Translocation of phospholipid/ Ca^{2+} -dependent protein kinase in B-lymphocytes activated by phorbol ester or cross-linking of membrane immunoglobulin

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Stimulation of peripheral-blood B-lymphocytes with phorbol ester or anti-immunoglobulin demonstrated intracellular translocation of phospholipid/ Ca^{2+} -dependent protein kinase (C-kinase) activity from cytosol to membrane fractions. This phenomenon, which was dose- and time-dependent, was found in both normal and chronic-lymphocytic-leukemia B-cells. This suggests that C-kinase-dependent protein phosphorylation may be related to membrane receptor occupation and may therefore be important in B-lymphocyte responses.

INTRODUCTION

Phospholipid/ Ca^{2+} -dependent protein kinase (C-kinase) has been shown to be involved in stimulus–response coupling in several cells (Nishizuka, 1984), including pancreatic endocrine and exocrine cells (De Pont & Fleuren-Jakobs, 1984; Zwalich *et al.*, 1983), platelets (Kaibuchi *et al.*, 1983; Yamanishi *et al.*, 1983) and neutrophils (Helfman *et al.*, 1983; McPhail *et al.*, 1984). This enzyme is also present in large amounts in lymphoid tissue (Kikkawa *et al.*, 1982; Kuo *et al.*, 1980; Minakuchi *et al.*, 1981). In B-cells, C-kinase might be activated upon intracellular Ca^{2+} release (Braun *et al.*, 1979) and diacylglycerol generation (Coggeshall & Cambier, 1984), which are known to be stimulated by membrane immunoglobulin (Ig) interaction with ligand such as cross-linking antibody. We were therefore interested in the possible effects upon phospholipid/ Ca^{2+} -dependent phosphorylation of cross-linking with anti-Ig.

The direct demonstration of C-kinase activation is technically difficult in whole cells, but it appears that translocation of C-kinase activity from the cytosol to the surface membrane may provide one indirect index (Ashendel *et al.*, 1983; Kraft *et al.*, 1982; Kraft & Anderson, 1983; Sando & Young, 1983; Wooten & Wrenn, 1984, 1985). Therefore protein-phosphorylation assays were performed in normal and CLL B-cells by means of both exogenous and endogenous substrate assays. PMA was also used for the purposes of comparison, since this agent is a known activator both of human B-lymphocytes (Totterman *et al.*, 1980; Ashman, 1984; Cossman *et al.*, 1984; Gordon *et al.*, 1984; Nel *et al.*, 1985) and of C-kinase activity (Castagna *et al.*, 1982; Niedel *et al.*, 1983; Nishizuka, 1984). The results indicated that C-kinase translocation was induced by both stimulating agents, although the effect was less marked with anti-Ig than with PMA.

MATERIALS AND METHODS

Preparation, activation and extraction of cells

B-cell-enriched peripheral blood lymphocytes were obtained from patients with CLL and healthy donors undergoing leukaphoresis (Petrini *et al.*, 1984). From the latter, only non-activated small lymphocytes ($< 170 \text{ mm}^3$) separated by elutriation (Stevenson *et al.*, 1983) were used. Portions (2×10^8 CLL or 5×10^7 normal B-cells) were treated with various concentrations of PMA or anti-Ig, or medium only, for 20 min at 37°C . The anti-Ig reagent was a goat IgG fraction which recognized F(ab')_2 of all classes of human Ig (Cappel, a division of Cooper Biologicals, Malvern, PA, U.S.A.). In order to exclude Fc-receptor-mediated effects, results were confirmed with F(ab')_2 fragments of this antiserum. After washing, each cell pellet was then treated with ice-cold extraction buffer [20 mM-Tris/HCl (pH 7.5)/ 2 mM-EDTA/50 mM-mercaptoethanol/aprotinin ($100 \mu\text{g ml}^{-1}$)]. After sonication, homogenates were spun at $100\,000 \text{ g}$ for 1 h at 4°C and the supernatant fractions, designated ‘cytosol’, were desalted on Sephadex G-25 columns at 4°C (Wrenn & Wooten, 1984). The corresponding pellets were divided into two portions. The first was treated with extraction buffer containing 0.1% Triton X-100/10 mM-EGTA for 1 h at 4°C , and re-centrifuged at $100\,000 \text{ g}$ for 1 h at 4°C to obtain Triton-soluble material, which included membrane-bound protein (Weber & Osborn, 1979). These fractions were also desalted as described above. The second, designated ‘crude pellet’, was resuspended in extraction buffer by sonication and used for endogenous-phosphorylation assays.

Phosphorylation assays

Qualitative assessment of C-kinase activity involved examination of endogenous substrates for phospho-

lipid/ Ca^{2+} -dependent phosphorylation (Nel *et al.*, 1985; Wrenn & Wooten, 1984; Wooten & Wrenn, 1984, 1985). The reaction mixture (total volume $200\ \mu\text{l}$) contained $25\ \text{mM}$ -Tris/HCl, pH 7.5, $10\ \text{mM}$ - MgCl_2 , $25\ \mu\text{M}$ -EGTA, 80 – $100\ \mu\text{g}$ of sample protein and $20\ \mu\text{M}$ - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ ($\sim 2 \times 10^7$ c.p.m.) in the presence or absence of PtdSer ($10\ \mu\text{g}$) and various concentrations of Ca^{2+} as indicated. After 3 min incubation at $30\ ^\circ\text{C}$, reactions were terminated by the addition of stop solution [$30\ \text{mM}$ -Tris/HCl (pH 7.8)/ 9% (w/v) SDS/ 15% (v/v) glycerol/ 0.05% Bromophenol Blue], and samples were boiled for 3 min. SDS/polyacrylamide-gel electrophoresis [12% (w/v)-polyacrylamide gels] was performed after addition of mercaptoethanol (Laemmli, 1970); gels were stained with Coomassie Blue, destained, dried under vacuum, and autoradiography was performed with Kodak XAR film and intensifying screens.

Quantification of C-kinase activity was performed by means of a defined exogenous substrate as previously described (Nel *et al.*, 1985; Wrenn & Wooten, 1984; Wooten & Wrenn, 1984, 1985) and expressed as pmol of ^{32}P incorporated $\cdot\text{min}^{-1}\cdot\mu\text{g}$ of protein $^{-1}$. The assay system contained $20\ \text{mM}$ -Pipes, pH 6.5, $10\ \text{mM}$ - MgCl_2 , $250\ \mu\text{M}$ -EGTA, 20 – $30\ \mu\text{g}$ of cytosol or Triton-soluble material protein and $40\ \mu\text{g}$ of histone Type III-S in a final volume of $200\ \mu\text{l}$, with the addition of $10\ \mu\text{g}$ of PtdSer and various concentrations of CaCl_2 . Controls consisted of parallel tubes lacking Ca^{2+} , PtdSer or both. Reactions were initiated by incubation with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ at $30\ ^\circ\text{C}$ for 5 min, stopped with 6% (w/v) trichloroacetic acid, followed by washing and counting of radioactivity.

RESULTS

Quantitative assays of phosphorylation

Initial experiments with desalted cytosol obtained from unstimulated B-cells from a healthy control showed low background levels of kinase activity in the absence of Ca^{2+} or PtdSer [$2.66 (\pm 0.21)$ pmol $\cdot\text{min}^{-1}\cdot\mu\text{g}$ of protein $^{-1}$; mean $\pm$ S.D.], with small increases after addition of PtdSer (3.98 ± 0.01) and Ca^{2+} (4.76 ± 0.36) individually. However, upon addition of both, incorporation of ^{32}P increased progressively, with maximal phosphorylation being attained at $10\ \mu\text{g}$ of PtdSer and $50\ \mu\text{M}$ - Ca^{2+} (43.43 ± 0.67). A similar pattern of phospholipid/ Ca^{2+} -dependent phosphorylation was observed in all subjects tested (four normal subjects, two CLL patients), with C-kinase activity being in the range of 15.6 – 60.86 pmol $\cdot\text{min}^{-1}\cdot\mu\text{g}$ of protein $^{-1}$. Examination of these cytosol fractions after treatment with increasing amounts of PMA (1.62 – $162\ \text{nM}$) showed a dramatic and dose-dependent reduction in C-kinase activity to values less than 15% of pretreatment levels (Fig. 1). Parallel experiments with increasing amounts of anti-Ig showed a similar, but smaller, decrease in cytosol activity to 67% of control values (Fig. 1). The disappearance of C-kinase activity induced by maximal doses of PMA ($162\ \text{nM}$) and anti-Ig ($100\ \mu\text{g}\cdot\text{ml}^{-1}$) was also time-dependent (Fig. 2). The decrease was quantitatively smaller with anti-Ig, and was also less sustained in that C-kinase levels showed a return almost to pretreatment levels within 1 h (Fig. 2). These findings were consistent in all subjects tested ($n = 6$), and the decrease in cytosol levels was statistically significant ($P < 0.05$ by Wilcoxon's rank sum sign test).

To determine if the observed decrease in cytosol

Fig. 1. Dose-response of C-kinase disappearance from the cytosol

Cells were allowed to react with various doses of PMA (1 – $100\ \text{ng}\cdot\text{ml}^{-1}$ or 1.62 – $162\ \text{nM}$) or various dilutions ($10^4 = 0.1\ \mu\text{g}\cdot\text{ml}^{-1}$ and $10^1 = 100\ \mu\text{g}\cdot\text{ml}^{-1}$) of anti-Ig for 30 min and C-kinase activity was determined as described for Table 1. The Figure shows a representative experiment, performed in normal B-cells in triplicate (mean $\pm$ S.D.).

Fig. 2. Time course of C-kinase disappearance from the cytosol

Cells were allowed to react with maximally stimulating doses of PMA ($162\ \text{nM}$) or anti-Ig ($100\ \mu\text{g}\cdot\text{ml}^{-1}$) and C-kinase activity was determined as for Table 1 after stimulation of cells for increasing time periods (5–60 min). The Figure shows a representative experiment performed in triplicate; the S.D. values were in every case less than 10% of the mean.

Table 1. Translocation of C-kinase activity from cytosol to membrane

Results are from representative experiments with two normal subjects and one CLL patient (mean $\pm$ s.d., $n = 3$). Cells were treated with anti-Ig at 1 (subjects 1 and 2) or 10 (subject 3) $\mu\text{g} \cdot \text{ml}^{-1}$, or PMA (16.2 nM). Cytosol and membrane (Triton-soluble) fractions were obtained, and C-kinase activity was measured ($\text{pmol} \cdot \text{min}^{-1} \cdot \mu\text{g}^{-1}$) by exogenous-substrate phosphorylation assays as described in the text, and calculated as: (samples containing Ca^{2+} and PtdSer) – (samples containing Ca^{2+} only). The significance of the results was the same if activity in the presence of PtdSer only was subtracted rather than that in the presence of Ca^{2+} only.

Treatment . . .	Subject 1 (Normal)			Subject 2 (Normal)			Subject 3 (CLL)	
	Control	Anti-Ig	PMA	Control	Anti-Ig	PMA	Control	Anti-Ig
Cytosol	15.60 $\pm$ 0.08	17.25 $\pm$ 0.12	2.16 $\pm$ 0.14	46.34 $\pm$ 3.80	46.80 $\pm$ 2.51	1.48 $\pm$ 0.18	18.92 $\pm$ 0.37	15.54 $\pm$ 0.58
Membrane	0.76 $\pm$ 0.07	2.22 $\pm$ 0.21	18.70 $\pm$ 0.47	2.22 $\pm$ 0.04	8.74 $\pm$ 0.14	60.86 $\pm$ 5.66	0.15 $\pm$ 0.16	1.48 $\pm$ 0.36
Cytosol/mem- brane ratio	20.52 $\pm$ 2.84	7.75 $\pm$ 1.12	0.12 $\pm$ 0.02	20.87 $\pm$ 2.42	5.35 $\pm$ 0.41	0.024 $\pm$ 0.004	126.10 $\pm$ 3.40	10.50 $\pm$ 0.55

activity was due to translocation of enzyme activity to the membrane, Triton-soluble material was examined in parallel. Initial experiments verified that the use of this detergent at 0.1% still allowed clear detection of C-kinase activity. Comparison of C-kinase activity in cytosol and Triton-soluble material yielded a ratio that, under resting native conditions, was more than 20 (cytosol/membrane) in both normal B- and CLL cells (Table 1). However, stimulation of cells with either anti-Ig (1 or 10 $\mu\text{g} \cdot \text{ml}^{-1}$) or PMA (162 nM) resulted in detectable alteration of the ratio; this fell after PMA stimulation to less than 1.0, and after anti-Ig treatment to between 5 and 11. (Table 1). At these concentrations of anti-Ig, the altered ratio was found to be related more to increased C-kinase activity associated with the Triton-soluble-material than decreased cytosol activity, whereas with a larger dose (100 $\mu\text{g} \cdot \text{ml}^{-1}$) both cytosol- and membrane-associated activity were altered. Decreases in the cytosol/membrane ratio of C-kinase activity were found in cells from all subjects tested ($n = 6$), although, as noted above, the absolute levels of enzyme activity were variable.

Qualitative assays of phosphorylation

Further conformation of possible translocation of C-kinase activity was sought by examination of the distribution and phosphorylation of endogenous C-kinase protein substrates in cytosol and crude pellet membrane fractions. In untreated normal and CLL B-cells, several cytosol proteins were phosphorylated in phospholipid/ Ca^{2+} -dependent fashion (Fig. 3). The major substrates had apparent molecular masses of approx. 94, 66, 60, 56, 50, 43, 38, 35, 28–30, 20–23 and 15–18 kDa, together with a series of ill-defined proteins of <100 kDa (Fig. 3). No similar PtdSer/ Ca^{2+} -dependent labelling was seen in Triton-soluble material corresponding to such samples (Fig. 4). In contrast, PMA-stimulated cytosol fractions no longer contained any PtdSer/ Ca^{2+} -dependent phosphorylated species, and parallel studies of Triton-soluble material showed a major 29 kDa C-kinase substrate (Fig. 4). The apparently displaced enzyme in cells treated with anti-Ig also led to phosphorylation *in vitro* of a similar substrate in Triton-soluble material obtained from such cells. Sufficient C-kinase activity, however, was retained in the cytosol fractions to allow phosphorylation of endogenous substrates *in vitro*. Parallel examination of these gels by protein staining revealed no evidence of

Fig. 3. Phospholipid- and Ca^{2+} -dependence of cytosol C-kinase activity determined by the endogenous-substrate-phosphorylation assays described in the text

This Figure shows an autoradiogram of a representative experiment with CLL cells, and the lanes shown contain respectively: (a) no Ca^{2+} or PtdSer; (b) 50 μM - Ca^{2+} only; (c) 5 μg of PtdSer only; (d)–(h) 5 μg of PtdSer and Ca^{2+} at 1, 2.5, 5, 10 and 50 μM respectively; (i) and (j) 10 μM - Ca^{2+} and respectively 10 and 20 μg of PtdSer; (k)–(m) addition of PMA to cytosol fractions at 162 nM (k and l) and 16.2 nM (m), in the absence (k) or presence (l, m) of 10 μM - Ca^{2+} .

comparable intracellular shift of protein substrates from cytosol to crude pellet membrane fractions.

DISCUSSION

Previous studies have indicated a number of potentially important biological effects of PMA upon lymphocyte function. Thus, in normal resting B-cells, phorbol esters can stimulate limited activation and proliferation similar to that induced by anti-Ig, and in CLL cells can induce terminal differentiation as assessed by Ig synthesis (Ashman, 1984; Cossman *et al.*, 1984; Gordon *et al.*, 1984; Totterman *et al.*, 1980). The mechanisms involved are unclear, but it has been suggested that C-kinase

Fig. 4. Membrane endogenous C-kinase phosphorylation after cell stimulation with PMA or anti-Ig

Reactions were performed as for Fig. 3, except that membrane fractions were examined. (a)–(f) normal B-cells; (g)–(l) CLL cells. Cells were treated either with control buffer (a, b, g, h), with anti-Ig (c, d, i, j) or PMA (e, f, k, l) before preparation of Triton-soluble material. One sample from each pair received neither added Ca^{2+} nor PtdSer (a, c, e, g, i and k), and the other contained both Ca^{2+} and 10 μg of PtdSer (b, d, f, h, j and l). Notice PtdSer/ Ca^{2+} -dependent labelling of 29 kDa substrate in anti-Ig- and PMA-treated samples.

activity may be involved (Coggeshall & Cambier, 1984; Cossman *et al.*, 1984; Monroe *et al.*, 1984; Nel *et al.*, 1985). Although C-kinase activity is present in large amounts in lymphocytes (Kikkawa *et al.*, 1982; Kuo *et al.*, 1980; Minakuchi *et al.*, 1981; Nel *et al.*, 1985), possible correlations between stimulation of normal B-cells and alterations in the activity or subcellular distribution of this phospholipid/ Ca^{2+} -dependent kinase system have not been systematically explored.

Our results with exogenous-substrate assays ($n = 6$) indicate that stimulation of normal and CLL cells with both PMA and anti-Ig can cause translocation of C-kinase activity in a dose- and time-dependent fashion, a finding that is consistent with the effects of PMA in other cells studied previously (Ashendel *et al.*, 1983; Kraft *et al.*, 1982; Kraft & Anderson, 1983; Sando & Young, 1983; Wooten & Wrenn, 1984, 1985). Furthermore, although direct physical evidence for redistribution of the structural C-kinase protein is lacking, translocation of enzyme activity was confirmed by analysis of endogenous-substrate phosphorylation. This not only showed a loss of activity from the cytosol and a gain to the membrane fraction, but the profile in these fractions was also distinct and was not accompanied by any comparable shift or redistribution of substrates.

Previous studies of enzyme displacement focused upon disappearance of activity from the cytosol rather than appearance on the membrane. More recently, the use of non-ionic detergent in exogenous-substrate assays has allowed direct assessment of membrane-associated PtdSer/ Ca^{2+} activity in parallel, and has permitted the demonstration, in pancreatic acinar cells, of evidence of translocation of C-kinase activity from cytosol to

membrane-associated fractions (Wooten & Wrenn, 1984, 1985). Studies of the latter fractions are particularly important from a practical standpoint, since more physiological ligand-induced displacement (e.g. anti-Ig) may be comparatively much smaller than PMA-induced translocation.

In B-cells this phenomenon may explain differences in cell response to anti-Ig and PMA. Cross-linking membrane Ig with anti-Ig might therefore initiate a series of events at the plasma membrane proximal to a major pool of C-kinase activity in the cytoplasm; enzyme then becomes translocated to the membrane. On the other hand, PMA can pass rapidly into, or through, the membrane, and is known to bind directly to C-kinase (Castagna *et al.*, 1982; Niedel *et al.*, 1983). It is therefore not surprising that the effects of PMA on C-kinase displacement are of greater extent, and more sustained, than those of anti-Ig, and this may explain the greater biological potency of this phorbol ester in B-lymphocytes, as judged, for example, from the effective induction of Ig synthesis in CLL cells by PMA but not necessarily by anti-Ig (Fu & Kunkel, 1984). In summary, these observations indicate that translocation of C-kinase activity may constitute a normal biological response of B-cells to stimulation.

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