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THE ANGIOTENSIN AT_2 RECEPTOR STIMULATES PROTEIN TYROSINE PHOSPHATASE ACTIVITY AND MEDIATES INHIBITION OF PARTICULATE GUANYLATE CYCLASE

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Summary: The signalling mechanism and cellular targets of the AT_2 receptor are still unknown. We report that angiotensin II (Ang II) inhibits basal and atrial natriuretic peptide stimulated particulate guanylate cyclase (pGC) activity through AT_2 receptors in rat adrenal glomerulosa and PC12W cells. This inhibition is blocked by the phosphotyrosine phosphatase (PTPase) inhibitor orthovanadate but not by the Ser/Thr phosphatase inhibitor okadaic acid, suggesting the involvement of a PTPase in this process. Moreover, Ang II induces a rapid, transient and orthovanadate sensitive dephosphorylation of phosphotyrosine containing proteins in PC12W cells. Our findings suggest that AT_2 receptors signal through stimulation of a PTPase and that this mechanism is implicated in the regulation of pGC activity. This observation is also the first example of hormonal inhibition of basal pGC activity.

Two pharmacologically distinct Ang II receptor subtypes have been defined based on affinity for synthetic ligands and sensitivity of ligand binding to sulphydryl reducing agents (1-3). The primary structure of the AT_1 receptor subtype, which has recently been determined following cDNA cloning (4,5), and extensive pharmacological data (6-10), indicates that it is a G-protein coupled receptor. Characteristic of the AT_1 receptor is its ability to activate phospholipases A_2, C (7) and D (8), mobilize intracellular Ca^{++} stores (9) and inhibit adenylate cyclase activity (10). Much less is known regarding the AT_2 receptor subtype, which is also expressed in many Ang II responsive organs, but does not appear to function via classical signalling pathways (11,12).
Ang II has recently been suggested to decrease the cellular concentration of cGMP in neuron cultures through AT_2 receptors by a mechanism involving Ca^{++} and activation of a phosphodiesterase (PDE) (13). However, identical findings involving the same mechanisms had previously been shown in cultured vascular smooth muscle cells (14), known to express only AT_1 receptors (1,2). This apparent discrepancy as well as the contamination of the neuron cultures by

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astroglial cells expressing AT_1 receptors (13), led us to investigate whether the AT_2 receptor mediated effect of Ang II on cellular cGMP levels might not instead occur through regulation of pGC activity.

For this study we have used plasma membrane particulate isolated from either rat adrenal glomerulosa (RAG), which expresses both Ang II receptor subtypes (1), PC12W cells, which express only the AT_2 receptor (15) and from cultured rat aorta smooth muscle cells (VSMC) which express only AT_1 receptors (1).

Materials and Methods

RAG and VSMC were obtained as described previously (11). PC12W cells were cultured as described by Speth and Kim (15). Plasma membrane particulate was prepared by homogenizing tissue or freshly harvested cells in ice-cold buffer (20 mM Tris pH 7.4, 1 mM EDTA, 0.1% BSA, 1 mM benzamidin and 0.01% bacitracin) with a Dounce homogenizer. Homogenates were centrifuged twice at 1000 x g for 10 min and the supernatant 40000 x g for 45 min. The resulting pellet was resupended and washed twice in homogenization buffer containing 0.6 M KCl for the first wash. The final pellet was resuspended in 20 mM Tris pH 7.6, 1 mM EDTA at a concentration of 1 mg/ml and snap-frozen in liquid nitrogen.

pGC assay: 5 µg. of particulate were incubated in buffer (50 mM Tris pH 7.6, 125 mM NaCl, 0.1% BSA and 0.5 mM IBMX) in the presence of 1 nM Ang II where indicated, at 37°C for 20 min. Guanylate cyclase activity was measured at 37°C, in the same buffer containing also 15 mM creatine phosphate, 20 U/ml creatine phosphokinase, 5 mM $MgCl_2$ and where indicated, 100 nM rat ANP and/or 1 nM Ang II in a final volume of 90 µl. The reactions were started by the addition of 10 µl of 10 mM GTP and 4 mM $MnCl_2$, continued for 10 min and stopped by the addition of 900 µl 50 mM sodium acetate pH 5.8 followed by boiling for 3 min. Generated cGMP was quantified by radioimmunoassay using a kit from IBL (Hamburg, Germany). Cross reactivity with other nucleotides was less than 0.0005%.

Immunoblotting: Cells at approximately 70% confluency were washed with PBS and incubated in Krebs-Ringer solution with or without 10 nM Ang II in a CO_2 incubator at 37°C for various periods of time. Incubations were stopped by aspiration of the incubation solution and addition of boiling SDS sample buffer containing 1 mM Na_3VO_4. A similar procedure was to particulate: 100 µg aliquots were incubated in Krebs-Ringer buffer alone or with 10 nM Ang II and the reaction was stopped by the addition of 1 mM Na_3VO_4 and 10 µM staurosporine. After boiling and centrifugation the supernatants were subjected to SDS-PAGE on 9% polyacrylamide slab gels. Proteins were transferred to PVDF membranes and blots were probed with anti-phosphotyrosine mAb (21) and anti-mouse IgG-alkaline phosphatase conjugate and developed with NBT/BCIP.

Results

As shown in Fig. 1, Ang II inhibits basal pGC activity by more than 40% in membrane particulate from both RAG and PC12W cells. In order to characterize this pGC activity, its modulation by ANP was examined. ANP was found to stimulate pGC activity in both membrane preparations (Fig. 1), indicating that at least part of this activity can be accounted for by the A type ANP receptor (ANPR-A or GC-A), which has been shown to have intrinsic GC activity (16). However, ANP had no effect on Ang II mediated inhibition of pGC activity (Fig. 1).

All experiments were performed in the presence of 0.5 mM 3-isobutyl-1-methylxanthine and in the absence of Ca^{++} in order to exclude any possible interaction of a Ca^{++}/calmodulin dependent PDE. The absence of a significant contribution of soluble GC to our results is indicated by the lack of stimulation (< 5%) of cGMP production by 100 mM nitroprusside (data not shown).

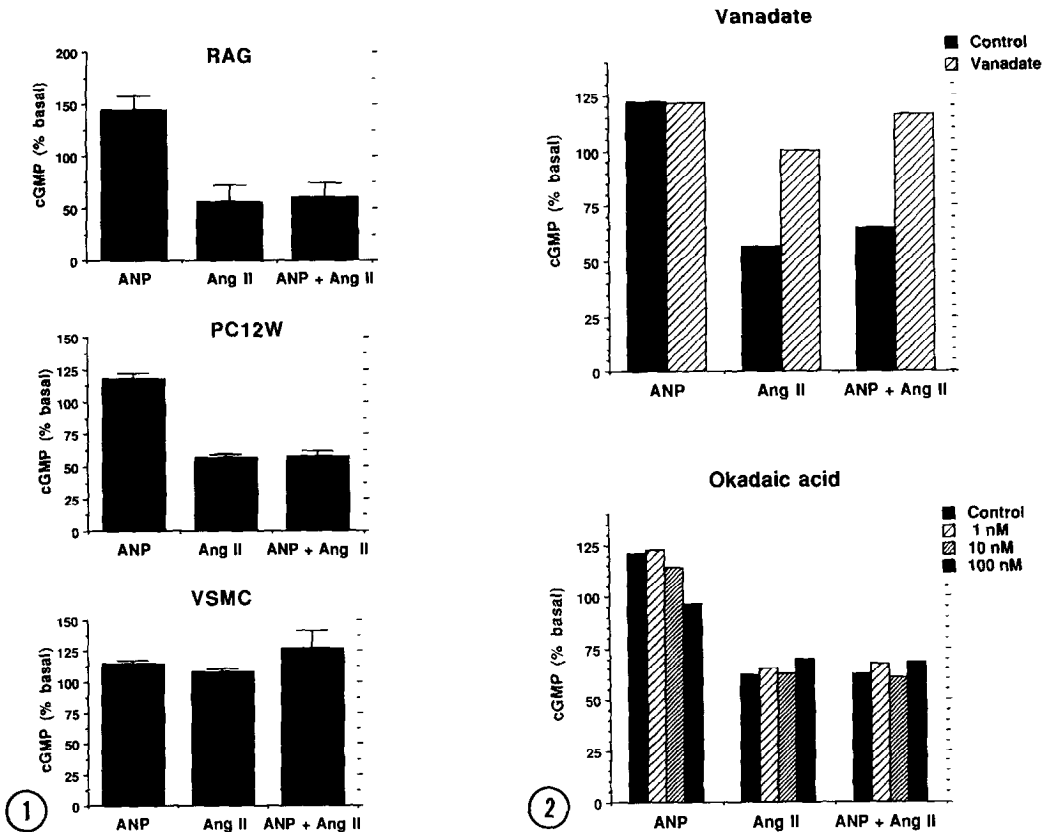

Fig.1. Effect of ANP and Ang II on particulate guanylate cyclase activity.
Guanylate cyclase activity in was measured in plasma membrane particulate from rat adrenal glomerulosa (RAG, n = 10), PC12W cells (PC12W, n = 7) and cultured rat aorta smooth muscle cells (VSMC, n = 4) was prepared as described above. The concentrations of rat ANP and Ang II were resp. 100 nM and 1 nM. Values are expressed as the percentage +/- S.D. of cGMP generated in the absence of hormones.

Fig.2. Effect of Na_3VO_4 and okadaic acid on ANP stimulated and Ang II inhibited particulate guanylate cyclase activity in PC12W particulate
Incubations and guanylate cyclase assays were performed as indicated under fig.1 but the samples were preincubated for 10 min at 37 °C in the presence of 100 µM Na_3VO_4, or 1 nM, 10 nM and 100 nM okadaic acid as indicated. Values are expressed as the percentage of cGMP generated in the absence of hormone and are the mean of 3 experiments.

In order to exclude the involvement of the AT_1 receptor subtype in Ang II mediated inhibition of pGC, the effect of Ang II on pGC activity was investigated in plasma membrane particulate prepared from cultured aorta smooth muscle cells (VSMC), which express only AT_1 receptors. As indicated in Fig. 1, Ang II did not inhibit basal or ANP stimulated cGMP generation, indicating the absence of AT_1 receptor involvement in the inhibition of pGC activity. This was further confirmed by the lack of effect of the AT_1 antagonist Losartan (DuP 753) at 1µM on the modulation of pGC activity by Ang II and ANP in RAG and PC12W cell particulate (data not shown). These findings imply that inhibition of pGC by Ang II is mediated via the AT_2 receptor.

It has been reported that phosphorylation of pGC's is important for their stimulation by ANP (17) or other stimulatory peptides (18). We therefore hypothesized that Ang II might modulate the ANPR-A pGC activity by a mechanism involving dephosphorylation. In order to test this

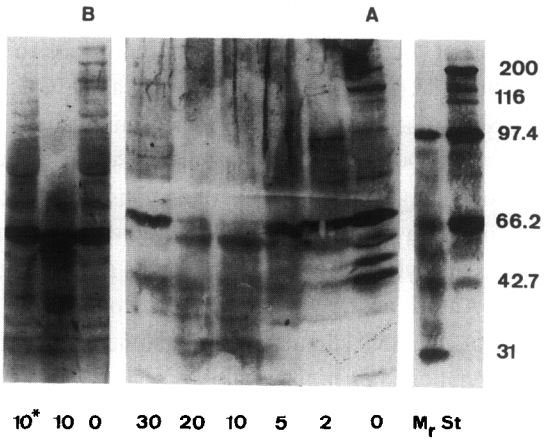

Fig.3. Effect of Ang II on the phosphorylation pattern of tyrosine residues in PC12W cells.
Panel A: Cells were washed with PBS and incubated in Krebs-Ringer with 10 nM Ang II for 2, 5, 10, 20 and 30 min. Controls were incubated for 30 min. in the absence of Ang II. **Panel B:** control, Ang II 10 min. and (*) Ang II in the presence of 1 mM Na_3VO_4 10 min. Blots were processed as described above.

hypothesis, we examined the effect of inhibitors of both tyrosine and serine/threonine phosphatase on pGC activity. Sodium orthovanadate, a selective inhibitor of PTPases (19), did not affect pGC stimulation by ANP (Fig. 2). However, Ang II induced inhibition of pGC was completely abolished (Fig. 2), suggesting the involvement of a PTPase in the AT_2 receptor mediated inhibition of pGC. Okadaic acid, a selective inhibitor of protein phosphatase 2A and 1 (PP2A and PP1) (20), was used to investigate the possible role of serine/threonine protein phosphatase. In vitro, the purified catalytic subunits of PP2A and PP1 are inhibited with IC_{50} values of 0.1 nM and 10 - 15 nM respectively (20). At a concentration of 1 nM, okadaic acid did not affect either activation of pGC by ANP or inhibition of pGC by Ang II (Fig.2). At higher inhibitor concentrations (10 and 100 nM), ANP stimulated pGC activity was progressively inhibited. The inhibitory effect of Ang II however, was not affected.

These observations strongly suggest that a PTPase is involved in the Ang II mediated inhibition of pGC via the AT_2 receptor. To further investigate this hypothesis, PC12W cells, which express only AT_2 receptors, were treated with Ang II. The phosphotyrosine pattern in Ang II stimulated cells was compared to non treated controls cells by immunoblotting of whole cell lysates using an antiphosphotyrosine monoclonal antibody (21). As shown in Fig. 3, Ang II induces a rapid dephosphorylation of tyrosine phosphorylated proteins in PC12W cells.
This effect was apparent within 2 min. and reached a maximum between 10 and 20 min. Partial rephosphorylation of these proteins was visible after 30 min. exposure to Ang II, indicating that this observation is not merely due to proteolysis. As expected, Ang II stimulated tyrosine dephosphorylation was inhibited by vanadate (Fig.3).

Discussion

The ANP receptor (ANPR-A), in addition to containing a guanylate cyclase domain, also encodes a domain with sequence similarity to the catalytic domain of protein kinases (16). A 256

aa region of the intracellular domain of the ANP receptor shows approximately 30% sequence identity to the kinase domain of the PDGF receptor and conforms to the kinase consensus signature sequence in 30 out of 33 positions. This includes the G X G X X (X) G sequence, which in the case of the ANPR-A sequence, has one more amino acid than is found in known protein kinases. This sequence is postulated to contribute to the nucleotide binding domain of protein kinases (22). Deletion mutagenesis studies have demonstrated that the kinase homology domain of the ANP receptor has a regulatory function. When this domain is removed, the resulting ANP receptor retains guanylate cyclase activity, but its activity becomes independent of ANP (23). Our finding that Ang II, acting via the AT_2 receptor, results in both activation of a PTPase and inhibition of basal and ANP stimulated pGC activity, led us to search for possible regulatory tyrosine phosphorylation sites in the ANPR-A receptor sequence. It was especially intriguing to find a tyrosine residue within the G R G S N Y G motif. This tyrosine residue is located in the same position of this kinase consensus sequence as Tyr^{15} of the $p34^{cdc2}$ protein kinase (24). Dephosphorylation of Tyr^{15} in $p34^{cdc2}$, which leads to activation of the $p34^{cdc2}$ /cyclin B kinase, results in onset of mitosis (25). Tyrosine phosphorylation of this residue presumably inhibits kinase activity by blocking ATP from binding to the ATP binding site (24). One may speculate that a similar situation could exist for the ANPR-A kinase domain, in which regulation of pGC activity could be modulated via the phosphorylation state of this tyrosine residue. This hypothesis is supported by the recent observation that ANPR-A specifically binds ATP and that, in the presence of Mn^{++}, ATP inhibits basal and ANP stimulated pGC activity (26). We observed a similar phenomenon in RAG and PC12W membrane particulate with an IC_{50} of approximately 0.1 mM for ATP when Mn^{++} and Mg^{++} were both present (data not shown).

In conclusion, our findings indicate that the AT_2 receptor mediates inhibition of basal and ANP stimulated pGC activity. This effect may involve the activation of a membrane associated PTPase. It is hypothesized that by analogy to the cdc2 kinase, this PTPase may regulate ATP binding to the kinase homology domain of the ANPR-A which has been shown to modulate its GC activity. Whether the AT_2 receptor molecule has intrinsic PTPase activity, like the lymphocyte antigen receptor family (27), will require purification or cloning of this receptor. Ang II mediated PTPase activation provides a first example of a novel signal transduction mechanism for peptide hormones and opens new perspectives for the physiological role of Ang II.

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