Mechanisms of Action of Corticotropin-releasing Factor and Other Regulators of Corticotropin Release in Rat Pituitary Cells*

(Received for publication, September 27, 1982)

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The role of cyclic AMP in the stimulation of corticotropin (ACTH) release by corticotropin-releasing factor (CRF), angiotensin II (AII), vasopressin (VP), and norepinephrine (NE) was examined in cultured rat anterior pituitary cells. Synthetic CRF rapidly stimulated cyclic AMP production, from 4- to 6-fold in 3 min to a maximum of 10- to 15-fold at 30 min. Stimulation of ACTH release by increasing concentrations of CRF was accompanied by a parallel increase in cyclic AMP formation, with ED₅₀ values of 0.5 and 1.3 nM CRF for ACTH and cyclic AMP, respectively. A good correlation between cyclic AMP formation and ACTH release was also found when pituitary cells were incubated with the synthetic CRF(15–41) fragment, which displayed full agonist activity on both cyclic AMP and ACTH release with about 0.1% of the potency of the intact peptide. In contrast, the CRF(21–41) and CRF(36–41) fragments were completely inactive. The other regulators were less effective stimuli of ACTH release and caused either no change in cyclic AMP (AII and VP) or a 50% decrease in cyclic AMP (NE). Addition of the phosphodiesterase inhibitor, methylisobutylxanthine, increased the sensitivity of the ACTH response to CRF but did not change the responses to AII, VP, and NE. In pituitary membranes, adenylate cyclase activity was stimulated by CRF in a dose-dependent manner with ED₅₀ of 0.28 nM, indicating that the CRF-induced elevation of cyclic AMP production in intact pituitary cells is due to increased cyclic AMP biosynthesis. The intermediate role of cyclic AMP in the stimulation of ACTH release by CRF was further indicated by the dose-related increase in cyclic AMP-dependent protein kinase activity in pituitary cells stimulated by CRF with ED₅₀ of 1.1 nM. These data demonstrate that the action of CRF on ACTH release is mediated by the adenylate cyclase-protein kinase pathway and that the sequence requirement for bioactivity includes the COOH-terminal 27 amino acid residues of the molecule. The other recognized regulators of ACTH release are less effective stimuli than CRF and do not exert their actions on the corticotroph through cyclic AMP-dependent mechanisms.

The ability of cyclic AMP to stimulate ACTH¹ release in normal pituitary cells (1) suggests that cyclic AMP could act as a second messenger for one or more of the factors known to regulate ACTH secretion. In cultured pituitary cells, ACTH release can be stimulated by a number of factors, including the recently characterized 41-residue hypothalamic peptide, CRF (2), vasopressin, angiotensin II (3–5), and norepinephrine (1, 6). Of these regulators, angiotensin II, vasopressin, and norepinephrine have been shown to exert specific actions in other tissues without stimulating cyclic AMP production. For example, each of the three hormones can increase hepatic glycogenolysis through a cyclic AMP-independent mechanism (7). Similarly, angiotensin II stimulates aldosterone production in the adrenal glomerulosa cell (6, 9) and contraction of smooth muscle cells (10) by cyclic AMP-independent mechanisms that appear to be initiated by a rise in cytosolic calcium concentration (7, 11). Thus, it is possible that the individual regulators of ACTH secretion stimulate the pituitary corticotrophs by different mechanisms that involve either cyclic AMP or calcium, according to the hormonal stimulus. The present studies were undertaken to analyze the role of cyclic AMP-dependent processes in the stimulation of ACTH release from cultured pituitary cells. The results indicate that the action of CRF on the corticotroph is mediated by cyclic AMP, whereas angiotensin II, vasopressin, and norepinephrine stimulate ACTH release by a cyclic AMP-independent mechanism and may serve as modulatory ligands in the process of corticotropin secretion.

MATERIALS AND METHODS

The 41-amino acid sequence of ovine CRF (2) and its fragments, CRF(15–41), CRF(21–41), and CRF(36–41) were synthesized on benzhydrylamine polystyrene 1% &vinyl benzene copolymer resin prepared by a modification of the method of Matsueda and Stewart (12) at a loading of 0.094 meq/g. Protecting groups used were Boc,N-tosyl imidarol (His), β-2-Cl-Z (Lys), O-Bzl (Thr and Ser), w-Bzl esters (Asp and Ghu), and N₅-tosyl (Arg). The protected amino acids were purchased from Veqa Biochemicals, Tucson, AZ, and when necessary were recrystallized until their physical constants matched literature values. DCC and all solvents were distilled trifluoroacetic acid from indole, diisopropylethylamine from CaH₂, and methylene chloride from anhydrous Na₂CO₃. Coupling was performed using symmetrical anhydrides (13) except for Boc-Glu and Boc-Asn which were coupled as their nitrinophenyl esters (14). Couplings were repeated until negative ninhydrin tests (15) were obtained. The NH₂-terminal Boc group was removed by trifluoroacetic acid containing 1% D,L-methionine before reaction of the resin peptide with HF. Peptide resins were treated with HF distilled from CoF₃ in solutions 20% v/v in anisole, 5% w/v in n,L-methionine for 45 min at ~20 °C and 45 min at 0 °C. The HF was removed in vacuo, and the peptides were extracted into 30% acetic acid and freeze-dried, treated with NH₄HCO₃ buffer to reverse N₁,O-acyl shifts, redissolved in 30% acetic acid, and passed through reverse phase C₁₈-HPLC.

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¹ The abbreviations used are: ACTH, corticotropin; CRF, corticotropin-releasing factor; Pipers, piperazine-N,N'-bis(2-thanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N'-'tetraacetic acid; Z, benzoyloxycarbonyl; Bzl, benzyl; Boc, t-butoxycarbonyl.
over a Sepharose G-25 column with 0.2 N acetic acid as eluent. Amino acid analysis of peptides obtained at this stage agreed with theory within experimental error (Table I). Final purification was accomplished by countercurrent distribution and high performance liquid chromatography.

Synthetic CRF was also purchased from Peninsula Laboratories, Inc. (San Carlos, CA). [3H]vasopressin and [3H]angiotensin II were obtained from Beckman Bioproducts, Palo Alto, CA. Norepinephrine, 8-Br-cyclic AMP, and methylisobutylxanthine were purchased from Sigma, and cholera toxin was from Schwarz/Mann.

Isolated anterior pituitary cells were prepared from 200-g female Sprague-Dawley rats by a modification of the technique of DeneY et al. (16). After 48 h of suspension culture (5,17), cells were transferred to fresh medium 199 (M. A. Bioproducts, Walkersville, MD) containing 0.2% bovine serum albumin and aprotinin (Sigma). For measurement of cyclic AMP, unless specified, incubations were performed for 10 min in the presence of 10 mM theophylline, 100 mM MgCl₂, and 1 mM cyclic AMP. The membrane preparation was separated on Dowex and alumina columns (20). Results were expressed as picomoles of cyclic AMP formed per mg of protein per min.

For determination of cyclic AMP-dependent protein kinase activity, 1-ml aliquots of pituitary cell suspension containing 8 × 10⁶ cells were incubated for 30 min at 37°C in the presence or absence of CRF, norepinephrine, vasopressin, and angiotensin II. The incubation tubes were then rapidly placed in an ice water bath at 0°C, centrifuged at 1500 g in a refrigerated centrifuge for 10 min, and the cell pellets were sonicated for 10 s in 20 mM Tris-HCl buffer, pH 7.4, containing 10 mM theophylline and 1.6 mM dithiothreitol. Protein kinase was determined as described by Huang and Robinson (21) after 3-min incubation at 30°C in a total volume of 100 µl of 25 mM Tris-HCl buffer, pH 7.4, containing 10 mM theophylline, 100 µg/ml of histone II-A (Sigma), 10 mM MgCl₂, 1 mM EGTA, 200 µM [γ-32P]ATP (1000 cpm/pmol), and 10 µl of cell sonicate. The reaction was started by the addition of the [γ-32P]ATP. After incubation, 10-µl aliquots were spotted on strips of chromatographic paper ITLC, type SG (Gelman Instrument Co.) to which 20 µl of 20% trichloroacetic acid containing 50 mM unlabeled ATP had been applied previously. By this procedure, 32P-labeled protein was precipitated at the origin, and unreacted ATP was separated by ascending chromatography with 200 mM KCl in 5% trichloroacetic acid. The radioactivity at the origin was determined in a β-scintillation counter after placing the paper in vials containing 10 ml of Aquasol (New England Nuclear). The cyclic AMP dependence of phosphorylation was assessed by the ratio of 32P incorporation obtained in the presence and absence of 5 µM cyclic AMP in the assay (activity ratio).

Maximal ACTH and cyclic AMP responses and the concentrations of the stimulators producing a 4-fold maximal response (ED₄₀) were determined by computer analysis, using a four-parameter logistic function (22). Data are expressed as the mean and S.E. of the pooled values of several experiments, and statistical analysis of the data was determined by Student's t test.

RESULTS

The kinetics of cyclic AMP production and ACTH release were first examined with concentrations of the stimulators shown in preliminary experiments to produce maximum ACTH release. As shown in Fig. 1A, CRF rapidly stimulated cyclic AMP accumulation, which was significantly increased within 3 min, the earliest time point measured (p < 0.001, n = 3) and reached a maximum at 30 min. A smaller cyclic AMP response to CRF, though equally significant, was observed when the incubation was performed in the absence of methylisobutylxanthine (not shown). Cholera toxin caused a slower cyclic AMP response, being detectable between 30 and 60 min of incubation, and reaching similar levels to those produced by CRF after 3 h. In contrast, cyclic AMP levels remained at the basal value in experiments when the cells were incubated with 10 nM angiotensin II and vasopressin (n = 2) and 1 experiment with 1 µM norepinephrine. As shown in Fig. 1A, CRF evoked an early and significant ACTH response at 5 min. In the pooled data of 3 experiments, ACTH release rose by 94.7 ± 5.7% (p < 0.001) at 5 min, followed by a linear increase in ACTH release from 90 to 180 min. Consistent with the slower cyclic AMP response to cholera toxin, ACTH release in the cells stimulated with cholera toxin was first apparent at 60 min and increased linearly between 120 and 180 min to reach levels similar to those observed during CRF stimulation. The ACTH responses to angiotensin II, vasopressin, and norepinephrine were smaller in magnitude than those elicited by CRF. Although not statistically significant, a minor ACTH response was detected in each of three experiments after 5-min incubation with the 3 stimuli. A significant increase was apparent at 60 min with norepinephrine (48.2 ± 12.3%, p < 0.05) and at 90 min with angiotensin II (28.5 ± 8.2%, p < 0.05) and vasopressin (25.9 ± 5.3, p < 0.05), increasing linearly until 180 min.

The ACTH responses of pituitary cells incubated with increasing concentrations of CRF and CRF fragments were slightly less potent than our synthetic peptide. The 15-
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Fig. 1. Time course of cyclic AMP production (A) and ACTH release (B) in rat anterior pituitary cells in response to hormonal stimuli and choleratoxin. The concentrations of CRF, angiotensin II (AII), vasopressin (VP), norepinephrine (NE), and choleratoxin (CT) are as shown in A. Points represent the mean of data from duplicate incubations in one of three similar experiments.

Fig. 2. Stimulation of ACTH release (A) and cyclic AMP production (B) by CRF and CRF fragments in cultured anterior pituitary cells. Points are the mean of data from duplicate incubations in a representative experiment.

Fig. 3. Stimulation of ACTH release (A) and cyclic AMP production (B) by CRF, angiotensin II (AII), vasopressin (VP), and norepinephrine (NE) in cultured rat anterior pituitary cells. Points are the mean of data from duplicate incubations in a representative experiment.

41 CRF fragment was 1200 times less active than CRF in stimulating ACTH release, while the 21–41 CRF and 36–41 CRF fragments were inactive at concentrations up to 10 μM. In all experiments, the stimulation of ACTH release by CRF was accompanied by parallel increases in cyclic AMP production (Fig. 2B). The maximum cyclic AMP response of 3.8 ± 0.9-fold was elicited by 13.3 ± 5.1 nM CRF, and the ED₅₀ was 1.3 ± 0.5 nM CRF (n = 3). The active 15–41 CRF fragment also stimulated cyclic AMP production, being 1400-fold less potent than CRF (n = 2). Incubation of the pituitary cells with angiotensin II and vasopressin caused much smaller but significant and dose-dependent increases in ACTH release (Fig. 3A). The maximum ACTH increment of 52.3 ± 4.8% above the basal value was produced by 300 pm angiotensin II (p < 0.001), and the ED₅₀ was 28.9 ± 7.2 pm angiotensin II (n = 4). The maximum ACTH response to vasopressin was similar to that elicited by angiotensin II (48.2 ± 8.2% above the basal value, p < 0.01), but the sensitivity of the response was lower (ED₅₀ = 1.8 ± 1.0 nM vasopressin, n = 2). In agreement with previous reports on the action of α-adrenergic agonists on ACTH release, a consistent stimulatory effect of norepinephrine on ACTH was observed only in the presence of 0.1 mM ascorbic acid. At this concentration, ascorbic acid did not alter basal ACTH release or the response to CRF. The ACTH responses to norepinephrine were variable in magnitude, ranging from 1.2- to 4.5-fold the basal value, and the mean ED₅₀ for ACTH release was 48.2 ± 1.5 nM norepinephrine (n = 4).

In contrast with the consistent increase in cyclic AMP during incubation of pituitary cells with CRF (Fig. 3B), no changes in cyclic nucleotide production were observed in the presence of increasing concentrations of angiotensin II (n = 4) and vasopressin (n = 2). In both experiments in which norepinephrine was included, the stimulation of ACTH release was accompanied by a decrease in cyclic AMP production in the presence of 1 μM norepinephrine to 47.3 ± 6.8% of the basal value. These results suggest that cyclic AMP
serves as a second messenger in the action of CRF, while angiotensin II, vasopressin, and norepinephrine increase ACTH release by a different mechanism(s). In support of this possibility, addition of the phosphodiesterase inhibitor methylisobutylxanthine to cultured pituitary cells (Fig. 4) increased basal ACTH release from 0.41 ± 0.02 to 1.1 ± 0.1 ng/10⁵ cells and increased the sensitivity of the ACTH response to CRF, with a fall in the ED₅₀ from 287.2 ± 13.1 to 97.8 ± 12.0 pm CRF (n = 3, p < 0.02, by paired t test). In the same experiments, methylisobutylxanthine did not change the sensitivity of the ACTH response to angiotensin II.

In another approach to examine the possibility that the actions of angiotensin II, vasopressin, and norepinephrine are mediated by a mechanism that differs from that of CRF, the interaction between stimuli of ACTH release was studied (Fig. 5). In each of 4 experiments, no additivity in the maximum ACTH responses to CRF, 8-Br-cyclic AMP, and choleragen was observed, whereas a completely additive effect was observed between the maximum ACTH responses to CRF and angiotensin II (n = 4), vasopressin (n = 2), and norepinephrine (n = 3).

To study the mechanism of the stimulatory action of CRF on cyclic AMP accumulation, the effect of the synthetic peptide on adenylate cyclase activity was studied in anterior pituitary homogenates. In this system, basal, fluoride- and CRF-stimulated adenylate cyclase activities were linear during incubation for up to 15 min. After 15 min adenylate cyclase activity was increased by 100% in the presence of 10 mM NaF and by 30% in the presence of 10 nM CRF (Fig. 6). A linear relationship was observed between the amount of protein added in the assay (up to 250 µg) and cyclic AMP formation from [α-³²P]ATP, under basal conditions as well as in the presence of NaF and CRF (Fig. 7). The effect of increasing concentrations of CRF on adenylate cyclase activity is shown in Fig. 8. In each of 3 experiments, 0.1 nM CRF caused a significant increase in adenylate cyclase activity. The maximum increment in adenylate cyclase activity of 33.2 ± 4.7% was obtained with 4.5 ± 1.8 nM CRF, with ED₅₀ of 0.28 nM CRF. Addition of 10 µM of the GTP analog, guanosine 5'-β,γ-mimino)triphosphate to the assay evoked a small increase in basal enzyme activity (+25.2 ± 19.8%) without changing the sensitivity or the magnitude of the CRF-induced stimulation of adenylate cyclase activity.

The increases in cyclic AMP caused by CRF were accompanied by parallel activation of cyclic AMP-dependent protein kinase. Preliminary experiments showed that incorporation of ³²P into histone by pituitary cell sonicates was rapid and linear for up to 9 min of incubation; therefore, all subsequent studies were performed at 3 min. As shown in Fig. 9A, prein-
could be involved in the process of corticotropin secretion, it is not yet clear whether the incorporation of nM and norepinephrine up to 0.1 pM were without effect on the activity of pituitary cells exposed to the hypothalamic peptide and observed that the inhibitory action of glucocorticoids was exerted at a step subsequent to cyclic AMP formation. The present studies have demonstrated that the action of CRF upon ACTH release is mediated by the adenylyl cyclase-protein kinase system, with cyclic AMP serving as the second messenger. In cultured pituitary cells, synthetic CRF caused prominent and concomitant increases in cyclic AMP formation and ACTH release, and in dose-response studies, there was a close correspondence between the concentrations of CRF that stimulated cyclic AMP production and ACTH release. The observations that methylisobutylxanthine potentiates the effects of CRF on both cyclic AMP and ACTH production and that cholera toxin elicits ACTH release gave further indication of the intermediate role of cyclic AMP in CRF action. That the effect of CRF on cyclic AMP is due to stimulation of cyclic nucleotide synthesis rather than inhibition of phosphodiesterase activity was confirmed by the demonstration that CRF activates adenylyl cyclase in pituitary homogenates. Although the magnitude of enzyme stimulation by CRF was not large, it was consistently observed, and the nanomolar concentrations of CRF required for activation of adenylyl cyclase were comparable with those necessary to stimulate cyclic AMP and ACTH production in intact cells. The relatively small degree of stimulation of pituitary adenylyl cyclase by CRF is probably a reflection of the multiplicity of cell types in the anterior pituitary, of which less than 10% correspond to corticotrophs (26). The limited effect of guanyl nucleotides on adenylyl cyclase activity may be the consequence of a high content of endogenous guanosine triphosphate in the pituitary homogenate, which would also contribute to the high basal rates of enzyme activity. Further evidence for the role of cyclic AMP in the stimulation of ACTH release by CRF was provided by the ability of the hypothalamic peptide to stimulate cyclic AMP-dependent protein kinase at doses similar to those required to activate adenylyl cyclase in pituitary homogenates.

The biological activity of the CRF molecule has been shown by Vale and colleagues (2) to be dependent upon the integrity of the COOH-terminal region and to be reduced to less than 0.1% by deletion of residues 40–41 or the COOH-terminal amide group. On the other hand, CRF(4–41) and N-acetyl CRF were fully active in vitro. The full activity of the molecule was also dependent upon the maintenance of the Met residue in its reduced state, since conversion to the sulfoxide derivative, a reduced biological potency by about 90% (2), Vale et al. also noted that the CRF molecule contains potential sites for cleavage by renin-like enzymes at Leu-Leu bonds at positions 14-15 and 37-38, as well as for tryptic cleavage at Arg-Lys, raising the possibility that smaller fragments of the molecule may contain the active region responsible for activation of the corticotroph. To evaluate this possibility, we determined the activities of synthetic CRF(15–41), CRF(21–41), and CRF(36–41) on cyclic AMP production and ACTH release. The smaller COOH-terminal fragments CRF(21–41) and (36–41) were completely devoid of biological activity. However, the (15–41) sequence corresponding to a cleavage product at the (15-41) sequence corresponding to a cleavage product at the (15-41) sequence corresponding to a cleavage product at the corticotroph. To evaluate this possibility, we determined the activities of synthetic CRF(15–41), CRF(21–41), and CRF(36–41) on cyclic AMP production and ACTH release. The smaller COOH-terminal fragments CRF(21–41) and (36–41) were completely devoid of biological activity. However, the (15–41) sequence corresponding to a cleavage product at

cubation of the cells with CRF caused an increase in incorporation into histone, which was detectable at 0.1 nM CRF and approached cyclic AMP-stimulated values at 10–100 nM CRF, with ED50 of 1.1 ± 0.4 nM CRF (n = 3). When expressed as the activity ratio measured in the absence and presence of cyclic AMP (Fig. 9B), protein kinase was stimulated by CRF from the basal value of 0.39 to a maximum of 0.76 ± 0.05 in 5 experiments. In contrast with the ability of CRF to activate cyclic AMP-dependent protein kinase activity, vasopressin and angiotensin II at concentrations up to 10 nM and norepinephrine up to 0.1 μM were without effect on the incorporation of 32P into histone in 3 experiments.

**DISCUSSION**

Although earlier reports have indicated that cyclic AMP could be involved in the process of corticotropin secretion, it is not yet clear whether the availability of pure CRF has permitted a more complete evaluation of the mechanisms involved in hormone release from the corticotroph. The possibility that cyclic AMP has a role in corticotropin secretion was suggested by the stimulatory effects of phosphodiesterase inhibitors and cyclic AMP derivatives on ACTH release (1, 23) and by observations such as the ability of hypothalamic extract (presumably containing CRF) to cause parallel increases in cellular cyclic AMP and ACTH release in ectopic ACTH-producing tumors (24). Using synthetic CRF, Giguere et al. (25) recently described rapid increases in cyclic AMP in cultured pituitary cells exposed to the hypothalamic peptide and observed that the inhibitory action of glucocorticoids was exerted at a step subsequent to cyclic AMP formation. The present studies have demonstrated that the action of CRF upon ACTH release is mediated by the adenylyl cyclase-protein kinase system, with cyclic AMP serving as the second messenger. In cultured pituitary cells, synthetic CRF caused prominent and concomitant increases in cyclic AMP formation and ACTH release, and in dose-response studies, there was a close correspondence between the concentrations of CRF that stimulated cyclic AMP production and ACTH release. The observations that methylisobutylxanthine potentiates the effects of CRF on both cyclic AMP and ACTH production and that cholera toxin elicits ACTH release gave further indication of the intermediate role of cyclic AMP in CRF action. That the effect of CRF on cyclic AMP is due to stimulation of cyclic nucleotide synthesis rather than inhibition of phosphodiesterase activity was confirmed by the demonstration that CRF activates adenylyl cyclase in pituitary homogenates. Although the magnitude of enzyme stimulation by CRF was not large, it was consistently observed, and the nanomolar concentrations of CRF required for activation of adenylyl cyclase were comparable with those necessary to stimulate cyclic AMP and ACTH production in intact cells. The relatively small degree of stimulation of pituitary adenylyl cyclase by CRF is probably a reflection of the multiplicity of cell types in the anterior pituitary, of which less than 10% correspond to corticotrophs (26). The limited effect of guanyl nucleotides on adenylyl cyclase activity may be the consequence of a high content of endogenous guanosine triphosphate in the pituitary homogenate, which would also contribute to the high basal rates of enzyme activity. Further evidence for the role of cyclic AMP in the stimulation of ACTH release by CRF was provided by the ability of the hypothalamic peptide to stimulate cyclic AMP-dependent protein kinase at doses similar to those required to activate adenylyl cyclase in pituitary homogenates.

**FIG. 9. Stimulation of cyclic AMP-dependent protein kinase by CRF.** A, effect of preincubation of pituitary cells with increasing concentrations of CRF upon histone phosphorylation by [γ-32P]ATP in cell sonicates. B, phosphotransferase activity expressed as a ratio of 32P incorporation measured in the absence and presence of 5 μM cyclic AMP. Points are the means of data from triplicate incubations in 1 of 3 similar experiments.
the Leu 14-Leu 15 residues displayed full intrinsic activity, with potency equivalent to about 0.1% of that of the intact molecule. Despite the relatively low potency of this portion of the CRF molecule, its ability to elicit full cyclic AMP and ACTH responses at high concentrations suggests that the receptor activation process requires the 15–21 region as well as the COOH-terminal of the molecule. Also, it is likely that the 4–14 region is important for enhancement of bioactivity by influencing the conformational stability and/or the receptor binding affinity of the CRF molecule.

In contrast with the actions of CRF and its active fragment on cyclic AMP-related processes, we observed no stimulatory effects of angiotensin II, vasopressin, or norepinephrine on cyclic AMP production or cyclic AMP-dependent protein kinase activity. The absence of such changes was observed under conditions in which angiotensin II, vasopressin, and norepinephrine caused significant increases in ACTH release, equivalent in magnitude to those elicited by low concentrations of CRF that stimulated both adenylyl cyclase and protein kinase activities. These differences suggest that the mechanisms by which angiotensin II, vasopressin, and norepinephrine exert effects upon ACTH release are independent of the adenylyl cyclase-protein kinase pathway. Although the biochemical events that mediate the pituitary actions of these hormones have yet to be clarified, they are likely to involve increases in cytosolic calcium and phospholipid turnover, as demonstrated for the actions of these hormones in other tissues (7, 10, 11, 27) and those of other cyclic AMP-independent peptide hormones such as gonadotropin-releasing hormone (28–30). Further evidence for the existence of two pathways was provided by the additivity observed between the ACTH responses to CRF and angiotensin II, vasopressin, or norepinephrine and the lack of additivity between CRF and 8-Br-cyclic AMP or cholera toxin. Alternatively, the additivity between CRF and angiotensin II, vasopressin, and norepinephrine could be due to the action of the individual hormones releasing ACTH from different populations of corticotropes. This possibility is unlikely, at least for angiotensin II and CRF, since no significant differences in the relative stimulation of ACTH release by both peptides have been observed in different populations of pituitary corticotropes separated by centrifugal elutriation.  

Interactions between several regulators that act on the same target cell via different mechanisms, either cyclic AMP-dependent or cyclic AMP-independent, are not uncommon. In the adrenal zona glomerulosa, aldosterone secretion is influenced by ACTH, a hormone recognized to act through cyclic AMP, as well as by more important cyclic AMP-independent regulators such as angiotensin II and potassium (8, 31, 32). A similar situation is observed in the regulation of hepatic glycogenolysis, which is stimulated by glucagon through a cyclic AMP-mediated pathway (33), as well as by angiotensin II, vasopressin, and alpha-adrenergic agonists through a noncyclic AMP-dependent mechanism (7). The cyclic AMP and calcium-mediated pathways are not mutually exclusive, because in certain cases calcium has been demonstrated to be necessary for the action of cyclic AMP-dependent hormones (34, 35). In this regard, the in vitro stimulation of ACTH release by CRF has also been shown to be calcium dependent, as indicated by the ability of the calcium antagonist Co<sup>2+</sup> to abolish the ACTH response to the hypothalamic peptide (2). Although the interactions between calcium and cyclic AMP in the mechanism of action of CRF need further clarification, these studies provide strong evidence for a functional correlation between the adenylyl cyclase-cyclic AMP-protein kinase pathway and ACTH release in CRF-stimulated cells.

The nature of the cellular substrate for the protein kinase, as well as the subsequent chain of biochemical events that results in processing of the pro-opiomelanocortin molecule and ACTH release, will need to be elucidated in future studies.

Stimulation of pituitary cells by CRF results in rapid release of ACTH, with a change in the rate of secretion after 60 to 90 min, probably due to ACTH release from different peptide pools in the corticotroph. Such early increases in ACTH release could be important in the rapid elevations of plasma ACTH in response to acute stress, while the delayed response may be more relevant to the maintenance of high ACTH levels during prolonged stress situations.

The extent to which the several hormones capable of stimulating ACTH release are involved in the physiological control of ACTH secretion is uncertain. Our results, and those of others (2), favor the proposal that CRF functions as a major regulator of ACTH. However, while the magnitude of the ACTH response to angiotensin II and vasopressin is much less than that elicited by CRF, the sensitivity of the corticotroph to such factors is quite high, as indicated by their ED50 values. The recent demonstration that vasopressin (36) and angiotensin II (37) potentiate the stimulatory effect of CRF on ACTH release in perfused pituitary cells suggests that such peptides may be important as physiological modulators of the action of CRF. It is, therefore, possible that interactions between CRF and other hormones are involved in the regulatory mechanisms that operate in the physiological control of ACTH secretion.

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