Acetylcholine, melatonin, and potassium depolarization stimulate release of luteinizing hormone-releasing hormone from rat hypothalamus in vitro

(Hypothalamic organ culture/hypothalamic releasing factors/neurotransmitters/indolamines)

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ABSTRACT We have examined the release of radioimmunoassayable luteinizing hormone-releasing hormone (LH-RH) from fragments of rat medial basal hypothalamus. These fragments were cultured overnight in medium containing serum and then preincubated in groups of three for 10 min in medium resembling cerebrospinal fluid in its electrolyte constituents and containing bacitracin. This was followed by 30-min incubation periods during which some of the hypothalami were exposed to test substances. Potassium depolarization, effected by the addition of 56 mM potassium chloride to the incubation medium, caused a marked stimulation in LH-RH release, but only in the presence of calcium. Acetylcholine at 10 nM and the parasympathomimetic anticholinesterase agent neostigmine at 1 μM markedly stimulated LH-RH release. Hexamethonium, a nicotinic antagonist, at 1 μM abolished the acetylcholine-induced increment in LH-RH release. Melatonin, a pineal indoleamine, caused significant stimulation of LH-RH release at a concentration as low as 10 nM. Bacitracin (21 μM) was employed in all these experiments. It had no effect on LH-RH release but did prevent the degradation of LH-RH in this system. We conclude that acetylcholine and melatonin are capable of inducing LH-RH release from the rat medial basal hypothalamus. These actions may account for some of the progonadotropic properties previously ascribed to these agents.

Although the control of hypothalamic luteinizing hormone-releasing hormone (LH-RH) secretion is complex and as yet incompletely understood, there is accumulating evidence that neuromodulators and neurotransmitters play a major role in this regulatory process. For example, prostaglandins (1–3), catecholamines (4–6), and opioid peptides (7) have documented effects on LH-RH secretion. In addition, acetylcholine (AcCho) (8) and melatonin (9) as well as their degradative enzymes (10, 11) are present in the hypothalamus, suggesting physiological roles at this site. AcCho has been reported to influence the release of both somatostatin (12) and corticotropin-releasing factor (13) from the hypothalamus. Atropine, an anticholinergic, when administered systemically in large doses, abolishes ovulation in rats (14). This finding was confirmed after intracerebral injection of atropine (15). Subsequently, it has been shown that AcCho stimulated gonadotropin release in vivo and in vitro through mechanisms dependent on the hypothalamus (16). In studies with short-term pituitary culture, AcCho stimulated gonadotropin secretion only when hypothalamic segments were coincubated in the medium (17). These data point to a hypothalamic site as the locus of action of AcCho's stimulatory progonadotropic effects.

The effects of melatonin on reproductive function have been examined in many animal species (18–22). The results of these studies attest to the antgonadotropic effect of melatonin. Some evidence suggests an effect at the level of the pituitary gonadotrophs (23), and direct gonadal effects may also occur (24). However, another locus of melatonin action appears to be at the level of the central nervous system (25), possibly within the hypothalamus. On the other hand, progonadotropic effects of chronically available melatonin have been demonstrated in the hamster (26), mouse (27), and rat (28). Two fairly recent reports document stimulation of LH-RH release from rat hypothalamic fragments in vitro, in response to supraphysiological concentrations of melatonin (100 μM or greater) (1, 29).

We have measured the secretion of radioimmunoassayable LH-RH from medial basal hypothalamic segments maintained in short-term organ culture. Both melatonin and AcCho at concentrations of 10 nM stimulated LH-RH release. Neostigmine, which potentiated AcCho by inhibiting cholinesterase activity, also stimulated LH-RH release, whereas hexamethonium, a ganglion-blocking anticholinergic, abolished the AcCho-induced stimulation of LH-RH secretion. Membrane depolarization (56 mM KCl) also stimulated LH-RH release via a calcium-dependent mechanism.

METHODS

Rat hypothalamic organ culture was carried out as described in studies of the release of thyrotropin-releasing hormone and somatostatin (12, 30, 31). In essence, the method entails rapid dissection of medial basal hypothalamic fragments from the brains of decapitated male 250-g Sprague–Dawley rats, under sterile conditions, and primary culture in groups of three hypothalami per dish for 24 hr in Hepes-buffered F-12 medium containing 10% heat-inactivated fetal calf serum (both obtained from GIBCO) and 21 μM bacitracin (Sigma). This was followed by a 10-min preincubation in a protein-free medium, pH 7.4, that resembles cerebrospinal fluid in its electrolyte constituents but contains 6 mM potassium and glucose at 2 mg/ml, and finally by a 30-min incubation in the fresh medium with or without test substances in a volume of 0.8 ml.

Melatonin (Sigma) was soluble in the medium at a concentration of 1 mM or less after warming and careful Vortex mixing [confirmed by melatonin radioimmunoassay of the sample (32)], or it could be dissolved in ethanol and diluted into the medium. The former method was used in these experiments because we demonstrated in pilot studies that ethanol itself at these concentrations may alter the release of LH-RH (unpublished observations). AcCho, neostigmine, hexamethonium, atropine (Sigma), and potassium chloride were all soluble in the medium, and the experimental design was slightly modified during these

Abbreviations: LH-RH, luteinizing hormone-releasing hormone; AcCho, acetylcholine.
experiments. After preincubation for 10 min, the hypothalamic segments were incubated for two successive 30-min periods and test substances were added for the duration of the second incubation (12, 31). The ratios of immunoreactive LH-RH released during the second and first incubation periods in test groups were compared to the same ratios in control groups (which were incubated twice in plain medium). Experimental samples were immediately frozen and stored at −20°C at the conclusion of each experiment until assayed for LH-RH. LH-RH was radioimmunoassayed by the methods of Nett et al. (33) with a specific antibody kindly provided by them. Synthetic LH-RH was purchased from Calbiochem and used as the standard as well as for iodination by the chloramine-T method. The lower limit of sensitivity of the assay was 0.5 pg per tube and the useful range was generally between 2 and 32 pg. Experimental results have been expressed as the mean ± SEM. Statistical significance for all experiments was determined by Student's t test after arc sine transformation of those values expressed as percentage of control and further corroborated by a comparison of the actual amounts of LH-RH released among the various treatments by one-way analysis of variance followed by Duncan's new multiple range test (34).

RESULTS

Methodological Results. Authenticity of radioimmunoassayable LH-RH is suggested by immunological criteria. The high degree of specificity of this antibody has been demonstrated (33). Subsequently, it has been documented that the antibody reacts specifically only with the complete intact LH-RH molecule and exhibits no measurable crossreaction with fragments of the LH-RH molecule that may present in vivo (35). We have shown that there was no interference with the antigen–antibody reaction by the experimental medium. In addition, there was parallelism of several dilutions of immunoreactive LH-RH present in the experimental medium in which hypothalami had been incubated (released by hypothalamic fragments in vitro) with serial dilutions of both synthetic LH-RH and hypothalamic extract that was prepared in our laboratory. This appearance of parallelism was confirmed by logit-log transformations of the data and statistical comparison of the resultant regression lines (34, 36). In order to exclude an immunological artifact related to melatonin, AcCho, neostigmine, hexamethonium, or potassium chloride, we also examined the effect of these agents upon the LH-RH standard curve. In the presence of concentrations of these substances that were used in this study, the LH-RH standard curves were superimposable on those obtained in their absence. Basal LH-RH release in this group of experiments was well within the range of sensitivity of this assay: mean ± SEM values for consecutive control incubations were 40 ± 3.6 pg per three hypothalami per 30 min (n = 29).

In light of reports that bacitracin stabilizes or prevents the degradation of LH-RH (37), this compound was employed during these experiments. We examined the effect of bacitracin on the stability of exogenous LH-RH at 37°C in medium in which hypothalamic fragments had been previously incubated. In the presence of 21 μM bacitracin, exogenous LH-RH was stable and fully recoverable from the medium at 10, 20, and 30 min. In the absence of bacitracin but in the presence of either 10 nM AcCho or 10 nM melatonin less than 20% of exogenous LH-RH was recoverable at these times (Fig. 1).

These findings suggest that the determination of LH-RH concentrations that form the basis of this communication are not falsely increased due to stabilization or prevention of LH-RH degradation by melatonin, AcCho, or both.

Experimental Results. Potassium depolarization (56 mM KCl) markedly stimulated LH-RH release to 254 ± 32% of control, a statistically significant change. When calcium (normally 1.45 mM) was omitted from the experimental medium, release of LH-RH did not differ significantly from control values (Fig. 2) either in regular medium or in medium containing 56 mM KCl.

Effects of AcCho. AcCho at 10 nM significantly stimulated LH-RH release to 233 ± 21% of control, whereas 1 μM likewise augmented LH-RH release to 221 ± 48% of control, as shown in Fig. 3. Significant stimulation was also observed with 100 nM AcCho, to 194 ± 39% of control, whereas no significant effect was seen with 1 nM AcCho. Also shown in this figure is the abolition of the stimulatory effect of AcCho by 1 μM hexamethonium. In contrast, 1 μM atropine did not significantly alter the stimulatory effect of 10 nM AcCho.

Effects of Melatonin. After an initial preincubation with medium for 10 min, cultured hypothalami were incubated for 30 min with medium alone (control) or with melatonin at various concentrations in the medium. As shown in Fig. 4, melatonin induced a significant increment in release of LH-RH as compared to control at concentrations of 10 nM (140 ± 15% of control) and 100 nM (177 ± 33% of control). In contrast, melatonin

![Fig. 1. Effect of melatonin, AcCho, and bacitracin on the in vitro stability of LH-RH. The stability of exogenous LH-RH in incubation medium in which hypothalami had been previously incubated was assessed at 37°C for 30 min. LH-RH was stable in the presence of 21 μM bacitracin (○) but not in the presence of 10 nM AcCho (●) or 10 nM melatonin (▲) or in medium containing no additives ().](image1)

![Fig. 2. LH-RH response to potassium depolarization. The significant stimulatory effect of 56 mM KCl on LH-RH release was seen only in the presence of calcium (1.45 mM) in the experimental medium. The bars represent mean ± SEM (n = 6 for each bar). The bar with an * above differs significantly from control (P < 0.01).](image2)
had no significant effect on LH-RH release at a concentration of 1 nM.

DISCUSSION

Membrane depolarization has been demonstrated to be a potent stimulus for hormonal release from many endocrine tissues—e.g., adrenal (38), hypothalamus (39), hypophysis (40), and pancreas (41). Potassium depolarization markedly stimulates LH-RH release from hypothalamic fragments in vitro, but only in the presence of calcium ions in the experimental medium, stressing the importance of this ion in the process of stimulus-secretion coupling. Although the exact mechanisms involved in the process remain to be elucidated, it has been shown that potassium depolarization triggers intracellular influx of calcium and sodium ions, leading eventually to the exocytosis of hormone. In addition to effects of potassium depolarization on LH-RH release we report stimulatory effects of cholinergic input and melatonin.

Our direct studies of LH-RH release from hypothalami in short-term culture suggest that AcCho’s progonadotrophic effects are indeed mediated at a hypothalamic level via stimulation of LH-RH secretion.

AcCho has been localized in many areas of the central nervous system—e.g., hypothalamus, brain stem, basal ganglia, limbic system, cerebral cortex, and cerebellum—and its distribution parallels that of its degradative enzyme, acetylcholinesterase (10). Acetylcholinesterase activity in the preoptic and suprachiasmatic area of the hypothalamus fluctuates in a cyclic fashion in relation to the estrous cycle in the rat (42). The anticholinesterase neostigmine potentiates the actions of AcCho by inhibiting cholinesterases that degrade AcCho. Our observation that neostigmine stimulates LH-RH release is compatible with a physiological role for endogenous AcCho in the control of hypothalamic hormone release.

The stimulation of LH-RH release by AcCho was blocked by 1 μM hexamethonium, a nicotinic blocker, but not by 1 μM atropine, a muscarinic blocker. The failure of atropine to block LH-RH stimulation does not provide an explanation for the ability of atropine to block ovulation. However, our findings with atropine in vitro in male rats would not be expected to be predictive of in vivo findings in females, in whom regulation of LH-RH release is obviously different and dependent upon fluctuant levels of estrogen, progesterone, and other hormones during the menstrual cycle. Moreover, despite its selectivity as an antagonist of muscarinic agents at the corresponding receptors of smooth and cardiac muscle and exocrine gland cells, atropine under appropriate experimental conditions may also block responses to histamine, serotonin, and norepinephrine (43). Indeed, with regard to the central nervous system, atropine has been shown to depress the effects of noncholinergic stimuli applied locally to the cerebral cortex and spinal cord. These findings clearly indicate that the drug has central actions other than blocking cholinergic synapses (44, 45).

We have previously reported observations that atropine does block AcCho’s effects on somatostatin release (12), suggesting a muscarinic site of action, whereas others have found that corticotropin-releasing factor secretion (13), which is stimulated by AcCho, is blocked only partially by atropine but is blocked more completely by the nicotinic blocker hexamethonium. In aggregate these findings are intriguing and point to an important role for AcCho in hypothalamic hormone release. However, further studies are necessary to clarify the precise site and mode of these actions of AcCho, particularly in light of the caveats previously raised with regard to selectivity of antagonism of atropine in particular and cholinergic agents in general in the central nervous system.

With regard to melatonin, our findings are qualitatively similar to those of Kao and Weisz (29) and Bigdeli and Snyder (1), but we discerned a significant response at 10 nM, a concentration roughly of the same order of magnitude as peak levels of endogenous melatonin in the plasma and cerebrospinal fluid (46). Both these authors reported stimulatory effects of melatonin only when employed at supraphysiological concentrations of 0.1 mM or greater. * The former group also observed no response of LH-RH secretion to AcCho between 0.1 and 100 mM. Direct comparisons between the results of the three groups are difficult because of significant methodological differences. Thus, Kao and Weisz utilized a perfusion system and did not add bacitracin to prevent LH-RH degradation. Moreover, both other groups employed larger hypothalamic fragments, a procedure that might accentuate diffusion barriers inherent in the organ culture system. The differences might tend to obscure potential stimulatory responses in the earlier studies at lower concentrations of added melatonin. Our observations are important because they suggest that melatonin, perhaps directly or via other neurotransmitters or modulators, but at any rate
at relatively physiological concentrations, acts at the level of the hypothalamus to stimulate LH-RH release. It is of interest that no negative effects of melatonin on the hypothalamus have been observed by us or previous investigators. These findings are compatible with mediation of the antigonadotropic effects of melatonin at a pituitary or gonadal level as previously reported (23, 24). Definitive assessment of the physiological relevance of these observations, obtained in organ culture of rat hypothalamus, is clearly premature, particularly in the light of findings that melatonin can exert pro- or anti-gonadotropic effects in hamsters, depending upon the dose and timing of administration, and must await further in vitro and in vivo studies in male and female animals of different species and at different times of day. However, our findings do raise the possibility that both AcCho and melatonin may exert a physiologically relevant stimulatory effect on LH-RH release, specifically at a hypothalamic level, and that these actions may mediate the progonadotropic effects of the neuroactive factors.

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