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CORTICOTROPIN RELEASING FACTOR STIMULATION OF PROTEIN CARBOXYLMETHYLATION IN MOUSE PITUITARY TUMOR CELLS

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Abstract—A putative role for the protein carboxylmethylase (PCM) enzyme has been suggested in exocytotic secretion. The involvement of ^{3}H-methyl incorporation into protein carboxylmethyl esters during corticotropin releasing factor (CRF)-induced ACTH secretion from AtT-20/D16-16 mouse pituitary cells was investigated. Protein carboxylmethylation and ACTH secretion both increased as a function of extracellular CRF concentration, and both processes were temporally parallel up to 60 min incubation. The less potent [Met(O)^{21}]-CRF also stimulated increases in protein carboxylmethylation and ACTH secretion. The free acid analogue of CRF did not alter either process. A combination of the PCM inhibitors, 3-deazaadenosine and L-homocysteine thiolactone, reduced both CRF-stimulated protein carboxylmethylation and ACTH release. Dexamethasone, known to inhibit ACTH secretion and synthesis, inhibited both CRF-stimulated protein carboxylmethylation and ACTH secretion.

Methyl groups can be transferred from *S*-adenosylmethionine (SAM) to free carboxyl groups of glutamyl and/or aspartyl residues of protein substrates by the enzyme protein carboxylmethylase (PCM; EC 2.1.1.24, *S*-adenosylmethionine:protein carboxyl-*O*-methylase) [1–4]. Since the methyl esters formed are labile [3, 5–8], it has been suggested that PCM can produce rapid and reversible alterations in activity of enzymes, hormones or other protein substrates [5]. One of the functions ascribed to PCM is a role in exocytotic secretion from endocrine [7, 9–12] and exocrine [8, 13] glands.

The AtT-20 mouse pituitary cell line, derived from an anterior pituitary tumor, has been used to study the regulation of synthesis, storage and secretion of ACTH, β-endorphin and related peptides [14–21]. Recent studies in our laboratory [22] have demonstrated that in this cell line the synthetic 41-residue corticotropin releasing factor (CRF) [23] induces secretion of ACTH and β-endorphin, as it does in other *in vivo* and *in vitro* systems [24, 25]. In view of a putative role for PCM in exocytotic secretion, the relationship between protein carboxylmethylation and ACTH secretion in AtT-20 cells was investigated.

MATERIALS AND METHODS

Materials. Synthetic CRF, the 21-methoxy derivative of CRF ([Met(O)^{21}]-CRF), and thyrotropin releasing hormone (TRH) were purchased from Peninsula Laboratories (San Carlos, CA). The free acid analogue of CRF (CRF-OH) was a gift from Dr. W. Vale (Salk Institute, La Jolla, CA). Bacitracin, dexamethasone acetate and L-homocysteine thiolactone (HC) were from the Sigma Chemical Co. (St. Louis, MO). 3-Deazaadenosine (3-DA) was obtained from the Southern Research Institute (Birmingham, AL). Dulbecco modified Eagle's basal medium (DMEM) and basal Eagle's medium containing Hank's salts (BEMH) were obtained from GIBCO (Grand Island, NY). Trypsin was from Worthington Diagnostics (Freehold, NJ). Fetal calf serum was from North American Biologicals (Miami, FL). ACTH standard and antiserum were donations of the National Pituitary Agency (Baltimore, MD). [^{125}I]-ACTH (1 μCi/10–20 pg) was from the Immunonuclear Corp. (Stillwater, MN) and L-[methyl-^{3}H]methionine (15 Ci/mmole) was obtained from the New England Nuclear Corp. (Boston, MA).

Culture methods. AtT-20/D16-16 cells, obtained from Dr. S. Sabol (N.I.H.), were grown and subcultured in DMEM containing 10% fetal calf serum as previously described [22].

Incubation procedures. Cells were innoculated into 35 mm diameter culture wells (six wells/dish, CoStar) at an initial density of 1.0 to 1.5 $\times$ 10^5 cells/well and grown to 60–80% confluency (5–7 days after passage) before use. Immediately prior to a secretion and methylation study, the culture medium was aspirated and the cells were equilibrated at 37° for 30 min in 1 ml of L-methionine-free BEMH containing 4.5 mg/ml glucose, 2% fetal calf serum and 3 μg/ml bacitracin. This medium was replaced with 1 ml of the same medium to which 50–100 μCi [^{3}H]-methylmethionine had been added, and the cells were incubated for an additional 30 min. CRF (prepared freshly in phosphate buffered saline, pH 7.4)

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was added, and the cells were incubated as indicated below. When used, 3-DA and HC were added 30 min prior to CRF. Dexamethasone (10^{-2} M stock in ethanol), when used, was added 20 hr prior to the start of the secretion and methylation study and was present throughout the incubation periods preceding CRF addition. The highest ethanol concentration to which cells were exposed (0.001%) had no effect on basal secretion of ACTH.

At the end of the incubation period, the medium was aspirated and centrifuged at 10,000 rpm (Beckman microfuge) for 1 min to remove detached and broken cells. The supernatant fraction was removed and stored at −20° until used in the ACTH radioimmunoassay. The cells were washed gently three times with 1 ml BEMH and lysed with the addition of 1 ml of ice-cold hypo-osmotic 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes) (pH 6.5) buffer containing 0.1 mM *S*-adenosyl-L-homocysteine (SAH). After freezing and thawing in methanol/dry ice, aliquots of the broken cell suspension were removed for measurement of protein concentration and ^{3}H-protein methyl esters formed.

ACTH radioimmunoassay. ACTH immunoactivities in the secreted medium were assayed with an antiserum specific for the 14-24 region of ACTH according to Hook *et al.* [22]. No cross reactivity with either α-melanocyte stimulating hormone (α-MSH) or β-endorphin was observed with the antiserum. ACTH values were calculated from three to four points on the linear section of the standard curves for reference ACTH (synthetic human ACTH 1-39). Radioimmunoassays of replicate samples of culture medium gave a coefficient of variation of less than 10%. Results are expressed as ng ACTH/per well.

Protein carboxylmethylation. An aliquot of the cell suspension in Hepes buffer was precipitated with 1 ml of ice-cold 15% trichloroacetic acid and centrifuged at 20,000 *g* for 20 min. Protein carboxylmethylation was measured [9] by subjecting the pellet to alkaline hydrolysis, and the resultant [^{3}H]-methanol formed from ^{3}H-protein methyl esters was extracted into a mixture of toluene–isoamylalcohol (3:2, v/v). Results are expressed as cpm ^{3}H-methyl incorporated into protein methyl esters per mg cell protein. There was little variability in protein levels among different culture wells.

Protein determination. Protein content in the Hepes–SAH cell suspension was measured by the method of Lowry *et al.* [26] using bovine serum albumin as standard.

Results are presented as means ± S.E. for triplicate cultures.

RESULTS

The effect of various concentrations of CRF on ACTH secretion and protein carboxylmethylation is illustrated in Fig. 1. Both ACTH secretion and protein carboxylmethylation were stimulated maximally by 10^{-8} M CRF. The half-maximal CRF concentration for ACTH secretion and carboxylmethylation was about 3 nM. At the lowest concentration tested (10^{-10} M), CRF did not alter basal release of ACTH from the cultured cells and did not change ^{3}H-methyl incorporation into carboxylmethylated proteins.

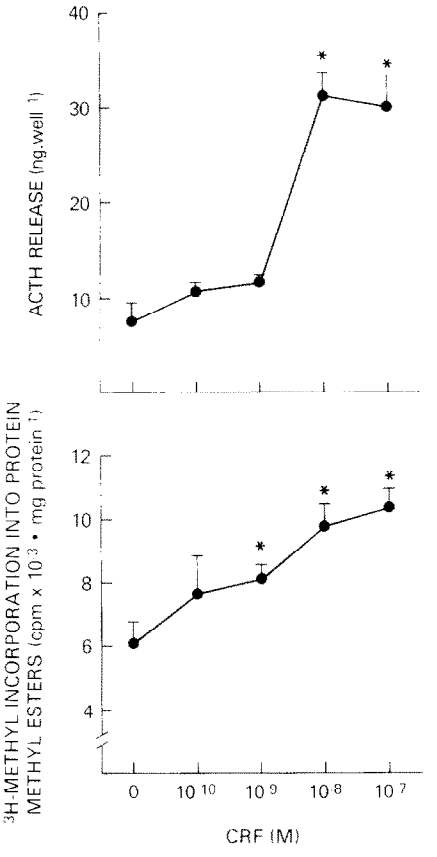

Fig. 1. Effect of various concentrations of synthetic 41-residue CRF on protein carboxylmethylation and ACTH release in AtT-20/D16-16 cells incubated for 90 min. Each point represents the mean ± S.E. of three separate cultures. Asterisks indicate $P < 0.05$ or better compared to 0 CRF.

The time–course study of basal and CRF-induced ACTH release and carboxylmethylation is illustrated in Fig. 2. CRF-elicited ACTH release was linear over the 90-min time course and was 2 to 3-fold higher at each of the time points studied in comparison to basal values. CRF consistently increased protein carboxylmethylation throughout the time course; the greatest difference in carboxylmethylation between stimulated and non-stimulated cells was observed at 60 and 90 min (35–40%). Protein carboxylmethylation in both CRF-stimulated and control cells increased linearly between 15 and 45 min and reached a plateau at 50–90 min.

The effects of two CRF analogues were tested on ACTH secretion and protein carboxylmethylation. [Met(O)^{21}]-CRF increased ACTH release and protein carboxylmethylation in a dose-dependent fashion. At 10^{-7} M, [Met(O)^{21}]-CRF increased ACTH release 3.6-fold above basal values (Fig. 3). CRF at a 10-fold lower concentration had a similar effect (3.7-fold increase in ACTH release). Both [Met(O)^{21}]-CRF (10^{-7} M) and CRF (10^{-8} M) increased protein carboxylmethylation by about

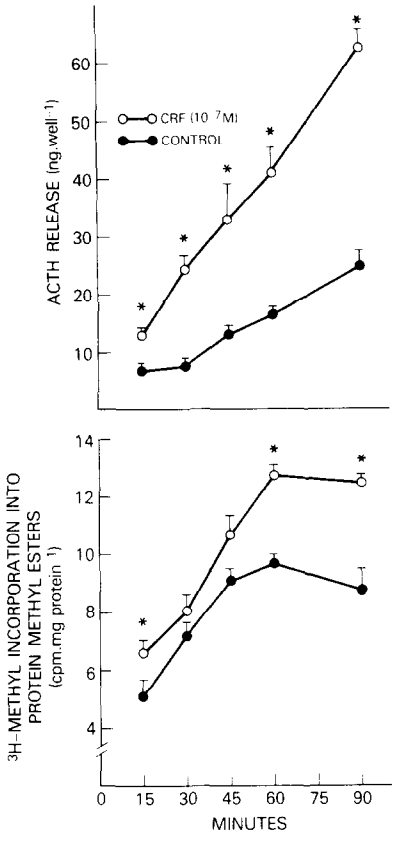

Fig. 2. Protein carboxylmethylation and ACTH secretion in AtT-20/D16-16 cells as a function of time of incubation. CRF was added (time 0) after 30 min of preincubation with [^{3}H]-methylmethionine. Each point represents the mean ± S. E. of three separate cultures. Asterisks indicate $P < 0.05$ or better compared to no CRF at each respective time point.

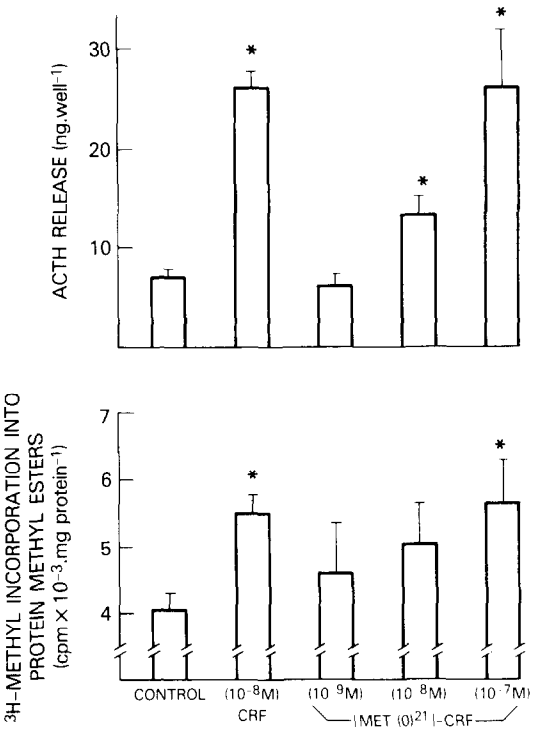

Fig. 3. Effect of the 21-methoxy derivative of synthetic 41-residue CRF [Met(O)^{21}]-CRF) on protein carboxylmethylation and ACTH release in AtT-20/D16-16 cells incubated for 90 min. Each value represents the mean ± S.E of three to six separate cultures. Asterisks indicate $P < 0.05$ or better compared to control cells.

38%. The C-terminal free acid of CRF, on the other hand, had no effect on either basal ACTH release or ^{3}H-methyl incorporation into protein methyl esters (90-min incubation) when tested at concentrations between 10^{-9} and 10^{-6} M (data not shown).

Similarly, TRH (10^{-7}M; 90-min incubation) did not affect basal ACTH release or protein carboxylmethylation in the AtT-20 cells (data not shown).

3-DA and HC increase the intracellular level of SAH, the endogenous competitive inhibitor of protein carboxylmethylase, and thereby inhibit protein carboxylmethylation [27]. The effect of these substances on PCM activity and ACTH secretion was assessed in the pituitary cells. 3-DA and HC reduced CRF-stimulated protein carboxylmethylation to control levels and reduced CRF-induced ACTH secretion by about 40% (Fig. 4). 3-DA and HC reduced carboxylmethylation in unstimulated cells by about 20% without concomitantly affecting ACTH release.

We reported [22] that dexamethasone inhibited ACTH and β-endorphin secretion in AtT-20 cells stimulated by CRF. The effect of dexamethasone treatment was evaluated on protein carboxylmethylation activity and ACTH secretion (Fig. 5).

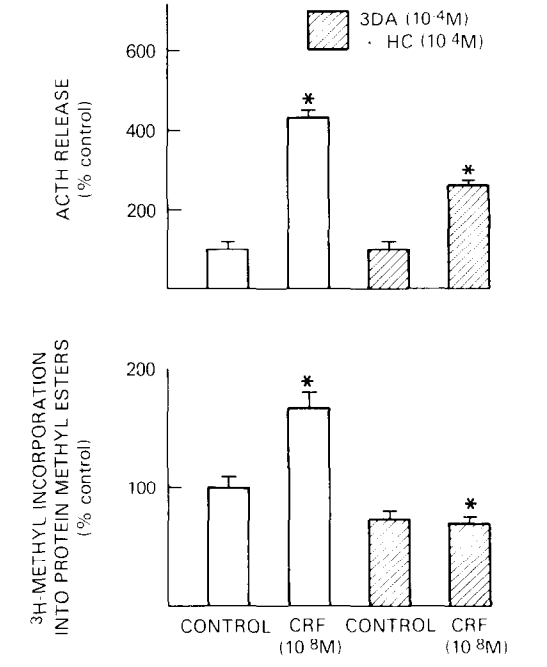

Fig. 4. Inhibitory effect of 3-deazaadenosine (3-DA) and L-homocysteine thiolactone (HC) pretreatment (30 min) on CRF-stimulated protein carboxylmethylation and ACTH secretion in AtT-20/D16-16 cells following 90 min of incubation. Results are expressed as percent of control. Values are means ± S.E. of three separate cultures. Asterisks indicate $P < 0.05$ or better compared to control cells.

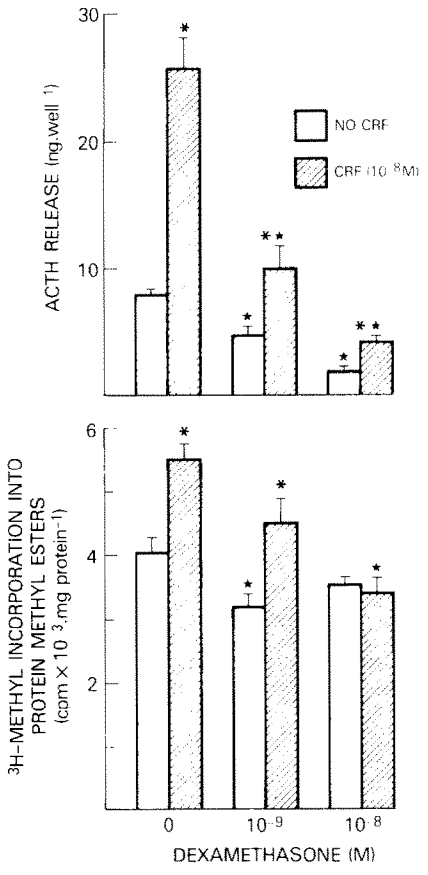

Fig. 5. Inhibitory effect of dexamethasone pretreatment (20 hr) on CRF-stimulated protein carboxylmethylation and ACTH secretion in AtT-20/D16-16 cells following 90 min of incubation. Values are means ± S.E. of three to six separate cultures. Asterisks indicate $P < 0.05$ or better compared to no CRF ± dexamethasone. Stars indicate $P < 0.05$ or better compared to no dexamethasone ± CRF.

Dexamethasone had an inhibitory effect on the amount of ACTH released in both unstimulated and CRF-stimulated cells. When cells were pretreated with 10^{-8} M dexamethasone, ACTH secretion from stimulated and unstimulated cells was reduced by as much as 80–90%. CRF-stimulated protein carboxylmethylation was reduced by 18% when cells were treated with 10^{-9} M dexamethasone, and 10^{-8} M dexamethasone reduced CRF-stimulated protein carboxylmethylation to unstimulated control levels.

DISCUSSION

Post-translational modification of protein structure by carboxylmethylation appears to be a ubiquitous reaction in both eukaryotes and prokaryotes [28, 29]. The enzyme catalyzing the reaction, PCM, has been identified in many mammalian tissues; its high specific activity in the cytoplasm of secretory organs [5] prompted the suggestion that the enzyme might be involved in exocytotic secretion. An increase in protein carboxylmethylation activity has been observed in adrenal medulla following insulin administration [10], in posterior pituitary glands of salt-loaded rats [30], and pancreatic lobules incubated with cholecystokinin or carbachol [13]. In the latter study, a correlation between carboxylmethylation activity and secretion of amylase was reported.

We have reported [22] that AtT-20/D16-16 mouse pituitary cells, grown in culture, respond to the synthetic 41-residue CRF by secreting ACTH and β-endorphin. In this study, CRF was also shown to stimulate an increase in the incorporation of ^{3}H-methyl into protein methyl esters. The following observations suggest that CRF stimulation of protein carboxylmethylation and ACTH secretion may be associated with one another.

Both ACTH secretion and protein carboxylmethylation in the pituitary cells increased as a function of extracellular CRF concentration, and the half-maximal concentration of the peptide required to stimulate both processes was similar. Unstimulated cells exhibited basal ACTH release [16, 20], and protein carboxylmethylation activity was also observed under these conditions.

Increases in secretion of ACTH and ^{3}H-methyl incorporation into protein methyl esters were temporally associated up to 60 min of incubation in unstimulated or CRF-stimulated cells. The subsequent plateau at longer incubation times seen in the time–course study of carboxylmethylation suggests that the ACTH secreted may not be completely associated with changes in protein carboxylmethylation.

If protein carboxylmethylation and ACTH secretion are related events, then analogues of CRF of varying potencies should have similar effects on ACTH release and protein carboxylmethylation. [Met(O)^{21}]-CRF which was reported to be 5- to 10-fold less effective in stimulating ACTH release than CRF [22, 23] was 10-fold less potent than CRF in stimulating ACTH secretion and ^{3}H-protein methyl ester formation. The C-terminal free acid of CRF had no secretory effect on the cells and also did not alter basal levels of protein carboxylmethylation. TRH, structurally unrelated to CRF or its analogues, also did not affect either ACTH secretion or protein carboxylmethylation.

For ACTH secretion and protein carboxylmethylation to be interrelated events, agents which inhibit the activity of PCM should be expected to block ACTH secretion. A combination of 3-DA and HC, indirect methylation inhibitors [27], reduced both CRF-induced ACTH release and protein methyl ester formation; however, each process was inhibited to a different degree. 3-DA and HC reduced CRF-stimulated carboxylmethylation to levels observed in unstimulated cells, but blocked CRF-stimulated ACTH secretion by only 40%. These data suggest that part of the CRF-stimulated ACTH secretory pathway may be related to changes in ^{3}H-protein methyl ester formation.

When cells were treated with dexamethasone, there was a reduction in basal and CRF-stimulated protein carboxylmethylation accompanied by a more marked reduction in basal and CRF-stimulated ACTH secretion. These data suggest that CRF stimulation of ACTH secretion may be partially associated with protein carboxylmethylation.

Dexamethasone is known to inhibit both ACTH release and synthesis [17, 19, 31, 32]. Inhibition of carboxylmethylation by dexamethasone suggests that protein carboxylmethylation may be involved in the synthesis and storage of ACTH, as well as ACTH release. This possibility is interesting in view of a recent report showing that the α-1-38 ACTH fragment can be carboxylmethylated *in vitro* [33].

From a number of studies [7–13] a putative role for PCM has been described in exocytotic secretion. It has been hypothesized that, following hormonal or neurohormonal stimulation of a receptive cell, PCM catalyzes the neutralization of negative charges of protein substrates localized on the surface of secretory vesicles, thereby facilitating the interaction and eventual fusion between these vesicles and the negatively charged plasma membrane. This study supports the idea that CRF-stimulated protein carboxylmethylation may be related to ACTH secretion but also suggests that, in addition to protein carboxylmethylation, other biochemical events are probably involved in CRF stimulation of ACTH secretion.

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REFERENCES

1. M. Liss, A. M. Maxam and L. J. Cuprak, *J. biol. Chem.* **244**, 1617 (1969).
2. S. Kim and W. K. Paik, *J. biol. Chem.* **245**, 1806 (1970).
3. S. Kim and W. K. Paik, *Biochemistry* **10**, 3141 (1971).
4. E. J. Diliberto, Jr. and J. Axelrod, *Proc. natn. Acad. Sci. U.S.A.* **71**, 1701 (1974).
5. E. J. Diliberto, Jr. and J. Axelrod, *J. Neurochem.* **26**, 1159 (1976).
6. S. Kim and W. K. Paik, *Experientia* **32**, 982 (1976).
7. C. Gagnon, O. H. Viveros, E. J. Diliberto, Jr. and J. Axelrod, *J. biol. Chem.* **253**, 3778 (1978).
8. S. Heisler and H. Lambert, in *Biochemistry of S-Adenosylmethionine and Related Compounds* (Eds. E. Usdin, R. T. Borchardt and C. R. Creveling), pp. 71–74. Macmillan, London (1982).
9. E. J. Diliberto, Jr. O. H. Viveros and J. Axelrod, *Proc. natn. Acad. Sci. U.S.A.* **73**, 4050 (1976).
10. O. H. Viveros, E. J. Diliberto, Jr. and J. Axelrod, in *Synapses* (Ed. G. A. Cottrell and P. N. A. Usherwood), p. 368. Blackie & Son, Glasgow (1977).
11. R. T. Borchardt, J. Olsen, L. E. Eiden, R. L. Schowen and C. O. Rutledge, *Biochem. biophys. Res. Commun.* **83**, 970 (1978).
12. L. E. Eiden, R. T. Borchardt and C. O. Rutledge, in *Transmethylation* (Eds. E. Usdin R. T. Borchardt and C. R. Creveling), p. 539. Elsevier-North Holland, New York (1979).
13. V. Povilaitis, C. Gagnon and S. Heisler, *Am. J. Physiol.* **240**, G199 (1981).
14. R. E. Mains and B. A. Eipper, *J. biol. Chem.* **251**, 4115 (1976).
15. R. G. Allen, E. Herbert, M. Hinman, H. Shibuya and C. B. Pert, *Proc. natn. Acad. Sci. U.S.A.* **75**, 4972 (1978).
16. J. L. Roberts, M. Philips, P. A. Rosa and E. Herbert, *Biochemistry* **17**, 3609 (1978).
17. S. L. Sabol, A. Ling and M. P. Daniels, in *Hormones and Cell Culture, Cold Spring Harbor Conference on Cell Proliferation* (Eds. R. Ross and G. Sato), p. 843. Cold Spring Harbor Laboratory, New York (1979).
18. B. A. Eipper and R. E. Mains, *Endocrine Rev.* **1**, 1 (1980).
19. S. L. Sabol, *Archs Biochem. Biophys.* **203**, 37 (1980).
20. R. E. Mains and B. A. Eipper, *J. Cell Biol.* **89**, 21 (1981).
21. B. Gumbiner and R. B. Kelly, *Cell* **28**, 51 (1982).
22. V. Y. H. Hook, S. Heisler, S. L. Sabol and J. Axelrod, *Biochem. biophys. Res. Commun.* **106**, 1364 (1982).
23. W. Vale, J. Speiss, C. Rivier and J. Rivier, *Science* **213**, 1394 (1981).
24. C. M. Tukelson, A. Arimura, M. D. Culler, J. B. Fishback, K. Groot, M. Kanda, M. Luciano, C. R. Thomas, D. Chang, J. K. Chang and M. Shimizu, *Peptides* **2**, 425 (1981).
25. C. Rivier, M. Brownstein, J. Speiss, J. Rivier and W. Vale, *Endocrinology* **110**, 272 (1982).
26. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
27. P. K. Chiang, K. Venkatasubramanian, H. H. Richards, G. L. Cantoni and E. Schiffmann, in *Transmethylation* (Eds. E. Usdin, R. T. Borchardt and C. R. Creveling), p. 165. Elsevier-North Holland, New York (1979).
28. C. Gagnon and S. Heisler, *Life Sci.* **25**, 993 (1979).
29. E. J. Diliberto, Jr., in *Cellular Regulation of Secretion and Release* (Ed. M. Conn). Academic Press, New York (in press).
30. C. Gagnon, J. Axelrod and M. J. Brownstein, *Life Sci.* **22**, 2155 (1978).
31. H. Watanabe, W. E. Nicholson and D. N. Orth, *Endocrinology* **93**, 411 (1973).
32. E. Herbert, R. G. Allen and T. L. Paquette, *Endocrinology* **102**, 218 (1978).
33. S. Kim and C. H. Li, *Int. J. Pept. Protein Res.* **13**, 282 (1979).