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Angiotensin II receptor type 1 on granulosa and thecal cells of rabbit preovulatory follicles

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Abstract

Specific, high-affinity angiotensin II (A II) receptors were observed on granulosa and thecal cells of preovulatory ovarian follicles from immature PMSG-treated rabbits. Scatchard analysis of ^{125}I-[Sar^1,Ile^8]A II binding to freshly prepared cells was indicative of only one class of binding sites. K_d values were 0.26 ± 0.11 nM and 0.18 ± 0.02 nM, densities of A II receptors were 0.06 ± 0.02 fmol/10^5 cells and 0.08 ± 0.01 fmol/10^5 cells for granulosa and thecal cells, respectively. When cells were incubated for 48 h with hCG, K_d values were of the same order of magnitude, but the amount of A II receptors was increased 2-fold in granulosa and 4-fold in theca. Using subtype specific ligands (Losartan for AT_1 and PD 123319 for AT_2) in competitive binding experiments, A II receptors were found to be of the AT_1 type on both granulosa and thecal cells freshly prepared or incubated 48 h in vitro. These results establishing the existence of high affinity AT_1 receptors on the two cell types of the rabbit preovulatory follicles contrast with previous observations showing the presence of AT_2 receptors on granulosa or theca from several species.

Keywords: Angiotensin II receptor; Granulosa; Theca; (Rabbit ovary)

1. Introduction

Angiotensin II (A II), the biologically active peptide in the renin-angiotensin system (RAS) elicits a variety of actions, related mainly to the regulation of blood pressure and fluid osmolarity [1]. The presence of RAS has been reported in a wide variety of tissues, including kidney, heart, adrenal, brain and reproductive organs [2]. A local RAS is present in the ovary [3,4]. A role for A II has been suggested in oocyte maturation [5] or ovulation [6–9] and several data support the hypothesis that A II might modulate steroidogenesis and play a role in follicular atresia [4,10–13].

A II receptors have been separated into two major subtypes using non-peptide selective antagonists [14,15]. Those defined by their sensitivity to Losartan are known as AT_1 subtype and those sensitive to PD 123319 are designated as AT_2 subtype. Most of the well-known physiological functions of A II are mediated by AT_1, whereas there is little information regarding the role and the transducing pathways of AT_2. In the ovary, conflicting data has been presented concerning the type of A II receptor. In the bovine ovary, radiolabeled A II bound to thecal cells but not granulosa cells and the receptor type was AT_2 [16]. In contrast, in the rat ovary A II receptors were found mainly on granulosa cells of atretic follicles and were shown to be AT_2, while receptors in the residual ovarian tissue after removal of the granulosa were of AT_1 type [10,17].

In a previous in vitro study on the rabbit ovary, we have shown that under hCG stimulation A II has the ability to enhance the production of androgens in theca and to reduce the synthesis of estrogens in granulosa and thecal cells suggesting that A II could induce locally an increase in follicular fluid androgen/estrogen ratio and possibly participate in causing atresia [13]. The purpose of the present study was now to identify biochemically and to characterize the receptor site for A II using selective antagonists of A II receptor subtypes on isolated granulosa and thecal cells from rabbit follicles under the conditions of our previous study.

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2. Materials and methods

2.1. Reagents

Culture products and fetal calf serum, were purchased from GIBCO (Eragny, France). PMSG (Chrono Gest) was obtained from Intervet (Angers, France), hCG from Organon (Sérifontaine, France) and T61 from Distrivet (Paris, France). Angiotensin II, Tris-HCl, bovine serum albumin (BSA) (fraction V) were from Sigma (St-Quentin Fallavier, France). ^{125}I-[Sar^1,Ile^8]A II (2000 Ci/mmol) was purchased from Amersham (Les Ulis, France). The non-peptide antagonists of A II, Losartan and PD 123319 were a gift from Dr. R.D. Smith (Du Pont Merck Pharmaceutical Company, Wilmington, DE, USA) and from Dr. D.G. Taylor (Park Davis, Ann Arbor, MI, USA), respectively.

2.2. Animals

Immature HY white rabbits, 12-weeks old, 2.5–3 kg body weight (Elevage Lemonnier, Avenay, France) were used throughout the study. They were housed individually for about 2 weeks with a 14 h-light and 10 h-dark cycle and allowed free access to water and food.

The development of numerous preovulatory follicles was induced by intramuscular injection of 200 IU PMSG daily for two days. The animals were killed by intracardiac injection of 2 ml T61 four days and a half after the second injection of PMSG.

2.3. Preparation of ovarian crude membrane fractions

Ovaries of rabbit were rapidly removed, immediately placed in 0.9% NaCl (wt/vol), then cleaned of fat and connective tissue and weighed. Membrane homogenate was obtained using a procedure described by Ziecik et al. [18]. Briefly, ovaries were minced in ice-cold THS buffer (25 mM Tris-HCl, pH 7.4, containing 0.25 M sucrose) and homogenized at 4°C with a Teflon-glass Potter homogenizer. The homogenate was centrifuged for 30 min at $500 \times g$ at 4°C, and the supernatant further centrifuged for 1 h at $25\,000 \times g$ at 4°C. The resulting crude membrane pellet was suspended in ice-cold THM buffer (25 mM Tris-HCl and 5 mM $MgCl_2$, pH 7.2). An aliquot of the membrane preparation was assayed for protein determination using the method of Bradford [19] with BSA (fraction V) as standard. After addition of BSA to a final concentration of 0.1%, the crude membrane suspension was stored at −80°C for further measurement of A II receptors.

2.4. A II binding studies using crude membrane fractions

^{125}I-[Sar^1,Ile^8]A II binding was analyzed by the method of Scatchard to determine the maximum A II binding capacity (B_{max}) of ovary membrane fractions and the dissociation constant (K_d) of the binding sites for A II. ^{125}I-[Sar^1,Ile^8]A II (range 4–560 pM, final concentrations) was incubated at 22°C for 60 min in THM buffer-0.1% BSA with 50 μl membrane suspension (20 μg proteins) in a final volume of 200 μl with or without 10^{-6} M unlabeled A II. At the end of the incubation, separation of receptor-bound from free ^{125}I-A II was performed by two successive washings with 4 ml ice-cold incubation buffer and subsequent centrifugations. Radioactivity of membrane pellets was determined by γ counting at an efficiency of 75%.

2.5. Binding studies on freshly prepared granulosa and thecal cells

Granulosa and thecal cells were isolated by a procedure previously described which allows to obtain granulosa cells devoid of thecal cell contamination and thecal cells essentially free of adherent granulosa cells [20]. The final cell suspensions were diluted with medium (MEM with Earle's salts, 20 mM Hepes, 50 IU penicillin and 50 μg streptomycin/ml, 0.1% BSA) and aliquots of cell suspensions ($5 \cdot 10^5$ cells/50 μl) distributed in tubes.

^{125}I-[Sar^1,Ile^8]A II binding on freshly prepared cells was analyzed by the method of Scatchard. Cells were incubated for 1 h at 37°C with varying concentrations of ^{125}I-A II (range 35–700 pM) with or without $2 \cdot 10^{-7}$ M unlabeled A II in a final volume of 200 μl. The reaction was stopped by addition of ice-cold medium followed by centrifugation at $300 \times g$ for 10 min at 4°C. The supernatant was aspirated and discarded. Cells were washed and centrifuged five times with MEM medium-0.1% BSA to separate cell-bound from free ^{125}I-A II. Radioactivity in each tube was determined by γ counting.

2.6. Binding studies on cultured granulosa and thecal cells

Isolated cells were incubated in Costar 24 well-plates in MEM medium-5% fetal calf serum at a concentration of $8 \cdot 10^5$ cells/ml with 10 mIU/ml hCG for 48 h under 5% CO_2 in air at 37°C before binding assay.

The procedure for the ^{125}I-[Sar^1,Ile^8]A II binding assay to primary cultures of granulosa and thecal cells was carried out essentially as described by Brunswig-Spickenheir and Mukhopadhyay [16] for bovine thecal cells. After 48 h of incubation the cultured cells were washed three times with fresh culture medium-0.1% BSA. They were then incubated for 1 h at 37°C with varying concentrations of ^{125}I-[Sar^1,Ile^8]A II (range 25–400 pM) with or without $2 \cdot 10^{-7}$ M unlabeled A II in a final volume of 200 μl. At the end of incubation, medium was aspirated off, then cells were washed five times with ice-cold culture medium-0.1% BSA and removed from the plates. Cell-bound radioactivity was determined by γ counting.

2.7. Characterization of A II receptor subtype on cultured cells

In order to characterize the A II receptor subtype on cultured cells ^{125}I-[Sar^1,Ile^8]A II was used as a ligand

(final concentration 70 pM) and incubated with increasing concentrations of unlabeled A II, Losartan (selective for AT_1 subtype) or PD 123319 (selective for AT_2 subtype).

2.8. Data analysis

The results are expressed as the mean ± S.E.M. Statistical analysis was performed using the Student's *t*-test to compare the number of binding sites.

Equilibrium binding experiments were analyzed using the linear regression analysis program Sigma Plot 4.0 (Jandel Scientific). IC_{50} values were obtained by linear regression of log–logit plots of competition curves (program Securia, Packard).

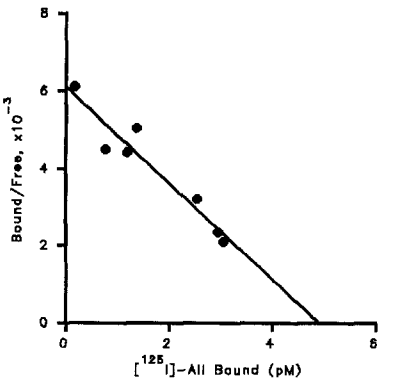

Fig. 1. Representative Scatchard plots of ^{125}I-[Sar^1,Ile^8]A II specific binding to ovarian crude membrane fractions. Values are the mean of triplicate incubations. In this experiment, the K_d was 0.3 nM.

3. Results

3.1. Density and binding characteristics of angiotensin II receptors in ovarian crude membranes

A II receptors (estimated by the specific cell binding of the A II receptor antagonist ^{125}I-[Sar^1,Ile^8]A II) were present on ovarian membrane homogenates from PMSG-treated rabbits. Scatchard analysis (Fig. 1) of the saturation binding experiments seems to indicate the existence of a single class of binding sites with a concentration of 43.6 ± 17.6 fmol/mg protein (B_{max}), and a high binding affinity (K_d = 0.27 ± 0.04 nM) (n = 4).

3.2. High-affinity angiotensin II receptors on freshly prepared granulosa and thecal cells

Next we determined the characteristics of ^{125}I-[Sar^1,Ile^8]A II binding sites in each two cell type of the

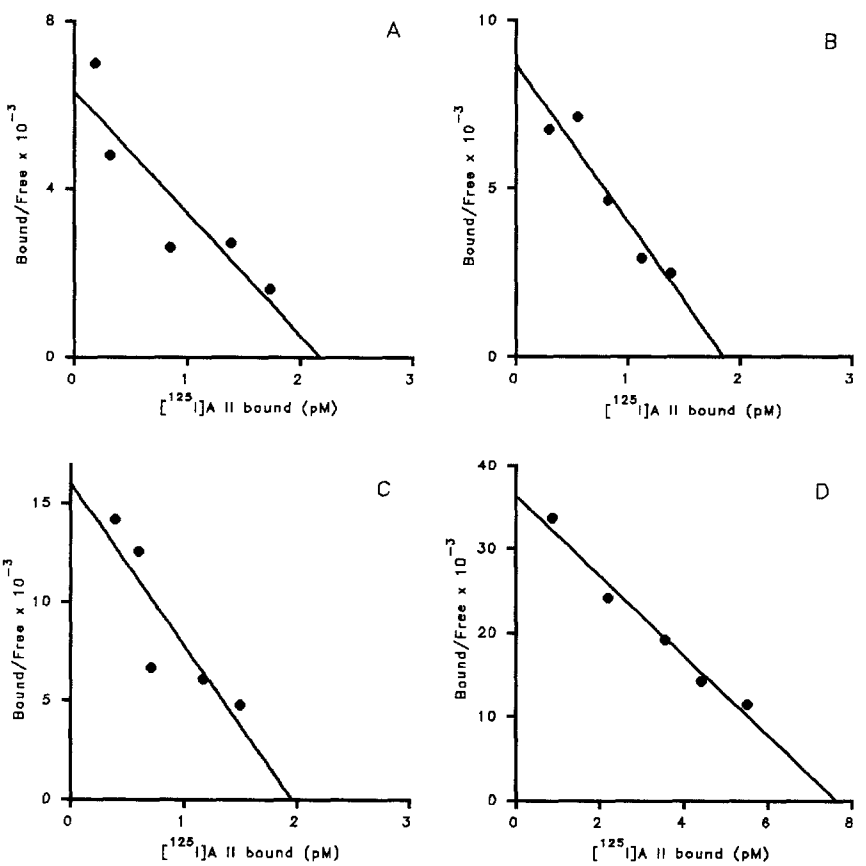

Fig. 2. Representative Scatchard analysis of ^{125}I-[Sar^1,Ile^8]A II specific binding to freshly prepared ovarian granulosa cells (A) and thecal cells (B) or to cultured granulosa cells (C) and thecal cells (D). Values are the mean of triplicate incubations. In these experiments, the K_d was 0.37 nM (A) and 0.12 nM (C) for granulosa cells and 0.16 nM (B) and 0.21 nM (D) for thecal cells.

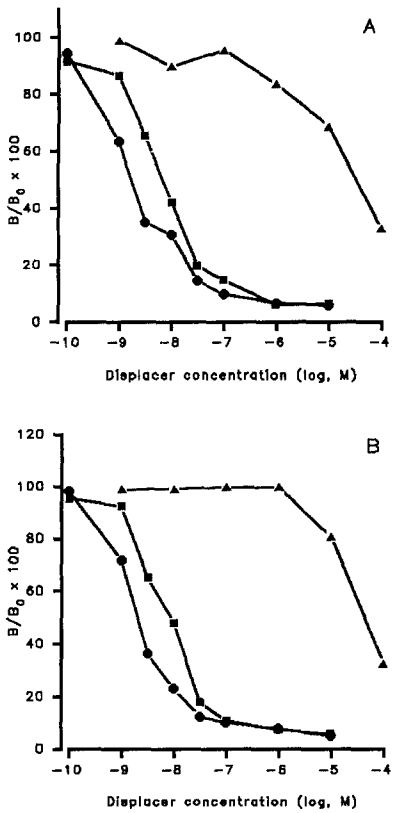

Fig. 3. Displacement of the binding of ^{125}I-[Sar^1,Ile^8]A II by (●) unlabeled A II, (■) Losartan (type 1 receptor antagonist) and (▲) PD 123319 (type 2 receptor antagonist) to cultured granulosa cells (A) and thecal cells (B). Values are the mean of triplicate incubations.

ovarian follicle. Granulosa and thecal cells were isolated and used immediately for binding studies.

Both granulosa and thecal cells exhibited specific binding for ^{125}I-[Sar^1,Ile^8]A II. Scatchard analysis of the results obtained (Fig. 2) showed an apparent straight line indicative of only one class of binding sites with high affinity on each one of the two cell types. K_d values were 0.26 ± 0.11 nM for granulosa cells and 0.18 ± 0.02 nM for thecal cells ($n = 2$). Density of A II binding sites (B_{max}) was found to be 0.06 ± 0.02 fmol/10^5 cells for granulosa and 0.08 ± 0.01 fmol/10^5 cells for theca.

3.3. Angiotensin II receptors on cultured granulosa and thecal cells

In a previous report, we have shown that under hCG stimulation, A II had the ability to influence steroidogenesis in vitro both in granulosa and theca. So, it was of interest to characterize A II receptors on primary cultures of granulosa and thecal cells under conditions of our previous study. Fig. 2 shows Scatchard analysis of ^{125}I-[Sar^1,Ile^8]A II binding to granulosa and thecal cells incubated for 48 h in the presence of 10 mIU/ml hCG. The resulting data showed that K_d values were 0.14 ± 0.04 nM for granulosa cells and 0.28 ± 0.06 nM for thecal cells

Table 1
Inhibitory constants (IC_{50}) of specific antagonists for displacement of ^{125}I-A II binding to cultured granulosa and thecal cells

	IC_{50} (nM)		
	A II	Losartan	PD 123319
Granulosa cells	8.6	5.5	20880
Thecal cells	1.3	4.7	22580

($n = 3$). These values were comparable to values obtained on freshly prepared cells. Density of A II binding sites (Bmax) was found to be 0.14 ± 0.04 fmol/10^5 cells for granulosa and 0.38 ± 0.04 fmol/10^5 cells for theca ($n = 3$). Culture of thecal cells in the presence of hCG resulted in a 4-fold significant increase in the amount of ^{125}I-A II specifically bound versus freshly prepared cells ($P < 0.05$). The 2-fold increase observed on granulosa cells was not statistically significant.

3.4. Angiotensin II receptor subtype on granulosa and thecal cells

Fig. 3 shows displacement curves of the binding of ^{125}I-[Sar^1,Ile^8]A II to cultured cells by unlabeled A II or by the two antagonists. The AT_1 receptor antagonist, Losartan inhibited binding of ^{125}I-[Sar^1,Ile^8]A II to granulosa and thecal cells in a concentration-dependent manner as did unlabeled A II while the AT_2 receptor antagonist PD 123319 exerted little inhibitory effect, indicating the occurrence of AT_1 receptors on each one of the two cell types. This was confirmed by analysis of IC_{50} values for the three ligands in granulosa and thecal cells (Table 1). The AT_1 receptor antagonist caused a substantial displacement of ^{125}I-A II binding ($IC_{50} = 5.5$ nM and 4.7 nM for

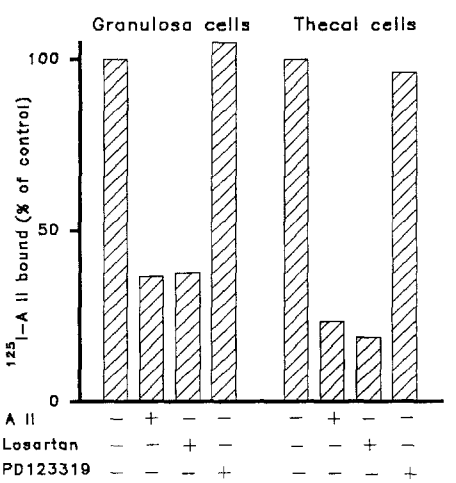

Fig. 4. Effect of subtype-selective A II antagonists on ^{125}I-[Sar^1,Ile^8]A II binding to freshly prepared ovarian granulosa and thecal cells. Cells were incubated with ^{125}I-[Sar^1,Ile^8]A II in the presence or absence of A II ($2 \cdot 10^{-7}$ M), Losartan ($2 \cdot 10^{-7}$ M) or PD 123319 ($2 \cdot 10^{-7}$ M). Results are given as the percent of control binding measured in the absence of the competitors.

granulosa and theca, respectively) whereas the AT_2 antagonist resulted in minimal displacement (IC_{50} = 20 880 nM and 22 580 nM for granulosa and thecal cells, respectively). This observation confirmed that A II receptors on rabbit granulosa and thecal cells are of the AT_1 type.

We also determined whether the receptors on fresh granulosa and thecal cells were of the same subtype as in cultured cells. As shown in Fig. 4, Losartan caused a 62% inhibition in ^{125}I-A II binding to granulosa and a 76% inhibition to thecal cells whereas PD 123319 was ineffective. As on cultured cells, A II receptors on freshly prepared follicular cells were of the type 1.

4. Discussion

The results obtained in this study clearly provide evidence for the existence of high-affinity specific A II receptors belonging to the AT_1 type on both granulosa and thecal cells isolated from preovulatory follicles of immature PMSG-treated rabbits. These findings are consistent with our previous observations of an effect of A II on aromatase activity of granulosa cells and on production of androgen by theca and with other studies which have demonstrated the presence of A II receptors in the ovary of the rat [21] and of the cow [22]. The K_d for rabbit ovarian A II receptors on cell membranes or each type of follicular cells determined in this study, indicated high-affinity binding with a K_d (0.2 nM) of the same order of magnitude as the K_d determined in rat and cow ovaries [16,21].

Our study demonstrated in the rabbit, the presence of A II receptors on both granulosa and thecal cells while in other species they were found either mainly on granulosa [10,23] or only on theca [16,24]. Human luteinized granulosa cells from IVF did not exhibit A II receptor expression [25]. Thus it appears that there is considerable variation between species in the distribution of A II receptors within the ovary. However, we can notice that A II receptors were observed on thecal cell layer from all studied species [16,24,26]. On the other hand, the presence of A II receptors on granulosa cells is variable from one species to another. A reason which could explain this variability is that A II receptors may display a cyclical pattern of variations during the ovarian cycle as observed in the rat [10]. The data obtained in PMSG-stimulated rabbits showed that the number of A II binding sites was quite similar in granulosa and in theca of freshly prepared cells (0.06 ± 0.02 vs. 0.08 ± 0.01 fmol/10^5 cells). A II receptor density was about 0.2 fmol/10^5 cells on freshly prepared immature DES-treated rat granulosa cells [27]. A course of 48 h incubation of rabbit follicular cells in the presence of hCG led to a marked increase in the number of binding sites for A II without a change in its binding affinity. The same results were obtained on bovine thecal cells [16].

As demonstrated in a previous study [20] the studied thecal cell population was a mixture of steroidogenic/fibroblast-like non-steroidogenic cells. The presence in the thecal preparation of non-steroidogenic cells bring about the possible existence of A II receptors on that type of cells. Nevertheless our previous study [13] showed a physiological action of A II on steroidogenesis in theca, which implies that A II receptors are expressed on steroidogenic cells. Moreover, the 4-fold increase in the amount of binding sites on thecal cells cultured in the presence of hCG versus freshly prepared cells seems to show that essentially steroidogenic cells are concerned.

Two main A II receptor subtypes have been identified: AT_1 and AT_2 [14]. In the rabbit ovary, we showed that only AT_1 receptors are expressed on granulosa and thecal cells. This observation differs from results obtained in other species. On bovine thecal cells A II receptors, were of the AT_2 subtype [16] as in rat granulosa cells while on other rat ovarian structures, such as thecal cell layer, interstitium, corpus luteum, blood vessels and the surface epithelium, they were found to be of the AT_1 subtype [17]. However, in a study on the effect of dithiothreitol on A II receptor in granulosa of DES-treated rats, Ohnishi et al. [28] detected a small population of AT_1 receptors. The presence of AT_1 receptors on granulosa is also supported by results obtained by Wang et al. [29] and Currie et al. [30] who reported that A II was able to increase intracellular calcium in respectively 40 and 25% of granulosa cells from PMSG-treated rats, an effect which is characteristic of AT_1 receptors. Such a species difference appears to exist in the distribution of A II receptors within the uterus, since rat uterus express both AT_1 and AT_2 receptors whereas human uterus express only the AT_2 receptor subtype [31]. In the same manner, the AT_2 subtype is predominant in the rabbit placenta (AT_1/AT_2 = 25:75) whereas in human placenta, only AT_1 receptor is present [32,33]. So, the relative amount and distribution of AT_1 and AT_2 subtypes may vary greatly in the same tissues obtained from different species [15] and results obtained with the ovary could illustrate these species differences. However one could wonder wether hormonal pretreatment of animals to obtain larger amounts of follicular cells (e.g. DES, PMSG) could explain the variability of the ratio AT_1/AT_2, particularly in the same species.

Recent studies have suggested a role for A II in ovulation in rats [6–8] and rabbits [5,9] while others have shown that A II could be involved in follicular atresia. In the rat ovary, A II receptors type 2 have been localized on granulosa cells of atretic follicles [10]. In a recent report, Tanaka et al. [12] observed in vitro that AT_2 content of rat granulosa cells increased with time while cells underwent internucleosomal DNA fragmentation characteristic of the apoptosis which occurs during follicular atresia. In our model, the PMSG-stimulated rabbit ovary, granulosa and thecal cells expressed AT_1 receptors. In a previous study [13] we were able to show that aromatase activity of granulosa cells increased in vitro in response to FSH

showing that the follicles were healthy. However, the same study suggested that the follicles had kept some potentiality to become atretic since under A II treatment their steroid secretions were modified in a way similar to that observed in vivo in atretic follicles but the exact nature of the follicles which express AT_1 receptors in the rabbit remains to be determine. Is the hypothesis of a role of A II in atresia compatible with the alternative hypothesis suggested by others of a role in ovulation? Clearly data at hands at the moment do not allow to decide.

In conclusion, this study reveals the existence of angiotensin II binding sites of the AT_1 type only on both granulosa and thecal cells of rabbit ovaries; this result contrasts with previous observations showing the presence of AT_2 receptors on follicular cells from several species. Further studies will be necessary to explain this differential expression of A II receptor subtypes among species and to elucidate the role of A II in ovarian physiology.

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