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PROTEIN TYROSINE PHOSPHATASE INHIBITION BY ANGIOTENSIN II IN RAT PHEOCHROMOCYTOMA CELLS THROUGH TYPE 2 RECEPTOR, AT_2

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SUMMARY: Two major isoforms of angiotensin II receptors, AT_1 and AT_2, have been defined on the basis of their ligand selectivity. While AT_1 is known to mediate typical biological actions of angiotensin II as a cardiovascular regulator, the biological function of AT_2 has not yet been established. In the present study using a rat pheochromocytoma cell line, which expresses AT_2 exclusively, we found that angiotensin II inhibits phosphotyrosine phosphatase activity *in vivo* as measured by the inhibition of hydrolysis of [^{32}P]-phosphate from the ^{32}P-labeled synthetic peptide substrate, Raytide. This phosphotyrosine phosphatase inhibition was completely reversed by pertussis toxin, which indicates a G-protein coupled mechanism. In SDS-polyacrylamide gel electrophoresis we found that the phosphotyrosine group of an 85 kDa protein was a substrate mainly preserved, presumably as a consequence of the plausible intracellular phosphotyrosine phosphatase inhibition by angiotensin II.

Angiotensin II (Ang II) has diverse actions in cardiovascular regulation, which include vasoconstriction, aldosterone release from adrenal glomerulosa, facilitation of peripheral adrenergic outflow, and vascular smooth muscle cell hypertrophy and hyperplasia to mention a few examples (1-5). Isoform specific ligands for Ang II receptors revealed the presence of more than two major isoforms of Ang II receptor, AT_1 and AT_2 (6-7). AT_1 is widely distributed in various tissues and is known to mediate most of the actions hitherto attributed to Ang II, whereas, the biological functions mediated by AT_2 have not yet been established (8).

Extensive studies on the tissue distribution of AT_2 revealed its presence in fetal tissues (9), several nuclei of the thalamus and brain stem (10-13), and healing wounds (14), which suggested its importance in as yet unidentified processes, such as tissue growth or neuronal functions. AT_2 is also expressed in several cell lines which include rat ovarian cells (15), rat fibroblast-derived R3T3 cells (16), cultured neuronal cell isolates from neonatal rats (17) and rat pheochromocytoma cells (PC12w) (18). Since PC12w cells are devoid of AT_1 (18), they

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have been used to characterize the properties of AT_2 without purification or cloning. Although it was reported that Ang II attenuated the guanylyl cyclase activity of the atrial naturetic factor (ANF) receptor in PC12w cells as a consequence of the activation of protein tyrosine phosphatase (19,20), these observations have not been reproduced (21-23).

To define the biological function of AT_2, we examined the effect of Ang II on the phosphotyrosine phosphatase (PTP) activity in PC12w cells. Using the tyrosine-phosphorylated peptide Raytide as a substrate, we have found that the phosphotyrosine phosphatase activity was inhibited rather than stimulated by a G-protein-mediated mechanism. In addition, we were able to demonstrate that the Ang II-induced PTP inhibition results in the retention of phosphate bound to tyrosyl residues of a cellular 85 kDa protein. It may be a specific substrate for the PTP inhibited by Ang II-activated AT_2. Here we report a new signal transduction mediated by AT_2.

Materials and Methods

Materials: Losartan and PD123319 were generous gifts from DuPont-Merck and Warner-Lambert Parke Davis, respectively. Raytide and $p60^{c\text{-}src}$ were purchased from Oncogene Science. Anti-phosphotyrosine antibody was from Upstate Biotechnology Inc., [γ-^{32}P]-ATP and [^{32}P]sodium orthophosphate (3000 *Ci*/mmol) were from DuPont-NEN, pertussis toxin (PTX), guanosine 5'-[β-thio]diphosphate (GDPβS) and other reagents were from Sigma Chemical Co.
Cell culture: PC12w cells were generously provided by Drs. R.C. Speth and K.H. Kim of Washington State University, and cultured according to their established method (18). The cells of 3 to 8 passages were used.
Membrane preparation: All procedures described below were conducted at less than 4°C. The PC12w cells were homogenized in 20 mM HEPES-NaOH (pH 7.4) containing 1 mM EDTA and protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride (PMSF), 20 μg/ml leupeptin and 10 μg/ml aprotinin) (buffer A) and then centrifuged at 48,000 x *g* for 30 min. The pellet was resuspended in buffer A and the centrifugation was repeated. Plasma membranes thus obtained were suspended in 140 mM KH_2PO_4-KOH (pH 7.4) containing 14 mM NaCl, 0.5 mM Mg Cl_2 and the same mixture of the protease inhibitors as above (buffer B). The protein concentration was determined by a Bio-Rad assay kit.
Phosphatase assay: Before the phosphatase assay plasma membranes (20 μg protein) were incubated with or without 100 nM Ang II for 30 min at 23°C in buffer B containing 20 μM GTP unless otherwise stated. The assay was performed according to the established method (24). The activity was determined as amount of radioactivity (cpm) released from ^{32}P-Raytide in 15 min and normalized in reference to the control which was taken as 100%.
Analysis of tyrosine-phosphorylated cellular protein: PC12w cells (> 10^7 cells/10 cm dish) were incubated with 20 μ*Ci*/ml [^{32}P]-sodium orthophosphate at 37 °C for 16 h in the Eagle's minimum essential medium lacking sodium phosphate, which was added with insulin (6.25 μg/ml), transferrin (6.26 μg/ml), sodium selenite (6.25 μg/ml, bovine serum albumin (BSA) (1.25 mg/ml) and linoleic acid (5.35 μg/ml). After the pre-labeling, medium was removed, cells were incubated with or without Ang II or its antagonists in Dulbecco's modified Eagle's medium containing 0.1% BSA for 1 h, then incubated on ice for 10 min with 1 ml of the lysis buffer containing 20 mM HEPES-NaOH (pH 7.4), 0.5% Nonidet P-40, 50 mM α-glycerophosphate, 0.1 mM sodium orthovanadate 10 mM sodium molybdate and the protease inhibitor mixture. After centrifugation at 14,000 x *g* for 5 min, an aliquot of the supernatant (1.5 mg protein) was incubated for 18 h at 4°C with anti-phosphotyrosine antibody and protein A-Sepharose CL-4B (20 mg) which had been swollen in 1 ml of the lysis buffer. The protein A-sepharose CL-4B matrix was washed three times with 1 ml of the lysis buffer and tyrosine-phosphorylated proteins were released from the matrix with the lysis buffer containing 10 mM phenylphosphate.

Radioactivity was measured by Cerenkov's method. The eluted proteins were subjected to SDS-PAGE according to Laemmli (25), transferred to a nitrocellulose membrane (Amersham) by electroblotting, then autoradiographed with a Kodak X-OMAT x-ray film.
Statistics: Statistical analysis was carried out by one-way ANOVA followed by Duncan's new multiple range comparison.

RESULTS AND DISCUSSION

The angiotensin II receptor in PC12w membranes, previously identified as AT_2 isoform (12), bound Ang II rapidly attaining the maximum binding in 20 min at 23°C. We examined the effect of Ang II on the PTP activity of plasma membranes of PC12w cells after preincubation with various ligands for 20 min as shown in Figure 1. Ang II (100 nM) inhibited the total basal PTP activity by 25%. This inhibition was not affected by pretreatment of membranes with the AT_1-specific antagonist, losartan (1 μM), but was completely abolished when treated with the AT_2-specific ligand, PD123319 (1 μM). Thus, it was clear that Ang II inhibits the membrane-associated PTP activity of PC12w cells through AT_2, but not AT_1. Further support for the AT_2-mediated inhibition of PTP was obtained with CGP42112A, another AT_2-specific ligand. Interestingly, however, this ligand worked as an agonist, similar to Ang II, and inhibited the PTP activity by about 32% of control. The present observation that CGP42112A is an AT_2-specific agonist is in good agreement with a previous report on its effect on T-type calcium channel activity (26).

In the course of this study we were able to clone the rat AT_2 cDNA from a cDNA library from PC12w, and showed it as a new member of the seven-transmembrane domain-receptor superfamily (27). To date, no functional features characteristic to a G-protein coupled receptor have been reported for AT_2. For example, GTP or its analogs did not reduce the ligand binding affinity of AT_2 (12,15,16,18,19,21-23). However, studies on the effects of PTX and GDPβS on the Ang II-induced PTP inhibition revealed evidence for the involvement of a G-protein in the action of AT_2. As shown in Figure 2, pretreatment with either PTX (0.2 μg/ml) or GDPβS (250 μM) completely reversed the attenuation of the PTP activity elicited by Ang II. These effects do not seem to be artifacts or nonspecific actions of the reagents used. Greater than 80% of the PTP activity in the plasma membranes were inhibited by a mixture of sodium orthovanadate (100 μM) and molybdate (10 mM). The inhibition of PTP by Ang II was observed in the orthovanadate-molybdate sensitive activity.

Interestingly, earlier studies (12,15,16,18,19,21-23) revealed that AT_2 has some properties which are not commonly shared by many of the seven-transmembrane-domain receptors, such as the lack of action of stable analogs of GTP. Yet, our recent cloning studies demonstrated that AT_2 has the structural feature of a seven-transmembrane-domain receptor (27), and the receptor mediated action is PTX sensitive. Thus, sensitivity to GTP analogs does not

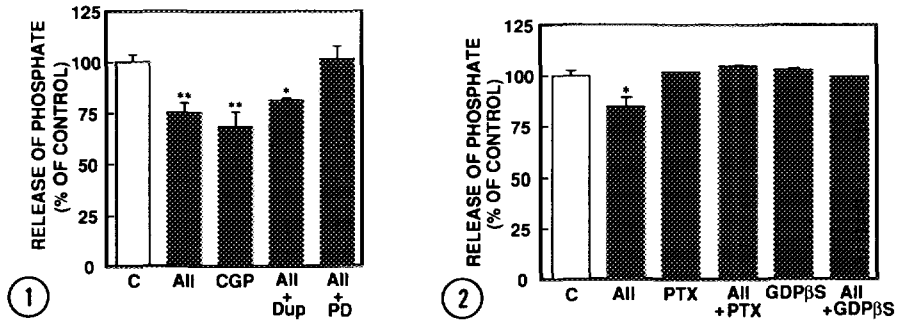

Fig. 1. ***Inhibition of phosphotyrosine phosphatase by angiotensin II and its related-compound in the plasma membranes of PC12w cells.*** Plasma membranes (25 μg) were incubated with 100 nM Ang II (AII), 100 nM CGP42112A (CGP), 100 nM Ang II in the presence of 1 μM losartan (AII + Dup) or 100 nM Ang II in the presence of 1 μM PD123319 (AII + PD) at 23°C and were subjected to the PTP assay. The PTP activity is shown as percent of control (C). Data are expressed as mean ± SEM of six experiments. Asterisks indicate significant difference versus control (*, $p<0.05$, **, $p<0.01$).

Fig. 2. ***Effects of pertussis toxin and GDPβS on the inhibition of the phosphotyrosine phosphatase activity by angiotensin II.*** Plasma membranes of PC12w cells were incubated alone (C), with 100 nM Ang II (AII), 0.2 μg/ml pertussis toxin (PTX), 100 nM Ang II plus 0.2 μg/ml PTX (PTX + AII), 250 μM GDPβS (GDPβS) or 100 nM Ang II plus 250 μM GDPβS (GDPβS + AII). PTX treatment was carried out as described previously (27). The phosphotyrosine phosphatase activity is shown as percent of control. Asterisks indicate significant difference versus control (*, $p<0.05$, **, $p<0.01$).

appear to be the absolute criterion of the G-protein dependence, particularly in the PTP-regulating receptors.

To extend the *in vitro* observations of the Ang II-induced PTP inhibition in plasma membranes to intact cells, we examined the effect of Ang II treatment on the cellular phosphotyrosine containing proteins in PC12w cells pre-labeled with ^{32}Pi. As shown in Figure 3, Ang II significantly increased the amount of tyrosine-phosphorylated protein by 138 ± 5% over the basal level. This increase can be explained by the retention of tyrosine phosphoryl group as the result of inhibition of endogenous PTP action. CGP42112A increased the amount of tyrosine phosphoryl protein by 70 ± 7%. In agreement with results of the experiments with membranes discussed above, the effect of Ang II was abolished by pretreatment with PD123319 (1 μM) in experiments with intact PC12w cells. These results indicate that Ang II inhibits the PTP both in plasma membrane preparations and in intact cells, and that the PTP inhibition seen with ^{32}P-Raytide represents a cellular function of AT_2 of physiological importance.

The fact that only a limited portion of the total PTP activity is inhibited by Ang II (Figs 1 and 2) suggests that the inhibition is specific to limited species of PTP's. To test this hypothesis we attempted to identify a tyrosine phosphorylated protein(s) whose dephosphorylation is suppressed by Ang II. Figure 4 shows autoradiograms of the tyrosine phosphorylated protein in SDS-polyacrylamide gel electrophoresis. The control sample gave

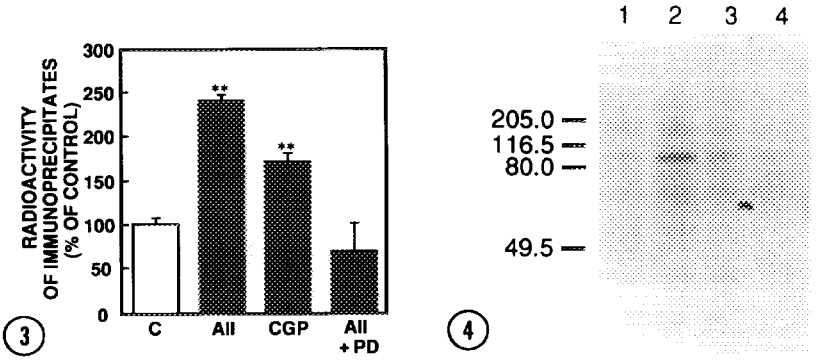

Fig. 3. ***Effects of angiotensin II and related-compounds upon the amount of tyrosine-phosphorylated proteins in intact PC12w cells in culture.*** After prelabeled with [^{32}P]-sodium orthophosphate, PC12w cells were incubated alone (C), with 100 nM Ang II (AII), 100 nM CGP42112A (CGP), or 100 nM Ang II plus 1 μM PD123319 (AII + PD) for 1 h at 37°C. The amounts of tyrosine-phosphorylated proteins were determined as the radioactivity eluted from immunoprecipitates with anti-phosphotyrosine antibody. The radioactivity was normalized in reference to the control (C).

Fig. 4. ***Increased retention of ^{32}P-labeling in phosphotyrosine proteins caused by Ang II and CGP42112A.*** PC12w cells were treated with vehicle (Lane 1), 100 nM Ang II (Lane 2), 100 nM CGP42112A (Lane 3), 100 nM Ang II in the presence of PD 123319 (Lane 4).

only a faint band, but the Ang II-treated sample gave a single band of 85 kDa with a much greater intensity of ^{32}P-radioactivity together with some smeared bands. A similar result was observed with the sample treated with CGP42112A. The radioactivity of this protein was attenuated by pretreatment with PD123319, an AT_2-specific antagonist. Thus, tyrosine-phosphorylated form of the 85 kDa protein (p^{85}) is a substrate for the PTP whose activity is affected by the AT_2-associated G-proteins. The identity of the p^{85} is not yet clear.

Recently, activation of PTP by somatostatin and dopamine through their seven-transmembrane-domain receptors has been reported (28,29). The present study demonstrates that AT_2, which has recently been cloned as a seven-transmembrane-domain receptor (27), mediates the inhibition of PTP activity. This observation represents a new mechanism in the regulation of PTP. These findings implicate that both activation and inhibition of some PTP are regulated by receptor-associated G-proteins, and that the positive and negative regulation of PTP by the G-proteins can provide a possible means for cross-talk between the seven-transmembrane-domain receptors and the protein phosphotyrosine system.

Using the same PC12w cell line, Bottari et al. have reported that Ang II caused a decrease in cGMP content and a concomitant decrease in several protein phosphotyrosine bands, indicating stimulation of the PTP activity (19,20). Other investigators, however, have not been able to reproduce the Ang II-induced decrease in cGMP in the same or other cell lines (21-23, 27). We now have evidence that Ang II inhibits the PTP in contrast to its activation as reported by Bottari et al. (19). Possible reasons for the discrepancy between these results may be due

to a difference in the condition of the cells. The present study was conducted under the basal conditions without any particular stimulation such as ANF and EGF. Since PC12w cells possess receptors for these peptide hormones, ANF-stimulated cells used by other investigators (19-22) may respond differently to Ang II than cells under basal conditions. Thus, intriguing possibilities exist in which both inhibitory and stimulatory mechanisms for PTP by AT_2-mediated signalling under different culture conditions.

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