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Short communication

Evidence against a role for protein kinase C in the regulation of the angiotensin II (AT_{1A}) receptor

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Abstract

Three putative protein kinase C phosphorylation sites in the carboxyl-terminal region of the angiotensin II AT_{1A} receptor suggest that protein kinase C is involved in the regulation and desensitisation of this receptor. We investigated this possibility by measuring angiotensin II induced Ca^{2+} transients in cultures of neonatal rat cardiac fibroblasts which express predominantly the angiotensin AT_{1A} receptor. Stimulating or inhibiting protein kinase C activity had no effect on angiotensin II stimulated Ca^{2+} transients. In addition, in situ and in vitro kinase assays revealed that a peptide, corresponding to the region of the angiotensin AT_{1A} receptor containing the protein kinase C sites, was a poor substrate for protein kinase C. Thus, a heterologous desensitising role for this kinase on angiotensin AT_{1A} receptors in these fibroblasts appears unlikely.

Keywords: Angiotensin AT_{1A} receptor; Protein kinase C; Receptor phosphorylation; Ca^{2+}, intracellular free; Angiotensin II

1. Introduction

Plasma membrane angiotensin II receptors mediate the diverse cellular functions of the vasoactive peptide angiotensin II. Two main types of angiotensin II receptors have been identified (AT_1 and AT_2) with two subtypes of AT_1 (AT_{1A} and AT_{1B}) (Sandberg, 1994). The angiotensin AT_{1A} receptor is the predominant subtype with respect to tissue distribution and control of cellular angiotensin II responses. Analysis of the amino acid sequence, deduced from the cloned angiotensin AT_{1A} receptor, reveals seven hydrophobic stretches of amino acids which represent the seven transmembrane spanning regions characteristic of G-protein coupled receptors. It also has an intracellular carboxyl-terminal tail, rich in serine and threonine residues, some of which are presumably phosphate acceptor sites. In particular, the motifs $S^{331}TK$, $S^{338}YR$ and $S^{348}AK$ present in the rat angiotensin AT_{1A} receptor are consensus sites for phosphorylation by protein kinase C (Sandberg, 1994).

The angiotensin AT_{1A} receptor has been reported to be phosphorylated on serine and tyrosine residues (Kai et al., 1994), but the functional role for phosphorylation, as well as the identity of the phosphorylated amino acids and the kinases responsible, is unresolved. For other G-protein coupled receptors, phosphorylation has been linked to the uncoupling of the receptor from signal transduction pathways, by interfering with G-protein interaction directly or through recruitment of other proteins which functionally uncouple the receptor-G-protein complex. The phosphorylation of G-protein coupled receptors is catalysed by specific receptor kinases and more general cellular kinases such as cAMP-dependent kinase and protein kinase C (Premont et al., 1995). For many G-protein coupled receptors, activation of these kinases has been reported to attenuate agonist mediated responses. Because angiotensin II stimulates protein kinase C activation and translocation to the plasma membrane (Dixon et al., 1994; Booz et al., 1994), and given the presence of three protein kinase C consensus sites in the angiotensin AT_{1A} receptor, we hypothesised that protein kinase C would phosphorylate the carboxyl-terminus of the angiotensin AT_{1A} receptor and modulate the cellular signalling functions of this receptor.

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2. Materials and methods

2.1. Tissue culture

Cultures of ventricular fibroblasts were prepared from the hearts of 0- to 3-day-old Sprague-Dawley rat pups, and maintained as previously described (Schorb et al., 1993). These cultures express high affinity (K_d 1 nM; B_{max} 778 fmol/mg protein) angiotensin AT_1 receptors (Schorb et al., 1993) predominantly of the AT_{1A} subtype, as determined by Northern blot analysis (data not shown) and polymerase chain reaction (Matsubara et al., 1994). Angiotensin II stimulates G-protein coupled responses in these cells, including the mobilisation of intracellular Ca^{2+}, the activation of mitogen-activated protein kinases, the tyrosine phosphorylation of cellular proteins and the activation of protein kinase C (Booz et al., 1994; Schorb et al., 1995). Subconfluent cultures were changed to serum-free media 24 h prior to experimentation.

2.2. Measurement of intracellular Ca^{2+} concentration

Changes in intracellular Ca^{2+} of neonatal fibroblast cultures were measured using the fluorescent dye, fura-2 acetoxy-methyl ester (fura-2 AM), as previously described (Schorb et al., 1993). Briefly, serum-starved fibroblasts were washed twice with Hanks' buffered salt solution (HBSS) and incubated for 1 h at 37°C in 5 μM of fura-2 AM in 'Ca^{2+} buffer': HBSS containing 20 mM Hepes (pH 7.4), 12.6 mM D-glucose and 0.1% BSA. After two washes in HBSS, the cells were harvested by trypsinisation and centrifugation, and resuspended in 2.5 ml of Ca^{2+} buffer. Cell suspensions were transferred to cuvettes for spectrophotometric measurement of fluorescence, at excitation wavelengths of 340 and 380 nm, and an emission wavelength of 505 nm. To determine the role of protein kinase C activation on angiotensin II induced signalling, 100 nM of the phorbol ester PMA (phorbol, 12-myristate, 13-acetate) was added to the cell suspension 10 min prior to Ca^{2+} measurements in response to angiotensin II. Downregulation of protein kinase C activity was achieved by PMA treatment (400 nM; 24 h) of fibroblast cultures. cAMP-dependent kinase activity was stimulated by the addition of 10 μM forskolin to the cell suspensions for 10 min prior to angiotensin II stimulation.

2.3. Protein kinase C assays

A peptide fragment of the carboxyl-terminal region of the angiotensin AT_{1A} receptor (hereafter termed ARCTP for AT_{1A} *R*eceptor *C*arboxyl-*T*erminal *P*eptide) corresponding to amino acids 325–351 and containing three consensus protein kinase C phosphorylation sites (see Fig. 2A) was synthesised using an automatic 431A peptide synthesiser (Applied Biosystems, Foster City, CA, USA) and purified by high pressure liquid chromatography. Cellular protein kinase C activity towards ARCTP was assessed in situ using permeabilised fibroblast cultures essentially as described by Sadoshima and Izumo (1993) and in vitro using cytosolic extracts of fibroblast cultures (Booz et al., 1994). For the in situ assay, kinase activity in the presence of no peptide (negative control), 100 μM glycogen synthase peptide (positive control) and 100 μM ARCTP was determined in cells pretreated, or not, with PMA (100 nM) for 15 min. For the in vitro assay, kinase activity was measured for the positive control peptide [Ser^{25}]protein kinase C-(19–31) and ARCTP in the presence and absence of Ca^{2+} and lipid to activate protein kinase C (Booz et al., 1994).

3. Results

We have previously shown that PMA (a phorbol ester) treatment of neonatal rat cardiac fibroblasts for 10 min or for 24 h causes maximal stimulation and downregulation of protein kinase C activity, respectively (Booz et al., 1994). Fig. 1 shows representative

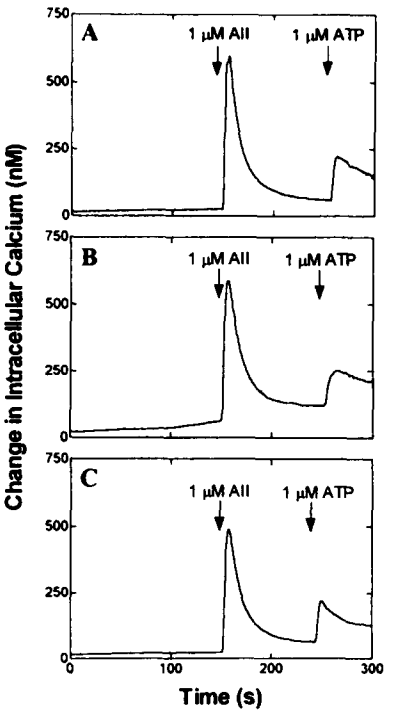

Fig. 1. Intracellular Ca^{2+} transients stimulated by angiotensin II (1 μM) in (A) untreated cardiac fibroblasts, (B) cells pretreated with 100 nM PMA for 10 min to maximally stimulate protein kinase C activity, or (C) cells treated with 400 nM PMA for 24 h to downregulate protein kinase C levels. Stimulation with 1 μM ATP was included as an internal positive control.

angiotensin II-induced intracellular Ca^{2+} transients from untreated and protein kinase C activated and downregulated cardiac fibroblasts. In untreated cultures, angiotensin II treatment caused a rapid maximal increase in intracellular Ca^{2+} of 535 ± 133 nM (mean ± S.D., $n = 4$). This level of Ca^{2+} mobilisation was not significantly different from that of protein kinase C activated fibroblasts (489 ± 144 nM; $n = 4$) or of protein kinase C inhibited cells (529 ± 161 nM; $n = 4$). Treatment of cells with 10 μM forskolin for 10 min to activate cAMP-dependent kinase also had no significant effect on the Ca^{2+} transients elicited by 1 μM angiotensin II.

To further investigate the role of protein kinase C in angiotensin AT_{1A} receptor function, we tested the ability of PMA-activated protein kinase C in cardiac fibroblast cell cultures to phosphorylate a 27 amino acid peptide corresponding to residues 325–351 of the carboxyl-terminus of the angiotensin AT_{1A} receptor. As shown in Fig. 2, despite the presence of three consensus phosphorylation sites, this peptide was a poor substrate for protein kinase C, in both in vitro and in situ protein kinase C assays. In contrast, the positive control peptides, glycogen synthase peptide and [Ser^{25}]-protein kinase C-(19–31), were readily phosphorylated under the same conditions.

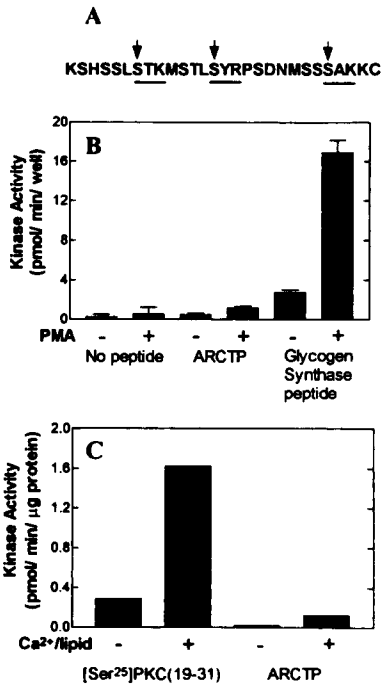

Fig. 2. In situ and in vitro kinase assays for protein kinase C activity towards the carboxyl-terminus region of the angiotensin AT_{1A} receptor. (A) shows the amino acid sequence of ARCTP (for AT_{1A} *R*eceptor *C*arboxyl-*T*erminal *P*eptide) with the putative protein kinase C consensus sites underlined and the phosphate acceptor residues indicated by arrows. (B) In situ kinase activity of cardiac fibroblast cultures towards ARCTP (100 μM) and the positive control substrate, glycogen synthase peptide (100 μM), in cells pretreated, or not, for 15 min with 100 nM PMA to maximally activate protein kinase C. Note, the kinase activity towards ARCTP is no different from the activity measured in the absence of added peptide. Data are expressed as the means ± S.D. for three separate experiments. (C) In vitro kinase assay of cardiac fibroblast extracts in the presence and absence of Ca^{2+} and lipid to activate protein kinase C (Booz et al., 1994). Substantial phosphorylation of the positive control peptide 5 μM [Ser^{25}]protein kinase C-(19–31) ([Ser^{25}]PKC(19–31)) was observed, but 100 μM ARCTP was poorly phosphorylated. Data are expressed as the means from two separate experiments.

4. Discussion

For growth factor and cytokine receptors, phosphorylation is an established phenomenon which promotes activation and interaction with intracellular signalling pathways. In contrast, for the superfamily of G-protein coupled receptors, phosphorylation appears to rapidly uncouple these receptors from downstream signalling pathways. There is evidence that both non-specific kinases such as cAMP-dependent and protein kinase C, as well as specific receptor kinases, play a central role in this uncoupling process through recognition, and phosphorylation, of motifs within the intracellular regions of the receptor. The carboxyl-terminal of the angiotensin AT_{1A} receptor contains three protein kinase C phosphorylation motifs, but the evidence for modulation of angiotensin AT_{1A} receptor function by this kinase is controversial (see Samasura et al., 1994). To investigate the role of protein kinase C in the regulation of angiotensin AT_{1A} receptor function, experiments were designed to test whether activation or inhibition of protein kinase C was capable of modulating angiotensin AT_{1A} receptor signalling in cultures of rat neonatal fibroblasts. We observed no effect of altering cellular protein kinase C activity on the capacity of these cells to mobilise intracellular Ca^{2+} in response to angiotensin II. Angiotensin AT_{1A} receptor mediated Ca^{2+} transients were also resistant to stimulation of cAMP-dependent kinase by forskolin. Thus, heterologous desensitisation of the angiotensin AT_{1A} receptor by these two kinases appears unlikely.

In the second part of this study, we synthesised a peptide corresponding to a portion of the carboxyl-tail of the receptor, incorporating three putative protein kinase C phosphorylation sites. Using in vitro and in vivo kinase assays optimised for protein kinase C activity, we showed that this peptide is a poor substrate, suggesting that protein kinase C probably does not target the carboxyl-terminal sequence of the angiotensin AT_{1A} receptor. Given that the conformation of the synthetic peptide fragment may not be identical to the carboxyl-terminus of the full length angiotensin AT_{1A} receptor, we cannot entirely rule out the possibility that protein kinase C may phosphorylate this region of the receptor. However, a variety of small peptide

substrates containing protein kinase C consensus motifs (including the [Ser^{25}]protein kinase C-(19–31) used in this study) are readily phosphorylated by protein kinase C, suggesting that complex secondary structure is not required for recognition and phosphate transfer.

We have shown for the first time that these putative protein kinase C phosphorylation sites are probably not utilised for heterologous desensitisation of the angiotensin AT_{1A} receptor, highlighting the possibility that the desensitisation observed following angiotensin II stimulation (Thekkumkara et al., 1995) is homologous, likely involving the activation of a specific kinase(s). Such receptor kinases are typified by the β-adrenergic receptor kinase which has been shown to phosphorylate a number of G-protein coupled receptors in addition to the prototypical β_2-adrenergic receptor (Premont et al., 1995). Molecular cloning has identified multiple members of this receptor kinase family (Premont et al., 1995), but the specificity of individual members for certain amino acid motifs and G-protein coupled receptors has yet to be resolved. Studies investigating the recognition and phosphorylation of the AT_{1A} carboxyl-terminus by members of the G-protein receptor kinase family are in progress.

Despite the inability of protein kinase C to phosphorylate the carboxyl-terminus of the angiotensin AT_{1A} receptor, the presence of 13 serine and threonine residues and three tyrosines within the carboxyl-terminus is highly suggestive of phosphorylation. Indeed, following agonist stimulation, the angiotensin AT_{1A} receptor is rapidly phosphorylated on serine and tyrosine residues (Kai et al., 1994), presumably within the carboxyl-terminus. Perhaps this phosphorylation plays a role in agonist-stimulated receptor internalisation, because mutations in the carboxyl-terminus region, which remove potential phosphorylation sites, cause significant reduction in receptor endocytosis (Hunyady et al., 1994; Thomas et al., 1995). Alternatively, phosphorylation of the angiotensin AT_{1A} receptor may permit coupling to recently identified novel signal transduction pathways (Bhat et al., 1994; Marrero et al., 1995) in a manner analogous to that described for many growth factor and cytokine receptors.

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