Vol. 137, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 29, 1986 Pages 128-134

PHYTOHEMAGGLUTININ TREATMENT OF T LYMPHOCYTES STIMULATES RAPID INCREASES IN ACTIVITY OF BOTH PARTICULATE AND CYTOSOLIC PROTEIN KINASE C

Anthony R. Mire, R. Gitendra Wickremasinghe , and A. Victor Hoffbrand

Department of Haematology, Royal Free Hospital & School of Medicine, Pond Street, Hampstead, London, NW3 2QG, U.K.

Received March 6, 1986

SUMMARY: We have measured the activity of protein kinase C in particulate and cytosolic fractions prepared from lymphocytes following stimulation with phytohemagglutinin. Activity in the particulate fraction increased approximately three-fold within 5 min, and declined to nearly zero between 20 and 60 min. Cytosolic activity increased in a biphasic manner, with an initial increase at 5 min, a decline at 10 min, and a further increase by 20 min, which was sustained for at least 60 min. By contrast, 12-O-tetradecanoylphorbol-13-acetate caused a rapid translocation of protein kinase C from cytosol to the particulate fraction which was sustained for at least 1 h. The results suggest that agents, such as phytohemagglutinin, which both generate diacylglycerol and mobilize intracellular Ca^{2+} stores, result in changes in subcellular distribution and activity of protein kinase C which are different from those elicited by 12-O-tetradecanoylphorbol-13-acetate.

In a wide variety of biological systems the transduction of external signals is mediated via the receptor-stimulated breakdown of the rare membrane lipid phosphatidyl inositol 4,5 bisphosphate (PIP_2)[1] resulting in the generation of the second messengers, diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP_3) [1,2]. DAG is responsible for the activation of protein kinase C (PKC), which also requires Ca^{2+} ions and phospholipid for activity [1,2]. The phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) mimics the action of DAG, stimulating PKC activity [3] and promoting its association with the particulate fraction [4,5]. Activation of PKC by the growth factors interleukin-2 and interleukin-3 is associated with

Abbreviations: PIP_2, phosphatidylinositol 4,5 bisphosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5 trisphosphate; PKC, protein kinase C; PHA, phytohemagglutinin; TPA, 12-O-tetradecanoylphorbol-13-acetate.

0006-291X/86 $1.50

translocation of the enzyme from cytosol to the particulate fraction [6, 7]. IP_3 mediates the release of Ca^{2+} ions from intracellular stores, and cascades of biochemical events triggered by the resultant increase in cytoplasmic Ca^{2+} levels are thought to synergize with events initiated by PKC activation in producing the ultimate biological response [1,2].

This signal transduction pathway is activated in response to the binding of platelet-derived [8] and epidermal [9] growth factors to their respective receptors. PIP_2 breakdown is also an early response to the mitotic activation of T lymphocytes by phytohemagglutinin (PHA) [10]. PHA also stimulates rapid increases in cytoplasmic Ca^{2+} ions [11]. The ability of TPA and Ca^{2+} ionophores to synergize in producing a mitotic response [12] provides additional evidence that activation of protein kinase C, together with elevation of cytosolic Ca^{2+} levels, synergize in securing the commitment of T lymphocytes to mitosis. We therefore investigated the subcellular distribution of PKC in T lymphocytes following addition of either PHA or TPA. We found that PHA addition resulted in a rapid, transient association of PKC with the particulate fraction. Cytosolic PKC levels also increased, but in a biphasic manner. By contrast, TPA treatment of lymphocytes resulted in a sustained translocation of PKC from cytosol to the particulate fraction.

MATERIALS AND METHODS

Preparation, incubation and fractionation of lymphocytes

Human peripheral blood lymphocytes, essentially free of platelets and monocytes were prepared as described [13]. By immunological criteria, 92% of these cells were T lymphocytes. They were suspended at 5 x 10^6 cells.ml^{-1} in RPMI 1640 medium containing 5% fetal calf serum and antibiotics. The viability of cells used in these experiments was in excess of 98%, as judged by trypan blue exclusion. PHA or TPA were added at zero time and aliquots (50 x 10^6 cells) were taken at intervals, washed twice in Hanks' salts and lysed hypotonically as described [14]. Nuclei were removed by centrifugation at 700 xg. The post-nuclear supernatant was then centrifuged at 150,000 xg to obtain cytosolic and particulate fractions.

Partial purification of PKC by DE52 column chromotography

Triton X-100 (1%) was added to all samples prior to chromatography on 1 ml columns of DE52, equilibrated in 20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 2 mM EDTA, 10% glycerol, 5 mM 2-mercaptoethanol. Columns were rinsed with 5 ml of the above buffer, and PKC was eluted with 0.2 M NaCl dissolved in the same. PKC activity was recovered only in the 0.2 M NaCl eluate.

Enzyme assay

PKC was assayed by its ability to catalyze incorporation of radiolabel from [γ-^{32}P]ATP (Amersham International) into lysine-rich histone (Sigma) in the presence of phosphatidylserine. Activity in the absence of phosphatidylserine was substracted in each assay [15]. Results were expressed as pmol [^{32}P] incorporated into histone per 5 min per μg protein. Protein was estimated by a sensitive modification of the method of Lowry [16].

RESULTS

Figure 1A shows the DEAE cellulose elution profiles of particulate-bound PKC extracted from lymphocytes at various times following addition of PHA. This data, expressed as pmol $^{32}PO_4^{2-}$ incorporated per 5 min per μg protein is presented graphically in Fig. 2. A three-fold elevation of particulate-bound PKC was evident within

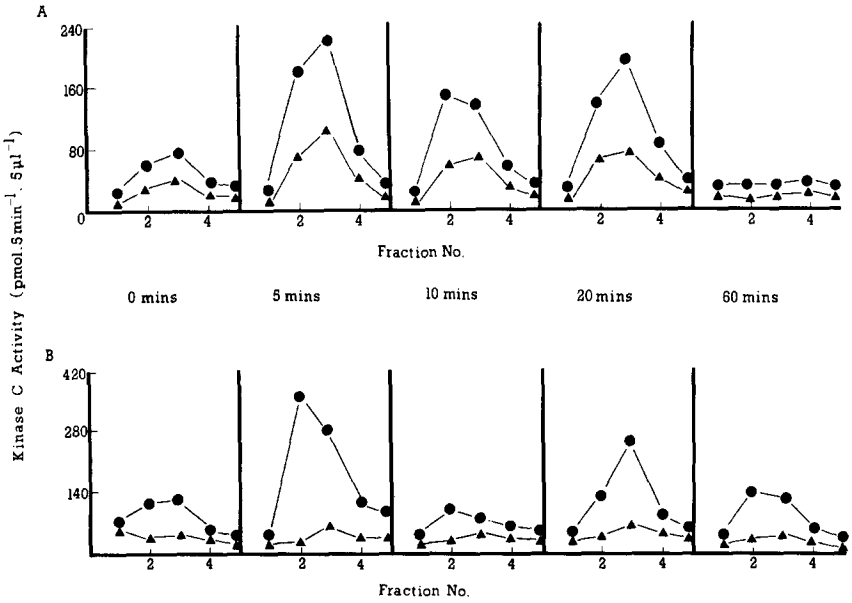

Fig. 1 Elution from DEAE cellulose of PKC activity from the particulate (A) and cytosolic (B) fractions of lymphocytes. Time following addition of PHA is indicated in each panel. Incorporation of $^{32}PO_4^{2-}$ into histone in the absence (▲) or presence (●) of phosphatidylserine is shown.

5 min following PHA addition. This level was sustained for 20 min, and then declined to nearly zero by 60 min. This increase was seen consistently in five separate experiments, with an average increase at 5 min of 244 (± 36)% of control levels.

Figure 1B shows the DEAE cellulose elution profiles of cytosolic PKC from PHA-treated lymphocytes. An initial rapid, three-fold increase in cytosolic PKC activity at 5 min was followed by a return to the control level by 10 min (shown graphically in Fig. 2). By 20 min, cytosolic PKC activity rose again, to about twice control level, and remained at this level for at least 60 min. This biphasic increase of PKC activity was observed in six experiments, with a mean initial rise in activity of 188 (± 23)%. In these experiments the total cellular PKC activity increased about four-fold 5 min following PHA addition.

For comparison, similar experiments were carried out using TPA. The data shown in Fig. 3 indicates that this reagent induced a rapid, sustained translocation of the bulk of the cytosolic PKC to the particulate fraction. Assay of a mixture of the cytosolic fraction

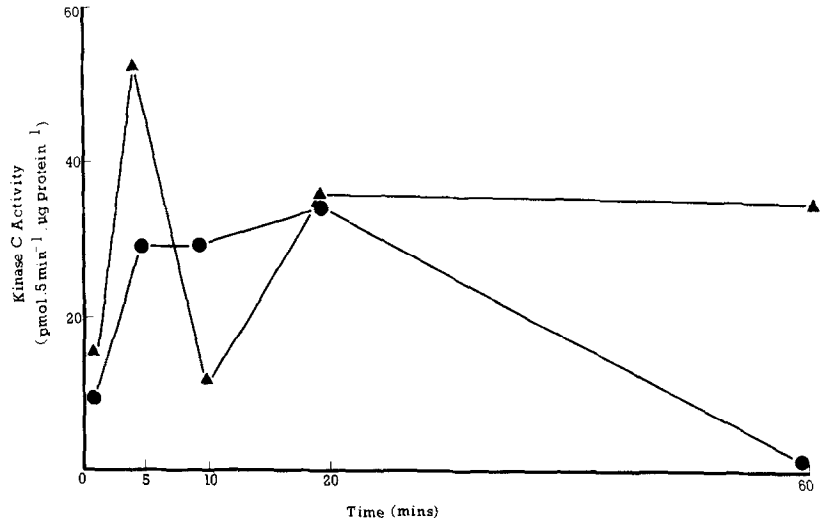

Fig. 2 Specific activity of PKC in particulate (●) and cytosolic (▲) fractions of lymphocytes following addition of PHA.

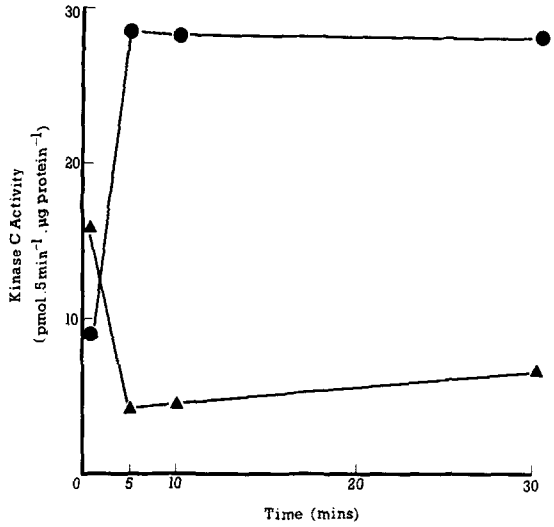

Fig. 3 Specific activity of PKC in particulate (●) and cytosolic (▲) fractions of lymphocytes following addition of TPA.

from lymphocytes treated for 5 min with TPA combined with the cytosol from untreated lymphocytes gave additive results, indicating that the reduced PKC level in the cytosol of TPA-treated lymphocytes was not due to the presence of inhibitors in these fractions. By contrast with PHA, TPA treatment of lymphocytes did not result in an increase in total PKC activity.

DISCUSSION

Certain mitogens stimulate the breakdown of PIP_2 in fibroblast plasma membranes with consequent activation of PKC [8,9]. PIP_2 breakdown also occurs as a consequence of addition of PHA to lymphocytes [10]. Furthermore, addition of mitogens to lymphocytes stimulates rapid phosphorylation of specific cellular proteins [17,18]. We therefore sought direct evidence for the involvement of PKC in the mitogenic response of lymphocytes to PHA. We observed a rapid two- to three-fold increase in particulate PKC, following PHA addition. Increase in particulate PKC activity, with a concomitant fall in cytosolic PKC was previously observed in response to binding of the growth factors, interleukin 2 [6] and interleukin 3 [7] to

cells carrying receptors for these growth factors, suggesting a growth factor-stimulated translocation of the enzyme. By contrast, we have observed in the present study that the cytosolic PKC activity also increased in response to PHA, in a biphasic manner, such that total cellular PKC increased following stimulation. Therefore we cannot state unequivocally that the increase in membrane PKC was due to translocation of cytosolic enzyme, although this is the most likely explanation, in view of current models for the activation of PKC. These suggest that a synergy between DAG generation and increase in intracellular Ca^{2+} promote association of PKC with membranes [19,20]. The rapid increase in cytosolic PKC is not due to the proteolytic generation of kinase M from kinase C, since enzyme activity was measured by its dependence on phospholipid, and kinase M is phospholipid-independent [1]. However, proteolytic activation of an inactive (or less active) precursor of PKC remains a possible explanation. It is of interest that treatment of Xenopus oocytes with insulin resulted in a similar rapid increase in S6 protein kinase, which was independent of protein synthesis [21].

By contrast, TPA treatment of lymphocytes induced a rapid, sustained translocation of PKC from cytosolic to particulate fractions. This response was similar to that observed following treatment of parietal yolk-sac cells with TPA [4]. TPA activates PKC directly by substituting for DAG [1,2] without a concomitant increse in cytosolic Ca^{2+} [11]. We suggest that the biphasic increase in cytosolic PKC elicited by PHA (but not by TPA) is a result of the generation of DAG concomitant with the elevation of the cytosolic Ca^{2+}.

ACKNOWLEDGEMENTS

This work was supported by the Cancer Research Campaign of Great Britain, who were also responsible for the personal support of R.G.W. We thank Mrs. Jackie Wilkinson for the preparation of the manuscript.

REFERENCES

1. Nishizuka, Y. (1984) Nature 308, 693-698.
2. Berridge, M.J. (1984) Biochem. J. 220, 345-360.
3. Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847-7851.
4. Kraft, A.S. and Anderson, W.B. (1983) Nature 301, 621-623.
5. Wickremasinghe, R.G., Piga, A., Campana D., Yaxley, J.C. and Hoffbrand, A.V. (1985) FEBS Lett. 190, 50-54.
6. Farrar, W.L. and Anderson, W.B. (1985) Nature 315, 233-235.
7. Farrar, W.L., Thomas, T.P. and Anderson, W.B. (1985) Nature 315, 235-237.
8. Habenicht, A.J.R., Glomset, J.A., King, W.C., Nist, C., Mitchell, C.D. and Ross, R. (1981) J. Biol. Chem. 256, 12329-12335.
9. Sawyer, S.T. and Cohen, S. (1981) Biochemistry 20, 6280-6286.
10. Fisher, D.B. and Mueller, G.C. (1968) Proc. Natl. Acad. Sci, USA. 60, 1396-1402.
11. Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) Nature 295, 68-77.
12. Truneh, A., Albert, F., Golstein, P. and Schmitt-Verhulst, A.-M. (1985) Nature 313, 318-320.
13. Mire, A.R., Wickremasinghe, R.G., Michalevicz, R. and Hoffbrand, A.V. (1985) Biochim. Biophys. Acta 847, 159-163.
14. Kuo, J.F., Anderson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, I., Brackett, N.L., Katoh, N., Shoji, M. & Wrenn, R.N. (1980) Proc. Natl. Acad. Sci. USA. 77, 7039-7043.
15. Parker, P., Stabel, S. and Waterfield, M.D. (1984) EMBO J. 3, 953-959.
16. Schleif, R.F. and Wensink, P.C. (1981) Practical Methods in Molecular Biology (Springer Verlag, New York, USA.) 75-76.
17. Samelson, L.E., Harford, J., Schwartz, R.H. and Klausner, R.D. (1985) Proc. Natl. Acad. Sci. USA. 82, 1969-1973.
18. Mire, A.R., Wickremasinghe, R.G. and Hoffbrand, A.V. (1986). Biochim. Biophys. Acta. (submitted).
19. Wolf, M., Le Vine III, H., May, W.S. jr., Cuatrecasas, P. and Sahyoun, N. (1985) Nature 317, 546-549.
20. May, W.S. jr., Sahyoun, N., Wolf, M. and Cuatrecasas, P. (1985) Nature 317, 549-551.
21. Stefanovic, D., Erikson, E., Pike, L.J. and Maller, J.L. (1986) EMBO J. 5, 157-160.
22. Gelfand, E.W., Cheung, R.K., Mills, G.B. and Grinstein, S. (1985) Nature 317, 419-420.
23. Hasegawa-Sasaki, H. and Sasaki, T. (1983) Biochim. Biophys. Acta 754, 305-314.