REVIEW ARTICLE

Toward the Mechanism of Phosphoinositide-Specific Phospholipases C

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Introduction

Receptor-mediated turnover of inositol phospholipids and inositol phosphates has received unparalleled attention during the last decade (ca 2000 papers a year) owing to its importance in cellular signal transduction. The key enzyme responsible for triggering the cascades of such metabolic events is phosphatidylinositol-specific phospholipase C (PL-PLC, EC 3.1.4.10; CA Registry Number 63551-76-8).1-14 This review is aimed at understanding molecular details of the structure, function, and catalytic mechanism of PL-PLC from mammalian and bacterial sources. Another very important aspect of PL-PLC—the mechanism of regulation—is not included since it has already been addressed in many recent reviews.7,9,11-21

The common feature of the various forms of PL-PLC is that they cleave the phosphodiester moiety of PI and its phosphorylated or glycosylated derivatives (1a-d) to produce the corresponding cyclic 1,2-inositol phosphates (2a-d) or the mixtures of cyclic (2) and acyclic 1-phosphates (3a-d) (Figure 1). Several products of the enzymatic cleavage of inositol phospholipids, including inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), have been proposed to act as calcium-mobilizing second messengers delivering the original extracellular message carried by a hormone, neurotransmitter or growth factor inside the cell.

Abbreviations: DAG, 1,2-diacyl-sn-glycerol; DDPI, 1,2-dipalmitoyl-sn-glycero-(1,3-dioleoylphospho-1-myo-inositol); DPFs, 1,2-dipalmitoyl-sn-glycero-(1-oleoyl-2-thiophospho-1-myo-inositol); GIP, glycosylated IP; GlcP, glycosylated GlcP; GPl, glycosylphosphatidylinositol; Glu, glycerol; IP, (1,1-P) 1-myo-inositol 1-phosphate; IC50, inhibitor concentration causing 50% inhibition of activity; IEP, 1,6-myo-inositol 1,2-cyclic phosphophosphatidylethanolamine; IP3, 1,6-myo-inositol 1,4,5-trisphosphate; mPVS, membrane form Variant Surface Glycoprotein; PA, phosphatidic acid; Pal, palmitoyl; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PI-4,5-P2 (PIP2), phosphatidylinositol-4,5-bisphosphate; PI-4-P (PIP), phosphatidylinositol-4-phosphate; PL-PLC, phosphatidylinositol-specific phospholipase C; SDC, sodium deoxycholate.

A subclass of PI-PLC, glycosylphosphatidylinositol-specific phospholipase C (GP-PLC), catalyzes cleavage of the spacing arm of GPI-anchored proteins to release extracellular enzymatic activities of these proteins.22-34 Structural variations of GPI (4, Figure 2), include the presence of glucosamine35 or galactosamine36,37 and the attachment of a varying number of phosphoethanolamine and oligosaccharide branches of the glycan main chain.24,33,35,39 Although the majority of naturally occurring GPs are derivatives of myo-inositol as shown in Figure 2, some chiro-GPs have also been found in nature.36-38 Analogous cleavage of the membrane constituent GPs generates water-soluble phosphoinositol glycans implicated in the regulation of a variety of insulin-sensitive enzymes.35-40 The substrate preferences and other characteristics of various enzyme types are summarized in Table 1.

PI-PLCs show no structural or functional similarity to the nonspecific PLC. The latter prefers phosphatidylcholine and also accepts a variety of phospholipids. PI-PLCs, on the other hand, do not hydrolyze phospholipids other than PI at any substantial rate. B. cereus PLC is a zinc metalloenzyme with three Zn2+ ions.41 No sequence homology is found between three related enzymes from B. cereus: PI-PLC, nonspecific (or PC-specific) PLC, and sphingomyelin-specific PLC.42

Types of PI-PLC

1) Mammalian PI-PLC

Mammalian PI-PLC1-15,42-70 cleave PI (1a) and its phosphorylated forms PI-4-P (1b) and PI-4,5-P2 (1c) to produce a mixture of inositol 1,2-cyclic (2a-c) and acyclic phosphates (3a-c).64,65,79,80 The preference of PI-PLCs for phosphorylated or nonphosphorylated substrates depends on the protein type and the level of calcium (Table 1). Typically, PIP2 is a preferred substrate at low (physiological) concentrations of calcium ion while PI becomes a preferred substrate at millimolar calcium concentrations. Several PI-PLCs such as the enzymes from guinea pig58 and melanoma cell line73 are active in the...
Absence of calcium and are only mildly activated by calcium. These enzymes either prefer the nonphosphorylated PI (melanoma PI-PLC\(^{73}\)) or hydrolyze various PI derivatives with similar efficiency (guinea pig PI-PLC\(^{81}\)). The preference for a specific type of fatty acid is low.\(^{82}\)

(2) Bacterial PI-PLC

Bacterial PI-PLC\(^{82,83-93}\) cleave nonphosphorylated (1a) and glycosylated forms (1d) of phosphatidylinositol and lysophosphatidylinositol.\(^{30,85,87,88}\) They are much more efficient catalysts than mammalian enzymes (with \(V_{max}\) 1000–2000 versus 15–30 \(\mu\)mol·mg\(^{-1}·\)min\(^{-1}\);\(^{57,88}\) see Table 1).

Figure 2. Structure of glucosamine glycan containing GPL (4).

Figure 3. Time course of the hydrolysis of \([\text{H}]^{16}O\), \(^{17}O\)DPP at bovine brain PI-PLC-\(\beta\) at pH 8, followed by \(^{31}P\) NMR (from ref. 94 with permission).
### Table 1. Properties of purified and partially purified PI- and GPI-PLC from various sources

<table>
<thead>
<tr>
<th>Source</th>
<th>Enzyme type</th>
<th>Size (kDa)</th>
<th>Substrate preference</th>
<th>K$_{m,app}$ (mM)</th>
<th>Specific Activity (μmol·mg$^{-1}$·min$^{-1}$ or half-max. effect)</th>
<th>Metal ion (K$_{m}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>bovine brain</strong></td>
<td>α</td>
<td>60$^d$</td>
<td>PIP$_2$/PIP</td>
<td>nd</td>
<td>8$^i$</td>
<td>Ca$^{2+}$</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>α</td>
<td>57</td>
<td>PIP$_2$/PI</td>
<td>0.09/0.26</td>
<td>0.005/0.25</td>
<td>Ca$^{2+}$</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>β$_1$</td>
<td>154</td>
<td>PIP$_2$/PIP&gt;PI</td>
<td>0.16/0.25$^h$</td>
<td>&gt;100</td>
<td>Ca$^{2+}$ (5-10 μM)$^i$</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>γ$_1$</td>
<td>85</td>
<td>PIP$_2$/PI$^a$</td>
<td>nd</td>
<td>30-40</td>
<td>Ca$^{2+}$ (10-100 μM)$^i$</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>δ$_1$</td>
<td>85</td>
<td>PIP$_2$/PIP$^a$</td>
<td>0.096/0.144$^h$</td>
<td>24</td>
<td>Ca$^{2+}$ (0.6 μM)</td>
<td>46, 243</td>
</tr>
<tr>
<td><strong>bovine heart</strong></td>
<td>PIC3</td>
<td>nd</td>
<td>PIP$_2$/PIP/PI</td>
<td>0.095/0.0001$^h$</td>
<td>6.1/0.075$^h$</td>
<td>Ca$^{2+}$ (230 nM)</td>
<td>115</td>
</tr>
<tr>
<td></td>
<td>δ</td>
<td>85</td>
<td>PIP$_2$/PI$^a$</td>
<td>0.095/0.0001$^h$</td>
<td>6.1/0.075$^h$</td>
<td>Ca$^{2+}$ (9.1/1.7 nM)$^h$</td>
<td>149</td>
</tr>
<tr>
<td><strong>bovine liver</strong></td>
<td>β</td>
<td>150</td>
<td>(PI-PLC-M$_3$)$^j$</td>
<td>0.015-0.05 (sat. $^i$)</td>
<td>nd</td>
<td>Ca$^{2+}$ (0.025-0.1 μM)</td>
<td>75</td>
</tr>
<tr>
<td><strong>bovine ROS</strong></td>
<td></td>
<td>nd</td>
<td>PIP$_2$/PI$^a$</td>
<td>nd</td>
<td>nd</td>
<td>Ca$^{2+}$ (0.1-0.01 mM)</td>
<td>146, 250</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nd</td>
<td>PIP$_2$/PI$^a$</td>
<td>nd</td>
<td>nd</td>
<td>Ca$^{2+}$ (0.1-0.01 mM)</td>
<td>250</td>
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<tr>
<td><strong>bovine smooth muscle cells</strong></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>α</td>
<td>58</td>
<td>PIP$_2$/PI$^a$</td>
<td>nd</td>
<td>204/29</td>
<td>Ca$^{2+}$ (0.17/0.3 mM)</td>
<td>151a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>157</td>
<td>PIP$_2$/PI$^a$</td>
<td>nd</td>
<td>204/29</td>
<td>Ca$^{2+}$ (0.09 mM)</td>
<td>151a</td>
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<tr>
<td><strong>bovine spleen</strong></td>
<td>γ$_2$</td>
<td>145</td>
<td>PIP$_2$/PI$^a$</td>
<td>0.16/0.11</td>
<td>12.8/18.1</td>
<td>Ca$^{2+}$ (0.1 mM)</td>
<td>67</td>
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<tr>
<td><strong>guinea pig</strong></td>
<td>α</td>
<td>58</td>
<td>PIP$_2$/PIP/PI</td>
<td>0.1/0.04/0.01</td>
<td>7.1/3.2/0.7</td>
<td>Ca$^{2+}$</td>
<td>58</td>
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<tr>
<td><strong>human spleen</strong></td>
<td></td>
<td>56</td>
<td>P$_k^j$</td>
<td>0.005</td>
<td>nd</td>
<td>none</td>
<td>74</td>
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<td></td>
<td>18</td>
<td>P$_k^k$</td>
<td>0.0015</td>
<td>nd</td>
<td>Ca$^{2+}$ (0.5-1 μM)$^i$</td>
<td>74</td>
</tr>
<tr>
<td>Human melanoma</td>
<td>Platelets</td>
<td>Porcine brain</td>
<td>Porcine lymphocyte</td>
<td>Rabbit brain</td>
<td>Rat brain</td>
<td>Rat liver</td>
<td>Sheep seminal vesicles</td>
</tr>
<tr>
<td>----------------</td>
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<td>-------------------</td>
<td>--------------</td>
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</tr>
<tr>
<td>platelets</td>
<td>mPLC-II 63 (m)</td>
<td>PIP₂&gt;Pi</td>
<td>0.5/0.7</td>
<td>8.6/1.3</td>
<td>Ca²⁺ (5-10 μM)</td>
<td>44</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td></td>
<td>mPLC-I 69 (m)</td>
<td>PIP₂&gt;Pi</td>
<td>0.5/0.7</td>
<td>9.3/1.3</td>
<td>Ca²⁺ (5-10 μM)</td>
<td>44</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td></td>
<td>PLC-II 57 (c)</td>
<td>PIP₂&gt;Pi</td>
<td>0.4/0.07</td>
<td>7.8/2.6</td>
<td>Ca²⁺ (5-10 μM)</td>
<td>44</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td></td>
<td>mPLC-II 61 (m)</td>
<td>PIP₂&gt;Pi</td>
<td>nd</td>
<td>nd</td>
<td>Ca²⁺ (0.1-10 μM)</td>
<td>251</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td>β₂</td>
<td>145 (c)</td>
<td>PIP₂, Pi</td>
<td>nd</td>
<td>nd</td>
<td>Ca²⁺ (10 μM, 1 mM)</td>
<td>252</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td>β</td>
<td>150 (m)</td>
<td>PIP₂&gt;Pi</td>
<td>0.12/0.29</td>
<td>38.9/1.6</td>
<td>Ca²⁺ (10-100 μM)</td>
<td>150</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td>β, fragment</td>
<td>100 (c)</td>
<td>PIP₂&gt;Pi</td>
<td>0.11/0.23</td>
<td>40.5/1.8</td>
<td>Ca²⁺ (10-100 μM)</td>
<td>150</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td></td>
<td>140 (c)</td>
<td>PIP₂, PIP, Pi</td>
<td>nd</td>
<td>nd</td>
<td>Ca²⁺ (10-100 μM)</td>
<td>56</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td></td>
<td>98</td>
<td>PIP₂/Pi</td>
<td>0.01-0.05°</td>
<td>1.2-2.5°</td>
<td>Ca²⁺ (Mg²⁺, °)</td>
<td>62</td>
<td>67 (c), 120 (c), 70 (c)</td>
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<td></td>
<td>440 (3x146)</td>
<td>PIP₂</td>
<td>nd</td>
<td>nd</td>
<td>Ca²⁺</td>
<td>69</td>
<td>67 (c), 120 (c), 70 (c)</td>
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<tr>
<td>Porcine brain</td>
<td>β</td>
<td>145 (c)</td>
<td>PIP₂</td>
<td>nd</td>
<td>Ca²⁺ (0.0001-0.01)</td>
<td>68</td>
<td>67 (c), 120 (c), 70 (c)</td>
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<tr>
<td>Lymphocyte</td>
<td>175 (m)</td>
<td>PIP₂/PIP</td>
<td>nd</td>
<td>nd</td>
<td>Ca²⁺ (1 mM)</td>
<td>49</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td>Rabbit brain</td>
<td>βₘ</td>
<td>155 (c)</td>
<td>PIP₂&gt;Pi</td>
<td>nd</td>
<td>Ca²⁺ (0.5 μM)</td>
<td>51</td>
<td>67 (c), 120 (c), 70 (c)</td>
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<tr>
<td>IV</td>
<td>66, 61, 54 (m)</td>
<td>PIP₂&gt;Pi</td>
<td>nd</td>
<td>nd</td>
<td>Ca²⁺ (0.5 μM)</td>
<td>51</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td>Rat brain</td>
<td>δ</td>
<td>85</td>
<td>PIP₂</td>
<td>0.08°</td>
<td>15.3°</td>
<td>50</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PIP</td>
<td>0.075°</td>
<td>3.3°</td>
<td>Ca²⁺ (1-10 μM)</td>
<td>50</td>
<td>67 (c), 120 (c), 70 (c)</td>
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<tr>
<td></td>
<td></td>
<td>PIP</td>
<td>&gt;0.2°</td>
<td>2.7°</td>
<td>Ca²⁺ (1 mM)</td>
<td>50</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PIP (not Pi)</td>
<td>0.13°</td>
<td>12.9°</td>
<td>Ca²⁺ (ca. 10 μM)</td>
<td>50</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PIP₂, PIP, Pi</td>
<td>0.08°</td>
<td>6.5°</td>
<td>Ca²⁺</td>
<td>50</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PIP₂ (not Pi)</td>
<td>nd</td>
<td>nd</td>
<td>Ca²⁺</td>
<td>50</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td>Rat liver</td>
<td>87 (c)</td>
<td>PIP₂=PIP₂&gt;Pi</td>
<td>0.087°</td>
<td>2390°</td>
<td>Ca²⁺ (2 μM)</td>
<td>59</td>
<td>67 (c), 120 (c), 70 (c)</td>
</tr>
<tr>
<td>Sheep seminal vesicles</td>
<td>α</td>
<td>65 (c)</td>
<td>PIP&gt;Pi</td>
<td>0.02-0.05</td>
<td>24.7, 28.7</td>
<td>Ca²⁺</td>
<td>234</td>
</tr>
<tr>
<td>Turkey erythrocytes</td>
<td>85</td>
<td>PIP&gt;Pi</td>
<td>0.02-0.05</td>
<td>7.5</td>
<td>Ca²⁺</td>
<td>234</td>
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Table 1. Continued

<table>
<thead>
<tr>
<th>Species</th>
<th>PIP&lt;sub&gt;2&lt;/sub&gt;/PIP (not PIP)</th>
<th>Bacterial PI-PLC</th>
<th>Ca&lt;sup&gt;2+&lt;/sup&gt; (Mg&lt;sup&gt;2+&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>wheat</strong></td>
<td></td>
<td></td>
<td>113</td>
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<tr>
<td><strong>B. cereus</strong></td>
<td>34.5</td>
<td>PI/GPI-AP&lt;sup&gt;q&lt;/sup&gt; 1.3 (PI)</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PI/GPI-AChE&lt;sup&gt;r&lt;/sup&gt; 2&lt;sup&gt;2&lt;/sup&gt;/0.017&lt;sup&gt;r&lt;/sup&gt;</td>
<td>1660&lt;sup&gt;s&lt;/sup&gt;/56&lt;sup&gt;s&lt;/sup&gt;/0.3&lt;sup&gt;r&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>B. thuringiensis</strong></td>
<td>34.5</td>
<td>PI</td>
<td>1850</td>
</tr>
<tr>
<td><strong>C. novyi</strong></td>
<td>30</td>
<td>PI/GPI-AP&lt;sup&gt;q&lt;/sup&gt; nd</td>
<td>nd</td>
</tr>
<tr>
<td><strong>Listeria monocytogenes</strong></td>
<td>32.9</td>
<td>mIVSG/PI          0.15&lt;sup&gt;r&lt;/sup&gt;</td>
<td>nd</td>
</tr>
<tr>
<td><strong>Cytophaga sp.</strong></td>
<td>17</td>
<td>PI, GPI-prot.     2</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>PI, GPI-AChE&lt;sup&gt;r&lt;/sup&gt; 0.2</td>
<td>nd</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td>33&lt;sup&gt;l&lt;/sup&gt;</td>
<td>PI, GPI-prot.     nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

**GPI-PLC**

<table>
<thead>
<tr>
<th>Trypanosoma brucei</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>rat liver</td>
<td>39 (m)</td>
<td>mIVSG, PI&lt;sup&gt;q&lt;/sup&gt; 0.004&lt;sup&gt;n&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>37 (m)</td>
<td>mIVSG, lipid A&lt;sup&gt;p&lt;/sup&gt; PI</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>40 (m)</td>
<td>GPI, mIVSG        nd</td>
<td>nd</td>
</tr>
<tr>
<td>rat serum</td>
<td>52 (m)</td>
<td>GPI, mIVSG        nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

<sup>a</sup>as defined in ref. 4; <sup>b</sup>c and m in parentheses stand for cytosolic and membrane-bound, respectively; <sup>c</sup>determined with substrates shown; <sup>d</sup>native protein is a trimer; <sup>e</sup>dimeric protein is also detected; <sup>f</sup>PI is utilized only at high [Ca<sup>2+</sup>]; <sup>g</sup>PIP<sub>2</sub> hydrolysis is inhibited at high [Ca<sup>2+</sup>]; <sup>h</sup>values for PIP<sub>2</sub> and PI, respectively; <sup>i</sup>saturating conc; <sup>j</sup>PI-PLC-M<sub>1</sub> and M<sub>2</sub> were shown to be of β type but more responsive to stimulation; <sup>k</sup>activity with PIP<sub>2</sub> was not determined; <sup>l</sup>complex with a regulatory protein; <sup>m</sup>several activities with similar molecular weight; <sup>n</sup>three other proteins with molecular weights of 95 and 270 and 400 kDa with similar substrate and calcium specificity were also isolated; <sup>o</sup>with PIP<sub>2</sub> as substrate; <sup>p</sup>at 0.1 mM Ca<sup>2+</sup>; <sup>q</sup>GPI-anchored alkaline phosphatase; <sup>r</sup>GPI-anchored acetylcholinesterase as a substrate; <sup>s</sup>PI as a substrate; <sup>t</sup>molecular weight of 20 kDa was also reported.
While mammalian PI-PLCs produce both cyclic and acyclic inositol phosphates simultaneously (Figure 3), bacterial enzymes produce only Ip and diacylglycerol. However, PI-PLC from B. cereus and B. thuringienis have been shown to further hydrolyze Ip at low rates to give inositol 1-phosphate (IP)\(^{95,94}\) (Figure 4) which may explain an earlier finding of both cyclic and acyclic products.\(^{83}\) Another difference between bacterial and mammalian PI-PLC is that phosphorylated PIs are not substrates for bacterial PI-PLC.\(^{88,91}\) Furthermore, bacterial PI-PLC may be used as a tool to release GPI-anchored proteins from the cell surface.\(^{30}\) The primary products of the cleavage of GPI is presumed to be the corresponding 1,2-cyclic phosphate and diacylglycerol\(^ {29,95}\) therefore palmitoylation at the 2-hydroxyl group of the inositol makes GPI-anchors resistant to PI-PLC cleavage.\(^ {99-101}\)

With PI-PLC from B. cereus, binding of GPI anchor is ca 50–100 times stronger than that of PI while their turnover is 10\(^ 2\) times slower thereby maintaining almost constant V\(_{\text{max}}/K_m\).\(^ {102}\)

(3) GPI-specific PLC (GPI-PLC) from Trypanosoma brucei\(^ {52,103-106}\)

This variant of PLC is involved in the removal of the variant surface glycoprotein during the life cycle of the parasite.\(^ {23,24}\) The preferred substrates for these enzymes are GPI (1d) and GPI-protein conjugates such as membrane form of Variant Surface Glycoprotein (mVSG)\(^ {105}\) or their corresponding lyso-derivatives.\(^ {106}\)

(4) Insulin-stimulated mammalian GPI-PLC

This enzyme\(^ {107-110}\) is implicated in the mechanism of the transduction of the insulin signal.\(^ {23,39,40,107}\) GPI-PLC appears to have a very stringent structural requirement for glycosylated derivatives of phosphatidylinositol. Marginal cleavage of PI (1a) has been observed with the trypanosomal enzyme,\(^ {105,110}\) but not with rat liver enzyme.\(^ {107}\) Phosphorylated PIs (1b,c) are not substrates either.\(^ {107}\) The fact that GPI-PLCs utilize structurally heterogeneous mVSG as a substrate\(^ {95,105}\) suggests that some modifications in the glycine chain are allowed.

In addition to these four types, PI-PLC has also been isolated from other sources such as Dictyostelium,\(^ {111}\) Drosophila\(^ {112}\) and plants.\(^ {113-115}\) The PI-PLC activity has also been detected and characterized in many animal tissues without isolation. The enzymes in the first two categories constitute the majority of PI-PLCs. Many of these proteins have been isolated, sequenced and characterized\(^ {5,30}\) and a few have been overexpressed.\(^ {104,105,116}\)

**Chemical Mechanism of PL-PLC Reactions**

(1) Parallel versus sequential formation of Ip and Ip

The most intriguing mechanistic question for PI-PLCs is why mammalian PI-PLCs produce both cyclic and acyclic products simultaneously\(^ {117}\) (Figure 3) while in the case of bacterial PI-PLCs the reaction is clearly sequential (Figure 4).\(^ {93,94}\) Two other enzymes, annexin III, which utilizes glycerol-1-phosphoinositol,\(^ {118,119}\) and the nonspecific phospholipase C from Listeria,\(^ {120}\) which utilizes phosphatidylinositol, produce exclusively acyclic inositol 1-phosphate as their final product. Whether the cyclic 1,2-phosphate is involved as an intermediate in the reactions of these two enzymes remains to be determined. For mammalian PI-PLC the ratios between cyclic and acyclic products depend on the substrate (1a-c), isozyme (1–5),
pH, and calcium concentration.\textsuperscript{79} In the case of isoforms from bovine brain the percentage of the cyclic phosphates decreases in the order PLC-\(\beta\) > PLC-\(\delta\) > PLC-\(\gamma\) and PI > PIP > PIP\(_2\).\textsuperscript{79} The latter is also true for the \(\alpha\)-isozyme from sheep seminal vesicles.\textsuperscript{64} The proportion of cyclic phosphates increases at lower pH.\textsuperscript{64,121} The increase of calcium concentration above the threshold value of 2 mM abruptly increases the percentage of cyclic phosphates when PIP and PIP\(_2\) (but not PI) are the substrates.\textsuperscript{79}

The most straightforward explanation for the simultaneous formation of the dual product (ICP + IP) by mammalian PI-PLC is a competitive attack of the water molecule or the inositol 2-hydroxyl group at the phosphorus atom\textsuperscript{122} (Figure 5, Scheme A). This could be the consequence of several mechanistic possibilities: (i) there is an actual competition between water molecule and the hydroxyl group in the single active site, which would require an unlikely scenario with simultaneous activation of the water molecule and the hydroxyl group; (ii) there are two conformational states of the enzyme–substrate complex, one using water and the other using hydroxyl group as the attacking nucleophile; and (iii) there are two active sites on the same protein each carrying out a different reaction. An alternative explanation is that the two products are formed in two consecutive steps (Figure 5, Scheme B) as in the case of bacterial enzymes, but part of the ICP intermediate is released from mammalian enzymes. In Schemes A and B we have assumed direct nucleophilic displacements. In principle, the reactions could also go through an enzyme–IP intermediate S as shown in Schemes C and D in Figure 5.

Figure 5. Possible mechanisms of PI-PLC.
Distinction between the four different mechanisms in Figure 5 was made by determining separately the steric courses of the formation of IcP and IP from the P-stereogenic analogs of phosphatidylinositol 6 and 7 in Figure 6.\textsuperscript{127,128} The underlying stereochemical principle is that, barring pseudorotation, a single displacement should result in the inversion of configuration at the phosphorus atom while an even number of steps proceeding with inversion should lead to overall retention.\textsuperscript{127,128} As shown in the Scheme A of Figure 7, cleavage of \( R_p \)-6 (a preferred substrate over \( S_p \)-6) by both guinea pig PI-PLC-\( \alpha \) and \( B. \) cereus PI-PLC occurs with inversion of configuration at phosphorus to give trans-IcPs (8).\textsuperscript{123,124} In a separate series of experiments (Figure 7, Scheme B), cyclization of P-chiral diastereomers of \([^{15}O,^{17}O]DPPI \) (7) to \([^{15}O,^{17}O]IcP \) (9) catalyzed by PI-PLC-\( \beta_1 \) was also shown to proceed with inversion of configuration.\textsuperscript{94} These results rule out mechanisms C and D in Figure 5.

Hydrolysis of diastereomers of \([^{16}O,^{17}O]IcP \) (9) to \([^{16}O,^{17}O,^{18}O]IP \) (10) by \( B. \) cereus PI-PLC proceeded with inversion of configuration as expected for a single displacement step.\textsuperscript{94} Since IcP is an incompetent substrate for mammalian PI-PLC, the same reaction cannot be examined directly. However, formation of \([^{16}O,^{17}O,^{18}O]IP \) from \([^{15}O,^{17}O]DPPI \) catalyzed by bovine PI-PLC-\( \beta_1 \) was shown to proceed with overall retention. This result strongly suggests that in spite of the deceptively simultaneous formation of IcP and IP by mammalian...
enzymes this process is likely to be sequential (Scheme B in Figure 5), in agreement with the mechanism of bacterial enzymes. The only difference between mammalian and bacterial enzymes could be in the relative rates of the microscopic steps. The kinetic incompetence of IcP for mammalian PI-PLC could be explained by its inability to partition into the micelles or bilayers of the substrate or to bind productively due to the lack of a hydrophobic tail. Even with bacterial enzymes IcP is hydrolyzed only at a very low rate.

(2) Transesterification of IcP with primary alcohols

The reversibility of enzymatic reactions is frequently utilized for synthetic purposes (e.g. base exchange activity of phospholipase D). For bacterial PI-PLC it has been demonstrated that formation of IcP from PI is a reversible process in which primary alcohols can substitute for diacylglycerol to form the acyclic diester (Figure 8). Formation of acyclic diesters was observed with a number of primary alcohols such as methanol, propanol, Tris buffer, ethylene glycol, propane 1,2-diol, glycerol, D-mannitol, L-serine, and choline, but not with secondary alcohols, as components of the reaction mixture. The new diester 11 is a much poorer substrate than PI and therefore accumulates in the course of the process. The product from glycerol consists of an equimolar mixture of sn-1 and sn-3 derivatives. The broad scope of acceptable primary alcohols and the low stereospecificity of the transesterification reaction are consistent with the lack of glycerol C-2 stereospecificity of the PI cleavage and suggests that the alcohol binding site is relatively flexible.

Catalytic Properties and Cofactors

(1) Interfacial binding and detergents

PI-PLC are enzymes which utilize membrane-bound substrates in their natural environment. Most PI-PLC (with notable exception of the species from S. aureus) prefer micellar substrates dispersed with detergents such as sodium deoxycholate, Triton X-100 or octyl glucoside. However, the majority of the kinetic results on PI-PLC obtained to date have not dealt with the problem of 'interfacial binding'. Furthermore, the results obtained under different substrate conditions may be difficult to compare, particularly since some of the PI-PLCs are cytosolic proteins and some are membrane-bound. Several PI-PLCs show different pH maxima when acting on micellarly dispersed, vesicular and monolayer substrates or substrates dispersed with different detergents. The cytosolic sheep seminal PI-PLC is activated by diglycerides and fatty acids, but inhibited by PC. On the other hand, the human platelet enzyme (membrane-bound) is greatly activated by phosphatidic acid (PA), but not by diglycerides nor by other phospholipids. The effect of PA on PI-PLC-γ depends on whether the enzyme is in its activated (tyrosine phosphorylated) or latent form. The effect of PA on the phosphorylated form is to decrease the K_m while it is acting as an allosteric modifier on the unphosphorylated form. The minimal concentration of substrates required to observe hydrolysis with PI-PLC-δ decreases in the order: di-C_4-PI > di-C_6-PI > di-C_8-PI, consistent with the decrease in their critical micelle concentrations.

Figure 8. The transesterification reaction catalyzed by PI-PLC.
\( \delta_1 \) binds strongly to negatively charged \( \text{PIP}_2/\text{PC} \) vesicles (\( K_a = 10^6 \text{ M}^{-1} \)) but not to vesicles composed of only zwitterionic lipids.\(^{139} \) The activity of PI-PLC-\( \delta_1 \) decreases with the increase of surface pressure in \( \text{PIP}_2 \) and \( \text{PI}^1 \) monolayers. This result is consistent with a necessity to insert a small part (ca 1%) of the protein molecule into a monolayer prior to hydrolysis.\(^{140} \)

Bacterial PI-PLC display little activity with pure PI as a substrate,\(^{89,90,91} \) but are activated when PI is presented to the enzyme as mixed micelles with other phospholipids.\(^{102,143,144} \) The enzyme from \( B. \) \textit{thuringiensis} is activated by short-chain PC by a rate factor of \( 10^3 \) to \( 10^4 \). The highest degree of activation by detergents typically occurs at their low concentration (ca 0.1% SDC).\(^{89,90,146} \) However, even the relatively small family of bacterial PI-PLCs is not uniform with regard to the effect of phospholipids and detergents on activity. For example, the \textit{Cytophage} enzyme is deactivated by SDC and is not stimulated by PC,\(^{147} \) while the \textit{Staphylococcus} enzyme is readily deactivated by nonionic, positively and negatively charged detergents.\(^{89} \)

(2) Metal ions

Most mammalian PI-PLCs require calcium ions for activity, but not for binding to bilayers.\(^{1,119} \) The calcium requirement and the concentrations for maximal activation are listed in Table 1. In some instances, however, binding of PI-PLC to membranes is calcium independent (e.g. PI-PLC from chromaffin granules\(^{148} \) and rat brain\(^{122} \)). The half-maximal concentrations of calcium are much lower with \( \text{PIP}_2 \) as a substrate than with PI,\(^{63,79,150} \) but these parameters vary for different types of PI-PLC from \( 30 \text{ mM}^{149} \) to several millimolar.\(^{47,50,59,73} \) Higher Ca\(^{2+} \) concentrations could become inhibitory for \( \text{PIP}_2 \).\(^{15,79,151} \)

Some PI-PLC-\( \alpha \) are only slightly stimulated by calcium ions.\(^{61} \) A species of PI-PLC from smooth muscle cell is also different from other types in that the hydrolysis of both \( \text{PIP}_2 \) and PI is stimulated even at millimolar Ca\(^{2+} \).\(^{151} \) Interestingly, murine B lymphocytes contain another type of PI-PLC specific for PI which is exclusively activated by Mg\(^{2+} \).\(^{152} \) The 98 kDa PI-PLC from platelet cytosol is activated by both Ca\(^{2+} \) and Mg\(^{2+} \).\(^{52} \) The activation by Mg\(^{2+} \) could function as a permanent 'on' switch of PI-PLC in the absence of agonist stimulation when the level of intracellular Ca\(^{2+} \) is too low for PI-PLC activation.\(^{52} \)

Many types of mammalian PI-PLC are reversibly inhibited by EDTA, and heavy metals such as La\(^{3+} \), Hg\(^{2+} \), Cu\(^{2+} \), Zn\(^{2+} \) and Cu\(^{2+} \).\(^{1,47,149} \)

Two types of GPI-PLC from rat liver differ in their metal cofactor requirements.\(^{107,109} \) Both enzymes are membrane-bound; the 52 KDa protein\(^{107} \) does not require calcium, while the second type (of an unknown molecular weight) is activated by calcium at concentrations slightly higher than physiological.\(^{109} \) The latter is also activated by metal chelating agents. The above properties are shared by two subtypes of membrane-associated GPI-PLC activities in bovine brain, one of which is calcium cation-chelator sensitive and the other one is calcium-independent.\(^{108} \)

In contrast to mammalian enzymes, bacterial PI-PLCs and trypanosomal GPI-PLCs do not require metal ions for activity,\(^{90} \) nor are they inactivated by EGTA, EDTA,\(^{87,90} \) or \( \alpha \)-phenanthroline.\(^{153} \) The inactivation by some metal ions takes place only at high (submicellar) concentrations.\(^{154} \) These results clearly indicate that metal ions are not cofactors of bacterial PI-PLC.

Assays of PI-PLC Activity

Major assay methods used for phospholipases have been reviewed recently;\(^{155} \) however, many of the reviewed methods are not applicable to PI-PLC. In particular, titrimetric assays have not been used for PI-PLC since bacterial enzymes do not liberate acids during the formation of ICP. Mammalian enzymes liberate acids (acrylic phosphates), but it is not a good measure of the total activity due to simultaneous formation of ICP.

(1) Assays employing radiolabeled substrates

These methods utilize \([\text{H}]\)-labeled PI\(^{156} \) or \([\text{H}]\)-labeled \( \text{PIP}_2 \) as substrates.\(^{32} \) Labeled phosphoinositides\(^{157} \) are less suitable due to a rapid decay of radioactivity. Polar products are separated from substrates by extraction of assay mixtures with organic solvents. The extent of the reaction is determined from the radioactivity remaining in the organic or aqueous phase after phase separation. Alternatively, the assay mixture is passed through the reversed-phase C\(_18\) resin to remove the lipid. The water soluble phosphate is then quantified by counting radioactivity after filtering off and washing the resin.\(^{158} \) Separation can also be achieved by thin-layer chromatography. These methods are laborious and discontinuous, but they are very sensitive and are by far the most commonly used.

(2) Spectrophotometric assays

Dye release. \( \alpha \)-Nitrophenyl-1-phosphoinositol (12, Figure 9) was used as an assay substrate for \( B. \) \textit{cereus} and \( B. \) \textit{thuringiensis} PI-PLCs.\(^{158,159} \) Formation of ICP is accompanied by the release of \( \alpha \)-nitrophenol which is quantified by UV spectrophotometry. This assay has the advantage of being continuous. However, the unaggregated substrate 12 binds only very weakly to bacterial PI-PLC (K\(_m\) > 15 mM).

Thiol assay. Hexadecylthio-1-phosphoinositol (13), a phosphorothiolate analog of PI, releases hexadecylthiol upon the cleavage to ICP by \( B. \) \textit{cereus} PI-PLC.\(^{160,161} \) In the presence of 4,4'-dipyridyl disulfide the thiol is converted to mixed pyridylalkyl disulfide thereby producing an equivalent amount of highly chromophoric pyridyl-4-thiol which is then quantified by UV spectrophotometry.\(^{162-165} \) This assay offers continuity and ensures relatively good interfacial binding of the substrate to the enzyme. However, the V\(_{\text{max}}\) of 13 is only about 1% of that with natural PI.\(^{161} \) The corresponding phosphorothiolate analogs of PI (14) have been synthesized\(^{166} \) but the kinetic parameters are comparable to those of 13.\(^{166} \)
Application of safranine. Metachromatic properties of a dye, safranine, were used as a basis of another PI-PLC assay. Interaction of safranine with negatively charged molecular aggregates brings about a decrease in the absorbance at 520 nm. The change is proportional to charge density at the interface and therefore also to the concentration of PI. This relatively simple assay allows determination of PI-PLC activity at the level of several milliunits.

(3) Coupled enzymatic assay

This method has been used for the determination of inositol phosphates in biological tissues, but it can be adapted for a continuous PI-PLC assay. Inositol phosphate released from PI-PLC reaction is dephosphorylated with alkaline phosphatase to give myo-inositol, which is further oxidized to scyllo-inosose with inositol dehydrogenase. The unfavorable equilibrium of this process can be shifted by oxidation of NADH by diaphorase, the enzyme that converts nonfluorescent dye resazurin into highly fluorescent resorufin. Resorufin is then continuously determined with a fluorometer.

(4) Fluorometric assays

(A) A discontinuous method utilizing 4-(1-pyrene)butyl-1-phospho-myoinositol as a substrate. The substrate upon hydrolysis gives pyrenebutanol which is quantified by fluorometry after it is separated from the mixture by HPLC. This assay is quite laborious, and the activity of B. cereus PI-PLC with this substrate analog is only 4% of that with natural substrate. The more promising substrate analogs (16 and its phosphate derivatives, and 17) have been obtained by a chemical–enzymatic method, but their use in kinetic studies has not yet been reported. (B) A continuous fluorometric assay utilizing β-naphthyl-1-phospho-myoinositol as substrate. The measurement is based on the batochromic shift of the emission maximum of product β-naphthol as compared to the substrate phosphodiester. A severe disadvantage of this method is the extremely slow turnover (0.003% of the rate with PI) of the fluorescent substrate.

(5) Monolayer assay

The radiolabeled substrate such as PI or PIP2 is spread as a lipid monolayer at the water–air interface followed by the addition of the enzyme preparation to the subphase. The progress of the reaction is monitored by means of a Geiger–Mueller tube suspended just above the monolayer or by counting water soluble phosphates in the subphase. Using the barostat technique and the so-called zero-order Langmuir trough, it
is possible to use the unlabeled phospholipids as substrates. The contraction of the monolayer area as a function of time is a measure of the reaction rate. Monolayer assays offer many advantages over the bulk substrate assays among which minimization of the enzyme and substrate usage, and mimicking the conditions existing in the natural membrane are the most important. As a disadvantage, partial loss of activity has been reported with some enzymes due to surface denaturation or adsorption into the hydrophobic trough walls.

(6) Release of GPI-anchored proteins

Rat kidney tissue or bovine erythrocytes are incubated with PI-PLC and the released alkaline phosphatase (AP) or acetylcholinesterase (ACHE), respectively, are quantified using chromophore-producing substrates such as pnitrophenyl phosphate and acetylthiocholine. Both methods are discontinuous.

(7) Phosphorus-31 NMR

$^{31}$P NMR is probably the most versatile method, allowing for simultaneous quantification and identification of hydrolytic products of PI-PLC. The distinction between the substrate, the cyclic and the acyclic inositol phosphates is easily made due to the low-field chemical shift of the cyclic phosphates (ca 16 ppm for IcP, 4 ppm for IP, -1.5 ppm for PI). The best utility of this method is in semi-quantitative measurements and in verification of the structure of products. In order to obtain spectra with sufficiently narrow line-shapes monomeric or micellar substrates have to be used, with detergent-phospholipid ratios substantially higher than those typically recommended for maximal activity.

(8) Mass spectrometry

This technique is used more frequently for structural assignments than for absolute product quantification. Quantification of nonvolatile inositol phosphates requires prior derivatization into persilyl or perfluoroaryl derivatives. Determination of the ratio of cyclic/acyclic inositol phosphate can be achieved by acidic hydrolysis of cyclic phosphates in $H_2^{18}O$ followed by removal of the phosphate with alkaline phosphatase, conversion of the phosphate into its tris(tert-butylimidethylsilyl) ester and measurement of $^{18}O$ enrichment by mass spectrometry.

(9) Miscellaneous assays

A number of useful methods of quantification of inositol phospholipids and inositol phosphates derived therefrom is described in the collective work "Methods in Inositol Research." A large effort has been devoted to developing alternative methods for detection of inositol phosphates (other than radioassay). HPLC chromatography in conjunction with the post-column complexometry or ion chromatography with conductometric detection are among the best choices.

Substrate Analogs and Inhibitors

Isozyme-specific inhibitors of PI-PLCs are of enormous therapeutic potential, especially as anti-inflammatory agents. They may also be important tools in establishing the correlations between various branches of phosphoinositide metabolism and in elucidation of the mechanism and structure of PI-PLCs. However, most of the synthetic activity in the area of phosphoinositides has been devoted to analogs of inositol polyphosphates rather than phosphatidylinositides (PI and its derivatives). Inhibition of inositol 1-phosphate phosphatase, phosphatidylinositol kinases, and IP$_3$-receptor binding have attracted more attention.

To date the inhibition studies of PI-PLC have not tried to resolve the interfacial binding and active site binding steps. Furthermore, various forms of PI-PLC (α-ε) have different origins (cytosolic or membrane bound) and thus probably different interfacial binding properties. It is thus important that the enzyme preparations tested be resolved into subtype species. Unfortunately this is not the case in many of the works cited below. For these reasons the substrate specificity and inhibition properties described in the following sections should be interpreted with great caution.

(1) Modification of the diacylglycerol moiety

The diacylglycerol moiety appears not to be an important determinant for catalysis, but it may be important for the partition of substrates into micelles or bilayers. Neither mammalian nor bacterial PI-PLCs discriminate between compounds having L or D configurations at the glycerol C-2 carbon. Lysophosphatidylinositol, other single chain primary alkyl, alkylthio, and aryl esters of inositol 1-phosphate are also cleaved, though at reduced rates. The deacylated glycerophosphoinositol is hydrolyzed by B. thuringiensis PI-PLC at a rate lower than that of IcP. The preference for a certain type of fatty acids is only several fold. The deacylated analog of PIP is an inhibitor of PI-PLC whereas single chain alkyl phosphoinositol analogs (in which the diacylglycerol is replaced by a long chain alcohol) inhibit guinea pig PI-PLC-α with IC$_{50}$ = 14–50 μM.

(2) Modification of the phosphodiester moiety

The phosphodiester moiety is important for catalysis. The phosphorothioate analogs, RS- and (S)$_2$-DPPs (6), show substantially decreased activities. In addition, both mammalian and bacterial PI-PLCs display a high degree of stereoselectivity toward the $R_p$ isomer. A series of phosphonate sulfate or methyleneoxide analogs of PI, (19–22, Figure 10) have been synthesized but no follow up study on PI-PLC inhibition with these analogs has been reported. The related single chain phosphonate analogs 23 were found to be weak inhibitors of PI-PLC from B. cereus with IC$_{50}$ in the range 4 to 7 mM.
The inhibitory properties of the analogs in which the phosphodiester group is replaced by nonionic groups vary. Carboxylate derivatives of myo-inositol,204,205 palmitoyl derivatives of myo-inositol,191 and palmitoyloxymethylene derivatives of myo- and chiro-inositol (24 and 25, respectively)191,197 are all poor inhibitors; however, introduction of phosphates or sulfates at the inositol ring increases the inhibitory capability of some of these analogs to crude platelet PI-PLC, with IC50 in the range of 10 to 100 μM.191 On the other hand, the PI-PLC-α from guinea pig is inhibited, with IC50 in the same range, by non-phosphorylated palmitoylinositol, palmitoylinositol, and the alkylosulfonyl derivative of inositol (26).188

(3) Modification of the inositol moiety

Interactions with the inositol moiety of PI are important for recognition. Both substrate and inhibitor activities are affected by such changes as inositol phosphorylation, substitution of hydroxyl groups, deoxygenation and configurational changes of inositol. The results are summarized as follows: (i) The 1D-configuration of myo-inositol is absolutely required for bacterial PI-PLC.93,159,172 1D-Insitol-1,4,5-trisphosphate was found to inhibit binding of PI-PLC-α to lipid bilayers while the corresponding enantiomer showed no effect;194 (ii) according to our proposed sequential mechanism94 the axial hydroxyl group at the 2-position of inositol should be absolutely required for catalysis. Supporting this view is the fact that 2-deoxy-PI is not a substrate of human melanoma PI-PLC198 and bovine brain PI-PLC-β1, -γ1 and -δ1,199,200 It is only a weak inhibitor of the melanoma enzyme (IC50 > 2 mM) implying that the 2-OH group is also important for overall binding.198 Likewise, GPl species acylated with fatty acid at 2-position are resistant to PI-PLC from S. aureus.99-101,201 (iii) The orientation of the 3-OH group is an important determinant for the substrate, since inversion of the 3-OH group of the natural 1D-mylo-PI (the resulting analog is 1L-chiro-PI, see Figure 12 for structures and numbering) causes a 103-fold decrease in the activity towards bacterial PI-PLCs.196 The fact that its enantiomer, 1D-chiro-PI, shows no detectable activity (<10−6 relative to 1D-mylo-PI) is consistent with the requirement of 1D-configuration for myo-PI mentioned in (i). This result, however, contradicts an earlier report that PI-PLC from S. aureus cleaves GPI-anchor containing D-chiro-inositol;97,98,195 (iv) in agreement with the importance of the 3-OH group, phosphorylation at the 3-position makes PI resistant to mammalian enzymes;202,203 (v) although the structural requirement for catalysis is stringent at the inositol ring, that for binding or inhibition is not. The 2,3,5,6-tetrahydroxy analog of palmitol PI (27) inhibits guinea pig PI-PLC-α with an IC50 of 25 μM; however, the corresponding 3,4,5,6-tetrahydroxy analog (28) is a poor inhibitor.188 These results suggest that deoxygenation can be tolerated in binding to PI-PLC-α, except at the 4-position.
Figure 11. Inactivation of PI-PLC (B. thuringiensis) by the epoxide analog 31 monitored by $^3$P NMR. (A) 2.5 µmol of DPPC in 0.4 ml of 0.1 M Tris-HCl, pH 7.0, containing 0.1 M sodium deoxycholate; (B) 90 min after addition of 3.5 µg of PI-PLC reincubated with 0.4 mM of the epoxide analog; (C) same as B, after 12 h; (D) control experiment in the absence of the inhibitor, 5 min after addition of PI-PLC. The remaining enzyme activity in B-C is <1% relative to that in D.

1D-myO-PI

1L-myO-PI or 3D-myO-PI

1L-chiro-PI

1D-chiro-PI

Figure 12. Structures of 1D-myO-PI (natural substrate for PI-PLC), 1L-myO-PI (no detectable activity), 1L-chiro-PI (the 3-epimer of 1D-myO-PI, poor substrate), and 1D-chiro-PI (no detectable activity). The numbering conventions are different between the derivatives of myo- and chiro-inositol according to 1976 IUPAC rules [Biochem. J. 153, 23–31 (1976)].

(4) Analogs of 1C

The fluorophosphonate analogue of 1C 29 was found to be a modest inhibitor of B. cereus PI-PLC. According to recent calculations the fluoromethylene group prefers an apical position in the phosphorus trigonal bipyramid of a transition state or an intermediate (30) in a substitution reaction; therefore fluoromethylene group in 1C analog should stabilize a trigonal bipyramid intermediate (or a transition state).

(5) Epoxide analogs

In an attempt to develop active site directed irreversible inhibitors we have synthesized an epoxide analog of palmitoyl PI, 31. Despite the lack of the phosphatidylinositol moiety, this analog inhibits B. thuringiensis PI-PLC as shown in Figure 11 (Bruzik and Tsai, unpublished results). Although preliminary, such results suggest that epoxy analogs of PI and its derivatives are likely to be good inactivators of PI-PLC.

(6) Nonspecific inhibitors

Most of the inhibitors described in this section either interact with the enzymes nonspecifically or do not interact with the enzymes directly. For the latter group, the term 'inhibitor' is used in a broad sense. Phenothiazine-type drugs including methylazide, promethazine, chlorpromazine, chloroquine, quinacrine and Cibacron Blue $^{27,205,211}$ are inhibitors of PI-PLC with IC$_{50}$ in the range 3 to 300 µM. Various aminoglycoside antibiotics are also good inhibitors of PI-PLC.$^{212}$ The potency of streptomycin, amikacin, kanamycin, tobramycin, gentamycin and neomycin as inhibitors of renal PI-PLC correlates positively with their nephrotoxicity (basicty) with IC$_{50}$ in the range 30 to 380 µM.$^{212}$ The inhibition presumably arises from the ionic interaction between
positively charged protonated aminoglycoside and the negatively charged phospholipid aggregates. Consistently, neomycin is a stronger inhibitor of the hydrolisis of PIP₂ than of PIP₂.23 The inhibition of human platelet PI-PLC by aliphatic alkanediamines24,25 and polyamines26 is probably based on the same mechanism. The effect of polyamines and aminoglycoside on the activity of human amnon PI-PLC appears to be dependent on the concentration of divalent metal ions. Amines are inhibitory at low concentrations of Ca²⁺, but are stimulatory at high Ca²⁺.24 These complex dependencies probably result from a competitive interaction of divalent metal ions and protonated amino-compounds with negatively charged phospholipid bilayers.

A diether analog of PC with stearyl ether at sn-1 and methyl ether at sn-2 is one of the most potent inhibitors of fibroblast and adenocarcinoma cell PI-PLC (IC₅₀ = 0.4 μM).27 Recently a polycyclic xanthone derivative, vinaxanthone, has been found to have a strong inhibitory effect on rat brain PI-PLC with IC₅₀ at the level 5.4 μM.28 The mechanism of the inhibition by these two compounds remains unclear. Manoside, a terpenoid shown earlier to inhibit phospholipase A₂,29 is also inhibitory towards PI-PLC from guinea pig uterus.21 The inhibition is irreversible and time-dependent with IC₅₀ 3-6 μM, and occurs due most likely to covalent modification of the enzyme. Treatment of platelet PI-PLC with thiol-specific reagents such as 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) or methyl methanethiosulfonate strongly inhibits its activity.22

**Sequences and Structures**

The sequences of many bacterial and mammalian PI-PLCs have been reported as summarized in Table 2. However, except for the secondary structure of PI-PLC-α,51 no structural study of bacterial or mammalian enzymes has been reported to date. Mammalian PI-PLCs are difficult to obtain in a quantity large enough for crystallization study; the instability of many PI-PLC isoforms over a longer period of time may also be a problem. The Oregon group has succeeded in crystallization of PI-PLC from *B. cereus*. Crystals of two types with hexagonal and orthorhombic packing were obtained; the latter diffracted to 2.5 Å.224 However, the molecular structure remains to be solved. In the absence of tertiary structures, sequence analysis is important in understanding the structure–function relationship of PI-PLC.

**(1) Mammalian PI-PLC**

Analysis of sequences of mammalian PI-PLCs now available (Table 2) indicates that although all PI- and GPLC catalyze the cleavage of the phosphodiester bond in structurally closely related substrates, only a limited sequence homology exists between these proteins.4,9,11,13,23,3 Molecular sizes of PI-PLC range from 17 kDa for PI-PLC from human spleen to 150 kDa for PI-PLC from bovine brain. Based on the molecular size, sequence, and immunological cross-reactivity, mammalian PI-PLCs have been divided into several categories: PI-PLC-α (MW 56-68 kDa)10,58,234-236 PI-PLC-β (150-154 kDa)4,47,48,233,237,238 PI-PLC-γ (143-148 kDa)45,47,48,238,240-242 PI-PLC-δ (85-88 kDa)16,239,243,234 and PI-PLC-ε (86 kDa).245 Each β, γ and δ category of mammalian PI-PLC is further divided into several related subcategories differentiated by the substrates such as B₁, B₂, B₃, γ₁, γ₂, δ₁, δ₂ and δ₃. The enzymes from β, γ and δ categories differ in the mechanism of regulation and do not cross react with antibodies raised against species from another category. The overall sequence homology between the β-γ-δ-PI-PLC is also low. On the other hand, the enzymes belonging to the same category show immuno-crossreactivity, are regulated by the same mechanism and share high sequence homology. The differences between the enzymes of the same subcategory from various tissues, animal genuses or species are minimal. As an example, rat, bovine and human brain PI-PLC-γ₁ show more than 95% sequence homology.11

**Syntheses of Substrates and Substrate Analogs**

Although the synthesis of inositol phosphates and their analogs has been well developed as reviewed recently,184-186 synthesis of phosphatidylinositides such as PI, PIP, PIP₂ and their analogs is a much more challenging task due to the need not only to protect the hydroxyl groups, but also to protect them differentially to allow introduction of the phosphatate and the phosphates. Moreover, the presence of the alkali-labile diacylglycerol precludes application of the acyl-protection groups. In addition, certain analogs of phosphatidylinositols (e.g. those containing unsaturated fatty acids or phosphorothionates) preclude the use of hydrogenolytically removable protective groups. Therefore most of the enzymatic work has been performed using naturally occurring PI, PIP and PIP₂. Early synthetic efforts were summarized by Shvets221,222 and Gieg.223 Recently, total chemical syntheses of PIP₁,187,224-226,231 PIP₂,226 PIP₂,227,228 and chiro-PI196 have been described. PIP₁,190,229,230, PIP₂ and some analogs with altered fatty acids170,171,229 have also been obtained semisynthetically using naturally available phosphatidylinositols.

The synthetic work summarized above involves a variety of approaches for the synthesis of protected myo-inositol precursors. Many of the precursors are aimed at a specific target compound. Furthermore, most of the procedures involve numerous protecting and optical resolution steps, which lead to low overall yield. To overcome such problems, we have developed a systematic and efficient route for the synthesis of enantiomerically pure and regiospecifically protected myo-inositol.25 The key strategies are the use of camphor as a protecting group and a chiral auxiliary, and the development of regiospecific controls in various steps. These precursors can in principle lead to most, if not all, of the naturally occurring phosphatidylinositols, their phosphate derivatives, and inositol phosphates. The procedures can also be modified to synthesize various unnatural analogs and inhibitors.
<table>
<thead>
<tr>
<th>Source</th>
<th>Type</th>
<th>Other names</th>
<th>Calculated FW (number of amino acids)</th>
<th>Accession Number in EMBL/GenBank</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. cereus</em></td>
<td></td>
<td></td>
<td>34,466 (298, mature)</td>
<td>M28549</td>
<td>42</td>
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<tr>
<td><em>B. thuringiensis</em></td>
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<td></td>
<td>(330, precursor)</td>
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<td><em>Listeria monocytogenes</em></td>
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<td></td>
<td>36,299 (317, precursor)</td>
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<td><em>T. brucei</em></td>
<td></td>
<td>VSG lipase</td>
<td>40,760 (358)</td>
<td>J04124</td>
<td>103</td>
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<td><em>T. brucei</em></td>
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<td>VSG lipase</td>
<td>40,660 (358)</td>
<td>X13292</td>
<td>104</td>
</tr>
<tr>
<td>Guinea pig uterus</td>
<td>α</td>
<td>PI-PLC-I</td>
<td>56,559 (504)</td>
<td></td>
<td>235</td>
</tr>
<tr>
<td>Rat brain</td>
<td>β1</td>
<td>PI-PLC-I</td>
<td>138,225 (1216)</td>
<td></td>
<td>239</td>
</tr>
<tr>
<td>Bovine brain</td>
<td>β1</td>
<td>PLC-154</td>
<td>138,600 (1225)</td>
<td>J03137</td>
<td>52</td>
</tr>
<tr>
<td>Human promyelocyte</td>
<td>β2</td>
<td>(1181)</td>
<td></td>
<td>M95678</td>
<td>15</td>
</tr>
<tr>
<td>Human fibroblast</td>
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<td>Z16411</td>
<td>15</td>
</tr>
<tr>
<td>Rat thyroid cells</td>
<td>β3</td>
<td>norpA</td>
<td>(1095)</td>
<td>J03138</td>
<td>260</td>
</tr>
<tr>
<td>Drosophila</td>
<td>β</td>
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<td>1305 (1320)</td>
<td>M60452 (M60453)</td>
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<tr>
<td>Drosophila (2 seqs.)</td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Rat brain</td>
<td>γ1</td>
<td>PI-PLC-II</td>
<td>148,431 (1289)</td>
<td>J03806</td>
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<td>PLC-148</td>
<td>148,300 (1291)</td>
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<tr>
<td>Human leukocyte</td>
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<tr>
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<td></td>
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<tr>
<td>Dictyostelium discoideum</td>
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<td>91,000 (801)</td>
<td>M95783</td>
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</table>
The low overall homology between the proteins belonging to different categories (β-δ) increases up to 60% and 40% in the two domains designated as X (ca 170 amino acids) and Y (ca 240 amino acids), respectively.11,12 (Figure 13). Significant sequence homology outside regions X and Y exists only within the same category. For instance, β₁ and β₂ isozymes exhibit 54% identity in the amino-terminal region (before X box) and 31% identity in C-terminal fragment (after Y box). The X domains in 16 eucaryotic PI-PLC have the following stretch of absolutely conserved amino acids: Met—GlnProLeu—HisTyr—Ile—SerSerHisAsnThrTyrLeu—Glu—SerSer—Glu—Tyr—Leu—GlyCysArgCysValGluLeuAsp—Trp—Gly; the Y domains contain LeuSerArgIleTyrPro—Gly—Arg—AspSerSerAsnTyr—Pro—Trp—Gly—GlnMetValAlaLeuAsp—PheGluThr (dashes mean nonconserved amino acids). It is likely that these amino acids play important roles in catalysis.

The α-form of bovine PI-PLC, which is similar to bacterial enzymes in terms of metal ion requirement (can catalyze turnover without Ca<sup>2+</sup>, albeit at a lower rate), does not contain X or Y domain and has no sequence homology with other mammalian species.13 The relationship of PI-PLC-α to other isozymes is even less clear in view of the fact that cDNA cloned is highly homologous to a number of unrelated proteins and does not code for a functional PI-PLC.12

There are other types of mammalian PI-PLCs which do not fall into the α–δ categories, such as a 157 kDa protein from rat smooth muscle,151 a 150 kDa protein from human melanoma cells,72 high molecular weight PI-PLC from platelets,56 a 17 kDa protein from human spleen,74 and an 11 kDa PLC activity from platelets,49 and a Mg<sup>2+</sup>-dependent PI-PLC.115 It is possible that the smaller proteins are proteolytic fragments of other PI-PLCs, but no direct evidence has been reported.

(2) Bacterial PI-PLC and trypanosomal GPI-PLC

The molecular weights of bacterial enzymes are ca 35 kDa. The sequences of PI-PLCs from <i>B. cereus</i> and <i>B. thuringiensis</i> differ by only eight amino acids.42 PI-PLC from <i>B. cereus</i> and trypanosomal GPI-PLC, though catalyzing different reactions, are similar in size and highly homologous in the N-terminal region (37%) and in a small central core (50%).42 Another bacterial enzyme from <i>Listeria monocytogenes</i> is also very similar in size and is 24% homologous to <i>B. cereus</i> and trypanosomal enzymes.246 A relatively high degree of homology (26% identity, 52% conservative) exists between bacterial enzymes and segments X of eucaryotic PI-PLCs in positions which are also conserved between eucaryotic enzymes.42 It is therefore anticipated that these peptide fragments contain elements of the active site.

(3) Active site

Binding experiments of trypic fragments from <i>B. cereus</i> PI-PLC to monoclonal antibodies raised against the intact protein suggest that the active site is located in the peptide stretch between Gin-45 and Lys-122.247 This tryptic fragment, which contains 9 positively and 9 negatively charged residues, also displays the highest homology to the X-domain of eucaryotic PI-PLCs (Kuppe et al. 1990) and is probably important functionally. The possible candidates for active site residues in <i>B. cereus</i> PI-PLC include Gin-45, Glu-52, Arg-56, Arg-64, Asp-67 or Gln-67, His-83 and Gln-93 or Asp-93 which are conserved among <i>B. cereus</i>, <i>B. thuringiensis</i>, <i>T. brucei</i>, <i>Drosophila</i> norp A, rat β1 and δ1, and bovine γ1 PI-PLCs.42 Sequence deletion study of PI-PLC-γ2 showed that a large segment of the protein (up to 1/3 of the total length) residing between the X and Y boxes can be removed with only a small effect on the activity (10-fold decrease) while deletions within these boxes results in a complete loss of activity.241 It was therefore concluded that both X and Y boxes contribute to the structure of the active site, and that the median fragment which has homology to regulatory domains of tyrosine kinases is probably only important for regulation. Analogously, C-terminal or N-terminal truncation of PI-PLC-γ1 completely abolishes activity but the removal of the regulatory domain results only in a threelfold drop of activity.45 Limited proteolysis of PI-PLC-δ1 which removes the 60 amino acid N-terminal segment affords a 77 kDa peptide which retains 10% activity of the native protein but has a lower binding affinity to PIP2.
vesicles. The protein remains active even after further cleavage into a heterodimer of 45 kDa and 32 kDa. Therefore, it was postulated that the surface binding site resides in the N-terminal part of PI-PLC-δ. Deletion of a 45 kDa C-terminal fragment of PI-PLC-β affords a fully catalytically active 100 kDa polypeptide. The C terminus is therefore not necessary for catalysis.

Concluding Remarks

Although a vast amount of information has been summarized in this review, it is clear that our understanding of the mechanisms of PI-PLCs and G-PLCs is still at the infant stage. No tertiary structure is yet available, a standard and accurate assay method is yet to be developed, and more substrate analogs and inhibitors are yet to be developed. However, it is also clear that significant progress has been made in each of these areas, and that major developments are imminent.

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200. Schedler, D. J. A.; Rhee, S. G.; Baker, D. C. to be published.


Biographical Sketch of Karol S. Bruzik

Karol S. Bruzik became Assistant Professor of Medicinal Chemistry at the University of Illinois at Chicago in 1993. He received a PhD degree working with Wojciech J. Stec at the Polish National Academy of Sciences in 1980. Between 1981 and 1983 he had a postdoctoral position in Ming-Daw Tsai's lab. He became Assistant Professor at the Center of Molecular and Macromolecular Studies in Lodz, Poland in 1988, but returned to The Ohio State University in 1990 as a Research Scientist. His research interests include the synthesis of analogs of biomolecules as probes and inhibitors of enzymes, the mechanism of enzyme reactions, NMR of biomolecules, and generalization of self-assembly of amphiphilic molecules.
Biographical Sketch of Ming-Daw Tsai

Ming-Daw Tsai was born in Taiwan, Republic of China in 1950, and received a BS in chemistry from the National Taiwan University in 1972. After two years of military service, he went to Purdue University; received a PhD working with Heinz G. Floss in 1978. He joined the Chemistry Department at The Ohio State University in 1981 where he is now Professor of Chemistry and Biochemistry. He has also spent research leaves in the laboratories of Eric Oldfield, Sture Forsen and John Markley. His research interest lies in the interface between chemistry and biology, particularly in phosphorus stereochemistry, phospholipid biochemistry, structure–function relationships of phospholipases and kinases, and the use of NMR in biological systems.

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