Corticotropin Releasing Factor Increases Proopiomelanocortin Messenger RNA in Mouse Anterior Pituitary Tumor Cells*

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The ability of corticotropin releasing factor (CRF) to stimulate adrenocorticotropic (ACTH) synthesis in corticotrophs was assessed by measuring total cell content of ACTH and the levels of proopiomelanocortin (POMC) mRNA in a cloned tumor cell line of the mouse anterior pituitary (AtT-20/D16-16). CRF treatment caused a time-dependent increase in POMC mRNA levels (measured using a hybridization technique) as well as elevating total ACTH content in AtT-20 cells. The increase in POMC mRNA levels preceded changes in ACTH content and slowly returned toward control levels after CRF withdrawal. The rise in POMC mRNA levels following CRF stimulation appeared to be specific since β-actin mRNA levels were not affected by CRF treatment. Both S-bromo-cAMP and phorbol ester increased POMC mRNA levels in AtT-20 cells, suggesting that CRF may act through different protein kinases to regulate the POMC gene. CRF appears to activate the POMC gene since treatment of the AtT-20 cells with the peptide increased the levels of an RNA species in the nuclei having the expected molecular weight of the transcript of the POMC gene. The results indicate that continued exposure of corticotrophs to CRF induces long term increases in the ACTH synthetic capacity of those cells.

Adrenocorticotropic is present both in the anterior and intermediate lobe of the pituitary (1). ACTH is derived from a larger precursor, proopiomelanocortin (2-4). In the intermediate lobe, mature ACTH is processed to α-melanocyte-stimulating hormone and corticotropin-like intermediate lobe peptide, both of which are secreted from this tissue (5). ACTH itself, rather than these other metabolites, is the secreted product of the anterior lobe of the pituitary (6, 7).

The release of ACTH from the anterior lobe is under multihormonal control (8). Corticotropin releasing factor is the most potent and effective natural stimulant of ACTH release in vivo and in vitro (9). Other hypothalamic factors and peripherally derived hormones also induce ACTH secretion (8). Glucocorticoids, synthesized in the adrenal cortex, block ACTH release and thus counterbalance the stimulatory influence of the hypothalamus on corticotroph activity (10-12).

While much is known concerning the hormonal regulation of ACTH release, less data are available on the regulation of corticotropin synthesis. Glucocorticoids inhibit ACTH synthesis, possibly by affecting the rate of transcription of the POMC gene (11-14). Removal of circulating glucocorticoids by adrenalectomy elevates POMC mRNA levels in the rat anterior pituitary (11-14). This increase may be due to the removal of the inhibitory influence of glucocorticoids on the corticotrophs or to a facilitation of hypothalamic release of CRF which could, in turn, act on the corticotrophs to stimulate ACTH synthesis (11, 12). Information is lacking, however, on whether stimulatory hormones like CRF can directly increase POMC mRNA levels in the corticotroph.

The present series of experiments were initiated to answer two questions. Is ACTH synthesis stimulated by CRF and, if so, are the intracellular mechanisms by which this agent regulates ACTH synthesis and release similar or distinct? For these studies, a tumor cell line (AtT-20/D16-16) derived from the mouse anterior pituitary was used. These cells are a homogenous population of corticotrophs, CRF was applied to these tumors and its effect on total ACTH immunoreactivity and POMC mRNA were determined. The results indicate that CRF can increase POMC gene activity and produce long term increases in ACTH levels and suggest that cAMP-dependent and phospholipid-dependent protein kinases could be involved in mediating these effects.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium (4500 mg/l of glucose) was obtained from Grand Island Biologies (Grand Island, NY). Fetal calf serum was from North American Biologicals (Miami, FL) and human ACTH (synthetic) and antisera were gifts from the National Pituitary Agency (Baltimore, MD). 125-I-Human ACTH was from Immuno Nuclear Corp. (Stillwater, MN) and goat anti-rabbit immunoglobulin was from Cappel Laboratories (Cochranville, PA). Guanidine thiocyanate was from Fluka Co. (Switzerland) and SDS was obtained from Bio-Rad Laboratories (Richmond, CA). CRF was obtained from Peninsula Laboratories (San Carlos, CA) and the various restriction enzymes (EcoRI and HindIII) and Escherichia coli polymerase I were purchased from BRL (Bethesda, MD). [32P]dCTP was from New England Nuclear (Boston, MA) and PDBu was a gift from Dr. S. Jaken (NCI, Bethesda, MD).

Cell Cultures and ACTH Immunoreactivity—The cloned mouse anterior pituitary cell line, AtT-20/D16-16, was grown and subcultured as previously described (15). The cells were plated at an initial density of 2 x 10^6 cells/75-cm² Falcon flask and used 5-6 days afterward (80-90% confluent). ACTH immunoreactivity in the medium was measured by radioimmunoassay using an antibody directed against the 14-24 segment of ACTH (15). For determination of ACTH immunoreactivity in the cells, the medium was removed, the cells were washed twice with phosphate-buffered saline (pH 7.4), and 0.5 N HCl was added. The tissue was then sonicated, the acid was removed by vacuum centrifugation, and ACTH immunoreactivity was assessed by radioimmunoassay.

Isolation of Total Cellular RNA—The cells from one Falcon flask were washed twice with 25 ml of ice-cold phosphate-buffered saline (pH 7.4) and lysed in 3.5 ml of a solution containing 5.5 M guanidine...
thiocyanate (16), 2.5 μM EDTA, 0.5% Sarcosyl, and 20 mM Tris-HCl (pH 7.4). The resulting slurry was passed three times through a 23-gauge needle and layered on top of a 1.5-M CaCl2-trifluoroacetic acid (Pharmacia) cushion (density: 1.51 with 250 mM EDTA). Centrifugation was for 16-18 h in a Beckman SW 50.1 rotor at 36,000 rpm. At the end of the spin, the supernatant RNA was dissolved in 0.3 ml of SDS (0.2%) at 88 °C for 5 min and precipitated with 3 volumes of ethanol in the presence of 0.4 M sodium acetate (pH 5.5). The RNA was reprecipitated once and stored at -20 °C in 50% formamide, 2.2 M formaldehyde in a mixture of 40 mM morpholinopropanesulfonic acid (pH 7.0), 10 mM sodium acetate, and 1 M sodium dithionite (FRB).

Quantitation and Hybridization of RNA (18, 19)—The RNA was size-fractionated on 1.2% agarose gels containing formaldehyde (17) and stained with ethidium bromide (10 μg/ml) for 30 min. The gel was then soaked for 12-16 h at 4 °C in FRB to remove the formaldehyde and photographed (Polaroid type 55 positive/negative film). The 18S and 28S rRNA bands were scanned using a soft laser densitometer and the amount of RNA per sample was determined by comparison to serial dilutions of known amounts of rat liver RNA. For quantitative hybridization of POMC mRNA, a second gel was run with equal amounts of RNA per lane and then electrophoretically transferred onto GeneScreen hybridization membrane (New England Nuclear) according to the manufacturer’s instructions (New England Nuclear). The filters were then washed for 4-12 h in a total volume of 2 liters (3 changes) of 0.1% sodium pyrophosphate, 0.05% SDS at 55 °C, blotted dry, and exposed to Kodak X-AR-2 x-ray film using intensifier screens.

For studies involving β-actin mRNA, the RNA bands were first hybridized with a 32P-labeled probe for β-actin mRNA kindly provided by Dr. B. Patterson (National Cancer Institute). This probe was subcloned into the pUC9 plasmid from the original clone described by Cleveland et al. (21). Following the washing and drying and exposure of the filters, the RNA blots were hybridized with the 32P-labeled probe for POMC mRNA. The filters were again washed, dried, and then exposed to x-ray film. The relative amounts of POMC mRNA hybridized to the probe were determined by soft laser densitometry.

Cell Fractionation and Isolation of Nuclear and Cytoplasmic RNA—Nuclei were isolated according to Kole et al. (22) and the nuclear RNA was extracted using hot phenol (23). The cytoplasmic fraction was extracted twice with phenol saturated with 20 mM Tris base and once with chloroform. The ratio of 32S to 28S rRNA in the nuclear preparation was always at least 1.0 as determined by soft laser scanning.

Construction of a POMC mRNA Specific Probe—The purely exonic, translated EcoRI-HindIII fragment obtained from the bovine genomic clone λ-AL12 (24, 25) was purified and subcloned in pUC9 (26) using standard subcloning procedures (17). The 1.2-kb fragment was excised by cutting the plasmid DNA with the restriction enzymes EcoRI and HindIII, gel-purified using low melting point agarose, and nick-translated according to Rigby et al. (20) using [32P]dCTP. The probe proved to be specific for quantitative analysis of mouse POMC mRNA under the conditions described. λ-DNA and φX174 DNA was restricted with the appropriate enzymes and labeled using the large fragment of the E. coli polymerase I (17).

RESULTS

CRF Increases Total ACTH Content of AtT-20 Cells—CRF treatment induces a time-dependent increase in the amount of ACTH immunoreactivity produced by the AtT-20 cells (Fig. 1). Significant elevation in total ACTH levels occurred within 16 h of CRF exposure and reached 3 times control levels after 24 h of peptide treatment. The enhanced production of ACTH is mainly reflected in a greater amount of immunoreactivity secreted into the medium (Fig. 1). Cell content of ACTH initially declined upon CRF treatment but returned to control levels after 24 h (Fig. 1). This enhanced production of ACTH immunoreactivity is dependent on the concentration of CRF used (Fig. 2). CRF has a similar potency to stimulate ACTH release acutely as it does to elevate total ACTH immunoreactivity (15) suggesting a specific receptor-mediated action of CRF in the present series of experiments.

Time Course for CRF to Increase POMC mRNA Levels in AtT-20 Cells—To determine whether the increased content of ACTH was the direct result of CRF stimulating ACTH synthesis, experiments were conducted to measure the effect of CRF treatment on POMC mRNA levels. The mRNA for POMC was quantitated by hybridization with a radiolabeled probe derived from bovine tissue (see “Materials and Methods”). A messenger RNA of 1.2 kb was detected with this probe which is in accordance with the reported size of the mouse POMC mRNA (11). Exposure of AtT-20 cells to CRF for 24 h increased the amount of POMC mRNA extracted from these corticotrophs about 3-fold (Fig. 3A). The increased amount of POMC mRNA was not the result of differences in cell number or overall RNA synthesis since equal amounts of total RNA were applied to each lane of the gels. Thus, a greater percentage of total RNA was POMC mRNA in CRF-treated cells.

The increase in POMC mRNA in the AtT-20 cells was dependent on the time of exposure of the corticotrophs to CRF (Fig. 3, B and C). Significant increases in POMC mRNA content were observed after 4 h of CRF treatment with a 3-fold elevation seen after 8 h of exposure.

AMP and Phorbol Esters Increase POMC mRNA Content in AtT-20 Cells—To investigate cellular mechanisms by which CRF stimulates an increase in POMC mRNA in AtT-20 cells, putative “second messengers” for CRF’s actions were tested for their ability to alter POMC mRNA levels in these tumors. 8-Bromo-cAMP is a cAMP analogue that activates cAMP-dependent protein kinase in AtT-20 cells and stimulates ACTH release (27, 28). The analogue also elevated POMC mRNA levels (Fig. 4). Another enzyme potentially involved in signal transduction in AtT-20 cells is protein kinase C. Protein kinase C is present in corticotrophs and phorbol esters, activators of this enzyme release ACTH from AtT-20 cells (29, 30). Treatment of these cells with PDBu, a relatively water-soluble phorbol ester, for 24 h caused a 3.0-fold increase in POMC mRNA (Fig. 4). The magnitude of increase in POMC mRNA was similar after a 24-h treatment with CRF, 8-bromo-cAMP, or PDBu (Figs. 3 and 4).

CRF and 8-Bromo-cAMP Do Not Affect β-Actin mRNA Levels—To determine the specificity in the rise in POMC mRNA levels following CRF and 8-bromo-cAMP treatment, the levels of β-actin mRNA, another product of RNA polymerase II activity, was measured. Following 6 h of CRF (10-8 M) or 8-bromo-cAMP (10-4 M) exposure to AtT-20 cells, the levels of β-actin mRNA were not different from controls (Fig. 5B). The amounts of total RNA, as assessed by the levels of 18S and 28S rRNA, were the same in each lane (Fig. 5A). However, CRF and 8-bromo-cAMP did increase POMC mRNA levels in these same samples (Fig. 5, B and C).

CRF Treatment Increases Nuclear POMC Precursor mRNA Levels—The increase in total cell POMC mRNA content following CRF treatment may have been due to an activation of the POMC gene. To investigate this possibility, nuclei from control and CRF-treated AtT-20 cells were isolated and the RNA was subjected to Northern blot analysis in order to detect POMC mRNA (Fig. 6). In the CRF-treated cells, 2 different RNA species were detected in the nuclear fraction. A 1.2-kb RNA was seen that corresponded to the POMC mRNA observed in the whole cell preparations. RNA of 5.6 kb in the nuclei of CRF-treated cells was also detected with our probe. The size and location of this RNA strongly suggests that it is indeed a primary transcript of the POMC gene (31, 32). This
FIG. 1. CRF causes a time-dependent increase in ACTH content of AtT-20 cells. AtT-20 cells were exposed to medium with (C) or without (D) CRF (10^{-7} M) for varying periods of time. At the end of each time point, ACTH immunoreactivity in the medium and cells was determined. The results are the mean ± S.E. of 3 separate experiments, each done in triplicate flasks.

FIG. 2. CRF causes a dose-dependent increase in ACTH content of AtT-20 cells. AtT-20 cells were treated for 24 h with different concentrations (abscissa represented as molarity of CRF used) of CRF and total (O) ACTH content or ACTH immunoreactivity in the medium (C) or cells (D) was determined. The results are the mean ± S.E. of 3 separate experiments, each done in triplicate flasks.

Higher molecular weight RNA was not found in the cytoplasmic fraction of both treated and untreated cells and is presumably expressed at levels too low to be detected in the nuclei of untreated cells under the conditions used.

Effect of CRF Withdrawal on the Elevated Levels of POMC mRNA—It was of interest to determine whether the increase in POMC mRNA levels seen during the continued exposure of AtT-20 cells to CRF persisted following the removal of peptide from the medium. To test this, cells were first exposed to CRF for 24 h. This manipulation resulted in a 3-fold increase in POMC mRNA levels in the corticotrophs (Fig. 7). Removal of CRF from the cells for 1 h after such treatment did not diminish the elevated levels of POMC mRNA. However, after 4 h of withdrawal, the POMC mRNA were significantly lower than cells only stimulated with CRF (no recovery period) and after 24 h of withdrawal, POMC mRNA content was just 60% above the levels in untreated cells.

**DISCUSSION**

Previous studies have suggested that CRF regulates the POMC gene in the anterior pituitary (12, 33, 34). Injection of CRF into rats for 3–8 days elevated POMC mRNA levels in the adenohypophysis (33, 34). Shorter periods of stimulation were reported not to cause such increases (33). Adrenalectomy raised anterior pituitary POMC mRNA levels (12, 33) and ablation of the paraventricular nucleus, which projects CRF neurons to the median eminence, prevented this rise (33). Since adrenalectomy increases CRF immunostaining in the paraventricular nucleus (35, 36), it was suggested that CRF mediates, in part, the effects of adrenalectomy on POMC gene expression.

In the present study, evidence is presented to indicate that CRF directly acts upon the corticotroph to specifically stimulate the POMC gene. An activation of the POMC gene is suggested by the appearance of a second, higher molecular weight RNA species in the nuclei of CRF-treated cells that is detected by the radiolabeled probe for POMC mRNA. This RNA has the expected size (5.6 kb) of the primary transcript of the POMC gene (31, 32). As anticipated for a primary transcript, the higher molecular weight RNA species was not detected in the cytoplasm of CRF-treated cells. Furthermore, this RNA was not seen in the nuclei of control cells in which the POMC gene is more quiescent than in the CRF-stimulated state. The specificity of CRF's actions is suggested by the lack of change in mRNA levels for β-actin, like POMC mRNA a product of RNA polymerase II activity, in AtT-20 cells after CRF treatment. At present, it is not clear whether the observed POMC gene activation is due to promotor affinity changes, higher transcription rates, or other regulatory events occurring downstream from the start site of transcription (37). Further studies including in vitro transcription assays in isolated nuclei or chromatin and the use of intronic hybridization probes will be necessary to determine the nature of the events leading to the POMC gene activation.

8-Bromo-cAMP, as well as CRF, increased POMC mRNA content in AtT-20 cells. These findings support the possibility that cAMP is involved in regulating both ACTH release and synthesis. Precedent for such a dual role of cAMP has been established in another tumor cell line (GH) of the anterior pituitary, where cAMP rapidly activates prolactin gene transcription and stimulates prolactin release (38). Furthermore, cAMP increased the phosphorylation of nuclear substrates in GH4 cells, suggesting a role for cAMP-dependent protein kinase in regulating the prolactin gene (38). CRF activates adenylate cyclase and cAMP-dependent protein kinase in corticotrophs (27, 28). Recent studies have shown that incorporation of cAMP-dependent protein kinase inhibitor into AtT-20 cells abolishes both CRF and cAMP-directed ACTH release implying a direct link between cAMP-dependent protein kinase and the ACTH release process (39). Experiments are in progress using a similar approach to directly determine whether cAMP mediates CRF’s effects on POMC gene expression.

Interestingly, a phorbol ester also enhanced POMC mRNA
CRF Activates the POMC Gene

Fig. 3. CRF increases POMC mRNA levels in AtT-20 cells. POMC mRNA levels were quantitated as described under "Materials and Methods." Northern blot analysis revealed that the RNA extracted from untreated AtT-20 cells had a size of 1.2 kb (Aa, RNA of control cells; Ad, DNA size markers: X-DNA and φX174 DNA were digested with HindIII and TarI, respectively, end-labeled, and, prior to electrophoresis, denatured in the presence of formamide and formaldehyde at 65 °C). (Larger X fragments not shown.) CRF treatment of two different cell batches increased the intensity of the bands representing POMC mRNA (Ab, Ac). AtT-20 cells were subsequently treated for different times with CRF (10⁻² M) and RNA-extracted and POMC mRNA was determined by Northern blot analysis (B). The positions of 18 S and 28 S rRNA are indicated (arrows). Quantitative analysis of the levels of POMC mRNA after the different times of CRF treatment was determined by densitometry (C). In A, a representative example of 3 experiments is depicted. The results of B were reproduced in 3 other experiments. The mean ± S.E. relative amount of POMC mRNA in the cells of those different experiments is presented in the bar graph of C. In each experiment, 3 different flasks were used.

Fig. 4. 8-Bromo-cAMP and phorbol ester increase POMC mRNA in AtT-20 cells. AtT-20 cells were exposed for 24 h to control medium (C), or medium containing 8-bromo-cAMP (10⁻⁴ M) (8-Bromo) or PDBu (10⁻⁶ M) (Phorbol). The POMC mRNA was determined by Northern blot analysis (A) and quantitated by densitometry (B). A is a representative Northern blot of 3 different experiments whose mean ± S.E. relative change in POMC mRNA compared to controls is depicted in B.

levels in AtT-20 cells. The possibility that both cAMP-dependent protein kinase and protein kinase C are involved in regulating the same biologic phenomena is not unprecedented. Albert et al. (40) observed that both kinases were involved in phosphorylating the same amino acid residue on tyrosine hydroxylase, the rate-limiting enzyme in dopamine synthesis. It will be of interest to determine whether these different kinases regulate POMC mRNA levels by similar mechanisms and whether they mediate the effect of the same or different hormones on ACTH synthesis.

In the AtT-20 cell, CRF first increased POMC mRNA levels significantly 4 h after exposure. Increases in ACTH content in the AtT-20 cells in response to CRF treatment lagged behind these POMC mRNA changes. Similar delays in anterior pituitary POMC mRNA levels and ACTH content were observed after adrenalectomy (12). The levels of POMC mRNA gradually return toward control levels after removal of CRF from the medium. Twenty-four h after CRF withdrawal, POMC mRNA levels fell from 300% to only 60% above untreated controls. The return of POMC mRNA toward control levels following CRF withdrawal could be due solely to inactivation of the POMC gene, specific degradation of the POMC mRNA, or a combination of both processes.
The physiologic consequences of long term increases in ACTH synthesis remain to be established. It will be interesting to determine whether such adaptive changes may lead to some of the clinical symptoms of chronic stress.

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REFERENCES


Fig. 6. CRF activates the POMC gene. AtT-20 cells were treated for 24 h with CRF (10^{-7} M) and then nuclei and a cytoplasmic fraction were prepared. RNA hybridizing with the radiolabeled probe in the nuclear (a,b,c) and cytoplasmic (a',b',c') fractions were revealed by Northern blot analysis. The RNA from untreated (a,b,c) and CRF-treated (b,c,b',c') (two different batches of treated cells) cells is shown. The size of the larger nuclear RNA hybridizing with the radiolabeled probe in lanes b and c was measured to be 5.6 kb. The migration distance of 18 S and 28 S RNA was determined after ethidium bromide staining of the bands (arrows). In lanes L, 12 μg of total mouse liver RNA was included as control.

Fig. 7. Recovery of POMC mRNA levels after CRF treatment. AtT-20 cells were treated with CRF (10^{-7} M) for 24 h, washed, and then incubated with control medium for 0, 1, 4, 8, and 24 h (panel A: B, C, D, E, F, respectively) and then POMC mRNA levels were analyzed. Control cells (panel A: A) were not exposed to CRF. The entire experimental protocol was for 48 h and the cells were lysed and RNA was purified for all the cells at the same time. Quantitative analysis of the mean ± S.E. relative changes in POMC mRNA in 3 separate experiments is shown in panel B.

CRF is known to mediate the effect of acute stress on raising plasma ACTH levels (8, 9). The long term increases in POMC mRNA content and ACTH levels in the AtT-20 cells and anterior pituitary following extensive CRF treatment may indicate that chronic stress results in a long term elevation in the ACTH synthetic capacity of the corticotroph.