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Corticotropin-releasing factor-induced adrenocorticotropin hormone release and synthesis is blocked by incorporation of the inhibitor of cyclic AMP-dependent protein kinase into anterior pituitary tumor cells by liposomes

(proopiomelanocortin/gene regulation/neural cell adhesion molecule/ACTH release)

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ABSTRACT **Corticotropin-releasing factor (CRF) is the most potent and effective natural stimulant of corticotropin (ACTH) secretion. In a tumor cell line of the mouse anterior pituitary (AtT-20/D16-16) consisting of a homogenous population of corticotrophs, CRF is known to increase adenylate cyclase and cAMP-dependent protein kinase activities as well as to release ACTH. To determine whether activation of cAMP-dependent protein kinase is essential for CRF to evoke the secretion of ACTH, an inhibitor (PKI) of this kinase was inserted into AtT-20 cells. This was accomplished by first encapsulating PKI into liposomes and then covalently coupling them to protein A for binding to antibodies directed against an AtT-20 cell surface antigen, N-CAM (neural cell adhesion molecule). The binding of the liposomes to the anti-N-CAM antibodies led to the internalization of the PKI into the tumor cells. The PKI treatment greatly attenuated CRF-stimulated ACTH release as well as the secretory response to β-adrenergic agonists. However, ACTH release in response to caerulein, an agonist of cholecystokinin 8 receptors, was not altered by the PKI treatment. CRF treatment also increased the levels of mRNA for proopiomelanocortin (POMC), the precursor for ACTH in AtT-20 cells. Application of liposomes containing PKI to AtT-20 cells blocked the ability of CRF and 8-bromo-cAMP, but not phorbol ester, to increase POMC mRNA levels. The results revealed an essential role for cAMP in mediating the effect of CRF on ACTH release and POMC gene expression.**

Adrenocorticotropin (ACTH) release is stimulated by a variety of hormones including corticotropin-releasing factor (CRF) (1, 2), vasopressin (3, 4), and catecholamines (5). These hormones may evoke secretion by initiating multiple events within the corticotroph. CRF activates both adenylate cyclase and cAMP-dependent protein kinase in rat anterior pituitary membranes (6) and homogenates of a tumor cell line of the mouse anterior pituitary (AtT-20/D16-16) consisting of a homogenous population of corticotrophs (7, 8). These data suggest that cAMP is involved in the receptor-mediated release of ACTH. β-Adrenergic agonists, which stimulate cAMP-dependent protein kinase activity and the secretion of ACTH from AtT-20 cells (7), also depolarize AtT-20 cells and increase the frequency of action potentials in these corticotrophs (9, 10). This electrical activity has been associated with transient calcium currents (9, 10), and β-adrenergic agonists and forskolin, a direct activator at adenylate cyclase, increase cytosolic calcium levels in AtT-20 cells (11). In addition to cAMP and calcium, activators of protein kinase C evoke ACTH release (12), and recent studies (13) have suggested a role for phosphatidylinositol turnover in hormone secretion from corticotrophs.

The synthesis of ACTH is also under hormonal control. Infusion of CRF into rats for 3 days increases the levels of mRNA for proopiomelanocortin (POMC), the precursor of ACTH, in the adenohypophysis (14). Application of CRF onto AtT-20 cells for 6–8 hr also increases POMC mRNA levels (15), and recently CRF has been speculated to activate the POMC gene (16).

The intracellular mechanisms mediating hormonal stimulation of ACTH release and controlling the expression of the POMC gene may be similar or different. To precisely determine the cascade of events initiated by physiological stimulants in regulating hormone secretion and synthesis, it would be ideal to incorporate selective agents into endocrine cells so as to block the actions of one, but not other, intracellular second messengers. In a previous study, we (17) showed that the inhibitor (PKI) of cAMP-dependent protein kinase could be encapsulated into liposomes and directed into AtT-20 cells by using antibodies against the cell surface adhesion molecule (N-CAM). Anti-N-CAM antibodies bound to AtT-20 cells in culture. By covalently coupling *Staphylococcus aureus* protein A to the liposome surface, it was possible to bind the liposomes containing PKI to all the AtT-20 cells. After liposome binding, the PKI was inserted into the AtT-20 cell cytoplasm. Subsequently, neither forskolin nor 8-bromo-cAMP could stimulate ACTH release (17). The ability of forskolin to increase intracellular cAMP levels was not attenuated by the treatment, indicating that PKI predominantly blocked cAMP-dependent protein kinase activity to inhibit forskolin-evoked hormone secretion. Forskolin-stimulated ACTH release was not blocked by pretreatment of AtT-20 cells with empty liposomes or with liposomes containing PKI but not coupled to protein A. The pretreatment of the cells with anti-N-CAM antibodies was also essential for the insertion of the PKI. Finally, the PKI treatment, while blocking forskolin evoked ACTH release, did not prevent the secretion response initiated by K^+ or phorbol ester, secretagogues that do not activate cAMP-dependent protein kinase (17).

In the present study, we incorporated PKI into AtT-20 cells to determine whether physiological stimulants of ACTH release or POMC gene expression primarily act through cAMP or through some other intracellular mechanism.

MATERIALS AND METHODS

Materials. Forskolin was purchased from Calbiochem-Behring. 8-Bromo-cAMP, KCl, isoproterenol, human serum albumin, and phorbol 12-myristate 13-acetate (PMA) were from Sigma. Dulbecco's modified Eagle's medium (DMEM)

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Abbreviations: ACTH, corticotropin; CRF, corticotropin-releasing factor; POMC, proopiomelanocortin; N-CAM, neural cell-adhesion molecule; PKI, inhibitor of cAMP-dependent protein kinase; CCK-8, cholecystokinin 8; PMA, phorbol 12-myristate 13-acetate.

(4500 mg per liter of glucose) was obtained from GIBCO. Fetal calf serum was from North American Biologicals (Miami, FL) and human ACTH (synthetic) and antiserum were gifts from the National Pituitary Agency (Baltimore, MD). ^{125}I-labeled human ACTH was from Immuno Nuclear (Stillwater, MN), and goat anti-rabbit immunoglobulin was from Cappel Laboratories (Cochranville, PA). Guanidine thiocyanate was from Fluka, and $NaDodSO_4$ was obtained from Bio-Rad. CRF and caerulein were obtained from Peninsula Laboratories (San Carlos, CA), and the restriction enzymes (*Eco*RI and *Hin*dIII) and *Escherichia coli* polymerase I were purchased from Bethesda Research Laboratories. [^{32}P]dCTP was from New England Nuclear.

In the present study, partially purified PKI (crude, from rabbit muscle) obtained from Sigma was used. Liposomes with or without PKI and covalently coupled to protein A were prepared as described (17). Polyclonal anti-N-CAM antibodies were obtained from a rabbit immunized with an antigen preparation purified as described (18) on a monoclonal H28 anti-N-CAM column from mouse brain. The antibodies obtained through such a procedure revealed on immunoblots only the bands detected by the monoclonal antibody H28.

Cell Culture Technique. Mouse AtT-20/D16-16 tumor cells (originally subcloned by S. Sabol, National Institutes of Health) were grown and subcultured in DMEM with 10% fetal calf serum as described (19). Cells were plated in 24- or 96-well flat-bottom tissue culture clusters at an initial density of 5×10^5 and 5×10^4 cells per well, respectively. The cells were grown for 48–96 hr to near confluency in 0.1–1.0 ml of culture medium.

Procedures for ACTH Release. Before the experiments, the AtT-20 cells (plated in 96-well plates) were washed twice with 100 μl of DMEM containing 25 mM Hepes and 2% fetal calf serum. The cells were then incubated with 50 μl of medium with the anti-N-CAM antibody (final dilution, 1:300) for 1 hr at 25°C. The cells were then washed twice and incubated for 3 hr with 100 μl of medium with or without PKI-containing liposomes coupled to protein A. At the end of this time, the medium was removed and fresh DMEM containing forskolin, K^+, PMA, CRF, caerulein, or isoproterenol was applied to the cells and incubated for 60 min at 37°C. For the time-course studies, the period of drug stimulation was varied. An aliquot of the medium was removed and frozen at −20°C for later analysis of ACTH immunoreactivity. ACTH immunoreactivity was measured as described (19). The antibody used was specific for the 14–24 segment of ACTH.

Procedures for ACTH Synthesis. For these studies, AtT-20 cells were plated in 24-well plates at an initial density of 5×10^5 cells and were used 4 days after subculturing. Cells were treated in the same manner as described above except that after the 3 hr of liposome treatment, stimulants such as CRF, 8-bromo-cAMP, or PMA were added to the liposome-containing medium and the cells were further incubated for 6 hr. Therefore, the liposome-containing medium was in contact with the cells for a total of 9 hr in these experiments. At the end of the treatments, an aliquot of medium was removed for analysis of ACTH levels.

Isolation, Hybridization, and Quantitation of RNA. The cells in each well were then washed twice with 2 ml of ice-cold phosphate-buffered saline (pH 7.4) and lysed at 37°C for 15 min in 0.5 ml of a solution of 150 mM NaCl/10 mM EDTA/1% $NaDodSO_4$/1 mg of proteinase K per ml/20 mM Tris·HCl, pH 7.4. The solution was extracted once with phenol and once with phenol/chloroform (1:1), and the nucleic acids were precipitated from the aqueous phase with 2.5 vol 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 (FRB) of 40 mM morpholinopropane sulfonic acid, pH 7.0/10 mM sodium acetate/1 mM EDTA].

The RNA was size-fractionated on 1.2% agarose gels containing formaldehyde and stained with ethidium bromide (10 μg/ml) for 30 min. The gel was soaked for 12–16 hr at 4°C in FRB to remove the formaldehyde and then photographed (Polaroid type 55 positive/negative film). The 18S and 28S rRNA bands were scanned by 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 it was either electrophoretically transferred onto GeneScreen hybridization membrane (New England Nuclear) or stained as described above and then blotted and hybridized. Preincubation and hybridization of the RNA blots with ^{32}P-labeled probe (15, 20) were carried out at 42°C for 6 and 16 hr, respectively, according to the manufacturer's instructions (New England Nuclear). The filters were then washed for 4–12 hr in a total vol of 2 liters (three changes) of 0.2× standard sodium citrate (SSC; 1× SSC = 0.15 M NaCl/0.015 M Na citrate)/0.1% sodium pyrophosphate/0.05% $NaDodSO_4$ at 55°C, blotted dry, and exposed to Kodak XAR-2 x-ray film with intensifier screens. The relative amount of POMC mRNA hybridized to the probe was determined by soft laser densitometry or the areas on the nitrocellulose paper for which the radiolabeled probe bound to POMC mRNA were cut out, the pieces were placed in counting vials with Hydrofluor (National Diagnostics, Somerville, NJ), and the amount of ^{32}P per sample was quantitated by liquid scintillation spectroscopy.

RESULTS

Effect of PKI on Hormone-Stimulated ACTH Release. Both CRF and the β-adrenergic receptor agonist isoproterenol stimulate ACTH release from AtT-20 cells in a dose- and time-dependent manner (Figs. 1 and 2). To determine whether cAMP mediates these responses, PKI was incorporated into AtT-20 cells. As previously reported (17) and also shown for this series of experiments (Fig. 1), the PKI treatment inhibited ACTH release initiated by forskolin but not secretion induced by K^+ or PMA—agents that do not stimulate cAMP-dependent protein kinase activity—suggesting that the procedure specifically blocked cAMP-directed ACTH release. The PKI treatment attenuated the ACTH release response to both CRF and isoproterenol (Figs. 1 and 2). In contrast, the treatment did not block CRF- or isoproterenol-stimulated cAMP accumulation (control, basal = 1.3 ± 0.2, CRF = 7.2 ± 0.4, isoproterenol = 6.3 ± 0.7; treated, basal = 1.5 ± 0.2, CRF = 6.9 ± 0.5, isoproterenol = 6.7 ± 0.5; values expressed as pmol per well and are the mean ± SEM of three experiments, assays performed as described in ref. 19), indicating that the manipulation itself did not block hormone-stimulated ACTH release by sequestering the stimulants or by affecting the coupling of hormone receptors to adenylate cyclase.

To determine whether all CRF-like hormones act through cAMP to stimulate ACTH release, the effects of PKI treatment on cholecystokinin 8 (CCK-8)-evoked ACTH secretion from AtT-20 cells were tested. In preliminary studies (unpublished results), CCK-8 and caerulein, a potent CCK-8 receptor agonist, were shown to stimulate ACTH release both from AtT-20 cells and from primary cultures of the rat anterior pituitary through a specific receptor-mediated action. CCK-8, however, does not raise cAMP levels in corticotrophs. Pretreatment of AtT-20 cells with the liposomes containing PKI did not reduce the ability of caerulein to release ACTH (Fig. 3).

PKI Blocks the Increase in POMC mRNA Levels Induced by CRF. To examine the effects of ACTH secretagogues on ACTH synthesis, POMC mRNA levels were measured in

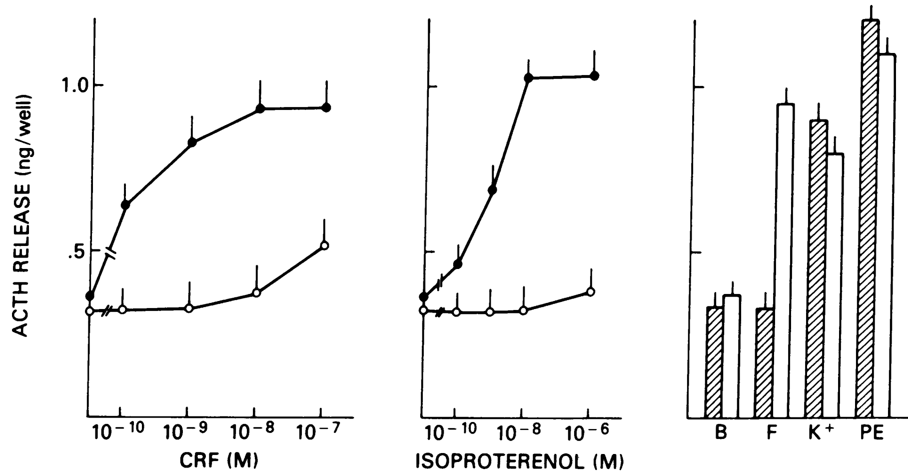

FIG. 1. PKI treatment blocks CRF and isoproterenol-stimulated ACTH release. AtT-20 cells were plated in 96-well culture plates at a density of 5×10^4 cells per well and were used 2 days after plating. The cells were exposed to medium containing 2% fetal calf serum and anti-N-CAM antibody (1:300 dilution) for 1 hr at room temperature in an atmosphere of 10% CO_2/90% air. The medium was removed and cells were exposed for 3 hr at 37°C in 10% CO_2 either to medium having liposomes containing PKI (0.2 μg per 100 μl) coupled to protein A (open circles and hatched bars) or medium alone (solid circles and open bars). At the end of this time, the medium was removed, and the cells were washed twice with fresh medium and then exposed to medium with a different concentration of CRF, isoproterenol (in 0.1% ascorbic acid), or either forskolin (100 μM) (F), K^+ (50 mM), or PMA (50 μM) (PE) or no stimulant (B). Aliquots of medium were then removed and analyzed for ACTH immunoreactivity. The results are the means ± SEM of three different experiments each done on quadruplicate wells.

AtT-20 cells. POMC mRNA was detected and quantitated by hybridization with a radiolabeled probe as described (15). RNA blot analysis showed that the probe hybridized with one RNA species of 1.2 kilobases (not shown), which is the size of mouse POMC mRNA previously reported (27). Treatment of AtT-20 cells for 6 hr with CRF increases the amount of POMC mRNA in AtT-20 cells ≈2-fold (Fig. 4). Total RNA content represented by the amounts of 18S and 28S rRNA was not affected by the CRF treatment (15). The CRF effect in raising POMC mRNA levels is relatively specific because mRNA for β-actin (measured by hybridization with a ^{32}P-labeled probe) was not changed in AtT-20 cells after a similar CRF treatment (15). 8-Bromo-cAMP as well as phorbol ester increases POMC mRNA levels in AtT-20 cells after the same stimulation period as CRF.

Application of PKI-containing liposomes coupled to protein A onto AtT-20 cells pretreated with anti-N-CAM antibodies decreased the levels of POMC mRNA to 50% of control cells (Fig. 4). This manipulation prevented either CRF or 8-bromo-cAMP from increasing POMC mRNA levels. In contrast, phorbol ester still increased POMC mRNA levels after this treatment. The PKI treatment did not change the amounts of 18S and 28S rRNA in the cells (not shown). Furthermore, exposure of anti-N-CAM-labeled AtT-20 cells to liposomes coupled to protein A but not containing PKI did not affect the increase in POMC mRNA induced by CRF, 8-bromo-cAMP, or phorbol ester, nor did it lower POMC mRNA levels (Fig. 4).

DISCUSSION

Insertion of PKI into AtT-20 cells through the use of liposomes allowed us to demonstrate that cAMP has an

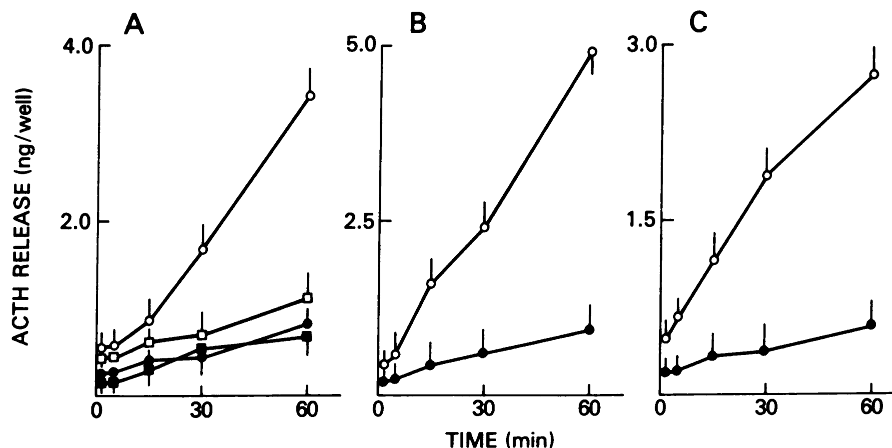

FIG. 2. PKI treatment blocks the time course of CRF-, isoproterenol-, and forskolin-stimulated ACTH release. AtT-20 cells were plated in 96-well culture plates at an initial density of 5×10^4 cells per plate and were used for experimentation 4 days after plating. Cells were treated for 1 hr at room temperature in 10% CO_2/90% air with anti-N-CAM antibodies, washed, and then treated for 3 hr at 37°C in 10% CO_2 with (solid symbols) or without (open symbols) liposomes containing PKI (0.2 μg per 100 ml) coupled to protein A. At the end of this time, the cells were washed twice and stimulated for different times with CRF (100 nM) (*A*), isoproterenol (1 μM) in 0.1% ascorbic acid (*B*), or forskolin (50 μM) (*C*). Open circles represent stimulation from control cells and closed circles are stimulation from cells pretreated with the PKI. Basal release from treated (■) and control (□) is shown in *A*. At the end of the stimulation period, aliquots of medium were analyzed for ACTH immunoreactivity. These results are the means ± SEM of three different experiments done in triplicate wells.

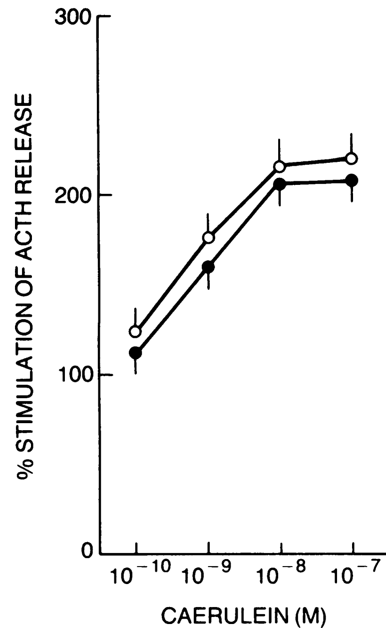

FIG. 3. Lack of effect of PKI treatment on caerulein-stimulated ACTH release. AtT-20 cells were treated with anti-N-CAM antibodies and liposomes containing PKI as described in Fig. 1 legend. Control (●) and treated (○) cells were then exposed to different concentrations of caerulein for 1 hr. Samples of medium were then removed and analyzed for ACTH immunoreactivity. The results are the means ± SEM of three experiments done in triplicate wells.

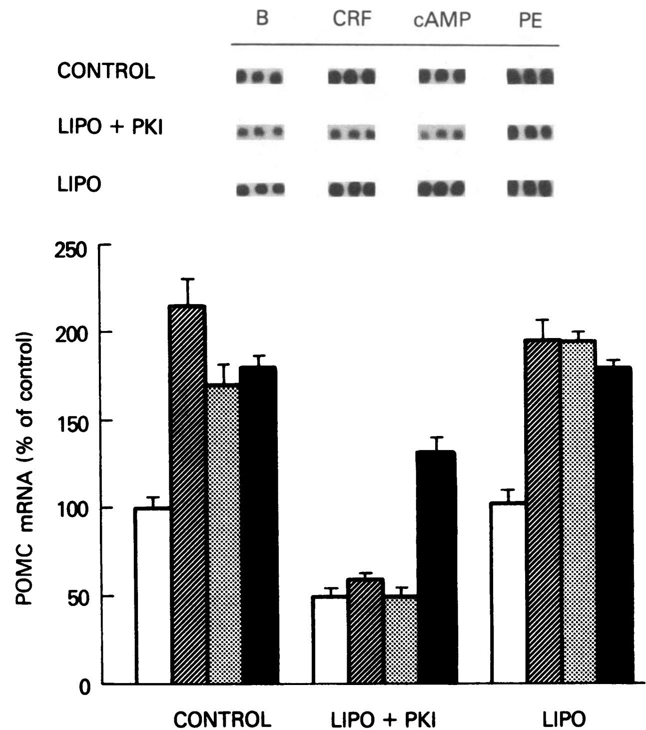

FIG. 4. Effect of PKI treatment on POMC mRNA levels. AtT-20 cells were plated in 24-well culture plates at an initial density of 5 × 10^5 cells and were used 4 days after plating. All cells were treated with anti-N-CAM antibodies (1:300 dilution) in 450 μl of medium for 1 hr. Cells were washed and then exposed for 3 hr to medium (450 μl) (control), medium containing PKI-encapsulated in liposomes coupled to protein A (LIPO + PKI), or medium containing liposomes coupled to protein A but without PKI (LIPO). Either 50 μl of CRF (final concentration, 100 nM), 8-bromo-cAMP (final concentration, 100 μM) (cAMP), PMA (final concentration, 100 nM) (PE), or phosphate-buffered saline containing 0.1% ethanol (*B*) was added to the treatment medium for 6 hr. At the end of this time, samples of medium were removed and analyzed for ACTH immunoreactivity. The remaining medium was removed and POMC mRNA levels were measured. (*Upper*) Autoradiograms of the ^{32}P-radiolabeled probe hybridized to POMC mRNA. The area on the nitrocellulose paper for which the ^{32}P-labeled probe hybridized to POMC mRNA for each sample was cut out and radioactivity was assessed by scintillation spectroscopy. Control basal levels were 839 ± 46 dpm. This value was taken as 100% of control and the values in each experimental condition were calculated as a percentage of this value. (*Lower*) The different conditions presented are control (□), CRF-treated (▨), 8-bromo-cAMP-treated (▩), or PMA-treated (■) cells. These results are the means ± SEM of three different experiments, each done on different subcultures. The ACTH immunoreactive content in the medium of each group is as follows: control, B = 12.5 ± 1.0; CRF = 23.1 ± 1.4; cAMP = 22.4 ± 1.5; PMA = 23.2 ± 1.4. LIPO + PKI, B = 12.2 ± 1.2; CRF = 14.3 ± 0.9; cAMP = 13.5 ± 1.2; PMA = 24.5 ± 1.5. LIPO, B = 12.8 ± 0.8; CRF = 23.5 ± 1.3; cAMP = 23.9 ± 1.5; PMA = 24.1 ± 1.5. Values are expressed as ng per well.

essential role in mediating the actions of CRF in stimulating ACTH release and synthesis. Both CRF and forskolin, an activator of adenylate cyclase, induce the phosphorylation of multiple proteins in AtT-20 cells (7, 8). Preliminary studies (21) indicate that the phosphorylation events initiated by forskolin are attenuated by the PKI treatment. The functional role of these phosphoproteins is not known. One site of their action may be the calcium channel. Recent studies by Curtis and Caterall (22) have shown that cAMP-dependent protein kinase regulates the phosphorylation of a component of the calcium channel in transverse tubular membranes. CRF, forskolin, and 8-bromo-cAMP all increase intracellular calcium levels in AtT-20 cells, and using patch clamp techniques, 8-bromo-cAMP was shown to directly activate calcium conductance channels in these cells (11). Thus, CRF activation of cAMP-dependent protein kinase could catalyze the phosphorylation of a voltage-sensitive calcium channel in AtT-20 cells so as to enhance calcium mobilization and hormone secretion.

In addition, β-adrenergic agonists enhance the frequency of action potential generation in AtT-20 cells, which has been associated with an increase in transient calcium currents across the cell membrane (9, 10). This increase in calcium currents has been correlated with the ability of β-adrenergic agonists to release ACTH from these tumor cells (9, 10). Isoproterenol-evoked ACTH release was also prevented by the PKI treatment. Thus, β-adrenergic agonists must initially increase cAMP levels to induce ACTH secretion. Whether this increase in cAMP levels is responsible for changes in membrane potential is not known.

The PKI treatment did not block the ACTH release response to the CCK-8 agonist caerulein. This is not unexpected because CCK-8 does not increase cAMP formation in corticotrophs. In pancreas, CCK-8 has been shown to induce amylase secretion through an increase in calcium utilization (23). A similar mechanism of action may occur in corticotrophs. Interestingly, PKI did not affect K^+-evoked ACTH release. K^+ also does not increase cAMP levels or activate cAMP-dependent protein kinase in AtT-20 cells (8), but it does greatly increase Ca^{2+} mobilization (11). Thus, it is possible that K^+ and CCK-8 stimulate ACTH release by altering cytosolic Ca^{2+} levels through a mechanism independent of the actions of cAMP.

CRF also stimulates ACTH synthesis through a cAMP-dependent mechanism. CRF increases the levels of POMC mRNA in AtT-20 cells in a time-dependent manner, which is reversible (15). This stimulation most likely is due to an activation of the POMC gene, since CRF treatment increases the levels of a nuclear RNA species larger than mature POMC mRNA and having the expected size of the primary transcript of the POMC gene (15). Furthermore, a recent preliminary

report[‡] indicated that CRF increases POMC gene transcription rates in AtT-20 cells as measured by nuclear runoff transcription assays. The ability of the PKI treatment to inhibit the increase in POMC mRNA levels, induced by both CRF and 8-bromo-cAMP, supports the role of cAMP-dependent protein kinase in regulating the POMC gene. The reduction in POMC mRNA but not total RNA levels after PKI treatment suggests that cAMP may tonically regulate the POMC gene.

CRF may regulate the POMC gene by inducing the phosphorylation of nuclear proteins. In preliminary experiments on AtT-20 cells, we showed that forskolin stimulated the phosphorylation of nuclear as well as other cellular proteins (21). The manner by which these phosphorylation events enhance gene expression is not known. cAMP-regulated phosphoproteins could induce promoter affinity changes, higher transcription rates, or other regulatory events occurring downstream from the start site of transcription.

Phorbol esters also increase POMC mRNA levels and ACTH release, and these effects are not blocked by the PKI treatment. That two different protein kinases could regulate the same biological event is not unprecedented. Both cAMP-dependent protein kinase and protein kinase C affect tyrosine hydroxylase activity in the corpus striatum and catalyze the phosphorylation of the same serine residue on this enzyme (24). In AtT-20 cells, phorbol esters induce the phosphorylation of both the same and different phosphoproteins as forskolin (21). Interestingly, Barinaga *et al.* (25) found in primary cultures of the anterior pituitary that cAMP but not phorbol esters increases growth-hormone gene transcription, whereas both agents stimulate growth-hormone release. The presence of phosphoproteins regulated by one or the other of these protein kinases in different cell types of the anterior pituitary may therefore be a major factor determining the mode of gene regulation in these cells by hormones and second messengers.

In few cells has a direct link between cAMP-dependent protein kinase activity and a biological response been established (26). Injection of PKI into a single *Aplysia* neuron has allowed for a direct demonstration of the role of cAMP in mediating the effect of serotonin on membrane potential (26). By using the liposome technique, it will be possible to insert agents, otherwise impermeable to cell membranes, into multicellular systems in order to determine the relationship between intracellular second messengers and physiological responses, such as hormone or neurotransmitter release. Furthermore, with the development of antibodies against unique cell-surface antigens, it will be possible to selectively affect the signal transduction mechanisms in subpopulations of cells within an organ of heterogenous cellular nature. This may allow for the precise determination of the manner by which hormones or neurotransmitters produce their physiological effects.

[‡]Knight, R., Blum, M., Roberts, J., Farah, J., Bishop, J. & O'Donohue, T., Fifth International Washington Spring Symposium on Neural and Endocrine Peptides and Receptors, July, 16, 1985, Washington, DC, abstr. 147.

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1. Vale, W. W., Spiess, J., Rivier, C. & Rivier, J. (1981) *Science* **213**, 1394–1395.
2. Spiess, J., Rivier, J., Rivier, C. & Vale, W. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 6517–6521.
3. Yates, F., Russell, S., Dallman, M., Hedge, G., McCann, S. & Dhariwal, A. (1971) *Endocrinology* **88**, 3–11.
4. Gilles, G., Linton, E. & Lowry, P. (1982) *Nature (London)* **299**, 355–356.
5. Tilders, F., Berkenbosch, F. & Smelik, P. (1982) *Endocrinology* **110**, 114–120.
6. Aguilera, G., Harwood, J., Wilson, J., Morell, J., Brown, J. & Catt, K. (1983) *J. Biol. Chem.* **258**, 8039–8045.
7. Miyazaki, K., Reisine, T. & Kebabian, J. (1984) *Endocrinology* **115**, 1923–1945.
8. Litvin, Y., Pasmantier, R., Fleischer, N. & Erlichman, J. (1984) *J. Biol. Chem.* **259**, 10296–10305.
9. Suprenant, A. (1982) *J. Cell Biol.* **95**, 559–566.
10. Adler, M., Wong, B., Sabol, S., Busis, N., Jackson, M. & Weight, F. F. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2086–2090.
11. Luini, A., Lewis, D., Guild, S., Corda, D. & Axelrod, J. (1985) *Proc. Natl. Acad. Sci. USA*, in press.
12. Phillips, M. & Tashijian, A. (1982) *Endocrinology* **110**, 892–903.
13. Zatz, M. & Reisine, T. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1286–1290.
14. Bruhn, T., Sutton, R., Rivier, C. & Vale, W. (1984) *Neuroendocrinology* **39**, 170–175.
15. Affolter, H. U. & Reisine, T. (1985) *J. Biol. Chem.*, in press.
16. Birnberg, N. C., Lissitzky, J.-C., Hinman, M. & Herbert, E. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 6982–6986.
17. Reisine, T., Rougon, G. & Barbet, J. (1985) *J. Cell Biol.*, in press.
18. Rougon, G., Deagostini-Bazin, H., Hirn, M. & Goridis, C. (1982) *EMBO J.* **1**, 1239–1244.
19. Reisine, T. (1984) *J. Pharmacol. Exp. Ther.* **229**, 14–20.
20. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251.
21. Rougon, G., Barbet, J., Affolter, H. U. & Reisine, T. (1985) *Soc. Neurosci. Abst.* **11**, 1093.
22. Curtis, B. & Caterall, W. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 2528–2532.
23. Gardner, J. D. & Jensen, R. J. (1981) in *Physiology of the Gastrointestinal Tract*, ed. Johnson, L. (Raven, New York), pp. 831–871.
24. Albert, K., Helmer-Matyjek, E., Nairn, A., Muller, T., Haycock, J., Greene, L., Goldstein, M. & Greengard, P. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 7713–7717.
25. Barinaga, M., Bilezikijian, L., Vale, W., Rosenfeld, M. & Evans, R. (1985) *Nature (London)* **314**, 279–281.
26. Nestler, E. & Greengard, P., eds. (1984) *Protein Phosphorylation in the Nervous System* (Wiley, New York).
27. Civelli, O., Birnberg, N., Comb, M., Douglass, J., Lissitzky, J., Uhler, M. & Herbert, E. (1983) *Peptides* **4**, 651–656.