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Stimulation of cyclic AMP accumulation and corticotropin release by synthetic ovine corticotropin-releasing factor in rat anterior pituitary cells: Site of glucocorticoid action

(adenylate cyclase/adenohypophysis/feedback/adrenocorticotropin/dexamethasone)

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ABSTRACT **A 2.5-fold stimulation of cyclic AMP cellular content is measured 60 sec after addition of 100 nM synthetic ovine corticotropin-releasing factor (C-RF; corticoliberin) to rat anterior pituitary cells in culture. A maximal response of cyclic AMP content at 400% above control is observed between 2 and 30 min after addition of the peptide, whereas an 8-fold stimulation of cyclic AMP released into the incubation medium is measured between 10 and 180 min. A linear 7-fold increase of corticotropin release is observed for up to 3 hr. Preincubation for 18 hr with the potent glucocorticoid dexamethasone has no effect on C-RF-induced cyclic AMP accumulation. The same treatment with dexamethasone causes an 80% inhibition of corticotropin release induced by both C-RF and the cyclic AMP derivative 8-bromoadenosine 3',5'-cyclic monophosphate. The present data show that ovine C-RF is a potent stimulator of cyclic AMP accumulation in rat anterior pituitary cells and that the process is insensitive to the action of dexamethasone. The marked inhibition by dexamethasone of corticotropin secretion induced by a cyclic AMP derivative indicates that glucocorticoids exert their potent inhibitory effects on corticotropin secretion at a step distant to cyclic AMP formation.**

The first evidence suggesting the presence of hypothalamic substances controlling anterior pituitary functions was that of a corticotropin-releasing factor (C-RF; corticoliberin) (1, 2). Recent elucidation of the structure of ovine C-RF (3, 4) opens new possibilities for studies of the mechanisms involved in the control of adrenocortical activity. The 41-amino acid peptide is a potent stimulator of corticotropin secretion *in vivo* in the rat and in adenohypophyseal cells in culture (3, 5–7).

Early studies were suggestive of a role of cyclic AMP as mediator of corticotropin secretion. These studies pertain to the stimulatory effect of theophylline, an inhibitor of cyclic nucleotide phosphodiesterase, on corticotropin release in intact pituitary glands (8, 9). In agreement with these data, cyclic AMP derivatives were found to be potent stimulators of corticotropin secretion in intact pituitaries (8, 9) and in rat anterior pituitary cells in primary culture (10, 11). However, definitive proof of the role of cyclic AMP as mediator of the action of C-RF could be obtained only by measurements of adenohypophyseal adenylate cyclase activity or changes in cyclic AMP concentrations under the influence of the peptide.

Glucocorticoids are potent inhibitors of corticotropin secretion (10–15). However, their site of action in corticotrophs is still unknown. This paper shows that synthetic ovine C-RF leads to a rapid and marked increase of cyclic AMP cell content and to a parallel stimulation of corticotropin and cyclic AMP release in anterior pituitary cells in primary culture. It also demonstrates that glucocorticoids exert their inhibitory action at a step subsequent to cyclic AMP formation.

MATERIALS AND METHODS

Materials. C-RF was prepared by solid-phase methods and purified by preparative reverse-phase HPLC. Homogeneity was determined by analytical HPLC on Vydac silica column (9 × 250 mm) of C_{18} (10 μm; 300-Å pore size) and by peptide mapping of enzymatic digests on HPLC. Dexamethasone from Steraloids was prepared in 0.9% NaCl/1% ethanol and was used at a dilution of 1:100 in the incubation medium. Theophylline and 8-bromoadenosine 3',5'-cyclic monophosphate (8-Br-cyclic AMP) were from Sigma. Dulbecco's modified Eagle's medium (DME medium) was obtained from GIBCO, and the sera were purchased from Flow Laboratories, McLean, VA. In order to remove endogenous steroids, sera were incubated overnight at 4°C with 1% activated charcoal (Norit A) and 0.1% Dextran T-70 (from Fisher and Pharmacia, respectively). A 2-hr supplementary adsorption was performed at 25°C to further remove protein-bound steroids. Sera were then inactivated by a 20-min incubation at 56°C.

Preparation of Dispersed Anterior Pituitary Cells. Adult female Sprague–Dawley rats (obtained from Canadian Breeding Farms, St. Constant, Quebec, Canada) at random stages of the estrous cycle were used for the preparation of primary cultures of anterior pituitary cells as described (16).

Cells ($3–5 \times 10^5$) in 1.0 ml of DME medium containing 10% horse serum and 2% fetal calf serum, both steroid-free through adsorption on dextran-coated charcoal, were plated in Linbro multiwell Petri dishes.

Incubation Procedure. Three to 4 days after plating, cells were washed four times with DME medium without serum, and incubation was carried out in triplicate for various time intervals after addition of synthetic C-RF or 8-Br-cyclic AMP. For measurement of the effect of dexamethasone, the steroid was added to the medium 18 hr before initiation of the experiment. At the end of incubation, the medium was removed and centrifuged at 100 × *g* for 7 min at 4°C, and the supernatant was acidified with 100 μl of 1 M HCl and frozen at −20°C until assayed for corticotropin by radioimmunoassay. In order to measure cyclic AMP cell content, culture medium was aspirated at the end of incubation, and intracellular cyclic AMP was extracted with 1.0 ml of 0.1 M acetic acid for 30 min at 4°C. The extract was then

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Abbreviations: C-RF, corticotropin-releasing factor (corticoliberin); DME medium, Dulbecco's modified Eagle's medium; 8-Br-cyclic AMP, 8-bromoadenosine 3',5'-cyclic monophosphate.

lyophilized and resuspended in 50 mM sodium acetate (pH 6.2) before acetylation (17). When cyclic AMP was measured in the culture medium, 50 μl of 10 mM theophylline was added to 450 μl of culture medium before boiling for 10 min.

Corticotropin and Cyclic AMP Assays and Calculations. Corticotropin and cyclic AMP were measured with radioimmunoassays developed in our laboratory (18, 19). Data were calculated and analyzed with a Hewlett–Packard calculator, model 9845, using a program based on model II of Rodbard and Lewald (20). Dose–response curves and 50% effective doses (ED_{50}) were calculated by using a weighted iterative nonlinear least-squares regression (21). Statistical significance was determined by the multiple-range test of Duncan–Kramer (22). All data are shown in figures as means ± SEM of duplicate measurements of triplicate dishes, except when the SEM is smaller than the symbol used (where only the symbol is shown).

RESULTS

Time Course of C-RF-Induced Corticotropin Release. A linear 7-fold stimulation of corticotropin release was observed up to 3 hr after addition of 100 nM C-RF to rat adenohypophyseal cells in culture, followed by a decrease (≈50%) of the rate of stimulated release during the next hour (Fig. 1). A 3-hr incubation period was then adapted for the following studies on the effect of C-RF on corticotropin secretion.

Time Course of C-RF-Induced Cyclic AMP Accumulation and Secretion. A 2.5-fold stimulation of cyclic AMP cellular content (0.99 ± 0.07 to 2.49 ± 0.03 pmol per dish) was observed 60 sec after addition of 100 nM C-RF (Fig. 2). The rise in cyclic AMP content was maximal at 4 min (400% above control) and decreased slowly during the next hour to reach a plateau at ≈100% above control for the next 3 hr. After these results, a 10-min incubation period was chosen for all subsequent experiments on the effect of C-RF on cyclic AMP cell content.

The rapid changes in cyclic AMP content induced by C-RF were accompanied by a rapid and marked stimulation of cyclic AMP accumulation in the incubation medium (Fig. 3). An 8-fold stimulation of cyclic AMP concentration in the medium was observed 10 min after addition of C-RF, with a 6-fold increase being measured after 4 hr.

Effect of Increasing Concentrations of C-RF on Cyclic AMP Cell Content and Corticotropin Release. Increasing concentrations of C-RF up to 1 μM led to a maximal 6-fold increase of cyclic AMP content at an ED_{50} for C-RF of 20 nM (Fig. 4*B*). The same concentrations of the peptide caused a maximal 9-fold increase of corticotropin release at an ED_{50} for C-RF of 3 nM after a 3-hr incubation period (Fig. 4*A*).

Differential Effects of Dexamethasone on C-RF-Induced Corticotropin Secretion and Cyclic AMP Accumulation. Fig. 4 also shows the effect of an 18-hr preincubation with 10 nM dexamethasone on the dose–response relationship of corticotropin secretion and cyclic AMP accumulation to C-RF. Pretreatment with dexamethasone reduced the maximal corticotropin response to C-RF by 65%, whereas the ED_{50} for C-RF action was slightly increased from 3 to 10 nM. Contrary to the marked inhibitory effect of dexamethasone on corticotropin secretion, the dose–response curve of cellular cyclic AMP accumulation to increasing concentrations of C-RF was not affected by the glucocorticoid (Fig. 4*B*). To further assess the possibility of an action of dexamethasone at a step subsequent to cyclic AMP, we studied the effect of preincubation with dexamethasone on the dose–response curve of corticotropin secretion to a cyclic AMP derivative, 8-Br-cyclic AMP. Increasing doses of

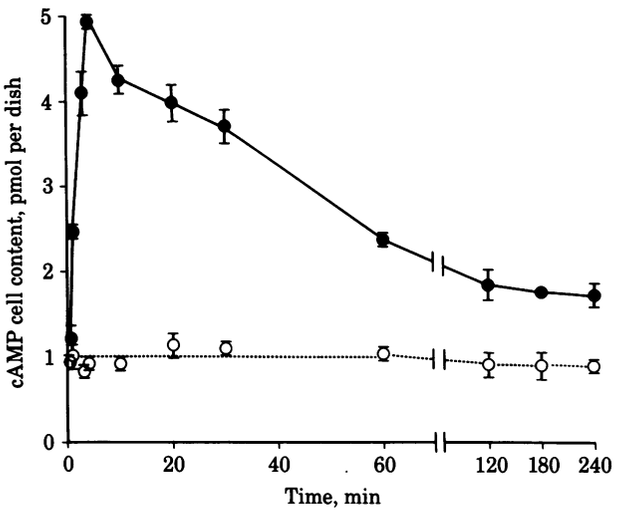

FIG. 2. Time course of the effect of 100 nM C-RF on intracellular cyclic AMP accumulation in rat adenohypophyseal cells in culture. The basal level of intracellular cyclic AMP was 0.95 ± 0.02 pmol per dish. ○, Control; ●, 100 nM C-RF (same experiment as in Fig. 1).

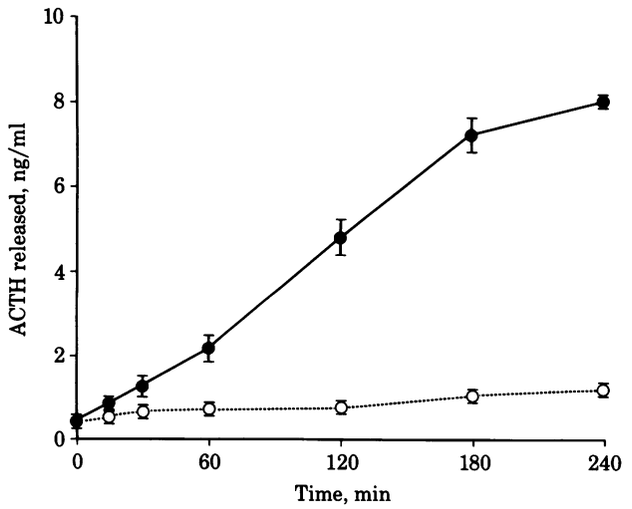

FIG. 1. Time course of the effect of 100 nM C-RF on corticotropin release by rat anterior pituitary cells in culture. ○, Control; ●, 100 nM C-RF.

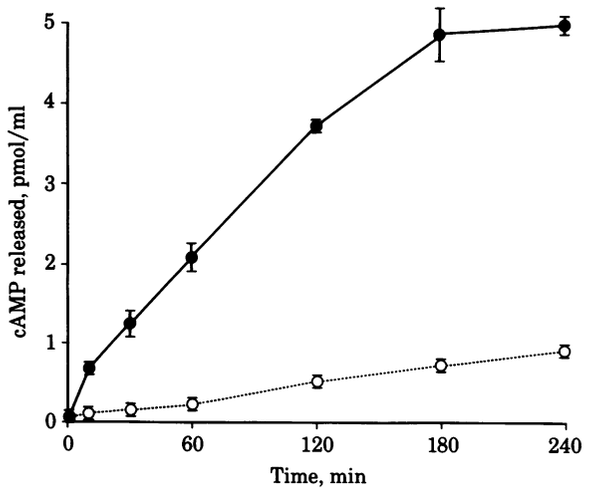

FIG. 3. Time course of the effect of 100 nM C-RF on cyclic AMP release in the culture medium. ○, Control; ●, 100 nM C-RF (same experiment as in Fig. 1).

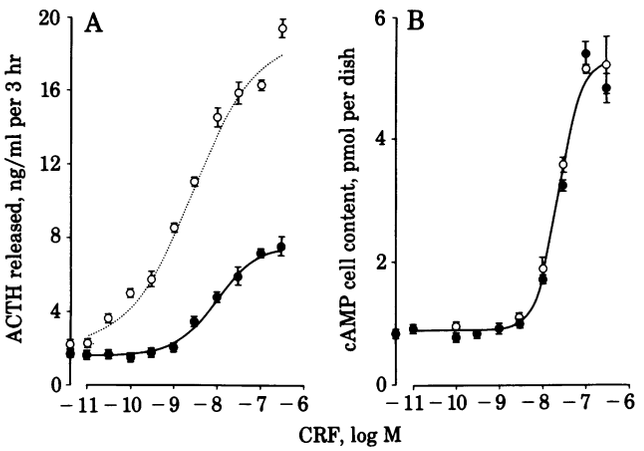

FIG. 4. Effect of an 18-hr preincubation with 10 nM dexamethasone on corticotropin release (*A*) and cyclic AMP accumulation (*B*) induced by increasing concentrations of C-RF during a 3-hr incubation period. Basal corticotropin secretion was 2.21 ± 0.07 ng/ml per 3 hr. Pretreatment with dexamethasone reduced basal corticotropin release to 1.67 ± 0.07 ng/ml per 3 hr. Basal cyclic AMP cell content was similar in the presence or absence of dexamethasone pretreatment: 0.84 ± 0.02 pmol per dish. ○, Control; ●, 10 nM dexamethasone.

the cyclic AMP derivative induced a maximal 14-fold stimulation of corticotropin release (Fig. 5). Pretreatment with dexamethasone caused an 80% inhibition of the maximal response to 8-Br-cyclic AMP, with no significant effect on the ED_{50} for 8-Br-cyclic AMP action.

DISCUSSION

The present data clearly show that the marked inhibitory effect of glucocorticoids on C-RF-induced corticotropin secretion is exerted at a step subsequent to cyclic AMP formation. This is well supported by the complete lack of effect of dexamethasone pretreatment on the stimulation of pituitary cyclic AMP content

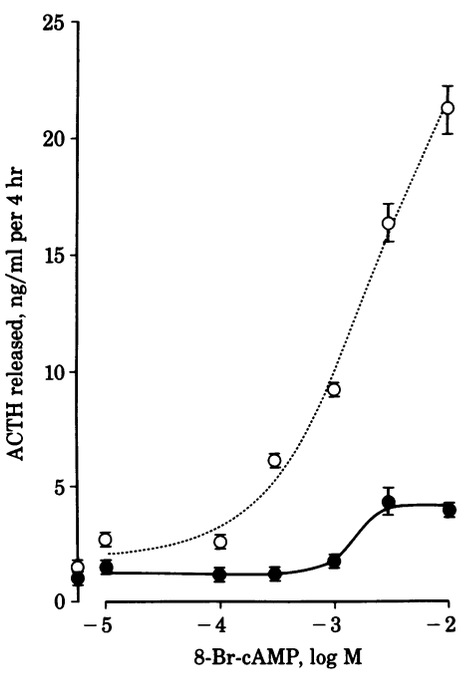

FIG. 5. Effect of an 18-hr preincubation with 10 nM dexamethasone on corticotropin release induced by increasing concentrations of 8-Br-cyclic AMP in rat anterior pituitary cells in culture. ○, Control; ●, 10 nM dexamethasone.

by C-RF and by the marked inhibition by C-RF of 8-Br-cyclic AMP-induced corticotropin release.

The site of action of glucocorticoids in corticotrophs subsequent to cyclic AMP formation is analogous to the action of estrogens on luteinizing hormone-releasing hormone (LH-RH; luliberin)-induced secretion of luteinizing hormone (lutropin) and follicle-stimulating hormone (follitropin) in gonadotrophs (23). In fact, using the same pituitary cell culture system, we have found that pretreatment with 17β-estradiol causes a similar stimulation of the gonadotropin responses induced by luteinizing hormone-releasing hormone and 8-Br-cyclic AMP (23). This is quite distinct from the effect of androgens, which are exerted at steps both prior and subsequent to cyclic AMP formation in gonadotrophs (23, 24). Although glucocorticoids were well known to exert inhibitory effects on corticotropin secretion (10–15, 25), this study provides evidence for their intracellular site of action. The present data are at variance with the hypothesis that glucocorticoids might act exclusively at the level of the C-RF receptor (26).

Although previous studies with cyclic AMP derivatives and theophylline have suggested cyclic AMP as a possible intracellular mediator in the control of corticotropin secretion (8–11), this study extends the findings of a preliminary report (7) and clearly shows that synthetic C-RF causes a parallel stimulation of corticotropin and cyclic AMP release into the culture medium. Moreover, it demonstrates that intracellular cyclic AMP content is increased within the first 60 sec of incubation in the presence of C-RF.

The finding of a 2.5-fold stimulation of cyclic AMP cellular content measured as early as 1 min after addition of C-RF (Fig. 2) strongly suggests that the changes of cyclic AMP accumulation coincide with or precede corticotropin secretion induced by the peptide. Because corticotrophs correspond to ≈10% of the entire population of adenohypophyseal cells, it is likely that the 4-fold increase of cyclic AMP content measured in total pituitary cells corresponds to a much higher increase in cellular cyclic AMP content in the specific cell population stimulated by C-RF.

The observation that C-RF increases corticotropin secretion at doses lower than those required for cyclic AMP accumulation, suggests compartmentalization of the cyclic nucleotide into pools of different biological significance. Similar observations have been reported with thyrotropin-releasing hormone (thyroliberin) and lysine-vasopressin on thyrotropin and corticotropin release, respectively (27). Although the present data leave little doubt about the role of the adenylate cyclase system as mediator of the action of C-RF in corticotrophs, the possible involvement of other potential intracellular mechanisms and their interactions with cyclic AMP remain to be determined.

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