The role of lysophosphatide acyltransferases and protein kinase C isoforms in the regulation of lymphocyte responses

M. Szamel', V. Kaever, H. Leufgen and K. Resch
Institute of Molecular Pharmacology, Medical School, D-30625 Hannover, Germany

T lymphocyte activation represents a pleiotropic set of cellular responses that includes cell cycle entry. Stimulation of T-cells results in transcription of several genes and expression of a variety of molecules, including cytokines and their specific receptors such as interleukin-2 (IL-2) and its receptor [1].

Since the late 1970s the involvement of protein kinase C (PKC) in the course of T-cell activation has been well established. Activation of lymphocytes via the T-cell antigen receptor (TCR)/CD3 complex leads to the increased hydrolysis of inositol phospholipids and to subsequent production of inositol phosphates and diacylglycerols (DAGs) that result in elevation of intracellular calcium concentration and activation of PKC, respectively [2]. PKC represents a family of serine/threonine-specific protein kinases; at present 11 different PKC isoenzymes are known, which were classified according to their structure and cofactor requirements for activation. Although all PKC isoforms are activated by phospholipids and (with notable exceptions) DAG particular isoenzymes differ markedly in their sensitivity towards calcium. PKC-α, -β1, -β2 and -γ are dependent on calcium for activation, whereas PKC-δ, -ε, -η and -θ are not. A third group of PKC isoenzymes (PKC-λ, -ι, -ζ) belongs to the PKC family structurally but is not activated by DAG or phorbol esters [3].

Upon activation by monoclonal antibodies directed against the TCR/CD3 complex (e.g. OKT3), PKC isoenzymes are activated with remarkably differential kinetics. As shown in our recent work, PKC-α (and -δ) were activated within 10 min of TCR stimulation; this activation of these PKC isoenzymes proved to be transient. In contrast, a late and sustained activation of the PKC isoforms PKC-β, -δ and -ε was observed [4–6]. These data suggested that: (1) different signalling mechanisms were involved in the activation of PKC isoenzymes upon TCR stimulation; and (2) different PKC isoenzymes regulated different cellular functions of stimulated T-cells.

Stimulation via the TCR/CD3 complex elicits sustained release of DAG from dual phospholipid origin

Stimulation of [3H]oleic acid-labelled human Jurkat T-cells via the TCR/CD3 complex resulted in an elevation of DAG release after
5 min; this increased level of DAG was maintained up to 4 h. Upon labelling cells with \[^{14}C\]lysophosphatidylcholine, which was reacylated and integrated into the phosphatidylcholine (PC) pool a long-lasting increase in DAG release was observed. These data implied that, besides phosphatidylinositol, PC was a major source of DAG in stimulated T-cells.

In order to differentiate between phospholipases involved in DAG release, we made use of potassium tricyclo-[5.2.1.0^2.6]-decyl-(9\(^{8J}\))-xanthogenate (D609), the specific inhibitor of PC-specific phospholipase C (PLC). While initial TCR/CD3 stimulation of DAG release was observed in D609-treated cells also, elevation of DAG production was completely inhibited by D609 after 30 min of activation. As in OKT3-stimulated cells, the high level of DAG release was maintained up to 1 h; these results suggested that the initial burst was due to the breakdown of phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P\(_2\)] and long-lasting DAG was catalysed by a PC-specific PLC (Figure 1A). This finding is supported by the notion that phosphatidylethanol (PtdEth) synthesis catalysed by phospholipase D (PLD) was not detectable in OKT3-stimulated cells. In contrast, in PMA-treated cells a significant increase in PtdEth synthesis was observed, suggesting that Jurkat T-cells contained PLD catalysing transphosphatidylation and thus PtdEth synthesis. PLD, however, was not regulated by the TCR and thus not responsible for long-lasting DAG release in stimulated Jurkat T-cells. Instead, increased DAG release seemed to be the consequence of activation of a PC-specific PLC.

Activation of Jurkat T-cells via the TCR led to a rapid and sustained translocation of protein kinase(s) C as measured by phosphorylation of peptide GS, a specific substrate of PKC and by increased \[^{3}H\]phorbol-dibutyrate binding in plasma membranes of stimulated cells. PKC translocation was observed after 5 min of stimulation and maintained for at least 60 min. Pre-incubation of cells with D609 was without any influence on PKC translocation up to 15 min, whereas the long-lasting activation of PKC was completely abolished upon pretreatment with the inhibitor (Figure 1B). Immunoblotting with PKC-isoenzyme-specific antibodies demonstrated that it was the PKC-\(\alpha\) which translocated rapidly upon TCR stimulation, while sustained translocation of PKC-\(\beta\) was observed upon activation via the TCR. These results suggested that phosphatidylinositol-derived DAG might be responsible for activation of the PKC-\(\alpha\) isoform; in contrast, PKC-\(\beta\) could be activated by DAG derived from PC.

**Figure 1**

Inhibition by D609 of PC-PLC-catalysed DAG release (A) and sustained PKC translocation (B) in stimulated Jurkat T-cells

(A) Cells (5 x 10^6/ml) were labelled with \[^{3}H\]oleic acid (10 Ci/mmol) for 16 h in RPMI 1640 medium supplemented with 2% fatty acid-free BSA. \[^{3}H\]oleic acid-labelled cells were stimulated in the presence (●) or absence (○) of D609 with the monoclonal antibody, OKT3, raised against the TCR/CD3 complex. At each of the indicated times aliquots of 1 x 10^7 cells were stopped by the addition of ethanol. Lipids were extracted and subjected to TLC. Lipid spots were visualized by iodine staining and scraped from the plates separately. Radioactivity was determined by liquid scintillation counting. Results are means ± S.D. of quadruplicates from two independent experiments. (B) Cells were incubated in RPMI 1640 medium at a density of 2 x 10^6 cells/ml in the presence and absence of D609 and stimulated with 5 μg/ml OKT3 for the indicated times. Cytosolic and membrane fractions were isolated and \[^{3}H\]phorbol dibutyrate binding measured as described in reference [6]. Results are means ± S.D. of triplicates from two independent experiments.
TCR-stimulated lysophosphatide acyltransferases (LAT): relevance of LAT-catalysed incorporation of polyunsaturated fatty acids into PC for PKC activation

In the light of the studies on PC-derived DAG in sustained activation of PKC, our experiments on TCR-mediated, LAT-catalysed elevated incorporation of polyunsaturated fatty acids become more significant. We have shown previously that in resting T-cells phospholipase A₂ and LAT were associated with the TCR itself, although the exact mechanism of receptor/effector coupling has not been defined precisely. Plasma membrane-bound acyltransferases become activated upon TCR stimulation resulting in elevated incorporation of polyunsaturated fatty acids into plasma membrane phospholipids, especially PC. As PC carries predominantly saturated fatty acids in resting T-cells, exchange of fatty acid chains may lead to more unsaturated PC species in activated T-cells that might represent an appropriate substrate for PC-specific PLC, resulting in elevation of DAG species carrying polyunsaturated fatty acids [5–8]. As shown recently, PKC was activated only by 1,2-DAG carrying polyunsaturated fatty acids. This metabolic pathway has been suggested to be involved in sustained activation of PKC and thus in regulation of IL-2 synthesis and proliferation of T-cells ([7]; Figure 2).

To date, the exact role of LAT remains obscure for two major reasons. The enzyme has not been purified to homogeneity so far and, concomitantly, no specific inhibitors are available. Purification has been hampered by the fact that LAT, which is a strictly membrane-bound enzyme, rapidly loses its activity after solubilization with various detergents. We have, therefore, chosen a systematic approach for the solubilization of LAT using a two-step protocol. First, conditions have been optimized to extract the LAT from crude membranes of pig spleen or human placenta in protein–lipid–detergent-mixed micelles with enzyme activity maintained using the non-ionic detergent n-octyl glucopyranoside in combination with solutions of high ionic strength [9,10]. Additional experimental conditions have now been established that allow the dissection of these mixed micelles by CHAPS thereby yielding protein–detergent complexes that are free of lipid and accessible to protein purification. The finding that palmitoyl-CoA was a poor substrate as well as an inhibitor of the partially purified LAT was the basis for affinity chromatography using a palmitoyl-CoA–agarose matrix [10]. Unfortunately, a substantial inactivation of LAT activity became obvious.

Figure 2

Model for the TCR/CD3-stimulated signalling pathways in the plasma membrane

PTKs, protein tyrosine kinases; PLC-γ, phospholipase C-γ; PdIns-P₂, phosphatidylinositol (4,5)-bisphosphate; Ins-P₁, inositol (1,4,5)-trisphosphate; DAG, 1,2-diacyl-sn-glycerol; PKC, protein kinase C; PLA₂, phospholipase A₂; LAT, lysophosphatide acyltransferase; PC, phosphatidylcholine with acyl chains saturated; PCₚᵤ, phosphatidylcholine with acyl chains unsaturated; PC-PLC, phosphatidylcholine-specific phospholipase C; IL-2, interleukin-2; IL-2-R, interleukin-2 receptor.
under these conditions. To overcome this drawback in monitoring enzyme purification we synthesized the unsaturated fatty acid analogue 18-(4'-azido-2'-hydroxybenzoylaminoo)oleoyl-CoA (ASO-CoA) and investigated its interaction with LAT [11]. It could be shown that ASO-CoA acts in the dark as a reversible inhibitor of LAT activity, but photolysis of the label results in irreversible inactivation of the enzyme. Several proteins from partially purified LAT fractions could be specifically visualized using 125I-labelled ASO-CoA. At present putative LAT candidate proteins are being microsequenced following gel electrophoresis.

PC-specific PLC-regulated signalling pathways regulate activation of activated T-cell nuclear factor (NFAT) and IL-2 gene expression in stimulated T-cells

Preincubation of Jurkat T-cells with the specific PC-PLC inhibitor, D609, led to a concentration-dependent and reversible inhibition of TCR-stimulated IL-2 synthesis. At a concentration of 30 μg/ml activation of both the IL-2 promoter and of IL-2 secretion was completely inhibited. These data suggest that inhibition of DAG release catalysed by PC-specific PLC and subsequent activation of PKC-β might be critically involved in regulation of the IL-2 promoter. This assumption is strengthened by the results showing that the PKC-specific inhibitor, bis-indolylmaleimide, resulted in inhibition of IL-2 promoter activity to a similar extent as that measured in D609-treated cells.

More importantly, pretreatment of cells with D609 resulted in inhibition of activation and nuclear binding of NFAT, measured by the lack of luciferase reporter gene activity in cells transfected with multiple copies of the NFAT binding site (Table 1). To our knowledge these data show for the first time that activation of NFAT might be impaired upon inhibition of plasma membrane phospholipid metabolism. This supports the concept that PC-derived DAG and subsequent activation of PKC-β were critically involved in activation of the IL-2-specific transcription factor, NFAT. These results are in accordance with our recent findings showing that PKC-β was the major PKC isoenzyme regulating IL-2 synthesis in stimulated lymphocytes [4].

In recent years evidence has been collected showing that PKC isoenzymes might regulate different cellular functions in stimulated T-lymphocytes. Our own data showed that transient activation of PKC-ε (and -δ) were sufficient for upregulation of high affinity IL-2 receptors [4,12]. In contrast, sustained activation of PKC-β and to a lesser extent that of PKC-δ (and -ε) was an absolute requirement for regulation of IL-2 gene expression and secretion in stimulated T-cells [4–8].

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>D609 (μg/ml)</th>
<th>BIM* (ng/ml)</th>
<th>NFAT activation (arbitrary units)</th>
<th>IL-2 secreted (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>−</td>
<td>152</td>
<td>&lt;15</td>
</tr>
<tr>
<td>+</td>
<td>0</td>
<td>−</td>
<td>38356</td>
<td>200±17</td>
</tr>
<tr>
<td>+</td>
<td>10</td>
<td>−</td>
<td>22149</td>
<td>140±15</td>
</tr>
<tr>
<td>+</td>
<td>15</td>
<td>−</td>
<td>5169</td>
<td>102±12</td>
</tr>
<tr>
<td>+</td>
<td>30</td>
<td>−</td>
<td>1988</td>
<td>40±4</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>100</td>
<td>155</td>
<td>&lt;15</td>
</tr>
</tbody>
</table>

*BIM, Bis-indolylmaleimide.
Taken together, the results provide strong evidence for a functional relevance of PC-specific PLC in the course of T-cell activation. PC-specific PLC can provide the DAG necessary for long-lasting activation of PKC, especially for that of PKC-β. In this context it can be envisaged that the role of LAT is to catalyse the enhanced incorporation of polyunsaturated fatty acids and thus generate PC species which are precursors of DAG with genuine PKC-activating properties. The data suggest that PKC-β-regulated signal transduction pathways might be critically involved in the regulation of NFAT activation and finally in activation of IL-2 gene expression in stimulated T-lymphocytes.


Received 2 March 1998

The non-specific lipid transfer protein (sterol carrier protein 2) acts as a peroxisomal fatty acyl-CoA binding protein

K. W. A. Wirtz*, F. S. Wouters*†, P. H. Bastiaens‡, R. J. A. Wanders*, U. Seedorf§ and T. M. Jovin†

*Department of Biochemistry of Lipids, Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands, †Department of Molecular Biology, Max Planck Institute for Biophysical Chemistry, D-37078 Göttingen, The Netherlands, ‡Department of Pediatrics, Academic Medical Centre, University of Amsterdam, NL-1105 AZ Amsterdam, The Netherlands, and §Institute for Arteriosclerosis Research, Westfalian-Wilhelms-University, D-48129 Münster, Germany

Introduction

The non-specific lipid transfer protein (nsL-TP) identical to sterol carrier protein 2 (SCP2) is a small basic protein which stimulates the transport of a great variety of lipids in vitro including cholesterol and phospholipids between membranes [1]. It was also reported that this protein stimulates the enzymic conversion of 7-dehydrocholesterol into cholesterol in microsomes [2], acyltransferase-mediated esterification of microsomal cholesterol [3], formation of pregnenolone in mitochondria of steroid hormone-producing cells [4] and dolichol biosynthesis by microsomes [5]. At present, this wide range of activities is difficult to reconcile with the intracellular localization of nsL-TP which is predominantly, if not exclusively, peroxisomal [6,7]. In view of this discrepancy between function and localization we favour the idea that due to an exposure of its low-affinity lipid binding site at the membrane interface, nsL-TP facilitates the mobilization of lipid monomers thereby stimulating lipid-metabolizing processes, supposedly in a non-specific way [8]. Given that peroxisomes contain enzymes involved in cholesterol and alklyphospholipid biosynthesis, β-oxidation of long-chain and branched-chain fatty acids and the side-chain cleavage reaction in bile acid synthesis (reviewed in [9]), nsL-TP may well have a role...