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PHORBOL MYRISTATE ACETATE STIMULATION OF LYMPHOCYTE GUANYLATE CYCLASE

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SUMMARY: Human lymphocyte guanylate cyclase activities are increased in a dose-dependent fashion by incubation of intact cells with phorbol myristate acetate, a tumor promoter and lymphocyte mitogen. Increased activity is detectable after 1 minute, and peak membrane-bound and soluble forms of guanylate cyclase occur after 10- and 30-minute exposure to phorbol myristate acetate, respectively. The soluble form is stimulated much more than the membrane form. Enzyme activities measured in the presence of either Ca^{2+}, Mg^{2+}, or Mn^{2+} are elevated to similar degrees. Comparisons of phorbol and a series of its diesters revealed a good correlation between the capacities for guanylate cyclase stimulation, lymphocyte mitogenesis, and tumor promotion.

Phorbol-12-myristate-13-acetate (PMA)[1] is a potent stimulant of lymphocyte transformation and DNA synthesis (1-3). This agent is derived from croton oil and acts as a tumor promoter in the two-stage carcinogenesis model in mouse skin (4,5). PMA is a complete mitogen in human (1-3) and other primate (6) peripheral blood T lymphocytes, but functions only as a co-mitogen in bovine (7,8), mouse (6), and guinea pig (9) lymphocytes. PMA binds selectively to plasma membranes of lymphocytes (3,10) and the primary site of action is believed to be at the cell surface (1,3-5,10). We (11,12) have shown that the plant lectin mitogens, phytohemagglutinin (PHA) and concanavalin A, which also act at the cell surface (13), stimulate increases in human lymphocyte cyclic GMP within minutes. Similarly, we (14) have found that PMA increases lymphocyte levels of cyclic GMP by 2-fold at 1 minute. PHA-induced elevations in cyclic GMP are due to enhanced activity of guanylate cyclase (E.C. 4.6.1.2), measured in both membrane and cytoplasmic fractions after incubation of intact

[1] Abbreviations: PMA, phorbol-12-myristate-13-acetate; PHA, phytohemagglutinin.

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lymphocytes with the mitogen (15). This report describes the effects of PMA, a terpene diester structurally unrelated to PHA or Con A, to stimulate guanylate cyclase activity in human peripheral blood lymphocytes.

MATERIALS AND METHODS

PMA was obtained from Consolidated Midland Corporation, Brewster, NY. Phorbol, phorbol diacetate, phorbol dibutyrate, phorbol dibenzoate, and phorbol didecanoate were obtained from Sigma Chemical Co., St. Louis, MO. Methoxyverapamil (D-600) was a generous gift from Knoll, A. G., Ludwigshafen am Rhein, W. Germany.

Peripheral blood lymphocytes were purified from heparinized venous blood of healthy adults as described (12) and suspended in Hank's Balanced Salt Solution at a concentration of 10 x 10^6/ml. Cells were equilibrated at 37°C for at least 30 minutes prior to the addition of PMA or other agents. Phorbol and phorbol diesters were dissolved in dimethylsulfoxide and added in 1-2 µl aliquots to 2-4 ml cells. The final concentration of dimethylsulfoxide did not exceed 0.1% and had no effect on the activities measured. Lymphocytes were incubated for 10 minutes with an optimum (1-3) mitogenic concentration (1 µg/ml) of PMA unless otherwise stated. They were then centrifuged at 800 x g for 2 minutes, washed, homogenized, and membrane and soluble (48,000 x g) components were prepared as described (15). Guanylate cyclase activity was assayed by incubation in duplicate at 37°C for 6 minutes in a total volume of 0.2 ml containing 20-50 µg lymphocyte protein, 0.5 mM GTP, 50 mM Hepes·Na (pH 7.6), 0.5 mM phosphocreatine, 3 U of creatine phosphokinase (E.C. 2.7.3.2), 0.5 mM 3-isobutyl-1-methylxanthine, and either 5 mM $CaCl_2$, 5 mM $MgCl_2$, or 2 mM $MnCl_2$ unless otherwise stated. The reaction was terminated with 1 ml of cold 0.5 M perchloric acid, and cyclic GMP was purified and measured by radioimmunoassay (15,16). Reaction rates were calculated as pMol cyclic GMP formed/min/mg protein and were linear with the amount of protein stated above and with time up to 6 minutes. Lymphocyte DNA synthesis was determined by [^{3}H]-thymidine incorporation (12).

RESULTS

Preincubation of lymphocytes with PMA stimulated both membrane and soluble forms of guanylate cyclase activity, measured in the presence of optimal concentrations of Ca^{2+}, Mg^{2+}, or Mn^{2+}, as shown in Table I. The degree of stimulation of the soluble guanylate cyclase was consistently found to be much greater than that of the membrane enzyme. Similar results were obtained with lymphocyte preparations rendered free of monocytes by glass adherence. In other experiments it was found that V_{max} was increased by PMA in all guanylate cyclase activities. Values for the apparent K_m (GTP) were reduced by PMA in the soluble fraction but not the membranes. No significant changes in the activating effects of the cations were induced by PMA.

TABLE I

STIMULATION OF LYMPHOCYTE GUANYLATE CYCLASE BY INCUBATION OF INTACT CELLS WITH PMA

	Membrane			Soluble		
	Ca 5 mM	Mg 5 mM	Mn 2 mM	Ca 5 mM	Mg 5 mM	Mn 2 mM
Controls						
Specific Activity	0.73	2.81	9.09	0.36	1.38	4.19
S.E.	0.16	0.62	2.18	0.09	0.44	0.95
Ratio						
PMA ÷ control	1.64	1.67	1.71	2.89	3.15	3.22
S.E.	0.27	0.12	0.14	0.21	0.28	0.23
p	<0.05	<0.001	<0.001	<0.001	<0.001	<0.001
n	7	12	11	9	13	13

Lymphocytes were incubated with or without 1 μg/ml of PMA for 10 minutes at 37°C, and the membrane and soluble fractions were prepared and assayed for guanylate cyclase activity in the presence of the indicated cations as described in Methods. Specific activities (pMol cGMP/mg protein/min) and S.E. (standard error of the mean) are indicated for the control incubations, and the means of the ratio of specific activity in samples from PMA-treated lymphocytes to the specific activity in control cells are given for the number of experiments (n). p values indicate significance of the increases as calculated by student's two-tailed test.

The responses of the Mg^{2+}-dependent guanylate cyclases to increasing concentrations of PMA are shown in Figure 1. Both membrane and soluble activities progressively increased from 0.01 to 10 μg/ml PMA, with greater increases in the soluble form at the lower concentrations. Similar results were obtained with the Ca^{2+}- and the Mn^{2+}-dependent enzyme activities (not shown). Increased guanylate cyclase was detected after 1-minute incubation with PMA, as shown in Figure 2. The peak response of the membrane enzyme occurred at 10 minutes, while the soluble enzyme continued to increase for 30 minutes.

The capacity of PHA to stimulate guanylate cyclase was largely inhibited by omission of Ca^{2+} from the preincubation medium, and totally inhibited by addition of EGTA (15). In similar experiments with PMA we observed no inhibition by omission of Ca^{2+}, and partial (56-66%) inhibition with 5 mM EGTA.

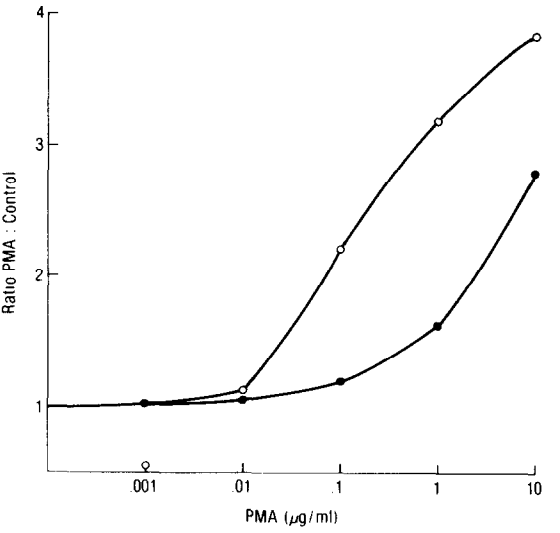

Fig. 1: Effect of increasing concentrations of PMA on lymphocyte guanylate cyclase activity. Mg^{2+}-dependent guanylate cyclase activity was assayed in lymphocyte membrane (●) and soluble (o) fractions after incubation of intact cells at 37°C for 10 minutes with or without 1 μg/ml PMA. Mean values for two experiments are shown.

However, almost complete (82-87%) inhibition of PMA stimulation was obtained in two experiments with the intracellular Ca^{2+} inhibitor D-600 (17) added at 10 μM levels 30 minutes before PMA.

Good correlations between mitogenic and tumor-promoting activities of PMA and several analogs have been reported in mouse epidermis (4,18) and in lymphocyte (3) and other cell cultures (18). We therefore compared the effects of phorbol and a series of phorbol diesters to stimulate lymphocyte guanylate cyclase. The compounds are listed in Table II in the approximate order of effectiveness as tumor promoters, with phorbol and phorbol diacetate as completely inactive, and PMA as the most active (4,18). The rank order of effectiveness as lymphocyte mitogens is similar (3; E. M. Hadden, unpublished observations). We found that PMA was the most potent stimulant of guanylate cyclase; phorbol didecanoate, phorbol dibenzoate, and phorbol dibutyrate were of intermediate effectiveness. Phorbol diacetate caused a slight stimulation in membrane but not soluble guanylate cyclase, and phorbol was without effect in both enzyme preparations.

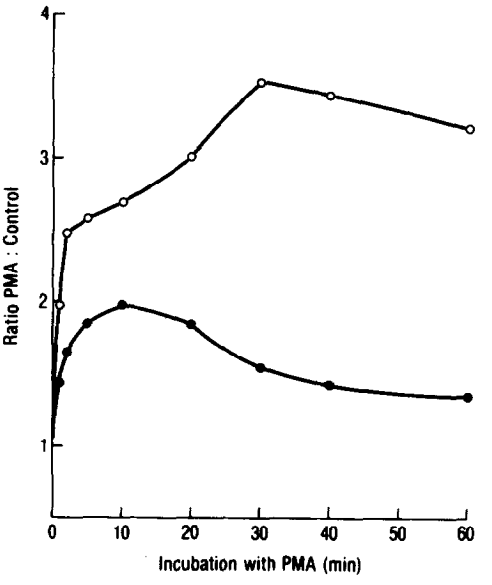

Fig. 2: Time course of PMA stimulation of lymphocyte guanylate cyclase activity. Mg^{2+}-dependent guanylate cyclase activity was assayed in lymphocyte membrane (•) and soluble (o) fractions after incubation of intact cells at 37°C for the indicated times, dilution with cold Hank's Balanced Salt Solution, centrifugation and homogenization as described in Methods. Mean values for two experiments are shown.

DISCUSSION

We (11,19) have proposed that cyclic GMP and Ca^{2+} are key elements in the mitogenic signal sequence leading to lymphocyte proliferation. PHA increases lymphocyte cyclic GMP by stimulating guanylate cyclase activity within

TABLE II

EFFECTS OF PHORBOL DIESTERS ON LYMPHOCYTE GUANYLATE CYCLASE

	Membrane		Soluble	
	S.A.	% C	S.A.	% C
Control	1.31		0.69	
Phorbol	1.21	92	0.63	91
Phorbol diacetate	1.64	125	0.66	96
Phorbol dibutyrate	1.64	125	2.19	317
Phorbol dibenzoate	1.76	134	2.04	296
Phorbol didecanoate	1.83	140	1.77	257
Phorbol myristate acetate	2.00	153	2.37	344

Lymphocytes were incubated in duplicate at 37°C for 10 minutes with phorbol or phorbol diester at a concentration of 1.6 μM, which is equal to 1 μg/ml for PMA. The specific activities (S.A.) of Mg^{2+}-dependent guanylate cyclase and the effects of the experimental compounds as percent of control (% C) are indicated.

minutes of mitogen addition (15). PMA also stimulates guanylate cyclase in a brisk, dose-dependent fashion. One difference between the mitogens involves the subcellular locus of the stimulated guanylate cyclase: the membrane enzyme is increased to a much greater extent by PHA, while the soluble enzyme is markedly activated by PMA. Like the lectin mitogens (13,19), PMA has been reported to require Ca^{2+} for its effects on thymic lymphoblast (20) as well as mouse fibroblast (21) DNA synthesis. The Ca^{2+} requirement for PHA is met in part by extracellular sources, while that for PMA appears to involve primarily intracellular Ca^{2+} stores. The PMA-induced release of granule enzymes from human neutrophils also requires only intracellular Ca^{2+}, as demonstrated by inhibition with an intracellular Ca^{2+} antagonist (22).

PMA-induced changes in cyclic nucleotide metabolism were first reported by Goldberg et al. (23) who found a 4-fold increase in cyclic GMP levels in human platelets within 30 seconds in association with induction of aggregation. Estensen et al. (1) also reported 20-fold increases in cyclic GMP in mouse fibroblasts induced to proliferate with PMA. Others have shown marked increases by PMA in the levels of cyclic GMP in Chinese hamster and human amnion cell cultures (24) and in myoblast cultures (25), and increased guanylate cyclase activity in rat embryo fibroblasts (26). In addition, decreased cyclic AMP levels (27) and desensitization of β-adrenergic stimulation of adenylate cyclase (28) also occur soon after PMA application to mouse skin or rat embryo fibroblasts. We have also observed reduced adenylate cyclase activity coincident with enhanced guanylate cyclase activity in PMA-treated lymphocytes (unpublished). It will be of great interest to explore the mechanisms by which these changes occur and whether they are interdependent on one another, or whether both result from some prior event such as phospholipid changes (5,29) induced by PMA.

REFERENCES

1. Estensen, R. D., Hadden, J. W., Hadden, E. M., Touraine, F., Touraine, J. L., Haddox, M. K., and Goldberg, N. D. (1974) in Control of

Proliferation in Animal Cells (ed. Clarkson, B., and Baserga, R.) pp. 627-634, Cold Spring Harbor Laboratory, New York.
2. Touraine, J. L., Hadden, J. W., Touraine, F., Hadden, E. M., and Good, R. A. (1977) J. Exp. Med. 145, 460-465.
3. Estensen, R. D., Drazich, B. F., and Hadden, J. W. (1978) in Carcinogenesis, Vol. 2. Mechanisms of Tumor Promotion and Co-carcinogenesis (ed. Slaga, T. J., Sivak, A., and Boutwell, R. K.) pp. 379-388, Raven Press, New York.
4. Boutwell, R. K. (1974) CRC Crit. Rev. Toxicol. 2, 419-445.
5. Balmain, A., and Hecker, E. (1974) Biochim. Biophys. Acta 362, 457-468.
6. Abb, J., Bayliss, G. J., and Deinhardt, F. (1979) J. Immunol. 122, 1639-1642.
7. Mastro, A. M., and Mueller, G. C. (1974) Exp. Cell Res. 88, 40-46.
8. Wertz, P. W., and Mueller, G. C. (1978) Cancer Res. 38, 2900-2904.
9. Rosenstreich, D. L., and Mizel, S. B. (1979) J. Immunol. 123, 1749-1754.
10. Estensen, R. D., DeHoogh, D. K., and Cole, C. F. (1980) Cancer Res. 40, 1119-1124.
11. Coffey, R. G., Hadden, E. M., and Hadden, J. W. (1977) J. Immunol. 119, 1387-1394.
12. Hadden, J. W., Hadden, E. M., Sadlik, J. R., and Coffey, R. G. (1976) Proc. Natl. Acad. Sci. USA 73, 1717-1721.
13. Hadden, J. W. (1977) in Immunopharmacology (ed. Hadden, J. W., Coffey, R. G., and Spreafico, F.) pp. 1-28, Plenum Press, New York.
14. Coffey, R. G., and Hadden, J. W. (1981) Fed. Proc. 40, 1728.
15. Coffey, R. G., and Hadden, J. W. (1981) J. Biol. Chem. 256, 4418-4424.
16. Steiner, A. L., Parker, C. W., and Kipnis, D. M. (1972) J. Biol. Chem. 247, 1106-1113.
17. Fleckenstein, A. (1977) Annu. Rev. Pharmacol. Toxicol. 17, 149-166.
18. Yuspa, S. H., Lichti, U., Ben, J., Patterson, E., Hennings, M., Slaga, T. J., Colburn, N., and Kelsey, W. (1976) Nature 262, 402-404.
19. Hadden, J. W., Coffey, R. G., Ananthakrishnan, R., and Hadden, E. M. (1979) Ann. N.Y. Acad. Sci. 332, 241-254.
20. Whitfield, J. F., MacManus, J. P., and Gillan, D. J. (1973) J. Cell. Physiol. 82, 151-156.
21. Boynton, A. L., Whitfield, J. F., and Isaacs, J. R. (1976) J. Cell. Physiol. 84, 25-34.
22. Smith, R. J., and Iden, S. S. (1979) Biochem. Biophys. Res. Commun. 91, 263-271.
23. Goldberg, N. D., Haddox, M. K., Dunham, E., Lopez, C., and Hadden, J. W. (1974) in Control of Proliferation in Animal Cells (ed. Clarkson, B., and Baserga, R.) pp. 609-626, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
24. Trosko, J. E., Yager, J. D., Jr., Bowden, G. T., and Butcher, F. R. (1975) Chem.-Biol. Interact. 11, 191-205.
25. Grotendorst, G. R., and Schimmel, S. D. (1980) Biochem. Biophys. Res. Commun. 93, 301-307.
26. Rochette-Egly, C., and Castagna, M. (1979) Biochem. Biophys. Res. Commun. 86, 937-944.
27. Belman, S., and Troll, W. (1974) Cancer Res. 34, 3446-3455.
28. Garte, S. J., and Belman, S. (1980) Nature 284, 171-173.
29. Levine, L., and Hassid, A. (1977) Biochem. Biophys. Res. Commun. 79, 477-484.