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Receptor- and G-Protein-Regulated 150-kDa Avian Phospholipase C: Inhibition of Enzyme Activity by Isoenzyme-Specific Antisera and Nonidentity with Mammalian Phospholipase C Isoenzymes Established by Immunoreactivity and Peptide Sequence

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SUMMARY

A 150-kDa phospholipase C previously was purified from turkey erythrocytes and shown to be a P_{2Y}-purinergic receptor- and guanine nucleotide-binding protein-regulated enzyme [*J. Biol. Chem.* **265**:13508–13514 (1990)]. The relationship of this enzyme to the 150-kDa mammalian phospholipase C isoenzymes, termed phospholipase C-β and -γ, has been examined. Four antisera to the turkey erythrocyte phospholipase C recognized the avian enzyme in immunoblots but failed to recognize phospholipase C-γ; one of the these weakly recognized phospholipase C-β. Antibodies to phospholipase C-β and -γ failed to recognize the turkey erythrocyte phospholipase C. However, two antibodies raised against peptide sequence in regions of conserved sequence common to mammalian phospholipase C isoenzymes recognized the 150-kDa turkey erythrocyte phospholipase C. Antisera against the native form of the turkey erythrocyte phospholipase C inhibited the activity of this enzyme against phosphatidylinositol 4,5-bisphosphate presented as a component of mixed phospholipid vesicles or of mixed phospholipid and sodium cholate micelles; inhibition occurred as a decrease in V_{max}, with no apparent change in K_m for substrate or in the Ca^{2+} dependence of phospholipase C activity. Catalytic activity of phospholipase C-β or -γ against exogenous substrate was unaffected by antisera to the turkey erythrocyte enzyme. Antisera against the native form of the turkey erythrocyte phospholipase C also partially inhibited (50–60% inhibition) the capacity of AlF_4^- or adenosine 5′-*O*-(β-thio) diphosphate plus guanosine 5′-*O*-(γ-thio) triphosphate to stimulate phosphoinositide hydrolysis in ghosts prepared from [^{3}H]inositol-prelabeled turkey erythrocytes. Moreover, the capacity of the purified 150-kDa enzyme to reconstitute receptor and G-protein-regulated phospholipase C activity in purified turkey erythrocyte plasma membranes devoid of this activity was completely inhibited by antisera to the turkey erythrocyte enzyme. Five peptides that were purified by high performance liquid chromatography from a tryptic digest of the turkey erythrocyte 150-kDa phospholipase C had no recognizable sequence homology with any deduced sequence of the mammalian phospholipase C isoenzymes. One turkey erythrocyte phospholipase C-derived peptide had clear homology with sequence in the first (X-domain) conserved region common to at least three of the mammalian phospholipase C isoenzymes, and another 16-amino acid peptide had partial sequence homology with the second (Y-domain) conserved region common to the mammalian enzymes. An 8-amino acid peptide from the tryptic digest had 75% homology with a sequence near the carboxyl terminus of mammalian phospholipase C-β. Thus, the 150-kDa turkey erythrocyte phospholipase C contains homology with two stretches common to mammalian phospholipase C isoenzymes, but it diverges in sequence elsewhere. Homology with sequence in phospholipase C-β outside the conserved X- and Y-domains suggests some similarity between the turkey 150-kDa phospholipase C and the phospholipase C-β family of isoenzymes.

Polyphosphoinositide-specific phospholipase C-catalyzed production of second messengers is obligatory in the signaling response to a broad range of hormones, neurotransmitters, growth factors, chemoattractants, and other stimuli (1). At least four classes (phospholipase C-α, -β, -γ, and -δ) of phospholipase C isoenzymes exist, and multiple forms of phospholipase C may occur in each class (2).

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ABBREVIATIONS: G protein, guanine nucleotide-binding regulatory protein; BSA, bovine serum albumin; PtdIns, phosphatidylinositol; ADPβS, adenosine 5′-*O*-(β-thio)diphosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-*O*-(γ-thio)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; HPLC, high performance liquid chromatography; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns4P, phosphatidylinositol 4-phosphate.

Turkey erythrocyte membranes have proven to be a useful model in which to study the kinetics of receptor- and G protein-regulated phospholipase C and to begin to identify and purify the protein cohorts in the inositol lipid signaling cascade (3–6). A 150-kDa phospholipase C recently has been purified to homogeneity (7). By reconstitution of the purified enzyme into membranes essentially devoid of phospholipase C activity, this protein has been shown to express kinetic and regulatory properties indistinguishable from those of the native receptor- and G protein-regulated enzyme (8). As such, we have concluded tentatively that the purified 150-kDa enzyme is the protein that is under P_{2Y}-purinergic receptor and G protein regulation in the turkey erythrocyte. Although this protein is similar in apparent size to the mammalian phospholipase C isoenzymes termed phospholipase C-β and phospholipase C-γ (2, 9), its relationship to these and other phospholipase C isoenzymes is unknown. In this study, we report internal amino acid sequence and reactivity with phospholipase C-specific antisera in a comparison of the turkey erythrocyte phospholipase C and mammalian phospholipase C isoenzymes.

Experimental Procedures

Purification and assay of phospholipase C. Phospholipase C was purified from turkey erythrocytes as we have previously reported (7). Enzyme activity was quantitated as previously described in detail (7), with substrate presented as mixed phospholipid vesicles of phosphatidylserine/phosphatidylethanolamine/[^{3}H]PtdIns(4,5)P_2 in a molar ratio of 1:1:1. The concentration of PtdIns(4,5)P_2 was 100 μM under standard assay conditions. The final assay mixture contained 120 mM KCl, 10 mM NaCl, 2 mM EGTA, 10 mM HEPES, pH 7.4, 4 mM $MgSO_4$, and $CaCl_2$ to give a range of free Ca^{2+}. Under standard assay conditions, the Ca^{2+} concentration was 1 μM. In some experiments, assays were in the presence of 0.2% cholate. In most experiments examining the effects of phospholipase C antibodies, purified turkey erythrocyte 150-kDa phospholipase C was incubated with antisera for 1 hr on ice in the presence of 1% BSA in 10 mM HEPES, pH 7.4, before addition of substrate and assay of polyphosphoinositide hydrolysis.

Preparation of turkey erythrocyte ghosts and purified plasma membranes. Turkey erythrocyte ghosts were prepared from [^{3}H]inositol-labeled cells (3), and stimulated hydrolysis of membrane [^{3}H]phosphoinositides was measured as we have discussed in detail (4). Purified plasma membranes were prepared from [^{3}H]inositol-labeled erythrocytes as follows. Fresh turkey erythrocytes were washed (3), and 1 ml of packed cells was incubated overnight with 2 mCi of [^{3}H]inositol in Dulbecco's modified Eagle's medium, in an atmosphere of 8% CO_2/92% air at 37°. Forty milliliters of 154 mM NaCl containing 100 μM PMSF and 100 μM benzamidine were added to the labeled cells, which were centrifuged at 2000 × *g* for 10 min and resuspended in 10 ml of 154 mM NaCl/PMSF/benzamidine. The cells were disrupted by incubation for 30 min in an atmosphere of N_2 (1100 psi), using a Parr cell disruption bomb (no. 4639; Parr Instruments, Moline IL), followed by rapid discharge into a vessel containing 10 ml of 5 mM EGTA, 5 mM $MgCl_2$, 20 mM Tris, pH 7.4 (see Ref. 7). The lysate was centrifuged for 10 min at 4000 × *g*, and the supernatant was collected. The pellet was resuspended in 20 ml of the Mg^{2+}-containing buffer indicated above, and the 4000 × *g* centrifugation step was repeated. The 4000 × *g* supernatants were pooled (total volume, 40 ml) and centrifuged at 20,000 × *g* for 10 min at 4°. The resultant pellet was resuspended in 1 ml of 20 mM Tris, pH 7.4, 5 mM EDTA, and centrifuged at full speed in a tabletop Eppendorf microcentrifuge at 4° for 10 min. The white plasma membrane fragments sedimented by this procedure were resuspended in 1 ml of 20 mM Tris, pH 7.4, 5 mM EDTA, and stored on ice until required. Immediately before their use, the membranes were sedimented again by centrifugation in the Eppendorf microfuge, at top speed, and were resuspended in 10 mM HEPES, pH 7.4.

Production of polyclonal antibodies. Preimmune serum was prepared from blood collected from the marginal ear vein of four female New Zealand white rabbits. Two rabbits, D246 and D807, were subsequently immunized with approximately 100 μg of purified 150-kDa protein prepared as follows. Purified turkey erythrocyte phospholipase C (7) was subjected to SDS-PAGE on a preparative slab gel of 8.5% acrylamide. The material migrating with an apparent size of 150 kDa was excised from the wet gel, briefly washed in deionized water, and dispersed in a small volume of deionized water by repeated passage through a 21-gauge needle. Each rabbit received ten 250-μl subcutaneous injections of the homogenized gel solution along the back. Two other rabbits, N465 and N858, were immunized with approximately 100 μg of native phospholipase C purified from turkey erythrocytes as described (7). The purified enzyme was dialyzed into phosphate-buffered saline, and a water-in-oil emulsion was prepared consisting of 1 part complete Freund's adjuvant and 1 part purified enzyme. Each rabbit received six 250-μl subcutaneous injections along the back. Booster injections of 20–50 μg of enzyme prepared from SDS-PAGE gel fragments or in emulsions of 1 part incomplete Freund's adjuvant to 1 part enzyme were administered at 6-week intervals. Blood was collected from the marginal ear vein 12–14 days after the booster injection. Serum was prepared by allowing the blood to clot for 30 min at 37°, followed by overnight incubation at 4°. The insoluble material was removed by a 10-min centrifugation at 10,000 × *g*. The supernatant was collected and stored frozen at −70°.

Polyclonal and monoclonal antibodies against phospholipase C-β and phospholipase C-γ and these two isoenzymes purified from bovine brain were generous gifts from Dr. Sue Goo Rhee, National Heart, Lung, and Blood Institute (Bethesda, MD). Antipeptide antibodies generated against the phospholipase C sequence Gly-Cys-Arg-Cys-Ile-Glu-Leu-Asp-Cys-Trp in the so-called X-domain and against the sequence Leu-Ser-Arg-Ile-Tyr-Pro-Lys-Gly in the so-called Y-domain also were provided generously by Dr. Rhee.

Immunoprecipitation. Immunoprecipitation of turkey erythrocyte phospholipase C was initiated by addition of 5 μl of antiserum to either 1 ml of turkey erythrocyte cytosol (prepared by N_2 cavitation followed by centrifugation at 20,000 × *g* for 10 min at 4°) or 1 ml of 1% BSA containing 100 ng of purified enzyme, followed by incubation for 1 hr at 4°. The immune complexes were collected by addition of 100 μl of a 10% (v/v) suspension of Protein A-Sepharose and incubation for 1 hr at 4° on an orbital rocker. The beads were collected by a 30-sec centrifugation at 10,000 × *g*. The pellet was washed three times by centrifugation in 50 mM Tris, pH 8.5, 1% Nonidet P-40, 1 mM EDTA, 150 mM NaCl. The final pellet was resuspended in 50 μl of Laemmli (10) sample buffer, heated to 85° for 10 min, and centrifuged, and the supernatant was subjected to SDS-PAGE on 8.5% acrylamide gels.

SDS-PAGE and immunoblots. SDS-PAGE on 8.5% gels was according to the method of Laemmli (10). Immunoblots were generated after electrophoretic transfer, to nitrocellulose sheets, of the proteins separated by SDS-PAGE. The transblots were blocked with 3% BSA, incubated with the indicated dilution of primary antiserum for 2 hr, washed three times with 0.05% Tween-20, and incubated with an alkaline phosphatase-conjugated secondary antibody for 1 hr. The immunoblots were washed three times with 0.05% Tween-20 and developed with a solution containing 3 mg/ml *p*-nitro-blue tetrazolium, 1.5 mg/ml 5-bromo-4-chloro-3-indoyl phosphate, 1 mM $MgCl_2$, and 0.1 M $NaHCO_3$, pH 9.8.

Peptide purification and amino acid sequence analysis. Phospholipase C was purified from turkey erythrocytes as previously described in detail (7). The purified enzyme (approximately 100 μg) was desalted, concentrated, and denatured by treatment with 6 M guanidine HCl. The protein then was reduced in the presence of 0.1 M dithioerythreitol and alkylated by incubation with 0.1 M *N*-[^{3}H]ethylmaleimide. Digestion was accomplished with 10 μg of trypsin. The resultant peptides were resolved by reverse phase HPLC, and purified peptides were sequenced using an Applied Biosystems 470A protein microsequencer. The sequences of tryptic peptides derived from the purified 150-kDa turkey erythrocyte phospholipase C were compared with the

deduced amino acid sequences of phospholipase C-β, -γ, and -δ, and the putative *Drosophila* norpA gene-encoded phospholipase C, using the FASTA variation of the algorithm originally developed by Lipman and Pearson (11).

Materials. 5-Bromo-4-chloro-3-indoyl phosphate and *p*-nitro-blue tetrazolium were obtained from Bio-Rad. Alkaline phosphatase-conjugated goat anti-rabbit IgG and goat anti-mouse IgG were purchased from Boehringer-Mannheim. Complete and incomplete Freund's adjuvant, BSA, and Nonidet P-40 were obtained from Sigma Chemical Company, and Protein A-Sepharose CL-4B was purchased from Pharmacia. New Zealand rabbits were obtained from Hazelton. [^{3}H] PtdIns(4,5)P_2, [^{3}H]PtdIns4P, unlabeled PtdIns(4,5)P_2, and PtdIns4P were prepared as previously described in detail (7). All other reagents were obtained from sources previously identified (4, 7).

Results

Polyclonal antibodies were raised against the 150-kDa turkey erythrocyte phospholipase C as described in Experimental Procedures. Antibodies against native enzyme (N465 and N858) and against the denatured 150-kDa enzyme (D246 and D807) excised from SDS-polyacrylamide gels recognized the purified 150-kDa protein in dot blots at serum dilutions at least as low as 1/2000 (data not shown). Not only did the panel of antibodies recognize the native form of the phospholipase C in dot blots, but all four antisera, at dilutions at least as low as 1/2000, identified a 150-kDa protein in Western blots of SDS-polyacrylamide gels that coincided in electrophoretic mobility with the silver-stained 150-kDa phospholipase C (immunoblot shown for D246 at 1/500 dilution in Fig. 1, *lane 1, extreme left*). D246 also recognized protein species of apparent size of <150 kDa with some enzyme preparations (Fig. 2). Because this antibody was raised against the 150-kDa protein excised from gels and because the incidence of immunoreactivity with proteins of <150 kDa increased with prolonged storage of the purified 150-kDa enzyme at 4°, these proteins of <150 kDa recognized by D246 probably represent degradation products of the purified enzyme.

Based on the results discussed above, the four antibody preparations recognize the native form of the 150-kDa receptor- and G protein-regulated turkey erythrocyte phospholipase C. Thus, it is not surprising that a 150-kDa protein recognized in immunoblots (generated with D246) of SDS-polyacrylamide gels could be immunoprecipitated with Protein A-sepharose after incubation of the purified turkey erythrocyte phospholipase C or turkey erythrocyte cytosol with D246, D807, N465, or N858 (data shown for immunoprecipitation with N858 in Fig. 2).

The immunoreactivity of the purified turkey erythrocyte phospholipase C was compared with that of the two approximately 150-kDa mammalian phospholipase C isoenzymes (phospholipase C-β and -γ). D246 recognized the purified turkey erythrocyte phospholipase C, as described above, but also weakly recognized phospholipase C-β purified from bovine brain (Fig. 1). The other three antisera to turkey erythrocyte phospholipase C failed to recognize phospholipase C-β. None of the antibodies to the turkey erythrocyte enzyme recognized phospholipase C-γ purified from bovine brain.

Two antibodies raised against a peptide sequence (see Experimental Procedures) in either the so-called X- or Y-domains that are common to phospholipases C-β, -γ, and -δ (2) recognized the purified turkey erythrocyte phospholipase C with a sensitivity similar to that with which they recognized the mammalian 150-kDa proteins (Fig. 1). Finally, both a polyclonal and a mixture of monoclonal antibodies to phospholipase

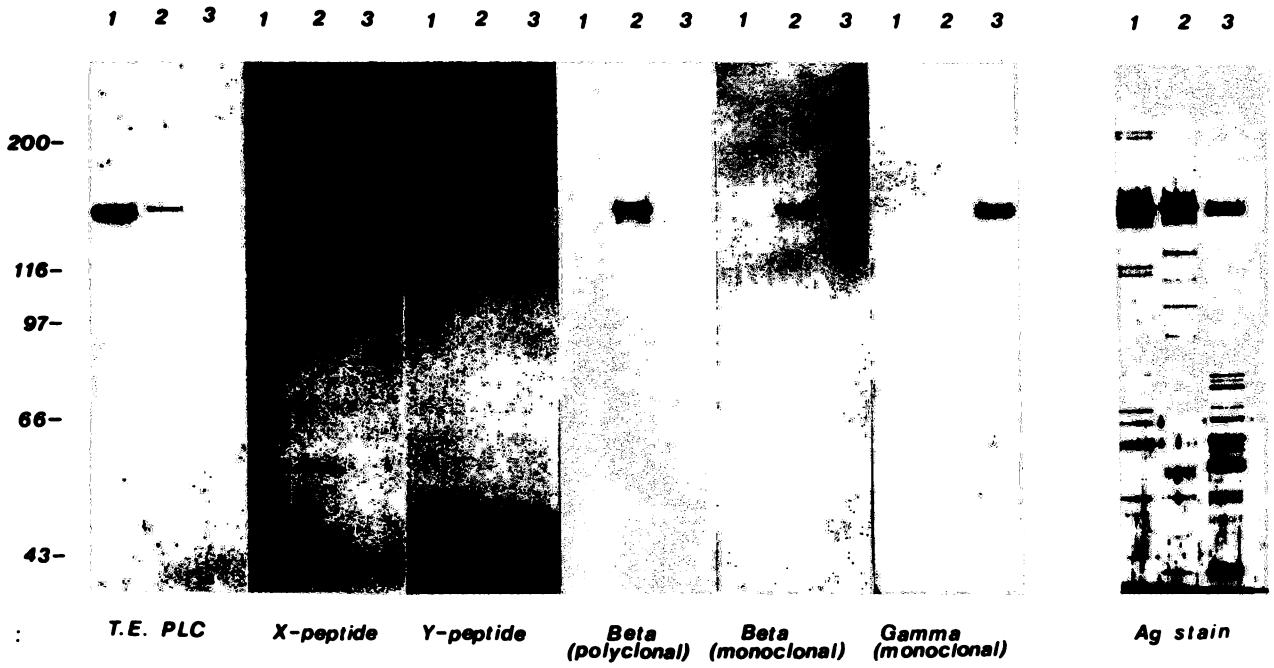

Fig. 1. Comparison of turkey erythrocyte phospholipase C with other 150-kDa phospholipase C isoenzymes by immunoblot analysis. Purified turkey erythrocyte phospholipase C (*lanes 1*), bovine brain phospholipase C-β (*lanes 2*), and bovine brain phospholipase C-γ (*lanes 3*) were subjected to SDS-PAGE and stained with silver (the three lanes on the extreme right) or transferred to nitrocellulose sheets and immunoblotted with the indicated antisera (the 18 lanes beginning on the left), as described in Experimental Procedures. The turkey erythrocyte phospholipase C (*T. E. PLC*) antiserum used was D246, at a 1/500 dilution, and the sources of the other antisera (diluted 1/500) are indicated in Experimental Procedures. The data are representative of three or more immunoblots obtained with each antiserum and with multiple preparations of turkey erythrocyte phospholipase C.

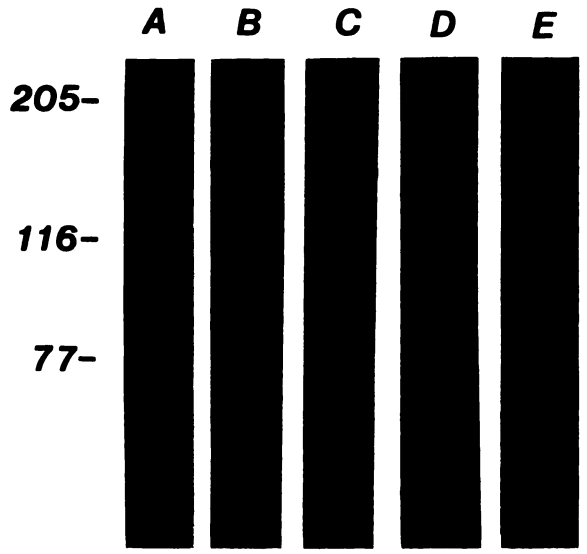

Fig. 2. Immunoprecipitation of a 150-kDa protein by antisera raised against turkey erythrocyte phospholipase C. Turkey erythrocyte phospholipase C was immunoprecipitated with antiserum N858 (*lanes C* and *E*) or preimmune serum (*lanes B* and *D*), as described in Experimental Procedures. The immunoprecipitate was subjected to SDS-PAGE on 8.5% acrylamide gels and transferred to nitrocellulose, and immunoblots with antiserum D246 (1/500) were developed as described. *Lanes B* and *C*, results of immunoprecipitation from 1 ml of a solution containing 1% BSA and 200 ng of purified turkey erythrocyte phospholipase C. *Lanes D* and *E*, results of immunoprecipitation from 0.5 ml of turkey erythrocyte cytosol. *Lane A*, purified turkey erythrocyte phospholipase C was loaded directly onto the gel without immunoprecipitation. Molecular mass standards (kDa) are indicated on the *left*.

C-β failed to recognize the purified turkey erythrocyte phospholipase C, and a mixture of monoclonal antibodies to phospholipase C-γ also failed to recognize the purified turkey erythrocyte enzyme (Fig. 1).

The capacity of antisera against turkey erythrocyte phospholipase C to modify phospholipase C-catalyzed phosphoinositide hydrolysis also was examined using previously characterized assay systems employing substrate-containing mixed phospholipid vesicles or phospholipid and cholate mixed micelles (7). Preimmune serum from the four rabbits used to generate these phospholipase C antibodies had little or no effect on phospholipase C activity at concentrations as high as a 1/10 dilution (Fig. 3; data shown for preimmune serum relevant to N465). The two antibodies (N465 and N858) generated against the native 150-kDa turkey erythrocyte protein inhibited in a concentration-dependent manner the activity of the purified turkey erythrocyte phospholipase C (Fig. 3). The concentration dependence for inhibition by N465 was the same irrespective of whether [^{3}H]PtdIns(4,5)P_2 or [^{3}H]PtdIns4P was used as substrate (data not shown). Essentially complete inhibition was obtained at 1/300 to 1/100 dilutions. In contrast, the antibodies to the denatured form of the enzyme, which, based on the dot blot and immunoprecipitation studies described above, clearly interact with the protein in its native form, either had no effect or reproducibly produced an increase in phospholipase C activity (at high concentration D246 inhibited enzyme activity).

The nature of the inhibition of phospholipase C activity by N465 and N858 was examined further. Ca^{2+} markedly increases phospholipase C activity measured against exogenous phosphoinositide substrate, although the mechanism involved and physiological relevance, if any, are unknown (2, 12). As we have previously reported (7), Ca^{2+} enhances activity of the purified

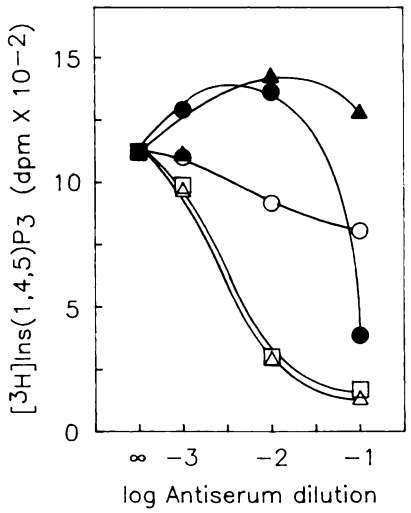

Fig. 3. Inhibition of phospholipase C activity by antisera raised against the turkey erythrocyte 150-kDa phospholipase C. Purified turkey erythrocyte phospholipase C (approximately 2 ng/assay) was incubated for 1 hr in the presence of the indicated dilutions of preimmune serum or four different antisera (●, D246; ▲, D807; △, N465; □, N858; ○, preimmune) generated against the purified enzyme. Phospholipase C activity was then measured using exogenous [^{3}H]PtdIns(4,5)P_2 in the presence of cholate, as described in Experimental Procedures. The data are the mean of triplicate determinations and are representative of results obtained in at least three experiments with each antisera.

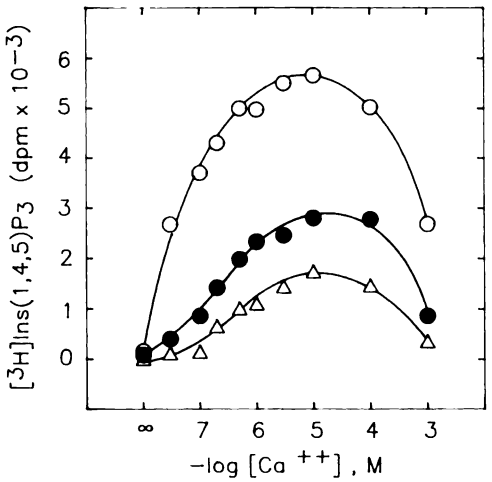

Fig. 4. Ca^{2+} dependence of phospholipase C activity in the absence or presence of phospholipase C antisera. Purified turkey erythrocyte phospholipase C (approximately 2 ng/assay) was preincubated, in the absence (○) or presence of a 1/300 (●) or 1/100 (△) dilution of turkey erythrocyte phospholipase C antiserum N465, for 1 hr. Phospholipase C activity was then measured at the indicated Ca^{2+} concentrations using exogenous [^{3}H]PtdIns(4,5)P_2 in the presence of cholate, as described in Experimental Procedures. The data are the mean of triplicate determinations and are representative of results obtained in two separate experiments.

150-kDa phospholipase C, and maximal phosphoinositide hydrolysis is observed at concentrations of Ca^{2+} of 1 μM or greater (Fig 4). N465 inhibited phospholipase C activity at all concentrations of Ca^{2+} examined, with no obvious change in the concentration dependence for modification of enzyme activity by Ca^{2+}. We have noted previously that, when incubated with increasing concentrations of its polyphosphoinositide substrates in the presence of 0.2% cholate, activity of the turkey erythrocyte phospholipase C is substrate concentration depend-

ent and saturable (7). The effect of N465 on phospholipase C activity of the purified 150-kDa protein was due to a decrease in the apparent V_{max} of [^{3}H]PtdIns(4,5)P_2 hydrolysis, with no change occurring in the apparent K_m for substrate (Fig. 5).

The data illustrated in Figs. 3, 4, and 5 were generated in a phospholipase C assay with exogenous [^{3}H]PtdIns(4,5)P_2 substrate presented as mixed phospholipid and cholate micelles. However, N465 was equally effective as an inhibitor of phospholipase C activity under conditions in which [^{3}H]PtdIns(4,5)P_2 was presented in phospholipid vesicles consisting of equimolar concentrations of [^{3}H]PtdIns(4,5)P_2, phosphatidylserine, and phosphatidylethanolamine in the absence of cholate (Table 1).

Preliminary findings suggest that, when presented with mixed phospholipid vesicles containing equimolar phosphatidylserine and phosphatidylethanolamine in combination with polyphosphoinositides, the turkey erythrocyte phospholipase C displays surface dilution kinetics[1] (see Ref. 12 and references therein). Our results support a model in which the association of phospholipase C with the substrate-containing phospholipid vesicles precedes substrate hydrolysis, with one piece of evidence for this proposal being that, under standard assay conditions, 70–80% of added phospholipase C activity co-sediments with substrate-containing phospholipid vesicles upon ultracentrifugation. Because all of the data presented to this point arose from experiments in which antibody was preincubated with the purified 150-kDa phospholipase C before addition of substrate, inhibition of enzyme activity by N465 could occur either as a consequence of a direct inhibition of enzyme activity or as a result of prevention of association of the purified enzyme in a functional configuration with substrate-containing phospholipid vesicles. The data presented in Table 1 indicate that N465 inhibited phospholipase C activity irrespective of whether it was incubated with enzyme before combination with substrate-containing vesicles or added to substrate-containing vesicles that had been preincubated at 4° with the purified enzyme. These data are consistent with the idea, but by no means prove, that N465 inhibits phospholipase C activity with a mechanism that is independent of an effect on the association of the enzyme with substrate-containing phospholipid vesicles.

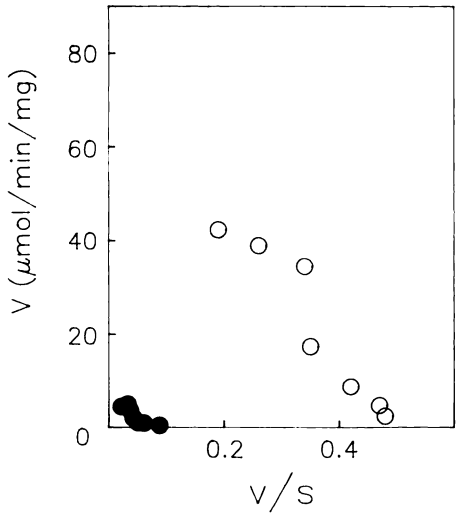

Fig. 5. Kinetic analysis of phospholipase C activity against PtdIns(4,5)P_2 substrate and effect of phospholipase C antisera. Purified turkey erythrocyte phospholipase C (approximately 1 ng/assay) was preincubated in the absence (○) or presence (●) of antiserum N465, at a dilution of 1/300, for 1 hr at 4°. Phospholipase C activity was then measured in the presence of cholate at PtdIns(4,5)P_2 concentrations ranging from 2.5 to 200 μM. Substrate was present with an equimolar concentration of phosphatidylserine and phosphatidylethanolamine at each concentration tested. The data are plotted as initial velocity versus initial velocity divided by substrate concentration and are representative of results obtained in three separate experiments. V_{max} values were 78 and 8 μmol/min/mg and K_m values were 40 and 34 μM for control and antiserum-treated conditions, respectively.

[1]A. J. Morris, unpublished observations.

TABLE 1

Influence of the order of addition of reactants on the inhibitory effects of antisera against turkey erythrocyte phospholipase C

Purified turkey erythrocyte phospholipase C (E) (approximately 2 ng/assay) was preincubated on ice with phospholipid vesicles (V) containing [^{3}H]PtdIns(4,5)P_2, for 1 hr, before addition of the indicated dilutions of antiserum N465 (Ab), and then phospholipase C activity was assayed at 30° as described in Experimental Procedures (left column). Alternatively, purified enzyme was preincubated on ice for 1 hr with the indicated dilutions of antiserum N465 before the addition of phospholipid vesicles containing [^{3}H]PtdIns substrate and then assay of phospholipase C activity for 5 min at 30° (right column). The data are presented as dpm of [^{3}H]inositol phosphates formed over background. All assays were in the absence of sodium cholate. The data are the mean of triplicate determinations, and the results are representative of those of two experiments.

Serum dilution	[^{3}H]Inositol phosphates	
	V + E → Ab	Ab + E → V
	dpm	
0	495	615
1/1000	246	176
1/316	122	9
1/100	0	0

The selectivity of the antibodies against the turkey erythrocyte phospholipase C for inhibition of the turkey erythrocyte enzyme versus phospholipase C-β and -γ was examined. As illustrated in Fig. 6, N465 inhibited activity of the turkey erythrocyte 150-kDa protein against exogenous [^{3}H]PtdIns(4,5)P_2 substrate at concentrations at which it had little or no effect on the activity of phospholipase C-β or -γ. Antisera N858, D246, and D807 were also inactive against the two bovine brain 150-kDa enzymes (data not shown).

The capacity of antibodies raised against the 150-kDa phospholipase C purified from turkey erythrocytes to inhibit the activity of this enzyme *in situ* also was examined. As illustrated in Fig. 7, preincubation of turkey erythrocyte ghosts with a 1/100 dilution of N465 or N858 but not D246, D807, or preimmune serum caused a partial inhibition (average of 50–60% in three experiments) of GTPγS plus ADPβS- and AlF_4^--stimulated phospholipase C activities. It is unclear why complete inhibition of activity was not observed, although inaccessibility of antibody to all of the phospholipase in the ghost preparation is a feasible explanation (see data below obtained with purified plasma membranes).

We recently have reported (8) that the activity of the purified 150-kDa turkey erythrocyte phospholipase C can be quantitated by reconstitution of the purified enzyme into ghosts prepared from [^{3}H]inositol-prelabeled cells in such a way as to be essentially devoid of phospholipase C activity. As illustrated in Fig. 8, the interaction of the purified phospholipase C with the turkey erythrocyte P_{2Y}-purinergic receptor and phospholipase C-related G protein also can be studied by reconstitution of purified enzyme into purified plasma membranes prepared from N_2-cavitated [^{3}H]inositol-labeled turkey erythrocytes. We have employed turkey erythrocyte plasma membranes prepared in this way to investigate guanine nucleotide-sensitive agonist

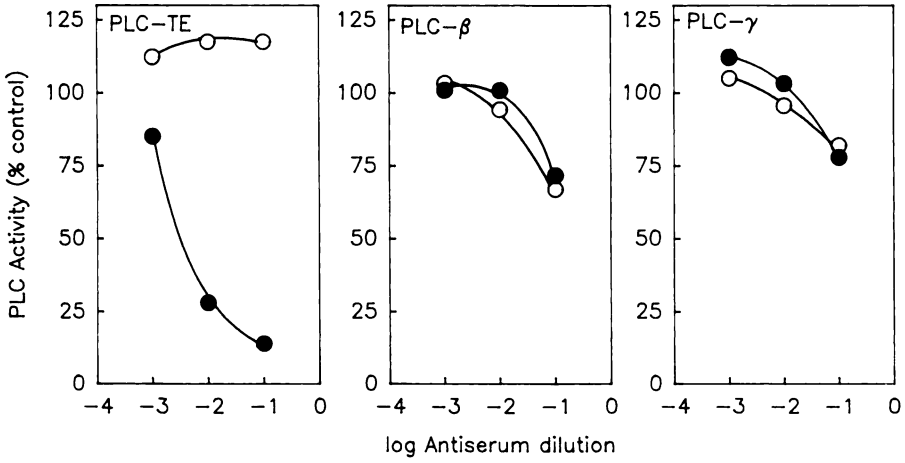

Fig. 6. Phospholipase C isoenzyme-specific inhibitory effects of antisera raised against the 150-kDa turkey erythrocyte phospholipase C. Turkey erythrocyte phospholipase C (*left*), bovine brain phospholipase C-β (*middle*), or bovine brain phospholipase C-γ (*right*) was incubated with preimmune antiserum (○) or antiserum N465 (●) against the turkey erythrocyte phospholipase C, as described in Experimental Procedures. The hydrolysis of [^{3}H]PtdIns(4,5)P_2 was then measured using exogenously provided substrate, as described above. Assays of phospholipase C-β and phospholipase C-γ contained 0.05% cholate, whereas assays of turkey erythrocyte phospholipase C contained 0.2% cholate. The data are plotted as the percentage of phospholipase C activity observed in the absence of added serum in each case. The data are representative of results obtained in two experiments.

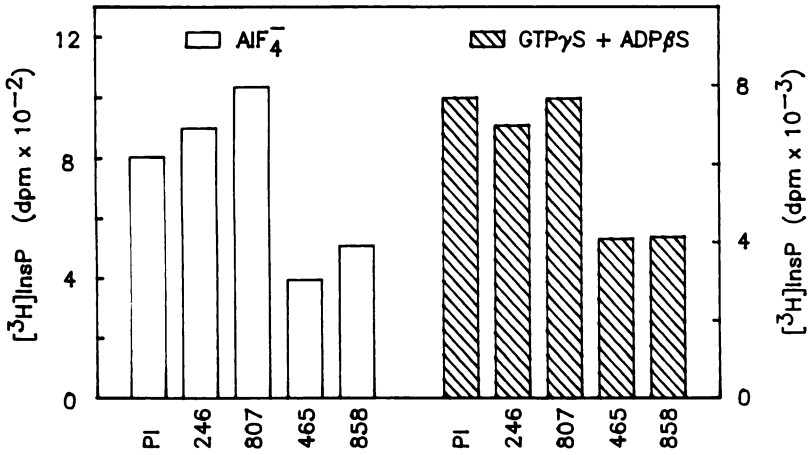

Fig. 7. Effect of turkey erythrocyte phospholipase C antisera on phosphoinositide hydrolysis in turkey erythrocyte ghosts. Turkey erythrocytes were labeled overnight with [^{3}H]inositol, and washed ghosts were prepared as described in Experimental Procedures. The labeled ghosts were incubated on ice for 1 hr with preimmune serum (*PI*) or D246, D807, N465, or N858 at 1/100 dilution. Phosphoinositide hydrolysis was then measured in the presence of 10 μM GTPγS plus 10 μM ADPβS (▨) or 10 mM/20 μM NaF/AlCl$_3$ (□) for 5 min at 30°. The results are the mean of triplicate determinations and are representative of results obtained in two experiments.

binding to the phospholipase C-linked P_{2Y}-purinergic receptor (13). As we have previously reported (13), before reconstitution of the 150-kDa phospholipase C these membranes display no phospholipase C activity either in the absence or in the presence of activators of the receptor and/or G protein (Fig. 8). Combination of the purified membranes with the purified enzyme conferred phospholipase C activity to the membranes, and this activity was sensitive to regulation by GTPγS, ADPβS plus GTPγS, or AlF_4^- (Fig. 8). Maximal effects were observed with 100 ng of purified 150-kDa protein reconstituted with approximately 10 μg of purified plasma membranes (Fig. 9). In contrast to the situation observed with reconstitution of the purified phospholipase C with acceptor ghosts (8), reconstitution of the 150-kDa enzyme with purified plasma membranes resulted in maximal responsiveness to AlF_4^- that exceeded that for agonist plus GTPγS. This difference may be explained by considerable "uncoupling" of receptor/G protein interaction that might be expected to occur during the extensive steps required for plasma membrane purification. Alternatively, loss of a factor important for efficient GDP/GTP exchange at the guanine nucleotide binding site of the relevant G protein is a conceivable explanation for these observations. Consistent with the previously observed inhibitory effects of the phospholipase C antisera on phospholipase C activity, preincubation of phospholipase C with antisera N465 or N858 before reconstitution resulted in failure to observe restoration of AlF_4^--stimulated phospholipase C activity in these membranes (Fig. 10). Preimmune serum had no effect.

Based on examination of immunoreactivity, the turkey erythrocyte 150-kDa phospholipase C expresses some similarity to the purified/cloned mammalian enzymes, in that two domains of amino acid sequence conserved across these proteins also appear to be present in the turkey enzyme (see Fig. 1). However, cross-reactivity among polyclonal and monoclonal antibodies against the various enzymes suggests that overall homology is low between the turkey erythrocyte enzyme and the other phospholipase C isoenzymes, although cross-reactivity of the phospholipase C-β enzyme with one of the antibodies to turkey erythrocyte phospholipase C suggests some structural similarity. To examine this idea further, peptide sequence of the turkey erythrocyte phospholipase C was obtained. The enzyme was purified, denatured, and digested, and resultant peptides were purified by reverse phase HPLC, as described in Experimental Procedures. Amino acid sequences determined for eight of these peptides are shown in Table 2. The sequences of these eight peptides were compared with the deduced amino acid sequences of phospholipase C-β from bovine brain (14, 15), phospholipase C-γ from bovine brain (16, 17), phospholipase C-δ [two sequences from rat brain (15) and bovine adrenal gland (17)], and the *Drosophila* norpA gene product (18). Within the regions of homology identified by this first search, comparisons were also made with the amino acid sequence of a phospholipase C isoenzyme related to phospholipase C-γ, called phospholipase C-γ_{II}, cloned from a human lymphocyte cDNA library (19). Peptides I, II, V, VI, and VII had no detectable homology with

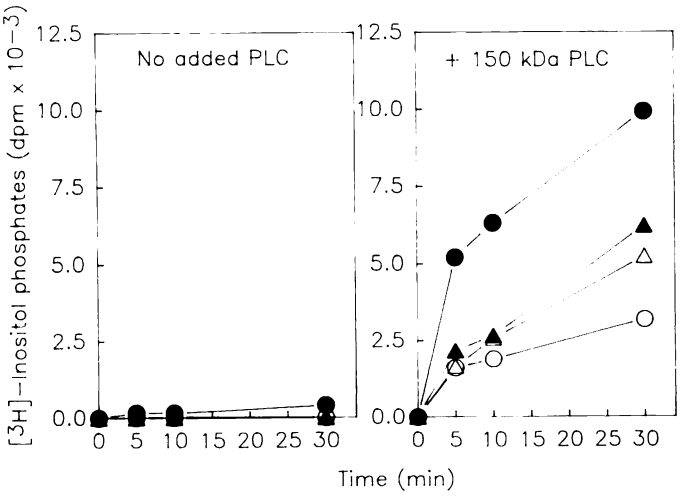

Fig. 8. Reconstitution of purified turkey erythrocyte phospholipase C with purified turkey erythrocyte plasma membranes. Turkey erythrocytes were incubated overnight with [^{3}H]inositol, to radiolabel endogenous phosphoinositides, and plasma membranes were purified as described in Experimental Procedures. Phospholipase C activity was measured as the stimulant-induced (○, no addition; ●, 10 mM NaF/20 μM $AlCl_3$; △, 10 μM GTPγS; ▲, 10 μM GTPγS plus 10 μM ADPβS) release of [^{3}H]inositol phosphates. *Left*, data for plasma membranes assayed in the absence of reconstituted purified phospholipase C; *right*, data for membranes reconstituted with purified 150-kDa turkey erythrocyte phospholipase C. The data are representative of results obtained in at least five experiments.

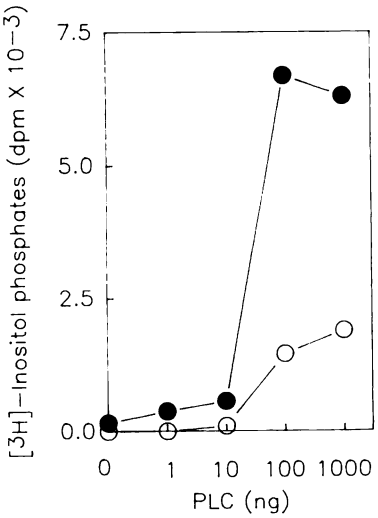

Fig. 9. Labeled plasma membranes were prepared as described for Fig. 8, and the indicated amount of purified turkey erythrocyte 150-kDa phospholipase C was reconstituted with approximately 10 μg of these acceptor membranes. [^{3}H]Inositol phosphate release was measured in the absence (○) or presence (●) of AlF_4 during a 5-min assay at 30°, as described in Experimental Procedures.

any deduced amino acid sequence in phospholipase C-β, -γ, or -δ. Peptide VIII had similarity with sequence in the first (X-domain) conserved region common to phospholipase C-β, -γ, and -δ (Table 3). In addition, a 16-amino acid peptide (peptide III) from the digested turkey erythrocyte phospholipase C had clear sequence homology to the second (Y-domain) conserved region common to phospholipase C-β, -γ, and -δ (Table 3). Finally, peptide IV from the turkey erythrocyte enzyme was 75% homologous to a sequence near the carboxyl terminus of phospholipase C-β and 50% homologous to an equivalent se-

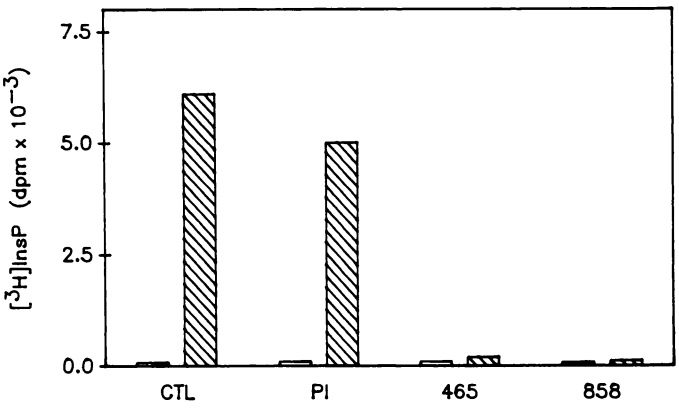

Fig. 10. Inhibition of reconstitution of phospholipase C activity in turkey erythrocyte plasma membranes by antisera raised against the 150-kDa turkey erythrocyte protein. Purified turkey erythrocyte phospholipase C was incubated with vehicle (*CTL*) or vehicle containing a 1/100 dilution of preimmune serum (*PI*) or a 1/100 dilution of either N465 or N858 antisera. The phospholipase C was then reconstituted with plasma membranes purified from [^{3}H]inositol-prelabeled erythrocytes and [^{3}H] inositol phosphate accumulation was measured in the absence (□) or presence of 10 mM NaF/20 μM $AlCl_3$ (▨), as described in Experimental Procedures. The data are from triplicate determinations and are representative of results from two experiments.

TABLE 2

Sequences of turkey erythrocyte phospholipase tryptic peptides

Peptide number	Sequence
I	IPGAEVPR
II	YVFPDGPE
III	VIPIIAVHSGYHTVCL
IV	DYVPDAVA
V	IVALEEG(GK)
VI	EVVISSEPQTASLA
VII	SY(T/H)QLPFTXL
VIII	GQLSPEGMVXFLCR

quence in the *Drosophila* norpA gene-encoded putative phospholipase C (Table 3) that is not homologous with any sequence in the deduced sequences of phospholipase C-α, -γ, or -δ.

Discussion

Although multiple phospholipase C isoenzymes have been purified and their cDNA cloned from a broad range of mammalian tissues (2, 14–19), alignment of any one of these proteins with G protein-regulated function has been difficult to establish. We previously have purified a 150-kDa phospholipase C from turkey erythrocytes that, when reconstituted with membranes devoid of phospholipase C, restores receptor- and G-protein-regulated activities analogous to those of native membranes (8). Although such data do not of themselves prove that the purified 150-kDa protein is the form of phospholipase C under regulation by the P_{2Y}-purinergic receptor and G protein *in situ*, they are clearly consistent with such an idea and provide identity, at least in a model avian cell, of a G protein-regulated form of phospholipase C. In light of this activity, it is important that the similarity of the turkey erythrocyte phospholipase C to those previously studied in detail from mammalian tissues be compared.

Considerable difficulty of interpretation is inherent in measurements of phospholipase C activity using exogenous phosphoinositide substrate presented either in phospholipid vesicles

TABLE 3

Partial amino acid sequence comparison of turkey erythrocyte phospholipase C and other phospholipase C isoenzymes

Isoenzyme	Sequence	Sequence conservation
		%
PLC-TE[a] (VIII)	G Q L S P E G M V X F L C R	
PLC-β (288)	G Q M S V D G F M R Y L S G	36
PLC-norpA (292)	V Q M S L D G F K R Y L M S	29
PLC-γ (291)	P Y F F L D E F V T F L F S	14
PLC-γII (283)	P F L L V D E F L T Y L F S	14
PLC-δa	NO HOMOLOGY	
PLC-δb	NO HOMOLOGY	
PLC-TE (III)	V I P I I A V H S G Y H T V C L	
PLC-β (761)	I L P V Q A I R P G Y H Y I C L	44
PLC-norpA (771)	I L P L D G L Q A G Y R H V S L	31
PLC-γ (1176)	T F P V K G L K T G Y R A V S L	31
PLC-γII (1026)	A L F S L N G R T G Y V L Q P E	13
PLC-δa (720)	T I P W N S L K Q G Y R H V H L	38
PLC-δb (720)	T I P L K S L K Q G Y R H I H L	31
PLC-TE (IV)	D Y V P D A V A	
PLC-β (797)	D Y V P D T Y A	75
PLC-norpA (807)	I Y V P D G F E	50
PLC-γ	NO HOMOLOGY	
PLC-γII	NO HOMOLOGY	
PLC-δa	NO HOMOLOGY	
PLC-δb	NO HOMOLOGY	

[a] PLC-TE, phospholipase C, turkey erythrocyte. The numbers in parentheses refer to the position in the overall deduced sequence for the first amino acid in the sequence presented.

or in mixed detergent and phospholipid micelles. Nevertheless, our previous analyses suggested divergence in catalytic properties of the purified turkey erythrocyte 150-kDa phospholipase C from those of the approximately 150-kDa mammalian phospholipase C-β and -γ (7). Activities are differentially sensitive to sodium cholate concentration and, in contrast to the mammalian enzymes, the turkey erythrocyte phospholipase C hydrolyzes polyphosphoinositides considerably more rapidly than PtdIns under the assay conditions that have been examined (7, 20).

Information presented in the current work indicates that the purified turkey erythrocyte enzyme, although similar in size to two of the major forms of mammalian phospholipase C, differs in structure and, thus possibly in function, from at least the members of the phospholipase C-β and -γ families that have been cloned. A cadre of isoenzyme- and peptide-specific antibodies generated against mammalian phospholipase C-β and -γ failed to recognize the turkey erythrocyte enzyme. Further, with one exception, antisera raised against the 150-kDa turkey erythrocyte phospholipase C failed to recognize phospholipase C-β and -γ. Contribution of tissue and/or species specificity of the antisera does not seem to apply. Antibodies against mammalian phospholipase C-β and -γ recognized, along with other proteins, a species of approximately 150 kDa in turkey brain. Antisera against the turkey erythrocyte phospholipase C also recognized a species of approximately 150 kDa in turkey brain, lung, and kidney.[2] Amino acid sequences of five peptides resolved from a tryptic digest of the turkey erythrocyte phospholipase C showed no homology with any of the deduced sequences of the mammalian and *Drosophila* phospholipase C isoenzymes.

In spite of major differences in immunoreactivity and likely sequence in the remainder of the molecule, the turkey erythrocyte 150-kDa phospholipase C apparently contains stretches of amino acid sequence similar to the so-called X- and Y-domains of shared sequence reported for at least five cloned mammalian phospholipase C isoenzymes (2). That is, antipeptide antibodies generated against stretches of sequence in these two domains strongly reacted with the purified turkey erythrocyte enzyme. Sequence similarity is supported further by the identification of amino acid sequences in peptides from the turkey erythrocyte phospholipase C that have 30–40% homology with sequences reported from within both the conserved X- and Y-regions common to the mammalian phospholipase C-β, -γ, and -δ isoenzymes. The function of these regions of highly conserved sequence across putative phospholipase C families has not been determined, although a common function, such as phosphoinositide binding/hydrolysis, has been proposed.

There is a potentially provocative similarity between the turkey erythrocyte enzyme and phospholipase C-β. Bovine brain phospholipase C-β is recognized by one of the antisera against the turkey erythrocyte phospholipase C, and a peptide sequenced from the 150-kDa turkey erythrocyte enzyme has 75% homology with a sequence in phospholipase C-β (14, 15) and 50% homology with an equivalent sequence in the *Drosophila* norpA phospholipase C (19). This sequence in the mammalian and *Drosophila* enzymes is located in an extension of these proteins, relative to phospholipase C-γ and -δ, between the conserved Y-domain and the carboxyl terminus. Function of this portion of the phospholipase C protein has not been established, but such conservation of sequence could be indicative of a common regulatory function, e.g., coupling to a G protein. The complete predicted sequence of the turkey erythrocyte phospholipase C is needed, and isolation of cDNA for the turkey erythrocyte enzyme represents an important goal that is currently compromised by the fact that turkey erythrocytes are not transcriptionally active. Efforts to identify phospholipase C cDNA in fetal turkey blood cDNA libraries are underway.

It is important to note that progress in identification of the G protein-regulated forms of phospholipase C has been accompanied recently by progress in identification of the G protein

[2] G. L. Waldo, unpublished observations.

that regulates phospholipase C. A 42-kDa phospholipase C activator has been purified from GTPγS-preactivated liver membranes by Taylor *et al.* (21). Antisera against a sequence common to a number of G protein α subunits reacts with this protein. Pang and Sternweis (22) have applied affinity chromatography with immobilized G protein βγ subunits to purify a G protein α subunit from bovine brain. This protein has internal amino acid sequence identical to sequence predicted by a cDNA for a G protein, called G_q, cloned from mouse brain (23). Purified G_q markedly activates phospholipase C obtained from bovine brain (24). Recently, Taylor and co-workers (25) have shown that the activating protein from liver also reacts with antisera against the G_q class of proteins. Using purified turkey erythrocyte phospholipase C in a reconstitution assay with cholate extracts, an aluminum fluoride-dependent phospholipase C-activating protein has been purified from turkey erythrocyte plasma membranes (26). This approximately 43-kDa protein also shows strong immunoreactivity with antisera generated against a peptide sequence found in G_q and G_{11}. Based on these observations, it is reasonable to anticipate that the phospholipase C-regulating G proteins will be identified and their specificity of interaction with phospholipase C isoenzymes will soon be established. Apropos to the work described here, Taylor *et al.* (25) have shown recently that the phospholipase C-activating 42-kDa G protein purified from bovine liver activates phospholipase C-β but not phospholipase C-γ or -δ purified from bovine brain. Similarly, G_q purified from bovine brain markedly activates brain phospholipase C-β,[3] as well as the 150-kDa phospholipase C purified from turkey erythrocytes.[2]

Finally, the capacity to inhibit phospholipase C activity by antisera against the turkey erythrocyte phospholipase C could prove very useful. The specificity of the antisera has not been established broadly, but their utility for studies of at least the turkey erythrocyte G protein-regulated phospholipase C is clear. It will be important to determine whether these antisera have similar activity with at least a subset of G protein-regulated phospholipase C isoenzymes in mammalian tissues. Monoclonal antibodies to bovine brain phospholipase-β and -γ previously have been shown to inhibit activity of these enzymes against exogenously provided PtdIns (27). Moreover, Carter *et al.* (28) recently have shown that an anti-phospholipase-β antibody partially inhibits muscarinic receptor-stimulated guanine nucleotide-dependent phospholipase C activity in rat brain membranes. Because receptor-mediated regulation of phospholipase C apparently occurs through at least two different classes of G proteins (29, 30), it will be important to establish whether multiple phospholipase C isoenzymes are involved in receptor/G protein-activated inositol lipid signaling.

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[3]A. V. Smrcka and P. C. Sternweis, personal communication.

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