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Evidence of an important and direct role for protein kinase C in agonist-induced phosphorylation leading to desensitization of the angiotensin AT_{1A} receptor

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1 The role of protein kinase C (PKC) in the mechanism underlying rapid agonist-induced desensitization of angiotensin AT_1 receptors remains unresolved. A major problem has been to isolate these receptors in a sufficiently purified form to allow study of their phosphorylation state.

2 A cleavable $(His)_6$ affinity tag was introduced into the N-terminus of the recombinant AT_{1A} receptor and stably expressed in human embryonic kidney cells. This affinity tag allowed rapid isolation, purification and determination of the phosphorylation state of the AT_{1A} receptor. Using these cells, we determined the role of PKC in both agonist-induced receptor phosphorylation and desensitization under identical conditions.

3 Agonist-induced phosphorylation of the AT_{1A} receptor was observed at both low and high concentrations of angiotensin II (AII). Preincubation of cells with Ro-31-8220 (a PKC specific inhibitor) revealed that at low concentrations of AII (1 nM), PKC appeared to be the main kinase involved in receptor phosphorylation. In contrast, at high concentrations of AII (100 nM), although PKC-mediated phosphorylation of the receptor was observed, this was overshadowed by a second kinase.

4 In preliminary desensitization studies we observed that at a low concentration of AII, preincubation with Ro-31-8220 attenuated desensitization, whilst at high concentrations of AII (100 nM) it had little or no effect on the level of desensitization observed.

5 These data directly demonstrate an association between PKC-induced receptor phosphorylation and desensitization at low concentrations of AII. Since circulating concentrations of AII are in the picomolar range, we propose that PKC is the physiologically relevant mediator of AT_1 receptor desensitization.

Keywords: AT_{1A} receptor; phosphorylation; protein kinase C; G protein-coupled receptor kinase; desensitization; inositol 1,4,5 trisphosphate; angiotensin II; $(His)_6$ affinity tag; HEK 293 cells; stable expression

Introduction

Angiotensin II (AII) plays an important role in the regulation of blood pressure, vascular tone and electrolyte homeostasis and has been implicated in cardiovascular disease. Through the development of selective non-peptide antagonists (Timmermans *et al*., 1991) two distinct AII receptor subtypes, AT_1 and AT_2 have been identified. Both have subsequently been cloned and are members of the super family of G-protein coupled receptors which possess seven transmembrane α-helices (Murphy *et al*., 1991; Sasaki *et al*., 1991; Kambayashi *et al*., 1993; Mukoyama *et al*., 1993). In some species two subtypes of the AT_1 receptor have been cloned and termed AT_{1A} and AT_{1B}, respectively (Iwai & Inagami, 1992; Sasamura *et al*., 1992). The classical physiological actions of AII are mediated through AT_1 receptors (for review see Timmermans *et al*., 1993). In many cells activation of AT_1 receptors, which are coupled to the phospholipase C signal transduction pathway, results in the generation of the second messengers inositol 1,4,5-trisphosphate and diacylglycerol, which in turn mobilize Ca^{2+} from intracellular stores and activate protein kinase C, respectively (Timmermans *et al*., 1993).

Like many G-protein coupled receptors, persistent or repetitive stimulation of the AT_1 receptor decreases its biological response, that is, it undergoes agonist-induced desensitization. Cumulative data from previous studies suggest that AT_1 receptor desensitization involves an initial uncoupling of the receptor from its G-protein, which may then be followed by sequestration from the cell surface (Bouscarel *et al*., 1988; Abdellatif *et al*., 1991; Boulay *et al*., 1994; Tang *et al*., 1995). Although the mechanism underlying the uncoupling of the AT_1 receptor has proved difficult to demonstrate, studies of other G-protein coupled receptors suggest phosphorylation of the receptor itself either directly or through recruitment of other proteins is likely to inhibit the interaction of receptor with G-protein (for reviews see Hausdorff *et al*., 1990; Sterne-Marr & Benovic, 1995). Phosphorylation of G-protein coupled receptors is mediated by second messenger-dependent enzymes such as protein kinase A and protein kinase C and by specific second messenger-independent G-protein coupled receptor kinases (GRKs). The targets for phosphorylation are the serine and threonine residues located in the intracellular loops and/or carboxyl-terminal regions (for examples see Hausdorff *et al*., 1989; Lattion *et al*., 1994; Eason *et al*., 1995).

Although agonist-induced phosphorylation of AT_{1A} receptors expressed by rat vascular smooth muscle cells has been demonstrated recently, the sites of phosphorylation, the kinases involved and the effect of phosphorylation on receptor coupling were not resolved (Kai *et al*., 1994). The carboxyl-terminal region of the AT_{1A} receptor contains multiple serine and threonine residues, three of which are present in potential consensus sites for protein kinase C phosphorylation (S^{331}TK, S^{338}YR and S^{348}AK). Since stimulation of phospholipase C leads to activation of protein kinase C, it is speculated that protein kinase C may be analogous to protein kinase A in the regulation of adenylate cyclase coupled receptors, and thus, provides a negative feedback mechanism of desensitization. There is substantial indirect evidence for this mechanism (Brock *et al*., 1985; Pfeilschifter, 1986; Pfeilschifter *et al*., 1989; 1990; Abdellatif *et al*., 1991; Ochsner *et al*., 1993; Tang *et al*., 1995) and that this leads to an uncoupling of the AII receptors from their G-proteins (Pfeilschifter & Bauer, 1987; Abdellatif *et al*., 1991). Furthermore, using a truncated AT_{1A} receptor lacking 41 amino acids from the C-terminus which included

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the three putative protein kinase C phosphorylation sites, we recently demonstrated that at least one of these sites was actively involved in the uncoupling of the receptor by protein kinase C (Balmforth *et al*., 1995).

The object of this study was to establish further the role of protein kinase C in the regulation of the AT_{1A} receptor. In particular, we wished to demonstrate directly if the AT_{1A} receptor was a substrate for protein kinase C and to establish the relevance of this phosphorylation event to agonist-induced desensitization. A major problem in applying conventional techniques in direct study of the angiotensin II receptor has been the difficulty of adequate identification and purification of the receptor following solubilization from membrane preparations (see Discussion). To study the phosphorylation state of the receptor we, therefore, stably expressed recombinant AT_{1A} receptors, into which we had introduced a six histidine N-terminal affinity tag, in the human embryonic kidney cell line HEK293. This affinity tag allowed us to isolate, purify and determine rapidly the phosphorylation state of the AT_{1A} receptor.

Methods

Site-directed mutagenesis

The entire coding region of the rat AT_{1A} receptor (a HindIII-NotI fragment of ~2 kb) was removed from the polylinker sites of pBluescript KS^+, gel purified and further digested with EarI. The resultant EarI-EarI (~1275 bp) and EarI-NotI (~600 bp) fragments were gel purified. A synthetic HindIII-EarI fragment containing a $(His)_6$/Factor X cleavage site element after the amino-terminal methionine of the receptor was designed (sense strand; 5′ AGCTTGCTTGTTC-TTTTTG CAGAAGCTCAG AATAAACG CTCAACTTTG-GCAGATCTACCATGCAC C ACCACCACCACCACATC-GAGGGCCGCGCCCTTAACTCTTCT 3′). This fragment is composed of (a) nucleotides 1 through 57, 'Xenopus β-globulin 5′-UTR', (b) nucleotides 58 through 64, 'Kozak sequence', (c) nucleotides 63 through 80, $(His)_6$ coding region, and (d) nucleotides 81 through 92, Factor X cleavage site. The synthetic HindIII-EarI fragment was co-ligated into HindIII-NotI cut pBluescript KS^+ along with the AT_{1A} receptor EarI-EarI and EarI-NotI fragments. Identification of a clone containing the $(His)_6XaAT_{1A}$ cDNA construct was confirmed by dideoxy sequencing with Sequenase II. This construct was subsequently subcloned into the mammalian expression vector pCEP4 by use of the restriction enzymes HindIII and NotI.

Permanent expression of $(His)_6XaAT_{1A}$ receptors in HEK293 cells

Human embryonic kidney cells (HEK293) were cultured in modified Eagles medium (MEM) containing Earle's salts, supplemented with 10% (v/v) foetal calf serum, 1% non-essential amino acids, 50 μg ml^{-1} gentamicin, 100 u ml^{-1} penicillin G, 100 μg ml^{-1} streptomycin and 0.25 μg ml^{-1} amphotericin at 37°C in a humidified atmosphere of air/CO_2 (19 : 1). Cells were transfected with 12 μg of pCEP4 containing the cDNA for the $(His)_6XaAT_{1A}$ receptor by use of Transfectam, according to the manufacturer's instructions. Stable expression of $(His)_6XaAT_{1A}$ receptors in HEK293 cells was achieved by addition of 200 μg ml^{-1} hygromycin B to the medium 3 days after transfection and for all subsequent passages of the cells.

Radioligand binding with intact cells

Cells were grown to confluence in 6-well plates containing MEM supplemented with 200 μg ml^{-1} hygromycin B. Radioligand binding with [^{3}H]-angiotensin II was performed as previously described (Wintersgill *et al*., 1992).

Photoaffinity labelling of the $(His)_6XaAT_{1A}$ receptor

Cells were grown to confluence in 6-well plates. Each well was washed with assay medium (2 ml; MEM containing 20 mM HEPES, 0.25% w/v bacitracin, 0.25% w/v bovine serum albumin, pH 7.4). After washing, 1 ml of assay medium containing the photoaffinity ligand [^{125}I]-[Sar^1,Val^5,L-(4′N_3)Phe^8]angiotensin II (1.25 nM, prepared by the method of Guillemette *et al*., 1986) was added and the cells incubated for 3 h at 4°C. Binding was terminated by washing the cells with ice-cold phosphate buffered saline containing 0.25% w/v bacitracin (2 × 2 ml) and performing all subsequent manipulations at 4°C. Following removal of unbound ligand, the cells were covered with 1 ml of wash buffer and irradiated for 15 min with u.v. light (Phillips mercury vapour lamp HPW 125). Following aspiration the cells were incubated for 90 min at 37°C in Laemmli sample buffer (0.6 ml/well) and the resulting solubilized photolabelled receptors analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS–PAGE; see below).

Phosphorylation of the $(His)_6XaAT_{1A}$ receptor

Cells were grown to confluence in 6-well plates. The cells were washed 3 times (2 ml) with phosphate-free medium DMEM (Dulbecco's modified Eagle's medium) containing 20 mM HEPES (pH 7.4) and supplemented with arginine, cystine, leucine, methionine, 1-inositol, glucose and glutamine and then incubated in 700 μl of the same medium containing 1 mCi ml^{-1} of [^{32}P]-orthophosphate for 90 min at 37°C. Cells were then incubated in medium (1 ml) containing the substance under test at the appropriate concentration and for the appropriate time (see figure legends). The stimulation was terminated by removal of the medium followed by 2 washes in ice-cold phosphate buffer (20 mM sodium phosphate buffer pH 7.8 containing 0.5 M sodium chloride, 0.2 mM AEBSF, 1 μg ml^{-1} aprotinin, 1 mM benzamidine hydrochloride, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin A, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 1 mM ethylenediaminetetraacetic acid). This buffer was then aspirated and the cells snap-frozen in an ethanol-dry ice slurry. The cells were thawed on ice, a further 1 ml of the same buffer was added and the cells resuspended. They were then centrifuged for 15 min at 12,000 *g* to pellet the membranes. The supernatant was discarded and the crude membrane preparations stored frozen until use.

Purification of the $(His)_6XaAT_{1A}$ receptor

The phosphorylated membrane preparations were solubilized in 500 μl of phosphate buffer (pH 7.8) containing 1% triton X-100 for 2 h on ice, with occasional agitation. They were then centrifuged for 15 min at 12,000 *g* and the supernatants retained. The supernatant containing the solubilized receptor was loaded under gravity, 250 μl at a time, on to a Ni-NTA agarose column (1.0 × 1.3 cm) equilibrated with phosphate buffer (pH 7.8) containing 0.1% triton X-100. Each 250 μl aliquot was allowed to adsorb to the column for 15 min. The column was then washed with a further 10 ml of the above buffer to remove non-binding material. It was then washed with 10 ml of phosphate buffer (pH 6.3) containing 0.1% triton X-100 to remove weakly binding proteins and then with 12 ml of the same buffer containing 30 mM imidazole to elute the $(His)_6$Xa-tagged receptor and any other strongly binding material. Fractions (1 ml) were collected at a flow rate of 20 ml h^{-1}. Fractions containing the eluted $(His)_6$Xa-tagged receptor were pooled and concentrated to ~50 μl by use of centricon 30 concentrators. Concentrates were mixed with 1.95 ml of phosphate buffer (pH 7.8) and re-concentrated to reduce the imidazole concentration. The volume of the resulting concentrate was measured and factor Xa protease was added, as supplied, to an end concentration of 100 μg ml^{-1}. Samples were then incubated overnight (15 h) at 23°C to allow

removal of the $(His)_6$ tag of the receptor to occur. After overnight incubation, the samples were made up to 100 μl with phosphate buffer (pH 7.8) containing 0.1% triton X-100 and loaded under gravity on to a second Ni-NTA agarose column (1.0×1.3 cm) equilibrated with phosphate buffer (pH 7.8) containing 0.1% triton X-100. The column was then left for 30 min to allow the sample to adsorb to the column. An initial 300 μl of the equilibration buffer was run on to the column under gravity and the eluate discarded. Then a further 2 ml of buffer was run on to the column under gravity and the eluate retained. This eluate was concentrated by use of centricon 30 concentrators and retained for analysis by SDS–PAGE, as described below.

Deglycosylation of $(His)_6XaAT_{1A}$ receptors

Purified $(His)_6XaAT_{1A}$ receptors were mixed with the endoglycosidase PNGase F, as supplied, to an end concentration of 1.9 u ml^{-1} and incubated for 2 h at 37°C. The reaction was terminated by preparation of the sample for SDS–PAGE, as described below.

SDS–PAGE and autoradiography

Samples were prepared for electrophoresis by addition of an equal volume of 125 mM Tris-HCl (pH 6.7), 6% sodium dodecyl sulphate, 20 mM dithiothreitol, 40% (v/v) glycerol, 0.02% (w/v) bromophenol blue followed by incubation for 2 h at 37°C. The sample constituents were resolved by use of SDS–PAGE with a 10% resolving gel. The gels were silver stained (Morrissey, 1981), dried and then subjected to autoradiography. Autoradiography was carried out by means of REFLECTION autoradiography film and intensifying screens at −70°C. For quantitative comparison of band intensities, the portions of the gels corresponding to the radioactive bands were cut out and subjected to scintillation counting.

Measurement of inositol-1,4,5-trisphosphate (IP_3) production in transfected HEK293 cells

Cells were subcultured on 30 mm petri dishes and used at confluence. All procedures were performed at 37°C, unless otherwise stated. Each dish was washed three times with assay medium (inositol free-DMEM containing 20 mM HEPES, 0.25% BSA, 0.25% bacitracin, pH 7.4; 2 ml) and then incubated in assay medium with and without 10 μM Ro-31-8220 for 10 min. Desensitization was induced by incubation in the absence or presence of 1 nM or 100 nM AII for 3 min, and 100 nM TPA for 10 min. Following removal of stimuli, cells were treated for 1 min with ice-cold 50 mM glycine buffer, pH 3, containing 150 mM NaCl (1 ml) to remove bound AII. After being rinsed three times with assay medium (2 ml), the cells were incubated for 15 s in assay medium (1 ml) with and without 100 nM AII to stimulate IP_3 production. All assays were stopped by the addition of ice-cold 20% trichloroacetic acid (0.5 ml). Subsequent extraction and measurement of IP_3 production was undertaken by use of an inositol-1,4,5-trisphosphate ^3H radioreceptor assay kit according to the manufacturer's instructions.

Protein determination

The method of Lowry *et al.* (1951) was followed with bovine serum albumin as the standard.

Data analysis

The equilibrium dissociation constant (K_D) and concentration of receptor sites (B_{max}) were determined from Scatchard plots by means of linear regression analysis. All data are quoted as the mean of three or more experiments ± s.e.mean.

Materials

[^{3}H]-angiotensin II, Sequenase II, carrier free [^{32}P]-orthophosphate and carrier free [^{125}I]-iodide were purchased from Amersham International plc (Little Chalfont, Bucks, U.K.); REFLECTION autoradiography film and intensifying screens from DuPont (U.K.) Ltd. (DuPont, Stevenage, Herts, U.K.); angiotensin II, TPA (phorbol 12-myristate 13-acetate), bacitracin, benzamidine hydrochloride, aprotinin, leupeptin, pepstatin A and triton X-100 (t-octylphenoxypolyethoxyethanol) from Sigma-Aldrich Company Ltd. (Poole, Dorset, U.K.); [Sar^1(4′-N_3)L-Phe^8]angiotensin II from Peptide Products Ltd. (Downton, Wilts, U.K.); centricon 30 concentrators were from Amicon (Beverly, MA, U.S.A.); Iodo-Gen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) was from Pierce and Warriner (U.K.) Ltd. (Chester, Cheshire, U.K.); Ro-31-8220 ([1-[3-(amidinothio)propyl-1H-indoyl-3-yl]-3-(1-methyl-1H-indoyl-3-yl)-maleimide-methane sulphate), hygromycin B and AEBSF hydrochloride (4-(2-aminoethyl) benzenesulphonylfluoride, hydrochloride) from Calbiochem-Novabiochem (U.K.) Ltd. (Nottingham, U.K.); PNGase F (N-glycosidase F) and factor Xa protease were from New England Biolabs (U.K.) Ltd. (Hitchin, Herts, U.K.); Ni-NTA agarose was from Qiagen Ltd. (Dorking, Surrey, U.K.); all cell culture reagents were purchased from Gibco BRL (Paisley, Scotland, U.K.); foetal calf serum from Sera Lab (Crawley, Sussex, U.K.); human embryonic kidney (HEK293) cells were obtained from the European Collection of Animal Cell cultures (ECACC No 85120602, Porton Down, Salisbury, U.K.); transfectam from Integra Biosciences Ltd. (St. Albans, U.K.); pBluescript KS^+ from Stratagene Ltd. (Cambridge, U.K.); the mammalian expression vector pCEP4 from Invitrogen (San Diego, CA). A synthetic HindIII-EarI fragment containing a $(His)_6$/Factor Xa cleavage site element was designed and synthesized by R & D Systems Europe Ltd. (Abingdon, U.K.). The cDNA clone-encoding the rat angiotensin AT_{1A} receptor was kindly provided by Glaxo Group Research Limited (Greenford Road, Middlesex, U.K.). Losartan (2-n-butyl-4-chloro-5-hydroxymethyl-1-[2′-(1H-tetrazol-5-yl)biphenyl-4-yl)methyl]imidazole, potassium salt) was generously donated by DuPont (Wilmington, U.S.A.).

Results

Stable expression of the $(His)_6XaAT_{1A}$ receptor in HEK 293 cells

The pCEP4 plasmid construct containing the rat angiotensin $(His)_6XaAT_{1A}$ receptor cDNA was stably transfected in HEK 293 cells through selection of a hygromycin B-resistant cell population. The saturability of specific binding of [^{3}H]-angiotensin II was examined as a function of free radioligand (0.5–50 nM). Scatchard analysis revealed a single class of binding site with a B_{max} value of 1897 ± 166 fmol mg^{-1} of total cell protein and an equilibrium dissociation constant (K_D) of 5.9 ± 0.3 nM (Figure 1). Non-specific binding did not exceed 3% of the total radioactivity bound at the K_D. Furthermore, no specific binding was observed in non-transfected cells (data not shown). A comparison of the K_D values of the $(His)_6$-$XaAT_{1A}$ ($K_D = 5.9 \pm 0.3$ nM) and wild type AT_{1A} receptors ($K_D = 4.4 \pm 0.2$ nM) indicates that addition of the affinity tag to the N-terminus does not change the affinity of the receptor for its ligand.

Photoaffinity labelling of $(His)_6XaAT_{1A}$ receptor

Photoaffinity labelling of intact HEK 293 cells either untransfected or expressing $(His)_6XaAT_{1A}$ receptors with [^{125}I]-[Sar^1(4′-N_3)L-Phe^8]angiotensin II was performed to assess the mobility of the receptors on SDS-polyacrylamide gels. No labelling was detected in non-transfected cells (data not shown), whereas in cells expressing the $(His)_6XaAT_{1A}$ receptor, a single

peptide with an apparent molecular weight of 81.5 ± 3.4 kDa ($n = 10$) was specifically labelled (Figure 2a, lane 1). To characterize further the $(His)_6XaAT_{1A}$ receptor, photolabelled receptors were treated with the endoglycosidase PNGase-F, which cleaves N-linked saccharides, since angiotensin II receptors have been previously shown to be highly glycosylated.

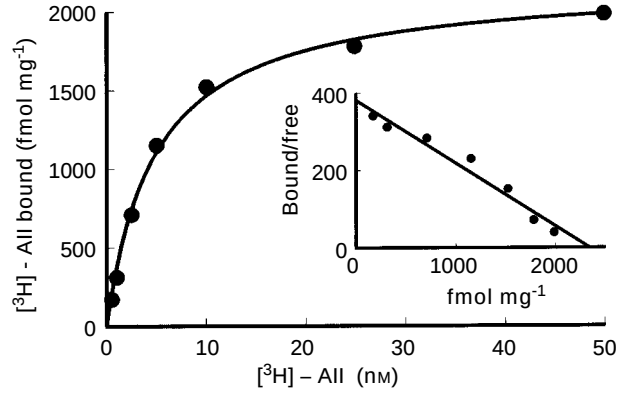

Figure 1 Saturation binding of [^{3}H]-angiotensin II (AII) to intact HEK293 cells stably expressing $(His)_6XaAT_{1A}$ receptors. Specific binding (●), defined as total minus non-specific (binding in the presence of 10 μM AII), was determined following incubation of cells with ligand for 25 min at 37°C. Each point is the mean of triplicate determinations of a single experiment, which is representative of nine separate experiments. The inserts show a Scatchard plot of the data.

Endoglycosidase treatment of photolabelled receptors led to the loss of the single peptide product and the appearance of two new peptide products with reduced molecular weights, indicating the glycoprotein nature of the native receptor. The major peptide product migrated with an apparent molecular weight of 37.7 ± 0.5, whilst the minor product migrated with an apparent molecular weight of 32.1 ± 0.7 kDa ($n = 3$; Figure 2a, lane 3).

Phosphorylation state of $(His)_6XaAT_{1A}$ receptors

To investigate the phosphorylation state of the $(His)_6XaAT_{1A}$ receptor we developed a purification method which took advantage of the high affinity between the $(His)_6$Xa-tag engineered into the N-terminus of the AT_{1A} receptor and a Ni-NTA agarose column (see methods). HEK 293 cells expressing $(His)_6XaAT_{1A}$ receptors were equilibrated with ^{32}P to label their ATP pool and then stimulated with 100 nM AII (3 min). Following purification and SDS-polyacrylamide gel electrophoresis, on autoradiography of unstimulated cells there was no observable phosphorylation band which migrated to the same position on the gel as the photolabelled $(His)_6XaAT_{1A}$ receptor. However, following 100 nM AII stimulation, a readily identifiable phosphorylation band was observed (Figure 2b, lane 2). This phosphorylation event was AT_1 receptor-mediated since it could be blocked by co-incubation of AII with losartan, the AT_1 receptor selective non-peptide antagonist (data not shown). This phosphorylation band was believed to represent the phosphorylated receptor since the apparent molecular weight (82.5 ± 1.4 kDa, $n = 9$) was identical to that

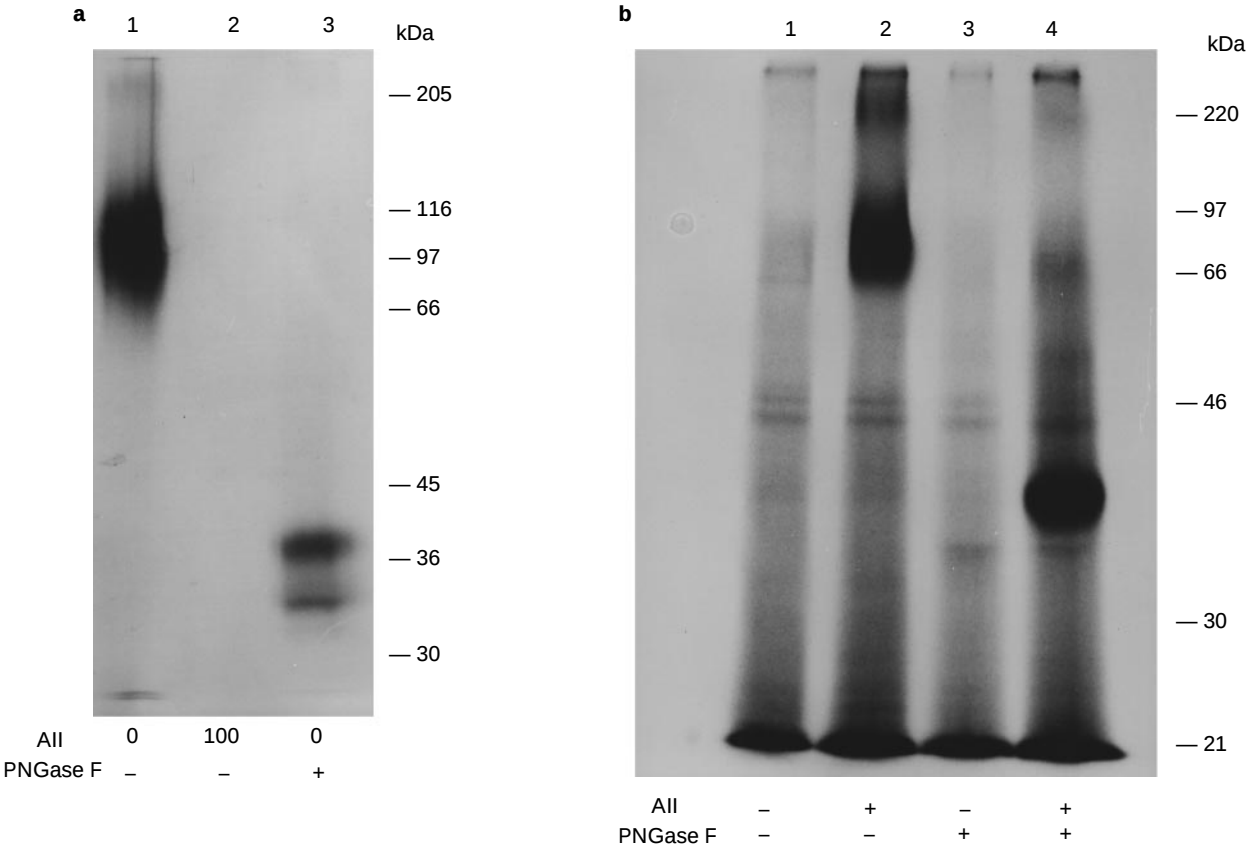

Figure 2 (a) Photoaffinity labelling of $(His)_6XaAT_{1A}$ receptors. HEK 293 cells stably expressing $(His)_6XaAT_{1A}$ receptors were incubated with [^{125}I]-[Sar^1,Val^5, L-(4′N$_3$)Phe^8]angiotensin II (1.25 nM), in the absence (lanes 1 and 3) or presence (lane 2) of 100 nM AII for 3 h at 4°C. Washed, then irradiated for 15 min with u.v. light. Irradiated cells were then either (lanes 1 and 2) incubated for 90 min at 37°C in Laemmli sample buffer (0.6 ml/well) or (lane 3) purified and degycosylated (as described in Methods), and then subjected to 10% SDS–PAGE under reducing conditions. (b) Deglycosylation of phosphorylated $(His)_6XaAT_{1A}$ receptors. HEK 293 cells stably expressing $(His)_6XaAT_{1A}$ receptors were metabolically labelled with ^{32}P and then incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 100 nM AII for 3 min at 37°C. Following receptor solubilization and purification, receptors were incubated in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of PNGase F for 2 h at 37°C, and then subjected to 10% SDS–PAGE under reducing conditions.

of the photolabelled receptor. Further proof of its identity was established through treatment of the purified receptor preparation with PNGase-F. Deglycosylation resulted in the simultaneous substantial loss of the 82 kDa putative phosphorylated receptor and the appearance of a phosphorylation band which migrated on the SDS-polyacrylamide gel with an apparent molecular weight of 37.4 ± 0.3 kDa ($n=3$), identical to that of the major deglycosylated photolabelled receptor product (Figure 2b, lane 4). Finally, omission of the factor Xa cleavage step during purification, which results in retention of the receptor on the second Ni-NTA agarose column, also eliminated the phosphorylation band (data not shown). In addition to 100 nM AII, phosphorylation of the receptor was also detectable following stimulation of cells with 1 nM AII (Figure 3a, lane 3).

To investigate the role of protein kinase C (PKC) in the mechanism underlying AII-induced phosphorylation of the AT$_1$ receptor, we treated cells with both AII and the phorbol ester TPA (a direct activator of PKC) in the presence and absence of the PKC inhibitor Ro-31-8220. Treatment of cells with 100 nM TPA also resulted in the appearance of a phosphorylated receptor (Figure 3b, lane 5; 80.1 ± 2.3 kDa, $n=7$), which was reduced to 36.2 ± 0.6 kDa ($n=4$) following PNGase-F treatment (data not shown). Direct measurement of ^{32}P incorporation into the receptor revealed that PKC-stimulated phosphorylation amounted to $60 \pm 14\%$ ($n=4$) of that stimulated by 100 nM AII. Furthermore, and of particular interest, was the observation that pretreatment of cells with the PKC-specific inhibitor, Ro-31-8220 (10 μM), resulted in almost complete loss of 1 nM AII- and TPA-stimulated phosphorylation but only partially blocked (reduced by $28 \pm 8\%$, $n=4$) that stimulated by 100 nM AII (Figure 3a, b).

Relationship between mechanisms underlying receptor phosphorylation and desensitization

In order to study receptor desensitization we first established that the recombinant (His)$_6$XaAT$_{1A}$ receptor expressed in HEK293 cells coupled to the phospholipase C signal transduction pathway. Using a radioreceptor binding assay to measure IP$_3$ formation, we established that treatment of cells with 100 nM AII resulted in a rapid increase in IP$_3$ production. The increased production of IP$_3$ was maximal within 15–20 s and represented an 8.2 ± 0.3 ($n=4$) fold increase in respect to untreated cells. We then proceeded to investigate the relationship between AT$_{1A}$ receptor phosphorylation and agonist-induced desensitization by determining the effects of pretreating cells, under the conditions previously found to induce receptor phosphorylation, on their subsequent ability to generate an IP$_3$ response to 100 nM AII (Figure 4). Desensitization of the subsequent AII response was observed following pretreatment of cells with either 1 nM (reduced to $39.6 \pm 6.5\%$ of untreated cell response, $n=4$) or 100 nM AII (reduced to $27.8 \pm 5.9\%$ of untreated cell response, $n=4$). In addition to AII, pretreatment of cells with 100 nM TPA also resulted in receptor desensitization (reduced to $36.7 \pm 5.3\%$ of untreated

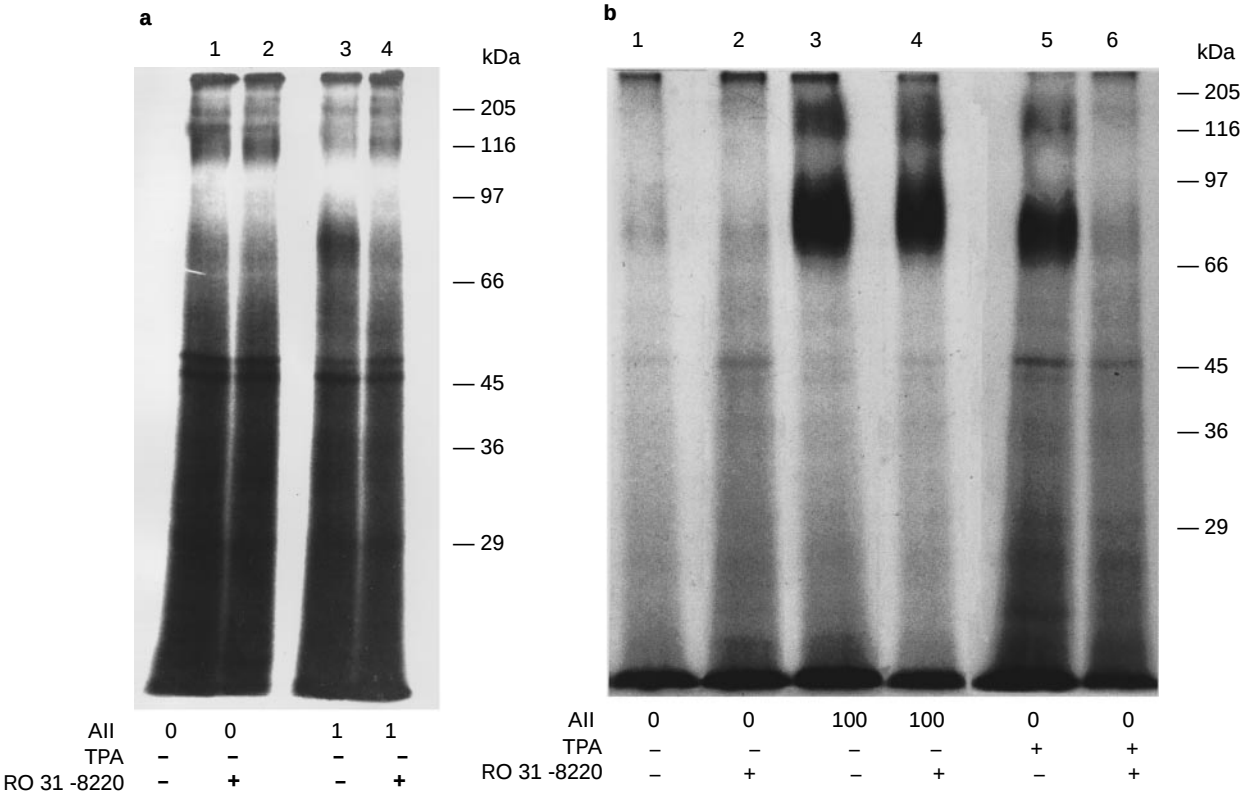

Figure 3 (a) Attenuation of 1 nM AII-induced (His)$_6$XaAT$_{1A}$ receptor phosphorylation by PKC inhibition. HEK 293 cells stably expressing (His)$_6$XaAT$_{1A}$ receptors were metabolically labelled with ^{32}P and then preincubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 10 μM Ro-31-8220 for 10 min at 37°C before cellular stimulation. Cells were then stimulated at 37°C, in the continued presence or absence of 10 μM Ro-31-8220, with medium alone (lanes 1 and 2,) or 1 nM AII (lanes 3 and 4) for 3 min. Following receptor solubilization and purification, receptor preparations were subjected to 10% SDS–PAGE under reducing conditions. (b) Attenuation of 100 nM AII- and TPA-induced (His)$_6$XaAT$_{1A}$ receptor phosphorylation by PKC inhibition. HEK 293 cells stably expressing (His)$_6$XaAT$_{1A}$ receptors were metabolically labelled with ^{32}P and then preincubated in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of 10 μM Ro-31-8220 for 10 min at 37°C before cellular stimulation. Cells were then stimulated at 37°C, in the continued absence or presence of 10 μM Ro-31-8220, with medium alone (lanes 1 and 2) or with 100 nM AII for 3 min (lanes 3 and 4) or 100 nM TPA for 10 min (lanes 5 and 6). Following receptor solubilization and purification, receptor preparations were subjected to 10% SDS–PAGE under reducing conditions.

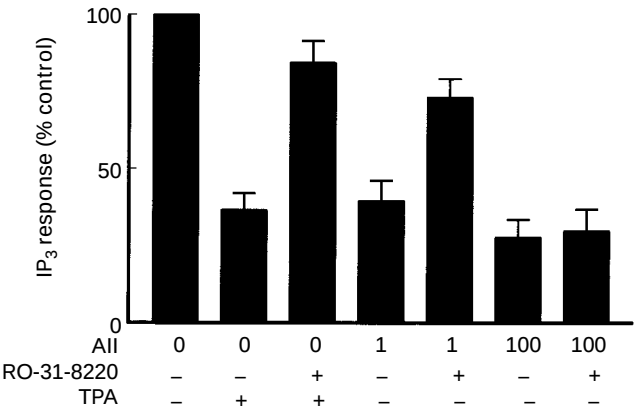

Figure 4 The effect of PKC inhibition on agonist-induced AT_{1A} receptor desensitization mediated by low and high concentrations of angiotensin II. HEK 293 cells stably expressing $(His)_6XaAT_{1A}$ receptors were preincubated in the absence or presence of 10 μM Ro-31-8220 for 10 min at 37°C, before receptor desensitization. Cells were then stimulated at 37°C, in the continued presence or absence of 10 μM Ro-31-8220, with either medium alone, 100 nM TPA for 10 min, 1 nM AII for 3 min or 100 nM AII for 3 min. Cells were subsequently acid-washed, then stimulated with 100 nM AII for 15 s and IP_3 levels measured as described under Methods. The results represent the means ± s.e.mean of three or four independent experiments. Each experiment was performed in triplicate. The IP_3 response produced in each experiment by 100 nM AII in cells not desensitized was taken as 100% and the IP_3 responses in the desensitized cells are expressed as a percentage of this maximal response.

cell response, $n=3$), implicating PKC in AT_{1A} receptor desensitization. To test this hypothesis, we also pretreated cells in the presence of the PKC-specific inhibitor Ro-31-8220. AT_{1A} receptor desensitization by TPA pretreatment was almost completely inhibited by Ro-31-8220 (84.5 ± 6.9% of untreated cell response, $n=3$), indicating PKC as the major mediator of this effect. In contrast, the presence of Ro-31-8220 during 100 nM AII pretreatment did not appear to alter the resulting level of AT_{1A} receptor desensitization observed (30.3 ± 6.8% of untreated cell response, $n=4$). However, in the presence of Ro-31-8220, AT_{1A} receptor desensitization induced by 1 nM AII was markedly inhibited (73.4 ± 5.9% of untreated cell response, $n=4$).

Discussion

The biochemical mechanisms underlying the rapid desensitization of G-protein coupled receptors have been the focus of intense investigation. Studies of the β_2-adrenoceptor have established two particular mechanisms, one second messenger-dependent and the other second messenger-independent (for review see Hausdorff *et al*., 1990). Both involve uncoupling of the receptor from its G protein through phosphorylation of serine and/or threonine residues within the intracellular loops and carboxyl-terminal region, either by protein kinases A and C or the second messenger-independent G-protein-coupled receptor kinases (GRKs; for review see Sterne-Marr & Benovic, 1995). The relative contributions of these two mechanisms to the rapid agonist-induced desensitization of the angiotensin AT_1 receptor remain unclear in spite of considerable research effort. However, most recently Oppermann *et al*. (1996) have argued that GRK mediated phosphorylation plays the dominant role in such regulation. In a previous study we established that consensus sites for PKC, located in the carboxyl-terminal region of the AT_{1A}, were actively involved in receptor uncoupling (Balmforth *et al*., 1995). The results of the present study support our earlier findings and demonstrate directly a pivotal role for PKC-induced AT_1 receptor phosphorylation in desensitization at agonist concentrations of AII likely to occur under physiological circumstances.

$(His)_6$ affinity tag purification and identification of AT_{1A} receptors

The first essential step of our work was to establish a method to isolate and purify rapidly the AT_{1A} receptor. To circumvent problems with traditional purification strategies (Capponi & Catt, 1980; Marie *et al*., 1990) we introduced a six histidine affinity tag into the N-terminus of a recombinant AT_{1A} receptor. The histidine tag allows a rapid purification of proteins by means of Ni^{2+}-NTA affinity chromatography under both denaturing (Hochuli *et al*., 1988) and native conditions (Janknecht *et al*., 1991). In preliminary studies we established conditions to rapidly purify a $(His)_6AT_{1A}$ receptor and established that incorporation of the histidine tag did not affect receptor function (Balmforth *et al*., 1994a,b). However, subsequent studies established that in spite of the high level of purification achieved it was insufficient to allow identification of phosphorylated receptors. We therefore introduced a further four amino acids (IleGluGlyArg) into the N-terminus, to permit removal of the $(His)_6$ tag by factor Xa cleavage, and a second Ni^{2+}-NTA affinity chromatography step. This allowed us to establish a technique in which factor Xa treated contaminating proteins eluted from the first Ni^{2+}-NTA affinity chromatography column, bound to the second, whilst factor Xa treated $(His)_6XaAT_{1A}$ receptors were collected in the flow through. An important factor during the establishment of this methodology was the use of photoaffinity labelling of the $(His)_6XaAT_{1A}$ receptor to follow reliably the receptor through the purification steps and later to identify the phosphorylated receptor on the SDS–PAGE gel.

Application of photoaffinity labelling identified the recombinant $(His)_6XaAT_{1A}$ receptor expressed in HEK 293 cells as a single broad radioactive band which migrated in a SDS–PAGE gel with an apparent molecular weight of 81 kDa. This observation is in keeping with previous AII receptor photoaffinity labelling studies (Guillemette *et al*., 1986; Carson *et al*., 1987; Tence & Petit, 1989; Rondeau *et al*., 1990). Although significantly higher than the predicted molecular weight of 42 kDa, treatment of the photolabelled receptor with endoglycosidase PNGase-F established that this difference was caused by postranslational glycosylation (Carson *et al*., 1987; Desarnaud *et al*., 1993).

Receptor phosphorylation by angiotensin II

We observed that stimulation of a HEK 293 cells with 100 nM AII (3 min) resulted in a readily identifiable phosphorylation band which migrated to the same position on the gel as the photolabelled $(His)_6XaAT_{1A}$ receptor. This phosphorylation event was AT_1 receptor-mediated since it could be blocked by co-incubation of AII with losartan, the AT_1 receptor selective non-peptide antagonist. That this band was the phosphorylated AT_{1A} receptor was validated by comparing its characteristics to those previously established for the photolabelled receptor. In addition to its identical molecular weight in the native form, treatment of the purified receptor preparation with PNGase-F also resulted in the simultaneous substantial loss of the 82 kDa phosphorylation band and the appearance of a new phosphorylation band with an apparent molecular weight of 37 kDa, identical to that of the major deglycosylated photolabelled receptor product. Furthermore, omission of the factor Xa cleavage step during purification, which results in retention of the photolabelled receptor on the second Ni-NTA agarose column, also eliminated the phosphorylation band.

Receptor phosphorylation by the phorbol ester TPA

A major observation was that phosphorylation of the AT_{1A} receptor was also observed following exposure to the phorbol ester TPA. Furthermore, pretreatment of cells with the PKC

inhibitor Ro-31-8220 abolished the effect of TPA on AT_{1A} receptor phosphorylation and partially attenuated the effect of AII (100 nM). These data not only firmly establish that the AT_{1A} receptor is a substrate for PKC, but also indicate that PKC participates in agonist-induced phosphorylation. Similar findings have been described for other receptors coupled to phospholipase C (Ali *et al*., 1993; 1994; Alaluf *et al*., 1995). However, such findings are not universal. A series of receptors have been observed to be phosphorylated by phorbol esters and their respective agonists, but inhibition of PKC activity was found only to attenuate the effect of the phorbol esters (Ali *et al*., 1993; Tobin & Nahorski, 1993; Ishii *et al*., 1994; Lattion *et al*., 1994; Kroog *et al*., 1995). These studies therefore concluded that the mechanism underlying agonist-induced phosphorylation did not include PKC.

The importance of PKC-induced receptor phosphorylation

The inability of Ro-31-8220 to attenuate completely agonist-induced phosphorylation of the AT_{1A} receptor suggests that, in addition to PKC, a second distinct kinase, also participates. By analogy to other G protein-coupled receptors, the most likely candidate is a member of the novel family of G protein coupled receptor kinases (GRKs; for review see Sterne-Marr & Benovic, 1995). These kinases have the unique feature of only phosphorylating agonist occupied receptors and thus the extent of their participation in agonist-induced receptor phosphorylation is ultimately controlled by the concentration of the stimulating agonist (Palczewski & Benovic, 1991). This is an important issue since in many studies pharmacological, rather than physiological concentrations of agonists have been used to address the importance of GRKs in both agonist-induced phosphorylation and desensitization. In the present study, the K_D of the $(His)_6XaAT_{1A}$ receptor was 5.9 nM and thus at a concentration of 100 nM AII, over 90% of the receptors would be occupied. This would result in near maximal phosphorylation by one or more GRKs whatever the state of activation of PKC. The observation that much of the receptor phosphorylation induced by 100 nM AII was PKC-independent is not therefore surprising and would tend to favour GRKs as the dominant underlying mechanism at maximal occupation. In contrast, at a sub-saturating concentration of 1 nM AII, only some 10% of the receptors would be occupied. At this concentration, we observed receptor phosphorylation was almost completely attenuated by Ro-31-8220, suggesting PKC as the major mechanism. Thus the extent of participation of PKC and GRKs in phosphorylation of the AT_{1A} receptor is governed by the level of AII presented at the cell surface.

The relationship between phosphorylation of the receptor and its desensitization

A second aim of this study was to determine the relationship between receptor phosphorylation and agonist-induced desensitization. To relate these we performed preliminary desensitization studies under conditions identical to those previously established in the phosphorylation studies. The extent of desensitization after exposure to either a low non-saturating or a large saturating concentration of AII was quantified by measuring mass IP_3 production induced by a subsequent 15 s application of a saturating level of AII (100 nM) to the cells. In keeping with the phosphorylation studies, the mechanisms underlying desensitization at low (1 nM) and high (100 nM) AII concentrations appeared different. Pretreatment of cells with 1 nM AII or 100 nM TPA produced similar levels of desensitization, which were markedly attenuated in the presence of Ro-31-8220, indicating a common mechanism of desensitization involving PKC. However, the attenuation of 1 nM AII desensitization by Ro-31-8220 tended to be slightly lower than that by TPA, suggesting that unlike TPA, a minor PKC-independent mechanism was also involved. In contrast, pretreatment of cells with 100 nM AII produced similar levels of desensitization in the absence or presence of Ro-31-8220. An initial response to this observation might be that this lack of attenuation by PKC inhibition means that at high concentrations of AII the PKC-dependent mechanism is not functioning. However, a more logical interpretation is that PKC uncouples the receptor at low and high concentrations of stimulating AII, but at high concentrations the GRK-induced uncoupling masks any role of PKC.

The initial step involved in rapid desensitization is an uncoupling of the AT_1 receptor from its G-protein (Abdellatif *et al*., 1991; Tang *et al*., 1995). Our evidence here supports phosphorylation of the receptor as the mechanism responsible for this uncoupling. The involvement of PKC in uncoupling has been speculated for some time, since activation of PKC is part of the signalling pathway of the AT_1 receptor. However, previous data have been both for (Pfeilschifter, 1988; Sakuta *et al*., 1991; Ochsner *et al*., 1993; Sasamura *et al*., 1994; Tang *et al*., 1995) and against (Abdellatif *et al*., 1991; Thekkumkara *et al*., 1995). Of particular relevance to this study are the essentially identical observations with recombinant mouse AT_{1A} receptors (Sasamura *et al*., 1994) and rat AT_{1B} receptors (Tang *et al*., 1995). At similar low concentrations of AII (0.1 – 1 nM) as used by us, both PKC depletion and PKC specific inhibitors attenuated desensitization, whilst at high concentrations of AII (100 nM) these interventions had little or no effect on the level of desensitization observed.

There are only two previous studies of AT_1 receptor phosphorylation. The first (Kai *et al*., 1994) examined AT_1 receptors expressed endogenously by rat aortic vascular smooth muscle cells. Using a polyclonal antibody raised against amino acids 15 through 24 of the rat AT_{1A} receptor, they demonstrated immunoprecipitation of the AT_1 receptor as a 52 kDa phosphoprotein. AII induced rapid phosphorylation of this protein. However, they did not observe any phosphorylation through PKC activation. In the second study of AT_{1A} receptor phosphorylation (Oppermann *et al*., 1996), published near to the completion of our work, transiently expressed HA-epitope tagged recombinant AT_{1A} receptors were used and immunoprecipitation showed clearly that GRKs phosphorylate the AT_{1A} receptor and induce desensitization. In keeping with our findings they also observed AT_{1A} receptor phosphorylation through AII stimulation of PKC. However, they gave little credence to a role for PKC in agonist-induced desensitization as they found inhibition of this enzyme had no effect on desensitization, but used only a high (100 nM) concentration of AII. Our studies, taken together with the desensitization findings of two other groups (Sasamura *et al*., 1994; Tang *et al*., 1995) confirm this, but more importantly illustrate clearly that at lower concentrations (0.1 – 1 nM) of AII, the PKC-dependent mechanism is predominant. Furthermore, our findings directly demonstrate an association between PKC-induced receptor phosphorylation and desensitization at low concentrations of AII.

In summary, using a cell line stably expressing affinity tagged AT_{1A} receptors we have shown agonist-dependent receptor phosphorylation results in homologous desensitization. This phosphorylation is predominantly through PKC at low concentrations of AII. In contrast, at high concentrations of AII, although PKC still contributes, receptor phosphorylation is dominated by GRK activity. Circulating concentrations of AII are in the picomolar range, though higher local concentrations may be present in tissues. The PKC mechanism, predominant in the desensitization at low concentrations of AII (1 nM), seems likely to be the physiologically relevant mediator of AT_1 receptor desensitization.

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