Phorbol Ester-Induced Reversible Inactivation of Cytotoxic T Cell Function: Correlation with Down-Regulation of Protein Kinase C Activity

Hitoshi Ohmori, Toshitaka Shimada, Masaki Hikida and Toshiyuki Takai

Department of Biotechnology, Faculty of Engineering, Okayama University, Tsushima-Naka, Okayama 700, Japan

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ABSTRACT—When an H-2d-specific cytotoxic T lymphocytes (CTL) clone, FC1, was incubated in the presence of $10^{-7}$ M phorbol myristate acetate (PMA) for 10–12 hr, the cytolytic activity of the CTL against H-2d target cells was abrogated, but was reversibly restored to the normal level after subsequent incubation of the cells in PMA-free medium for more than 10 hr. These effects of PMA have been reported (Russell, J.H.: J. Immunol. 133, 907-912 (1984)), but the mode of its action has not been fully investigated. Here, we analyzed the biochemical basis of the PMA-induced loss of cytolytic activity. Cycloheximide completely blocked the restoration of the PMA-suppressed cytolytic activity, suggesting that protein synthesis was required in this process. PMA-treatment did not affect the levels of CD3 and CD8 molecules expressed on the CTL, nor was the level of a CTL-specific serine esterase, BLT esterase, affected by this treatment. However, the target cell-induced release of BLT esterase from the CTL was suppressed if the cells were pretreated with PMA. PMA-treatment of the CTL led to the down-regulation of protein kinase C (PKC) activity by about 50%. On the other hand, staurosporin, an inhibitor of PKC, completely blocked the target cell lysis when added at $10^{-6}$ M. These results suggest that the down-regulation of at least some isoform(s) of PKC is responsible for the PMA-induced loss of the cytolytic activity of CTL.

Keywords: Cytotoxic T cell, Phorbol ester, Protein kinase C, Serine esterase, Target cell lysis

Cytotoxic T lymphocytes (CTL) play a pivotal role in cellular immunity including the rejection of allografts or tumors and the killing of virus-infected cells. The development of technologies controlling CTL activity is of great importance, for instance, in the successful performance of transplantation. The immunosuppressive drugs like cyclosporin A and FK506 have been reported to be effective in suppressing CTL activity at the induction phase (1). It is also considered to be valuable to down-regulate the established CTL activity. For this purpose, one must elucidate the whole profile of the signal transduction pathway that is initiated from the antigen-recognition by the T cell receptor (TcR) and finally leads to the exocytosis of cytolytic factors. Generally, T cell activation via the TcR has been shown to result in the stimulation of phospholipase C, which subsequently yields inositol phosphates and diacylglycerol. These compounds in turn induce a rise in intracellular Ca$^{2+}$ and the activation of protein kinase C (PKC), respectively (2). However, the roles of PKC in the cytolytic function of CTL have not been fully investigated.

Orósz et al. (3) and Russell (4) have independently reported that the treatment of cloned CTL with phorbol myristate acetate (PMA) for over 12 hr results in the complete abrogation of the cytolytic activity in a reversible manner. These authors, however, have not described the mode of action of PMA in detail. In the present report, we analyzed the biochemical changes caused by PMA-treatment of cloned CTL and their correlation with the loss of CTL activity.

MATERIALS AND METHODS

Cell lines

NS-1, a BALB/c-derived myeloma cell line, was obtained from the Riken Cell Bank, Tsukuba. NS-1 was transfected with the Escherichia coli β-galactosidase (β-Gal) gene by the DEAE-dextran/osmotic shock method developed in our laboratory (5). An NS-1 transfectant that permanently expresses β-Gal was selected and designated as NS-1/Z, which was used as the target of H-2d-specific CTL as described previously (6). A CD8$^+$, CD4$^-$ CTL clone, FC1, was established by repeated stimulation of C3H (H-2b) spleen cells with mitomycin C (MMC)-
treated BALB/c (H-2d) spleen cells according to the procedure of Gillis and Smith (7). FC1 was maintained without loss of cytotoxicity against NS-1/Z cells in the culture medium containing MMC-treated BALB/c spleen cells and 10% (v/v) of the conditioned medium of concanavalin A-stimulated rat spleen cells.

**Chemicals, antibodies and other materials**

PMA, staurosporin, N-benzyloxycarbonyl-L-lysine thiobenzylester (BLT), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 4-methyl-umbelliferyl-β-D-galactoside (4-MUG) were obtained from Sigma (St. Louis, MO, USA). Fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies against CD3 and CD8 were purchased from Cosmo Bio (Tokyo). Cell cultures were carried out in RPMI-1640 medium containing 50 µg/ml streptomycin, 100 U/ml penicillin G, 1 x 10⁻⁵ M 2-mercaptoethanol and 10% fetal calf serum (FCS) (Gibco, Grand Island, NY, USA).

**Enzyme release assay of CTL activity**

CTL activity was assayed by the release of β-Gal from the target cell line NS-1/Z as described previously (6). Briefly, varying numbers of FC1 cells (0.25–1.0 x 10⁵) were mixed with 2 x 10⁴ NS-1/Z in 0.2 ml of RPMI 1640 medium containing 2% FCS in a V-bottomed 96 well microculture plate (Nunc, Roskilde, Denmark). Usually, triplicate experiments were done at each effector/target (E/T) ratio. The plate was centrifuged for 2 min at 800 rpm and incubated at 37°C for 4 hr in a humidified atmosphere of 5% CO₂ and 95% air. The supernatants were collected and assayed for the released β-Gal activity as follows: A 0.1-ml aliquot of each supernatant was incubated for 30 min at 37°C with 0.3 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM MgCl₂, 0.1% bovine serum albumin and 0.1 mM 4-MUG, a fluorescent substrate. The reactions were stopped by the addition of 2 ml of 0.1 M glycine buffer (pH 10.3). Fluorescent intensity was determined with the excitation at 360 nm and the emission at 450 nm using a fluorescence microplate reader MTP-32 (Corona Electric, Tokyo). The enzyme release in the absence of effector cells was designated as spontaneous release (S). Total enzyme activity (T) of the target cells was measured after disrupting the cells with 0.0425% Triton X-100. A 0.1 ml aliquot of each supernatant was incubated for 30 min at 37°C with 0.3 ml of 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM MgCl₂, 0.1% bovine serum albumin and 0.1 mM 4-MUG, a fluorescent substrate. The reactions were stopped by the addition of 2 ml of 0.1 M glycine buffer (pH 10.3). Fluorescent intensity was determined with the excitation at 360 nm and the emission at 450 nm using a fluorescence microplate reader MTP-32 (Corona Electric, Tokyo). The enzyme release in the absence of effector cells was designated as spontaneous release (S). Total enzyme activity (T) of the target cells was measured after disrupting the cells with 0.0425% Triton X-100. This concentration of the detergent had no significant effects on the assay of β-Gal. Percent cytolysis was defined as follows:

\[
\% \text{ cytolysis} = \frac{R - S}{T - S} \times 100
\]

where R is the observed enzyme release by CTL. We have confirmed that this enzyme release assay gives parallel results with those in the ⁵¹Cr-release assay (6). Data are usually presented as the mean of triplicate experiments. Experimental variation was always less than 10% of the mean and is not usually shown. Typical data from several repeated experiments are presented.

**Assay of BLT esterase activity**

A serine esterase named BLT esterase expressed in CTL was assayed as described by Pasternack and Eisen (8). Briefly, 20 µl of a test sample was mixed with 180 µl of 0.2 M Tris-HCl buffer (pH 8.1) containing 0.22 mM DTNB and 0.2 mM BLT in flat-bottomed, 96-well microculture plates (Nunc). After incubation for 20–30 min at 37°C, the absorbance at 415 nm was measured by a microplate reader (MPR A4i; Tosoh, Tokyo).

**Assay of PKC activity**

For the extraction of PKC, FC1 cells were suspended at 5 x 10⁶/ml in 50 mM Tris-HCl buffer (pH 7.5) containing 0.5 mM EDTA, 1 mM EGTA, 10 mM MgCl₂ and 0.1% Triton X-100. After it was kept at 4°C for 5 min, the suspension was centrifuged at 12,000 x g for 15 min. The supernatants were collected and assayed for PKC activity by the PKC assay system (Amersham, Tokyo) according to the manufacturer’s protocol.

**RESULTS**

**Reversible abrogation of cytolytic activity of CTL treated with PMA**

When the cells of the CTL clone FC1 were incubated in the culture medium containing 10⁻⁷ M PMA, the cytolytic activity against NS-1/Z target cells was reduced as a function of the incubation time. As shown in Fig. 1A, the CTL activity was almost completely abrogated in 12 hr. At least 10⁻⁷ M PMA was required for this inactivation process (data not shown). Figure 1B shows that PMA-induced inactivation of the CTL was reversible. When PMA-treated CTL that lost their cytolytic activity were washed and then cultured for 10 hr in the PMA-free medium, it was clearly shown that the CTL activity was restored to the original level. This restoration process is thought to require protein synthesis because cycloheximide completely blocked the reexpression of the cytolytic activity (Fig. 1B). Figure 2 shows that the CTL activity induced by the mixed culture of C3H and MMC-treated BALB/c spleen cells was also abrogated by a similar treatment with PMA, thus indicating that PMA-induced inactivation of the cytolytic activity is not limited to particular CTL clones.

**PMA-induced biochemical changes in the CTL**

To understand the mode of action of PMA, we analyzed the biochemical changes induced in PMA-treated FC1 cells. As shown in Fig. 3, flow cytometric analysis
was performed to examine if CD3 and CD8 molecules expressed on the CTL are down-regulated in the PMA-treated cells. The results show that the PMA-treatment had no significant effects on the level of these molecules that are involved in the recognition of target cells by the CTL.

It has been shown that CTL usually possess a specific serine esterase named BLT esterase that is thought to be one of the cytotoxic factors, and is released after antigen-stimulation (9, 10). We confirmed that a high level of BLT esterase is present in FC1 cells and examined whether the intracellular level of BLT esterase was reduced after the PMA-treatment. As shown in Fig. 4, PMA-treated CTL contained a comparable level of the enzyme to that of the untreated cells. The target cell line NS-1/Z did not possess a significant level of the esterase. When the CTL was stimulated by target cells, BLT esterase was released into the medium concomitant with the target cell lysis as shown in Fig. 4B (lane 1). However, BLT esterase-release was strongly suppressed in the CTL that were pretreated with PMA for 12 hr (lane 2). When the PMA-treated CTL were cultured in the absence of PMA, the ability to release BLT esterase was restored nearly to the control level concomitant with the reexpression of the cytolytic activity (lane 3). On the other hand, the enzyme-release remained suppressed in the CTL if they were exposed to PMA persistently (lane 4). These results suggest that PMA-treatment causes some disorders in the exocytotic machinery of the CTL.

Down-regulation of PKC activity in the PMA-treated CTL

The short term exposure of various cells to PMA usually results in the transient enhancement of PKC activity (11). However, chronic PMA-treatment is known to result in the down-regulation of PKC level in some cell lines (12, 13). Thus, we investigated the effects of PMA-treatment on the level of PKC activity in FC1 cells. As shown in lane 3 of Fig. 5, persistent PMA-treatment for 12 hr resulted in approximately 50% reduction of PKC

Fig. 1. Reversible abrogation of the cytolytic function of the CTL clone FC1 by PMA-treatment. A: Effect of incubation time on the loss of the CTL activity. FC1 cells were suspended in RPMI-1640 medium at 1 x 10^6 cells/ml, and they were incubated for the indicated time in the presence of 10^{-7} M PMA. B: Restoration of the PMA-suppressed CTL activity in the PMA-free medium. FC1 cells (1 x 10^6 cells/ml) were incubated for 12 hr in the presence () or absence () of 10^{-7} M PMA. A portion of the PMA-treated cells were washed and further cultured with () or without () 20 µg/ml cycloheximide in the PMA-free medium for 10 hr. The CTL activity against NS-1/Z target cells was measured by the β-Gal release assay at the indicated effector/target (E/T) ratio as described in Materials and Methods. Each point represents the mean of triplicate experiments.

Fig. 2. Effect of PMA-treatment on the cytolytic activity of CTL induced in the mixed lymphocyte culture. C3H spleen cells (5 x 10^6) were cultured with MMC-treated BALB/c spleen cells (1.25 x 10^6) in 1 ml of the culture medium for 5 days. Harvested cells were incubated at 1 x 10^6 cells/ml with () or without () 10^{-7} M PMA for 12 hr and then assayed for the CTL activity.
activity compared with that of untreated cells (lane 1). Under these conditions, we confirmed that the cytolytic activity was reduced by more than 90% (data not shown). On the other hand, short term PMA-treatment of the cells for

![Graphs showing CD3 and CD8 expression](image)

**Fig. 3.** Effect of PMA-treatment on the expression of CD3 and CD8 in FC1 cells. FC1 cells were incubated with (B and D) or without (A and C) $10^{-7}$ M PMA for 12 hr as described in the legend of Fig. 1. Flow cytometric analysis was done after staining the cells with FITC-labeled anti-CD3 (A and B) or anti-CD8 (C and D), by using FACScan flow cytometer (Becton-Dickinson and Company). The vertical and horizontal axes represent relative cell number and relative fluorescence intensity, respectively.

![Graphs showing BLT esterase activity](image)

**Fig. 4.** PMA-pretreatment leads to the suppression of the target cell-induced BLT esterase release from CTL, but not of the intracellular level of the enzyme. A: FC1 cells were treated with PMA as described in Fig. 1. BLT esterase activity from $1 \times 10^5$ treated cells was measured and compared with that of the control cells or NS-1/Z cells. B: FC1 cells were incubated with (lane 2) or without (lane 1) $10^{-7}$ M PMA for 12 hr. A portion of the PMA-treated cells were washed and further cultured for 12 hr in the presence (lane 4) or absence (lane 3) of PMA. Each group of cells were tested for the cytotoxicity and the ability to release BLT esterase after incubation for 4 hr with the target cells at the E/T ratio of 5. Data are presented as the mean ± S.E. from triplicate experiments.
10 min enhanced PKC activity significantly (lane 2). Figure 6 shows that staurosporin, an inhibitor of PKC, strongly blocked the cytolysis when it was present during the contact of the CTL with the target cells, indicating that PKC activation is critical for the cytolytic function. These data suggest that the down-regulation of some, but not all, isoforms of PKC is at least responsible for PMA-induced loss of the CTL activity.

DISCUSSION

Orosz et al. first reported that the cytolytic function of cloned CTL was inactivated when the cells were cultured in the presence of PMA for 3 days (3, 14). They also found that this inactivation process was reversible since the cytolytic activity returned to the normal level when the PMA-treated cells were incubated in the PMA-free medium containing cytokines derived from the mixed lymphocyte reaction. On the other hand, Russell made similar observations, although he stated that no added cytokines were necessary for the restoration of the PMA-suppressed cytolytic activity (4). We confirmed that cytokines are not essential in this process (Fig. 3), although the addition of IL2 slightly accelerated the restoration (data not shown).

It has been reported that the cytolysis of nonspecific (third party) target cells by cloned CTL was augmented in the presence of PMA and Ca\(^{2+}\)-ionophore (15, 16). Generally, it has been shown that the antigen-stimulation via TcR leads to the formation of inositol phosphate and diacylglycerol, the latter of which is known to be an activator of PKC (2). O'Rourke and Mescher have suggested that PKC is involved in the dissociation process of CTL from target cells rather than the association step (2). However, the detailed roles of PKC in the CTL function have not been fully elucidated. In rat basophilic leukemia (RBL) cells, six isoforms of PKC have been identified: \(\alpha, \beta I, \beta II, \delta, \epsilon\) and \(\zeta\) (13, 17). Of these isoforms, it has been shown that the former three isoforms are Ca\(^{2+}\)-dependent, and the latter three are Ca\(^{2+}\)-independent (13). It has been reported that the \(\alpha, \beta I, \beta II\) isoforms of PKC in RBL cells are subjected to the down-regulation by chronic PMA-treatment, while the \(\delta, \epsilon\) and \(\zeta\) isoforms are resistant to the same treatment. The antigen-induced exocytosis of chemical mediators from RBL cells was not affected by PMA-treatment, thus suggesting Ca\(^{2+}\)-independent, but not Ca\(^{2+}\)-dependent isoforms are involved in this exocytotic process (17).

The murine T cell line C19 was shown to possess at least the \(\epsilon\)-type (Ca\(^{2+}\)-independent) PKC that was shown
to play a central role in the expression of IFN-γ and IL2-receptor genes in this cell line (18). Compared with the isoforms of PKC in rats (17), their counterparts in murine cells have not been fully investigated. Our experiments showed that PMA-treatment of the CTL resulted in a partial down-regulation (ca. 50%) of PKC concomitant with almost complete loss of the cytolytic activity, suggesting that CTL possess both PMA-sensitive and PMA-resistant isoforms of PKC. Moreover, PKC activity is thought to be essential for the CTL function since staurosporin strongly inhibited the target cell lysis by the CTL. Thus, it is considered that PKC isoforms that are sensitive to PMA-induced down-regulation might be involved in the CTL activity.

Hidaka et al. have developed monoclonal antibodies directed to the α, β and γ isoforms of rabbit brain PKC (19). By using these monoclonal antibodies, it has been shown that only the β-isofrom was detected in murine Langerhans cells, but keratinocytes did not possess PKC reactive with these antibodies (20). In our preliminary experiments, we failed to detect PKC isoforms in the extracts of the CTL clone by Western blot analysis using these monoclonal antibodies (unpublished results). Further studies are necessary to identify the PMA-sensitive and PMA-resistant isoforms that are expressed in murine CTL.

Taken together, PMA-induced abrogation of the cytolytic activity of CTL may not be due to the reduced expression of CD3 and CD8 that are involved in antigen-recognition by CTL, but at least partially due to the down-regulation of some PKC isoform(s) that are responsible for the signal transduction pathway from TcR to the exocytotic machinery.

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