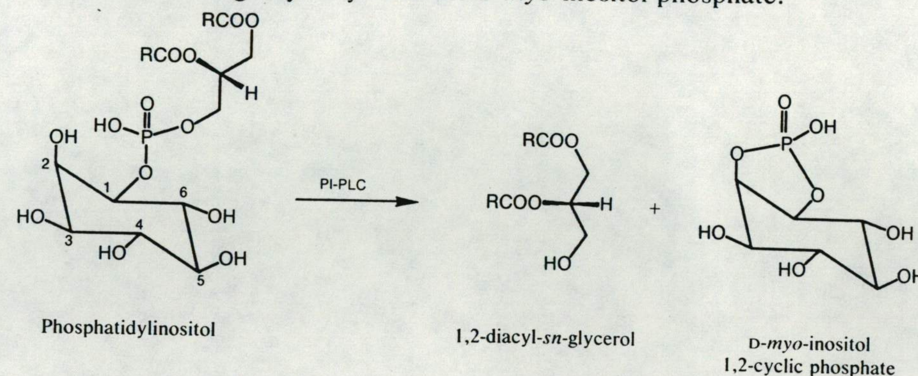
[47] Phosphatidylinositol-Specific Phospholipases C from *Bacillus cereus* and *Bacillus thuringiensis*

By O. HAYES GRIFFITH, JOHANNES J. VOLWERK, and ANDREAS KUPPE

Introduction

Phosphatidylinositol-specific phospholipase C (PI-PLC; EC 3.1.4.10, 1-phosphatidylinositol phosphodiesterase) from *Bacillus cereus* and *Bacillus thuringiensis* catalyzes the cleavage of the *sn*-3 phosphodiester bond of phosphatidylinositol (PI). Under some conditions the cyclic phosphate may then undergo hydrolysis to the D-*myo*-inositol phosphate.



Phosphatidylinositol 1,2-diacyl-*sn*-glycerol D-*myo*-inositol 1,2-cyclic phosphate

Bacteria of the genus *Bacillus* secrete a variety of hydrolytic enzymes including proteases, amylases, glucanases, and lipases, depending on the specific strain.[1] *Bacillus cereus* secretes three phospholipases, one specific for PI (the PI-PLC), a second hydrolyzing sphingomyelin, and a third with a preference for hydrolyzing phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine.[2-4] *Bacillus cereus* and *B. thuringiensis* are closely related rod-shaped gram-positive bacteria that are nonpathogenic to man. The enzymes are excreted in relatively large quantities across the single cell membrane into the growth medium. This facilitates the purification of PI-PLC because intracellular proteins are removed with the cells during an initial centrifugation step. PI-PLC is isolated from the

[1] A. Krieg, *in* "The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria" (M. P. Starr, ed.), p. 1743. Springer-Verlag, New York, 1981.

[2] H. Ikezawa, M. Mori, T. Ohyabu, and R. Taguchi, *Biochim. Biophys. Acta* **528,** 247 (1978).

[3] C. Little, this series, Vol. 71 [83].

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[1] A. Priest, *in* "The Prokaryotes: A Handbook on Habitats, Isolation, and Identification of Bacteria" (M. P. Starr *et al.*, eds.), p. 1713. Springer-Verlag, New York, 1981.
[2] H. Ikezawa, M. Mori, T. Ohyabu, and R. Taguchi, *Biochim. Biophys. Acta* 528, 247 (1978).
[3] C. Little, this series, Vol. 71 [83].
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supernatants of *B. cereus* and *B. thuringiensis* cultures in milligram quantities by an extension of the procedure of Ikezawa and Taguchi.[5] The final product is homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE), exhibits high specific activity, and was used to raise monoclonal antibodies and provide polypeptide sequence information for the cloning and DNA sequencing of PI-PLC from *B. cereus*.

Purification of Phosphatidylinositol-Specific Phospholipase C from Culture Media of *Bacillus cereus* and *Bacillus thuringiensis*

The same procedure is used to purify PI-PLC from *B. cereus* and *B. thuringiensis*.

Growth Conditions

The growth medium for *B. cereus* (ATCC 6464) and *B. thuringiensis* (ATCC 10792) contains 40 g Bacto-peptone, 40 g yeast extract, 20 g NaCl, 1 g Na_2HPO_4, and water to reach a final volume of 4 liters, adjusting the pH to 7.0 with 1 *M* NaOH. Stock cultures are prepared from single colony isolates by inoculating 100 ml of medium and growing overnight on a shaker at 37°. Stock cultures are stored in 4-ml aliquots containing 50% glycerol at −20°.

To generate starting material for the purification of PI-PLC, *B. cereus* or *B. thuringiensis* is grown in 4-liter batches in a jar fermentor. A preculture is prepared by inoculating 100 ml of medium with 4 ml of stock culture followed by incubation on a shaker at 37° for 12 hr. The preculture is then added to 4 liters medium in a jar fermentor and incubated at 37° with the stirrer set at 400 rpm and the air flow at 10 liters/min, while the pH is maintained at 7.0 by addition of NaOH and H_3PO_4. Incubation is continued for 3.5 hr until the culture is in the early stationary phase. The increase of PI-PLC activity in the culture medium closely parallels cell growth and is optimal in the late logarithmic/early stationary phase.[6]

Purification Procedure

Ammonium Sulfate Precipitation and Dialysis. The *B. cereus* or *B. thuringiensis* culture is cooled to 4°, and the cells are removed by centrifugation for 30 min at 13,000 *g*. Solid ammonium sulfate is added

[5] H. Ikezawa and R. Taguchi, this series, Vol. 71 [84].

[6] H. Ikezawa, M. Yamanegi, R. Taguchi, T. Miyashita, and T. Ohyabu, *Biochim. Biophys. Acta* **450,** 154 (1976).

slowly to the supernatant to 90% saturation (576 g/liter), and the solution is kept overnight at 4°. The dark-brown precipitate is collected by centrifugation for 30 min at 13,000 *g*, dissolved in a small volume of cold 5 m*M* Tris–maleate buffer (pH 6.5), and dialyzed against 3 changes of the same buffer at 4°. Subsequent chromatography and dialysis steps are carried out at 4°.

CM-Sephadex Column Chromatography. The dialyzed material is collected, and a small precipitate is removed by centrifugation for 30 min at 4500 *g*. The clear supernatant is applied to a column of CM-Sephadex (3.5 × 10 cm) and eluted with Tris–maleate buffer at a flow rate of 50 ml/hr. The dark-colored break-through fractions, which contain the PI-PLC activity, are pooled and dialyzed against 3 changes of 20 m*M* Tris-HCl (pH 8.5).

DEAE-Cellulose Column Chromatography. Following dialysis, the solution is applied to a DEAE-cellulose column (3.5 × 10 cm) equilibrated in 20 m*M* Tris-HCl (pH 8.5) and eluted with a linear gradient (2 × 750 ml) from 0 to 0.3 *M* NaCl in the same buffer. The fractions containing PI-PLC activity are pooled and concentrated to approximately 10 ml by ultrafiltration [Amicon (Danvers, MA) cell with a YM10 membrane].

Phenyl-Sepharose Column Chromatography. The concentrated DEAE-cellulose fractions are loaded onto a Phenyl-Sepharose column (2 × 7 cm) equilibrated in 20 m*M* Tris-HCl (pH 7.5). The column is rinsed exhaustively with the same buffer (at least 50 ml) until the UV monitor gives a baseline reading. PI-PLC activity is then eluted with the same buffer containing 50% ethylene glycol (by volume). Fractions containing PI-PLC activity are pooled, and the ethylene glycol is removed and the solution concentrated by ultrafiltration. Enzyme solutions are stored in 20 m*M* Tris-HCl (pH 7.5) at 1–2 mg protein/ml at −20° and are stable for at least 1 year, withstanding repeated freezing and thawing.

Comments. The results of a typical purification of the enzymes from *B. cereus* and *B. thuringiensis* are summarized and compared in Table I. Figure 1 shows an SDS–polyacrylamide gel[7] of the material obtained at the various stages of purification of both preparations. The purity of the final enzyme preparations is generally better than 95%, with similar specific activities for the *B. cereus* and *B. thuringiensis* enzymes in the range of 1200 to 1500 units/mg[4,8] using the PI/deoxycholate assay described below. During the early steps of the purification, the specific enzyme activities are affected by the presence of colored contaminants that con-

[7] U. K. Laemmli, *Nature (London)* **227,** 680 (1970).

[8] J. J. Volwerk, J. A. Koke, P. B. Wetherwax, and O. H. Griffith, *FEMS Microbiol. Lett.* **61,** 237 (1989).

TABLE I
PURIFICATION OF PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C FROM *Bacillus thuringiensis*[a] AND *Bacillus cereus*[b]

Step	Total protein (mg)		Total activity (units)		Specific activity (units/mg)		Yield (%)	
	Bt	Bc	Bt	Bc	Bt	Bc	Bt	Bc
$(NH_4)_2SO_4$ (90% saturated)	145	194	6176	6900	43	36	100	100
CM-Sephadex	71	73	4132	5920	58	80	67	86
DEAE-cellulose	6.4	3.8	2106	2180	332	580	34	32
Phenyl-Sepharose	0.9	1.4	1420	1850	1515	1280	23	27

[a] Bt, ATCC 10792.
[b] Bc, ATCC 6464.

tribute strongly in the protein determinations. These contaminants are completely removed by the final Phenyl-Sepharose step. The Bradford protein assay[9] is preferred since it is the least sensitive to the colored material. In some of our *B. thuringiensis* preparations we observe a second minor polypeptide component of slightly higher molecular weight than the main PI-PLC band.[8] Western blot experiments using monoclonal antibodies specific for the *B. cereus* and *B. thuringiensis* PI-PLCs indicate that this polypeptide is immunologically related to the enžyme (A. Kuppe, unpublished observations).

The initial steps of the purification procedure described here are essentially the same as those reported earlier for *B. cereus*.[5] However, addition of the final Phenyl-Sepharose step greatly improves the purity of the preparation and is partly responsible for the significantly higher specific enzyme activities both of the *B. cereus* and *B. thuringiensis* preparations compared to those reported previously.[5] A different purification procedure for the *B. thuringiensis* enzyme has been reported more recently and also yields enzyme with a high specific activity.[10] We find that the procedure described here works well both for the *B. cereus* and *B. thuringiensis* PI-PLCs, yielding preparations of similar quality for these highly homologous enzymes.

[9] M. Bradford, *Anal. Biochem.* **72,** 248 (1976).
[10] M. G. Low, J. Stiernberg, G. L. Waneck, R. A. Flavell, and P. W. Kincade, *J. Immunol. Methods* **113,** 101 (1988).

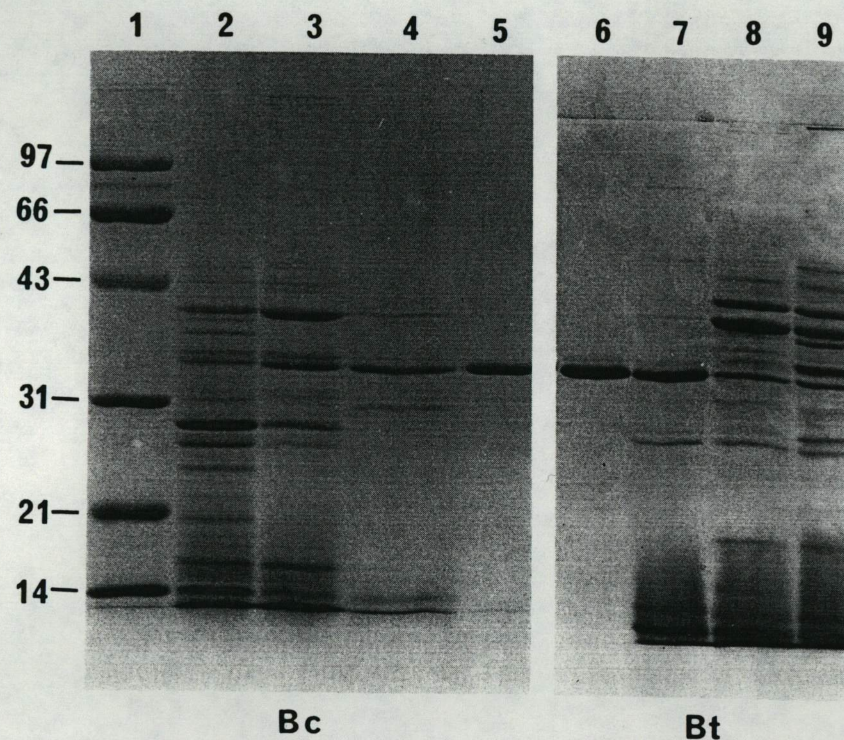

FIG. 1. SDS–polyacrylamide gel electrophoresis of *B. cereus* and *B. thuringiensis* PI-PLC (Bc and Bt, respectively) during the various stages of purification. Acrylamide gels (12%) were run with a 6% acrylamide stacking gel and the discontinuous buffer system of Laemmli.[7] Lane 1, molecular weight markers [molecular weights ($\times 10^{-3}$) are indicated at left]; lanes 2 and 9, after ammonium sulfate precipitation; lanes 3 and 8, after CM-Sephadex chromatography; lanes 4 and 7, after DEAE-cellulose chromatography; lanes 5 and 6, after Phenyl-Sepharose chromatography.

Assay Methods

Two different types of assays are currently available to measure enzyme activities of PI-PLC from *B. cereus* and *B. thuringiensis*. The first method is based on the enzyme-catalyzed cleavage of the substrate PI in the presence of detergent and quantitation of the water-soluble product, inositol phosphate. The procedure we describe below is an adaptation of an assay method reported previously[5] but employs radiolabeled PI instead of a colorimetric phosphorus determination. The second method is based on the ability of bacterial PI-PLC to release into the medium a number of enzymes tethered to membranes by means of a glycosylphosphatidylinosi-

tol(GPI)-containing membrane anchor.[11] Quantitation of the released enzyme by means of its own specific reaction thus provides a measure of the PI-PLC activity.

Method A: Cleavage of Phosphatidylinositol

Reagents

0.1 *M* sodium borate/HCl (pH 7.5)
0.8% (w/v) sodium deoxycholate
Chloroform/methanol/concentrated HCl (66 : 33 : 1, by volume)
10 m*M* radiolabeled PI [specific radioactivity 70,000 cpm (counts/min)/μmol] in aqueous suspension is prepared by mixing the appropriate amounts of cold PI (Avanti, Birmingham, AL, from bovine brain) and [*inositol*-2-^{3}H]PI (NEN, Wilmington, DE), in chloroform, followed by evaporation of the solvent and resuspension in water by bath sonication for 5 min. The lipid suspension is stored at −20° in 1-ml aliquots.

Assay Procedure. The reaction mixture is prepared by mixing (in this order) 0.1 ml PI suspension, 0.1 ml deoxycholate solution, and 0.2 ml borate buffer. The reaction is initiated by addition of 0.1 ml enzyme solution containing up to 0.005 units of PI-PLC appropriately diluted in 0.1% bovine serum albumin (pH 7.5). Incubation is at 37° for 10 min, and the reaction is terminated by addition of 2.5 ml of the chloroform/methanol/HCl mixture and vigorous stirring. After brief centrifugation to separate the layers, 0.5 ml of the aqueous upper layer (~1 ml total) is transferred to a scintillation vial, cocktail is added, and the sample is counted with automatic quench correction. Counts are multiplied by a factor of 2 to estimate the total amount of substrate cleaved in the assay mixture. One unit is defined as the amount of enzyme converting 1 μmol of substrate per minute. Specific enzyme activities are expressed in units/milligram protein. Total protein is determined using the Bradford protein assay.[9]

Comments. A strict proportionality between the amount of enzyme added and the counts in the aqueous layer is observed for up to 0.005 units of PI-PLC. Use of radiolabeled PI significantly improves both the ease and sensitivity of the assay. A drawback is the high cost of cold and radiolabeled PI. However, for comparative purposes, for example, finding the active peak in column fractions, the assay can be performed with as little as 0.025 ml PI suspension.

[11] M. G. Low and A. R. Saltiel, *Science* **239,** 268 (1988).

Method B: Enzyme Release Assay

Detailed procedures for determining the PI-PLC-catalyzed release of alkaline phosphatase from rat kidney slices and acetylcholinesterase from bovine erythrocytes have been described by Ikezawa and Taguchi.[5] We use the latter procedure to compare the enzyme-release activities of the *B. cereus* and *B. thuringiensis* preparations.[8]

Properties of the Enzymes

The PI-PLCs purified from *B. cereus* and *B. thuringiensis* are nearly identical in their physicochemical properties examined thus far. We did not observe any (substantial) differences in systematic comparisons of several properties of the purified enzymes: specific activity toward substrate (PI, GPI), molecular weight and electrophoretic mobility, sensitivity to certain effectors, and interaction with inhibitory monoclonal antibodies.

Amino Acid Sequence. Inspection of the amino acid sequences of the enzymes, derived by translation of the sequenced genes,[12,13] has shown that there are only eight amino acid substitutions apparent between the proteins (Fig. 2). These amino acid substitutions do not seem to alter the enzymatic properties of the enzyme. There is an amino acid sequence similarity of the *B. cereus* enzyme with a GPI-specific phospholipase C from *Trypanosoma brucei*.[13] The bacterial enzyme shows limited sequence similarity with eukaryotic PI-PLCs presumed to be involved in the signal transduction of Ca^{2+}-mobilizing hormones.[13]

Molecular Weight and Isoelectric Point. The PI-PLCs purified by the above method comigrate in SDS–PAGE (on Laemmli-type mini slab gels of 12% acrylamide/0.32% *N,N'*-methylenebisacrylamide) and run at about 35,000 apparent molecular weight. This value agrees with the molecular weights calculated from the sequences of the enzymes (e.g., 34,466 for the *B. cereus* PI-PLC). The value is higher than that observed by gel filtration (23,000–29,000)[5]; preliminary results from our laboratory (J. J. Volwerk, unpublished observation) indicate that this discrepancy can be ascribed to interaction of the proteins with the column matrix. The isoelectric point of the *B. cereus* PI-PLC is reported to be 5.4 using ampholite (pH 5–8).[2]

pH Optimum. The pH optimum appears to be rather broad and does

[12] D. J. Henner, M. Yang, E. Chen, R. Hellmiss, H. Rodriguez, and M. G. Low, *Nucleic Acids Res.* **16,** 10383 (1988).
[13] A. Kuppe, L. M. Evans, D. A. McMillen, and O. H. Griffith, *J. Bacteriol.* **171,** 6077 (1989).

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-31                          M SNKKLILKLF ICSTIFITFV FALHDKRVVA

                      N
  1 ASSVNELENW SKWMQPIPDS IPLARISIPG THDSGTFKLQ NPIKQVWGMT

 50 QEYDFRYQMD HGARIFDIRG RLTDDNTIVL HHGPLYLYVT LHEFINEAKQ

                                 G          N
100 FLKDNPSETI IMSLKKEYED MKGAEDSFSS TFEKKYFVDP IFLKTEGNIK

                      S                                V
150 LGDARGKIVL LKRYSGSNEP GGYNNFYWPD NETFTTTVNQ NANVTVQDKY

     N
200 KVSYDEKVKS IKDTMDETMN NSEDLNHLYI NFTSLSSGGT AWNSPYYYAS

            D        T
250 YINPEIANYI KQKNPARVGW VIQDYINEKW SPLLYQEVIR ANKSLIKE
```

FIG. 2. Amino acid sequence of the PI-PLC from *B. cereus*. The protein is synthesized as a larger precursor carrying an N-terminal signal peptide (position −31 to −1) in addition to the mature enzyme of 298 residues. Amino acid replacements in the PI-PLC from *B. thuringiensis* are indicated above the sequence.

not show much variation between pH 5 and 8.5 in the PI-cleavage assay using Tris–acetate buffers. The enzyme remains active after short exposure to low pH (pH 2.5, <60 min).

Substrate Specificity. Enzyme preparations from *B. cereus* and *B. thuringiensis* show no proteolytic activity and cleave only lipids containing the *myo*-inositol group. The specific activities toward cleavage of PI and the GPI anchors are very similar for the two enzymes.[8] Diacylglycerol is the only product detectable in the organic phase after prolonged incubation of PI with the *B. cereus* enzyme. The enzymes do not recognize as substrate the more highly phosphorylated inositol phospholipids phosphatidylinositol phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2). No hydrolysis of phosphatidylcholine (PC), phosphatidylethanolamine (PE), or sphingomyelin is observed.[4]

Influence of Effectors. Treatment of the enzyme with a reducing agent (dithiothreitol) or the thiol reagents iodoacetamide and *N*-ethylmaleimide has no effect on enzyme activity. The enzymes contain no disulfide bonds, and, in fact, the mature protein shows no Cys residue in the amino acid sequence. As with many phospholipases, the presence of detergent in the assay activates the enzyme. At the detergent/PI ratios of 2 : 1 used in the standard assay, deoxycholate activates most strongly. Under these conditions, several other detergents activate to a lesser degree: cholate

(30% of the maximal deoxycholate activation), Triton X-100 (18%), and *n*-octylglucoside (12%).[4]

Metal ions exert an inhibitory effect on enzyme activity, possibly due to interaction with the substrate rather than enzyme. Divalent cations inhibit at lower concentrations (1–10 m*M*: Ca, Mg < Mn, Zn) than NaCl (100 m*M*). The inhibitory effect of metal ions is pH dependent.[14]

Inhibitors. Several nonhydrolyzable enzyme inhibitors have been synthesized, based on the structure of the substrate PI.[15,16] In these molecules, the PI-PLC-sensitive P—O bond is replaced by a P—C bond (phosphonate). In place of the diacylglycerol moiety, single chains of varying lengths were introduced in the inhibitors. The best inhibitor in this series, 1-*myo*-inositol (4-palmitoyloxybutan-1)phosphonate, reduces PI-PLC activity to half-maximum at 5 m*M* concentration under standard assay conditions. PI-PLC-specific monoclonal antibodies almost completely inhibit the enzymes at low antibody/PI-PLC ratios.[17] This effect is observed in both of the commonly used assay systems, PI cleavage and release of acetylcholinesterase from the surface of bovine erythrocytes through cleavage of the membrane anchor. The PI-PLCs from *B. cereus* and *B. thuringiensis* are identical in their interaction with the inhibitory monoclonal antibodies. The lipid inhibitors and antibodies should prove useful in the study of structure–function relationships of the bacterial PI-PLCs and in the investigation and characterization of membrane proteins containing glycosylphosphatidylinositol anchors.

Enzyme Stability. As mentioned above, the purified PI-PLCs from *B. cereus* and *B. thuringiensis* remain active after many freeze–thaw cycles. This is observed for enzyme preparations stored in 20 m*M* Tris-HCl (pH 7.5) buffer at concentrations between 0.1 and 3 mg protein/ml. PI-PLC is also fairly stable during prolonged incubations at room temperature and at 37°. We suspect that PI-PLC is only partially denatured by SDS–PAGE dissociation buffer (3% SDS in stacking gel buffer, no mercaptoethanol) or renatures rapidly on removal of the SDS. This notion stems from results of experiments where enzyme activity is measured in gel slices after gel electrophoretic separation of the (ammonium sulfate-precipitated) secreted proteins of *B. cereus*.[4]

In summary, the PI-PLCs of *B. cereus* and *B. thuringiensis* are very nearly identical. These PI-PLCs are stable enzymes which can be purified

[14] R. Sundler, A. W. Alberts, and P. R. Vagelos, *J. Biol. Chem.* **253,** 4175 (1978).

[15] M. S. Shashidhar, J. F. W. Keana, J. J. Volwerk, and O. H. Griffith, *Chem. Phys. Lipids* **53,** 103 (1990).

[16] M. S. Shashidhar, J. J. Volwerk, J. F. W. Keana, and O. H. Griffith, *Biochim. Biophys. Acta* **1042,** 410 (1990).

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to high specific activity by the method described here without contamination by other phospholipases or proteases. The enzymes are used in the study of novel cell surface proteins containing the glycosylphosphatidylinositol anchors and may serve as model systems for the eukaryotic phospholipases involved in signal transduction.

Acknowledgments

We are pleased to acknowledge useful discussions with our colleagues Dr. M. S. Shashidhar, Dr. H. Stewart Hendrickson, and Mr. John A. Koke. This work was supported by U.S. Public Health Service Grant GM 25698.

[48] Assays of Phosphoinositide-Specific Phospholipase C and Purification of Isozymes from Bovine Brains

By SUE GOO RHEE, SUNG HO RYU, KEE YOUNG LEE, and KEY SEUNG CHO

Introduction

It is well established that in a variety of cells, receptor-mediated phosphoinositide-specific phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] to yield diacylglycerol and inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]. Both products act as intracellular messengers, the former by activating protein kinase C and the latter by mobilizing intracellular Ca^{2+} pools. PLC is present in most mammalian cells[1] as well as in plants[2] and various microorganisms.[3] Multiple forms of PLC enzymes have been purified from both particulate and soluble fractions of a variety of mammalian tissues. An examination of the molecular weights of PLC isozymes determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, the amino acid sequences deduced from cDNA, and the immunocross-reactivity indicates that PLC enzymes purified from mammalian tissues fall into four groups[1]: PLC-α with a molecular weight of 60,000–70,000,[4-6] PLC-β with 140,000–155,000,[7-10] PLC-γ with

[1] S. G. Rhee, P.-G. Suh, S. H. Ryu, and S. Y. Lee, *Science* **244,** 456 (1989).
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145,000–148,000,[7-9] and PLC-δ with 85,000–88,000.[9,11-13] These isoforms are expressed differently between tissues, between individual cells, and during development.[1]

Despite these differences, all the purified isozymes exhibit similar catalytic properties. (1) They are specific for inositol phospholipid and do not hydrolyze other phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS). (2) They hydrolyze three inositol-containing lipids, namely, phosphatidylinositol (PtdIns), phosphatidylinositol 4-phosphate [PtdIns(4)P], and PtdIns(4,5)P_2. (3) The hydrolyses of these three substrates are dependent on Ca^{2+}. (4) Optimal Ca^{2+} concentrations required for the hydrolysis of PtdIns(4)P and PtdIns(4,5)P_2 are significantly less than that for PtdIns. (5) Recently, new phosphoinositides, PtdIns(3)P and PtdIns(3,4)P_2, have been found in cells stimulated with growth factors and transformed by certain oncoproteins. None of the four types of PLC isozymes can hydrolyze these phosphoinositides containing phosphate at the 3-OH position of the inositol ring.[14,15]

In the past several years it has become evident that guanine nucleotide-binding proteins (G proteins) are involved in linking receptor activation to PLC. However, neither the nature of G protein nor whether all PLC isozymes require G protein for activation is known. In addition, various metal-chelating reagents,[16] nucleotides,[7] proteins,[9] and lipids[17-19] might differently affect PLC activity. Since all three substrates for PLC are

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