Endothelin-1: a new autocrine/paracrine factor in rat testis

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Fantoni, Guido, Patricia L. Morris, Gianni Forti, Gabriella Barbara Vannelli, Claudio Orlando, Tullio Barni, Roberta Sestini, Giovanna Danza, and Mario Maggi. Endothelin-1: a new autocrine/paracrine factor in rat testis. *Am. J. Physiol.* 265 (*Endocrinol. Metab.* 28): E267–E274, 1993.—Cultured Sertoli cells of 20-day-old rats were found to produce and release endothelin-1-like immunoreactivity (ET-1-LI) under follicle-stimulating hormone control. The elution profile of ET-1-LI from extracts of spent Sertoli cell culture medium corresponds to that of synthetic ET-1, suggesting a testicular production of authentic ET-1. In contrast, the conditioned medium from rat Leydig cells did not contain ET-1-LI. Immunohistochemical studies confirmed that, in 20-day-old rats, the positive staining was confined to some Sertoli cells, whereas interstitial cells were negative. In the adult rat testis the positivity was not limited to the tubular compartment (Sertoli cells) but was also present in the interstitium. A high concentration (13 pmol/mg protein) of high-affinity (dissociation constant = 0.6 nM) ^{125}I-labeled ET-1 binding sites was present in Leydig cells. These sites bind ET-1 and ET-2 with 1,000-fold higher affinity than ET-3, suggesting that they correspond to the subtype ET_A of the ET receptors. Specific ^{125}I-ET-1 binding sites are present also in Sertoli cells but are 50-fold less concentrated than in Leydig cells. Our results suggest an autocrine/paracrine role for ET-1 in rat testis.

Leydig cell; Sertoli cell; endothelin receptors; follicle-stimulating hormone

ENDOTHELINS (ETs) are a family of 21-amino acid peptides including four distinct isoforms (ET-1, ET-2, ET-3, and vasoactive intestinal contractor) that specifically interact with two populations of receptors (ET_A and ET_B; see Refs. 23, 27). ET_A and ET_B receptors have been recently cloned and sequenced from bovine (2) and rat (24) lung, and both belong to the superfamily of G protein-coupled receptors. The two ET receptors differ in their ligand selectivities; ET_B receptor binds with virtually equal affinity all of the ET isopeptides, whereas ET_A does not bind ET-3.

Because either the peptides or their receptors are distributed widely in mammalian tissues, a large variety of biological actions have been attributed to ETs, including the control of reproductive functions (3, 10). We previously demonstrated that ET-positive cells and ET receptors are present in the rabbit uterus (14, 19). In uterus, ETs are produced by endometrium (19) and are biologically active on myometrial smooth muscle cells (14). Therefore, a role for ETs in the physiological control of myometrial contractility was suggested (20). In addition to the well-characterized stimulatory effect of ETs on different types of smooth muscle cells (26), several reports indicated that ETs might also regulate steroidogenesis. Indeed, ET-1 stimulates aldosterone secretion in bovine (5, 21), rabbit (16), rat (31), frog (6), and human (32) adrenal glomerulosa cells. Because in calf adrenal cells ET-3 (a selective ligand for ET_B receptor) was 100-fold less potent than ET-1 in either radioligand binding studies or aldosterone stimulation (5), one can assume that ET_A receptors are involved in the ET-induced stimulation of adrenocorticoid steroids. Bovine adrenal, indeed, expresses ET_A receptor mRNA (2). Conversely, in porcine granulosa cells, both ET-1 and ET-3 equally inhibited luteinizing hormone (LH)-induced adenosine 3′,5′-cyclic monophosphate (cAMP) formation and progesterone production, suggesting the presence of an ET-unselective receptor (i.e., ET_B) that negatively affects ovarian steroidogenesis (9). Because in the rat the ovarian (29) and adrenal (15) tissue concentration of ET-1 exceeds by one or two orders of magnitude the levels present in peripheral circulation a local production of ETs is suggested.

The presence of elevated concentrations (100 pM) of immunoreactive ET-1 (15), together with the relative abundance of preproendothelin-1 mRNA (22) in rat testis, strongly indicates that the male gonad should be considered a new source of ET-1. Because several distinct populations of cells coexist and interact within testis, we initiated studies to localize the testicular cells immunopositive for ET-1 and the cells that express the receptor for ETs.

MATERIALS AND METHODS

Chemicals. ^{125}I-labeled endothelin (ET-1) was purchased from Amersham (Amity, Milan, Italy). Radiochemical purity of the tracer was assessed by reverse-phase high-pressure liquid chromatography (HPLC). The labeled ligand was separated into aliquots in plastic tubes, sealed under nitrogen, and frozen at −80°C until used. ET-1, ET-2, ET-3, and sarafotoxin S6b were obtained from either NovaBiochem or Peninsula Laboratories (San Carlos, CA). The polyclonal antibody to ET-1 (RAS-6901) was purchased from Peninsula Laboratories (San Carlos, CA); the monoclonal antibody to ET-1 (clone TR.ET.48.5) was purchased from Affinity Bioreagents (Nashanic Station, NJ). Bovine serum albumin (BSA), bovine insulin, bacitracin, benzamidine, and soybean trypsin inhibitor were from Sigma (St. Louis, MO). Purified human chorionic gonadotropin (hCG, 13,000 IU/mg) was a gift of Y.-Y. Tsong (the Population Council, NY). 3,4,3′,4′,Tetraaminodiphenyl hydrochloride (Diaminobenzidine) was obtained from BDH Chemical. Universal immunoperoxidase staining kits were obtained from Cambridge Research Laboratories. Murine epidermal growth factor (EGF) was purchased from Biomedical Technologies (Cambridge, MA). Ovine follicle-stimulating hormone [FSH; National Institutes of Health (NIH) FSH S17; FSH activity: biological potency = 20 × NIH FSH S1 U/mg] was a gift from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseaes, NIH. Forskolin, human

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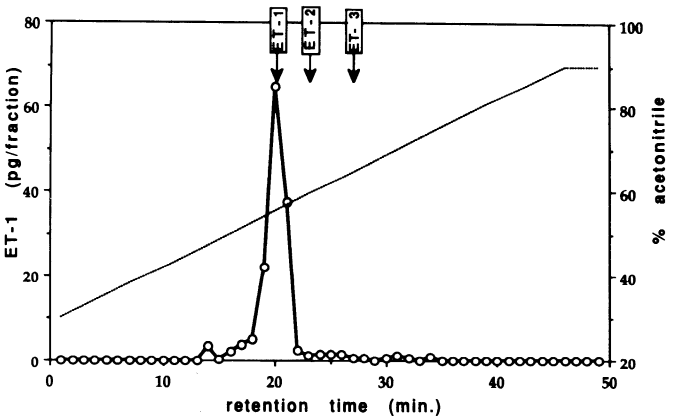

Fig. 1. Reverse-phase high-pressure liquid chromatography (HPLC) profile of extract from conditioned medium of Sertoli cells. Concentrations of endothelin-1-like immunoreactivity (ET-1-LI) in each 1-ml fraction are shown by open circles. Retention times of standard ET-1, ET-2, and ET-3 are indicated by arrows. Peak of ET-1-LI emerged at position corresponding to that of authentic ET-1.

transferrin, and 17β-estradiol (17βE_2) were obtained from Calbiochem-Behring (La Jolla, CA). Collagenase-dispase was purchased from Boeringher Mannheim (Indianapolis, IN). Powdered F-12/Dulbecco's modified Eagle's (DME) medium was obtained from Irvine Scientific (Irvine, CA). Serum-free (SF) medium was prepared from reconstituted F-12-DME medium (F-12-DME) containing 1.2 g/l sodium bicarbonate, 15 mM HEPES (Research Organics, Cleveland, OH), and 20 mg/l gentamycin (GIBCO, Grand Island, NY). Low insulin-containing medium contained (in μg/ml) 2.5 insulin, 1 human transferrin, and 5 bacitracin.

Sertoli and Leydig cell cultures. Sprague-Dawley rats (20 days old) were killed after asphyxiation in a CO_2-precharged chamber. Testes were removed and placed in sterile F-12-DME. SF cultures of Sertoli and Leydig cells were prepared from decapsulated testes as reported previously (11, 17).

For membrane preparation from cultured cells, the Sertoli cell-enriched (SCE) suspension was plated in 100-mm dishes in SF medium supplemented with bovine insulin (10 μg/ml), human transferrin (1 μg/ml), and bacitracin (5 μg/ml) and allowed to attach. After 4 days, the SF medium was replaced with charcoal-stripped serum (5% horse serum and 2.5% newborn calf serum) with or without 20 nM 17βE_2 to evaluate the effects of estrogens on ^{125}I-ET-1 binding, as previously shown in rabbit myometrium (14). Particulate membranes were prepared after 48 h of treatment. Leydig cell-enriched (LCE) preparations were cultured in 100-mm dishes in SF medium supplemented with bovine insulin (10 μg/ml), human transferrin (1 μg/ml), bacitracin (5 μg/ml), and hCG (100 ng/ml). Membranes were prepared on *day 7* of culture.

For the determination of ET secretion, SCE preparations were plated in 100-mm dishes in low-insulin-containing medium. The medium was removed after 24 h and replaced with fresh medium containing the following treatments: FSH (50 and 300 ng/ml), forskolin (1 and 10 μM), EGF (2.5 ng/ml), or vehicle [phosphate-buffered saline (PBS)]. The conditioned medium was removed after 24 or 48 h and saved at −20°C. LCE preparations were plated as before, but the following treatments were added after 30 min: hCG (100 ng/ml), prolactin (PRL; 100 ng/ml), EGF (2.5 ng/ml), or vehicle (PBS). Media were collected after 24, 65, or 96 h and stored at −20°C.

Membrane preparation. At the time of membrane preparation the cells were washed carefully with ice-cold PBS, harvested by scraping in PBS, and centrifuged at low speed. Membranes were prepared as described previously (13). Briefly, cells were homogenized in *buffer 1* [10 mM tris(hydroxymethyl)aminomethane (Tris)·HCl, pH 7.4, 1.5 mM EDTA, 0.5 mM dithiothreitol, 1 mM benzamidine, 0.01% bacitracin, 0.002% soybean trypsin inhibitor], and the homogenate was centrifuged at 1,000 *g* for 10 min at 4°C. The supernatant containing the crude membrane fraction was then ultracentrifuged at 160,000 *g* for 30 min at 4°C. The resulting pellets were washed one time in *buffer 2* (50 mM Tris-maleate, pH 7.6, 10 mM $MgSO_4$, 1 mM benzamidine, 0.01% bacitracin, 0.002% soybean trypsin inhibitor) and centrifuged again at 160,000 *g* for 30 min at 4°C. The final pellets were then resuspended in *buffer 2* and divided into small aliquots. The membrane preparations were frozen in solid CO_2 and stored at −80°C until assayed. Protein concentrations were determined using the Pierce protein assay reagent (Pierce Chemical, Rockford, IL), according to the "standard assay procedure" recommended by the manufacturer.

Binding studies. Binding studies were carried out as described previously (14). Aliquots of membranes (0.075 mg/ml) were incubated for 120 min at 4°C in *buffer 2* in the presence of 0.01% BSA, with increasing concentrations (10–70 pM) of ^{125}I-ET-1, with or without increasing concentrations of unlabeled ligands. All measurements were obtained in triplicate. After incubation, membranes were filtered through Whatman GF/B filters (Clifton, NJ), presoaked in ice-cold 50 mM Tris, pH 7.4, in 0.1% BSA, using the Brandel M-48R 48-well cell harvester (Gaithersburg, MD). Filters were washed two times with 3 ml ice-cold 50 mM Tris, pH 7.4. Radioactivity retained by filters was measured in a gamma counter at 70% efficiency. The binding data were evaluated quantitatively with non-linear least-square curve fittings using the computer programs LIGAND (18) and ALLFIT (7).

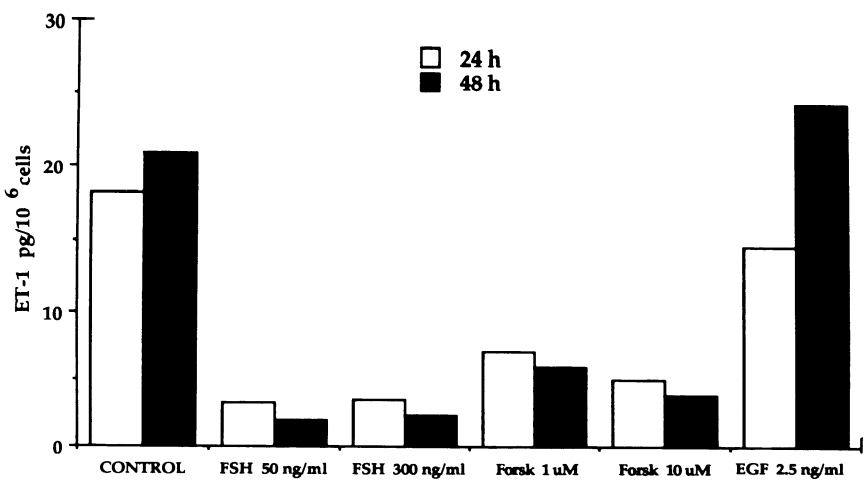

Fig. 2. Effects of follicle-stimulating hormone (FSH; 50–300 ng/ml), forskolin (Forsk; 1–10 μM), and epidermal growth factor (EGF; 2.5 ng/ml) on ET-1-LI secretion from rat Sertoli cells in primary culture. Sertoli cells were incubated for 24 or 48 h with hormones.

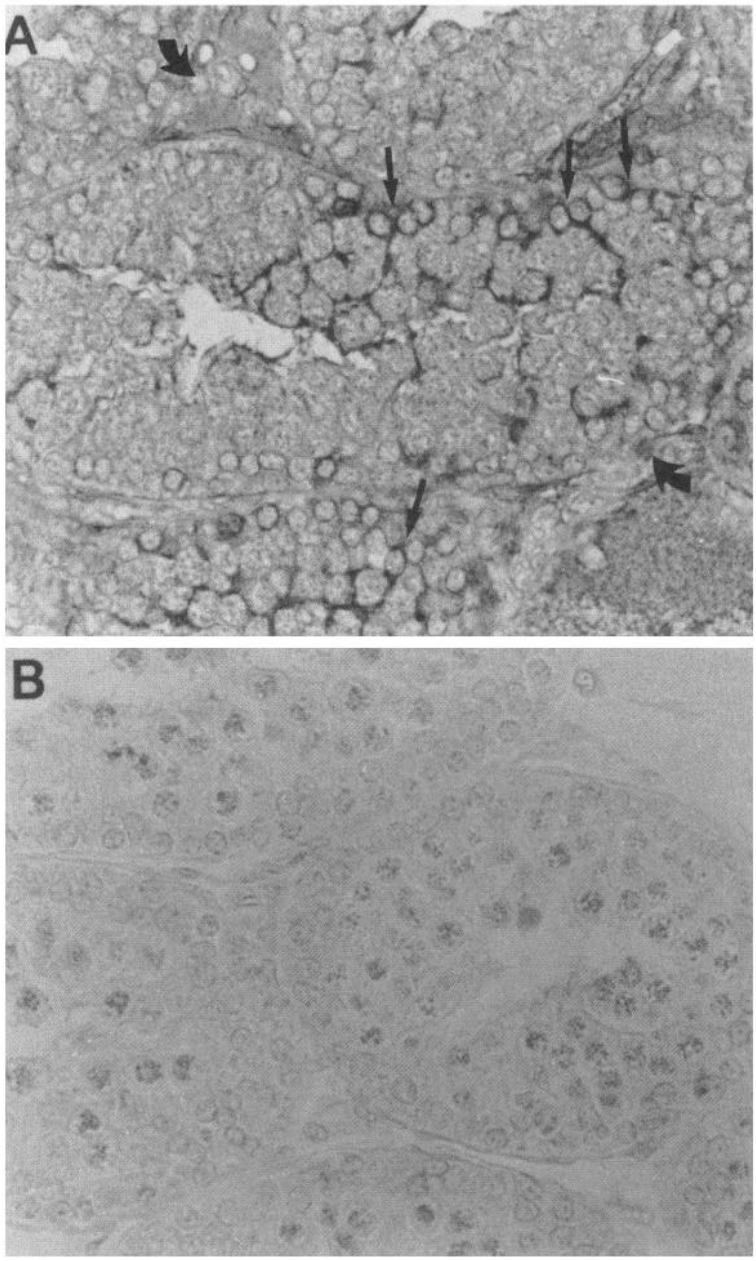

Fig. 3. Photomicrographs of cross sections of seminiferous tubules from 20-day-old rats (×200 magnification). *A*: section immunostained with monoclonal antiserum to ET-1. Some Sertoli cells were positively stained (thin arrows), whereas interstitial cells were negative (thick arrows). Similar results were obtained using polyclonal antibody to ET-1 (not shown). *B*: control section (hematoxylin counterstained); immunoabsorption of antiserum with synthetic ET-1 (100 nM) has completely prevented staining.

ET-1 radioimmunoassay. ET-1 radioimmunoassay (RIA) was performed essentially as previously reported (19). ET-1 was extracted from conditioned media (1.5–2.0 ml) using Sep-Pak C_{18} cartridges (Waters, Millipore, MA) previously activated with acetonitrile, trifluoroacetic acid (TFA), and water (60:0.1:39.9 vol/vol/vol) and washed with water TFA (99.9:0.1 vol/vol). Immunoreactive ET-1 was eluted with acetonitrile, TFA, and water, evaporated to dryness by a centrifugal concentrator (Univapo 150 H; Uni Equipe, Martinsned, Germany), and reconstituted in assay buffer for RIA or HPLC analysis.

The assay buffer for ET-1 RIA was a 100 mM PBS, pH 7.4 (0.1% Triton X-100, 0.1% BSA, and 0.01% NaN_3). Samples or standards (0.1 ml) were preincubated with rabbit anti-ET-1 antiserum (0.1 ml, final dilution 1:60,000) at 4°C overnight and further incubated with ^{125}I-ET-1 (0.1 ml, 10 pM) in the same condition. Bound-to-free separation was performed by the second antibody technique. The sensitivity of the assay was 0.4 pg/tube.

HPLC. Reverse-phase HPLC was performed using a Waters Resolve C_{18} 5-μm column (150 × 3.9 mm). ETs were eluted with a linear gradient of acetonitrile (from 30 to 90% in 0.1% TFA) for 40 min, with a flow rate of 1 ml/min. Fractions (1 ml) were collected, evaporated to dryness, reconstituted in assay buffer, and subjected to RIA.

Immunohistochemistry. Immunohistochemical studies were performed as described previously (14). Briefly, testes derived from 20-day-old and adult Sprague-Dawley rats were fixed in Bouin's solution for 4 h, embedded in paraffin, and sectioned. At the time of the experiment the sections, after being deparaffinized and rehydrated, were incubated for 24 h with the two ET-1 antiserums (working dilutions: 1:1,500 and 1:100 for the polyclonal and monoclonal antiserum, respectively). Sections were rinsed in PBS and then incubated with the specific immunoglobulin G peroxidase conjugates for 30 min. The dilutions of the second antibodies were 1:1,000 and 1:500 for sections incubated with the polyclonal or the monoclonal antiserum, respectively. The sections were rinsed again in PBS and finally washed in 50 mM Tris. Demonstration of peroxidase activity

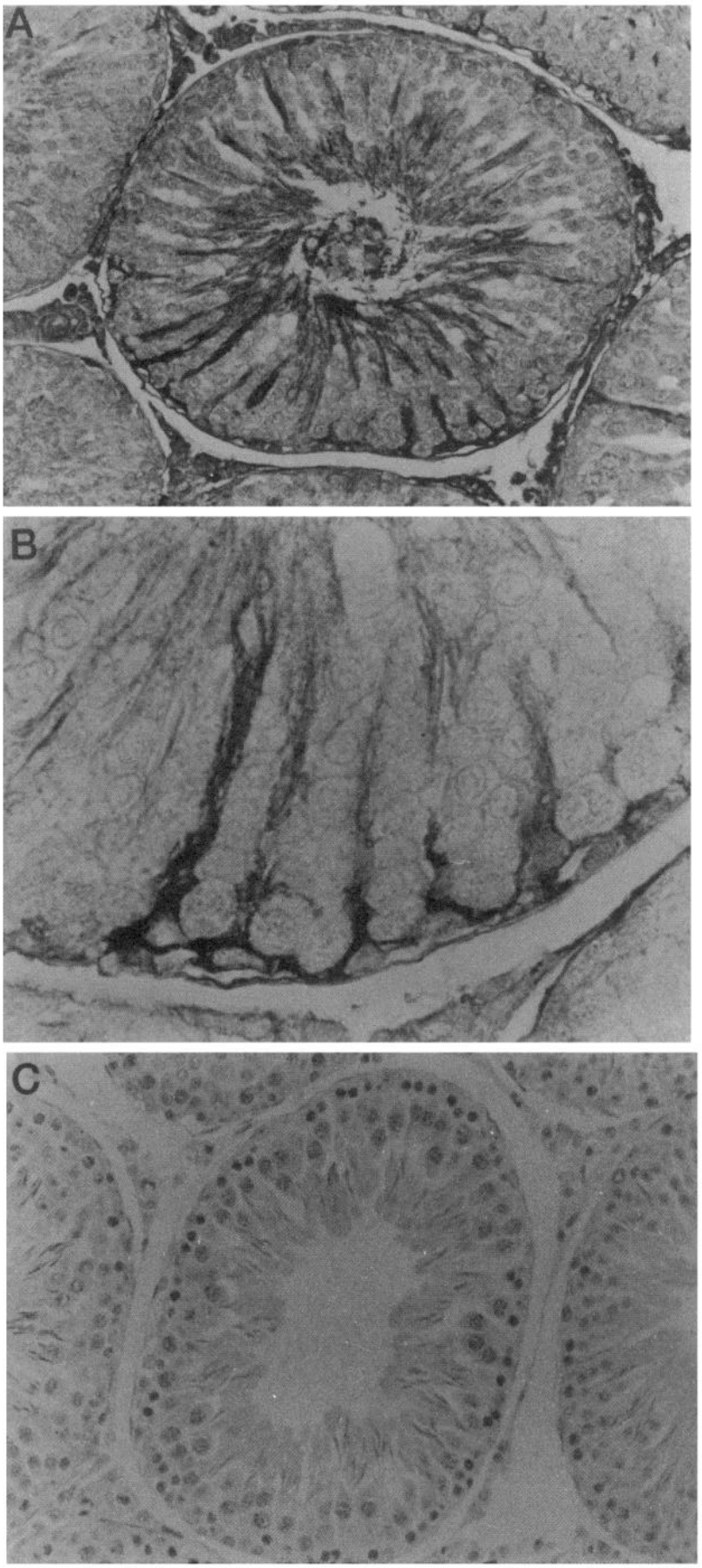

Fig. 4. Sections from adult rat testis immunostained with polyclonal antibody against ET-1 . *A*: strong staining can be detected in cytoplasm of some Sertoli cells. Weak staining is present also in interstitium (×200 magnification). *B*: higher magnification of *A*. Note intense cytoplasmic staining of Sertoli cells (×750 magnification). *C*: control section incubated with polyclonal antiserum preincubated with ET-1 (100 nM); no staining was seen (hematoxylin counterstained, ×200 magnification).

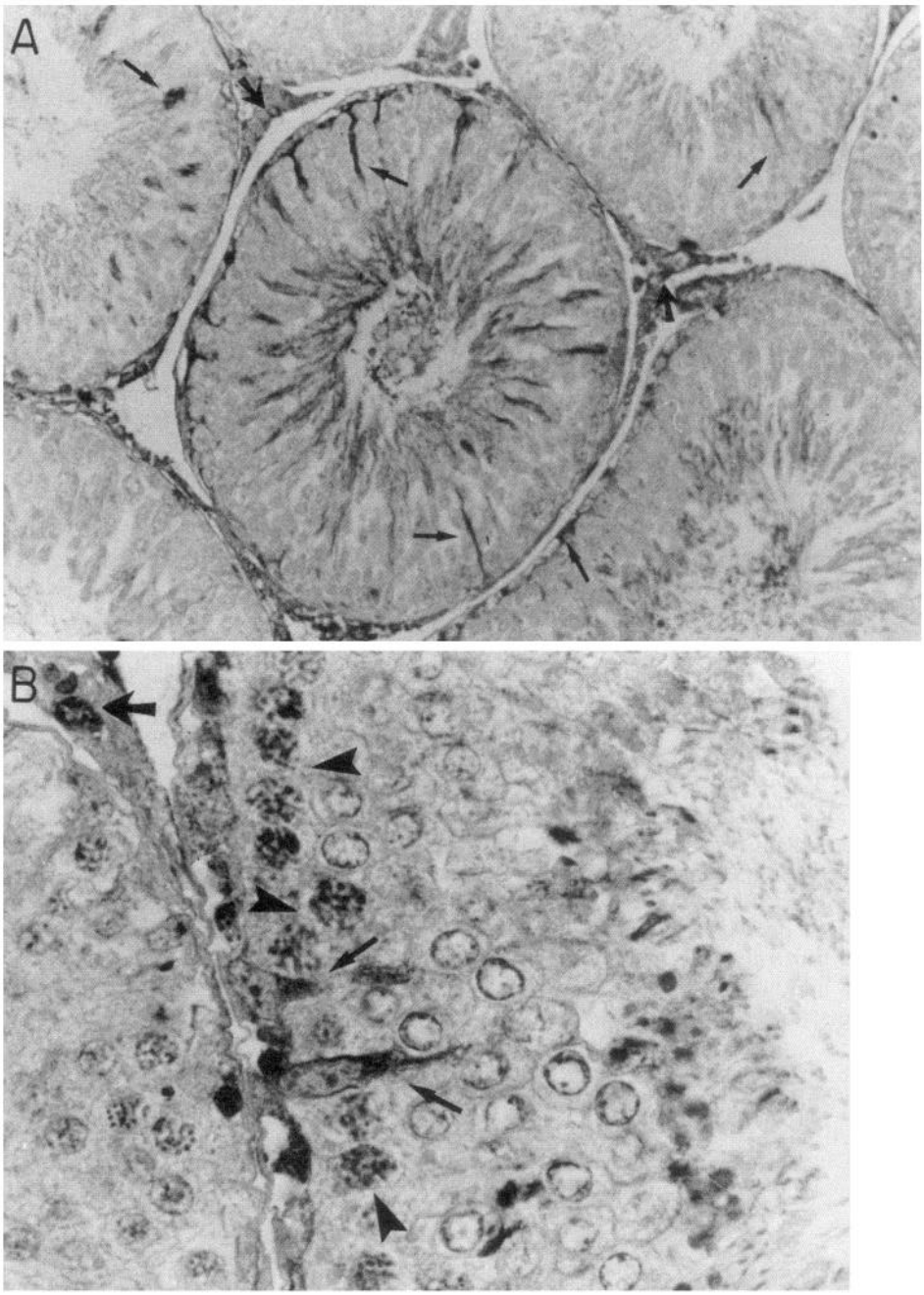

Fig. 5. Seminiferous tubules from adult rat testis immunostained with monoclonal antibody against ET-1. *A*: Sertoli cells with stained cytoplasm are seen in tubules (thin arrows). Minority of interstitial cells shows positive staining for ET-1 (thick arrows; ×200 magnification). *B*: hematoxylin-counterstained higher magnification of *A* (×750 magnification) showing positive staining in cytoplasm of Sertoli cells (thin arrows) and in 1 interstitial cell (thick arrow). Arrowheads indicate unstained spermatocytes.

and controls for specificity of the antisera were carried out as previously described (14).

The slides were evaluated and photographed using a Nikon MICROPHOT-FX microscope (Nikon, Kogaku, Tokyo, Japan).

RESULTS

RIA and HPLC studies. ET-1-like-immunoreactivity (ET-1-LI) was found in the extracts from Sertoli cell- but not from Leydig cell-conditioned media. The dilution curves of the conditioned medium from Sertoli cells was parallel to that of standard ET-1, suggesting that the ET-like material present in the extracts is immunologically indistinguishable from authentic ET-1 (data not shown). An elution profile of Sertoli cell media extract on reverse-phase HPLC is shown in Fig. 1. A major component of ET immunoreactivity was eluted in the position corresponding to authentic ET-1.

Either FSH or forskolin decreased the secretion of ET-1-LI in a dose-dependent manner. After the addition of the highest (2 days) dose of FSH (300 ng/ml) or forskolin (10 μM) the secretion of ET-1-LI was reduced 10- and 5-fold, respectively. Conversely, treatment with EGF (2.5 ng/ml) did not affect ET-1-LI release (Fig. 2). ET-1-LI was undetectable in extracts from Leydig cell media either in the basal condition or after different hormonal treatment (hCG, EGF, PRL) for various times (24, 65, 96 h).

Immunohistochemical studies. In 20-day-old rat testis, immunostaining for ET-1 was exclusively found in Sertoli cells using either the monoclonal (Fig. 3*A*) or the

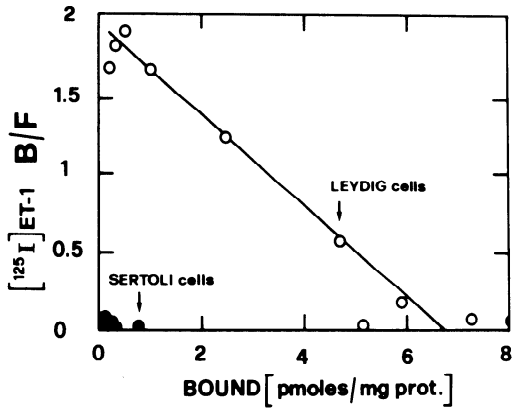

Fig. 6. Equilibrium binding of ^{125}I-labeled ET-1 to Leydig cells (open circles) or Sertoli cells (filled circles). Ordinate: B/F, bound-to-free ratio; abscissa: concentration of bound ligand (pmol/mg protein). Note dramatic difference in concentration of ET-1 binding sites in 2 populations of testicular cells.

polyclonal antiserum (data not shown). Similar results were also obtained in the adult rat testis using both antibodies (Fig. 4, *A* and *B* and Fig. 5, *A* and *B*). However, in the adult testis the staining was not confined to the seminiferous tubules but was also present in the interstitium (Fig. 4*A* and Fig. 5, *A* and *B*). In the labeled cells, staining was observed in the cytoplasm. When the antiserum was previously absorbed with synthetic ET-1 (100 nM), no staining could be detected in both tubular and interstitial compartments (Figs. 3*B* and 4*C*).

Binding studies. Binding of ^{125}I-ET-1 to membranes from Leydig cells reached apparent equilibrium within 120 min at 4°C (data not shown). Hence, subsequent binding studies were carried out at this time point. In Leydig cells, Scatchard analysis of equilibrium binding curves for ET-1 revealed a single class of high-affinity high-capacity binding sites [dissociation constant (K_d) = 0.63 ± 0.13 nM; binding capacity = 13 ± 5 pmol/mg protein; mean ± SE of 3 separate experiments from a single membrane preparation]. A representative Scatchard plot is shown in Fig. 6. Also shown in Fig. 6 is a Scatchard plot obtained with Sertoli cell membranes. In these cells, ET-1 still binds with high affinity (K_d = 0.33 nM). However, the density of ET-1 receptors was 50-fold lower than in Leydig cells (0.2 pmol/mg protein). The subacute treatment (48 h) of Sertoli cells with 20 nM 17βE$_2$ did not affect either the receptor density or the affinity constant of ET-1 (K_d = 0.28 nM; maximal binding = 0.25 pmol/mg protein). Figure 7 shows a family of competition curves for ^{125}I-ET-1 binding in Leydig cells. Among the different ET isopeptides tested, ET-1 and ET-2 displaced with the highest affinity ^{125}I-ET-1 binding, whereas ET-3 was 1,000-fold less potent than ET-1, suggesting the presence of ET$_A$ receptors.

DISCUSSION

Testicular activity is mainly regulated by the pituitary hormones LH and FSH. However, there is now a considerable body of physiological evidence suggesting that Leydig cell function is also modulated by factors derived from the seminiferous tubules (28, 30). This is the first demonstration that Sertoli cells from immature rats contain and release, under FSH and cAMP control, an ET-related peptide that is apparently indistinguishable from authentic ET-1. We suggest that ET-1 represents a new paracrine factor produced by Sertoli cells and involved in the regulation of interstitial Leydig cell activity. Indeed, receptors for ET-1 are present in high density in Leydig cells. The pharmacological characterization of ^{125}I-ET-1 binding to Leydig cells indicates that ET$_A$ receptors are predominantly present in these cells. The concentration of ET$_A$ receptors in Leydig cells is 10-fold higher than in aorta and comparable to the density found in the uterus (14, 12), suggesting a role in testicular physiology. Recent reports from other groups indicate that ET-1 might indeed affect Leydig cell activity, increasing basal and hCG-induced steroidogenesis (4, 8). These findings, together with the evidence of ET$_A$ mRNA in primate testis (1), strongly suggest a positive role for ET-1 in the regulation of testicular steroidogenesis, as previously reported for adrenal steroidogenesis (5, 6, 16, 21, 31, 32).

The immunohistochemical results obtained in rat testis using a polyclonal or a monoclonal antiserum against

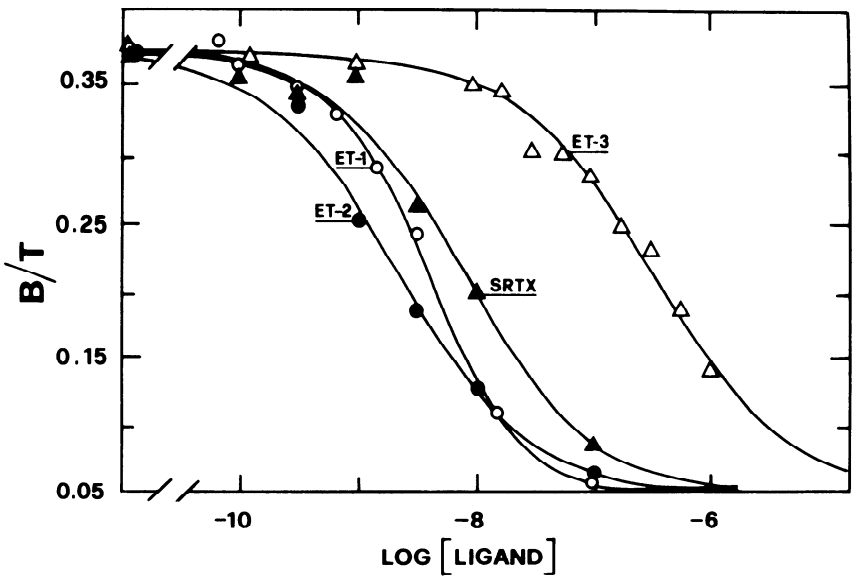

Fig. 7. Displacement of specific ^{125}I-ET-1 binding to Leydig membranes by ET isopeptides. Membranes (0.075 mg/ml) were incubated for 120 min at 4°C with 50 pM ^{125}I-ET-1 in presence or absence of increasing concentrations of the following peptides: ET-1, ET-2, ET-3, and sarafotoxin S6b (SRTX). B/T, bound-to-total ratio.

ET-1 are relatively consistent. In the immature rat testis, immunoreactive ET-1 is limited to Sertoli cells. This is in perfect agreement with results derived from testicular cells in culture. In adult rat testis, the immunostaining pattern of ET-1 in the seminiferous tubule is similar to immature rats, and positive staining was also present in the interstitium, suggesting an age-dependent change in the immunohistochemical localization of ET-1. We also found that either in adult or in immature rat testis not all of the Sertoli cells are labeled in adjacent tubules. This might indicate a stage-specific production of ET-1 within the seminiferous tubules.

^{125}I-ET-1 binding sites are present also in Sertoli cells, although the density of sites in these cells is dramatically lower (i.e., 50-fold) than in Leydig cells. Furthermore, the ET-1 receptor concentration in Sertoli cells is not apparently affected by estradiol, as previously reported in the rabbit female genital tract (12, 14). Because it has been shown that, in rat Sertoli cells, ET-1 increases intracellular calcium and modulates both the cAMP and estradiol response to FSH (25), an autocrine role for ET-1 within the seminiferous tubule should also be considered.

In conclusion, we report that Sertoli cells from immature rats produce and release ET-1, whereas receptors for this peptide are present in both Leydig cells and Sertoli cells. Hence, ET-1 represents a new factor probably involved in the local control mechanism for the exchange of information within the testis.

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