Volume 301, number 3, 310–314 FEBS 10972 April 1992

Development and characterization of a protein kinase C β-isozyme-deficient T-cell line

Dermot Kelleher and Aideen Long

Department of Clinical Medicine, Trinity College Dublin, Dublin 8, Ireland

Received 10 March 1992

In the human T-cell lymphoma line, HuT 78, proliferation and phorbol ester-induced growth arrest and differentiation were inhibited by the protein kinase C (PKC) inhibitor, staurosporine. By contrast, an alternative PKC inhibitor, H-7, inhibited proliferation but not phorbol ester-induced growth arrest. The cell line was found to contain both α and β isoforms of PKC by Western blot techniques. A cell line, K-4, was cloned from HuT 78 in the presence of H-7 and this clone was found to be positive for PKC-α only. PKC-β did not return on cultivation in the absence of H-7. Proliferation of K-4 was insensitive to inhibition with both H-7 and staurosporine. However, phorbol ester-induced growth arrest remained staurosporine sensitive. Phorbol-stimulated IL-2 secretion was minimal in the PKC-β-deficient cell line. These data suggest that PKC-β may be a regulatory enzyme for proliferation and stimulated interleukin-2 secretion in HuT 78 cells. Heterogeneity of responses to PKC activation may reflect the use of different isozymes in different intracellular pathways. The K-4 cell line should provide a useful tool in the dissection of involvement of PKC isozymes in cellular function.

Protein kinase C; T lymphocyte; Interleukin 2; Staurosporine; H-7; Differentiation; Proliferation

1. INTRODUCTION

The protein kinase C (PKC) family of enzyme is involved in a wide range of cellular functions including regulation of cell proliferation and stimulus–secretion coupling. PKC appears to be coupled to a multiplicity of receptors via the generation of diacylglycerols, the physiological ligands for PKC [1]. In addition, the so-called tumour promoters, including phorbol esters and others, appear to function by activation of protein kinase C [2]. Thus, resting T-cells can be induced to proliferate in the presence of phorbol esters [3], and this proliferation may be blocked by protein kinase C inhibitors [4]. It is notable, however, that tumour cells undergo growth arrest and differentiation when stimulated with phorbol esters. K-562 cells differentiate to become erythrocytes [5] while HL-60 cells differentiate to become mature cells of the monocyte macrophage series [6]. Similarly, complex effects may be noted in the regulation of IL-2 secretion [7]. We have previously reported that PMA-induced IL-2 secretion was mediated through a pathway insensitive to the protein kinase C inhibitor, H-7 in HuT 78 cells. IL-2 secretion was inhibited by H-7 in the Jurkat cell line, suggesting the existence of multiple pathways for the generation of IL-2 secretion [4]. Thus, protein kinase C isozymes may mediate diverse effects, both up-regulating and down-regulating control of proliferation and secretion, either by utilization of different isozymes, transacting elements or substrates. Studies based on cloning of the PKC gene have demonstrated that PKC comprises a family of related enzymes comprising three conventional isoforms, α, β and γ, and four novel isoforms some of which are calcium independent (δ, ε, η, ζ) [8]. However, specific roles have not as yet been assigned to individual isozymes.

In this study we have generated a clone of the HuT 78 cell line deficient in the PKC-β isozyme expressed in the parent line. We reasoned that the selection of the cell line in the presence of the protein kinase C inhibitor H-7 would permit the selection of variants which would not require one or more PKC isozymes for proliferation. This cell line, K-4, provides a useful tool for the study in more detail of the heterogeneity of enzyme utilisation in proliferation, PMA-induced growth arrest and interleukin-2 secretion.

Correspondence address: D. Kelleher, Dept. of Clinical Medicine, Trinity College Dublin, St. James's Hospital, Dublin 8, Ireland.

2. MATERIALS AND METHODS

2.1. *Antibodies and cell lines*

The HuT 78 cell line was obtained from ATCC. Antibodies to CD3 (Leu 4), CD4 (Leu 3), CD8 (Leu 2a), T-cell receptor αβ (WT31) were obtained from Beckton Dickinson. Antibodies to PKC isozymes were obtained from Seikagaku America, Rockville, MD. All cell cultures were performed in RPMI 1640 containing 10% foetal calf serum, 2 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol (complete medium).

2.2. *Derivation of the K-4 clone*

HuT 78 cells were cultivated in the presence of H-7 (50 μM). Surviving cells were then cultivated in complete medium in the presence of

 Published by Elsevier Science Publishers B.V.

H-7 (100 μM). A cell line derived from these cultures was cloned at a concentration of 1/3 cell per well in complete medium containing 100 μM H-7 in the absence of feeder cells. One clone, K-4, was selected for further study based on its rapid growth rate in H-7-containing medium. Following selection in H-7 this cell line was maintained in complete medium. Cells used for further studies were maintained in culture in the absence of H-7.

2.3. *Phenotype analysis*

Cells were surface-labelled with murine monoclonal antibodies using fluoresceinated rabbit anti-mouse immunoglobulin (Dakopatts) as the second antibody. Data were gathered using a Beckton Dickinson FACSCAN fluorescent cell analyser and analysed using Consort 30 software.

2.4. *Thymidine incorporation assay*

HuT 78 or K-4 cells were incubated in 96-well flat-bottomed microtitre plates at a concentration of 2×10^4 cells/well for 24 h and pulsed with tritiated thymidine (0.5 μCi/well) for the last 6 h of incubation. PMA at varying concentrations was added at the start of incubation and inhibitors added 30 min prior to the addition of PMA. Samples were harvested on a multiple automated cell harvester and counted in a Beckman liquid scintillation counter.

2.5. *Interleukin-2 assay*

HuT 78 or K-4 cells plated at 10^6/ml in 0.1 or 0.2 ml volumes in 96-well U-bottomed plates were stimulated with titrated concentrations of PMA (Sigma, St Louis, MO) in the presence or absence of the enzyme inhibitor, H7 (Seikagaku America Inc, Rockville, MD), or staurosporine (Calbiochem, La Jolla, CA). After 18 h supernatants were harvested, diluted and assayed for IL-2 using a CTLL line bioassay as described previously [4]. Specificity of the assay for IL-2 was verified by blocking of proliferation with antibody to the IL-2 receptor (7D4) and antibody to IL-2 (DMS-1).

2.6. *Immunoblot studies*

Immunoblot analysis of PKC isozymes using monoclonal antibodies was performed as follows. HuT 78 or K-4 cells were washed in phosphate-buffered saline, pH 7.2, centrifuged at $250 \times g$ for 5 min and resuspended in 1 ml of buffer containing 0.5% NP40, 0.5 mM EGTA, 0.1 mM PMSF, leupeptin 10 μg/ml. This lysate was gently agitated at 4°C for 30 min and centrifuged at $200 \times g$ for 10 min to provide a total cellular PKC extract. The resultant supernatant was then spun at $100{,}000 \times g$ for 30 min at 4°C using a Beckman L5-50 ultracentrifuge equipped with a SW 41 spin-out rotor. Proteins were separated by polyacrylamide gel electrophoresis (10%) and electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked using Blotto Tween in phosphate-buffered saline at pH 7.2 and then incubated with monoclonal antibodies to PKC-α, β and γ isozymes in PBS/Blotto Tween for 18 h followed by biotinylated sheep anti-mouse antibody (1 h) and streptavidin–biotinylated horseradish peroxidase complex in 1% gelatin for 30 min. Immunoreactive bands were visualised by treatment with 3-amino-9-ethylcarbimazole. Molecular weight marker proteins were localised by staining with 0.2% Ponseau-S in 3% trichloroacetic and 3% sulphosalicylic acid.

3. RESULTS

3.1. *Phenotype of K-4*

Clone K-4, derived from the HuT 78 line, was chosen for further study on the basis of its growth rate in medium containing H-7. K-4 proliferated in RPMI containing 100 μM H-7 at rates of growth similar to that in H-7 free medium. The phenotype of K-4 was qualitatively identical with that of the parent HuT 78 cell line (Table I). It expressed CD3, T-cell receptor αβ form and HLA Class II molecules. In addition, HLA typing by microcytotoxicity showed K-4 to have the same HLA phenotype as the parent HuT 78 line. However, there were clear differences between HuT 78 and K-4 with respect to PKC isozyme expression (Fig. 1). In HuT 78, both α and β isozymes were present. Trace amounts of a 78 kDa fragment reactive with antibody to the γ isozyme were seen on some immunoblots. However in K-4 cells there was no detectable β or γ isozyme and the α isozyme was detected as a doublet with a second band of slightly lower mol. wt. This low mol. wt. species was found to be associated with the plasma membrane (data not shown).

Table I

Phenotype of cell lines used in this study

Antibody	Cell lines	
	HuT 78	K-4
CD3 (OKT3)	78	77
TCR αβ	90	76
TCR γδ	0	0
HLA DR	91	95
HLA DQ	89	91
HLA DP	92	99
LFA-1	99	NT
TfR	99	90

Phenotype of HuT 78 and clone K-4. Data represent percentage positive staining cells on indirect immunofluorescence. The second antibody used was a fluoresceinated rabbit anti-mouse Fab2 fragment. Fluorescence was analysed on a Beckton Dickinson FACSCAN using Consort 30 software. All antibodies used are as referred to in the text. TfR represents transferrin receptor. NT, not tested.

3.2. *Proliferation characteristics of the K-4 cell line*

It is apparent from Fig. 2 that proliferation of HuT 78 was inhibited to approximately 50% of baseline values by high concentrations of the protein kinase C inhibitor, staurosporine. When PMA was added differentiation of HuT 78 took place with loss of expression of transferrin receptor (data not shown) and the development of dendritic processes [9]. Significantly there was virtually complete growth arrest with consequent reduction in thymidine incorporation. This process of growth arrest was also antagonised by staurosporine, and this resulted in restoration of proliferation to approximately 50% of normal background proliferation. These data indicate that staurosporine inhibits both resting proliferation and PMA-induced growth arrest in HuT 78 cells. Similar effects on proliferation were seen with H-7 (Fig. 2b) but H-7 was not able to over-ride the down-regulatory effect of PMA on proliferation.

K-4 cells had been selected for their ability to grow in the presence of a potent PKC inhibitor H-7. Thus, not surprisingly, K-4 cells were capable of proliferating in inhibitory concentrations of staurosporine (Fig. 2c). However, PMA was still capable of inducing growth

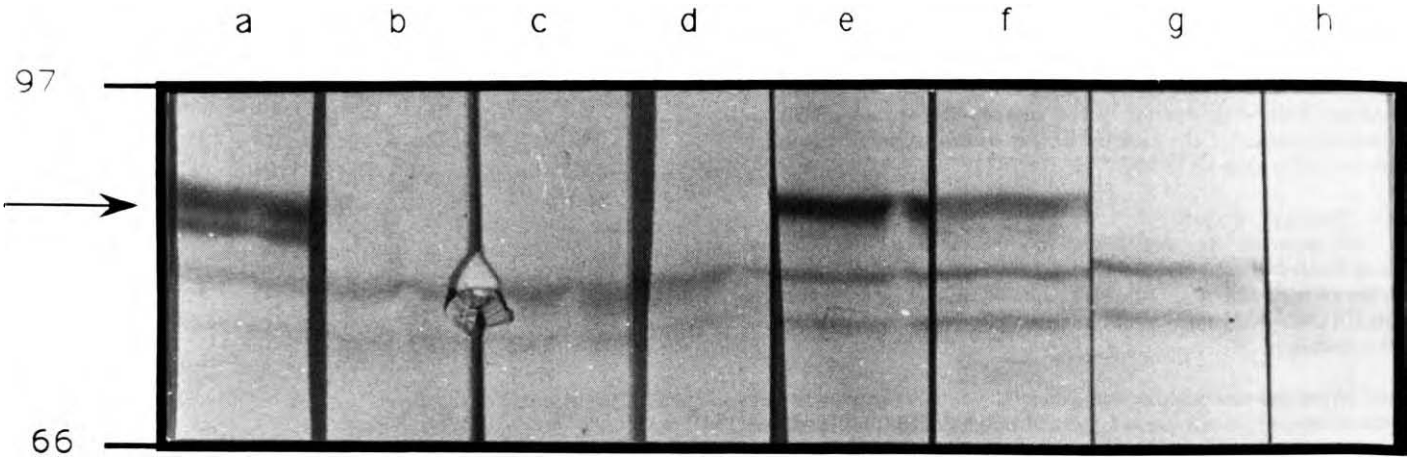

Fig. 1. Immunoblotting of protein kinase C isozymes from the K-4 clone (a–d) and from the HuT 78 parent line (e–h). a and e are stained with a monoclonal antibody to PKC-α, b and f with anti-PKC-β, c and g with anti-PKC-γ and d and h are controls. The dominant bands at 82–84 kDa are arrowed.

arrest in this cell line. Growth inhibition was reversed by staurosporine suggesting that the enzyme involved in PMA-induced growth arrest is distinct from that involved in staurosporine-sensitive proliferation of the parent line. Proliferation was no longer H-7 sensitive in this cell line; however, growth arrest was now somewhat H-7 sensitive (Fig. 2d), possibly suggesting that H-7 may have been inducing growth arrest in the parent line in a manner analogous to that described in neuroblastoma cells [10].

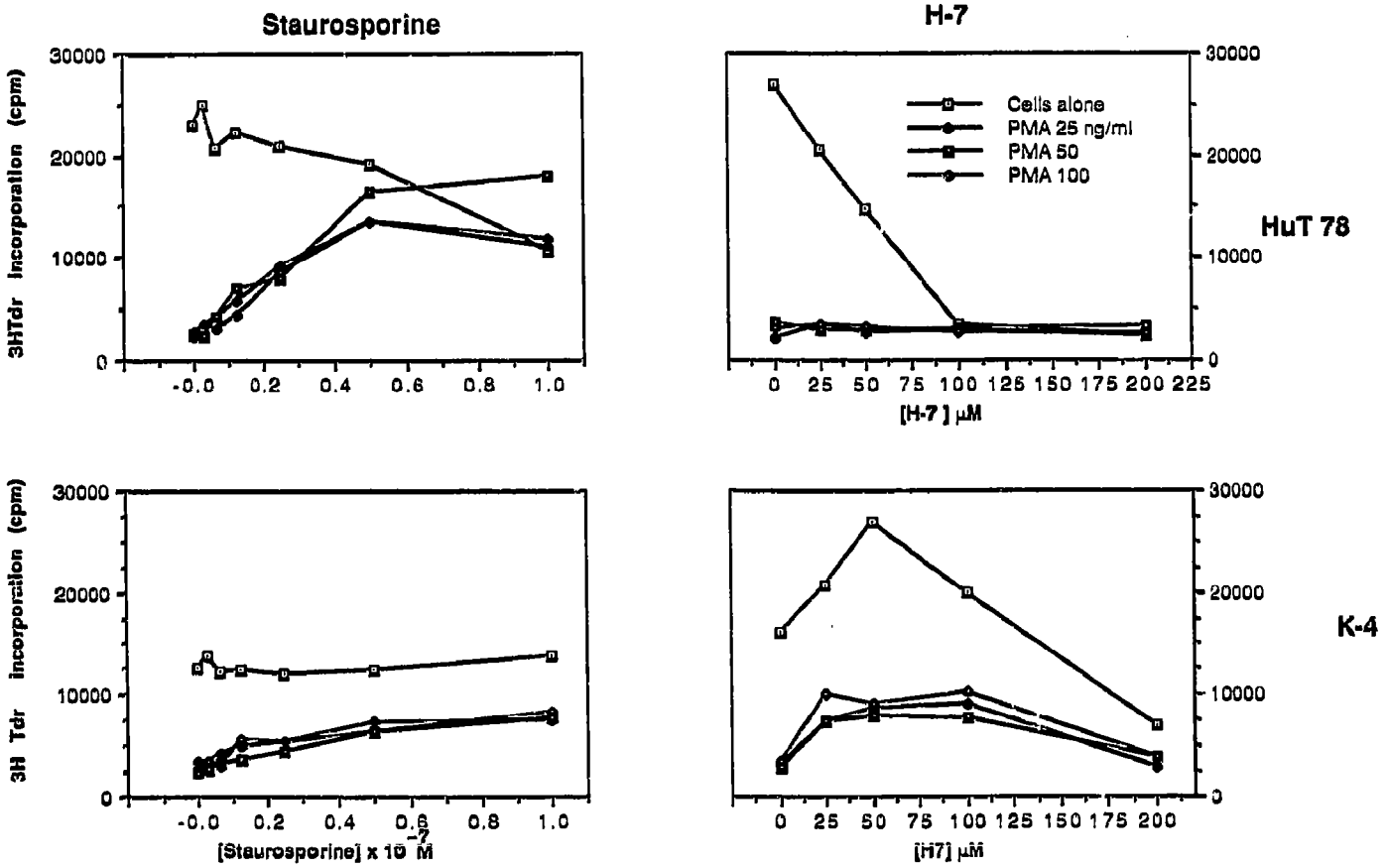

Fig. 2. Proliferation and PMA-induced differentiation measured by tritiated thymidine incorporation of HuT 78 (a and b) and K-4 (c and d) in the presence of inhibitors, staurosporine (a and c) and H-7 (b and d). PMA dosage key is given in the figure. These data are representative of three experiments.

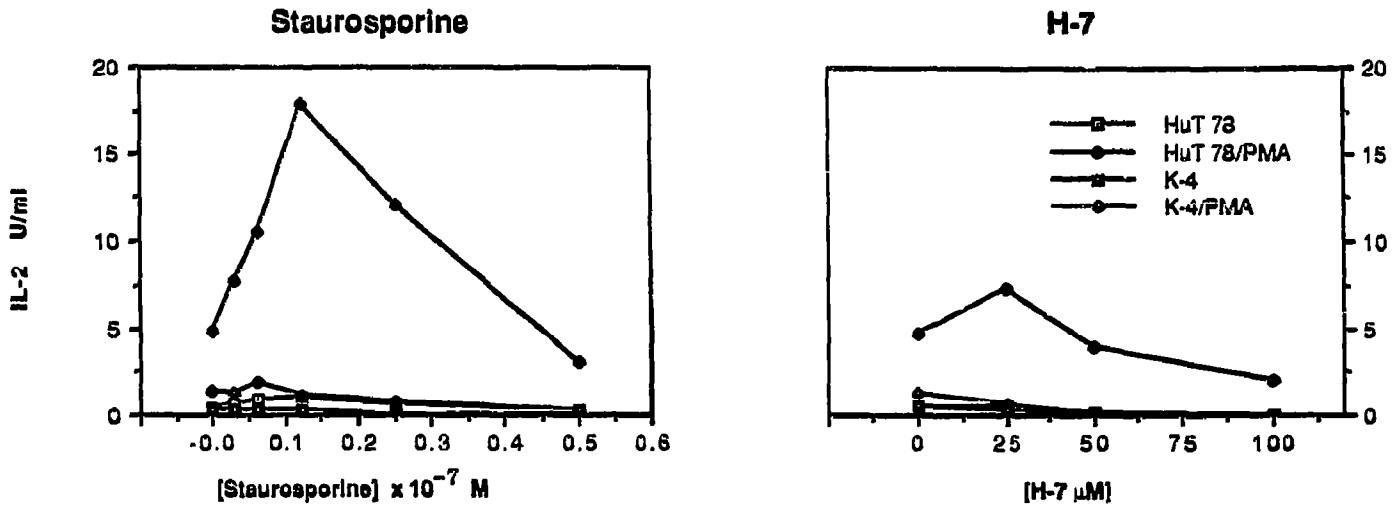

Fig. 3. Interleukin-2 production by HuT 78 and K-4 in the presence of staurosporine (a) and H-7 (b). IL-2 production is measured using a bio-assay based on thymidine incorporation by the CTLL cell line. These data are representative of three experiments.

3.3. *IL-2 secretion*

In HuT 78, PMA-stimulated IL-2 secretion exhibited a biphasic pattern of response to inhibition with staurosporine. At high concentrations of staurosporine IL-2 secretion was partially blocked (Fig. 3a). However, at lower concentrations of staurosporine PMA-stimulated IL-2 secretion was considerably enhanced. These data could be explained on the basis of two PMA-responsive pathways involved in the regulation of IL-2 secretion in this cell line, one of which is H-7 insensitive (Fig. 3b) and is involved in the stimulation of IL-2 secretion. Down-regulation of IL-2 secretion may represent a separate pathway sensitive to inhibition with lower doses of staurosporine. Stimulated IL-2 release was considerably reduced in K-4 cells which lack PKC-β suggesting an obligate requirement for PKC-β for stimulated IL-2 release in this line. This was somewhat paradoxical, since IL-2 secretion is not eliminated by H-7 in the parent line. However, it is entirely possible that the mechanism of stimulated IL-2 release, albeit H-7 insensitive, requires prior priming in the cycling cell by a mechanism dependent on PKC-β.

4. DISCUSSION

Previous studies have demonstrated heterogeneity in expression of PKC isozymes in human T-cells. In one such study [11], two isozymes were characterised using hydroxyapatite chromatography. While one of these was shown to be a β isozyme using a monoclonal antibody, the second was not characterised. In another study utilising T-cell lines, some cells expressed message for PKC-α while others expressed message for both PKC-α and -β [12]. However, individual functions have not previously been assigned to PKC isozymes in human T lymphocytes or in lymphomas. The aims of this study were to select cell lines deficient in PKC or individual isozymes with the ultimate objective of utilising such lines to examine individual functions of PKC isozymes. We elected to use immunoblot analysis of protein product to detect the presence or absence of PKC isozymes. Native PKC-β protein product was clearly undetectable in K-4 cells even when cultured in the absence of H-7. While we have not examined this cell line for message, if inhibition was at the level of message it would be expected to recover on withdrawal of H-7. K-4 cells have similar growth characteristics and phenotype to HuT 78. However, they exhibit clear differences in that proliferation is insensitive to inhibition with the PKC inhibitors, H-7 and staurosporine, in K-4 cells. These data suggest that while PKC-β may be involved in mitogenesis of the parent line, this isozyme is not essential for proliferation. However, neither H-7 nor staurosporine are specific PKC inhibitors and it is possible that their interference with growth characteristics of HuT 78 is due to inhibition of enzymes other than PKC. PMA-induced growth arrest occurred in both HuT 78 and K-4 providing suggestive evidence that the growth arrest function is mediated by the α isozyme. This view is supported by the finding that clones selected for growth in PMA at high concentrations were found to be deficient in the α isozyme (unpublished observations). Furthermore, in B-cell studies differentiation of B-cells is accompanied by loss of PKC-β and expression of increased levels of α suggesting a specific role for this isozyme in differentiation [13].

These data in total demonstrate a clear separation in the nature of the PKC isozymes involved in cell proliferation and in PMA-induced growth arrest. Since we have previously demonstrated that all calcium- and phospholipid-dependent kinase activity was blocked by H-7 in HuT 78 [4], these data suggest that the normal forms of

PKC-α, -β and -γ were not essential for proliferation in the K-4 cell line. These findings may be important since it has been suggested that PKC inhibitors may be potentially useful agents in chemotherapy [14]. Our findings suggest that their use may be limited by the development of alternative pathways of mitogenesis. An additional lower mol. wt. species of PKC-α was found to be associated with the plasma membrane in the K-4 cell line. It is notable that a mutant PKC-α molecule containing 4 point mutations preferentially associated with the plasma membrane and was found to function as an oncogene in transforming human fibroblasts [15] although this was not confirmed by other workers [16]. Hence it is possible that proliferation of the K-4 cell line is regulated by an inhibitor-insensitive membrane-associated transforming variant of PKC-α. Our findings are complementary to data from Isakov et al. [17]. In their study, a T-cell line adapted to survive in PMA was found to have reduced expression of PKC-α and to have lost expression of CD3. In K-4 cells, loss of PKC-β did not result in loss of PMA-induced differentiation and did not result in absence of CD3 expression, supporting the hypothesis that PMA-induced differentiation may be regulated by PKC-α. Stimulated IL-2 release was considerably reduced in K-4 cells which lack PKC-β suggesting an obligate requirement for PKC-β in this function. However, in order to confirm this requirement it will be necessary to perform transfections of the PKC-β isozyme back into the isozyme-deficient variants.

While we have examined only three isoforms of PKC in this study, recent work has demonstrated further isoforms of PKC [18], including calcium-independent forms [19]. These data suggest that cellular responses to stimulation with phorbol esters are complex and represent the summation of effects on multiple isoforms which may operate on mutually antagonistic pathways. Furthermore, these data indicate that different enzymes were involved in proliferation and growth arrest in HuT 78 and suggest that PKC-α may be the critical isozyme in PMA-induced growth arrest. The reduction in IL-2 secretion and the loss of sensitivity to inhibition of proliferation seen in K-4 cells suggest that these functions may be mediated by PKC-β in the parent line. Definitive confirmation will require transfection of K-4 cells with the PKC-β gene to confirm involvement of this isozyme in proliferation and IL-2 secretion. Thus, the K-4 line should prove to be a useful tool in the dissection of individual functions of PKC isozymes.

This work was supported by a grant from the Cancer Research Advancement Board. D.K. is a Wellcome Senior Fellow in Clinical Science.

REFERENCES

[1] Nishizuka, Y. (1986) Science 233, 305–310.
[2] Horowitz, A.D., Fujiki, H., Weinstein and I.B. et al. (1983) Cancer Res. 43, 1529–1535.
[3] Isakov, N. and Altman, A. (1987) J. Immunol. 138, 3100–3107.
[4] Kelleher, D., Pandol, S.J. and Kagnoff, M.F. (1988) Immunology 65, 351–355.
[5] Melloni, G.F., Michetti, M., Sacco, O., Cakiroglu, A.G., Jackson, J.F., Rufkind, R.A. and Marks, P.A. (1987) Proc. Natl. Acad. Sci. USA 84, 5282–5287.
[6] Bories, D.R.M., Solomon, D.H., Darzynikiewicz, Z. and Cayre, Y.E. (1989) Cell 59, 959–964.
[7] Weiss, A., Manger, B. and Imboden, J. (1986) J. Immunol. 137, 819–825.
[8] Nishizuka, Y. (1988) Nature 334, 661–665.
[9] Kelleher, D., Murphy, A. and Cullen, D. (1990) Eur. J. Immunol. 20, 2351–2354.
[10] Felipo, V., Minana, M. and Grisolia, S. (1990) J. Biol. Chem. 265, 9599–9601.
[11] Beyers, A.D., Hanekom, C., Rheeder, A., Strachan, A.F., Wooten, M.W. and Nel, A.E. (1988) J. Immunol. 141, 3463–3470.
[12] Koretzky, G.A., Wahl, M., Newton, M.E. and Weiss, A. (1989) J. Immunol. 143, 1692–1695.
[13] Mischak, H., Kolch, W., Goodnight, J., Davidson, W.E., Rapp, U., Rose-John, S. and Mushinski, J.F. (1991) J. Immunol. 147, 3981–3987.
[14] Minana, M.D., Felipo, V., Cortes, F. and Grisolia, S. (1991) FEBS Lett. 284, 60–62.
[15] Megidish, T. and Mazurek, N. (1989) Nature 342, 807–811.
[16] Borner, C., Filipuzzi, I., Weinstein, I.B. and Imber, R. (1991) Nature 353, 78–81.
[17] Isakov, N., McMahon, P. and Altman, A. (1990) J. Biol. Chem. 265, 2091–2097.
[18] Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. and Nishizuka, Y. (1988) J. Biol. Chem. 263, 6927–6932.
[19] Ohno, S., Akita, Y., Konno, Y., Imajoh, S. and Suzuki, K. (1988) Cell 53, 731–741.