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Protein kinase C isotypes θ, δ and η in human lymphocytes: differential responses to signalling through the T-cell receptor and phorbol esters

C. KEENAN, A. LONG, Y. VOLKOV & D. KELLEHER *Department of Clinical Medicine, Trinity College Dublin, Ireland*

SUMMARY

The repertoire of novel and atypical protein kinase C (PKC) isotypes present in human T cells and their subcellular localization have not been fully characterized. We detected calcium-independent PKC activity in whole cell fractions from unstimulated peripheral blood lymphocytes (PBL). Towards an understanding of the role of PKC isoforms in lymphocyte activation we have studied the expression of calcium-independent PKC isoforms θ, δ and η in PBL. With isoform-specific antibodies we detected the presence of PKC θ and δ in whole cell fractions from unstimulated human PBL by Western blot analysis. In addition, immunocytochemical analysis confirmed the presence of the novel PKC isoform PKC η in PBL. Using immunocytochemistry, PKC θ, δ and η had distinct patterns of redistribution following activation by phorbol myristate acetate (PMA). However, signalling through the T-cell receptor (TCR) did not appear to induce such changes in isoenzyme redistribution. These findings indicate that activation of lymphocytes either through the TCR–CD3 complex or with PMA induces different signalling pathways with respect to calcium-independent isoenzymes. Signalling through receptors other than the CD3 complex may be involved in activation of these isotypes.

INTRODUCTION

The protein kinase C (PKC) signal transduction pathway is intimately linked with T-lymphocyte activation. Cross-linkage of antigen, presented in the context of major histocompatibility complex (MHC), with T-cell receptor (TCR) stimulates membrane phospholipid turnover which ultimately gives rise to PKC activation. Several lines of evidence have implicated PKC as being important in T-cell activation.[1] PKC inhibitors block T-lymphocyte activation and PKC activators induce interleukin-2 (IL-2) secretion and IL-2 receptor expression – features associated with T-cell proliferation.[2] Activation of this family of enzymes in T cells also leads to phosphorylation of lymphocyte surface molecules CD3, CD4, CD8, lymphocyte function-associated antigen-1 (LFA-1) and additional endogenous substrates that are involved in cell growth and differentiation.[3]

The PKC family has been classified into three subgroups based on different enzyme properties and structural features. The conventional PKCs (cPKC) α, β1, β2, and γ are dependent on calcium and phosphatidylserine (PS) for activity and can be activated by diacylglycerol (DAG) or phorbol esters. Additional endogenous activators include *cis* unsaturated fatty acids and lysophosphatidylcholine.[4] The new PKCs ε, δ, η, and θ are known as the novel or new PKCs (nPKC). The distinguishing feature of nPKC from traditional cPKC is they lack a C_2 domain which allows them to function in a calcium-independent manner. Individual members of this class are activated by different combinations of lipids, for example, PKC ε is activated by *cis*-unsaturated lipids but PKC δ is not. Similarly, different patterns of tissue expression have been observed for PKC ε and δ.[4] The final group of atypical PKCs ζ, ι and λ do not require calcium or DAG for activation. PKC ζ possesses only one cysteine-rich zinc finger motif compared to the other cPKC and nPKC group members which have two. This isoform is also dependent on PS for activation but does not require DAG for activity and is insensitive to phorbol esters.[4] PKC μ is one of the most recently described isoforms which has similar characteristics to the atypical isoforms but has been shown to be sensitive to activation by phorbol esters.[5]

Some studies have described the distribution of PKC isoforms in lymphocytes. Jurkat cells, a human T leukemic cell line, possess PKC proteins α, β, δ, ε, ζ[6] and θ.[7] However, the expression of novel and atypical isoforms in normal human T cells is less well characterized. Previous studies examining the expression of the three groups of PKC isoforms in a number of different tissues have indicated that PKC θ, δ and η are the major PKC isoforms in murine T cells and human T-cell lines.[6–9] It was for this reason that we decided to examine the expression and distribution of the novel isoforms

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Abbreviations: DAG, diacylglycerol; PBL, peripheral blood lymphocyte; PKC, protein kinase C; PMA, phorbol myristate acetate; PS, phosphatidyl serine.

Correspondence: Dr D. Kelleher, Department of Clinical Medicine, Trinity Centre for Health Sciences, St James's Hospital, Dublin 8, Ireland.

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PKC θ, δ, and η in human lymphocytes. Increasing evidence exists to suggest that subcellular localization of PKC isoforms serves to define the activator requirements and specific cellular functions by directing the enzyme to its target substrates. We also set out to examine subcellular localization of these isoforms by immunocytochemistry following PBL activation with PMA and following T-cell stimulation through the TCR–CD3 complex.

MATERIALS AND METHODS

Materials and cells

Monoclonal mouse antibodies to PKC θ and PKC δ used for Western blotting were obtained from Affiniti, Nottingham, UK. PKC θ, δ and η immunofluorescent assay kits were obtained from R&D Systems, Berkeley, CA. Secondary biotinylated anti-rabbit/anti-mouse antibodies and streptavidin alkaline phosphatase were obtained from Amersham Life Sciences, UK. Nitroblue tetrazolium (NBT), and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were obtained from Promega Corp., Madison, WI. Phorbol myristate acetate (PMA), myelin basic protein (MBP), histone and kemptide were obtained from Sigma, Poole, Dorset, UK. The anti-CD3 (OKT3) and anti-IE (Y-17) producing hybridomas were obtained from the American Type Culture Collection (ATCC; nos CRL 8001 and HB 179 respectively). PKC ε peptide was obtained from Dr Pat Harriot, Queen's University, Belfast. Peripheral blood lymphocytes (PBL) were obtained via venepuncture followed by Ficoll–Hypaque sedimentation from healthy normal volunteers.

Cell extract preparation

Total cell extracts were prepared by solubilizing the lymphocytes in 0·5% (w/v) Nonidet P-40 in phosphate buffered saline (PBS) containing 0·5 mM ethylene glycol-bis(β-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA), 1 mM phenyl methyl sulphonyl fluoride (PMSF) and 10 μg/ml leupeptin. Cells were resuspended at a concentration of 1×10^7 cells/ml of lysis buffer. The lysate was agitated for 30 min at 4° and centrifuged at 200 ***g*** for 10 min. The resultant supernatant was spun at 100 000 ***g*** for 30 min using a Beckman L5-50 ultracentrifuge with a SW41 spin-out rotor. Whole cell lysates were stored at −70° for kinase assay analysis and −20° for Western blotting. Protein concentration was determined according to the method of Bradford.[10]

Peptide kinase assay

This assay was adapted from a previously described protocol[11] with some alterations. A peptide derived from the pseudosubstrate site of PKC ε (ERMRPRKRQG<u>S</u>VRRRV) was used as specific kinase substrate.[12] PKC activity was measured in total cell extracts by incorporation of ^{32}P into the PKC-selective peptide substrate. The reaction mixture consisted of 10 μl of cell lysate (0·5–1 mg/ml protein), 10 μl of 10 mM peptide substrate (in 50 mM Tris buffer), 20 μl of component mix, 5 μl of 10 mM PMA and 10 μl of 100 mg/ml phosphatidyl serine (PS). The calcium-independent component mix consisted of 1·25 mM ethylenediamine tetra-acetic acid (EDTA), 5·0 mM EGTA, 6·0 mM $MgCl_2$ with 0·18% 2-mercaptoethanol added fresh each day while the calcium-dependent component mix contained 1·25 mM EDTA, 1·25 mM EGTA, 30 mM $MgCl_2$ and 5·0 mM $CaCl_2$. The total volume was adjusted to 90 μl with 50 mM Tris buffer, pH 7·5. The reaction was initiated by adding 10 μl of 250 mM [^{32}P]ATP (0·5 μCi/tube). Following incubation for 10 min at 25°C, the reaction was terminated by addition of 10 μl of 5% acetic acid. Individual samples at 90 μl were spotted onto 2·5 × 2·5 cm pieces of P81 Whatman paper which were washed twice for 10 min with 5% acetic acid. Radioactivity was measured by liquid scintillation (1500 Tri-Carb Liquid Scintillation Counter) using Ecoscint scintillation cocktail. Non-specific binding of PKC was determined by carrying out the reaction in the absence of activators PS and PMA.

Immunoblot studies

Proteins (10–20 μg/well) from total cell lysates were separated by 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. 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 was subsequently incubated with monoclonal antipeptide antibodies to specific isoforms for 18 hr. Negative controls were incubated with isotype-matched irrelevant antibody from mouse. The membrane was then incubated with biotinylated rabbit anti-mouse antibody for 1 hr, followed by streptavidin–alkaline phosphatase complex for 30 min. Specific bands were visualized using NBT and BCIP.

Immunocytochemistry

PBL were isolated by Ficoll–Hypaque gradient centrifugation. Cells, at a concentration of 1×10^6 cells/ml, were incubated alone (resting) or in the presence of PMA (25 ng/ml) or OKT3 (1 : 50 dilution of hybridoma supernatant) in flasks for 60 min at 37°. T lymphocytes were purified from PBL; firstly monocytes were removed by adherence to plastic, T cells were then isolated by panning with antibodies against CD20, CD56, CD16, CD11c and anti-DR. Purity of lymphocytes was confirmed by flow cytometry. Lymphocytes were pelleted and washed twice in Hanks' balanced salt solution with 2% HEPES, and once in PBS. Cytospins were prepared of resting or activated PBL/T lymphocytes at a concentration of 5×10^5 cells/ml. Cells were fixed in cold acetone for 10 min. Non-specific binding sites were blocked by incubation of cells for 15 min with normal goat serum. Isoform-specific antisera was applied to the cells for 2 hr. Negative controls consisting of PKC isoform-specific antibodies with immunogenic peptide to block specific staining were also incubated for 2 hr. Fluorescein isothiocyanate (FITC)-conjugated secondary antibody was then applied for 1 hr. Cells were mounted in medium containing 1,4-diazabicyclooctane (DABCO). Fluorescence was observed using a Leitz microscope. Photographs were taken using a Wild Heerburg camera under water immersion × 500 or oil immersion × 1000 magnification using 1600 ASA Agfachrome slide film. The exposure time for all photographs was 3 min.

RESULTS

Protein kinase C activity

In a previous study we have demonstrated the presence of calcium-independent activity in a T-cell lymphoma cell line

K-4.[13] In this study we examine the calcium-independent kinase activity in human PBL. Using a peptide derived from the pseudosubstrate sequence of PKC ε as specific substrate we observed calcium-independent activity in the total cell preparations from PBL isolated from healthy volunteers. Table 1 illustrates the levels of calcium-independent activity compared to calcium-dependent activity in unactivated PBL. The results indicate that PBL express substantial calcium-independent activity. We then compared the calcium-independent kinase activity from whole cell fractions using histone IIIS, a peptide derived from myelin basic protein (MBP), kemptide – a PKA substrate, and a peptide derived from PKC ε as substrates (Table 2). Previous studies have reported that the traditional PKC substrate histone is a poor substrate for the calcium-independent PKC isoforms.[14] The results verify that this is the case for human PBL calcium-independent activity. Substrate specificity is greatest for the peptide derived from PKC ε, with activity using histone, peptide from MBP and kemptide substantially lower. Calcium-dependent activity was also higher when the PKC ε peptide was used as substrate compared to histone (data not shown).

Western blot analysis

Total cell preparations from resting PBL were examined for expression of novel isotypes using peptide-specific antibodies to specific PKC isoforms. The expression of PKC isoforms θ and δ in whole cell fractions from unstimulated PBL is illustrated in Fig. 1. PKC θ detected in PBL (Fig. 1a) had been previously identified in the T-cell leukaemic cell line Jurkat.[7] Subsequent studies comparing mRNA and protein expression in various tissues have identified this isoform as the major PKC polypeptide in haematopoietic cells and T cells in particular.[15] This isoform migrates at a molecular weight of 85 000 Da in PBL (Fig. 1a). We also detected a lower

Table 1. Human PBL express calcium-independent PKC activity

Activity	Substrate	pmol/min/mg protein
Calcium-independent	Peptide ε	5379
	Histone	119
Calcium-dependent	Peptide ε	3581
	Histone	1643

For assay conditions refer to the Materials and Methods. Substrate used is specific PKC ε peptide. Results shown are a representative experiment, all assays were carried out in duplicate.

Table 2. Substrate specificity of calcium-independent activity from human PBL

Substrate	pmol/min/mg protein
Histone IIIS	72
MBP	35
Kemptide	49
PKC ε	4287

Assay conditions are described in the Materials and Methods. All assays were carried out in duplicate. Substrate concentrations used were – histone IIIS and MBP, 200 μg/ml; Kemptide, 100 μM; and PKC ε peptide, 10 μg/ml.

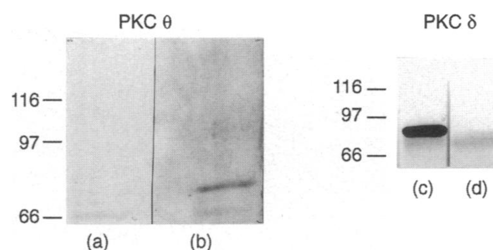

Figure 1. Expression of PKC θ and δ in whole cell fractions from human PBL. Whole cell lysates from human PBL were separated by 10% SDS–PAGE and transferred to PVDF as described in the Materials and Methods. An anti-murine MHC (IE) was used as an isotype control (a). The expression of PKC θ was examined using specific monoclonal antibody (b). Similarly the expression of PKC δ is examined (c) with isotype control (d).

molecular weight species of approximately 54 000 Da in these cells. Previous studies have suggested a role in signal transduction for the catalytic fragment of PKC known as PKM.[16] It is possible that the lower molecular weight species detected is a catalytic fragment of PKC θ or an alternatively spliced polypeptide. A lower molecular weight species was also detected by Baier *et al.*[15] who suggested that it was a degradation product. PKC δ was also detected using a specific monoclonal antibody and migrated at a molecular weight of 86 000 Da (Fig. 1c). Specificity of immunoreactive species is confirmed using an isotype-matched irrelevant antibody Figures 1(b) and (d). The PBL expression of PKC θ and δ was confirmed by flow cytometry (data not shown). Results for the expression of PKC η by Western blot analysis are not presented due to lack of a suitable PKC η antibody.

Immunocytochemistry

The distribution of PKC isoforms in PBL was also examined by immunocytochemistry. In this study we focus on the distribution of PKC θ, δ and η and their response to activation with PMA or anti-CD3. The ability of the OKT3 supernatant to induce lymphocyte activation and proliferation was confirmed using a thymidine incorporation assay. Cells in the presence of soluble OKT3 (1/50 dil.) incorporated 38 300 c.p.m. compared to 3301 c.p.m. for control cells. In resting PBL PKC θ is expressed in a diffuse cytoplasmic staining pattern (Fig. 2a). Specific staining is abrogated by incubating the cells with specific antibody in the presence of immunogenic peptide (Fig. 2b). The resting staining pattern is not radically altered, however, following 60 min activation with anti-CD3 – Fig. 2(c) or following 60 min stimulation with PMA – Fig. 2(d). Specific PKC θ staining was blocked in negative controls for activated cells (data not shown). PKC δ was expressed in a cytoplasmic staining pattern – Fig. 3(a). Anti-CD3 activated cells express PKC δ in a cytoplasmic staining pattern – Fig. 3(c). Following activation with PMA for 60 min PKC δ redistributes to a discrete spot – (Fig. 3d). Negative controls are presented for unactivated cells only, similar backgrounds were obtained for activated cells. The resting, anti-CD3- and PMA-activated expression of PKC η is illustrated in Fig. 4. PKC η expression is diffuse cytoplasmic before activation (Fig. 4a). Anti-CD3-activated cells express PKC η in a staining pattern similar to resting cells – Fig. 4(c).

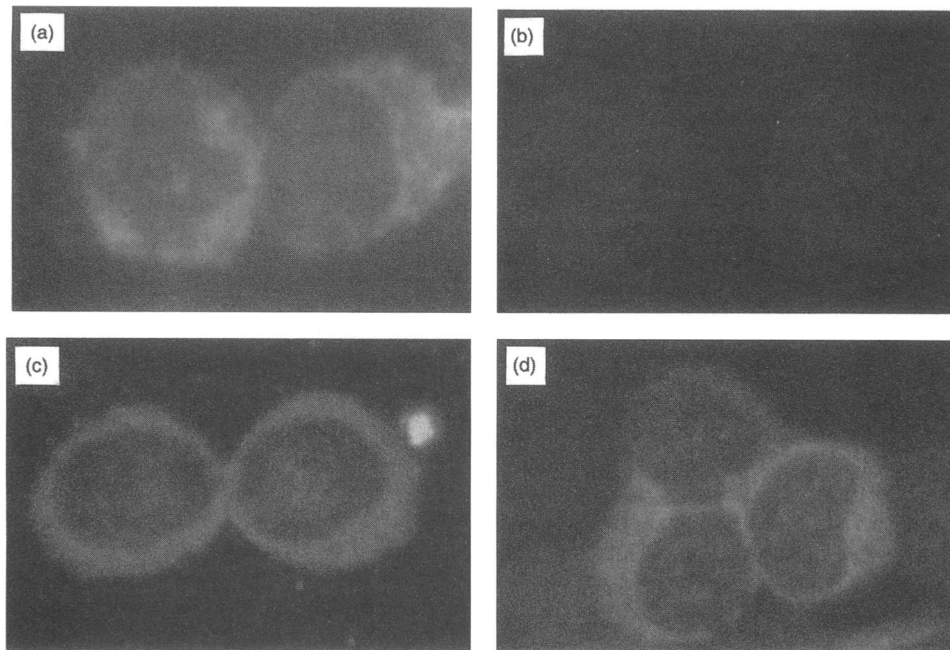

Figure 2. Localization of PKC θ by immunofluorescence microscopy. PBL were fixed and stained as described in the Materials and Methods. Unstimulated cells were stained in the absence (a) and presence (b) of immunogenic peptide. Cells were also stained following stimulation with anti-CD3 for 1 hr (c) and following stimulation with PMA for 1 hr (d).

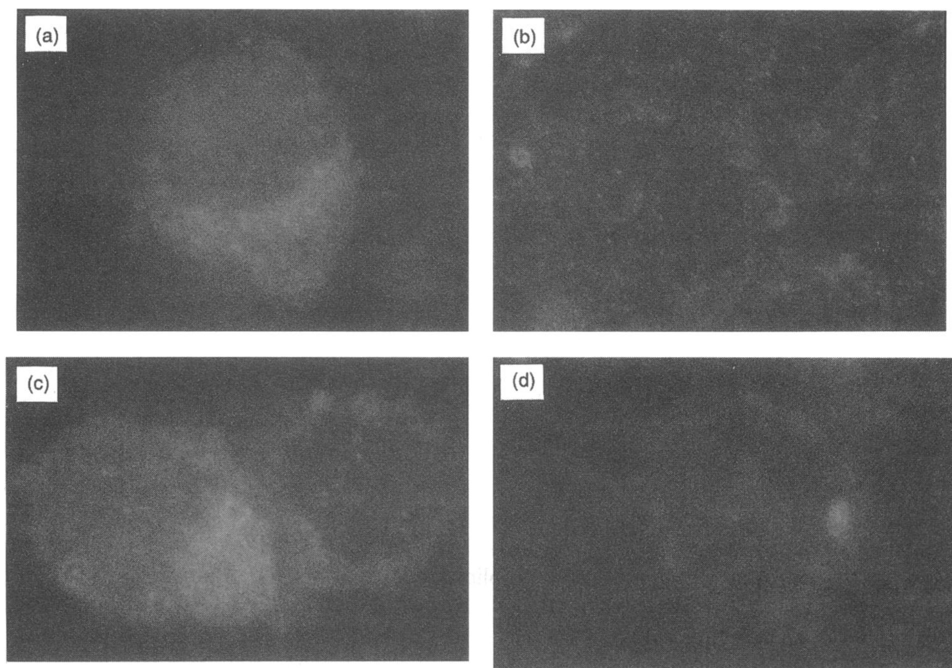

Figure 3. Localization of PKC δ by immunofluorescence microscopy. PBL were fixed and stained as described in the Materials and Methods. (a) Unstimulated cells; (b) negative control – cells stained in the presence of immunogenic peptide; (c) cells stained following stimulation with anti-CD3 for 1 hr; and (d) following stimulation with PMA for 1 hr.

PMA induces a redistribution of the isoform to a polar cap and spot – Fig. 4(d). Similar immunocytochemical staining patterns were observed using purified T cells with the exception of PKC θ. Following panning, PKC θ aggregated to a spot upon stimulation with PMA. This may possibly be an indirect effect arising from the process of negative selection.

DISCUSSION

The results indicate that human PBL possess calcium-independent PKC activity. The substrate specificity of calcium independent isoforms has previously been examined in isoform-specific insect cell transfectants.[14] In our system we

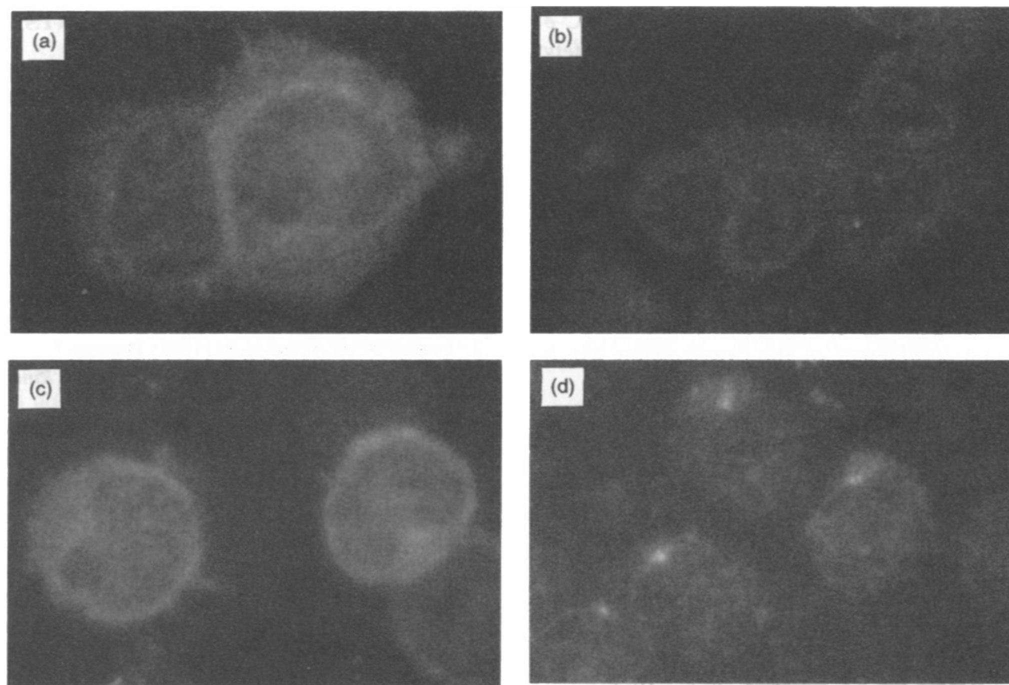

Figure 4. Localization of PKC η by immunofluorescence microscopy. PBL were fixed and stained as described in the Materials and Methods. (a) Unstimulated cells; (b) negative control – cells stained in the presence of immunogenic peptide; (c) cells stained following stimulation with anti-CD3 for 1 hr; and (d) following stimulation with PMA for 1 hr.

showed that calcium-independent activity derived from a heterogeneous sample of PKC isoforms from human lymphocytes utilises the pseudosubstrate peptide from PKC ε more effectively as a substrate than the traditionally used histone. Calcium-dependent isoforms from PBL also phosphorylate the peptide efficiently as has been previously described.[14] Resting cells display substantial kinase activity. The activity being measured may be due to a population of activated T cells, or alternatively may be due to a constitutively active isoform. PKC ζ has been reported to be constitutively active in insect cells transfected with human PKC ζ.[17] Thus the kinase activity detected could be due to one or more constitutively active isoform(s) that are expressed in PBL.

Using two separate techniques we have shown that human PBL express the PKC isoforms θ, δ and η at the protein level. The expression of these isoforms has been described in murine lymphocytes and human T-cell lines.[6–9] The expression of PKC θ has also been previously examined at the mRNA level in human PBL.[7] The PKC θ polypeptide migrates at a molecular weight of 85 000 Da which is in close agreement with the PKC θ polypeptide detected in Jurkat T cells as a 82 kDa protein. A lower molecular weight species of PKC θ was detected in whole cell fractions. Other investigators have reported the presence of a lower molecular weight protein detected by the PKC θ antisera. This protein could be a degradation product of PKC θ. Alternatively it could be the catalytic fragment of this isoform. PKC δ migrates at a molecular weight of 86 000. This isoform has been reported to be one of the major PKC isoforms in murine haematopoietic cells.[9] The PKC isoform η was not detected by Western blot due to lack of suitable antibody.

The cytochemical staining pattern of the three isoforms examined – θ, δ and η is similar in resting cells with subtle variations. All resting cell distribution of these isoforms is cytoplasmic with some perinuclear staining. In a recent study which examined the localisation of PKCs overexpressed in NIH 3T3 fibroblasts, the resting cell distribution of eight PKC isoforms was very similar,[18] being predominantly cytoplasmic. However in our study, the detailed architecture of the lymphocyte is difficult to observe using simple microscopy. The majority of the cell is occupied by the nucleus, i.e. distinct definition of the cytoskeleton is difficult to observe.

In our study the individual isoform responses to activation by anti-CD3 antibodies and the classical direct PKC activator PMA are examined. We examine the distribution of θ, δ and η isoforms following 60 min activation of PBL with OKT3 or PMA. We chose this time-point as preliminary experiments which examined the subcellular redistribution of calcium-independent kinase activity in cytosol, membrane and cytoskeletal fractions indicated that the most significant changes in kinase activity occurred at this time-point (submitted for publication). PKC is traditionally thought to be localised in the cytosol and translocate to the membrane following activation.[19] Activation of T cells through the T-cell receptor complex has been shown to cause translocation of the calcium-dependent PKC activity from cytosol to membrane.[1] Activation of PBL with anti-CD3 for 60 min does not appear to radically alter the resting distribution pattern of PKC θ, δ or η. It is possible that the isoforms are already localised at target substrates and are activated *in situ*. Alternatively the activation time point examined may not be optimal for detection of changes induced by the anti-CD3 antibody in these cells.

PMA, however, induces distinct changes in the distribution patterns of PKC δ and η. PMA induces redistribution of PKC δ to a distinct spot from a cytoplasmic location in unactivated cells. The staining of the δ spot is discrete and well defined.

The observed translocation may be required to bring PKC δ into closer proximity with a specific substrate ultimately allowing it to perform its target function. PMA has been shown to increase the association of PKC β with a cytoskeletal protein spectrin[20] with both proteins staining as a polar spot in lymphocytes. The immunofluorescent staining pattern of centrin, a recently described component of the cytoskeleton thought to be involved in cell division, is also similar.[21] Previous studies have reported that PKC δ is associated with the cytoskeleton[22,23] and is involved in regulation of the cell cycle.[24] Thus in human PBL it is possible that PKC δ may be associating with some component of the cytoskeleton which may be important in cell division/the cell cycle.

The PMA responsiveness of PKC θ has been verified in a previous study.[15] Evidence that PKC θ plays a role in IL-2 gene transcription has also been presented further outlining the importance of this isoform in T-cell function.[15] In PKC θ-transfected COS cells, PMA induces a cytosol to membrane translocation of PKC θ after 15 min stimulation with 100 nM PMA.[15] In our study we did not observe redistribution of this isoform following 60 min activation with PMA. It is possible that in resting T cells this PKC isoform is already associated with its target substrates in an inactive state. PKC η is expressed in unactivated PBL in a pancytoplasmic diffuse staining pattern. PMA induces a dramatic redistribution of this isoform to a polar plasma membrane spot and cap. In T cells PMA has been shown to induce the capping of various lymphocyte surface markers.[25] The cap and spot seen in PMA activated PBL stained with PKC η may represent this isoform colocalizing with capped surface receptors. The compartmentalization of this isoform in close proximity to these structures may play a role in augmenting the relative concentration of PKC η and its target substrate and thus driving the phosphorylation of these proteins. PKC-induced phosphorylation of lymphocyte surface receptors may facilitate their capping, a process that is believed to be important for transmigration of lymphocytes and cell:cell interactions.[25]

Novel and atypical PKC isoforms may have an important role in T-cell function. Our results indicate that the PKC isoforms θ, δ and η are expressed in human PBL and have distinct responses to PMA and antibodies directed against the TCR–CD3 complex. The PKC response mediated through PMA could reflect alternative signalling pathways to the TCR–CD3 associated complex. In this regard it has been shown that PKC θ colocalizes with CD28 during T cell–antigen presenting-cell interactions.[26] CD28 has been shown to be associated with phosphatidylinositol-3-kinase which hydrolyses lipids that have been shown to activate PKC η and δ.[27,28] In addition, the observed PMA-induced changes may also be important for signalling through adhesion molecules such as LFA-1 and CD44. In this regard we have previously shown that signalling via LFA-1 and CD44 can induce morphological changes in T cells. This required prior activation of the cells with PMA.[29] Identification of the cellular components with which individual PKC isoforms colocalize by double labelling and confocal microscopy is important to further characterise the exact location of these isoforms. Our results have provided further evidence that T cells express a number of PKC isoforms at the protein level which have distinct responses to activators.

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