Subcellular localization and translocation of protein kinase C isoforms ζ and ε in human peripheral blood lymphocytes

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Abstract

The calcium-independent members of the protein kinase C (PKC) family may play a significant role in T cell function. We have characterized the subcellular localization and redistribution of calcium-independent kinase C activity and of two specific members of this family (ζ and ε) in response to activation of human peripheral blood lymphocytes with phorbol myristate acetate (PMA) or through the TCR–CD3 complex. Both PMA and OKT3, an antibody against the TCR-associated CD3 complex, induce an increase in membrane and cytoskeletal activity with a concomitant decrease in cytosolic activity. By Western blot analysis, PKC ε is present in resting cytosol and membrane fractions, and is detected in the membrane following activation with PMA and in both the membrane and cytoskeleton following OKT3 activation. By contrast, PKC ζ is progressively lost from the cytoskeleton following activation with anti-CD3. Immunocytochemistry reveals distinct redistribution patterns for these enzymes in response to activation through anti-CD3 and by PMA. These findings demonstrate that signaling through the CD3 complex induces significant changes in calcium-independent PKC activity and in the intracellular distribution of specific isoenzymes, and support a role for specific functions for individual isoenzymes in T cell activation. Lastly, changes in the cytoskeletal distribution of these isoenzymes suggest a potential role in the modulation of cell structure in response to activation.

Introduction

Activation of T cells results in a variety of responses including cytokine production, redistribution of surface molecules, gene transcription and cytoskeletal reorganization (1–3). Several lines of evidence have implicated the protein kinase C (PKC) family of isoenzymes as having a role in these processes. PKC comprises a family of 12 isoenzymes that can be divided into three subgroups based on cofactor requirements and enzymatic properties. Conventional isoforms PKC α, β1, β2 and γ are dependent on calcium, diacylglycerol (DAG) and phosphatidylserine (PS) for activation, and are sensitive to phorbol esters. Novel isoforms ε, δ, θ, η and μ are calcium independent, require DAG and PS for activation, and are also sensitive to phorbol esters. The third subgroup of atypical PKC isoforms ζ and λ/ι are calcium and DAG independent, and do not bind phorbol esters (4). In human T cells PKC isoforms α,

β, γ, ζ and θ have been detected at the mRNA or protein level (5,6). Isoforms δ and ε have also been detected in human T cell lines (7). Little is known, however, regarding the subcellular localization of the calcium-independent isoforms in human T cells and the responses of these isoforms to various activators.

In this study we focus on characterizing the responses to activation of two non-conventional isoforms PKC ζ and ε. It is likely, given the differences that exist in cofactor requirements for these isoforms, that they play different roles in T cell function. A recent study looking at the effects of constitutively active mutants of these two isoforms in a T cell line has indicated that PKC ε and ζ have different downstream effectors following activation with phorbol myristate acetate (PMA) (8). It is also becoming evident that subcellular localization of isoforms may

define cellular function, by directing the enzyme to target substrates. Thus the traditional cytosol to membrane translocation of PKC upon activation may not hold true for all cell types and activators. It is therefore important to define for each cell type the subcellular distribution of individual isoforms and their responses to various activators.

In this study we chose to characterize the responses of PKC ε and ζ to activators for a number of reasons. In a previous study we have shown, using immunocytochemistry, that PKC ζ exhibited a distinct distribution pattern when a T cell lymphoma cell line was activated by OKT3, an antibody against the TCR-associated CD3 complex when compared to activation using PMA (9). In addition, studies using a dominant negative mutant of PKC ζ have implicated this isoform as having a role in the function of the ubiquitous transcription factor NFκB (10) which is involved in the regulation of transcription of T cell cytokines. A study using the Jurkat T cell line has also implicated PKC ε as having a role in the regulation of the transcription factors NF-AT and AP-1 which are involved in the regulation of IL-2 expression (8). In addition, PKC ε exhibits different responses to PMA activation in thymocytes (11) and T lymphoma cell lines (7). Both PKC ζ and ε have also been implicated as having a role in IL-2 signaling in a murine T cell line (12). PKC ζ has also been shown to be involved in regulation of the actin cytoskeleton in IL-2-stimulated T cells (13). Thus these two isoforms appear to have important and distinct roles in T cell function. We therefore set out to examine the pattern of subcellular localization of these two isoforms in response to T cell activation using PMA or OKT3.

Methods

Materials and cells

A monoclonal PKC ε antibody (Transduction Laboratories, Lexington, KY) was obtained from Affiniti Research Products (Nottingham, UK). The PKC ζ isoform was detected using a polyclonal antibody obtained from Gibco/BRL (Gaithersburg, MD). PKC ε and ζ immunoflourescent assay kits were obtained from R&D Antibodies (Berkeley, CA). Secondary biotinylated anti-rabbit or anti-mouse antibodies and streptavidin–alkaline phosphatase were obtained from Amersham Life Sciences (Amersham, UK). PMA was obtained from Sigma (Poole, UK). Nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) were obtained from Promega (Madison, WI). The OKT3 and IE (isotype antibody control) producing hybridomas were obtained from ATCC (Rockville, MD). The PKC ε peptide used as substrate in the kinase assay was obtained from Dr Pat Harriot, Queen's University Belfast. The PKC inhibitor Ro 31-8220 was a kind gift of Dr Trevor Hallum, Roche Pharmaceuticals. Go 6976 was purchased from Calbiochem (Nottingham, UK). Peripheral blood lymphocytes (PBL) were obtained via venepuncture followed by Ficoll-Hypaque sedimentation from healthy volunteers.

Subcellular fractionation

Cytosol and membrane fractions were prepared according to a previously published method with alterations (14). Lymphocytes were isolated by Ficoll-Hypaque gradient centri-

fugation. Cells (5×10^7) were washed once in ice-cold PBS and suspended in 1.0 ml of an ice-cold hypotonic lysis medium (1 mM $NaHCO_3$, 5 mM $MgCl_2$, pH 7.5). Protease inhibitors, phenylmethylsulfonyl fluoride (PMSF) (1 mM) and leupeptin (10 μg/ml) were added to the cells. The cell suspension was then vortexed for 2 min and sonicated for 2 min. The lysate was centrifuged at 100,000 *g* for 10 min at 4°C in a Beckman L5-50 ultracentrifuge. The resulting supernatant was used as the cytosol fraction, and the pellet was washed with PBS and resuspended in 0.5% (w/v) Nonidet P-40 in PBS containing 0.5 mM EGTA, 1 mM PMSF and 10 μg/ml leupeptin. The particulate pellet was agitated for 30 min at 4°C, centrifuged at 200 *g* for 10 min and then spun at 100,000 *g* for 30 min. The supernatant was used as the membrane fraction. The cytoskeletal fraction was prepared according to a previous method with alterations (15). Cells were washed in modified Hanks medium (130 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 0.2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.8 mM Na_2HPO_4, 10 mM glucose and 20 mM HEPES, adjusted to pH 7.4 with NaOH). Cells at a concentration of 5×10^7 cells were lysed in 2 ml of 0.5% Triton-X cytoskeletal lysis buffer (50 mM NaCl, 0.1 mM Na_2VO_4, 1 mM EGTA and 20 mM HEPES, pH 7.5) with protease inhibitors PMSF (1 mM) and leupeptin (10 μg/ml) for 30 min with agitation. Following lysis the Triton-X insoluble fraction was pelleted. The pellet was washed 3 times with cytoskeletal lysis buffer containing Triton-X and 3 times with the cytoskeletal lysis buffer without Triton-X. The washed pellet was resuspended in assay buffer (50 mM Tris–HCl, pH 7.5). Cytosolic and membrane contamination of the cytoskeletal fraction was examined by measuring lactate dehydrogenase levels and by Western blot analysis of the lymphocyte membrane marker CD2 respectively. Subcellular fractions were stored at –70°C. Protein content was measured according to the method of Bradford (16).

PKC activity assay

This assay was adapted from a previously described protocol with some alterations (14). PKC ε peptide was used as specific kinase substrate (17). PKC activity was measured in total cell extracts by incorporation of γ-^{32}P label 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 (1.25 mM EDTA, 5.0 mM EGTA, 30 mM $MgCl_2$ with 0.18% 2-mercaptoethanol added fresh each day), 5 μl of 10 μM PMA and 10 μl of 100 μg/ml PS. 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 [γ-^{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 (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 serine/ threonine kinase activity was determined by carrying out the reaction in the absence of activators PS and PMA, and in the presence of the PKC inhibitor Ro 31-8220. Non-specific binding was characterized in this way due to the presence

of an unidentified kinase activity in reactions carried out in the absence of PS and PMA.

Western blot analysis

Proteins (15 μg /well) from activated or resting cell fractions were separated by 10% SDS–PAGE and transferred to a 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 PVDF was subsequently probed with either anti-PKC ζ or ε for 18 h. Specificity of the immunoreactive species was confirmed for PKC ζ by inclusion of immunogenic peptide. Specificity of the PKC ε antibody was confirmed by incubation with an isotype-matched control antibody (anti-murine MHC-IE). The membrane was then incubated with biotinylated anti-rabbit antibody (PKC ζ) or anti-mouse antibody (PKC ε) for 1 h, followed by streptavidin–alkaline phosphatase complex for 30 min. Specific bands were visualized using NBT and BCIP. Densitometric measurements of specific bands were made using NIH 1.60 image software.

Immunocytochemistry

PBL isolated by Ficoll-Hypaque gradient centrifugation were washed twice in PBS. Cytospins were prepared of PBL at a concentration of 5×10^5 cells/ml. Cells were fixed in cold acetone for 10 min. Non-specific binding was blocked by incubation with normal goat serum for 15 min. Isoform-specific antiserum was applied to the cells for 2 h. Negative controls were incubated with isoform-specific antisera in the presence of immunogenic peptides for 2 h. An FITC-conjugated secondary antibody was then applied to the cells for 1 h. Cells were mounted in medium containing DABCO. Fluorescence was observed using a Leitz microscope. Photographs were taken using a Wild Heerbrug camera under ×100 magnification using 1600 ASA Kodak slide film. All photographs were exposed for the same amount of time.

To investigate a role for other PKC isoforms in the translocation of PKC ζ in response to PMA, cells were pre-incubated in the presence of the PKC inhibitors Ro 31-8220 or Go 6976 (both at 10 μM) for 1 h, stimulated with PMA (25 ng/ml) and stained for PKC ζ as described above at 60 min post-stimulation.

Results

PKC activity in subcellular fractions

In this study we examined the calcium-independent PKC activity in human PBL. Using a peptide derived from the pseudosubstrate site of PKC ε as substrate we measured calcium-independent activity in membrane, cytosolic and cytoskeletal fractions prepared from resting and PMA- or OKT3-activated PBL. Figure 1(A) shows the kinetics of the subcellular redistribution of calcium-independent activity in OKT3-activated PBL over a 120 min period. Following activation with OKT3 a decrease in cytosolic calcium-independent activity with a concomitant increase in membrane and cytoskeletal activity was observed. Maximal activity in membrane and cytoskeletal fractions occured following activation with OKT3 for 30 min. After an initial fall in cytosolic calcium-
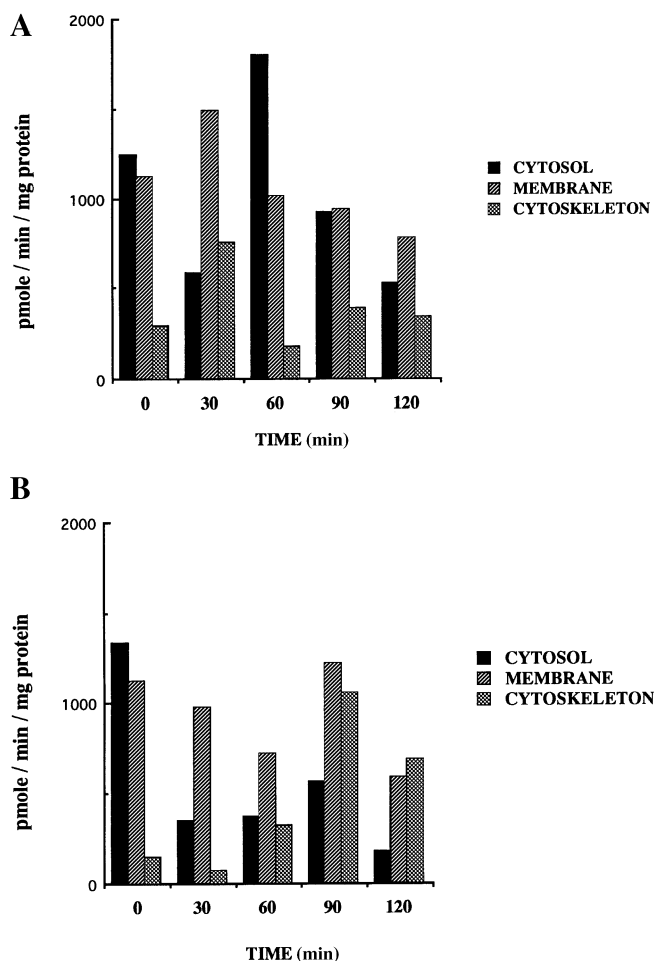

Fig. 1. Kinetics of redistribution of calcium-independent PKC phosphotransferase activity in PBL subcellular fractions following activation with either anti-CD3 (OKT3) (A) or PMA (B). Phosphotransferase activity was measured using a PKC ε pseudosubstrate site peptide in the presence of EGTA and EDTA. Background activity was measured in the presence of the PKC inhibitor Ro 31-8220. Activity is expressed as pmol/min/mg protein and was calculated from the average of duplicate experiments. Data is representative of three separate experiments.

independent activity there was a subsequent increase in activity at 60 min which was paralleled by a decrease in membrane and cytoskeletal activity. Figure 1(B) illustrates the changes in calcium-independent activity that occur during activation of PBL with PMA. Following 30 min activation with PMA, cytosolic activity decreased. After an initial fall in activity, membrane and cytoskeletal activity increased while maximal activity occured at 90 min activation in both of these fractions. Activation of PBL with PMA induced a more prolonged increase in membrane and cytoskeletal calcium-independent activity than activation of PBL with OKT3. In both PMA and OKT3 activated cells a late fall in cytosolic and membrane calcium-independent activity was observed. With respect to the cytosolic PKC activity this may reflect redistribution to an alternative subcellular site. Decreased membrane activity may
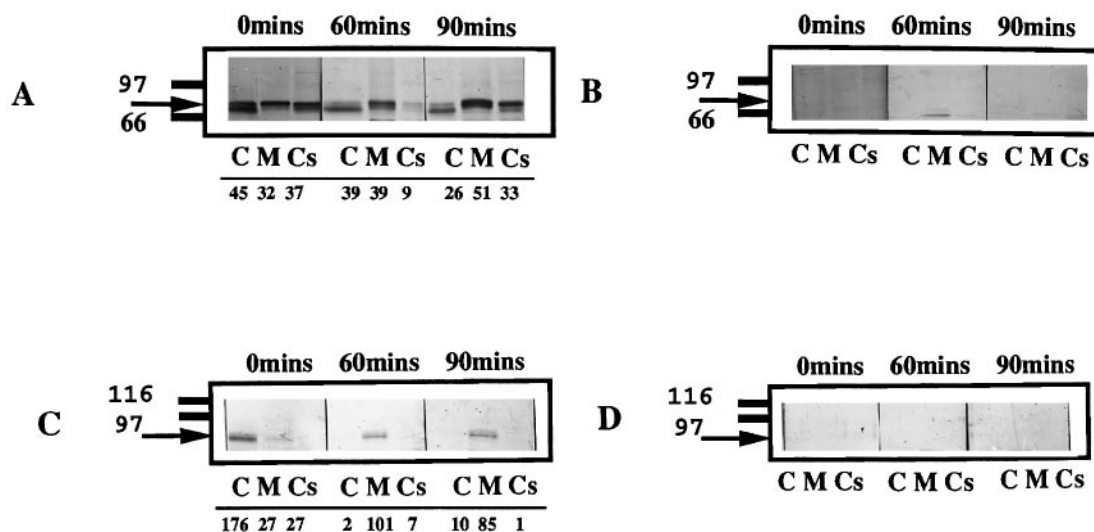
Fig. 2. Immunoblot analysis of the subcellular distribution of PKC ζ and ε following activation of PBL with PMA. At the time points indicated, subcellular fractions were prepared from PMA-activated human PBL as described under experimental procedures. These fractions were subjected to Western Blot analysis and probed with PKC ζ- (A) or ε- (C) specific antisera. Times of activation with PMA (0, 30, 60 and 90 min) are indicated at the top of each lane with subcellular fraction [cytosol (C), membrane (M) and cytoskeleton (Cs)] indicated at the base. Densitometric measurements for specific bands are listed beneath each fraction. The PKC ζ-specific immunoreactive species is blocked in the presence of immunogenic peptide (B) and the specific immunoreactive band of PKC ε is absent when an isotype control is used (D).

possibly be due to increased susceptibility of the activated enzyme to proteolysis.

Subcellular localization of PKC ζ and ε

Having examined the distribution of PKC activity in the three subcellular fractions we went on to determine the localization of two calcium-independent isoforms in these fractions. We had previously determined that a T cell line expressed these isoforms in whole cell fractions by Western blot analysis (9). PKC ζ was detected as a doublet in resting cytosol, membrane and cytoskeletal fractions (Fig. 2A). In cells stimulated with PMA (25 ng/ml), there was a decline in cytosolic and cytoskeletal PKC ζ expression at 60 min with some heterogeneity of expression between the two bands (Fig. 2A). At 90 min the cytosolic decline was sustained but there was an increase in membrane and cytoskeletal PKC ζ levels. Specific bands were blocked in negative controls (Fig. 2B). In unstimulated cells PKC ε was localized in both the cytoplasm and in the membrane (Fig. 2C). Following stimulation with PMA, this isoform was lost from the cytoplasm (Fig. 2C). The PKC ε-specific bands were absent in the presence of an isotype-matched control antibody (Fig. 2D). Figure 3 depicts a time-course of PKC ζ and ε expression in response to PBL activation through the TCR-associated complex CD3. Expression was measured at 0, 30, 60 and 90 min anti-CD3 activation. These time points were chosen to reflect the times where the greatest changes in kinase activity occured. At 30 min, a decrease in cytoskeletal and cytosolic PKC ζ was observed (Fig. 3A). At 60 min there was a significant decrease in cytoskeletal PKC ζ expression with a concomitant increase in the plasma membrane. The decline in cytoskeletal-associated PKC ζ was sustained over 90 min whereas cytosolic expression returned to resting levels. The specificity of the immunoreactive species was confirmed by the absence of specific bands when
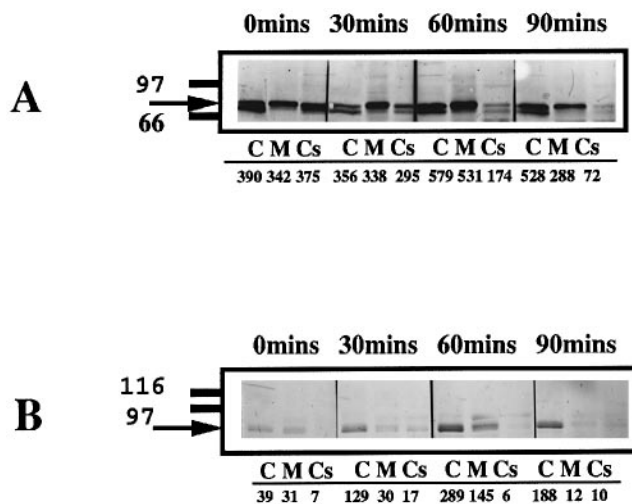

Fig. 3. Immunoblot analysis of the subcellular distribution of PKC ζ and ε following activation of PBL with anti-CD3 (OKT3). At the time points indicated, subcellular fractions were prepared from OKT3-activated human PBL as described under experimental procedures. These fractions were subjected to Western Blot analysis and probed with PKC ζ- (A) or ε- (B) specific antisera. Times of activation with OKT3 (0, 30, 60 and 90 min) are indicated at the top of each lane with subcellular fraction [cytosol (C), membrane (M) and cytoskeleton (Cs)] indicated at the base. Densitometric measurements for specific bands are listed beneath each fraction. Specific bands are absent in negative controls for OKT3-activated cells (data not shown).

immunogenic peptide was included in the antibody solution (data not shown). Activation of PBL with OKT3 induced a small translocation of PKC ε to the cytoskeleton at 30 min activation (Fig. 3B). At 60 min PKC ε was detected at increased levels in both membrane and cytosolic fractions. Specificity
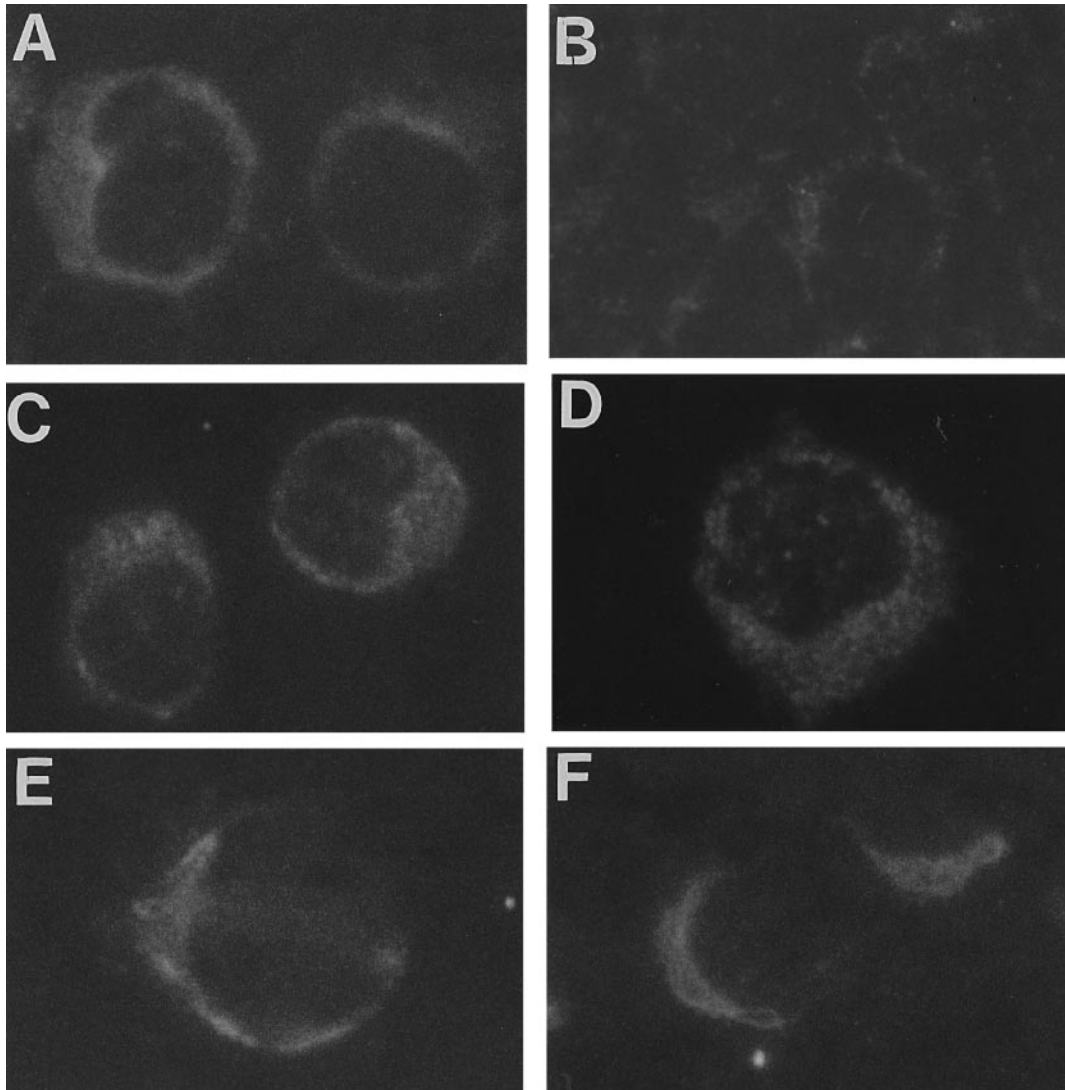
Fig. 4. Immunocytochemical analysis of PKC ζ localization in PBL. Cytospins were prepared from resting or activated cells and stained with isozyme-specific sera as indicated under Methods. Resting PBL were stained with antibody against PKC ζ (A) and also with PKC ζ antisera pre-incubated with immunogenic peptide (B). The distribution of PKC ζ in cells activated with OKT3 for 60 (C) and 90 (D) min is illustrated. Similarly the pattern of PKC ζ staining was examined in cells activated with PMA for 60 (E) and 90 (F) min. Staining patterns for PKC ζ in OKT3- and PMA-activated cells were abrogated in negative controls (data not shown).

of the immunoreactive species was confirmed by absence of specific bands in isotype-matched irrelevant controls (data not shown).

Immunocytochemistry of PKC ζ and PKC ε

We have previously reported that the distribution of isoforms ζ and ε change in response to different methods of activation in a T cell line as measured by immunocytochemistry (9). We examined the distribution of these two calcium-independent isoforms in resting and activated PBL by immunocytochemistry. Using the same activators and time-courses of activation as for the PKC assay and Western blot analysis we observed that the two isoforms had distinct patterns of distribution. Cells were stimulated with either OKT3 or PMA and PBL were stained for PKC ζ and ε following 60 and 90 min activation.

The changes in resting PKC ζ staining pattern that occur following activation through the TCR and with PMA at two time points are illustrated in Fig. 4. The resting distribution of this isoform is diffuse cytoplasmic (Fig. 4A). Figure 4(B) indicates that background staining is minimal when cells are stained for PKC ζ in the presence of immunogenic peptide. In cells activated with OKT3 for 60 min staining is cytoplasmic and granular (Fig. 4C). PKC ζ was also expressed in a granular cytoplasmic staining pattern following activation for 90 min with OKT3 (Fig. 4D). Activation of PBL with PMA appears to induce redistribution of this isoform to a polar plasma membrane cap at 60 (Fig. 4E) and 90 (Fig. 4F) min activation. To investigate the role of other PKC isoforms in this redistribution of PKC ζ in response to PMA, PBL were pre-incubated with the PKC inhibitors Ro 31-8220 and Go
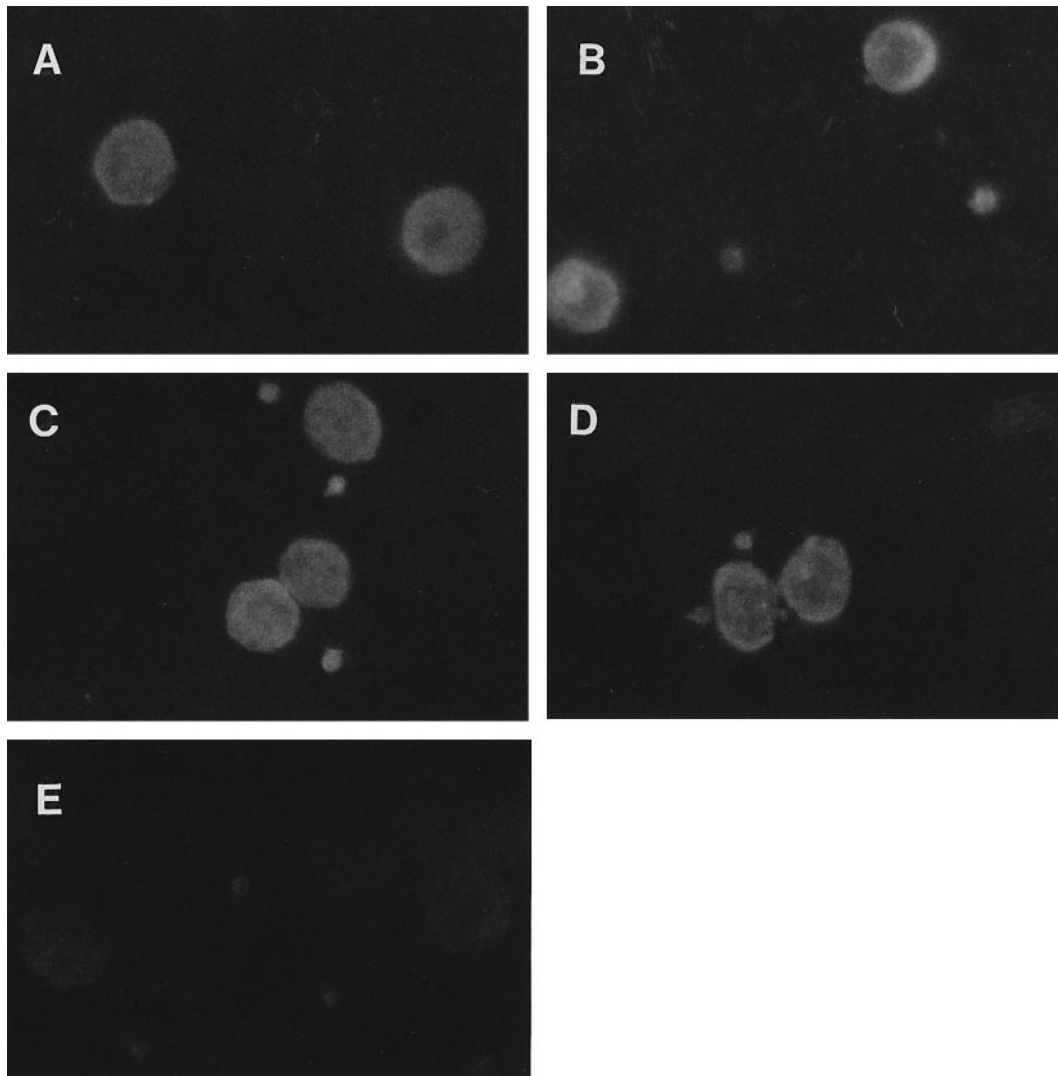
Fig. 5. Immunocytochemical localization of PKC ζ in response to PMA stimulation and in the presence of PKC inhibitors. Cells were prepared and stained as above. (A) Resting PBL. (B) B cells activated with PMA (60 min). (C) Cells pre-incubated with Ro 31-8220 and stimulated with PMA (60 min). (D) Cells pre-incubated with Go 6976 and stimulated with PMA as in (C). (E) Negative control.

9676 prior to stimulation with PMA (Fig. 5). Go 9676 has high selectivity for the classical isoforms α and β. As before, PKC ζ displayed a cytosolic distribution in resting cells (Fig. 5A) with this isoform migrating to a polar cap when cells were activated with PMA (Fig. 5B). When cells were pre-incubated with Ro 31-8220 PKC ζ did not redistribute in response to PMA (Fig. 5C) while pre-incubation with Go 6976 resulted in a granular pattern of PKC ζ staining without the characteristic polar cap (Fig. 5D). These results suggest that the observed redistribution of PKC ζ in response to PMA is indeed an indirect effect mediated perhaps by classical isoform(s). Immunocytochemical subcellular localization of PKC ε was also examined (Fig. 6). Resting expression was diffuse and appeared to be predominantly cytoplasmic (Fig. 6A). Specific staining was blocked in the presence of immunogenic peptide (Fig. 6B). When PBL were activated with OKT3 the distribution of PKC ε shows a more aggregated pattern following 60 min activation (Fig. 6C). This isoform redistributes to a polar

aggregate following 90 min stimulation with OKT3 (Fig. 6D) with additional peripheral granular staining. In PMA-stimulated cells, PKC ε translocates to a polar plasma membrane location with concentrated staining in a discrete spot at 60 min (Fig. 6E) and 90 min (Fig. 6F) activation. Similar staining patterns were also observed in purified T cell populations (data not shown).

Discussion

In previous studies we have reported distinct distribution patterns of PKC ε and ζ and isoform-specific responses to different activators in a human T cell lymphoma line (9). These data suggested that activation of T cells through the TCR resulted in redistribution of PKC ζ to a putative cytoskeletal location. In further studies we have demonstrated that cytoskeletal rearrangement in T cells following activation through the TCR and through adhesion molecules was PKC dependent
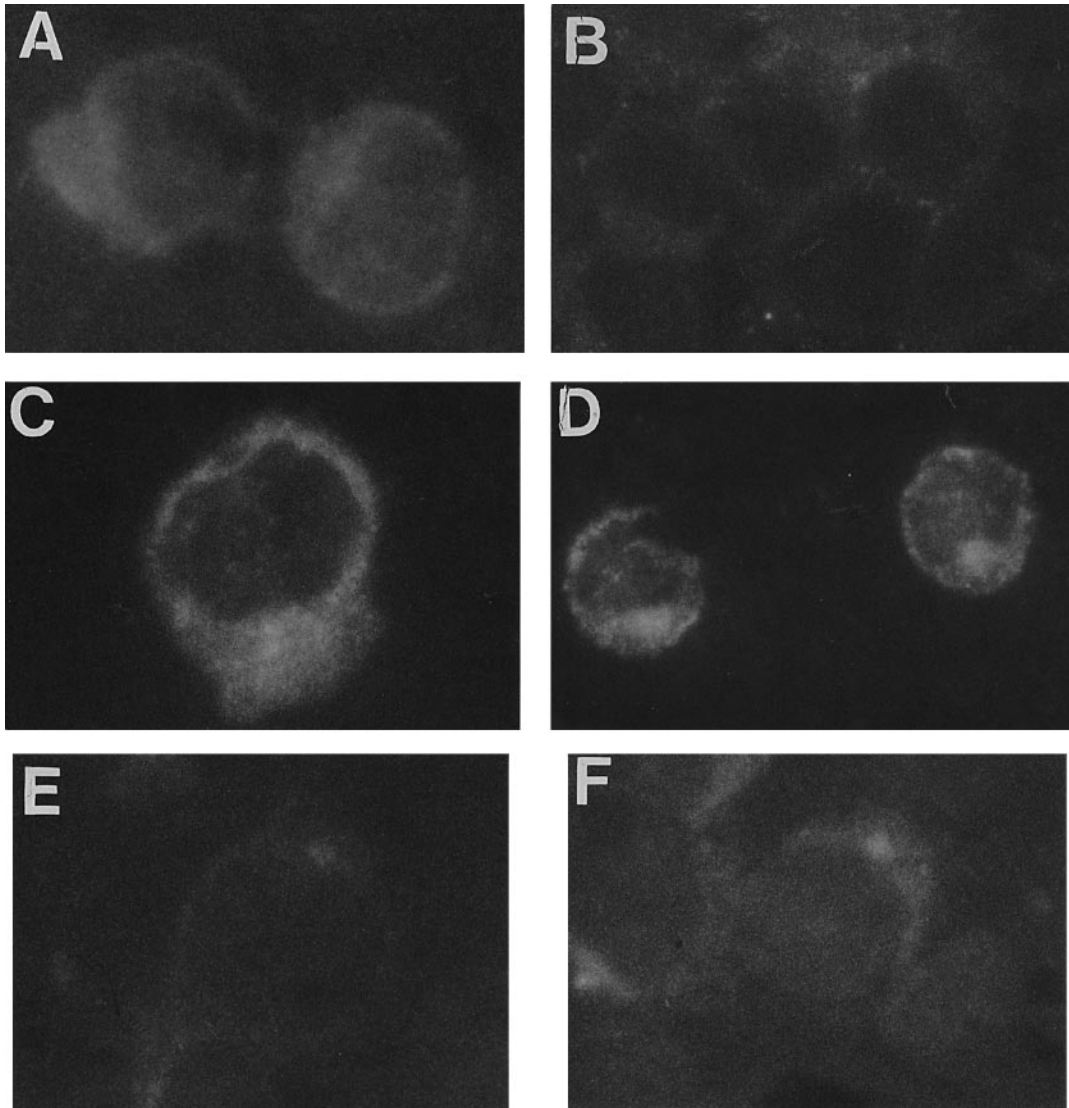
Fig. 6. Immunocytochemical analysis of PKC ε localization in PBL. Cytospins were prepared from resting or activated cells as indicated under experimental procedures. Resting PBL were stained with antibody against PKC ε (A) and also with PKC ε antisera pre-incubated with immunogenic peptide (B). The distribution of PKC ε in cells activated with OKT3 for 60 (C) and 90 (D) min is illustrated. Similarly, the pattern of PKC ε staining was examined in cells activated with PMA for 60 (E) and 90 (F) min. Staining patterns for PKC ε in OKT3- and PMA-activated cells were abrogated in negative controls (data not shown).

(19). T cell clones deficient in PKC ε did not undergo such cytoskeletal rearrangement (20). In these studies we have characterized the expression and responses to activation of calcium-independent PKC isoforms ε and ζ in human PBL. Activation of human PBL through the TCR-associated CD3 complex or using the phorbol ester PMA gave rise to distinct changes in subcellular localization of calcium-independent activity and distribution of calcium-independent isoforms PKC ζ and ε.

Increases in phosphotransferase activity were observed in membrane and cytoskeletal fractions following both methods of activation, and this may reflect a translocation of calcium-independent activity from cytosol to membrane/cytoskeleton or may reflect a stimulation of inactive enzyme already localized in these fractions. Activation of PKC may occur in the absence of translocation, e.g. in fibroblast growth factor-activated 3T3 cells (21) and in the IL-2-dependent murine T cell line CTLL-2 (22). Thus the traditional view of PKC activation being equivalent to translocation from cytosol to membrane does not hold true in all circumstances.

Western blot analysis showed that PKC ζ is present in cytosol, membrane and cytoskeletal fractions. This isoform was detected as a doublet in all three fractions with a degree of heterogeneity of expression between fractions. It is possible that the two bands are differentially phosphorylated forms of ζ. The level of phosphorylation may be related to the profile of PKC regulatory proteins in specific fractions. Nishikawa *et al.* describe high and low salt eluates of PKC from mouse epidermal cells which exist as doublets on Western blot analysis and differ in their response to phorbol ester (23).

Activation with anti-CD3 induced a decrease in expression of both bands in the cytoskeletal fraction with a concomitant increase in expression in the membrane fraction. The findings on immunochemistry demonstrate that PKC ζ is detected in an aggregated form possibly associated with subcellular signal transduction complexes. This is in contrast to the K-4 cell line where PKC ζ appeared to redistribute to a putative cytoskeletal location on immunocytochemistry. However, K-4 cells are a malignant cell line and as PKC ζ has been implicated in the regulation of the cell cycle the responses of this isoform to activation may be abnormal in these cells. The sustained loss from the cytoskeleton is of considerable interest, particularly in the light of recent work demonstrating that PKC inhibitors could induce increased T cell motility (24). Loss of this isoform from a cytoskeletal location may be important for activation-induced shape changes and induction of lymphocyte motility.

In anti-CD3-activated cells PKC ε redistributes from a cytosol and membrane location to a cytosol, membrane and cytoskeletal location. These findings are maximal at 30 min and are consistent with the observed translocation of phosphotransferase activity. This redistribution may play a role in bringing the isoform into close proximity with its target substrate. Anti-CD3- and PMA-induced cytoskeletal rearrangements in lymphocytes have been documented, and PKC has also been shown to associate with intermediate filaments and stress fibers in a number of different cell types including a human T cell line (25). Following activation with anti-CD3, PKC ε progresses from a diffuse staining pattern to a focal aggregate after 90 min activation. Further cytochemical studies are required to delineate the exact cellular location of this aggregate. However, these studies underscore the observation that translocation studies present a simplistic view of PKC activation and that further ultrastructural studies will be required to identify patterns of PKC redistribution on cellular activation.

It is possible that some of the cytoskeletal changes mediated by anti-CD3 antibodies are due to PKC-induced phosphorylation of target cytoskeletal substrates. Of equal importance may be the reduction of cytoskeletal phosphorylation, particularly as we see a decrease in PKC ζ expression in the cytoskeletal fraction. Increasing evidence has suggested that components of the cytoskeleton may be important sites of translocation for activated PKC isoforms. PKC β has been shown to co-localize with ankyrin and spectrin in PMA-stimulated PBL (26). PKC δ has been shown to associate with vimentin (27) and a catalytically active fragment of PKC ε has been found to associate with cytokeratins in epithelial cells (28). PKC ε has also been shown to be associated with the contractile elements of cardiac myocytes (29). Known substrates of PKC have been shown to act as cytoskeletal–membrane cross-bridges (MARCKS) and have a punctate distribution in macrophages (30).

PMA induced a decrease in the expression of PKC ε in the cytosol of PBL and was not detected in the cytoskeletal fraction of these PMA-activated cells. Phorbol esters have previously been shown to alter the cytosol to membrane ratio of PKC ε in GH_4C_1 rat pituitary cells (31). The decrease in cytosolic expression may reflect translocation of PKC ε to the membrane and/or proteolysis of the isoenzyme in this fraction

following activation. This could be important for the regulation of PKC-mediated processes in the cytosol. The expression of PKC ζ in the membrane and cytoskeleton is altered following activation with PMA with expression altering in the three fractions at distinct time points. A decrease in expression of the higher mol. wt form of ζ in the cytosol is also observed. The issue of phorbol ester sensitivity of PKC ζ is controversial. However, a recent study has described the PMA-induced translocation of PKC ζ from cytosol to membrane in human PBL (5). PMA-induced translocation of ζ has also been reported for other cell types. In another study, PKC ζ was significantly down-modulated by long-term incubation in phorbol esters (32). In view of the fact that PKC ζ lacks a phorbol response element, our results indicate that the translocation of PKC ζ in response to PMA stimulation is an indirect effect, possibly involving the activation of the classical isoforms α and/or β. The Go 9676 inhibitor has high selectivity for these classical isoforms and pretreatment of PBL with this substance was sufficient to prevent PMA-induced redistribution of PKC ζ.

This study provides evidence of a role for two calcium-independent isoforms in lymphocyte signaling. In this study we examine the responses of PKC isoforms over a 120 min period following cell activation. Generation of more prolonged cell responses may be mediated by the calcium-independent isoforms which may be activated by DAG species derived from phosphatidylcholine or alternative lipid cofactors (4). The different subcellular locations of the two isoforms is likely to be a reflection of their substrate specificities and may also reflect the localization of isoform-specific binding proteins/receptors. The relationship of these isoforms to the cytoskeleton on T cell activation could suggest a role in T cell locomotion and/or spreading. In addition, cytoskeletal changes may be relevant for T cell effector functions including CD3 down-regulation, T cell cytotoxicity, and the export of cellular proteins and cytokines. Hence, further characterization of the substrate specificities of the individual isoforms and the subcellular components with which they are associated is required to fully elucidate the role of this family of enzymes in T cell function.

Acknowledgements

Abbreviations

BCIP	5-bromo-4-chloro-3-indolyl phosphate
DAG	diacylglycerol
NBT	nitroblue tetrazolium
PBL	peripheral blood lymphocyte
PKC	protein kinase C
PMA	phorbol myristate acetate
PMSF	phenylmethylsulfonyl fluoride
PS	phosphatidylserine

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