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IMMUNOHISTOCHEMICAL DETECTION OF INHIBIN IN THE GONAD

Pedro Cuevas, Shao-Yao Ying, Nicholas Ling, Naoto Ueno,
Frederick Esch, and Roger Guillemin

Laboratories for Neuroendocrinology,
The Salk Institute for Biological Studies,
10010 North Torrey Pines Road, La Jolla, California 92037

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SUMMARY: Antiserum to inhibin was produced in rabbits by immunization with a synthetic [Tyr^{30}]α-chain(1-30)NH_2 fragment of porcine inhibin coupled to bovine serum albumin, and the elicited antiserum was used in conjunction with the avidin-biotin immunoperoxidase procedure to localize inhibin-reactive cells in various rat tissue preparations. In the testes, only the Sertoli cells revealed immunoreactivity with the antiserum. Intense staining was also observed in ovarian follicular granulosa cells but not in the theca layer outside the basement membrane. In addition, the luteal cells in the corpus luteum were also stained by the antiserum. The positive staining in the gonadal tissues could be blocked completely by pre-adsorbing the serum with either the synthetic peptide or native inhibin. Immunostaining was not detected in brain, pituitary, thymus, stomach, pancreas, kidney and adrenal sections, thus confirming that inhibin is a polypeptide originating only from specific cells of the gonad.

Inhibin is a polypeptide hormone produced in the gonad that feeds back to the anterior pituitary gland to inhibit specifically the secretion of follicle stimulating hormone (1). The inhibin concept has been in existence for over 50 years but it was only recently that this molecule was isolated and characterized. Two forms of inhibin, A and B, with a molecular weight of 32,000 daltons were isolated (2-4) and characterized (5) from porcine ovarian follicular fluid, each containing a common glycosylated α-chain of 18,000 daltons and a distinct, but homologous β-chain of 14,000 daltons. From the bovine ovarian follicular fluid, the 32,000-dalton inhibin of form A (6,7) as well as its 56,000-dalton precursor were also isolated (8) and characterized (9). Availability of the protein sequences for the inhibins has enabled us to generate antibodies against synthetic peptide fragments that recognize the parent molecule. Specifically, we have produced a polyclonal antibody in rabbits using a synthetic [Tyr^{30}]α-chain(1-30)NH_2 fragment of porcine inhibin coupled to bovine serum albumin as immunogen. This antiserum recognizes both native in-

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hibins A and B. To ascertain what cell types in the gonad produce inhibin, we have used this antiserum to localize inhibin-like immunoreactivity in the male and female gonads by immunohistochemistry. We report here the visualization of immunoreactive inhibin in the gonad.

MATERIALS AND METHODS

Tissue Preparation. Male and female Sprague-Dawley rats (200-300 g, Charles River) were perfused transcardially under Nembutal anesthesia (50 mg/Kg i.p.) with 100 ml phosphate buffered-saline (PBS) (pH 7.4) at 37°C, followed by 200 ml ice-cold phosphate-buffered paraformaldehyde-picric acid fixative (pH 7.4). The organs were removed and post-fixed with the same fixative for 3-18 hr and then equilibrated in 18% sucrose-PBS overnight. The testes and ovaries were then mounted on microtome chucks with O.C.T. compound (Miles Scientific, Elkhart, IN) on dry ice, and 10 µm cryostat sections were prepared at -20° on gelatin-coated microscope slides. The sections were dried in a vacuum dessicator at room temperature and stored at 4°C.

Induction of Inhibin Antiserum. The inhibin α-subunit N-terminal fragment, $[Tyr^{30}]\alpha$-chain(1-30)NH_2, was synthesized by solid-phase methodology (10) and purified by gel filtration, cation-exchange chromatography and partition chromatography, using procedures similar to the purification of synthetic growth hormone releasing factor (11). The purified material has the correct amino acid composition and greater than 95% purity by high performance liquid chromatography analysis. It was conjugated to bovine serum albumin with bis-diazotized benzidine as described for the coupling of β-endorphin (12). Five rabbits were each immunized with 2 mg of the protein conjugate in 1 ml of solution, emulsified with an equal volume of Freund's complete adjuvant. Monthly booster injections were administered with 0.5 mg of conjugate, emulsified with an equal volume of Freund's incomplete adjuvant. After the third booster injection, one of the rabbits produced an antiserum which could bind the labelled peptide fragment at a final dilution of 1/20,000. In addition, the label could be displaced in a dose-dependent manner by the synthetic peptide as well as both native inhibins A and B.

Immunohistochemistry. Immunohistochemical localization of inhibin was performed by incubating tissue sections with the porcine inhibin α-chain antiserum and visualization of the antigen-antibody complex was carried out by the avidin-biotin immunoperoxidase procedure (13). Briefly, tissue sections were pre-incubed in buffer (PBS plus 0.1% Triton X-100 and 1 mg/ml bovine serum albumin) containing 3% normal goat serum for 30 min at 4°C. The medium was removed and buffer-diluted primary antiserum (1:1000) was added for overnight incubation at the same temperature. The sections were then processed through the avidin-biotin immunoperoxidase procedure (Vector Lab., Burlingame, CA) with 0.5% diaminobenzidine and 0.003% hydrogen peroxide as substrate. The sections were then rinsed, dehydrated and protected with cover slips for microscopic examination. In control experiments, the specificity of the inhibin antiserum was ascertained by pre-adsorbing the diluted antiserum with either 200 µg/ml of the synthetic inhibin fragment or 100 µg/ml native inhibin B, before applying it to the immunohistochemical study as described above.

RESULTS AND DISCUSSION

In cyrostat sections of rat seminiferous tubules, Sertoli cells can be identified by the irregularly shaped nuclei with prominent nucleoli. The cytoplasm of Sertoli cells extends from the basement membrane of the seminiferous tubule up to the lumen. Fine Sertoli cell extensions surround the germinal cells at the lumen of the tubule. In the rat testes, Sertoli cells reveal immunoreactivity with the anti-inhibin serum, with more intense staining in the portion of the cytoplasm near the nucleus (Fig. 1a). The supranuclear

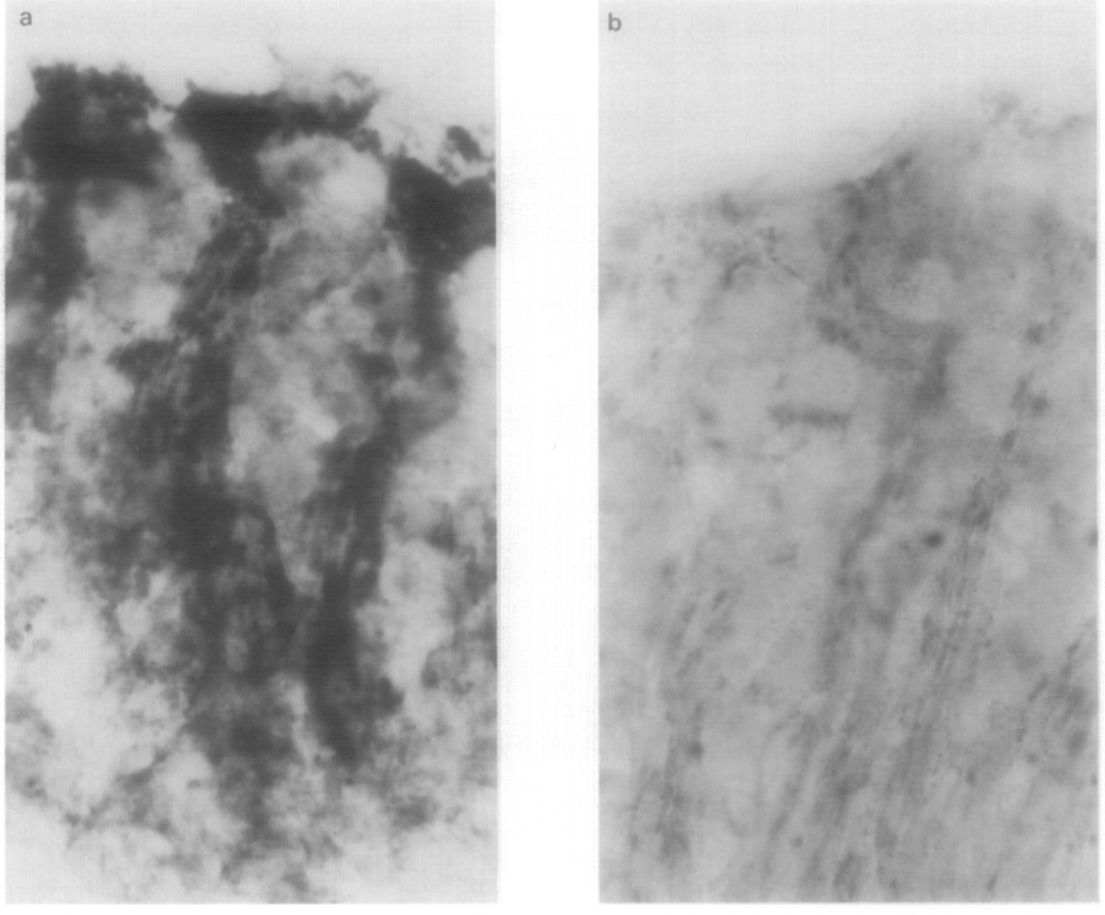

Fig. 1. (a) Immunohistochemical detection of inhibin in a seminiferous tubule section of rat testes, showing immunoreactivity in three Sertoli cells. Magnification: 380x

(b) Absence of staining in an adjacent seminiferous tubule section when the antiserum was pre-treated with the synthetic [Tyr^{30}]α-chain (1-30)NH_2 or native Inhibin B. Magnification: 380x

portion of the cytoplasm also shows immunoreactivity as thin cytoplasmic prolongations up to the tubular lumen. In control experiments, antiserum pre-incubated with either the synthetic α-chain [Tyr^{30}](1-30)NH_2 fragment or native inhibin no longer revealed immunoreactivity in Sertoli cells (Fig. 1b).

In rat ovaries, intense staining with the antiserum was observed in the follicular granulosa cells but not in the theca layer outside the basement membrane. Fig. 2a shows the staining of one ovarian follicle. Clearly visible are several layers of granulosa cells, an antrum filled with fluid and well differentiated theca layers outside the basement membrane. The maturing oocyte that projects into the follicular fluid is surrounded by immunoreactive

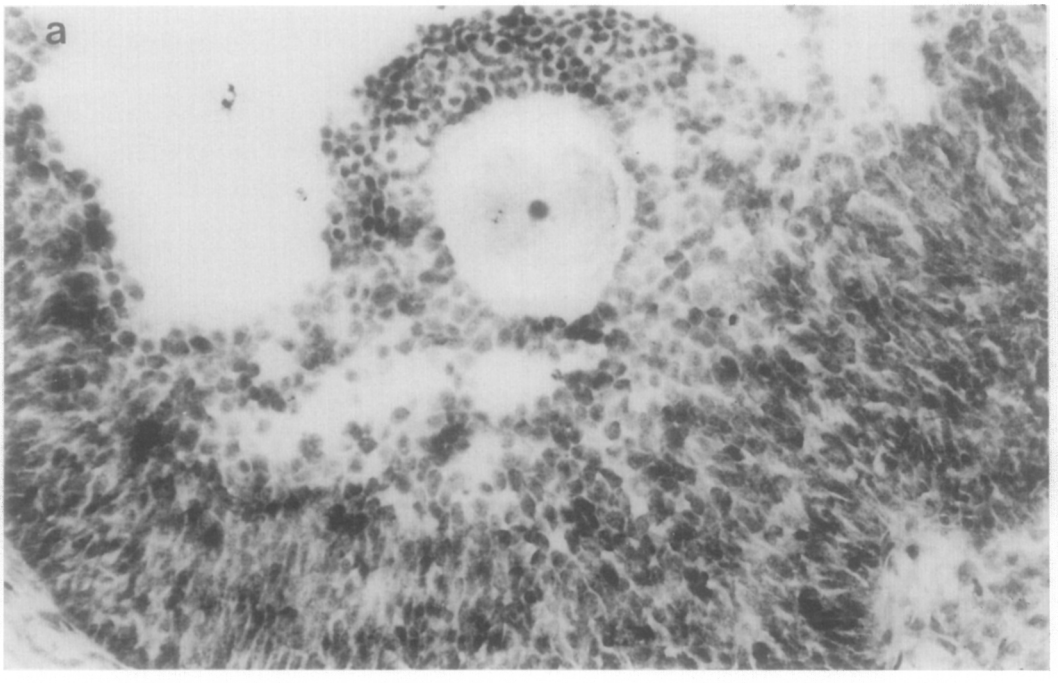

Fig. 2. (a) Immunohistochemical detection of inhibin in a maturing follicle in a section of rat ovary. Intense staining is exhibited by the granulosa cells on the follicular wall as well as in the cumulus oophorus surrounding the oocyte. Magnification: 275x

(b) Immunostaining of the granulosa cells on the internal portion of the follicular wall. Note that the theca layers outside the wall are not stained. Magnification: 300x

(c) Absence of staining in an adjacent follicular wall section when the antiserum was pre-adsorbed with either the synthetic peptide fragment or native inhibin. Magnification: 300x

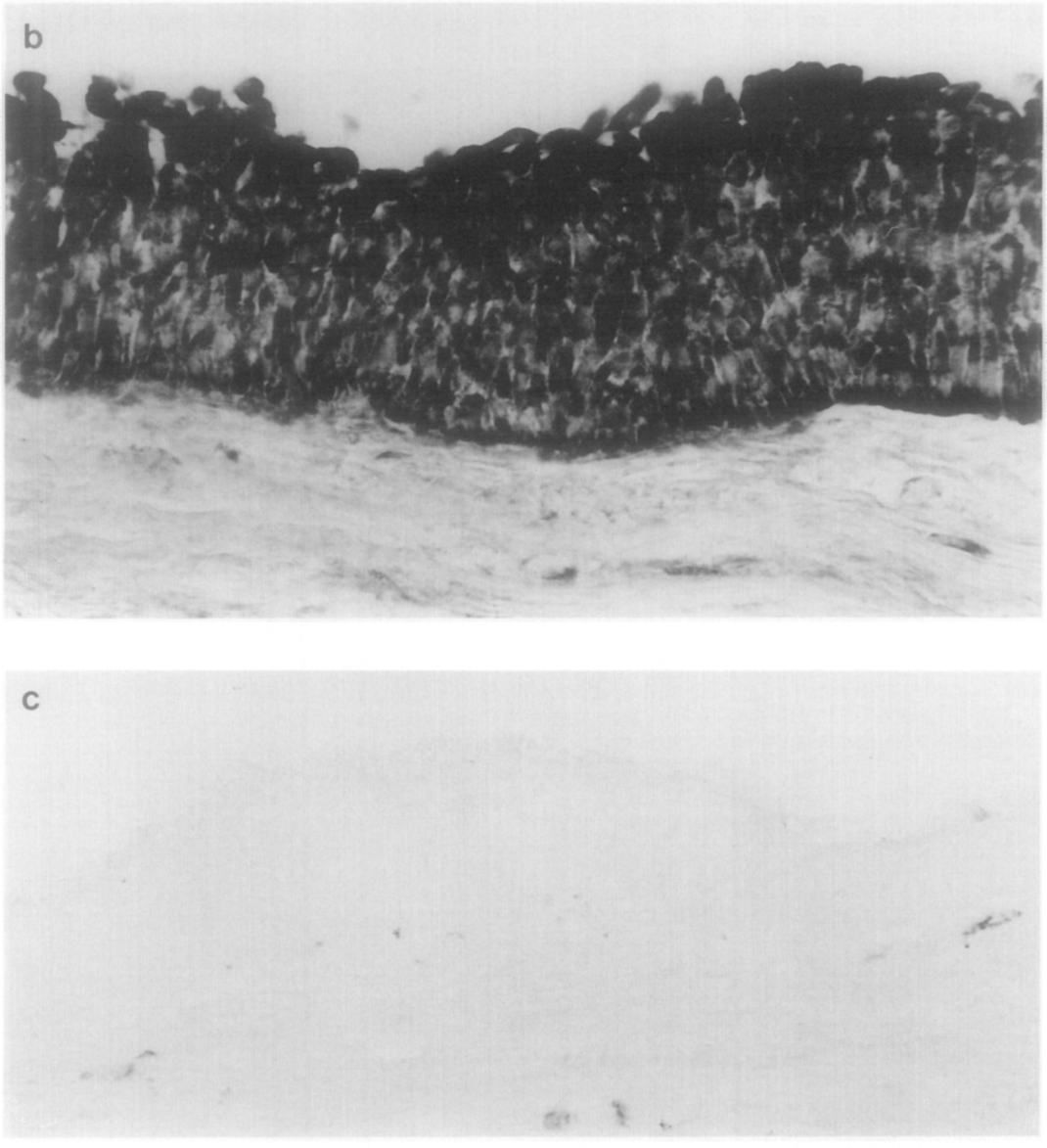

Fig. 2– Continued.

granulosa cells that form the cumulus oophorus. Fig. 2b presents a higher magnification of the ovarian follicle, showing the staining of the granulosa cells by the antiserum on the luminal side of the follicular wall. As in the Sertoli cells of testes, the staining is completely blocked by pre-adsorbing the serum with either the synthetic peptide or native inhibin (Fig. 2c). In addition, immunostaining was not observed in brain, pituitary, thymus, stom-

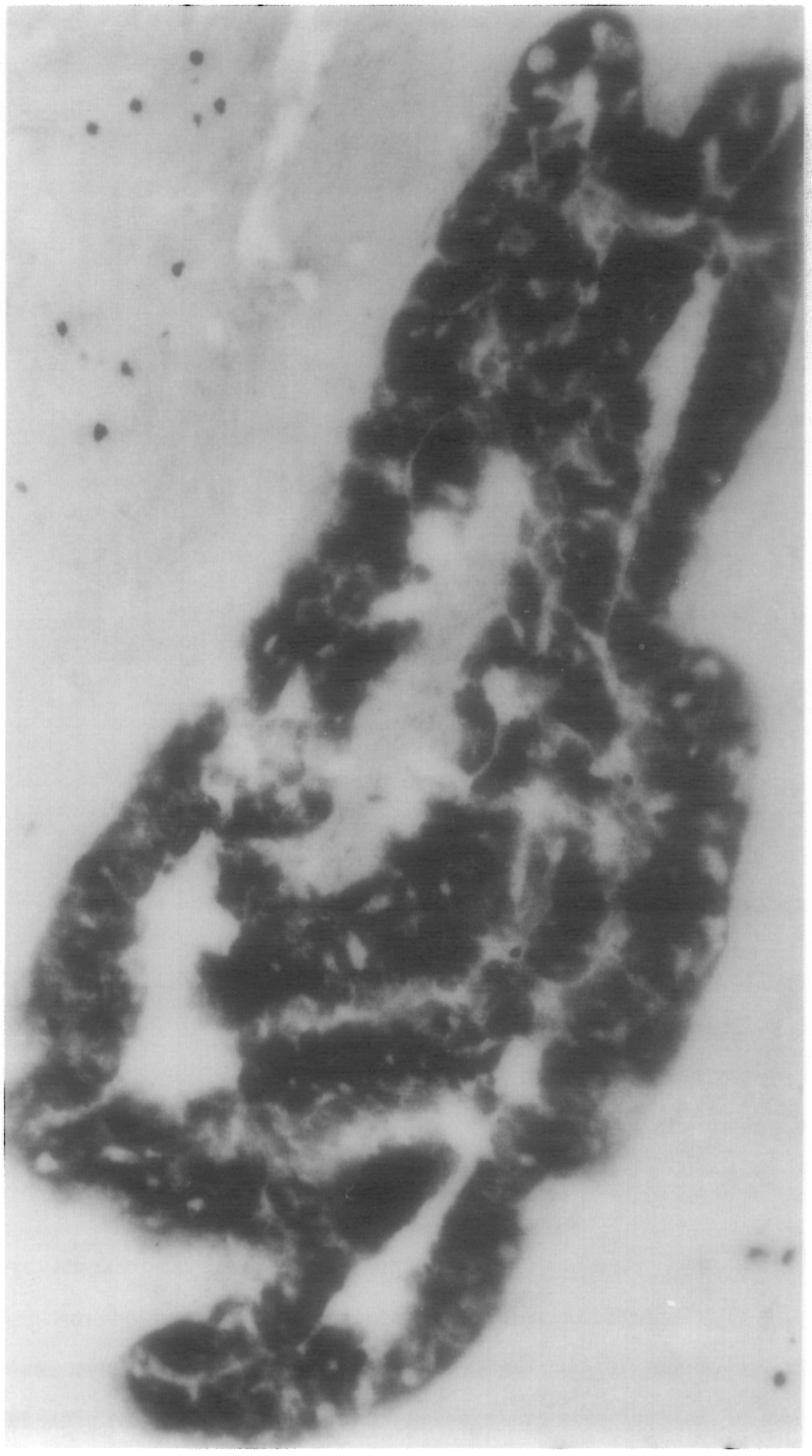

Fig. 3. Immunohistochemical detection of inhibin in rat corpus luteum. Magnification: 140x

ach, pancreas, kidney and adrenal sections. However, the luteal cells in the corpus luteum were stained by the inhibin antiserum as shown in Fig. 3.

Thus, immunohistochemical data obtained from this study confirms that inhibin is a peptide derived from the gonad. The inhibin-like immunoreactivity observed in rat Sertoli cells is in agreement with several studies, demonstrating that the Sertoli cells in the male gonad are the site of production of inhibin (14-16). These cells have been shown to secrete increasing amounts of inhibin in response to FSH stimulation *in vitro* (17). The data obtained from the ovarian follicles is in accord with the *in vitro* studies on the secretion of inhibin by murine granulosa cells (18). The unexpected immunohistochemical demonstration of inhibin in corpus luteum is in agreement with a recent report that showed significant inhibin-mRNA levels in the rat corpora lutea (19). Taken together, the results obtained in these studies confirm that inhibin is a polypeptide produced by specific cells in the gonad.

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