Identification of protein kinase C phosphorylation sites in the angiotensin II (AT_{1A}) receptor

Hongwei QIAN, Luisa PIPOLLO and Walter G. THOMAS¹

Molecular Endocrinology Laboratory, Baker Medical Research Institute, PO Box 6492, St. Kilda Road Central, Melbourne, Victoria 8008, Australia

Protein kinase C (PKC) phosphorylates the C-terminus of the type 1 angiotensin II receptor (AT₁), although the exact site(s) of phosphorylation are unidentified. In the present study, we examined the phosphorylation of epitope-tagged wild-type AT_{1A} receptors, transiently expressed in Chinese hamster ovary K1 cells, in response to angiotensin II (AngII) and following selective activation and inhibition of PKC. This phosphorylation was compared with mutant receptors where C-terminal serine residues (Ser³³¹, Ser³³⁸ and Ser³⁴⁸) within three putative PKC consensus sites were replaced with alanine, either individually or in combination. Stimulation by AngII or the phorbol ester PMA to activate PKC induced an increase in phosphorylation of the wild-type AT_{1A} receptor, which was prevented by truncation of the receptor C-terminus to remove the last 34 amino acids, including Ser³³¹, Ser³³⁸ and Ser³⁴⁸. Whereas single alanine mutation (Ser³³¹Ala, Ser³³⁸Ala and Ser³⁴⁸Ala) resulted in decreased receptor phosphorylation, no single mutant completely inhibited either AngII- or PMA-induced phosphorylation. Combined mutation of the three PKC consensus sites caused an $\approx 70\%$ reduction in PMA-mediated phosphorylation. The $\approx 60\%$ reduction in AngII (1 μ M)-induced phosphorylation of this triple mutant and the partial inhibition of wild-type receptor phosphorylation by bisindolylmaleimide I, a specific PKC inhibitor, suggest a significant contribution of PKC to agonist-

stimulated regulation. The ratio of PKC to total receptor phosphorylation was greatest at low doses of AngII (1 nM), consistent with the idea that PKC phosphorylates and regulates receptor function at low levels of stimulation, whereas phosphorylation by other kinases is more prevalent at high levels of agonist stimulation. To determine if a single PKC site is favoured when the contribution of PKC varies, the phosphorylation of wild-type and mutant receptors was examined over a range of AngII concentrations (0, 1, 10 and 100 nM). At all AngII concentrations, single mutation of Ser³³¹, Ser³³⁸ or Ser³⁴⁸ was incapable of completely preventing receptor phosphorylation, suggesting no clear preference for PKC consensus-site utilization. Together, these results indicate a redundancy in PKC phosphorylation of the AT_{1A} receptor, whereby all three consensus sites are utilized to some degree following homologous (AngII) and heterologous (PMA) stimulation. The contribution of PKC phosphorylation to receptor regulation is unclear, but multiple PKC phosphorylation of the AT_{1A} receptor may allow independent and/or complementary events to occur at the three separate sites of the C-terminus.

Key words: desensitization, G-protein-coupled receptor, kinases, receptor regulation, site-directed mutagenesis.

INTRODUCTION

The peptide hormone angiotensin II (AngII) regulates vasoconstriction, water and salt balance, neuromodulation and cellular growth through actions on two types of AngII receptor (type 1, AT₁, and type 2, AT₂) [1,2]. The AT₁ receptor, which has two subtypes (AT_{1A} and AT_{1B}) in rodents, is the principal mediator of the biological actions of AngII. AT₁ receptors are members of the seven-transmembrane-spanning G-protein-coupled receptor (GPCR) superfamily and activate G-proteins through membrane-proximal regions of the third cytoplasmic loop [3,4] and C-terminus in the receptor [5,6]. AT₁ activation by AngII results in the generation of inositol trisphosphate and diacylglycerol, via a phospholipase C β -coupled G-protein (G α q/11), to cause release of calcium from intracellular stores and activation of protein kinase C (PKC), respectively. AngII also activates mitogen-activated protein kinases, tyrosine phosphorylation of cellular substrates and the JAK-STAT (Janus kinase-signal transduction and activators of transcription) pathway [7]. AT₁-receptor signalling is rapidly desensitized by mechanisms that, by analogy to other GPCRs [8,9], probably

involve phosphorylation of the receptor, the association of proteins termed arrestins with the phosphorylated receptors and the removal of activated receptors from the cell surface by internalization. The exact molecular mechanism of AT₁-receptor desensitization and internalization is, however, unclear and seems to differ significantly from that reported for the β -adrenergic receptor [10–12].

The phosphorylation of GPCRs is catalysed by two main types of kinase: GRKs (GPCR kinases, six members of which have been identified [13]) or second-messenger-activated kinases, such as protein kinase A and PKC [14]. GRKs are thought to phosphorylate GPCRs when activated by agonist and therefore result in an agonist-specific or homologous desensitization [13]. In contrast, PKC, which is activated by diacylglycerol, can phosphorylate receptors in the absence of agonist, and hence any stimulus that activates PKC can potentially lead to phosphorylation and a heterologous uncoupling of receptor from signalling pathways.

The phosphorylation of AT_{1A} receptors, and the kinases involved, has been investigated recently. In a variety of cell lines transfected with epitope-tagged AT_{1A} receptors [15–19], and in

Abbreviations used: AngII, angiotensin II; PKC, protein kinase C; AT₁, AT_{1A}, AT_{1B}, AT₂, AngII receptor subtypes; CHO-K1 cells, Chinese-hamster ovary K1 cells; GPCR, G-protein-coupled receptor; GRK, GPCR kinase; HA, influenza haemagglutinin antigen; α -MEM, α -minimal essential medium; HBSS, Hanks buffered salt solution; BIM, bisindolylmaleimide I hydrochloride.

¹ To whom correspondence should be addressed (e-mail walter.thomas@baker.edu.au).

adrenal glomerulosa cells expressing endogenous AT₁ receptors [19], AngII stimulation caused phosphorylation of the AT_{1A} receptor, which was resolved by SDS/PAGE as a broad band in the range 60000–150000 Da. The AT₁ receptor can be phosphorylated by GRKs and PKC [15], although the exact sites of phosphorylation are not known. PKC appears to be the dominant kinase at low levels of agonist stimulation, whereas at concentrations that cause maximal phosphorylation, GRKs are evoked [16]. The rat AT_{1A} receptor C-terminus contains 13 serine/threonine potential phosphorylation sites, three of which are present in consensus sites for PKC phosphorylation (Ser³³¹-Thr-Lys, Ser³³⁸-Tyr-Arg and Ser³⁴⁸-Ala-Lys). Coincident reports from Smith et al. [19] and ourselves [18] have shown that truncation of the AT₁ C-terminus (to remove all C-terminal serines and threonines) abolishes AngII-induced phosphorylation, identifying the C-terminus as the major, if not sole, site of AT₁ phosphorylation.

In this study, the putative PKC phosphorylation sites for the AT_{1A} receptor were examined using N-terminal influenza haemagglutinin antigen (HA)-tagged AT_{1A} receptors, which were constructed with individual or combined alanine mutations at Ser³³¹, Ser³³⁸ and Ser³⁴⁸ and transiently expressed in Chinese-hamster ovary K1 cells (CHO-K1 cells). Using varying concentrations of AngII to promote AT_{1A} phosphorylation, in combination with direct activators and inhibitors of PKC, we show that PKC is capable of phosphorylating the AT_{1A} receptor at all three consensus sites.

METHODS AND MATERIALS

Reagents and cell-culture materials

¹²⁵I-AngII (specific radioactivity > 2000 Ci/mmol) was kindly supplied by Dr. Conrad Sernia (Department of Physiology and Pharmacology, University of Queensland, Brisbane, Australia). CHO-K1 cells were obtained from the American Type Culture Collection. The ExSite Mutagenesis kit was purchased from Stratagene; DNA-modifying enzymes from Promega; and 5'-phosphorylated oligonucleotides and [³²P]orthophosphate from Geneworks. α -Minimal essential medium (α -MEM), OPTI-MEM, Hanks buffered salt solution (HBSS), okadaic acid and lipofectAMINE were purchased from Life Technologies, and Protein A-agarose was from Boehringer Mannheim. The 12CA5 monoclonal antibody was purified from hybridoma culture media using an HA-peptide affinity column. The PKC inhibitor, bisindolylmaleimide I hydrochloride (BIM, GF109203X), was purchased from Calbiochem. All other chemicals were from Sigma or BDH Laboratory Supplies.

Receptor constructs, epitope tagging and mutagenesis

The cloning and incorporation of the full-length rat AT_{1A} receptor (coding for 359 amino acids) into the pRc/CMV mammalian expression vector (pRc2A/AT_{1A}) has been described previously [20]. The HA epitope (YPYDVPDYA), which allows immunoprecipitation of AT_{1A} receptors by the monoclonal antibody 12CA5, was subsequently inserted at the N-terminus of the AT_{1A} receptor (pRcNHA/AT_{1A}) [18].

Substituting Ser³³¹, Ser³³⁸ and Ser³⁴⁸ with alanine, either individually or in combination, generated four mutated versions of this N-terminally HA-tagged receptor (NHA-AT1A), i.e. S331A, S338A and S348A, and S331/338/348A, respectively (see Figure 1). These various mutations were introduced into the wild-type HA-tagged AT_{1A} receptor expression vector (pRcNHA/AT_{1A}) using PCR-based site-directed mutagenesis (ExSite, Stratagene). 5'-Phosphorylated oligonucleotides used

for mutagenesis were (5'-3'): S338SW, ^p-ACGAAATGAG-CACGCTTKCTTACCGG (sense); S331ASW, ^p-AGMCAG-GCTCGAGTGGGACTTGGCC (antisense); S348AS, ^p-GCGG-CCAAAAAGCCTGCGTC (sense); and S348AAS, ^p-GGAG-CTCATGTTATCCGAAGG (antisense).

The first pair of primers, S338SW and S331ASW, contained 'wobble' nucleotides (K = T or G, M = A or C) that were designed to generate two point mutants, S331A and S338A, or one double mutant, S331/338A. The second pair of primers, S348AS and S348AAS, was used to generate either a point mutant (S348A) or a triple mutant (S331/338/348A) when the double mutant (S331/338A) was used as template. A silent *Xho*I (bold; primer S331ASW) or *Sac*II (bold; formed after ligation of PCR product generated by primers S348AS and S348AAS) restriction site was introduced to assist with the screening for mutated clones. Mutations from the original sequence are underlined. The major 6.7-kb PCR bands, representing the linearized mutated plasmids, were in-gel purified and blunt-ended ligated to circularize and reform the expression plasmids. After transformation into XL1-blue *Escherichia coli* and plating on Luria broth/ampicillin plates, plasmid-bearing colonies were screened for the relevant silent restriction site. Positive clones for each receptor mutant were sequenced to confirm the entire coding region and the relevant nucleotide mutations.

Expression of epitope-tagged wild-type and mutated AT_{1A} receptors in CHO-K1 cells

CHO-K1 cells were maintained in α -MEM containing 10% foetal bovine serum, penicillin G sodium (100 μ g/ml), streptomycin sulphate (100 μ g/ml) and amphotericin B (0.25 μ g/ml) (complete media), seeded in 12-well culture plates and grown in complete media until 70–80% confluent. Cells washed in serum-free OPTI-MEM were transiently transfected with 0.6 μ g/well of either epitope-tagged wild-type or mutated AT_{1A}-receptor plasmid DNA using lipofectAMINE, as described previously [21]. After a 5-h exposure to DNA-lipofectAMINE complexes, cells were washed and grown in complete media for a further 48 h.

Determination of receptor-binding affinity and expression

The cell-surface expression and affinity of wild-type receptor and the PKC-phosphorylation-site mutants (S331A, S338A, S348A and S331/338/348A) was determined by homologous competitive binding analysis using ¹²⁵I-AngII as described previously [18]. In brief, transiently transfected cells in 12-well plates were washed twice with ice-cold HBSS and incubated (5 h at 4 °C) in a receptor-binding-assay buffer [21] containing 30 pM ¹²⁵I-AngII and various concentrations of unlabelled AngII (0, 10⁻¹⁰, 10⁻⁹, 3 $\times$ 10⁻⁹, 10⁻⁸ and 10⁻⁷ M). At equilibrium, the cells were washed three times with ice-cold binding buffer and harvested with a 0.25 M NaOH/0.25% SDS solution. Cell-associated radioactivity was plotted against the concentration of unlabelled AngII added and the data fitted by non-linear regression using GraphPad Prism (GraphPad Software). Dissociation constants (K_d) and cell-surface receptor densities (B_{max}) were determined as described by Swillens [22].

Phosphorylation and immunoprecipitation of AT_{1A} receptors

The procedure for phosphorylation and immunoprecipitation of transiently transfected HA-tagged AT_{1A} receptors in CHO-K1 cells was as described previously by Thomas et al. [18], with

Figure 1 Schematic representation of the AT_{1A} receptor and mutagenesis of putative PKC phosphorylation sites in the C-terminus

An N-terminal HA epitope was engineered into the seven-transmembrane spanning AT_{1A} receptor (NHA-AT_{1A}) to allow immunoprecipitation of expressed receptors. Shown in detail (single-letter amino acid code) is the entire cytoplasmic AT_{1A} receptor C-terminus from Leu³⁰⁵ to Glu³⁵⁹. Brackets at positions Ser³³¹, Ser³³⁸ and Ser³⁴⁸ highlight the three PKC consensus phosphorylation sites, and the location of a truncation (after Lys³²⁵) to remove all C-terminal serine/threonine residues is indicated. Single- and triple-point mutations of Ser³³¹, Ser³³⁸ and Ser³⁴⁸ to alanine and the truncated mutant (TK325) are listed.

minor modification. In brief, the transfected cells in 12-well plates were serum-starved for 16 h, loaded with [³²P]P_i (200 µCi/ml) and stimulated by the agonist AngII (1 nM–1 µM, 10 min) or PMA (2 µM, 8 min) at 37 °C. When required, the PKC inhibitor, BIM (2 µM), was added for 30 min at 37 °C prior to agonist stimulation. After stimulation, the plates were placed on ice, washed twice with 1 ml/well of HBSS (4 °C), and solubilized by the addition of lysis buffer (0.3 ml/well) containing phosphatase inhibitors. The cell lysates were harvested and clarified by centrifugation (14000 *g* for 15 min) and precleared by the addition of Protein A-agarose and BSA (1 h, 4 °C). Precleared lysates were incubated with 2 µg of affinity-purified 12CA5 antibody and 20 µl of Protein A-agarose, and agitated overnight at 4 °C to immunoprecipitate the epitope-tagged AT_{1A} receptors. The immunoprecipitates were washed five times, resuspended in 55 µl of a urea-based SDS sample buffer, heated at 60 °C for 15 min and resolved by SDS/10% PAGE. Gels were fixed, dried and then placed against Fuji-type BAS-III PhosphorImaging plates for overnight exposure. The plates were subsequently read in a FUJIX Bio-imaging Analyser BAS 1000 (Fuji Photo Film) and the data were analysed using MacBAS version 1.0 software. For autoradiography, gels were exposed against Biomax MS film (Eastman Kodak, Rochester, NY,

U.S.A.) and a Biomax TranScreen-HE (High Energy) for 3–10 h at –80 °C.

In all experiments, the quantitative PhosphorImaging data were normalized for receptor expression at the cell surface by transfecting parallel plates of CHO-K1 cells with the various constructs and performing AngII radioreceptor-binding assays. Binding assays were performed at 4 °C for 5 h to prevent receptor internalization.

Changes in receptor phosphorylation for the wild-type and mutant AT_{1A} receptors are described in terms of fold-increase, determined by subtracting the normalized PhosphorImaging units of unstimulated lanes from stimulated lanes and then dividing by the unstimulated value. This standardization was performed because arbitrary units and basal levels of receptor phosphorylation vary for the various receptor constructs and between studies.

Determination of AT_{1A}-receptor internalization

Internalization kinetic assays on wild-type and mutated receptors were done as described previously [21]. Briefly, transfected CHO-K1 cells in 12-well plates were exposed to [¹²⁵I]-AngII (0.4 nM) in receptor-binding buffer for 2, 5, 10 and 20 min at 37 °C. Internalization was terminated, and cell-surface-bound [¹²⁵I]-AngII was removed by acid washing, whereas internalized [¹²⁵I]-AngII-receptor complexes were harvested with a 0.25 M NaOH/0.25% SDS solution. An index of internalization was obtained by expressing the acid-insensitive radioactivity (internalized receptors) as a percentage of the total binding (acid-insensitive + acid-sensitive) for each well. The percentage of internalized receptors was plotted against time and analysed as a one-phase exponential association using GraphPad Prism. The *t*_{1/2} (in min) to reach a *Y*_{max} value (as a percentage) was determined for each associated curve.

RESULTS AND DISCUSSION

The AT_{1A} receptor is phosphorylated in response to AngII stimulation, and truncation of its serine/threonine-rich C-terminus abolishes this phosphorylation [18,19]. Direct activation of PKC by phorbol esters also causes phosphorylation of the AT_{1A} receptor and inhibition of PKC with staurosporine reduces AngII-induced phosphorylation, suggesting a role for PKC in regulating receptor function. The aim of the present study was to identify the PKC phosphorylation site(s) within the C-terminus of the AT_{1A} receptor. To accomplish this, we constructed mutants of the HA-tagged AT_{1A} receptor where serine residue(s) within putative PKC consensus sites (Ser³³¹-Thr-Lys, Ser³³⁸-Tyr-Arg and Ser³⁴⁸-Ala-Lys) of the AT_{1A} C-terminus were substituted with alanine, either individually or in combination (see Figure 1). The HA-tagged wild-type AT_{1A} receptor and the various single and triple mutants were transiently expressed in CHO-K1 cells, which are devoid of endogenous AngII receptors, and receptor phosphorylation determined. To confirm that these mutations did not affect receptor expression and binding affinity, homologous competition binding assays using [¹²⁵I]-AngII were performed. Figure 2 shows competition binding curves for the wild type and each of the mutant receptors. All receptors displayed similar dissociation constants (*K*_d; 2.6 nM for wild-type, 2.4 nM for S331A, 2.7 nM for S338A, 2.6 nM for S348A and 2.1 nM for S331/338/348A), with an inter-assay variation of less than 20% among three separate experiments. The number of receptor-binding sites at the cell surface (*B*_{max}) was similar for

Figure 2 Mutation of putative PKC phosphorylation sites has no effect on receptor expression or affinity

CHO-K1 cells were transfected with HA-tagged wild-type AT_{1A} receptor (NHA-AT1A) or single (S331A, S338A and S348A) and triple (S331/338/348A) alanine-substituted mutants. Competitive binding assays were performed and the data expressed as percentages of bound [¹²⁵I]-AngII in the absence of unlabelled AngII (Bo) for wild-type and mutated receptors. Data were fitted using non-linear regression analysis (GraphPad Prism). Estimated dissociation constants (K_d) and receptor-binding sites (B_{max}) were similar for wild-type and mutated receptors. This representative Figure was one of three separate experiments.

each construct (1060 fmol/mg of protein for wild type, 980 fmol/mg for S331A, 1140 fmol/mg for S338A, 1040 fmol/mg for S348A and 860 fmol/mg for S331/338/348A, with an inter-assay variation of less than 15 % between three separate experiments). The similarity of binding parameters indicates that alanine mutations of the putative PKC phosphorylation sites, either individually or in combination, have negligible effect on the AT_{1A}-receptor ligand-binding conformation and expression.

To examine the phosphorylation of AT_{1A} receptors, CHO-K1 cells transiently expressing the wild-type N-terminally-tagged AT_{1A} receptor, or the truncated (TK325), single (S331A, S338A and S348A) and triple (S331/338/348A) alanine mutants (see Figure 1), were loaded with [³²P]P_i and treated with the agonist (AngII, 1 μ M). Following solubilization and immunoprecipitation, the receptor was resolved on SDS/PAGE and relative levels of phosphorylation quantified by Phosphor-Imaging. As observed previously [18,19], wild-type N-terminally epitope-tagged AT_{1A} receptors are phosphorylated in response to AngII challenge, whereas the receptor truncated to remove 34 amino acids from the C-terminus, including all serine and threonine residues, is not phosphorylated. As shown in Figure 3, AngII stimulation (1 μ M) induced a 1.6-fold increase in phosphorylation of wild-type AT_{1A} receptor, whereas no AngII-mediated phosphorylation was observed for the truncated mutant. AngII stimulation of the single alanine mutants resulted in 1.1-, 0.7- and 0.7-fold increases in phosphorylation for S331A, S338A and S348A, respectively. When the level of phosphorylation in the absence of stimulation is subtracted from that observed in the presence of agonist, these fold-increases correspond to approx. 22 % (S331A), 52 % (S338A) and 47 % (S348A) decreases, compared with the wild-type AT_{1A} receptor. Interestingly, no single mutation was found to abolish the phosphorylation, although Ser³³⁸ and Ser³⁴⁸ appeared to be favoured over Ser³³¹. Moreover, when all three putative PKC phosphorylation sites (Ser³³¹, Ser³³⁸ and Ser³⁴⁸) were mutated together (S331/338/348A), AngII-induced phosphorylation was decreased by 62 % compared with the wild-type AT_{1A} receptor, suggesting that PKC is mediating a significant proportion

Figure 3 AngII-stimulated phosphorylation of HA-tagged wild-type and mutated AT_{1A} receptors

CHO-K1 cells were transiently transfected with HA-tagged wild-type AT_{1A} receptor (NHA-AT1A), a truncated AT_{1A} receptor (NHA-TK325) or the single (S331A, S338A and S348A) and triple (S331/338/348A) alanine-substituted mutants. [³²P]-Loaded cells were stimulated with AngII (1 μ M for 10 min at 37 °C), solubilized, and the receptors immunoprecipitated with 12CA5 antibody and Protein A-agarose. Phosphorylated receptors were resolved by SDS/PAGE, analysed by autoradiography and quantified by PhosphorImaging. Top panel: a representative autoradiogram illustrating the AngII-induced phosphorylation of wild-type and mutated receptors. The phosphorylated AT_{1A} receptor was resolved as a major broad band of 60–110 kDa. Middle panel: comparison of cell-surface-receptor expression for the various receptor mutants expressed as a percentage of the wild-type receptor. Receptor expression was determined for each phosphorylation experiment by radioligand-binding assay to allow normalization of PhosphorImaging data. Bottom panel: PhosphorImaging data (the means $\pm$ S.D. from three experiments) corrected for receptor expression.

of agonist-stimulated phosphorylation. Alternatively, additional agonist-induced kinases may be phosphorylating one or more of the PKC consensus sites as well as other residues within the C-terminus.

To examine the capacity of PKC to phosphorylate the AT_{1A} receptor in the absence of agonist activation, we treated CHO-

Figure 4 PMA-induced phosphorylation of HA-tagged wild-type and mutated AT_{1A} receptors

Phosphorylation was performed as described in the legend to Figure 3 except that cells were stimulated with PMA (2 μ M for 8 min at 37 °C) to activate PKC in the absence of agonist activation. Phosphorylated receptors were resolved by SDS/PAGE, analysed by autoradiography (top panel) and quantified by PhosphorImaging (bottom panel; data are the means $\pm$ S.D. from four experiments) normalized for receptor expression shown in the middle panel.

K1 cells expressing epitope-tagged wild-type, TK325 and various alanine mutants (S331A, S338A, S348A and S331/338/348A) with the phorbol ester, PMA, to directly activate PKC. As shown in Figure 4, maximal PKC activation by PMA resulted in a consistent, but modest, 0.7-fold elevation of wild-type receptor phosphorylation, which was less than that observed with AngII stimulation (1.6-fold; see Figure 3). This indicates that, in addition to PKC, AngII activates other kinases. PMA-stimulated phosphorylation was decreased by individual mutation of S331A (28 %), S338A (61 %) and S348A (57 %) and phosphorylation of the triple mutant S331/338/348A showed a 66% decrease compared with wild-type. As was the case for AngII stimulation, Ser³³⁸ and Ser³⁴⁸ seem to be slightly favoured over Ser³³¹ for

Figure 5 Effect of the PKC inhibitor (BIM) on AngII-induced AT_{1A}-receptor phosphorylation

CHO-K1 cells transiently expressing wild-type AT_{1A} receptor were treated with the specific PKC inhibitor BIM (2 μ M for 30 min at 37 °C) prior to stimulation with varying concentrations of AngII (1, 10 and 100 nM; 10 min at 37 °C) and processing for receptor phosphorylation. A representative autoradiogram is shown in the upper panel, and the quantification of receptor phosphorylation by PhosphorImaging from four separate experiments (means $\pm$ S.D.) is shown in the bottom panel. BIM pretreatment had little effect on receptor expression or binding (middle panel).

direct PKC phosphorylation. Unexpectedly, we observed a small residual PMA-stimulated phosphorylation in the triple mutant where all three consensus sites were removed. This was not seen with the truncated TK325 mutant, indicating that this residual phosphorylation was confined to the C-terminus, and may reflect minor PKC phosphorylation of non-consensus sites or the PKC-mediated activation of other kinases that then phosphorylate the receptor C-terminus.

A direct role of PKC in AT_{1A} receptor phosphorylation has been reported [15,16,19]. Using an N-terminal (His)₆-tagged AT_{1A} receptor expressed in human embryonic kidney cells, Balmforth et al. [16] provided evidence that the contribution of PKC to receptor phosphorylation was dependent upon the degree of AngII stimulation. At low concentrations of AngII (1 nM), AT_{1A} phosphorylation could be inhibited by a PKC inhibitor, whereas at higher concentrations (> 100 nM), phosphorylation could not be blocked by a PKC inhibitor,

Figure 6 Dose-dependent phosphorylation of the wild-type AT_{1A} receptor and S331/338/348A triple mutant by AngII

The wild-type HA-tagged AT_{1A} receptor and the triple alanine mutant (S331/S338/S348/A) were transiently expressed in CHO-K1 cells and phosphorylated by increasing concentrations of AngII (0, 1, 10 and 100 nM; 10 min at 37 °C). Top panel: representative autoradiogram showing a dose-dependent increase in wild-type-receptor phosphorylation, which was reduced by mutation of all three putative PKC phosphorylation sites. Bottom panel: PhosphorImager quantification of data from three separate experiments (means $\pm$ S.D.) normalized for receptor expression (middle panel).

suggesting the contribution of an unidentified GRK. Similarly, Tang et al. [23] examined the AngII-induced (homologous) and PMA-induced (heterologous) desensitization of full-length and truncated AT_{1A} receptors and found that desensitization was dependent on the region between Ser³²⁸ and Ser³⁴⁷ of the AT_{1A} -receptor C-terminus and the activity of a GRK. The observed desensitization was mediated by PKC at low doses of AngII (1 nM) and the GRK at higher levels of agonist stimulation (100 nM). To examine the contribution of PKC to AT_{1A} -receptor phosphorylation over a range of AngII concentrations, we treated CHO-K1 cells expressing wild-type AT_{1A} receptors with BIM, a specific inhibitor of PKC [23], before AngII stimulation (0, 1, 10 and 100 nM). BIM treatment (2 μ M, 30 min) had no significant effect on binding of AngII by the AT_{1A} receptor (Figure 5, middle

Figure 7 Dose-dependent phosphorylation of the wild-type AT_{1A} receptor and the S331A, S338A and S348A single mutants by AngII

The wild-type HA-tagged AT_{1A} receptor and the single alanine mutants (S331A, S338A and S348A) were transiently expressed in CHO-K1 cells and phosphorylated by increasing concentrations of AngII (0, 1 and 10 nM; 10 min at 37 °C). Top panel: representative autoradiogram showing a dose-dependent increase in wild-type and mutant receptor phosphorylation. Bottom panel: PhosphorImager quantification of data from three separate experiments (means $\pm$ S.D.) normalized for receptor expression (middle panel).

panel). As shown in Figure 5, the phosphorylation of the AT_{1A} receptor, in the presence and absence of BIM, increased in a dose-dependent fashion, although the phosphorylation at all concentrations was lower in the BIM-treated cells. The inhibition of phosphorylation by BIM, as a percentage of that in the absence of BIM, was greatest at 1 nM AngII (47% reduction), intermediate at 10 nM (25% reduction) and least at 100 nM AngII (4% reduction). This result confirms that a proportion of AngII-stimulated AT_{1A} receptor phosphorylation is mediated by PKC, especially at low agonist concentration ($\approx$ 1 nM).

As our initial experiments for examining AT_{1A} receptor phosphorylation used a maximal dose (1 μ M) of AngII, we may have masked a selective PKC phosphorylation at one of the PKC consensus sites by the robust activation of GRKs. To address this possibility, we compared the phosphorylation of the triple mutant (S331/338/348A) over a range (1, 10 and 100 nM) of AngII concentrations. As shown in Figure 6, there was a dose-

dependent increase (0.4-fold at 1 nM, 0.7-fold at 10 nM and 1.1-fold at 100 nM) in AngII-induced phosphorylation of the S331/338/348A mutant, which was lower at all levels of stimulation compared with the wild-type receptor (0.8-fold at 1 nM, 1.0-fold at 10 nM and 1.5-fold at 100 nM). The proportion of phosphorylation that was inhibited was 62% with 1 nM AngII and 47% in 100 nM AngII (Figure 6). Comparison of this with the 47 and 4% inhibition by BIM at 1 and 100 nM AngII, respectively, indicates that PKC is a participating kinase at low agonist stimulation, but that at higher levels of agonist stimulation a putative GRK is evoked that is able to phosphorylate Ser³³¹, Ser³³⁸ and Ser³⁴⁸ in addition to other residues.

In another series of experiments, we also examined the phosphorylation of each of the three single mutants (S331A, S338A and S348A) in response to 1 and 10 nM AngII stimulation. Our aim was to determine if any of the individual PKC phosphorylation sites was phosphorylated exclusively at low concentrations of AngII, where the contribution of PKC to total receptor phosphorylation is highest (see above and [16]). As shown in Figure 7, compared with the wild-type, single mutation caused either no change in phosphorylation (S331A, 0% reduction) or a slight decrease in phosphorylation (S338A, 10% reduction; S348A, 36% reduction) at 1 nM AngII. At 10 nM AngII, which causes near-maximal stimulation of wild-type AT_{1A}-receptor phosphorylation, individual mutation of S331A, S338A or S348A caused slight decreases in receptor phosphorylation. Thus mutation of single PKC consensus sites was ineffective at completely inhibiting AngII-mediated phosphorylation and this inability appears unrelated to the degree of receptor activation. Hence, we propose that PKC, whether activated homologously via receptor activation by AngII, or heterologously via direct stimulation of PKC by PMA, is capable of phosphorylating the AT_{1A} receptor at each of the three consensus PKC sites, perhaps with slight preference for Ser³³⁸ and Ser³⁴⁸. These data are also consistent with a redundancy in AT_{1A} PKC-mediated phosphorylation such that a mutation at one consensus site is compensated for by increased utilization of the remaining sites.

Our results can be compared most directly with the recent findings of Smith et al. [19]. We have reported previously [18] that serial truncation of the AT_{1A} receptor C-terminus results in a graded decrease in the level of AngII-stimulated phosphorylation, suggesting phosphorylation at multiple sites along the entire cytoplasmic tail. Using Cos-7 cells transiently transfected with HA-epitope-tagged mutant AT_{1A} receptors, Smith et al. [19] observed a similar correlation between the degree of receptor truncation and the loss of receptor phosphorylation. In addition, they reported that agonist-induced phosphorylation of the AT_{1A} receptor was confined to an 11-amino acid segment (Ser³²⁶ to Thr³³⁶) of the C-terminus, in particular Ser³³⁵/Thr³³⁶, which agrees with our observation [18] that about 50% of AngII-induced phosphorylation occurs in the region Thr³³² to Ser³³⁸. Based on the capacity of the phorbol ester PMA to phosphorylate a receptor mutant truncated at Ser³³⁵ (leaving only the Ser³³¹ PKC consensus site intact), Smith et al. [19] predicted that Ser³³¹ is the major, if not sole, PKC phosphorylation site. The results of the present study, however, argue against this conclusion. Whereas some phosphorylation certainly occurs on Ser³³¹ following AngII and PMA stimulation, it is not the sole site of PKC-mediated phosphorylation; if anything, Ser³³¹ is the least-utilized site and some preference exists for Ser³³⁸ and Ser³⁴⁸. These discrepancies may be explained by considering a possible deleterious effect of large truncations of the C-terminus on kinase recognition and overall receptor function. Interestingly, the truncated mutants displayed an

enhanced signal or, more likely, a diminished desensitization, due to the removal of multiple phosphorylation sites. For example, the Ser³³⁵ truncated mutant shows a 3–4-fold increase in inositol phosphate generation in response to AngII stimulation compared with the wild-type receptor [19]. Thus the degree of PKC activation following AngII stimulation of the truncated mutants would be expected to be comparatively greater than the wild-type receptor, and hence PKC phosphorylation of any available remaining sites (i.e. Ser³³¹ in the case of the Ser³³⁵ truncated mutant) may be 'artificially' enhanced as a consequence of super-activation. However, even at normal levels of PKC activation, our proposal of a redundancy in PKC site usage would predict that in the Ser³³⁵ truncated mutant, where Ser³³⁸ and Ser³⁴⁸ are absent, the sole remaining PKC site (Ser³³¹) would be targeted for compensatory phosphorylation.

The role of PKC phosphorylation with respect to AT_{1A} receptor function is unclear. By analogy with other GPCRs, where second-messenger kinases have been implicated in receptor desensitization and/or switching of receptor-signalling pathways [9], it is tempting to speculate that PKC phosphorylation may have an important impact on receptor function. Some investigators [16,23–28], but not others [15,29–31], have reported a role for PKC in the desensitization of AT₁ receptors. Balmforth et al. [16] demonstrated that the capacity to induce inositol phosphate production in response to AngII stimulation, in cells expressing the wild-type AT_{1A} receptor, was reduced by activation of PKC with PMA. This desensitization was not observed in a truncated mutant (at Tyr³¹⁹), which lacked the three putative C-terminal PKC phosphorylation sites. Similarly, Conchon et al. [28] reported a small (20–30%) desensitization to PMA in the full-length AT_{1A} receptor, but not in a truncated version (at Ser³²⁸). Tang et al. [23,26] reported that PKC causes a rapid desensitization of AT_{1A}- [23] and AT_{1B}- [26] receptor-mediated inositol phosphate responses and also provided evidence, using truncated versions of the AT_{1A} receptor [23], for a role of the region Ser³²⁸ to Ser³⁴⁷ of the C-terminus in both homologous (AngII-induced) and heterologous (PMA-induced) desensitization. In agreement with Balmforth et al. [16], Tang et al. [23] observed that desensitization to low doses of AngII (1 nM) are mediated by PKC, whereas at higher agonist concentrations an unidentified GRK is responsible. PKC activation and phosphorylation may also be important for cross-talk between AT₁ receptors and other GPCRs. For example, [Arg]vasopressin-receptor activation of PKC in cardiomyocytes attenuates responses to AT₁, and vice versa [27], by a PKC-dependent mechanism. Also, activation of the endothelin type-A (ET_A) receptor by endothelin caused the plasma-membrane translocation of a green fluorescent protein-conjugated PKC β II and the subsequent desensitization of AT₁, which occurred at the level of the receptor rather than more distal sites in the signalling cascade [32]. Most likely, desensitization resulted from a PKC-induced phosphorylation of the AT₁ receptor, but this needs to be confirmed and the sites of phosphorylation determined.

Another possibility is that PKC phosphorylation may impinge on receptor internalization and trafficking. Truncation of the AT_{1A} receptor (removing all three PKC sites) results in vastly reduced receptor internalization [17,31,33]. Smith et al. [19] showed a general correlation between the degree of receptor phosphorylation and receptor internalization, and we have demonstrated [18], using acidic mutations to substitute for putative phosphorylation sites, that phosphorylation within this region maximizes AT_{1A}-receptor internalization. Although inhibition of PKC by staurosporine or activation by PMA [34] had little effect on AT₁ internalization, the role of single or multiple mutations of the putative PKC phosphorylation sites (Ser³³¹,

Figure 8 Similar internalization kinetics for the wild-type AT_{1A} receptor and the single and triple PKC phosphorylation-site mutants

CHO-K1 cells were transfected with HA-tagged wild-type AT_{1A} receptor and the single (S331A, S338A and S348A) and triple (S331/338/348A) alanine-substituted mutants. The kinetics of [¹²⁵I]AngII internalization were determined as described in the Methods and materials section and the averaged data from three separate assays plotted as percentages of receptors internalized versus time (min).

Ser³³⁸ and Ser³⁴⁸) on AT_{1A} receptor endocytosis has not been investigated directly. Shown in Figure 8 are internalization kinetic curves for the wild-type AT_{1A} receptor and each of the single point and triple PKC consensus-site mutants. As observed previously, wild-type AT_{1A} receptors displayed a rapid ($t_{1/2}$ 1.6 min) and robust ($Y_{\max}$ 70% at 20 min) internalization of [¹²⁵I]-AngII. Compared with the wild-type, no significant reduction in receptor internalization was observed with each single point mutation (S331A, $t_{1/2}$ 1.6 min, $Y_{\max}$ 70%; S338A, $t_{1/2}$ 1.7 min, $Y_{\max}$ 70%; S348A, $t_{1/2}$ 1.7 min, $Y_{\max}$ 68%) or with the triple mutant (S331/S338/S348A, $t_{1/2}$ 1.6 min, $Y_{\max}$ 70%). Hence, it appears likely that PKC activity, either at the level of the receptor, or generally, has little effect on AT_{1A}-receptor endocytosis.

Conclusions

We have quantified the effect of mutating, either singly or in combination, the three putative PKC consensus sites in the C-terminus of the AT_{1A} receptor. Our data indicate that all three consensus sites are phosphorylated to some degree, although Ser³³⁸ and Ser³⁴⁸ may be favoured over Ser³³¹, in response to both AngII and PMA stimulation. The lack of clear preference for PKC consensus site suggests a multiplicity of phosphorylation or some degree of redundancy in their usage. Given the proposed role of PKC in regulating receptor desensitization [16,23–28,32], the mutants described in this paper should prove useful in investigating the contribution of each PKC site to both homologous and heterologous AT_{1A}-receptor desensitization. Such studies are underway.

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