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PREVENTION OF THE TPA-MEDIATED DOWN-REGULATION OF PROTEIN KINASE C

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Summary: Protein kinase C activity in lymphocytes was down-regulated upon exposure of the cells to 12-O-tetradecanoylphorbol-13 acetate. This down-regulation was prevented by preincubating the cells with sphingosine, a reported protein kinase C inhibitor. Two other protein kinase C inhibitors, palmitoylcarnitine and phloretin, were ineffective in preventing down-regulation by the phorbol ester.

TPA added simultaneously with mitogenic lectins potentiates lymphocyte proliferation. In contrast, pretreatment of lymphocytes with TPA for 16 to 24 h before addition of lectins, depresses the proliferative response (1). With the more recent report by Rodrigues-Pena and Rozengurt (2) that TPA down-regulated protein kinase C in Swiss 3T3 cells, we asked whether the down-regulation of protein kinase C by TPA occurs in lymphocytes. Furthermore, in order to more clearly define the role that protein kinase C plays in the proliferation process, we also looked for compounds which would prevent the down-regulation of protein kinase C by TPA. Several effectors of protein kinase C activity were tested: sphingosine, phloretin, and palmitoylcarnitine. Sphingosine and palmitoylcarnitine, reported inhibitors, compete with ^{3}H-PDBU binding to protein kinase C (3-5). Phloretin is also reported to inhibit protein kinase C activity but through a less specific process (5). We found that sphingosine but not palmitoylcarnitine or phloretin prevented the down-regulation of protein kinase C by TPA. To our knowledge, this is the first report that down-regulation of protein kinase C was prevented by sphingosine *in vivo*.

MATERIALS AND METHODS

TPA was obtained from Midland Chemical Co., Brewster, NY. Sphingosine, Sphingosine sulfate, histone II-AS (calf thymus mixture), palmitoylcarnitine,

ABBREVIATIONS: TPA, 12-O-Tetradecanoylphorbol-13-acetate; Con A, Concanavalin A IL2, Interleukin 2; ^{3}H-PDBU, [20-^{3}H(N)]7-phorbol-12,13,dibutyrate

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leupeptin, protamine sulfate, and phloretin were obtained from Sigma, St. Louis, MO. Calf serum was obtained from Gibco, Long Island, NY. Triton X-100 and γ-[^{32}P]ATP (10 Ci/mmol) were purchased from NEN, Boston, MA. Kodak XRP-5 film was from Picker, Cleveland, OH.

Lymphocytes were isolated from bovine lymph nodes (6) and cultured as described previously (7). Cells were treated with TPA (10^{-9} to 10^{-6} M) dissolved in dimethyl sulfoxide (final concentration in culture medium, 0.1%). Sphingosine (5×10^{-6} to 1×10^{-5}) was dissolved in ethanol (final concentration in medium, 0.1%). In some experiments, sphingosine sulfate powder was added to the medium.

Lymph node cells were suspended in buffer C (20 mM Tris-Cl pH 7.5, 50 mM 2-mercaptoethanol, 2 mM EDTA, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride) and leupeptin to a final concentration of 25 μg/ml. They were lysed by sonication with a cup horn sonicator (W-220 sonicator, Ultrasonics Inc., Farmingdale, NY) as described previously (7). To extract both the soluble and particulate fractions, Triton X-100 was added to 0.25 % and lysates were held on ice for 1/2 h prior to centrifugation (100,000 rpm, 10 min, 435,000 x g, Beckman TL100, TLA-100.2 rotor). Supernatants were collected, aliquoted, and frozen at -80°C.

Kinase activity was identified by phosphorylation in a gel as described previously (7). Briefly, 25 μg and 50 μg of protein were electrophoresed in a non-denaturing gel. After incubation of the gels for 30 min with the substrates histone or protamine sulfate, γ-[^{32}P]ATP was added for an additional 30 min. Finally, the gels were fixed, washed, dried, and exposed to Kodak XRP-5 film. The radioautogram was scanned with a laser densitometer (LKB, Model 2202 Ultrascan, Gaithersburg, MD) to quantitate the bands representing kinase activity.

RESULTS

Treatment of primary lymphocyte cell cultures with TPA (10^{-7} M) led to a detectable loss of protein kinase C activity when assayed with histone as a substrate (Fig. 1). For example in one experiment after 1 h of treatment of

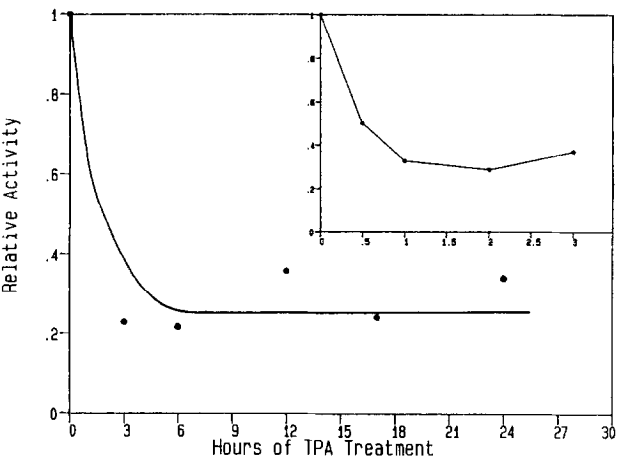


Figure 1. Down-regulation of protein kinase C activity by TPA. Protein kinase C activity was measured in extracts of lymphocytes treated from 3 to 24 h with TPA (10^{-7} M). Activity is expressed as relative kinase activity of treated cells over the activity of untreated cells. Cells were extracted as described in the methods section. Extracts were electrophoresed into a non-denaturing gel, and the gel was phosphorylated in situ with histone as substrate. The inset graph shows the results of a second experiment in which protein kinase C activity was determined in lymphocytes treated from 1/2 to 3 h with TPA (10^{-7} M).

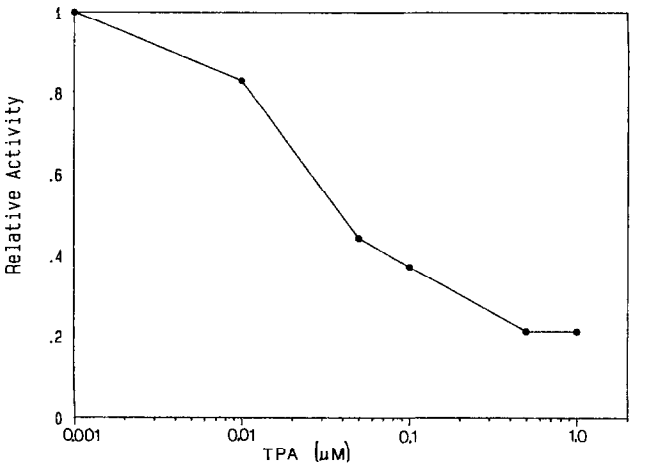
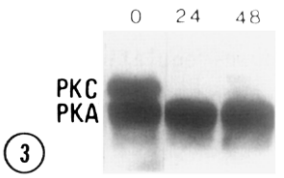


Figure 2. Concentration-dependent down-regulation of protein kinase C by TPA. Lymphocytes were treated with various concentrations of TPA for 1/2 h, and extracts were treated as described in the legend to Fig. 1.

cells there was a decrease of 70 to 80% of the protein kinase C activity compared with untreated cells. The percentage loss of activity varied in 5 experiments from 33 to 80% but averaged 46% (S.D. +/- 17). Higher concentrations of TPA did not cause 100% down-regulation (Fig. 2). With the shortest time tested, 30 min, the activity was reduced to 50%. Down-regulation of activity persisted up to 72 h in the presence of TPA (Fig. 1 and data not shown). However, protein kinase C activity in extracts from cells which were treated with TPA (10^{-7} M) for 24 h, washed with fresh medium, and incubated an additional 24 h in TPA-free medium, returned to 100% after being down-regulated by 50%. Protamine kinase activity (measured by using protamine as an alternate substrate of protein kinase C) as well as the autophosphorylating activity of protein kinase C were also lost to the same extent as was the histone kinase activity (data not shown).

Because 100% down-regulation of protein kinase C was never seen in lymphocytes, extracts of quiescent Swiss 3T3 cells were tested to determine if residual levels of protein kinase C could be seen after phorbol ester treatment. The cells were treated according to the protocol of Rodrigues-Pena and Rozengurt (2) but assayed with the gel assay for PKC activity. In these cells complete loss of protein kinase C was seen (Fig. 3). Note that cAMP-dependent protein kinase activity did not change during this treatment. Therefore, we concluded that lymphocytes resembled human epidermal cells, growing 3T3 cells, and transformed fibroblasts (8,9), in that complete loss of protein kinase C activity after TPA treatment does not occur.

In order to determine if reported effectors of protein kinase C could prevent down-regulation, the lymph node cells were preincubated with



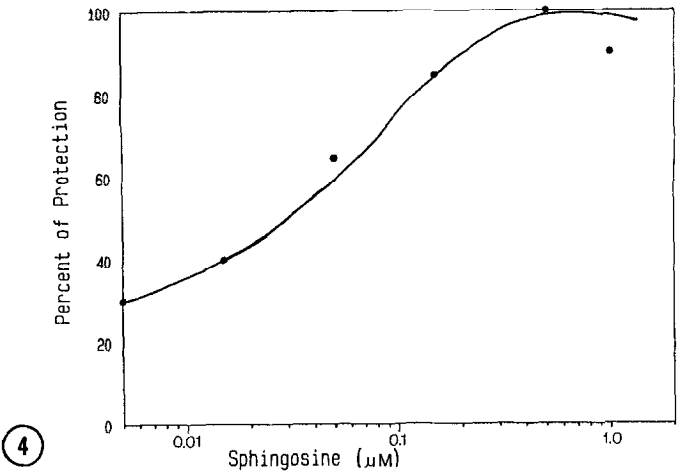


Figure 3. Down-regulation of protein kinase C in Swiss 3T3 cells. Cells were treated with phorbol-12,13-dibutyrate (4 x 10^{-7} M) for 24 or 48 h, or left untreated. Extracts were assayed as described in the legend to Fig. 1 and the resulting radioautogram is shown. Protein kinase C (PKC) and cyclic AMP-dependent protein kinase (PKA) are shown for untreated (0) or treated samples (24 h, 48 h).

Figure 4. Concentration dependent prevention by sphingosine of the down-regulation of protein kinase C. Various concentrations of sphingosine were added to cells for 30 min before the addition of TPA (10^{-7} M) for a further 45 min incubation. Cells were extracted and protein kinase C activity assayed.

sphingosine, palmitoylcarnitine, or phloretin for 20 to 30 min before the addition of TPA (10^{-7}M). Sphingosine prevented the down-regulation of protein kinase C by TPA (Fig. 4). Concentrations of 5 and 10 x 10^{-6} M

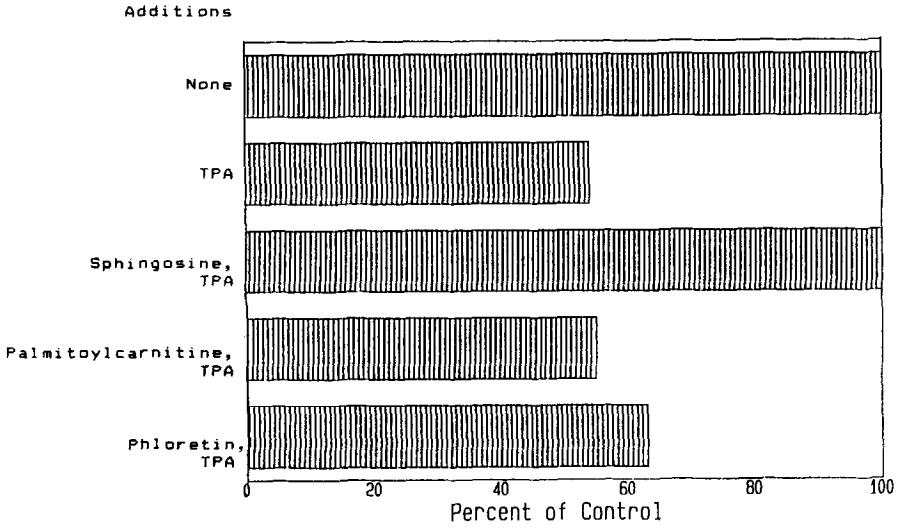


Figure 5. Prevention of down-regulation of protein kinase C. Lymphocytes were treated with either 10^{-7} M TPA alone for 1/2 h or various compounds were added with TPA to determine if down-regulation were prevented. Concentrations of the compounds tested were sphingosine (1 x 10^{-5} M)), palmitoylcarnitine (7 x 10^{-5} M), and phloretin (1.4 x 10^{-4} M).

sphingosine completely prevented down-regulation of protein kinase C after 1 h of TPA (10^{-7} M) treatment (Fig. 5). At a sphingosine concentration of 5 x 10^{-8} M and a TPA concentration of 10^{-7} M about 30% protection still occurred. Sphingosine added to cells alone had no effect on kinase activity (data not shown). When sphingosine was mixed directly with cell extracts and incubated at 30°C for 30 min prior to electrophoresis, there was no effect on protein kinase activity in the in situ gel phosphorylation assay (data not shown).

Palmitoylcarnitine and phloretin did not affect the down-regulation by TPA (Fig. 4). These compounds were used at concentrations reported to inhibit protein kinase C activity by 50% (5).

DISCUSSION

Sphingosine at concentrations of 5 and 10 x 10^{-6} M prevented down-regulation of protein kinase C by TPA (10^{-7} M). These concentrations of sphingosine are 50 to 100-fold higher than the concentration of TPA added. However, the amount of sphingosine that actually entered the cells is unknown.

Because sphingosine has been reported to inhibit protein kinase C the difference between the effects of down-regulation by TPA and inhibition by sphingosine would ordinarily be difficult to distinguish in a standard activity assay. In this respect the in situ gel assay provided a distinct advantage because the expected inhibition of protein kinase C by sphingosine was not observed. In fact, when sphingosine was added directly to the extract prior to electrophoresis, inhibition of protein kinase C was not seen apparently because sphingosine separated from protein kinase C during electrophoresis.

Neither palmitoylcarnitine or phloretin prevented down-regulation of protein kinase C by TPA. These compounds either did not compete with TPA binding to protein kinase C, they were not specific enough for protein kinase C in the in vivo environment, or they could not enter the cell.

Regulation of protein kinase C activity by TPA and sphingosine can be used to determine at what stage in the signalling process TPA can affect proliferation of lymphocytes. Treatment of lymphocytes with TPA leads to depressed proliferation when the cells are subsequently exposed to Con A (1). The major block appears to be in IL2 production (manuscript in preparation). The correlation between the block in IL2 production and the down-regulation of protein kinase C is being studied using TPA and sphingosine.

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