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Modulation of Angiotensin II Binding Affinity by Allosteric Interaction of Polyvinyl Sulfate with an Intracellular Domain of the DuP-753-Sensitive Angiotensin II Receptor of Bovine Adrenal Glomerulosa

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SUMMARY

Angiotensin II (AII) is an important regulator of aldosterone secretion by adrenal glomerulosa cells. AII interacts with a specific receptor coupled to a guanine nucleotide-binding protein that controls the activity of phospholipase C. Recently, novel AII nonpeptide antagonists (DuP-753 and PD-123319) have been shown to discriminate between two subclasses of AII receptors in many different tissues. Our studies confirmed that ^{125}I-AII specifically labeled two classes of binding sites for AII in a membrane preparation of bovine adrenal glomerulosa cells. The first class (DuP-753 sensitive) represented approximately 85% of the total binding sites for AII and possessed a high affinity (IC_{50} of 92.9 ± 19.5 nM) for DuP-753. PD-123319 did not have any effect on ^{125}I-AII binding to this site. The second class of binding sites was more sensitive to PD-123319, with an IC_{50} of 6.9 ± 3.7 nM, and had a much lower affinity for DuP-753 (IC_{50} around 10 μM). The two classes of receptors had different affinities for AII. AII showed an affinity around 2 nM for AII type 1 receptor (AT_1) (DuP-753 sensitive) and a higher affinity, around 0.3 nM, for AII type 2 receptor (AT_2) (PD-123319 sensitive). AII-induced steroidogenesis was completely abolished in the presence of 3 μM DuP-753, indicating that this activity was mediated through a DuP-753-sensitive receptor. We also found that polyvinyl sulfate (PVS), a polyanion, could partly inhibit the binding of ^{125}I-AII to bovine adrenal glomerulosa cell membranes, with half-maximal efficiency at 17.3 ± 8.2 nM. The inhibitory effect of PVS was selective for AT_1. The inhibitory effect of PVS was due to a change in the affinity state of the receptor. Unexpectedly, PVS had no effect on AII-induced steroidogenesis or on AII binding to intact bovine adrenal glomerulosa cells. However, the inhibitory effect of PVS on AII binding was recovered after permeabilization of cells. Direct interaction of polyanions with AT_1 was suggested by the capacity of solubilized photoaffinity-labeled ^{125}I-AT_1 to adsorb to heparin-agarose gels. The adsorption of ^{125}I-AT_1 to heparin-agarose was inhibited by prior incubation of solubilized receptor with heparin or PVS. These results suggest that AII-induced steroidogenesis is mediated by a DuP-753-sensitive receptor and that PVS decreases the affinity of this receptor by interacting with an intracellular domain (possibly the positively charged domain responsible for coupling with guanine nucleotide-binding proteins).

AII is an important regulator of aldosterone synthesis and secretion by adrenal glomerulosa cells (1). This effect is due to the activation of specific receptors at the surface of the cells (2, 3). The primary effector mechanism controlled by these receptors is hydrolysis of polyphosphoinositides by phospholipase C, thus generating the second messengers inositol 1,4,5-trisphosphate and diacylglycerol (4–8). This mechanism is common to a wide variety of cells, in response to Ca^{2+}-mobilizing hormones (9–11). A G protein, different from G_s and G_i, is involved in the molecular mechanism by which AII-receptor complex formation activates phospholipase C (12), as also observed in many other receptor systems (13–17). This G protein is presumably the one recently isolated by protein purification (18, 19) and identified by molecular cloning (20) as G_q.

The recent development of nonpeptide AII antagonists has revealed the presence of more than one type of AII receptors in various tissues (21–23). AT_1 displays a high affinity for DuP-753 [2-*n*-butyl-4-chloro-5-hydroxymethyl-1-[[2'-(1*H*-tetrazol-5-yl)biphenyl-4-yl]methyl]imidazole] and no affinity at all for PD-123319 [1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid, 1-[[4-(dimethylamino)-3-methylphenyl]methyl]-5-(diphenylace-

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ABBREVIATIONS: AII, angiotensin II; AT_1, angiotensin II type 1 receptor; AT_2, angiotensin II type 2 receptor; PVS, polyvinyl sulfate; BSA, bovine serum albumin; G protein; guanine nucleotide-binding protein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTPγS, guanosine-5'-*O*-(3-thio)triphosphate; Gpp(NH)p, guanosine-5'-(2,3-imido)triphosphate.

tyl)-4,5,6,7-tetrahydro-(*S*)-trifluoroacetate (1:2)]. AT_2 displays a low affinity for DuP-753 and a relatively high affinity for PD-123319. The two types of receptors display similar affinities for peptide analogues of AII. DuP-753 is a potent inhibitor of AII-induced aldosterone release by rat adrenal glomerulosa cells (23). DuP-753 is also a potent antagonist of AII-stimulated inositol phosphate accumulation in cultured clone 9 cells and of AII-induced rabbit aorta and uterus contraction (24). The affinity AT_1 is modulated by the nonhydrolyzable GTP analogue Gpp(NH)p, whereas AT_2 is unaffected by this compound, suggesting that only AT_1 is coupled to a G protein. So far, there has been no report on PD-123319 being able to antagonize any physiological action of AII, suggesting that AT_2 is not involved in the known physiological actions of AII. At the present time, the significance of this silent receptor remains unknown.

Heparin and other polyanions were reported to uncouple the α_2-adrenoreceptor and opioid receptor from G proteins and to inhibit agonist-induced adenylate cyclase inhibition (25, 26). It was suggested that the effects of polyanions were due to an action on the G_i protein of the adenylate cyclase system. More recently, a series of polyanionic compounds were shown to inhibit the coupling of the α_2-adrenoreceptor and β_2-adrenoreceptor to the G proteins G_i and G_s, respectively, which are negatively and positively involved in the activation of adenylate cyclase (27).

Knowing that the AII receptor is coupled to a G protein (G_q) that is responsible for activation of phospholipase C, the purpose of the present study was to look for an effect of PVS, a polyanion, on the mechanism of AII signal transduction. We show that a membrane preparation of bovine adrenal zona glomerulosa contains two types of AII receptors (AT_1 and AT_2). In this preparation, PVS modulates the affinity of AT_1 and does not affect AT_2 significantly. PVS does not inhibit the binding of AII to a primary culture of bovine adrenal glomerulosa cells. The effect of PVS is restored after cell permeabilization. Our results suggest that PVS is regulating the affinity of AT_1 through allosteric interaction with an intracellular domain of the receptor protein.

Experimental Procedures

Preparation of adrenal zona glomerulosa microsomes. Slices (0.5 mm) of the bovine adrenal cortex (containing cells of the zona glomerulosa) were scraped and homogenized with eight strokes of a Dounce homogenizer (loose pestle), in a medium containing (in mM) 100 NaCl, 5 $MgCl_2$, 1 dithiothreitol, and 25 Tris·HCl, pH 7.3. After stirring for 5 min and centrifugation at 500 × *g* for 20 min, the supernatant was centrifuged at 35,000 × *g* for 20 min. The pellet was resuspended in the same medium at a concentration of 10–20 mg of protein/ml. These microsomes were stored at −70°.

Preparation and culture of adrenal zona glomerulosa cells. Glomerulosa cells were prepared from bovine glands and cultured as described by Boulay *et al.* (8). Briefly, 0.5-mm slices of the bovine adrenal cortex were minced into ≈1.0-mm^2 fragments and digested with 2 mg/ml collagenase and 0.2 mg/ml DNase, followed by mechanical dispersion. This procedure was repeated five times. After two washes, the cells were plated (5 × 10^5 cells/well or 2.5 × 10^5 cells/well) in 1.0 ml of Dulbecco's minimal essential medium supplemented with 2% fetal bovine serum, 1% GMS-A (fetal calf serum supplement), 50 units/ml penicillin, 60 μg/ml streptomycin, and 2 mM L-glutamine. Cells were kept in culture at 37°, in a CO_2 incubator, and the medium was changed daily. All the experiments described in this study were performed with cells that had been cultured for 2 days.

Measurement of aldosterone production. After the second day of culture, glomerulosa cells (5 × 10^5 cells/well) were washed twice and incubated in 1 ml of M199 with 3.5 mM K^+, 25 mM HCO_3^-, 0.1% (w/v) BSA, and 0.01% bacitracin, for 2 hr at 37°. The aldosterone content of the medium was measured by radioimmunoassay (28).

AII binding assay with microsomes. Bovine zona glomerulosa microsomes (70–200 μg of protein) were incubated in medium containing (in mM) 100 NaCl, 5 $MgCl_2$, and 25 Tris·HCl, pH 7.3, with 0.1% BSA. Incubations were performed for 45 min at 25°, in a final volume of 1 ml, with ^{125}I-AII (≈0.3 nM) and selected concentrations of AII, DuP-753, PD-123319, or PVS. Nonspecific binding was determined in the presence of 1 μM AII. Incubations were terminated by vacuum filtration through presoaked glass fiber filters (Whatman GF/C) and rapid washing with 3 ml of cold medium. The receptor-bound radioactivity was analyzed by γ counting.

AII binding assay with intact cells. After the second day of culture, glomerulosa cells (2.5 × 10^5 cells/well) were washed twice and incubated in M199 containing 3.5 mM K^+, 25 mM HCO_3^-, 15 mM HEPES, pH 7.3, 0.1% (w/v) BSA, and 0.01% bacitracin. Incubations were performed for 20 min at 37°, in a final volume of 200 μl, with ^{125}I-AII (≈0.3 nM) and selected concentrations of AII, DuP-753, or PVS. Nonspecific binding was determined in the presence of 1 μM AII. Incubations were terminated by immersion of the whole culture plates into two successive baths containing ice-cold phosphate-buffered saline-glucose (5.5 mM). The cells were then scraped from the plates, and the associated radioactivity was determined by γ counting.

Photoaffinity labeling and solubilization of AII receptors. The AT_1 subclass of AII receptors was photolabeled according to the method of Guillemette *et al.* (29). In brief, bovine adrenal cortex membranes (2 mg of protein) were incubated with 2 μCi of [Sar^1, ^{125}I-Tyr^4, (N_3)D-Phe^8]AII (a photosensitive antagonist analogue of AII), in 1.0 ml of medium containing (in mM) 100 NaCl, 5 $MgCl_2$, and 25 Tris·HCl, pH 7.3, with 0.1% BSA. PD 123319 (3 μM) was added in order to prevent the labeling of the AT_2 subclass of receptors. After 45 min at room temperature, the membranes were washed by centrifugation (three times) and irradiated with UV light for 30 min, at 0°. Noncovalently bound ligand was removed by successive washes with an acidic medium (pH 5.5). ^{125}I-labeled receptors were solubilized by incubating the membranes for 45 min at 0° in 1% Triton X-100. After incubation with detergent, a nonsedimentable fraction was obtained by centrifugation for 10 min at 15,000 × *g*. Fresh preparations of solubilized labeled receptors were used for heparin-agarose affinity chromatography.

Adsorption on heparin-agarose. Solubilized labeled receptors (15 fmol) were incubated under gentle agitation, for 15 min at room temperature, with heparin-agarose (50–500-μl wet bed volume), in 1.0 ml of a medium containing 25 mM Tris·HCl, pH 7.3, 100 mM $MgCl_2$, and 0.1% Triton X-100. The incubation was terminated by centrifugation at 200 × *g* for 1 min, and the radioactivity associated with the pellet was determined by γ counting.

Materials. [^{3}H]Aldosterone was purchased from Amersham Corp. (Arlington Heights, IL). Heparin-agarose, DNase, and bacitracin were from Sigma Chemical Co. (St. Louis, MO). Collagenase, culture media, and GMS-A (fetal calf serum supplement) were from GIBCO (Grand Island, NY). Aldosterone antiserum was from ICN Biomedicals (Montreal, Quebec, Canada). DuP-753 and PD-123319 were generous gifts from DuPont and Warner-Lambert, respectively. All other reagents were from Sigma or Fisher (Montreal, Quebec, Canada). ^{125}I-AII (specific activity, 400 Ci/mmol) was prepared with Iodogen as described by Fraker and Speck (30). [Sar^1, ^{125}I-Tyr^4,(N_3)D-Phe^8]AII was synthetized in our laboratory. Scatchard data were analyzed by using the computer-assisted iterative curve-fitting program SCAFIT (31).

Results

Specific and saturable binding sites for ^{125}I-AII were characterized in our microsomal preparation of bovine adrenal glo-

merulosa. Dose-displacement experiments showed that ^{125}I-AII binding was significantly reduced in the presence of 0.1 nM AII and was completely abolished by a concentration around 100 nM (Fig. 1). The IC_{50} value (concentration inhibiting 50% of tracer binding) was 1.6 ± 0.2 nM (mean ± standard deviation of three experiments). The nonpeptide antagonists DuP-753 and PD-123319 also inhibited the binding of ^{125}I-AII to the microsomal preparation (Fig. 1). DuP-753 exhibited a biphasic effect. ^{125}I-AII binding was progressively reduced in the presence of increasing concentrations of DuP-753 (from 3 nM to about 1 μM). At concentrations of DuP-753 between 1 μM and 10 μM, ^{125}I-AII binding remained relatively stable, at a level corresponding to about 15% of maximal binding. In three independent experiments, 3 μM DuP-753 inhibited 86.5 ± 3.4% of ^{125}I-AII binding, and the IC_{50} for this first portion of the dose-displacement curve was 92.9 ± 19.5 nM (mean ± standard deviation). Addition of higher concentrations of DuP-753 (10 μM and above) completely abolished ^{125}I-AII binding (data not shown). These results, suggesting that DuP-753 is interacting with two classes of AII binding sites, are in agreement with recent observations in many tissues (21–23). PD-123319 was also a potent inhibitor of ^{125}I-AII binding to our microsomal preparation (Fig. 1). Although it could inhibit only 12.1 ± 4.1% of maximal binding, its IC_{50} was 6.9 ± 3.7 nM (mean ± standard deviation of three experiments). These observations suggest that ^{125}I-AII interacts with at least two classes of sites in bovine adrenal glomerulosa microsomes and that DuP-753 and PD-123319 selectively recognize different sites. It must be mentioned that a mixture of 10 μM DuP-753 and 0.3 μM PD-123319 completely inhibited ^{125}I-AII binding (data not shown).

Fig. 2 shows the inhibition of ^{125}I-AII binding by increasing concentrations of AII. In the control experiment, AII displaced tracer binding with an IC_{50} of 1.6 ± 0.2 nM. In the presence of 0.3 μM PD-123319, AII was slightly less potent in inhibiting tracer binding, as demonstrated by the rightward shift of the dose-displacement curve. The IC_{50} observed in the presence of 0.3 μM PD-123319 was 2.2 ± 0.5 nM (mean ± standard deviation of four experiments). In the presence of 3 μM DuP-753, the dose-displacement curve was shifted significantly to the left. The IC_{50} observed under these conditions was 0.29 ± 0.21 nM (mean ± standard deviation of three experiments performed

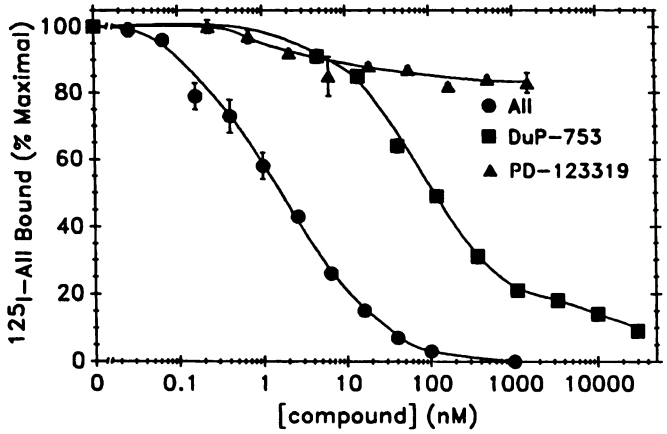

Fig. 1. Inhibition of ^{125}I-AII binding. Typical dose-displacement curves obtained with bovine adrenal glomerulosa microsomes (70 μg of protein) incubated in the presence of 0.3 nM ^{125}I-AII and increasing concentrations of AII, DuP-753, or PD-123319. *100%*, ≈9000 cpm. Each *point* is the mean ± standard deviation of triplicate determinations.

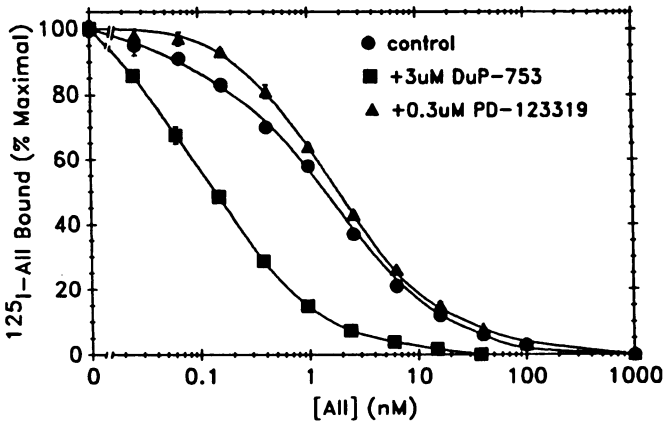

Fig. 2. Inhibition of ^{125}I-AII binding to each class of AII receptors. Typical dose-displacement curves obtained with bovine adrenal glomerulosa microsomes (70 μg of protein) incubated in the presence of 0.3 nM ^{125}I-AII and increasing concentrations of AII. For the experiment in the presence of DuP-753, a larger amount of microsomes (200 μg of protein) was used. *100%*, specific binding in the presence of DuP-753 (≈3,000 cpm) or PD-123319 (≈10,000 cpm). Specific binding in control experiments, ≈12,000 cpm. Each *point* is the mean ± standard deviation of triplicate determinations.

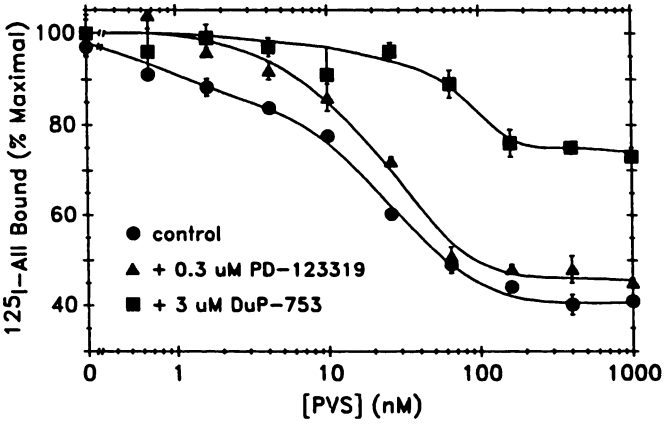

Fig. 3. Effect of PVS on ^{125}I-AII binding. Typical dose-displacement curves obtained with bovine adrenal glomerulosa microsomes (70 μg of protein in the control experiment or in the presence of PD-123319; 200 μg of protein in the presence of DuP-753) incubated in the presence of 0.3 nM ^{125}I-AII and increasing concentrations of PVS. Each *point* is the mean ± standard deviation of triplicate determinations.

with two different membrane preparations). These data suggest that bovine adrenal glomerulosa microsomes contain at least two classes of binding sites for AII. The first class (AT_1) is blocked by low concentrations of DuP-753, does not recognize PD-123319, and displays an affinity of 2.2 nM for AII. The second class (AT_2) is blocked by low concentrations of PD-123319 and by very high concentrations of DuP-753 and displays a high affinity (0.3 nM) for AII.

Under the same experimental conditions, PVS was also a potent inhibitor of ^{125}I-AII binding (Fig. 3). In dose-displacement experiments, tracer binding was reduced by about 10% in the presence of 1 nM PVS and was progressively inhibited by increasing concentrations up to 100 nM PVS. The IC_{50} was 17.3 ± 8.2 nM (mean ± standard deviation of three experiments). PVS could not completely inhibit the binding of ^{125}I-AII. The maximal effect obtained was an inhibition of 56.3 ± 4.6% of ^{125}I-AII binding. In the presence of 0.3 μM PD-123319 (a concentration high enough to saturate AT_2 sites), PVS had sub-

stantially the same effect on ^{125}I-AII binding. However, in the presence of 3 μM DuP-753 (a concentration high enough to occupy most AT_1 sites), only 25% of ^{125}I-AII binding was inhibited by PVS, and this effect was observed at relatively high concentrations of PVS (IC_{50} around 80 nM). To better define the kinetic aspects of the interaction between PVS and AII receptors, AII dose-displacement experiments were performed in the absence or the presence of 3 μM PVS (Fig. 4). Because AT_1 seemed to be the principal target of PVS action, these experiments were performed in the continuous presence of 0.3 μM PD-123319, in order to block AT_2. Fig. 4 shows that, in the presence of 3 μM PVS, ^{125}I-AII binding was reduced by >50%, compared with the control experiment (in the absence of PVS). The two curves, however, indicate that, under both conditions, ^{125}I-AII binding was abolished in a dose-dependent manner by increasing concentrations of unlabeled AII. Fig. 4, *inset*, shows the Scatchard analysis of the same data. In the absence of PVS, Scatchard analysis yielded a curvilinear plot, which could be resolved (using the curve-fitting program SCAFIT) into two specific binding components, probably reflecting two affinity states of AT_1. The values found for the two affinity states were K_{d_1} = 1.1 ± 0.6 nM, B_{max_1} = 1.44 ± 0.48 pmol/mg of protein (100.8 ± 33.6 pM), K_{d_2} = 7.1 ± 4.2 nM, and B_{max_2} = 3.21 ± 0.30 pmol/mg of protein (224.7 ± 21.0 pM). The proportion of high affinity sites was about 31%. In the presence of PVS (3 μM), the two specific binding components were still present and their affinities were only slightly altered (K_{d_1} = 1.1 ± 0.3 nM and K_{d_2} = 9.6 ± 4.2 nM). Under these conditions, however, the number of high affinity sites was significantly reduced (0.91 ± 0.04 pmol/mg of protein) and the number of low affinity sites was significantly increased (4.98 ± 0.73 pmol/mg of protein). The total number of binding sites was not significantly different under the two conditions.

The inhibitory effect of PVS was not selective for AII receptor binding, because PVS could also inhibit the binding of ^{125}I-endothelin (which is known to interact with a G protein-linked receptor) to the same microsomal preparation (IC_{50} of 270 nM) (data not shown).

Because PVS interfered with AII binding, we were interested in looking at the physiological significance of this effect on whole bovine adrenal glomerulosa cells. Fig. 5A shows that PVS (1 μM) had no effect on AII-induced steroidogenesis. On the other hand, DuP-753 (3 μM) inhibited the steroidogenic effect of AII. Consistently, PVS (1 μM) did not inhibit the binding of AII to intact glomerulosa cells (Fig. 5B). Interestingly, however, PVS was able to inhibit the binding of AII to cells that had previously been broken (by freezing and thawing). DuP-753 had a similar inhibitory effect on ^{125}I-AII binding to intact or broken cells. These data suggest that PVS does not interact directly with the recognition site of AII but that the site of action of PVS is intracellular.

Fig. 6 shows that GTPγS, a nonhydrolyzable GTP analogue, inhibited ^{125}I-AII binding. In dose-displacement experiments, tracer binding was reduced by about 10% in the presence of 1 nM GTPγS and was progressively inhibited by increasing concentrations of up to 100 nM GTPγS. The IC_{50} was 9.2 ± 1.3 nM (mean ± standard deviation of three experiments). GTPγS could not completely inhibit the binding of ^{125}I-AII. The maximal effect obtained was an inhibition of 47.8 ± 4.0% of ^{125}I-AII binding. Under the same experimental conditions, PVS had essentially the same effect as GTPγS. It is important to note that concomitant addition of maximal doses of PVS and GTPγS did not produce any additive effect (data not shown),

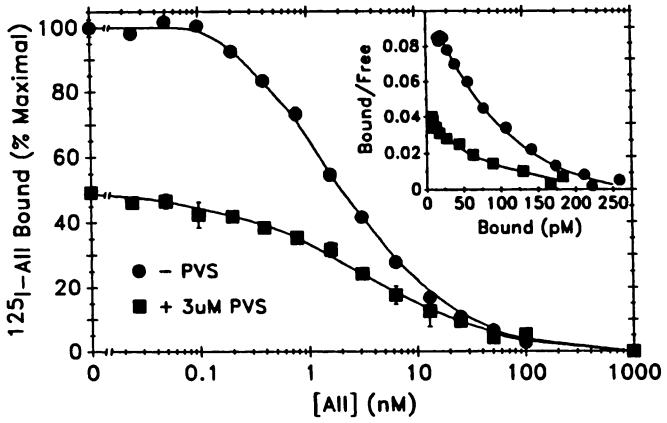

Fig. 4. Nature of PVS inhibitory effect on AT_1. Bovine adrenal glomerulosa microsomes (70 μg of protein) were incubated in the presence of 0.3 nM ^{125}I-AII, 0.3 μM PD-123319, and increasing concentrations of unlabeled AII. The incubation buffer contained no PVS (●) or 0.3 μM PVS (■) *Inset*, Scatchard plot of the same binding data. *100%*, specific binding (≈9000 cpm). Each *point* is the mean ± standard deviation of triplicate determinations.

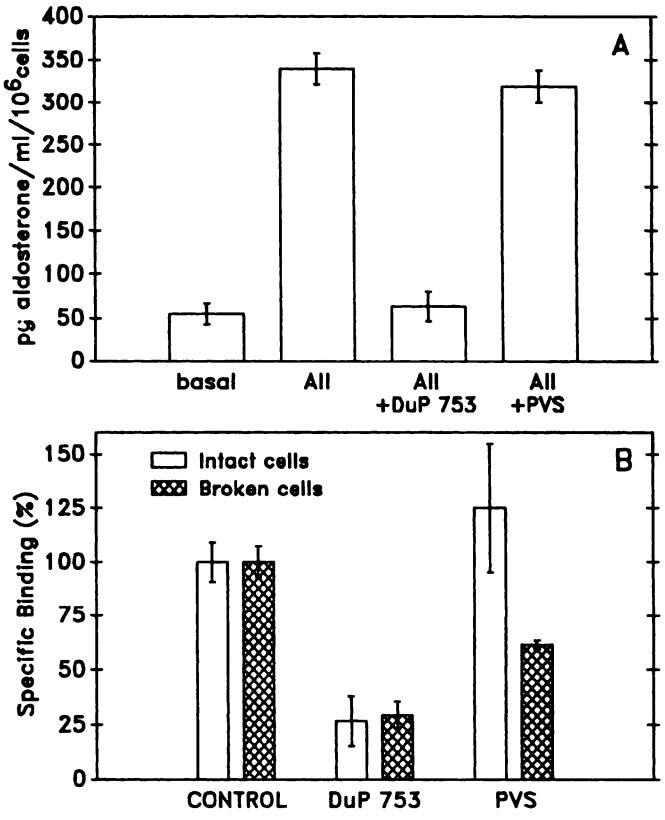

Fig. 5. Effect of PVS and DuP-753 on AII-induced steroidogenesis and AII binding to bovine adrenal glomerulosa cells. A, Glomerulosa cells (4 × 10^5 cells/well) were incubated for 2 hr in the presence of different combinations of the following agents: 1 nM AII, 3 μM DuP-753, and 1 μM PVS. Aldosterone secretion was evaluated by radioimmunoassay. Values are the mean ± standard deviation of triplicate determinations. B, Intact bovine adrenal glomerulosa cells or broken cells were incubated with 0.3 nM ^{125}I-AII in the absence or presence of 1 μM DuP-753 or 0.3 μM PVS. After 45 min at 22°, total binding of the tracer was evaluated, as indicated in Experimental Procedures. Values are the mean ± standard deviation of triplicate determinations.

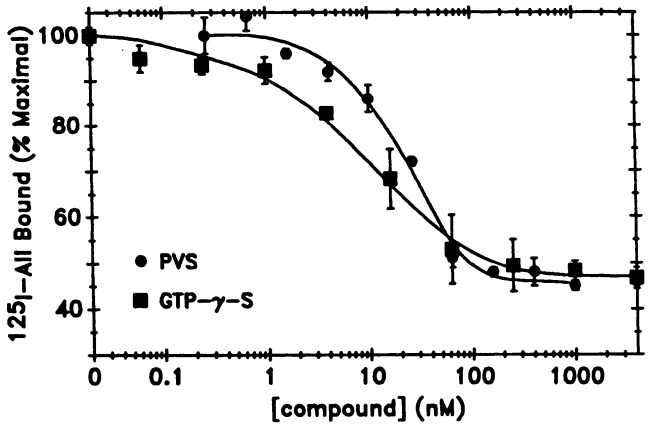

Fig. 6. Effect of GTPγS on ^{125}I-AII binding. Typical dose-displacement curves obtained with bovine adrenal glomerulosa microsomes (50 μg of protein) incubated in the presence of 0.3 nM ^{125}I-AII, 0.3 μM PD-123319, and increasing concentrations of GTPγS or PVS. Each *point* is the mean ± standard deviation of triplicate determinations.

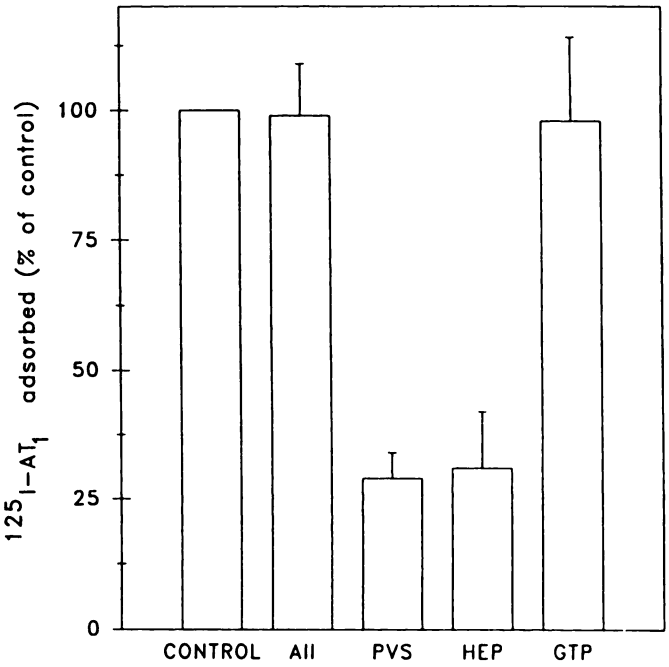

Fig. 7. Adsorption of solubilized AT_1 on heparin-agarose. Solubilized photolabeled AT_1 (25 fmol) was preincubated for 1 hr at room temperature in 1.0 ml of a binding medium containing no further addition (*CONTROL*), 10 μM AII (*AII*), 10 μM PVS (*PVS*), 1 mg/ml heparin (*HEP*), or 10 μM GTPγS (*GTP*). Heparin-agarose gel (50-μl wet bed volume) was then added, and the incubation lasted for 15 min, under gentle agitation. After centrifugation at 200 × *g* for 1 min, gel-associated radioactivity was determined by γ counting. The results are expressed as percentage of radioactivity associated with the gel under control conditions (100% = 2319 cpm). Nonspecific association (in the presence of 1 M NaCl) was around 200 cpm. Each value is the mean ± standard deviation of triplicate determinations. This typical experiment is representative of three similar experiments.

suggesting that the two inhibitors are acting on different components of a common mechanism.

Because our results suggested that PVS was interacting with the receptor protein, we hypothesized that solubilized AT_1 would be retained on a heparin-agarose gel. AT_1 was covalently labeled with a photosensitive analogue of AII and solubilized as described in Experimental Procedures. When 15 fmol of solubilized receptor were incubated with 500 μl of heparin-agarose (wet bed volume) for 15 min at room temperature, about 80% of labeled receptor remained adsorbed on the gel and was readily eluted with 1 M NaCl (data not shown). Fig. 7 shows that prior incubation of solubilized receptor for 1 hr with PVS (10^{-5} M) on heparin (1 mg/ml) inhibited its adsorption on heparin-agarose gel. On the other hand, prior incubation with high concentrations of AII (10^{-5} M) or GTPγS (10^{-5} M) did not interfere with the adsorption of solubilized AT_1 on the gel, indicating that polyanions were interacting directly with the receptor protein at a site different from the AII binding site (as expected, because this extracellular site was occupied by the radioactive photoligand). These results also clearly indicated that PVS and GTPγS were not interacting with the same regulatory element.

Discussion

The octapeptide AII mediates a variety of responses in several tissues, including contraction of vascular smooth muscle, excretion of salt and water from the kidney, release of prolactin from the anterior pituitary gland, and stimulation of aldosterone secretion from the adrenal cortex (1, 32, 33). Recent development of nonpeptide AII antagonists permitted identification of different types of AII binding sites in several tissues (21–24, 34). The sites that were sensitive to DuP-753 (or analogues) were termed AT_1, and those sensitive to PD-123319 (or analogues) were termed AT_2.

The present study revealed the presence of two distinct binding sites for ^{125}I-AII in bovine adrenal glomerulosa microsomes. This view is supported by the demonstration that DuP-753 inhibited ^{125}I-AII binding in a biphasic manner and PD-123319 could displace only 12% of specific ^{125}I-AII binding. Moreover, ^{125}I-AII binding was abolished in the simultaneous presence of 10 μM DuP-753 and 0.3 μM PD-123319 (data not shown). Another indication that bovine adrenal glomerulosa microsomes contained at least two distinct AII binding sites was their residual ability to bind AII in the presence of high concentrations of DuP-753 or PD-123319. Similar results were obtained with partially purified plasma membranes prepared according to the protocole of Glossmann *et al.* (2), indicating that the AT_2 site was present in plasma membrane and not in another membrane fraction. We demonstrated that AII had a significantly higher affinity for AT_2 than for AT_1. Other studies (21–24) have reported that AII had similar affinities for the two sites, but in most of these studies the affinity of AII for AT_2 was higher than for AT_1, as we observed. The affinity and the proportion of AT_1/AT_2 vary considerably according to tissues and species. Dudley *et al.* (24) reported that the proportion of AT_1 was very high in rat liver and rabbit adrenal. In rabbit uterus, AT_2 represented about 70% of total AII binding sites (24). Whitebread *et al.* (22) reported that the ratio of AT_1 to AT_2 was 55:45 in human adrenal and 60:40 in rat uterus. In human uterus only AT_2 was found, whereas in smooth muscle cells of human renal artery and rat aorta only AT_1 was found. In rat adrenal, a tissue that was studied extensively, Chiu *et al.* (21) reported a ratio of AT_1 to AT_2 of 72:28, whereas Whitebread *et al.* (22) reported a ratio of 60:40 and Chang and Lotti (23) found a ratio of 50:50. Our own observations indicated that different microsomal preparations from bovine adrenal glomerulosa contained different proportions of AT_1, varying from 60% to 90% of total AII binding sites. This wide variation

in the proportion of receptor subtypes might explain why a recent study reported the absence of AT_2 in a membrane preparation of the whole bovine adrenal cortex (45). It is known that AT_1 mediates most AII effects on many tissues and isolated cells (23, 24, 35, 36). On the other hand, no functional role has been ascribed to AT_2. In the search for a physiological relevance for AT_2, it should be considered that the high affinity and low capacity of this receptor in bovine adrenal glomerulosa imply that, upon AII stimulation, AT_2 will be more responsive because it will become saturated more quickly than AT_1.

We demonstrated that PVS was a potent inhibitor of AII binding. This inhibitory effect of PVS showed some selectivity for AT_1. The mechanism by which AII binding is inhibited by PVS involves a shift of AT_1 from a high to a low affinity state. We showed that in the absence of PVS the ratio of low affinity to high affinity sites was 2.2, whereas in the presence of 3 μM PVS this ratio was increased to 5.5. In these experiments, the total amount of AT_1 (low affinity plus high affinity) did not significantly change upon addition of PVS. Similar affinity shifts are also observed when agonist-receptor complexes are incubated in the presence of GTP (or analogues) (for review, see Ref. 37). Such an inhibitory effect of GTP (or analogues) on AII binding was observed in the present study (Fig. 6) and in other independent studies (38–40). Because AT_1 is known to stimulate phospholipase C via a G protein (24), our results suggest that PVS might act through a mechanism similar to that of GTP (uncoupling the G protein and the receptor).

The potent inhibitory effect of PVS on AII binding to bovine adrenal glomerulosa microsomes should be reflected in AII-induced steroidogenesis by bovine adrenal glomerulosa cells. Unexpectedly, PVS could inhibit neither AII-induced steroidogenesis nor AII binding to intact bovine adrenal glomerulosa cells. The inhibitory effect of PVS on AII binding was observed, however, if glomerulosa cells had previously been broken by freezing and thawing. These results strongly suggest that PVS interferes with an intracellular domain of AII receptor. Indeed, its polyanionic nature does not allow PVS to cross the plasma membrane, in order to reach the interior of the cell and to produce its inhibitory effect. Obviously, this restriction does not hold when the cell is broken. The effect of PVS on AII binding seems to be due to its polyanionic nature, because other polyanions, such as heparin (IC_{50} of 1.5 μg/ml), dextran sulfate (M_r 8,000) (IC_{50} of 65 nM), and dextran sulfate (M_r 500,000) (IC_{50} of 3 nM), were also able to inhibit ^{125}I-AII binding (data not shown). This suggestion is further supported by the fact that polyvinyl chloride, which lacks a polyanionic group, did not show any inhibitory effect at a concentration as high as 1 μM (data not shown). The results shown on Fig. 7 argue for a direct interaction of PVS with the receptor protein.

Recent studies have shown that polyanions uncouple some receptors from G_s or G_i, thus interfering with agonist-induced activation or inhibition of adenylate cyclase. Willuweit and Aktories (25) reported that heparin blocked the adrenalin-induced inhibition of adenylate cyclase and stimulation of GTP hydrolysis in membranes of human platelets. Their results showed that the affinity of the α-adrenoceptor for adrenalin was decreased in the presence of heparin. Butler *et al.* (26) showed that suramin, a polyanion with antihelmintic properties, completely prevented the opioid peptide-stimulated increase in GTP hydrolysis, by a mechanism other than direct inhibition of opioid peptide binding to its receptor, in neuroblastoma × glioma hybrid (NG 108–15) cell membranes. More recently, Huang *et al.* (27) showed that a series of polyanionic compounds could inhibit the coupling of the α_2-adrenergic receptor and β_2-adrenergic receptor to the G proteins G_i and G_s, respectively. They showed that polyanionic compounds decreased the affinity of these receptors for their agonists. In the same study, they showed that the effects of polyanion binding to a mutant β_2-adrenergic receptor were blunted. This mutant receptor binds agonists and antagonists normally but is lacking the specific domain responsible for coupling to G_s protein.

Interestingly, Bueb *et al.* (41) showed that mastoparan and other cationic amphiphilic peptides (such as substance P, neurotensin, bradykinin, and their analogues) could activate a GTPase activity by interacting directly with a polyanionic domain of a G protein. Finally, it was also observed that the third intracellular loop of many receptors (a domain postulated to be responsible for interaction with G proteins) is positively charged (42). Taken together, these observations strongly suggest that PVS decreases the binding of AII by an allosteric interaction with an intracellular domain of the AII receptor, possibly the positively charged domain responsible for coupling with G proteins. This suggestion is further supported by the recent work of two groups that have successfully used expression cloning strategies to purify an AT_1-encoding cDNA (43, 44). The corresponding protein sequence possessed many cationic amino acids in the third intracellular loop. PVS can thus be considered as a new pharmacological tool that may have useful application in the study of the mechanisms involved in signal transduction.

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