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A Receptor and G-protein-regulated Polyphosphoinositide-specific Phospholipase C from Turkey Erythrocytes

I. PURIFICATION AND PROPERTIES*

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Eighty-three percent of polyphosphoinositide-specific phospholipase C activity was recovered in a cytosolic fraction after nitrogen cavitation of turkey erythrocytes. This activity has been purified approximately 50,000-fold when compared to the starting cytosol with a yield of 1.7–5.0%. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the phospholipase C preparation revealed a major polypeptide of 150 kDa. The specific activity of the purified enzyme was 6.7–14.0 μmol/min/mg of protein with phosphatidylinositol 4,5-bisphosphate or phosphatidylinositol 4-phosphate as substrate. Phospholipase C activity was markedly dependent on the presence of Ca^{2+}. The phospholipase C showed an acidic pH optimum (pH 4.0). At neutral pH, noncyclic inositol phosphates were the major products formed by the phospholipase C, while at pH 4.0, substantial formation of inositol 1:2-cyclic phosphate derivatives occurred. Properties of the purified 150-kDa turkey erythrocyte phospholipase C were compared with the approximately 150-kDa phospholipase C-β and -γ isoenzymes previously purified from bovine brain (Ryu, S. H., Cho, K. S., Lee, K. Y., Suh, P. G., and Rhee, S. G. (1987) *J. Biol. Chem.* 262, 12511–12518). The turkey erythrocyte phospholipase C differed from the two mammalian phospholipases with respect to the effect of sodium cholate on the rate of polyphosphoinositide hydrolysis observed. Moreover, when presented with dispersions of pure inositol lipids, phospholipases C-β and -γ displayed comparable maximal rates of polyphosphoinositide and phosphatidylinositol hydrolysis. By contrast, the turkey erythrocyte phospholipase C displays a marked preference for polyphosphoinositide substrates.

The *trans*-plasma membrane signalling response to many extracellular stimuli involves activation of an inositol lipid-specific PLC.[1] Two hydrolysis products of this enzyme, inositol 1,4,5-trisphosphate and *sn*-1,2-diacylglycerol act as second messengers, releasing intracellularly stored calcium and activating protein kinase C (1, 2). The established role of PLC in signal transduction has recently stimulated much progress in defining a family of PLC isoenzymes that consists of a minimum of five structurally and immunologically distinct proteins (3–8). A nomenclature recently proposed by Rhee *et al* (9) terms these proteins PLC-α, -β, -γ, -δ, and ϵ with respective sizes determined by SDS-PAGE analysis of 65, 150, 145, 85, and 85 kDa. The entire primary sequence of PLC-α, -β, -γ, and -δ has been determined by cDNA cloning (10–14). Although overall homology is low, PLC-β, -γ, and -δ contain two domains of significant sequence similarity, possibly involved in catalysis. The isoenzymes share some common properties. When assayed with substrates presented as components of phospholipid vesicles or of mixed phospholipid and detergent micelles, each isoenzyme displays dependence on calcium for activity and can hydrolyze all three inositol lipids. However, substrate selectivity is critically dependent on both the composition of the substrate preparation used and on the concentration of Ca^{2+} and pH of the assay medium. The polyphosphoinositides are better substrates for these enzymes than is phosphatidylinositol under physiologically relevant conditions of Ca^{2+} and pH. The molecular properties of members of the family of PLC isoenzymes identified to date recently have been reviewed by Rhee *et al* (9).

The mechanism(s) by which extracellular signals increase intracellular PLC activity are not yet fully understood. G-proteins play established roles in the stimulatory and inhibitory coupling of cell surface receptors to adenylate cyclase and the regulation of cGMP phosphodiesterase by rhodopsin in retinal rod outer segments (Ref. 15). A substantial body of evidence supports the idea that a G-protein couples cell surface receptors to PLC (Ref. 16). This proposal does not exclude alternative regulatory mechanisms: certain growth factor receptors may modulate PLC activity through a re-

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[1] The abbreviations used are: PLC, phospholipase C; G-protein, guanine nucleotide binding protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns, phosphatidylinositol; Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; Ins(1:2-cyclic,4,5)P_3, D-*myo*-inositol 1:2-cyclic 4,5-trisphosphate; Ins(1,4)P_2, D-*myo*-inositol 1,4-bisphosphate; Ins(1:2-cyclic,4)P_2, D-*myo*-inositol 1:2-cyclic, 4-bisphosphate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; DTPA, diethylenetriaminepentaacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography.

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TABLE I
Purification of turkey erythrocyte PLC

PLC activity was purified from turkey erythrocyte cytosol. Enzyme activity was determined as described in the text.

Step	Protein	PLC activity	Specific activity
	mg	*μmol/min*	*μmol/min/mg protein*
Cytosol	1,330,000[a]	188	0.000141
Resuspended $(NH_4)_2SO_4$ precipitate	24,000[b]	119	0.00496
Q-Sepharose	3,313[b]	46	0.0139
Hydroxylapatite	73.4[b]	24.3	0.331
Heparin-Sepharose	18.8[b]	17.6	0.936
Sephacryl S-300	3.0[b]	10.6	3.53
Mono Q	1.1[c]	6.9	6.27

[a] Protein was determined by colorimetric hemoglobin assay.
[b] Protein was determined by the method of Bradford (29).
[c] Protein was determined by determination of protein absorbance at 280 nm.

cently demonstrated receptor-catalyzed tyrosine-specific phosphorylation (17).

Ghosts prepared by hypotonic lysis of turkey erythrocytes are an excellent model cell-free system for studying P_{2Y}-purinergic receptor and G-protein-mediated regulation of PLC (18–21). None of the identified isoenzymes of PLC has been directly implicated in receptor-stimulated generation of inositol lipid-derived second messengers. Turkey erythrocytes offer a homogeneous and readily available source from which we are currently attempting to identify and purify components of the PLC-dependent signalling system. In this paper, we describe the purification and properties of a polyphosphoinositide-specific PLC from the cytosolic fraction of turkey erythrocytes and demonstrate differences in substrate selectivity and detergent dependence between the purified PLC and two mammalian PLCs of similar molecular weight. In the following paper, we demonstrate that, when reconstituted with [^{3}H]inositol-labeled turkey erythrocyte ghosts, the activity of this enzyme can be regulated by P_{2Y}-purinergic receptor agonists in a guanine nucleotide-dependent manner.

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Results related to the purification and general catalytic properties of a 150-kDa PLC from turkey erythrocytes are presented below in Miniprint form. Table 1 summarizes results from a typical purification. A silver-stained SDS-polyacrylamide gel of fractions from a final step of Mono Q anion exchange chromatography and corresponding measurements of PLC activity for each fraction is shown in Fig. 6. On preliminary characterization of the enzyme, we obtained results that were in some respects markedly different from those reported for two similarly sized PLC isoenzymes (PLC-β and -γ) previously purified from bovine brain (6). We therefore undertook a direct comparison of the turkey erythrocyte PLC with PLC-β and -γ.

Effect of Ca^{2+} on Turkey Erythrocyte PLC Activity

The purified PLC was incubated with either PtdIns(4,5)P_2, PtdIns4P, or PtdIns under standard assay conditions except that the free ionized calcium concentration in the incubations was varied using calcium EGTA buffers. For the lower values (0.05–0.15 mM), the actual Ca^{2+} concentration was measured in parallel incubations using the fluorescent indicator, Fura-

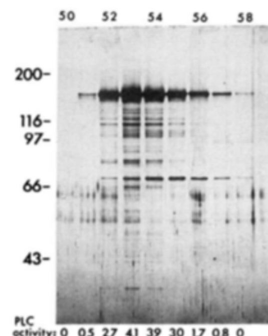

FIG. 6. **Examination of the purified PLC by SDS-PAGE.** Fractions 50–60 from a final step of Mono Q anion exchange chromatography were examined by SDS-PAGE, and proteins were detected by silver staining as described under "Experimental Procedures." The *lower panel* shows PLC activity (nmol/2 μl/5 min) determined in each fraction using the methods described under "Experimental Procedures."

2 (33). Activity against the polyphosphoinositides was markedly dependent on Ca^{2+}, with half-maximal activation occurring at approximately 70 nM Ca^{2+} with both substrates (Fig. 9). The PLC displayed some activity in the absence of added Ca^{2+}. This activity was 8% and 26% of the maximal Ca^{2+}-stimulated activity observed with PtdIns4P and PtdIns(4,5)P_2, respectively. Under the range of assay conditions used, the rate of phosphatidylinositol hydrolysis observed with the purified PLC was at most one-hundredth of that displayed with the polyphosphoinositides as substrates.

Comparison of the Turkey Erythrocyte PLC with PLC-β and PLC-γ

Ca^{2+} Dependence and Substrate Selectivity—The high degree of substrate selectivity observed with the 150-kDa turkey erythrocyte PLC differs from that observed for PLC-β and -γ (6), although it should be noted that Katan and Parker (5) reported that a preparation of PLC (subsequently shown to be PLC-β, Ref. 11) purified from detergent extracts of bovine brain membranes hydrolyzed pure polyphosphoinositides some 30-fold faster than PtdIns. Since these earlier observations were made using dispersions of pure inositol lipids and no detergent, it was important to compare directly the turkey erythrocyte PLC under conditions identical with those previously used. As such, substrate was prepared as previously described (6) and consisted of dispersed sonicates of pure PtdIns or PtdIns(4,5)P_2 with no detergent or other phospholipids present, each at a concentration of 0.1 mM. The activity of the purified turkey erythrocyte PLC was compared with PLC-β and -γ (Fig. 10). Hydrolysis of PtdIns by all three

[2] Portions of this paper (including "Experimental Procedures" and certain "Results" including Figs. 1, 2, 3, 4, 5, 7, and 8 and Table 2) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

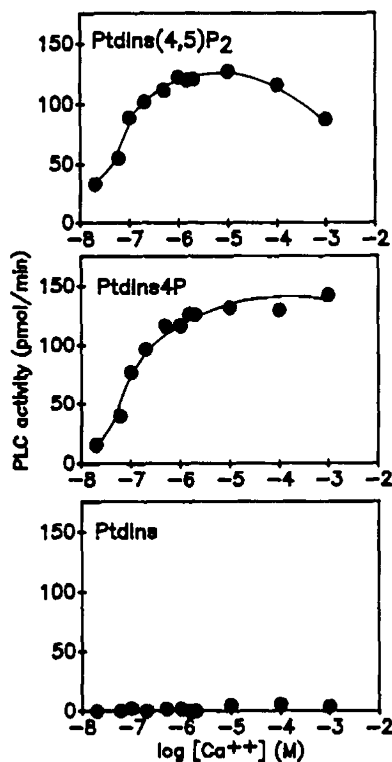

FIG. 9. **Ca^{2+} dependence of PLC activity.** The hydrolysis rates of PtdIns, PtdIns4P, or PtdIns(4,5)P_2 by the purified PLC were determined as a function of Ca^{2+} concentration as shown using the methods described under "Experimental Procedures." The incubations contained an equal amount of the purified PLC (approximately 100 ng).

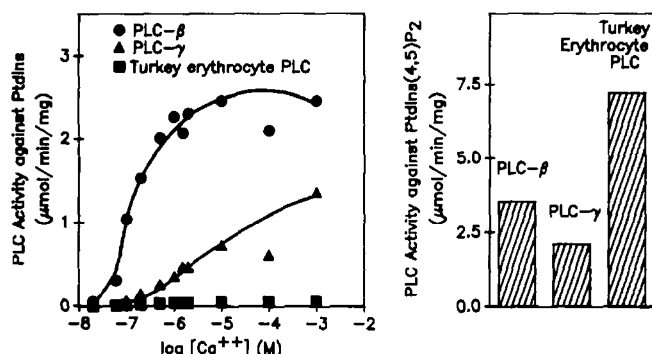

FIG. 10. **Ca^{2+} dependence of PtdIns *versus* PtdIns(4,5)P_2 hydrolysis catalyzed by the turkey erythrocyte PLC and by PLC-β and PLC-γ.** The hydrolysis of sonicated dispersions of PtdIns by the purified turkey erythrocyte PLC (■——■) and by PLC-β (●——●) and -γ (▲——▲) was determined at a series of increasing Ca^{2+} concentrations as described under "Experimental Procedures." The rates of PtdIns(4,5)P_2 hydrolysis were determined at a Ca^{2+} concentration of 10^{-6} M.

PLC preparations was Ca^{2+}-dependent. However, the maximum specific activities displayed by PLC-β and PLC-γ against this pure substrate were substantially greater than that observed with the turkey erythrocyte PLC (2.45 and 1.36 *versus* 0.0631 μmol/min/mg, respectively). By contrast, when presented with pure PtdIns(4,5)P_2 at an experimentally determined optimal Ca^{2+} concentration of 10^{-6} M, PLC-β, PLC-γ, and the turkey erythrocyte PLC showed similar specific activities for substrate hydrolysis (3.54, 2.10, and 6.7 μmol/min/mg). Similar results were obtained with PtdIns4P as substrate. Thus, while the two mammalian PLC isoenzymes hydrolyze PtdIns, PtdIns4P, and PtdIns(4,5)P_2 with essentially equivalent effectiveness, the turkey erythrocyte PLC displays high selectivity for polyphosphoinositides over PtdIns.

Effect of Sodium Cholate on PLC Activity—The effect of increasing concentrations of sodium cholate on the activity of purified PLC-β, PLC-γ, and the turkey erythrocyte PLC was investigated. The substrate preparation used for these experiments was phospholipid vesicles composed of PtdIns(4,5)P_2, phosphatidylserine, and phosphatidylethanolamine in a molar ratio of 1:1:1 prepared as described above. The concentration of PtdIns(4,5)P_2 was 0.1 mM, and the concentration of Ca^{2+} was 10^{-6} M. As previously reported by Ryu *et al.* (6) using dispersions of pure PtdIns as substrate, the activity of PLC-β and PLC-γ sharply increased in activity as the concentration of detergent was increased with maximal activity attained at 0.05% w/v (Fig. 11). Activity declined rapidly at higher detergent concentrations. By contrast, no effect of 0.05% Na^+ cholate on the turkey erythrocyte PLC was observed. Higher concentrations of cholate increased activity of the turkey erythrocyte PLC with half-maximal activation occurring at around 0.2% w/v and maximal activity attained at 0.4% w/v. The absolute magnitudes of the maximal increases in enzymic activity observed for PLC-β, PLC-γ, and the turkey erythrocyte PLC were similar (3.11-, 4.21-, and 4.42-fold, respectively).

DISCUSSION

A 150-kDa PLC has been purified from the cytosolic fraction of turkey erythrocytes. When assayed with exogenously provided substrates, the properties of this enzyme are broadly similar to those described for the other identified isoenzymes of PLC (9 and references therein). That is, activity is dependent on Ca^{2+} and displays an acidic pH optimum, and the enzyme can form both cyclic and noncyclic inositol phosphates although only the latter products are detectable at neutral pH. The specific activity of the turkey erythrocyte PLC when assayed with exogenously provided polyphosphoinositide substrates is comparable to that reported for the other isoenzymes of PLC (9). Two isoenzymes of PLC with molecular weights similar to the turkey erythrocyte PLC (PLC-β and PLC-γ) have been purified and their cDNAs have been cloned (5, 6, 10, 11). In accord with published findings (6), we find that these isoenzymes of PLC display similar specific activities for hydrolysis of pure PtdIns and PtdIns(4,5)P_2. However, when directly compared with these isoenzymes of PLC under identical conditions, the turkey erythrocyte PLC hydrolyzes PtdIns(4,5)P_2 approximately 100

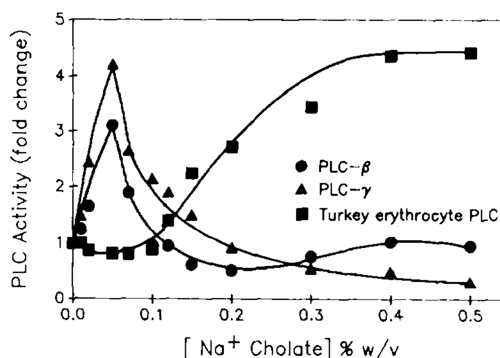

FIG. 11. **Effect of sodium cholate on PtdIns(4,5)P_2 hydrolysis catalyzed by the turkey erythrocyte PLC, PLC-β, and PLC-γ.** The effect of increasing concentrations of sodium cholate on the hydrolysis rate of PtdIns(4,5)P_2 in mixed phospholipid vesicles with phosphatidylserine and phosphatidylethanolamine catalyzed by the purified turkey erythrocyte PLC (■——■) and by PLC-β (●——●) and PLC-γ (▲——▲) was determined as described under "Experimental Procedures."

times faster than PtdIns. Further divergence in properties between the 150-kDa turkey erythrocyte PLC, PLC-β, and PLC-γ is also observed with the effect of sodium cholate on rates of substrate hydrolysis. Thus, when added to assay mixtures containing substrates in mixed phospholipid vesicles, low concentrations of sodium cholate (0.05% w/v) stimulate PLC-β and -γ and have little effect on the turkey erythrocyte PLC, while stimulation of the turkey erythrocyte enzyme occurs with higher concentrations (>0.15% w/v) of detergent that are non-stimulatory or inhibitory to PLC-β and PLC-γ. It should be noted that, when dispersed in aqueous solution, the polyphosphoinositides have been observed to form micellar structures while PtdIns forms larger vesicular structures (4, 42) so catalytic discrimination between substrates in these forms may represent a substrate aggregation state preference of the PLCs rather than an absolute substrate selectivity. The basis of the effect of anionic bile salt detergents on PLC activity is also poorly understood. Differential effects of detergent have been noted for isoenzymes of phospholipase A_2 and variously ascribed to differential susceptibility to inhibition by detergent monomers, to differences in enzyme binding to substrate-containing detergent and phospholipid micelles, and to effects on "surface dilution" of substrate in the micelle (see Ref. 43 for review). Nevertheless, in the comparative experiments described above, the turkey erythrocyte PLC can be distinguished from PLC-β and- γ, and further studies of the structure and function of these enzymes will be required to uncover the basis of this distinction.

As an initial step in this process, by immunoblotting, we have found that the turkey erythrocyte PLC does not react with mixtures of monoclonal antibodies raised against either of these proteins.[3] Based on a number of considerations, the existence of further isoenzymes of PLC with certain sequence similarities to, yet significant divergence from, PLC-β and -γ is not unexpected. Ohta *et al.* (39) report the sequence of a cDNA derived from a human lymphocyte cDNA library that potentially encodes a protein with some homology to PLC-γ. We have found that antibodies raised against a recombinant form of the protein encoded by this cDNA would not react with the turkey erythrocyte PLC.[4] The genetic lesion of the *Drosophila* visual mutant NorpA involves a mutation of a gene that potentially encodes a protein with considerable sequence similarity to PLC-β (40). Finally, an isoenzyme of PLC with an estimated size of 143 kDa has been purified from bovine platelets (41). No information is available to suggest the relationship of this isoenzyme of PLC to the turkey erythrocyte-derived PLC or indeed to the other identified isoenzymes of PLC.

Previous work from our laboratory has investigated the properties of a receptor- and G-protein-regulated PLC present in turkey erythrocyte ghosts (18-20). The substrate selectivity and propensity to form cyclic inositol phosphate products displayed by this receptor- and guanine nucleotide-activated PLC when acting on endogenously labeled substrates are similar to those of the purified PLC determined using exogenously provided substrates. However, the significance of this similarity is questioned by a series of original observations by Irvine and co-workers (35-37) made using a crude preparation of pig brain cytosolic PLC. Their findings established that the physicochemical state and phospholipid composition of the substrate preparation can profoundly influence the catalytic activity of PLC. In particular, PLC activity against phospholipids presented as components of a natural phospholipid bilayer was much reduced when compared with that displayed toward vesicles of pure phospholipid substrate. Under the latter assay conditions, inclusion of certain phospholipids (notably phosphatidylserine, phosphatidylethanolamine, and phosphatidic acid) in the substrate-containing vesicles stimulated PLC activity while phospholipids containing a choline head group were potent inhibitors of the enzyme. Similar results have been reported for one purified preparation of PLC (38, see also Ref. 9 for review) and further studies on the effect of substrate phospholipid composition on the activity of the 150-kDa turkey erythrocyte PLC are clearly warranted. In broad agreement with the results discussed above, we note that although it shows high activity against substrates presented as mixed phospholipid and detergent micelles, the turkey erythrocyte PLC is considerably less active when incubated with turkey erythrocyte ghosts containing radiolabeled substrates. However, under appropriate conditions, we have found that hormonal activators can stimulate the purified turkey erythrocyte PLC to hydrolyze polyphosphoinositide components of turkey erythrocyte ghost membranes, and this phenomenon is discussed in the following paper (44).

In summary, further work is required to establish the relationship of the turkey erythrocyte PLC to the other members of the family of PLC isoenzymes. The generation of antibodies directed against the turkey erythrocyte PLC and ultimately molecular cloning and the determination of its primary sequence will be important steps in this process.

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[3] G. L. Waldo, A. J. Morris, S. G. Rhee, and T. K. Harden, unpublished observations.

[4] J. Knopf, G. L. Waldo, A. J. Morris, and T. K. Harden, unpublished observations.

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SUPPLEMENTARY MATERIAL TO
A RECEPTOR AND G-PROTEIN REGULATED POLYPHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C FROM TURKEY ERYTHROCYTES.

1. Purification and properties.

By Andrew J. Morris, Gary L. Waldo, C. Peter Downes and T. Kendall Harden.

EXPERIMENTAL PROCEDURES.

Materials. Neomycin-linked glass beads were prepared from glyceryl-coated controlled pore glass beads (pore size 240 angstroms, 200-400 mesh, Sigma Chemical Co, St. Louis, MO.). [^{3}H]-inositol was from American Radiolabeled Chemicals, St. Louis, MO. Fast flow Q-Sepharose, heparin-Sepharose-4B, Sephacryl S-300 HR and Mono-Q chromatography resins were from Pharmacia, Piscataway, N.J. Hydroxylapatite (BioGel HTP) was from Bio Rad Labs, Richmond, Ca. Unlabeled PtdIns (from bovine liver), phosphatidylserine, and phosphatidylethanolamine (both from bovine brain) were purchased from Sigma Chemical Co, St. Louis, MO or from Avanti Polar lipids, Birmingham, AL.

Preparation of phospholipids. [^{3}H]-PtdIns(4,5)P_2 and PtdIns4P (specific radioactivity 0.8-1.0 Ci/mmol) were prepared from lipid extracts of turkey erythrocytes that had been labeled with [^{3}H]-inositol under conditions described previously except that chicken serum was omitted from the incubation (20). The lipids were purified by affinity chromatography on neomycin-linked glass beads as described (22) with minor modifications. A 1 ml column of this resin was used for the purification of lipids extracted from 4 ml of packed erythrocytes. The lipids were applied to the column in 1:1 $CHCl_3$:MeOH and the column washed with 0.2 M NH_4COOH in 5:10:2 $CHCl_3$:MeOH:H_2O. Ten column volumes of 0.6 M NH_4COOH in 5:10:2 $CHCl_3$:MeOH:H_2O and of 1.2 M NH_4COOH in 5:10:2 $CHCl_3$:MeOH:H_2O were used to elute PtdIns4P and PtdIns(4,5)P_2, respectively. The purified polyphosphoinositides were extracted from these eluants and stored in $CHCl_3$ at -20°C. [^{3}H]-PtdIns was purified from the fraction of the [^{3}H]-Inositol labeled turkey erythrocyte lipid extract not absorbed to the neomycin-linked glass beads by two steps of precipitation in absolute ethanol at -20°C followed by chromatography on alumina (23). The purity of these lipids was assessed by deacylation with methylamine (24) followed by anion-exchange HPLC analysis of the [^{3}H]-glycerophosphorylinositol phosphates formed (25). All of the [^{3}H]-labeled lipids used in this study had a radiochemical purity of greater than 95% when analyzed in this way. Unlabeled PtdIns(4,5)P_2 and PtdIns4P were purified from lipid extracts of bovine brain (Folch fraction I, Sigma Chemical Co., St. Louis, MO) by large scale neomycin affinity chromatography using the elution protocol described above for the preparation of radioactive polyphosphoinositides. A 50 ml column of the neomycin-linked glass beads was used to process 2 g of the Folch fraction. The purity of these lipids was assessed by thin layer chromatography on potassium oxalate-impregnated silica gel-coated plastic-backed plates (PE SIL G/UV, Whatman, U.K.) as described (26). Phospholipids were visualized by iodine staining. PtdIns(4,5)P_2 prepared by the above method was pure by this analysis; our preparations of PtdIns4P typically contained small amounts of another phospholipid, probably phosphatidylserine. For some experiments, the PtdIns4P used was generously provided by Dr R.F. Irvine, AFRC Institute of Animal Physiology, Babraham, England. The mass of the purified lipids was quantitated by phosphate determination after wet digestion in 70% perchloric acid (27).

PLC assay. Mixed phospholipid vesicles of phosphatidylserine :phosphatidylethanolamine: [^{3}H]-PtdIns(4,5)P_2 in a molar ratio of 1:1:1 were prepared by drying lipids from $CHCl_3$ under a stream of N_2 and resuspending them in 10 mM HEPES, pH 7.4, followed by sonication with a probe type sonicator (Virsonic 50, Virtis Instrument Co, Gardiner NY) set at 0.75 x full power for 1 minute during which time the sample was kept on ice. The final PLC assay mixture contained 120 mM KCl, 10 mM NaCl, 2 mM EGTA, 10 mM HEPES, pH 7.4, 4 mM $MgSO_4$ and added $CaCl_2$ to give the required free Ca^{++} concentrations. Under the standard assay conditions used for determination of PLC activity during purification, incubations contained 0.2% Na cholate (purified as described in ref 28), the [Ca^{++}] was 0.1 mM and the concentration of PtdIns(4,5)P_2 was 0.1 mM in a total assay volume of 0.1 ml. Incubations were at 30°C and were terminated by the addition of 0.375 ml of $CHCl_3$:MeOH:HCl 20:40:1 followed by 0.125 ml of $CHCl_3$ and 0.125 ml of 0.1 M HCl. Radioactivity released into the upper aqueous phase was quantitated by liquid scintillation spectrometry.

Protein assay. Protein was determined by the method of Bradford (29) using bovine serum albumin as standard. In some cases, protein concentrations were calculated by measuring absorbance at 280 nm.

Hemoglobin assay. Hemoglobin was determined colorimetrically (absorbance at 540 nm) after conversion to cyanomethemoglobin. Turkey hemoglobin (Sigma Chemical Co., St. Louis, MO.) was used as standard.

Collection of turkey blood. Whole blood was collected directly from freshly killed turkeys into anticoagulant containing 95 mM EDTA, 5 mM EGTA and 5 mM DTPA, pH 7.4 (100 ml anticoagulant per 2 l blood). The blood was filtered through cheese cloth and kept on ice during transit. The volume of blood collected varied between 20 and 40 l. The purification scheme outlined below used 20 l of whole blood.

Preparation of turkey erythrocyte cytosolic fraction. All operations were performed at 4°C and all buffers used contained 0.1 mM benzamidine and 0.1 mM PMSF. Erythrocytes were sedimented from whole blood by centrifugation (Beckman J-6 centrifuge, 525 x g_{av}, brake setting 5, using 1000 ml bottles). The supernatant and buffy coat were removed by aspiration. The cells were washed twice by resuspension in approximately three volumes of 2.5 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA followed by further centrifugation and removal of the supernatant.

The cells were disrupted by nitrogen cavitation using a modified version of a previously characterized method for small scale subcellular fractionation of turkey erythrocytes (30). One liter of the washed packed erythrocytes was placed in the pressure chamber of a Parr cell disruption bomb (model 5309, Parr Instrument Co, Moline Il). 1 M $MgCl_2$ was added to a final concentration of 10 mM and the bomb pressurized to 1300 psi with N_2 gas. The packed cells were stirred during this process. After 30 min of equilibration, the contents of the bomb were discharged into 1 l of 20 mM Tris, pH 7.4, 5 mM EGTA, 5 mM $MgCl_2$. The pressure within the bomb was maintained during discharge. The resultant cell lysate was centrifuged (3250 x g_{av}) for 10 min, (Beckman J-6 centrifuge, brake setting 10), and the pellet, which contained predominantly nuclei, was discarded. The supernatant was collected and centrifuged at 36,000 x g_{av} for 20 min (Beckman J2-21 centrifuge, JA-14 rotor, brake setting 5). The supernatant from this step constituted the cytosolic fraction. Starting with 20 l of whole blood, the volume of cytosol obtained was approximately 10 l.

Purification of PLC.

PLC has been purified through the first six steps of the following scheme on nine separate occasions with similar results each time. The volumes, fraction numbers and values in table 1 are from a typical preparation.

Step 1: Ammonium sulphate precipitation. For efficient and reproducible precipitation of PLC activity, it was necessary to dilute the hemoglobin content of the cytosolic fraction to approximately 100 mg/ml. Cytosol was therefore added quickly and with constant stirring to an equal volume of lysis buffer containing dissolved $(NH_4)_2SO_4$ at twice its final concentration of 226 g/l. Precipitation was allowed to proceed for 10 min and the resultant solution was centrifuged at 36,000 x g_{av} for 10 min. The supernatant was removed and the precipitated protein washed from the tubes with 20 mM Tris, pH 7.4, 5 mM EDTA containing 226 g/l $(NH_4)_2SO_4$. The precipitated protein was homogenized in 6 l of this buffer using a plexiglass homogenizer (31), sedimented by repeated centrifugation (36,000 x g_{av} for 10 min), and the supernatant discarded. The washed, precipitated protein was resuspended by homogenization in 9 l of 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM benzamidine, and 0.1 mM PMSF (Buffer A). The resultant solution was clarified by centrifugation (36,000 x g_{av} for 30 min) and the supernatant obtained after this step diluted with buffer A to yield a final conductivity equivalent to 100 mM NaCl (final volume 24 l).

Step 2: Anion-exchange chromatography on Q-Sepharose. The resuspended protein solution was applied to 600 ml of Q-Sepharose in a 3 l glass funnel with a 15 cm diameter coarse porosity sintered base at a flow rate of 6 l/hr. The resin was then poured into a 5 cm diameter column and packed to a final bed height of 32 cm with 1 l of buffer A. Protein was eluted from the column with a 2 l linear gradient of 0-1 M NaCl at a flow rate of 5 ml/min. The eluant was collected in 12 ml fractions. Fractions (99-111) containing PLC activity from the Q-Sepharose column were pooled (146 ml) and desalted by gel filtration chromatography on a 570 ml (5 x 29 cm) column of Sephadex G-25 (flow rate 7 ml/min) equilibrated with buffer A containing 0.1 mM EDTA.

Step 3: Hydroxylapatite chromatography. The desalted pool of fractions containing PLC activity from the Q-Sepharose column (total volume 230 ml) was applied to a column of hydroxylapatite (2.6 x 34.5 cm) at a flow rate of 4 ml/min. The column was washed with 100 ml of buffer A containing 0.1 mM EDTA and eluted with a 1 l linear gradient of 200-600 mM KH_2PO_4. The eluant was collected in 7 ml fractions.

Fractions (55-64) containing PLC activity from the hydroxylapatite column were pooled and exchanged into buffer A containing 100 mM NaCl by gel filtration chromatography on Sephadex G-25 as described above.

Step 4: Heparin-Sepharose chromatography. The desalted pooled fraction containing PLC activity from the hydroxylapatite column (190 ml) was applied to a (1 x 18 cm) column of Heparin-Sepharose 4B at a flow rate of 0.4 ml/min. The column was washed with 20 ml of buffer A containing 100 mM NaCl and eluted with a 120 ml linear gradient of 100-1000 mM NaCl. The eluant was collected in 1.2 ml fractions.

Step 5: Gel filtration chromatography on Sephacryl S-300 HR. Fractions (63-72) containing PLC activity from the Heparin-Sepharose column were pooled (11 ml) and applied to a column of Sephacryl-S300 HR (2.6 x 91 cm). The column was eluted with buffer A containing 100 mM NaCl at 2 ml/min. The eluant was collected in 5 ml fractions.
Step 6: Mono-Q anion-exchange chromatography. Fractions (51-57) containing PLC activity from the Sephacryl S-300 column were pooled (31 ml) and applied to a Mono-Q HR 10/10 anion-exchange column at a flow rate of 0.5 ml/min. The column was washed with 5 ml of buffer A containing 100 mM NaCl and eluted with a 20 ml linear gradient of 100-600 mM NaCl followed by 5 ml of 1 M NaCl. The eluant was collected in 0.3 ml fractions.
Step 7: Second gel filtration chromatography on Sephacryl S-300 HR. Fractions (40-44) containing PLC activity from the Mono-Q column were pooled (1.3 ml) and applied to a second column of Sephacryl S-300 HR (1.6 x 64 cm). The column was eluted with buffer A containing 100 mM NaCl at a flow rate of 0.5 ml/min. The eluant was collected in 1 ml fractions.
Step 8: Second anion-exchange chromatography on Mono-Q. Fractions (72-85) containing PLC activity from the second gel filtration column were pooled (10.5 ml) and applied to a Mono-Q HR 10/10 analytical anion-exchange column at a flow rate of 0.5 ml/min. The column was washed with 5 ml of buffer A containing 100 mM NaCl and eluted with a 20 ml linear gradient of 100-400 mM NaCl followed by 5 ml of 1000 mM NaCl. The eluant was collected in 0.3 ml fractions.
We employed several different strategies to remove contaminating proteins from our final preparations of the PLC. The most routinely used method involved repeated sequential steps of gel filtration chromatography on Sephacryl S-300 and anion-exchange chromatography using a Mono-Q FPLC column as described above. In certain cases, the first anion-exchange FPLC step was performed using a prepacked column of Hydropore AX resin (100 x 4.6 mm, Ranin Instrument Co, Woburn, MA) and the second anion-exchange step was replaced by chromatography on a BioRad BioGel HPHT hydroxylapatite HPLC column (100 x 7.8 mm, BioRad Labs, Richmond, CA) eluted with a gradient of KH_2PO_4.
PLC-β and PLC-γ: PLC-β and PLC-γ were generously provided by Dr Sue Goo Rhee, National Heart, Lung and Blood Institute, National Institutes of Health Bethesda, Maryland. The PLC-β used in the experiments was purified from bovine brain while the PLC-γ was from overexpressed cells (6). Both preparations of PLC were stored frozen until required.
SDS polyacrylamide gel electrophoresis. Protein preparations were analyzed on 8.5% SDS polyacrylamide gels by the method of Laemmli (32). Proteins were detected by silver staining.

RESULTS.

Subcellular distribution of PLC in turkey erythrocytes. Turkey erythrocytes were disrupted by N_2 cavitation. Cytosolic, nuclear and plasma membrane-containing fractions were prepared by differential centrifugation of the N_2 cavitated cell suspension (30). Eighty three percent of the cellular PLC activity was associated with the cytosolic fraction. Although the plasma membrane fraction obtained after the first centrifugation step contained 8% of cellular PLC activity, this value had diminished to 1% after three washes in lysis buffer. Plasma membranes prepared in this manner do, however, show a parallel enrichment in β-adrenergic and P_{2Y}-purinergic receptors (10). Based on the abundance of enzyme activity, we elected to purify PLC from the cytosolic fraction.
Purification of PLC. The very large amounts of hemoglobin present in the cytosol of turkey erythrocytes placed some constraints on the methodologies available for use at the early stages of the purification. However, PLC activity could be selectively precipitated from the cytosol with a concentration of 226 g/l $(NH_4)_2SO_4$ leaving the hemoglobin in solution provided that the initial concentration of hemoglobin was kept below 100 g/l. Above this concentration, the high viscosity of the solution apparently made sedimentation of precipitated proteins inefficient. An alternative strategy of isoelectric precipitation by reduction of the pH of the cytosol with acetic acid was not effective for resolving PLC activity from the hemoglobin. Recoveries through this first step were never greater than 70 % (ranging from 53 to 68% for 5 separate purifications). Because of the large volumes in the initial steps, it was not possible to include a comprehensive battery of antiproteolytic agents in our solutions. We noted that PLC activity in the cytosol and resuspended $(NH_4)_2SO_4$ precipitate rapidly declined during storage at 4°C. For these reasons we cannot exclude the possibility that turkey erythrocytes contain multiple species of PLC and that our purification scheme is selective for one of these proteins. A direct chromatographic analysis of the PLC activity present in unfractionated turkey erythrocyte cytosol has not been attempted.

When the resuspended $(NH_4)_2SO_4$ precipitated proteins were applied to Q-Sepharose, PLC activity bound to the resin and could be eluted with a gradient of NaCl (fig 1). In certain purifications we noted lesser amounts of PLC activity (2-5% of the total) which eluted at a lower concentration of NaCl than the major peak of PLC activity that was pooled for further purification. This minor PLC activity has not been investigated further.

PLC behaved as a single activity during all of the subsequent chromatographic steps. These comprised chromatography on hydroxylapatite, heparin-sepharose and repeated sequential chromatography on Sephacryl S-300 and anion-exchange FPLC using a pre-packed Mono-Q column (figs 2,3,4 and 5). Table 1 summarizes the results obtained from a typical purification. Starting with 20 l of turkey blood we obtained approximately 0.4 mg of purified PLC. The purified protein had a specific activity of 7-14 umol/min/mg when assayed with PtdIns(4,5)P_2 under standard conditions. The degree of purification achieved was consistently around 50,000-fold although the large quantity of hemoglobin present in the starting preparation makes this value somewhat misleading. The purified enzyme was most stable when stored at 4°C with no significant loss in activity observed over 4 weeks.
SDS-PAGE analysis of the purified PLC. Fractions (50 - 60) from the second step of anion-exchange chromatography were subjected to SDS-PAGE and proteins detected by silver staining (Figure 6). The purified preparation contained a major polypeptide of approximately 150 kDa. The chromatographic behavior of PLC activity was identical to that of the 150 kDa protein over the Mono Q column step (Figure 6) as well as over the heparin-sepharose and gel filtration steps in each purification (data not shown). The maximal specific activities of the final PLC preparation determined against both polyphosphoinositides (see below) are similar to those values obtained by other workers for purified PLC isoenzymes (9). However, in addition to the 150 kDa protein our final preparation of the PLC does contain considerably lesser quantities of other proteins. It is possible that, as has been reported for PLC-β (6), certain of these represent fragments derived by proteolysis of the 150 kDa turkey erythrocyte PLC.

Properties of the purified PLC.

Substrate concentration-dependence of PLC activity: The purified PLC was incubated with increasing concentrations of PtdIns(4,5)P_2 or of PtdIns4P under standard assay conditions and the initial rate of formation of Ins(1,4,5)P_3 or of Ins(1,4)P_2 determined (figs 7a,b). The apparent Km for PtdIns(4,5)P_2 was 5.7 ± 0.33 μM while that for PtdIns4P was 8.0 ± 2.1 μM. Similar Vmax values were obtained for PtdIns(4,5)P_2 (8.9 ± 0.18 mmoles/min/mg) and PtdIns4P (10.7 ± 1.2 mmoles/min/mg). All of the above values are means ± sem of at least three separate determinations made using two independent preparations of the PLC.
Effect of pH on PLC activity: The purified PLC was incubated with PtdIns(4,5)P_2 or PtdIns4P under standard assay conditions. The incubations contained no sodium cholate and the Ca^{++} concentration was 0.1 mM. PLC activity was determined between pH 3.5 and pH 9.0 using a mixed buffer system of Tris, Hepes, imidazole and acetate. PLC activity against both substrates displayed a broad pH dependence with a pH optimum at around 4.0 (fig 8).
Structure of the products formed by the PLC: PLC was incubated with PtdIns(4,5)P_2 or PtdIns4P under the conditions used to investigate pH dependence of enzyme activity. Incubations were conducted at pH 4.0 or pH 7.0 and terminated as described in Experimental Procedures except that the solvents used contained water instead of HCl. The water soluble products formed were analyzed by anion-exchange HPLC using a Whatman Partisil 10-SAX column and gradient elution (25). Radioactivity in the eluant was monitored by in-line liquid scintillation spectrometry. At neutral pH, the major products were Ins(1,4,5)P_3 and Ins(1,4)P_2 formed from PtdIns(4,5)P_2 and PtdIns4P, respectively, with Ins(1;2cyclic,4,5)P_3 and Ins(1;2cyclic,4)P_2 comprising only 1 and 2% of the total products in each case. By contrast, at pH 4.0 32 and 53% Ins(1;2cyclic,4,5)P_3 and Ins(1;2cyclic,4)P_2 were formed from their respective precursors (table 2). The structures of the cyclic and non cyclic inositol trisphosphates were confirmed by examining the polyols formed from these compounds following periodate oxidation, reduction, and dephosphorylation before and after subjection to acidic hydrolysis of the phosphodiester bond (data not shown, see 34 for details of the methods used).

Table 2. Products formed by the PLC at pH 4.0 and pH 7.5

		pH of incubation	
		4.0	7.5
Substrate	Product	cpm (% total products)	
PtdIns4P	Ins(1,4)P_2	27107 (47%)	10319 (99%)
	Ins1;2cyclic,4P_2	30143 (53%)	134 (1%)
PtdIns(4,5)P_2	Ins(1,4,5)P_3	11608 (68%)	3250 (98%)
	Ins(1;2cyclic,4,5)P_3	5365 (32%)	106 (2%)

PLC was incubated with either PtdIns4P or with PtdIns(4,5)P_2, the incubations were terminated under neutral conditions, and the inositol phosphates formed analyzed by anion exchange HPLC as described in the text. The incubations contained 111357 cpm of [^{3}H]-PtdIns4P and 41170 cpm of [^{3}H]-PtdIns(4,5)P_2. The data shown are from a single experiment.

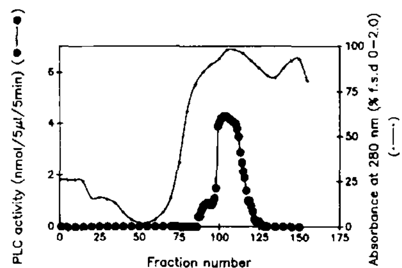

Figure 1. Purification of PLC by chromatography on Q-Sepharose. Proteins were precipitated from 20 l of turkey erythrocyte cytosol with 226 g/l $(NH_4)_2SO_4$ and resuspended in running buffer. The resultant solution was applied to 600 ml of Q-Sepharose resin, the resin was packed into a column which was washed and eluted with a gradient of NaCl. Fractions of the eluant were collected and assayed for PLC activity (-●-) as described in the Experimental Procedures. The elution of protein determined by absorbance at 280 nm is also illustrated (-○-). PLC activity was eluted from the column at a conductivity equivalent to 272 mM NaCl.

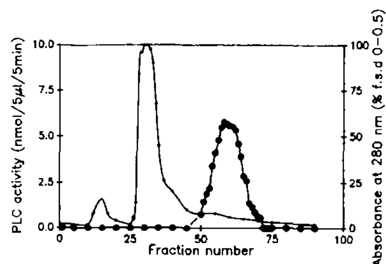

Figure 2. Purification of PLC by chromatography on Hydroxylapatite. Fractions containing PLC activity from the Q-Sepharose column were pooled, desalted and applied to a column of hydroxylapatite. The column was washed, eluted with a gradient of KH_2PO_4 and fractions of the eluant collected and assayed for PLC activity (-●-) as described in the Experimental Procedures. The elution of protein determined by absorbance at 280 nm is also illustrated (-○-). PLC activity was eluted from the column at a conductivity equivalent to 335 mM KH_2PO_4.

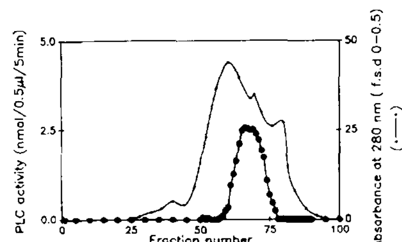

Figure 3. Purification of PLC by chromatography on Heparin-Sepharose. Fractions containing PLC activity from the hydroxylapatite column were pooled, desalted and applied to a column of heparin-Sepharose. The column was washed, eluted and fractions collected and assayed for PLC activity (-●-) as described in Experimental Procedures. The elution of protein determined by absorbance at 280 nm is also illustrated (-○-). PLC activity was eluted from the column at a conductivity equivalent to 454 mM NaCl.

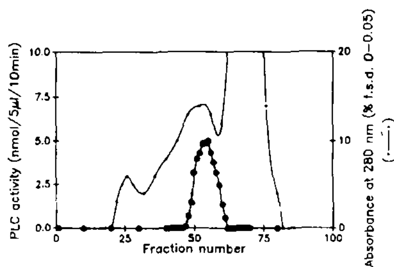

Figure 4. Purification of PLC by chromatography on Sephacryl S-300 HR.
Fractions containing PLC activity from the heparin-Sepharose column were pooled, applied to a column of Sephacryl S-300 HR, the column was eluted and fractions collected and assayed for PLC activity (-●-) as described in Experimental Procedures. The elution of protein determined by absorbance at 280 nm is also illustrated (-○-).

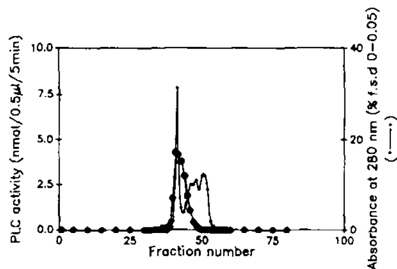

Figure 5. Purification of PLC by chromatography on Mono-Q.
Fractions containing PLC activity from the Sephacryl S-300 HR column were pooled and applied to a Mono-Q HR 10/10 column. The column was washed, eluted, and fractions collected and assayed for PLC activity (-●-) as described in the Experimental Procedures. The elution of protein determined by absorbance at 280 nm is also illustrated (-○-). PLC activity eluted from the column at a conductivity equivalent to 270 mM NaCl.

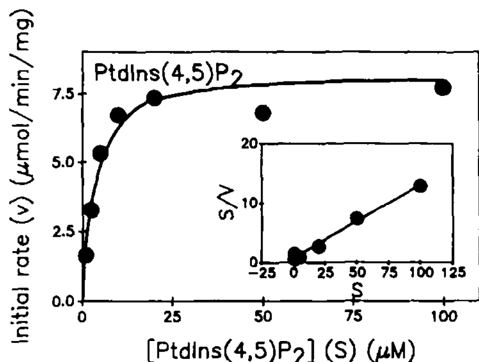

Figure 7 (a). Determination of the apparent Km of the purified PLC for PtdIns(4,5)P_2.
Purified PLC was incubated with PtdIns(4,5)P_2 as described in the text and the initial rate of formation of Ins(1,4,5)P_3 determined. The inset is a plot of substrate concentration divided by initial rate plotted against substrate concentration.

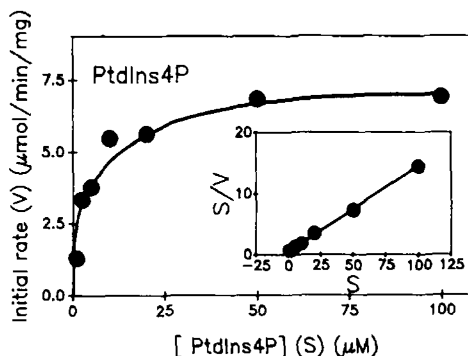

Figure 7 (b). Determination of the apparent Km of the purified PLC for PtdIns4P.
Purified PLC was incubated with (a) PtdIns4P or (b) PtdIns(4,5)P_2 as described in the text and the initial rate of formation of Ins(1,4)P_2 determined. The inset is a plot of substrate concentration divided by initial rate plotted against substrate concentration.

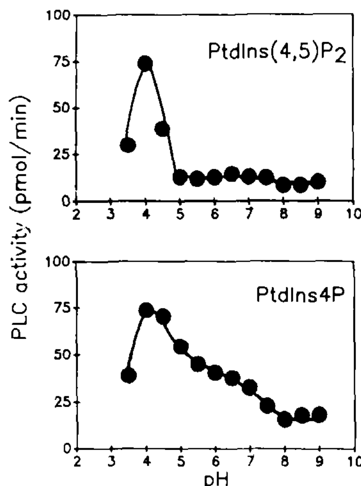

Figure 8. pH dependence of PLC activity.
PLC activity against either PtdIns4P (top panel) or PtdIns(4,5)P_2 (bottom panel) was determined as the pH of the incubations was varied under the conditions described in Experimental Procedures. The assays contained an equal amount of the purified PLC.