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PHORBOL ESTERS ENHANCE BASAL AND STIMULATED ADENYLATE CYCLASE ACTIVITY IN A PITUITARY CELL LINE

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SUMMARY: We reported in anterior pituitary cells that hormone stimulation of cyclic AMP levels is amplified by agents that activate protein kinase C (e.g., phorbol esters). We utilized the 235-1 pituitary cell line to explore the mechanism of this response. PGE1- and forskolin-stimulated cyclic AMP accumulation and adenylate cyclase activity are enhanced by exposing viable cells to phorbol esters. Adenylate cyclase activity in the presence of PGE1 demonstrated a biphasic stimulatory, then inhibitory response to increasing GTP concentrations; phorbol esters attenuated this inhibition. These data support the hypothesis that protein kinase C can covalently change the functional state of the adenylate cyclase holoenzyme, amplifying its response to certain hormones.

Covalent modification of hormone-sensitive adenylate cyclase by protein kinases is a potential mechanism by which cells can control the strength of an incoming hormonal signal. In this context, we were intrigued to find that phorbol esters and other agents which activate protein kinase C enhance the ability of maximal levels of growth hormone releasing factor (1) and beta-adrenergic agonists (2) to stimulate cAMP accumulation in anterior pituitary cells. In order to understand the mechanism of this phenomenon in pituitary cells, we utilized the rat 235-1 cell line cloned from a anterior pituitary tumor (3,4). After establishing that phorbol esters enhance PGE_1-stimulated cAMP accumulation in the 235-1 cell, we addressed the following series of questions: A) Do phorbol esters enhance cAMP accumulation through increased synthesis or decreased breakdown of the nucleotide? B) If cAMP synthesis is increased, does exposure of viable cells to phorbol esters result in an

Abbreviations: cAMP, cyclic AMP; GTP, guanosine triphosphate; IBMX, isobutylmethylxanthine; N_s (stimulatory) and N_i (inhibitory) coupling subunit of adenylate cyclase; PBS, phosphate buffered saline; PGE_1, prostaglandin E_1; PMA, phorbol 12-myristate 13-acetate.

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increased adenylate cyclase activity in crude membrane preparations? 3. Can judicious application of various known modulators of adenylate cyclase reveal the component of the cAMP generating complex which is modified by exposure to phorbol esters (i.e., hormone receptor, N_s or N_i coupling proteins, catalytic moeity)?

MATERIALS AND METHODS

The supplies and drugs were purchased from commercial sources: phorbol esters (LC Services Corp, Woburn, MA), media and sera (GIBCO, Grand Island, NY), Falcon culture plates (Oxnard, CA), [^{3}H]-cAMP (Amersham, Arlington Hgts, IL), Dowex AG 50W-X4 and Bradford dye reagent (Biorad, Richmond, CA), forskolin (Calbiochem, La Jolla, CA); all other materials were purchased from Sigma (St. Louis, MO). The [alpha-^{32}P]-ATP was synthesized by the University of Virginia Diabetes Core Facility.

The 235-1 cell line (passages 45-70) was maintained as previously described (5) with the exception that cells were passaged with 0.1% trypsin. When cellular cAMP content was determined, cells were cultured to 70-90% confluency in 24 well Primaria plates. The culture medium was removed and the cells incubated 30 min in RPMI-H (RPMI-1640, 20 mM HEPES without bicarbonate or serum). This medium was removed and replaced by RPMI-H containing the test substances. In experiments using 1 mM IBMX, the drug was present during both the 30 min preincubation and the 2-15 min test incubation at 37°C. To stop the reaction, the medium was removed and cAMP extracted by a 15 min exposure to 0.1 N HCl. The cAMP concentration was then measured by radioimmunoassay (6). The HCl insoluble protein (approx 50% of total) was then extracted (0.2 N NaOH, 4°C, 24 hr).

To determine adenylate cyclase activity, cells were cultured to 70-90% confluency in Corning 100 mm or 150 mm dishes. The culture medium was removed and serum-free RPMI-1640 medium containing phorbol esters or vehicle (0.01% ethanol) was added for 2-60 min. To terminate the pretreatment, the medium was removed, cells were rinsed with ice cold PBS and then scraped off of the dishes and homogenized at 4°C with a Dounce homogenizer in buffer A (20 mM Tris, pH 7.5; 6 mM $MgCl_2$, 3 mM dithiothreitol, 1 mM NaF, 1.2 mM ethyleneglycol-bis-(beta-aminoethyl ether) N,N'-tetraacetic acid, 25 ug/ml leupeptin). The homogenate was centrifuged for 5 min (328 x g, 4C), and the resulting supernatant was centrifuged 15 min [12,200 x g, 4C]. The pellet was resuspended in buffer B (buffer A with 250 mM sucrose) and either analyzed immediately or frozen at -70°C. Adenylate cyclase activity was assayed in triplicate in a 100 µl reaction volume containing 0.5 mM ATP, 500,000 cpm [alpha-^{32}P]-ATP, 50 mM Tris, pH 8.0; 4 mM IBMX, 0.1 mg/ml bovine serum albumin, 1 mM ethylenediaminetetraacetic acid, 10 mM $MgCl_2$, 10 IU/ml creatine phosphokinase, 10 mM phosphocreatine, 5 to 20 ug protein sample and test agents. The reaction was carried out for 10 min at 30°C and stopped by the addition of 90 µl of stop buffer (4% SDS, 50 mM ATP, 0.175 mM cAMP, and 100 cpm/µl [^{3}H]-cAMP to monitor recovery) and heating to 90°C. The generated cAMP was separated by the procedure of Soloman (7). The assay was linear for at least 10 min at the protein concentrations used. All proteins were measured by the method of Bradford (8). Statistical assessment was by analysis of variance; a difference denoted as significant has a P value less than 0.05.

RESULTS AND DISCUSSION

PMA (100 nM) enhanced the ability of maximal concentrations of PGE_1 to stimulate cAMP accumulation in 235-1 cells (Table 1). PMA also increased

Table 1. The effect of PMA on cAMP levels in 235-1 cells

	Cellular cAMP (pmoles/well)	
	Vehicle	PMA (100 nM)
Basal	2.17 ± 0.04	3.05 ± 0.11
PGE1 (1 μM)	36.5 ± 1.4	79.9 ± 3.7
Forskolin (1 μM)	7.99 ± 0.40	27.2 ± 1.2

235-1 cells were incubated for 10 min in the presence of 1 mM IBMX with the indicated agents. Data representative of experiments repeated 3 times is listed as the mean ± SEM (4 wells/group). Protein concentrations did not vary significantly between groups.

basal and forskolin stimulated cAMP levels. Phorbol-12,13-dibutyrate (100 nM) was as effective as PMA, while 4-alpha-phorbol, which does not activate protein kinase C, had no effect (data not shown). The increase in basal cAMP mediated by phorbol esters, required the cAMP phosphodiesterase inhibitor IBMX. This observation is reminiscent of a study using the GH_3 pituitary cell line (9). In contrast, the phorbol ester effect after PGE_1 and forskolin was apparent with or without IBMX. These data indicate that PMA enhances the action of cAMP generating agents by an action on cAMP synthesis. Furthermore, the target(s) appears to be distal to the hormone receptor because PMA also affected the action of forskolin, a direct stimulator of the catalytic subunit of adenylate cyclase (10), and because the ED_{50} for PGE_1 was not significantly altered by PMA (unpublished observation).

These results led us to the hypothesis that phorbol esters induce a covalent modification of the adenylate cyclase holoenzyme. When we examined this enzyme in crude membrane preparations, both basal and stimulated adenylate cyclase activity were enhanced by PMA (Table 2). The maximal effect of PMA was apparent after 5 min and was stable for up to 1 hr of pretreatment, the longest time tested (data not shown). The ED_{50} for PMA was 1 - 10 nM, similar to the ED_{50} of PMA for the activation of protein kinase C (11). Adding GTP to the assay, while essential for PGE_1 stimulation of adenylate cyclase, was unnecessary for the increase in basal and forskolin-stimulated activity mediated by phorbol esters. This suggests that an effect of PMA may be independent of the guanine nucleotide binding subunits, N_s and N_i.

Table 2. Ratio of adenylate cyclase activity in membranes from PMA/vehicle pretreated cells

	No Added GTP	GTP Added (100 μM)
Basal	1.26 ± 0.06 (7)	1.30 ± 0.06 (16)
Forskolin (10 μM)	1.32 ± 0.06 (8)	1.38 ± 0.06 (15)
PGE1 (1 μM)	ND	1.24 ± 0.06 (9)

The ratios of adenylate cyclase activity in membranes from PMA (100 nM) pretreated cells divided by the activity in membranes from vehicle pretreated cells were significantly different from 1.00 for all groups. The conversion was necessary because the specific activity of the membrane preparations varied by as much as 2-4 fold between days. The specific activity of membrane fractions prepared simultaneously from independent dishes of cells never varied by more than 10%. Mean ± SEM is represented for (n) independent studies. Basal activities were 50-200 pmoles cAMP/min/mg protein whereas stimulated activities were 600-2000 pmoles cAMP/min/mg protein. ND = not done

We next established that the fractional enhancement of activity induced by PMA was identical at all concentrations of forskolin (Figure 1). One interpretation of these data is that N_s was activated by the phorbol ester in the 235-1 cell. This concept also appealed to Bell and colleagues (12) who studied S49 lymphoma membranes stimulated by cholera toxin and fluoride. To further assess the contribution of the guanine nucleotide coupling proteins N_s

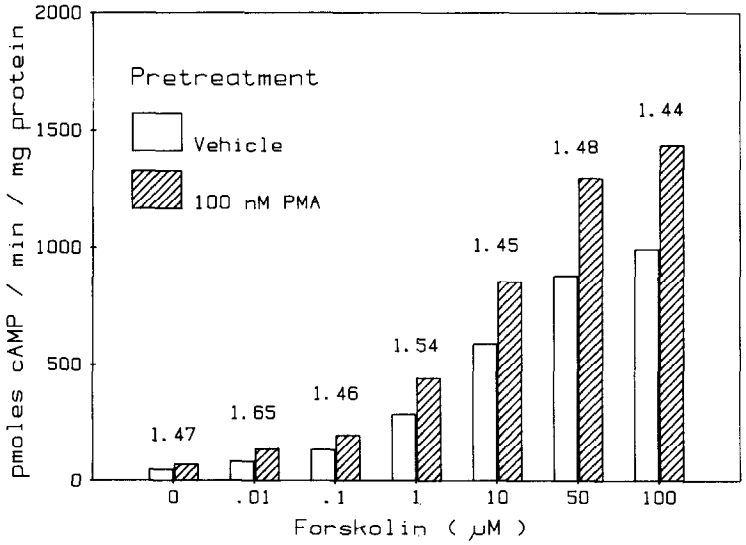

Figure 1. The adenylate cyclase activity in membranes from cells pretreated with PMA (100 nM) or vehicle for 30 min at the indicated concentrations of forskolin with GTP (100 μM). Membranes were isolated as described in Methods. The SEM varied from 1-9% of the mean. The data are the mean of 6 independent experiments. The activity of PMA pretreated membranes was significantly greater than the activity of control membranes for every condition tested.

and N_i, we examined the effect of GTP on PGE_1 stimulated activity. N_s requires GTP to couple the membrane receptor to catalytic adenylate cyclase, while GTP greater than 1 μM can activate N_i in the absence of inhibitory receptor occupation (13,14). In 235-1 cells, maximal PGE_1 stimulation occurred at approximately 1 μM GTP (data not shown), while 10 μM and 100 μM GTP significantly reduced this maximal effect on adenylate cyclase activity by 18 + 2% and 26 + 6%, respectively. In the membranes from PMA pretreated cells, however, there was no apparent inhibition at 10 μM GTP (4 ± 2%) and an attenuated inhibition at 100 μM GTP (12 ± 3%: mean ± SEM of 4 independent experiments). Our interpetation of these data is similar to that of Jakobs and collegues using the platelet (15,16), that protein kinase C inactivates N_i by phosphorylating one or more of its subunits. This concept could also be used to explain an increase in adenylate cyclase activity, both basal and forskolin-activated, if N_s behaves like a proportional amplifier in the absence of functional N_i.

In conclusion, the activation of protein kinase C results in increased basal and stimulated levels of adenylate cyclase activity in the 235-1 pituitary cell line. This appears to represent a widely conserved response that has been noted in a variety of cells, including the S49 lymphoma (12), brain slices (16), anterior pituitary (1,2), pineal (17), platelets (14), and vascular smooth muscle (18). In the 235-1 cell, the alteration induced by PMA appears to occur at a site distal to the hormone receptor. The effect of PMA at all forskolin concentrations supports the notion that protein kinase C activation leads to increased N_s or decreased N_i activity in 235-1 cells. The PMA-mediated attenuation of GTP inhibition of adenylate cyclase activity favors the concept that inactivation of N_i may be an important target of protein kinase C. Finally, resolution of PMA-stimulated adenylate cyclase activity [assayed in the absence of added GTP] suggests that a direct effect of protein kinase C on the catalytic subunit, or some other as yet uncharacterized modulator of adenylate cyclase, cannot be exluded as a mechanism by which phorbol esters affect the adenylate cyclase holoenzyme.

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REFERENCES

1. Cronin, M.J., and Canonico, P.L. (1985) Biochem. Biophys. Res. Commun. 129, 404-410.
2. Perkins, S.N., Summers, S.T., and Cronin, M.J. (1986) Endocrine Res. (in press).
3. Reymond, M.J., Nansel, D.D., Burrows, G.H., Neaves, W.B., and Porter, J.C. (1984) Acta Endocrin. 106, 459-470.
4. Cronin, M.J., Myers, G.A., MacLeod, R.M., and Hewlett, E.L. (1983) J. Cyc. Nuc. Prot. Phosphoryl. Res. 9, 245-258.
5. Cronin, M.J., Perkins, S.N., Keefer, D.A., and MacLeod, R.M. (1982) Mol. Cell. Endocrin. 28, 229-246.
6. Brooker, G., Terasaki, W.L., and Price, M.G. (1976) Science 194, 270-276.
7. Solomon, Y., Londos, C., and Rodbell, M. (1974) Anal. Biochem. 58, 541-548.
8. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
9. Quilliam, L.A., Dobson, P.R.M., and Brown, B.L. (1985) Biochem. Biophys. Res. Comm. 129, 898-903.
10. Seamon, K., and Daly, J.W. (1981) J. Biol. Chem. 256, 9799-9801.
11. Castagna, M., Takai, Y., Kaibuchi, K, Sano, K., Kikkawa, U, and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851.
12. Bell, J.D., Buxton, I.L.O., and Brunton, L.L. (1985) J. Biol. Chem. 260, 2625-2628.
13. Hildebrandt, J.D., Hanoune, J., and Birnbaumer, L. (1982) J. Biol. Chem. 257, 14723-14725.
14. Jakobs, K.H., Bauer, S., and Watanabe, Y. (1985) Eur. J. Biochem. 151, 425-430.
15. Katada, T., Gilman, A.G., Watanake, Y., Bauer, S., Jakobs, K.H., (1985) Eur. J. Biochem. 151, 431-437.
16. Hollingsworth, E.B., Sears, E.B., and Daly,J.W. (1985) FEBS Lett. 184, 339-342.
17. Sugden, D., Vanecek, J., Klein, D.C, Thomas, T.P., and Anderson, W.B. (1985) Nature 314, 359-361.
18. Nabika, T., Nara, Y., Yamori, Y, Lovenberg, W, and Endo, J. (1985) Biochem. Biophys. Res. Commun. 131, 30-36.