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PHORBOL ESTER EFFECT IN PLATELETS, LYMPHOCYTES, AND LEUKEMIC CELLS (HL-60) IS ASSOCIATED WITH ENHANCED PHOSPHORYLATION OF CLASS I HLA ANTIGENS

Coprecipitation of myosin light chain

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SUMMARY: Phorbol-12-myristate-13-acetate (PMA) is a potent tumor promoter. However, the mechanism of its effect is still unknown. In the present study we use two dimensional gel electrophoresis to show that the PMA effect on platelets is associated with enhanced phosphorylation of a series of polypeptides at 44K which migrate close to but distinct from a previously reported 47K protein. We identified these proteins as the class I molecules of the human histocompatibility antigens (HLA A,B). We further demonstrate that the PMA effect is also associated with a dramatic phosphorylation of HLA antigens in HL-60 leukemic cells and in human lymphocytes, showing that an increase in phosphorylation of HLA antigens is intimately related to the signal of PMA in various cellular systems. Immunoprecipitation of HLA proteins resulted in coprecipitation of phosphorylated myosin light chain (20K). HLA antigens are transmembrane proteins which interact with cytoskeletal elements, probably via their intracellular region, which has been previously shown to be phosphorylated. It is suggested that phosphorylation of HLA membrane proteins may represent an important mechanism in the effects induced by PMA.

PMA[1] has profound effects on a variety of cellular functions (review 1, 2). It has been suggested that these effects are mediated through a membrane-associated mechanism (1-4). Most significantly, treatment of intact cells with PMA involves rapid and tight association with the plasma membrane of the putative receptor of PMA (5-8), Ca-PL-PK (3). This suggests that phosphorylation of membrane-associated proteins might play an important role in mediation of the PMA signal. In the present study, we used two-dimensional gel electrophoresis to analyze the effect of PMA on phosphorylation of proteins in human

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[1]Abbreviations used in this manuscript: PMA: phorbol-12-myristate-13-acetate ; Ca-PL-PK: Ca^{+2} activated phospholipid dependent protein kinase ; MLC: myosin light chain (20K) ; HLA: histocompatibility antigens .

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platelets. We show that PMA enhances the phosphorylation of a series of polypeptides at 44K which migrate close to, but distinct from a previously reported 47K protein (9). We identified this series of 44K phosphoproteins immunologically as the class I molecules of the human histocompatibility antigens (HLA A, B). We further demonstrate that PMA effect is also associated with a dramatic phosphorylation of HLA antigens in HL-60 leukemic cells and in human lymphocytes, suggesting that an increase in phosphorylation of HLA antigens is intimately related to the signal of PMA in various cellular systems.

METHODS:

Cell culture: Human peripheral lymphocytes (10) and platelets (11) were prepared from fresh blood of normal donors as previously described (10,11). HL-60 leukemic cells were grown in culture as described (12).

HLA immunoprecipitation: Immunoprecipitation was done with monoclonal antibody to a framework specificity of HLA A, B and C (W6/32) as described (13,14).

Two-dimensional gel electrophoresis: Two-dimensional gel electrophoresis was done as described (15). First dimension isoelectrofocusing gels were ampholytes pH 3.5 to 10. Second dimension gels were 12% acrylamide. Radiography was done between Kodak XAR intensifying screens.

RESULTS AND DISCUSSION:

In the course of studies on the mechanism of action of PMA in leukemic cells (12,16,17,10,18) we also examined the effect of this ligand on human platelets. This experiment (Fig. 1) revealed that in addition to the increase of phosphorylation of MLC (20K) and pp47 previously described (9,4,19), the PMA effect was also associated with a specific increase in phosphorylation of another series of polypeptides (outlined in Fig. 1). These proteins migrate close to, but distinct from pp47 (M_r 45-44K, pI 5.3-6). Apparently this phosphorylation event could only be distinguished from the phosphorylation of pp47 by use of two-dimensional gel electrophoresis. The electrophoretic mobility of this chain of polypeptides is similar to that of the proteins of human class I HLA antigens (13,14). The HLA antigens (14,20) are transmembrane glycoproteins whose intracellular region has been shown to be phosphorylated in vivo and in vitro at serine residue(s) in normal human lymphocytes and lymphoblastoid cell lines (21,22,23) and in mouse lymphocytes (24,25).

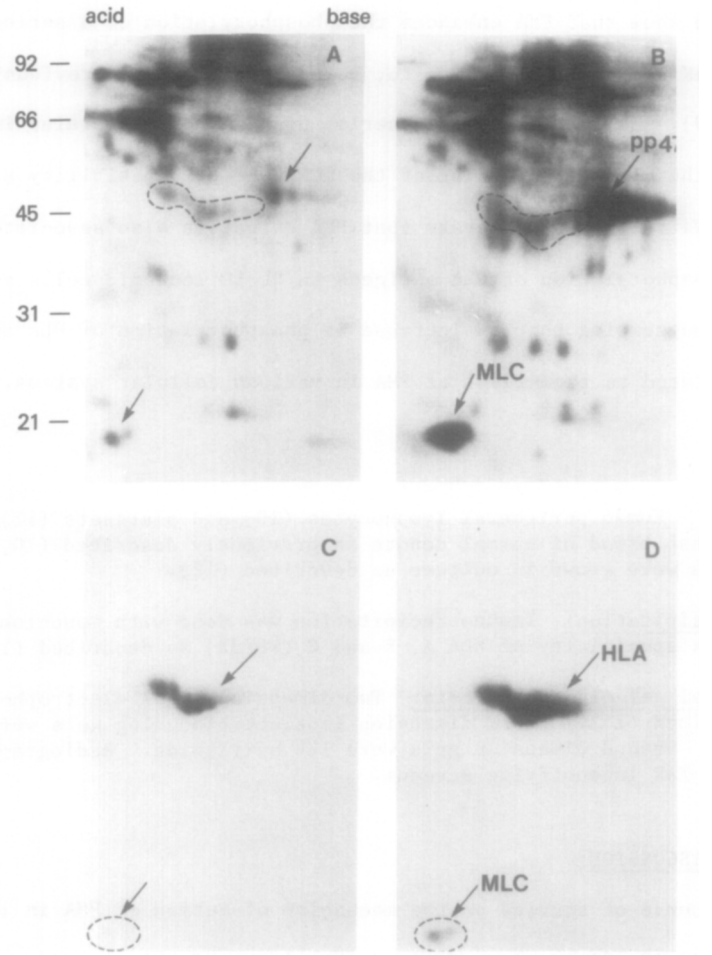

Fig. 1. Identification of a series of phosphoproteins at 44K, pI 5.3-6 in human platelets as the class I HLA antigens and demonstration of an increase in phosphorylation by PMA. Platelets were separated from human blood, washed with phosphate-buffered saline, pH 7.4 (supplemented with EDTA, 0.5 mM), and suspended in Tyrode-HEPES (pH 7.4) solution (11). The platelets were labeled with $^{32}PO_4$ (0.25 mCi/ml) for 60 min at 37°C, treated with PMA ($5x10^{-8}$M, 30 min). At the end of the experiment, a small portion of the platelet sample was lysed with iso-electrofocusing solution (12) for analysis of total phosphoproteins by two-dimensional gel electrophoresis (15) and the rest of the sample was used for immunoprecipitation as described (13). In brief, the platelets were spun down and suspended in detergent lysis solution (0.01 M NaCl, 0.01 M Tris-HCl, pH 7.4, 3 mM $MgCl_2$, 0.2 mM PMSF, 0.4% Triton N-101) and further homogenized in Dounce homogenizer fitted with a tight teflon pestle (15 strokes). The lysate was centrifuged (3,000 RPM, 4°C, 5 min) and stored at -80°C. Immunoprecipitation was done with monoclonal antibody to a framework specificity of HLA A, B and C (W6/32) as described (13,14). The immunoprecipitates were mixed with unlabeled platelet proteins (from the same donor, to be used as internal markers) and analyzed by two-dimensional gel electrophoresis. Gels were autoradiographed for 4 days. Using the Coomassie-blue stained pattern of proteins as internal markers it was seen that the immunoprecipitated phosphoproteins in C and D migrate identically with the series of phosphoproteins outlined in A and B. A: total phosphoproteins from control platelets, B: total phosphoproteins from PMA-treated platelets, C: immunoprecipitation of HLA proteins from control platelets, D: immunoprecipitatin of HLA proteins from PMA-treated platelets. Arrows indicate the location of HLA proteins, pp47 and myosin light chain (MLC).

Immunoselection of HLA molecules from PO_4-labeled platelets (Fig. 1) clearly identified the chain of phosphoproteins (outlined in Fig 1A,B) with the HLA proteins. Identical results were obtained when immunoprecipitation of HLA proteins was done with anti- β_2 microglobulin antibody (data not shown). This further supports the identification of these proteins as the class I HLA antigens (13).

In further kinetic studies we found that the effect of PMA on phosphorylation of HLA in platelets could be detected following 1 min exposure to PMA (2-fold increase) and was further increased after 10 min exposure to PMA (3-4 fold). This suggests that phosphorylation of HLA by PMA is an early event associated with the signal of PMA in platelets.

We further examined whether the effect of PMA on phosphorylation of HLA antigens could be demonstrated in other cell types. We found that phosphorylation of HLA is dramatically enhanced in human lymphocytes (Fig. 2) and promyelocytic leukemic cells, HL-60 (Fig. 3), following exposure to PMA. These results clearly demonstrate that an increase in phosphorylation of HLA proteins is intimately related to the signal of PMA in a variety of cellular systems.

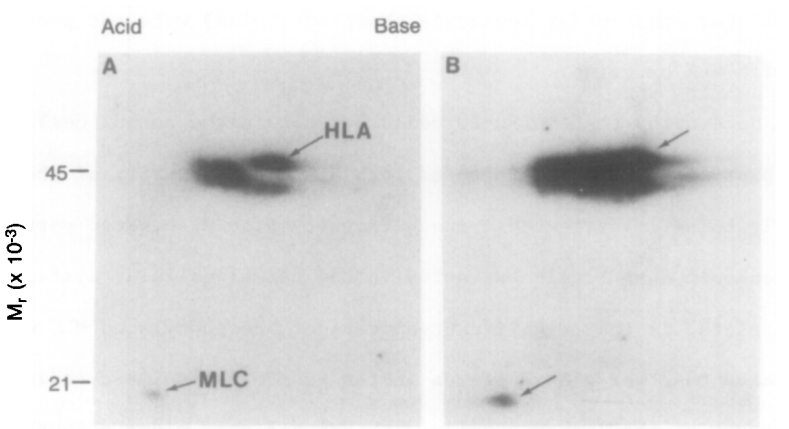

Fig. 2. Effect of PMA on phosphorylation of HLA antigens in human lymphocytes

Lymphocytes were purified from peripheral blood as described (10), labeled with ^{32}Pi (0.25 mCi/ml) for 2 h (26) and then exposed to PMA (10^{-7}M) for 30 min. At the end of the experiment the cells were spun down, lysed and immunoprecipitation for HLA proteins was done as described in legend for Fig. 1. A: immunoprecipitate of HLA in control lymphocytes, B: Immunoprecipitation of HLA in PMA-treated lymphocytes.

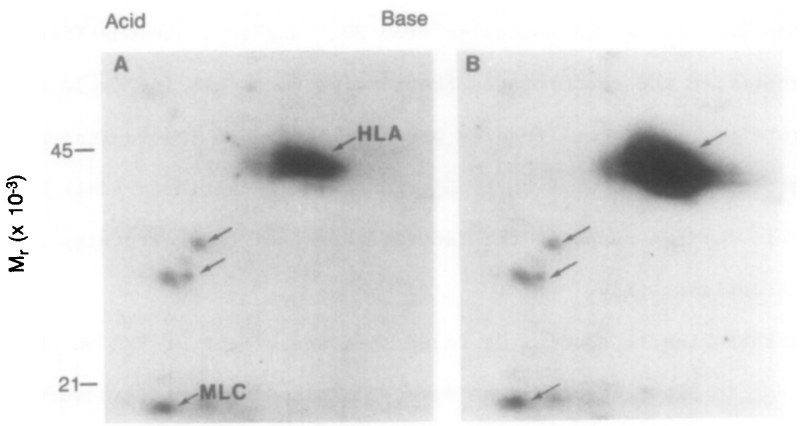

Fig. 3. Effect of PMA on phosphorylation of HLA antigens in HL-60 leukemic cells.

HL-60 leukemic cells (12) were washed and labeled with $^{32}P_i$, as described (12,16) and then exposed to 10^{-8}M of PMA (30 min). At the end of the experiment the cells were spun down, lysed and immunoprecipitation for HLA proteins was done as described in legend for Fig. 1. A: Immunoprecipitation of HLA in control HL-60 cells, B: Immunoprecipitation of HLA in PMA-treated HL-60 cells. Arrows indicate the location of HLA, myosin light chain (MLC) and 2 other proteins that were coprecipitated together with HLA antigens.

An observation of importance is that myosin light chain was coprecipitated with HLA proteins in PMA-stimulated platelets (Fig. 1) as well as in HL-60 cells (Fig. 3) and lymphocytes (Fig. 2). The specificity of this event is shown by the fact that other major platelet phosphoproteins and especially the heavily labeled pp47 (or other major leukocyte phosphoproteins) were not precipitated together with HLA proteins.

It is noteworthy that in HL-60 cells but not in the normal cellular systems two other proteins were coprecipitated with HLA. The identity of these proteins is currently being investigated. Another observation associated particularly with the leukemic HL-60 cells but not with the normal cellular systems (lymphocytes, platelets) is that significant amounts of phosphorylated MLC were coprecipitated together with HLA even in the non-PMA-stimulated cells. Furthermore, treatment of the leukemic cells with PMA had only minor effect on the amount of coprecipitated-phosphorylated MLC although the phosphorylation of the HLA proteins was dramatically increased under these conditions (Fig. 3). This is in agreement with our previous report (26) that PMA does not enhance the phosphorylation of MLC in certain malignant cells such as leukemic HL-60 cells

and A431 epidermoid carcinoma, while it does in normal cellular systems such as platelets, lymphocytes, fibroblast and epithelial cells. The significance of these observations should await further investigation.

HLA proteins have been shown to interact with the actin-myosin complex (21,27,30). It has been suggested that this interaction is regulated via the intracellular region of the HLA (27) which is phosphorylated (23,22). Thus, phosphorylation of HLA proteins by the tumor promoter, PMA may induce a change in their association with the cytoskeletal elements. Interference in the normal assembly of cytoskeletal proteins has been reported to be associated with the effect of tumor promoters (1,2) as well as with malignancy induced by oncogenes (36). In this regard it is pertinent that PMA-induced phosphorylation of MLC has been shown to be associated with inhibition (rather than activation which is induced by the physiological ligands) of actin-activated MgATPase, suggesting that PMA may induce an of interference in the normal interaction between actin and myosin (31). It will be of importance to examine possible relationships between PMA effects on HLA and PMA effect on MLC and whether these events are associated with the mechanism by which PMA induce tumor promotion.

A question of major importance is the identity of the enzyme which is involved in the phosphorylation of HLA by PMA. The putative receptor of PMA is a protein kinase at 80K which depends for its activation on Ca^{+2} and phospholipids (Ca-PL-PK) (5-8). Interestingly, an 80K protein was found in the actin-rich fraction which showed an enhanced interaction with HLA antigens (21). Exposure of cells to PMA results in disappearance of Ca-PL-PK from the cytosol (32) and its tight association with the plasma membrane (3). This position favors this enzyme as a possible candidate to be directly involved in phosphorylation of the intracellular COOH-terminus of the HLA proteins. also be involved (33). In this regard it is noteworthy that HLA proteins were recently shown to be phosphorylated <u>in vitro</u> by Rous sarcoma virus kinase ($pp60^{v-src}$) at a tyrosine residue (34).

Elucidation of the precise interaction between actin-myosin complex, HLA proteins and the protein kinases involved in their normal and abnormal interaction will promote our understanding of the nature of association of contractile components with the plasma membrane and might also provide insight into a biochemical mechanism involved in tumor promotion by PMA.

REFERENCES

1. Blumberg, P.M. (1980) CRC Crit. Rev. Toxicol. 8, 153-234.
2. Blumberg, P.M., Dunn, J.A., Jaken, S., Jeng, A.Y., Leach, K.L., Sharkey, N.A., and Yeh, E. (1984) in Mechanism of Tumor Promotion, vol. 3, (ed. Slaga, T.J.) (CRC Press, Boca Raton, in press).
3. Kraft, A.S. and Anderson, W.B. (1983) Nature 301, 621-623.
4. Yamanishi, J., Takai, Y., Kaibuchi, K., Sano, K., Castagna, M., and Nishizuka, Y. (1983) Biochim. Biophys. Res. Commun. 112, 778-786.
5. Niedel, J.E., Kuhn, L.J., and Vandenbark, G.R. (1983) Proc. Natl. Acad. Sci. USA 80, 36-40.
6. Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 11442-11445.
7. Sando, J.J. and Young, M.C. (1983) Proc. Natl. Acad. Sci. USA 80, 2642-2646.
8. Ashendel, C.L., Staller, J.M., and Boutwell, R.K. (1983) Cancer Res. 43, 4333-4337.
9. Imaoka, T., Lynham, J.A., and Haslam, R.J. (1983) J. Biol. Chem. 258, 11404-11414.
10. Feuerstein, N. and Cooper, H.L. (1984) Biochim. Biophys. Acta 781, 239-246.
11. Timmons, S. and Hawiger, J. (1978) Thrombosis Res. 12, 297-306.
12. Feuerstein, N. and Cooper, H.L. (1983) J. Biol. Chem. 258, 10786-10793.
13. Monos, D.S. and Cooper, H.L. (1983) J. Immunol. 131, 341-346.
14. Monos, D.S., Tekolf, W.A., Shaw, S., and Cooper, H.L. (1984) J. Immunol. 132, 1379-1385.
15. O'Farrel, P.H. (1975) J. Biol. Chem. 250, 4007-4021.
16. Feuerstein, N. and Cooper, H.L. (1984) J. Biol. Chem. 259, 2782-2790.
17. Feuerstein, N., Nishikawa, M., and Cooper, H.L. (submitted).
18. Feuerstein, N. and Cooper, H.L. (1984) Biochim. Biophys. Acta 781, 247-251.
19. Naka, M., Nishikawa, M., Adelstein, R.S., and Hidaka, H. (1983) Nature, 306, 4942, 490-492.
20. Mittal, K.K. (1979) J. Sci. Indus. Res. 38, 37-46.
21. Pober, J.S., Guild, B.C., Strominger, J.L., and Veatch, W.R. (1981) Biochemistry 20, 5633-5642.
22. Loube, S.R., Owen, M.J., and Crumpton, M.J. (1983) Biochem. J. 210, 79-87.
23. Pober, J.S., Guild, B.C., and Strominger, J.L. (1978) Proc. Natl. Acad. Sci. USA 75, 6002-6006.
24. Rathbard, J.B., Hopp, T.P., Edelman, G.M., and Cunningham, B.A. (1980) Proc. Natl. Acad. Sci. USA 77, 4239-4243.
25. Johnstone, A.P., DuBois, J.H., and Crumpton, M.J. (1981) Biochem. J. 194, 309-318.
26. Feuerstein, N., Sahai, A., Anderson, W.B., Salomon, D.S., and Cooper, H.L. Cancer Res. (in press).
27. Mescher, M.F., (1982) In Histocompatibility antigens: Structure and function (eds. Parham, P. and Strominger, J.L., Champman and Hall, London), 58-83.
28. Koch, G.L.E. and Smith, M.J. (1978) Nature 273, 274-278.
29. Ash, J.F., Louvard, D., and Singer, S.J. (1977) Proc. Natl. Acad. Sci. USA 74, 5584-5588.
30. Huet, C., Ash, J.F., and Singer, S.J. (1980) Cell 21, 429-438.
31. Nishikawa, M., Hidaka, H., and Adelstein, R.S. (1983) J. Biol. Chem. 258, 14069-14072.

32. Kraft, A.S., Anderson, W.B., Cooper, H.L., and Sando, J.J. (1982) J. Biol. Chem. 257, 13193-13196.
33. Moon, S.O., Palfrey, C.H., and King, C. (1984) Proc. Natl. Acad. Sci. USA 2298-2308.
34. Guild, B.C., Erikson, R.L., and Strominger, J.L. (1983) Proc. Natl. Acad. Sci. USA 80, 2894-2898.
35. Bishop, J.M. and Varmus, H. In: RNA Tumor Viruses. Ed: R. Weiss, N. Teich, H. Varmus, J. Coffin. (1982) CSH Lab, N.Y., pp 999-1108.
36. Maness, P.F. (1981) In: Cell and Muscle Motility. Vol. 1. Ed: R. Dowben and J. Shay Plenum Press, N.Y. pp 335-373.