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Purification and Characterization of Bovine Heart Phosphoinositide-Specific Phospholipase C: Kinetic Analysis of the Ca^{2+} Requirement and La^{3+} Inhibition†

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ABSTRACT: Bovine heart contains multiple phosphoinositide-specific phospholipase C (PIC) activities separable by ion-exchange chromatography. One PIC activity was purified to apparent homogeneity and migrated as a single band of M_r 85 000 on SDS-PAGE. The purified PIC was characterized with sonicated suspensions of either pure phosphatidylinositol 4,5-bisphosphate (PIP_2) or phosphatidylinositol (PI) as substrates. At pH 7, apparent V_{max} and K_m values were higher for PIP_2 than for PI, but the value of V_{max}/K_m was similar for the two substrates. PIC required Ca^{2+} for the hydrolysis of either PI or PIP_2, and increasing free Ca^{2+} concentrations from 20 to 300 nM saturated PIC activity. The requirement of Ca^{2+} for PIC activity and the sensitivity of PIC to Ca^{2+} concentrations in the physiological range suggested the ion may be a cofactor. The PIC reaction mechanism was determined by two-substrate kinetic analysis; the data fit a model in which PIC contained single sites for Ca^{2+} and phosphoinositide, and utilized a rapid-equilibrium, random-order ternary mechanism for phosphoinositide hydrolysis. The K_{Ca} value for either PI or PIP_2 hydrolysis was approximately 30 nM, suggesting resting intracellular free Ca^{2+} concentrations are sufficient to saturate the Ca^{2+} site of PIC. La^{3+} was used as a calcium analogue to modulate PIC activity. Low concentrations of $LaCl_3$ (0.01-0.3 μM) inhibited PIC activity competitively with respect to calcium, consistent with a Ca^{2+} binding site on the enzyme.

Phosphoinositide-specific phospholipase C (PIC)[1] is the intracellular enzyme that hydrolyzes phosphoinositides upon stimulation of cells with Ca^{2+}-mobilizing agonists (Hirasawa & Nishizuka, 1985). PIC is a family of enzymes; multiple isoforms are present in most tissues (Rhee et al., 1989), including the heart (Low & Weglicki, 1983). Several distinct PIC enzymes have been purified from mammalian tissues, ranging from 60 to 150 kDa (Rhee et al., 1989). Isozymes of 85-kDa PIC (PIC δ and ϵ) have been purified from brain

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[1] Abbreviations: BSA, bovine serum albumin; DG, 1,2-diacylglycerol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate; IP_3, inositol trisphosphate; MOPS, 3-(*N*-morpholino)propanesulfonate; PI, phosphatidylinositol; PIC, phosphoinositide-specific phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; SD, standard deviation; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SEM, standard error of the mean.

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and liver (Fukui et al., 1988; Homma et al., 1988; Ryu et al., 1987).

During agonist-directed phosphoinositide turnover, PIC initially hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2), creating inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG). However, in some cases, more DG is created than can be accounted for by the hydrolysis of the minor membrane component PIP_2 (Majerus et al., 1985). The additional accumulation of DG is due, in certain cases, to a prolonged hydrolysis of the more abundant substrate PI (Hokin-Neaverson & Sadeghian, 1984; Imai & Gershengorn, 1986; Majerus et al., 1985).

Components of the phosphoinositide turnover second-messenger system have been identified in heart, including PIC (Low & Weglicki, 1983; McDonald & Mamrack, 1988; Schwertz et al., 1987, 1989). In heart, PIC is activated by endothelin, muscarinic, α_1-adrenergic, or purinergic agonists, as well as by electrical stimulation (Ambar et al., 1989; Brown & Brown, 1983; Haggblad & Heilbronn, 1988; Poggioli et al., 1986).

PIC activity may be regulated, in part, by changes in intracellular calcium concentration (Eberhard & Holz, 1988). Increased intracellular calcium stimulates PIC in some cell types including cultured cardiomyocytes (Godfrey & Putney, 1984; Martin et al., 1986; McDonough et al., 1988; Monaco, 1987a), but not in other cell types (Fisher et al., 1985; McMillian et al., 1988; Murayama & Ui, 1987; Nakamura & Ui, 1985; Renard et al., 1987; di Virgilio et al., 1985), indicating that PIC activity is not under identical control in all tissues. Heart PIC activity measured in vitro is Ca^{2+} dependent, demonstrated by a sensitivity to the chelator EGTA (McDonald & Mamrack, 1988; Schwertz et al., 1987, 1989). Purified PIC isozymes are activated by Ca^{2+} concentrations from 0 to 1 μM (Bennett & Crooke, 1987; Hofmann & Majerus, 1982; Homma et al., 1988; Ryu et al., 1987).

In this report, an 85-kDa PIC from bovine heart cytosol was purified to apparent homogeneity and characterized by using PI and PIP_2 as substrates. The activity of purified PIC was sensitive to Ca^{2+} concentrations in the physiological range, suggesting Ca^{2+} was a cofactor for PIC activity. PIC hydrolyzed phosphoinositides via a ternary mechanism, with single sites for both phosphoinositide and Ca^{2+}. Competitive inhibition of PIC by the Ca^{2+} analogue La^{3+} provided further evidence for a Ca^{2+} site on the enzyme.

Materials and Methods

Preparation of [^{3}H]Inositol-Labeled Phosphatidylinositol. The microsome-associated inositol exchange activity of liver was used to synthesize radiolabeled PI, which was prepared and purified as described previously (McDonald & Mamrack, 1988). Upon storage at −20 °C, some degradation of the purified PI was observed, creating neutral lipid contaminants. The substrate was therefore routinely repurified by silica gel column chromatography; PI was loaded in chloroform solution, washed with chloroform to remove neutral lipid contaminants, and then eluted with methanol. For some experiments, the substrate for PIC was [^{3}H]inositol-labeled PI purified from metabolically labeled human fibroblasts. PI was extracted from cultured fibroblasts using methanol and 0.1 M HCl (Monaco, 1987b) and then purified by the same procedure used with liver microsome PI.

Purification of PIC. Fresh bovine heart obtained from a local slaughterhouse was immediately placed on ice; subsequent steps were performed at 0–4 °C. Ground ventricular tissue from one heart (approximately 500 g) was homogenized in 4 volumes of buffer A (10 mM Na-MOPS, 1 mM EDTA, 0.5 mM DTT, and 0.01% NaN_3, pH 6.8) supplemented with 350 mM NaCl, 3 mM EDTA, 2 mM EGTA, and 0.5 mM PMSF. The homogenate was centrifuged at 16000*g*, and the supernatant was further centrifuged at 50000*g*. A 30–70% ammonium sulfate fraction of the cytosol was applied to a 5 × 26 cm column of phosphocellulose resin in buffer A at ionic strength 0.4 M. One form of PIC bound the column under these conditions (McDonald & Mamrack, 1988); however, the majority of heart PIC activity did not bind. The phosphocellulose column flow-through was bound to a 5 × 26 cm column of Affigel Blue–agarose in buffer A at ionic strength 0.5 M, adjusted with NaCl. The column was washed with buffer A containing 0.5 M NaCl and then eluted with 2.4 L of buffer A containing 2 M NaCl.

Eluant from the Affigel Blue column was precipitated with solid ammonium sulfate at 70% saturation, resolubilized in buffer B (10 mM Tris-HCl, 1 mM EDTA, 0.5 mM DTT, and 0.01% NaN_3, pH 7.4), and dialyzed 24 h against two changes of buffer B. A copious precipitate formed and was removed by centrifugation at 50000*g* for 80 min.

Column Chromatography. The postdialysis supernatant was adjusted to 100 mM NaCl and applied to a 2.5 × 35 cm column of phosphocellulose resin. The column was washed with buffer A containing 100 mM NaCl, and then PIC activity was eluted with a 1.2-L linear gradient of 0.1–0.5 M NaCl in buffer A. Fractions containing PIC 2 activity were pooled and dialyzed overnight against buffer B. The solution was applied to a 2.5 × 7 cm column of aminohexylagarose, washed with buffer B, and eluted with a 400-mL linear gradient of 0–0.3 M NaCl in buffer B. PIC activity was pooled, adjusted to pH 6.8 with 1 M Na-MOPS, and applied to a 0.9 × 9 cm column of heparin–agarose. The column was washed with buffer A containing 0.25 M NaCl, and then PIC activity was eluted with a gradient containing 0.25–0.75 M NaCl in buffer A.

Fractions with PIC 2 activity from the heparin–agarose column were pooled and applied to a 1.4 × 1.3 cm column of hydroxylapatite. The column was washed with buffer A containing 0.5 M NaCl and then with buffer C (25 mM potassium phosphate, 100 mM NaCl, 0.5 mM DTT, and 0.01% NaN_3, pH 7.0). PIC was eluted with a 100-mL gradient of buffer C containing 25–250 mM potassium phosphate. Fractions with PIC activity were pooled and dialyzed against two changes of 4 L of buffer containing 10 mM Tris-HCl, 100 mM KCl, 0.5 mM DTT, and 0.01% NaN_3, pH 7.4, prepared with fresh high-quality water (greater than 17 MΩ cm^{-1}) in acid-washed glassware. PIC was stored in aliquots in acid-washed glass tubes at 4 °C and retained at least 90% of its activity for 4 weeks.

PIC Assay. PIC activity was measured by using sonicated suspensions of phosphoinositides, as described (McDonald & Mamrack, 1988). Standard assay conditions were approximately 10 μM phosphoinositide (approximately 5000 ^{3}H cpm), 50 mM Na-MOPS, 0.1 M KCl, and 50 μM $CaCl_2$, pH 7.0. Conditions for individual experiments are provided in the figure legends. Reactions were initiated with the addition of enzyme, typically 20–40 ng (approximately 0.3–0.6 pmol), run at 37 °C, and terminated after 30–90 min. Reaction rates were linear for 120 min or up to 35% of the substrate hydrolyzed. PIC was characterized by using data obtained when less than 20% of the substrate was hydrolyzed.

Determination of Two-Substrate Kinetic Parameters. Two-substrate kinetic experiments were performed with six concentrations each of EGTA-buffered free Ca^{2+} and the phosphoinositide substrate. Averages of duplicate measure-

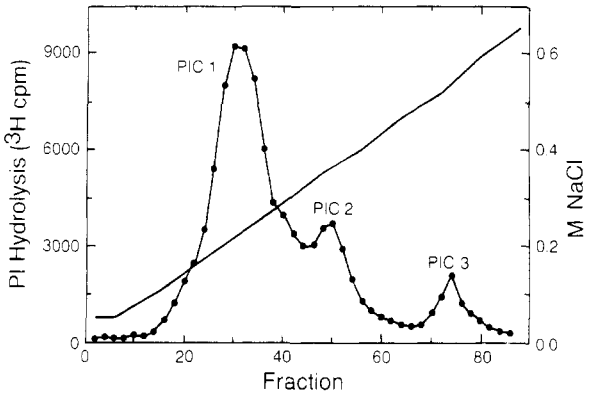


FIGURE 1: Phosphocellulose chromatography of bovine heart cytosol. Cytosol from 5 g of ventricle (wet weight) was diluted to approximate ionic strength 0.05 M and applied to a 2.5 × 20 cm column of phosphocellulose resin. All PIC activity bound to the column. PIC activities were eluted with a 500-mL linear gradient of 0.05–0.65 M NaCl in buffer A (Materials and Methods). Alternate fractions were assayed for the hydrolysis of [^{3}H]inositol-labeled PI (●) for 20 min at 37 °C in reactions containing 50 mM Na-MOPS, 1.5 mM $CaCl_2$, and 0.07% sodium deoxycholate, pH 6.8. Deoxycholate and a higher concentration of calcium were included in this experiment to maximize sensitivity of the PIC assay. The three major PIC activities resolved were denoted PIC 1, PIC 2, and PIC 3. The NaCl gradient, estimated by using a conductivity meter, is shown in the solid line.

ments were cast as Lineweaver–Burk plots, and linear regression best-fit lines were determined with the plotting program Cricket Graph on a Macintosh II microcomputer. Slopes and intercepts from the primary plots of one substrate were plotted against the inverse concentration of the other substrate as described (Engel, 1981). Dalziel parameters (Dalziel, 1957) for two-substrate rate equations were obtained from the slopes and intercepts of the secondary plots. Secondary plots were created from both the calcium and phosphoinositide primary plots; Dalziel parameters derived from either set of secondary plots were in agreement. For determination of the PIC kinetic parameters, the Dalziel parameters were averaged from the secondary plots originating from both the Ca^{2+} and phosphoinositide primary data. Alberty parameters for ternary mechanism rate equations were derived from the Dalziel parameters (Engel, 1981).

Other Methods. During the purification of PIC, the ionic strength of solutions was estimated with a conductivity meter. SDS–PAGE was performed using 8% polyacrylamide gels (Laemmli, 1970). Protein was assayed by the method of Bradford (1976) using BSA as standard. Lipid phosphorus was measured as reported (Organisciak & Noell, 1976). Inositol phosphate products of PIC reactions were analyzed by Dowex-1 anion-exchange chromatography as described (Berridge et al., 1983). The reaction pH was buffered from 4 to 9 using acetate (pH 4–5.5), Bis-Tris (pH 5.5–7.5), and Tris (pH 7.5–9), prepared with NaOH, HCl, and NaCl to achieve the appropriate pH at 37 °C, and equivalent ionic strengths. Free calcium was buffered from 0.02 to 2 μM using 5 mM EGTA under reaction conditions of pH 7.0, and ionic strength 0.1 M (Raaflaub, 1956). In the equilibrium equations, an EGTA–Ca^{2+} association constant of $10^{11.0}$ and EGTA pK_a values of 9.38 and 8.77 were used (Durham, 1983; Martell, 1971).

Materials. [^{3}H]Inositol–PIP_2 (1 Ci mmol^{-1}) was obtained from American Radiolabeled Chemicals, St. Louis, MO. PIP_2 (ammonium salt) was obtained from Calbiochem, San Diego, CA, or from Boehringer Mannheim, Indianapolis, IN. EGTA was obtained from Fluka Chemicals. Affigel Blue–agarose and hydroxylapatite were obtained from Bio-Rad, Richmond, CA. Phosphocellulose, aminohexylagarose, and heparin–

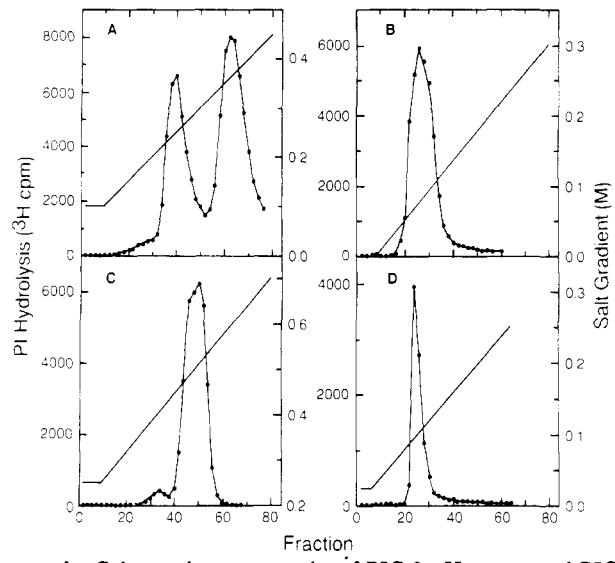


FIGURE 2: Column chromatography of PIC 2. Heart cytosol PIC 2 was partially purified through the dialysis step as described under Materials and Methods. PIC 2 was then purified by consecutive column chromatography steps. (A) Phosphocellulose chromatography: PIC 1 and PIC 2 were sequentially eluted with increasing NaCl. Fractions 58–72 were pooled. (B) Aminohexylagarose chromatography: fractions 21–34 were pooled. (C) Heparin–agarose chromatography: remaining PIC 1 was eluted prior to PIC 2. Fractions 44–55 were pooled. (D) Hydroxylapatite chromatography: fractions 23–28 were pooled. PIC hydrolysis of [^{3}H]inositol-labeled PI was determined (●) as described in the legend to Figure 1. NaCl gradients in panels A–C and the phosphate gradient in panel D are denoted by the solid lines.

agarose were obtained from Sigma, St. Louis. Other reagents were obtained either from Sigma, from Fisher, or from Research Organics, Cleveland, OH, and were reagent grade or better.

RESULTS

Purification of PIC. Multiple forms of PIC from bovine heart cytosol were identified by analytical ion-exchange chromatography on phosphocellulose resin (Figure 1). Three major peaks of PIC activity eluted from the column, denoted PIC 1, PIC 2, and PIC 3. Characterization of partially purified PIC 3 was described in a previous report (McDonald & Mamrack, 1988). For the current studies, PIC 2 was purified to apparent homogeneity by a multistep procedure.

An ammonium sulfate fraction of bovine heart cytosol was initially applied to a preparative phosphocellulose column under conditions whereby PIC 3 bound the column but PIC 1 or PIC 2 did not. Subsequent steps in the purification of PIC 2 included step elution from Affigel Blue–agarose and dialysis against low ionic strength buffer (I <0.02 M), during which approximately half of the protein precipitated but PIC activity remained soluble. Four column chromatography steps were then used to purify PIC 2 (Figure 2). PIC 2 was resolved from PIC 1 on the two cation-exchange columns, phosphocellulose and heparin–agarose. Finally, hydroxylapatite chromatography with a phosphate gradient elution produced a highly purified PIC.

The purification scheme for PIC 2 is shown in Table I. The final yield of PIC 2 was typically 40–60 μg from a single heart (approximately 500 g of ventricle, wet weight). With the assumption that PIC 2 comprises 25% of heart cytosol PIC activity (based on profiles such as Figure 1), the yield of PIC 2 activity was approximately 1.5%. Analysis of the purification of PIC 2 by SDS–PAGE revealed a predominant single band at M_r 85 000 from the final step in the purification procedure (Figure 3). The purity of PIC 2 was estimated at greater than

Table I: Purification of PIC 2[a]

step	total protein (mg)	total act. (μmol min^{-1})	sp act. [nmol (min·mg)$^{-1}$]	purification (x-fold)
(1) cytosol	16000	70	4.4	1
(2) ammonium sulfate	8400	48	5.7	1.3
(3) phosphocellulose 1	7600	45	5.9	1.4
(4) Affigel Blue	790	18	23	5.2
(5) dialysis	420	18	42	9.6
(6) phosphocellulose 2	13	3.6	280	63
(7) aminohexyl-agarose	3.6	2.2	620	140
(8) heparin–agarose	0.26	0.62	2400	550
(9) hydroxylapatite	0.052	0.27	5100	1160

[a] The purification is described in the text. PIC activity was determined at 37 °C with 60 μM PI, 50 mM Na-MOPS, 100 mM KCl, and 50 μM $CaCl_2$, pH 7.0. The data shown are representative of 11 preparations of PIC 2.

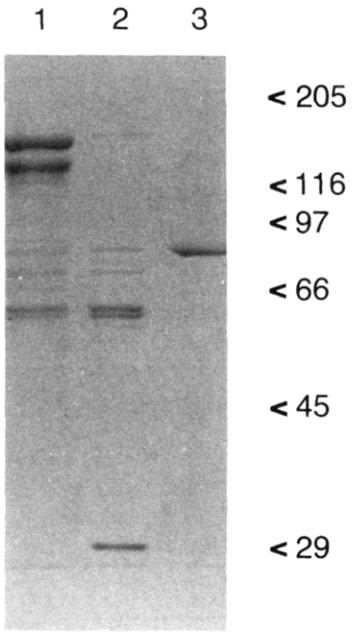


FIGURE 3: SDS–PAGE of purified PIC 2. Approximately 10 μg of protein from each of the final three column chromatography steps of the PIC 2 purification procedure was run on SDS–PAGE (8% polyacrylamide) and stained with Coomassie blue. Lane 1, aminohexylagarose chromatography peak. Lane 2, heparin–agarose chromatography peak. Lane 3, hydroxylapatite chromatography peak. Purified PIC 2 migrated as a single band of M_r 85000 and was estimated to be greater than 95% pure. Molecular weight standards are indicated.

95% on the basis of Coomassie blue stained gels.

Characterization of PIC 2. Sonicated suspensions of PI or PIP_2 were used as substrates for the characterization of PIC 2 activity. Confirmation of the enzyme activity as a phospholipase C was obtained by anion-exchange chromatography of the water-soluble reaction products. [^{3}H]Inositol monophosphate and [^{3}H]inositol trisphosphate were the products obtained from the hydrolysis of labeled PI and PIP_2, respectively.

The pH optimum of PIC 2 for the hydrolysis of PI and PIP_2 is shown in Figure 4, where acetate, Bis-Tris, and Tris buffered the reaction from pH 4 to 9. The pH optimum for PIP_2 hydrolysis was near neutrality (pH 7–7.5) whereas the optimum pH for the hydrolysis of PI was substantially lower, at pH 5–5.5. Similar results were obtained with Tris–maleate buffers over the same pH range. Maximal PIC activity was obtained with either NaCl or KCl over the range 100–300 mM. Below 100 mM, monovalent salt stimulated PIC activity.

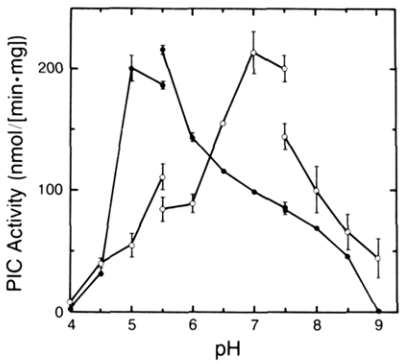


FIGURE 4: pH optima of PIC 2 hydrolysis of PI or PIP_2. PIC 2 was assayed with 4.2 μM PI (●) or 1.3 μM PIP_2 (○) in reactions buffered from pH 4 to 9 using 50 mM acetate (pH 4–5.5), Bis-Tris (pH 5.5–7.5), or Tris (pH 7.5–9). Data points are means ± SEM of triplicate measurements from one experiment representative of seven performed.

Table II: Cation Requirement of PIC 2 Activity[a]

treatment (0.2 mM)	activity [nmol (min·mg)$^{-1}$] PI hydrolysis	PIP_2 hydrolysis
none	0.6 ± 1.0	5.7 ± 12
$CaCl_2$	68 ± 8.7	113 ± 26
$LaCl_3$	1.9 ± 0.5	0
$MgCl_2$	1.2 ± 0.4	ND
$BaCl_2$	1.2 ± 0.4	ND
$SrCl_2$	9.5 ± 1.2	ND

[a] Reactions were performed in the presence of 0.1 mM EGTA with 4.5 μM PI or 5.6 μM PIP_2, at pH 7 and 37 °C. In the absence of PIC, approximately 1% of the PI or 5–10% of the PIP_2 was detected in the aqueous phase of the chloroform extractions; these values were subtracted as background from assays. Data are means ± SD of triplicate measurements. ND, not determined.

Calcium ion was required for PIC 2 catalyzed hydrolysis of either PI or PIP_2 (Table II). In the presence of 0.1 mM EGTA, PIC activity was not significantly above background for the hydrolysis of either phosphoinositide. Addition of excess $CaCl_2$ stimulated the hydrolysis of either PI or PIP_2, while other ions tested did not. Only $SrCl_2$ activated PIC significantly above background, but only 15% of the activity at equimolar $CaCl_2$.

Ca^{2+} and Phosphoinositide Substrate Kinetics. EGTA-buffered free Ca^{2+} was used to examine the dependence of PIC 2 activity on Ca^{2+} concentrations over the range 20–300 nM. With increasing free Ca^{2+}, the hydrolysis of either PI or PIP_2 was saturated with linear kinetics. The sensitivity of PIC activity to approximately physiological Ca^{2+} concentrations suggested Ca^{2+} was possibly a metal ion cofactor for PIC.

To determine the role of Ca^{2+} in PIC activity, two-substrate kinetic analysis was performed, using six concentrations of both free Ca^{2+} and phospholipid. Under all the conditions tested, covering Ca^{2+} concentrations from 20 to 300 nM and phosphoinositide concentrations from 0.4 to 500 μM, the sonicated suspensions of PI or PIP_2 were hydrolyzed with Michaelis–Menten kinetics. In the kinetics experiments, the concentrations of PI and PIP_2 were kept below saturating to limit the possibility of substrate inhibition. The data were cast initially as Lineweaver–Burk plots for phosphoinositide saturation. Linear plots were observed for either phosphoinositide substrate, and the sets of lines from several Ca^{2+} concentrations were convergent, albeit with only slightly different slopes. Corresponding Lineweaver–Burk plots for the putative cofactor Ca^{2+} were obtained for a range of concentrations of PI (0.4–5 μM) or PIP_2 (8–100 μM), and linear, convergent plots with similar but distinguishable slopes were observed. In all the primary plots, increasing the concentration of one substrate

Table III: Parameters for PIC 2 Two-Substrate Kinetics[a]

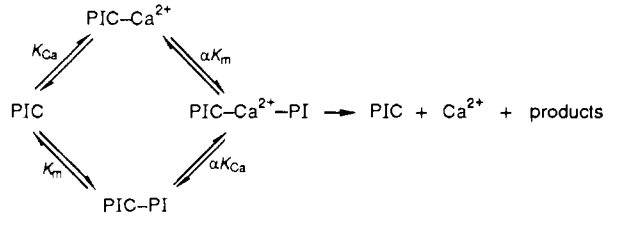
	PI hydrolysis	PIP_2 hydrolysis
Dalziel parameters		
Φ_0 (s)	13 ± 3.1	0.16 ± 0.054
Φ_{Ca} (μM s)	0.47 ± 0.18	0.0036 ± 0.0006
Φ_{PI} (μM s)	24 ± 3.8	42 ± 9.0
Φ_{CaPI} (μM^2 s)	0.041 ± 0.010	0.33 ± 0.076
Alberty parameters		
V_{max} (s^{-1})	0.076 ± 0.018	6.1 ± 2.0
K_{Ca} (μM)	0.0017 ± 0.00032	0.0091 ± 0.0036
K_m (μM)	0.11 ± 0.036	95 ± 26
αK_{Ca} (μM)	0.042 ± 0.016	0.029 ± 0.011
αK_m (μM)	2.2 ± 0.8	460 ± 98
K_{CaPI} (μM^2)	0.0035 ± 0.00076	2.4 ± 0.7
$V_{max}/\alpha K_m$ [(μM s)$^{-1}$]	0.035 ± 0.021	0.013 ± 0.0072

[a] Dalziel and Alberty parameters are the coefficients in the rate equations for two-substrate kinetics (Dalziel, 1957; Engel, 1981). Kinetics data were obtained from a six by six matrix of free Ca^{2+} and phosphoinositide concentrations, and analyzed by primary and secondary plots as described under Materials and Methods. Data represent means ± SEM (n = 3).

increased the apparent V_{max} and apparent K_m of the other substrate.

The data were further analyzed in secondary plots, where the slopes and intercepts of the primary plots were cast against the reciprocal concentration of the constant substrate. The slopes and intercepts of the secondary plots represent the Dalziel parameters for two-substrate reaction mechanisms (Dalziel, 1957). The experiments were performed 3 times with both PI and PIP_2, and a compilation of Dalziel parameters is presented in Table III. Though the change in slope among the lines in the primary plots was small, a nonzero Φ_{CaPI} parameter was obtained for either PI or PIP_2 hydrolysis. The presence of a Φ_{CaPI} parameter in the two-substrate rate equation suggests PIC utilized a ternary complex mechanism and not a ping-pong mechanism.

Also shown in Table III are the Alberty parameters for two-substrate rate equations, derived arithmetically from the Dalziel parameters (Engel, 1981). The V_{max} and K_m values for PIP_2 hydrolysis were much higher than those for PI hydrolysis, while the specificity constant, V_{max}/K_m, was comparable for the two substrates. Since increasing the concentration of either Ca^{2+} or phosphoinositide increased the K_m value for the other, PIC 2 was apparently following a random-ordered ternary mechanism for the hydrolysis of phosphoinositides:



The changes in apparent K_{Ca} or K_m due to the presence of the other substrate are expressed as the constants αK_{Ca} and αK_m, also derived from the Dalziel parameters and listed in Table III. The K_{Ca} values estimated from the data were in the range 2–20 nM Ca^{2+}, below the range of Ca^{2+} concentrations buffered by EGTA. In these experiments, then, the K_{Ca} value was not bracketed by the experimental data, and the results were obtained with Ca^{2+} concentrations above the K_{Ca}.

Inhibition by Lanthanum. The Ca^{2+} ion analogue La^{3+} was used to probe the PIC 2 metal ion site. For examination of the effect of La^{3+} on PIC activity, unbuffered ion conditions were used, since the association constant of EGTA for La^{3+} is approximately 10^5-fold higher than that for Ca^{2+} (Martell, 1971). Several precautions were taken to minimize contamination by ions or chelators in the unbuffered ion experiments. The substrate phosphoinositide was extracted with an EDTA solution and then several times without chelator, and the enzyme was dialyzed to remove endogenous Ca^{2+} and chelator. With these precautions, PI was hydrolyzed with linear $CaCl_2$ kinetics over the range 0.7–10 μM $CaCl_2$. Below approximately 0.7 μM $CaCl_2$, PIC activity was not sensitive to added $CaCl_2$. With increasing $CaCl_2$ (over 10 μM), a secondary stimulation of PIC 2 activity was observed. Under similar unbuffered calcium conditions, hydrolysis of PIP_2 was maximal without the addition of $CaCl_2$, precluding other experiments with PIP_2 in unbuffered conditions.

PI hydrolysis was inhibited by $LaCl_3$ (0.01–0.3 μM) at low concentrations of $CaCl_2$. To determine the mechanism of inhibition, $CaCl_2$ kinetics of PI hydrolysis were performed at several $LaCl_3$ concentrations, holding the PI concentration constant (10 μM). Analysis of the Lineweaver–Burk plots suggested $LaCl_3$ was a competitive inhibitor of PIC 2 catalyzed PI hydrolysis. With no $LaCl_3$ present, the apparent K_{Ca} value was 173 ± 18 nM, and V_{max} was 456 ± 73 nmol (min·mg)$^{-1}$ (SEM, n = 4); addition of $LaCl_3$ from 10 to 300 nM increased the apparent K_{Ca} value without affecting the apparent V_{max} value. For example, at 100 nM $LaCl_3$, the apparent K_{Ca} value was 720 ± 160 nM, and the V_{max} was 467 ± 79 nmol (min·mg)$^{-1}$ (n = 4). In experiments with low concentrations of unbuffered ions, scatter in the data was greater, but the pattern of competitive inhibition was consistent. An apparent K_{La} value was determined from secondary analysis of the inhibition data, in which increasing $LaCl_3$ concentration (four $LaCl_3$ concentrations from 10 to 300 nM) linearly increased the apparent K_{Ca}. The slope of this plot, representing the ratio apparent K_{Ca}/apparent K_{La}, was 4.5 ± 0.4 (SEM, n = 4). The resulting apparent K_{La} value was 39 ± 8 nM. These data suggest La^{3+} was a competitive inhibitor of PIC 2 activity with respect to the enzyme's Ca^{2+} binding site.

Discussion

Multiple isoforms of PIC exist in mammalian heart (Low & Weglicki, 1983). Although PIC has not previously been purified from heart, 85-kDa isozymes of PIC have been purified from brain and liver (Fukui et al., 1988; Homma et al., 1988; Ryu et al., 1987). Recently, a quantitative immunoassay for PIC was developed using monoclonal antibodies prepared against three PIC isoforms from brain (PIC β, γ, and δ) (Suh et al., 1988). Anti-PIC δ antibodies detected an 85-kDa PIC in heart homogenate, suggesting the possibility the 85-kDa heart PIC 2 purified here may be the same isozyme as PIC δ from brain.

Heart PIC 2 had an acidic pH optimum (pH 5–5.5) for hydrolysis of PI and a neutral pH optimum (pH 7–7.5) for hydrolysis of PIP_2, in agreement with a recent report using 85-kDa PIC from brain (Homma et al., 1988). The difference in pH optima for hydrolysis of the two substrates may be due to a required protonation state of the substrate PIP_2, the phosphomonoester groups of which have pK_a values around 7.0 and 7.7 (Toner et al., 1988).

Hydrolysis of both substrates PI and PIP_2 followed linear Michaelis–Menten saturation kinetics over a wide range of phosphoinositide concentrations (0.4–500 μM), indicating PIC 2 probably contains a single phospholipid substrate site. The phosphoinositide substrate was presented to the enzyme as a sonicated suspension in these experiments. Since the sonicated micelles or vesicles are not equivalent to a soluble substrate, the physical meaning of derived kinetic values for the lipid

substrates is uncertain. Also, direct comparisons of the phospholipid kinetic constants for PI versus PIP_2 may not be valid, since the structures of the two substrate suspensions are not necessarily equivalent. Sonicated PI forms vesicles (Hofmann & Majerus, 1982), whereas sonicated PIP_2 forms micellelike aggregates (Sugiura, 1981). The higher K_m and V_{max} values obtained for PIP_2 hydrolysis are consistent with other purified PIC isozymes (Bennett & Crooke, 1987; Ryu et al., 1987; Wilson et al., 1984). Additionally, turnover of PIC 2 is dependent on the assay conditions. For example, PI hydrolysis by PIC 2 can be stimulated 8–10-fold by certain cations or bile detergents (unpublished experiments).

The role of Ca^{2+} in phosphoinositide hydrolysis was analyzed by substrate kinetics, in which linear and convergent Lineweaver–Burk plots were observed. The data suggested PIC catalyzed the hydrolysis of either PI or PIP_2 by a rapid-equilibrium, random-order ternary mechanism in which the binding of one substrate decreased the affinity of the enzyme for the other substrate. The data obtained were inconsistent with other possibilities for the mechanism of PIC 2 catalysis. A ping-pong mechanism was ruled out by the presence of the Φ_{CaPI} parameter (Engel, 1981). The Theorell–Chance mechanism was not a viable alternative, because of the approximately 100-fold difference in apparent V_{max} between hydrolysis of the two substrates assayed under similar conditions.

The possibility that the true substrate of PIC 2 is a complex of Ca^{2+} and phosphoinositide [(Ca^{2+}–PI)] was also eliminated. Segel (1975) outlines several possible mechanisms, none of which are supported by our data. In one case, PIC 2 would bind only (Ca^{2+}–PI), but neither free Ca^{2+} nor free phosphoinositide. In this mechanism, the initial equilibrium process of (Ca^{2+}–PI) formation results in equivalent apparent K_{Ca} and phosphoinositide K_m values [system B1 in Segel (1975)]. This was not observed with PIC 2, where the K_{Ca} value was approximately 30 nM and the K_m values for PI or PIP_2 were at least 100-fold higher. A second case includes the possibility that in addition to binding (Ca^{2+}–PI), PIC also binds free Ca^{2+} and/or free phosphoinositide. Excess Ca^{2+} or phosphoinositide would inhibit PIC activity competitively at the enzyme's (Ca^{2+}–PI) site in these mechanisms [systems B2, B3, and B4 in Segel (1975)], but this was not observed in our data. Other possible mechanisms were ruled out by the linear nature of our data, and by the absolute requirement of PIC 2 activity for Ca^{2+} [systems B5, B6, and B7 in Segel (1975)]. Furthermore, the stimulation of PIC 2 by Ca^{2+} occurred at much lower concentrations than the reported affinities of PI or PIP_2 for calcium, which are in the range 1–100 μM (Toner et al., 1988; Williams & Schacht, 1986).

The value αK_{Ca}, the apparent affinity for Ca^{2+} in the presence of saturating phosphoinositide, was similar for the two substrates used, PI (αK_{Ca} = 42 ± 16 nM) or PIP_2 (αK_{Ca} = 29 ± 11 nM). This finding suggests a model in which PIC 2 contains a single Ca^{2+} site used in the hydrolysis of either PI or PIP_2. Other isozymes of PIC have properties consistent with the random-order kinetic mechanism proposed for PIC 2. For example, purified PIC α binds either phosphoinositide or Ca^{2+} in the absence of the other (Herrero et al., 1988), properties predicted by a random-order binding kinetic mechanism for PIC.

Further evidence supporting a PIC 2 Ca^{2+}-binding site was provided in studies with the Ca^{2+} analogue La^{3+}. PIC 2 activity was inhibited by submicromolar $LaCl_3$, attributed to a competition with Ca^{2+} for the putative calcium site on PIC 2. The apparent inhibition constant for lanthanum (39 ± 8 nM, n = 4) was on the same order as the kinetically derived Ca^{2+} constant (approximately 30 nM). A similar competitive inhibition by low concentrations of La^{3+} was observed using partially purified bovine heart PIC 3 (unpublished experiments).

The stated K_{Ca} values have a large uncertainty associated with them, mainly due to the choice of an association constant for the calcium–EGTA complex, values for which range from $10^{10.67}$ to $10^{11.39}$ in the literature (Raaflaub, 1956; Martell, 1971; Durham, 1983). An intermediate value of $10^{11.0}$ was used for these experiments. Additional error is due to the choice of pK_a values for EGTA, and together these sources of error created an estimated total uncertainty in the stated Ca^{2+} constants of approximately ±0.4 log. Thus, for example, the stated K_{Ca} value of approximately 30 nM for either PI or PIP_2 hydrolysis may be as low as 10 nM or as high as 80 nM.

Heart intracellular free Ca^{2+} concentration is similar to that reported for many other tissues, ranging from 0.1 μM in the resting state to greater than 1 μM during stimulation (Marban et al., 1988). Assuming the catalytic mechanism and Ca^{2+} kinetics values presented here can be extrapolated to conditions in vivo, PIC 2 may be saturated with Ca^{2+} in the resting cell, when the enzyme is presumably inactive. This situation would be consistent with the accepted model in which PIC is activated, at the resting intracellular free Ca^{2+} concentration, by a receptor-mediated process.

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Registry No. PIC, 63551-76-8; Ca^{2+}, 7440-70-2; La^{3+}, 7439-91-0.

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Bleomycin·Iron Can Degrade DNA in the Presence of Excess Ethylenediaminetetraacetic Acid in Vitro†

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ABSTRACT: The antineoplastic drug bleomycin, when complexed to Fe(II), causes both single- and double-stranded lesions in DNA in vitro. EDTA is commonly used to inhibit the reaction of bleomycin·Fe with DNA, presumably by removing the metal cofactor. In this study, we utilized a simple assay involving the conversion of supercoiled plasmid DNA to the nicked or linear forms to further investigate the ability of bleomycin·Fe to degrade DNA in the presence of EDTA. We found that a 1:1 complex of bleomycin and Fe can degrade plasmid DNA even in the presence of a 10^6 molar excess of EDTA over bleomycin. Furthermore, we found that the half-life for inactivation of bleomycin·Fe by excess EDTA is about 1.5 h. Finally, we demonstrate that excess bleomycin associated with the outer plasma membranes of cells can damage DNA after the cells are lysed in buffers containing EDTA and SDS. These results suggest that EDTA may not be an efficient inhibitor of the reaction of bleomycin·Fe with DNA.

Bleomycins, a group of glycopeptide antibiotics isolated from *Streptomyces verticillus* (Umezawa et al., 1966), are commonly used for the treatment of several different carcinomas (Bennett & Reich, 1979; Sikic et al., 1985). When complexed to a reduced transition metal such as Cu(I) or Fe(II), bleomycins have been shown to degrade DNA both in vitro and in vivo. This degradation includes both single-stranded lesions and double-stranded breaks in the DNA backbone (Hecht, 1986). The single-stranded lesions or "nicks" are caused by both direct breaks in the DNA backbone and release of bases from the deoxyribose moiety. Regions in which the bases are removed are then susceptible to alkaline lysis (Lloyd et al., 1978). Double-stranded breaks in the DNA are thought to be due to two independent breaks on opposite strands of the DNA (Mirabelli et al., 1982), although it is likely that one single-stranded nick will increase the chance of a second break on the opposite strand (Keller & Oppenheimer, 1987). The ability of bleomycin to degrade DNA is thought to be the basis for its antineoplastic activity [for a review, see Sikic (1986)].

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