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Isolation of protein kinase C subspecies from a preparation of human T lymphocytes

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Using a preparation of purified human T lymphocytes, we were able to resolve a partially purified protein kinase C (PKC) enzyme fraction into two distinct subspecies, of approximately equal activity. Biochemical and immunocytochemical analysis revealed that these fractions closely resembled the type II(β) and type III(α) PKC subspecies previously identified and characterised from brain tissue. These results provide valuable information for further studies on the role of individual PKC subspecies in T lymphocyte proliferation.

Protein kinase C; T lymphocyte; Cell proliferation

1. INTRODUCTION

Information transduction into cells via the bifurcating signal pathway involving calcium mobilization and PKC activation is a mechanism utilized by a large number of hormones, neurotransmitters and growth factors (reviews [1–3]). The diverse physiological responses that can be promoted by such a variety of stimuli acting through a common pathway appears to result, in part, from molecular heterogeneity within the individual components of the signal transduction machinery. For PKC, realisation of this phenomenon occurred relatively recently, and can be attributed primarily to molecular cloning analysis of the enzymes isolated from the brain tissue of several mammalian species (see [4] for references to molecular cloning studies, and also [5–7]. PKC is now known to exist as a family of multiple subspecies, which may be provisionally grouped according to common structural features [7,8]. The complete primary structures of one group of subspecies, having α-, βI-, βII- and γ-sequences, have been deduced by analysis of the cDNA sequences obtained from brain and spleen libraries. These sequences have been shown to encode the enzyme fractions designated type I(γ), type II(βI + βII) and type III(α), resolved from a purified preparation of rat brain enzyme by hydroxyapatite column chromatography [9–11]. (The system of nomenclature used herein for the PKC subspecies has been explained previously [8], as have the experimental approaches that allowed the identification of the cDNA clones that correspond to the enzyme fractions resolved from rat brain by hydroxyapatite column chromatography [10–12].) In addition, a second group of enzyme subspecies, designated δ, ϵ and ζ, have been identified [4,7], that are closely related to, but clearly different from, the first group. The enzyme subspecies that comprise the known PKC enzyme family appear to have a slightly different mode of activation and to exhibit a differential distribution among various tissues and cells [12–19]. These

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Abbreviations: PKC, protein kinase C; FPLC, fast-protein liquid chromatography

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properties per se are likely to be important factors in determining the functional specificity of the enzyme within particular cell types.

In human T lymphocytes, activation of PKC by either phorbol ester or diacylglycerol analogues has been shown to act synergistically with calcium ionophore to induce cell proliferation [20–22]. Human T lymphocytes represent, therefore, a convenient non-clonal cell type suitable as a model for studying the role of the individual PKC subspecies in the molecular events underlying cell proliferation. As a preliminary approach to such studies, this paper reports a biochemical and immunocytochemical investigation of the PKC subspecies expressed in a pure population of resting human T lymphocytes.

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

Calf thymus H1 histone was prepared as previously described [23]. Phosphatidylserine and 1,2-diolein were from Serdary Research Laboratories. [γ-^{32}P]ATP was purchased from Amersham.

2.2. *Purification of human T lymphocytes from whole blood*

Peripheral venous blood (400 ml) was collected from healthy volunteers and depleted of erythrocytes by mixing with 0.75 vols of 1% (w/v) dextran in normal saline and incubating at room temperature for 45 min. Mononucleocytes were then separated by centrifugation of the plasma over Ficoll-Hypaque (Nycomed, Norway) and depleted of B lymphocytes and macrophages, firstly, by passage through a nylon wool column, as described by Dixon and Uyemura [24] (except that all procedures were carried out at room temperature), and then by incubation in plastic culture dishes (2×10^7 cells/dish) containing 20 ml RPMI 1640 (Gibco) and 10% (v/v) autologous serum, for 30 min at room temperature. Finally, the T lymphocyte preparation was treated with T cell 'Lymphokwik' (One Lambda Inc., CA), at 1×10^7 cells/0.8 ml reagent, to eliminate contaminating B lymphocytes, macrophages, granulocytes and pre-activated T lymphocytes. Using this method, 400 ml of blood yielded approx. 5×10^7 resting T lymphocytes, that were >99% positive for the CD3 antigen and 0% positive for the HLA-DR antigen, as determined by immunocytochemical staining with the appropriate monoclonal antibodies. Likewise, immunocytochemical staining revealed that the preparation consisted of approx. 60% CD4 antigen positive cells and 40% CD8 antigen positive cells.

2.3. *Partial purification of PKC from human T lymphocytes*

All procedures were carried out at 4°C unless otherwise stated. The preparations of purified T lymphocytes (approx. 5×10^7 cells) were washed once in 10 ml of 20 mM Tris-HCl (pH 7.5 at room temperature), containing 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 20 μg/ml leupeptin, before being disrupted by sonication on ice (4×15 s; Kontes cell disrupter) in 5 ml of the same buffer (pH 7.5 at 4°C), and then homogenized using a Potter-Elvejhem-type teflon/glass homogenizer. The homogenate was centrifuged at $1500 \times g$ for 10 min, and the pellet was resuspended in 2 ml of the buffer and re-centrifuged as above. The supernatant fractions from these two steps were pooled, and the buffer was adjusted to 1% (v/v) Triton X-100. The suspension was stirred for 30 min at 4°C, sonicated (4×15 s), and then centrifuged at $100000 \times g$ for 30 min. The supernatant from this centrifugation step was then applied to a TSKgel DEAE-5PW column (0.75 × 7.5 cm, Toyo Soda, Tokyo) connected to an FPLC system (Pharmacia), that had been pre-equilibrated with 20 mM Tris-HCl at pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA and 10 mM 2-mercaptoethanol (buffer A). Protein was eluted from the column by the application of a linear concentration gradient of NaCl in buffer A (0–400 mM NaCl, in 20 ml buffer), at a flow rate of 0.5 ml/min. Fractions (0.5 ml) were collected and assayed for PKC activity as described below. The fractions containing PKC activity were pooled (see fig.1A), and then subjected to chromatography on a hydroxyapatite column (0.78 × 10 cm, type S, Koken Co. Ltd, Tokyo), as described previously [25]. For characterisation of the enzyme subspecies that were eluted from the hydroxyapatite column, the fractions containing peak enzyme activity were pooled and dialyzed against buffer A containing 10% (v/v) glycerol.

2.4. *Assay of PKC activity*

Enzyme activity was assayed by measuring the incorporation of $^{32}P_i$ into H1 histone from [γ-^{32}P]ATP, as described previously [15]. To the standard reaction mixture were added 8–20 μg/ml phosphatidylserine, 0.8–2 μg/ml 1,2-diolein and calcium chloride (as indicated), and 20 μl enzyme fractions. Non-PKC kinase activity was measured by replacing phospholipid with 20 mM Tris-HCl, and by replacing calcium chloride with 0.5 mM EGTA.

2.5. *Immunocytochemical staining of human T lymphocytes*

Paraformaldehyde (0.05%, w/v) fixed, Triton X-100 (0.1%, v/v) permeabilized cells were subjected to immunocytochemical staining with polyclonal antibodies raised against synthetic oligopeptides. The polyclonal antibodies designated CKpV5βI-a, CKpV5βII-a and CKpC1α-a, were prepared against the sequence-specific oligopeptides SYTNPEFVINV (type II(βI) enzyme subspecies, residues 661–671, C_4–V_5 region), SFVNSEFLKPEVKS (type II(βII) enzyme subspecies, residues 660–673, C_4–V_5 region) and VYPANDSTASQDVANR (type III(α) enzyme subspecies residues 4–19, V_1 region), respectively. Incubation with these antibodies was carried out overnight at 4°C, in 0.1 M Tris-HCl at pH 7.6, containing 4% (v/v) normal human serum, at a dilution that produced no positive immunoreaction when the antibodies had been pre-adsorbed by the appropriate peptide. Immunoreactive material was subsequently visualised using an anti-rabbit immunoglobulin streptavidin/biotin-peroxidase immunostaining kit (Biogenex, CA), employing 3,3′-diaminobenzidine as substrate. Cells were finally washed, dehydrated, mounted in Entellan (Merck), and photographed under a Nikon Optiphot microscope, using Fuji Minicopy 32 film.

3. RESULTS AND DISCUSSION

3.1. *Partial purification and resolution of PKC subspecies*

Chromatography of the T lymphocyte extract on a TSK gel DEAE-5PW column produced a single major peak of calcium- and phospholipid-dependent protein kinase activity (fig.1A), which, when applied to a hydroxyapatite column, could be resolved into two distinct enzyme fractions (fig.1B), eluting at approx. 90 mM (peak I) and 130 mM (peak II) potassium phosphate concentration, respectively. These fractions were estimated to contain approx. equal levels of enzyme activity (peak I, 49%; peak II, 51%). The resolution of only two distinct PKC subspecies from human T lymphocytes is consistent with the results of similar studies from this laboratory [10,11,15,19] and others [9,12,16], using rat brain and various other tissue sources. These studies reported that, whereas central nervous tissue expressed types I(γ), II(β) and III(α) enzyme subspecies, other tissues such as liver, spleen and kidney, expressed types II(β) and III(α) in varying ratios, but not type I(γ). In this study, the concentration of potassium phosphate at which the two enzyme fractions from human T lymphocytes eluted from the hydroxyapatite column was found to correspond closely to the type II(β) and type III(α) enzyme subspecies from rat brain. Further analysis was needed, however, to confirm their identity.

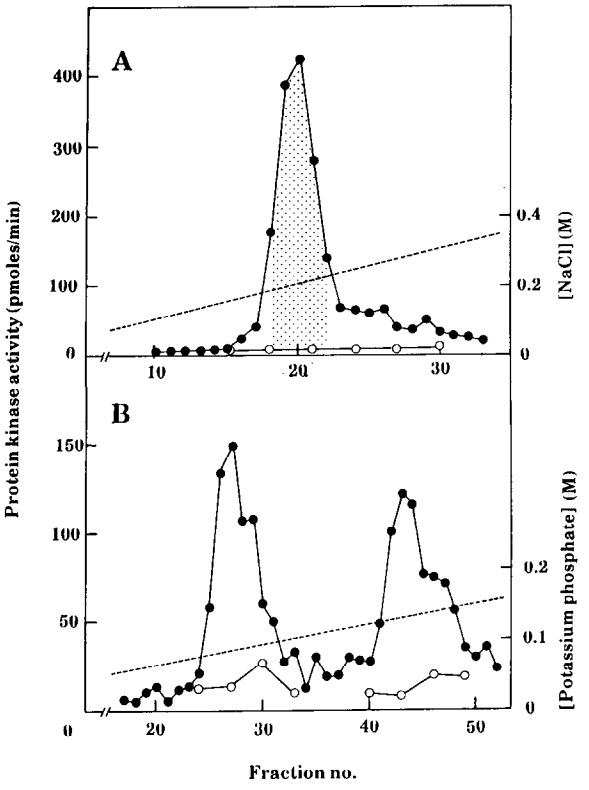

Fig.1. Partial purification and resolution of PKC subspecies from human T lymphocytes. Protein kinase activity was assayed with 0.3 mM calcium chloride, 20 μg/ml phosphatidylserine and 2 μg/ml 1,2-diolein (●) or in the presence of 0.5 mM EGTA (○), as described in section 2. (A) DEAE-5PW profile. Fraction = 0.5 ml. Shaded area subjected to chromatography on a hydroxyapatite column. (B) Hydroxyapatite profile. Fraction = 1.0 ml.

3.2. *Calcium-, diacylglycerol- and phospholipid-sensitivity of the resolved PKC subspecies*

Fig.2 shows the calcium-dependent activation, in the presence of 1,2-diolein (diacylglycerol) and phosphatidylserine, of the peak I and peak II PKC enzyme fractions obtained from human T lymphocytes. In the presence of 1,2-diolein alone, neither subspecies showed calcium-dependent protein kinase activity toward H1 histone. In contrast, in the presence of phosphatidylserine alone, both

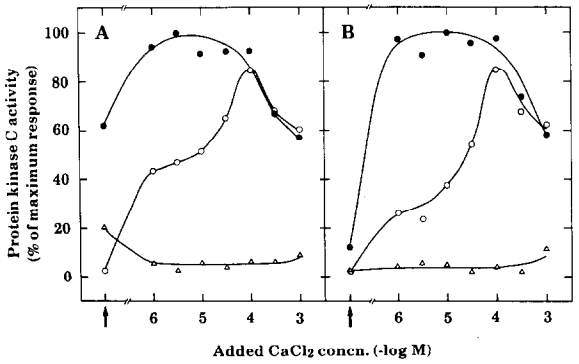

Fig.2. Activation of PKC subspecies isolated from human T lymphocytes by 1,2-diolein and phosphatidylserine, at various concentrations of calcium chloride. Protein kinase C activity was assayed for each enzyme subspecies in the presence of 0.8 μg/ml 1,2-diolein (△), 8 μg/ml phosphatidylserine (○) or both lipids (●), at various concentrations of calcium chloride, as described in section 2. Where indicated with arrows, 0.5 mM EGTA was added instead of calcium chloride. (A) Peak I; (B) peak II. Data are the mean values obtained from two independent enzyme preparations, expressed as a percentage of the maximum response obtained in the presence of diolein and phosphatidylserine.

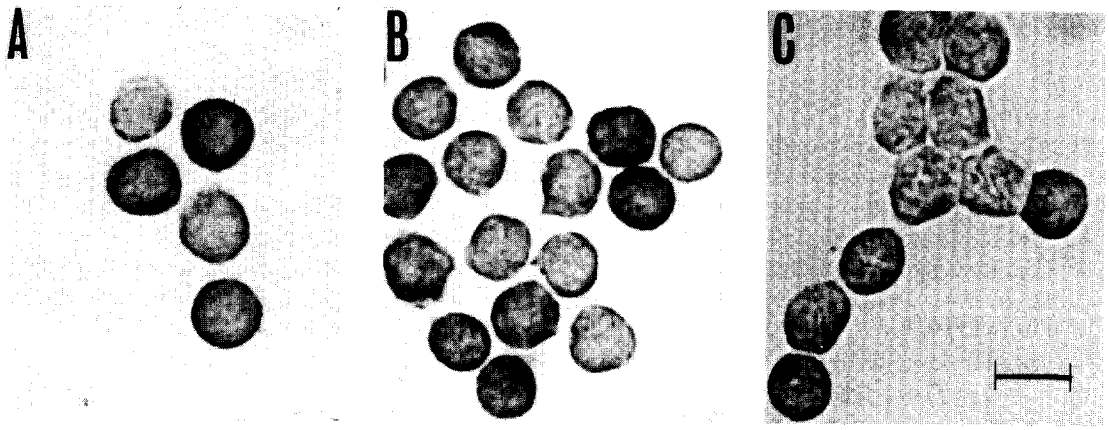

Fig.3. Immunocytochemical staining of human T lymphocytes with subspecies-specific anti-PKC polyclonal antibodies. Human T lymphocytes were stained with polyclonal antibodies as described in section 2. (A) CKpC1α-a, anti-type III(α) antibody; (B) CKpV5βII-a, anti-type II(βII) antibody; (C) CKpV5βI-a, anti-type II(βI) antibody. Control cells, where the primary antibody was replaced with Tris-buffered saline, did not show a positive immunoreaction. In this photograph, oil immersion was used under a $\times$ 100 objective. Bar = 20 μm.

enzyme subspecies were activated in a calcium-dependent manner. Addition of 0.1 mM calcium ions resulted in approx. 80% of maximal activity, with concentrations higher than this having an inhibitory effect. The addition of both 1,2-diolein and phosphatidylserine had a marked effect on the enzyme activity, and revealed a characteristic difference between peak I and peak II enzyme fractions. The peak I enzyme activity could be stimulated to >60% of maximal activity in the nominal absence of calcium ions, whereas the peak II enzyme showed <20% of maximal activity. With the addition of only micromolar concentrations of calcium ions, however, both enzymes showed nearly full activity. This mode of activation of peak I and II enzymes is very similar to type II(βI+βII) and type III(α) enzyme subspecies purified from rat whole brain [14] and bovine cerebellum (Shearman, M.S., unpublished), and provides further evidence that the two enzyme subspecies isolated from human T lymphocytes are closely related to these brain enzymes. The calcium-independent activation of the type II(β) enzyme subspecies in the presence of diacylglycerol and phosphatidylserine is of considerable interest, as it suggests that extracellular stimuli, which can promote the formation of diacylglycerol in the absence of calcium mobilization (for example, by the hydrolysis of phosphatidylcholine) may preferentially activate this PKC subspecies.

3.3. *Immunocytochemical staining of human T lymphocytes*

The expression, in human T lymphocytes, of PKC subspecies corresponding to the brain type II(β) and type III(α) enzymes was further investigated by immunocytochemical staining of the cells with polyclonal antibodies raised against peptide sequences uniquely present in the deduced primary structure of the type II(βI), type II(βII) and type III(α) enzymes (fig.3). All three of these antibodies gave a positive immunoreaction in the T lymphocytes, whilst an antibody raised against a peptide sequence uniquely present in the type I(γ) PKC sequence (CKpV5γ-a; DARSPTSPVPVPV, residues 684–696, V_5 region), gave no positive staining (not shown). For each antibody, the staining was predominantly cytoplasmic, although some membrane localization was noted in the more intensely staining cells, and was heterogeneous within the T cell population. A comparison of the immunocytochemical staining obtained with the anti-PKC antibodies with that obtained with antibodies against the CD4 and CD8 antigens, suggests that the heterogeneity observed is not related to the helper/suppressor T lymphocyte subsets (Berry, N., unpublished). Western blotting analysis with the antibody against the type II(βII) enzyme subspecies has confirmed that the epitope against which it was raised is present in the peak I enzyme fraction, but not in the peak II fraction. In

addition, the protein containing this amino acid sequence has a molecular mass very similar to that of the rat whole brain and bovine cerebellum type II(βI+βII) enzymes, and is present at a much higher ratio than the type II(βI) enzyme, in the peak I fraction.

The results presented above show that the PKC activity isolated from resting human T lymphocytes can be resolved into two distinct enzyme fractions, which – as assessed by biochemical and immunocytochemical criteria – correspond closely to type II(βI+βII) and type III(α) PKC subspecies that have been previously isolated and characterised from brain tissue. Partial characterisation of the T lymphocyte enzymes suggests that, in certain cellular environments, one of the subspecies may become preferentially activated. The possibility that the different enzyme subspecies may fulfil defined temporal or spatial functions in the biochemical mechanisms underlying T lymphocyte proliferation is an important focus of future studies.

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