ヒトTリンパ芽球細胞株MOLT-3におけるprotein kinase Cの活性化の役割

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Activation of protein kinase C induces differentiation in the human T-lymphoblastic cell line MOLT-3

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Summary
We attempted to determine whether or not activation of calcium phospholipid-dependent protein kinase C (PKC) is associated with the induction of differentiation by 12-O-tetradecanoylphorbol-13-acetate (TPA) in the human T-lymphoblastic cell line MOLT-3. PKC activities were assessed in MOLT-3 and its subclones, resistant to TPA-induced cell differentiation. The cytosolic PKC activities of TPA-resistant subclones were 10-15% of that of the parental MOLT-3 cells. TPA treatment led to a rapid decrease in PKC activities in the cytosol, together with a concomitant increase in PKC activities in the particulate fraction, in both MOLT-3 and a TPA-resistant subclone. Thus, translocation of PKC from the cytosol to the membrane occurred following treatment with TPA, in both cell lines. However, the amount of PKC translocated from the cytosol to the particulate fraction for 60 min in a TPA-resistant subclone was about 20% of that of the parental MOLT-3 cells. These findings suggest that the quantity of cytosolic PKC activity and the extent of translocation may relate to responses to TPA-induced cell differentiation in this T-cell line.

Materials and methods

Chemicals

Histone H1 (type HI-3), phosphatidylserine (PS), TPA and ATP were purchased from Sigma Chemical Co. (St Louis, MO). [3H]-ATP (5.00 Ci mmol^-1), obtained from Amersham Japan Ltd was diluted with non-radioactive ATP to 100 μm, 100 μm just before use. TPA was dissolved at 2 mg ml^-1 in dimethylsulphoxide (DMSO) or 100 mg ml^-1 in acetone and stored at -20°C.

Cell culture

The human T-lymphoblastic cell line MOLT-3 was obtained from E. Gelfand (Hospital for Sick Children, Toronto, Canada). MOLT-3 subclones (R01, R02, R03, R04 and R05) resistant to the growth inhibition effect by TPA were obtained from colonies formed in 0.8% methylcellulose containing 16 μM TPA and 15 μM fetal calf serum (FCS), as described (Mayumi et al., 1988). These cells were maintained in RPMI-1640 medium supplemented with 10% FCS, 100 μg ml^-1 streptomycin and 100 μg ml^-1 penicillin. The concentration of acetone used in the cell culture did not exceed 0.01%.

Terminal deoxynucleotidyl transferase assay

The terminal deoxynucleotidyl transferase (TdT) activities in MOLT-3 cells and its subclones were measured by biochemical assay, as described (Okamura et al., 1978).

Subcellular fractionation

MOLT-3 or TPA-resistant cells (3-6 x 10^7 cells) maintained without TPA for at least a month were used in the following study. All subsequent steps were done at 4°C. The cells were centrifuged twice with divalent calcium-free PBS, resuspended in 3 ml homogenising buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 0.33 M sucrose, 2 mM phenylmethylsulphonyl fluoride (PMSF) and 50 μg ml^-1 2-mercaptoethanol) and sonicated with a Branson Model Sonifier for 45 s at 20 W. The homogenates were centrifuged for 60 min at 100,000 g and the supernatant served as the cytosol fraction. The pellets were washed with homogenising buffer and re-centrifuged for 60 min at 100,000 g. The pellet was used for

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TPA, a most potent tumour promoter phorbol ester, exerts its effect on tissues and cultured cells (Diamond et al., 1980). The initial event in its action involves binding to the specific receptors on cell membrane, now identified to be calcium phospholipid-dependent PKC (Berridge, 1984; Niedel et al., 1983; Nishizuka et al., 1984; Parker et al., 1984; Sande & Young, 1983; Shoyab & Todaro, 1980). We reported TPA-induced differentiation in human malignant T-cell lines MOLT-3 and Jurkat and analysed the processes in the differentiation, using T-cell differentiation markers such as cell proliferation, E rosette formation, terminal deoxynucleotidyl transferase activity, monoclonal OKT antigen expression and morphological changes (Nagasawa & Mak, 1980; Nagasawa et al., 1981a,b). Using MOLT-3 and its subclones resistant to TPA induction, we also found that the receptors for phorbol ester play an important role at the initial process of induction of cell differentiation (Mayumi et al., 1988). Our main interest has been whether activation of PKC mediates the signals for differentiation as it does in the process of stimulation or proliferation in various cell systems (Kraft et al., 1982; Kajikawa et al., 1983; Malaise et al., 1983; Rozengurt et al., 1984), including human mature T-lymphocytes (Hakov et al., 1987; Manger et al., 1987).

In the induction of differentiation in the promyelocytic leukaemia cell line HL-60, a line most often used as a model of differentiation, some investigators suggested that TPA exerts an induction effect through PKC as a signal mediator (Anderson et al., 1985; Vandenberg et al., 1984), whereas others refuted this (Kreutter et al., 1985; Vandenberg et al., 1984) first proposed that the TPA-induced maturation of HL-60 might be mediated by the activation of intracellular PKC whereas Kreutter et al. (1985) observed a dissociation of the activation of PKC and the induction of cell maturation, determined using 1-octanol-2-acetylgluceral, a synthetic compound which also directly activates PKC. However, little is known of the role of PKC in the induction of T-lymphoblast differentiation.

Activation of PKC is associated with its translocation from the cytosol to the membrane (Kraft et al., 1982; Kraft & Anderson, 1985). Kraft et al. (1982) and Kraft and Anderson (1985) demonstrated that TPA caused a translocation of PKC from the cytosol to the membrane in intact cells and these data were supported by other investigations (Shoji et al., 1987). Horrma et al. (1986) also found that a translocation of PKC from the cytosol to the membrane fractions occurred in HL-60 cells, in response to TPA, whereas it did not occur in the TPA-resistant HL-60 variant cells, hence the translocation of PKC was presumed to be closely related to the TPA-induced differentiation in HL-60.

We have now examined the role of PKC, in particular its activation and subcellular distribution, in the TPA-induced differentiation of MOLT-3.
PKC activity was determined by measuring the incorporation of 3^2P-ATP (100 c.p.m. pmol^-1) at the particulate fraction. PKC activity of five TPA-resistant subclones ROI was assayed and expressed as nmol of 3^2P transferred to histone H1 per min per 10^8 cells. PKC activity is expressed as mean ± s.e. of three separate experiments. PKC activity is expressed as nmol of 3^2P transferred to histone H1 per min per 10^8 cells.

### Results

#### Characteristics of TPA-resistant subclones

The proliferation of MOLT-3 in the presence of 16nM TPA was reduced by 3 days of culture, after which the cells grew slowly up to day 6 with a viability extending 90%, as determined by trypan blue dye exclusion. Thus, the TPA was not toxic to these MOLT-3 cells (Figure 1a). The five TPA-resistant subclones of MOLT-3 obtained in methotrexate containing TPA grew equally well in suspension cultures, with or without 16nM TPA (Figure 1b). The form and size of these TPA-resistant subclones did not differ from those of the parental MOLT-3. The resistance of these clones to TPA was not lost for up to several months, even in continuous culture without TPA.

The presence of TdT is characteristic of prothymocytes and is absent in mature T-lymphocytes (Bellum, 1979). The level of TdT activity was determined by assaying the incorporation of 5^2P-ATP into acid-precipitable material at 37°C, using oligo(dA)_18 as a primer. The values are expressed as mean ± s.e. of three separate experiments.

#### Elution profile of cytosolic PKC of MOLT-3 and TPA-resistant subclones

Cytosolic preparations of MOLT-3 and TPA-resistant subclones were fractionated on DEAE-sepharose columns using a linear gradient of salt concentration. PKC activity was eluted at a concentration of 0.06-0.16M NaCl with a peak at 0.10M NaCl (Figure 2a). Cytosolic preparations of ROI showed an elution profile of PKC, in a similar fashion and with a peak at 0.10M NaCl (Figure 2b). However, the amount of PKC activity in ROI was 80% less than that of the parental MOLT-3. The baseline of PKC activity in ROI was not already activated. To determine whether PKC activities of TPA-resistant subclones were less than those of parental MOLT-3, we assayed the cytosolic PKC activities in MOLT-3 and TPA-resistant subclones by partial purification on DEAE-sepharose columns with a one step with 0.15M NaCl in 4 ml of buffer A. The amount of cytosolic PKC activity of five TPA-resistant subclones ROI, R02, R03, R04 and R05 in 0.1M NaCl and 50% of 2.60 nmol min^-1 per 10^8 cells, respectively, all being significantly low compared to 106 nmol min^-1 per 10^8 cells, respectively, all being significantly higher than 106 nmol min^-1 per 10^8 cells.

The presence of TdT is characteristic of prothymocytes and is absent in mature T-lymphocytes (Bellum, 1979). The level of TdT activity was determined by assaying the incorporation of 5^2P-ATP into acid-precipitable material at 37°C, using oligo(dA)_18 as a primer. The values are expressed as mean ± s.e. of three separate experiments. PKC activity is expressed as nmol of 3^2P transferred to histone H1 per min per 10^8 cells.

#### Effect of TPA on subcellular distribution of PKC

To investigate the activation of PKC, the effect of TPA on the subcellular distribution of PKC in MOLT-3 and the TPA-resistant subclone ROI was examined (Figure 3). Most (99-95%) of the PKC activity was found in the cytosol and there was little PKC activity in the particulate fraction, in both cell lines. The stimulation with 16nM TPA in the parental MOLT-3 resulted in a 50% decrease in cytosolic PKC activity within 5 min, followed by a gradual decline to 0% of the initial level at 60 min (Figure 3a) and a concomitant increase in PKC activity in the particulate fraction occurred (Figure 3b). A similar change of subcellular distribution also occurred in ROI. Thus, the PKC activities in the cytosol and particulate fraction changed inversely, in both cell lines and in a time dependent manner, indicating that the translocation of PKC from the cytosol to the particulate fraction was caused by TPA stimulation. However, the amount of decrease for 60 min was 0.57 nmol min^-1 per 10^6 cells in ROI, 24% of 2.40 nmol min^-1 per 10^6 cells in MOLT-3 (Figure 3a) and the amount of increase for 60 min was 0.52 nmol min^-1 per 10^6 cells in R01, 20% of 2.60 nmol min^-1 per 10^6 cells in MOLT-3 (Figure 3b). There was no detectable PKC activity in the cytosol and particulate fractions of both MOLT-3 and R01 at 24, 72 and 120h after addition of 16nM TPA (data not shown). DMSO (0.005%) had no apparent effect on translocation in MOLT-3 and the TPA-resistant subclones (data not shown).

#### Discussion

To investigate the role of protein kinase C (PKC) in this case of TPA-induced differentiation, a comparison between TPA-sensitive and TPA-resistant subclones was performed. The TPA-resistant subclones from MOLT-3 proliferated either in the presence or absence of TPA. The TPA-resistant subclone was also confirmed by the assay of TdT activities. TdT activities in these subclones remained high even in the presence of TPA.
presence of TPA, whereas they were reduced in the sensitive parental MOLT-3 cells in 3 days' culture with TPA. These results differ from those observed by Homma et al. (1986); in the TPA-resistant MOLT-3 cells, the amount of phorbol ester binding to the cytosolic PKC in parental MOLT-3 was about half that of the parental MOLT-3, presumably due to a deficiency of the specific phorbol ester receptors. In contrast, as assessed by Scatchard analysis (Mayumi et al., 1988), the number of phorbol ester receptors for phorbol esters was approximately the same in the cytosol in both cells. These results may reflect differences in the number of PKC in the cytosol and in the membrane. Several studies have shown that the levels of PKC in the cytosol and in the membrane in both TPA-treated and control cells were very similar. These results may reflect the continued translocation of PKC in both TPA-treated and control cells, as assessed by Scatchard analysis (Mayumi et al., 1988). The precise relationship between the down regulation of phorbol ester binding and PKC translocation is unclear, but both events may be related.

The role of PKC after the onset of cell differentiation is also unclear. There was no detectable PKC activity in the cytosol and partial cell fractions for up to 120 hours after treatment with 16-lysophorleic acid in both TPA-treated and control cells. These results may reflect the continued translocation of PKC from the cytosol to the membrane and the degradation of membrane-associated PKC (Ballester & Rosen, 1985). While this study provides some evidence that PKC translocation from the cytosol to the membrane causes differentiation, the existence of differences in the amount of cytosolic PKC, the amount of PKC translocation and the number of phorbol ester receptor sites between TPA-resistant subclones and the parental MOLT-3 cells do suggest that PKC plays an important role in the induction of differentiation by TPA in this T-lymphoblastic cell line MOLT-3. This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (no 6257209). We thank Mrs. Ohara for pertinent comments.

References


