Rapid down-regulation of protein kinase C and membrane association in phorbol ester-treated leukemia cells

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Peripheral blood lymphocytes from patients with chronic lymphocytic leukemia (CLL) acquire after several days of exposure to 12-*O*-tetradecanoylphobol-13-acetate (TPA) several morphological, immunological and histochemical features of hairy cell leukemia. We have investigated the short term effects of TPA treatment on protein kinase C and its subcellular distribution. Within minutes of addition of TPA to CLL cells 20% of the cytosolic protein kinase C had associated with the particulate fraction. The remaining 80% of protein kinase C activity was down-regulated. The association with the membrane dramatically increased the resistance of the enzyme to inhibition by the non-ionic detergent, Triton X-100. These results suggest that activation of protein kinase C causes multiple biological changes in CLL cells.

Chronic lymphocytic leukemia *Protein kinase C* *Tumor promoter* *Differentiation*

1. INTRODUCTION

The tumor-promoting phorbol esters cause a variety of biological effects in different systems [1–4]. Treatment with TPA stimulates conversion of cells from patients with CLL to plasmacytoid cells showing many of the characteristics of hairy cell leukemia [5,6]. The biological effects of TPA are mediated via protein kinase C, a phospholipid- and calcium-dependent enzyme [7]. This enzyme is activated by diacylglycerol produced by receptor-stimulated breakdown of membrane inositol lipids. Phorbol esters bypass this step by substituting for diacylglycerol [7,8]. We show here that TPA treatment of cells from CLL patients results in the rapid disappearance of the bulk of the cytosolic kinase C activity. The remainder (approx. 20%) associates transiently with the particulate fraction. This association dramatically increases the resistance of the enzyme to inhibition by the non-ionic detergent, Triton X-100.

Abbreviations: CLL, chronic lymphocytic leukemia; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

2. MATERIALS AND METHODS

Peripheral blood lymphocytes from patients with CLL were isolated by centrifugation on Lymphoprep gradients (Nyegaard, Norway) and cultured in RPMI 1640 medium containing 10% fetal calf serum. Cytosolic and $100\,000 \times g$ particulate fractions were prepared as described [9]. Protein kinase C assays were carried out according to Parker et al. [10]. Kinase C was quantitated by its ability to transfer radiolabel from [γ-^{32}P]ATP to histone in the presence but not in the absence of phosphatidylserine.

3. RESULTS AND DISCUSSION

In each experiment described here, TPA-induced conversion of CLL cells was routinely monitored by morphological and immunological criteria

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described previously [5,6]. After 3 days of treatment with 16 nM TPA, CLL cells had lost their characteristic lymphocytoid appearance. Over 90% of the cells exhibited protrusions and membrane ruffles. Twenty to 50% of the cells showed one or two protrusions which were 5–8-times the length of the cell. After 24 h treatment with TPA, >80% of the cells had lost surface immunoglobulin, whereas cytoplasmic immunoglobulin (of the same light chain class present on untreated cells) was clearly detected. None of these changes were seen in cultures to which TPA was not added. In addition, B lymphocytes from non-leukemic subjects did not undergo these changes when treated with TPA.

Cytosolic protein kinase C disappeared rapidly when CLL cells were treated with 16 nM TPA (fig.1a). The specific activity of this enzyme was 16300 units · mg^{-1} protein in untreated cells and fell to 60 units · mg^{-1} within 10 min of TPA addition (1 unit is the incorporation of 1 pmol $^{32}PO_4$ into histone per 5 min). To determine the fate of the enzyme, we assayed particulate fractions prepared from the same cell samples. No activity was detected at any time using the standard assay. However, the inclusion of 0.25% Triton X-100 in the reaction enabled the detection of kinase C activity in particulate fractions from TPA-treated cells [11]. Fig.1b shows a rapid rise in particulate-bound kinase C, peaking at 30 min, then declining slowly. 24 h after TPA addition, no activity remained in this fraction (not shown). The increase in specific activity in the particulate fraction was rapid and dramatic, rising from 200 units · mg^{-1} in untreated cells to 12300 units · mg^{-1} 30 min after TPA addition. Similar results were obtained using cells from three different CLL patients. We also observed that less than 5% of cellular kinase C activity was recovered in the nuclear fraction, both before and after TPA treatment (not shown).

However, when absolute amounts of protein kinase C activity (expressed as nmol · min^{-1} · 10^8 cell equiv.$^{-1}$) as opposed to specific activity (expressed as units · mg $protein^{-1}$) in the particulate and cytosolic fractions were quantitated, only 20% of the kinase C activity lost from the cytosol was recovered in the particulate fraction (cf. fig.1a and b). Since we observed that the cytosolic form of the enzyme was totally inhibited in the presence of 0.25% Triton X-100 (table 2, expt 1), we wished to investigate whether this detergent exerted a dual effect on the particulate enzyme, increasing its access to substrates but at the same time inhibiting in the assay by a separate mechanism. Therefore, Triton X-100 treated particulate kinase C was separated from the detergent and solubilized membrane lipid by chromatography on a column of DEAE cellulose. Fig.2 shows that the enzyme was bound by

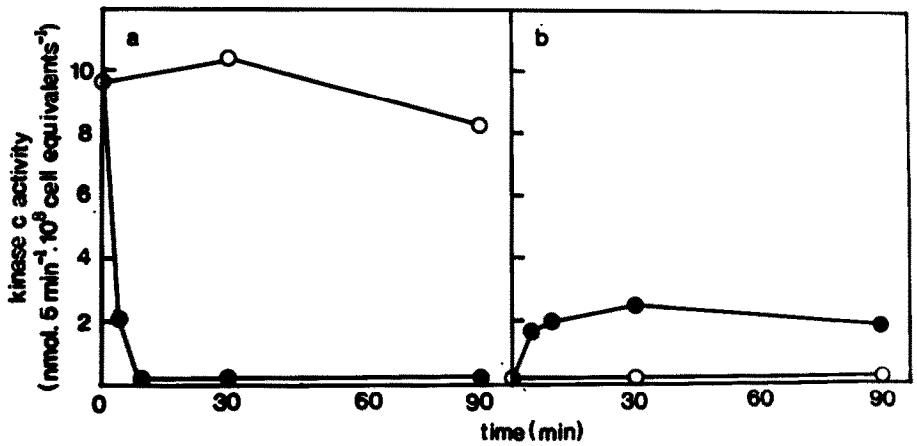

Fig.1. Changes in cytosolic and particulate forms of protein kinase C following TPA treatment of CLL cells. Kinase C activity was assayed in cytosolic (panel a) and particulate (panel b) fractions prepared from the cells of the indicated intervals following addition of TPA to cultures: Triton X-100 (0.25%) was inclused in assays of particulate fractions. ●, 16 nM TPA added; ○, control.

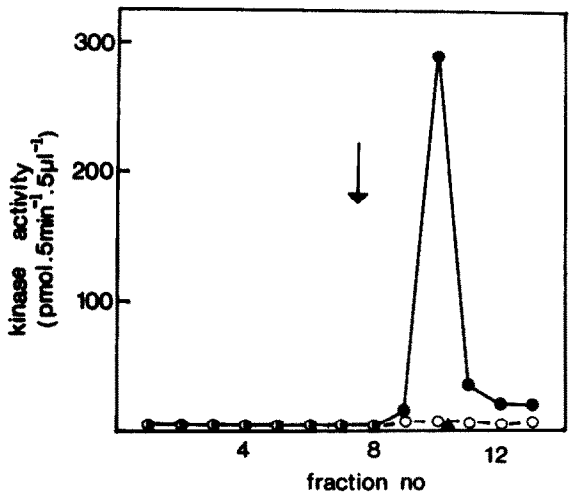

Fig.2. DEAE cellulose chromatography of protein kinase C from the particulate fraction of TPA-treated CLL cells. A particulate fraction was incubated for 1 h at 4°C in the presence of 1% Triton. The enzyme was adsorbed to a column of DEAE cellulose and eluted with buffer containing 0.1 M NaCl [10,11]. The arrow indicates commencement of this elution. Aliquots (5 µl) were assayed for kinase C activity in the presence (●) or absence (○) of phosphatidylserine (▲), assay carried out in the presence of 0.25% Triton X-100.

the column and was eluted by 0.1 M NaCl. The column load contained 10634 units of activity (assayed in the presence of 0.25% Triton X-100) whereas 14300 units were recovered in the eluate (134% recovery). Since there was no dramatic increase in enzyme activity after the column step and since cytosolic kinase C inhibition by Triton X-100 is fully reversible by DEAE cellulose chromatography (table 1, expt 1), we conclude that assay of particulate fractions in the presence of Triton X-100 gives a true value for this form of the enzyme. Fig.2 also confirms the identity of the par-

Table 1

Properties of cytosolic and particulate forms of protein kinase C

Enzyme fraction	Protein kinase C activity (pmol · 5 min^{-1})	
	−Triton X-100	+0.25% Triton X-100
Experiment 1		
(a) (0.25 ml cytosol from untreated cells	4100	112
(b) as (a) above, after incubation with 0.25% Triton X-100 and subsequent chromatography on DEAE cellulose	5200	0
Experiment 2		
(a) 2.5 µl cytosol from control cells	31	ND
(b) 2.5 µl cytosol from TPA-treated cells	4	ND
(c) (a) plus (b)	39	ND
Experiment 3		
(a) 2.5 µl particulate fraction from control cells	ND	1.2
(b) 2.5 µl particulate fraction from TPA-treated cells	ND	49
(c) (a) plus (b)	ND	48
Experiment 4		
(a) 2.5 µl cytosol from control cells	46	1.5
(b) 2.5 µl particulate fraction from TPA-treated cells	2	50
(c) (a) plus (b)	51	42

ticulate form of the enzyme as protein kinase C since the elution behaviour and the total dependence of activity on phosphatidylserine are characteristics of authentic kinase C [10,11].

We then eliminated the possibility that the TPA-induced effects were due to changes in the concentration of inhibitors or to changes in their subcellular distribution. Mixing of either active and inactive cytosolic fractions or active and inactive particulate fractions gave additive yields in the assay (table 1, expts 2 and 3). Furthermore, DEAE cellulose chromatography of a cytosolic preparation from TPA-treated cells failed to reveal any cryptic kinase C activity, also suggesting that no inhibitors were present in this preparation (not shown).

The rapid appearance of kinase C activity in the particulate fraction of TPA-treated cells might be explained if the drug bound to the particulate fraction and activated kinase C molecules already present (but undetectable) in this fraction. However, addition of TPA to 16 nM or to 160 nM to the in vitro assay did not activate kinase C activity in either particulate or cytosolic fractions from control or TPA-treated cells (not shown). This was not surprising, since TPA activates kinase C by reducing its calcium requirement so that it can function at the low intracellular calcium levels (about 10^{-7} M) existing in normal cells [7,8]. At the levels of Ca^{2+} used in our assay (1.5 mM) no further activation of kinase C by TPA would thus have been expected.

We then investigated the pharmacological specificity of the effect of phorbol esters. Phorbol-13-monoacetate, which unlike TPA, lacks a diacylglycerol-like moiety, failed to induce the long-term biological responses produced by TPA. No changes in the subcellular distribution or activity of protein kinase C occurred, confirming the absolute dependence of the TPA effect on its diacylglycerol structure.

The results described here show that treatment of CLL cells with TPA leads to a rapid and extensive decrease of protein kinase C activity. Almost all of the kinase activity in untreated CLL cells was recovered in the cytosolic fraction. Upon treatment with TPA a small but reproducible proportion (approx. 20%) of this activity was translocated to the $100\,000 \times g$ particulate fraction, whereas the remainder was down-regulated. Our results differ significantly from those reported earlier by Kraft and Anderson [12], since in their study treatment of parietal yolk sac cells led to almost complete translocation of kinase C from the cytosol to the particulate fraction with no evidence of down-regulation. It must be pointed out that in their study Kraft and Anderson did not report any biological consequence of TPA treatment on the target cells. In this work conversion of CLL cells to cells with many of the phenotypic characteristics of hairy cells was clearly demonstrated. Rapid down-regulation of proteins involved in signal transduction following receipt of the signal is a well documented phenomenon. For example, the receptors for several growth factors are rapidly internalized and degraded following the binding of the appropriate factors [13–15]. This is consistent with a role for protein kinase C in transducing the TPA signal in the present system.

Protein kinase C activity of 3T3 mouse fibroblasts was also shown to be down-regulated in response to treatment of the intact cells with phorbol ester [16]. However, down-regulation in this case was slow, with a half-life of 6–7 h, whereas the effects reported in the present work were complete within 10 min. Furthermore, no redistribution of enzyme between cellular compartments could be demonstrated in the 3T3 system, since detergent lysates of whole cells were used for assay. The marked difference in the kinetics of phorbol ester-induced kinase C down-regulation in 3T3 cells and in human CLL cells may be related to the differences in biological responses elicited in the two systems; phorbol esters are co-mitogenic with growth factors in the 3T3 system [17], whereas they induce differentiation-like changes in CLL cells.

Finally, we observed a striking difference in properties between the cytosolic and particulate forms of the enzyme. The cytosolic form of kinase C was totally inhibited by the presence in the assay of 0.25% Triton X-100. This was not due to an irreversible action of detergent on the enzyme, since DEAE cellulose chromatography of Triton-treated cytosolic kinase C led to complete recovery of the enzyme (table 2, expt 1). In contrast, the particulate form of the enzyme was dramatically activated by 0.25% Triton X-100, probably due to the solubilization of membranes. The resistance of the particulate enzyme cannot be explained simply

by the presence in the reaction mixture of solubilized components from the particulate fraction, since cytosolic kinase C mixed with a particulate fraction and then assayed in the presence of Triton X-100 was also inhibited (table 1, expt 4). It is thus likely that the detergent resistance of particulate kinase C was due to a specific association with a component of the particulate fraction. This component can be separated from particulate kinase C by ion-exchange chromatography since the resultant enzyme fraction was completely inhibited by Triton X-100 (fig.2). We will attempt in future studies to identify the stabilizing component with the expectation that this will help to elucidate the mechanism by which protein kinase C mediates the phorbol ester signal for differentiation of CLL cells to cells resembling hairy cells. An understanding of this mechanism may have therapeutic consequences, since hairy cell leukemia appears to be amenable to treatment with interferon [18], whereas CLL typically is not responsive to interferon [19].

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REFERENCES

[1] Lotem, J. and Sachs, L. (1979) Proc. Natl. Acad. Sci. USA 76, 5158–5163.
[2] Dicker, P. and Rozengurt, E. (1981) Biochem. Biophys. Res. Commun. 100, 433–441.
[3] Rozengurt, E., Rodriguez-Pena, M. and Smith, K.A. (1983) Proc. Natl. Acad. Sci. USA 80, 7244–7248.
[4] Truneh, A., Albert, F., Golstein, P. and Schmitt-Verhulst, A.-M. (1985) Nature 313, 318–320.
[5] Totterman, T.H., Nilsson, K. and Sunstrom, C. (1980) Nature 288, 176–178.
[6] Caligaris-Cappio, F., Janossy, G., Campana, D., Chilosi, M., Bergui, L., Foa, R., Delia, D., Giubellino, M.C., Biagini, G. and Gobbi, M. (1984) Leuk. Res. 8, 567–578.
[7] Nishizuka, Y. (1984) Nature 308, 693–698.
[8] Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851.
[9] Kuo, J.F., Anderson, R.G.G., Wise, B.C., Mackerlova, L., Salomonsson, I., Brackett, N.L., Katoh, N., Shoji, M. and Wrenn, R.N. (1980) Proc. Natl. Acad. Sci. USA 77, 7039–7043.
[10] Parker, P.J., Stabel, S. and Waterfield, M.D. (1984) EMBO J. 3, 953–959.
[11] Kikkawa, Y., Takai, Y., Minakuchi, R., Inohara, S. and Nishizuka, Y. (1982) J. Biol. Chem. 257, 13341–13348.
[12] Kraft, A.S. and Anderson, W.B. (1983) Nature 301, 621–623.
[13] Stoschek, C.M. and Carpenter, G.J. (1984) J. Cell Biol. 98, 1048–1053.
[14] Beguinot, L., Lyall, R.M., Willingham, M.C. and Pastan, I. (1984) Proc. Natl. Acad. Sci. USA 81, 2384–2388.
[15] Heldin, C.H., Westermark, B. and Wasteson, A. (1981) Proc. Natl. Acad. Sci. USA 78, 3664–3668.
[16] Rodriguez-Pena, A. and Rozengurt, E. (1984) Biochem. Biophys. Res. Commun. 120, 1053–1059.
[17] Dicker, P. and Rozengurt, E. (1978) Nature 276, 723–726.
[18] Quseada, J.R., Reuben, J., Manning, J.T., Hersh, E.M. and Gutterman, J.U. (1984) New Eng. J. Med. 310, 15–18.
[19] Foon, K.A., Bottino, G.C., Abrams, P.G., Fer, M.F., Longo, D.L. Schoenberger, C.S. and Oldham, R.K. (1984) Blood 64, suppl., p. 164a, abstr. no. 566.