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PHORBOL ESTERS INCREASE ADENYLATE CYCLASE ACTIVITY AND STABILITY IN PITUITARY MEMBRANES

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In this report, we demonstrate that calcium and phorbol esters enhance cAMP production in GH_4C_1 cell homogenates. The mechanism for this is a reduction in the rate of decay of adenylate cyclase activity over the course of the assay. Purified protein kinase C can reconstitute calcium- and phorbol ester-dependent adenylate cyclase. Phorbol ester-activated protein kinase C increases both the initial rate of cAMP synthesis and reduces the time-dependent decay of adenylate cyclase activity in membrane preparations. The rate of cAMP production is fit to an equation derived from a model which assumes that adenylate cyclase initially exists in a high activity state which decays exponentially into a low activity state. We suggest that protein kinase C can both prevent the decay of the high activity state and convert the low activity state into the high activity state.

Phorbol esters increase basal and hormone-stimulated cAMP accumulation in pituitary (1-7) and a number of other tissues (8-11). In addition, several groups have demonstrated that membrane preparations from cells exposed to phorbol esters exhibit increased basal and hormone-stimulated adenylate cyclase activity (7,10,12,13). Thus it appears that protein kinase C activation can increase the rate of cAMP synthesis in some cell types. However, the exact mechanism by which protein kinase C increases adenylate cyclase activity has not been established.

In most studies to date, protein kinase C is activated in the intact cell and the effects on adenylate cyclase activity are characterized in subsequently prepared plasma membranes. In our previous studies, addition of phorbol 12-myristate, 13-acetate (PMA) directly to the adenylate

The abbreviations used are PMA, phorbol 12-myristate, 13-acetate; PDB, phorbol 12,13-dibutyrate; EDTA, ethylenediamine-tetraacetic acid; EGTA, ethylene glycol bis-β-aminoethyl ether-N,N,N',N'-tetraacetic acid; ATP, adenosine 5' triphosphate.

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cyclase assay of membrane preparations had little effect on the rate of cAMP synthesis (7,14). The membrane fractions used in those studies contained very little protein kinase C because the membranes were prepared in the presence of a calcium chelator as demonstrated in pituitary (15) and neutrophil (16) membranes. Thus, it is possible that PMA had no direct effect on adenylate cyclase activity in these experiments because there was insufficient protein kinase C in the membrane preparation.

Brostom, *et al.* (17) demonstrated that PMA increased cAMP synthesis in GH_3 cell homogenates when added directly to the adenylate cyclase assay. Recently, several groups have reported that the addition of partially purified protein kinase C to membrane preparations can modify adenylate cyclase activity (18-20). In this report we suggest that protein kinase C regulates the equilibrium between two or more activity states of adenylate cyclase. We also present a two state mathematical model which predicts the effect of protein kinase C on cAMP production.

MATERIALS AND METHODS

Cell Culture. The prolactin- and growth hormone-secreting GH_4C_1 anterior pituitary tumor cell line was kindly provided by Dr. Patty Hinkle (Univ of Rochester, Rochester NY). The cells were cultured as described previously for GH_3 cells (15) except that they were passaged with 1 mM EGTA instead of 0.1% pancreatin.

Cell Fractionation. Membrane fractions were prepared as previously described (7) and frozen at -70°C. The frozen membranes were generally assayed within 4 weeks of preparation. In studies using cell homogenates, 20-30 million cells were scraped, homogenized in 2-3 ml buffer, and immediately assayed. In the experiment of Figure 2B, the amount of endogenous protein kinase C present in the membrane preparations was manipulated by pretreating the cells with or without phorbol 12,13-dibutyrate (PDB, 100 nM) and homogenizing them in the presence or absence of calcium (2 mM). Protein kinase C content (assayed by phorbol ester binding, see below) increased from control to PDB-treated to Ca^{2+}-homogenized to PDB-treated and Ca^{2+}-homogenized preparations.

Adenylate cyclase assay. Adenylate cyclase activity was assayed in a 100 µl reaction volume containing 0.5 mM ATP, 0.5-1.5 x 10^6 cpm [α-^{32}P]-ATP (synthesized by the Univ. of Va. Diabetes Core Facility), 50 mM Tris, pH 7.4, 0.4 mM isobutylmethylxanthine, 0.1 mg/ml bovine serum albumin, 5 mM $MgCl_2$, 0.5 mM EDTA, 200 mM KCl, 1 mM dithiothreitol, 10 IU creatine phosphokinase, 10 mM phosphocreatine, 2-10 µg of homogenate or membrane protein and test agents. Dithiothreitol was not included in assays of membrane preparations. The reaction was carried out for 10 min at 37°C and stopped by the addition of 100 µl of stop buffer (4% sodium dodecyl sulfate, 50 mM ATP, 0.175 mM cAMP) and heating to 90°C. [^{3}H]-cAMP (50-100 cpm/µl) was included in the stop buffer to monitor the subsequent recovery of product. Generated cAMP was separated from ATP by the procedure of Salomon (21). Time course experiments were initiated by adding ice cold membranes to prewarmed reaction volume containing test agents. One reaction was carried out for each condition and 100 µl aliquots were removed and mixed with 100 µl of stop buffer at the indicated times.

Protein kinase C was added to the assay in a solution containing 20 mM Tris, pH 7.4, and 50% v/v glycerol. All dilutions of protein kinase C were made in this solution such that 10 µl were

added to all assay tubes. The amount of protein kinase C is expressed as volume of the highly purified stock preparation added. Calcium concentrations given represent the total calcium; all assays included 240 μM EGTA.

Phorbol ester binding. The [^{3}H]-PDB binding was performed by the method of Sando and Young (22) as previously described (15). Effective exchange binding was obtained in fractions from cells which had been previously treated with unlabeled PDB.

Purification and assay of protein kinase C. Protein kinase C was purified from Sprague-Dawley rat brains by sequential chromatography on DEAE-cellulose, threonine-sepharose, and phenyl-sepharose accorded to the method of Kikkawa, *et al.* (23). The use of Triton was specifically avoided so as not to interfere with adenylate cyclase activity in subsequent experiments. Kinase activity was determined by the ability of the preparation to phosphorylate lysine-rich histone in a reaction mixture (150 μl) containing histone H1 (0.2 mg/ml), 300 μM $CaCl_2$, 100 μM [γ-^{32}ATP] (10 mCi/mol), 5 mM Mg acetate, 20 mM Tris, pH 7.4 at 4°C, and 100-200 ng of the purified enzyme preparation with or without phosphatidylserine (42 μg/ml) and 2.6 μM dioctanoylglycerol; the addition of lipids enhanced the activity 3.1-fold (see Figure 2 legend). The reaction was terminated after 5 min at 30° by spotting 75 μl onto P81 ion exchange paper (Whatman) and papers were washed in 50 mM NaCl to remove unreacted ATP.

Data Analysis. Data are reported as the means ± standard deviations. The *p* values were determined by an unpaired, two-sided Student's t test. P values of less than 0.05 were considered significant. Kinetic parameters were determined by nonlinear, least-square regression analysis (24) and are reported with a 65% confidence interval.

RESULTS AND DISCUSSION

Protein kinase C activators increase cAMP synthesis in homogenates by decreasing the decay of adenylate cyclase activity. In GH_4C_1 cell homogenates, the addition of phorbol esters significantly increased adenylate cyclase activity. In contrast, phorbol esters which do not activate protein kinase C, such as 4-α PMA and 4-α phorbol had no such effect (data not shown). Both thyrotropin releasing hormone, which stimulates phosphoinositide hydrolysis in GH cells (25, 26), and calcium also stimulate cAMP production in homogenate assays.

The conditions under which adenylate cyclase activity is assayed influence the extent of phorbol ester- and calcium-stimulated cAMP production. Under optimal assay conditions, similar to those used by Brostrom, *et al.* (17, see Methods), kinetic analysis of cAMP production by GH_4C_1 homogenates revealed that the rate of cAMP production decayed rapidly over the course of the assay. The ability of PMA and calcium to increase cAMP synthesis in 10 min assays appears to result primarily from a reduction in the decay of adenylate cyclase activity over time (Figure 1).

The decay of enzymatic activity can be modeled using a 2 state system with a high activity

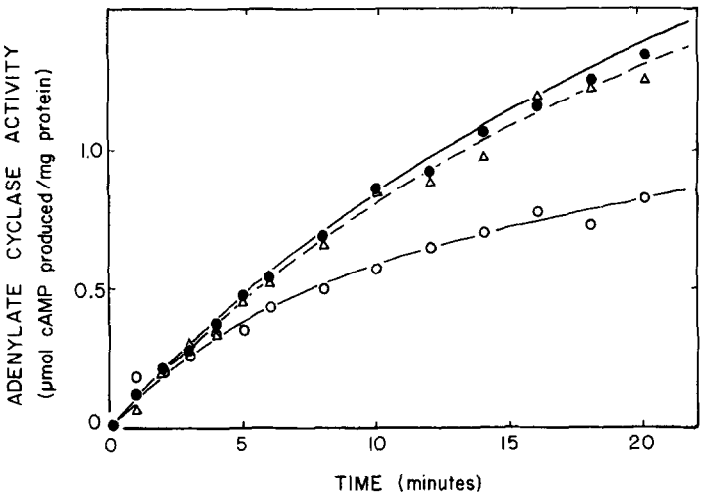

Figure 1. The effect of phorbol esters and calcium on decay of adenylate cyclase activity in GH_4C_1 homogenates is shown. The rate of cAMP formation in GH_4C_1 homogenates was determined in the presence of vehicle (○), 300 μM calcium (Δ), or 100 nM PMA (●). Each point represents the amount of cAMP in a single 100 μl aliquot removed from the incubation at the indicated time. All of the points for a given condition are taken from a single incubation. The lines are predicted by nonlinear regression analysis using a two state decay model. The values for the initial rate of cAMP production (pmol/min), final rate of cAMP production (pmol/min) and the rate of decay (min^{-1}) are as follows: vehicle= 107, 17, 0.18; calcium= 103, ND, 0.05; PMA= 106, ND, 0.05. ND indicates that regression analysis could not determine a value which was significantly different from 0.

state, E_1, having an activity of k_1 and a low activity state, E_2, having an activity of k_2. The rate of product formation, dP/dt at time t is

$$\frac{d[P]}{dt} = k_1 [E_1](t) + K_2[E_2](t) \quad (1)$$

If it is assumed that only E_1 exists initially, and that it decays exponentially into the E_2 state with a rate constant of k_3, the equation for $\frac{d[P]}{dt}$ is

$$\frac{d[P]}{dt} = [E_T](k_1 e^{-k_3 t} + k_2(1 - e^{-k_3 t})), \text{ where } E_T = E_1 + E_2 \quad (2)$$

Assuming that E_T remains constant, this rate equation can be integrated to yield:

$$[P](t) = (k_1 - k_2)/k_3 \, (1 - e^{k_3 t}) + k_2 t \quad (3)$$

where [P](t) is the amount of product (cAMP) at time t and k_1, k_2, and k_3 are the initial activity, final activity, and decay rate constants, respectively. The half time of the decay process is given by $1/k_3$.

Use of this model to analyze the experiment shown in Figure 1 gave an initial calculated rate of cAMP production of 107 ± 8 pmol cAMP/min in the control homogenate. This initial rate was reduced to a calculated final rate of 17 ± 6 pmol cAMP/min with a decay constant of 0.18 ± 0.5

min^{-1}. The impeded decay in the presence of calcium or PMA made it impossible to predict the final rate (k_2) for these conditions with any confidence. Under these conditions regression analysis reproducibly predicted k_1 and k_3 when k_2 was set equal to a constant much less than k_1; for convenience k_2 was set equal to zero. Calcium or PMA did not affect the initial rate of cAMP systhesis (k_1 = 103 ± 7 and 106 ± 2 pmol cAMP/min, respectively) but the decay constant, k_3, was reduced to 0.051 ± 0.01 min^{-1} and 0.05 ± 0.004 min^{-1}, respectively.

Protein kinase C increases adenylate cyclase activity in membrane preparations. To determine whether the activation/stabilization of adenylate cyclase could be caused by protein kinase C activation, we added highly purified protein kinase C (Figure 2A inset) to GH_4C_1 membrane preparations and assayed cAMP production (Figure 2A). Higher concentrations of brain protein kinase C increased cAMP sythesis in the absence of PMA or calcium. Lower concentrations of protein kinase C only increased adenylate cyclase activity in the presence of protein kinase C stimulators. Increased adenylate cyclase activity was also observed in GH_4C_1 membranes enriched for endogenous protein kinase C by pretreating the cells with PDB and

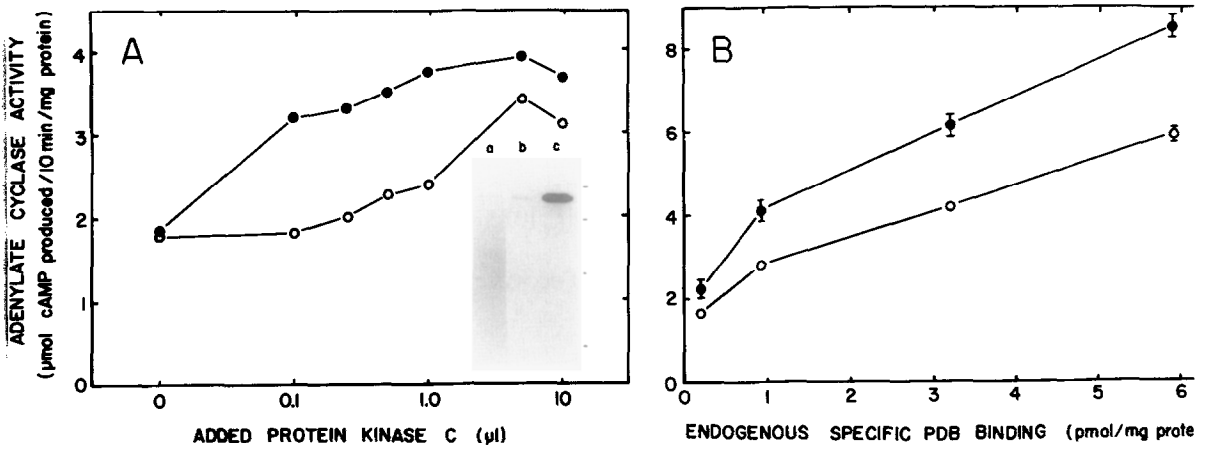

Figure 2. Protein kinase C increases adenylate cyclase activity in GH_4C_1 membrane preparations. The adenylate cyclase activity of GH_4C_1 cell membrane preparations was determined in the presence (●) or absence (○) of 100 nM PMA. A) The indicated amounts of protein kinase C purified from rat brain were added to the adenylate cyclase assay. The histone kinase activity of the preparation was 81 nmol phosphate transferred/min/μl in the absence of lipid and 254 nmol phosphate transferred/min/μl in the presence of lipid. Each point represents the mean of duplicate determinations and the data are representative of 3 independent studies. The inset shows a Coomassie-stained sodium dodecylsulfate-10% polyacrylamide gel of the purified protein kinase C (1.3 μl, lane a), and autoradiographs of gels of the the enzyme autophosphorylated (20 min, 30°C) in the absence (lane b) or presence (lane c) of 300 μM calcium plus phosphatidyl serine (42 μM) and dioctanoyl glycerol (2.6 μM, lane c). B) GH_4C_1 membranes were prepared under conditions designed to vary the content of endogenous protein kinase C (see "Methods"). The protein kinase C content, indicated on the x axis, was determined by [^{3}H]-PDB binding. Each point represents the mean ± SD of triplicate determinations. The data are representative of 3 independent experiments.

homogenizing them in the presence of calcium (see "Methods"). The increased content of protein kinase C in the membrane preparation, as measured by ^{3}H-PDB binding capacity, was accompanied by increased basal cAMP production and larger PMA-stimluated adenylate cyclase activity (Figure 1B).

Brostrom, *et al.* (17) proposed that GH_3 cells contain a calcium - calmodulin dependent adenylate cyclase similar to the enzyme which has been characterized in brain tissue (27-29). In this study, addition of 1 to 50 μg/ml of calmodulin to GH_4C_1 membrane preparations did not restore the ability of calcium to increase cAMP production (data not shown). In the presence of 0.2 μl of exogenous protein kinase C, however, 100 nM PMA or 200 nM calcium (maximal stimulating concentration in this kinase preparation) increased cAMP production to equal extents. This concentration of protein kinase C (0.2 μl) only modestly stimulated adenylate cyclase activity in the absence of PMA or calcium. Thus, the calcium-stimulated adenylate cyclase activity observed in cell homogenates in the present study is very likely due to stimulation by calcium of endogenous protein kinase C.

Protein kinase C increases adenylate cyclase stability and initial activity in membrane preparations. Activation of protein kinase C also leads to the stabilization of an active form of adenylate cyclase in membrane preparatations. This occurs whether the cells are exposed to PMA prior to membrane preparation (data not shown) or exogenous protein kinase C is added directly to the assay (Figure 3).

In contrast to the results in homogenates, where stimulation of protein kinase had no effect on the initial rate (k_1) of cAMP production, addition of protein kinase C to membrane preparations of GH_4C_1 cells increased the initial rate of cAMP production to 126 ± 16 % of control (mean ± SD, $n = 6$, $p = 0.01$). This increase in cAMP production is similar to that in membranes from GH_4C_1 cells (data not shown), GH_3 cells (14,29) and 235-1 pituitary cells (7) exposed to PMA prior to cell fractionation, and assayed under conditions giving linear adenylate cyclase activity.

The loss of basal adenylate cyclase activity in the absence of protein kinase C is not consistent with irreversible denaturation of the adenylate cyclase; addition of protein kinase C, after the initial activity has decayed, increased the rate of cAMP production. This suggests that in addition to reducing the decay of the high activity (E_1) state of adenylate cyclase into the low activity (E_2) state, protein kinase C can convert E_2 into a higher activity form.

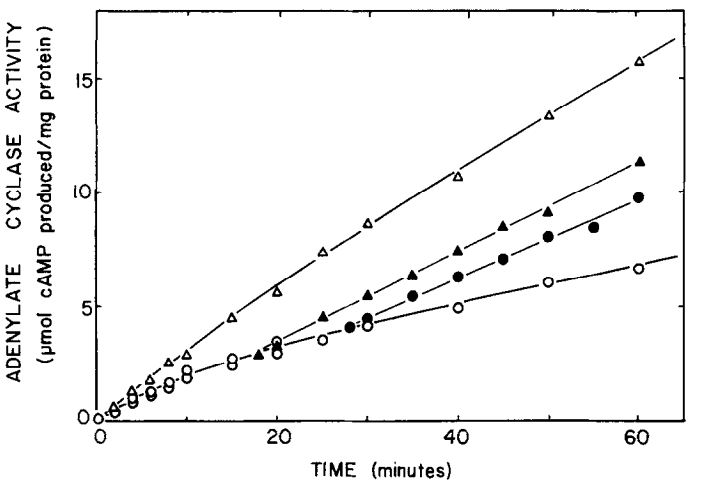

Figure 3. Protein kinase C increases and stabilizes adenylate cyclase activity. The rate of cAMP production in GH_4C_1 membrane preparations was determined in the presence of vehicle (○), or 0.02 μl of protein kinase C plus 100 nM PMA added at time 0 (Δ), at 15 min (▲) or at 25 min (●). The initial rate of cAMP production, the final rate of cAMP production, and the decay rate, determined by nonlinear regression analysis with a two activity state model, gave the following values (pmol/min, pmol/min, min^{-1}, respectively): control was 264, 85, 0.10; protein kinase C from t = 0 was 334, 231, 0.05.

It has been previously reported that the presence of ATP and an ATP regenerating system can preserve (31) or increase (32) adenylate cyclase activity in membrane preparations incubated for long periods of time (hours) on ice. We have demonstrated that protein kinase C induces an apparent increase and stabilization of adenylate cyclase activity in membrane preparations from GH_4C_1 pituitary cells. These effects on cyclase activity can be explained by a protein kinase C mediated conversion of a low activity to a high activity state. Stimulation is only observed when the initial or control preparation has significant amounts of the low activity state present. The decay of adenylate cyclase activity and the restoration of its activity upon addition of protein kinase C occurred rapidly, on the order of seconds to minutes. Thus, the regulation of cAMP production by protein kinase C and possibly other kinases may be of physiological relevance.

The protein kinase C substrate responsible for the increase in adenylate cyclase activity is currently unknown. One possibility is that protein kinase C phosphorylates and inactivates the inhibitory coupling protein, G_i (33). This could result in increased cAMP production through the withdrawal of a tonic inhibitory input (13,34,35). Alternatively, protein kinase C may directly phosphorylate and activate the catalytic subunit of adenylate cyclase (36). Although the exact

molecular mechanism has yet to be established, we suggest that protein kinase C and perhaps other protein kinases may regulate cAMP production by altering an equilibrium between high and low activity states of adenylate cyclase.

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