPA for 5 min were subjected to immunoprecipitation with the anti-HA antibody before treatment for the indicated times with TFMS. In each panel, precipitated proteins were resolved by SDS-PAGE. Az, ^{125}I-[Sar^1,(4-N_3)Phe^8]Ang II photoaffinity-labeled HA-AT_2-Rs. Results in this and subsequent figures are representative of data obtained from at least three independent experiments.

tor, and no intermediate forms were present (Fig. 1D). The intensity of the deglycosylated TFMS-treated photoaffinity-labeled receptor was considerably less than that of the untreated receptor. However, TMSF treatment of membranes from photoaffinity-labeled HA-AT_2-R-expressing COS-7 cells for increasing times before immunoblotting with the anti-HA antibody did not change the intensity of the immunoreactive deglycosylated HA-AT_2-R band, whereas the intensity of photoaffinity labeling decreased markedly (data not shown). These findings suggest that prolonged exposure of photoaffinity-labeled HA-AT_2-Rs to TFMS causes loss of the coupled photoaffinity ligand, possibly due to cleavage of its covalent attachment to the receptors' carbohydrate residues.

Consistent with this effect, exposure of photoaffinity-labeled HA-AT_2-Rs to TFMS for only 5 min gave a greater yield of deglycosylated receptor than after acid for 1 h (Fig. 1E). Interestingly, shorter TFMS treatment times gave rise to receptors that retained some attached carbohydrate and migrated more slowly than the fully deglycosylated receptor but faster than the partially deglycosylated intermediates observed after PNGase F treatment. Increasing the duration of TFMS treatment caused a slow time-dependent deglycosylation of this minimally glycosylated receptor, and its progression to the faster-migrating fully deglycosylated form (Fig. 1E). However, treatment times longer than 5 min also caused a marked reduction in the intensity of photoaffinity labeling.

The progressive increase in the migration of the photoaffinity-labeled receptor during TFMS treatment corresponded with the time-dependent increase in migration of the PKC-phosphorylated HA-AT_2-R (Fig. 1F), confirming the latter's identity as the deglycosylated phospho-HA-AT_2-R. However, whereas the intensity of the photoaffinity-labeled receptor decreased markedly with TFMS treatment periods greater than 5 min, that of the phospho-HA-AT_2-R was unchanged for up to 1 h and showed some reduction at 90 min (Fig. 1F). In subsequent experiments, the TFMS treatment time was 5 min. The appearance of the minimally glycosylated phospho-HA-AT_2-R varied between experiments (being one, two, or three distinct bands), but always corresponded in appearance to, and comigrated with, the deglycosylated photoaffinity-labeled receptor subjected to identical treatment.

Agonist-Induced Phosphorylation of the HA-AT_2-R. Ang II-stimulated cells showed an increase in receptor phosphorylation that was similar to that induced by TPA (Fig. 2A), whereas the partial AT_2-R agonist, CGP42112, caused a much smaller increase in phosphorylation. HA-AT_2-R phosphorylation showed a progressive elevation with increasing agonist concentrations and reached a maximum at around 10 nM Ang II (Fig. 2B). Pretreatment with the specific AT_2-R antagonist, PD123177, abolished the HA-AT_2-R phosphorylation observed after 5 min stimulation with 10 μM Ang II (Fig. 2B), indicating the specificity of the agonist-stimulated HA-AT_2-R phosphorylation.

Site(s) of Phosphorylation of the HA-AT_2-R by PKC. The rat AT_2-R contains three residues (Ser^{152}, Ser^{348}, and Ser^{354}) that are situated within consensus sequences (Ser-Xaa-Lys/Arg) for phosphorylation by PKC (Pearson and Kemp, 1991). We next determined whether the HA-AT_2-R is phosphorylated on one or more of these residues following TPA or Ang II treatment of cells expressing mutant HA-AT_2 receptors with alanine replacements at each of the three candidate serine residues. Scatchard analysis of ^{125}I-[Sar^1,Ile^8]Ang II displacement data from five independent experiments revealed similar dissociation constants for the wild-type and mutant receptors (K_d, 0.65 ± 0.12 nM for wild-type; 0.55 ± 0.08 nM for S152A; 0.66 ± 0.11 nM for S348A; and 0.84 ± 0.11 nM for S354A). The expression levels of the mutant receptors at the cell surface were not significantly different from that of the wild-type receptor (B_{max}, 2.3 ± 0.4 pmol/mg of protein for wild-type; 2 ± 0.2 pmol/mg of protein for S152A; 2.2 ± 0.1 pmol/mg of protein for S348A; and 2.7 ± 0.2 pmol/mg of protein for S354A). Nevertheless, B_{max} values obtained from Scatchard analysis of ^{125}I-[Sar^1,Ile^8]Ang II binding to intact replicate transfected COS-7 cells were routinely used to normalize ^{32}P-labeled membrane lysates to an

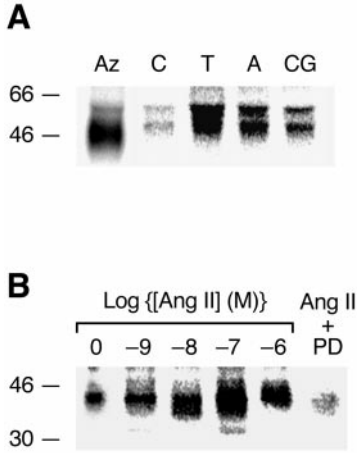

Fig. 2. Agonist-induced phosphorylation of the HA-AT_2-R. A, HA-AT_2-R-expressing COS-7 cells were exposed to vehicle (C), 200 nM TPA (T), 100 nM Ang II (A), or 100 nM CGP42112 (CG) for 5 min as indicated. B, ^{32}P-labeled HA-AT_2-Rs immunoprecipitated from cells exposed to the indicated concentrations of Ang II for 5 min or pretreated for 10 min with 10 μM PD123177 (PD), before the addition of 10 μM Ang II for an additional 5 min, were treated with TFMS for 5 min. Immunoprecipitated phospho-HA-AT_2-Rs were treated with TFMS for 5 min before SDS-PAGE. Az, photoaffinity-labeled HA-AT_2-Rs.

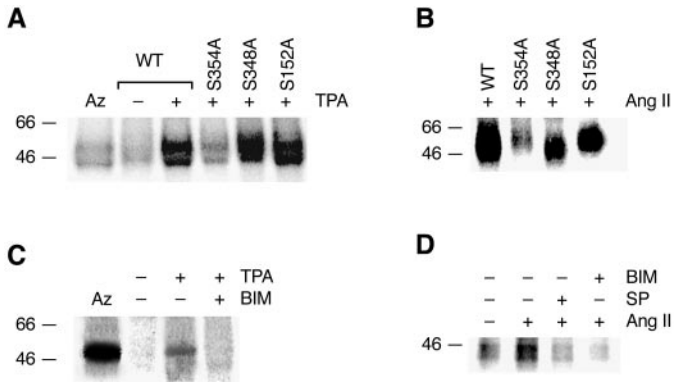

Fig. 3. Protein kinase C-mediated phosphorylation of wild-type and mutant HA-AT_2-Rs. ^{32}P-Labeled COS-7 cells expressing the indicated HA-AT_2-Rs (which were normalized to an equal number of receptors as required), were exposed to vehicle or 200 nM TPA (A) or 100 nM Ang II (B) for 5 min as indicated. C, wild-type HA-AT_2-R-expressing cells were pretreated for 10 min with vehicle or 1 μM BIM before the addition of vehicle or 200 nM TPA for an additional 5 min as indicated. D, wild-type HA-AT_2-R-expressing cells were pretreated for 10 min with vehicle, 1 μM BIM, or 500 nM SP before the addition of vehicle or 100 nM Ang II for an additional 5 min as indicated. In each panel, phospho-HA-AT_2-Rs were immunoprecipitated from membrane lysates (which were normalized to an equal number of receptors as required) and treated with TFMS for 5 min before SDS-PAGE. Az, photoaffinity-labeled HA-AT_2-Rs.

equal number of HA-AT_2-Rs before immunoprecipitation, to assess the relative degree of phosphorylation of each mutant receptor.

Neither TPA-induced (Fig. 3A) nor Ang II-stimulated (Fig. 3B) phosphorylation of the HA-AT_2-R was affected by mutation of Ser^{152} or Ser^{348} to alanine. However, both responses were markedly reduced by mutation of Ser^{354} to alanine, indicating that TPA- and Ang II-activated PKC phosphorylation of the HA-AT_2-R occur predominantly at this residue. This was confirmed by the ability of the PKC inhibitor, BIM (1 μM, which inhibits the α, βI, βII, γ, δ, and ϵ subtypes of PKC), to abolish both TPA- (Fig. 3C) and Ang II-stimulated phosphorylation of the HA-AT_2-R (Fig. 3D). Furthermore, the less specific serine-threonine protein kinase inhibitor, SP, also completely inhibited the HA-AT_2-R phosphorylation induced by Ang II (Fig. 3D).

Heterologous Ligand-Mediated Phosphorylation of the HA-AT_2-R via the AT_{1a}-R. Because the HA-AT_2-R can be phosphorylated in response to PKC activation by TPA, we determined whether it was also phosphorylated during heterologous ligand-mediated activation of PKC. For this purpose, COS-7 cells were cotransfected with both the HA-AT_2-R and the wild-type (non-HA-tagged) G_q-coupled rat AT_{1a}-R. In preliminary experiments, photoaffinity-labeled AT_{1a} receptors were not immunoprecipitated by the anti-HA antibody (data not shown). Exposure of the cotransfected cells to 100 nM Ang II caused prominent phosphorylation of the HA-AT_2-R (Fig. 4A) that was inhibited by preincubation of the cells with 1 μM BIM or 500 nM SP (Fig. 4B). Receptor phosphorylation was reduced by preincubation with either 10 μM PD123177 or 10 μM Dup753, an AT_1-R-specific antagonist (Chiu et al., 1989), and was abolished by combined treatment with both antagonists (Fig. 4A). Hence, the HA-AT_2-R can be phosphorylated by ligand-mediated activation of PKC via the AT_{1a}-R, as well as directly during agonist activation.

To determine which HA-AT_2-R residue(s) is phosphorylated in response to AT_{1a}-R activation, COS-7 cells were cotransfected with both the wild-type AT_{1a}-R and each of the HA-AT_2-R mutants described above. Samples were normalized to an equal number of HA-AT_2-Rs before immunoprecipitation, using B_{max} values derived from ^{125}I-[Sar^1,Ile^8]Ang II binding to intact replicate transfected cells in the presence of 10 μM DuP753 (to prevent radioligand binding to the coexpressed AT_{1a}-Rs). It is evident from Fig. 4C that agonist activation of the AT_{1a}-R resulted in phosphorylation of the same residue (Ser^{354}) of the HA-AT_2-R that is phosphorylated in response to TPA treatment.

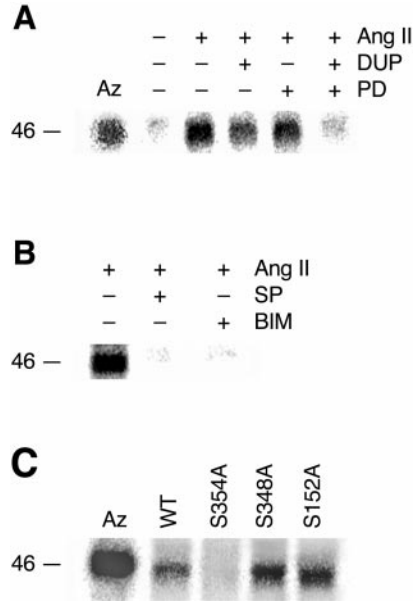

Fig. 4. Heterologous phosphorylation of the HA-AT_2-R via the rat AT_{1a}-R. A, COS-7 cells coexpressing the HA-AT_2-R and the (non-HA-tagged) rat AT_{1a}-R were pretreated for 10 min with vehicle, 10 μM PD123177 (PD), 10 μM DuP753 (DuP), or both antagonists, before the addition of 100 nM Ang II for an additional 5 min as indicated. B, COS-7 cells coexpressing the HA-AT_2-R and the AT_{1a}-R were pretreated for 10 min with vehicle, 500 nM SP, or 10 μM BIM as indicated before the addition of 100 nM Ang II for an additional 5 min. C, COS-7 cells expressing the indicated receptors were exposed to vehicle or 100 nM Ang II for 5 min as indicated. Solubilized membrane lysates were normalized to an equal number of receptors before immunoprecipitation with the anti-HA antibody. Az, photoaffinity-labeled HA-AT_2-Rs.

Discussion

These studies have demonstrated that the AT_2-R undergoes PKC-dependent phosphorylation at Ser^{354} in its cytoplasmic tail during agonist stimulation by Ang II. It is also phosphorylated at the same site during heterologous ligand-mediated PKC activation via the G_q-coupled AT_{1a}-R. Many GPCRs are coupled through G_q-mediated phosphoinositide hydrolysis to the generation of inositol 1,4,5-trisphosphate, which elevates intracellular [Ca^{2+}] and diacylglycerol (DG), which act in conjunction with phosphatidylserine to activate PKC. However, because AT_2-R activation does not lead to inositol 1,4,5-trisphosphate production or Ca^{2+} elevation (Mukoyama et al., 1993), this pathway cannot be responsible for the PKC-dependent phosphorylation observed during agonist stimulation of AT_2-R-expressing COS-7 cells. In the absence of AT_2-R-induced calcium signaling, the inhibitory effect of BIM on AT_2-R phosphorylation suggests that the Ca^{2+}-independent or novel isoforms (PKCδ and ϵ) are involved in this process.

An alternative source of DG production could be AT_2-R-mediated hydrolysis of phosphatidylcholine by phospholipases other than PLCβ. Because DG is also the source of arachidonic acid, and the AT_2-R has been reported to mediate Ang II-stimulated arachidonic acid release in certain cell types (Lokuta et al., 1994), it is possible that activation of the AT_2-R leads to DG production (possibly from phosphatidylcholine), with resulting release of eicosanoids. This may in turn lead to the activation of a Ca^{2+}-independent PKC isoform. In this regard, Ang II stimulates AT_2-R-mediated intracellular alkalinization in cardiac myocytes by a mechanism that is independent of phosphoinositide signaling, but appears to depend on arachidonic acid formation and activation of PKC (Kohout and Rogers, 1995). The AT_2-R has also been reported to mediate Ang II-induced activation and translocation of PKC from cytosol to membrane in cardiac myocytes (Rabkin, 1996).

Agonist-induced receptor phosphorylation via GRKs and/or second messenger-activated kinases has been implicated in the desensitization and internalization mechanisms of many GPCRs (Bohm et al., 1997; Krupnick and Benovic, 1998; Pitcher et al., 1998). The angiotensin AT_1 receptor is phosphorylated at a serine/threonine-rich region of its cytoplasmic tail during agonist activation, and this appears to be mediated largely by GRKs and to a lesser extent by PKC

(Smith et al., 1998b; Thomas et al., 1998). Phosphorylation of the AT_1 receptor has been implicated in desensitization and endocytosis of the AT_1 receptor, features that are not associated with activation of the AT_2 receptor.

The functional role of PKC-mediated phosphorylation in AT_2-R action remains to be determined, but does not involve receptor endocytosis because the AT_2-R does not undergo agonist-induced internalization (Hein et al., 1997; Hunyady, 1999). It is possible that phosphorylation of the AT_2-R participates in the initiation or desensitization of its signaling pathways, which are not yet well defined. The ability of the AT_2-R to stimulate SHP-1 tyrosine phosphatase activity (Nouet and Nahmias, 2000) might provide the basis for an assay to detect desensitization of AT_2-R-mediated signaling, comparable to the indices for desensitization of G_s- and G_q-coupled GPCRs provided by measurements of cyclic AMP and inositol phosphate, respectively (Freedman et al., 1995). By analogy with the role that protein kinase A-mediated phosphorylation has been reported to play in switching the G protein coupling of β_2-ARs from G_s to G_i (Daaka et al., 1997), it is also possible that agonist-induced, PKC-mediated phosphorylation of the AT_2-R could influence its G protein coupling specificity. Furthermore, variations in the cell-specific expression of PKC isoforms might explain the apparent differences in G protein coupling observed for AT_2-Rs in different tissues.

The existence of Ang II-mediated homologous and heterologous phosphorylation of the AT_2-R could have significant implications for the understanding of AT_2-R function. Several studies have shown that activation of the AT_2-R counteracts the growth effects of Ang II mediated by the AT_1-R, suggesting that the AT_2-R could provide a brake for the AT_1-dependent hormonal signal. However, little is known about possible converse mechanism(s) for short-term attenuation of the AT_2-R intracellular signal by AT_1-R. It is possible that the interaction between AT_1 and AT_2 receptors has a regulatory role in the physiological actions of Ang II. In this context, the ability of the AT_1-R to transphosphorylate the AT_2-R via PKC in cells expressing both Ang II receptor subtypes reveals a potential mechanism for the modulation of AT_2 receptor-mediated counteraction of AT_1 receptor function. We have recently observed that activation of the endogenous histamine receptor in COS-7 cells also stimulates phosphorylation of the AT_2-R (J. Olivares-Reyes and R. Smith, unpublished data), suggesting that this mechanism could also apply to other G_q-coupled receptors.

References

Bedecs K, Elbaz N, Sutren M, Masson M, Susini C, Strosberg AD and Nahmias C (1997) Angiotensin II type 2 receptor mediate inhibition of mitogen-activated protein kinase cascade and functional activation of SHP-1 tyrosine phosphatase. *Biochem J* **325:**449–454.

Bohm SK, Grady EF and Bunnett NW (1997) Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. *Biochem J* **322:**1–18.

Bottari SP, King IN, Reichlin S, Dahlstroem I, Lydon N and de Gasparo M (1992) The angiotensin AT_2 receptor stimulates protein tyrosine phosphatase activity and mediates inhibition of particulate guanylate cyclase. *Biochem Biophys Res Commun* **183:**206–211.

Bottari SP, Taylor V, King IN, Bogdal Y, Whitebread S and de Gasparo M (1991) Angiotensin II AT_2 receptors do not interact with guanine nucleotide binding proteins. *Eur J Pharmacol* **207:**157–163.

Chiu AT, Herblin WF, McCall DE, Ardecky RJ, Carini DJ, Duncia JV, Pease LJ, Wong PC, Wexler RR, Johnson AL and Timmermans PBMWM (1989) Identification of angiotensin II receptor subtypes. *Biochem Biophys Res Commun* **165:**196–203.

Daaka Y, Luttrell LM and Lefkowitz RJ (1997) Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature (Lond)* **390:**88–91.

de Gasparo M and Siragy HM (1999) The AT_2 receptor: Fact, fancy and fantasy. *Regul Pept* **81:**11–24.

Freedman NJ, Ligget SB, Drachman DE, Pei G, Caron MG and Lefkowitz RJ (1995) Phosphorylation and desensitization of the human endothelin A an B receptors. *J Biol Chem* **270:**17953–17961.

Gohlke P, Pees C and Unger T (1998) AT_2 receptor stimulation increases aortic cyclic GMP in SHRSP by a kinin-dependent mechanism. *Hypertension* **31:**349–355.

Hein L, Meinel L, Pratt RE, Dzau VJ and Kobilka BK (1997) Intracellular trafficking of angiotensin II and its AT_1 and AT_2 receptors: Evidence for selective sorting of receptors and ligand. *Mol Endocrinol* **11:**1266–1277.

Horvath E, Edwards AM, Bell JC and Braun PE (1989) Chemical deglycosylation on a micro-scale of membrane glycoproteins with retention of phosphoryl-protein linkages. *J Neurosci Res* **24:**398–401.

Huang XC, Richards EM and Sumners C (1996) Mitogen-activated protein kinases in rat brain neuronal cultures are activated by angiotensin II type 1 receptors and inhibited by angiotensin II type 2 receptors. *J Biol Chem* **271:**15635–15641.

Hunyady L (1999) Molecular mechanisms of angiotensin II receptor internalization. *J Am Soc Nephrol* **10:**S47–S56.

Hunyady L, Bor M, Balla T and Catt KJ (1994) Identification of a cytoplasmic Ser-Thr-Leu motif that determines agonist-induced internalization of the AT_1 angiotensin receptor. *J Biol Chem* **269:**31378–31382.

Jayadev S, Smith RD, Jagadeesh G, Baukal AJ, Hunyady L and Catt KJ (1999) N-linked glycosylation is required for optimal AT1a angiotensin receptor expression in COS-7 cells. *Endocrinology* **140:**2010–2017.

Kambayashi Y, Bardhan S, Takahashi K, Tsuzuki S, Inui H, Hamakubo T and Inagami T (1993) Molecular cloning of a novel angiotensin II receptor isoform involved in phosphotyrosine phosphatase inhibition. *J Biol Chem* **268:**24543–24546.

Kang J, Sumners C and Posner P (1993) Angiotensin II type 2 receptor-modulated changes in potassium currents in cultured neurons. *Am J Physiol* **265:**C607–C616.

Kohout TA and Rogers TB (1995) Angiotensin II activates the NA+/HCO3-symport through a phosphoinositide-independent mechanism in cardiac cells. *J Biol Chem* **270:**20432–20438.

Krupnick JG and Benovic JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol* **38:**289–319.

Lee KB, Pals-Rylaarsdam R, Benovic JL and Hosey MM (1998) Arrestin-independent internalization of the m1, m3, and m4 subtypes of muscarinic cholinergic receptors. *J Biol Chem* **273:**12967–12972.

Lemp D, Haselbeck A and Klebl F (1990) Molecular cloning and heterologous expression of N-glycosidase F from Flavobacterium meningosepticum. *J Biol Chem* **265:**15606–15610.

Lokuta AJ, Cooper C, Gaa ST, Wang HE and Rogers TB (1994) Angiotensin II stimulates the release of phospholipid-derived second messengers through multiple receptor subtypes in heart cells. *J Biol Chem* **269:**4832–4838.

Malecz N, Bambino T, Bencsik M and Nissenson RA (1998) Identification of phosphorylation sites in the G protein-coupled receptor for parathyroid hormone. Receptor phosphorylation is not required for agonist-induced internalization. *Mol Endocrinol* **12:**1846–1856.

Mukoyama M, Nakajima M, Horiuchi M, Sasamura H, Pratt RE and Dzau VJ (1993) Expression cloning of type 2 angiotensin II receptor reveals a unique class of seven transmembrane receptor. *J Biol Chem* **268:**24539–24542.

Nahmias C, Cazaubon SM, Briend-Sutren MM, Lazard D, Villageois P and Strosberg AD (1995) Angiotensin II AT_2 receptors are functionally coupled to protein tyrosine dephosphorylation in N1E-115 neuroblastoma cells. *Biochem J* **306:**87–92.

Neill JD, Sellers JC, Musgrove LC and Duck LW (1997) Epitope-tagged gonadotropin-releasing hormone receptors heterologously-expressed in mammalian (COS-1) and insect (Sf9) cells. *Mol Cell Endocrinol* **127:**143–154.

Nouet S and Nahmias C (2000) Signal Transduction from the angiotensin II AT_2 Receptor. *Trends Endocrinol Metab* **11:**1–6.

Oliverio MI, Kim HS, Ito M, Le T, Audoly L, Best CF, Hiller S, Kluckman K, Maeda N, Smithies O and Coffman TM (1998) Reduced growth, abnormal kidney structure, and type 2 (AT_2) angiotensin receptor-mediated blood pressure regulation in mice lacking both AT_{1A} and AT_{1B} receptors for angiotensin II. *Proc Natl Acad Sci USA* **95:**15496–15501.

Pearson RB and Kemp BE (1991) Protein kinase phosphorylation site sequences and consensus specificity motifs: Tabulations. *Methods Enzymol* **200:**62–81.

Pitcher JA, Freedman NJ and Lefkowitz RJ (1998) G protein-coupled receptor kinases. *Annu Rev Biochem* **67:**653–692.

Rabkin SW (1996) The angiotensin II subtype 2 (AT_2) receptor is linked to protein kinase C but not cAMP-dependent pathways in the cardiomyocytes. *Can J Physiol Pharmacol* **74:**125–131.

Smith RD, Baukal AJ, Zolyomi A, Gaborik Z, Hunyady L, Sun L, Zhang M, Chen HC and Catt KJ (1998a) Agonist-induced phosphorylation of the endogenous AT_1 angiotensin receptor in bovine adrenal glomerulosa cells. *Mol Endocrinol* **12:**634–644.

Smith RD, Hunyady L, Olivares-Reyes JA, Mihalik B, Jayadev S and Catt KJ (1998b) Agonist-induced phosphorylation of the angiotensin AT_{1a} receptor is localized to a serine/threonine-rich region of its cytoplasmic tail. *Mol Pharmacol* **54:**935–941.

Thomas WG, Motel TJ, Kule CE, Karoor V and Baker KM (1998) Phosphorylation of the angiotensin II (AT_{1a}) receptor carboxyl terminus: A role in receptor endocytosis. *Mol Endocrinol* **12:**1513–1524.

Tsuzuki S, Matoba T, Eguchi S and Inagami T (1996) Angiotensin II type 2 receptor inhibits cell proliferation and activates tyrosine phosphatase. *Hypertension* **28:**916–918.

Yamada T, Horiuchi M and Dzau VJ (1996) Angiotensin II type 2 receptor mediates programmed cell death. *Proc Natl Acad Sci USA* **93:**156–160.

Zhang J, Ferguson SSG, Barak LS, Menard L and Caron MG (1996) Dynamin and β-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *J Biol Chem* **271:**18302–18305.

Zhang J and Pratt RE (1996) The AT_2 receptor selectively associates with $G(_i$-α-2) and $G(_i$-α-3) in the rat fetus. *J Biol Chem* **271:**15026–15033.

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