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ALUMINUM AFFECTS PHOSPHOINOSITIDE HYDROLYSIS BY PHOSPHOINOSITIDASE C

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A single phosphoinositidase C (PIC) activity has been purified 500-fold from bovine heart cytosol to a specific activity of 3μmol phosphatidylinositol (PtdIns) hydolyzed / (min • mg protein). The enzyme required Ca^{2+} for the hydrolysis of polyphosphoinositides or PtdIns; PIC activity was saturable at micromolar free Ca^{2+} concentrations with an apparent K_{Ca} of 230nM. None of the multivalent cations tested could effectively substitute for Ca^{2+} in the activation of PIC, although several ions inhibited PIC activity in the presence of Ca^{2+}. $AlCl_3$ had a differential effect on PIC activity, stimulating PtdIns hydrolysis while inhibiting PtdIns-(4,5)P_2 hydrolysis in a concentration-dependent manner from 1 - 100μM. The effect of $AlCl_3$ was attributed to the free ion, Al^{3+}. Complexation of Al^{3+} with phosphate, citrate, fluoride, or hydroxide could block the stimulatory effect on PtdIns hydrolysis. Only fluoride or hydroxide could partially reverse the inhibition of PtdIns-(4,5)P_2 hydrolysis by $AlCl_3$.

The turnover of phosphoinositides is a ubiquitous eukaryotic mechanism by which the second-messengers diacylglycerol and inositol trisphosphate are generated by the hydrolysis of membrane phospholipid (1). Phosphoinositide hydrolysis is catalyzed by a phosphoinositide-specific phospholipase C called phosphoinositidase C (PIC). The initial agonist-directed event appears to be the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PtdIns-(4,5)P_2), forming inositol-1,4,5-trisphosphate. Additional breakdown of phosphatidylinositol (PtdIns) may be a secondary event kinetically, leading to a prolonged, enhanced production of diacylglycerol (2,3).

PIC is present in multiple forms in most mammalian tissues studied (4), including heart (5). Different forms of PIC have been purified from a variety of tissues (6,7,8). Divalent calcium is required for PIC activity (1). Most of the biochemical characterization of PIC has been performed at millimolar concentrations of Ca^{2+}, though the enzyme is active at sub-micromolar concentrations of free calcium *in vitro* (6,7). Although other multivalent ions generally do not support PIC activity in the absence of calcium, they may be inhibitory to the enzyme when calcium is present (8,9,10). Aluminum complexed with fluoride has been widely used to stimulate polyphosphoinositide hydrolysis in whole cells (11) and in cell-free systems (12). In this report, a direct effect of Al^{3+} on phosphoinositide hydrolysis *in vitro* is presented.

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Abbreviations Used DTT dithiothreitol, PE phosphatidylethanolamine, PIC phosphoinositidase C, PMSF phenylmethylsulfonyl fluoride, PtdIns phosphatidylinositol, PtdIns-(4,5)P_2 phosphatidylinositol-(4,5)-bisphosphate

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In this study, the effect of cations on the hydrolysis of phosphoinositides is examined, using a highly purified PIC, free of guanine-nucleotide binding activity. PIC activity required Ca^{2+}, and no other cations tested could substitute for Ca^{2+}. At 1μM $CaCl_2$, 1-100μM $AlCl_3$ had potent, contrasting effects on PIC activity; hydrolysis of PtdIns could be stimulated up to 10-fold, while hydrolysis of PtdIns-(4,5)P_2 was inhibited up to 90%.

Materials and Methods

Materials. L-myo-[1,2-^{3}H]-inositol (56.6Ci / mmol) was obtained from New England Nuclear. L-3-phosphatidyl-[2-^{3}H]-inositol 4,5-bisphosphate (1Ci / mmol) was from Amersham. EGTA was obtained from Fluka Chemicals, Ronkonkoma, N.Y. Affigel-Blue agarose and Dowex AG1-X8 were obtained from BIO-RAD, Richmond, CA. Other reagents were obtained from Fisher, Fair Lawn, N.J or from Sigma, St. Louis, USA.

Preparation of Radiolabelled Phosphatidylinositol. [^{3}H]-inositol-labelled PtdIns was prepared utilizing the Mn^{2+}-stimulated inositol exchange activity of bovine liver microsomes (13). A phosphoinositide-enriched fraction (Folch Fraction 1) of a chloroform extract of the labelled microsomes, containing 100 to 150μCi [^{3}H]-PtdIns, was precipitated with the addition of ethanol (14). PtdIns was purified by preparative thin-layer chromatography (15). The silica gel of the PtdIns band was scraped, neutral lipids were eluted with chloroform, and then PtdIns was eluted with 3:1 methanol: chloroform (v / v), dried under nitrogen, and redissolved in chloroform. The PtdIns solution was washed once with one volume methanol : one volume 1M-KCl, 10mM-EDTA, 10mM-EGTA, 50mM-Tris-HCl, pH 7.4, and then twice without the EDTA or EGTA. The lower phase of this extraction was pure PtdIns as judged by analytical thin-layer chromatography (16). The specific activity of purified PtdIns prepared in this manner was 2000 to 6000 d.p.m. / nmol lipid phosphorous.

Purification of PIC. Fresh bovine heart obtained from a local slaughterhouse was immediately placed on ice and subsequent steps were performed at 0 - 4°C. Ground ventricular tissue from one heart was homogenized at 20 to 25% (w / v) in Buffer A (10mM-Bistris pH 6.8, 1mM-EDTA, 0.5mM-DTT, 1mM-benzamidine-HCl, 0.1mM-PMSF, 0.01%-NaN_3) supplemented with 350mM-NaCl, 3mM-EDTA, 2mM-EGTA, and 0.4mM-PMSF. The homogenate was centrifuged at 16000***g***; the supernatant was further centrifuged at 50000***g***. A 30 - 70% $(NH_4)_2SO_4$ precipitate fraction was prepared using solid $(NH_4)_2SO_4$.

The precipitate was dissolved in buffer A supplemented with 20mM-NaCl, 2mM-EDTA, 1mM-EGTA, 0.4mM-PMSF, and the protease inhibitors leupeptin, antipain, and aprotinin, each at 0.2μg / ml, and loaded onto a 5 x 40cm column of phosphocellulose resin. The column was eluted with a 2.4 liter gradient of Buffer A containing from 350 to 800mM-NaCl. Fractions with PIC activity were pooled and precipitated with $(NH_4)_2SO_4$. The resolubilized sample was applied by gravity to a 1.5 x 5cm column of amino-hexyl agarose at 50mM NaCl. PIC activity was eluted with a gradient from 50 to 400mM-NaCl in Buffer A. Fractions with PIC activity were immediately applied to a 0.9 x 1.5cm column of Affigel-blue agarose resin. The column was washed exhaustively with 10mM-Bistris pH6.8, 0.5M-NaCl, 0.5mM-DTT, 0.01%-NaN_3, and then step-eluted with 20ml of the same buffer containing 2M-NaCl. Fractions with PIC activity were pooled and stored at 4°C in glass tubes.

PIC Assay. Radiolabelled PtdIns (4 -10nmol) as prepared above or PtdIns-(4,5)P_2 (7 - 14pmol) was dried under nitrogen and sonicated into a buffer for 3 minutes on ice. The total reaction volume was 200μl for PtdIns and 50μl for PtdIns-(4,5)P_2, containing 25mM-Tris-HCl, pH 7. $CaCl_2$ was 1mM for the PIC purification assays, or as indicated for the other studies. The reactions were performed at 37°C for 10 to 30 minutes, and were stopped with the addition of 500μl cold 2:1 methanol: chloroform (v / v), followed by 100μl 2M-HCl and 200μl chloroform. Tubes were capped, vortexed vigorously, and centrifuged briefly. The upper (aqueous) phases of the extractions were analyzed for radioactivity.

Other Methods. The pH optima of PIC activity was determined with a series of Tris-maleate buffers (50mM final concentration) balanced with NaOH and NaCl to give equivalent conductivity readings. Free Ca^{2+} was buffered from 50nM to 5μM with EGTA (17). Water-soluble hydrolysis products of PIC were analyzed by Dowex-1 anion-exchange chromatography as described (18). Protein concentration was determined by the method of Bradford (19), with bovine serum albumin as the standard. Lipid phosphorus was determined by the method of Organisciak & Noell (20). Guanine nucleotide-binding activity was determined by the method of Evans *et al.* (21).

Results and Discussion

Purification of PIC. Phosphoinositidase C is the Ca^{2+}-dependent effector enzyme of the phosphoinositide turnover second-messenger system. Multiple PIC activities were detected in bovine

Table 1. Purification of Bovine Heart Cytosolic PIC

Step	Protein (mg)	Specific Activity [μmol / (min • mg protein)]	Purification
1. Cytosol	15200	0.0063	1
2. $(NH_4)_2SO_4$	9200	0.010	1.6
3. Phosphocellulose	3.2	0.34	53
4. Amino-hexyl agarose	0.044	1.4	220
5. Affi-gel Blue agarose	0.020	3.0	480

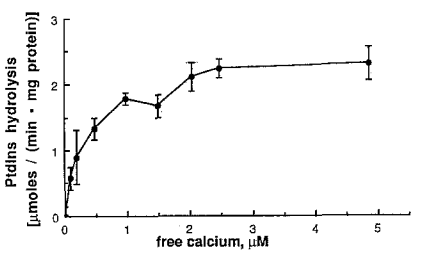
Protein was measured as described (19). PIC activity was measured with 100μM-PtdIns and 1μM-$CaCl_2$, pH 7.

heart cytosol (data not shown), in agreement with a previous report (5). A single PIC activity was partially purified, shown in Table 1. Single peaks of PIC activity were eluted from phosphocellulose and amino-hexyl agarose. Step elution from Affi-gel blue agarose yielded a PIC activity purified 500-fold (with respect to heart cytosol) to a specific activity of 3μmol PtdIns hydrolyzed / min / mg protein at 37°C with 100μM-PtdIns and 1μM-$CaCl_2$. PIC activity was separated from guanine nucleotide-binding activity at the phosphocellulose chromatography step (data not shown). Protein yield was 10 - 20μg from 400g heart tissue, and enzyme activity was stabile for at least one month at 4°C. Enzyme at this level of purity was used in the characterization studies.

Characterization of PIC. The water-soluble hydrolysis products of this enzyme activity were analyzed by anion-exchange chromatography (18). Hydrolysis of radiolabelled PtdIns, PtdIns-4*P*, or PtdIns-(4,5)P_2 in separate reactions yielded the products inositol monophosphate, inositol bisphosphate, and inositol trisphosphate, respectively, as would be expected for a phospholipase C-type activity (data not shown). The pH dependence of PIC hydrolysis of [^{3}H]-inositol-PtdIns revealed a moderately broad pH optimum around pH 6, in agreement with reports for heart PIC (5,10). PIC activity depended strongly on the ionic strength of the reaction. Adjusting the monovalent salt concentration with either NaCl or KCl revealed a monovalent salt optimum from 80 to 140mM (data not shown). All characterization of PIC was performed at pH 7 with 100mM-NaCl and total ionic strength at 0.13.

Ca^{2+} Dependence of PIC Activity. Ca^{2+} is necessary for mammalian cytosolic PIC-catalyzed hydrolysis of phosphoinositides (1). Since the phosphoinositides bind cations, including Ca^{2+} (22), the substrate PtdIns was extracted with EDTA and EGTA prior to the characterization of PIC. Figure 1 shows the Ca^{2+}-dependence of PIC activity from 50nM to 5μM free Ca^{2+} using $CaCl_2$ buffered with EGTA. No PtdIns hydrolysis occured with only EGTA present, while PIC activity increased with increasing free Ca^{2+} with saturable kinetics. Double reciprocal analysis of PIC activity at these Ca^{2+} concentrations revealed an apparent K_{Ca} value of 230 ± 55nM (mean ± S.D., 3 separate experiments). Hydrolysis of PtdIns-(4,5)P_2 was also sensitive to EGTA and was saturable at similar free Ca^{2+} concentrations. Free Ca^{2+} concentrations as high as 100μM produced no further activation of PIC. Thus, this PIC could be active at the sub-micromolar free Ca^{2+} of mammalian cell cytoplasm. This high-affinity Ca^{2+} effect is distinct from the affinity of the substrate PtdIns for Ca^{2+}, which is approximately 1mM (22).

Specificity of the PIC high-affinity metal site for Ca^{2+} was shown in that none of the other multivalent ions tested (Mg^{2+}, Mn^{2+}, Ba^{2+}, Zn^{2+}, or Al^{3+}) at 0.1mM could effectively substitute for Ca^{2+}. In contrast, with Ca^{2+} present at 10μM, some of the ions tested modulated PtdIns hydrolysis. $AlCl_3$ was the



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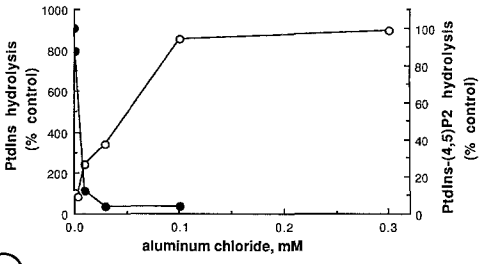

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Figure 1. Ca^{2+} Dependence of PtdIns Hydrolysis by PIC.

Free Ca^{2+} was buffered with 2mM-EGTA and various amounts of $CaCl_2$ as described (Raaflaub, 1956). Reactions contained 50μM-PtdIns, pH 7.0. Total ionic strength was maintained at 0.1. Points represent the means ± S.D. of triplicate measurements.

Figure 2. Effect of $AlCl_3$ on PtdIns and PtdIns-(4,5)P_2 Hydrolysis by PIC.

PIC activity was determined in the presence of 100μM-PtdIns or 200nM-PtdIns-(4,5)P_2 at pH 7 with 1μM-$CaCl_2$. O—O, PtdIns, control (100%) activity was 1.5μmol / (min • mg protein); ●—●, PtdIns-(4,5)P_2, control activity was 48nmol / (min • mg protein).

only salt tested which stimulated PtdIns hydrolysis. $MgCl_2$ and $BaCl_2$ were without effect at 0.1mM, while similar concentrations of $MnCl_2$ and $ZnCl_2$ inhibited PIC activity by 77% and 97%, respectively. Differential Effect of Al^{3+} on PtdIns and PtdIns-(4,5)P_2 Hydrolysis. The effect of $AlCl_3$ was investigated further using both PtdIns and PtdIns-(4,5)P_2 as substrates. $AlCl_3$ stimulated PIC hydrolysis of PtdIns, but inhibited hydrolysis of PtdIns-(4,5)P_2 in a concentration-dependent manner from 1 - 100μM-$AlCl_3$ (Figure 2). PIC hydrolysis of PtdIns was stimulated up to 8-fold at 100μM-$AlCl_3$, whereas PtdIns-(4,5)P_2 hydrolysis was inhibited 80 - 90% at concentrations over 30μM-$AlCl_3$. From 0.1nM to 0.1μM, $AlCl_3$ had esentially no effect on the hydrolysis of either PtdIns or PtdIns-(4,5)P_2 (data not shown). The effects of $AlCl_3$ were not confined solely to sonicated vesicles of pure negatively charged phosphoinositides. If sonicated vesicles contained PtdIns or PtdIns-(4,5)P_2 mixed with 5 mole-equivalents of phosphatidylethanolamine, 0.1mM-$AlCl_3$ had virtually the same effects (Table 2).

Table 2. Effect of $AlCl_3$ on PIC Activity with Either Pure Phosphoinositide or Phosphoinositide - Phosphatidylethanolamine Vesicles

Vesicles	PIC Activity	
	no $AlCl_3$	100μM-$AlCl_3$
	PtdIns Hydrolysis [μmol / (min • mg protein)]	
PtdIns	1.1 ± 0.06	4.7 ± 0.55
PtdIns + PE	0.60 ± 0.02	1.9 ± 0.14
	PtdIns-(4,5)P_2 Hydrolysis [nmol / (min • mg protein)]	
PtdIns-(4,5)P_2	45 ± 4.3	6.2 ± 3.5
PtdIns-(4,5)P_2 + PE	67 ± 1.9	4.9 ± 1.3

Reactions contained 40μM-PtdIns or 250nM-PtdIns-(4,5)P_2 sonicated in pH 7 buffer with 1μM-$CaCl_2$. Phosphoinositide - phosphatidylethanolamine vesicles were prepared by resonicating phosphoinositide vesicles with 5 mole-equivalents dried phosphatidylethanolamine (PE). Data is presented as the means ± S.E.M. (n=3) of a single experiment representative of three replicates.

Table 3. Effect of $AlCl_3$ on PtdIns or PtdIns-(4,5)P_2 Hydrolysis in the Absence or Presence of Agents Known to Complex Al^{3+}

	PtdIns Hydrolysis [μmol / (min · mg protein)]		PtdIns-(4,5)P_2 Hydrolysis [nmol / (min • mg protein)]	
Treatment	no $AlCl_3$	100μM- $AlCl_3$	no $AlCl_3$	100μM- $AlCl_3$
control	1.4 ± 0.05	5.4 ± 0.51	24 ± 2.2	4.9 ± 2.1
20mM-KPO_4	1.1 ± 0.07	1.1 ± 0.02	34 ± 7.3	4.3 ± 2.2
20mM-citrate	0.36 ± 0.02	0.36 ± 0.03	32 ± 6.5	6.5 ± 1.9
20mM-NaF	1.3 ± 0.04	1.3 ± 0.10	41 ± 6.0	26 ± 3.9

Reactions contained 50μM-PtdIns or 270nM PtdIns-(4,5)P_2 at pH 7 with 1μM-$CaCl_2$. Phosphate was added as potassium phosphate, pH 7.0. Citrate was added as sodium citrate, pH 7.4. Data is presented as the means ± S.E.M. (n=3) of a single experiment representative of three replicates.

It was of interest to determine whether the species responsible for the contrasting effects on phosphoinositide hydrolysis was an aluminum complex or free Al^{3+}. Levels of free Al^{3+} in biological systems are held very low by complexation with a variety of molecules, such as hydroxide, phosphate, or citrate (23). Fluoride also complexes free Al^{3+}, and such complexes have been used to activate enzymes (24). Complexation of Al^{3+} by phosphate, citrate, or fluoride abolished the stimulatory effect of $AlCl_3$ on PtdIns hydrolysis (Table 3), suggesting the agent responsible for modulation of PIC activity was free Al^{3+}. The $AlCl_3$-induced inhibition of PtdIns-(4,5)P_2 hydrolysis, however, was more resistant to the complexation of Al^{3+}. Inhibition of PtdIns-(4,5)P_2 hydrolysis by $AlCl_3$ was only partially reversed by 20mM fluoride, and was unaffected by phosphate or citrate (Table 3).

The hydroxide complexes $Al(OH)_3$ and $Al(OH)_4^-$, which predominate in aqueous solution near neutral pH, also limit the concentration of free Al^{3+} intracellularly (23). The $AlCl_3$ effect on PIC activity was tested as a function of reaction pH. Below pH 7, the stimulatory effect of $AlCl_3$ on PtdIns hydrolysis was enhanced. Above pH 8, the effect of $AlCl_3$ as an inhibitor of PtdIns-(4,5)P_2 hydrolysis or as a stimulator of PtdIns hydrolysis was diminished (data not shown). Therefore, the aluminum effect was most pronounced under pH conditions where the Al^{3+} free ion predominates in solution. The aluminum effects on PIC activity are probably not due to modulation of a PIC-associated G-protein, as guanine-nucleotide binding activity was separated from PIC activity at an early step in the PIC purification procedure.

Human tissues may contain enough aluminum to create these effects. Total serum aluminum is approximately 4μmol / liter (25). Tissue aluminum has been most thoroughly studied in brain, where aluminum may be a factor in dementia (26). Brain aluminum levels are approximately 0.1 - 0.2mmol / kg in normal humans and up to 1mmol / kg in dementia patients (26).

Since aluminum is known to bind phosphate-rich compounds such as DNA and nucleotide triphosphates with high affinity (23), the aluminum effects on PIC activity may be due to a direct Al^{3+} – phosphoinositide interaction, although an aluminum – PIC interaction cannot be ruled out. Al^{3+} does not usually replace Ca^{2+} in specific protein binding sites, due to the large difference in sizes between the ions (23). $AlCl_3$ did not support PIC activity in the absence of calcium. The Al^{3+}–PtdIns-(4,5)P_2 affinity may be strong enough to occur intracellularly, since normal biological chelators of Al^{3+}, such as citrate and

phosphate, apparently were unable to compete with PtdIns-(4,5)P_2 for the Al^{3+} in solution. Assessing the possible physiological influence of Al^{3+} on phosphoinositide metabolism awaits determination of intracellular aluminum levels, Al^{3+}-PtdIns-(4,5)P_2 binding affinity, and the interaction of aluminum with phosphoinositides *in vivo*.

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