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Studies on the Gonadotropin-Releasing Activity of Thymulin: Changes With Age

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We assessed the ability of thymulin, a zinc-dependent nonapeptide produced by the thymic epithelial cells, to influence the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from dispersed anterior pituitary (AP) cells from young, adult, and senescent female rats. Perifusion of young and senescent AP cells with thymulin doses of 10^{-6} to 10^{-5} M gave a significant stimulatory response for LH but not FSH. Gonadotropin release was always lower in the senescent cells. AP cells from both age groups incubated with 10^{-8} to 10^{-3} M thymulin showed a time- and dose-dependent response for both gonadotropins, with a maximal stimulation at 10^{-7} M. Preincubation of thymulin with an antithymulin serum completely quenched the secretagogue activity of the hormone. Coincubation of thymulin with the secretagogue gonadotropin-releasing hormone (GnRH) revealed a synergistic effect on LH release and an additive effect on the release of FSH. The calcium chelator EGTA blocked the gonadotropin-releasing activity of thymulin in AP cells. The cAMP enhancers, caffeine, NaF, and forskolin significantly increased the thymulin-stimulated release of gonadotropins. The inositol phosphate enhancer LiCl potentiated the action of thymulin on gonadotropins. It is concluded that the gonadotropin-releasing activity documented here for thymulin is an age- and receptor-dependent effect mediated in part by calcium, cAMP, and inositol phosphates.

THE immune system is functionally linked to the nervous and endocrine systems, thus constituting an integrated homeostatic network (1). Within this network, the interaction between the endocrine thymus and the reproductive system appears to be particularly significant. Such interaction became evident after the discovery of the congenitally athymic (nude) mouse in the mid 1960s (2). In this mutant, the homozygous (nu/nu) females have severe deficiencies in reproductive function in comparison with their phenotypically normal heterozygous littermates (nu/+). The times of vaginal opening and first ovulation are delayed (3), fertility is reduced (4), and follicular atresia is increased such that premature ovarian failure results (5). Similar abnormalities result from neonatal thymectomy of normal female mice (6,7). Nude female mice show significantly reduced levels of circulating and pituitary gonadotropins, a fact that seems to be causally related to the reproductive derangements in these mutants (4). These and other early life dysfunctions have led some investigators to consider the nude mouse as a suitable model of thymus-dependent accelerated aging.

Interestingly, all of the above reproductive deficiencies can be prevented in nude mice by neonatal grafting of a normal age-matched thymus (8,9). It has been also shown that the administration of certain thymic peptides can restore, at least in part, gonadotropin production in nude mice (10).

Among the thymic peptides with a reported ability to modulate luteinizing hormone (LH) release in vitro, thymulin is of particular interest (11). Thymulin is a thymic hormone involved in several aspects of intra- and extrathymic T-cell differentiation (12). Thymulin consists of a biologically inactive nonapeptide component (facteur thymique sérique or FTS) coupled in an equimolecular ratio to the ion zinc (13), which confers biological activity to this molecule (14). Thymulin is exclusively produced by the thymic epithelial cells (TEC) and its secretion declines steadily with age (15–17).

The control of thymulin secretion by TEC seems to be dependent on a complex network of events. Initial studies showed that the hormone itself exerts a controlling feedback effect on its own secretion both in vivo and in vitro (18,19). Additionally, thymulin production and secretion is influenced directly or indirectly by the neuroendocrine system and gonadal steroids (reviewed in ref. 20).

In order to determine whether thymulin may influence follicle stimulating hormone (FSH) secretion (in addition to LH) and whether the gonadotropin-releasing activity of thymulin is due to a ligand-specific, second messenger–mediated action, we undertook to study the mechanism of action of thymulin *in vitro.* Also, it was of interest to investigate whether the chronic hypothymic condition of old rats would be associated with altered gonadotropin responses to thymulin. The present report describes our findings.

Methods

Test Substances and Antibodies

Ionophore A23187, EGTA, caffeine, and rat gonadotropin-releasing hormone (GnRH) were purchased from Sigma Chemical Company, St. Louis, MO, USA. FTS was synthesized by Laboratoires Choay, Paris, France. A specific rabbit antiserum against thymulin, raised and previously characterized, was used (21).

Preparation of Rat Median Eminence Extracts

Young animals were sacrificed by decapitation, their median eminence (ME) dissected, and homogenized in 1 mL

0.05M HCl plus 1% *w/v* ascorbic acid per ME. After thorough homogenization, the extract was frozen and thawed three times and then submitted to three 20-second sonication bursts, with 10-second intervals between bursts. Finally, the homogenate was centrifuged at 10,000 *g* for 15 minutes, and the clear supernatant, considered as undiluted, was aliquoted and stored at −70°C until use.

Cultures of TEC and Preparation of Supernatants

The BALB/c mouse and Wistar rat TEC lines, IT-76M1 and IT-45R1, respectively, used in this study, were spontaneously obtained after continuous cultures of thymic stromal cells. The epithelial nature of these TEC lines was demonstrated by the presence of cytokeratin filaments and desmosomes (22,23). After trypsinization, all cultures were settled in RPMI 1640, pH 7.2, supplemented with 5% fetal calf serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin, at 37°C in an atmosphere containing 5% CO_2 and incubated for 7 days. Twenty-four hours before supernatant collection, culture medium was removed and replaced by serum-free medium. Supernatants were collected and frozen at −20°C until use.

Animals

Young (4 months), adult (10 months), and senescent (29–33 months) female Sprague-Dawley rats, raised and kept in our animal breeding facilities, were used. Animals were housed in a temperature-controlled room (22 ± 2°C) on a 14:10 hour light/dark cycle. Food and water were available ad libitum.

Cell Dispersion

Animals were sacrificed by decapitation and their anterior pituitaries (AP) rapidly dissected. Ten to 12 AP were cut into 8 to 10 pieces each with a razor blade and placed together in a Petri dish where they were washed twice with Earle's Balanced Salt Solution (EBSS) containing 1 g/L glucose, 1 g/L $NaCO_3H$, 0.5% bovine serum albumin, 30 μg/mL ascorbic acid, 1.8×10^{-3} M $CaCl_2$ and 50 IU/mL aprotinin (perifusion medium, PM). The pieces were transferred to a plastic tube containing 10 mL PM (without aprotinin) with 30 mg collagenase type IV and 1 mg DNase type I (Sigma). After 1 hour's incubation at 37°C under constant shaking, the cell suspension was repeatedly flushed with a Pasteur pipette to complete the dispersion process. The suspension was centrifuged at 500 *g* for 20 minutes at 4°C. The cell pellet was gently resuspended in 4 mL PM, an aliquot was mixed with an equal volume of 0.4% Trypan Blue in saline, and the mixture used for cell viability assessment. Cell viability ranged from 87 to 95% and was similar for young and senescent animals. Another aliquot was homogenized and used to determine cell DNA content by a microfluorometric method using *bis*-benzimidazole (Hoechst H33258) as a fluorophor (24).

Cell Perifusion

Dispersed AP cells obtained as described above were packed with Biogel P-2 into short columns kept in a water bath at 37°C and continuously perifused with PM. The substances to be tested (stimuli) were dissolved in PM and, at appropriate times, a pulse of 2.5 mL per stimulus was pumped through the perifusion circuit, at the end of which 1.5-mL fractions were collected with two synchronized fraction collectors every 2 minutes. ME extract was routinely used at the beginning and at the end of each experiment as a system performance control. The quantity of LH or FSH released by each stimulus was assessed by radioimmunoassay (RIA) with the materials obtained through the National Hormone and Pituitary Program (NHPP), NIDDK, NICHD, USDA. Hormone secretion was expressed as ng LH or FSH/μg cell DNA.

Cell Incubation

Dispersed AP cells were obtained as described above but from six to seven AP per experiment, and were placed into Eppendorf tubes (200 μL/tube). The tubes were placed in a metabolic incubator and preincubated at 37°C for 20 minutes under continuous shaking. At the end of the preincubation period, the tubes were centrifuged at 1,000 *g* for 2 minutes. Pellets were gently resuspended in PM containing the appropriate concentration of the different stimuli (tested in duplicates). Cells were incubated for the indicated times and centrifuged at 1,000 *g* for 2 minutes. Gonadotropins were determined in supernatants.

Statistics

Assessment of the level of significance of differences between stimulated and basal levels of gonadotropins released by AP cells was carried out by one-way analysis of variance (ANOVA). When more than one age group was studied, data were assessed by two-way ANOVA, taking "age" as factor A and "stimulus" as factor B. When appropriate, ANOVAs were followed by the Duncan's multiple range test to assess the significance of differences between means. A p value lower than .05 was considered to be significant in all cases.

Results

Dynamic Studies

A perifusion system was first used in order to evaluate AP cell response to test substances under conditions (continuous flux of medium and short secretagogue pulses) that minimize paracrine influences on gonadotrophic cells. Median eminence extract was used in the perifusion experiments as a reference secretagogue in order to check that AP cells at the beginning and at the end of the perifusion experiments had comparable responses to a strong physiological stimulus. A 2 × 9 two-way ANOVA for each hormone revealed that basal LH and FSH secretion of AP cells from senescent rats was lower than that of their young counterparts ($p < .01$ for factor Age in both ANOVAs; for factor Stimulus, $p < .01$ and $< .05$ for LH and FSH, respectively; for interaction, $p > .05$ and $< .05$ for LH and FSH, respectively; also see Figure 1). Thymulin stimulated LH but not FSH release at 10^{-6} M ($p < .05$ [Duncan's test]) and 10^{-5} M ($p < .01$), whereas its separate components, FTS (10^{-5} M) and $ZnCl_2$ (0.86 μg/mL), were inactive by themselves. TEC supernatants from both rat and mouse origin were also effective in stimulating LH ($p < .01$), but not FSH release. The gonadotropin secretory responses to ME extracts run through

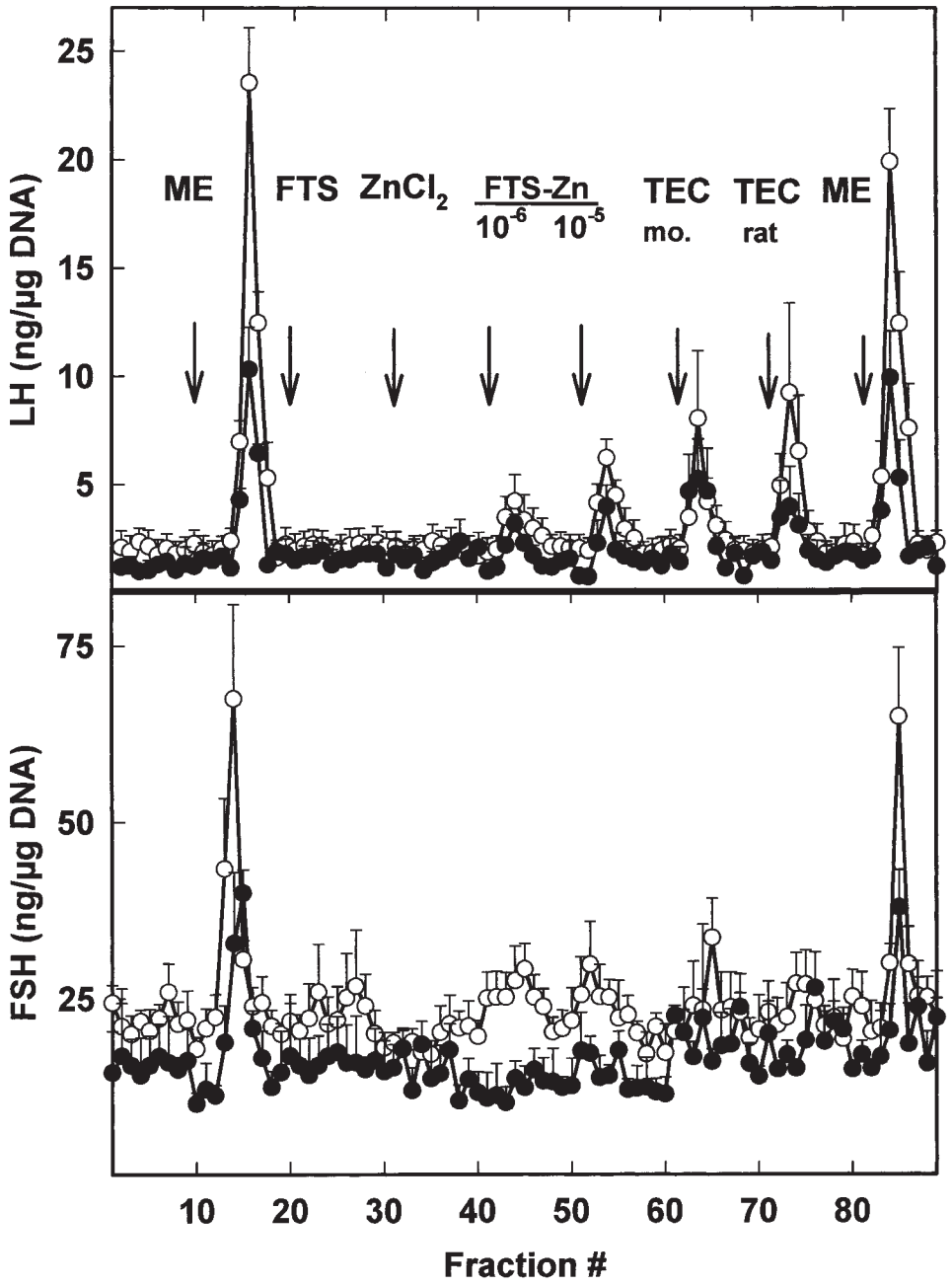

Figure 1. Profiles of LH (upper) and FSH (lower) release in perifusates of pituitary cells from young and senescent female rats. Cells were perifused with 10^{-5} M FTS, 0.86 µg/mL $ZnCl_2$, the indicated doses of thymulin, undiluted mouse and rat thymic epithelial cell (TEC) supernatants, and median eminence (ME) extract 1/30 *v/v*; the latter used as a positive control. Arrows indicate the point at which the corresponding stimuli were applied. Data points and bars represent the mean ± *SEM* of 12 and 6 perifusion experiments for young and senescent pituitary donors, respectively. (○—○) young group; (●—●) senescent group.

the columns at the beginning and at the end of the experiments were comparable. The gonadotropin responses of the cells from senescent rats were always lower than those of their young counterparts.

Time Course and Dose-Response Studies

Incubation experiments were carried out in order to expose cells for longer times than in perifusion studies to different doses of thymulin. In young rats, thymulin stimulated LH and FSH release in a time-dependent manner (data not shown). AP cells from senescent rats also showed a time-dependent response to thymulin, although the magnitude was lower than in young cells (data not shown). The dose-response pattern of gonadotropins to thymulin was bell-shaped with a maximum around 10^{-7} M in both young and senescent cells, with the latter displaying lower responses at all doses tested ($p < .01$ for factor Age in the 2×7 two-way ANOVAs for LH and FSH; also see Figure 2).

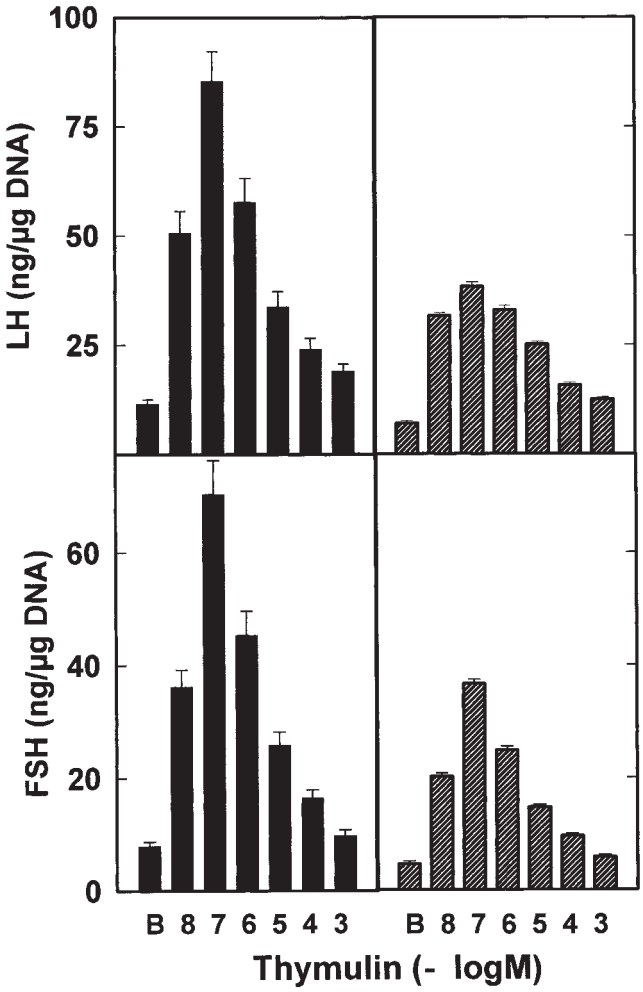

Figure 2. Dose-response profiles of thymulin concentration versus LH or FSH release after 40 minutes' incubation of young (solid bars) and senescent (hatched bars) AP cells. Data points and bars represent the mean ± *SEM* of six sets of incubation experiments. M, thymulin molarity (mol/L).

Combined Effects of Thymulin and GnRH on Gonadotropin Secretion

When AP cells obtained from young, adult, or senescent rats were incubated with thymulin plus the natural gonadotropin secretagogue GnRH, the LH responses were synergistic, whereas the FSH responses were approximately additive (Table 1). The response of both young and adult pituitary cells to the separate stimuli or their combinations were similar, but the release of LH and FSH from senescent cells was significantly diminished in all cases.

Blocking Experiments

Preincubation of thymulin or rat TEC supernatants with antithymulin rabbit serum (1/500) completely blocked the gonadotropin-releasing activity of these stimuli in young pituitary cells (Figure 3). The same results were obtained using concentrations of thymulin ranging from 10^{-8} to 10^{-3} M (data not shown). The antithymulin serum per se did not influence the basal secretion of gonadotropins, whereas normal rabbit serum failed to display any blocking activity.

Signal Transduction Pathways Involved in the Gonadotropin-Releasing Activity of Thymulin

The incubation of young AP cells with the calcium chelator EGTA (5 mM) completely blocked the stimulatory effect of thymulin on gonadotropin release, whereas the presence of the calcium ionophore A23187 (5 µM) had no effect on thymulin-stimulated gonadotropin secretion (Figure 4). On the other hand, LiCl, an inhibitor of inositol phosphate phosphatases, did not affect gonadotropin release per se (data not shown), but was able to potentiate the stimulatory effect of thymulin on gonadotropin release. The protein kinase C inhibitor trifluoperazine (TFP) did not modify the thymulin-stimulated release of gonadotropins (Figure 4).

Caffeine and NaF, two intracellular cAMP enhancers which act at proximal and distal sites, respectively, of the cAMP pathway, did not affect, per se, gonadotropin release (data not shown), but potentiated the stimulatory effect of thymulin on LH and FSH secretion (Figure 4). The adenylate cyclase activator forskolin, but not its inactive derivative 1,9-dideoxyforskolin, increased gonadotropin release in nonstimulated AP cells (data not shown). Forskolin potentiated the stimulatory effect of thymulin on gonadotropin release (Figure 4).

DISCUSSION

The present study documents that the metallopeptide thymulin possesses gonadotropin-releasing activity on AP cells in vitro under dynamic and stationary conditions. In perifusion, the clearance rate is equal to the flow rate of the system and is the same for all secreted products. Therefore, there is no influence of cell-secreted substances (25). Paracrine interactions appear at flow rates of 1–5 mL/h (26), which is much slower than the flow rate used in our perifusion model (0.7–0.8 mL/min). It is therefore unlikely that the gonadotropin-releasing activity of thymulin and TEC supernatants on our perifused AP cell preparations may have been exerted through paracrine influences on gonadotrophs by factors released from nongonadotrophic cells in response to thymulin. Our findings are in line with a previous study in which thymulin was reported to stimulate LH release from perifused rat pituitary fragments. The glands responded in a dose-dependent manner with a maximal LH release at a thymulin concentration of 10^{-5} M and a minimal effective dose around 10^{-7} M thymulin (11). Although these doses are substantially higher than normal levels of circulating thymulin, approximately 70×10^{-14} M and 6×10^{-14} M in young and old rats, respectively, as determined by bioassay (16), it is interesting to note that TEC supernatants, which possess physiological concentrations of thymulin, were also effective in stimulating gonadotropin secretion. One possible explanation for this apparent inconsistency is that TEC may co-secrete factors that act synergistically with thymulin. Also, it is important to take into account that while the bioassay most likely measures free (bioactive) thymulin, which represents a very small fraction of total circulating thymulin, our data represent total thymulin, most of which is probably bound to albumin. In any case, it is un-

Table 1. Combined Effect of Thymulin With GnRH on LH and FSH Release by AP Cells From Female Rats of Different Ages

Hormone	Stimuli	Young (6)	Middle age (8)	Senescent (6)
LH	Medium (basal)	4.2 ± 0.8	3.6 ± 0.6	1.5 ± 0.3*
	ME extract	151.3 ± 11.8^{+}	160.0 ± 7.1^{+}	35.7 ± 3.2**$^{+}$
	GnRH	76.8 ± 3.5^{+}	71.5 ± 4.1^{+}	18.9 ± 1.8**$^{+}$
	Thymulin	59.3 ± 3.6^{+}	62.9 ± 3.1^{+}	14.3 ± 2.3**$^{+}$
	Thymulin plus GnRH	197.1 ± 9.0^{+}	191.4 ± 6.2^{+}	48.7 ± 3.4**$^{+}$
FSH	Medium (basal)	2.6 ± 0.5	2.1 ± 0.3	1.1 ± 0.2*
	ME extract	37.3 ± 4.2^{+}	35.8 ± 3.5^{+}	6.8 ± 0.3**$^{+}$
	GnRH	20.1 ± 2.8^{+}	22.6 ± 2.5^{+}	4.9 ± 0.8**$^{+}$
	Thymulin	17.8 ± 2.1^{+}	18.4 ± 1.9^{+}	4.4 ± 0.4**$^{+}$
	Thymulin plus GnRH	36.1 ± 4.4^{+}	35.7 ± 3.8^{+}	8.8 ± 0.5**$^{+}$

Notes: Numbers in parentheses represent the *n* value of each group. The numeric values represent the amount of either LH or FSH (mean ± SEM), in ng/μg cell DNA, released by the pituitary cells incubated for 40 minutes. For each gonadotropin, data were analyzed using a 3 × 5 two-way ANOVA followed by Duncan's multiple range test. Raw data were subjected to logarithmic transformation to achieve homogeneity of the variance.

$^{+}p < 0.05$; $^{+}p < .01$, when compared to their basal level.

$*p < .05$; $**p < .01$, when each stimulus is compared with the young counterparts.

likely that thymulin is a major stimulator of gonadotropin secretion because if so, a self-potentiating stimulatory loop would exist between thymulin and gonadotropins. A modulatory role of thymulin on gonadotropin secretion appears as a more plausible alternative.

The fact that FTS or Zn^{2+}, separately, were inactive is in line with previous studies showing that FTS requires Zn^{2+} to exert its biological effect on lymphocytes (3). The relative gonadotrophic cell number, but not the secretory activity, declines with age in Sprague-Dawley rats (27). Therefore, the lower LH responses to thymulin of the perifused AP cells from senescent animals, as compared to their young counterparts, is probably due to a reduced proportion of gonadotrophic cells in these preparations. On the other hand, perifused FSH cells appeared to be unresponsive to thymulin irrespective of the age of the donors. However, it should be pointed out that due to our small sample size, a minor sitimulatory effect of thymulin on FSH may have been missed. It also should be noted that the significance of the main 'age effect' detected by the statistical analysis does not imply that a significant difference exists at all doses.

It should be pointed out that at 10 months of age, a time at which thymus involution is significant and circulating thymulin levels have fallen markedly (28), incubated gonadotrophs showed the same response to thymulin (and to ME and GnRH) than the 4-month-old cells (Table 1). This indicates that the chronically low serum levels of thymulin to which gonadotrophic cells from adult rats were exposed did not modify their responsiveness to the thymic hormone.

Our time-course experiments showed that gonadotrophic cells responded to thymulin in a time-dependent manner at several doses and that age changed the magnitude but not the shape of this response. The same is true for the bell-shaped dose-response of gonadotropins to thymulin in incubated AP cells. Many immune-derived peptides, such as interleukins, are able to elicit bell-shaped dose-responses for the release of hypothalamic hormones (29). The facts that in the perifusion experiments the dose-response behavior of LH was logarithmic rather than bell-shaped and that FSH did not respond at all to thymulin were probably due to the lack of paracrine interactions in our perifusion system. Likewise, the observed displacement of the secretory LH curve towards higher concentrations in the perifusion system may be due to the continuous washing of the perifused cells and, consequently, to shortened interaction times of the secretagogues with their target cells.

The fact that the stimulatory effect of TEC supernatants was blocked by antibodies antithymulin indicates that most of the secretagogue activity of TEC supernatants is due to the presence of thymulin. The specificity of the action of thymulin on gonadotropin secretion becomes clear from the blocking experiments shown in Figure 3 as well as from the fact that FTS or Zn^{+2} per se were inactive on gonadotropin release. This specificity as well as the dose-dependency of the gonadotropin-releasing activity of thymulin suggest the existence of receptor sites for this metallopeptide on AP cells. In two tumor-derived human T cell lines, two types of receptor for thymulin were found. One of high affinity (K_d=3.5 nM) and another of low affinity (K_d=100 nM). No cooperative effects between receptors were detected (30,31).

The stimulation of gonadotropin release by GnRH is known to be mediated by increased intracellular calcium as well as by increased intracellular levels of diacylglycerol (DAG) and inositol triphosphate (IP_3). The latter induces a rhythmic release of calcium from an IP_3-sensitive store which is coupled to a GnRH-stimulated gonadotropin burst of exocytosis (32,33).

Our data showing a synergistic effect of thymulin with GnRH on LH release and an additive effect for FSH imply that thymulin and GnRH do not share proximal steps of their signal transduction pathways. Our pharmacological experiments with EGTA and EGTA plus the calcium ionophore A23187 indicate that the gonadotropin-releasing activity of thymulin is dependent on the presence of appropriate levels of extracellular calcium. The cation Li^{+}, known to increase the intracellular levels of inositol phosphates because of its ability to inhibit at least two inositol phosphate phosphatases, potentiated (this term is used because, whereas LiCl alone had no effect on gonadotropin release, thymulin plus LiCl had a significantly higher effect on gonadotropin release than thymulin alone) the effect of thymulin on gona-

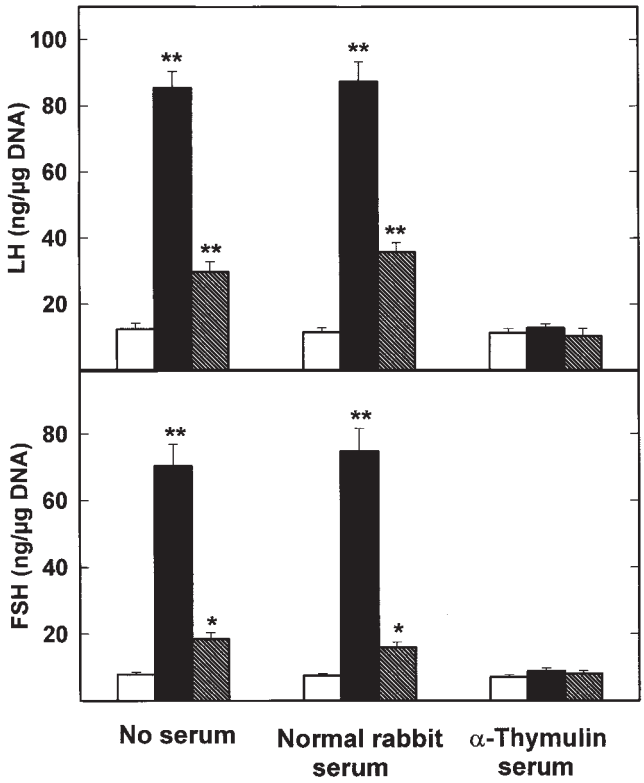

Figure 3. Immunoneutralization of the LH- and FSH-releasing activity of thymulin and TEC supernatants (upper and lower panels, respectively). Young AP cells were incubated for 40 minutes with either medium alone (open bars), 10^{-7} M thymulin (solid bars), or undiluted rat TEC supernatant (hatched bars). Stimuli were preincubated for 3 hours with the indicated sera (1/500). Data points and bars represent the mean ± *SEM* of six sets of incubation experiments. Six individual one-way ANOVAs were performed; $*p < .05$; $**p < .01$, for comparisons of stimuli with their corresponding controls (medium alone). When necessary, raw data were subjected to logarithmic transformation to achieve homogeneity of the variance.

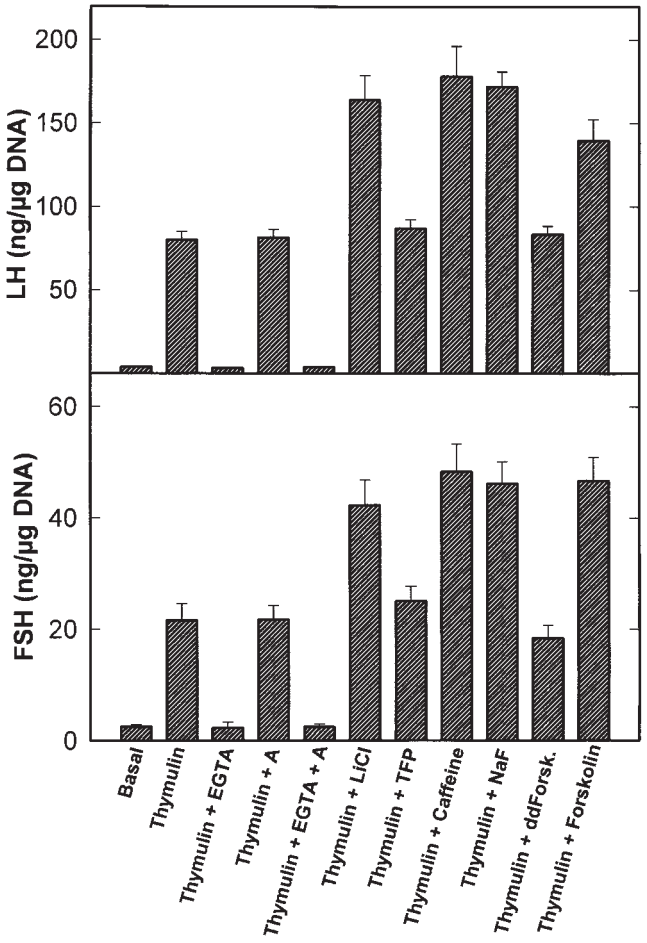

Figure 4. Effect of different pharmacologic substances on 10^{-7} M thymulin-stimulated LH and FSH release by AP cells incubated for 40 minutes in the presence of the indicated stimuli (A = ionophore A23187). LH data are shown in the upper panel whereas FSH data are shown in the lower panel. Data points and bars represent the mean ± *SEM* of six sets of incubation experiments (54 young rats). For each gonadotropin, data were analyzed using a one-way ANOVA across all conditions ($p < .001$ in both cases), followed by Duncan's multiple range test. Except for the groups involving EGTA, differences with the corresponding basals were highly significant ($p < .01$). Also, for both hormones, differences between thymulin alone and thymulin + LiCl (or caffeine or NaF or forskolin) were highly significant ($p < .01$).

dotropin release. This supports the idea that phosphoinositides are involved in the secretagogue action of thymulin. The potentiation (see above) of thymulin-stimulated release of gonadotropins induced by intracellular cAMP enhancers like caffeine, NaF, and forskolin suggests that cAMP may be involved in the signal transduction pathway activated by thymulin in AP cells and is in line with a previous report indicating that thymulin, at doses comparable to those in this report, increases intracellular cAMP in AP cells (34). In the immune system, indirect evidence has been reported suggesting that cAMP could be a second messenger of thymulin because cAMP could mimic the effect of this peptide on T lymphocytes (35).

The finding that TFP did not affect the gonadotropin response of pituitary cells to thymulin suggests that the diacylglycerol pathway is not involved in the action of this thymic hormone. Whether the action of thymulin involves other second messengers such as cGMP remains to be investigated. Nevertheless, there is indirect evidence that this nucleotide is not involved in the activation of T lymphocytes by thymic extracts (35).

Taken together, the present results suggest that the gonadotropin-releasing activity documented here for thymulin is a receptor-mediated effect which is effected in part by calcium, cAMP, and inositol phosphates. This mechanism of action of thymulin on gonadotrophs seems to be common to other pituitary cell populations which have been recently shown to be responsive to this thymic peptide (34,36,37).

Although the cloning of the gene for thymulin has remained elusive so far, it is unlikely that this molecule represents a cleavage product of some endogenous protein: a computer search of the protein database FASTA (38) which gives access to all known amino acid sequences through the protein databases Swiss-prot and NBRF/PIR, revealed that there are only eight proteins possessing any sequence in common with thymulin. None of these sequences corre-

sponds to mammalian proteins. Within this context, the hypophysiotrophic activity of thymulin probably reflects the existence of a modulatory feedback loop of the pituitary-thymic axis. This loop does not seem to be disrupted by the early onset of thymus involution. Only when pituitary cell function becomes compromised, in very old rats, does this circuit deteriorate.

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