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CHARACTERIZATION AND COUPLING OF ANGIOTENSIN-II RECEPTOR SUBTYPES IN CULTURED BOVINE ADRENAL FASCICULATA CELLS

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Summary—Angiotensin-II (A-II) receptor subtypes and their potential coupling mechanisms were investigated in bovine adrenal fasciculata cells (BAC) in culture, by the use of selective antagonists for AT1 (DUP 753 or Losartan) and AT2 (PD 123177 and CGP 42112A) sites. Competition for [^{125}I]A-II specific binding with AT1 or AT2 selective ligands produced a biphasic displacement curve, suggesting two distinct A-II binding sites. In the presence of PD 123177 (10^{-5} M), a concentration at which most of the AT2 sites were saturated, DUP 753 displaced [^{125}I]A-II specific binding in a monophasic manner with an IC_{50} of $6.2 \pm 1.4 \times 10^{-7}$ M. In the presence of DUP 753 (10^{-5} M), the displacement produced by CGP 42112A and PD 123177 was also monophasic, with IC_{50}s of $8 \pm 3 \times 10^{-10}$ and $4.6 \pm 2.1 \times 10^{-7}$ M, respectively. The reducing agent dithio-1,4-erythritol inhibited the binding of [^{125}I]A-II to AT1 (DUP 753 sensitive) sites, but increased its binding to AT2 sites 2-fold. The IC_{50} values for these two effects were about 0.5 and 3 mM, respectively. The biological effects of A-II in BAC, phosphoinositide hydrolysis and cortisol production, were inhibited in a dose-dependent manner by DUP 753, but not by AT2 antagonists. Similarly, the potentiating action of A-II on corticotropin-induced cAMP production was blocked by DUP 753, but not by AT2 antagonists. These data indicate that BAC contain both receptor subtypes, but that all the known effects of A-II in BAC were induced via the AT1 receptor subtype.

INTRODUCTION

Angiotensin-II (A-II), the principal biologically active hormone of the renin–angiotensin system, is a pleiotropic factor which elicits a variety of physiological responses in vascular and non-vascular tissues [1]. The possibility of multiple A-II receptor subtypes in peripheral tissues and in the central nervous system has been recognized for some time, but the heterogeneous forms have been established by A-II receptor binding studies using the recently developed series of peptide and non-peptide A-II receptor antagonists [2–4]. A-II receptors have been classified as type 1 or B (AT1), which are sensitive to non-peptide biphenylimidazole antagonists typified by DUP 753, and type 2 or A (AT2), which are sensitive to both non-peptide tetrahydroimidazopyridenes, typified by PD 123177, and to the peptide antagonist CGP 42112A [3, 4]. The existence of two subtypes of the A-II receptor was confirmed recently by the fact that COS cells transfected with a cDNA encoding the A-II receptor express only the AT1 receptor [5, 6].

Several recent studies have shown that the presence and proportion of AT1 and AT2 receptors vary greatly within different tissues of the same species and among the same tissues of different species [2–4, 7], and in some tissues have different developmental patterns [8]. In the adrenal cortex, human [3] and rat [2–4, 9], both subtypes of the A-II receptor are present, but pharmacological evidence has indicated that only the AT1 receptor subtype is involved in the steroidogenic effects of A-II.

In the present study, using cultured bovine adrenal fasciculata cells (BAC) which contain functional A-II receptors [10–12], we attempted to identify the subtypes of A-II receptors present, their coupling to intracellular effectors, and the potential role of each receptor subtype in the steroidogenic response of the cells to A-II.

MATERIALS AND METHODS

Synthetic A-II was obtained from Bachem (Bubendorf, Switzerland), corticotropin (ACTH

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Synacthen) from CIBA (Rueil-Malmaison, France), and insulin and transferrin from Sigma Chemicals Co. (St Louis, MO). D-myo-[2-^{3}H]inositol (16.3 Ci/mmol) was purchased from Amersham (Bucks., England), iodinated cAMP (1100 Ci/mmol) from Pasteur Production (France), and Ham's F-12 medium and Dulbecco's modified Eagle's medium (DMEM) from Gibco (Paris, France). The non-peptide antagonists DUP 753 and PD 123177 were provided by Dr R. D. Smith (Dupont Merck Pharmaceutical Company, Wilmington, DE) and the peptide antagonist CGP 42112A was a generous gift from Dr M. De Gasparo (Ciba-Geigy, Basel, Switzerland).

Cell culture

BAC were prepared by sequential treatment of adrenal cortical slices with trypsin (0.15%) [12]. The cells were cultured in a chemically defined medium, Ham's-F12–DMEM (1:1), containing transferrin (10 μg/ml), insulin (10 μg/ml) and antibiotics. On the second day of culture, the medium was removed and replaced with fresh medium alone. Most of the experiments were carried out on cells cultured for 4 days. In all the experiments, A-II antagonists were added 15 min before the addition of A-II.

cAMP and cortisol determination

Measurement of cAMP and cortisol content of culture medium was performed by specific RIA 1 and 2 h, respectively, after incubation with the hormones [11].

Measurement of the accumulation of [^{3}H]inositol phosphates

Inositol phosphate accumulation was determined as described previously [13]. Briefly, adrenal cells were incubated with myo-[^{3}H]inositol (5 mCi/ml) for 48 h at 37°C. At the end of the incubation period, the medium was removed and the cells were washed and incubated for an additional 10 min in medium containing 10 mM inositol. The medium was again removed, and the cells incubated for 30 min in medium containing 10 mM lithium chloride. The medium was once more removed and replaced by fresh medium containing 10 mM lithium chloride, with or without the indicated effectors. After 30 min, the reaction was terminated by adding ice-cold trichloroacetic acid at a final concentration of 10%. After centrifugation the supernatant was neutralized with NaOH (1 M). Inositol monophosphate (InsP1), inositol diphosphate (InsP2) and inositol triphosphate (InsP3) were separated on Dowex resin columns by the method of Berridge *et al.* [14]. In the present paper, only the accumulation of total inositol phosphates is given, since the inhibitory effect of A-II antagonist on A-II-induced accumulation of total inositol phosphates was parallel to its effect on each of the inositol phosphates mentioned above.

A-II receptor binding assay

A-II was radiolabeled by the iodogen method and then purified on a DEAE trisacryl column [10, 11]. The specific activity of the isolated monoiodinated A-II, as measured by self-displacement in radioreceptor assay, was 600–900 Ci/mmol.

On day 4 of culture, the receptor assay was performed on 12-well plates containing approx. 0.5×10^6 cells per well. Binding was carried out for 1 h at 37°C in 0.5 ml binding medium (DMEM–F12, 0.5% bovine serum albumin, 0.1% bacitracin, 10 mM Hepes pH 7.4) containing approx. 10^5 cpm of [^{125}I]A-II without (total binding) or with 10^{-6} M A-II (non-specific binding). Displacement curves were generated by adding increasing concentrations of unlabeled A-II (10^{-11} to 10^{-7} M), DUP 753 (10^{-10} to 10^{-4} M), PD 123177 (10^{-9} to 10^{-4} M) or CGP 42112A (10^{-11} to 10^{-6} M). To calculate IC_{50} values for high affinity sites, competition binding experiments were carried out with each antagonist while the low affinity sites were blocked with saturating concentrations of the corresponding antagonists. We used DUP 753 (10^{-5} M) to block AT1 sites, and CGP 42112A (5×10^{-8} M) or PD 123177 (10^{-5} M) to block AT2 sites.

Binding experiments, as described above, were also performed with dithioerythritol (DTT) in the binding medium and with cells pretreated with DTT for 15 min at 37°C.

Data are reported as the mean $\pm$ SEM for a minimum of three different experiments. Statistical significance was determinated by Student's t-test.

RESULTS

Effects of A-II or A-II antagonists on [^{125}I]A-II binding to BAC

Specific binding of [^{125}I]A-II to BAC was displaced by unlabeled A-II in a monophasic

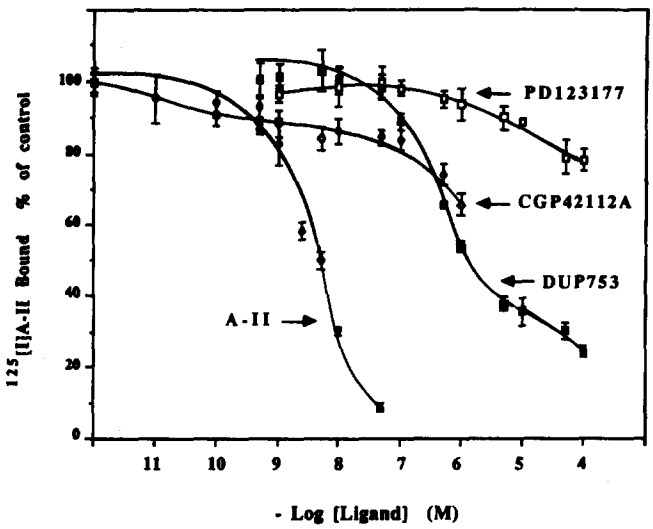

Fig. 1. Displacement of specific binding of [^{125}I]A-II to BAC by A-II (◆), DUP 753 (■), CGP 42112A (◇) and PD 123177 (□). Each point represents the mean ± SD of triplicate determinations from 3 experiments.

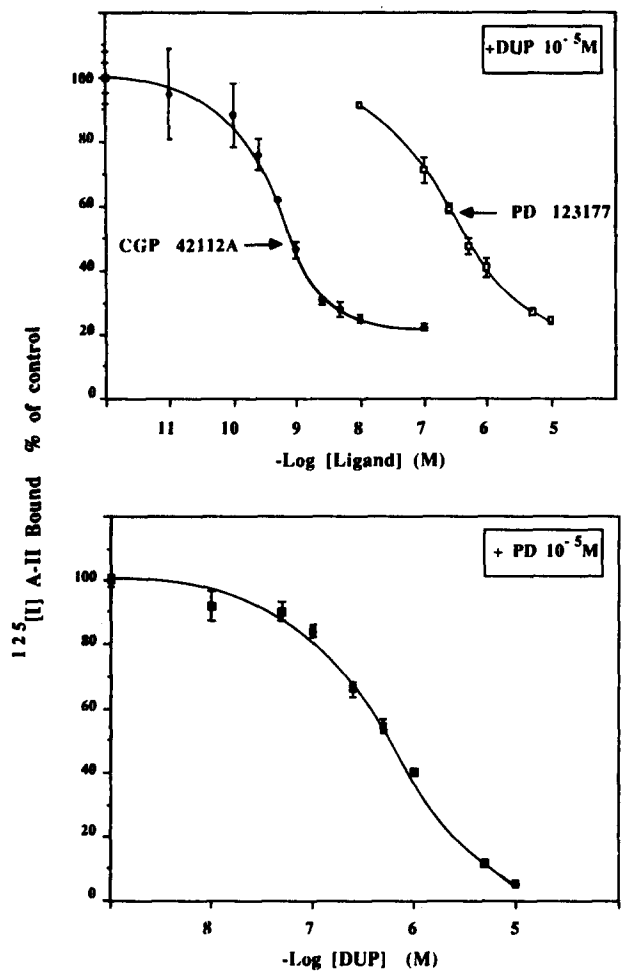

Fig. 2. Top: displacement of specific binding of [^{125}I]A-II by CGP 42112A and PD 123177 in the presence of DUP 753 (10^{-5} M). Bottom: displacement of specific binding of [^{125}I]A-II by DUP 753 in the presence of PD 123177 (10^{-5} M). The results are expressed as % of control, which represents 20% (top) and 75% (bottom) of the total [^{125}I]A-II specific binding. The data are expressed as mean ± SD of triplicate determinations from 3 experiments.

Table 1. Inhibition of the specific binding of [^{125}I]A-II to BAC in the presence of the antagonists or DTT

	[^{125}I]A-II bound % of control	n
Control	100	—
DUP 753 10^{-5} M	32.5 ± 4^{a}	11
DTT 5 mM	$43.7 \pm 3^{a,b}$	4
PD 123177 10^{-5} M	$83.7 \pm 9^{a,c}$	7
CGP 42112A 5×10^{-8} M	$82 \pm 12^{a,c}$	4

Results are expressed as the % of control binding, and represent the mean ± SD of the number of experiments (n) indicated.
$^{a}P < 0.05$ compared to control; $^{b}P < 0.05$ compared to DUP; $^{c}P < 0.05$ compared to DUP or DTT.

manner (Fig. 1). Scatchard analyses of the binding data (not shown) revealed a single class of binding sites with a K_d of $3.7 \pm 1.5 \times 10^{-9}$ M ($n = 10$). In contrast, the displacement curves produced by DUP 753, PD 123177, and CGP 42112A were biphasic (Fig. 1). The first plateau of inhibition with DUP 753 and CGP 42112A was observed at approx. 5×10^{-6} and 10^{-8} M, respectively. The plateau with PD 123177, although less clear, was observed at about 10^{-5} M. These results suggest that BAC, like many other A-II target tissues, contains two distinct A-II receptor subtypes (type AT1, DUP 753 sensitive, and type AT2, PD 123177 and CGP 42112A sensitive). In order to confirm this hypothesis, the displacement of [^{125}I]A-II by one type of antagonist was studied in the presence of saturating concentrations of the other antagonist (Fig. 2). In the presence of PD 123177 at 10^{-5} M, a concentration at which most of the AT2 receptors were saturated, DUP 753 displaced [^{125}I]A-II binding in a monophasic manner (Fig. 2) with an IC_{50} of $6.2 \pm 1.4 \times 10^{-7}$ M. Similar results were obtained in the presence of 5×10^{-8} M CGP 42112A (data not shown). On the other hand, when AT1 receptors were blocked by 10^{-5} M DUP 753, the displacements produced by PD 123177 and CGP 42112A were monophasic, with IC_{50}s of $4.6 \pm 2.1 \times 10^{-7}$ and $8 \pm 3 \times 10^{-10}$ M, respectively (Fig. 2). The residual A-II binding (≃20%) observed in the presence of 10^{-5} M DUP 753 with a maximal dose of CGP 42112A or PD 123 1777 (Fig. 2 top) represents about 4% of the total binding of A-II (20% of AT2 sites × 20%). It could be due to the fact that 10^{-5} M DUP 753 did not block all the AT1 receptors (Fig. 1).

Further evidence for the two receptor subtypes was obtained by the binding studies in the presence of DTT. The reducing agent inhibited binding of A-II to BAC (Table 1), but at high concentrations, the effect was less than that produced by DUP 753. This might be related to the fact that DTT has been reported to modify, in an opposite manner, the binding of A-II to the two receptor subtypes [4, 15]. The data in Fig. 3 clearly demonstrates that the binding of A-II to the AT1 subtype receptors was inhibited in a dose-dependent manner by DTT, whereas binding to the AT2 subtype receptors was enhanced. The concentration required to produce half-maximal inhibition of [^{125}I]A-II binding to AT1 sites (≃3 mM) appears higher than that required to increase [^{125}I]A-II binding to AT2 sites (≃0.5 mM).

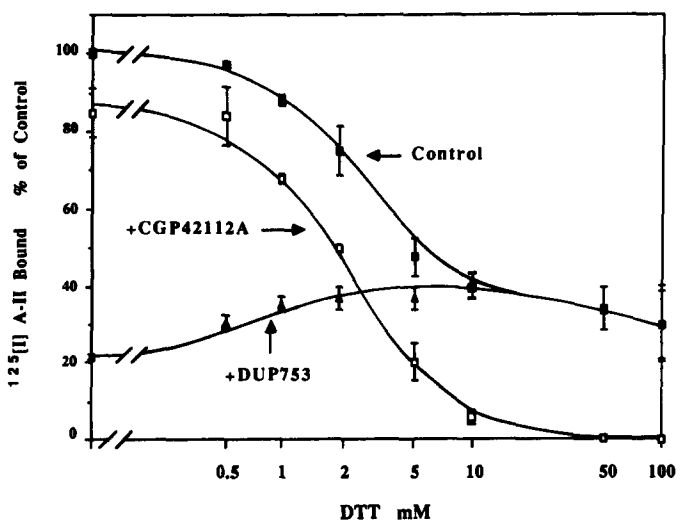

Fig. 3. Effects of DTT on binding of [^{125}I]A-II to BAC, in the absence (control), or in the presence of CGP 42112A (5×10^{-8} M) or DUP 753 (10^{-5} M). The results are expressed as the mean ± SD of triplicate determinations from 2 experiments.

Effects of A-II antagonists on A-II-induced phosphoinositide breakdown in BAC

In the present study, as shown previously [16], A-II induced a dose-dependent increase accumulation of total [^{3}H]inositol phosphates (Fig. 4). The accumulation of total inositol phosphates was increased approx. 7-fold with A-II at 10^{-6} M.

The three A-II antagonists tested, DUP 753 (10^{-5} M), PD 123177 (10^{-5} M) and CGP 42112A (5×10^{-8} M), were without effect by themselves (Fig. 5). However, DUP 753 (10^{-5} M) almost completely blocked the stimulatory effect of 10^{-7} M A-II, and shifted the dose–response to A-II (Fig. 4) to the right. In the presence of A-II at 10^{-7} M, the inhibitory effect of DUP 753 was dose-dependent, with an ID_{50} of $1.7 \pm 1 \times 10^{-6}$ M (Fig. 4).

In contrast, PD 123177 at 10^{-5} M and CGP 42112A at 5×10^{-8} M were unable to antagonize the effect of A-II on this parameter (Fig. 5). When BAC were pretreated with PD 123177 (10^{-5} M) or CGP 42112A (5×10^{-8} M) in conjunction with DUP 753 (10^{-5} M), the inhibitory effect was similar to that observed in the presence of DUP 753 at 10^{-5} M alone (Fig. 5).

DTT at 5 mM also significantly inhibited A-II-induced phosphoinositide breakdown, though at this concentration its effect was lower than that induced by DUP 753 at 10^{-5} M (Fig. 5).

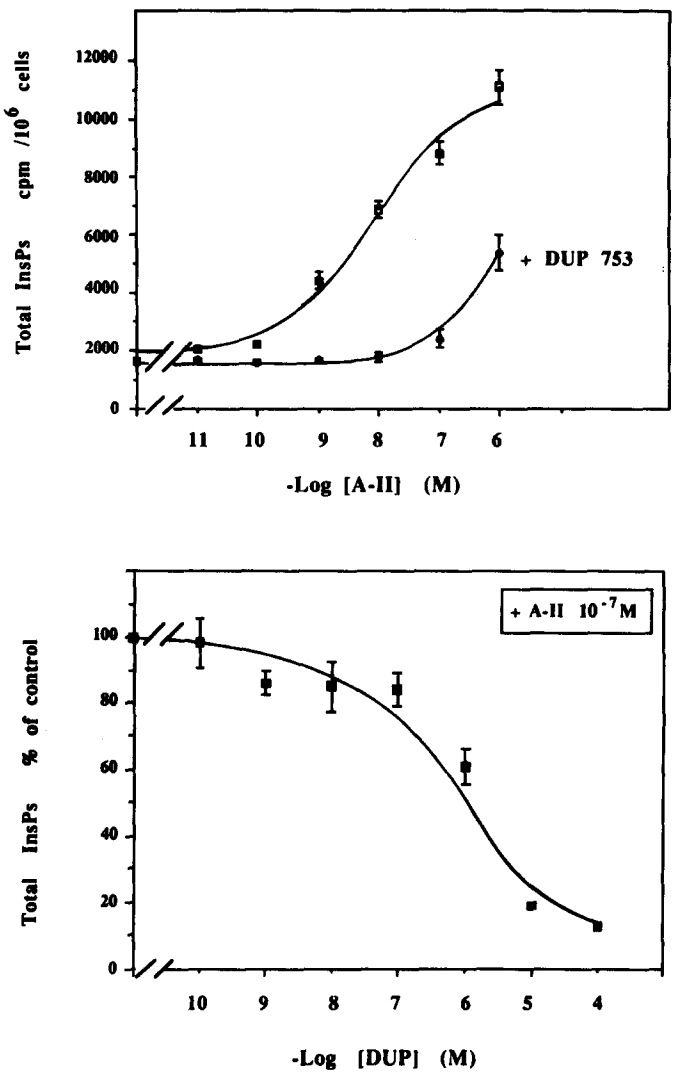

Fig. 4. Top: A-II-induced inositol phosphate (InsP) accumulation in the absence or in the presence of DUP 753 (10^{-5} M). Bottom: inhibition of A-II (10^{-7} M)-induced inositol phosphate accumulation by increasing concentrations of DUP 753. Results are expressed as the mean ± SD of 2 or 3 different experiments, each done in triplicate.

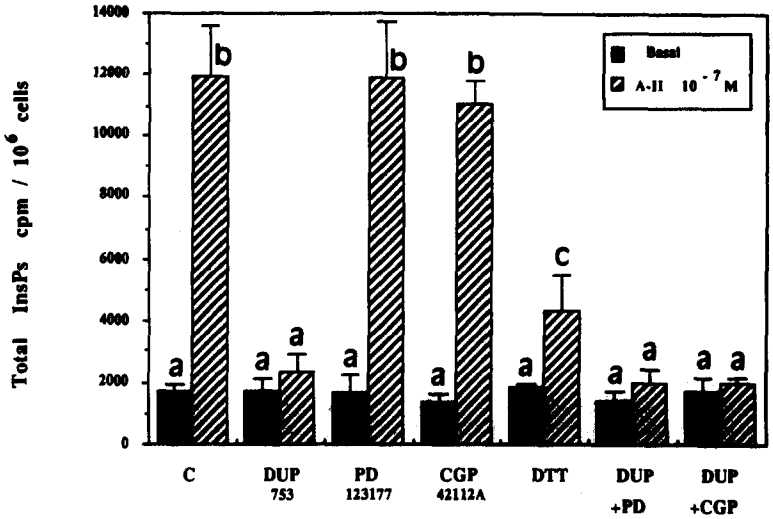

Fig. 5. Effects of A-II antagonists DUP 753 (10^{-5} M), PD 123177 (10^{-5} M), and CGP 42112A (5×10^{-8} M), alone or together with DTT (5 mM) on basal and A-II-stimulated inositol phosphate (InsP) accumulation by BAC. The results are expressed the mean ± SD of 2 to 6 experiments, each done in triplicate. Different superscript letters denote a statistical difference ($P < 0.05$).

Effects of A-II antagonists on the potentiating action of A-II on ACTH-induced cAMP production by BAC

In several cell systems [17, 18], including BAC [13], it has been shown that activation of protein kinase C by the phorbol ester PMA potentiates the effects on cAMP production of agonists coupled positively to adenylate cyclase. Similarly, A-II, which alone had a small but significant effect on cAMP production by BAC, potentiated the effects of ACTH [13]. Therefore, we investigated the effects of the A-II antagonists on cAMP production by BAC under several

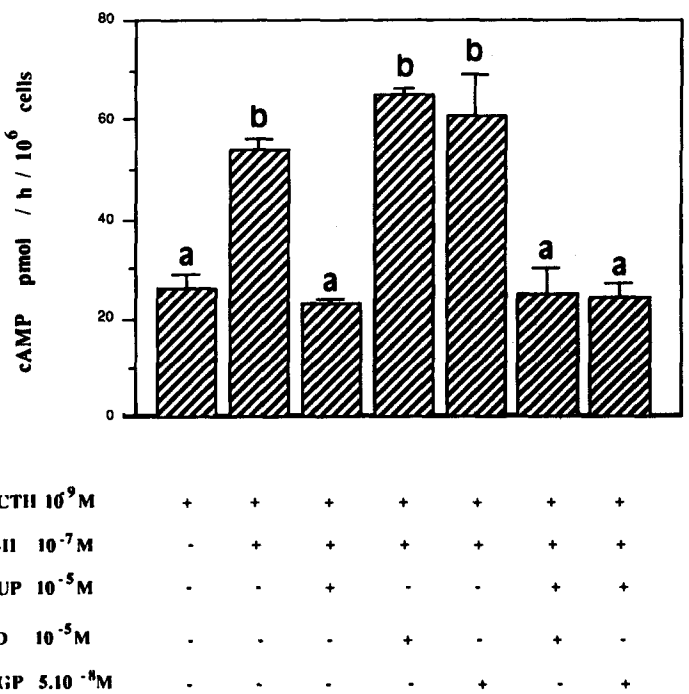

Fig. 6. Effects of A-II antagonists DUP 753 (10^{-5} M) and CGP 42112A (5×10^{-8} M), alone or together, on A-II-potentiation of ACTH-induced cAMP production by BAC. The results are expressed as the mean ± SD of 3 experiments, each done in triplicate. Basal and A-II-stimulated cAMP were 0.12 ± 0.03 and 0.68 ± 0.07 pmol/h/10^6 cells, respectively. Different superscript letters denote a statistical difference ($P < 0.05$).

experimental conditions. Neither DUP 753 at 10^{-5} M, PD 123177 at 10^{-5} M, nor CGP 42112A at 5×10^{-8} M had any effect on cAMP production. Similarly, these compounds were unable to modify ACTH-induced cAMP production (data not shown). As previously shown [13] A-II at 10^{-7} M enhanced ACTH-induced cAMP production 2-fold (Fig. 6), and this potentiating effect was abolished by DUP 753 (10^{-5} M), but not by PD 123177 or CGP 42112A at concentrations which blocked most of the AT2 receptors.

Effects of A-II antagonists on A-II stimulated cortisol production by BAC

A-II stimulated, in a dose-dependent manner, cortisol production by BAC, with an ED_{50} of $3.9 \pm 2 \times 10^{-9}$ M ($n = 3$) (Fig. 7). DUP 753 at 10^{-5} M shifted the response to A-II to the right (Fig. 7) suggesting competitive A-II antagonism. This hypothesis was confirmed by the fact that in the presence of A-II (10^{-7} M), the inhibitory effect of DUP 753 on A-II-induced cortisol production was dose-dependent, with an ID_{50} of $6.5 \pm 2 \times 10^{-7}$ M ($n = 3$; Fig. 7), which is similar to the IC_{50} (Fig. 2). DTT at 5 mM produced similar inhibition to that of DUP 753. In contrast, neither PD 123177 at 10^{-5} M, nor CGP 421124 at 5×10^{-8} M, concentrations at which these compounds occupied most of the AT2 receptors, had any effect on A-II-induced cortisol production (Fig. 8).

DISCUSSION

The present results show that specific [^{125}I]A-II binding in BAC is to two distinct sites. This view is supported by the finding that the A-II

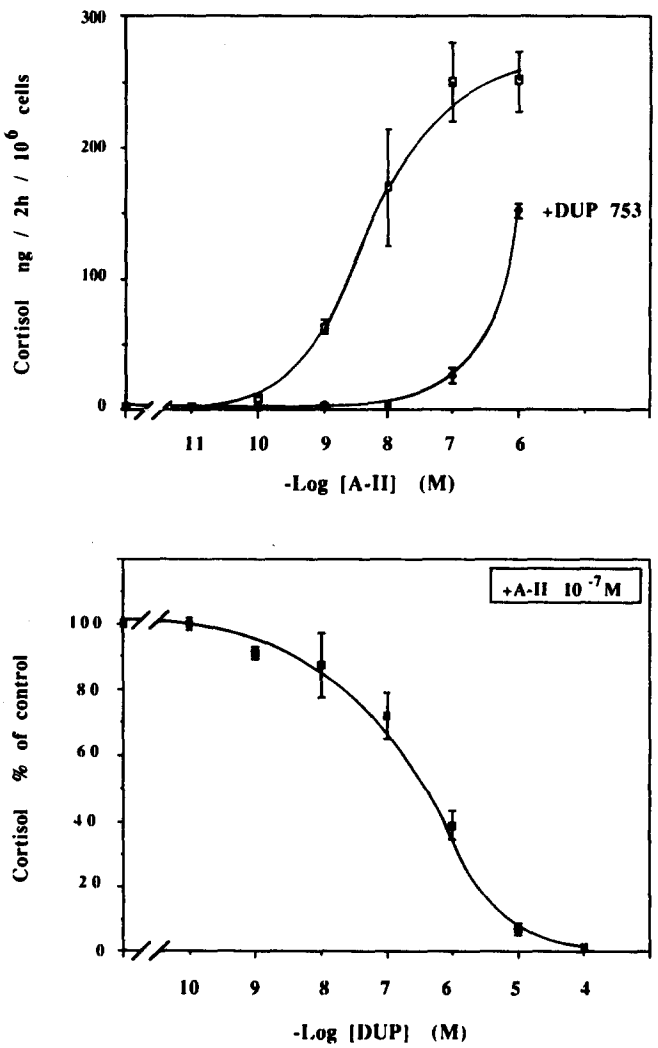

Fig. 7. Top: A-II-induced cortisol production by BAC in the absence (□) or in the presence (◆) of DUP 753 (10^{-5} M). Bottom: inhibition of A-II (10^{-7} M)-induced cortisol production by increasing concentrations of DUP 753. The results are expressed as the mean ± SD of 3 experiments each done in triplicate.

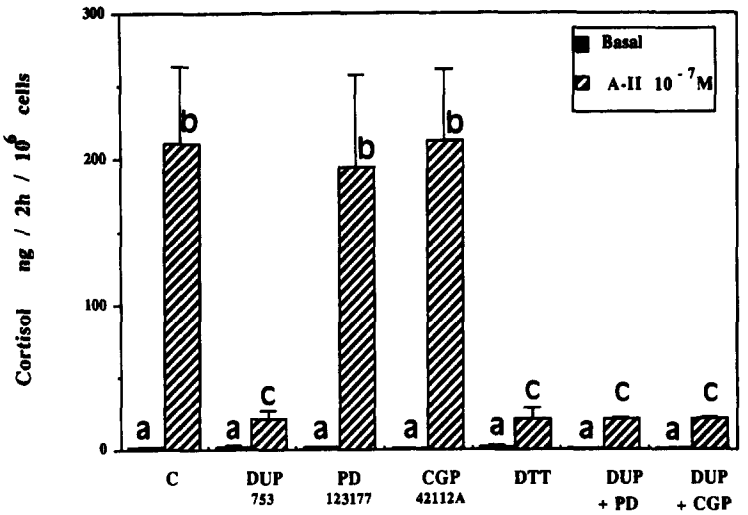

Fig. 8. Effects of A-II antagonists DUP 753 (10^{-5} M) PD 123177 (10^{-5} M), and CGP 42112A (5×10^{-8} M), alone or together, with and DTT (5 mM) on basal and A-II-induced cortisol production. The results are expressed as the mean ± SD of 2 to 6 experiments, each done in triplicate. Bars with different superscript letters differ from each other ($P < 0.05$).

antagonists DUP 753, PD 123177 and CGP 42112A displaced specific [^{125}I]A-II binding in a biphasic manner. In the presence of 10^{-5} M DUP 753, PD 123177 and CGP 42112A displaced [^{125}I]A-II binding in a monophasic manner; in the presence of PD 123117 at 10^{-5} M, DUP 753 produced a monophasic displacement of bound [^{125}I]A-II.

These binding data are in good agreement with those previously reported for human and rat adrenal membranes [2–4, 9], and indicate that BAC also contain the two A-II receptor subtypes, AT1 and AT2. However, the proportion of AT2 binding sites in BAC (≃20%) was lower than that reported for rat and human adrenal membranes (50%), but higher than that seen in rabbit adrenal cortex (5%) [15, 19]. Another difference is that the IC_{50} value for DUP 753 is about one order of magnitude higher for BAC than for rat or human adrenal. The binding sites were also differentiated by their sensitivity to the disulfide reducing agent DTT. As in other A-II-target tissues [2, 3], this agent inhibited the binding of A-II to AT1 sites in BAC, and increased its binding to the AT2 receptor subtype about 2-fold. Since bovine [5], rat [6] and human [20] AT1 receptors contain cysteine residues in the extracellular domains (N-terminal) and DTT has inhibitory effects on the binding of A-II to AT1 sites, the disulfide bridge between these cysteines might be involved in the binding. On the other hand, the mechanisms by which DTT enhances the binding affinity of A-II to AT2 receptors [4, 15, and our own unpublished results] are unknown, since the amino-acid sequence of this receptor has not yet been determined.

Recently, it has been reported that membranes from bovine adrenal cortex and medulla, as well as cultured bovine glomerulosa cells, contain only AT1 receptors [21]. We observed the AT2 receptor subtype in more than 15 different cell preparations; accordingly, the discrepancy between the previous results and our own may be related to a difference in breed and/or in culture conditions.

Since we have characterized many A-II-induced responses in BAC [10–13, 16, 22], we intended to use this model to explore the functional role of the two A-II receptor subtypes. In these cells, since A-II stimulates phosphoinositide hydrolysis in a dose-dependent manner [16], we investigated the potential inhibitory action of A-II antagonists on this parameter. We found that DUP 753 blocked the stimulatory effect of the hormone in a dose-dependent manner, whereas PD 123177 and CGP 42112A were without effect at concentrations selective for AT2 sites.

A-II inhibits both basal and ACTH-stimulated adenylate cyclase in membranes from rat and bovine adrenal [13, 23, 24]. However, in cultured BAC, A-II potentiates rather than inhibits ACTH-induced cAMP production. Although the mechanisms involved in this A-II action are unclear, we have investigated whether any of the agonists for the A-II receptor subtypes could inhibit this action of the

hormone. Again, only the AT1 receptor antagonist DUP 753 was able to block the effect of the hormone.

A-II is a very potent stimulator of BAC steroidogenesis [10–12, 22, 25]. Neither PD 123177 nor CGP 42112A, at concentrations which are selective for AT2 sites, had any effect. In contrast, the steroidogenic effects of A-II were effectively antagonized by DUP 753 in a manner consistent with competitive inhibition as seen by the shift in the A-II concentration–response curve in the presence of DUP 753, and in the dose–response inhibition by DUP 753 in the presence of A-II. DTT also inhibited the steroidogenic effects of A-II. Moreover, the inhibitory effects of DUP 753 on A-II-induced BAC cortisol production are similar to those obtained both *in vivo* and *in vitro* for aldosterone production by the adrenal glomerulosa [2, 21].

In our model there was good correlation between the estimated IC_{50} ($6.2 \pm 1.4 \times 10^{-7}$ M) and the half-maximal concentration of DUP-755 required to inhibit A-II-induced cortisol secretion ($ID_{50} \simeq 6.5 \pm 2 \times 10^{-7}$ M), but the ID_{50} for phosphoinositide breakdown was about 3-fold higher. The reasons for this discrepancy is unclear, but it must be noted that such discrepancy also exists for A-II: the K_d or the ED_{50} for cortisol ($\simeq 3 \times 10^{-9}$ M) are about 3 times lower than the ED_{50} for phosphoinositol hydrolysis. This implies that submaximal inositol phosphate accumulation is able to induce maximal cortisol secretion. It is interesting to note that in BAC we have also observed a discrepancy between ACTH receptor occupancy, cAMP production and cortisol secretion [26].

The present results demonstrated that BAC contain two A-II receptor subtypes. They confirm that the acute effects of A-II on adrenal cells are mediated by the AT1 receptor, which, as in other tissues, is probably coupled to a G-protein [27, 28]. Although the coupling of the AT1 receptor to phosphoinositide breakdown has been extensively studied in many A-II target tissues, and confirmed recently by the cloning and expression of the AT1 receptor [5, 6], the coupling as well as the physiological role of the AT2 receptor subtype remains to be elucidated. Some preliminary observations suggest that the AT2 receptor mediates the A-II-induced fall in cGMP levels in neonatal rat neurones [29], but the significance of this signaling pathway on the effects of A-II is not yet clear.

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