Multiple Feedback Regulatory Loops upon Rat Hypothalamic Corticotropin-releasing Hormone Secretion

Potential Clinical Implications

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Abstract

To examine whether the hypothalamic corticotropin-releasing hormone (CRH) neuron is regulated by CRH, by products of the proopiomelanocortin (POMC) gene, and/or by glucocorticoids, we used a rat hypothalamic organ culture system in which rat CRH secretion from single explanted hypothalami was evaluated by an RIA (iCRH) specific for rat CRH. The effects of graded concentrations of ovine CRH (oCRH), adrenocorticotropin hormone (ACTH), β-endorphin (β-EP), α-melanocyte-stimulating hormone (α-MSH), corticotropin-like intermediate lobe peptide (CLIP), ovine β-lipotropin (ovine β-LPH), and dexamethasone (DEX) upon unstimulated and serotonin- (5HT), acetylcholine- (ACh), and norepinephrine- (NE) stimulated CRH secretion were determined. oCRH and DEX inhibited unstimulated iCRH secretion with ID_{50} at the 10^{-8} M range. ACTH had no detectable suppressive effect at 10^{-8} M. oCRH, ACTH, and DEX inhibited 5HT-, ACh-, and NE-stimulated iCRH secretion in a dose-dependent fashion. β-EP, α-MSH, and CLIP also inhibited 5HT-induced iCRH secretion. Of the latter peptides, the strongest inhibitor was β-EP and the weakest was CLIP. Ovine β-LPH had only a weak inhibitory effect on 5HT-induced iCRH secretion. Generally, the concentrations required for 50% suppression of neurotransmitter-stimulated iCRH secretion were significantly lower than those required for a similar suppression of unstimulated iCRH secretion.

In conclusion, these data suggest the presence of multiple negative feedback loops involved in the regulation of the hypothalamic CRH neuron: an ultrashort CRH-mediated loop, a short, hypothalamic POMC-derived peptide loop, and a long, glucocorticoid-mediated negative feedback loop. The potency of these negative feedback loops may be determined by the state of activation of the CRH neuron.

Introduction

The activity of the hypothalamic-pituitary-adrenal (HPA)[1] axis is regulated by circadian and stress-related excitatory inputs, inhibitory neural modulation, and various negative feedback control loops. Ultrashort-loop feedback of hypothalamic releasing/inhibiting hormones on their own secretion was postulated by Martini and co-workers (1). Recently, such autoregulatory mechanisms were demonstrated for oxytocin, growth hormone-releasing hormone, somatostatin, and luteinizing hormone-releasing hormone (2–5). Moreover, Ono and co-workers proposed that although corticotropin-releasing hormone (CRH) exerts no ultrashort-loop negative feedback under basal conditions, it may stimulate rather than inhibit its own secretion in stress states (6). Direct evidence for a positive or a negative ultrashort CRH feedback loop would be necessary to clarify this issue.

Observations from animal studies have suggested that circulating ACTH may restrain its own secretion (7, 8). Whether this postulated ACTH autoregulation takes place directly at the pituitary level as an ultrashort negative feedback loop or is mediated via inhibition of CRH secretion from the hypothalamus, participating thus as a short negative feedback loop, remains uncertain. A direct pituitary self inhibition of ACTH has been suggested by in vitro studies using mouse pituitary tumor cells producing ACTH (9), and in a recent clinical study (10). This type of control, however, could not be demonstrated in dispersed rat anterior pituicyte cultures (11). The presence, on the other hand, of a short-loop negative feedback effect of ACTH on hypothalamic CRH secretion has been recently suggested (12, 13).

Long-loop negative feedback is exerted by glucocorticoids at the pituitary corticotroph cell, the hypothalamic CRH-secreting neuron and possibly at sites of the limbic system, like the amygdala and the hippocampus. A pituitary and a hypothalamic locus for glucocorticoid inhibition of HPA axis function have been demonstrated clearly in both in vivo and in vitro experiments (14–38).

This study was undertaken to determine whether or not basal and stimulated CRH secretion from isolated rat hypothalami is affected by CRH, products of the proopiomelanocortin (POMC) gene such as ACTH, β-endorphin (β-EP), α-melanocyte-stimulating hormone (α-MSH), corticotropin-like intermediate lobe peptide (CLIP), and ovine β-lipotropin (ovine β-LPH), or glucocorticoids. To accomplish this task, we examined the effects of each of these factors upon hypothalamic CRH secretion in vitro. The study was conducted using

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1. *Abbreviations used in this paper:* Ach, acetylcholine, α-MSH, α-melanocyte-stimulating hormone; ANOVA, analysis of variance; ARN, arcuate nucleus; AUC, area under the curve; β-EP, β-endorphin; β-LPH, β-lipotropin; CLIP, corticotropin-like intermediate lobe peptide; CRB, corticotropin-releasing bioactivity; CRH, corticotropin-releasing hormone; DEX, dexamethasone; DUN, Duncan multiple range test; 5HT, serotonin; HPA, hypothalamic-pituitary-adrenal; iCRH, radioimmunoassay for CRH; NE, norepinephrine; oCRH, ovine CRH; POMC, proopiomelanocortin; PVN, paraventricular nucleus; rCRH, rat CRH.

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a sensitive rat hypothalamic organ culture system in which CRH secretion from single explanted hypothalami was evaluated by a specific RIA for rat CRH (iCRH).

Methods

Materials. Serotonin (5HT) hydrochloride, acetylcholine (ACh) chloride, norepinephrine (NE) hydrochloride, human ACTH(1–39), α-MSH [ACTH(1–13)], CLIP [ACTH(18–39)], β-EP, and dexamethasone (DEX) were purchased from Sigma Chemical Co. (St. Louis, MO). Ovine CRH (oCRH) and rat CRH (rCRH) were purchased from Bachem, Inc. (Torrance, CA). Ovine β-LPH was a gift from Prof. C. H. Li, University of California, San Francisco.

Hypothalamic organ culture. Adult male Sprague-Dawley rats were decapitated. Using a sterile technique, we rapidly removed the hypothalami with fine-pointed curved scissors. The hypothalamic boundaries were the optic chiasm anteriorly, the mammillary bodies posteriorly, and the hypothalamic sulci laterally. The fragments were ~ 3 mm deep. Immediately after explantation, the hypothalami (one explant per incubation chamber, 48-multiwell plates, Costar Corp., Cambridge, MA) were incubated in medium 199 with modified Earle's salt (Gibco Laboratories, Grand Island, NY) containing 0.1% BSA (Sigma Chemical Co.) and 20 μmol/liter bacitracin (Aldrich Chemical Co., Milwaukee, WI), in a water-jacketed incubator at 37°C, under 5% CO_2 atmosphere. The experiments (see Experimental Protocols below) were performed after an overnight (18 h) preincubation. Hypothalami incubated overnight had iCRH content and histologic appearance, studied with both light and electron microscopies, similar to freshly explanted tissues, although they were more fragile. Cells of the paraventricular nucleus of the hypothalami preincubated overnight excluded trypan blue dye. Detailed biological and histologic studies on freshly explanted and overnight preincubated hypothalami have been previously reported (39).

After each experiment (see Experimental Protocols below), we indirectly confirmed tissue viability by exposing each hypothalamus to a depolarizing concentration of potassium chloride (60 mmol/liter KCl) for 20 min. Hypothalami that failed to respond to KCl with an increase of iCRH secretion of at least 90% (~ 10 intraassay coefficients of variation at the level of the unstimulated release) above baseline were excluded from analysis. Generally, ~ 20% of hypothalami fail to respond to KCl and are excluded from the analysis.

Experimental protocols. Two different experimental protocols were used for this study. Protocol 1 was designed to evaluate the effects of oCRH, ACTH, and DEX on unstimulated hypothalamic iCRH secretion. For this purpose, the explanted hypothalami (one hypothalamus per well) were serially incubated in 13 wells for 20 min per well, for a total period of 260 min. The hypothalami were transferred from one well to another using a 3 × 3 mm nylon mesh grid (Small Parts, Inc., Miami, FL). The hypothalami were incubated in plain medium in the first six wells to evaluate basal iCRH secretion, whereas they were exposed to either placebo or different concentrations of oCRH, ACTH, or DEX in the subsequent six wells. Each hypothalamus was exposed to KCl in the final well for 20 min.

Protocol 2 was designed to evaluate the effects of oCRH, POMC gene-derived peptides, and DEX on neurotransmitter-stimulated iCRH secretion. For this purpose, the hypothalami (one hypothalamus per well) were serially incubated in six wells for 20 min per well, for a total period of 120 min. Placebo or different concentrations of oCRH, ACTH, β-EP, α-MSH, CLIP, ovine β-LPH, or DEX were added in the medium of incubation during the last hour of preincubation and throughout the experiment. 2 h after exposure to the above mentioned substances, the hypothalami were stimulated with 10^{-9} M of 5HT, ACh, or NE (wells 4 and 5). The mean iCRH concentration in wells 1, 2, and 3 was used as basal secretion for a given hypothalamus. The mean iCRH concentration in wells 4 and 5 was used as stimulated secretion. Hypothalamic responsivity to KCl was tested in well 6.

rCRH radioimmunoassay. The concentration of CRH in the medium of incubation was measured directly without prior extraction, by RIA. We used two different CRH antisera (TS-3A and TS-3B) obtained from rabbits immunized with synthetic rCRH conjugated to BSA by the carbodiimide reaction (40). ^{125}I-rCRH, prepared by the chloramine T method (41), was stored at 4°C for up to 8 wk. An appropriate aliquot was purified through a 0.9 × 58-cm column of Sephadex G-50 fine (Pharmacia Fine Chemicals, Piscataway, NJ), before use.

100-μl aliquots of rCRH standard solutions or media samples were incubated for 48 h at 4°C with TS-3A antiserum or with TS-3B antiserum when the media sample contained added oCRH. TS-3A and TS-3B antisera were used in final dilutions of 1:18,000 and 1:48,000, respectively. After 48 h, 100 μl of [^{125}I]-rCRH (3,000–3,200 cpm) was added and left to incubate for 24 h. Separation of bound labeled rCRH from free hormone was achieved by incubating each tube for 12–16 h at 4°C with 50 μl of goat anti-rabbit serum, diluted 1:5 with assay buffer containing 1% BSA. The tubes were centrifuged at 2,000 *g* for 20 min. The supernatants were aspirated and the precipitates were counted in a gamma counter. Standards were measured in triplicate and samples in duplicate. Total and nonspecific binding were 32±3 (±SE) and 1.9±0.1% for TS-3A and 36±4 and 2.1±0.1% for TS-3B, respectively. Sensitivity (ED_{90}) was 2.0±0.1 pg/tube (20 pg/ml of media) for the assays using TS-3A and 2.8±0.2 pg/tube (28 pg/ml of media) for the assays using TS-3B. The intraassay and the interassay coefficients of variation were 8.1 and 17.2%, respectively, for TS-3A antiserum and 7.2 and 15.1%, respectively for TS-3B antiserum.

TS-3A and TS-3B antisera showed no significant cross-reactivity (< .01%) with the following peptides: luteinizing hormone-releasing hormone, growth hormone-releasing hormone, thyrotropin-releasing hormone, somatostatin, substance P, porcine vasoactive intestinal peptide, neuropeptide Y, hACTH(1–39), β-EP, α-MSH, β-MSH, CLIP, dynorpin(1–17), arginine vasopressin, and oxytocin. In addition, TS-3B showed no cross-reactivity (< 0.001%) with oCRH.

Analysis of data. The results are expressed as mean±SE throughout the study. The total area under the curve (AUC) was calculated by summing the amount of iCRH secreted in wells 1–6 (AUC_{1-120}) and in the wells 7–12 ($AUC_{121-240}$). Ratios between AUCs were obtained dividing the $AUC_{121-240}$ by the AUC_{1-120}. Δ-AUC were calculated as a difference between the $AUC_{121-240}$ and the AUC_{1-120}. The effects of all the peptides and DEX on the stimulated iCRH secretion were calculated as percent iCRH increase above baseline for each hypothalamus, applying the following formula: ΔCRH(%) = [(Stimulated iCRH concentration)/(unstimulated iCRH concentration) − 1] × 100.

Statistical evaluation was performed using one-way analysis of variance (ANOVA) followed by Duncan multiple range test (DUN). Logarithmic transformation of the data, applied to correct for heteroscedasticity (detected by the Bartlett test), was performed for data on neurotransmitter-stimulated iCRH secretion before ANOVA and DUN analyses. Significance was accepted at a P value < 0.05. For substances that caused a significant inhibition of iCRH secretion, the mean±SE concentration producing ID_{50} was calculated using the four-parameter logistic equation. A computer program "ALLFIT" developed by DeLean, Munson, and Rodbard was used for the computations (42).

Results

Mean (±SE) unstimulated iCRH secretion was 36±2 pg/0.4 ml/hypothalamus per 20 min (n = 32). iCRH secretion in plain medium was constant for 180–200 min of incubation, whereas there was a gradual increase thereafter. Accordingly, the total iCRH $AUC_{121-240}$ was about threefold greater than AUC_{1-120}. The effects of different concentrations of oCRH, ACTH, and DEX upon the unstimulated iCRH secretion are shown in Fig. 1. The AUC_{1-120} secreted in plain medium

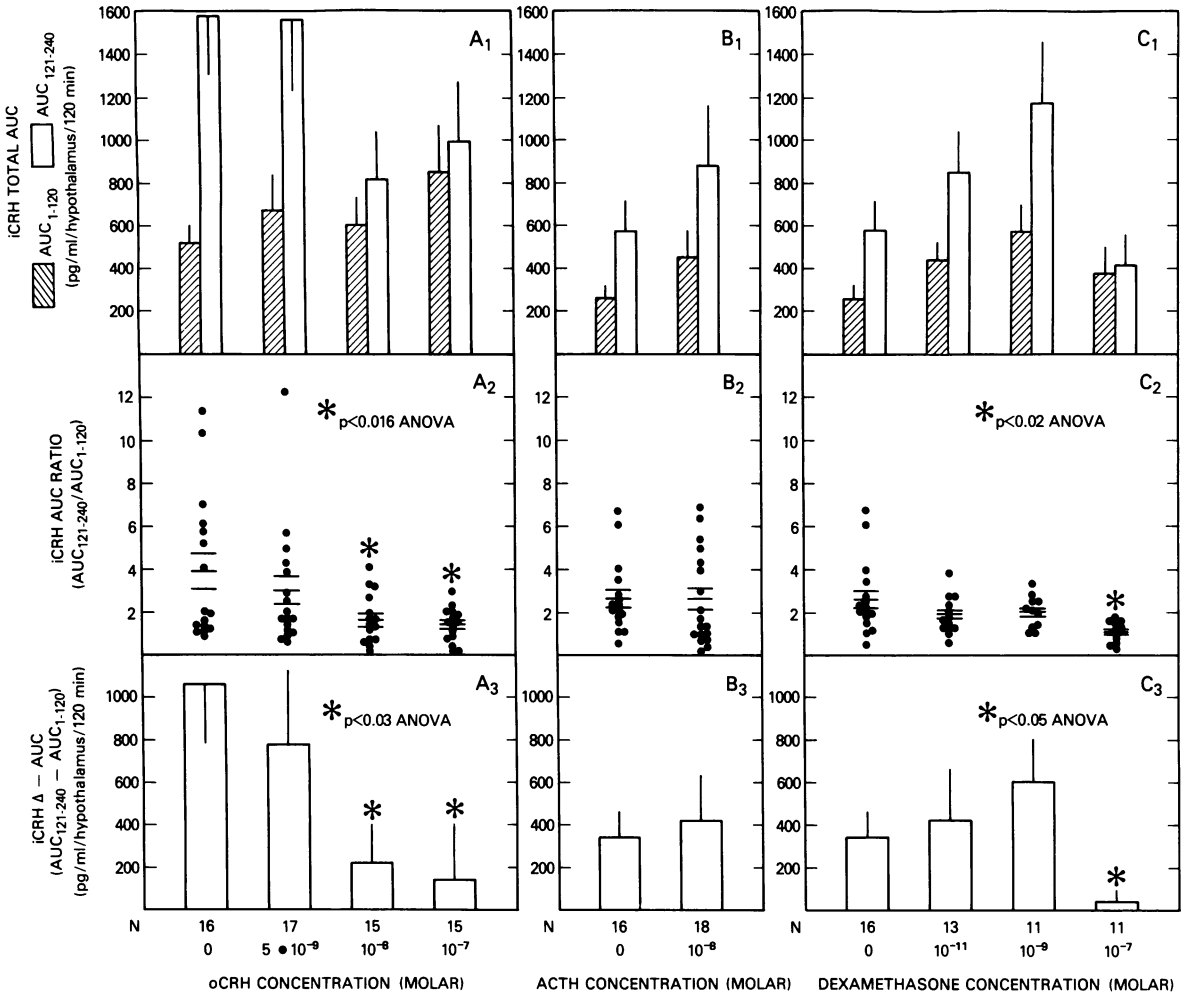

Figure 1. Effects of different concentrations of oCRH (A_1, A_2, and A_3), ACTH (B_1, B_2, and B_3), and dexamethasone (C_1, C_2, and C_3) upon unstimulated iCRH secretion by single explanted rat hypothalami. Mean±SE total AUC of iCRH secreted in plain medium during the first 120 min of incubation (AUC_{1-120}; shaded bars) and the AUC of iCRH secreted during placebo or hormonal treatment in the following 120 min of incubation ($AUC_{121-240}$) are reported in the upper panels. iCRH AUC ratio ($AUC_{121-240}/AUC_{1-120}$) are reported in the middle panels (horizontal lines represent mean±SE). The lower panels report the Δ-AUC obtained as difference between $AUC_{121-240}$ and AUC_{1-120}. *$P < 0.05$ vs. control, DUN. N = number of hypothalami tested.

(shaded bars) and the $AUC_{121-240}$ secreted during incubation with placebo or different concentrations of oCRH, ACTH, or DEX (open bars) are reported in the upper panels. Ratios and differences between iCRH $AUC_{121-240}$ and iCRH AUC_{1-120} are reported in the middle and lower panels, respectively. oCRH inhibited unstimulated iCRH secretion at 10^{-8} and 10^{-7} M, as indicated by the significant reduction in the mean ratio and mean Δ-AUC ($P < 0.05$ vs. placebo, DUN; A_2 and A_3). ACTH at 10^{-8} M did not have any detectable effect on unstimulated iCRH secretion (B_2 and B_3), whereas DEX was able to inhibit unstimulated iCRH secretion only at the highest concentration tested ($P < 0.05$ vs. placebo, DUN; C_2 and C_3). Concentrations of oCRH and DEX able to reduce the basal iCRH output by 50% were 1.2 (±0.9) × 10^{-8} M and 9.9 (±5.7) × 10^{-8} M, respectively (Table I).

The effects of oCRH, ACTH, and DEX on neurotransmitter-stimulated iCRH secretion were also tested (Fig. 2). Previously conducted dose-response studies have indicated that 5HT, ACh, and NE are capable of stimulating iCRH secretion in this system, with peak effect at 10^{-9} M (39, 43, 44). 5HT (*top*), ACh (*middle*), and NE (*bottom*), each at 10^{-9} M, were used as standard stimuli in this study. oCRH inhibited 5HT- and NE-induced iCRH in a similar fashion (*A* and *C*). oCRH concentrations required to produce 50% suppression of 5HT- and NE-stimulated iCRH secretion were 1.7 (±0.5) and 2.1 (±0.1) × 10^{-12} M, respectively. On the other hand, a higher concentration of oCRH was required to produce 50% inhibition of ACh-induced iCRH secretion at 1.3 (±0.2) × 10^{-8} M (B). ACTH inhibited 5HT-, ACh-, and NE-stimulated iCRH secretion (*D, E,* and *F,* respectively). Concentrations of ACTH required to inhibit 50% of NE-induced iCRH secretion were ~ 10 times lower than those necessary to suppress 5HT- or ACh-induced iCRH secretion (3.0 [±1.0] × 10^{-10} M vs. 2.3 [±1.0] × 10^{-9} M and 2.1 [±0.6] × 10^{-9} M, respectively). DEX suppressed 5HT-, ACh-, and NE-induced iCRH secretion ($P < 0.05$ vs. control, DUN; *G, H,* and *I,* respectively). DEX ID_{50} were 3.5 (±1.8) × 10^{-12} M, 7.6 (±2.0) × 10^{-14} M, and 1.7 (±0.2) × 10^{-14} M for 5HT-, ACh-, and NE-induced iCRH secretion, respectively. These concentrations were several orders of magnitude lower than the concentrations required to suppress basal iCRH secretion by 50%.

The effects of various concentrations of β-EP, α-MSH,

Table I. oCRH, ACTH, β-EP, α-MSH, CLIP, and DEX ID_{50} (±SE) on Basal and 5HT-, ACh-, and NE-stimulated Hypothalamic iCRH Secretion

	Basal	5HT-stimulated	ACh-stimulated	NE-stimulated
oCRH	1.2 (±0.9) × 10^{-8} M	1.7 (±0.5) × 10^{-12} M	1.1 (±0.2) × 10^{-8} M	2.1 (±0.1) × 10^{-12} M
ACTH	NDE	2.3 (±1.0) × 10^{-9} M	2.1 (±0.6) × 10^{-9} M	3.0 (±1.0) × 10^{-10} M
β-EP	ND	5.7 (±2.0) × 10^{-11} M	ND	ND
α-MSH	ND	2.1 (±0.3) × 10^{-9} M	ND	ND
CLIP	ND	1.9 (±1.3) × 10^{-8} M	ND	ND
DEX	9.9 (±5.7) × 10^{-8} M	3.5 (±1.8) × 10^{-12} M	7.6 (±2.0) × 10^{-14} M	1.7 (±0.2) × 10^{-14} M

NDE, no detectable effect.

CLIP, and ovine β-LPH on 5HT-induced iCRH secretion are shown in Fig. 3. β-EP inhibited 5HT-stimulated iCRH secretion from 10^{-10} to 10^{-7} M ($P < 0.05$ vs. 5HT alone, DUN; *A*). The concentration of β-EP required to inhibit 50% of 5HT-stimulated iCRH secretion was ~ 200 times lower than that required for ACTH to inhibit 5HT-stimulated iCRH secretion by the same extent: 5.7 (±2.0) × 10^{-11} M vs. 2.3 (±1.0) × 10^{-9} M, respectively (Table I). α-MSH inhibited 5HT-stimulated iCRH secretion with a potency similar to that of ACTH: 2.1 (±0.3) × 10^{-9} M vs. 2.3 (±1.0) × 10^{-9} M, respectively (*B*), whereas CLIP was ~ 10 times less effective in causing suppression of iCRH secretion (*C*). Ovine β-LPH was a weak inhibitor of 5HT-stimulated iCRH secretion ($P < 0.016$, ANOVA; *D*).

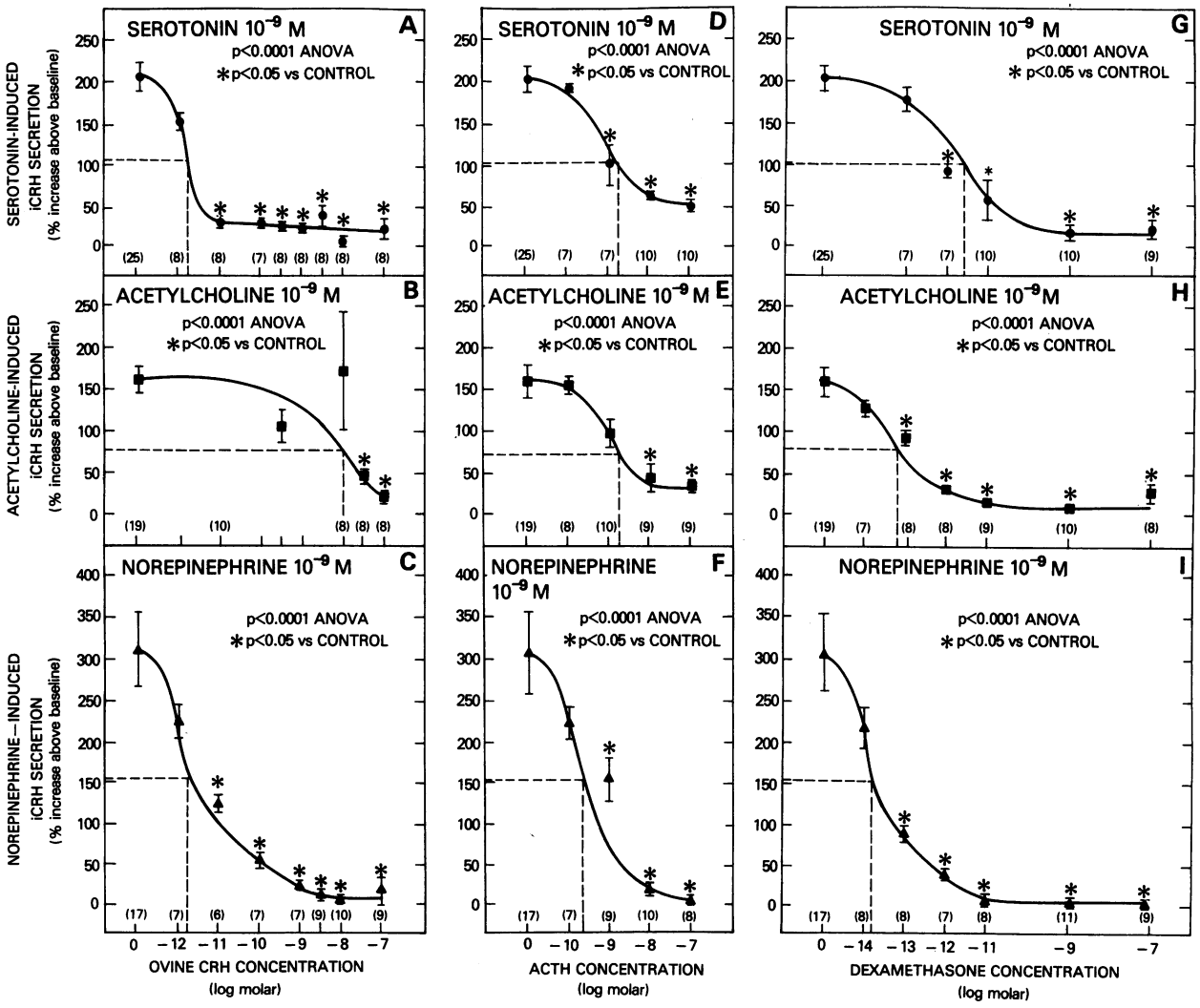

Figure 2. Effects of different concentrations of oCRH (*left*), ACTH (*center*) and DEX (*right*) on iCRH secretion by single explanted rat hypothalami stimulated by serotonin (*top*), acetylcholine (*middle*), or norepinephrine (*bottom*). Results (mean±SE) are expressed as percent increase above a baseline of 36±2 pg hypothalamus/20 min. *$P < 0.05$ vs. control, DUN. The number of hypothalami tested at each dose level is indicated in parentheses. Dotted lines intercept curves at ID_{50} level.

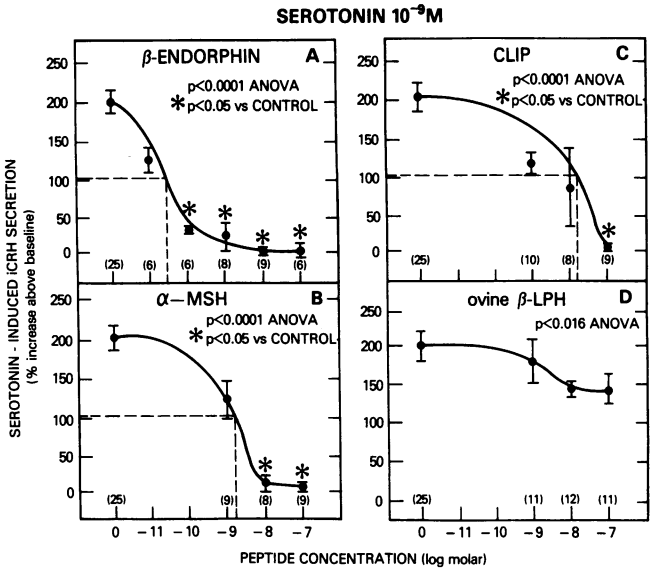

Figure 3. Effects of different concentrations of β-EP (*A*), α-MSH (*B*), CLIP (*C*), and ovine β-LPH (*D*) upon 5HT-induced iCRH secretion by single explanted rat hypothalami. Results (mean±SE) are expressed as percent increase above baseline. $^*P < 0.05$ vs. 5HT alone, DUN. The number of hypothalami tested is indicated in parentheses. Dotted lines intercept curves at ID_{50} level.

Discussion

Although the experimental procedure used in this study enabled us to evaluate the regulatory influence of several peptides and DEX on rat hypothalamic iCRH secretion in a direct and specific manner, several limitations of the system used should be mentioned. First, the CRH neurons involved in HPA axis regulation may represent a portion of the CRH neurons of the paraventricular nucleus (PVN) of the hypothalamus. CRH-stained cell bodies and fibers are present in the entire PVN as well as the suprachiasmatic nucleus, the lateral preoptic and hypothalamic areas, the dorsomedial and arcuate nuclei (ARN), and the median eminence (45). Since CRH may be released in the incubation medium from axons of diverse areas of the hypothalamus and since it is possible that some of these regions may be more or less sensitive to regulation by some or all of the substances used, one should extrapolate the findings of this study to in vivo regulation of CRH with caution. Second, the hypothalami in this study are examined 18 h after explantation, which could have resulted in altered sensitivity of the CRH neurons to stimulatory and/or inhibitory influences. This could be the result of denervation and/or demyelination. Third, basal iCRH secretion during the experiment remained stable for only 180–200 min. After this time point, CRH secretion gradually increased. This was reflected in the data on unstimulated CRH secretion, in which $AUC_{121-240}$ was considerably greater than AUC_{1-120}.

Despite the above limitations, however, our data are consistent with several older and recent in vivo and in vitro studies (46–51). Using our system, we have shown that 5HT, ACh, and NE stimulate iCRH secretion in a dose-dependent fashion with peak effect at 10^{-9} M. These effects can be antagonized by the respective antagonists and appear to be direct rather than mediated by interneurons (39, 43, 44). The stimulatory nature of 5HT and ACh has been suggested by older and recent in vivo and in vitro studies (46–48). According to our data, NE is a potent stimulatory neurotransmitter for the central component of the HPA axis (44), rather than inhibitory as previously suggested (51). This finding supports an increasing body of literature according to which the ascending noradrenergic projection to the PVN stimulates CRH secretion (49, 50).

Exogenous CRH was able to inhibit both unstimulated and neurotransmitter-stimulated iCRH secretion in vitro, a result compatible with the presence of an ultrashort negative feedback loop. 5HT- and NE-induced iCRH secretion were very sensitive to the suppressive effects of the ovine analogue of CRH, whereas suppression of basal and ACh-induced iCRH secretion required concentrations of oCRH that were four orders of magnitude higher. The concentrations of oCRH required to inhibit both unstimulated and neurotransmitter-stimulated iCRH secretion in vitro were of the same order of magnitude as those reported in the hypophysial portal blood system of stressed rats (27). Neither the source nor the site of action of endogenous CRH involved in an ultrashort-loop negative feedback are known with certainty. The median eminence that contains CRH in nerve terminals may be both a source of endogenous CRH and a target for the inhibitory effects of CRH. Compatible with the latter hypothesis is autoradiographic evidence for the presence of CRH receptors in the external zone of the median eminence (52). Alternatively and/or additionally, CRH may suppress hypothalamic CRH neuron activity by acting at the PVN level. There is electrophysiological evidence for the presence of recurrent CRH inhibitory collaterals to the PVN itself (53). Our data do not provide any evidence for the existence of a positive feedback loop of CRH on its own secretion as previously suggested (6), indicating that the in vivo effect observed by these investigators may be due to additional unknown mechanisms.

ACTH, β-EP, α-MSH, and CLIP inhibited neurotransmitter-stimulated iCRH secretion, a result compatible with the presence of a short POMC peptide-mediated negative feedback loop. Previous reported observations have indicated that both ACTH and β-EP inhibit hypothalamic CRH secretion. ACTH implanted in the median eminence of rat suppressed blood corticosterone levels (7). ACTH also was shown to inhibit unstimulated iCRH secretion in a dose-dependent fashion in vitro. This inhibitory effect was partially retained (~ 60%) by α-MSH, but not by CLIP (12). β-EP has been shown to suppress ACTH and cortisol levels in normal subject (54) and to inhibit both corticotropin-releasing bioactivity (CRB) and iCRH secretion in vitro (55, 56). The source of POMC gene-derived peptides involved in a possible short negative feedback loop in vivo has not been definitely elucidated. Pituitary ACTH, which is unable to cross the blood-brain barrier (57), might be able to suppress hypothalamic CRH secretion by acting at the median eminence, one of five brain structures not protected by the blood-brain barrier (58). Alternatively, POMC peptides of pituitary origin could be transported to the hypothalamus via retrograde blood flow. However, recent studies have questioned the existence of direct blood flow from the pituitary to the brain (59). Furthermore, the concentrations of POMC peptides in the hypophysial portal blood of adult monkeys are not affected by hypophysectomy (60), suggesting that the most likely source of these peptides is the brain (61). In this regard, it has been shown that POMC content is high in the hypothalamic ARN (62), and clinical studies also support a central origin for cerebrospinal fluid ACTH in man.

For example, our group has shown that patients with Cushing's disease manifest low to undetectable levels of cerebrospinal fluid ACTH despite high basal plasma ACTH levels (63). Recently, anatomical and functional data have been reported to suggest an interrelationship between the ARN and the PVN. Anatomically, it is well established that the ACTH-, β-EP-, and α-MSH-stained fibers found in the PVN originate from the ARN (64). Conversely, the ARN contains a small number of CRH-stained fibers, whose origin from the PVN, however, has not been conclusively shown (45). Functionally, direct application of CRH onto the ARN results in release of both ACTH and β-EP (65).

Our results suggesting the presence of a long glucocorticoid-mediated negative feedback loop are consistent with studies conducted with similar or different experimental paradigms. In this regard, it has been shown that CRB content of the hypothalamus and the sensitivity of CRB secretion to various stimuli are increased after adrenalectomy and both effects are reversed by glucocorticoid administration (29, 30, 33, 34). Furthermore, stress-induced and basal CRB content of the hypothalamus and the median eminence are suppressed by glucocorticoid administration (24–26) or by DEX implants in the median eminence (66). DEX treatment is also able to prevent the increase of CRH immunoreactivity in the hypophysial portal blood of rats undergoing hemorrhage-induced stress (27). Adrenalectomy has been shown capable to increase hypothalamic levels of prepro-CRH mRNA, although a more marked effect has been observed on pituitary POMC mRNA (31). Recently, a number of immunohistochemical studies have been done to clarify the brain site(s) of glucocorticoid action (67, 68). It seems that the PVN and the hippocampus could be the major targets for the glucocorticoid negative feedback. Local implantation of DEX in these regions, but not in the amygdala or cerebral cortex, can prevent the expected adrenalectomy-induced enhancement of CRH and vasopressin immunoreactivity in the PVN (67, 68). This conclusion is strengthened by data showing the presence of glucocorticoid receptors in the PVN and CA1 and CA2 subregions of the hippocampus (69).

The suppressive effects of each of the agents examined in this study, especially DEX, were apparently more pronounced on neurotransmitter-stimulated than on unstimulated iCRH secretion. Although it is not known whether this different sensitivity to inhibitory influences of the hypothalamic CRH neuron is present in vivo, it might be tempting to speculate that in states where the CRH neurons are activated, the brain eventually tries to shut off CRH secretion by rendering the CRH neuron more sensitive to a given level of glucocorticoid. It should be pointed out, however, that basal CRH secretion in vitro and in vivo may represent different types of secretion. Whereas the latter may be a result of stimulatory and inhibitory inputs brought to the PVN from higher central nervous system centers, the former may represent autonomous hypothalamic CRH secretion. In our in vitro system, an alternative and/or additional explanation is a possible nonspecific release of CRH from injured cells.

Our data may help explain some related clinical observations. It has long been known that injection of morphine to patients undergoing heart surgery suppresses the activity of the HPA axis (70). Our findings, showing suppression of the CRH neuron by β-EP, suggest that morphine may suppress the HPA axis via inhibition of hypothalamic CRH secretion. Although both morphine and β-EP interact with both μ and delta receptors (71), it seems that their effects on CRH secretion is μ receptor-mediated. This is supported by our findings that the specific δ agonist met-enkephalin (71) fails to inhibit 5HT-stimulated iCRH secretion (Calogero et al., unpublished information). Additional mechanisms for morphine-induced HPA axis suppression should be considered. Rittmaster et al. have shown that morphine blunts ACTH response to exogenous CRH in humans (72). This blunting could be due to suppression of endogenous CRH or vasopressin by morphine and/or to stimulation of hypothalamic factor(s) that inhibit ACTH secretion. Note that the presence of hypothalamic corticotropin-inhibiting factor(s) has been previously suggested (73). Efforts by Rittmaster et al. to show direct inhibition of rat dispersed anterior pituicytes by morphine failed to show any effect (72).

Patients with Cushing's disease develop secondary (central) adrenal insufficiency after successful transsphenoidal excision of their ACTH-secreting pituitary adenoma (74). This observation is compatible with our findings that neurotransmitter stimulation of hypothalamic CRH secretion is strongly inhibited by both ACTH and glucocorticoids. That the adrenal insufficiency observed in these patients is hypothalamic rather than pituitary is strengthened by the finding that most of these patients demonstrate a plasma ACTH response to exogenous ovine CRH (75).

A definitive role for CRH in any major disease process has not yet been elucidated. We have recently advanced several lines of data to suggest that hypercortisolism in major depression represents a defect at or above the hypothalamus, resulting in the hypersecretion of CRH. We have hypothesized that such a defect could reflect inadequate restraint of the CRH neuron by cortisol exerting its long-loop negative feedback (76). This study, showing the ultrashort negative feedback loop by CRH upon itself, as well as short negative feedback loop on CRH neurons by POMC-related peptides, further extends the list of possible feedback abnormalities that could be involved in hyperactivity of the hypothalamic CRH system.

In conclusion, our data suggest the presence of multiple negative feedback control loops involved in the regulation of hypothalamic CRH secretion. These include an ultrashort CRH-mediated loop, a short hypothalamic POMC-derived peptide loop, and a long, glucocorticoid-mediated negative feedback loop. All of these regulatory circuits could be clearly shown to inhibit neurotransmitter-stimulated CRH secretion by isolated rat hypothalami.

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