Phorbol 12-Myristate 13-Acetate and Vasopressin Potentiate the Effect of Corticotropin-releasing Factor on Cyclic AMP Production in Rat Anterior Pituitary Cells

MECHANISMS OF ACTION*

(Received for publication, June 3, 1986)

Abdul-Badi Abou-Samra, James P. Harwood, Vincent C. Manganiello, Kevin J. Catt, and Greet Aguilera

From the Endocrinology and Reproduction Research Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892

The potentiation of corticotropin-releasing factor (CRF)-stimulated cAMP production by vasopressin (VP) in the pituitary cell was investigated by studies on the interaction of CRF, VP, and the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA) on cAMP, adenylate cyclase and phosphodiesterase. Addition of VP or PMA (0.01–100 nM) alone did not alter cellular cAMP content, but markedly increased the effect of 10 nM CRF with ED50 of about 1 nM. Treatment of the cells with 200 ng/ml pertussis toxin for 4 h increased CRF-stimulated cAMP accumulation by 3.2-fold, an effect that was not additive to those of VP and PMA. Incubation of pituitary cells with 2 mM 1-methyl-3-isobutylxanthine increased CRF-stimulated cAMP accumulation and decreased the relative effect of VP and PMA, suggesting that the actions of VP and PMA are partially due to inhibition of phosphodiesterase. This was confirmed by the demonstration of a 30% inhibition of the low-affinity phosphodiesterase activity in cytosol and membranes prepared from cells preincubated with VP or PMA. In intact cells, following [H]adenine prelabeling of endogenous ATP pools, measurement of adenylate cyclase in the presence of 1-methyl-3-isobutylxanthine showed no effect of VP and PMA alone, but did show a 2-fold potentiation of the effect of CRF. Measurement of adenylate cyclase in pituitary homogenates by conversion of [α-32P]ATP to [32P]cAMP showed a paradoxical GTP-dependent inhibition by VP of basal and CRF-stimulated adenylate cyclase activity, suggesting that the VP receptor is coupled to an inhibitory guanyl nucleotide-binding protein. Pertussis toxin pretreatment of the cells prevented the VP inhibition of adenylate cyclase activity observed in pituitary cell homogenates. These findings indicate that besides inhibition of phosphodiesterase, VP has a dual interaction with the pituitary adenylate cyclase system; a direct inhibitory effect, manifested only in broken cells, that is mediated by a receptor-coupled guanyl nucleotide-binding protein, and a physiologically predominant indirect stimulatory effect in the intact cell, mediated by protein kinase C phosphorylation of one of the components of the CRF-activated adenylate cyclase system.

The multifactorial control of ACTH secretion from pituitary corticotrophs involves stimulatory effects of several hormones including corticotropin-releasing factor (CRF), vasopressin (VP), angiotensin II and norepinephrine, and feedback inhibition by glucocorticoids. CRF stimulates ACTH release through activation of adenylate cyclase, leading to cAMP formation and activation of cAMP-dependent protein kinase (1–3). Other secretagogues, such as VP, angiotensin II, and norepinephrine, do not stimulate adenylate cyclase (1), and recent evidence suggests involvement of phospholipid turnover and activation of protein kinase C in their mechanism of action (4, 5). Whereas these agonists alone are weak stimuli of ACTH secretion, they have been shown to potentiate markedly the stimulatory effect of CRF (6, 7). Vasopressin has also been shown to enhance the effect of CRF on cAMP production, which could contribute to the potentiation of ACTH release by this hormone (8). Recently, we observed that the potentiating effect of vasopressin on CRF-induced ACTH release (4) can be mimicked by phorbol esters. Since phorbol esters and VP alone do not stimulate cyclic AMP formation, it is possible that these agents act indirectly by modulating the effects of agonists on adenylate cyclase or phosphodiesterase activity. In the present work, the interactions between phorbol 12-myristate 13-acetate (PMA) or VP and CRF in the control of phosphodiesterase, adenylate cyclase, and cAMP production were analyzed in rat anterior pituitary preparations. The data demonstrate that VP potentiates the effect of CRF on cAMP production through a dual mechanism that involves protein kinase C-mediated inhibition of phosphodiesterase, and enhancement of CRF-stimulated adenylate cyclase activity.

EXPERIMENTAL PROCEDURES

Materials—CRF was synthesized by a solid phase method and purified by high performance liquid chromatography as previously reported (1). VP was purchased from Bachem, Torrance, CA; PMA from Sigma; [H]cAMP, [H]adenosine, and [α-32P]ATP from New England Nuclear; and pertussis toxin (PT) from List Biological Labs., Inc., Campbell, CA.

Cell Cultures—Anterior pituitary cells from male and female Sprague-Dawley rats were enzymatically dispersed and plated in multiwell culture dishes in bicarbonate-buffered medium 199, 1 μg/ml strepto-
mycin, 100 units/ml penicillin, and 10% horse serum and incubated at 37 °C under 5% CO₂, 95% air in a humidified incubator (9).

Incubation Procedure—After 3-5 days culture, the cells were washed with serum-free medium (199, Gibco, Grand Island, NY) containing 1 μg/ml streptomycin, 100 units/ml penicillin, 100 kalli- krein inhibitory units/ml aprotinin, 30 μg/ml ascorbic acid, and 0.1% bovine serum albumin. The cultures were then incubated with the serum-free medium containing the different stimulants for various time periods. At the end of the incubation period, the medium was removed, and the cells were frozen in a dry ice/acetone bath and lyzed by adding 0.05 N HCl.

cAMP Measurement—cAMP in the cell extract was determined by radioimmunoassay as previously described (1), using an antibody raised in the rabbit against the cAMP analogue, succinyl-cAMP, and [3H]-labeled succinyl-cAMP as tracer. Both the samples and standards were acetylated before assay. The assay sensitivity was 0.05 fmol/ml, and the concentration of cAMP which displaced 50% of bound tracer was 0.5 fmol/ml.

Determination of ACTH Release—ACTH was measured by direct radioimmunoassay of the incubation media, using a specific N-terminal antibody (IgG-ACTH-1, IgG Corp., Nashville, TN) which cross-reacted equally with ACTH(1-24) and ACTH(1-39). The [125I]-labeled tracer was prepared by iodogen radiiodination of [Phe2,Nle4]-ACTH(1-38), kindly provided by Dr. J. Rivier, Salk Institute (La Jolla, CA) and purified by high performance liquid chromatography (10). The assay sensitivity was 1 pg/tube. Intra- and interassay variations were less than 10%.

Determination of Phosphodiesterase Activity—Cells were cultured for 3-5 days and subsequently incubated for 30 min with VP or PMA in a serum-free medium in the presence or absence of CRF. The incubation was terminated by removing the medium and freezing the cells in a dry ice/acetone bath. The cells were thawed by adding 300 μl of 250 mM sucrose, 10 mM Tris, 5 mM MgCl₂, and 0.2 mM EGTA, and incubated on ice for 5 min at 30 °C. The cells were then homogenized in a Dounce homogenizer, and centrifuged at 100,000 x g in an Airfuge (Beckman). The supernatant (cytosol fraction) and the pellet (membrane fraction) were used for phosphodiesterase determination.

Measurement of Adenylate Cyclase Activity—Adenylate cyclase activity was measured as previously described (11) at two substrate concentrations (0.5 and 50 μM cAMP) to measure the low and high affinity enzymes. 50 μg of protein from membrane or cytosolic fractions were incubated with [3H]cAMP and the appropriate substrate concentration for 15 min at 30 °C. The reaction mixture consisted of 50 mM HEPES, 0.2 mM EGTA, 8.3 mM MgCl₂, 0.5 or 50 μM cAMP, 0.17 mg/ml ovalbumin, and 20,000 cpm [3H]cAMP. The reaction was terminated by adding 100 μl of 7.5 mM cAMP, 5 mM 5'-AMP in 0.25 M Tris, pH 8. The pH was neutralized with 100 μl of 0.25 M NaOH and 0.25 M Tris. The 5'-AMP formed in the reaction was hydrolyzed with snake venom 5'-nucleotidase (Crotalus atrox), and the resulting [3H]adenosine was separated from the nonhydrolyzed [3H]cAMP on DEAE-Sephadex columns. The reaction was linear with time for up to 15 min.

Measurement of Adenylate Cyclase Activity—Adenylate cyclase activity was determined in intact cells by measuring [3H]cAMP formation after prelabeling of the endogenous ATP pools and in pituitary homogenates by measuring the conversion of [α-32P]ATP to [32P]cAMP. The pituitary cells were labeled by overnight incubation with 10 μCi of [3H]adenosine, washed twice with incubation medium, and incubated in a volume of 1 ml with VP, PMA, and CRF for 30 min in the presence or absence of phosphodiesterase inhibitors. The medium was removed and cellular cAMP was extracted with 1 ml of 10% triiodoacetic acid after addition of 2000 cpm [14C]cAMP to correct for recovery. The cAMP was separated on Dowex/AL₂O₃ columns and the eluate analyzed for radioactivity in a Packard β-spectrometer.

For measurement of adenylate cyclase activity in pituitary homogenates, approximately 100 μg of protein were incubated with [α-32P]ATP in the presence or absence of CRF, NaF, VP, or PMA (1). Anterior pituitary glands were homogenized in 6.25 mM PIPES, pH 7.4, containing 0.5 mM hexylene glycol and 0.1 mM MgCl₂ in a Dounce homogenizer and filtered through nylon gauze. The reaction mixture consisted of 25 mM Tris·HCl, pH 7.4, 5 mM MgCl₂, 1 mM cAMP and 0.1 mM of [α-32P]ATP (700 cpm/mol), a creatine phosphate/creatine kinase regenerating system and 0.01 mM GTP, and 20 μl of the pituitary homogenate. The mixture was incubated for 10 min at 30 °C, and the reaction stopped by addition of 0.2 ml of 1 M HClO₃. [3H]cAMP was added to correct for recovery and the [32P]- and [3H]cAMP were separated by chromatography on Dowex/AL₂O₃ columns.

RESULTS

The time courses of the effects of VP and PMA on CRF-stimulated cAMP accumulation and ACTH release are shown in Fig. 1. Incubation with 100 nM PMA or 10 nM VP alone for 10-120 min had no effect on cAMP content (Fig. 1A), but increased ACTH release (Fig. 1B). However, incubation of pituitary cells with either 100 nM PMA or 10 nM VP in combination with 10 nM CRF significantly increased the effect of CRF on both ACTH release (Fig. 1B) and cAMP accumulation (Fig. 1A). The effect of VP and PMA on CRF-stimulated cAMP accumulation was rapid, with a peak at 15 min, and persisted for the 120-min incubation period (Fig. 1A). Incubation of the cells for 15 min with 100 nM PMA or 10 nM VP with increasing concentrations of CRF resulted in 3.8- and 5.7-fold increases in the maximal effect of CRF on cAMP accumulation with VP and PMA, respectively, without modifying the ED₅₀ for CRF on cAMP content (Fig. 2A). Incubation of the cells for 3 h with 100 nM PMA or 10 nM VP in the presence of increasing concentrations of CRF also caused a significant (p < 0.01) increase in the maximum CRF-stimulated ACTH release, without modifying the ED₅₀ for CRF (Fig. 2B). The effects of VP and PMA on CRF-induced cAMP accumulation and ACTH release were dose-dependent, with ED₅₀ values of about 1 nM (Fig. 3).

Since the effect of PMA suggested the involvement of protein kinase C in the potentiating effects of VP, the role of this enzyme was studied by analyzing the effects of the synthetic diacylglycerol, dioctanoylglycerol, and the protein kinase C inhibitor, retinal, on cAMP production. Similar to the effects of PMA and VP, 100 μM dioctanoylglycerol enhanced the action of CRF upon cAMP content (Fig. 4A). Treatment of the cells with 100 μM retinal markedly reduced the abilities of dioctanoylglycerol, PMA, and VP to enhance CRF-stimulated cAMP accumulation, whereas retinal alone had only a small effect on cAMP accumulation stimulated by CRF (Fig. 4B).

FIG. 1. Time course of the effects of VP and PMA on CRF-stimulated cAMP production (A) and ACTH release (B). The cells were incubated with 10 nM CRF, 10 nM VP, 100 nM PMA, alone or in combination. The data are means ± S.E. of measurements performed on triplicate incubations.
CRF, Vasopressin, and PMA Interactions on Pituitary cAMP

FIG. 2. Effects of VP and PMA on the dose-dependent stimulation of cAMP production (A) and ACTH release (B) by CRF. The cells were incubated for 15 (A) or 180 (B) min with 10^{-12}-10^{-7} M CRF in the presence or absence of 10 nM VP or 100 nM PMA. The data are means ± S.E. of results from triplicate incubations.

FIG. 3. Dose-dependent effects of VP and PMA on CRF-stimulated cAMP production (A) and ACTH release (B). The cells were incubated with increasing concentrations of VP or PMA in the presence or absence of 1 or 10 nM CRF for 15 (A) or 180 (B) min. The data are means ± S.E. of results from triplicate incubations.

FIG. 4. Effects of retinal on stimulated cAMP production (A) and ACTH release (B). The cells were treated with 100 μM retinal for 120 min, and then 10 nM CRF, 10 nM VP, 100 nM PMA, 100 μM dioctanoylglycerol, or their combinations were added and incubations continued for a further 15 (A) or 180 (B) min. The data are means ± S.E. of results from triplicate incubations. *, p < 0.01 versus CRF alone, **, p < 0.01 versus without retinal.

4A). As previously reported (4), retinal significantly decreased the effects of CRF, VP, and PMA, alone or in combination, upon ACTH release (Fig. 4B).

The potentiation of the effect of CRF on cAMP accumulation by PMA and VP could be due to inhibition of cAMP degradation by an effect on phosphodiesterase activity and/or sensitization of the adenylate cyclase complex to CRF stimulation. To test whether inhibition of phosphodiesterase activity might be involved, the interactions of VP and PMA with CRF on cAMP accumulation were evaluated in the presence of the phosphodiesterase inhibitor, methylisobutylxanthine (MIX). Addition of 2 mM MIX to the incubation medium increased both basal and CRF-stimulated increase in cAMP cell content to values similar to those obtained with PMA and VP (Fig. 5). However, there was still significant additivity between the effects of MIX and those of VP and PMA with CRF on cAMP accumulation, suggesting the participation of another mechanism in addition to the inhibition of phosphodiesterase activity. The ability of VP and PMA to inhibit phosphodiesterase activity was confirmed by direct measurement of the enzyme activity in pituitary cell membranes and cytosol. In three experiments, incubation of the cells with VP and PMA resulted in significant inhibition of the low affinity phosphodiesterase activity measured in the presence of 50 μM cAMP. VP inhibited the low affinity phosphodiesterase activity by 29.4 ± 6.3% (p < 0.01) in cytosol and 17.2 ± 3.5% (p < 0.05) in membranes, whereas PMA
CRF, Vasopressin, and PMA Interactions on Pituitary cAMP

Fig. 5. Effects of MIX on cAMP production. The cells were treated with or without 2 mM MIX for 15 min, and then 10 nM CRF, 10 nM VP, 100 nM PMA or their combinations were added for various time periods. The data are means ± S.E. of results from triplicate incubations. The effects of VP and PMA in the presence or absence of MIX were not different from basal values and are not shown.

TABLE I
Effect of CRF, VP, PMA, or their combination on cAMP phosphodiesterase activity

The pituitary cells were incubated with 10 nM CRF, 10 nM VP, 100 nM PMA, or their combinations for 15 min and then homogenized. Phosphodiesterase activity was measured at two substrate concentrations; 0.5 and 50 μM cAMP for the high and low affinity enzymes, respectively. The data are means ± S.E. of three experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Low affinity cAMP phosphodiesterase</th>
<th>High affinity cAMP phosphodiesterase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cytosol</td>
<td>Membranes</td>
</tr>
<tr>
<td>Control</td>
<td>136 ± 26</td>
<td>182 ± 33</td>
</tr>
<tr>
<td>CRF</td>
<td>129 ± 31</td>
<td>168 ± 29</td>
</tr>
<tr>
<td>VP</td>
<td>96 ± 22a</td>
<td>152 ± 35a</td>
</tr>
<tr>
<td>PMA</td>
<td>92 ± 19a</td>
<td>148 ± 30a</td>
</tr>
<tr>
<td>VP + CRF</td>
<td>95 ± 28a</td>
<td>150 ± 25a</td>
</tr>
<tr>
<td>PMA + CRF</td>
<td>88 ± 18a</td>
<td>145 ± 28a</td>
</tr>
</tbody>
</table>

*p < 0.01 versus control.

*p < 0.05 versus control.

inhibited the activity by 30.2 ± 5% (p < 0.01) in cytosol and 19 ± 6% (p < 0.05) in membranes (Table I). The high affinity enzyme measured in the presence of 10 μM cAMP was not significantly affected by either VP or PMA treatment (Table I).

The effects of VP and PMA on adenylate cyclase activity were investigated in intact cells after prelabeling the endogenous ATP pools with [3H]adenine, and in pituitary homogenates by conversion of [γ-32P]ATP to [32P]AMP. Fig. 6 shows the kinetics of [3H]cAMP accumulation following prelabeling of the endogenous ATP pools with [3H]adenine. In the presence of 5 mM MIX, CRF caused a linear increase in cAMP accumulation, up to 7-fold at 30 min. Consistent with the experiments on cAMP content of cultured cells, VP and PMA alone had no effect, but markedly potentiated the stimulation of [3H]cAMP accumulation by CRF. In three experiments, conducted in the absence and presence of phosphodiesterase inhibitors, incubation of [3H]adenine-prelabeled cells with CRF for 30 min caused a dose-dependent increase in [3H]cAMP content (Fig. 7). VP and PMA had no effect on basal, but potentiated the effect of CRF on [3H]cAMP accumulation.

In the absence of MIX, the maximum CRF-stimulated [3H]cAMP accumulation was increased by 333 ± 30% and 602 ± 50% by VP and PMA, respectively. This stimulatory effect of VP and PMA on CRF-stimulated activity was clearly evident during the blockade of phosphodiesterase by MIX, with a 189 ± 12.7 and 267 ± 9% increase in maximum activity, respectively. Similar results were obtained in one experiment using the phosphodiesterase inhibitor Ro 1724 which, in contrast
to MIX and theophylline, does not interact with adenosine receptors.

In pituitary homogenates, CRF increased adenylate cyclase activity by 1.6 ± 0.1-fold (n = 4) with an ED$_{50}$ of 40 ± 15 nM CRF (n = 2) in the presence of 10 μM GTP. Addition of PMA caused minor changes in CRF-stimulated [$^{32}$P]cAMP formation, with a small and not significant decrease in the ED$_{50}$ to 20 ± 10 nM CRF (n = 2) and no significant change in maximum CRF stimulation (1.7 ± 0.13-fold, n = 4). In contrast to the effects observed in intact cells, VP caused a paradoxical inhibition of basal and CRF-stimulated adenylate cyclase activity in the broken cell preparation. In two experiments, 100 nM VP caused an 18 ± 5% inhibition of basal adenylate cyclase activity. In the presence of increasing concentrations of CRF, adenylate cyclase activity remained inhibited below the basal values (Fig. 8). The inhibitory effect of VP on basal and CRF-stimulated adenylate cyclase activity was evident only in the presence of GTP, suggesting the participation of a guanyl nucleotide-binding protein. In two experiments, addition of adenosine deaminase (10 units/ml), did not modify the inhibitory effect of VP on adenylate cyclase activity. The observation that the potentiating effect of VP on CRF-stimulated adenylate cyclase requires an intact cell system suggests that the effect could be the result of indirect modification, e.g. phosphorylation of a component of the adenylate cyclase complex by vasopressin. In this case, the inhibitory effect of VP on adenylate cyclase in the broken preparation should be prevented by preincubation of the tissue with VP. As shown in Table II, the 34% stimulation of adenylate cyclase by CRF was inhibited by VP, but a 30-min preincubation of the tissue with VP (10$^{-7}$ M) resulted in loss of the inhibitory action of VP with no significant effect on CRF stimulation alone.

To determine the involvement of an N$_{1}$-like guanyl nucleotide-binding protein in the mechanism of the potentiation of CRF-stimulated cAMP accumulation by VP and PMA, pituitary cell cultures were treated with PT and then incubated with CRF and/or VP. Preincubation of the cells with 100, 200, and 400 ng/ml PT for 4 h increased the effect of 10 nM CRF upon cAMP accumulation by 2.1-, 3.2-, and 4.6-fold, respectively. Pretreatment of the cells with 200 ng/ml PT for 2, 4, and 24 h increased CRF-stimulated cAMP accumulation by 2.5-, 3.6-, and 3.9-fold, respectively. The potentiating effect of PT pretreatment on CRF-stimulated cAMP accumulation for 4 h (Fig. 9) or 24 h (data not shown) was not additive to the potentiating effects of VP and PMA. Further evidence for coupling of the VP receptor to an N$_{1}$-like guanyl nucleotide-binding protein, was provided by the effect of treatment of pituitary cell cultures with PT on adenylate cyclase activity, measured in cell homogenates (Table III). Preincubation of the cells with PT, 200 μg/ml, for 24 h caused a small increase in basal adenylate cyclase activity and prevented the direct inhibitory effect of VP on basal and CRF-stimulated adenylate cyclase activity.

**DISCUSSION**

These observations have shown that the potentiating action of VP on CRF stimulation of cAMP accumulation involves an increase in cAMP formation by stimulation of adenylate cyclase and a decrease in cAMP degradation by inhibition of phosphodiesterase. The present data also provide evidence for the involvement of protein kinase C in mediating both of these effects of VP. First, the effects of VP were mimicked by phorbol esters and the synthetic diacylglycerol, dioctanoylglycerol, and second, inhibition of protein kinase C activation by retinal attenuated the potentiating effect of VP and phorbol esters on CRF-stimulated cAMP accumulation.

The possibility that the potentiating effect of PMA and VP on CRF-induced cAMP accumulation could be due to inhibition of cAMP degradation was first suggested by the ability of phosphodiesterase inhibitors partially to simulate the potentiating effect of PMA and VP. Direct measurement of phosphodiesterase activity showed an inhibition of the low affinity enzyme during VP and PMA potentiation of CRF-induced cAMP accumulation. The lack of effect on the high affinity enzyme may explain the ability of VP and PMA to enhance cAMP production only in the presence of increased intracellular cAMP stimulated by CRF. This is in contrast to the effect of MIX, which inhibits both the low and the high affinity phosphodiesterases (12), resulting in increases in basal cAMP content. Although inhibition of phosphodiesterase partially accounts for the effects of VP and phorbol esters, the fact that they can still potentiate CRF action in the presence of phosphodiesterase inhibitors suggests the involvement of other mechanisms in addition to inhibition of phosphodiesterase.

The interactions of VP with adenylate cyclase are complex and involve both stimulatory and inhibitory actions, depending on the experimental conditions. In intact cells, VP and phorbol esters enhanced agonist-stimulated adenylate cyclase activity. This effect was evident in the presence of phospho-

![FIG. 8. Effects of VP and PMA on CRF stimulation of adenylate cyclase activity, measured as described under "Experimental Procedures" in the presence of 100 nM VP or 1 μM PMA and increasing concentrations of CRF. The data are means ± S.E. of three experiments.](image-url)
diesterase inhibitors at concentrations that completely block cAMP degradation. Similar results were obtained when phosphodiesterase activity was blocked with Ro 1724 which, in contrast to MIX, does not interact with adenosine receptors. These experiments suggested that adenosine is unlikely to be involved in the modulation of adenylate cyclase by VP. In contrast to the potentiating effect on CRF-stimulated adenylate cyclase in intact cells, VP had a paradoxical inhibitory effect in broken cell preparations. This inhibitory effect is also not likely to be associated with adenosine formation, since it was not modified by adenosine deaminase. The inhibitory effect of VP upon adenylate cyclase was evident only in the presence of GTP, suggesting the participation of a guanylnucleotide regulatory protein. The pituitary VP receptor has been classified as a variant of the V-1 vascular vasopressin receptor, which does not stimulate cAMP production, but in intact cell could cause phosphorylation of a component of the adenylate cyclase complex with consequent activation of the catalytic moiety, resulting in increased cAMP formation. In rat hepatocytes, however, a pertussis toxin-insensitive guanyl nucleotide-binding protein (27). Such phosphorylation might block the activity of the inhibitory subunit of the adenylate cyclase complex, and can inhibit adenylate cyclase. For example, activation of polymorphonuclear leukocytes by the chemotactic peptide f-Met-Leu-Phe is thought to be regulated by a pertussis toxin-sensitive substrate that is similar, if not identical, to N, (25, 26). In rat hepatocytes, however, a pertussis toxin-insensitive guanylnucleotide-binding protein is apparently involved in VP receptor-mediated hydrolysis of phosphatidylinositol bisphosphate, production of inositol triphosphate, mobilization of Ca²⁺, activation of phosphorylase, and glycogenolysis (15). In the case of the pituitary actions of VP, coupling of the hormone-activated receptor to N, or another guanylnucleotide-binding protein primarily associated with phospholipid turnover, could subsequently inhibit adenylate cyclase, producing the effect seen in broken cell preparations.

It is also possible that activation of protein kinase C in the intact cell could cause phosphorylation of a component of the adenylate cyclase complex with consequent activation of the catalytic moiety, resulting in increased cAMP formation. In this regard, purified protein kinase C has been reported to phosphorylate the purified α subunit of the N, protein (27). Such phosphorylation might block the activity of the inhibitory subunit of the adenylate cyclase complex, and augment the action of a stimulatory ligand such as CRF. On the other hand, PMA has been shown to enhance cyclase activation by factors that directly activate the stimulatory N, protein, such as cholera toxin, or the catalytic subunit, such as Mn²⁺ (28), suggesting an action distal to the N proteins.

Phorbol esters have been shown to potentiate or to inhibit

---

**TABLE III**

<table>
<thead>
<tr>
<th>Pretreatment conditions</th>
<th>Adenylate cyclase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaF, CRF, VP, CRF + VP, 10 mM</td>
</tr>
<tr>
<td>Experiment 1 0</td>
<td>44</td>
</tr>
<tr>
<td>PT, 18 h</td>
<td>49</td>
</tr>
<tr>
<td>Experiment 2 0</td>
<td>37</td>
</tr>
<tr>
<td>PT, 6 h</td>
<td>38</td>
</tr>
</tbody>
</table>

---

**FIG. 9. Effect of PT on stimulated cAMP accumulation.** The cells were preincubated with 200 ng/ml PT for 4 h, and then washed with serum-free medium and incubated for 15 min with 10 nM CRF, 10 nM VP, and 100 nM PMA either alone or in combination. Cellular cAMP content was extracted and measured by radioimmunoassay. The data are means ± S.E. of three experiments.
adenylate cyclase in several other systems. Thus, PMA potentiates cAMP accumulation in response to isoproterenol in rat pinealocytes (29) and in vascular smooth muscle cells (30), to 2-chloroadenosine in guinea pig cerebral cortical particulate cells (31), to human chorionic gonadotropin in ovarian granulosa cells (32), and to growth hormone-releasing factor in rat anterior pituitary cells (33). On the other hand, PMA inhibited cAMP accumulation in response to glucagon in rat hepatocytes (34), and to human chorionic gonadotropin in purified mouse Leydig cells (35). Such divergent effects of PMA could be the result of differences in the experimental conditions, with different time periods of exposure to the phorbol ester or variable degrees of preservation of the integrity of the protein kinase C pathway. It is also possible that differences in the responsiveness of various tissues to protein kinase C activation may vary according to the content of specific guanyl nucleotide-binding proteins susceptible to protein kinase C-dependent phosphorylation and their coupling to adenylate cyclase. The potentiating effect of PT on CRF-stimulated cAMP accumulation and the lack of additivity between the effect of PT and those of VP or PMA indicate that inactivation of the inhibitory component of adenylate cyclase by ADP ribosylation can, in fact, potentiate the agonist-activated enzyme, and suggests that protein kinase C activation may vary according to the content of specific guanyl nucleotide-binding proteins susceptible to protein kinase C-dependent phosphorylation and their coupling to adenylate cyclase. This mechanism could contribute to the modulation of adenylate cyclase activity in various tissues.

The CRF receptor is another potential site of modulation for the effect of VP on CRF-stimulation of cAMP accumulation. In this regard it has been shown that adrenergic receptor function is mediated by cAMP-dependent and independent phosphorylation (37). However, it seems unlikely that such a mechanism would be a significant factor in the VP/CRF system, since VP has been shown to cause a decrease rather than an increase in CRF receptors after injection in rats or preincubation of pituitary quarters (38).

Little is known about the molecular mechanisms for regulation of cellular cycle nucleotide phosphodiesterase. In intact cells, cAMP phosphodiesterase activity can be increased by agents that activate adenylate cyclase thereby increasing intracellular cAMP content. These increases in phosphodiesterase activity, which are thought to be mediated by activation of cAMP-dependent protein kinase, may represent a type of "feedback" mechanism to terminate the cAMP signal.


e stimulates adenylate cyclase and inhibits phosphodiesterase (39). Also, in 3T3-L1 adipocytes the inhibition of lipolysis by phenylisopropyladensinolide has been suggested to involve inhibition of adenylate cyclase as well as activation of cAMP phosphodiesterase (39). The present work shows that multiple mechanisms are involved in the potentiation by VP and phorbol esters of the action of CRF on cAMP accumulation, including protein kinase C-mediated inhibition of phosphodiesterase and enhancement of CRF-stimulated adenylate cyclase. Although the molecular mechanisms by which protein kinase C activation results in this dual regulation of cellular cAMP levels in the corticotroph remain to be elucidated, such modulation of CRF action by protein kinase C-dependent hormones provides a mechanism to facilitate the secretory response during physiological regulation of ACTH secretion.

REFERENCES


Am. J. Physiol., in press.