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Characterization and hormonal modulation of immunoreactive thiamin carrier protein in immature rat Sertoli cells in culture

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Abstract

Immature rat Sertoli cells synthesize and secrete a protein species which has immunological similarity with chicken egg thiamin carrier protein (TCP) as assessed by immunocytochemical localization, liquid phase radioimmunoassay (RIA), immunoprecipitation of [^{35}S]-methionine incorporated newly synthesized proteins by polyclonal antibodies (pAbs) to chicken TCP and tryptic peptide mapping of iodinated immunoprecipitated proteins. FSH and testosterone together bring about 4-fold induction of Sertoli cell TCP over the control levels which is inhibitable upto 75% by an aromatase inhibitor. Addition of optimal concentrations of exogenous estradiol-17β to the cultures causes 2-fold enhancement of secretion of TCP which can significantly be inhibited by tamoxifen, when added along with estradiol-17β. These results show that Sertoli cells produce estrogen-inducible TCP, presumably to transport the vitamin to the developing germ cells.

1. Introduction

During spermatogenesis in the mammalian testis, interaction between Sertoli cells and germinal cells in terms of provision of essential nutrients and growth factors is critical for germ cell survival and metabolism. This is primarily attributable to operation of the blood–testis barrier due to tight junctions between adjacent Sertoli cells resulting in sequestration of differentiating spermatocytes and spermatids in a serum-free microenvironment in the adluminal compartment of the seminiferous tubules [1,2]. Although some nutritional components and ions can be transported through these junctional complexes, several other nutrients require specific binding proteins to facilitate their availability at adluminal compartment. Functional identification of Sertoli cell-secreted proteins has revealed the production of a number of different transport proteins [3,4] including transferrin [5], ceruloplasmin [6], sulfated glycoprotein-1 (SGP-1) [7] etc. Available evidence favours the secretion of specific high affinity vitamin-binding proteins by Sertoli cells for retinol [8], folate and biotin [9] presumably to provide nutritional support to germ cells. We have recently shown that a specific carrier protein for riboflavin with physicochemical and immunological similarities with the chicken egg riboflavin carrier protein (RCP) is synthesized and secreted by immature rat Sertoli cells in culture which is modulated either by in situ produced or exogenous estradiol [10]. In the avian system, RCP is an estrogen-inducible phosphoglycoprotein obligatory for yolk deposition of the vitamin to support embryonic development [11]. Our earlier investigations have also revealed that a specific thiamin carrier protein (TCP) participates in oocyte deposition of the vitamin in the egg-laying birds and that the protein is evolutionarily conserved in mammals to support embryonic development [12,13]. It was therefore attractive to visualize that Sertoli cells also elaborate

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TCP as a stratagem to provide thiamin to differentiating germ cells. In the present communication, we provide immunohistochemical evidence for TCP in rat testicular tissue and show that the rodent Sertoli cells in culture synthesize and secrete TCP which is hormonally modulated.

2. Materials and methods

2.1. Materials

DME/F-12 medium powder, methionine free-DME/F-12 powdered medium, insulin, transferrin, epidermal growth factor, growth hormone, testosterone, progesterone, estradiol-17β, bovine serum albumin (fraction V), chloramine T, triton X-100, sodium deoxycholate, 1,4,6-androstatrien-3,17-dione, goat anti-rabbit IgG conjugated to horse radish peroxidase and diaminobenzidine were obtained from Sigma Chemical Co (St. Louis, MO). Tamoxifen was procured from Spuart Pharmaceuticals (Wilmington, DE). DEAE-Sephacel and S-Sepharose were purchased from Pharmacia Fine Chemicals (Sweden). Gentamycin was obtained from Pharmaceutical Company of India. [^{35}S]-methionine (specific activity 500 Ci/mmol) and carrier-free Na^{125}I were from Amersham International plc. (UK). Purified ovine FSH was a kind gift from Professor N.R. Moudgal of this Institute. Tissue cultureware was supplied by Costar (USA) or Nunc (Denmark). Other chemicals used were of analytical grade and obtained locally. Chicken TCP was isolated from egg yolk [13]. Polyclonal and monoclonal antibodies (mAbs) to chicken TCP used in the present study were raised and characterized as described earlier [12]. Immature male rats (Wistar) used for isolation of Sertoli cells were from Institute's Central Animal Facility.

2.2. Immunocytochemical localization

Testes from 20-day-old rats were dissected out and fixed for 24 h in Bouin's fluid composed of saturated picric acid:formalin:glacial acetic acid (15:5:1, v/v/v). The tissues were subsequently washed in 50% ethyl alcohol and dehydrated through alcohol series, cleared in toluene and infiltered with paraffin at 58°C. Tissue sections were taken at 6–8 μm thickness using a microtome (American optical company, NY). The sections were mounted on polylysine coated glass slides. For immunocytochemical staining, an indirect immunoperoxidase staining procedure was employed [14]. Briefly, the sections were deparaffinized in xylene and hydrated by passing through descending grades of alcohol and finally water. After blocking the endogenous peroxidase activity, the tissue sections were incubated with rabbit polyclonal anti-chicken TCP antiserum (1:1000) followed by goat anti-rabbit IgG conjugated to horse radish peroxidase. Suitably diluted (1:1000) non-immune rabbit serum was used as a negative control. The bound peroxidase was visualized with the chromogen diaminobenzidine and counterstained with haematoxylin. The slides were then washed with water, dehydrated through alcohol series, clarified in xylene and mounted in DPX mountant.

2.3. Isolation of Sertoli cells and maintenance in culture

Sertoli cells were isolated from 20-day-old rats by successive collagenase treatment [15]. The isolated cells were homogeneous to an extent of >90% as observed by phase contrast microscopy. These cells were maintained in DME/F-12 medium with the addition of 1.2 gm/L of $NaHCO_3$ and 20 mg/L of gentamycin at a density of 4×10^5 cells/cm^2 area in a humidified atmosphere of 5% CO_2 at 32°C. The culture medium was supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), EGF (1 ng/ml), FSH (25 ng/ml), growth hormone (100 ng/ml), retinol (50 ng/ml) and testosterone (10^{-6}M). To study the hormonal modulation, the isolated cells were first maintained in DME/F-12 medium alone for 24 h to deplete the endogenous hormones. Following this, control culture dishes contained the medium with the above mentioned concentrations of insulin, transferrin and EGF (3F). The test cultures, in addition to these factors, contained exogenous hormones or other compounds at specified concentrations. The spent media were collected and replenished with fresh media every 24 h. The viability of the cells at the end of the cultures was monitored by trypan blue (0.2% w/v in 20 mM phosphate buffer, pH 7.2 containing 0.9% NaCl) dye exclusion test. It was noted that the viability was not altered following treatments.

2.4. Radioimmunoassay

Chicken TCP was radiolabelled by iodogen method [16]. A liquid phase RIA was developed with rabbit anti-chicken TCP antiserum and ^{125}I-labelled chicken TCP as the tracer. Sertoli cell culture supernatants were 100-fold concentrated by ultrafiltration using YM-10 membranes (Amicon, USA). Varying amounts of the concentrated culture supernatants were incubated with ^{125}I-labelled chicken TCP (approx. 10^5 cpm) and its antiserum (1:40,000 dilution). The antibody bound radioactivity was quantitated by double antibody-polyethylene glycol precipitation [17]. The results were expressed in terms of chicken TCP as the standard.

2.5. Immunoprecipitation

Pooled Sertoli cell culture supernatant was subjected to DEAE-Sephacel chromatography using 20 mM phosphate buffer, pH 6.0. The bound proteins were eluted with 0.5 M NaCl, dialyzed and passed through S-Sepharose column pre-equilibrated with 20 mM acetate buffer, pH 4.8. The bound proteins were eluted with 0.5 M NaCl, dialyzed, concentrated and labelled with $Na^{125}I$ by iodogen method [16]. The labelled proteins (approx. 10^6 cpm) were immunoprecipitated with either pAbs or mAbs to chicken TCP as described earlier [10].

2.6. Two dimensional tryptic peptide mapping of ^{125}I-labelled proteins

As described above, the Sertoli cell secreted proteins were enriched, radiolabelled, immunoprecipitated with polyclonal anti-chicken TCP antibodies and the immune complexes were resolved on a 10% SDS-polyacrylamide gel under reducing conditions [18]. In a parallel lane, ^{125}I-labelled chicken TCP was electrophoresed to serve as positive control. After electrophoresis, the gel bands containing the radioactive protein species were excised, subjected to trypsinization and the digested peptides were separated by high voltage electrophoresis and thin layer chromatography as described by Elder et al. [19].

2.7. In vitro labelling and immunoprecipitation of Sertoli cell secreted proteins

Following preculture for 24 h, the cells were incubated for 1 h in the methionine-free medium. Subsequently, the medium was supplemented with 10 μCi/ml of [^{35}S]-methionine (specific activity 500 Ci/mmol) and the culture continued for another 10 h. The supernatant was collected and the total [^{35}S]-methionine incorporated into the secreted proteins was estimated following trichloroacetic acid (10%, w/v) precipitation [9]. The radiolabelled secreted proteins (approx. $3–4\times10^6$ cpm) were precipitated with polyclonal antibodies to chicken TCP and the immunoprecipitates resolved on SDS-PAGE were analyzed by fluorography as described earlier [10].

2.8. Statistical analysis

The interassay coefficient of variation for the RIA was found to be $<10\%$. The data obtained from each set of experiments which have been repeated at least four times were analyzed by Student's *t*-test. To avoid the interassay variation, samples obtained from a single set of experiments were assayed simultaneously.

3. Results and discussion

3.1. Immunohistochemical localization of TCP in testis

Earlier characterized from chicken egg yolk and pregnant rat sera, TCP is an estrogen-inducible, acidic, nonglycoprotein with M_r 70,000 which binds the vitamin with K_a of 0.3×10^9 M^{-1}. The vitamin carrier is obligatory for embryonic development since immunoneutralization of the maternal protein curtails pregnancy in rodents [12,13]. Taking advantage of the fact that the vitamin carrier is conserved in mammals, we examined the cellular localization of the putative thiamin carrier immunocytochemically in testis sections from immature rats using monospecific polyclonal anti-chicken TCP serum. Fig. 1A represents the staining pattern showing the presence of crossreactive TCP in Sertoli cells and developing spermatogenic cells in contrast to the tissue sections treated with pre-immune serum. (Fig. 1B). As majority of the proteins in the

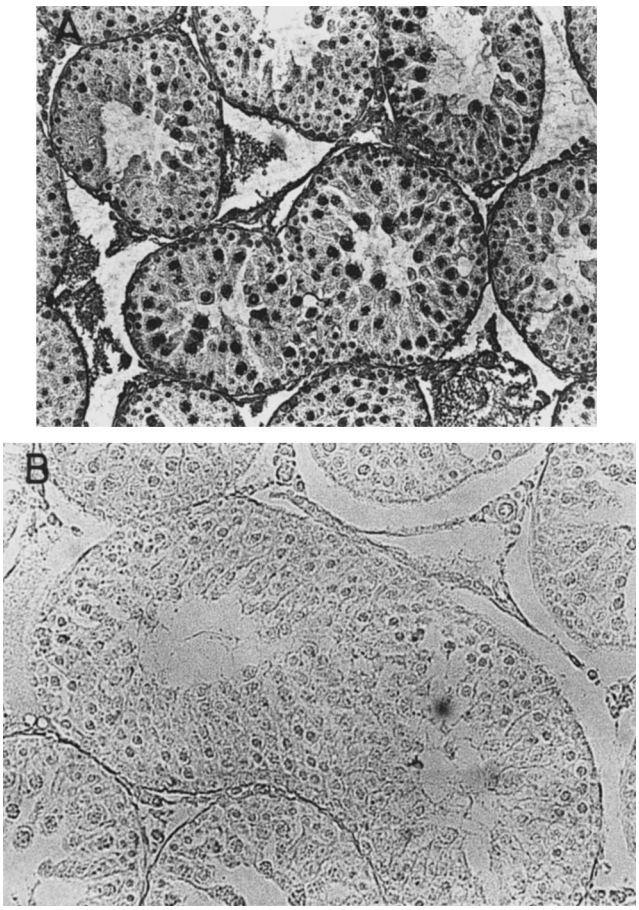

Fig. 1. Immunocytochemical localization of TCP in the rat testis. Sections of immature rat testis were incubated with either rabbit anti-chicken TCP antiserum (A) or normal rabbit serum (B) and further stained with immuno-peroxidase technique. Magnification: (A) ×200, (B) ×400.

testis are synthesized and secreted by Sertoli cells, it is reasonable to assume that TCP also is produced by these cells. To examine this premise further, Sertoli cells were cultured under defined in vitro conditions. We preferred the Sertoli cells from 20-day-old rats since around day 19, tight junctions between adjacent Sertoli cells form a blood–testis barrier [20].

3.2. Immunological and biochemical characterization of TCP from rat Sertoli cells in culture

To investigate whether Sertoli cells secrete thiamin carrier, these cells were cultured in serum-free DME/F-12 medium supplemented with growth factors and hormones. The spent medium was collected, 100-fold concentrated and heterologous RIA was carried out using specific polyclonal antiserum raised against chicken TCP in rabbits. Fig. 2 represents the competition between fixed amount of radioiodinated TCP and varying concentrations of either chicken TCP or Sertoli cell TCP to combine with 1:40,000 diluted rabbit polyclonal anti-chicken TCP serum. The assay had a sensitivity in the range of 40 ng–300 pg. Concentrated DME/F-12 medium and all the hormones and vitamins used in the cell culture did not show any interference in the assay. The parallelism between the displacement curves obtained with testicular TCP and chicken TCP indicates the extent of immunological similarity between the two. In terms of chicken protein standard, these cells secrete approx. 1.5 ng of TCP/10^6 cells/48 h into the culture medium.

The molecular size of Sertoli cell TCP was determined by analyzing immunoprecipitated proteins wherein the radioiodinated secreted proteins were precipitated with either the mAbs or pAbs raised against chicken thiamin carrier. The immunoprecipitates were resolved on a 10% SDS-gel and an autoradiogram was developed. The results obtained revealed that all the five mAbs as well as the pAbs to chicken TCP (Fig. 3A and B) could precipitate the testicular protein suggesting thereby that the overall immunotopological characteristics of the vitamin carrier from the rodent testis and the chicken egg are very similar. This similarity extends to their molecular size (M_r 70,000) as revealed by the autoradiography. Additional evidence for similarities stems from a comparison of tryptic peptide maps of ^{125}I-labelled and immunoprecipitated Sertoli cell TCP and chicken thiamin carrier (Fig. 4). This is in line with the premise that these two homologous proteins possess similar if not identical primary structure accounting for the extensive conformational commonality indicated by mAb crossreactivity.

Biochemical evidence for de novo synthesis of TCP by Sertoli cells stems from in vitro experiments wherein newly synthesized proteins were labelled in the presence of [^{35}S]-methionine followed by immunoprecipitation with the specific TCP antibodies (Fig. 5). The observation that the immunoprecipitated radioactive species from the Sertoli cell spent medium migrates to the position corresponding to the chicken vitamin carrier confirms that Sertoli cells indeed synthesize TCP of similar molecular mass.

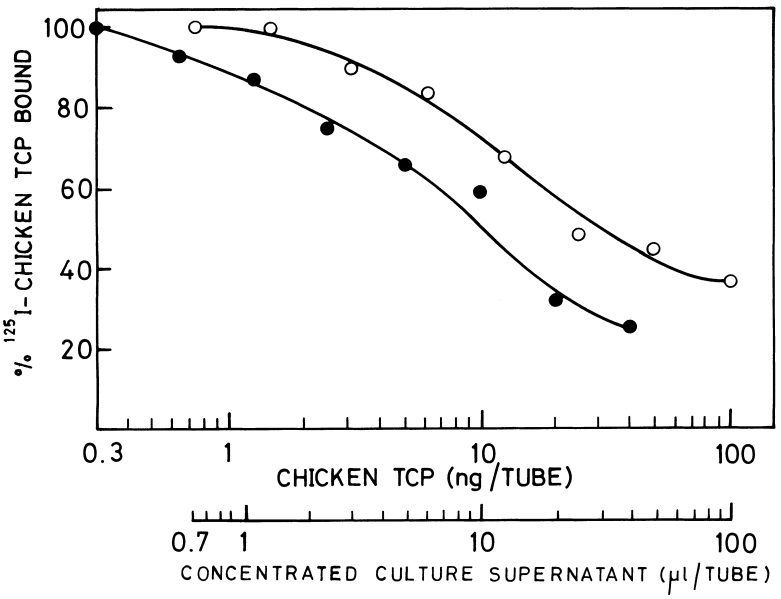

Fig. 2. Inhibition of binding of ^{125}I-labelled chicken TCP to its antiserum by unlabelled chicken TCP (●) or concentrated Sertoli cell culture supernatant (o). The percentage of specifically bound radioactivity is plotted against the concentration of the unlabelled competing antigen.

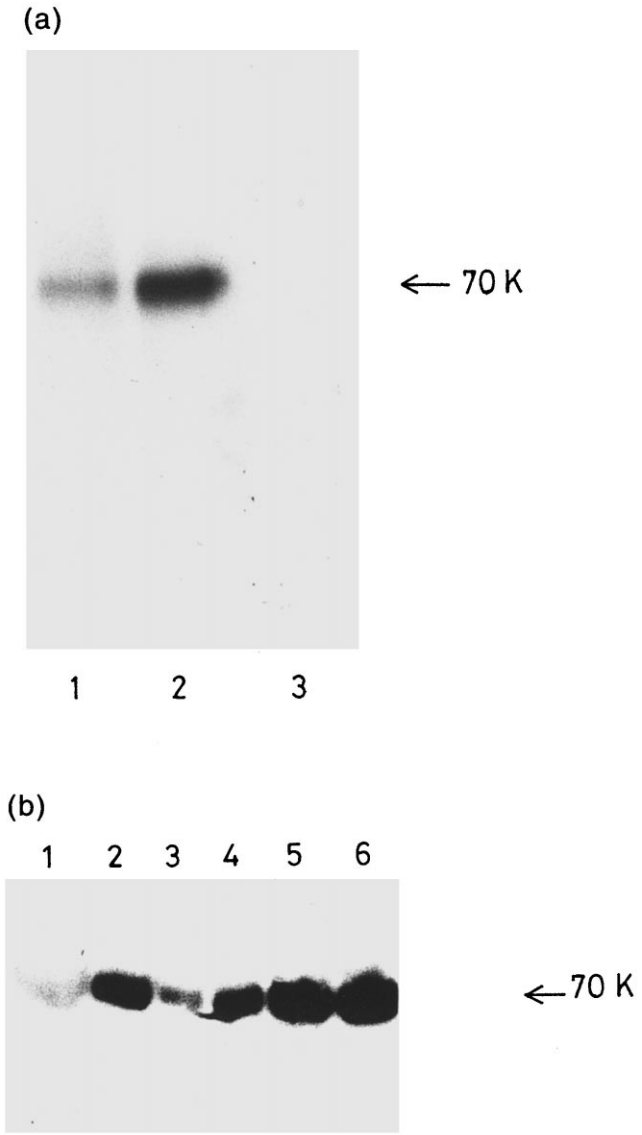

Fig. 3. Autoradiogram of Sertoli cell secreted proteins precipitated with antibodies to chicken TCP. The partially purified proteins from Sertoli cell spent medium were iodinated and precipitated with various antibodies to chicken TCP. The immune complexes were resolved by a 10% SDS-PAGE. The gel was dried and an autoradiogram developed. Panel (a) shows immuno-precipitation with pAbs to chicken TCP. Lane 1: proteins precipitated with polyclonal rabbit anti-chicken TCP serum; lane 2: ^{125}I-labeled chicken TCP (as a reference); lane 3: proteins precipitated with normal rabbit serum (as a negative control). Panel (b) shows immunoprecipitation with mAbs F3H6 (lane 1); A4C4 (lane 2); C8C1 (lane 3); H8H3 (lane 4); G7H10 (lane 5). ^{125}I-labelled chicken TCP was taken as a reference (lane 6).

3.3. Hormonal modulation of TCP secretion

Earlier investigations from this laboratory have demonstrated that the induction of chicken TCP is under estrogenic control [21]. Now the question arises as to whether the Sertoli cell TCP is similarly modulated by the steroid hormone. In recent years, the capacity of different cell types in the mammalian testis to synthesize estradiol-17β has widely been recognized [22]. Additionally, intratesticular loci of aromatization and the specificity of gonadotropins in regulating this enzyme complex are governed by the age, cell type and the species of the animal concerned [23]. In the immature rat testis, under the influence of FSH, the aromatase enzyme complex which catalyzes the conversion of testosterone to estradiol-17β is markedly stimulated in Seroli cells through enhanced intracellular accumulation of cyclic AMP [22]. It was therefore attractive to investigate the influence of FSH, testosterone, exogenous estradiol-17β, an aromatase inhibitor and the anti-estrogen tamoxifen on secretion of TCP by Sertoli cells in culture. In preliminary experiments, optimal concentrations of FSH and testosterone required for maximal stimulation of secretion of TCP was determined. At very low concentrations of FSH (< 2.5 ng/ml), the response of Sertoli cells was very minimal (Table 1) whereas with increase in FSH concentration, a progressive enhancement in secretion of TCP was observed. At ≥25 ng/ml of FSH, maximal stimulation (two-fold induction) of elaboration of the vitamin carrier over the basal values was observed. However, testosterone (10^{-9}–10^{-5} M) was marginally effective in this respect, vis-a-vis FSH, its stimulatory influence being confined to 120–125% of the control values (Table 2). Contrastingly, a combination of the optimal concentrations of FSH (25 ng/ml) and testosterone (10^{-6}M) acted synergistically since it elicited 4-fold increase in secretion of TCP (Fig. 6). In view of the fact that the aromatase inhibitor 1,4,6-androstatrien-3,17-dione could curtail (approx. 75%) enhanced TCP secretion due to FSH and testosterone combination, the observed stimulation of TCP secretion is apparently attributable to in situ synthesized estradiol. Interestingly, the aromatase inhibitor could not affect the basal secretion of TCP when added alone to the cultures.

To substantiate the above premise regarding involvement of endogenously produced estrogen in stimulated

Table 1
The secretion of thiamin carrier by Sertoli cells cultured in the presence of various concentrations of FSH[a]

Concentration of FSH (ng/ml)	ng TCP/10^6 cells/48 h
0 (14)	1.47±0.21
0.025 (4)	1.56±0.2
0.25 (4)	1.81±0.33
2.5 (4)	2.12±0.45
25 (8)	3.0±0.42
100 (4)	3.14±0.51

[a] Each value represents the mean±S.D. with the number of determinations shown in parentheses. Cells were maintained in DME/F-12 plus 3F and protein levels from spent medium in 48 h was determined.

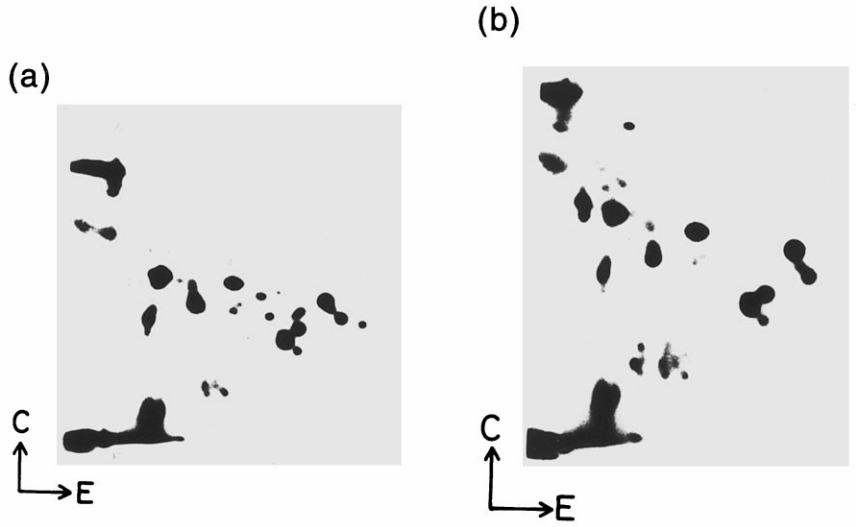

Fig. 4. Two-dimensional tryptic peptide map analysis of Sertoli cell secreted- and chicken TCP. Radiolabelled and immunoprecipitated Sertoli cell TCP (a) and iodinated chicken thiamin carrier (b) were trypsinized. The tryptic peptides were dissolved in 5 μl of electrophoresis buffer, spotted in one corner of the cellulose plate and electrophoresed in one dimension (E) followed by chromatography in the second dimension (C). The plates were air-dried and the autoradiograms developed.

TCP production intracellularly, graded concentrations of estradiol-17β were added to the cell cultures to study the influence, if any, of the exogenous steroid hormone in terms of carrier protein secretion (Table 3). Optimal concentration of estradiol required for maximal stimulation was around 1 μM. At this concentration, the steroid hormone could enhance TCP secretion to 2-fold over the control values (Fig. 7). The

Table 2
Effect of various concentrations of testosterone on TCP secretion by Sertoli cells[a]

Concentration of testosterone (μM)	ng TCP/10^6 cells/48 h
0 (14)	1.47±0.21
0.01 (4)	1.47±0.19
0.1 (4)	1.54±0.25
1 (8)	1.7±0.3
10 (4)	1.71±0.27

[a] Each value represents the mean±S.D. with the number of determinations shown in parentheses. Cells were maintained in DME/F-12 plus 3F and protein levels from spent medium in 48 h was determined.

Table 3
The secretion of thiamin carrier by Sertoli cells cultured in the presence of different concentrations of estradiol-17β[a]

Concentration of estrogen (μM)	ng TCP/10^6 cells/48 h
0 (6)	1.27±0.23
0.01 (4)	1.46±0.18
0.1 (4)	1.93±0.22
1 (8)	2.7±0.34
10 (4)	2.75±0.38

[a] Each value represents the mean±S.D. with the number of determinations shown in parentheses. Cells were maintained in DME/F-12 plus 3F and protein levels from spent medium in 48 h was determined.

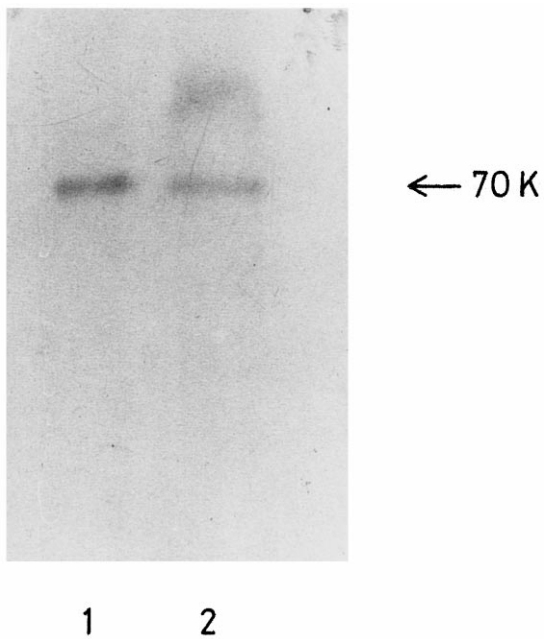

Fig. 5. Fluorogram of [^{35}S]-methionine labelled Sertoli cell proteins precipitated with polyclonal chicken TCP antiserum. Sertoli cells were incubated with [^{35}S]-methionine in culture. The conditioned medium was immunoprecipitated with polyclonal antiserum to chicken TCP and the immune complex was resolved by a 10% SDS-PAGE. The gel was dried and a fluorogram developed. Shown are the precipitated Sertoli cell protein (lane 1) and ^{125}I-labeled chicken TCP, taken as a positive control (lane 2).

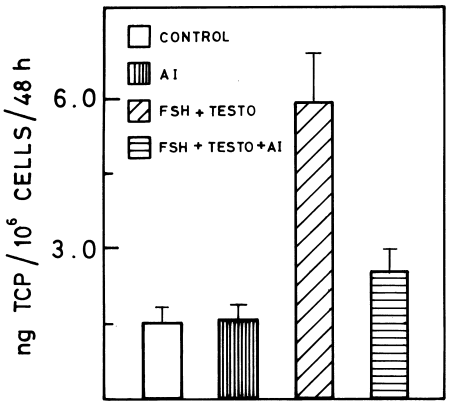

Fig. 6. The influence of FSH, testosterone (TESTO) and aromatase inhibitor (AI) on TCP secretion by Sertoli cells. The cells were maintained in DME/F-12 medium supplemented with the above mentioned combinations of substances (FSH:25 ng/ml; TESTO:1 μM; AI:100 μM) for 48 h and the amount of TCP secreted was measured by RIA. Each data point is the mean±S.D. of duplicate cultures from four experiments.

relatively lesser magnitude of stimulation of TCP by exogenous estradiol vis-a-vis that elicited by in situ produced steroid hormone remains unexplored at present and could be due to factors such as differential concentration at intracellular loci of action and/or catabolism. Additional support for the modulation of testicular TCP by estrogen stems from experiments wherein the influence of tamoxifen was investigated. This anti-estrogen could inhibit the enhanced TCP secretion due to FSH and testosterone by approx. 70% and the exogenous estrogen induced TCP levels by 60%. However, tamoxifen per se did not seem to affect the basal secretion of TCP by Sertoli cells. Thus, the

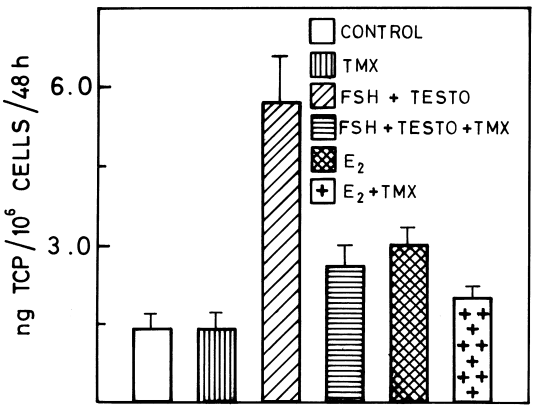

Fig. 7. The influence of tamoxifen (TMX) on estradiol (E_2) induced TCP secretion by Sertoli cells. The cells were cultured in the presence of the above mentioned combinations (FSH: 25 ng/ml; TESTO: 1 μM; E_2: 1 μM; TMX: 10 μM) for 48 h and the secreted TCP levels measured. Each data point is the mean±S.D. of replicate cultures from four experiments.

above lines of evidence clearly favour the view that Sertoli cell TCP secretion is regulated by estrogen produced by intracrine mode which in turn is modulated by FSH and testosterone. While the exact physiological relevance of this vitamin carrier in spermatogenesis is yet to be explored, it is conceivable that TCP mediates adequate accessibility of thiamin in a carrier mediated fashion to circumvent the physiological barrier offered by Sertoli cell tight junctions to ensure uninterrupted supply of the vitamin to rapidly proliferating and differentiating germ cells and its functions are analogous to those of the other micronutrient carriers referred to earlier.

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