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The Actions of Calcitonin on the TM_3 Leydig Cell Line and on Rat Leydig Cell-Enriched Cultures

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Studies demonstrating calcitonin receptors on Leydig cells have suggested that these cells may be one of the many sites affected by this peptide. To investigate this possibility, the effect of synthetic salmon calcitonin on the TM_3 Leydig cell line (derived from immature mouse Leydig cells) and on primary Leydig cell-enriched preparations was examined. Synthetic salmon calcitonin stimulated the conversion of [^{3}H]adenine to [^{3}H]cyclic AMP in TM_3 cells. In addition, the hormone stimulated the basal secretion of testosterone in both TM_3 cell- and Leydig cell-enriched cultures and potentiated the action of hCG on Leydig cell-enriched cultures. Synthetic salmon calcitonin also increased the concentration of androgen and estrogen receptors in cultured TM_3 Leydig cells by 2- and 4-fold, respectively, when added to the culture medium (1 μg/ml). The fact that 8-bromo-cyclic AMP decreased both androgen and estrogen receptor concentrations suggested that the effect of calcitonin on sex steroid receptors is not mediated by its effect on cyclic AMP in these cells. The possibility that the action of calcitonin on steroid receptors might be mediated by another messenger such as calcium (Ca^{2+}) was therefore considered. Progressively lowering the concentration of Ca^{2+} in the culture medium of the cells from 1.5 mM to less than 0.01 mM decreased the concentration of both androgen and estrogen receptors. Returning the Ca^{2+} concentration to normal levels (1.5 mM) restored steroid receptor levels. Receptor levels were also decreased when the extracellular Ca^{2+} concentration was lowered to 0.5 mM, and treatment with the Ca^{2+} ionophore, A23187 (1 μM), restored receptor levels to normal. The calcium channel blocker, verapamil, decreased the androgen receptor concentration but unexpectedly increased the concentration of estrogen receptors. It was concluded that calcitonin stimulates cAMP formation and testosterone secretion, and increases the concentration of sex steroid receptors. These observations provide evidence that the previously demonstrated calcitonin receptors on Leydig cells may be coupled to several biologic responses in this cell type.

Key words: Calcitonin, TM_3 cell line, Leydig, androgen receptor, estrogen receptor, cAMP.

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The presence of functional calcitonin receptors in bone and kidney are well documented and the action of this hormone on these organs has been extensively studied (for review see Queener and Bell,

1975). Calcitonin binding sites also have been demonstrated in membrane fractions of rat testicular cells (Chausmer et al, 1980; Fischer et al, 1981). Using autoradiographic techniques, the binding sites were localized on Leydig cells (Chausmer et al, 1982). Although the physiologic significance of these receptors is not yet known, their presence suggests that calcitonin could regulate Leydig cells. The purpose of this study was to examine the activity of calcitonin *in vitro* on primary Leydig cell-enriched cultures and on the TM_3 Leydig cell line. Since it has been suggested that calcitonin alters zinc and Ca^{2+} homeostasis in the testis (Chausmer et al, 1980) and since these ions are known to influence the activity of steroid receptors (Colvard and Wilson, 1982), we studied the effects of calcitonin on androgen and estrogen receptors in Leydig cells.

Materials and Methods

Chemicals

Synthetic salmon calcitonin was generously provided by Dr. R. C. Orlowski of Armour Pharmaceutical Company (Kankakee, IL) and by Sandoz Ltd. (Basel, Switzerland). Verapamil hydrochloride was supplied by Knoll Pharmaceutical Co. (Whippany, NJ). The Ca^{2+} ionophore, A23187, was purchased from Calbiochem-Behring Corp. (La Jolla, CA) and 8-bromoadenosine 3', 5'-cyclic monophosphate (8-BrcAMP) from Sigma Chemical Co. (St. Louis, MO). [17α-*methyl*-^{3}H]Methyltrienolone (SA, 87 Ci/mmol), [^{3}H]estradiol ([^{3}H]E_2; SA, 91.5 Ci/mmol) and [2-^{3}H]adenine (SA, 21.6 Ci/mmol) were purchased from New England Nuclear (Boston, MA). F12/DME powdered culture medium was supplied by Irvine Scientific Co. (Santa Ana, CA), and serum by Grand Island Biological Co. (Grand Island, NY). All other chemicals of the highest purity available were from Sigma Chemical Co. or Fisher Scientific Co. (Fair Lawn, NJ).

Culture of Leydig Cells

The TM_3 mouse testis cell line, which is believed to be derived from immature Leydig cells (Mather, 1980; Chen et al, 1984), was grown in serum-free F12/DME medium [Ham's F12 nutrient mixture and Dulbecco's modified Eagle's medium, 1:1 (v/v)] supplemented with 15 mM Hepes buffer (pH 7.2), 20 μg/ml gentamycin sulfate, with or without 5% (v/v) horse serum and 2.5% (v/v) newborn bovine serum. Serum-free medium was supplemented with insulin (10 μg/ml), transferrin (5 μg/ml), and epidermal growth factor (EGF) (2.5 ng/ml) (Mather, 1980; Mather et al, 1982). These substances were dissolved in phosphate-buffered saline solution (PBS) or 0.01 N HCl (insulin) and added to the culture medium. The Ca^{2+} ionophore, A23187, and verapamil were dissolved in dimethyl sulfoxide.

Since TM_3 cells have LH/hCG receptors that are difficult to measure, primary Leydig-enriched cultures were used in some experiments to determine whether synthetic salmon calcitonin altered the effect of hCG. Primary Leydig-enriched cultures were prepared from mature rat testis using a mixture of collagenase-dispase (Boehringer-Mannheim, Mannheim, West Germany) (0.03%, w/v), soybean trypsin inhibitor (0.001%, w/v), and DNAase (0.001%, w/v) for 15 min at 35 C. These cells were diluted to ~2.3×10^6/ml with M199 medium containing 0.1% (w/v) BSA. Samples (0.5 ml) of this preparation were employed for measuring testosterone (T) secretion (Dufau et al, 1974).

Assay of cAMP Formation

The ability of TM_3 Leydig cells to convert [^{3}H]adenine to [^{3}H]cAMP was determined as previously described (Salomon, 1979). Cells were grown to confluency in serum-containing medium. The cell monolayers were washed twice with serum-free F12/DME and preincubated in the same medium containing 5 μCi/ml [^{3}H]adenine for 2 h at 35 C. The preincubation mixture was aspirated and the cells were incubated with or without hormone for 20 min at 37 C in serum-free F12/DME medium containing 0.1 mM isobutyl-methylxanthine and BSA (100 μg/ml). At the end of the incubation period, the reaction was terminated by adding 1.0 ml of 2.5% perchloric acid solution, and [^{3}H]cAMP was extracted from the cells, separated by column chromatography and quantitated as described previously (Salomon, 1979). Since preliminary experiments showed that cAMP formation was maximal by 20 min, this time point was used in all studies.

Assay of Testosterone

Testosterone was measured in the culture media from the TM_3 cell line and primary Leydig cell-enriched cultures by specific radioimmunoassay. The sensitivity was 3.5 pg. Intra- and interassay coefficients of variation were 8.5% and 13.1%, respectively (Thau et al, 1983).

Preparation of Cytosol and Determination of Steroid Receptor

At confluency (4 to 5 days), the cells were washed with PBS and removed from the culture dish with a rubber spatula. All the subsequent procedures were performed at 2 to 4 C. The cells were sedimented at $800 \times g$ for 5 min and then disrupted in cytosol buffer [50 mM Tris/HCl, 0.1 mM EDTA, 5 mM dithiothreitol, 10 mM Na_2MoO_4, 10% (v/v) glycerol, pH 7.4, at 20 C] using 20 strokes of the tight pestle of a Dounce (Bellco, Vineland, NJ) homogenizer. The homogenate was centrifuged at $105,000 \times g$ for 60 min and the supernatant was designated cytosol. Androgen and estrogen receptor concentrations were measured by a modification (Nakhla et al, 1984) of a previously reported technique (Walters and Clark, 1977; Isomaa et al, 1982). Briefly, aliquots of cytosol (0.2 ml) were incubated with [^{3}H]methyltrienolone (15 nM) or [^{3}H]E_2 (10 nM) with and without a 200-fold molar excess of the corresponding nonradioactive steroid for 16 h at 4 C. Bound and free steroids were separated using hydroxylapatite suspension (Walters and Clark, 1977; Isomaa et al, 1982; Nakhla et

al, 1984). The bound [^{3}H]steroid was extracted from the hydroxylapatite pellet with 1.5 ml ethanol and added to 5 ml Liquiscint (National Diagnostics, Somerville, NJ). Radioactivity was measured in a Packard liquid scintillation counter (model Tri-Carb 460 C) with an efficiency of 35% for tritium. The low androgen concentrations possibly present in cytosols from TM_3 cells did not affect the measured androgen receptor levels since the same values were obtained whether or not the cytosol was extracted with charcoal. To assure that changes in androgen and estrogen receptor concentrations measured by the single-point assays were not due to altered binding affinities of the receptors, the binding parameters in several experiments were also determined using a wide range of [^{3}H]methyltrienolone (0.1 to 20 nM) and [^{3}H]E_2 (0.1 to 15 mM) concentrations and Scatchard analysis.

Other Methods

Cell number was determined using a Coulter counter following release of the cells from the culture dishes with a trypsin-EDTA solution [0.1% (w/v) trypsin and 0.1 mM EDTA]. Protein concentration was determined by the method of Bradford (1976). The results of each study were analyzed by one-way analysis of variance. When appropriate, differences between treated and control cultures were determined using Student's t-test.

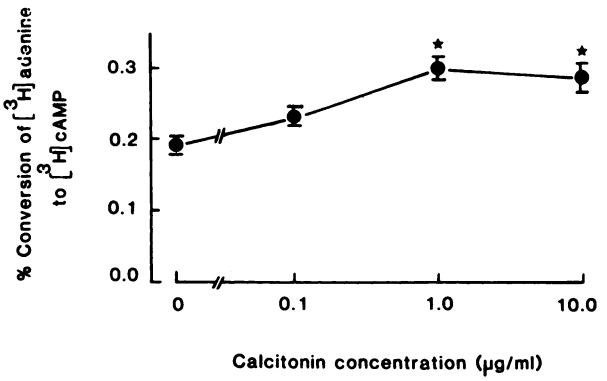

Fig. 1. Effect of synthetic salmon calcitonin on cAMP formation by TM_3 Leydig cells. The cells were grown in serum-supplemented F12/DME medium. Hormone-dependent conversion of [^{3}H]adenine to [^{3}H]cAMP was measured as described in the Methods. For comparison, the response of the cells to forskolin (20 μM) was measured (% conversion = 13.84 ± 2.41). Results are the mean ± SE of two representative experiments. In this and other figures, each point was determined from triplicate plates unless otherwise indicated. The asterisk indicates a significant difference from the control value ($P < 0.02$).

Results

Effect of Synthetic Salmon Calcitonin on the Growth of the Cell

The effect of synthetic salmon calcitonin on the growth of TM_3 Leydig cells was examined first. The cells were grown in serum-free F12/DME medium supplemented with insulin, transferrin, and EGF in the presence and absence of salmon calcitonin. After 4 days in culture, the cells were harvested and counted. No significant difference in cell number was observed in the groups treated with synthetic salmon calcitonin concentrations from 0.1 to 10 μg/ml (data not shown). As a consequence, none of the effects of calcitonin noted below are due to a change in cell number.

Effect of Calcitonin on cAMP Formation and Testosterone Secretion

A small but significant increase in the conversion of [^{3}H]adenine to [^{3}H]cAMP was observed in TM_3 cells in the presence of 1 μg/ml synthetic salmon calcitonin. No additional increase was observed with a larger dose (10 μg/ml; Fig. 1). When synthetic salmon calcitonin was added to the confluent TM_3 cells grown in serum-containing medium, an increased secretion of T was observed. The concentration of T in the medium rose by 1 h, plateaued, and increased again by 24 h (Fig. 2A). The reason for this biphasic response is unexplained at present but it was observed in three experiments. To study the effects of calcitonin on hCG-stimulated T secretion, primary Leydig cell-enriched cultures were employed because TM_3 cells do not contain hCG receptors. Time- and dose-response studies showed that the maximal response to calcitonin occurred at 1 μg/ml. Calcitonin stimulated basal T secretion in a pattern similar to that shown in TM_3 cells, except that the first peak occurred at 3 h. The results shown in Fig. 2B indicate that calcitonin enhanced hCG-stimulated T secretion in primary Leydig cell-enriched cultures. Since a major interest of our laboratory is the regulation of steroid receptors in testicular cells, the effects of calcitonin on androgen and estrogen receptors were examined next.

Effect of Synthetic Salmon Calcitonin on Androgen and Estrogen Receptor Concentrations in Leydig Cells

Confluent TM_3 cells were exposed to synthetic salmon calcitonin (1 μg/ml) and receptor levels were measured in the cytosol by an exchange method. In three separate experiments, the hormone increased the content of both androgen and estrogen receptors. Androgen receptors were 100% higher than the control (Fig. 3A) and estrogen receptors were 250% higher by 6 h after the treatment (Fig. 3B). Scatchard analysis of receptor binding showed that calcitonin changed the concentration of both

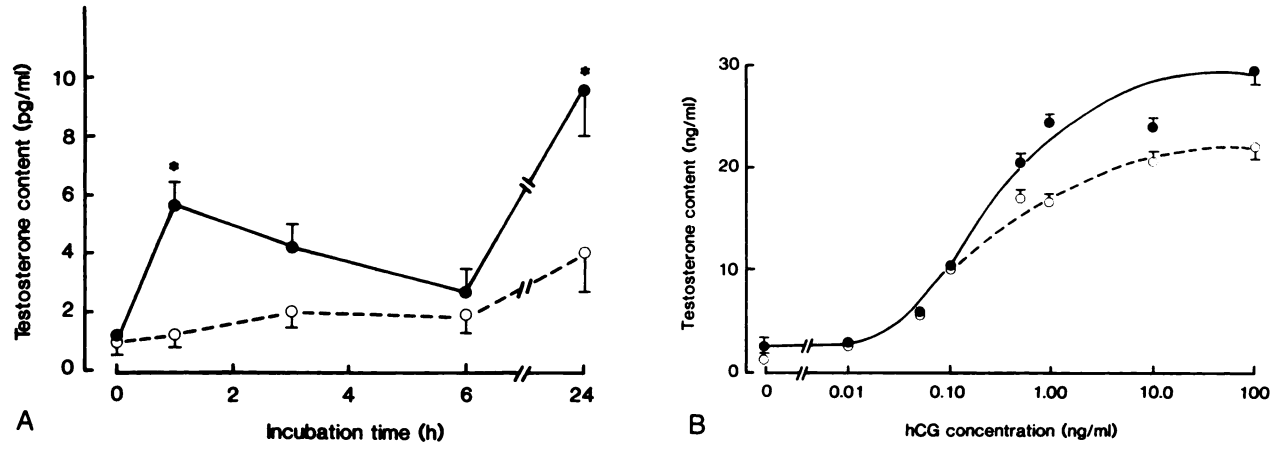

Fig. 2. Effect of calcitonin on T secretion by TM_3 cells and Leydig cell-enriched cultures. (A) The TM_3 cells were grown in F12/DME medium in 60-mm culture dishes (2.8×10^6 cells/dish). At confluency, fresh media were added with (●) or without (○) synthetic salmon calcitonin (1 μg/ml), and T was measured at different times. Results are the mean ± SE of three experiments each in triplicate. The asterisk indicates a significant difference from the control value ($P < 0.02$). (B) Leydig cell-enriched cultures were prepared by collagenase dispersion of rat testis. Aliquots (0.5 ml) of cell suspension (5.6×10^6 cells/ml) were stimulated for 3 h with different doses of hCG (hCG CR117; NIH) in the presence (●) and absence (○) of synthetic salmon calcitonin (1 μg/ml). Results are the mean ± SE of three assays. The last four points on the curse were significantly different from their corresponding controls ($P < 0.005$).

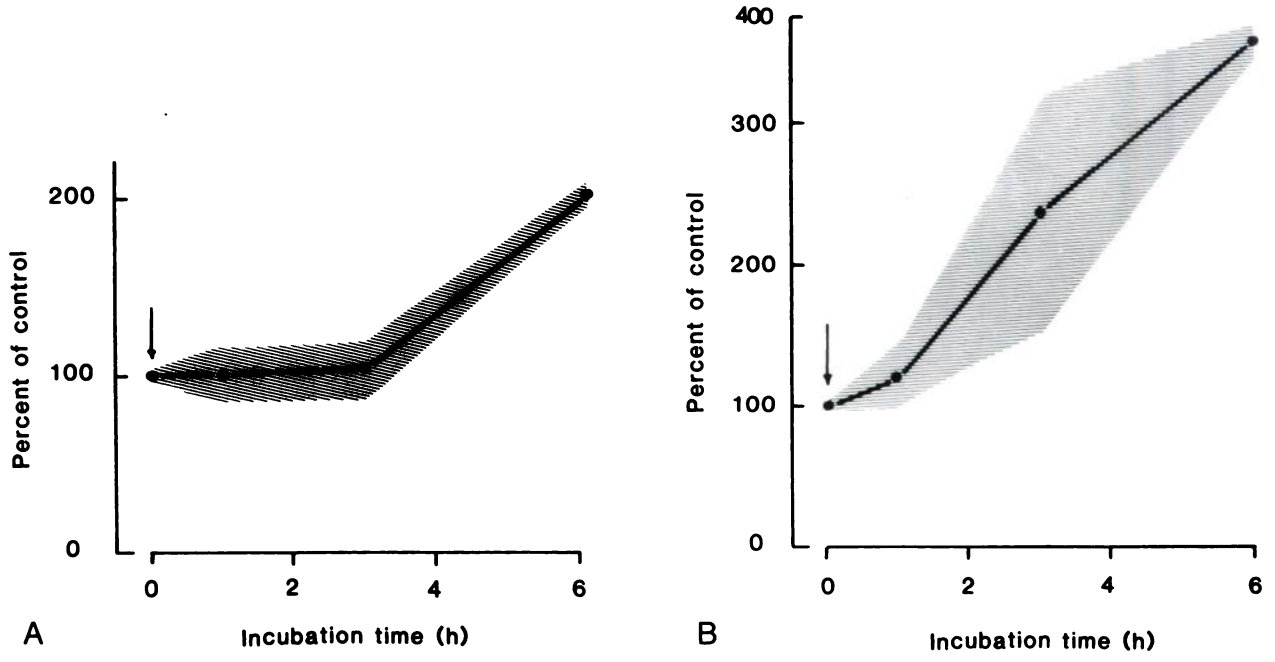

Fig. 3. Effect of calcitonin on androgen (A) and estrogen (B) receptor concentration in TM_3 cells. The cells were grown in F12/DME medium containing 7.5% serum. Synthetic salmon calcitonin was added (1 μg/ml) to confluent culture dishes and cells were collected at different time intervals. Results show the average of two experiments. The shaded area shows the range of the means. In this and subsequent experiments control receptor lvels are the mean of measurements at the beginning and end of the study. These values were 25 ± 2 fmol/mg protein for androgens and 24 ± 7 fmol/mg protein for estrogen receptors (m ± SE). Each assay was on triplicate plates. The arrow indicates addition of synthetic salmon calcitonin.

androgen and estrogen receptors rather than their binding affinities (Kd for [^{3}H]methyltrienolone: control 0.3 nM, synthetic salmon calcitonin 0.4 nM; Kd for [^{3}H]E_2: control 1.0 nM; synthetic salmon calcitonin 0.9 nM). Since the testis is regulated by both Ca^{2+} and cAMP (Mendelson et al, 1975; Janszen et al, 1976; Lin et al, 1980; Veldhuis and Klase, 1982), it is possible that calcitonin modulates Leydig cell function via one or both of these messengers.

Effect of cAMP on Sex Steroid Receptor Concentrations

Since synthetic salmon calcitonin increased cAMP formation in TM_3 cells, it was possible that this messenger mediated the action of calcitonin on steroid receptors. To study this possibility, TM_3 cells were treated with a stable cAMP derivative, 8-BrcAMP (Hillier and deZwart, 1982), using a dose that stimulated T 2 to 4 fold. In four different experiments, 8-BrcAMP diminished androgen receptors by 50% during the 6-h period of study without affecting cell number (Fig. 4). Estrogen receptor values decreased by 33% within 1 h and then rose to 50% above controls by 6 h. Similar effects were produced on sex steroid receptors by dibutryl cAMP. As the pattern of receptor changes produced by the cAMP derivatives were markedly different from that produced by calcitonin, it seemed unlikely that cAMP mediated this action of the peptide. Since calcitonin is known to influence intracellular Ca^{2+} concentrations (Borle, 1975), we studied changes in receptor concentrations produced by factors that alter the intracellular calcium ion concentration.

Change in Ca^{2+} Concentration in Relation to Steroid Receptor Levels

TM_3 cells were grown to confluency in medium containing 1.5 mM Ca^{2+}. The Ca^{2+} concentration in the medium was then reduced for 18 h to either 0.5 mM or to less than 0.01 mM (i.e., no added Ca^{2+}) (Sullivan and Cooke, 1984a). Reduction of extracellular Ca^{2+} for 18 h reduced both androgen and estrogen receptor concentrations in the cytosol by 70% without affecting cell number or viability (Table 1). Maximal reduction of androgen receptors occurred only when the Ca^{2+} concentration was reduced to < 0.01 mM; estrogen receptors were reduced maximally at a Ca^{2+} concentration of 0.5 mM. In another series of experiments, Ca^{2+} was reduced to < 0.01 mM for 18 h, the physiologic concentration of Ca^{2+} (1.5 mM) was then restored, and receptor concentrations were measured 1 h and 6 h later. Addition of Ca^{2+} restored the levels of both receptors. The effect of this ion was rapid since androgen and estrogen receptor levels returned to 110 and 60% of control values, respectively, within 1 h (Fig. 5). By 6 h estrogen receptors were 60% higher, whereas androgen receptors remained at the control levels.

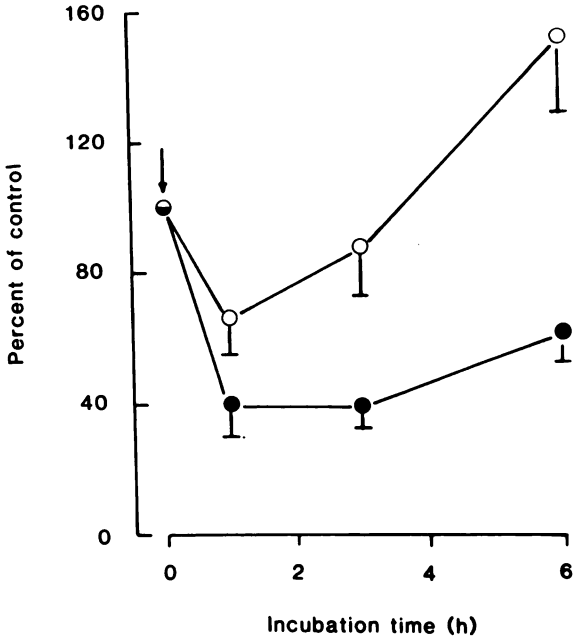

Fig. 4. Effect of 8-BrcAMP on androgen (●) and estrogen (○) receptor levels in TM_3 Leydig cells. The cells were grown to confluency in F12/DME medium containing serum. 8-BrcAMP (0.5 mM) was added and cells were collected at different time intervals. After the cells were washed with PBS, cytosol was prepared and used for measuring receptor concentrations. Results are the mean ± SE of three experiments, each in triplicate. Control values were 57 ± 15 fmol/mg protein for androgen receptors and 22 ± 6 fmol/mg protein for estrogen receptor. The arrow indicates the addition of 8-BrcAMP.

Effect of Ca^{2+} Regulating Agents on the Receptor Levels

To study further the possible role of Ca^{2+} on receptor concentrations in TM_3 cells, the effects of the Ca^{2+} ionophore, A23187, and the Ca^{2+} channel blocker, verapamil, were examined. To study the effect of Ca^{2+} ionophore, A23187 (Janszen et al, 1976; Lin et al, 1980; Veldhuis and Klase, 1982), TM_3 cells were maintained in 0.5 mM Ca^{2+} for 18 h. This concentration of Ca^{2+} in the medium was associated with a decline in receptor concentrations as noted in Table 1. When the cells were treated with the ionophore, receptor levels were returned to control values by 6 h (Figs. 6A, 6B).

TABLE 1. Androgen and Estrogen Receptor Concentrations in TM_3 Leydig Cells Grown in Different Concentrations of Ca^{2+} for 18 H

Calcium Concentration	Receptor Concentration (fmol/mg cytosol protein)*	
	Androgen Receptor	Estrogen Receptor
1.5 mM	70 ± 11.7 (19)	27 ± 6 (18)
0.5 mM	49 ± 3 (3)	8 ± 3 (3)†
0.01 mM	23 ± 10 (3)†	7 ± 3 (3)†

*Mean ± SE. The receptor levels in cells maintained in 1.5 mM Ca^{2+} were the same at the beginning and end of the experiment after a medium change. These times correspond to −18 h and 0 in Fig. 5. The number in parentheses indicates the number of assays, each of which was in triplicate.

†Differs from receptor concentration measured at 1.5 mM Ca^{2+}, $P < 0.05$.

Verapamil, which blocks the Ca^{2+} channels (Veldhuis and Klase, 1982; Moger, 1983), reduced the content of androgen receptors in the cytosol of TM_3 cells. In three separate experiments, verapamil decreased androgen receptor values by 75% within 1 h of its addition without changing cell number or viability. At 3 h and 6 h, the levels of this receptor returned toward those of control cells (Fig. 7). By contrast, the estrogen receptor concentration did not decrease significantly during the first hour, and by 6 h had increased to 170% of control (Fig. 7). This divergent effect of verapamil on androgen and estrogen receptors is unexpected, since other agents used tended to have the same effects on both receptors. It is therefore possible that some of the effects of this agent may be due to action by other than Ca^{2+} channel blockade.

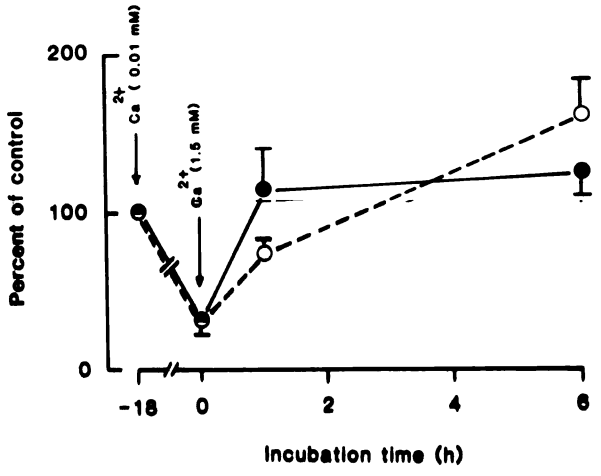

Fig. 5. The effect of changes in Ca^{2+} concentration on androgen (●) and estrogen (○) receptor concentrations in the cytosol of the TM_3 Leydig cell line. At 18 h before the start of the experiment, the medium was changed to serum-free F12/DME without added Ca^{2+} (0.01 mM). At time zero, a physiologic concentration of Ca^{2+} (1.5 mM) was added to the medium and cells were collected at different time intervals for steroid receptor assays. Results are the mean ± SE of three experiments, each in triplicate. Control values for androgen receptors were 78 ± 15 fmol/mg protein and 15 ± 2 fmol/mg protein for estrogen receptors.

Change in Steroid Receptors in Nuclei

Since a decrease in steroid receptors in cytosol may be associated with an increase in measurable nuclear receptors, it was pertinent to know whether compounds that decreased cytosol receptors would concomitantly increase nuclear receptors. The effects of 8-BrcAMP and verapamil on androgen receptors were studied. These agents did not increase receptor levels in the nuclei of TM_3 cells (not shown).

Discussion

Studies demonstrating calcitonin receptors on Leydig cells suggest that they might be one of the many sites affected by this peptide (Chausmer et al, 1980; 1982). The results of the present study show that synthetic salmon calcitonin stimulates T secretion and cAMP formation, and increases the content of sex steroid receptors in Leydig cell-enriched cultures and a Leydig cell line, thereby providing evidence that the calcitonin receptors are coupled to biologic responses.

Synthetic salmon calcitonin enhanced basal T secretion by TM_3 cells and primary Leydig cell-enriched cultures. This effect was similar to that of LH, LHRH analogs, and hCG on interstitial cells (Mendelson et al, 1975; Janszen et al, 1976; Veldhuis and Klase, 1982; 1983; Haour et al, 1983; Sullivan and Cooke, 1984b; Lin, 1984a; 1984b; Themen et al, 1984). The increase in T secretion was accompanied by a small increment in cAMP formation. This result is similar to the stimulatory effect of calcitonin on the adenylate cyclase system in rat

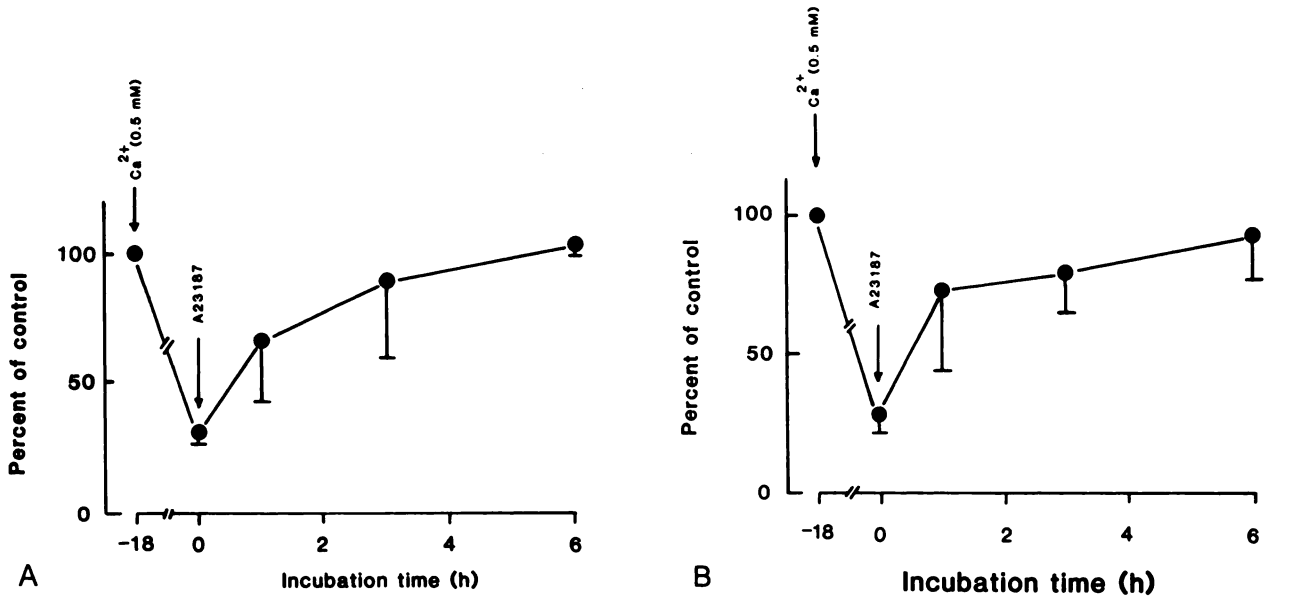

Fig. 6. Effect of the Ca^{2+} ionophore, A23178, on androgen (A) and estrogen (B) receptor concentrations in TM_3 cells. The cells were grown in normal F12/DME medium. At 18 h before the start of the experiment the medium was changed to serum-free F12/DME medium containing 0.5 mM Ca^{2+}. The Ca^{2+} ionophore A23187 was added to the culture medium (0.5 μg/ml; in dimethyl sulfoxide) at time zero and the cells were collected for measurement of androgen (A) and estrogen (B) steroid receptor concentrations. Results are the mean ± SE of two experiments. Control values (−18 h) for androgen receptors were 65 ± 13 fmol/mg protein and 23 ± 5 fmol/mg protein for estrogen receptors.

kidney (Chao and Forte, 1982; 1983), bone cells (Bell and Stern, 1970; Heersche et al, 1974), the FM-2 cell line derived from a human osteosarcoma (Eilon et al, 1983), human lymphocytes (Perry et al, 1983), and other human cancer cells such as T-47D (Ng et al, 1983; Moseley et al, 1983), MCF-7, 2R-7S and BEN (Michelangeli et al, 1983). Although an increase in cAMP levels in Leydig cells is known to be associated with an increase in steroidogenesis (Hedin and Rosberg, 1983; Sullivan and Cooke, 1984b), we did not establish that the stimulation of T secretion by calcitonin is cAMP-dependent. Although LH stimulates Leydig cell steroidogenesis via a cAMP-dependent mechanism, this is not so for LHRH (Themen et al, 1984). Furthermore, low concentrations of either LH or hCG have been found to stimulate T synthesis by rat interstitial cells *in vitro* without a detectable increase in cAMP formation (Catt and Dufau, 1973; Dufau et al, 1973; Moyle and Ramanchandran, 1973). These observations suggest that cAMP is not the only intracellular mediator of hormone-induced steroidogenesis in Leydig cells.

Janszen et al (1976) found that the maximum stimulation of T production in rat Leydig cells by LH could be obtained only in the presence of Ca^{2+}.

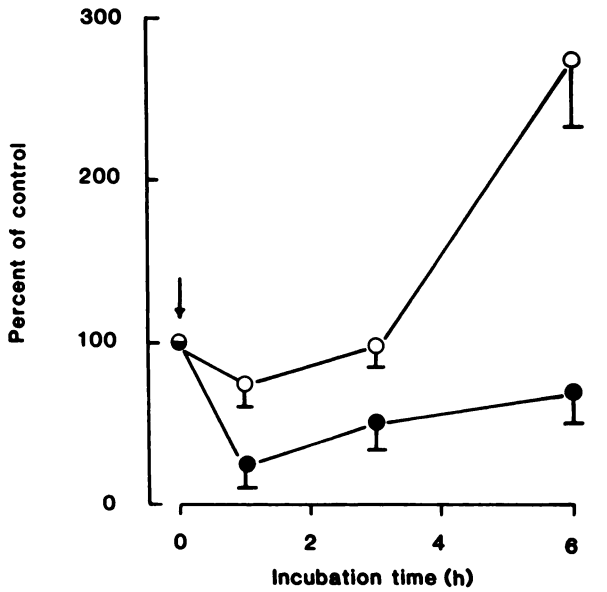

Fig. 7. Effect of verapamil on androgen (●) and estrogen (○) receptor concentrations in TM_3 Leydig cell line. The cells were grown in F12/DME medium. At confluency, the medium was changed and verapamil was added (50 μM; in dimethyl sulphoxide). The cells were collected at different time intervals for steroid receptor assays. Results are the mean ± SE of two experiments, each in triplicate. Control values were 56 ± 18 fmol/mg protein for androgen receptors and 19 ± 3 fmol/mg protein for estrogen receptors. The arrow indicates the time of addition of verapamil.

The Ca^{2+} ionophore, A23187, which facilitates Ca^{2+}-influx, was found to enhance T production by LH (Lin et al, 1980), whereas verapamil, which impedes Ca^{2+} influx, was found to diminish its secretion (Janszen et al, 1976). These observations suggest that transmembrane flux of Ca^{2+} may be an important regulator of steroidogenesis. Similar observations on the effects of Ca^{2+} on steroidogenesis have been made in Leydig (Mendelson et al, 1975; Janszen et al, 1976), granulosa (Veldhuis and Klase, 1982; Tsang and Carnegie, 1984), and adrenocortical cells (Bowyer and Kitabchi, 1974). These reports, together with the present results, suggest that calcitonin may stimulate T secretion in Leydig cells via a Ca^{+2}-mediated mechanism.

The present study confirms that Leydig cells contain estrogen receptors that are believed to mediate the action of these steroids (Kato et al, 1974; Abney, 1976; de Boer et al, 1977; Nozu et al, 1981; Lin et al, 1982; Nakhla et al, 1984). The present paper also confirms that Leydig cells contain androgen receptors (Sar et al, 1975; Wilson and Smith, 1975; Gulizia et al, 1983; Nakhla et al, 1984). It has been difficult to study the action of androgens on cells that produce T. Studies on androgen-resistant mice (Tfm/Y) suggested that androgens are necessary for Leydig cell differentiation (Bardin, 1973; Blackburn et al, 1973). Subsequent studies confirmed that androgens have a direct action on Leydig cells (Purvis and Hansson, 1978; Adashi and Hsueh, 1981; Darney and Ewing, 1981).

Synthetic salmon calcitonin increased the concentration of both androgen and estrogen receptors in the cytosol of TM_3 cells even though the testosterone produced by these cells was expected to decrease androgen receptor concentrations. It was therefore of interest to study the mechanism by which calcitonin affects steroid receptors. Because derivatives of cAMP diminished, while calcitonin increased, androgen and estrogen receptor concentrations in TM_3 Leydig cells, the effects of this peptide were probably not mediated by cAMP. These results are similar to those obtained on an endometrial adenocarcinoma cell line by Fleming et al (1982; 1983), who found that cAMP lowered the binding activity of estrogen receptors.

Van Bohemen et al (1983) observed that glucocorticoid receptors are sensitive to Ca^{2+} ions. In cytosols of rat and human cell lines, Ca^{2+} modulated the affinity of agonists and antagonists for glucocorticoid receptors. Kinetic, thermodynamic, and physicochemical studies suggested that Ca^{2+} alters receptor conformation at free Ca^{2+} ion concentrations (0.1 to 1 μM) known to occur within intact cells. A similar effect of Ca^{2+} on the kinetics of steroid binding in cytosol preparations was also found with the mineralocorticoid receptor from rat kidney, but not with the androgen, estrogen, or progesterone receptor (Rousseau and Hue, 1984). However, when rat hepatoma cells were loaded with Ca^{2+} using the ionophore, A23187, more receptor-bound dexamethasone was observed in the nuclei. Similar results were achieved with rat hepatocytes in primary suspension culture. These observations suggest that physiologic variation in Ca^{2+} homeostasis could modulate the cellular response to corticoids via a steroid receptor-mediated effect. The results of the present study show that depletion of Ca^{2+} from the cell growth medium diminishes the concentration of measurable androgen and estrogen receptors in TM_3 Leydig cells. Receptor levels were restored to control values when Ca^{2+} ions were replaced. Supporting results were obtained when Ca^{2+} ionophore, A23187, and verapamil were used as cellular Ca^{2+} regulators. Taken together, these findings suggest that a physiologic concentration of extracellular Ca^{2+} is required to maintain the binding activity of androgen and estrogen receptors in Leydig cells. It is not known at present whether changes in the concentration of Ca^{2+} modulate cellular responses to androgens and estrogens.

In conclusion, the present studies on TM_3 and Leydig cell-enriched cultures suggest that calcitonin plays a role in the regulation of Leydig cell function. Calcitonin also increases the levels of sex steroid binding to receptors. It is likely that this latter action is mediated by Ca^{2+} ions.

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References

Abney TO. A cytoplasmic estradiol receptor in rat testicular tissue. Endocrinology 1976;99:555–566.

Adashi EY, Hsueh AJW. Autoregulation of androgen production in a primary culture of rat testicular cells. Nature 1981;293:737–738.

Bardin CW. Sexual differentiation. In: Brobeck JR, ed. Best and Taylor's physiological basis of medical practice, vol. 7. Baltimore: Williams and Wilkins, 1973;88–94.

Bell NH, Stern PH. Effects of changes in serum calcium on hypocalcemic response to thyrocalcitonin in the rat. Am J Physiol 1970;218:64–68.

Blackburn WR, Chung KW, Bullock LP, Bardin CW. Testicular feminization in the mouse: studies of Leydig cell structure

and function. Biol Reprod 1973;9:9-23.
Borle AB. Regulation of cellular calcium metabolism and calcium transport by calcitonin. J. Membrane Biol 1975;21:125-146.
Bowyer F, Kitabchi AE. Dual role of calcium in steroidogenesis in the isolated adrenal cell of rat. Biochem Biophys Res Commun 1974;57:100-105.
Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-254.
Catt KJ, Dufau ML. Interactions of LH and hCG with testicular gonadotropin receptors. Adv Exp Med Biol 1973;36:379-384.
Chao WTH, Forte LR. Rat kidney cells in primary culture: hormone-mediated desensitization of the adenosine 3',5'-monophosphate response to parathyroid hormone and calcitonin. Endocrinology 1982;111:252-259.
Chao WTH, Forte LR. Rat kidney cells in primary culture: interaction of salmon calcitonin with receptor sites. Endocrinology 1983;112:745-752.
Chausmer A, Stuart C, Stevens M. Identification of testicular cell plasma membrane receptors for calcitonin. J Lab Clin Med 1980;96:933-938.
Chausmer AB, Stevens MD, Severn C. Autoradiographic evidence for a calcitonin receptor on testicular Leydig cells. Science 1982;216:735-736.
Chen C-L, Mather JP, Morris PL, Bardin CW. Expression of proopiomelanocortin-like gene in the testis and epididymis. Proc Natl Acad Sci USA 1984;81:5672-5675.
Colvard DS, Wilson EM. Nuclear uptake of the androgen receptor *in vitro* is potentiated by Zn^{2+} and inhibited by the 8S androgen receptor-promoting factor. Program of the 64th Endocrine Meeting, San Francisco, abstract no. 737 1982;264.
Darney Jr KJ, Ewing L. Autoregulation of testosterone secretion in perfused rat testes. Endocrinology 1981;109:993-995.
de Boer W, de Vries J, Mulder E, van der Molen HJ. Comparative study of nuclear binding sites for oestradiol in rat testicular and uterine tissue. Determination of low amounts of specific binding sites by an [^{3}H]oestradiol-exchange method. Biochem J 1977;162:331-339.
Dufau ML, Watanabe K, Catt KJ. Stimulation of cyclic AMP production by the rat testis during incubation with hCG *in vitro*. Endocrinology 1973;92:6-11.
Dufau ML, Mendelson CR, Catt KJ. A highly sensitive in vitro bioassay for luteinizing hormone and chorionic gonadotropin: testosterone production by dispersed Leydig cells. J Clin Endocrinol Metab 1974;39:610-613.
Eilon G, Perkins J, Viola MV. Characteristics of a calcitonin-responsive cell line derived from a human osteosarcoma. Cancer Res 1983;43:3763-3769.
Fischer JA, Sagar SM, Martin JB. Characterization and regional distribution of calcitonin binding sites in the rat brain. Life Sci 1981;29:663-671.
Fleming H. Blumenthal R, Gurpide E. Effects of cyclic nucleotides on estradiol binding in human endometrium. Endocrinology 1982;111:1671-1677.
Fleming H, Blumenthal R, Gurpide E. Rapid changes in specific estrogen binding elicited by cGMP or cAMP in cytosol from human endometrial cells. Proc Natl Acad Sci USA 1983;80:2486-2490.
Gulizia S, D'Agata R, Sanborn BM, Steinberger E. Evidence for the presence of androgen receptors in purified rat Leydig cells. J. Androl 1983;4:248-252.
Haour F, Bommelaer MC, Bernier AM, Sanchez P, Saez AJ, Mather JP. Androgen production in primary culture of immature porcine Leydig cells. Mol Cell Endocrinol 1983;30:73-84.
Hedin L, Rosberg S. Forskolin effects on the cAMP system and steroidogenesis in the immature rat ovary. Mol Cell Endocrinol 1983;33:69-80.
Heersche JN, Marcus R, Aurbach GD. Calcitonin and the formation of 3',5'-cAMP in bone and kidney. Endocrinology 1974;94:241-247.
Hillier SG, deZwart FA. Androgen/antiandrogen modulation of cyclic AMP-induced steroidogenesis during granulosa cell differentiation in tissue culture. Mol Cell Endocrinol 1982;28:347-361.
Isomaa V, Pajunen AEI, Bardin CW, Jänne OA. Nuclear androgen receptors in the mouse kidney: validation of a new assay. Endocrinology 1982;111:833-843.
Janszen FHA, Cooke BA, van Driel MJA, van der Molen HJ. The effect of calcium ions on testosterone production in Leydig cells from rat testis. Biochem J 1976;160:433-437.
Kato J, Onouchi T, Okinaga S, Ito N. Estradiol receptors in rat testis. Endocrinology 1974;94:902-907.
Lin T, Murono E, Osterman J, Nankin H. The effects of calcium ionophore A23187 on interstitial cell steroidogenesis. Biochem Biophys Acta 1980;627:157-164.
Lin T, Chen GCC, Murono EP, Osterman J, Nankin HR. Estradiol receptors of two distinct populations of Leydig cells. Steroids 1982;40:53-63.
Lin T. Mechanism of action of gonadotropin-releasing hormone-stimulated Leydig cell steroidogenesis I. The stimulatory effect is calcium dependent and not mediated by cyclic nucleotides. J Androl 1984a;5:193-200.
Lin T. Mechanism of action of gonadotropin-releasing hormone-stimulated Leydig cell steroidogenesis II. Gonadotropin-releasing hormone stimulates phospholipid labeling. J Androl 1984b;5:201-205.
Mather JP. Establishment and characterization of two distinct mouse testicular epithelial cell lines. Biol Reprod 1980;23:243-252.
Mather JP, Zhuang LZ, Perez-Infante V, Phillips DM. Culture of testicular cells in hormone-supplemented serum-free medium. Ann NY Acad Sci 1982;383:44-68.
Mendelson C, Dufau M, Catt K. Gonadotropin binding and stimulation of cyclic adenosine 3':5'-monophosphate and testosterone production in isolated Leydig cells. J. Biol Chem 1975;250:8818-8823.
Michelangeli VP, Findlay DM, Moseley JM, Martin TJ. Mechanisms of calcitonin induction of prolonged activation of adenylate cyclase in human cancer cells. J Cyclic Nucleotide Protein Phosphor Res 1983;9:129-142.
Moger WH. Effects of the calcium-channel blockers cobalt, verapamil, and D600 on Leydig cell steroidogenesis. Biol Reprod 1983;28:528-535.
Moseley JM, Findlay DM, Gorman JJ, Michelangeli VP, Martin TJ. The calcitonin receptor on T 47D breast cancer cells. Biochem J 1983;212:609-616.
Moyle WR, Ramanchandran J. Effect of LH on steroidogenesis and cyclic AMP accumulation in rat Leydig cell preparations and mouse tumor Leydig cells. Endocrinology 1973;93:127-134.
Nakhla AM, Mather JP, Jänne OA, Bardin CW. Estrogen and androgen receptors in Sertoli, Leydig, myoid, and epithelial cells: effects of time in culture and cell density. Endocrinology 1984;115:121-128.
Ng KW, Livesey Sa, Larkins RG, Martin TJ. Calcitonin effects on growth and on selective activation of type II isoenzyme of cyclic adenosine 3':5'-monophosphate-dependent protein kinase in T 47D human breast cancer cells. Cancer Res 1983;43:794-800.
Nozu K, Dufau ML, Catt KJ. Estradiol receptor-mediated regulation of steroidogenesis in gonadotropin-desensitized Leydig cells. J Biol Chem 1981;256:1915-1922.
Perry HM, Kahn AJ, Chappel JC, Kohler G, Teitelbaum SL, Peck WA. Calcitonin response in circulating human lymphocytes. Endocrinology 1983;113:1568-1573.
Purvis K. Hansson V. Androgens and androgen-binding protein

in the rat epididymis. J Reprod Fertil 1978;52:59–63.
Queener SF, Bell NH. Calcitonin: a general survey. Metabolism 1975;24:555–567.
Rousseau GG, Hue L. Calcium and steroid receptors. Program of the 7th International Congress of Endocrinology, Quebec, Abstract no. S141 1984;155.
Salomon Y. Adenylate cyclase assay. In: Brooker G, Greengard P, Robison GA, eds. Advances in cyclic nucleotide research, vol. 10. New York: Raven Press, 1979;35–55.
Sar M, Stumpf WE, McLean WS, Smith AA, Hansson V, Nayfeh SN, French FM. Localization of androgen target cells in the rat tests. Autoradiographic studies. In: French FS, Hansson V, Ritzen EM, Nayfeh SN, eds. Hormonal regulation of spermatogenesis. New York: Plenum Press, 1975;311–322.
Sullivan MHF, Cooke BA. LHRH agonist decreases LH- but not forskolin-stimulated cyclic AMP levels in rat Leydig cells in vitro. Mol Cell Endocrinol 1984a;36:115–122.
Sullivan MHF, Cooke BA. The effect of calcium on the potentiation of LH-stimulated steroidogenesis and inhibition of LH-stimulated cyclic AMP production by LHRH agonist (ICI 118630) in rat Leydig cells. Mol Cell Endocrinol 1984b;34:17–22.
Thau RB, Yamamoto Y, Sundaram K. Spinola PG. Human chorionic gonadotropin stimulates luteal function in Rhesus monkeys immunized against the β-subunit of ovine luteinizing hormone. Endocrinology 1983;112:277–283.
Themen APN, Hoogerbrugge JW, Rommerts FFG, van der Molen HJ. Comparison of LH and LHRH-agonist action on rat Leydig cell steroidogenesis. Program of the 7th International Congress of Endocrinology, Quebec. Abstract no. 1441 1984;981.
Tsang BK, Carnegie JA. Calcium-dependent regulation of progesterone production by isolated rat granulosa cells: effects of the calcium ionophore A23187, prostaglandin E_2, dl-isoproterenol and cholera toxin. Biol Reprod 1984;30:787–794.
van Bohemen CG, Eliard PH, Rousseau GG. Control by calcium ions of steroid binding to the glucocorticoid receptor. Mol Physiol 1983;4:95–110.
Veldhuis JD, Klase PA. Mechanisms by which calcium ions regulate the steroidogenic actions of luteinizing hormone in isolated ovarian cells *in vitro*. Endocrinology 1982;111:1–6.
Veldhuis JD, Klase PA. Mechanisms by which calcium ions modulate the steroidogenic actions of luteinizing hormone in ovarian cells: relation of the classical cyclic AMP effector system. Program of the 65th Endocrine Meeting. San Francisco. Abstract no. 207 1983;131.
Walters MR, Clark JH. Cytosol progesterone receptor of the rat uterus: assay and receptor characteristics. J Steroid Biochem 1977;8:1137–1144.
Wilson EM, Smith AA. Localization of androgen receptors in rat testis: biochemical studies. In: French FS, Hansson V, Ritzen EM, Nayfeh SN, eds. Hormonal regulation of spermatogenesis. New York: Plenum Press, 1975;281–286.