

## Purification, G Protein Activation, and Partial Amino Acid Sequence of a Novel Phospholipase C from Squid Photoreceptors<sup>†</sup>

Jane Mitchell\* and Linnia H. Mayeenuddin

Department of Pharmacology, University of Toronto, Toronto, M5S 1A8, Canada

Received November 10, 1997; Revised Manuscript Received March 20, 1998

**ABSTRACT:** Invertebrate visual signal transduction is initiated by rhodopsin activation of a guanine nucleotide binding protein, G<sub>q</sub>, which stimulates phospholipase C (PLC) activity. We have previously purified a 140-kDa PLC enzyme from squid photoreceptors that is regulated by squid G<sub>q</sub>. In these studies, an additional PLC enzyme was purified from the cytosol of squid photoreceptors and identified as a 70-kDa protein by SDS–polyacrylamide gel electrophoresis. Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) by PLC-70 was optimal at pH 5 in the presence of 100  $\mu$ M Ca<sup>2+</sup> with a specific activity of 10.3  $\mu$ mol min<sup>−1</sup> mg<sup>−1</sup>. A polyclonal antibody raised against purified PLC-70 did not recognize purified PLC-140, and proteolytic digestion of the two purified enzymes with trypsin or *Staphylococcus aureus* V8 protease showed distinct patterns of peptide fragments, indicating that PLC-70 is not a fragment of PLC-140. The partial amino acid sequence of the protein showed homology with PLC21 and *norpA* isozymes cloned from *Drosophila*, and mammalian PLC  $\beta$  isozymes. Reconstitution of purified GTP $\gamma$ S-bound soluble squid G<sub>q</sub> with PLC-70 resulted in significant enhancement of PIP<sub>2</sub> hydrolysis over a range of Ca<sup>2+</sup> concentrations and shifted the maximum activation by calcium to 1  $\mu$ M. These results suggest that cephalopod phototransduction is mediated by G<sub>q</sub> activation of more than one cytosolic PLC enzyme.

The molecular mechanism of photoexcitation in several invertebrate species has recently been established (1–6). In systems characterized thus far, photoactivated rhodopsin activates a heterotrimeric G protein<sup>1</sup> which in turn stimulates the hydrolysis of inositol phospholipids by PLC enzymes expressed in photoreceptor cells. The subsequent increase in inositol 1,4,5-trisphosphate (IP<sub>3</sub>) levels has been shown to activate release of Ca<sup>2+</sup> from subrhabdomeric cisternae, and subsequently increase membrane channel conductance, resulting in depolarization of the photoreceptor membrane.

Many of the component proteins of these signaling pathways have been isolated and/or cloned from several species. Invertebrate opsins are structurally related to their vertebrate counterparts and can be activated by light to form stable metarhodopsins (7, 8). The subunits of invertebrate retinal G proteins which are activated by these rhodopsins have also been identified in *Drosophila* and squid. The *Drosophila* *Dgq* gene product (9) and squid G<sub>q</sub> $\alpha$  proteins (10, 11) have both been cloned. The primary sequences of these two G protein  $\alpha$  subunits show homology to mammalian proteins of the G<sub>q</sub> family. The homology with G<sub>q</sub> $\alpha$

is greatest in the carboxyl terminus, a region of these G protein  $\alpha$  subunits which has been shown to interact with their target PLC enzymes (12–16). Squid G $\beta$  subunits and *Drosophila* G $\beta$ e are also highly homologous to their mammalian counterparts (17, 18); however, the invertebrate visual G protein  $\gamma$  subunits appear to be quite distinct and bear little overall sequence homology to mammalian  $\gamma$  subunits (19, 20). The role of invertebrate visual  $\beta\gamma$  subunits in signal transduction has not been well characterized although they clearly play some role in this pathway since *g $\beta$ e* *Drosophila* mutants have been reported to have reduced light sensitivity, delayed photoactivation, and delayed recovery (21–23).

The PLC isozymes which are activated by *Dgq* and squid G<sub>q</sub> have also been identified. In *Drosophila*, the *norpA* protein is abundantly expressed in the retina and has a deduced sequence that shows homology to mammalian PLC- $\beta$  isozymes (24–26), and isolated *norpA* protein demonstrated Ca<sup>2+</sup>-sensitive phospholipid hydrolysis (6). *Dgq* has not been demonstrated to activate *norpA* in vitro; however, studies using genetic manipulations of these two proteins demonstrate their involvement in light-activated increases in IP<sub>3</sub> in *Drosophila* eyes (27). Using squid photoreceptors, we have recently isolated a 140-kDa protein (PLC-140), that appears from partial sequence data to be related to *norpA* and PLC- $\beta$  subtypes (28). Purified PLC-140 is a Ca<sup>2+</sup>-sensitive inositolphospholipid hydrolase whose activation is profoundly enhanced by reconstitution with activated soluble squid G<sub>q</sub> (28). Others have also isolated a similar PLC protein from squid and shown that it can be activated by insoluble G<sub>q</sub> if this protein is first incorporated into lipid vesicles (29).

<sup>†</sup> This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada.

\* To whom correspondence should be addressed at the Department of Pharmacology, University of Toronto, 1 King's College Circle, Toronto, M5S 1A8, Ontario, Canada. Telephone: 416-978-0841. Fax: 416-978-6395. E-mail: jane.mitchell@utoronto.ca.

<sup>1</sup> Abbreviations: G protein, guanine nucleotide binding protein; PLC, phospholipase C; PIP<sub>2</sub>, phosphatidylinositol 4,5-bisphosphate; PE, phosphatidylethanolamine; PI, phosphatidylinositol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; AEBF, 4-(2-aminoethyl)benzenesulfonyl fluoride; GTP $\gamma$ S, guanosine 5'-3-*O*-(3-thiotriphosphate); PAGE, polyacrylamide gel electrophoresis.

We present evidence in this report for the isolation and characterization of a second PLC enzyme, PLC-70, from squid photoreceptors. This enzyme is also activated by  $\text{Ca}^{2+}$  and regulated by soluble squid  $\text{G}_q$  with characteristics that are distinct from those seen for PLC-140.

## EXPERIMENTAL PROCEDURES

**Materials.** CM-Sepharose, DEAE-Sephacel, and heparin-Sepharose were purchased from Pharmacia. HA Ultrogel, phosphatidylethanolamine (PE), PI, and  $\text{PIP}_2$  were purchased from Sigma. Phosphatidyl[2- $^3\text{H}$ ]inositol 4,5-bisphosphate (16.3 Ci/mmol), phosphatidyl[2- $^3\text{H}$ ]inositol (1 Ci/mmol), and anti  $\text{G}_{q11}\alpha$  antiserum were purchased from DuPont-New England Nuclear. Anti-PLC70 and anti-PLC140 antisera were produced in rabbits using 100  $\mu\text{g}$  of purified PLC-70 or PLC-140 protein by standard techniques (30). Crude antisera were purified over protein A-agarose (Schleicher & Schuell, Keene, NH) before use. Trypsin and *S. aureus* V8 protease were obtained from Boehringer Mannheim. Frozen squid eyes were originally purchased from Calamari Inc., Woods Hole, MA, and were a generous gift from John K. Northup, National Institute of Mental Health, Bethesda, MD.

**Phospholipase C Assay.** Phosphatidylinositol hydrolyzing activity was measured, unless otherwise stated, by incubation for 10 min at 30 °C in a mixture containing 10 mM Hepes, pH 7.0, 10 mM NaCl, 4 mM  $\text{MgSO}_4$ , 100 mM KCl, 0.1% deoxycholate, 50  $\mu\text{M}$   $\text{PIP}_2$ , 250  $\mu\text{M}$  PE containing 25 000 dpm of [ $^3\text{H}$ ] $\text{PIP}_2$ , 2 mM EGTA, and the concentration of free  $\text{Ca}^{2+}$  indicated in individual experiments. The total incubation volume was 100  $\mu\text{L}$ , and reactions were terminated as previously described (28). Free  $\text{Ca}^{2+}$  concentrations were calculated as described previously (31).

**Squid Photoreceptor Fractionation and PLC Purification.** Photoreceptor membranes were prepared from 50 frozen squid eyes as previously described (28) with the following modification. Crude homogenates were prepared by briefly vortexing frozen squid eyes in 100 mL of 10 mM Mops, pH 7.5, 2 mM EGTA, 1  $\mu\text{M}$  4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) and filtered to remove eye cups. Filtered material was further homogenized in a Dounce homogenizer, and crude membranes were separated from the cytosol by centrifugation at 30000g for 20 min at 4 °C. P2 membranes were further purified by flotation in 40% (w/w) sucrose solutions as previously described (28). For experiments in which PLC isozymes and  $\text{G}_q\alpha$  were localized to different retinal fractions, highly purified rhabdomeral membranes were prepared by the method of Kito et al. (32). PLC enzymes were purified from the cytosol fraction produced by the 30000g sedimentation step in the photoreceptor preparation, and rhabdomeric membranes were used as a source of  $\text{G}_q$ .

Cytosol was diluted with an equal volume of 100 mM Mes, pH 6.0, 100 mM NaCl, 1  $\mu\text{M}$  AEBSF, and proteins were fractionated over CM-Sepharose also as previously described (28). Fractions containing PLC activity from the CM-Sepharose column were pooled and diluted with an equal volume of 100 mM Hepes, pH 7.0, 1 mM EGTA, 1 mM dithiothreitol (HED buffer) and applied to a column (3.2  $\times$  25 cm) of heparin-Sepharose equilibrated in 100 mM NaCl in HED buffer. Proteins containing PLC activity were eluted

from the column in a 400 mL linear gradient of 200–500 mM NaCl in HED. Fractions containing predominantly PLC-140 or PLC-70 as assessed by SDS-polyacrylamide gel electrophoresis were pooled separately and concentrated by filtration using PM30 filters. Further purification of PLC-70 from contaminating smaller molecular weight proteins was achieved by chromatography on a 10 mL column of HA-Ultrogel preequilibrated with 10 mM Mops, pH 7.0, 100 mM NaCl (MN buffer). The concentrated PLC-70 pool from heparin-Sepharose was diluted 10-fold in MN buffer and applied to the column, the column was washed with 5 mL of equilibration buffer, and proteins were eluted with a 30 mL linear gradient of 100–300 mM potassium phosphate in MN buffer.

**Proteolytic Digestion and Amino Acid Sequencing of PLC Proteins.** Purified PLC-70 or PLC-140, 5  $\mu\text{g}$  each, were incubated in the presence of proteolytic enzymes at an enzyme:PLC molar ratio of 1:10 for trypsin and 1:50 for *S. aureus* V8. The final reaction mixture for digestion with trypsin contained 50 mM Tris-HCl, pH 7.6, 6 mM  $\text{MgCl}_2$ , 75 mM sucrose, 1 mM dithiothreitol, 1 mM EDTA, and 0.6% (v/v) Lubrol PX, in a final volume of 20  $\mu\text{L}$  and incubated for 10 min at 30 °C. When the reaction contained the V8 enzyme, the final reaction solution contained 10 mM Mops, pH 7.8, 1 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, and 200 mM NaCl also in 20  $\mu\text{L}$  volume, and the samples were incubated for 90 min at 30 °C. All reactions were stopped by the addition of 5  $\mu\text{L}$  of 24 mM AEBSF, and incubated for a further 15 min at 30 °C. Samples were then diluted into sample buffer and peptide fragments separated on an 11% SDS-polyacrylamide gel. Peptide bands were visualized by Coomassie blue staining.

For amino acid sequence analysis, 10  $\mu\text{g}$  samples of PLC-70 were digested with *S. aureus* V8 as detailed above. Peptides were then separated by electrophoresis on an 8% polyacrylamide gel and transferred to PVDF membranes (33) lightly stained with Coomassie blue, and predominant bands were excised and subjected directly to amino acid analysis using a Proton Gas-phase Microsequencer, Model 2090. Sequences were compared with known DNA and protein sequences in GenBank.

**Guanine Nucleotide Binding and G Protein Isolation.**  $\text{G}_q\alpha$  subunits were isolated from rhabdomeric membranes washed 3 times in 10 mM Mops, pH 7.5, 1 mM EGTA, 100 mM NaCl to remove peripheral proteins. GDP or  $\text{GTP}\gamma\text{S}$  was bound to the G protein  $\alpha$  subunits before extraction since squid  $\text{G}_q$  does not efficiently bind guanine nucleotides once it has been released from the photoreceptor membrane.<sup>2</sup> Membrane proteins (50 mg) were incubated in a buffer containing 20 mM Tris-HCl, pH 8.0, 1 mM EGTA, 100 mM NaCl, 1 mM  $\text{MgCl}_2$ , and either 1 mM GDP or 1 mM  $\text{GTP}\gamma\text{S}$  in a total volume of 40 mL on ice for 10 min. KCl was then added to a final concentration of 1 M; samples were incubated on ice for 30 min and then centrifuged for 20 min at 100000g at 4 °C to separate soluble from membrane bound proteins.  $\text{GDP-G}_q$  and  $\text{GTP}\gamma\text{S-G}_q$  were further purified by batch chromatography over DEAE-Sephacel by the following procedure. Soluble fractions were diluted with 20 mM Tris-HCl, pH 8.0, 1 mM EGTA, 5 mM  $\text{MgCl}_2$  (TEM) to reduce

<sup>2</sup> J. Mitchell, C. Bamsey, and R. Cheung, unpublished data.

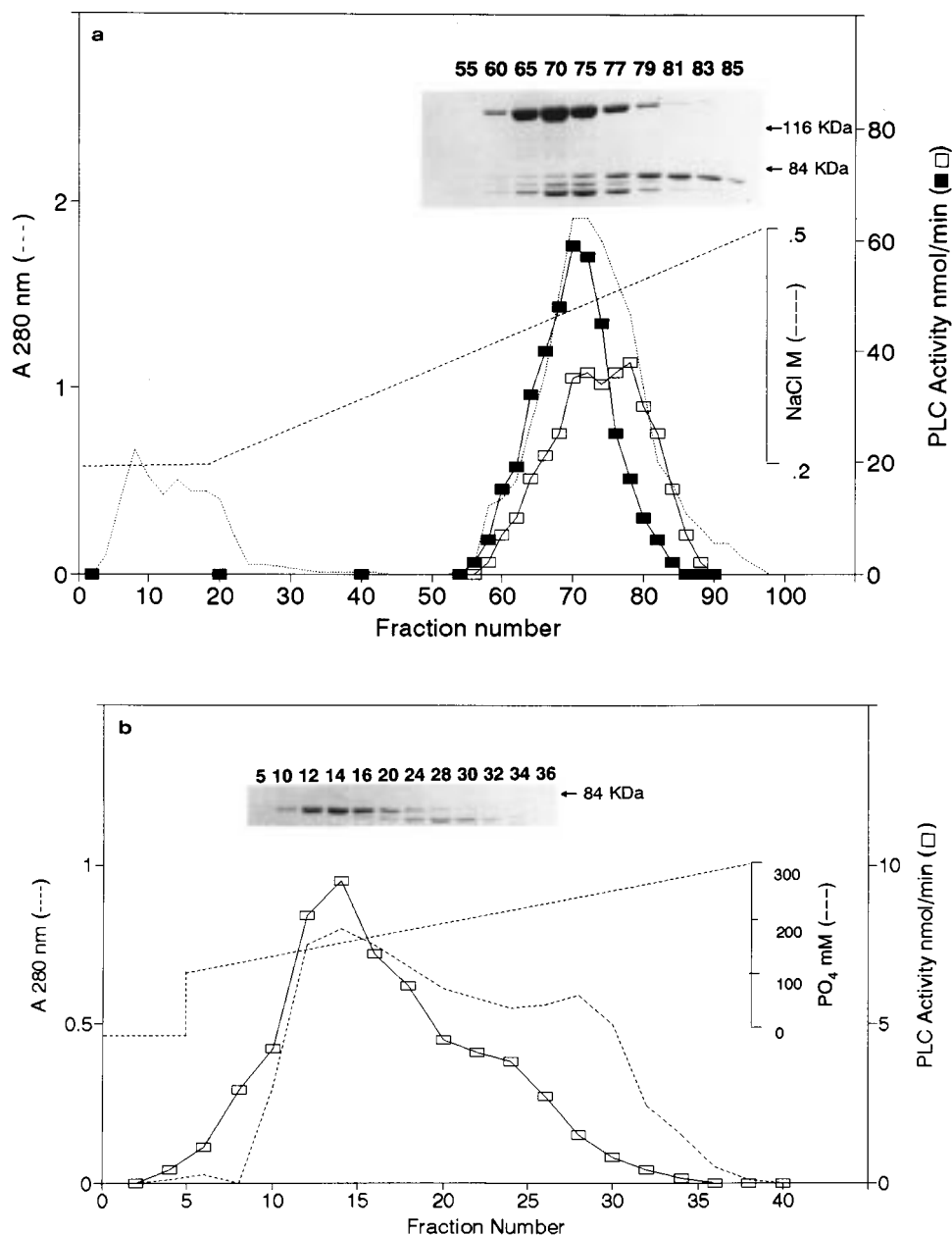


FIGURE 1: Purification of PLC-70 from squid retinal extract by sequential chromatography on heparin-Sepharose (a) and hydroxyapatite (b). PLC activity of each fraction was assayed using PE/PIP<sub>2</sub> mixed phospholipid vesicles in the presence of 1 μM (■), or 100 μM (□) free Ca<sup>2+</sup> as described under Experimental Procedures. (Insert) 10 μL samples of the indicated fractions were analyzed on 8% polyacrylamide gels and visualized with Coomassie blue. Numbers at the side of the inserts indicate the migration of protein standards.

the KCl concentration to 50 mM and applied to 10 mL columns of DEAE-Sephacel preequilibrated in TEM containing 50 mM NaCl. Columns were washed with 4 volumes of equilibration buffer, and then G protein α subunits were eluted in TEM containing 250 mM NaCl. Fractions collected from the columns were assayed by SDS-PAGE, and G<sub>q</sub>α was identified by immunoblot using an antibody to mammalian G<sub>q/11</sub>α. G protein samples were pooled and concentrated by filtration using a PM-10 filter.

**G<sub>q</sub>-PLC Reconstitutions.** For measurement of G protein regulated PLC activity, aliquots of GDP- or GTPγS-bound G<sub>q</sub> (5 μL, 10 ng) were mixed on ice with PLC-70 or PLC-140 (5 μL, 2 ng) for 10 min prior to addition of 5 μL of PIP<sub>2</sub>/PE vesicles (400 μM PIP<sub>2</sub>, 8 mM PE containing 25 000 dpm of [<sup>3</sup>H]PIP<sub>2</sub> in 10 mM Hepes, pH 7.0). The mixture was made to a final volume of 100 μL containing 10 mM

Hepes, pH 7.0, 100 mM NaCl, 4 mM MgSO<sub>4</sub>, 2 mM EGTA, 1 μM free Ca<sup>2+</sup> to start the reaction and continued for 10 min at 30 °C. Reactions were terminated as previously described (28).

**Other Methods.** SDS-PAGE was carried out by the method of Laemmli (34). Protein concentrations in extracts were determined using the amido black staining procedure (35). Proteins were transferred to nitrocellulose for identification by Western blotting with anti-G<sub>q</sub>α, anti-PLC70, or anti-PLC140 antisera as previously described (36), and developed with a horseradish peroxidase-conjugated secondary antibody and visualized using an enhanced chemiluminescent substrate (ECL, Amersham).

Quantitative assessment of PLC enzymes and G<sub>q</sub>α proteins was performed using methods previously described for G protein subunits (37). Briefly, crude eye homogenates were

Table 1: Purification of PLC-70 and PLC-140 from Squid Retina<sup>a</sup>

purification step	PLC	protein (mg)	total activity ( $\mu\text{mol}/\text{min}$ )	specific activity ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ )	purification (x-fold)
supernatant	total	140	65.6	0.5	0.5
CM-Sepharose	total	21.6	75.6	3.5	7.1
heparin-Sepharose	–140	5.9	61.9	10.5	21.4
	–70 <sup>b</sup>	1.39	7.9	5.1	10.4
hydroxyapatite	–70 <sup>b</sup>	0.63	4.7	7.4	15.1

<sup>a</sup> PLC activity in each step was determined using [<sup>3</sup>H]PIP<sub>2</sub> as a substrate in the presence of 1  $\mu\text{M}$  free Ca<sup>2+</sup> for total PLC activity and PLC-140.

<sup>b</sup> The PLC activity of PLC-70 was measured in the presence of 100  $\mu\text{M}$  free Ca<sup>2+</sup>.

run on SDS–PAGE gels using six different concentrations of membranes ranging from 0.1 to 10  $\mu\text{g}$  of total protein along with purified PLC enzymes or G<sub>q</sub> $\alpha$  in concentrations ranging from 0.1 to 5 pmol. Proteins were transferred to nitrocellulose membranes and visualized with appropriate antisera as outlined above. Specific protein bands were quantified by densitometry on an LKB 222-020 Ultrosan XL laser densitometer, and the area of each band was determined using Gel-Scan XL software (Pharmacia). The amount of each PLC enzyme or G<sub>q</sub> $\alpha$  subunit in each membrane sample was then calculated from a standard curve constructed from the densitometric values obtained with the known amounts of purified proteins run on the same gel. The calculated amount of each protein represents the mean of triplicate assessments from three different eye preparations.

## RESULTS

**Isolation of PLC-70 from Squid Photoreceptors.** Using an experimental procedure similar to that developed previously to isolate PLC-140 (28), a pool of PLC activity was separated from the majority of cytosolic proteins when chromatographed on CM–Sepharose. This pool of proteins was then applied to a heparin–Sepharose column, and the major pool of PLC activity was found to be associated with the elution of the 140-kDa protein when assayed using 1  $\mu\text{M}$  free Ca<sup>2+</sup> and PIP<sub>2</sub> in liposomes containing deoxycholate (Figure 1a, closed symbols). When the same samples were assayed in the presence of 100  $\mu\text{M}$  free Ca<sup>2+</sup> and the same substrate, the activity associated with the 140-kDa peak was diminished, and a second peak of activity was found to elute immediately after this protein (Figure 1a, open symbols). This second peak of PLC activity was associated with the upper band of a triplet of proteins that elute from CM-Sepharose with PLC-140. The molecular mass of this new PLC was estimated to be approximately 70 kDa from its migration on SDS–polyacrylamide gels and is therefore referred to as PLC-70. The pooled PLC-70 protein was further separated from residual contaminating smaller proteins using hydroxyapatite. PLC-70 bound less avidly to this matrix than the other proteins and could therefore be eluted with buffers containing a lower phosphate concentration (Figure 1b). No intact PLC-140 was found in the eluted fractions from the hydroxyapatite column. Using this combination of ion-exchange and affinity chromatography, 0.63 mg of PLC-70 and 5.9 mg of PLC-140 were purified from 50 squid eyes. A summary of the purification of both PLC proteins is presented in Table 1.

**Ca<sup>2+</sup> and pH Dependence of PLC-70 Activity.** The Ca<sup>2+</sup> dependence of hydrolysis of PIP<sub>2</sub> and PI by PLC-70 was examined (Figure 2). PIP<sub>2</sub> hydrolysis by the purified enzyme

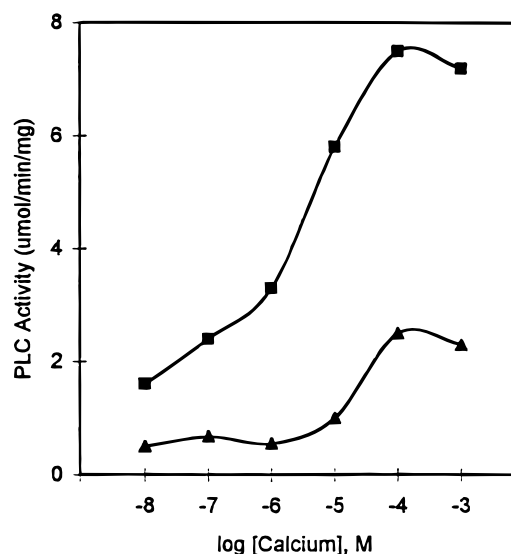


FIGURE 2: Ca<sup>2+</sup> dependence of phosphoinositide hydrolysis by purified PLC-70. Mixed phospholipid vesicles of PE/PIP<sub>2</sub> (■) or PE/PI (▲) in a molar ratio of 5:1 were used as substrates. Assays were performed using various free Ca<sup>2+</sup> concentrations at pH 7.0.

increased slowly at low Ca<sup>2+</sup> concentrations, reached a maximum at 100  $\mu\text{M}$  Ca<sup>2+</sup>, and then decreased slightly at millimolar concentrations (Figure 2). The effect of Ca<sup>2+</sup> on the hydrolysis of PI was similar to that seen for PIP<sub>2</sub>; however, this enzyme had much lower specific activity when PI was used as a substrate (Figure 2). PLC-70 showed no ability to hydrolyze either phosphatidylcholine or phosphatidylethanolamine when assayed under similar conditions (data not shown).

The effect of pH on the activity of PLC-70 was biphasic (Figure 3). Using PIP<sub>2</sub> as a substrate, PLC-70 enzyme activity was inhibited at very acidic pH but steeply increased to a maximum at pH 5, decreased as the pH reached neutral, and was steeply inhibited at higher pH levels.

**Proteolytic Digestion of PLC Isozymes and Peptide Sequence.** To determine if PLC-70 and PLC-140 are structurally related, the two proteins were compared by proteolytic analysis and for recognition by antibodies raised against the purified proteins. As shown in Figure 4, both enzymes were sensitive to trypsin and *S. aureus* V8 protease. The resultant peptide patterns from the two PLC proteins were, however, quite different. PLC-140 was cleaved by trypsin into one major fragment migrating with an apparent molecular mass of 90 kDa, whereas PLC-70 was digested by trypsin into very small fragments that were not visualized on the gel (Figure 4, panel B). Digestion of PLC-70 by V8 protease produced three major peptide fragments with molecular masses of 48, 40, and 36 kDa, whereas PLC-

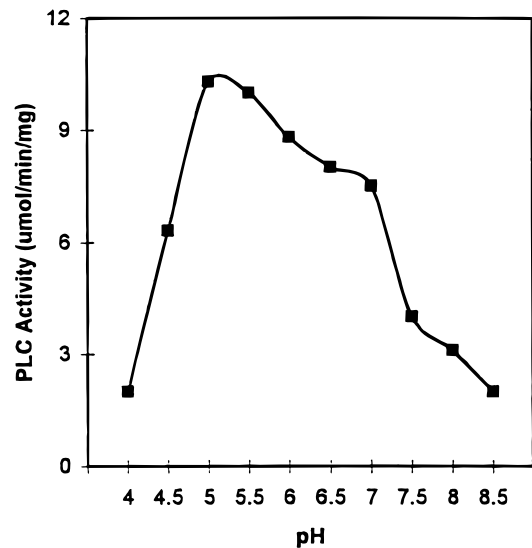


FIGURE 3: Effect of pH on hydrolysis of PIP<sub>2</sub> by PLC-70. PLC activity was determined using Bis-Tris propane buffer at various pH values. The reaction mixture contained 100  $\mu$ M free Ca<sup>2+</sup> and PE/PIP<sub>2</sub> phospholipid vesicles as substrate and were performed as described under Experimental Procedures.

140 digestion with this enzyme resulted in a set of six fragments none of which migrated with the same molecular mass as the peptides from PLC-70 (Figure 4, panel C). A polyclonal antibody raised against purified PLC-70 recognized two proteins migrating at approximately 70 kDa. The major protein in the PLC-70 preparation appeared as a single band with Coomassie blue staining using 5  $\mu$ g of purified PLC-70, and therefore the appearance of the second band when visualized using the anti-PLC70 antiserum likely represents a degradation product of the 70 kDa protein that we have noted to increase with storage of the purified proteins. The PLC-70 antiserum clearly did not recognize any protein bands in 5  $\mu$ g samples of purified PLC-140 (Figure 4, panel D). Similarly, an antiserum raised against purified PLC-140 did not recognize any protein bands using purified PLC-70 and recognized a single band migrating at

Table 2: Comparison of Amino Acid Sequences of Peptide Fragments from Squid PLC-70 with Those of Other PLC Isozymes

Isozyme	Amino acid sequence <sup>a</sup>
Peptide 1	I N F Y E E I K K F Q G
PLC21 806–817	I a a Y E E g g K l i G
Norpa 757–768	f g v Y E E s g K l i G
PLC- $\beta$ 1 747–758	I a a Y E E g g K i l G
PLC- $\beta$ 2 749–760	v a v m E E g n K F i G
PLC- $\beta$ 3 778–789	I a a f E E g g r F v G
PLC- $\beta$ 4 771–782	I a v Y d d n n K l i G
Peptide 2	A S K K I V L P D P
PLC21 792–801	v f K K v v L P e l
Norpa 743–752	v f r K v v L P D l
PLC- $\beta$ 1 733–742	v f K K v v L P s l
PLC- $\beta$ 2 734–743	v f e K I l m P e l
PLC- $\beta$ 3 764–773	d f p K v v L P t l
PLC- $\beta$ 4 759–768	v f r K v i L P D l
PLC-140 <sup>b</sup>	v f K K v v L P D l
Peptide 3 <sup>c</sup>	V L A K G V A Y D S S X E
PLC21 656–668	V y p a g t r f D S S n f
Norpa 607–619	i Y p K G y r a D S S d l
PLC- $\beta$ 1 597–609	i y p K G t r v D S S n y
PLC- $\beta$ 2 601–613	i y p K G t r v D S S n y
PLC- $\beta$ 3 629–641	i y p K G t r v D S S n y
PLC- $\beta$ 4 623–635	i y p K G g r v D S S n y
Peptide 4	I D R M T M T M N V E L P D S M S
PLC21 377–393	v h g y T f v p e i f a k D v i q
Norpa 378–394	t h g h a y c t e i l f k D c i q
PLC- $\beta$ 1 375–391	t h g f T M T t e i s f k e v i e
PLC- $\beta$ 2 372–389	t h g f T M T t d i f f k e a i e
PLC- $\beta$ 3 358–374	t h g f T M T t e v p l r D v l e
PLC- $\beta$ 4 372–389	t h g k a M c t d i l f k D v i q

<sup>a</sup> Identical residues are shown in uppercase letters; nonidentical residues in lowercase letters. <sup>b</sup> Sequence from peptide 3 in ref 28. <sup>c</sup> X indicates a residue that could not be identified.

approximately 140 kDa using purified PLC-140 (Figure 4, panel E). These results offer strong evidence that the two PLC enzymes differ in their primary structure and that PLC-70 is not a proteolytic fragment of PLC-140.

Amino acid analysis was performed on seven peptide fragments generated by *S. aureus* V8 protease digestion of purified PLC-70. The four longest independent polypeptides are shown in Table 2 with homologous amino acid sequences

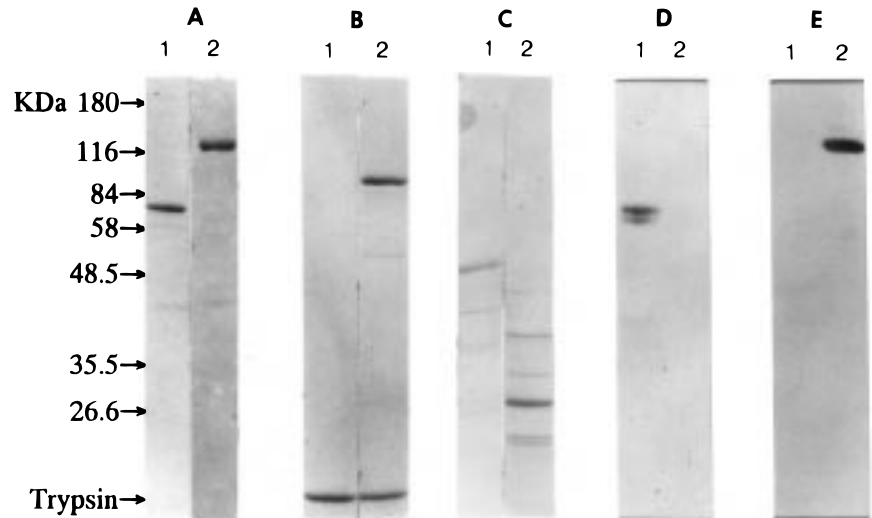


FIGURE 4: Proteolytic digestion and immunological recognition of PLC-70 and PLC-140. Five microgram aliquots of PLC-70 (lanes 1) or PLC-140 (lanes 2) were separated on 11% polyacrylamide gels following incubation without enzymes (A, D, and E) or following digestion with trypsin (B) or *S. aureus* V8 (C). Proteins or digested peptides were visualized by staining with Coomassie blue (A–C), or transferred to nitrocellulose filters and visualized using antisera raised against purified PLC-70 (D) or PLC-140 (E). Numbers at the side of the gel indicate the migration of protein standards. The protein band resulting from trypsin (B) is indicated on the left of the gel.

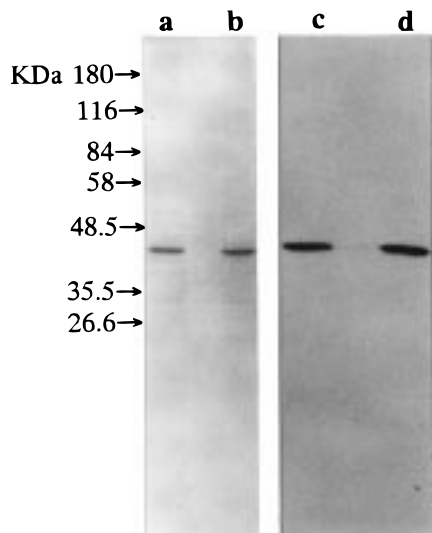


FIGURE 5: SDS-polyacrylamide gel and immunoblot analysis of purified squid retinal  $G_q\alpha$ . Guanine nucleotides were bound to squid  $G_q$  proteins in rhabdomic membranes prior to extraction in 1 M KCl. Extracts were purified by chromatography on DEAE-Sephacel; fractions containing  $G_q$  were pooled and subjected to electrophoresis on 11% polyacrylamide gels. The gels were stained with Coomassie blue (a and b) or immunoblotted with antipeptide antibody to the C-terminal region of mammalian  $G_{q11}$  (c and d). GDP- $G_q$ , lanes a and c; GTP $\gamma$ S- $G_q$ , lanes b and d. Numbers at the side of the gel indicate the migration of protein standards.

identified from other members of the PLC  $\beta$  family of proteins: mammalian PLC- $\beta$ 1–4, *Drosophila* visual norpA, and *Drosophila* neuronal PLC21. PLC-70 sequences 1–3 matched the two *Drosophila* proteins and mammalian PLC- $\beta$ 1 with highest homology. Peptide sequence 4 did not match any PLC enzyme when compared with the entire sequence database, and aligns with these proteins as shown. Peptide 2 in Table 2 aligns with sequences in mammalian and *Drosophila* PLC enzymes, and this sequence is similar but not identical to that of a fragment of PLC-140 included in Table 2 that we reported previously (28).

**G Protein Regulation of PLC Activity.** Activated (GTP $\gamma$ S-bound) and inactive (GDP-bound) preparations of soluble squid  $G_q$  were isolated from visual membranes and tested for their ability to activate PLC-70. The soluble fractions from both GDP and GTP $\gamma$ S preparations following purification appeared as homogeneous preparations composed of a single 44-kDa protein on SDS-PAGE that could be visualized by an antibody raised against a peptide encoding the C-terminal sequence common to mammalian  $G_{q11}$  proteins and squid  $G_q$  (Figure 5). As shown in Figure 6, reconstitution of PLC-70 with a molar excess of GTP $\gamma$ S-bound  $G_q$  increased enzymatic activity in the presence of 1  $\mu$ M  $Ca^{2+}$ . This enhancement of PLC activity was similar to that seen using purified PLC-140 in the same assay and comparable to the effect of AIF $^-$ -activated  $G_q$  on this enzyme as reported previously (28). In contrast, the GDP-bound form of the G protein was without effect on either PLC isozyme. Further examination of the activation of PLC-70 over a range of  $Ca^{2+}$  concentrations demonstrated that the presence of GTP $\gamma$ S- $G_q$  shifted the  $Ca^{2+}$  sensitivity of the enzyme such that it was now maximally active in the presence of 1  $\mu$ M  $Ca^{2+}$  (Figure 7).

**PLC-70 and PLC-140 Quantification and Localization by Immunoblotting.** To determine and compare the quantity of

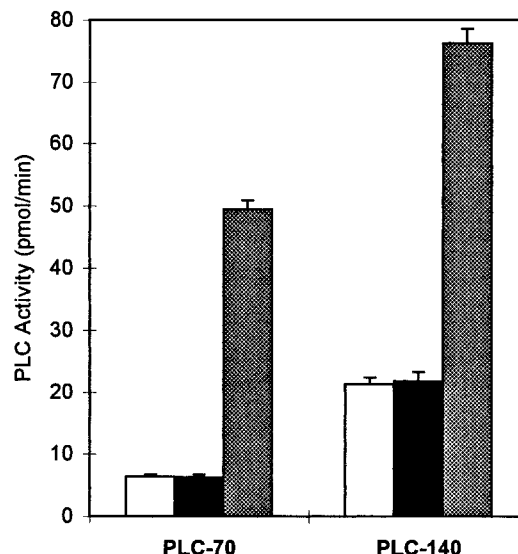


FIGURE 6: Effect of GDP- $G_q$  and GTP $\gamma$ S- $G_q$  on PLC-70 and PLC-140 activity. Purified PLC-70 and PLC-140 (2 ng each) were assayed alone (open bars), with 10 ng of purified GDP- $G_q$  (solid bars), or with 10 ng of purified GTP $\gamma$ S- $G_q$  (stippled bars). Assays were performed in the presence of 1  $\mu$ M free  $Ca^{2+}$  with PE/PIP $_2$  phospholipid vesicles. Results shown are the means of triplicate determinations  $\pm$  SE and are representative of three separate preparations of G proteins and PLC enzymes.

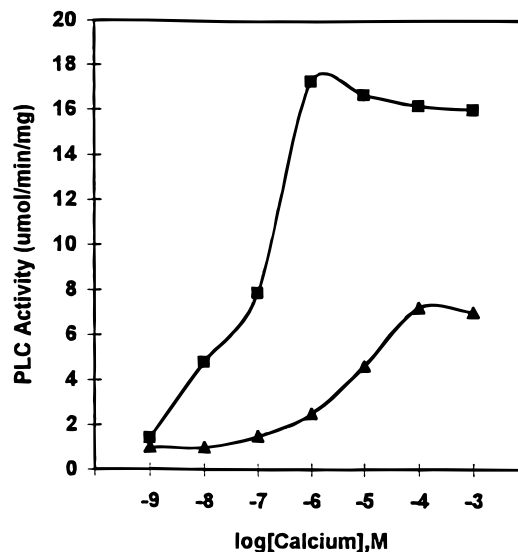


FIGURE 7: Effect of GTP $\gamma$ S- $G_q$  on PLC-70 activity at varying calcium concentrations. Purified PLC-70 (2 ng) was incubated with PE/PIP $_2$  phospholipid vesicles in the presence (■) or absence (▲) of 10 ng of GTP $\gamma$ S- $G_q$  and the indicated concentrations of free  $Ca^{2+}$ . Assays were performed as described under Experimental Procedures. Results shown are the means of triplicate determinations and are representative of two separate preparations of G proteins and PLC enzymes.

the two PLC isozymes in the squid eye, crude homogenates were subjected to quantitative immunoblotting techniques using the polyclonal antisera raised against each of the purified enzymes. PLC-70 was present at a concentration of 0.064 mg/mg of total protein while PLC-140 was found to be present at a concentration of 0.09 mg/mg of total protein. The two enzymes are therefore quite abundant proteins in the eye, representing approximately 6% and 9%, respectively, of the total protein in the crude homogenate. Examination of the localization of the two enzymes with various membranes and soluble fractions prepared from the

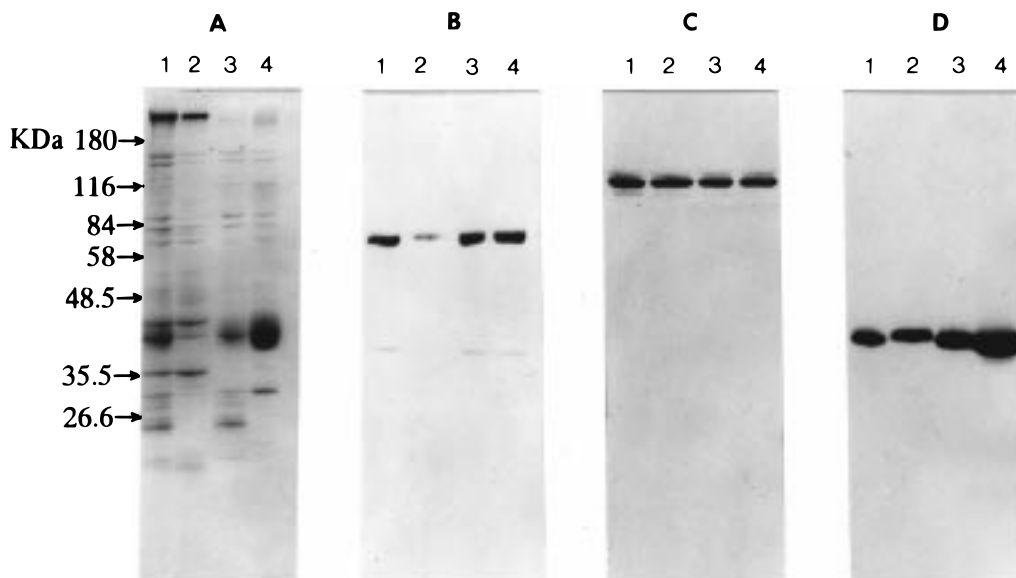


FIGURE 8: SDS-polyacrylamide gel and immunoblot analysis of the distribution of PLC-70, PLC-140, and  $G_q\alpha$  in squid retinal fractions. Samples of 10  $\mu$ g of crude retinal homogenate (lanes 1), soluble proteins (lanes 2), P2 membranes (lanes 3), and rhabdomeral membranes (lanes 4) were separated on 11% polyacrylamide gels. The gels were either stained with Coomassie blue (panel A) or transferred to nitrocellulose membranes and immunoblotted with antibodies to purified PLC-70 (panel B), purified PLC-140 (panel C), or antipeptide antibody to the C-terminal region of mammalian  $G_q/11\alpha$  (panel D). Numbers at the side of the gel indicate the migration of protein standards.

crude homogenate is shown in Figure 8. Immunoreactivity with anti-PLC70 antiserum shown in panel B was found in the crude homogenate (lane 1), and fractionated primarily with the membrane fraction (lane 3) with only small amounts found in the soluble fraction (lane 2). PLC-70 was clearly associated with the purified rhabdomeric membranes (lane 4), suggesting that this is the primary site of PLC-70 localization in the squid eye. PLC-140 (panel C), on the other hand, was found in higher concentrations in the soluble fraction (lane 2) but was also found associated with the P2 and rhabdomeric membrane fractions. This difference in distribution of the two PLC isozymes accounts for the greater abundance of PLC-140 found throughout our purification scheme from the cytosolic fraction (Table 1). As noted above, PLC-70 and PLC-140 are present in similar concentrations in the whole eye, and therefore PLC-70 is actually in molar excess over that of PLC-140. Neither of the PLC enzymes appear to be very tightly associated with the membrane as both could be removed by washing membranes in the presence of 1 M NaCl (data not shown).

$G_q\alpha$  was also found in both the soluble and membrane fractions as noted earlier. As shown in Figure 8, panel D,  $G_q\alpha$  is enriched in the rhabdomeric membrane fraction (lane 4). Quantification of this G protein in the total eye homogenate demonstrated a concentration of 0.11 mg/mg of total protein.

## DISCUSSION

The results of experiments presented here clearly demonstrate the presence of a 70-kDa enzyme in squid photoreceptors that shares biochemical characteristics with the PLC- $\beta$  family of proteins, namely, hydrolysis of phosphatidylinositol phospholipids, calcium dependence, and activation by a  $G_q$ -like protein. PLC-70 was functionally distinguished from the other major PLC enzyme expressed in this tissue, PLC-140, by differential sensitivity of the two enzymes to calcium. The activity of PLC-70 was maximal in the

presence of 100  $\mu$ M  $Ca^{2+}$ , similar to the calcium sensitivity of many mammalian PLC isozymes (38, 39), whereas PLC-140 was maximally stimulated with 1  $\mu$ M  $Ca^{2+}$ . PLC-70 also demonstrated a different activity profile from that found previously for PLC-140 when assayed under conditions of variable pH. The pH optimum of PLC-70 was pH 5.0, and activity diminished at more basic or acidic pH levels. This property resembles those described for many mammalian PLC isozymes including PLC- $\beta_4$  (38, 40–43), but differs from PLC-140 and mammalian PLC- $\beta_1$ –3 isozymes which are more active at neutral pH (28, 43–45). Also in keeping with other PLC- $\beta$  isozymes, PLC-70 showed a marked preference for  $PIP_2$  over PI as substrate at all concentrations of calcium.

We have previously reported the activation of PLC-140 by AIF $^-$ -activated  $G_q$  (28). In this report,  $G_q\alpha$  was prepared from photoreceptor membranes in an inactive (GDP-bound) and an active (GTP $\gamma$ S-bound) form by first binding the guanine nucleotides to the G protein in the membrane environment and then purifying the solubilized  $\alpha$  subunits. When these were used in reconstitution studies with both enzymes, only the GTP $\gamma$ S-bound form of the G protein activated the PLC enzymes. Activation of PLC-70 by GTP $\gamma$ S- $G_q$  was seen over the entire range of  $Ca^{2+}$  concentrations, similar to the effect of AIF $^-$ - $G_q$  on PLC-140 previously reported. The major difference between the effect of the G protein on the two enzymes was the shift of calcium sensitivity seen for PLC-70 which was not seen with PLC-140. This effect is reminiscent of that reported for mammalian  $G_q$  and PLC- $\beta$  (14), and may play a role in the activation of PLC-70 under physiological conditions. Intracellular calcium levels have been reported to rise from 0.6 to 50  $\mu$ M upon illumination in *Limulus* photoreceptors (46). If a similar range in cytosolic free  $[Ca^{2+}]$  exists in squid photoreceptors during illumination, then the activity of PLC-70 at low illumination (low  $[Ca^{2+}]$ ) would be greatly amplified by the presence of activated  $G_q$ , and thus tightly

coupled to rhodopsin activation of this G protein. Thus, the shift in calcium sensitivity of PLC-70 may play a role in maximizing the PLC response at low  $\text{Ca}^{2+}$  levels by targeting two enzymes with the same G protein. Estimates of the abundance of the two PLC enzymes in squid photoreceptors indicate that PLC-70 is approximately 1.5 times the molar concentration of PLC-140, based on the amounts found in crude eye homogenate.  $\text{G}_q\alpha$  protein levels in the squid eye suggest that this G protein is present in approximately the same molar concentration as that of PLC-140 and PLC-70 combined, and therefore could activate both enzymes in response to sufficient rhodopsin activation. It is not clear from our studies why this system has two PLC enzymes to perform apparently the same function, and further studies to inactivate the individual enzymes *in vivo* are needed in order to discern their individual roles (if any) in visual transduction.

Squid  $\text{G}_q$  exists in two forms, a membrane-bound form and a soluble form. In the membrane, the G protein interacts with rhodopsin to rapidly exchange guanine nucleotides. The G proteins can also be eluted into aqueous buffers in both an active and an inactive state. It is this soluble form of the  $\text{GTP}\gamma\text{S}$ -activated G protein that interacts with the PLC enzymes most efficiently in our reconstitution system. Experiments using G proteins extracted in several different detergents have yielded only limited activation of PLC-140 and PLC-70 by the insoluble  $\text{G}_q$  in our hands. However, this may be the result of detergent effects on the enzymes since Suzuki et al. have reported that detergent-extracted  $\text{G}_q$  from squid can activate soluble PLC if the G protein is first incorporated into lipid vesicles (29). Further studies using both preparations of  $\text{G}_q$  are needed to directly compare the potencies of the two forms of  $\text{G}_q$  in reconstitution systems with purified PLC enzymes. Nevertheless, the squid  $\text{G}_q$  protein appears to be unique among the  $\text{G}_q$  family of proteins in its ability to be solubilized and to activate PLC enzymes in a soluble form as shown here using  $\text{GTP}\gamma\text{S}$ - $\text{G}_q$  as well as previously using  $\text{AlF}^-$ -activated  $\text{G}_q$ . There is an intriguing parallel between the squid  $\text{G}_q$  and mammalian retinal  $\text{G}_t\alpha$  which is also soluble in aqueous buffers when activated. Indeed, the solubility of  $\text{G}_t$  is thought to facilitate the very large amplification of the visual signal from a rhodopsin molecule to hundreds of  $\text{G}_t$  proteins (47). The apparent solubility of visual  $\text{G}_q\alpha$  may therefore serve a similar role in allowing rapid activation of multiple G proteins in the invertebrate system.

In summary, a growing body of evidence has now demonstrated that light-dependent activation of PLC enzymes is the major signal transduction pathway in invertebrate photoreceptors. We have shown that two highly abundant PLC enzymes exist in squid photoreceptors, and that activation of both enzymes at low intracellular  $\text{Ca}^{2+}$  levels is amplified greater than 10-fold by activated  $\text{G}_q\alpha$ . It remains to be demonstrated what role these two PLC enzymes play in regulating  $\text{IP}_3$  levels *in vivo*, and how this mediates depolarization of the photoreceptor membrane.

## ACKNOWLEDGMENT

We are grateful to Dr. John K. Northup for the gift of squid eyes and for continuing encouragement of this work.

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BI972768A