Angiotensin II inhibits adenylate cyclase from adrenal cortex glomerulosa zone

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Angiotensin binding and the effects of angiotensin on adenylate cyclase activity were determined on purified membranes from the glomerulosa zone of bovine adrenal cortex. Angiotensin II inhibited adenylate cyclase activity in a dose-dependent manner with an A_{50}-value of 2 nM. The angiotensin effect required the presence of GTP. Angiotensin also inhibited ACTH-stimulated activity. Angiotensin binding was sensitive to the same effectors which influenced angiotensin-induced adenylate cyclase inhibition. In the presence of NaCl 100 mM and magnesium, angiotensin interacted with a single population of binding sites (K_d = 4 nM and maximal binding capacity of 440 fmol/mg protein). These results and published data suggest that the ability to inhibit adenylate cyclase might be a general property of angiotensin receptors.

Angiotensin II *Adenylate cyclase* *Receptor* *Adrenal cortex*

1. INTRODUCTION

It has been convincingly demonstrated that the angiotensin-induced glycogenolytic response of isolated hepatocytes is mediated by a rise in cytosolic calcium concentration [1]. Current experimental evidence indicates that calcium also plays a predominant role in several other actions of angiotensin such as its vasopressor [2] or steroidogenic effect on the adrenal cortex [3]. On the other hand, it was shown that angiotensin inhibits adenylate cyclase from rat liver [4,5], rat renal cortex [6], and rat anterior pituitary membranes (Gaillard et al., in preparation) in a GTP-dependent manner. Angiotensin receptors were found to be sensitive to GTP, as most receptors positively or negatively coupled to adenylate cyclase [7]. The above mentioned data might be interpreted as indicating the existence of two types of angiotensin receptors, receptors of one type being functionally coupled to the adenylate cyclase and receptors of the other type with the yet unidentified effector responsible for calcium mobilization. Alternatively, they might be interpreted by assuming that angiotensin receptors have the peculiar property of being functionally coupled to two distinct transduction mechanisms (adenylate cyclase inhibition and calcium mobilization). Indeed, it has been shown that receptors such as hepatic vasopressin and α_1-adrenergic receptors which can mediate calcium mobilization in hepatocytes are not negatively coupled to adenylate cyclase. To test the two above hypotheses we studied the effect of angiotensin on bovine and rat adrenal cortex adenylate cyclase, angiotensin-binding to adrenal cortex receptors was also determined. The choice of this material was justified by the fact that there is pharmacological evidence that angiotensin receptors from adrenal cortex are different from other angiotensin receptors at least with respect to their recognition patterns [8].

2. MATERIALS AND METHODS

2.1. *Membrane preparation*

Purified bovine adrenal cortex membranes were prepared as in [9] with minor modifications. Adrenals were collected at the slaughterhouse, cleaned from surrounding fat and kept in cold,

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calcium and magnesium-free phosphate-buffered saline (PBS). The outer portion of the adrenal cortex including the capsula and adhering cells from the glomerulosa zone were separated by dissection. Cells were scraped off from the capsula, rinsed in cold PBS and homogenized in $NaHCO_3$ 20 mM by 10 strokes in a loose Dounce homogenizer. A crude membrane fraction was prepared by two successive centrifugations at 1500 and 20000 × *g*. Membranes were purified using a discontinuous sucrose gradient (31.5%, 38.5%, w/w) centrifuged for 120 min at 25000 rev./min in a Beckman SW 28 rotor. Membranes collected at the top of the 31.5% sucrose layer were sedimented in 20 mM $NaHCO_3$ by centrifugation at 40000 × *g* for 40 min and suspended in 1 mM $NaHCO_3$. All experiments were performed using freshly prepared membranes. Crude membranes from rat (Wistar) adrenal cortex were prepared as follows: The cortical portions of 30 adrenals were homogenized as indicated for bovine adrenal membranes. Cell debris was eliminated by centrifugation at 600 × *g* for 15 min and a crude membrane fraction collected by centrifugation at 30000 × *g* for 30 min. Membranes were suspended in 1 mM $NaHCO_3$.

2.2. *Adenylate cyclase assay*

The incubation medium (100 μl final vol.) was composed of triethanolamine–HCl, 50 mM (pH 7.4); [α-^{32}P]ATP, 50 μM, 1 μCi/assay; ATP, 100 μM; isobutylmethylxanthine, 1 mM; creatine phosphate, 10 mM; creatine kinase, 0.2 mg/ml; adenosine desaminase, 10 units/ml; myokinase, 36 units/ml; GTP 10 μM; [^{3}H]cAMP, 0.01 μCi/assay; and various amounts of NaCl, $MgCl_2$ and hormonal agents tested. After thermal equilibration at 25°C the reaction was initiated by adding membranes (30–50 μg protein), allowed to proceed for 10 min, and stopped by adding 900 μl of a solution containing sodium dodecyl sulphate, 1% (w/w); ATP, 1 mM; and cAMP, 1 mM. Labeled cAMP was separated using the procedure in [10] as modified in [11]. All determinations were performed in triplicate or quadruplicate. Proteins were determined as in [12] using bovine serum albumin as a standard.

2.3. *[^{3}H]Angiotensin-binding assay*

[^{3}H]Tyr^4-labeled Asp^1, Val^5 angiotensin ([^{3}H]angiotensin) was prepared as in [13]. It was purified by high pressure liquid chromatography to a specific radioactivity of 14 Ci/mmol. The incubation medium (final vol. 250 μl) was composed of triethanolamine–HCl, 50 mM (pH 7.4); bovine serum albumin, 1 mg/ml; bacitracine, 0.5 mg/ml; and various amounts of NaCl, $MgCl_2$ and [^{3}H]angiotensin. When the effects of GTP were tested, the incubation medium contained a triphosphonucleotide regenerating system identical to that used for the adenylate cyclase assay. The reaction was initiated by the addition of membranes (30–50 μg protein), and allowed to proceed for 30 min at 22°C. Bound [^{3}H]angiotensin was separated from free [^{3}H]angiotensin as in [14]. Non-specific binding was determined in the presence of 1 μM unlabeled angiotensin. It represented about 10% of total binding at 5 nM [^{3}H]angiotensin.

3. RESULTS AND DISCUSSION

The results from [^{3}H]angiotensin binding experiments are summarized in fig.1. Under control conditions (absence of added magnesium, NaCl and GTP) determination of dose-dependent binding at equilibrium revealed an heterogeneity in the population of binding sites as indicated by a curvilinear Scatchard plot. Addition of magnesium (1 mM) increased the proportion of high affinity sites but did not completely suppress receptor heterogeneity. In the presence of NaCl, 100 mM, the dose-binding curve led to an almost linear Scatchard plot. Apparent K_d and maximal binding capacity were respectively, 5 nM and 550 fmol/mg protein. The mean corresponding values derived from a series of 8 experiments were 4.0 ± 0.2 nM and 440 ± 61 fmol/mg protein. Addition of GTP (100 μM) slightly reduced the maximal binding capacity and increased the apparent K_d (fig.1 right upper panel). The mean K_d in the presence of GTP was 11.7 ± 0.3 nM. The GTP effect was dose-dependent with an apparent K_m-value of 50 nM. 5′-Guanylimidodiphosphate (Gpp (NH)p) was as potent and as efficient as GTP. As illustrated in fig.1, left lower panel, the guanylnucleotide-induced decrease in hormone-binding was dependent on the NaCl concentration in the incubation medium. The maximal NaCl effect was observed between 100 and 200 mM. The effect of Mg^{2+} reached a maximal value at 3 mM; higher concen-

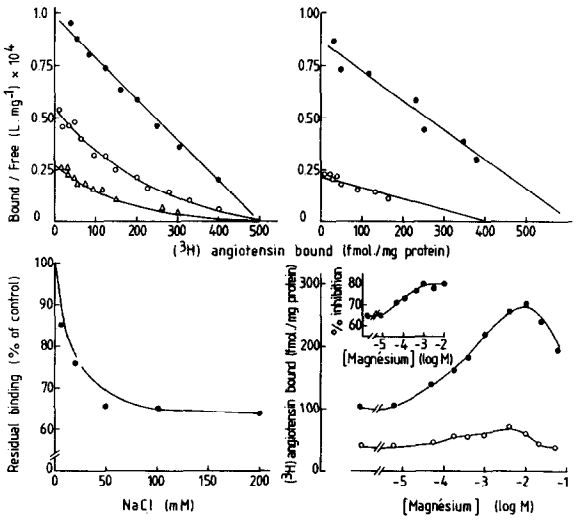

Fig.1. Effects of magnesium, sodium chloride and GTP on [^{3}H]angiotensin-binding to bovine adrenal cortex membranes. Left upper panel: Scatchard plots of dose-dependent [^{3}H]angiotensin-binding determined in the presence of NaCl, 100 mM (●), $MgCl_2$, 3 mM (○) and under control conditions (△). Data on the graph are means of 8 experiments. Right upper panel: Scatchard plots of dose-dependent [^{3}H]angiotensin-binding determined in the presence of NaCl, 100 mM alone (●) and in the presence of NaCl plus GTP (100 μM). The calculated K_d-values and maximal binding capacities were, respectively, 4.9 and 10 nM and 610 and 402 fmol/mg protein. Left lower panel: Specific [^{3}H]angiotensin (4 nM)-binding was determined in the presence (○) or absence (●) of GTP (100 μM) and in the presence of the indicated amounts of $MgCl_2$. Insert: the percent inhibition due to GTP is plotted as a function of magnesium concentration. Right lower panel: Specific [^{3}H]angiotensin (4 mM)-binding was determined in the presence of GTP, 100 μM, and the indicated amounts of NaCl. Data are expressed as % of control values (absence of NaCl).

trations were inhibitory. The relative inhibition of angiotensin binding by guanylnucleotides was slightly increased when increasing magnesium concentration (fig.1). The above described data on the guanylnucleotide and NaCl effect on angiotensin-binding confirm the data reported in [15]. In addition, they indicate that magnesium ions might also be a regulatory ligand of the receptor. The observation (not shown) that the binding of an agonist to the angiotensin receptor is not affected by magnesium, reinforces this interpretation. Thus it appears that angiotensin receptors from bovine adrenal cortex are sensitive to the 3 putative effectors (Mg^{2+}, guanylnucleotide and monovalent ions) involved in receptor-mediated inhibition of adenylate cyclase.

Preliminary experiments on the adenylate cyclase from bovine adrenal cortex indicated that enzyme activity was sensitive to Mg^{2+}, GTP and NaCl. GTP (100 μM) increased basal activity by about 50%. NaCl (100 mM) alone had a similar effect. A combination of NaCl, 100 mM and GTP, 100 μM led to a further increase in enzyme activity. As shown in fig.2, the NaCl component of the combined GTP plus NaCl stimulation was apparent at all magnesium concentrations tested between 0.1 to 10 mM. Magnesium ions increased in similar proportions activities determined in the presence of GTP and GTP plus NaCl. The effect of angiotensin II (1 mM) was tested in the presence and absence of NaCl and as a function of magnesium concentrations. The results obtained are shown in fig.3 and table 1. Both in the presence and absence of NaCl, angiotensin inhibited adenylate cyclase activity. The highest relative inhibition observed was obtained at low magnesium concentrations 47 and 53% at 0.5 mM, Mg^{2+} and 16 and 16% at 10 mM Mg^{2+} in the absence and presence of NaCl, respectively. In the absence of NaCl, ACTH induced a marked activation of adenylate cyclase. The relative activation was more

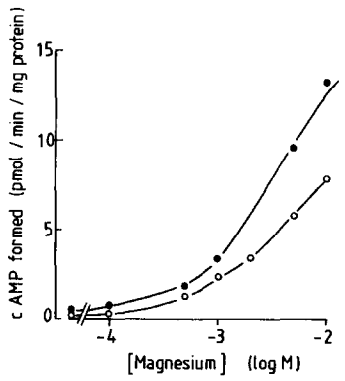

Fig.2. Effects of magnesium and sodium chloride on basal adenylate cyclase activity from bovine adrenal cortex membranes. Adenylate cyclase activity was determined in the presence of the indicated amounts of $MgCl_2$ with (●) or without (○) NaCl 100 mM. Values are means of 3 expt.

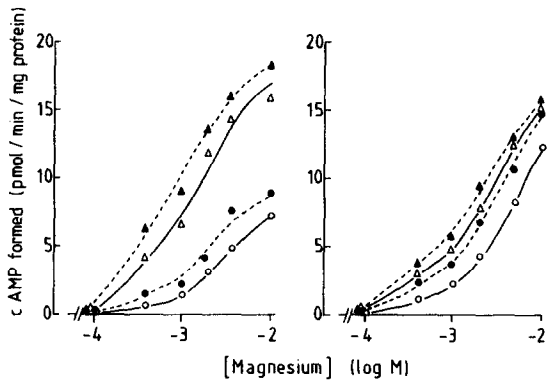

Fig.3. Effect of angiotensin on basal and ACTH-stimulated adenylate cyclase activity from bovine adrenal cortex membranes. The figure shows the results of a typical experiment. Determinations were performed in the presence (right panel) or absence (left panel) of NaCl 100 mM. Activities were determined under basal conditions (●), in the presence of 1 μM ACTH (▲), 1 μM angiotensin (○) and ACTH plus angiotensin (△).

pronounced at low magnesium concentration (364% at 0.5 mM Mg^{2+} as compared to 100% at 5 and 10 mM). Angiotensin inhibited ACTH-stimulated activity; however, the relative inhibition was less pronounced than observed in the absence of ACTH. In the presence of NaCl, stimulation of adenylate cyclase by ACTH was markedly reduced at low magnesium concentration and almost completely abolished at 10 mM magnesium. Inhibition by angiotensin at low magnesium concentration was still apparent.

Table 1

Inhibition by angiotensin of bovine adrenal cortex adenylate cyclase

Mg^{2+} (mM)	Adenylate cyclase activity (pmol.min^{-1}.mg protein^{-1})		Inhibition (%)
	Basal	Reduction due to angiotensin	
0.1	0.52 ± 0.10	0.18 ± 0.05[a]	35
0.5	1.96 ± 0.22	0.43 ± 0.25[b]	22
1.0	3.27 ± 0.17	0.71 ± 0.40[b]	22
5.0	8.99 ± 0.86	1.4 ± 0.8[b]	16
10.0	13.17 ± 1.17	2.3 ± 2.2[NS]	17

[a] $p < 0.01$; [b] $p < 0.05$; [NS], $p > 0.05$

The table summarizes the results of experiments similar to that described in fig.3. Values are means ± SD of 9 determinations. The mean differences between activities determined in the presence and absence of angiotensin (100 μM) were compared to zero using the Student's t-test for paired values

Angiotensin also inhibited adenylate cyclase activity from rat adrenal cortex (not shown). The inhibition was also dependent on the magnesium concentration and had a magnitude comparable to that observed for bovine adrenal cortex adenylate cyclase.

The angiotensin-induced inhibition of bovine adrenal cortex adenylate cyclase was dose-dependent (fig.4). The observed A_{50}-value of 2 nM is close to the K_d for angiotensin-binding (4–5 nM, see above).

It seems reasonable to conclude that angiotensin can inhibit adenylate cyclase activity from rat and bovine adrenal cortex. The inhibition is revealed in the presence of GTP. The requirement for a monovalent ion is less apparent than reported in the case of rat liver [4,5] and rat renal cortex membranes [6]. We show that inhibition by angiotensin of adrenal cortex adenylate cyclase is dependent on the magnesium concentration in the incubation medium. It is increased at low magnesium concentration and markedly reduced at 10 mM magnesium. The conditions (presence of GTP and NaCl, reduction of the magnesium concentration) which affect angiotensin-induced adenylate cyclase inhibition are also the conditions in which agonist-

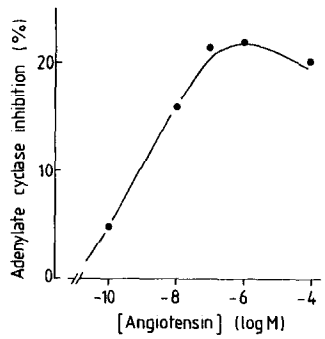

Fig.4. Dose-dependent inhibition of bovine adrenal cortex adenylate cyclase by angiotensin. Adenylate cyclase activity was determined in the presence of the indicated amounts of angiotensin. The incubation medium contained NaCl, 100 mM and $MgCl_2$, 0.5 mM.

specific changes in receptor affinity were demonstrated (not shown). This observation might indicate that, at least a large fraction of the detected angiotensin-binding sites are the receptors involved in adenylate cyclase inhibition. The comparable dose-dependencies for hormone binding and adenylate cyclase inhibition is an additional argument favoring this conclusion.

Our results, together with previously published data, are compatible with the hypothesis that the ability to inhibit adenylate cyclase might be a general property of angiotensin receptors. The physiological significance of this effect remains to be established. For hepatocytes and adrenal cells in which the same function (glycogenolysis and steroidogenesis) was found to be regulated both by cyclic AMP and calcium-dependent pathways, one might suggest that adenylate cyclase inhibition by a calcium mobilizing agent such as angiotensin might contribute to a switch from one regulatory pathway to the other.

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