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

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

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Contributed by Julius Axelrod, July 22, 1982

ABSTRACT The 41-residue synthetic ovine corticotropin-releasing factor (CRF; corticotropin) 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 mechanism for some receptor-induced events. CRF increased phospholipid methylation in pituitary tumor cells at concentrations that also stimulated immunoreactive ACTH secretion, and both processes increased linearly and in parallel with time. The methionine sulfoxide derivative of CRF was less potent than CRF in stimulating 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 methylation and ACTH secretion were reduced when cells were treated with the phospholipid methyltransferase inhibitors 3-deazaadenosine and L-homocysteine thiolactone. These CRF-stimulated effects were also blocked by the glucocorticoid dexamethasone. It is suggested that phospholipid methylation may be a CRF receptor-mediated event associated with ACTH release in pituitary tumor cells.

A hypothalamic peptide termed corticotropin-releasing factor (CRF; corticotropin), which stimulates the in vitro and in vivo 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 physiologic hypothalamic factor involved in the regulation of ACTH and β-endorphin secretion from corticotrophs. 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-adenosylmethionine (AdoMet) as methyl donor has been shown in several cellular systems to be involved as a possible membrane transduction 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 Biochemical 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% CO₂ 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 x 10⁶ cells per dish and grown to 60–80% confluency (≈10⁶ 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 x 10¹² becqurels) of L-[methyl-³H]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 thiolactone were added 30 min prior to addition of CRF. Stock solutions 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 x g (Beckman Microfuge) for 1 min to remove cellular debris. The resultant supernatant was stored at −20°C for subsequent 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 [³H]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 x 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 [³H]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). Bactritacin, 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 (Cochraneville, PA). [³⁵S]-Labeled ACTH (1 μCi/10–20 pg) was from Immunonuclear Corporation (Stillwater, MN), and L-[methyl-³H]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 ACTH 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 [³H]methylylated phospholipids after incubating the cells with L-[methyl-³H]methionine and extracting them with chloroform/methanol, the samples were subjected to TLC on silica gel G (Fig. 4). Peaks of [³H]methyl radioactivity corresponding to PtdCho and phosphatidylcholine (PtdCho) were found. CRF stimulated the incorporation of [³H]methyl groups into PtdCho and PtdCho by 90% and 44%, respectively, over nonstimulated controls. No peaks of radioactive phosphatidylmonomethylamine (PtdEmMe) or lyso phosphatidylcholine (lysoPtdCho) were seen. CRF also stimulated the incorporation of radioactivity into unidentified peaks with Rf 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 PtdCho to PtdCho can be inhibited by treating the cells with 3-deazaadenosine and L-homocysteine thiolactone. When incubated with these reagents, cells accumulate 5-adenosylhomocysteine and 3-deazaadenosylhomocysteine, which are competitive inhibitors of AdoMet-dependent methyltransferases (17, 18). Frencubation 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
methyltransferase inhibitors. After preincubation of the cells with methyltransferase 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
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 PtdEtnMe₉ and PtdCho, CRF also stimulated the incorporation of radioactivity into other unidentified products. These may represent methylation of unidentified compounds, and the peak 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 Ca²⁺-dependent activation of phospholipase A₂ to yield arachidonic acid and lysothecisin 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 methyltransferase 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.
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.

The authors thank Dr. F. Hirata, Dr. M. Brownstein, and Dr. M. Zatz for helpful discussions and Ms. S. Derdeyn and Ms. B. Holcomb for expert assistance in the preparation of this manuscript.