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Angiotensin AT_1 Receptor Phosphorylation and Desensitization in a Hepatic Cell Line. Roles of Protein Kinase C and Phosphoinositide 3-Kinase

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ABSTRACT

Desensitization and phosphorylation of the endogenous angiotensin II AT_1 receptor were studied in clone 9 liver cells. Agonist activation of AT_1 receptors blunted the response to subsequent addition of angiotensin II. Partial inhibition of the angiotensin II-induced calcium response was observed when cells were pretreated with dibutyryl cyclic AMP, tetradecanoyl phorbol acetate (TPA), vasopressin, or lysophosphatidic acid. All of these desensitization processes were associated with receptor phosphorylation. Angiotensin II-induced AT_1 receptor phosphorylation was partially blocked by the protein kinase C inhibitor bisindolylmaleimide I and by phosphoinositide 3-kinase inhibitors (wortmannin and LY294002); the actions of these inhibitors were not additive. Pertussis toxin pretreatment of cells also partially inhibited angiotensin II-induced AT_1 receptor phosphorylation. TPA-induced AT_1 receptor phosphorylation was completely blocked by bisindolylmaleimide I. AT_1 receptor phosphorylation was also induced by vasopressin and lysophosphatidic acid, and these effects were partially inhibited by bisindolylmaleimide I. Angiotensin II increased Akt/PKB (protein kinase B) phosphorylation and protein kinase C membrane association. The effect on Akt/PKB phosphorylation was blocked by phosphoinositide 3-kinase inhibitors. These findings indicate that clone 9 cells exhibit both homologous and heterologous desensitization in association with AT_1 receptor phosphorylation. In these hepatic cells, angiotensin II-induced receptor phosphorylation involves pertussis toxin-sensitive and -insensitive G proteins, and is mediated in part through protein kinase C and phosphoinositide 3-kinase.

The pressor octapeptide angiotensin II (Ang II) mediates a plethora of physiological processes in the cardiovascular system, brain, liver, kidney, adrenal glands, and pituitary gland, as well as in many other organs and systems (Jackson and Garrison, 1996). The actions of Ang II are initiated through its interaction with two seven-transmembrane domain receptors, the AT_1 and AT_2 receptor subtypes (Inagami et al., 1994).

AT_1 receptors (AT_1-Rs) mediate most of the known actions of Ang II, and they are GPCRs that activate phosphoinositide/Ca^{2+} signaling via pertussis toxin-insensitive G proteins. In some cell types, they are also coupled to other phospholipases and to inhibition of adenylyl cyclase via pertussis toxin-sensitive G proteins (García-Sáinz and Macías-Silva, 1990; Bauer et al., 1991). AT_1-Rs are integral membrane proteins (Murphy et al., 1991; Sasaki et al., 1991) and have a molecular mass of ~41 kDa. The intracellular carboxyl terminus (residues 305–359) of the AT_1-R contains numerous serine and threonine residues, some of which have been implicated in receptor activation and regulation (Hunyady et al., 1994; Thomas et al., 1995; Smith et al.,1998a; Thomas, 1998).

As with many other GPCRs, AT_1-Rs are subject to several forms of regulation. Desensitization of GPCRs is frequently associated with their covalent modification, typically by phosphorylation, which uncouples them from G proteins and initiates their internalization and recycling. Desensitization of AT_1-R-mediated signaling has been observed in cells transfected with cloned AT_1-Rs (Tang et al., 1995; Oppermann et al., 1996; Balmforth et al., 1997; Tang et al., 1998) and in cells that endogenously express them (Abdellatif et al., 1991; Boulay et al., 1994). AT_1-R phosphorylation by GRKs seems to be a major mechanism in homologous desensitization (Oppermann et al., 1996), whereas receptor phosphorylation by

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ABBREVIATIONS: Ang II, angiotensin II; AT_1-R, angiotensin AT_1 receptor; TPA, tetradecanoyl phorbol acetate; PKC and PKA, protein kinases C and A; PI3K, phosphoinositide 3-kinase; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; C9, clone 9; $[Ca^{2+}]_i$, intracellular calcium concentration; PAGE, polyacrylamide gel electrophoresis; PDK, phosphoinositide-dependent protein kinase; EGF, epidermal growth factor.

PKA and PKC appears to participate in heterologous desensitization. The carboxyl terminus region of AT_1-Rs contains an 11-amino acid serine/threonine-rich segment (Ser^{326}–Thr^{336}) that is phosphorylated in response to Ang II (Smith et al., 1998b). This segment has one putative PKC phosphorylation site at Ser^{331} and possibly a GRK phosphorylation site at Ser^{335} and/or Thr^{336}. Substitution of Ser^{335} and Thr^{336} in this segment by alanine not only impairs phosphorylation of the mutant receptor but also affects the receptor internalization process (Smith et al., 1998b). More recently, Qian et al. (1999) observed that mutation of the three putative PKC consensus sites (Ser^{331}, Ser^{338}, and Ser^{348}) of the AT_1 receptor caused a 70% reduction of PKC-mediated phosphorylation and a 60% reduction in Ang II-induced phosphorylation. In bovine adrenal glomerulosa cells, agonist-dependent phosphorylation of the endogenous AT_1-Rs, as well as receptor phosphorylation induced by activation of PKA and PKC, have been observed (Smith et al., 1998a).

Ang II is a modulator of hepatic metabolism, and its receptors and signaling have been extensively studied in isolated liver cells (García-Sáinz and Macías-Silva, 1990; Bauer et al., 1991). However, normal hepatocytes lose their ability to respond to Ang II due to a marked decrease in receptor density during culture (Bouscarel et al., 1990). In contrast, the clone 9 (C9) epithelial cell line isolated from normal rat liver has been found to express AT_1-Rs as detected by functional and binding studies (Kozlowski et al., 1993; García-Sáinz et al., 1998). Activation of AT_1-Rs in C9 cells induces phosphoinositide turnover via pertussis toxin-insensitive G proteins, with increased $[Ca^{2+}]_i$ and c-*fos* mRNA expression (García-Sáinz et al., 1998). In the present study, we observed that AT_1-R-mediated $[Ca^{2+}]_i$ responses in C9 cells exhibit both homologous and heterologous forms of desensitization that are associated with receptor phosphorylation. In addition, our studies also revealed that the AT_1-R phosphorylation induced by Ang II involved pertussis toxin-sensitive G proteins as well as PI3K and PKC activities.

Experimental Procedures

Materials. EGF, F-12K nutrient mixture (Kaighn's modification), phosphate-free Dulbecco's modified Eagle's medium, medium 199, fetal bovine serum, trypsin, and antibiotic/antimycotic solutions were from Life Technologies (Gaithersburg, MD) or Biofluids (Rockville, MD). Ang II, TPA, protease inhibitors, and all other analytical grade chemicals were from Sigma Chemical Co. (St. Louis, MO). Protein A-agarose, wortmannin, LY294002, bisindolylmaleimide I, okadaic acid, protease inhibitors, pepstatin A, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, and benzamidine were all from Calbiochem (San Diego, CA). Losartan (DuP 753) and PD123177 were generous gifts from DuPont (Wilmington, DE). ^{125}I-[Sar^1,(4-N_3)Phe^8]angiotensin II was from Covance Laboratories (Vienna, VA). [^{32}P]P_i (8500–9120 Ci/mmol) was from NEN Life Science Products (Boston, MA). Recombinant *N*-glycosidase F (*N*-glycanase) (EC 3.5.1.52) was from Roche Molecular Biochemicals (Mannheim, Germany) and from Glyco (Heyford, UK). Fura-2/acetoxymethyl ester was from Molecular Probes (Eugene, OR). Pertussis toxin was purified from vaccine concentrates (Sekura et al., 1983; García-Sáinz et al., 1992). The C9 cell line was obtained from the American Type Culture Collection (Manassas, VA). Rabbit polyclonal anti-PKCδ antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-phospho-Akt/PKB antibodies were from PharMingen (San Diego, CA), and secondary antibodies and the chemiluminescence kits were obtained from Pierce (Rockford, IL).

Cell Culture. C9 cells were cultured in F-12K medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.25 μg/ml amphotericin B at 37°C under a 95% air/5% CO_2 atmosphere. Cells were incubated with pertussis toxin (100 ng/ml) for 12 to 24 h where indicated.

Intracellular Calcium Measurements. To quantify $[Ca^{2+}]_i$, cells were loaded with 5 μM Fura-2/acetoxymethyl ester in Krebs-Ringer-HEPES buffer (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) containing 0.05% bovine serum albumin, pH 7.4, for 1 h at 37°C. Cells were detached by gentle trypsinization. Fluorescence measurements were carried out with an Aminco-Bowman (Urbana, IL) series 2 spectrometer with excitation monochromator set at 340 and 380 nm, with a chopper interval of 0.5 s, and the emission monochromator set at 510 nm. The intracellular calcium concentration was calculated according to the method of Grynkiewicz et al. (1985), using software provided by Aminco-Bowman; traces were directly exported to the graphs.

Immunoprecipitation of Photoaffinity-Labeled AT_1-Rs. Confluent C9 cells cultured in 10-cm dishes were washed three times with ice-cold medium 199 before overnight incubation at 4°C in the same medium containing the photoaffinity ligand, ^{125}I-[Sar^1,(4-N_3)Phe^8]Ang II. Cells were then washed three times with ice-cold phosphate-buffered saline and exposed to UV light for 10 s. Noncovalently bound ^{125}I-azido-Ang II was removed by incubating the cells for 10 min in ice-cold 150 mM NaCl containing 50 mM acetic acid. After further washes with ice-cold phosphate-buffered saline, dishes were drained and the cells were scraped into lysis buffer [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 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 1 μM okadaic acid] and probe-sonicated (Sonifier Cell Disruptor; Heat Systems Ultrasonics, Plainview, NY) for 2 × 20 s. After removal of nuclei by centrifugation for 10 min at 750*g*, membranes were collected by centrifugation for 45 min at 200,000*g*. Membrane pellets were solubilized by Dounce homogenization in ice-cold lysis buffer B [lysis buffer supplemented with 1% (v/v) Nonidet P-40, 1% (w/v) sodium deoxycholate, and 0.1% (w/v) SDS]. After centrifugation for 10 min at 10,000*g*, solubilized membranes were incubated for 4 h at 4°C with 2% (v/v) protein A-agarose. This precleared supernatant was then divided into aliquots and stored at −20°C before use. Immunoprecipitation was initiated by adding 5 μl of a selective anti-AT_1-R antibody and 2% (v/v) protein A-agarose overnight at 4°C with tumbling. The preparation and characteristics of the rabbit anti-AT_1-R polyclonal antibody are described in detail elsewhere (Smith et al., 1998a). Immune complexes were collected by centrifugation and washed three times with ice-cold lysis buffer B lacking protease inhibitors. After the final wash, immune complexes were eluted into Laemmli's sample buffer (Laemmli, 1970) for 1 h at 48°C. After resolution by SDS-PAGE (8–16% resolving gel), photoaffinity-labeled AT_1-Rs were visualized using a PhosphorImager (Molecular Dynamics, Sunnyvale CA).

AT_1-R Phosphorylation Assay. C9 cells cultured in 10-cm Petri dishes were metabolically labeled for 4 h at 37°C in phosphate-free Dulbecco's modified Eagle's medium containing 100 μCi/ml [^{32}P]P_i. After three washes in Krebs-Ringer-HEPES buffer, cells were further incubated in the same medium for 10 min at 37°C in a water bath and stimulated with hormones or phorbol 12-myristate 13-acetate for an additional 5-min period. This time was selected on the basis of preliminary experiments. When required, the PKC inhibitor, bisindolylmaleimide I (1 μM), or the PI3K inhibitors, LY294002 (15 μM) or wortmannin (100 nM), were added for 30 min at 37°C prior to the agonist stimulation. After stimulation, the plates were washed with ice-cold phosphate-buffered saline, and the cells were drained before being scraped into lysis buffer and probe-sonicated for 2 × 20 s. After removal of nuclei at 750*g*, membranes were pre-extracted by the addition of an equal volume of lysis buffer containing 2 M

NaCl and 8 M urea followed by overnight tumbling at 4°C. The membranes then were collected at 200,000*g* and solubilized in lysis buffer B by Dounce homogenization. After centrifugation at 14,000*g*, solubilized membranes were incubated with 2% (v/v) protein A-agarose for 1 h at 4°C. The precleared supernatant was incubated overnight at 37°C with 10 units/ml recombinant *N*-glycosidase F. The deglycosylated AT_1-R was immunoprecipitated by the addition of 1 μl of anti-AT_1 antibody and 2% (v/v) protein A-agarose and incubated overnight at 4°C. The agarose-bound immune complexes were washed with lysis buffer B lacking protease inhibitors, Laemmli's sample buffer was added, and the mixtures were incubated for 1 h at 48°C. The denatured complexes were resolved by SDS-PAGE on an 8 to 16% gradient resolving gel. Phosphorylated AT_1-Rs were visualized with a PhosphorImager and images were exported to the graphs. The level of receptor phosphorylation was assessed in the gels with the PhosphorImager ImageQuant software.

Akt/PKB and PKCδ Assays. Subconfluent C9 cells, in 6-well plates, were switched to serum-free medium overnight (16–18 h) and were stimulated. After three washes with ice-cold phosphate-buffered saline, cells were drained, scraped into 0.1 ml of Laemmli's sample buffer (Laemmli, 1970), and sonicated for 5 s. After boiling for 5 min, equal quantities of cell lysates were subjected to SDS-PAGE, and the separated proteins were transferred to membranes. A rabbit polyclonal anti-phospho-Akt/PKB antibody (0.3 μg/ml) was used to identify the active enzyme. Immunoreactive bands were detected using a horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence. Immunoreactive bands were visualized by autoradiography and quantified in a densitometer (model GS-700 imaging densitometer, Bio-Rad, Hercules, CA) using the Molecular Analyst software (Bio-Rad).

Membrane association of PKCδ was used as an index of enzyme activation, and it was performed as described previously (García-Sáinz and Alcántara-Hernández, 1998). In brief, cells were stimulated and the reaction stopped by the addition of lysis buffer and homogenization. Homogenates were centrifuged at 28,000*g* for 20 min. Supernatants were discarded and protein in the pellets was determined (Lowry et al., 1951). Samples (50 μg) were separated by SDS-PAGE followed by electrophoretic transfer to membranes. Immunoblotting was performed as described above using a PKCδ-selective antibody.

Results

In agreement with previous results (García-Sáinz et al., 1998), Ang II caused a rapid 2- to 3-fold increase of $[Ca^{2+}]_i$ in C9 cells (Fig. 1). This reached a maximum at 100 nM and had an EC_{50} of ~5 nM (data not shown; see García-Sáinz et al., 1998). When the cells were stimulated with Ang II, a transient increase of $[Ca^{2+}]_i$ was observed and the cells became less responsive or refractory to a second stimulation by 100 nM Ang II (Fig. 1, A and C). The desensitization to the second stimulation to Ang II was dependent on the concentration of the initial stimulus; the concentration-response curve showed an EC_{50} value of 2 nM for the desensitization process (Fig. 1B). Desensitization of the response to Ang II was not due to depletion of the relevant phosphoinositide or intracellular calcium pools, as evidenced by the ability of vasopressin, bradykinin, and lysophosphatidic acid to increase $[Ca^{2+}]_i$ in cells that exhibited refractoriness to Ang II (Fig. 1C).

Activation of PKC and PKA by treatment with 1 μM TPA or 100 μM dibutyryl cyclic AMP, respectively, also decreased the magnitude of the $[Ca^{2+}]_i$ response to Ang II (Fig. 2A). Again, this was not due to depletion of calcium pools as evidenced by the ability of other agents, such as lysophosphatidic acid or thapsigargin, to increase $[Ca^{2+}]_i$ (Fig. 2B). In these cases, the desensitization of the Ang II response was both partial (~30%) and very rapid, being evident after only

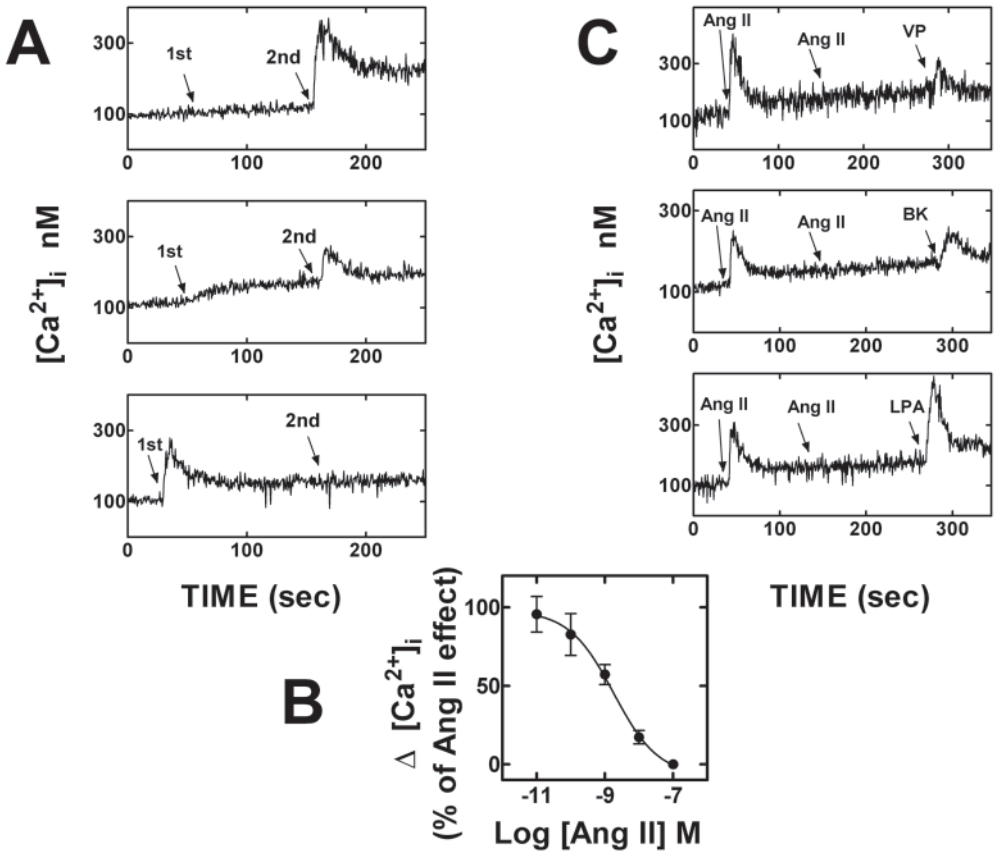

Fig. 1. Homologous desensitization of AT_1-Rs in clone 9 cells. A, cells were stimulated (1st) with 10 pM (upper trace), 1 nM (middle trace), or 100 nM Ang II incubated for ~2 min and challenged again with 100 nM Ang II (2nd). Intracellular calcium was measured as described under *Experimental Procedures*. B, concentration dependence of Ang II-induced desensitization. The concentration of Ang II used during the first stimulation is indicated in the abscissa, and in the ordinate, the response to 100 nM Ang II (second stimulation, as a percentage of the response of cells not previously stimulated) is plotted. The means are plotted, and vertical lines represent the S.E.M. of seven independent experiments. C, cells were stimulated with 100 nM Ang II, challenged again with the same concentration of Ang II, and further challenged with 10 nM vasopressin (VP), 10 nM bradykinin (BK), or 1 μM lysophosphatidic acid (LPA). In all cases traces are representative of five to seven determinations using different cell preparations.

1 min of exposure to the PKA and PKC activators (Fig. 2A). Initial stimulation of the cells with lysophosphatidic acid or vasopressin reduced the subsequent response to Ang II, and this was not due to depletion of calcium pools as evidenced by the ability of thapsigargin to increase $[Ca^{2+}]_i$ (Fig. 3). These data indicate that the Ang II-induced phosphoinositide/Ca^{2+} response is subject to both homologous and heterologous desensitization in C9 cells.

To study receptor phosphorylation, photoaffinity labeling and immunoprecipitation studies of the AT_1-R were performed. As shown in Fig. 4A, photoaffinity labeling with ^{125}I-[Sar^1,(4-N_3)Phe^8]Ang II gave a broad band with molecular mass in the 66- to 97-kDa range. Photoaffinity labeling intensity was markedly decreased by Ang II and losartan, a nonpeptide AT_1-R antagonist, but not by PD123177, a nonpeptide AT_2 receptor antagonist (Fig. 4A). The photolabeled band was effectively immunoprecipitated by the selective anti-AT_1-R antibody (Fig. 4B). When membranes were subjected to enzymatic deglycosylation, the labeled 66- to 97-kDa band was no longer evident, and components with lower molecular mass (46–41 kDa) were observed (Fig. 4B); with longer deglycosylation times, the ~41-kDa band was the

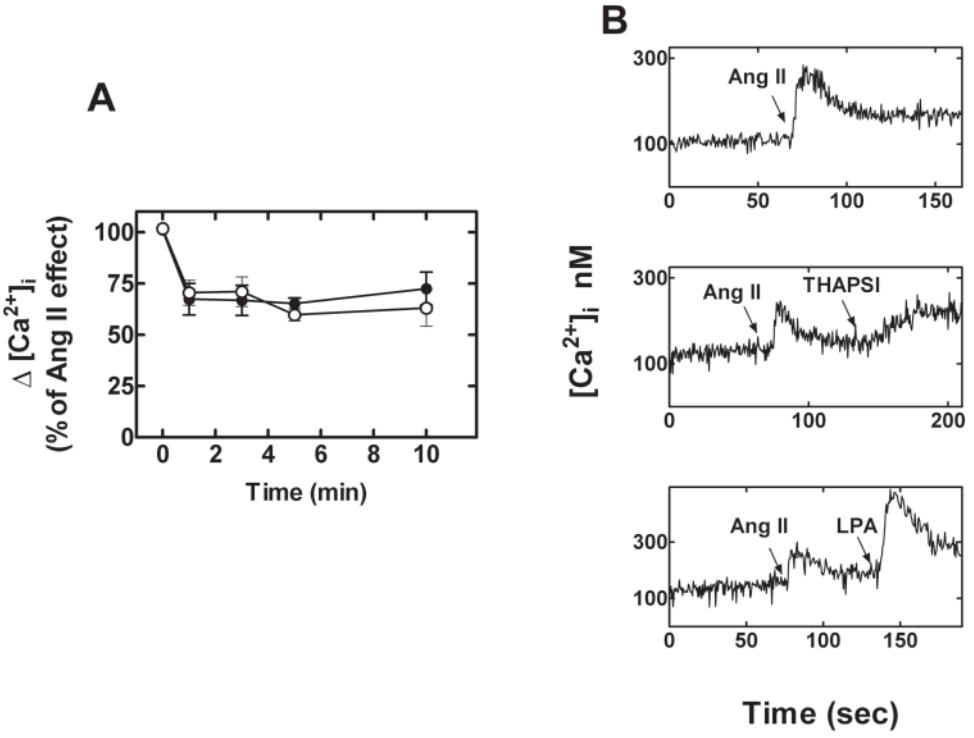

Fig. 2. Effect of TPA and dibutyryl cyclic AMP on Ang II-induced increase in $[Ca^{2+}]_i$. A, cells were incubated for the times indicated in the presence of 100 μM dibutyryl cyclic AMP (○) or 1 μM TPA (●) and challenged with 100 nM Ang II; data are expressed as a percentage of the response of cells not previously stimulated. The means are plotted, and vertical lines represent the S.E.M. of six to eight independent experiments. B, cells were incubated for 5 min in the absence of any agent (upper panel), with 1 μM TPA (middle panel), or with 100 μM dibutyryl cyclic AMP (lower panel) and challenged with 100 nM Ang II. Where indicated, 10 μM thapsigargin (THAPSI) or 1 μM lysophosphatidic acid (LPA) was added. In all cases, traces are representative of five to seven determinations using different cell preparations.

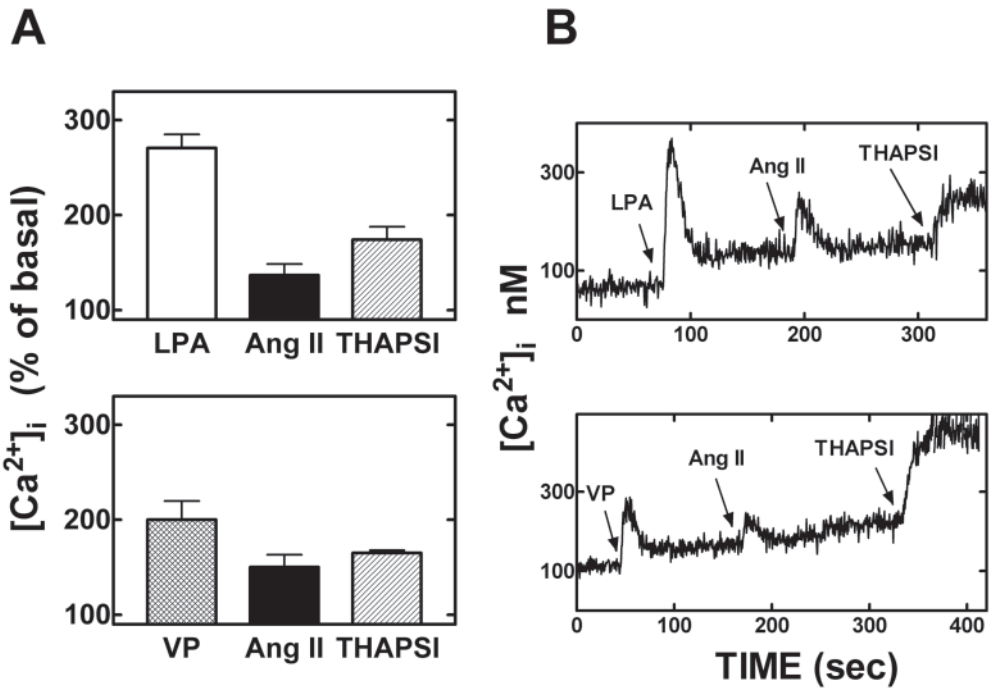

Fig. 3. Effect of lysophosphatidic acid and vasopressin on Ang II-induced increase in $[Ca^{2+}]_i$. A, cells were incubated with 1 μM lysophosphatidic acid (LPA; □) or 10 nM vasopressin (VP; ▨) for 2 min and then were challenged with 100 nM Ang II; where indicated, 1 μM thapsigargin (THAPSI) was added. The means are plotted, and vertical lines represent the S.E.M. of six to eight experiments using different cell preparations. B, representative tracings.

major band detected (data not shown). It is also evident in Fig. 4B that the antibody was able to immunoprecipitate the enzymatically deglycosylated receptor.

During phosphorylation studies, analysis of the deglycosylated samples revealed labeled bands in the 46- to 41-kDa range (Fig. 5). However, in the absence of glycosidase treatment only a broad labeled band in the 66- to 97-kDa range was observed (data not shown). These data indicate that the ^{32}P-labeled bands correspond to the AT_1-R detected in the photoaffinity-labeling studies. It should be noted that the basal labeling of the AT_1-R was very small and was almost undetectable in some experiments. Stimulation with Ang II markedly increased AT_1-R phosphorylation in a concentration-dependent manner (Fig. 5A) with an EC_{50} value of 0.4 nM. TPA also increased receptor phosphorylation in a concentration-dependent fashion (EC_{50}, 2.8 nM) (Fig. 5B). The receptor phosphorylation induced by Ang II was consistently greater than that induced by TPA. When both agents were used together to stimulate the cells, the effect was almost additive (Fig. 5C).

We next examined the effects of specific inhibitors to define the role of different protein and lipid kinases in these effects. As expected, the action of TPA was completely blocked by 1 μM bisindolylmaleimide I (Fig. 6). However, Ang II-induced AT_1-R phosphorylation was reduced to a more limited extent (~30%). Wortmannin and LY294002, inhibitors of PI3K activity, also reduced Ang II-induced AT_1-R phosphorylation (Fig. 7A). The effects of these inhibitors were concentration-dependent, with IC_{50} values for wortmannin and LY294002 of 8 nM and 125 nM, respectively (Fig. 7B). The inhibitory actions of wortmannin and bisindolylmaleimide I were not additive (data not shown). None of the inhibitors altered the low basal phosphorylation of the AT_1 receptor. Inhibitors of PKC and PI3K were unable to block the homologous desensitization of the calcium response to Ang II.

Pretreatment of the cells with pertussis toxin consistently

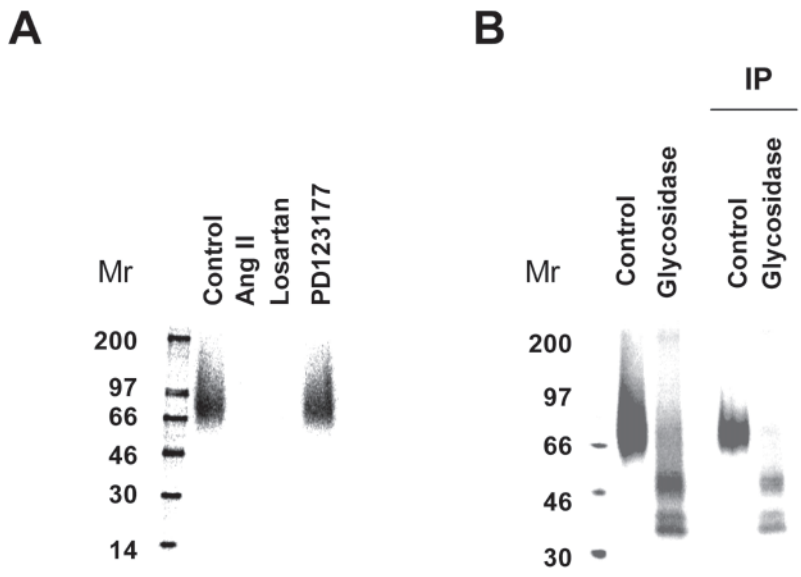

Fig. 4. Photoaffinity labeling and immunoprecipitation of AT_1-Rs. A, cells were incubated overnight at 4°C with ^{125}I-[Sar^1,(4-N_3)Phe^8]Ang II in the presence or absence of 1 μM Ang II, 10 μM losartan, or 10 μM PD123177. B, solubilized ^{125}I-[Sar^1,(4-N_3)Phe^8]Ang II photoaffinity-labeled membranes from clone 9 cells were incubated overnight at 37°C in the absence or presence of 10 U/ml glycosidase F; AT_1-Rs were immunoprecipitated and resolved by SDS-PAGE. A representative image is shown from three independent experiments.

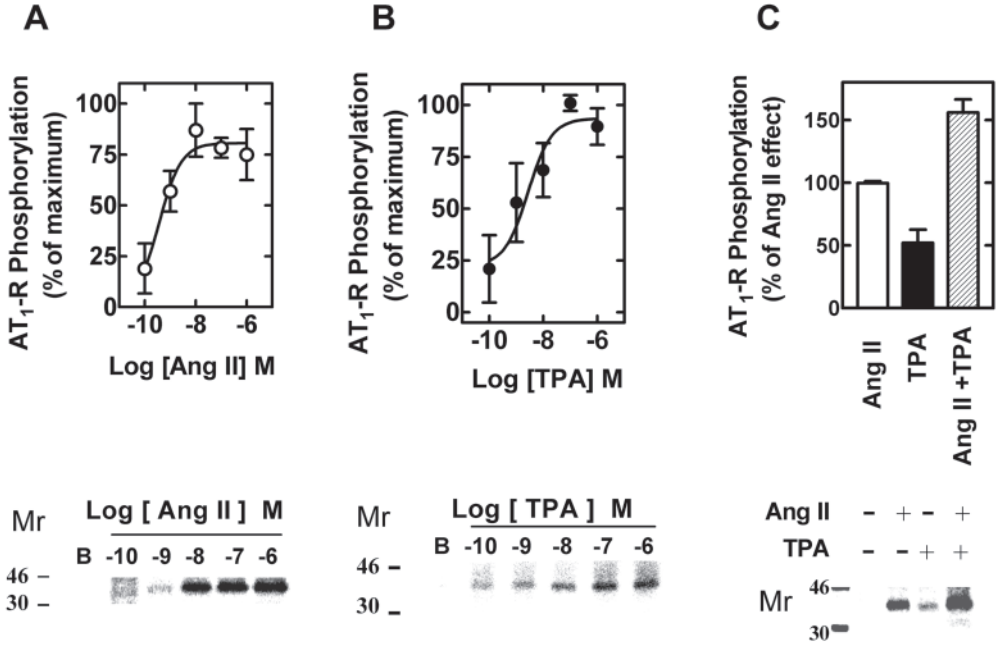

Fig. 5. Ang II- and TPA-induced AT_1-R phosphorylation. A and B, the indicated concentrations of Ang II (○) and TPA (●) were used. The maximum phosphorylation induced by each agent was considered as 100% and basal phosphorylation as 0%. C, cells were incubated with 100 nM Ang II (open column), 1 μM TPA (solid column), or both agents (hatched column) for 5 min. Representative images are presented below each graph. Data are expressed as the receptor phosphorylation induced by 100 nM Ang II. The means are plotted, and vertical lines represent the S.E.M. of three to four determinations using different cell preparations.

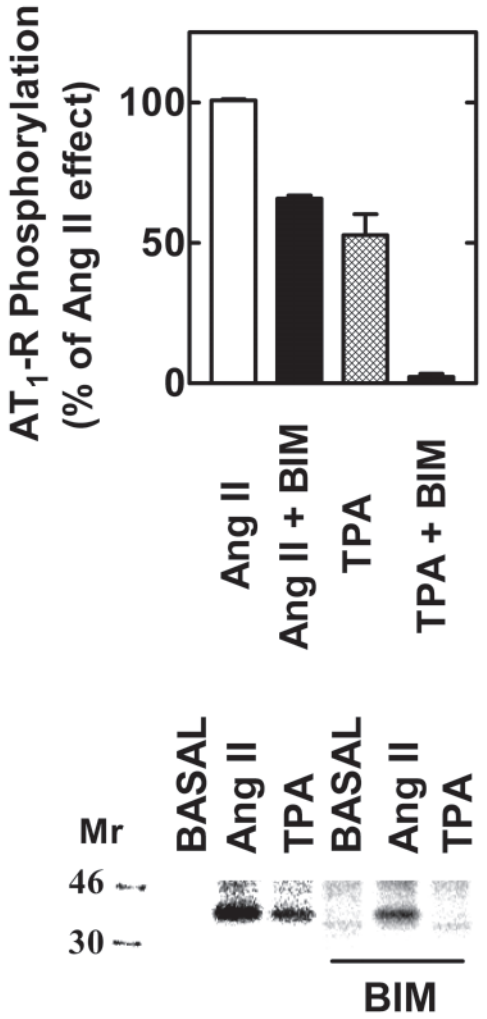

Fig. 6. Effect of bisindolylmaleimide I on AT_1-R phosphorylation. Cells were preincubated for 30 min without or with 1 μM bisindolylmaleimide I (BIM, ■) and then stimulated with 100 nM Ang II (□) or 1 μM TPA (▨). Data are expressed as the receptor phosphorylation induced by 100 nM Ang II. The means are plotted, and vertical lines represent the S.E.M. of three to four experiments using different cell preparations. A representative image is shown.

reduced Ang II-induced AT_1-R phosphorylation (Fig. 8), suggesting that G_i protein(s) participate in the mechanism of receptor phosphorylation. The possibility that such G proteins could mediate the effects of PI3K and PKC was tested. However, the abilities of wortmannin and bisindolylmaleimide I to decrease Ang II-induced AT_1-R phosphorylation were not affected in pertussis toxin-treated cells (data not shown).

The extent to which heterologous stimulation by lysophosphatidic acid and vasopressin or activation of PKA could induce AT_1-R phosphorylation was also examined. As shown in Fig. 9, upper panels, vasopressin, lysophosphatidic acid, and dibutyryl cyclic AMP significantly increased AT_1-R phosphorylation. To study the roles of PKC and PI3K in the AT_1-R phosphorylations induced by lysophosphatidic acid and vasopressin, the effects of wortmannin and bisindolylmaleimide I were examined. As shown in Fig. 9, lower panels, wortmannin did not alter the receptor phosphorylations induced by these agents, but the PKC inhibitor, bisindolylmaleimide I, markedly reduced such receptor phosphorylation. However, treatment with bisindolylmaleimide I did not affect the desensitization of the Ang II-mediated calcium response induced by lysophosphatidic acid or vasopressin (data not shown).

To obtain more evidence about the ability of Ang II to activate PKC and PI3K, Akt/PKB phosphorylation and membrane association of PKCδ were studied. Akt/PKB is a serine/threonine kinase substrate of PDK-1 whose activation has been shown to be mediated through PI3K activity (Alessi and Cohen, 1998; Chow et al., 1998; Stephens et al., 1998). As shown in Fig. 10A, Ang II (100 nM) increased Akt/PKB phosphorylation with a maximum at 5 min. The effect of Ang II was concentration-dependent (EC_{50}, 2 nM) (Fig. 10B) and was blocked by the AT_1 antagonist, losartan, and by the PI3K inhibitors, wortmannin (100 nM) and LY294002 (1 μM) (Fig. 10C). EGF (100 ng/ml) was used as a positive control. Activation of Akt/PKB by Ang II has previously been observed in smooth muscle cells (Takahashi et al., 1999). Figure 10D shows that Ang II increased membrane-associated PKCδ and that this effect was reduced by 1 μM bisindolylmaleimide I.

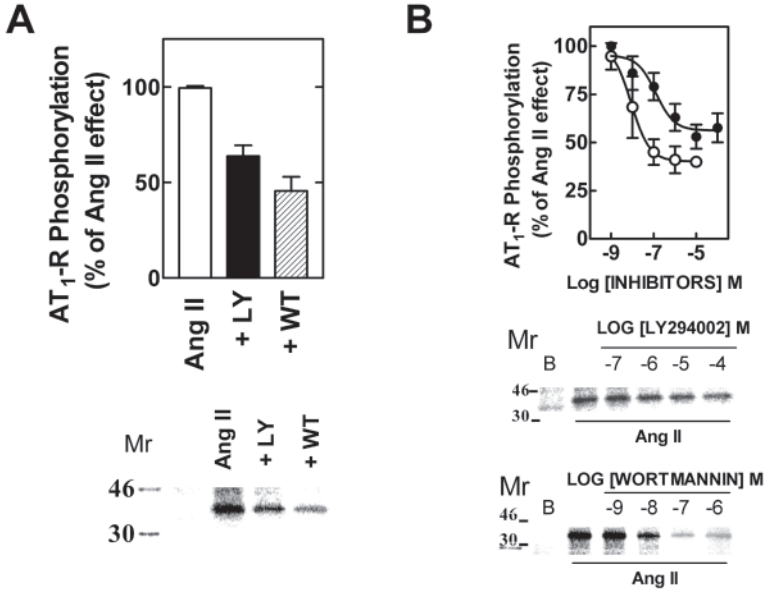

Fig. 7. Role of PI3K on Ang II-induced AT_1-R phosphorylation. A, cells were preincubated for 30 min with maximal 15 μM LY294002 (+LY) or 100 nM wortmannin (+WT) and then stimulated for 5 min with 100 nM Ang II. B, cells were preincubated with the indicated concentration of LY294002 (●) or wortmannin (○) for 30 min and then stimulated for 5 min with 100 nM Ang II. Data are expressed as a percentage of the receptor phosphorylation induced by 100 nM Ang II. Representative images are shown. The means are plotted, and vertical lines represent the S.E.M. of three to four determinations using different cell preparations.

Discussion

This study has shown that the endogenous AT_1-Rs expressed in C9 cells are subject to homologous and heterologous desensitization and that such desensitization is associated with receptor phosphorylation. Although C9 cells have a limited AT_1-R density (Kozlowski et al., 1993), these receptors were clearly identified by photoaffinity labeling and exhibited the anticipated pharmacological profile in competition studies. In bovine adrenal glomerulosa cells, Smith et al. (1998b) observed that phosphorylated AT_1-Rs run as a broad 60- to 65-kDa band in SDS-PAGE. This is shifted to a molecular mass of ~41 kDa after deglycosylation, consistent with the predicted size of the cloned AT_1 receptor protein (41 kDa). In the present study, phosphorylated AT_1-Rs ran in SDS-PAGE as a broader band of higher molecular mass (66–97 kDa); nevertheless, the deglycosylated receptors also shifted to a molecular mass of ~41 kDa. These data suggest that the AT_1 receptors in C9 cells may be more extensively glycosylated than those expressed in adrenal glomerulosa cells. The broad migration pattern of the endogenous AT_1-Rs in the C9 cell line, together with the possible presence of comigrating nonreceptor phosphoproteins, could interfere with the quantification of glycosylated phospho-AT_1-Rs. For this reason, the solubilized ^{32}P-labeled phospho-AT_1-Rs were subjected to enzymatic deglycosylation before SDS-PAGE analysis.

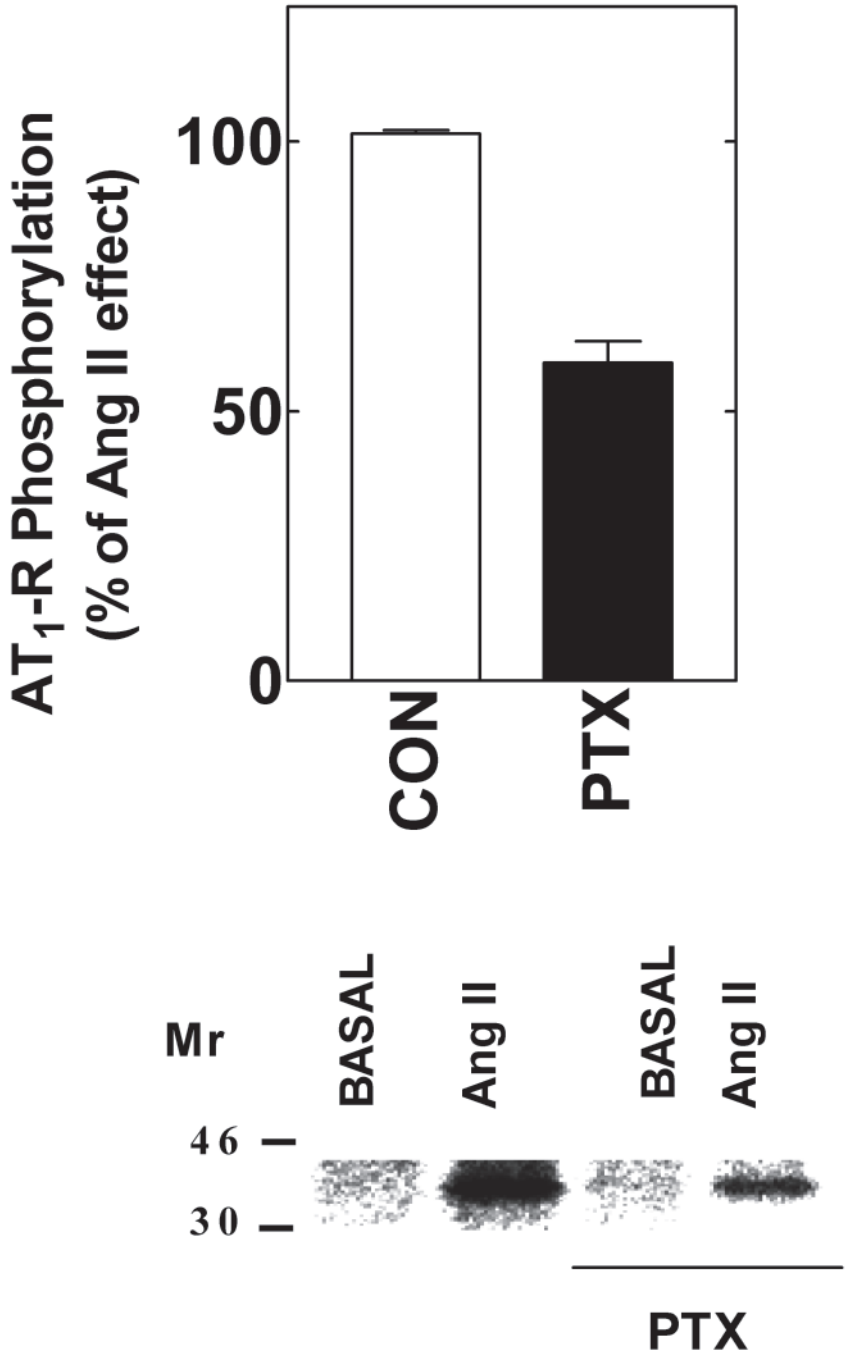

Fig. 8. Effect of pertussis toxin on Ang II-induced AT_1-R phosphorylation. Cells were preincubated or not with 100 ng/ml of pertussis toxin (PTX) overnight and then stimulated for 5 min with 100 nM angiotensin II (Ang II). Data are expressed as the receptor phosphorylation induced by 100 nM Ang II in control cells. A representative image is shown. The means are plotted, and vertical lines represent the S.E.M. of three determinations using different cell preparations.

In this study, we observed that activation of PKC with TPA stimulated receptor phosphorylation and decreased the responsiveness of the cells to Ang II. The effects of TPA on AT_1-R phosphorylation, and on the functional response, were of smaller magnitude than those induced by Ang II, the natural agonist. The carboxyl termini of AT_1-Rs contain several phosphorylation sites and motifs involved in receptor internalization (Hunyady et al., 1994; Thomas et al., 1995; Oppermann et al., 1996; Balmforth et al., 1997; Qian et al., 1999). Certain sites, including Ser^{331}, Ser^{338}, and Ser^{348}, have been identified as putative PKC phosphorylation sites (Smith et al., 1998b; Qian et al., 1999).

Our studies in Ang II-stimulated C9 cells showed that the natural agonist markedly desensitized the $[Ca^{2+}]_i$ response and induced a receptor-selective refractory period. In addition, Ang II induced prominent AT_1-R phosphorylation. Treatment with both Ang II and TPA caused an almost additive degree of receptor phosphorylation. These data initially suggested the possibility that these phosphorylations could represent two separate processes. However, the effects of bisindolylmaleimide I indicated that this was not the case and that Ang II-induced AT_1-R phosphorylation is in part attributable to PKC. It is possible that activation of AT_1-Rs partially stimulates certain PKC isoforms present in C9 cells, whereas TPA induces a full and sustained activation of all phorbol ester-sensitive isoforms expressed in the cells. These findings may reflect differences in the intensity, kinetics, and nature of the PKC isoforms affected by the actions of the

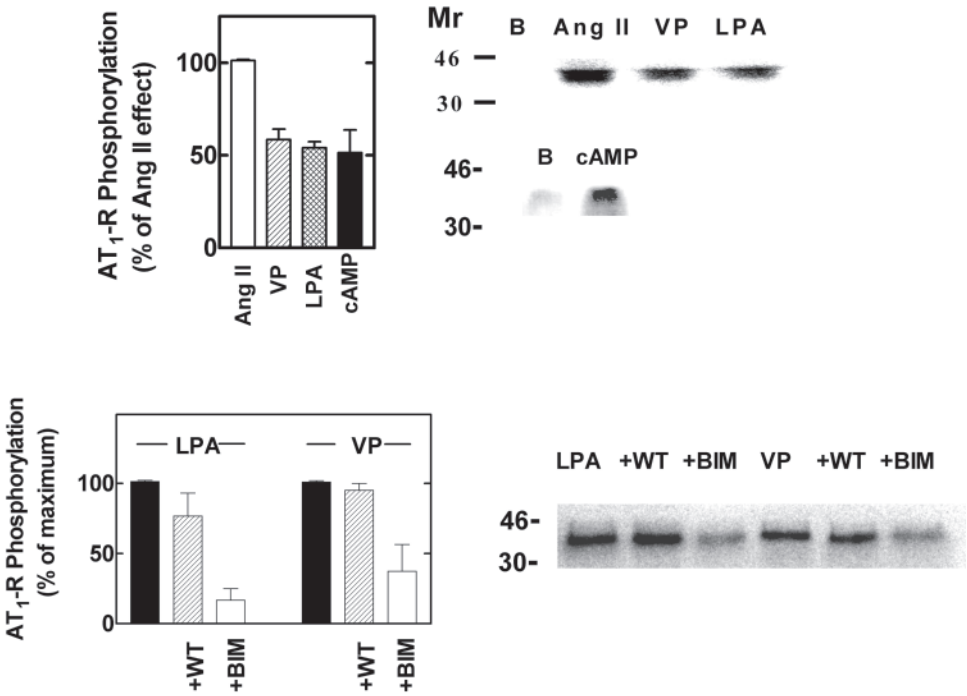

Fig. 9. Phosphorylation of AT_1-Rs induced by dibutyryl cyclic AMP, vasopressin, and lysophosphatidic acid. Upper panels, cells were stimulated with 100 nM Ang II, 10 nM vasopressin (VP), 1 μM lysophosphatidic acid (LPA), or 100 μM dibutyryl cyclic AMP (cAMP) for 5 min. Representative images are shown. Data are expressed as the receptor phosphorylation induced by 100 nM Ang II. Lower panels, cells were incubated in the absence of any agent, 100 nM wortmannin (+WT), or 1 μM bisindolylmaleimide I (BIM) for 30 min and then were stimulated with 1 μM lysophosphatidic acid (LPA) or 10 nM vasopressin (VP) for 5 min. A representative image is shown. Data are expressed as the percentage of phosphorylation induced by lysophosphatidic acid or vasopressin alone. The means are plotted, and vertical lines represent the S.E.M. of three to four determinations using different cell preparations.

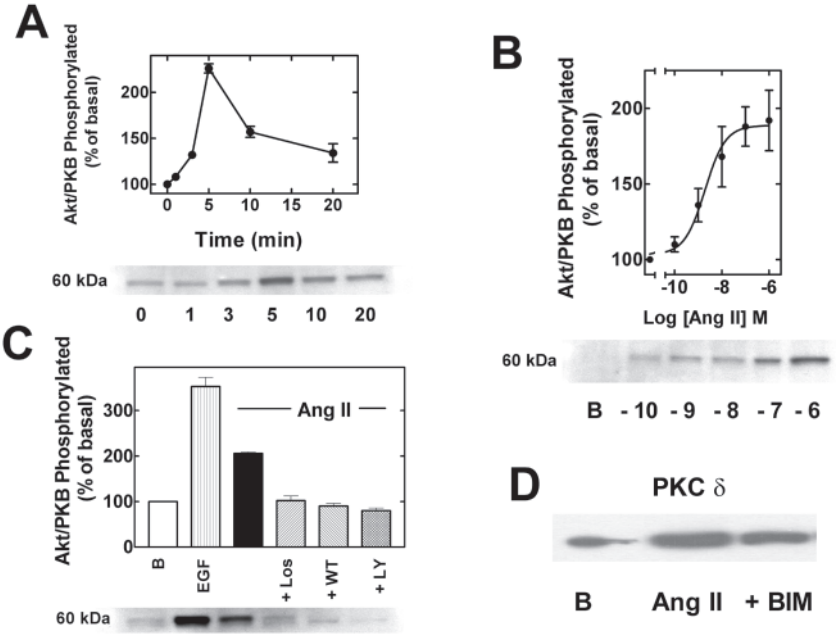

Fig. 10. Phosphorylation of Akt/PKB and membrane association of PKCδ. A, cells were incubated for the times indicated in the presence of 100 nM Ang II. A representative image is presented. B, cells were incubated for 5 min in the presence of different concentrations of Ang II. A representative image is presented. C, cells were incubated in the absence of any agent or presence of 10 μM losartan (+Los), 100 nM wortmannin (+WT), or 1 μM LY294002 (+LY) for 30 min and then challenged with 100 nM Ang II for 5 min; EGF (100 ng/ml for 5 min) was used as a positive control. A representative image is presented. D, representative image of PKCδ-membrane association; cells were incubated in the absence of any agent (B), with 100 nM Ang II for 5 min, or with bisindolylmaleimide I for 30 min followed by 100 nM Ang II for 5 min (+BIM). An image representative of three different experiments is shown. The means are plotted, and vertical lines represent the S.E.M. of three to four determinations using different cell preparations.

agonist and TPA. In transfected human embryonic kidney 293 cells, agonist-induced AT_1-R phosphorylation was partially blocked by the PKC inhibitor, staurosporine, and was completely suppressed by the concomitant expression of a dominant GRK2 mutant (Oppermann et al., 1996). Also, it has been observed that bisindolylmaleimide I partially blocked Ang II-induced AT_1-R phosphorylation (Qian et al., 1999). In addition, alanine replacement of putative PKC consensus sites (Ser^{331}, Ser^{338}, and Ser^{348}) markedly decreased (70%) TPA-induced AT_1-R phosphorylation and also reduced (60%) the receptor phosphorylation induced by Ang II (Qian et al., 1999). Our data also indicate that Ang II-induced AT_1-R phosphorylation and desensitization are partially dependent on PKC activity in clone 9 cells. It is likely that GRKs play a major role in agonist-induced receptor phosphorylation (Oppermann et al., 1996) and that this may depend on the agonist concentration and the cell type studied.

An interesting finding of the present study was the apparent role of PI3K in AT_1 receptor phosphorylation. We have previously observed that lysophosphatidic acid induces α_{1b}-adrenoceptor phosphorylation. This effect is mediated through LPA receptors coupled to pertussis toxin-sensitive G proteins via PI3K and PKC (Casas-González et al., 2000). To the best of our knowledge, this was the first evidence for a role of PI3K in the phosphorylation of a GPCR. In the present study, the use of selective inhibitors suggests that PI3K is also involved in Ang II-induced AT_1-R phosphorylation. The IC_{50} values obtained for wortmannin and LY294002 (8 and 125 nM, respectively) are comparable with those that inhibit lysophosphatidic acid-induced α_{1b}-adrenoceptor phosphorylation (4 and 60 nM, respectively). Interestingly, the effects of PI3K inhibitors and bisindolylmaleimide I are not additive, which suggests that these families of kinases act sequentially in the same pathway. Phosphorylation of Akt/PKB in response to Ang II and its sensitivity to PI3K inhibitors further suggest a role of the phospholipid kinase in this process.

The PI3K family of enzymes has been grouped into several classes. Class IA PI3K isoforms interact with phosphorylated tyrosine motifs of receptors with intrinsic tyrosine kinase activity, whereas PI3Kγ (a class IB isoform) interacts with heterotrimeric G proteins; such interactions seem to control PI3K activity (Wymann and Pirola, 1998).

PI3K can stimulate PKC through the phosphoinositides generated by its activity. Phosphatidylinositol (3,4)-bisphosphate and phosphatidylinositol (3,4,5)-trisphosphate have been reported to activate both novel (δ, ϵ, and η) and atypical (ζ and λ) PKC isoforms (Nakanishi et al., 1993; Wymann and Pirola, 1998; Rameh and Cantley, 1999). An intermediary kinase, such as the recently identified phosphoinositide-dependent protein kinase-1 (PDK1) (Stephens et al., 1998; Alessi and Cohen, 1998) may also participate in the control of PKC. PDK1, which binds with high affinity to phosphatidylinositol (3,4,5)-trisphosphate, phosphorylates the activation loop sites of PKCζ and PKCδ in vitro and in a PI3K-dependent manner in vivo (Chow et al., 1998; Le Good et al., 1998). Interestingly, the classical PKCα, -βI, and -βII isoforms bind directly to PDK1 coexpressed in human embryonic kidney 293 cells (Le Good et al., 1998), raising the possibility of general control of the PKC family by PDK1.

The partial inhibition of Ang II-induced AT_1-R phosphorylation by pertussis toxin was unexpected, since we previously observed in C9 cells that several Ang II-stimulated responses, including phosphoinositide turnover, calcium mobilization, and proto-oncogene expression, were mediated by pertussis toxin-insensitive G proteins (García-Sáinz et al., 1998). Nevertheless, it is well established that AT_1-R activation in liver cells inhibits adenylyl cyclase and stimulates the synthesis and secretion of angiotensinogen through pertussis toxin-sensitive G_i proteins (Klett et al., 1990; Bauer et al., 1991).

The present findings, and current concepts of receptor phosphorylation/desensitization, suggest the following sequence of events in Ang II-induced AT_1-R phosphorylation in the C9 liver cell line: 1) Ang II activation of AT_1-Rs stimulates both pertussis toxin-insensitive G proteins, probably of the $G_{q/11/14}$ family, and pertussis toxin-sensitive G proteins, probably of the G_i family; 2) activated G proteins catalyze the GDP-GTP exchange reaction that results in the dissociation of G_α subunits and $G_{\beta\gamma}$ complexes; 3) the released $\beta\gamma$ complexes recruit soluble GRKs that phosphorylate AT_1-Rs, promoting the binding of β-arrestin and stabilizing an uncoupled state (Krupnick and Benovic, 1998); and 4) the released $\beta\gamma$ complexes also activate PI3K, which leads to activation of PKC and additional AT_1-R phosphorylation that further contributes to receptor desensitization.

This work also provides evidence that AT_1-R phosphorylation can be induced by activation of other GPCRs, agents that act on different receptors, such as vasopressin and lysophosphatidic acid, and through activation of PKA. PKC was involved in the AT_1-R phosphorylations induced by lysophosphatidic acid and vasopressin, although other pathways also seem to participate. Such cross-talk between receptors could be physiologically important in the many cell types in which AT_1-R are coexpressed with a variety of others GPCRs.

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