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Protein kinase C isoform expression in CD45RA$^+$ and CD45RO$^+$ T lymphocytes

E. CORRIGAN,* D. KELLEHER,† C. FEIGHERY* & A. LONG† *Departments of *Immunology and †Clinical Medicine, St James's Hospital and Trinity College, Dublin, Ireland*

SUMMARY

Differences in levels of specific enzymes utilized in intracellular signalling could be a factor in the distinct signalling properties observed in memory and naive T cells. We have studied the expression of both classical and non-classical protein kinase of C (PKC) isoenzymes in CD45RA and CD45RO cells using a combination of Western blot and flow cytometric analysis. These data indicate that CD45RA cells express higher levels of PKCα, PKCβ and PKCδ than CD45RO cells. In addition, CD45RA$^+$ cells show greater proliferative activity when stimulated with phorbol myristate acetate (PMA) and calcium ionophore than their CD45RO$^+$ counterparts. Variations in the levels of these isoenzymes could be implicated in functional differences, such as proliferation and cytokine production, in these cell subsets.

INTRODUCTION

T lymphocytes may be divided into subsets according to their expression of CD45 (leucocyte common antigen; LCA) isoforms. Isoforms of CD45 are generated by alternative splicing of at least three exons (A, B and C) of a single gene.[1] The CD45 molecule is an integral membrane protein displaying phosphotyrosine phosphatase activity in its cytoplasmic domain, and appears to be a critical component in T-cell receptor (TCR)-mediated signalling.[2] CD45$^-$ mutants have been shown not to produce interleukin-2 (IL-2) or proliferate in response to antigenic stimulation.[3,4] Ligands for CD45 are not well characterized, although CD22 has been put forward as a putative ligand for CD45RO.[5] Following lymphocyte activation, CD45 molecules expressing the A exon (CD45RA) are lost and replaced by molecules expressing neither exon A, B nor C (CD45RO).[6] This is often referred to as the conversion from a naive to a memory T cell. Alloantigens and mitogenic lectins activate the CD45RO and CD45RA populations to a similar extent; however, CD45RO$^+$ cells are more responsive to recall antigen.[7] In addition, the two subsets exhibit different cytokine profiles[8] and the CD45RO$^+$ population expresses higher levels of the activation markers CD25, transferrin receptor, class II molecules and the adhesion molecule CD44.[9,10] Analysis of the signalling properties of these subsets has revealed that CD45RO$^+$ cells have higher basal levels of diacylglycerol (DAG) and higher CD3-induced elevation of intracellular calcium and protein kinase C (PKC) activity than their CD45RA$^+$ counterparts.[9]

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Correspondence: Dr A. Long, Department of Clinical Medicine, TCD Medical School, St James's Hospital, Dublin 8, Ireland.

PKC is a serine/threonine-specific kinase that participates in signal transduction in a wide variety of cell types.[11] It was originally described as a calcium- and phospholipid-dependent enzyme; however, molecular cloning studies have revealed it to be a family of at least 10 isoforms with individual enzymatic properties and tissue distribution.[11–14] The classical PKC isoforms α, β, γ require DAG, phosphatidylserine and calcium for activation, while both the novel (δ, ε, η, θ) and the less-well characterized atypical (ζ, λ, ι, μ) isoforms are calcium independent.[11] PKCα and PKCβ are widely expressed in cells of the immune system, including T and B lymphocytes and neutrophils.[15–17] T cells also express the calcium-independent isoforms ε, ζ, η and θ (C. Keenan, D. Kelleher & A. Long, unpublished observations). Activation of T cells results in PKC-mediated IL-2 secretion and proliferation concomitant with CD45 isoform switching.[18] Multiple T-cell proteins are phosphorylated by PKC, including the CD45 molecule itself.[19] Differences in responses of naive and memory T cells to various stimuli may reflect different levels of signalling molecules and/or their substrates in the two populations, or may even be a consequence of distinct second messenger pathways which couple the signal from the membrane to the nucleus and elicit a response in these cells. The aim of this study was to compare the expression of PKC isoforms in isolated CD45RO and CD45RA populations. Differences in specific PKC isoform expression, or even level of expression, could be an important factor in the differences in signalling properties observed in naive and memory T cells.

MATERIALS AND METHODS

Materials

Monoclonal antibodies to PKCα and PKCβ were obtained

from Seikagaku (Rockville, MD), and Leu-18 (CD45RA), Leu-2a (CD8), Leu-M3 (CD14; monocyte) and Leu-14 (CD22; B cell) from Becton Dickinson (Mountain View, CA). Anti-CD45RO (UCHL1) and FITC labelled $F(ab')_2$ of rabbit anti-mouse and swine anti-rabbit were obtained from Dako (High Wycombe, Bucks, UK), and polyclonal antibodies to PKCα, δ, ε and ζ from R&D Antibodies (Berkeley, CA). Polyclonal antibodies were raised against C-terminal peptide sequences specific to PKC isoforms. Polyclonal anti-δ was not suitable in our hands for detection of PKCδ by Western blot in human leucocytes.[20] However, this antibody was effective in immunofluorescence studies for both immunohistochemistry[21] and flow cytometric analysis. RPMI-1640 medium was from Gibco (Paisley, UK) and nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) from Promega Corp. (Madison, WI).

Separation of T-cell subsets

Peripheral blood mononuclear cells (PBMC) were isolated by Ficol–Hypaque density gradient centrifugation. Monocytes were removed by plastic adherence at 37°. $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ subpopulations were then isolated from the non-adherent cells by negative selection, following incubation for 45 min with saturating doses of antibody to either Leu-18 (CD45RA) or UCHL1 (CD45RO) together with Leu-2a, Leu-M3 and Leu-14 at 4° with mixing. Cells were then washed twice, resuspended with Dynabeads coated with goat anti-mouse IgG (1 : 10 ratio) and incubated at 4° for 50 min. Cells bound to the beads were removed using a Dynal magnetic particle concentrator, leaving the required purified T-cell subset. The purity of the $CD45RA^+$ population obtained was in the range 76–93%, with no greater than 2·5% $CD45RO^+$ cells contaminating ($n = 6$). Similarly, purity of $CD45RO^+$ subsets varied between 79% and 83% with an average of 2·1% $CD45RA^+$ cells detected ($n = 6$).

Western blot analysis

Western blot analysis was carried out as described previously.[21] Briefly, total cell extracts were prepared by solubilizing the cells in 0·5% (w/v) Nonidet P-40 in phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and 6 μg/ml leupeptin. Proteins (100 μg) were separated by 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose. Non-specific binding sites were blocked with Blotto–Tween (5% non-fat dried milk containing 0·05% Tween-20 in PBS) for 1 hr. The nitrocellulose membrane was then incubated with monoclonal antibody to the respective isozyme for 18 hr. The membrane was incubated with biotinylated sheep anti-mouse immunoglobulin for 1 hr, followed by streptavidin–alkaline phosphatase complex for 30 min. Immunoreactive bands were visualized using NBT and BCIP. Molecular weight marker proteins were localized by staining with 0·2% Ponceau-S (Sigma Chemical Co. Ltd, Poole, Dorset, UK) in 3% trichloroacetic acid, 3% sulphosalicyclic acid.

Flow cytometry

Surface marker analysis. The purity of the isolated CD45RA and CD45RO subsets was assessed by labelling the populations with the antibodies Leu-18 (CD45RA), UCHL1 (CD45RO), Leu-3a, Leu-2a, Leu-14 and Leu-M3, respectively. Cells were incubated with saturating amounts of the monoclonal antibody followed by fluorescien isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments of rabbit anti-mouse immunoglobulin. Labelled cells were analysed on a FACScan using Lysys II software (Becton Dickinson).

Cytoplasmic staining. Freshly isolated populations of $CD45RO^+$ and $CD45RA^+$ cells were fixed by incubating a paraformaldehyde (1% in PBS) on ice for 15 min. Cells were then permeabilized using Triton-X 100 (0·1%) in immunofluorescence assay (IFA) medium (RPMI with 4% fetal calf serum) and mixing on ice for 3 min. Non-specific binding sites were blocked by incubating cells in either normal rat serum (those to be incubated with anti-PKCα, ε, δ and ζ) or normal mouse serum (PKCβ). Intracellular PKC isoforms were then labelled by incubating the cells in 2·5 μg of anti-PKC isotype-specific antibody (90 min), followed by FITC-conjugated $F(ab')_2$ fragments of the relevant immunoglobulins (45 min). A polyclonal antibody against keyhole limpet haemocyanin (2·5 μg) and a monoclonal anti-murine major histocompatibility complex (MHC) (I-E) were used as controls. Normal human serum was added with each antibody. After each incubation cells were washed and finally resuspended in IFA medium for FACScan analysis.

Thymidine incorporation assay

The proliferative response of T-cell subsets to mitogen was detected by tritiated thymidine ($[^3H]$TdR) incorporation. Cells were resuspended at 5×10^5/ml in RPMI supplemented with 10% heat-inactivated fetal calf serum, 25 μg/ml gentamicin and 1% L-glutamine. Cells were incubated in triplicate in flat-bottomed microtitre wells (5×10^4 cells/well) in the presence of phorbol myristate acetate (PMA) (0·5 ng/ml) and/or calcium ionophore A23187 (0·4 μM) for 72 hr. Dosages of PMA and calcium ionophore used were determined from preliminary experiments. Cultures were incubated for 3 days at 37° and were pulsed with 0·3 μCi $[^3H]$TdR (specific activity 2·0 Ci/mmol; Amersham Int., Amersham, UK) 18 hr before termination of culture.

RESULTS

Western blot analysis

The expression of the classical PKC isoforms α, β and γ was compared in separated $CD45RO^+$ and $CD45RA^+$ populations by Western blot analysis (Fig. 1). An arrow indicates the PKC isoforms at their designated molecular weights (approximately 78 000). In some cases non-specific bands appeared

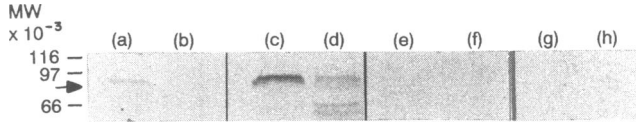

Figure 1. Expression of classical PKC isoforms α and β and γ in $CD45RO^+$ and $CD45RA^+$ T-cell subsets. PKCα, PKCβ and PKCγ were detected by Western blot analysis; 100 μg of protein was loaded in each lane. PKC isoforms are indicated by an arrow. PKCα in CD45RA (a) and CD45RO cells (b); PKCβ in CD45RA (c) and CD45RO (d); PKCγ in CD45RA (e) and CD45RO (f) populations. Anti-murine MHC (I-E) was used as an isotype control for CD45RA (g) and CD45RO (h). This blot is representative of six experiments.

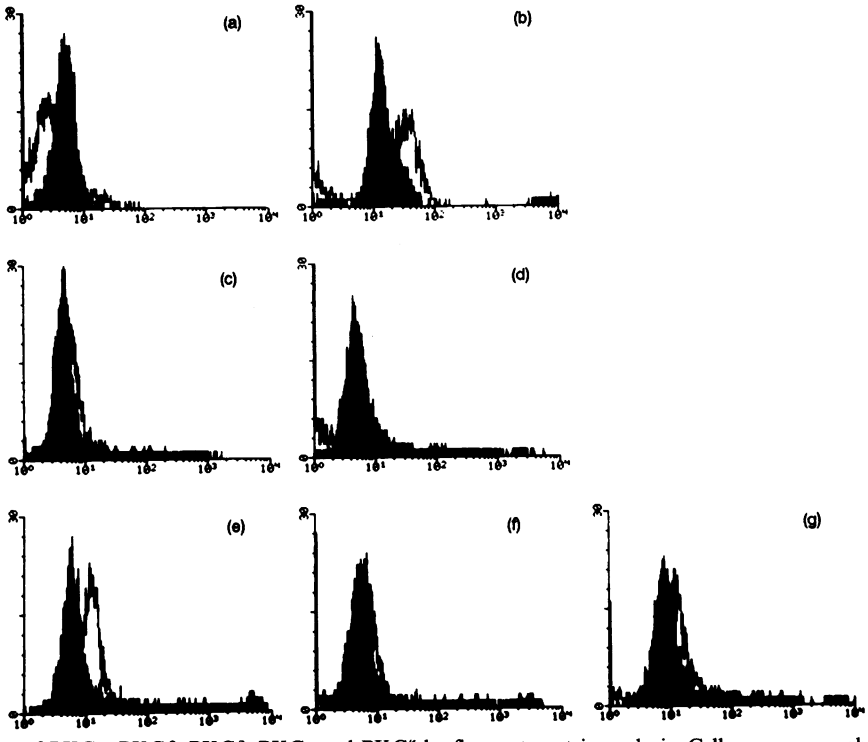

Figure 2. Detection of PKCα, PKCβ, PKCδ, PKCε and PKCζ by flow cytometric analysis. Cells were permeabilized using Triton X-100 (0·1%), stained with the appropriate PKC antibody followed by a FITC-labelled conjugate, as described in the Materials and Methods. PKC isoform expression was compared in CD45RA (unshaded histogram) and CD45RO (shaded) populations. A monoclonal antibody was used to detect PKCβ (b) with anti-I-E as an isotype control (a). PKCα (d), PKCδ (e), PKCε (f) and PKCζ (g) were detected using polyclonal antibodies, with anti-keyhole limpet haemocyanin used as an irrelevant control (c). These profiles are representative of six separate experiments.

below this molecular weight; they may have been proteolytic degradation products reactive with the monoclonal antibodies. Both $CD45RO^+$ and $CD45RA^+$ cells expressed the PKC isoforms α and β, with PKCβ present in both populations at significantly greater levels than PKCα. PKCα was present in extremely low levels and was difficult to quantify by densitometric means. However, when detected ($n = 5$), $CD45RA^+$ cells were observed to express higher levels of PKCα than $CD45RO^+$ cells from the same individual (Fig. 1). Similarly, $CD45RA^+$ cells expressed more PKCβ than their $CD45RO^+$ counterparts (Fig. 1). The difference in PKCβ expression was quantified by densitometry and it was found that PKCβ expression was reduced in five of six individuals in CD45RO cells (86%, 87%, 68%, 48%) and 2% of CD45RA PKCβ expression, respectively). In a single case, CD45RO PKCβ expression slightly exceeded CD45RA (106%). On average, $CD45RO^+$ cells expressed only 66% of the PKCβ expressed in $CD45RA^+$ cells (standard error = 15, $n = 6$). As expected, PKCγ was barely detectable in either $CD45RO^+$ or RA^+ cells. This isoform appears to be largely localized in central nervous tissue,[11] although it has been detected in very low amounts in peripheral blood lymphocytes.[22]

Flow cytometry

The expression of PKCα and PKCβ, in addition to the novel isoforms PKCδ and PKCε and the atypical PKCζ, was also compared in isolated $CD45RA^+$ and $CD45RO^+$ populations by a flow cytometric technique designed to detect cytosolic antigens (Fig. 2). Polyclonal antibodies against PKCα, δ, ε and ζ and a monoclonal anti-PKCβ were used. Again, PKCβ expression was found to be markedly higher in $CD45RA^+$ cells (Fig. 2b), a difference which may have been emphasized by the fact that the binding of the isotype control I-E to $CD45RA^+$ cells was lower than to cells expressing CD45RO (Fig. 2a). PKCα levels were extremely low in both cell populations by this technique (Fig. 2d). PKCε was not detected in either $CD45RA^+$ or $CD45RO^+$ cells (Fig. 2f) even though it has been detected at low levels with some donor variability on Western blots of whole PBMC preparations (C. Keenan, D. Kelleher & A. Long, unpublished observations). PKCζ was detected in both $CD45RA^+$ and $CD45RO^+$ cells in approximately equal amounts (Fig. 2g). However, there was a marked difference in the levels of PKCδ expressed in the two populations, with $CD45RA^+$ cells again showing higher levels (Fig. 2e).

Thymidine incorporation

Finally, we compared the rate of proliferation of the separated $CD45RA^+$ and $CD45RO^+$ populations in response to PMA and calcium ionophore (used singly and in combination). Neither population proliferated in response to calcium ionophore alone, while the $CD45RA^+$ population showed some proliferative activity in response to PMA alone (Fig. 3). The $CD45RA^+$ population proliferated at approximately three

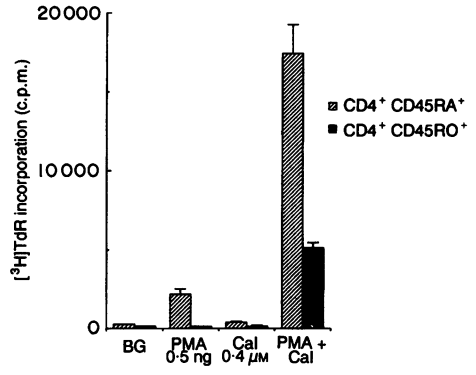

Figure 3. CD4$^+$ CD45RA$^+$ cells show higher levels of proliferation in response to PMA plus calcium ionophore (CaI) than CD4$^+$ CD45RO$^+$ cells. Cells were cultured for 3 days with medium alone (BG), with PMA (0·5 ng/ml), with calcium ionophore A23187 (0·4 μM), or with a combination of PMA and CaI. [^{3}H]TdR incorporation was measured during the last 18 hr of culture. A representative experiment is shown ($n = 4$).

times the rate of the CD45RO$^+$ cells in the presence of both PMA and calcium ionophore (0·5 ng/ml PMA and 0·4 μM A23187).

DISCUSSION

A number of previous studies has examined PKC activity in lymphocyte subpopulations. The higher levels of PKC isoforms (especially PKCβ and PKCδ) do not correlate with previous findings of higher PKC activity in the memory T-cell subset.[9,23] Robinson *et al.*[9] found higher PKC activity in a whole cell assay in anti-CD3-stimulated CD45RO$^+$ cells than CD45RA$^+$ cells activated in the same way. While these activated cells contained similar levels of DAG, 'resting' CD45RO$^+$ cells contained higher levels of DAG than their CD45RA$^+$ counterparts. This, they suggest, is a recently activated phenotype that has reduced activation requirements, perhaps explaining the greater responsivensss of memory cells to recall antigen. Hollsberg *et al.*[23] had similar findings with respect to PKC activity when they divided memory and naive T cells on the basis of CD26 expression (this does not correlate with CD45RO expression). However, when they performed dual immunocytochemical staining (CD26 and PKCα and PKCβ), they found no difference in the levels of either isoform between the two cell populations.

In this study, we found increased levels of PKC isoforms β and δ in CD45RA$^+$ cells. Increased expression of PKCβ was confirmed by both Western blot and flow cytometry. PKCδ was clearly increased by flow cytometry in CD45RA$^+$ cells. However, while the anti-PKCδ antibody used is suitable for immunofluorescence use,[21] this antibody has not been suitable for detection of PKCδ on Western blots of T lymphocytes. PKCα also appeared to be increased by Western blot in CD45RA$^+$ cells using a monoclonal anti-PKCα antibody. PKCα was detected only at low levels by flow cytometry using a polyclonal antibody preventing accurate quantification. Although PKCα levels were low on Western blot analysis, the finding of increased expression on CD45RA$^+$ cells was consistent. Finally, in agreement with a previous study[24] and with our data indicating increased PKC isoform levels, we have seen increased responsiveness of the CD45RA$^+$ population to the PKC activator PMA, either alone or in combination with calcium ionophore.

There is a number of potential reasons for differences between levels of PKC activity and immunodetection of the enzyme. Firstly, in measuring PKC activity in whole cells, several factors must be taken into account, including the distinct and very diverse cofactors required for activity of individual PKC isoforms. These cofactors may be a limiting factor in the determination of activity and may vary between individuals for unknown and unrelated reasons. Secondly, individual PKC isoforms vary in their response to different stimuli. In fact PKCζ does not translocate in response to DAG or the phorbol ester PMA.[11,25] Other PKC isoenzymes may be activated by agents other than diacylglycerol, such as lysophosphatidylcholine or cis unsaturated fatty acids.[11] In addition, PKCε has been found to associate with the cytoskeleton[26] and PKCα may translocate to the nucleus upon activation.[27] Thus, the spatiotemporal responses of the enzyme may not be taken into account in assays of PKC activity.

The prominence of PKCβ in cells of the immune system, and our previous work with a PKCβ-isozyme deficient mutant, suggests an important role for this isoform in T-cell function. Such functions include PKC-mediated IL-2 secretion,[28] CD3 down-regulation[29] and regulation of adhesion molecule expression (A. Long & D. Kelleher, unpublished data). The PKCβ-deficient mutant, K-4, failed to produce IL-2 in response to PKC activation.[28] Furthermore, antibodies to PKCβ blocked IL-2 secretion (and proliferation) in human T cells.[30] While it has been reported previously that CD45RA$^+$ cells are less responsive to activation, recent data suggest that there is no intrinsic defect in the ability of these cells to produce IL-2, and that the reported differences relate to the response to accessory signals. In fact, when CD45RO$^+$ and CD45RA$^+$ cells were co-cultured in the presence of alloantigen and then assayed individually for IL-2 production, it was found that CD45RA$^+$ cells produced significantly higher IL-2.[8] In this study we have demonstrated that proliferation of CD45RA$^+$ cells in response to activation through phorbol esters is significantly higher than that of CD45RO$^+$ cells. These data are in agreement with those of Schwinzer *et al.*,[24] who demonstrated that the proliferative response of CD45RA$^+$ cells to phorbol esters was greater than that of CD45RO$^+$ cells in the absence of accessory cells. Hence these data suggest that the major difference between CD45RO$^+$ and CD45RA$^+$ cells in relation to antigen or anti-CD3-driven proliferation may lie in the response to accessory signals rather than in any defect in signalling pathways.

Specific roles for PKCδ have not yet been well characterized. CHO cells overexpressing PKCδ have been shown to accumulate in the G_2/M phase of the cell cycle when activated with PMA.[31] This correlates with the finding that almost all peripheral blood T cells in the S, G_2/M phase are CD45RA$^+$,[32] which may be due to the higher levels of PKCδ in these cells. Thus, PKCδ is likely to be a factor in the increased proliferative response of the CD45RA$^+$ population.

While there is no absolute difference in the spectrum of PKC isoforms analysed in CD45RA$^+$ and CD45RO$^+$ T lymphocytes, the significantly higher levels of PKCβ and PKCδ in the CD45RA$^+$ population may play an important role in the distinct

functional properties of these cells. Furthermore, the distribution and levels of PKC isoforms in $CD45RA^+$ cells compared to $CD45RO^+$ cells would suggest that PKC is not a factor in the decreased responsiveness of these cells to certain stimuli.

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