TYROSINE PHOSPHORYLATION REGULATES PHOSPHOLIPASE C ACTIVATION IN HUMAN T-CELLS

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Introduction
One early transmembrane signal in response to stimulation of the T-cell antigen receptor (CD3/Ti) is the activation of phosphatidylinositol (PtdIns)-specific phospholipase C (PLC). The resulting formation of inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) and diacylglycerol (DAG) causes calcium release from internal stores, calcium influx through calcium channels, and translocation of protein kinase C (PKC) to the plasma membrane (for a review, see [1]). Although this signal is associated with T-cell responses, several studies have shown that PLC activation can occur without resulting in cellular proliferation. For example, when immobilized anti-CD3 monoclonal antibodies (mAbs) are used for stimulation of T-cells, the PLC activation signal is prolonged and results in interleukin 2 (IL-2) receptor α-chain (CD25) expression, production of cytokines including IL-2 and T-cell autocrine proliferation [2, 3]. In contrast, when anti-CD3 mAbs are used in solution, the PLC activation signal is transient and does not result in T-cell autocrine proliferation. Rather, desensitization occurs, resulting in a period of non-responsiveness to stimulation of other receptors [4].

Transient activation of PLC also occurs in T-cells when other receptors such as CD2, CD5 or CD28 are cross-linked on the cell surface with specific mAbs [5]. These molecules have been implicated as receptors for accessory signals in T-cell activation, since mAb-mediated ligation can augment responses to CD3/Ti stimulation [6]. The CD2 and CD28 receptors are known to mediate cell–cell adhesion with antigen-presenting cells by binding to their co-receptors, the LFA-3 and the B7/BB1 molecules, respectively [7, 8]. Both LFA-3 and the B7/BB1 deliver transmembrane signals to T-cells through the CD2 or CD28 molecules [9, 10]. A normal ligand for CD5, however, has not yet been identified. All of these accessory receptors that activate PLC depend upon expression of CD3/Ti for their functional activity [4].

How are these T-cell surface receptors coupled to activation of PLC in the plasma membrane? Several studies have implicated GTP-binding G-proteins in the process, because non-hydrolysable GTP analogues or fluoroaluminate activate PLC either in T-cells or in T-cell membrane fractions (see [1] for a review). Additional evidence derives from experiments where CD3/Ti-negative Jurkat T-cells were transfected with one subtype of the muscarinic acetylcholine receptor, resulting in PLC activation and IL-2 secretion after stimulation with carbachol [11]. Because the muscarinic acetylcholine receptor is a G-protein-linked receptor, these studies indicate that T-cells contain G-protein(s) that can activate PLC, and that a PLC exists in T-cells that can be regulated by G-proteins. These data, however, do not show that the endogenous surface receptors linked to PLC activation in T-cells use this mechanism.

A second early transmembrane signal associated with stimulation of CD3/Ti is the rapid induction of tyrosine phosphorylation of specific substrates [12, 13]. A relationship between the activation of protein-tyrosine kinases and the activation of PLC in T-cells has been suggested from several studies over the past year. Immunoblots using specific anti-phosphotyrosine antibodies have revealed increased tyrosine phosphorylation on multiple proteins, including those of 145, 135, 100, 75 and 40 kDa, after CD3/Ti ligation [14]. The tyrosine phosphorylation was detectable at 5 s and was maximal at 90 s. The tyrosine phosphorylation preceded activation of PLC, since Ins(1,4,5)P3 was not detectable until 30 s after stimulation [14]. When inhibitors of protein-tyrosine kinases, including herbinycin A and genistein were tested with T-cells, inhibition of CD3-mediated PLC activation was observed [15, 16]. Herbinycin A is a benzoquinonoid ansamycin antibiotic that was found to reverse oncogenic transformation induced by pp60 v-src [17], and to both inhibit src kinase activity and induce degradation of the src protein. Herbinycin A completely inhibits CD3-mediated PLC activation in T-cells without preventing PLC activation by aluminium fluoride, and without preventing T-cell responses to ionomycin plus phorbol 12-myristate 13-acetate (PMA) [15]. Genistein, an unrelated protein-tyrosine kinase inhibitor, gives partial and transient inhibition of T-cell-receptor-induced PLC activation in T-cells. These data therefore suggest

Abbreviations used: PLC, phospholipase C; mAbs, monoclonal antibodies; IL-2, interleukin-2.

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that tyrosine phosphorylation is required for antigen-specific PLC activation in T-cells.

In this communication, we address the PLC activation signals induced in T-cells by cross-linking accessory receptors including CD2, CD28 and CD4. In each case, tyrosine phosphorylation is required for PLC activation, although differential sensitivity to inhibition by herbimycin A is shown. Evidence for the regulation of protein-tyrosine kinase activation by stimulation of these receptors, obtained by anti-phosphotyrosine immunoblotting of whole cell lysates, is also presented.

Results and discussion
Measurement of cytoplasmic calcium concentration ([Ca\(^{2+}\)]\(_i\)) with the indo-1 dye and a flow cytometer provides a very sensitive assay for the activation of PLC in T-cells [18]. To activate PLC by CD2, CD28 or CD4 receptor ligation, we used biotin-conjugated mAbs followed by an excess of avidin (Fig. 1). In this experiment, the signal from CD2 cross-linking was very strong, reaching a peak of >2000 nM-[Ca\(^{2+}\)]\(_i\), within 3 min of avidin addition. This response is a combination of intracellular calcium release and extracellular calcium influx [19]. The signal from the interaction of the CD3 and CD4 receptors, generated by cross-linking CD3 and CD4 together with an antibody hetero-

![Fig. 1](image1.png)

Inhibition of calcium mobilization in T-cells by Herbimycin A

Resting (G0) T-cells from peripheral blood were incubated with (---) or without (-----) Herbimycin A (1 μg/ml) for 16 h in RPMI containing 5% (v/v) fetal calf serum. The cells were then loaded with indo-1 and assayed for responses in cytoplasmic calcium concentration ([Ca\(^{2+}\)]\(_i\)) with a flow cytometer. Cells were stimulated with ionomycin, CD2 cross-linking with biotin-9.6, followed by avidin added at the arrow; CD3 × CD4 cross-linking with 5 μg of a heteroconjugate of mAbs G19-4 × G17-2, CD3 cross-linking with an IgM anti-CD3 mAb 38.1; CD28 cross-linking with biotin-9.3 followed by avidin added at the arrow; or CD4 cross-linking with biotin-G17-2 followed by avidin added at the arrow.

![Fig. 2](image2.png)

Tyrosine phosphorylation of substrates in HPB-ALL T-cells is regulated by the CD3/Ti and CD4 receptors

HPB-ALL cells were stimulated with a CD3 × CD3 homoconjugate of mAb G19-4 (10 μg/ml), a CD3 × CD4 heteroconjugate of mAbs G19-4 × G17-2 (10 μg/ml), or a CD4 × CD4 homoconjugate of mAb G17-2 (10 μg/ml). At 0 (before stimulation), 2 and 10 min after stimulation, 5 × 10\(^6\) cells were rapidly pelleted and lysed in SDS sample buffer. Polyacrylamide gels were run and protein was transferred to a PVDF filter. Tyrosine-phosphorylated proteins were detected by immunoblotting with purified rabbit anti-phosphotyrosine followed by 125I-labelled protein A. Molecular mass markers were included and their migration positions are indicated.
Peripheral blood mononuclear cells (containing 80–90% T-cells) were isolated by centrifugation on Ficoll and were then incubated at 10^6 cell/ml in RPMI with 5% (v/v) fetal bovine serum for 16 h in the indicated concentrations of herbimycin A. Stimulation of the cells was with IgM anti-CD3 mAb 38.1 (CD3) at an optimal concentration, by cross-linking CD2 using biotin-conjugated mAb 9.6 followed by avidin (CD2), or by a heteroconjugate of anti-CD3 × anti-CD4 mAbs G19-4 and G17-2 at 5 μg/ml (CD3 × CD4). Calcium responses after stimulation were measured with indo-1 and a flow cytometer. Maximal responses are shown calculated from programs that measure the percentage of responding cells and the mean calcium concentration [Ca^{2+}],. Basal [Ca^{2+}], of unstimulated cells was 130 nM.

### Table I

<table>
<thead>
<tr>
<th>Herbimycin concentration (μg/ml)</th>
<th>Responding cells (%)</th>
<th>Calcium response</th>
<th>Peak mean [Ca^{2+}], (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD3</td>
<td>CD2</td>
<td>CD3 × CD4</td>
</tr>
<tr>
<td>0</td>
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</tr>
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<td>0.25</td>
<td>24</td>
<td>51</td>
<td>60</td>
</tr>
<tr>
<td>0.5</td>
<td>&lt;5</td>
<td>42</td>
<td>45</td>
</tr>
<tr>
<td>1.0</td>
<td>&lt;5</td>
<td>40</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>CD3</td>
<td>CD2</td>
<td>CD3 × CD4</td>
</tr>
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<td>1800</td>
<td>750</td>
</tr>
<tr>
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<td>310</td>
<td>605</td>
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</tr>
<tr>
<td>0.25</td>
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<td>580</td>
<td>490</td>
</tr>
<tr>
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<td>130</td>
<td>310</td>
<td>460</td>
</tr>
<tr>
<td>1.0</td>
<td>130</td>
<td>260</td>
<td>440</td>
</tr>
</tbody>
</table>

To examine the comparative sensitivity of these signals to Herbimycin A, a titration between 0.125 μg/ml and 1 μg/ml was used for the 16 h incubation. The CD3, CD2 and CD3 × CD4 signals were then compared at each Herbimycin concentration (Table I). A significant difference was readily apparent, since the CD3-induced signal was completely inhibited by 0.5 μg/ml, whereas the CD2 and CD3 × CD4-induced signals were only partially inhibited. In this experiment, the CD2 and CD3 × CD4 signals were still present even at 1 μg of Herbimycin A/ml. These results suggest that the accessory receptors on T-cells that are linked to PLC activation are not equally dependent on protein-tyrosine kinase activity, and that the strongest signals are more resistant to inhibitors of protein-tyrosine kinases.

Multiple isoenzymes of PLC have been characterized [20]. Although T-cells may contain a form such as PLCβ that is activated by a G-protein mechanism, it is clear that the CD3/Ti receptor-linked PLC must be closely related to PLCγ because the activity of this isoenzyme is directly regulated by tyrosine phosphorylation [21]. Our evidence suggests that the CD2, CD4, CD28 and other accessory receptors that are linked to PLC activation are also dependent upon first initiating protein-tyrosine kinase activation. Consistent with this idea are the observations that CD2 cross-linking induces tyrosine phosphorylation in T-cells [22], and that the CD4 and CD8 receptors are non-covalently associated with the protein-tyrosine kinase p56Lck [23]. Association of the CD4 or CD8 receptor with CD3/Ti enhances the activation of PLC [24], whereas similar CD3/Ti interaction with the CD45 protein-tyrosine phosphatase inhibits both the [Ca^{2+}], response and the activation of PLC [25].

Cross-linking of CD4 was shown to activate the associated p56Lck protein-tyrosine kinase and to induce tyrosine phosphorylation of specific cellular substrates [26]. Because the CD4 interaction with CD3/Ti results in enhanced PLC activation, we examined the effects of this interaction on induction of tyrosine phosphorylation. Fig. 2 shows that, in comparison with either CD4 or CD3/Ti cross-linking separately, the cross-linking of CD3 and CD4 together results in a much stronger signal. Tyrosine-phosphorylated proteins are detectable at 20 kDa, 35 kDa, 55 kDa, 67 kDa, 80 kDa, 105 kDa and 150 kDa in the HPB-ALL T-cells used for this experiment and are similar to the tyrosine-phosphorylated proteins seen in normal T-cells, Jurkat T-cells or CEM T-cells. PLC or regulators of PLC are likely to be included among these substrates of tyrosine kinases in T-cells.

Cross-talk between surface immunoglobulin and interleukin-4 receptors on murine B-lymphocytes

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Introduction

Interleukin 4 (IL-4) is a T-cell-derived lymphokine with pleiotropic effects on many different cell types (reviewed in [1]). One of the first activities ascribed to this factor was its capacity to act as a co-mitogen in B-lymphocytes, in conjunction with submitogenic concentrations of antibodies (anti-Ig) directed to the clonally distributed surface immunoglobulin receptors (sIgM and sIgD) on these cells. Ligation of sIg receptors provokes the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2], with consequent elevation of intracellular Ca2+ and activation of protein kinase C (PKC), and this signalling cascade involves uncharacterized guanine nucleotide regulatory protein (reviewed in [2]). Little is known about the signal transduction mechanisms utilized by IL-4 receptors (IL-4R), especially in murine B-cells, where various studies have shown that IL-4 does not provoke PtdIns(4,5)P2 hydrolysis or PKC activation, nor does it detectably modulate PtdIns(4,5)P2 hydrolysis induced by anti-Ig [3–5]. In contrast, IL-4 has been shown to induce a short-lived burst of Ins(1,4,5)P3 release, followed by a prolonged elevation of cyclic AMP, in human B-cells [6].

The following experiments were therefore undertaken in an attempt to gain more information...