Corticotropin-releasing factor stimulates phospholipid methylation and corticotropin secretion in mouse pituitary tumor cells

(corticotropin-releasing factor analogues/methyltransferase/adrenocorticotropic hormone)

VIVIAN Y. H. HOOK*†, SEYMOUR HEISLER‡§, AND JULIUS AXELROD*

*Section on Pharmacology, Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland 20205; and ‡Unite de Bioregulation Cellulaire et Moleculaire, Centre Hospitalier de l'Université, Laval, Quebec, Canada G1V 4G2

ABSTRACT   The 41-residue synthetic ovine corticotropin-re-
leasing factor (CRF; corticoliberin) has been shown to stimulate
release of corticotropin (adrenocorticotropic hormone; ACTH)
and β-endorphin from AtT-20/D16-16 mouse pituitary tumor
cells. Phospholipid methylation of phosphatidylethanolamine to
phosphatidylcholine with S-adenosylmethionine as methyl donor
has been suggested as a possible membrane transduction mech-
anism for some receptor-induced events. CRF increased phos-
pholipid methylation in pituitary tumor cells at concentrations that
also stimulated immunoreactive ACTH secretion, and both pro-
cesses increased linearly and in parallel with time. The methionine
sulfoxide derivative of CRF was less potent than CRF was in stim-
ulating both phospholipid methylation and hormone secretion,
and the COOH-terminal free acid analogue of CRF had no effect
on either process. CRF-induced increases in phospholipid meth-
ylation and ACTH secretion were reduced when cells were treated
with the phospholipid methyltransferase inhibitors 3-deazaaden-
osine and L-homocysteine thiolactone. These CRF-stimulated ef-
fects were also blocked by the glucocorticoid dexamethasone. It
is suggested that phospholipid methylation may be a CRF recep-
tor-mediated event associated with ACTH release in pituitary tu-
mor cells.

A hypothalamic peptide termed corticotropin-releasing factor
(CRF; corticoliberin), which stimulates the in vivo and in vitro
secretion of corticotropin (adrenocorticotropic hormone; ACTH)
and β-endorphin from the anterior pituitary, has been isolated,
sequenced, and characterized (1–3). This 41-residue peptide is
believed to be the physiological hypothalamic factor involved
in the regulation of ACTH and β-endorphin secretion from cor-
ticotrophs. It also has been shown to stimulate secretion of these
hormones from mouse pituitary tumor cells (4), which have
been used to study the synthesis and secretion of ACTH/β-
endorphin-related peptides (5–9).

Phospholipid methylation of phosphatidylethanolamine
(PtdEtn) to phosphatidylcholine (PtdCho) with S-adenosylme-
thionine (AdoMet) as methyl donor has been shown in several
cellular systems to be involved as a possible membrane trans-
duction mechanism for receptor-induced events (10–14). In this
study, phospholipid methylation has been examined in the
CRF-stimulated process of ACTH release in mouse pituitary
tumor cells.

METHODS AND MATERIALS

Cell Culture. A subclone of AtT-20/D16v cells designated
AtT-20/D16-16, provided by S. Sabol (Laboratory of Biochem-
ical Genetics, National Institutes of Health), was used in this
study. Cells were cultured in 90% Dulbecco's modified Eagle's

medium/10% fetal calf serum in humidified 90% air/10% CO_2
at 37°C as described (9).

Cell Incubation Procedures. Cells were plated in 35-mm-
diameter culture wells (six wells per plate; Costar) at a density
of 1.5×10^5 cells per dish and grown to 60–80% confluency
($\approx 10^6$ cells per dish at 6–7 days after plating). Immediately prior
to phospholipid methylation and ACTH secretion studies, the
culture medium was removed and the cells were incubated at
37°C for 30 min in 1 ml of medium B [L-methionine-free basal
Eagle's medium with Hanks' salts containing glucose (4.5 mg/
ml), fetal calf serum (2%), and bacitracin (3 μg/ml)] per well.
The cells were then incubated for an additional 30 min with
medium B containing 50–100 μCi (1 Ci = 3.7×10^{10} becque-
rels) of L-[methyl-^{3}H]methionine per ml. The test incubation
period was started by adding the test agent to each well, and
the cells were incubated for an additional 15–90 min. In some
experiments, 3-deazaadenosine and L-homocysteine thiolac-
tone were added 30 min prior to addition of CRF. Stock solu-
tions of 0.01 M dexamethasone were in 95% ethanol; the final
ethanol concentration had no effect on secretion. Incubation
was stopped by removing the medium and centrifuging it at
10,000 × g (Beckman Microfuge) for 1 min to remove cellular
debris. The resultant supernatant was stored at −20°C for sub-
sequent ACTH radioimmunoassay. The cells in each well were
gently washed three times with 1 ml of basal Eagle's medium
with Hanks' salts and lysed with the addition of 1 ml of ice-cold
25 mM Hepes buffer (pH 6.5) containing 0.1 mM S-adenosyl-
L-homocysteine. The suspension was frozen and thawed twice
in methanol/dry ice, and 0.4-ml aliquots were removed for the
measurements of protein and [^{3}H]methyl incorporation into
phospholipids. All experiments were conducted in triplicate
wells. Protein was measured with bovine serum albumin as the
standard (15).

Phospholipid Methylation. Ice-cold 15% trichloroacetic acid
was added to each aliquot of Hepes buffer containing the broken
cell suspensions. The mixture was kept on an ice bath for ≈15
min and centrifuged at 20,000 × g for 20 min. The resultant
pellets were extracted with 3-ml of chloroform/methanol, 2:1
(vol:vol), and 2 ml of 0.1 M KCl in 50% methanol as described
(16). Results are expressed as cpm of [^{3}H]methyl incorporation
into chloroform/methanol.

In order to identify the methylated phospholipids, an aliquot

of the chloroform/methanol phase was dried under a stream of nitrogen gas, and the residue was dissolved in 25 μl of chloroform/methanol, 2:1. The samples were applied to silica gel G TLC plates, and chromatograms were developed in chloroform/propionic acid/n-propyl alcohol/water, 30:20:60:10 (vol/vol). Radioactivity in 1-cm sections of each lane was assayed in a Beckman scintillation counter. Phospholipid standards were chromatographed, and the spots were visualized by exposure to iodine vapor.

ACTH Radioimmunoassay. The rabbit anti-porcine ACTH antiserum recognizes human ACTH-(1–39) and ACTH-(1–24) with equal affinity, and does not recognize α-melanotropin or β-endorphin. Assays were conducted as described (4) with human ACTH-(1–39) as standard.

Materials. Synthetic ovine CRF, the methionine sulfoxide derivative of CRF [Met(O)^{21}-CRF], and thyrotropin-releasing hormone were from Peninsula Laboratories (San Carlos, CA). The COOH-terminal free acid derivative of CRF (CRF-OH) was a generous gift from W. Vale (Salk Institute, La Jolla, CA). Bacitracin, dexamethasone, and L-homocysteine thiolactone were from Sigma. 3-Deazaadenosine was obtained from Southern Research Institute (Birmingham, AL). Media were from GIBCO. Fetal calf serum was from North American Biologicals (Miami, FL). Human ACTH standard and rabbit anti-porcine ACTH antiserum were provided by the National Pituitary Agency (Baltimore, MD). Goat anti-rabbit immunoglobulin was from Cappel Laboratories (Cochranville, PA). ^{125}I-Labeled ACTH (1 μCi/10–20 pg) was from Immunonuclear Corporation (Stillwater, MN), and L-[methyl-^{3}H]methionine (15 Ci/mmol) was from New England Nuclear. Silica gel G TLC plates were from Analtech (Newark, DE).

RESULTS

CRF Stimulates Phospholipid Methylation and ACTH Release. CRF stimulated both phospholipid methylation and release of ACTH immunoreactivity in a time-dependent manner (Fig. 1). Phospholipid methylation in CRF-stimulated and control cells increased linearly from 15 to 60 min and reached a plateau by 90 min of incubation. Basal and CRF-stimulated ACTH release was linear up to 90 min of incubation, the longest incubation period tested.

CRF stimulated both phospholipid methylation and release in a concentration-dependent manner (Fig. 2). Increases in ACTH release and phospholipid methylation were detected at nM CRF, and both processes appeared to be maximally stimulated by 10–100 nM CRF (half maximally effective concentration, 1.5–2 nM CRF). ACTH release was increased 4-fold over control values, and phospholipid methylation was increased by 45% over that of controls (10 nM CRF). From experiment to experiment, CRF consistently stimulated phospholipid methylation by 26–50%.

Met(O)^{21}-CRF was less potent than CRF in stimulating phospholipid methylation and ACTH release (Fig. 3). A concentration of 100 nM Met(O)^{21}-CRF was required to elicit a 12% increase in phospholipid methylation and a 5-fold stimulation of ACTH release, whereas a concentration of 10 nM CRF was required to maximally stimulate phospholipid methylation and ACTH release. CRF-OH had no effect on either ACTH-releasing activity or on phospholipid methylation (data not shown). Thyrotropin-releasing hormone, a peptide structurally unrelated to CRF, had no effect on either process at 100 nM (data not shown).

To identify the [^{3}H]methylated phospholipids after incubating the cells with L-[methyl-^{3}H]methionine and extracting them with chloroform/methanol, the samples were subjected to TLC
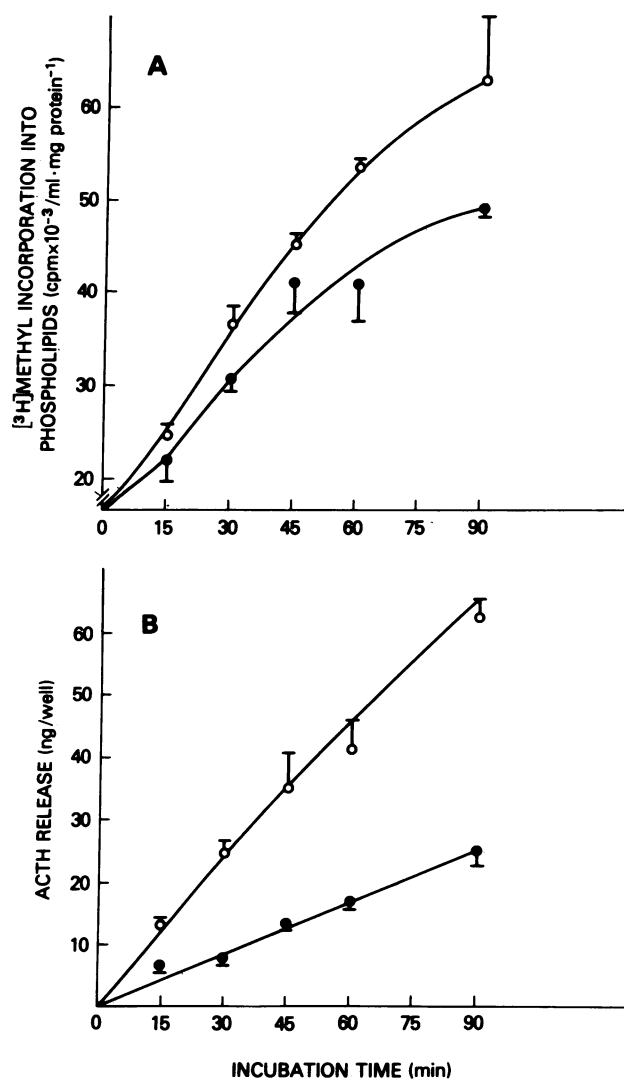

FIG. 1. Time course of phospholipid methylation (A) and ACTH release (B). Cells were incubated with (○) or without (●) CRF (10 nM) for 15 to 90 min. Phospholipid methylation and the amount of ACTH immunoreactivity were measured in the same sample. Each well contained 1 ml of medium.

on silica gel G (Fig. 4). Peaks of [^{3}H]methyl radioactivity corresponding to PtdCho and phosphatidyldimethylethanolamine (PtdEtnMe$_2$) were found. CRF stimulated the incorporation of [^{3}H]methyl groups into PtdEtnMe$_2$ and PtdCho by 90% and 44%, respectively, over nonstimulated controls. No peaks of radioactive phosphatidylmonomethylamine (PtdEtnMe) or lysophosphatidylcholine (lysoPtdCho) were seen. CRF also stimulated the incorporation of radioactivity into unidentified peaks with R$_f$ values 0 and 1.0, which correspond to the origin and front of the chromatogram, respectively.

Effects of Inhibitors. The methyltransferase enzymes responsible for the conversion of PtdEtn to PtdCho can be inhibited by treating the cells with 3-deazaadenosine and L-homocysteine thiolactone. When incubated with these reagants, cells accumulate S-adenosylhomocysteine and 3-deazaadenosylhomocysteine, which are competitive inhibitors of AdoMet-dependent methyltransferases (17, 18). Preincubation with 100 μM 3-deazaadenosine and 100 μM L-homocysteine thiolactone resulted in the inhibition of the CRF-induced increases in phospholipid methylation and ACTH release (Fig. 5); the two inhibitors reduced the CRF (10 nM) stimulation of phospholipid
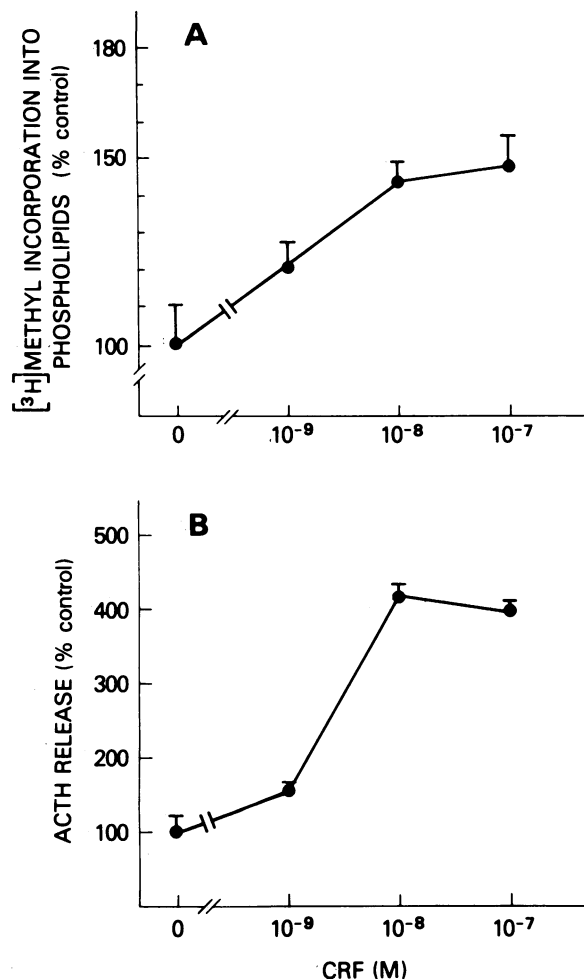
FIG. 2. Effect of various concentrations of CRF on phospholipid methylation (*A*) and ACTH release (*B*). Cells were incubated with CRF for 90 min. Phospholipid methylation and the amount of ACTH immunoreactivity were measured in the same sample.

FIG. 3. Effect of Met(O)^{21}-CRF on phospholipid methylation (*A*) and ACTH release (*B*). Cells were incubated with or without various concentrations of Met(O)^{21}-CRF for 90 min. Phospholipid methylation and ACTH immunoreactivity released were measured in the same sample.

methylation about 75% and reduced CRF-stimulated ACTH release by ≈50%. Basal levels of phospholipid methylation and ACTH release were not affected by the preincubation procedure.

Effect of Dexamethasone. The effect of the potent glucocorticoid dexamethasone on the CRF-elicited increases in phospholipid methylation and ACTH release was examined (Fig. 6). Preincubation with dexamethasone for 20 hr markedly reduced CRF-stimulation of phospholipid methylation and ACTH release in a dose-dependent manner. Partial inhibition of both processes occurred with 1 nM dexamethasone, the lowest concentration tested, and they were almost completely inhibited with 100 nM dexamethasone. Basal levels of phospholipid methylation and ACTH release were also reduced by dexamethasone in a dose-dependent manner.

DISCUSSION

Phospholipid methylation of PtdEtn to PtdCho with AdoMet as methyl donor has been implicated as a possible membrane transduction mechanism for receptor-induced events such as induction of lymphocyte mitogenesis by the lectin concanavalin A (10), IgE-mediated histamine release from rat basophilic leukemia cells (13), stimulation of chemotaxis by the chemotactic peptide fMet-Leu-Phe in rabbit neutrophils (14), and nerve growth factor-stimulated neurite outgrowth in ganglia (19). Evidence presented here suggests that phospholipid methylation
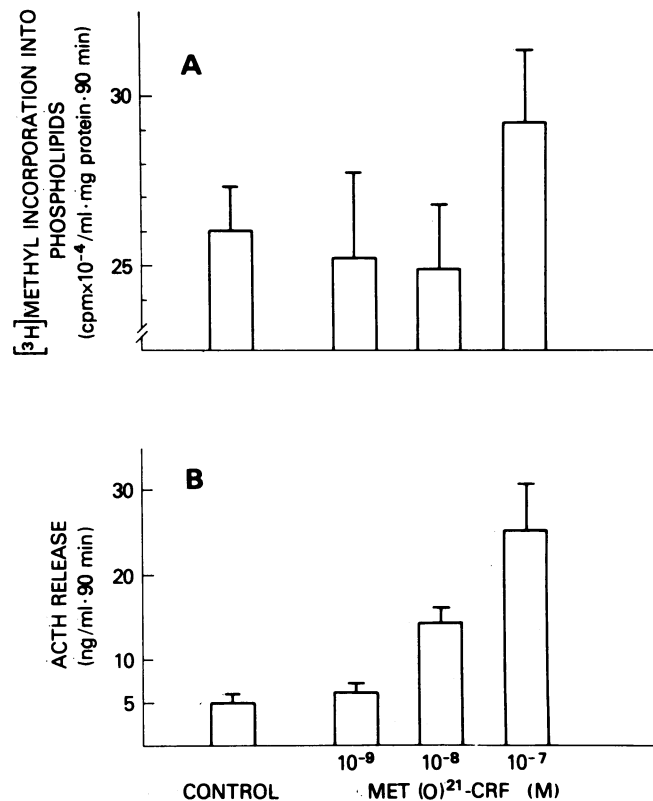
may be associated with CRF stimulation of ACTH secretion in mouse pituitary tumor cells under a variety of experimental conditions.

Stimulation of phospholipid methylation and ACTH release by CRF was concentration-dependent with a half-maximally effective concentration of 2 nM. Increases in phospholipid methylation and ACTH release occurred in parallel and in a linear fashion over a 15- to 60-min incubation with CRF. Analogues of CRF showed a similar relative order of potency in stimulating both phospholipid methylation and ACTH secretion. Met(O)^{21}-CRF was less potent than CRF was in stimulating phospholipid methylation and ACTH secretion in the tumor cells (4). CRF-OH had no effect on either ACTH release or phospholipid methylation.

Additional evidence for the involvement of phospholipid methylation in ACTH secretion was obtained with methyl transferase inhibitors. After preincubation of the cells with the methyl transferase inhibitors 3-deazaadenosine plus L-homocysteine thiolactone, both CRF-stimulated phospholipid methylation and CRF-stimulated ACTH release were considerably reduced. If phospholipid methylation is associated with CRF-induced ACTH secretion, agents that inhibit secretion of the hormone also should inhibit phospholipid methylation. The glucocorticoid dexamethasone reduced both basal and CRF-stimulated phospholipid methylation and ACTH release in a concentration-dependent manner.

Preliminary findings in primary cultures of dispersed rat anterior pituitary cells also showed that CRF stimulated phospholipid methylation and ACTH release (unpublished observation). However, because of the heterogeneity of the cell population and low cell number, it was difficult to adequately
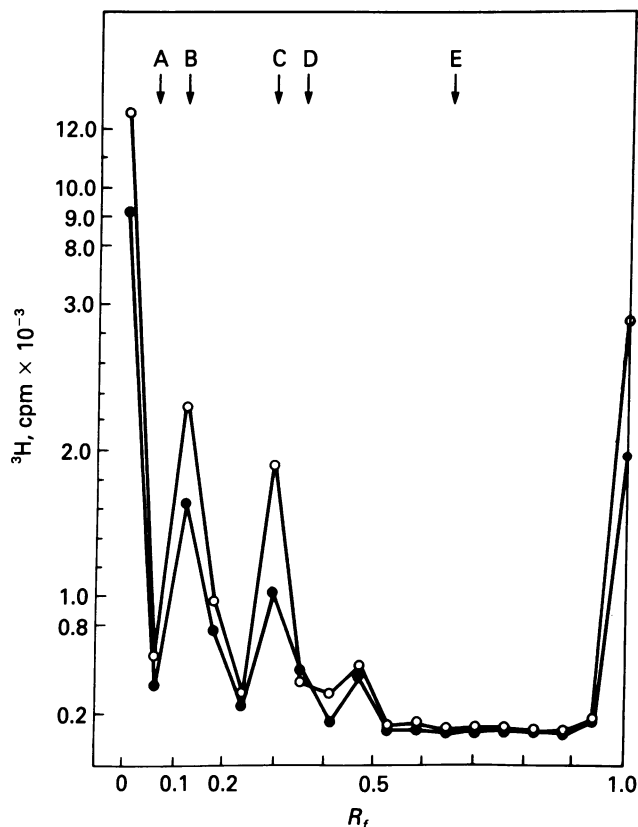
FIG. 4. TLC of [³H]methylated phospholipids. Cells were incubated with (○) or without (●) 10 nM CRF for 90 min and [³H]methyl-containing phospholipids were extracted. Chloroform/methanol extracts of phospholipids were chromatographed on silica gel G in chloroform/propionic acid/*n*-propyl alcohol/water, 30:20:60:10 (vol:vol). Each value on the chromatogram was normalized with regard to protein and total cpm in the sample and represents the mean from three culture wells. Arrows: A, lysoPtdCho; B, PtdCho; C, PtdEtnMe₂; D, PtdEtnMe; E, PtdEtn.

quantitate the CRF effect on phospholipid methylation. Nonetheless, the similar effect of CRF on phospholipid methylation in both the pituitary tumor cells and normal anterior pituitary cells support the use of the tumor cells as a model system to study the role of phospholipid methylation in CRF action.

CRF has been shown to stimulate the synthesis and the secretion of ACTH in the pituitary tumor cells (4). Although it is not known if CRF stimulates synthesis and secretion through similar or different mechanisms, phospholipid methylation may possibly play a role in CRF regulation of ACTH synthesis. Inhibition of CRF-stimulated phospholipid methylation by dexamethasone, a compound which inhibits ACTH synthesis and secretion (4, 9), also suggests that phospholipid methylation may be associated with ACTH synthesis.

Evidence presented also suggests that CRF stimulation of ACTH release may not be dependent solely on phospholipid methylation. The methyltransferase inhibitors 3-deazaadenosine and L-homocysteine thiolactone completely inhibited CRF stimulation of phospholipid methylation but only partially inhibited CRF stimulation of ACTH release. CRF was able to induce some ACTH secretion even without an increase in phospholipid methylation. Furthermore, TLC of the chloroform/methanol-extracted radioactive products showed that, in addition to stimulation of [³H]methyl incorporation into PtdEtnMe₂ and PtdCho, CRF also stimulated the incorporation of radioactivity into other unidentified products. These may represent methylation of unidentified compounds, and the peak
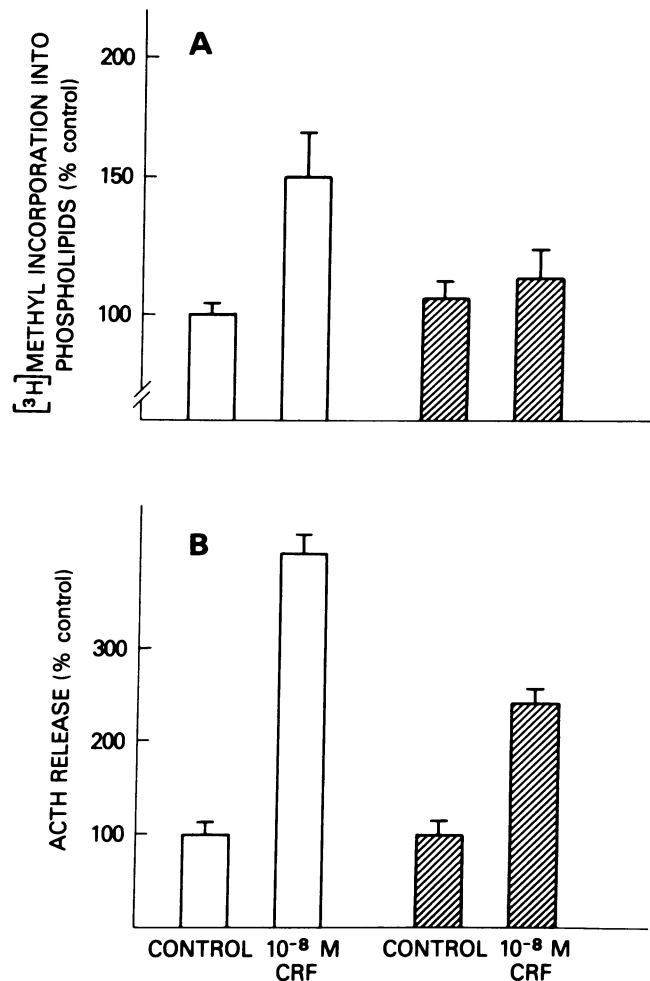

FIG. 5. Effect of 3-deazaadenosine and L-homocysteine thiolactone on CRF-stimulated phospholipid methylation (*A*) and ACTH release (*B*). Cells were preincubated without (□) or with (▨) 100 μM 3-deazaadenosine and 100 μM L-homocysteine thiolactone and then further incubated in the presence or absence of CRF.

at the origin may also include [³H]methionine incorporated into proteins. Thus, other chloroform/methanol-extracted compounds may be related to CRF action.

In some receptor-mediated cellular processes such as IgE-mediated histamine release (13) and chemotaxis in neutrophils (14), a cascade of biochemical events after phospholipid methylation has been hypothesized to occur as follows. Receptor-mediated synthesis of PtdCho through methylation of PtdEtn may be followed by Ca⁺⁺-dependent activation of phospholipase A₂ to yield arachidonic acid and lysolecithin from PtdCho. The arachidonic acid may be further metabolized to yield biologically active compounds such as prostaglandins and leukotrienes. Phospholipase A₂ activity has been found to be regulated by a protein lipomodulin (20, 21). Whether all or any part of this cascade is associated with CRF stimulation of ACTH release remains to be determined.

CRF has been found to stimulate other methyl transferase reactions besides phospholipid methylation. We have found that protein carboxyl-methylation is associated with CRF stimulation of ACTH secretion in the tumor cells (unpublished data). Preliminary findings have shown that cellular cAMP levels were increased by CRF, implying a role for cAMP-dependent protein kinase(s). Investigations on the complex interrelationships among these biochemical processes will be important to deduce the molecular mechanism of CRF action.
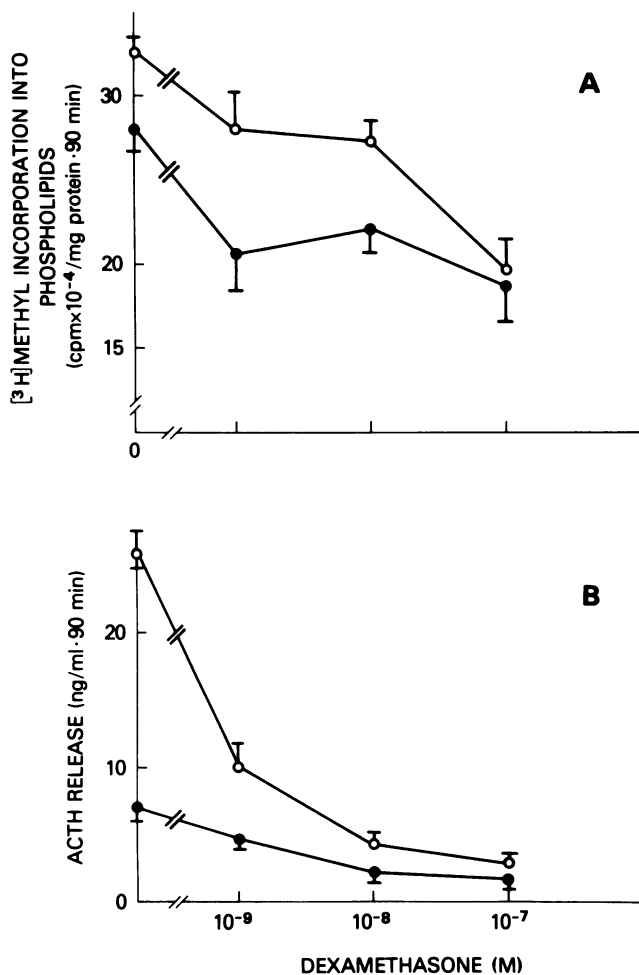
FIG. 6. Effect of dexamethasone on phospholipid methylation (A) and ACTH release (B). Cells were preincubated for 20 hr with various concentrations of dexamethasone in 90% Dulbecco's modified Eagle's medium/10% fetal calf serum and then incubated with (○) or without (●) 10 nM CRF. Phospholipid methylation and ACTH immunoreactivity released were measured in the same sample.

In conclusion, phospholipid methylation in conjunction with other biochemical processes may be involved in CRF receptor-mediated stimulation of ACTH release in pituitary tumor cells.

1. Vale, W., Spiess, J., Rivier, C. & Rivier, J. (1981) *Science* 213, 1394–1397.
2. Spiess, J., Rivier, J., Rivier, C. & Vale, W. (1981) *Proc. Natl. Acad. Sci. USA* 78, 6517–6521.
3. Rivier, C., Brownstein, M., Spiess, J., Rivier, J. & Vale, W. (1982) *Endocrinology* 110, 272–278.
4. Hook, V. Y. H., Heisler, S., Sabol, S. L. & Axelrod, J. (1982) *Biochem. Biophys. Res. Commun.* 106, 1364–1371.
5. Mains, R. E. & Eipper, B. A. (1976) *J. Biol. Chem.* 251, 4115–4120.
6. Roberts, J. L., Phillips, M., Rosa, P. A. & Herbert, E. (1978) *Biochemistry* 17, 3609–3618.
7. Eipper, B. A. & Mains, R. E. (1980) *Endocr. Rev.* 1, 1–27.
8. Sabol, S. L., Ling, A. & Daniels, M. P. (1979) in *Hormones and Cell Culture,* Cold Spring Harbor Conferences on Cell Proliferation, ed. Sato, G. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), Vol. 6, pp. 843–853.
9. Sabol, S. L. (1980) *Arch. Biochem. Biophys.* 203, 37–48.
10. Hirata, F., Toyoshima, S., Axelrod, J. & Waxdal, M. J. (1980) *Proc. Natl. Acad. Sci. USA* 77, 862–865.
11. Hirata, F., Axelrod, J. & Crews, R. T. (1979) *Proc. Natl. Acad. Sci. USA* 76, 4813–4816.
12. Ishizaka, T., Hirata, F., Ishizaka, K. & Axelrod, J. (1980) *Proc. Natl. Acad. Sci. USA* 77, 1903–1906.
13. Crews, R. T., Morita, Y., McGivney, A., Hirata, F., Siraganian, R. P. & Axelrod, J. (1982) *Arch. Biochem. Biophys.* 212, 561–571.
14. Bareis, D. L., Hirata, F. & Axelrod, J. (1982) *J. Cell Biol.* 93, 690.
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* 193, 265.
16. Hirata, F., Viveros, O. H., Diliberto, E. J. & Axelrod, J. (1978) *Proc. Natl. Acad. Sci. USA* 75, 1718–1721.
17. Zimmerman, T. P., Wolberg, G. & Duncan, G. S. (1978) *Proc. Natl. Acad. Sci. USA* 75, 6220–6224.
18. Richards, H. H., Chiang, P. I. & Cantoni, G. L. (1978) *J. Biol. Chem.* 253, 4476–4480.
19. Pfenninger, K. H. & Johnson, M. P. (1981) *Proc. Natl. Acad. Sci. USA* 78, 7797–7800.
20. Hirata, F., Sciffman, E., Venkatasubramanian, K., Saloman, D. & Axelrod, J. (1980) *Proc. Natl. Acad. Sci. USA* 77, 2533–2536.
21. Hirata, F. (1981) *J. Biol. Chem.* 256, 7730–7733.