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Conventional protein kinase C isoforms are not essential for cellular proliferation of a T cell lymphoma line

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The role of conventional protein kinase C (PKC) isoforms in the proliferation of a T cell lymphoma line was investigated. PKC isozyme-deficient cell lines were generated from HuT 78 which expresses PKC isoforms α, β, ε and ζ. The first of these, K-4, expresses PKC α, ε and ζ but not β. L-2, a mutant of K-4, expresses PKC α and ζ but expresses neither PKC α, β, γ, ε nor ζ when cultured continuously in PMA (L-2/PMA). All four cell lines continued to proliferate although at reduced rates in the presence of the PKC inhibitors staurosporine and H-7. K-4 and HuT 78 undergo growth arrest when activated with PMA (rate of proliferation decreases by 76 and 95%, respectively). While PMA-induced growth arrest occurs in L-2 cells which possess PKC α and ζ, PMA-induced growth arrest does not occur in L-2/PMA which is deficient in these isoforms. As these cell lines continue to proliferate in the presence of PKC inhibitors and since proliferation occurs in the absence of PKC isoforms (L-2/PMA), the results suggest that neither conventional PKC nor the ε and ζ isoforms are essential for proliferation of these cells. These data further suggest that PKC α and/or ζ may be involved in PMA-induced growth arrest.

Protein kinase C; Lymphoma; Proliferation; Apoptosis; Isozyme

1. INTRODUCTION

Protein kinase C (PKC) comprises a family of isoforms which may be divided into three major groups based on differences in enzymatic properties [1]. Many of these isoforms are activated by tumour promoters such as phorbol myristate acetate (PMA) which can mediate a range of intracellular functions [2,3]. Tumour promoters were first described by virtue of their ability to induce proliferation in resting cells in animal models of carcinogenesis [4]. Application of phorbol ester to mouse skin results in the development of cutaneous adenomas which can then be induced to malignant proliferation by the addition of a tumour induction agent. Resting T lymphocytes proliferate in the presence of phorbol esters and this proliferation is inhibited by PKC inhibitors [5]. However, many tumour cells undergo growth arrest when exposed to phorbol esters and this may be accompanied by differentiation. For example, in the HL60 cell line activation of protein kinase C through phorbol esters results in differentiation of the cell line to either a macrophage or a neutrophil lineage [6]. In addition, in K562 cells, the activation of PKC through phorbol esters results in differentiation to an erythrocyte lineage [7]. However, proliferation of certain tumours has been shown to be arrested by PKC inhibitors [8] and it has been suggested that PKC inhibitors may have a potential role as anti tumour agents [9]. The aim of this study was to investigate the role of PKC in proliferation of human lymphoma cells. For this purpose we used the human T cell lymphoma line HuT 78 and a mutant of this, K-4, which is deficient in PKC β [10]. From these cell lines we derived a number of variants which were cloned lines deficient in known conventional and novel PKC isoforms. These mutants have been used to study the role of PKC isoforms in tumour proliferation.

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2. MATERIALS AND METHODS

2.1. *Materials and cells*

The HuT 78 cell line was obtained from ATCC. Antibodies to PKC isozymes α, β and γ were obtained from Seikagaku America, St. Petersburg, FL. Antibodies to PKC ε and ζ and cell culture reagents were obtained from Gibco BRL, Gaithersburg, MD. PMA and Genestein were obtained from Sigma, UK, and Staurosporine and H-7 from Calbiochem, UK. Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were obtained from Promega Corp., Madison, WI.

2.2. *Cell culture*

Cells were grown in RPMI 1640 (CO_2 independent) supplemented with 10% heat-inactivated foetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and 5×10^{-5} M 2-mercaptoethanol at 37°C. K-4 cells were generated as previously described [10]. These cells were then cultivated in the presence of increasing concentrations of PMA (20–240 ng/ml) over a period of several weeks. Surviving cells were then cloned in complete medium containing 100 ng/ml PMA at a concentration of 1/3 cell per well in the absence of feeder cells. The resultant cell line (L-2) was either maintained in complete medium or cultured continuously in the presence of PMA (100 ng/ml).

2.3. *Flow cytometric analysis*

Cells were incubated with PMA (0, 25, 50 and 100 ng/ml) for 24 h

and fixed in 0.5% (w/v) paraformaldehyde. Data were gathered using a Beckton Dickinson FACScan fluorescent cell analyser and forward scatter was analysed using Lysis II software.

2.4. *Thymidine incorporation assay*

Cells were incubated in 96-well flat-bottomed microtitre plates at a concentration of 2×10^5 cells/well for 24 h and pulsed with tritiated thymidine (0.5 μCi/well) for the last 6 h of incubation. PMA (100 ng/ml), PKC inhibitors at varying concentrations were added at the start of the incubation. Samples were harvested on a multiple automated cell harvester and counted in a Packard liquid scintillation counter.

2.5. *Immunoblot studies*

Total cell extracts were prepared by solubilising the cells in 0.5% (w/v) Nonidet P-40 in PBS containing 1 mM PMSF and 10 μg/ml leupeptin. Proteins (100 μg) were separated by 10% SDS-PAGE and transferred to nitrocellulose [11]. Non-specific binding sites were blocked with Blotto-Tween (5% non-fat dried milk containing 0.05% Tween-20 in PBS) for 1 h. The nitrocellulose membrane was then incubated with monoclonal or polyclonal (anti-peptide) antibody to the respective isozyme for 18 h. The membrane was then incubated with biotinylated sheep anti-mouse or donkey anti-rabbit antibody for 1 h, followed by streptavidin-biotinylated alkaline phosphatase complex for 30 min. Immunoreactive bands were visualised using Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). Molecular weight marker proteins were localised by staining with 0.2% Ponseau-S in 3% trichloroacetic acid, 3% sulphosalicylic acid.

3. RESULTS

3.1. *Generation of PKC isozyme-deficient T cell lines*

A cell line K-4 was generated from HuT 78 as described previously [10]. The K-4 cell line was cultured in increasing concentrations of PMA (20–240 ng/ml). An additional cell line, L-2, was cloned from K-4 cells which were resistant to 240 ng/ml PMA. L-2 cells were then maintained in continuous culture in the presence or absence of PMA. L-2 cells which proliferate in the continuous presence of PMA are designated L-2/PMA. PKC isoform expression in these cell lines was determined by Western blot analysis (Fig. 1). The specific bands at approximately 90 kDa and 80 kDa for the polyclonal antibodies to ε and ζ were abrogated by preincubation of these antibodies in blocking peptide. HuT 78 cells express the PKC isoforms α, β, ε and ζ while K-4 express PKC α, ε and ζ but not PKC β. The L-2 cell line expressed only PKC α and ζ but L-2/PMA contain neither α, β, γ, ε nor ζ. Thus, the L-2/PMA line is deficient in all conventional and novel PKC isoforms tested. Unfortunately PKC δ could not be evaluated due to the unsuitability of current available antibodies for detection of PKC δ in these cell lines. While PKC δ could be detected clearly in rat brain, a persistent non-specific band at the appropriate molecular weight prevented detection in any of these cell lines.

3.2. *Proliferation of T cell lines*

Proliferation of the HuT 78 cell line was assessed by tritiated thymidine incorporation. Proliferation of HuT 78 was inhibited at concentrations of 150 μM H-7 (Fig. 2). Proliferation of HuT 78 was significantly inhibited at a concentration of 1×10^{-7} M staurosporine which inhibited proliferation to approximately 40% of baseline values (Fig. 3). The PKC β deficient K-4 cell line was not significantly inhibited by H-7 (Fig. 2), except at concentrations of 150 μM, when inhibition was approximately 40%. Staurosporine produced a similar inhibition of about 40%, which was considerably less than that induced in HuT 78 cells (Fig. 3). The rate of proliferation of the L-2 cell line was slightly reduced by H-7 (150 μM) with an inhibition of 20% (Fig. 2). Staurosporine (0.15 μM) produced very little inhibition of proliferation of L-2 with continuing proliferation at 73% of basal levels (Fig. 3). The L-2/PMA cell line, which is

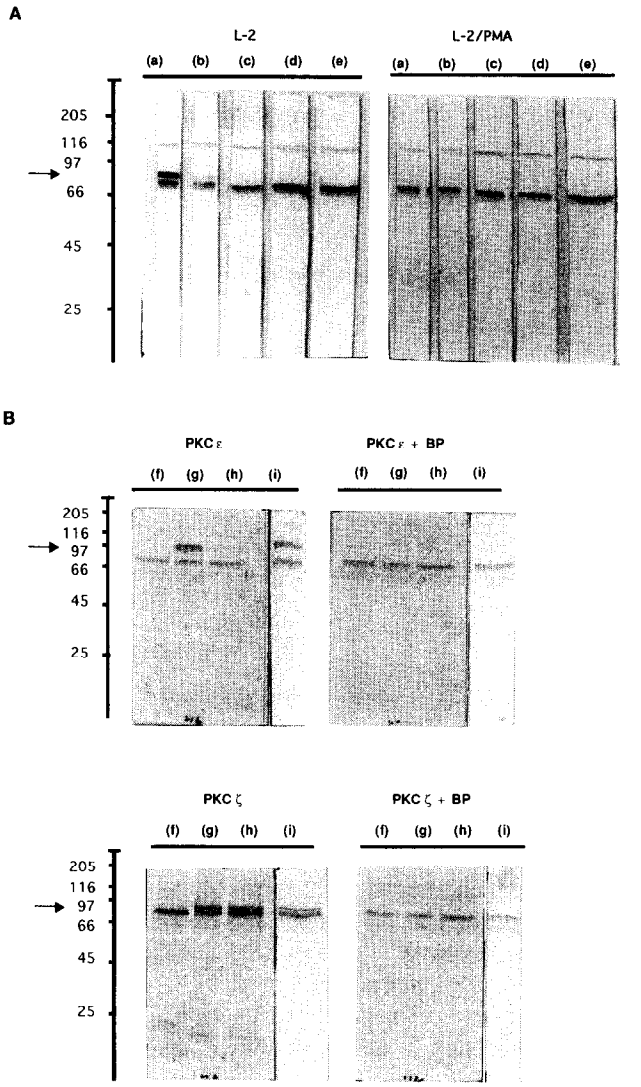

Fig. 1. Western blot analysis of PKC isoform expression in HuT 78, K-4, L-2 and L-2/PMA cells. (A) Classical PKC expression in L-2 and L-2/PMA cells; (a) PKC α, (b) PKC β, (c) PKC γ, (d) isotype control, and (e) no antibody control. (B) PKC ε and ζ expression in L-2/PMA (f), L-2 (g), K-4 (h) and HuT 78 (i) determined in the presence and absence of the immunogenic (blocking) peptide. Relevant bands are indicated with an arrow.

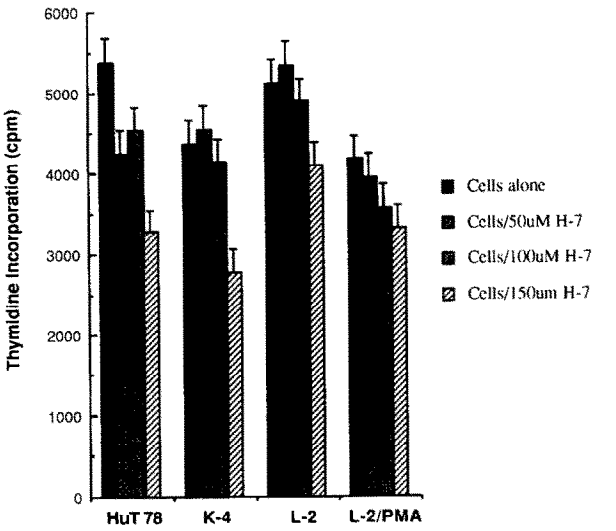

Fig. 2. Proliferation of T cell clones in the PKC inhibitor H-7. HuT 78, K-4, L-2 and L-2/PMA cells were incubated in the presence of either 0, 50, 100 or 150 μM H-7 for 24 h. Rate of proliferation was measured as described in section 2.4.

deficient in all PKC isoforms tested, was partially inhibited by H-7 and staurosporine (with PMA added to this medium). Proliferation continued at 80% and 66% of basal levels respectively (Figs. 2 and 3).

3.3. *Effects of PMA*

PMA produced growth arrest in HuT 78 cells, resulting in almost complete reduction of thymidine incorporation (Fig. 4). PMA similarly produced a reduction in thymidine incorporation in the K-4 cell line, although not to the same extent as HuT 78. In the L-2 cell line, the reduction of proliferation with PMA was only 40% of the total cellular proliferation. In the L-2/PMA cell line there was little if any further inhibition of proliferation with added PMA. The reduction in proliferation of the HuT 78 cell line and to a lesser extent K-4 and L-2 represented apoptotic cell death and was reflected in a decrease in forward scatter (FSC) on flow cytometry (Fig. 5). In HuT 78 cells there was a reduction in FSC observed in the majority of cells. In the K-4 cell line there was clearly two populations, one of which showed a forward scatter shift consistent with apoptosis and a second which was resistant to PMA-induced apoptosis. Little change in FSC was seen in either L-2 cells or L-2/PMA cells. Similar forward scatter profiles were observed over the PMA concentration range analysed (25–100 ng/ml).

4. DISCUSSION

The Protein kinase C family of enzymes comprises the conventional PKC isoforms α, β and γ, the novel PKC isoforms δ, ε, η and θ and the atypical isoforms ζ and λ [1]. PKC clearly plays a role in the proliferation of resting T cells, which can be blocked by incubation with PKC inhibitors [5]. Activators of PKC such as phorbol esters are also tumour promoters in multistage models of carcinogenesis. Calcium flux is certainly synergistic with the effects of phorbol esters on T cell proliferation [12], suggesting an involvement of conventional protein kinase C isoforms as the novel PKC isoforms are frequently calcium independent and may indeed be inhibited by calcium [13]. It has been suggested that protein kinase C inhibitors may be useful in cancer chemotherapy on the basis of inhibition of proliferation of tumour cell lines [9]. Proliferation of the HuT 78 lymphoma cell line is partially inhibited by PKC inhibitors. However proliferation of HuT 78 is inhibited to a greater extent by activation of the protein kinase C enzyme by phorbol esters. The aim of this study was to determine whether conventional protein kinase C isoforms are necessary for proliferation. We have generated a panel of cloned lines derived from HuT 78 which are deficient in one or more PKC isoforms. The cell line L-2 is deficient in PKC β and ε. Despite the fact that this cell line expresses PKC α, proliferation was not significantly inhibited by the PKC inhibitors, staurosporine or H-7. Furthermore the cell line L-2/PMA does not express PKC α, β, γ, ε or ζ. Despite this, L-2/PMA incorporated thymidine at levels comparable to the parent HuT 78 cell line. Thus we can conclude that none of these PKC isoforms are necessary for proliferation.

The molecular weight of PKC ζ expressed in these cells is approximately 80 kDa, higher than the deduced molecular mass of 67 kDa [1]. Characterisation of PKC ζ has shown it to lack a PMA-binding site and hence to be resistant to both translocation and down-regulation in response to PMA [14]. In our hands down-regu-

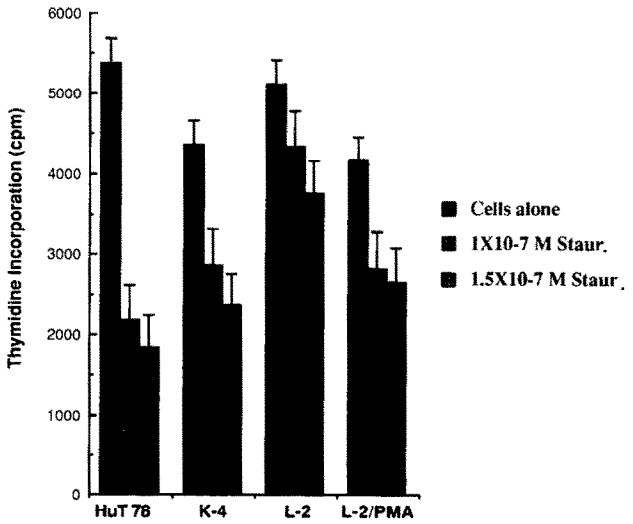

Fig. 3. Proliferation of T cell clones in the presence of the PKC inhibitor staurosporine. HuT 78, K-4, L-2 and L-2/PMA were incubated in the presence of 0, 1×10^{-7} M or 1.5×10^{-7} M staurosporine for 24 h. Rate of proliferation was assessed by [^{3}H]thymidine incorporation as described (section 2.4).

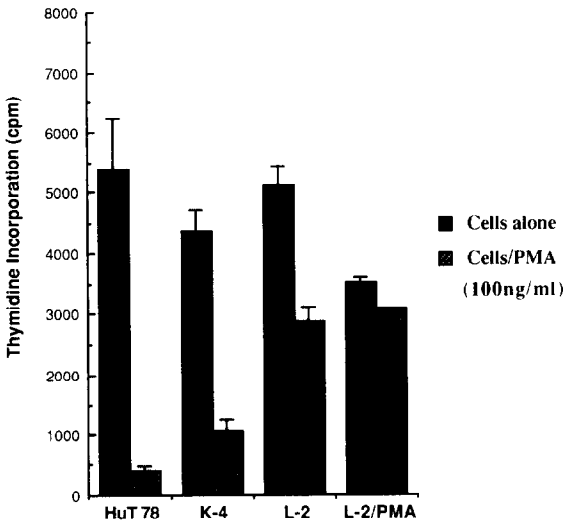

Fig. 4. Proliferation of T cell clones in phorbol myristate acetate (PMA). HuT 78, K-4, L-2 and L-2/PMA cells were incubated in the presence and absence of PMA (100 ng/ml) for 24 h. Rate of proliferation was assessed by the rate of [^{3}H]thymidine incorporation.

lation of PKC ζ was not observed in short term culture (24 h) with PMA (data not shown). However, Tsutsumi et al. [15] describe an isoform of PKC ζ (PKC ζ^{82}) in Jurkat cells which was down-regulated when cultured overnight in PMA and significantly reduced when these cells were cultured long term in PMA. Similarly, we have observed significant down-regulation of this isoenzyme on long-term culture of L-2 cells in the presence of PMA. It is unclear at what level this down-regulation occurs but it could conceivably represent a consequence of cross-talk between PKC isoforms. However, our data indicates that proliferation occurs in the absence of detectable PKC ζ. It is possible however that isoforms of PKC such as PKC δ, η or θ may be implicated in proliferation. Detection of PKC δ in these cell lines has been inconclusive due to the presence of a cross reactive band at the predicted MW of PKC δ using a commercial antibody. Since PKC δ, is partially resistant to inhibition with both staurosporine and H-7 [16,17] and is universally distributed, this may be a candidate for involvement in proliferation. However, experiments where PKC δ was overexpressed in CHO cells have suggested that this isoform may be involved in PMA-induced growth arrest rather than proliferation [18].

PMA-induced growth arrest occurs in a wide range of tumour types. It also occurs in thymocytes when this is manifested in apoptosis [19]. Striking growth arrest accompanied by cell death was observed in the HuT 78 cell line. The decrease in forward scatter seen on flow cytometry suggests that this is mediated by apoptosis. In K-4 cells there was less growth arrest than in HuT 78. Significantly the forward scatter plot shows two populations, one of which appears to be undergoing apoptosis while the second appears resistant. This is compatible with our finding that it was possible to sub-

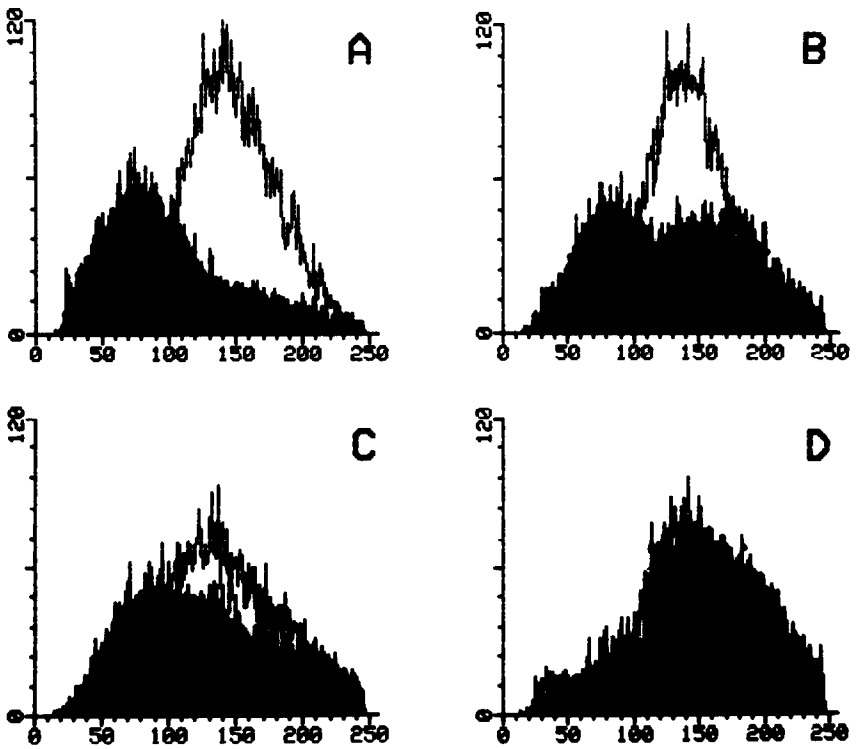

Fig. 5. Flow cytometric analysis. HuT 78 (A), K-4 (B), L-2 (C) and L-2/PMA (D) were incubated in the presence (shaded) and absence (unshaded) of PMA (50 ng/ml). Cells were fixed and their forward scatter pattern analysed by flow cytometry. The forward scatter profiles for L-2/PMA in the presence and absence of PMA (D) are super-imposed.

clone PMA-resistant cells from K-4. Little forward scatter shift and growth arrest is seen in L-2 cells which are deficient in PKC β and ε. However, the L-2/PMA cells, which are also deficient in PKC α and ζ, do not show any growth arrest in response to PMA. These data provide suggestive evidence that PKC α and ζ may be involved in growth arrest. The fact that these isoforms are universal lends further support for a common role in the regulation of the cell cycle.

A number of studies have used overexpression of individual PKC isoforms to study effects on cell growth patterns. Overexpression of PKC β results in altered pattern of growth in agar and increased tumourigenicity of fibroblast cell lines [20]. By contrast overexpression of this isoform resulted in reduced growth in the colonic tumour line HT29 [21]. Overexpression of PKC δ has been reported to result in growth arrest in CHO cells [18]. However increased expression of PKC δ has been reported in regenerating hepatocytes [22]. It is clear that there is considerable variation in the effects of PKC overexpression in different cellular systems. However, our data suggest that in replicating lymphoma cells, there is considerable redundancy in the requirements of individual enzymes in proliferation. While PKC isoenzymes may be implicated in cellular proliferation in the parent HuT 78 cell line, this is not an obligatory requirement. These data suggest that tumour therapy directed at inhibiting protein kinase C may be ineffective in the long term due to the selection of variant clones which do not utilise PKC in proliferation. Of greater interest however, may be the development of strategies based on PKC-induced growth arrest. Such strategies will require an expanded knowledge of the isoenzymes involved, their regulation and the receptors coupled to these isoenzymes.

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