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Estradiol and Plasminogen Activator Secretion by Cultured Rat Sertoli Cells in Response to Melanocyte-Stimulating Hormones

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Pro-opiomelanocortin-derived peptides, α-MSH and β-endorphin, are synthesized and secreted by Leydig cells, and are believed to have paracrine effects on Sertoli cells in the testis. Peptides with MSH activity stimulate adenylate cyclase and cAMP accumulation in Sertoli cell-enriched cultures. The purpose of the present study was to determine whether such peptides would affect Sertoli cell parameters, such as aromatase and plasminogen activator activities, that are known to be regulated by cAMP. α-MSH stimulated aromatase activity in Sertoli cell-enriched cultures prepared from 10-day-old rats and this effect was potentiated by methyl isobutylxanthine (MIX). The combination of α-MSH plus MIX was not as potent as FSH. α-MSH, des-acetyl-α-MSH, β-MSH, ACTH(1-13), and ACTH(1-24) stimulated aromatase activity to a similar extent, suggesting that Sertoli cells do not distinguish between the activities of these peptides. α-MSH potentiated the action of dbcAMP and forskolin on Sertoli cell aromatase, but unexpectedly had no effect on the action of either half-maximal or maximal doses of FSH. The regulation of plasminogen activator was examined next; urokinase was markedly suppressed by FSH in 10-day-old Sertoli cells. Although neither α-MSH nor MIX alone had an effect on urokinase secretion, in combination they were as effective as FSH. In 10-day-old Sertoli cells each of these peptides had little or no effect on tissue plasminogen activator. It was concluded that molecules such as the MSH/ACTH peptides modulate Sertoli cell function via cAMP, that there is a differential response depending more upon the Sertoli cell products examined rather than the peptide tested, and that the magnitude of the responses to α-MSH and MIX examined to date do not exceed those produced by FSH.

Key words: α-MSH, ACTH, Sertoli cell, plasminogen activator, aromatase, pro-opiomelanocortin

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There are at least two long-loop regulatory systems between the hypothalamo-pituitary unit and the testis. First, the seminiferous tubular epithelium is stimulated by FSH, which in turn is suppressed by inhibin, an FSH-dependent hormone secreted by the Sertoli cells. Second, testosterone (T) secretion is stimulated by LH, which in turn is suppressed by the steroids produced by Leydig cells. These long-loop systems are believed to be the major hormonal regulators of testicular function.

The demonstration that androgens are also important for spermatogenesis was the first of

several observations indicating that a product produced within the testis by Leydig cells can stimulate the seminiferous tubular epithelium (Fritz, 1978; Sharpe, 1984). Several studies also suggest that a variety of products from the seminiferous tubules may, in turn, regulate Leydig cell function (Sharpe, 1984; Parvinen et al., 1984; Bergh, 1985; Verhoeven and Cailleau, 1985). Taken together, the latter observations are compatible with the postulate that there are multiple regulatory molecules produced by the testis that have paracrine actions. The demonstration that pro-opiomelanocortin and its derivative peptides are produced by testicular cells suggested that this may be one such group of regulatory molecules (Shaha et al., 1984; Chen et al., 1984; Pintar et al., 1984; Gizang-Ginsberg and Wolgemuth, 1987; Kilpatrick et al., 1987; Fabbri et al., 1988). The possible function of such peptides was suggested by studies showing that α-MSH, des-acetyl α-MSH, and ACTH stimulate adenylate cyclase and cAMP accumulation in Sertoli cell-enriched cultures (Mather et al., 1985; Boitani et al., 1986). The present study was therefore conducted to define the extent to which α-MSH and the other MSH-related peptides could affect aromatase and plasminogen activator activities, two functional parameters of the Sertoli cell that are believed to be regulated by cyclic nucleotides.

Materials and Methods

Sertoli Cell-Enriched Culture

Sertoli cell monolayers were obtained from 10-day-old Sprague Dawley rats as previously described (Mather and Phillips, 1984), except that the tubular fragments resulting from the second collagenase treatment were not filtered through medium-sized Nitex in order to improve recovery of cells. After several washes, tubular fragments were plated on 12-well dishes in MEM with Earle's salts. Incubation was carried out at 32 C in a controlled atmosphere of 95% air-5% CO_2. In some experiments, Sertoli cell-enriched monolayers were exposed on day 3 of culture to hypotonic solution (Galdieri et al., 1981) to remove contaminating germ cells. The cells were treated 24 h after the removal of germ cells. On day 4 of culture, cells were washed twice with fresh medium and incubated for 24 h in the presence of 5×10^{-6} M androstenedione as aromatase substrate and various hormones at the concentrations indicated in the results. Bacitracin was added at a final concentration of 10 μg/ml to prevent degradation of the peptides during the course of treatment. In studies in which plasminogen activator activity was measured, hormone incubations were carried out in MEM with Earle's salts supplemented with bovine serum albumin (BSA) (0.1%).

Assay of Aromatase Activity

At the end of incubation, media were collected, centrifuged at 2000 rpm for 10 min and stored at −20 C for estrogen assay. Estrogen was measured by radioimmunoassay (RIA) as previously described (Boitani et al., 1981) with an antiserum that was a generous gift of Radim S.R.L., with the following cross-reactivities: 17β-estradiol, 100%; estrone, 1.2%; estriol, 0.5%; and progesterone, 0.1% (Monaco et al., 1984). Results were expressed as ng estradiol (E_2) produced per mg protein. Protein was measured using the method of Lowry (1951) with BSA as standard.

Assay of Plasminogen Activator Activity

Presence of plasminogen activator in the medium was measured by incubating the samples with plasminogen and a chromogenic substrate (Verheijen et al., 1982; Andrade-Gordon and Strickland, 1986). To characterize the type of plasminogen activator present in the medium, samples were preincubated with antisera specific for tissue-type plasminogen activator or urokinase for 30 min at room temperature before initiating the assay. The absorbance generated at 405 nm was related to the plasminogen activator activity. Activities, expressed as Ploug units (PU), were determined with reference to a standard preparation of urokinase. Assays performed in the absence of plasminogen did not reveal plasminogen-independent proteolytic activity.

Antiserum against mouse urokinase was raised in rabbits against urokinase purified from the culture medium of a Lewis lung carcinoma cell line; antiserum against human tissue plasminogen activator was prepared in rabbits against enzyme isolated from culture of HeLa cells. Both antisera have previously been shown to be specific in inhibiting either tissue plasminogen activator or urokinase (Marotti et al., 1982). Antisera tissue plasminogen activator and antisera urokinase were kindly provided by Drs. Dominique Belin and Edmund Waller, respectively.

Analysis of Results

Results from RIA and dose-response studies were analyzed with a program that uses a four-parameter logistic function, and unknowns were interpolated from the resultant curve. Each experiment was replicated at least three times. The results of each experiment were analyzed by one-way analysis of variance. When appropriate, differences between treated and untreated cultures were determined by Student's t test.

Chemicals

Eagle's minimum essential medium was obtained from Grand Island Biological Co. (Grand Island, NY); collagenase-dispase and deoxyribonuclease were purchased from Boehringer (Mannheim, West Germany); methylisobutylxanthine (MIX) was purchased from Aldrich (Milwaukee, WI); α-MSH, β-MSH, des-acetyl-α-MSH, γ-MSH, ACTH(1-13) were obtained from Peninsula Laboratories (Belmont, CA); ACTH(1-24), bacitracin and dibutyryl cAMP were purchased from

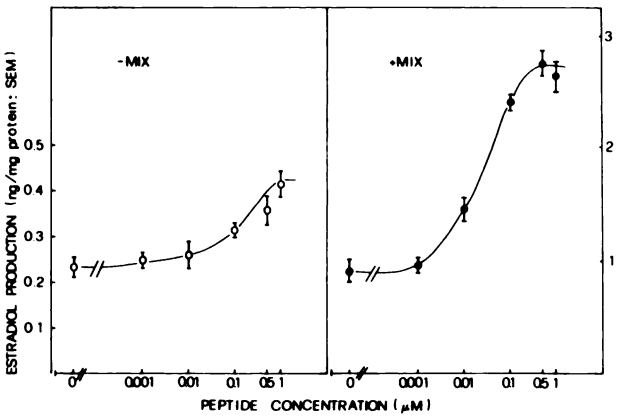

Fig. 1. The effect of α-MSH on aromatase activity as measured by Sertoli cell estradiol production from androstenedione. Cell monolayers were treated for 24 h with different concentrations of the peptide, in the absence (left) or in the presence (right) of 0.3 mM MIX. At the end of incubation, the E_2 accumulated in the medium was measured by RIA. Each point represents the mean (± SEM) of three plates, each assayed in duplicate.

Sigma (St. Louis, MO); forskolin was obtained from Calbiochem (San Diego, CA). Chromogenic plasmin substrate H-D-val-leu-lys-p-nitroanilide.2HCl (S2251) was obtained from Bachem Feinchemikalien AG (Basel, Switzerland). Ovine FSH-S16 was supplied by the NIDDK and National Hormone and Pituitary Program, NIH.

TABLE 1. Effect of Various Pro-Opiomelanocortin-Derived Peptides on Aromatase Activity in Sertoli Cell-Enriched Cultures*

Treatment	Estradiol Production (ng/mg protein, ± SEM)
None	0.23 ± 0.01
MIX	0.52 ± 0.06[a]
α-MSH	1.48 ± 0.10[b]
Des-acetyl-α-MSH	1.57 ± 0.11[c]
β-MSH	1.84 ± 0.05[d]
γ-MSH	0.96 ± 0.07[e]
ACTH (1-13)	1.80 ± 0.06[f]
ACTH (1-24)	1.81 ± 0.07[g]
β-endorphin	0.50 ± 0.01[h]

*All peptides were tested in the presence of MIX. Cells were incubated for 24 h in the presence of MIX (0.3 mM) and different pro-opiomelanocortin-derived peptides at the final concentration of 1 μM. At the end of the incubation, media were collected and E_2 levels were measured as described in Methods. Each point is the mean ± SEM of three different plates, each assayed in duplicate (a vs b,c,d,f,g: $P < 0.01$; a vs e: $P < 0.02$; b vs e: $P < 0.05$; a vs h: $P > 0.5$).

Results

The Effect of α-MSH on Aromatase Activity

The effect of α-MSH on Sertoli cell aromatase activity is shown in Fig. 1. Graded doses of α-MSH produced a significant dose-dependent increase of E_2 accumulated in the medium with a maximal 2-fold increase. When Sertoli cells were incubated in the presence of a phosphodiesterase inhibitor, methyl isobutylxanthine (MIX), the sensitivity and the maximal response to α-MSH was increased. The dose-response curve in the presence of MIX was shifted to the left by about 1 log.

To evaluate the specificity of this biologic effect of α-MSH, Sertoli cells were treated with other melanocyte-stimulating hormones and other peptides of the pro-opiomelanocortin family. The effects of these peptides on E_2 production are shown in Table 1. β-MSH and des-acetyl-α-MSH, the nonacetylated form of α-MSH that is present in the testis (Margioris et al., 1983), were as effective as α-MSH. Both ACTH(1-13) and ACTH(1-24) were also potent in stimulating aromatase activity while γ-MSH was significantly less potent ($P < 0.05$). During the 24-h incubation period used in this experiment, β-endorphin did not produce any effect consistent with the previously reported observation that several days are required for this peptide to influence adenylate cyclase in Sertoli cell cultures (Morris et al., 1987). Similar patterns of response to these peptides were obtained when accumulation of cAMP in the medium was measured in the presence of MIX (not shown).

The finding that γ-MSH was less potent than α-MSH prompted us to investigate the interaction of γ-MSH and α-MSH. Sertoli cells were incubated with the two hormones alone or in combination. As shown in Table 2, 0.01 μM γ-MSH did not produce a response significantly different from the control with MIX; significant stimulation ($P < 0.02$) was observed with 1 μM γ-MSH, which was less than that observed with the same amount of α-MSH. When γ-MSH was added together with α-MSH, the aromatase activity in Sertoli cells was similar to that observed with α-MSH alone. At the dose tested, γ-MSH was neither additive nor antagonistic to α-MSH.

The magnitude of the Sertoli cell response observed with maximally stimulating dose of α-MSH plus MIX was 2 × that observed with MIX alone, but only one half that with FSH + MIX (Fig. 2). The effects of α-MSH on the actions of FSH were

TABLE 2. Effect of α-MSH, γ-MSH, and α-MSH plus γ-MSH on Aromatase Activity by Sertoli Cell-Enriched Cultures*

Treatment	Estradiol Production (ng/mg protein, ± SEM)
None	0.23 ± 0.01
MIX (0.3 mM)	0.52 ± 0.06[a]
α-MSH (1 μM)	1.48 ± 0.13[b]
γ-MSH (0.01 μM)	0.68 ± 0.05[c]
γ-MSH (1 μM)	0.96 ± 0.07[d]
γ-MSH (0.01 μM) + α-MSH (1 μM)	1.61 ± 0.18[e]
γ-MSH (1 μM) + α-MSH (1 μM)	1.67 ± 0.17

*Cells were treated for 24 h with the indicated concentrations of the peptides in the presence of MIX. At the end of incubation the E_2 secreted into the medium was measured by RIA. Each point represents the mean ± SEM of three different plates, each assayed in duplicate (a vs d: $P < 0.02$; b vs d: $P < 0.05$; a vs c: $P > 0.05$; b vs e: $P > 0.5$).

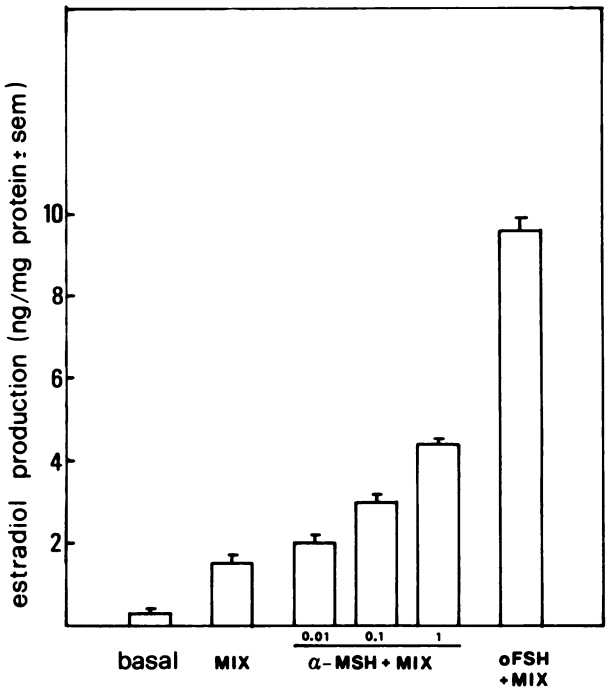

Fig. 2. The effect of α-MSH and FSH on Sertoli cell aromatase activity. Sertoli cell-enriched cultures were treated for 24 h in the absence or in the presence of MIX (0.3 mM) and FSH (50 ng/ml) or α-MSH (0.01, 0.1, or 1 μM). Estrogen levels in media from three plates were assayed in duplicate, mean ± SEM.

explored next. The concentrations of FSH chosen for these experiments, 5 ng/ml and 50 ng/ml, caused half-maximal and maximal stimulation, respectively, of E_2 production. Sertoli cells were incubated with FSH with or without α-MSH (1 μM). As shown in Fig. 3 (upper panel), α-MSH did not further potentiate the stimulatory effect of FSH although basal production of E_2 was significantly increased by the peptide (inset of the upper panel). Similar results were obtained in three other experiments with a variety of FSH doses. These observations were not expected since α-MSH potentiates the action of submaximal doses of FSH (= 5 ng) on cAMP production (Boitani et al., 1986). In view of this, we investigated the interaction of α-MSH with the cAMP analog, dibutyryl cAMP (dbcAMP), and forskolin, a stimulator of cAMP synthesis. When cells were stimulated with suboptimal doses of dbcAMP in the presence of MIX, α-MSH significantly enhanced ($P < 0.01$) the accumulation of E_2. The peptide had no effect on maximally stimulated Sertoli cells (Fig. 3, middle panel). A similar potentiating effect of α-MSH was also observed on a half-maximal, but not a maximal, dose of forskolin (Fig. 3, bottom panel).

The Effect of α-MSH on Plasminogen Activator Activity

The effects of α-MSH on the activities of urokinase and tissue plasminogen activator, two other parameters of Sertoli cell function, were studied. Sertoli cells were incubated with different concentrations of α-MSH in the presence and absence of MIX. The accumulation of urokinase and tissue plasminogen activator in the medium was measured at 24 h. Sertoli cell monolayers used in these experiments were treated with hypotonic solution before hormones were added to remove germ cells that produce tissue plasminogen activator (Vihko et al., 1988; Canipari, unpublished results). In the presence of MIX, α-MSH produced a dose-dependent decrease of urokinase activity released into the media (Fig. 4). Maximal effects were observed with α-MSH concentrations between 0.1 and 1 μM. The ED_{50} derived from these dose-response studies was approximately 0.2 nM. The tissue plasminogen activator activity was slightly stimulated but only by the highest concentration of α-MSH (1 μM; not shown). It is of note that α-MSH had no effect on plasminogen activator activity in the absence of MIX (Fig. 5).

When cells were incubated with a maximal dose

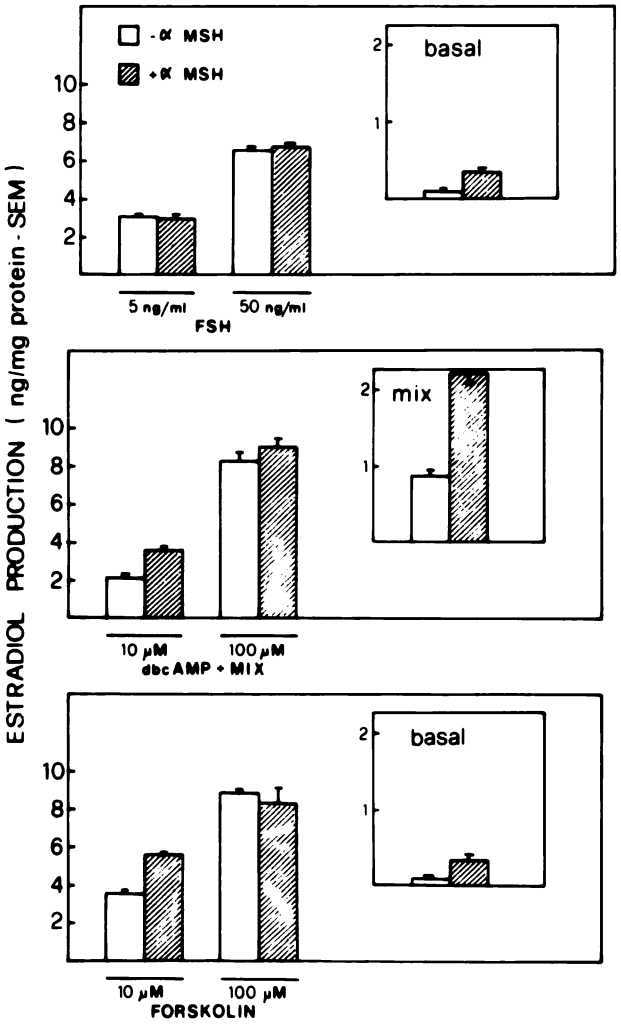

Fig. 3. The effects of α-MSH on FSH-, dbcAMP- or forskolin-dependent E_2 production by Sertoli cells in culture. Cells were incubated for 24 h in the presence of different concentrations of the stimulating factors with or without α-MSH (1 μM). At the end of the incubation, the E_2 accumulated in the medium was measured by RIA. Each point is the mean of three plates, each assayed in duplicate.

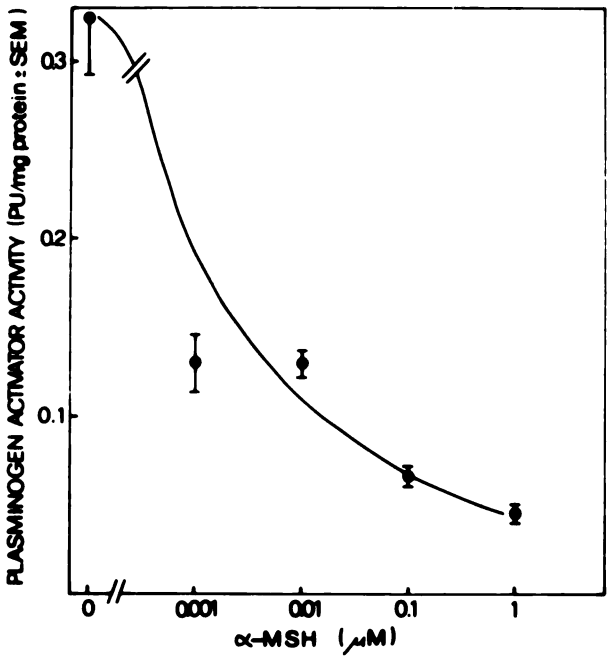

Fig. 4. The effects of α-MSH on urokinase plasminogen activator secretion into the medium by Sertoli cells. Cells were incubated for 24 h in the presence of α-MSH plus 0.3 mM MIX. Urokinase was measured in the presence of anti-tissue plasminogen activator (see Methods section). Each point is the mean of three plates, each assayed in duplicate.

of FSH (50 ng/ml), a marked decrease of urokinase levels in the medium was observed either in the absence or presence of MIX (Fig. 5). Tissue plasminogen activator was not stimulated by FSH in the absence of MIX (not shown). In this study and in those noted above, neither α-MSH nor MIX had effects alone but were synergistic when administered together. α-MSH plus MIX produced the same 10-fold decline in urokinase activity as FSH; this is the only Sertoli cell parameter that is stimulated to this extent by α-MSH.

Discussion

Immunoreactive pro-opiomelanocortin-derived peptides are present in testicular extracts (Tsong et al., 1982a; Margioris et al., 1983). In the testis, immunostainable ACTH, β-endorphin, and α-MSH are specifically localized in Leydig cells (Tsong et al., 1982b; Shaha et al., 1984), one of the extrapituitary sites where pro-opiomelanocortin messenger RNA is synthesized as demonstrated by Northern blot analysis (Chen et al., 1984) and *in situ* hybridization (Pintar et al., 1984; Gizang-Ginsberg and Wolgemuth, 1985). Recently, pro-opiomelanocortin transcripts also have been observed in enriched male germ cell populations (Gizang-Ginsberg and Wolgemuth, 1987; Kilpatrick et al., 1987).

The observation that pro-opiomelanocortin-derived peptides are released into interstitial fluid

in vivo (Valenca and Negro-Vilar, 1986) and into media by cultured Leydig cells suggests that these peptides are actually secreted by the Leydig cells in the testis (Fabbri et al., 1988). At present, there is no evidence that such peptides are secreted by germ cells. Several lines of evidence are consistent with the postulate that pro-opiomelanocortin-derived peptides may be local modulators of testicular function (Gerendai et al., 1984; Bardin et al., 1987). Injection of opiate antagonist into the testis suggested that testicular opiates inhibit Sertoli cell function (Gerendai and Halasz, 1981; Gerendai et al., 1986). This observation was supported by *in vitro* studies showing that β-endorphin inhibited Sertoli cell divison (O'Donohue et al., 1982), as well as ABP (Fabbri et al., 1985), and inhibin secretion (Morris et al., 1987). The latter study also suggested that some effects of β-endorphin may be mediated by inhibition of FSH receptor coupling to adenylyl cyclase.

A variety of *in vitro* studies also suggest that the MSH/ACTH group of peptides also modulates Sertoli cell cAMP production (Mather et al., 1985; Boitani et al., 1986). The present results provide further evidence for the possible regulatory role of these peptides in the male gonad by showing that they stimulate Sertoli cell parameters known to be regulated by cAMP. α-MSH and related peptides stimulate an increase of aromatase activity and a decrease of urokinase plasminogen activator activity in Sertoli cells.

α-MSH and ACTH are known to exert their effects by activation of adenylate cyclase, which increases intracellular levels of cAMP in melanocytes, adrenal cortex, and other cells (Christensen et al., 1976; Saez et al., 1984). Moreover, the role of cAMP as an obligatory mediator of steroid synthesis is generally accepted. In agreement with this is our finding that α-MSH stimulates E_2 production from androstenedione in Sertoli cells, both in the presence and the absence of MIX.

Among the MSH peptides tested, des-acetyl-α-MSH is similar to α-MSH in stimulating E_2 production by Sertoli cells. It is known that N-acetylation is an important determinant of the biologic activity of this peptide on some cells. For example, α-MSH is much more potent than des-acetylated-α-MSH in terms of grooming behavior (O'Donohue et al., 1981a; 1982) and melanosome dispersion in melanocytes of *Rana pipiens* (McCormack et al., 1982), whereas only des-acetyl-α-MSH is able to block opiate analgesia and opiate receptor

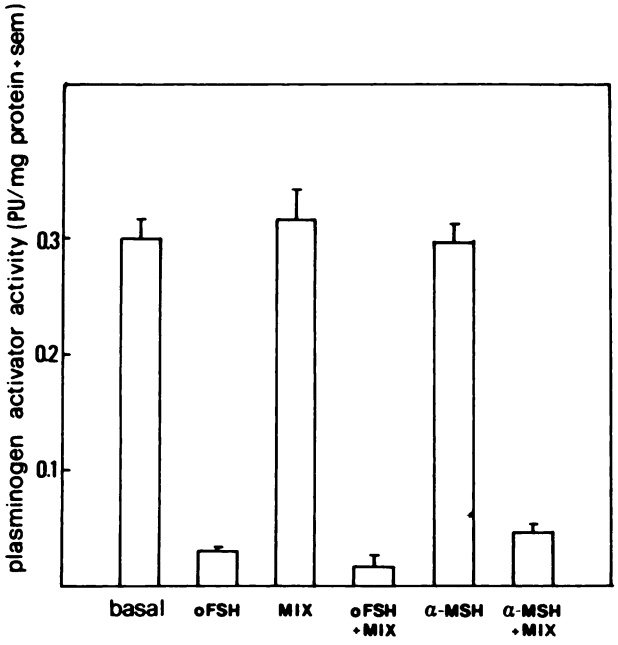

Fig. 5. Effect of FSH on urokinase secretion into the medium by Sertoli cells. Cells were incubated for 24 h in the presence of maximal doses of FSH (50 ng/ml) or α-MSH (1 μM) with or without MIX (0.3 mM). Urokinase levels in the media were measured as described in Fig. 4. Each point is the mean of three different plates, each assayed in duplicate.

binding in brain. By contrast, Sertoli cells do not distinguish between these two peptides. In addition, a variety of MSH-related peptides with diverse actions on melanocytes have similar effects on Sertoli cells (Sandman and Kastin, 1981). Thus, α-MSH, des-acetyl-α-MSH, β-MSH, ACTH(1-13) and ACTH(1-24) possess similar biologic activities consistent with the fact that they share a common sequence and/or conformations (Schwyzer and Eberle, 1977). On the other hand, γ-MSH, which shares some features with α-MSH, was less potent in stimulating Sertoli cell aromatase activity. Consistent with our results on testicular cells is the observation (O'Donohue et al., 1981b) that γ-MSH has only weak melanotropic activity and compared to α-MSH is ineffective in eliciting grooming.

The present report also shows that FSH produces a marked decrease in the release of urokinase activity by Sertoli cells from 10-day-old rats (Fig. 5). Little or no stimulation of tissue plasminogen activator was noted. These observations are of interest since they differ from those obtained from Sertoli cells isolated from 20-day-old rats where urokinase is not

under FSH control and tissue plasminogen activator is increased by FSH and dbcAMP (Lacroix et al., 1977; Lacroix and Fritz, 1982; Hettle et al., 1986). Changes of plasminogen activator activity in Sertoli cells during gonadal development have been noted. In this regard, an age-dependent pattern of plasminogen activator secretory activity by both rat seminiferous tubules (Vihko et al., 1986) and Sertoli cell cultures (Stefanini et al., 1988) has been observed. The reason for this age-dependent difference in Sertoli cells is not known. However, since an increase of cAMP occurs at both ages with either FSH or α-MSH (Means et al., 1980; Boitani et al., 1986), these observations imply that intracellular cAMP selectively inhibits the secretion of urokinase at 10 days while stimulating tissue plasminogen activator at 20 days of age.

Another notable observation of the present study was that in the presence of MIX, there was a more sensitive response to α-MSH when urokinase activity rather than cAMP accumulation or aromatase activity was used as an end point. In addition, α-MSH and MIX produced the same magnitude of change as FSH, although FSH produces a much larger effect on cAMP and aromatase than α-MSH plus MIX (Boitani et al., 1986). These observations indicate that α-MSH and FSH have differential effects on various parameters that cannot be explained by cAMP accumulations *per se*.

In summary, our results show that peptides with MSH/ACTH activity influence Sertoli cell functions *in vitro*. These results support the postulate that such peptides might be involved in the paracrine regulation of Sertoli cells *in vivo*.

Acknowledgments

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VIII International Congress of Hormonal Steroids

The VIII International Congress of Hormonal Steroids will be held September 17 to 21, 1990 in the Netherlands Congress Centre, The Hague, The Netherlands. The program will include plenary lectures, symposia and poster sessions emphasizing the most current developments in basic and clinical research related to steroids.

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