Cyclic Expression of Endothelin-converting Enzyme-1 Mediates the Functional Regulation of Seminiferous Tubule Contraction

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Abstract. The potent smooth muscle agonist endothelin-1 (ET-1) is involved in the local control of seminiferous tubule contractility, which results in the forward propulsion of tubular fluid and spermatozoa, through its action on peritubular myoid cells. ET-1, known to be produced in the seminiferous epithelium by Sertoli cells, is derived from the inactive intermediate big endothelin-1 (big ET-1) through a specific cleavage operated by the endothelin-converting enzyme (ECE), a membrane-bound metalloprotease with ectoenzymatic activity. The data presented suggest that the timing of seminiferous tubule contractility is controlled locally by the cyclic interplay between different cell types. We have studied the expression of ECE by Sertoli cells and used myoid cell cultures and seminiferous tubule explants to monitor the biological activity of the enzymatic reaction product. Northern blot analysis showed that ECE-1 (and not ECE-2) is specifically expressed in Sertoli cells; competitive enzyme immunoassay of ET production showed that Sertoli cell monolayers are capable of cleaving big ET-1, an activity inhibited by the ECE inhibitor phosphoramidon. Microfluorimetric analysis of intracellular calcium mobilization in single cells showed that myoid cells do not respond to big endothelin, nor to Sertoli cell plain medium, but to the medium conditioned by Sertoli cells in the presence of big ET-1, resulting in cell contraction and desensitization to further ET-1 stimulation; in situ hybridization analysis shows regional differences in ECE expression, suggesting that pulsatile production of endothelin by Sertoli cells (at specific "stages" of the seminiferous epithelium) may regulate the cyclicity of tubular contraction; when viewed in a scanning electron microscope, segments of seminiferous tubules containing the specific stages characterized by high expression of ECE were observed to contract in response to big ET-1, whereas stages with low ECE expression remained virtually unaffected. These data indicate that endothelin-mediated spatiotemporal control of rhythmic tubular contractility might be operated by Sertoli cells through the cyclic expression of ECE-1, which is, in turn, dependent upon the timing of spermatogenesis.

Key words: endothelin • endothelin-converting enzyme • spermatogenesis • peritubular myoid cells • seminiferous epithelium

ENDOTHELIN-1 (ET-1)[1] is a 21–amino acid (aa) vasoconstrictive peptide originally isolated from the supernatant of cultured porcine aortic endothelial cells (Yanagisawa et al., 1988). Subsequently, three distinct endothelin genes encoding three closely related peptides were identified: ET-1, ET-2, and ET-3 (Inoue et al., 1989). These endothelin isopeptides are each produced from corresponding preproETs of ~200 residues (Inoue et al., 1989) and act on two distinct subtypes of G-protein–coupled receptors termed ET_A and ET_B (Arai et al., 1990; Sakurai et al., 1990). Longer intermediates termed big endothelins (big ETs, 38–41 aa) are first excised from the preproETs by dibasic pair-specific endopeptidases (Seidah et al., 1993). Big ETs are then further cleaved at Trp^{21}-Val/Ile^{22} by the endothelin-converting enzyme (ECE) to produce the 21-residue mature peptides (Opgenorth et al., 1992). The fact that the biological activity of big ETs is negligible (Kimura et al., 1989) indicates that ECE is a key enzyme for the production of biologically active ETs.

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1. *Abbreviations used in this paper:* aa, amino acid; ECE, endothelin-converting enzyme; EIA, enzyme immunoassay; ET, endothelin; FSH, follicle-stimulating hormone; KHH, Krebs-Henseleit-Hepes; PR, phosphoramidon.

Complementary DNAs coding for two bovine ECEs have been isolated recently and the corresponding proteins have been termed ECE-1 (Xu et al., 1994) and ECE-2 (Emoto and Yanagisawa, 1995). Both enzymes are membrane zinc-binding metalloendopeptidases with a single transmembrane domain, a short NH_2-terminal cytoplasmic tail and a large extracellular COOH-terminal containing the catalytic domain (Shimada et al., 1996; Turner and Tanzawa, 1997). Analysis of the conversion of big ET-1 into ET-1 by ECE in vivo and in vitro (McMahon et al., 1991; Xu et al., 1994) has demonstrated that the conversion takes place on the cell surface. Recently, the presence of ECE on the plasma membrane has also been confirmed by ultrastructural immunolocalization showing that ECE and angiotensin-converting enzyme colocalize on the luminal membrane of endothelial cells (Barnes et al., 1998).

The abundance of ECE-1 mRNA in whole testis extracts favors the hypothesis that this enzyme plays an important role by mediating ET activation in the testis (Xu et al., 1994). In the mammalian testis, seminiferous tubules are ensheathed by a layer of smooth muscle-like cells, the peritubular myoid cells. In the adult rat, myoid cells are arranged to form a squamous epithelioid layer in which no major orientation is apparent (Hermo and Clermont, 1976; Palombi et al., 1992). The main biological function of peritubular contractility is the generation of impulses for the progression of spermatozoa (Hargrove et al., 1977). The transport of spermatozoa along the seminiferous tubule lumen towards the rete testis, is thought to result from forces that are not intrinsic to the sperm cells (Ellis et al., 1981; Eddy, 1988). In fact, seminiferous tubules have been reported to undergo rhythmic contraction; in the apparent absence of nerve endings, the fine regulation of contractility is presumably subject to paracrine control.

Recently we demonstrated that ET-1 is specifically able to induce contraction of rat myoid cells both in cell culture and in peritubular tissue (Tripiciano et al., 1996). In addition, we demonstrated the simultaneous presence of ET_A and ET_B endothelin receptors on individual myoid cells, both of which mediate contraction through distinct regulation of calcium-mediated signaling (Filippini et al., 1993; Tripiciano et al., 1997). The studies of Fantoni et al. (1993) and Maggi et al. (1995) have demonstrated that Sertoli cells produce and secrete ET-1 in rat and human testis. Sertoli cells, somatic cells of the testis that provide the structural framework of the seminiferous tubules and the milieu for germ cell proliferation and differentiation, are targets for the hormones (FSH and testosterone) responsible for the initiation and maintenance of spermatogenesis (Bardin et al., 1988). In the seminiferous epithelium, each Sertoli cell maintains an extensive surface relationship, along its apical sides, with germ cells at various stages of differentiation up to spermiation while the basal side faces the peritubular myoid cells.

Seminiferous tubule contractility represents a fundamental and potentially critical function in male fertility, controlling testicular output of both fluid and sperm. Therefore, any level of regulation mediating seminiferous tubule contractility may represent a specific control mechanism regulating the timing of the contraction-relaxation cycle. In this study, we have examined the in vivo and in vitro specific expression of ECE in rat Sertoli cells as well as the biological activity of this enzyme from intact cultured Sertoli cells. Furthermore, we highlight the functional relevance of ECE by showing that it can mediate regional seminiferous tubule contraction by converting big ET-1 into fully biologically active ET-1. We provide evidence of differential expression of ECE-1 in the testis during spermatogenesis that underlies a pulsatile production of ET-1, accounting for a novel mechanism controlling contractility.

Materials and Methods

Materials

Collagenase A from *Clostridium histoliticum* and DNase-I were obtained from Boehringer Mannheim. Trypsin was purchased from Difco Laboratories. MEM was obtained from GIBCO BRL. Percoll was purchased from Pharmacia. ET-1 and big ET-1 were obtained from Peninsula Laboratories, Inc.; other reagents, when not specified, were purchased from Sigma Chemical Co. Plastic culture dishes and multiwell plates were from Falcon.

Animals

The animals used were adult and three-week-old Wistar rats (Charles River), fed ad libitum until killed by CO_2 asphyxia or cervical disarticulation. Animals were kept in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Alkaline Phosphatase Cytochemistry

Selective myoid cell identification through alkaline phosphatase cytochemistry was performed as previously described (Palombi and Di Carlo, 1988), based on the method of Ackermann (Ackermann, 1962). In brief, the fixed cells were incubated in an alkaline solution containing 0.5 mg/ml Fast Blue RR in water and 40 μl/ml α-naphtol phosphate (0.25% solution, pH 8.6). After 30 min incubation in the dark, a purple-blue precipitate appeared specifically on the surface of myoid cells (Palombi and Di Carlo, 1988).

Cell Isolation and Culture

Sertoli Cells. Primary Sertoli cell cultures from 18–20-d-old Wistar rats were prepared as previously described (Dorrington et al., 1975). Seminiferous tubules obtained by trypsin dispersion of testicular parenchyma were subjected to collagenase digestion to remove the peritubulum. The resulting fragments of seminiferous epithelium, mainly composed of Sertoli cells, were cultured at 32°C in a humidified atmosphere of 5% CO_2 and 95% air in a chemically defined medium (MEM). After 3 d in culture, germ cells contaminating the Sertoli cell monolayer were selectively removed through hypotonic shock (Galdieri et al., 1981); the cells were used one day after the treatment.

Myoid Cell Cultures and Sertoli Cell/Myoid Cell Cocultures. The supernatant-mixed cell population resulting from the collagenase treatment of seminiferous tubules (see above) was centrifuged at 40 *g*, yielding mostly minute fragments of tubular wall (Sertoli cells and myoid cells): culturing of this preparation in MEM for 3 d at 37°C results in a mixed monolayer in which myoid cells can be identified (Tripiciano et al., 1996) by differences in their morphology in phase contrast and through alkaline phosphatase cytochemistry after fixation. For pure myoid cell cultures, the tubular wall fragments were digested in trypsin and EDTA to a single cell suspension, subsequently fractionated on a discontinuous Percoll density gradient (Palombi et al., 1988; Filippini et al., 1993). Percoll-purified myoid cells were cultured under serum-free conditions at 37°C. The assessment of myoid cell purity, performed routinely for each preparation on the basis of the presence of alkaline phosphatase activity, was never below 96%.

Germ Cell Preparations. Seminiferous tubules from 35–60-d-old rats were freed from interstitial tissue by collagenase treatment and dispersed into single cells, as previously described (Geremia et al., 1977). The resulting cell suspension, highly enriched in germ cells, was used as such ("mixed germ cells") or fractionated into several cell classes by velocity sedimentation at unit gravity in an albumin gradient (Lam et al., 1970;

Boitani et al., 1983). The two cellular fractions, composed, respectively, of middle-late pachytene spermatocytes and of round spermatids (steps 1–8 of spermiogenesis), were found to be ~90% pure; the fraction composed of intermediate spermatids (steps 9–14) was ~60% pure, also containing late spermatids (~10%) and residual bodies (~30%).

RNA Isolation and Northen Blot Analysis

RNA was extracted from testicular cells and different organs using the acid guanidine thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). Sertoli cell poly(A)$^+$ RNA was prepared by means of a Quick Prep mRNA purification kit (Pharmacia Biotech). Total RNA (10 μg) and mRNA (4 μg) were separated in a formaldehyde and 1.1% agarose gel, transferred to a nitrocellulose membrane Gene Screen Plus, and then hybridized in QuikHyb solution, as recommended by the manufacturer (Stratagene). Random-primed ^{32}P-labeled cDNA inserts (~4.7 and ~2 kb) encoding bovine ECE-1 and ECE-2 were used as probes (Xu et al., 1994). The membranes were washed in 2× SSC and 0.1% SDS at 55°C and were exposed to an x-ray film for 3 d at −80°C.

Measurement of [Ca^{2+}]i

[Ca^{2+}]i was measured by dual wavelength fluorescence in single cells loaded with the Ca^{2+}-sensitive indicator fura-2 (Grynkiewicz et al., 1985). Testicular myoid cells were plated onto coverslips in serum-free MEM. After 4 d in culture, the cells were incubated in MEM containing 3 mM fura-2-acetoxymethylester for 1 h at 37°C. The cells were then rinsed with Krebs-Henseleit-Hepes (KHH) buffer (140.7 mM Na^+, 5.3 mM K^+, 132.4 mM Cl^-, 0.98 mM PO_4^{2-}, 1.25 mM Ca^{2+}, 0.81 mM Mg^{2+}, 5.5 mM glucose, and 20.3 mM Hepes) supplemented with 0.2% fatty acid–free BSA. Measurements were performed in single cells, at 340- and 380-nm excitation wavelengths, with an AR-Sm microfluorimeter (Spex Industries) connected to a Diaphot TMD inverted microscope (Nikon Corp.) equipped with a CF ×40 objective. Emission was collected by a photomultiplier carrying a 510-nm cut-off filter and recorded by an ASEM Desk 2010 computer (ASEM SpA), which automatically calculated real-time 340/380 ratios. Calibration of the signal was obtained at the end of each observation by adding 5 μM ionomycin to saturate the dye maximal fluorescence, followed by 7.5 mM EGTA plus 60 mM Tris-HCl, pH 10.5, to release Ca^{2+} from fura-2 and obtain minimal fluorescence. [Ca^{2+}]i was calculated according to previously described formulas (Grynkiewicz et al., 1985).

Preparation and Treatment of Seminiferous Tubule Segments for Contraction Assay in Scanning Electron Microscopy

Seminiferous tubules were prepared as previously described (Tripiciano et al., 1996). In brief, testes from 2-mo-old rats were decapsulated and digested under gentle shaking at room temperature in MEM containing 1 mg/ml collagenase. After dispersion of the interstitium, the tubular mass was rinsed in MEM, then stretches of tubules were dissected by means of sharp needles and carefully transferred to 35-mm culture dishes in 300 μl of medium. For the dissection of homogeneous samples at precise stages of the seminiferous epithelium, the tubular segments were identified under transillumination (Parvinen and Ruokonen, 1982). The tubules were incubated for 10 min at 32°C in a humidified chamber under an atmosphere containing 5% CO_2. At the end of the incubation time, the medium was replaced by 600 μl of medium to be tested at different experimental times, as detailed in figure legends. Samples were fixed in 2.5% glutaraldehyde, postfixed in 1% OsO_4, dehydrated and critical point dried in ethanol, coated with gold, and then viewed in a Hitachi S-570 scanning electron microscope.

Evaluation of ECE Activity

ECE activity in Sertoli cell cultures was assayed through estimation of exogenous big-endothelin conversion (Little et al., 1994). The culture medium, conditioned for 30 min to 3 h in the presence of big ET (with or without ECE inhibitors), was purified on a Sep-Pak C18 solid phase cartridge (Waters). After drying by vacuum centrifugation and reconstitution in buffer, the samples were assayed for endothelin content by means of a commercial enzyme immunoassay (EIA) kit (Cayman Chemical Co.) according to the manufacturer's instructions.

Preparation of ^{35}S-labeled RNA Probes and In Situ Hybridization

Adult and 18-d-old Wistar rat testes were fixed in 4% paraformaldehyde in PBS at 4°C overnight. The fixed testes were dehydrated with ethanol and embedded in paraffin by standard procedures. 5-μm-thick paraffin sections were placed on slides pretreated with 3-amino-propyltriethoxysilane. Sections were analyzed by in situ hybridization using the procedure described by Davidson et al. (1988). In brief, before hybridization, sections were deparaffinized, rehydrated, partially digested with proteinase-K (20 μg/ml), and then treated with acetic anhydride. These last two steps were necessary to improve access of the probe to the mRNA and reduce nonspecific binding of the nucleic acid probes. Sections were dehydrated and then incubated at 55°C for ~18 h with ^{35}S-labeled RNA probes. For generation of RNA probes, the 0.5 Kb 5′ PstI-PstI fragment of bovine ECE-1 cDNA, nucleotides 214–751, was subcloned in pBluescript vector and transcribed in vitro with T7 (anti-sense) and T3 (sense) RNA polymerases. Unbound cRNA probe was removed by incubation in RNase solution (40 μg/ml) for 30 min at 37°C in 0.5 M NaCl, TE buffer and by two 20-min washes at 65°C in 2× saline sodium citrate (SSC). Autoradiography was performed with Ilford K2 liquid emulsion (Ilford). After exposure for the time periods indicated, sections were stained with carmalum and examined under a Zeiss microscope using dark- or brightfield illumination. The stages of the seminiferous epithelium were identified from adjacent sections using the criteria of Leblond and Clermont (1952).

Statistical Analysis

Data are presented as the mean ± SE of results from at least three independent experiments. Student's *t* test was used for statistical comparison between means where applicable.

Results

Determination of Expression Levels of ECE-1 and ECE-2 mRNAs in Different Testicular Cells and in Extragonadal Tissue

RNA blot analysis with the bovine ECE-1 cDNA as probe (Xu et al., 1994) showed that a ~4.7 kb ECE-1 mRNA is expressed abundantly in cultured Sertoli cells (Fig. 1 a). As shown, the expression of ECE-1 mRNA is much higher in the testis from 20-d-old rats than in the adult. Since the increase in weight of the testis depends mostly on germ cell multiplication, the evidence that homogenous preparations of specific types of germ cells exhibit no expression of ECE-1 indicates that the reported high expression of ECE-1 mRNA in the testis (Xu et al., 1994) could be attributed prevalently to Sertoli cells. Peritubular myoid cells express much lower levels than Sertoli cells (Fig. 1 a).

In addition, a 3.1-kb mRNA is expressed at a lower level. Since only the 4.7-kb ECE-1 mRNA is present in Northern blots of poly(A)$^+$ RNA from cultured rat Sertoli cells, the two different sizes of mRNA are presumably generated by alternative poly(A)$^+$ addition in the 3′ noncoding region (Fig. 1 b). Conversely, Northern blot analysis of testicular cells with the bovine ECE-2 cDNA as probe (Emoto et al., 1995) revealed no expression of ECE-2 mRNA in the seminiferous epithelium cells, while a 3.3-kb mRNA was detected in the control neural tissue and adrenal gland (not shown).

Time-dependent Conversion of Big ET-1 and Big ET-3 by Intact Sertoli Cells

To analyze ECE activity, we examined whether cultured Sertoli cells can convert synthetic rat big ET-1 exogenously added to the culture medium. We therefore assayed

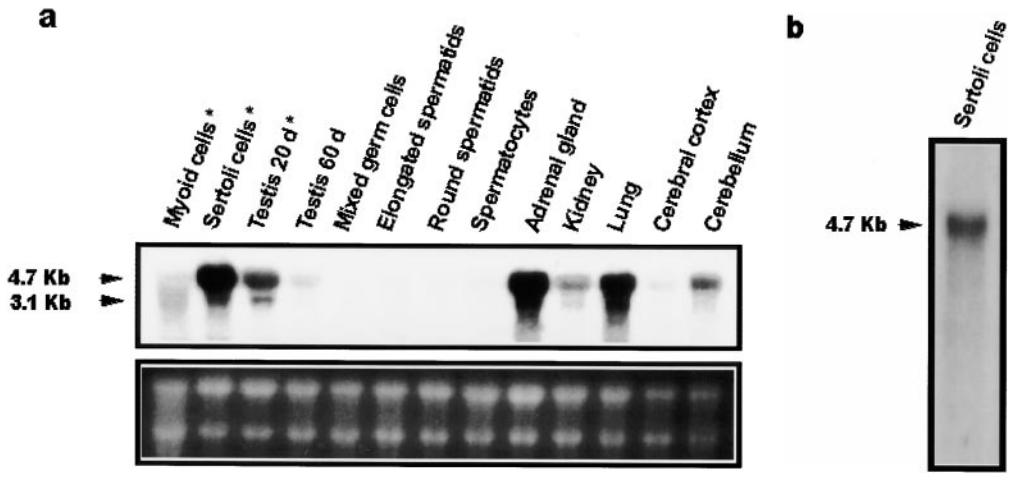

Figure 1. Northern blot analysis of ECE-1 transcripts in different compartments of three-week-old (a*, b) and adult testes. The blots were prepared with 10 μg total RNA (a) or 4 μg poly(A)$^+$ RNA (b) and hybridized to ^{32}P-labeled ECE cDNA probe; the integrity and equal loading of RNA were ascertained by ethidium bromide staining of the gel before transfer (a, lower panel). Sertoli cells appear to represent the main site of ECE-1 expression in the male gonad.

the generation of mature ET-1 by means of a competitive enzyme immunoassay (EIA) that does not cross-react with the substrate big ET-1. As shown in Fig. 2, big ET-1 was efficiently converted into ET-1 by intact Sertoli cells in a time-dependent fashion. At a substrate concentration of 1 μM, up to 69% of the added big ET-1 was converted into ET-1. The Sertoli cell ECE was more efficient in converting big ET-1 than big ET-3. When the metalloprotease inhibitor phosphoramidon (PR), known to specifically inhibit ECE activity (Xu et al., 1994), was present during incubation, it completely inhibited the production of mature ET-1 (Fig. 2). The analogue of big ET-1, [D-Val^{22}]big ET-1 [16-38], an inhibitor of ECE (Morita et al., 1994), strongly inhibited ECE activity and was as effective as PR in completely inhibiting the production of ET-1 by Sertoli cells incubated with big ET-1.

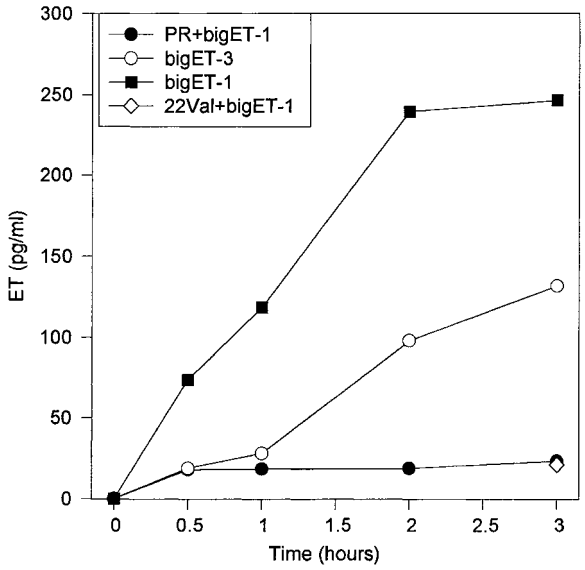

Figure 2. Immunoenzymatic assay of ET-1 production by intact Sertoli cell monolayers treated with inactive endothelin precursors, as a measure of ECE activity. 0.5 μM big ET-1 is more efficiently converted than 0.5 μM big ET-3, and the conversion is inhibited by 2 mM phosphoramidon (PR) and by the synthetic inhibitory peptide [D-Val^{22}]big ET-1[16-38] (22 val) administered, at a 1 mM concentration, 10 min before precursor addition.

Effect of Big ET-1 or Sertoli Cell–conditioned Medium on Calcium Mobilization in Isolated Myoid Cells

We have previously demonstrated that ET-1 is able to induce PI turnover and rapidly increase [Ca^{2+}]i in testicular myoid cells (Tripiciano et al., 1997). Cytofluorimetric analysis of intracellular calcium mobilization, measured by dual wavelength fluorescence in single cells loaded with the Ca^{2+}-sensitive indicator fura-2, indicate that the inactive precursor of ET-1, big ET-1, does not induce calcium mobilization in myoid cells; however, the same cells are able to respond to the addition of ET-1 with an increase in calcium levels (Fig. 3 a), which confirms the total biological inactivity of big ET-1. Conversely, when myoid cells were stimulated with medium conditioned by Sertoli cells for 30 min in the presence of 100 nM big ET-1 (SCMbig), a rapid [Ca^{2+}]i transient comparable to that induced by ET-1 was observed; subsequent stimulation with ET-1 was ineffective (Fig. 3 b). Therefore, medium conditioned by Sertoli cells in the presence of big ET-1 desensitizes myoid cells to the actions of ET-1, which clearly indicates that the biologically active molecule in SCMbig is ET-1 itself, converted from big ET-1 by ECE expressed in Sertoli cells. The observed slow calcium response is comparable to that obtained in response to 0.5–1 nM ET-1 which is below EC_{50}, but sufficient to desensitize to 100 nM ET-1 (not shown). When SCMbig were conditioned in presence of phosphoramidon (PR), an inhibitor of ECE, no effect on calcium response was observed even though myoid cells were still responsive to ET-1 (Fig. 3 c). Fig. 3 d shows the levels of ET-1–, SCMbig– (treated or untreated with phosphoramidon), and big ET-1–dependent [Ca^{2+}]i increases (both peak and plateau).

Morphological Response of Cultured Myoid Cells

To corroborate the presence of fully functional ECE activity from intact Sertoli cells, we have treated cultured myoid cells with SCMbig one day after plating. Treatment at this culture time with SCMbig resulted in an immediate

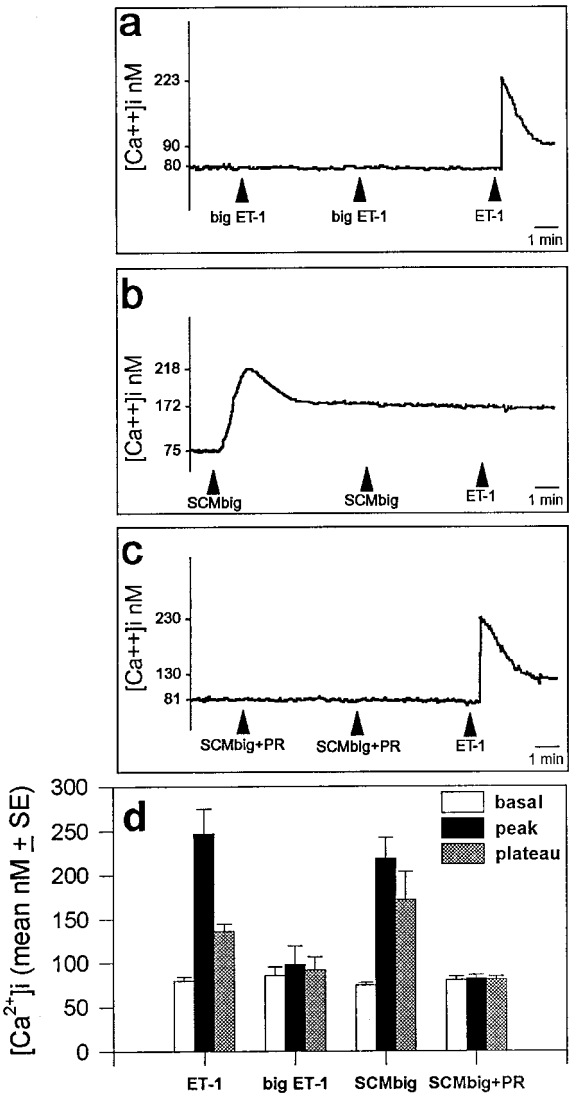

Figure 3. Pattern of $[Ca^{2+}]_i$ mobilization in Fura-2–loaded single myoid cells in response to: (a) 100 nM big-ET-1; (b) medium (KHH) preincubated for 30 min with a Sertoli cell monolayer in the presence of 100 nM big ET-1; (c) medium preincubated for 30 min with a Sertoli cell monolayer in the presence of 100 nM big ET-1 plus 2 mM phosphoramidon. Early myoid cell response followed by desensitization, observable in b, demonstrates that Sertoli cells convert big ET-1 into active ET-1; when indicated, 100 nM ET-1 has been added; (d) size of $[Ca^{2+}]_i$ increases evaluated in five independent experiments.

rounding-up with retraction of cytoplasm, which could be directly followed in an inverted microscope (not shown). To assess whether the observed myoid cell contraction was specific for this cell type and whether Sertoli cells express significant activity of ECE, which is able to process big ET-1 into an amount of ET-1 sufficient to determine the contraction of myoid cells, we used mixed cultures containing fragments of seminiferous epithelium and patches of myoid cells. This mixed population of tubular and peritubular tissue was treated with 100 nM big ET-1 and shape changes which occurred 10–20 min after treatment were photographically recorded (Fig. 4). In these cultures, myoid cells patches were observed to undergo contraction in response to big ET-1, while the morphology of adjacent Sertoli cells remained unmodified. We processed the same sample for the detection of alkaline phosphatase activity, a specific marker for testicular myoid cells (Palombi and Di Carlo, 1988), and found that the cells that contracted are stained for alkaline phosphatase (Fig. 4, c and d). Inhibition of ECE activity by 2 mM PR resulted in the block of contractile response to big ET-1 (Fig. 5). Since PR is a metabolically stable phosphorylated sugar derivative and is unlikely to enter cells at an appreciable rate within a short incubation time, our observation indicates that the conversion of big ET into ET is a plasma membrane event that occurs on the extracellular side, analogous to the production of the vasoconstrictor angiotensin II from angiotensin I.

Myoid Cell Contraction Induced by Big ET-1 in Peritubular Tissue

Fig. 6 a shows the surface of a seminiferous tubule as viewed in the scanning electron microscope. In the adult testis, the myoid cells appear arranged in a continuous monolayer of ephithelioid polygonal cells, particularly flat and wide and with bulging central nucleus. Addition of either 100 nM ET-1 (Fig. 6 b) or 100 nM big ET-1 (Fig. 6 c) results in dramatic contraction of the myoid peritubular cells, which display enhanced bulging of the central area and reduced distance between cell centers in most areas. From a morphological point of view treatment with either ET-1 or with its inactive precursor, big ET-1, induces a basically equal contraction of myoid cells; the only difference between these two treatments is in the timing required to achieve this effect. In fact, we observed myoid cell contraction within 15 s of ET-1 treatment, but only ~10 min after big ET-1 addition, presumably because more time is required for a sufficient amount of big ET-1 to be converted into biologically active ET-1 by ECE-1 expressed by adjacent Sertoli cells in the seminiferous tubule. When seminiferous tubules were challenged with big ET-1 in the presence of PR, we did not observe any contraction of myoid cells, which appeared as flat as in the control sample (Fig. 6 d). Furthermore, as a further control, we challenged seminiferous tubules with SCMbig. In this case, strong contraction of the myoid peritubular cells was observed within a few seconds (Fig. 7 a). When the seminiferous tubules were stimulated only with Sertoli cell–conditioned medium, the surface of myoid cells appeared to be unaffected, as in the control samples (Fig. 7 b).

In Situ Hybridization of ECE-1 mRNA in the Rat Testis

To explore the possibility that the production of ET within the seminiferous epithelium is a discontinuous, cyclically regulated process, we studied the transcription of ECE-1 by in situ hybridization. Sense and antisense RNA for ECE-1 mRNA was prepared as detailed in Materials and Methods. Interestingly, the ECE-1 probe showed striking regional differences in the level of signal (Fig. 8, a and c). The density of the grains was maximal at stages IX-X of the cycle and at the background level in all other stages. The control samples, hybridized with sense ECE-1 probe, dis-

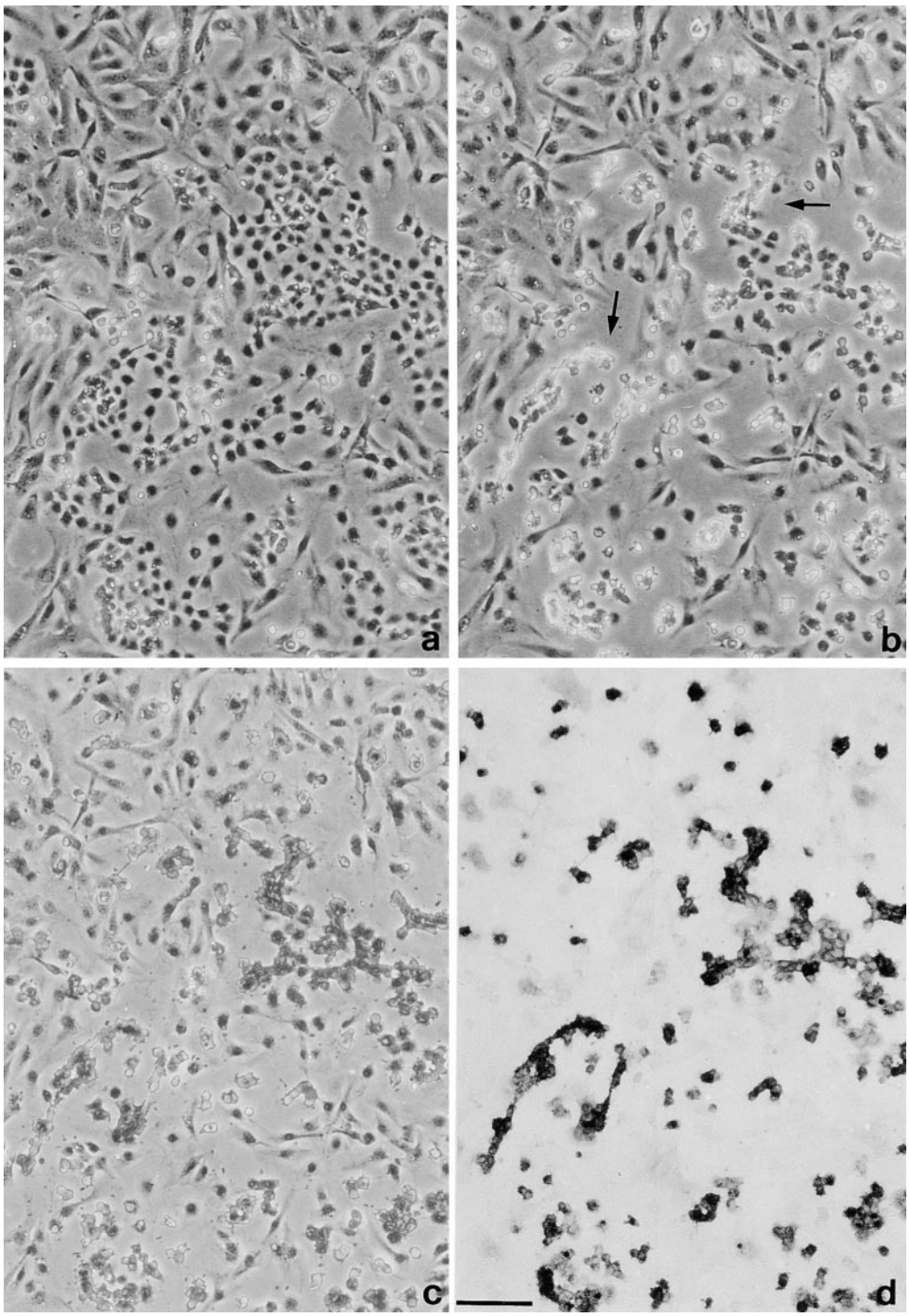

Figure 4. Mixed population of tubular (mostly Sertoli cells) and peritubular myoid cells photographed before (a) and 20 min after (b) addition of 100 nM big ET-1. Myoid cells (arrows) appear specifically contracted in response to treatment. To visualize the selective contractile response of myoid cells, the same cells were photographed after cytochemical detection of alkaline phosphatase activity (c and d); (a–c) phase contrast. Bar, 100 μm.

played a low level of background labeling, with no appreciable differences in grain density between seminiferous tubule profiles and interstitium, thus confirming the specificity of the hybridization signals. In tubules at stages IX-X, the bovine ECE-1 probe hybridized to a basal columnar region surrounding the germ cells (Fig. 8 c, left tubule). This indicates that the ECE-1 gene is expressed above all in Sertoli cells prevalently in the basal region. These findings are in agreement with and extend the above Northern blot analysis, indicating that ECE-1 is expressed in Sertoli cells from adult animal in a cyclical fashion during the seminiferous cycle in stages IX-X soon after spermiation. By contrast, testis from 20-d-old rats exhibits homogeneous labeling intensity in all seminiferous tubules (not shown).

Peritubular Myoid Cell Contractility Is Controlled by ECE Expression at Specific Stages of the Seminiferous Epithelium Cycle

To investigate whether the restricted expression of ECE-1 could be functionally related to the regulation of peritubular contractility induced by big ET-1, seminiferous tubule

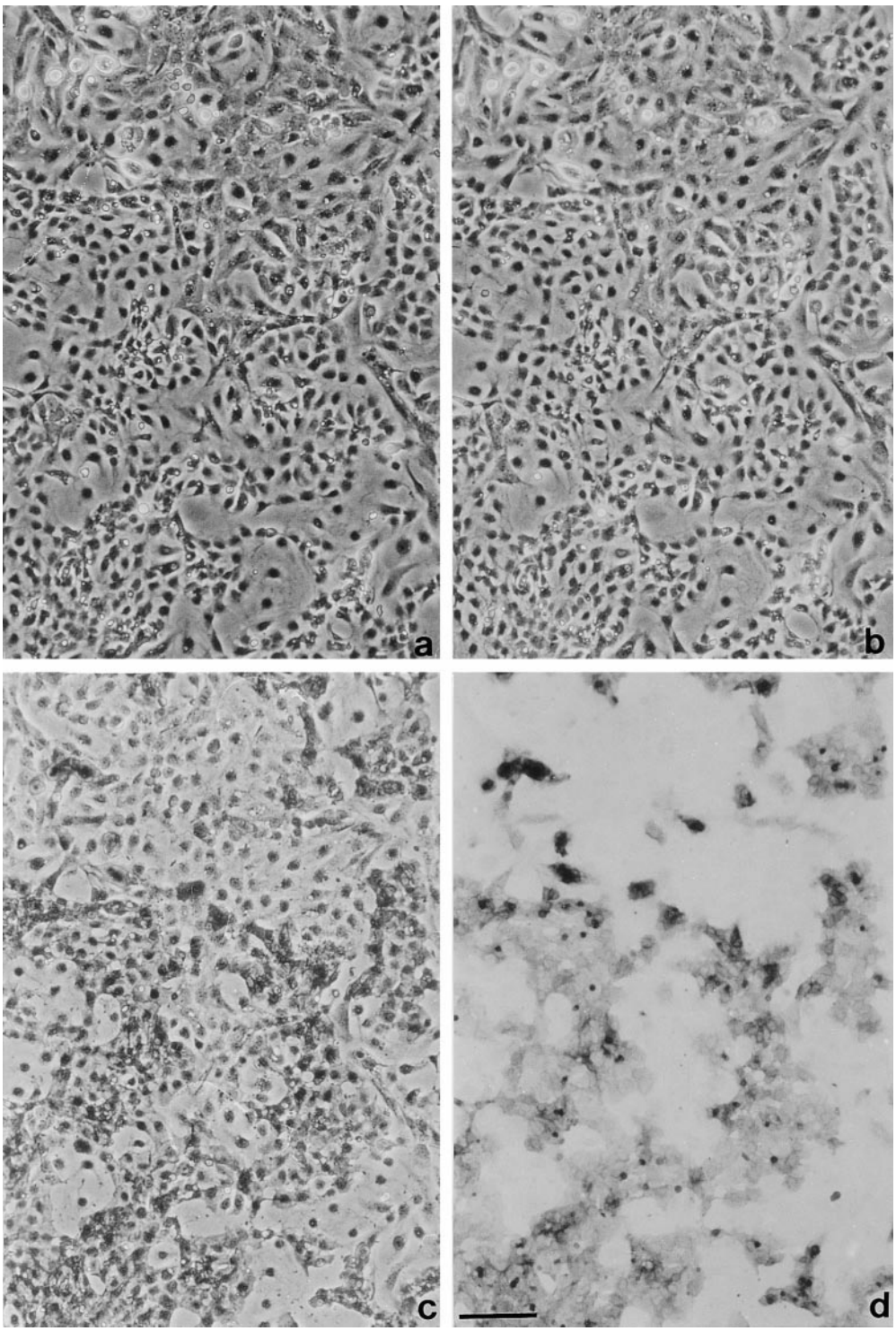

Figure 5. Mixed population of tubular and peritubular cells photographed before (a) and 20 min after (b) addition of 100 nM big ET-1 plus 2 mM phosphoramidon. Owing to inhibition of ECE activity, myoid cells (arrows) fail to respond to big ET-1 (see Fig. 4 for comparison); (c and d) same cells photographed after cytochemical detection of alkaline phosphatase activity in myoid cells. a-c: phase contrast. Bar, 100 μm.

segments from adult testis were microdissected to isolate specific "stages" of the seminiferous tubules (Parvinen and Ruokonen, 1982) and their ability to respond to either ET-1 or big ET-1 was studied at the scanning electron microscope. Fig. 9 a shows a transilluminated tubular segment in which the transition from stage VIII to stage IX is very apparent. The hatched line indicates the level at which the tubules were dissected. Two groups of specific stages of the seminiferous tubule were tested: VII-VIII and IX-XI, which showed low and high ECE expression, respectively. Fig. 9 shows that treatment with big ET-1 is able to induce a strong contraction of seminiferous tubules at stages IX-X in 10 min (Fig. 9 g); by contrast, the seminiferous tubule fragments containing stages VII-VIII are totally unaffected by the treatment with big ET-1 (Fig. 9 f). Furthermore, ET-1 was still active in inducing an immediate contraction of the myoid peritubular cells in both groups of seminiferous tubule fragments (Fig. 9, d and e).

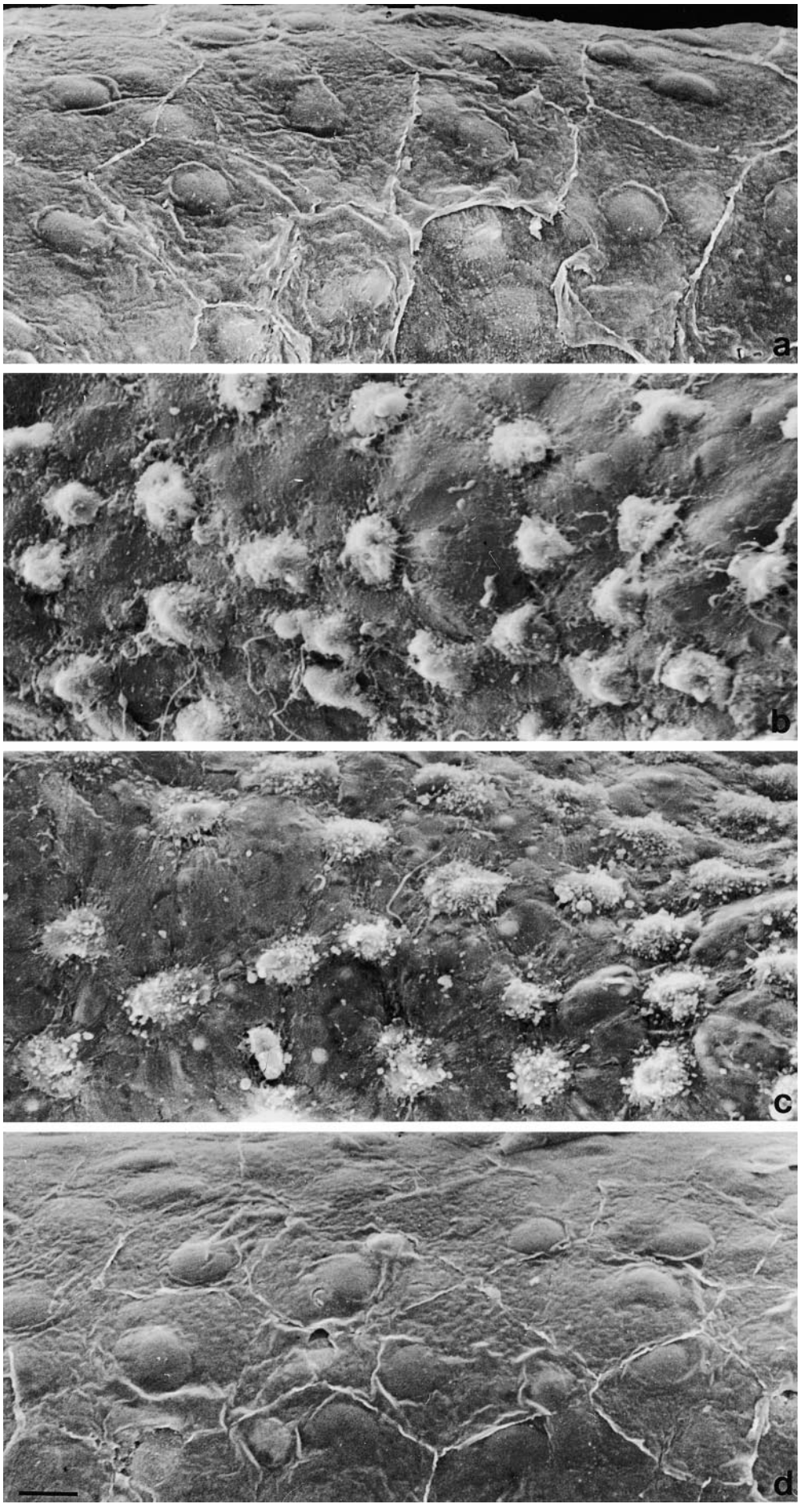

Figure 6. Scanning electron micrograph showing the peritubular surface of adult seminiferous tubules. (a) Control condition. Myoid cell contraction immediately after treatment with 100 nM ET-1 (b) and after 10 min treatment with 100 nM big ET-1 (c). (d) Lack of response after treatment with 100 nM big ET-1 plus 2 mM phosphoramidon. Bar, 10 μm.

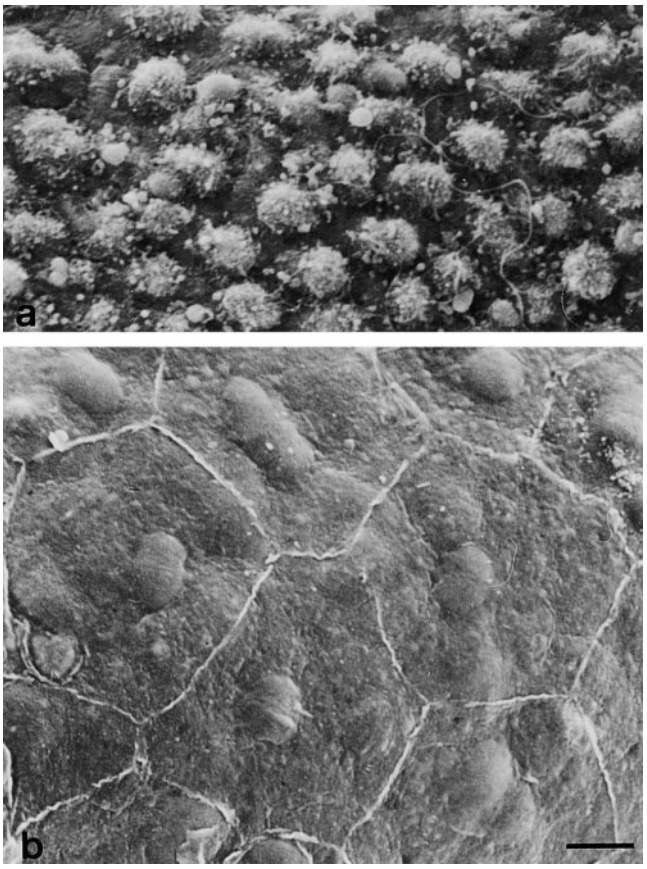

Figure 7. Scanning electron micrograph of the peritubular surface of adult seminiferous tubules immediately after treatment with medium preconditioned for 30 min by Sertoli cells in the presence (a) or the absence (b) of 100 nM big ET-1. Bar, 10 μm.

These data suggest a direct correlation between the restricted expression of ECE-1 and the functional regulation of seminiferous tubule contraction.

Discussion

Seminiferous tubule contractility is fundamental for sperm progression towards the rete testis and its regulation represents, therefore, a key point in male fertility. In this study, we focused on the paracrine communication between Sertoli cells and peritubular myoid cells as it represents an interesting model of cell–cell interactions between epithelial cells of the seminiferous tubule (which play a crucial role during spermatogenesis) and a particular class of nonvascular smooth muscle cells (responsible for seminiferous tubule contraction). Peritubular myoid cells express α-smooth muscle actin and desmin (Virtanen et al., 1986; Tung and Fritz, 1990), and specifically respond to endothelin undergoing cell contraction both in cell culture and in peritubular tissue (Filippini et al., 1995; Tripiciano et al., 1996, 1997).

Given the cyclicity that characterizes seminiferous epithelium activity, we wondered whether endothelin production might be cyclically regulated at the level of the maturation of its precursor by ECE.

In this report we describe the distribution of ECE-1 during the seminiferous epithelium cycle and present evidence that differential expression of ECE-1 in the Sertoli cells during spermatogenesis results in specific and regional seminiferous tubule contraction.

It has been shown that cultured Sertoli cells exhibit a basal production of ET-1 in the media (Fantoni et al., 1993). Preliminary observations, which showed that Sertoli cells incubated with ECE-1 specific inhibitors strongly reduced the secretion of ET-1 while increasing the accumulation of big ET-1 (not shown), prompted us to hypothesize a role for ECE-1 as a local regulator of ET-1 actions. Furthermore, the occurrence of phosphoramidon–sensitive ECE activity on Sertoli cells suggests that some processing of secreted big ET-1 may occur on the surface of ET-1–producing cells, adjacent to myoid cells. Since big ET-1 appears to be much more stable than ET-1 to generic proteolytic degradation (Murphy et al., 1994), this targeted conversion may allow more effective delivery of the active product in intact form to its receptors on myoid cells.

Since the prediction of its existence (Yanagisawa et al., 1988), ECE has been considered to be a potential site of regulation of endothelin production as well as a plausible target for therapeutic intervention in the endothelin system. Recently, the existence of three distinct ECE-1 isoforms has been demonstrated (Shimada et al., 1995; Valdenaire et al., 1995; Schweizer et al., 1997). These three isoforms (ECE-1a, ECE-1b, and ECE-1c) differ only in their N-terminal regions and are derived from a single gene through the use of alternative promoters. The three isoforms show similar kinetic rate constants, processing big ETs with similar velocities and have all been found to cleave the three big endothelins, but with a clear preference for big ET-1, which is in agreement with our results showing that intact Sertoli cell ECE-1 converts big ET-1 more efficiently than big ET-2 or big ET-3.

Recently, Yanagisawa et al. (1998) clearly demonstrated that the activity of ECE-1 is essential and that a physiologically relevant endothelin-converting enzyme exists for both big ET-1 and big ET-3 in vivo. In fact, ECE-1−/− mice (which all died within 30 min of birth) reproduced the phenotype resulting from the defects in both ET-1/ET_A– and ET-3/ET_B–mediated signaling pathways, which clearly shows that mature ET-1 and ET-3 are not synthesized in the relevant microenviroments without ECE-1 activity. Furthermore, a significant amount of mature ET-1/ET-2 still existed in the serum of ECE-1−/− embryos despite the absence of ECE-1, which suggests that other peptidases are responsible for the production of mature ETs. Intriguingly however, these remaining mature ETs completely failed to rescue the developmental phenotype of ECE-1−/− mice, which indicates that defined mature ETs must be produced at specific microenviroments in order to achieve a biological effect. The present study provides evidence that the restricted expression of ECE-1 might play a pivotal role in the control of peritubular contractility by providing a fine local modulation of biologically active ET levels.

If ET acts as a local regulator of seminiferous tubule contractility, it is conceivable that ECE is localized on the basal side of the Sertoli cells. In fact, Northern blot analy-

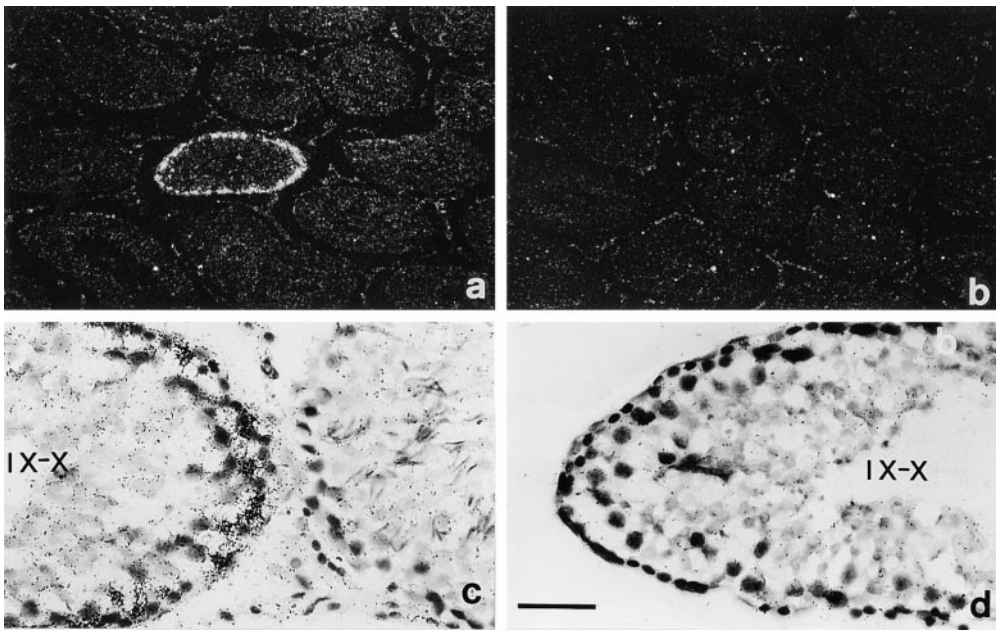

Figure 8. Localization of ECE-1 transcripts in testicular sections hybridized with ^{32}P-labeled antisense (a and c) and sense (b and d) RNA probes. Autoradiographies were exposed for 3 wk. (a and b) Dark field, (c and d) carmalum counterstain. (c) Stage IX-X (left side tubule) shows intense basal labeling, while in the adjacent tubule (at stage IV-V, right side) the labeling is not above background. Bar: (a and b) 200 μm; (c and d) 40 μm.

sis showed ECE-1 mRNA in cell extracts from purified Sertoli cells. Our in situ hybridization studies indicate that ECE-1 is predominantly localized in tubular areas where Sertoli cell bodies reside, particularly in the basal region. Sertoli cells are the only somatic cell type in the seminiferous epithelium; along the side of these elongated perennial elements, it is possible to observe, at any given time, several generations of germ cells, which flow radially to be eventually released as mature sperm into the tubular lumen. It has long been known that activities of the Sertoli cell, among which FSH responsiveness, vary according to the specific subset of differentiating germ cells with which it is associated ("stages" of the seminiferous epithelium) (Parvinen, 1982). In the prepuberal rat, in which the cyclicity of the epithelium has not been established yet, uniform expression of ECE was observed; in the adult, by contrast, ECE expression appears to be regulated in a temporal and spatial manner during spermatogenesis and the seminiferous epithelium cycle. Interestingly, expression of ECE-1 is exclusively restricted, in the adult rat, to stages IX-X of the cycle. These stages are characterized by the fact that they immediately follow spermiation and represent ~5% of the entire cycle length, which may explain why ECE expression was overlooked in a previous study (Takahashi et al., 1995).

When segments of seminiferous tubule at precise stages of the seminiferous epithelium cycle were dissected and individually exposed to the inactive precursor big ET-1 to test their ability to induce myoid cell contraction through the generation of active ET-1, fragments containing stages preceding IX were found to be unresponsive to the precursor. By contrast, in segments from stages after spermiation, normal contraction of myoid cells was observed in response to the inactive precursor, which indicates efficient processing of big ET-1. In parallel samples, directly stimulated with ET, no difference in responsiveness to the active peptide was observed, which suggests that myoid cells are constantly capable of responding. These experiments demonstrate a direct correlation between a restricted expression of ECE-1 and its biological function.

A perspective that warrants exploration is the mechanisms that regulate the expression of ECE-1 and the developmental transition from the diffuse to the restricted pattern of distribution of ECE-1, which may be connected to the known cyclic (Parvinen, 1982) and developmental changes in hormonal sensitivity Sertoli cells undergo (reviewed in Gondos and Berndston, 1993). Moreover, alterations in the pattern of ECE-1 and ET production might be involved in the pathogenesis of peritubular hyalinization, given the well-known role played by ET in fibrosis and matrix overproduction in a number of tissues (Hahn et al., 1993; Hocher et al., 1999).

In conclusion, our data could be used to outline a simplified model concerning the regulation of seminiferous tubule contractility, according to which the restricted expression pattern of ECE-1 would finely modulate local endothelin levels. In this model, ET-1 precursors produced by Sertoli cells are processed to biologically active ET-1 only in restricted areas of seminiferous tubule according to the spatiotemporal control of ECE-1 expression on the Sertoli cells (in turn, presumably dependent upon the spermatogenic cycle). Thus, seminiferous tubule contraction may originate in the specific tubular segments adjacent to those at which spermiation has just occurred, to be propagated as effective peristaltic waves by additional mechanisms that have yet to be identified.

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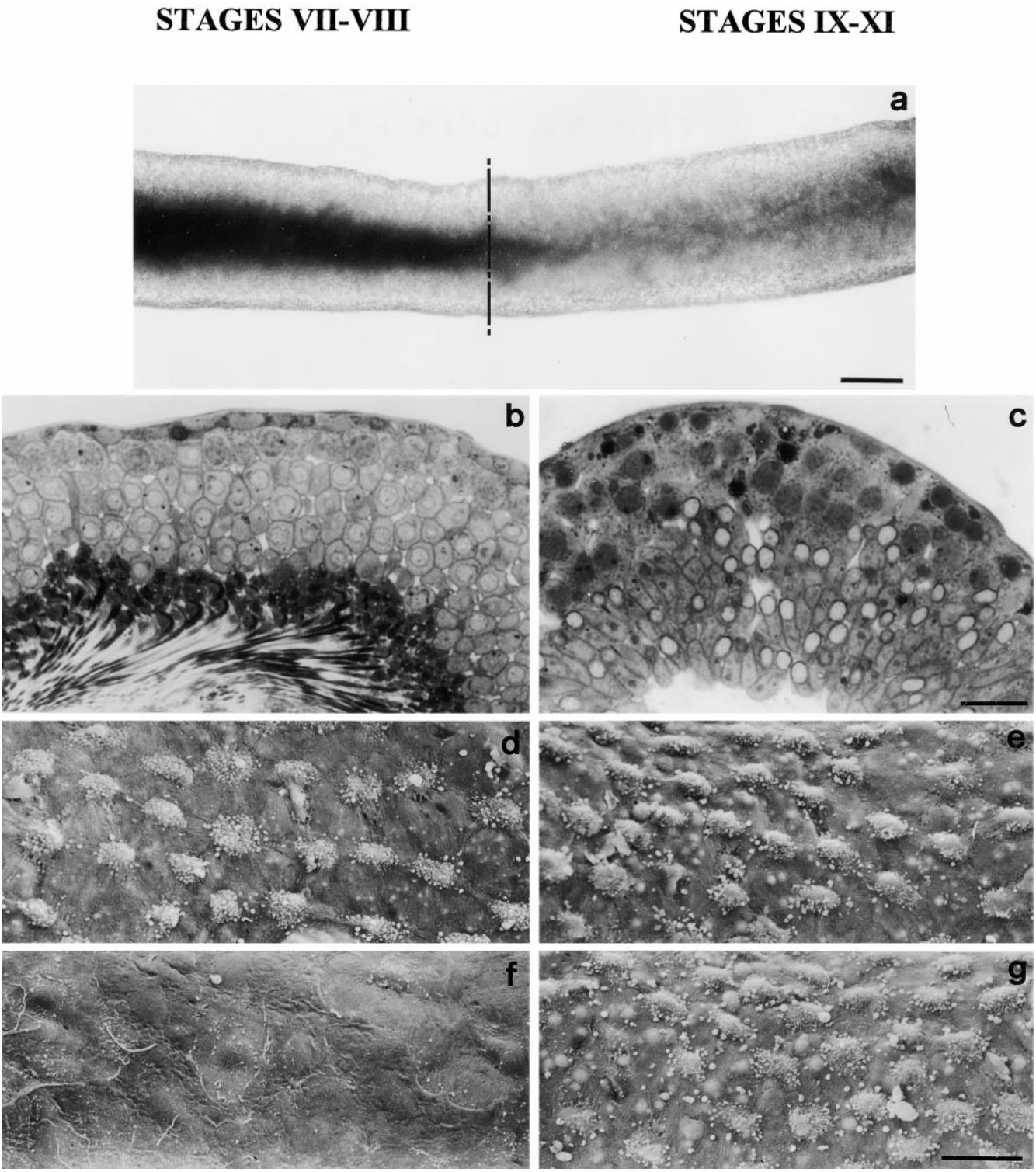

Figure 9. Different distribution of ECE activity along the seminiferous tubule. a: transilluminated tubular segment in which the transition from stage VIII (left side, dark) to stage IX (right side, light) is apparent. The hatched line indicates the level at which the tubules were dissected to yield segments with expected different ECE activity shown in d–g. (b and c) Toluidine blue–stained Epon sections showing the seminiferous epithelium composition at stage VII-VIII (b) and IX-X (c). Scanning electron micrograph of peritubular surface of seminiferous tubule segments isolated at precise stages of the seminiferous epithelium and treated as follows: (d) stage VII-VIII and (e) stage IX-XI, 10 nM ET-1 followed by immediate fixation; (f) stage VII-VIII and (g) stage IX-XI: 100 nM big ET-1, fixed after 15 min. Only stage IX-XI seminiferous epithelium shows an intrinsic ability to stimulate myoid cell contraction through conversion of big ET-1. Bars: (a) 100 μm; (b and c) 30 μm; (d–g) 25 μm.

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