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Localization of angiotensin II receptors in ovarian follicles and the identification of angiotensin II in rat ovaries

(ovarian peptides/receptor autoradiography)

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ABSTRACT **Specific, high-affinity ($K_d \approx 0.6$ nM), and saturable (3.3 fmol/mg of tissue, wet weight) binding of ^{125}I-labeled [Sar^1,Ile^8]angiotensin II to rat ovarian membranes was observed. Displacement of ^{125}I-labeled [Sar^1,Ile^8]angiotensin II binding to rat ovarian membranes by angiotensin II analogs and fragments resembled the potency order of these compounds on angiotensin II receptors in other tissues: [Sar^1,Ile^8]angiotensin II > angiotensin II > des-Asp^1-angiotensin II > angiotensin I > des-Asp^1,Arg^2-angiotensin II. Several unrelated peptides, including follicle-stimulating hormone at 10 μM, did not displace ovarian ^{125}I-labeled [Sar^1, Ile^8]angiotensin II binding. Autoradiograms of ^{125}I-labeled [Sar^1,Ile^8]angiotensin II binding to ovarian sections indicated that the angiotensin II receptor binding sites were localized exclusively to a subpopulation of follicles, occurring on the granulosa and theca interna cells. Other follicles were devoid of ^{125}I-labeled [Sar^1,Ile^8]angiotensin II binding sites. Angiotensin II immunoreactive material was also identified in the ovary. The concentration of ovarian Ang II immunoreactivity was 8- to 75-fold greater than that of plasma, was not reduced in bilaterally nephrectomized rats, and was shown by high-pressure liquid chromatographic analysis to be the native angiotensin II octapeptide. The presence of angiotensin II and its receptor binding sites in the ovary suggests a role for angiotensin II as a regulator of ovarian function.**

The sequential processing of circulating angiotensinogen by renin (EC 3.4.23.15) and angiotensin I converting enzyme (EC 3.4.15.1) to yield the effector peptide angiotensin II (Ang II) is initiated by the release of the enzyme renin, primarily from the kidney (1). Ang II formed in this way has multiple diverse physiological effects, including arteriolar vasoconstriction (1), aldosterone secretion (2, §), and induction of drinking behavior (3). In addition to kidney-dependent intravascular Ang II formation, several other tissues—for example, the brain (4) and adrenal gland (5)—are potentially important local sites of production of Ang II, since they also contain the components of the renin–angiotensin system. Furthermore, several tissues that contain renin–angiotensin system components also contain specific cell surface Ang II receptors (6). Thus, local Ang II production may be a significant autocrine or paracrine modulator of tissue function.

Prorenin, renin, angiotensinogen, and Ang II immunoreactivity (Ang II-ir) have been demonstrated in human ovarian follicular fluid (7, 8). The presence of angiotensin I converting enzyme has also been demonstrated on oocytes in the rabbit ovary (9). These studies point toward a potentially important role of Ang II in the ovary, and thus they prompted us to look for Ang II receptors in the ovary (10) and to evaluate the nature of ovarian Ang II-ir. Our data show that the rat ovary is a target organ for Ang II and demonstrate the presence of the Ang II octapeptide in the rat ovary.

MATERIALS AND METHODS

Measurement of Ovarian and Plasma Ang II-ir. Female Sprague–Dawley rats (≈200 g) were used in this study. Ang II-ir in ovaries and plasma (obtained from the initial 2 ml of trunk blood) was extracted by the method of Eng and Yalow (11). ^{125}I-labeled Ang II (^{125}I-Ang II; 1000 cpm; New England Nuclear) was added at the beginning of the extraction to all samples for the estimation of recovery. The dried extracts were resuspended in 0.1% trifluoroacetic acid and were applied to a C_{18} Sep-Pak cartridge (Waters Associates) and the fraction eluting between 10% and 30% acetonitrile (containing 0.1% trifluoroacetic acid) was used for the estimation by radioimmunoassay (12) of Ang II-ir. Recovery of Ang II and ^{125}I-Ang II during this procedure was 75 ± 6% (n = 18) and 85 ± 5% (n = 18), respectively. (All results are expressed as mean ± SEM.) Ovarian Ang II-ir was chromatographed on a C_{18} high-pressure liquid chromatography (HPLC) column (Nova-Pak; Waters Associates), using a 12-min concave gradient between 89% buffer A (25 mM sodium phosphate, pH 7.6, containing 5% acetonitrile)/11% buffer B (95% acetonitrile) and 68% buffer A/32% buffer B. The column was calibrated by using synthetic Ang II, des-Asp^1-Ang II [Ang-(2–8)], des-Asp^1,Arg^2-Ang II [Ang-(3–8)], and des-Asp^1,Arg^2,Val^3-Ang II [Ang-(4–8)]. These synthetic standards were provided by M. C. Khosla (The Cleveland Clinic Foundation). Ang II antiserum used for the radioimmunoassay showed 100% crossreactivity with all of these C-terminal Ang II fragments but less than 0.05% crossreactivity with angiotensin I, the C-terminal Ang II tetrapeptide, and N-terminal Ang II fragments.

Measurement of Ang II Receptors. Three-month-old female Sprague–Dawley rats were used for Ang II receptor analysis. The ovaries, as well as the adrenals and brains, were obtained from anesthetized rats perfused intracardially with ice-cold saline. The brains were further dissected to yield the hypothalamus–thalamus–septum–midbrain (HTSM) region, which was used for subsequent analysis. The tissue membranes were prepared and Ang II receptors were measured as described previously (14). Briefly, the incubation buffer contained 150 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM

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Abbreviations: Ang II, angiotensin II; Ang-(2–8), des-Asp^1-Ang II; Ang-(3–8), des-Asp^1,Arg^2-Ang II; Ang-(4–8), des-Asp^1,Arg^2,Val^3-Ang II; Ang II-ir, Ang II immunoreactivity; FSH, follicle-stimulating hormone; H&E, hematoxylin and eosin; HTSM, hypothalamus–thalamus–septum–midbrain.

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dithiothreitol, and 50 mM sodium phosphate, pH 7.1. Tissue membrane suspensions were incubated with ^{125}I-[Sar^1,Ile^8]-Ang II (0.07–1.0 nM; specific activity = 2200 Ci/mmol; 1 Ci = 37 GBq) to radiolabel Ang II receptor binding sites. [Sar^1,Ile^8]Ang II (Bachem Fine Chemicals, Torrance, CA) was radiolabeled with iodine-125 by using lactoperoxidase and purified by reverse-phase HPLC. Nonspecific binding was determined in the presence of 1 μM Ang II.

For autoradiographic studies, 20-μm-thick ovarian sections were cut in a cryostat at −16°C, thaw mounted onto slides, air-dried at room temperature, and stored at −23°C. Sections were thawed for about 0.5 min at 35°C, incubated for 30 min at 23°C in incubation buffer with 0.2% bovine albumin, and placed in incubation buffer containing 0.25 nM ^{125}I-[Sar^1,Ile^8]Ang II and 0.2% bovine albumin for 1 hr at 23°C. Alternate sections were placed in incubation buffer containing 1 μM Ang II in addition to the ^{125}I-[Sar^1,Ile^8]Ang II and 0.2% bovine albumin to serve as control sections for measurement of nonspecific binding. The sections were then sequentially rinsed in ice-cold distilled water, incubation buffer, and distilled water, and subsequently dried under a stream of hot air. The sections were first apposed to film (Ultrofilm, LKB) for 3 to 4 days and then stained with hematoxylin and eosin (H&E).

RESULTS

Ang II-ir in the Rat Ovary. The level of Ang II-ir in the rat ovary (450 ± 200 pg/g of tissue, wet weight; n = 5) exceeds the concentration of this hormone in the plasma (16 ± 1.6 pg/ml; n = 5) by 8- to 75-fold. In bilaterally nephrectomized rats the levels of Ang II-ir in the ovary (243 ± 100 pg/g of tissue, wet weight; n = 4) or the plasma (22 ± 6 pg/ml; n = 4) were not less than those found in control rats. The recovery of ^{125}I-Ang II added to the tissue at the time of extraction was consistently similar with ovaries (79 ± 1% for control; 83 ± 2% for nephrectomy) and plasma (74 ± 2% for control; 80 ± 3% for nephrectomy).

Under our chromatographic conditions, synthetic Ang II, Ang-(2–8), Ang-(3–8), and Ang-(4–8) were resolved as four clearly identifiable peaks (Fig. 1). Ang II-ir extracted from rat ovaries was analyzed by HPLC. Only one major Ang II-ir peak, occurring at the retention time of the [Ile^5]Ang II, constituting 90% of the total immunoreactivity, was observed. Three minor immunoreactive peaks were observed; one corresponded to the retention time of Ang-(4–8), and the other two minor Ang II-ir peaks were not identifiable. Recovery of ^{125}I-Ang II added to the ovarian extract in two HPLC analyses was 65% and 70%.

Characteristics of ^{125}I-[Sar^1,Ile^8]Ang II Binding Sites. Rat ovarian membranes displayed high-affinity binding sites for ^{125}I-[Sar^1,Ile^8]Ang II with a K_d of 643 ± 41 pM and a maximal binding capacity of 3.31 ± 0.72 fmol/mg of tissue, wet weight (n = 3). The binding of ^{125}I-[Sar^1,Ile^8]Ang II was highly specific: >90% of the binding at 1 nM was displaceable by 1 μM Ang II. ^{125}I-[Sar^1,Ile^8]Ang II binding density in the ovary was intermediate between that of membranes from the adrenal gland (17.7 ± 1.9 fmol/mg of tissue, wet weight) and the brain HTSM region (0.58 ± 0.09 fmol/mg of tissue, wet weight). Fig. 2 shows that Ang II and [Sar^1,Ile^8]Ang II were potent displacers of ovarian ^{125}I-[Sar^1,Ile^8]Ang II binding (IC_{50} = 3 and 0.9 nM, respectively). Angiotensin I, the decapeptide precursor of Ang II, was 1/100th as potent a displacer of specific ^{125}I-[Sar^1,Ile^8]Ang II binding as Ang II. Ang-(2–8) was approximately 10% as potent as a displacer as Ang II, while Ang-(3–8) was less than 0.1% as effective as Ang II in displacing ^{125}I-[Sar^1,Ile^8]Ang II binding to ovarian membranes. The assay conditions used to measure ovarian Ang II receptor binding are derived from those used to measure binding of ^{125}I-Ang II to adrenal membranes (13). These conditions minimize enzymatic degradation of ^{125}I-Ang II and cause no degradation of ^{125}I-[Sar^1,Ile^8]Ang II during a 2-hr incubation. However, degradation of Ang-(2–8) occurs rapidly under these conditions, precluding equilibrium binding evaluation of this analog. Therefore, to more accurately represent the binding affinity of Ang-(2–8) to these ovarian receptors, we used a 45-min incubation to determine inhibition of the initial rate of ^{125}I-[Sar^1,Ile^8]Ang II binding. Indeed, 2-hr incubation with Ang-(2–8) and Ang-(3–8) hexapeptide yielded IC_{50} values 3 times greater than those observed with 45-min incubation. There were no significant differences in IC_{50} values for Ang II, [Sar^1,Ile^8]Ang II, and Ang I with 45-min versus 2-hr incubations. FSH, neurotensin, [Leu^5]enkephalin, and [D-Ala^2,Met^5]enkephalin at 10 μM caused less than 5% displacement of ^{125}I-[Sar^1,Ile^8]Ang II binding to ovarian membranes.

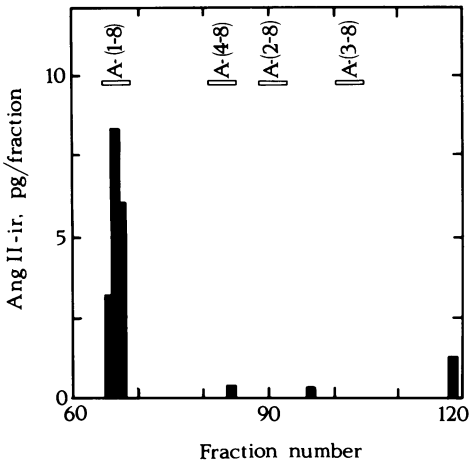

FIG. 1. Representative HPLC profile of Ang II-ir extracted from rat ovaries. See text for chromatographic conditions. Horizontal bars at the top of the figure identify fractions in which standards of Ang II [A-(1–8)], Ang-(2–8) [A-(2–8)], Ang-(3–8) [A-(3–8)], and Ang-(4–8) [A-(4–8)] elute. One hundred and twenty 150-μl fractions were collected and assayed for Ang II-ir. This figure depicts only fractions 60 to 120 since no Ang-ir was observed in the first 60 fractions.

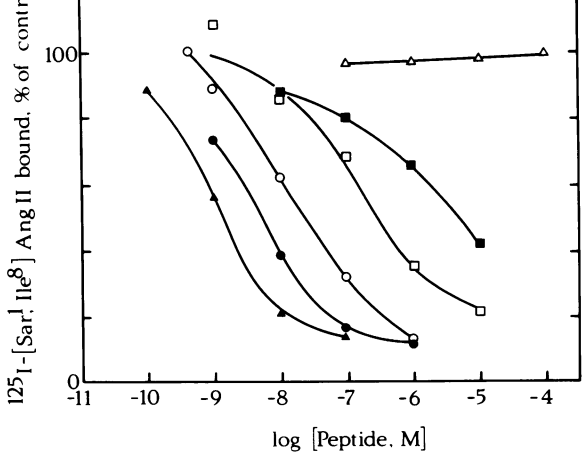

FIG. 2. Binding-inhibition activity of Ang II analogs and follicle-stimulating hormone (FSH) with ^{125}I-[Sar^1,Ile^8]Ang II as the radioligand. Each point represents the mean of duplicate determinations. Incubations were carried out over a period of 120 min for all peptides with the exception of Ang-(2–8) and Ang-(3–8), for which the incubation time was 45 min. ●, Ang II; ○, Ang-(2–8); ■, Ang-(3–8); □, angiotensin I; ▲, [Sar^1,Ile^8]Ang II; △, FSH.

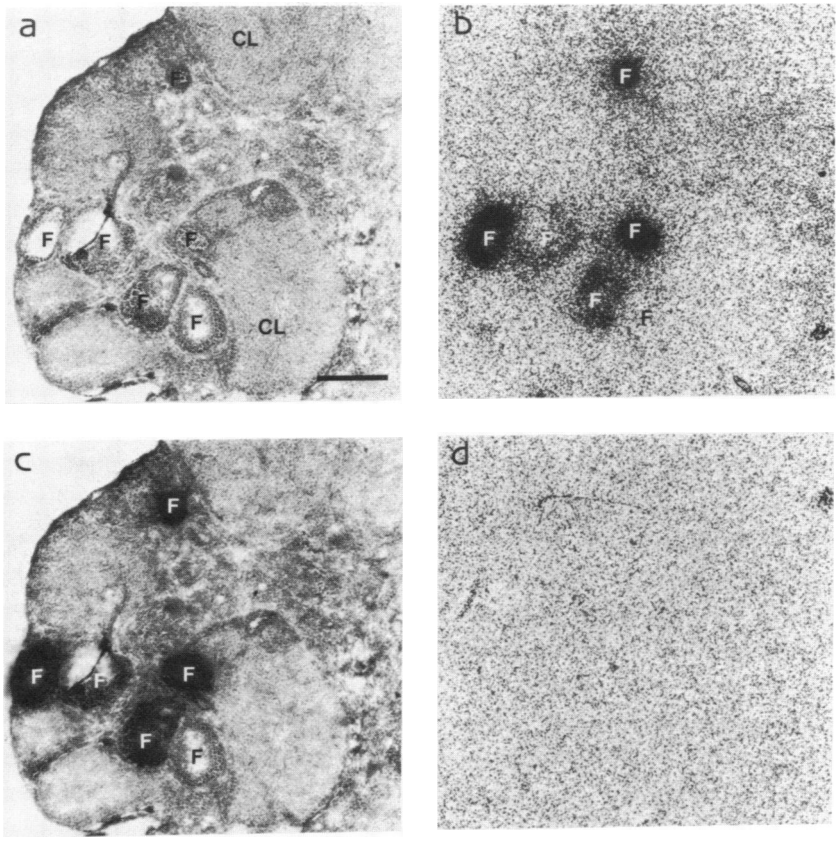

FIG. 3. ^{125}I-[Sar^1,Ile^8]Ang II binding sites in the rat ovary. (*a*) H&E-stained section of rat ovary. Note the location of follicles (F) and the corpora lutea (CL). (*b*) Autoradiogram produced by the tissue section in *a*. Note the high grain density corresponding to the follicular cell layers, with little specific grain density associated with the rest of the ovarian section. (*c*) H&E-stained section (in focus) shown in *a* was reapposed to the autoradiograph (out of focus) shown in *b* to determine cell types corresponding to the exposed areas of the film. (*d*) A section adjacent to that shown in *a* was incubated with the addition of 1 μM Ang II to produce the autoradiogram. The diffuse distribution of grains seen here indicates negligible nonspecific ^{125}I-[Sar^1,Ile^8]Ang II binding. Bar = 500 μm.

Autoradiographic Localization of ^{125}I-[Sar^1,Ile^8]Ang II Binding Sites in the Rat Ovary. Fig. 3 shows that specific (e.g., displaced by 1 μM Ang II) binding of ^{125}I-[Sar^1,Ile^8]Ang II was intense and localized in discrete regions of the rat ovary. Direct comparison of the autoradiograms with the H&E-stained sections demonstrated that the vast majority of the ^{125}I-[Sar^1,Ile^8]Ang II binding occurred over both small and large developing follicles (Figs. 3 and 4). Not all follicles, however, contained ^{125}I-[Sar^1,Ile^8]Ang II binding sites (Figs. 3 and 4). ^{125}I-[Sar^1,Ile^8]Ang II binding sites were conspicuously absent over luteinized granulosa cells of the corpora lutea (Fig. 4*a*). Fig. 4*b* shows that considerable autoradiographic exposure occurred over the theca interna and granulosa cell layers. On the basis of the size distribution of large follicles throughout the estrus cycle in the rat (14), the 600- to 700-μm-diameter follicles shown in Fig. 4 suggest that the ovary used to obtain the section was from a rat in diestrus-2 or proestrus.

DISCUSSION

These studies confirm and extend our preliminary observation of Ang II receptor binding in the rat ovary (10). The simultaneous demonstration of Ang II in rat ovary, independent of the renal renin–angiotensin system, suggests that Ang II is an autocrine or paracrine regulator of ovarian function.

Prorenin, renin, angiotensinogen, and Ang II-ir have been demonstrated in human follicular fluid, which suggests the presence of a local renin–angiotensin system in the human ovary (7, 8). The recent demonstration of angiotensinogen mRNA in the rat ovary by Ohkubo *et al.* (15) and our finding of high levels of Ang II-ir in the rat ovary support the concept of a local Ang II-forming system in the ovary of the rat. Chromatographic analysis of the total ovarian extract showed that over 90% of the Ang II-ir recovered eluted with the retention time of Ang II. Assuming similar recoveries of Ang II and its fragments, this indicates that ovarian Ang II-ir is primarily the octapeptide Ang II. The levels of Ang II-ir in the ovary were much higher than those found in an equivalent volume of plasma, indicating that Ang II-ir levels in the ovary are not due to simple contamination of this tissue with circulating Ang II. However, it is possible that the Ang II present in the ovary is sequestered by ovarian Ang II receptors and could therefore be derived in part from blood. Ang II-ir levels in the rat ovary ranged between 130 and 1210 pg/g of tissue, wet weight, and were much more variable than the levels found in plasma or those in other tissues such as the adrenal gland (data not shown). This variability was not due to differential recovery during the extraction procedures, since recovery of internal standard was consistent and high. It is known that several components of the renin–angiotensin system, including Ang II receptors, fluctuate during the estrus cycle in rats. For example, in rats, plasma angiotensinogen increases and brain angiotensinogen decreases at estrus (16). During diestrus, pituitary Ang II receptor density is highest and uterine Ang II receptor density is lowest (16, 17), compared with the rest of the estrus cycle. The female rats used in our study were not selected with reference to the stage of the cycle. Thus, the variability in ovarian Ang II levels may be due to variations associated with the estrus cycle.

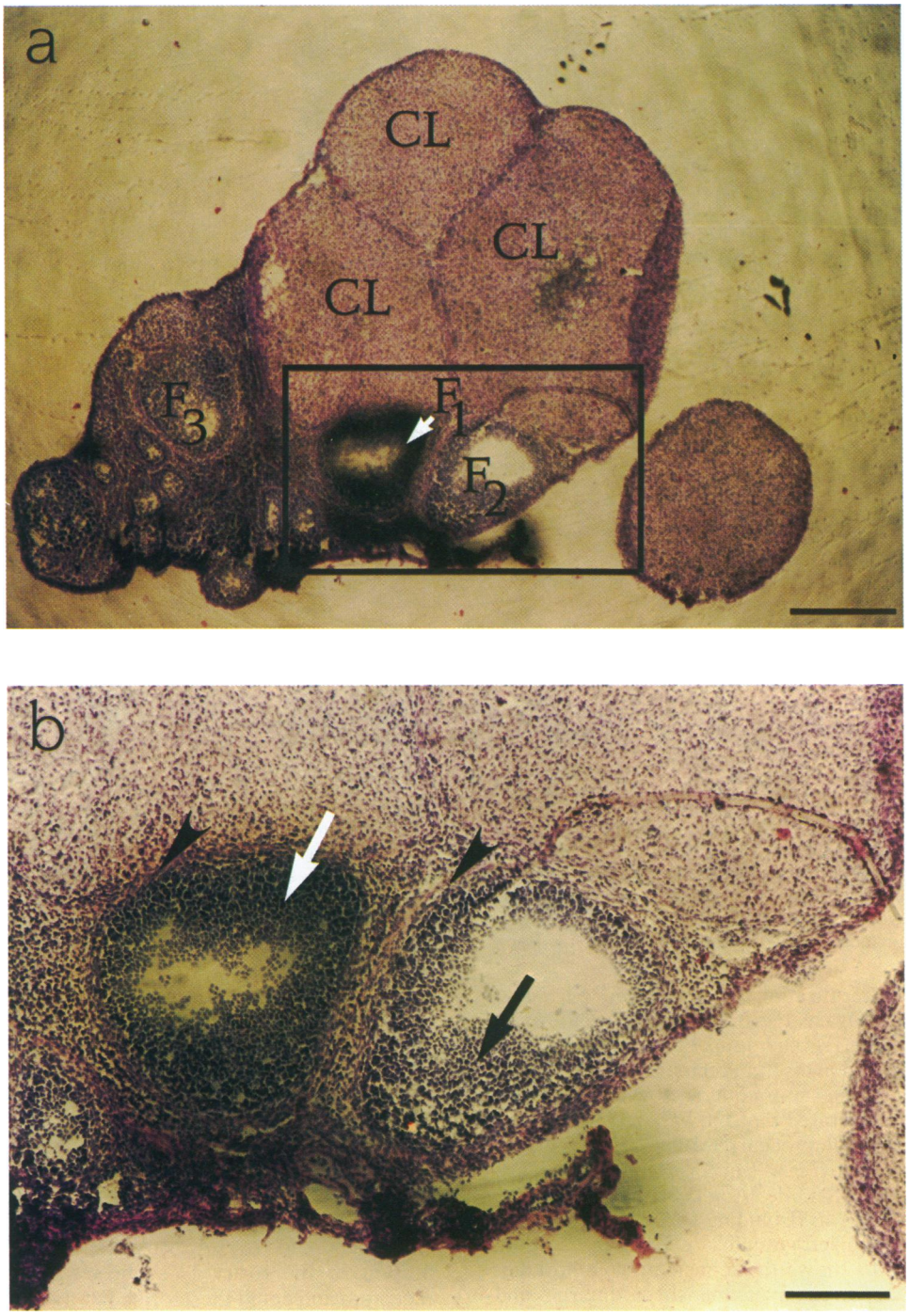

FIG. 4. Color plate showing the distribution of ^{125}I-[Sar^1,Ile^8]Ang II binding sites in the rat ovary. (*a*) H&E-stained section (in focus) and the autoradiogram generated by this section (not in focus) overlayed on the stained section. The black areas represent ^{125}I-[Sar^1,Ile^8]Ang II binding to putative Ang II receptors. Note that ^{125}I-[Sar^1,Ile^8]Ang II binding sites were present on follicle F_1 but absent from other follicles (F_2, F_3). ^{125}I-[Sar^1,Ile^8]Ang II binding sites were not present on corpora lutea (CL). Bar = 500 μm. (*b*) Enlargement of the portion framed in black in *a*, showing two antral follicles. ^{125}I-[Sar^1,Ile^8]Ang II binding sites were present over the granulosa and theca interna cells of the follicle shown on the left but were absent from the follicle shown on the right. In each case, the arrows point to the granulosa cells and the arrowheads point to the theca cells whether or not they bind ^{125}I-[Sar^1,Ile^8]Ang II. Bar = 200 μm.

To study Ang II receptor binding affinity, density, and localization, we used the potent Ang II receptor antagonist [Sar^1,Ile^8]Ang II (18). The radiolabeled antagonist identifies a single class of Ang II receptors with high affinity, is more stable than ^{125}I-Ang II, and dissociates at a slower rate from the Ang II receptor (19). ^{125}I-[Sar^1,Ile^8]Ang II binding site density in the rat ovary was intermediate between that of the adrenal gland and that of the brain. Ovarian ^{125}I-[Sar^1,Ile^8]Ang II binding sites probably represent Ang II receptors on the basis of the following criteria: (*i*) the ^{125}I-[Sar^1,Ile^8]Ang II binding sites showed high affinity ($K_d \approx$ 0.6 nM); (*ii*) it was saturable; and (*iii*) it could be displaced by Ang II analogs in a potency order that is the same as their potency on the well-characterized vascular and adrenal Ang II receptors (7).

Autoradiographic analysis localized ovarian ^{125}I-[Sar^1,Ile^8]-

Ang II binding sites to the theca interna and granulosa cell layers of the developing follicles. The granulosa cell layers of the ovarian follicles lack a direct blood supply (20). Thus, it is probable that the ^{125}I-[Sar^1,Ile^8]Ang II binding associated with this cell layer is located on the granulosa cell and does not represent vascular smooth muscle Ang II receptors. However, the theca interna cell layer is highly vascularized (20), and Ang II receptors in this cell layer may be associated with blood vessels in addition to the theca interna cells.

The granulosa and theca interna cells of the developing follicles are a major source of estrogens necessary for ovum development (21). The steroidogenic function of these cell types is primarily regulated by cyclic pituitary FSH, luteinizing hormone, and prolactin secretions. However, not all follicles in a given ovary respond to pituitary gonadotropins. Furthermore, only a limited number of selected follicles ovulate, while many other follicles become atretic (21). The autoradiographic distribution of Ang II receptors on the granulosa and theca interna cell layers of developing follicles of all sizes suggests a role for Ang II in modulating steroidogenesis. The dramatic absence of Ang II receptors on several other developing follicles of various sizes indicates that not all follicles respond to Ang II. Whether the presence or absence of responsivity to Ang II is related to the process of follicular atresia remains to be established. If some Ang II receptors in the theca interna cell layers are associated with vascular smooth muscle cells, neovascularization may be an important role of Ang II. The conspicuous lack of Ang II receptors from all corpora lutea indicates that once the granulosa cells have differentiated to the granulosa-lutein cells, angiotensinergic input to this cell type is not consequential.

The presence of Ang II receptors on follicular cells suggests that both circulating and locally produced Ang II are potentially important modulators of follicular development. A direct role for circulating Ang II in ovulation does not seem likely, however, since Steele *et al.* (22) have shown that intravenous infusion of an Ang II receptor antagonist, [Sar^1,Ala^8]Ang II, at a dose sufficient to block the effects of circulating Ang II, did not prevent ovulation. However, our studies show the presence of high levels of Ang II in the rat ovary. If a considerable portion of the total Ang II content of the rat ovary is present in follicular fluid, as in the human ovaries (7, 8), the physiological effect of intraovarian Ang II may not be easily antagonized by intravenous infusion of Ang II receptor antagonist. Thus, more studies are needed to unravel the role of intraovarian Ang II in ovulation.

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