Purification, G Protein Activation, and Partial Amino Acid Sequence of a Novel Phospholipase C from Squid Photoreceptors †

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Purification, G Protein Activation, and Partial Amino Acid Sequence of a Novel Phospholipase C from Squid Photoreceptors†

Jane Mitchell* and Linnia H. Mayeenuddin

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

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ABSTRACT: Invertebrate visual signal transduction is initiated by rhodopsin activation of a guanine nucleotide binding protein, G_q, which stimulates phospholipase C (PLC) activity. We have previously purified a 140-kDa PLC enzyme from squid photoreceptors that is regulated by squid G_q. 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_2) by PLC-70 was optimal at pH 5 in the presence of 100 μM Ca^{2+} with a specific activity of 10.3 μmol min^{-1} mg^{-1}. 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 aureaus* 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 β isozymes. Reconstitution of purified GTPγS-bound soluble squid G_q with PLC-70 resulted in significant enhancement of PIP_2 hydrolysis over a range of Ca^{2+} concentrations and shifted the maximum activation by calcium to 1 μM. These results suggest that cephalopod phototransduction is mediated by G_q 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[1] 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_3) levels has been shown to activate release of Ca^{2+} 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 $G_q\alpha$ proteins (*10*, *11*) have both been cloned. The primary sequences of these two G protein α subunits show homology to mammalian proteins of the G_q family. The homology with $G_q\alpha$ is greatest in the carboxyl terminus, a region of these G protein α subunits which has been shown to interact with their target PLC enzymes (*12*−*16*). Squid Gβ subunits and *Drosophila* G_βe are also highly homologous to their mammalian counterparts (*17*, *18*); however, the invertebrate visual G protein γ subunits appear to be quite distinct and bear little overall sequence homology to mammalian γ 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_βe *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_q 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-β isozymes (*24*−*26*), and isolated *norpA* protein demonstrated Ca^{2+}-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_3 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-β subtypes (*28*). Purified PLC-140 is a Ca^{2+}-sensitive inositolphospholipid hydrolase whose activation is profoundly enhanced by reconstitution with activated soluble squid G_q (*28*). Others have also isolated a similar PLC protein from squid and shown that it can be activated by insoluble G_q if this protein is first incorporated into lipid vesicles (*29*).

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* 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.

[1] Abbreviations: G protein, guanine nucleotide binding protein; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate; PE, phosphatidylethanolamine; PI, phosphatidylinositol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; GTPγS, guanosine 5′-3-*O*-(3-thiotriphosphate); PAGE, polacrylamide gel electrophoresis.

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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 Ca^{2+} and regulated by soluble squid 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 PIP_2 were purchased from Sigma. Phosphatidyl[2-^{3}H]inositol 4,5-bisphosphate (16.3 Ci/mmol), phosphatidyl[2-^{3}H]inositol (1 Ci/mmol), and anti $G_{q/11}\alpha$ antiserum were purchased from DuPont−New England Nuclear. Anti-PLC70 and anti-PLC140 antisera were produced in rabbits using 100 μg 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. aureaus* 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 $MgSO_4$, 100 mM KCl, 0.1% deoxycholate, 50 μM PIP_2, 250 μM PE containing 25 000 dpm of [^{3}H]PIP_2, 2 mM EGTA, and the concentration of free Ca^{2+} indicated in individual experiments. The total incubation volume was 100 μL, and reactions were terminated as previously described (*28*). Free 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 μM 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 30000*g* 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 $G_q\alpha$ 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 30000*g* sedimentation step in the photoreceptor preparation, and rhabdomeric membranes were used as a source of G_q.

Cytosol was diluted with an equal volume of 100 mM Mes, pH 6.0, 100 mM NaCl, 1 μM 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 × 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 μg 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. aureaus* V8. The final reaction mixture for digestion with trypsin contained 50 mM Tris-HCl, pH 7.6, 6 mM $MgCl_2$, 75 mM sucrose, 1 mM dithiothreitol, 1 mM EDTA, and 0.6% (v/v) Lubrol PX, in a final volume of 20 μL 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 $MgCl_2$, 1 mM dithiothreitol, and 200 mM NaCl also in 20 μL volume, and the samples were incubated for 90 min at 30 °C. All reactions were stopped by the addition of 5 μL 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 μg samples of PLC-70 were digested with *S. aureaus* 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. G_q α 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 GTPγS was bound to the G protein α subunits before extraction since squid G_q does not efficiently bind guanine nucleotides once it has been released from the photoreceptor membrane.[2] 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 $MgCl_2$, and either 1 mM GDP or 1 mM GTPγS 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 100000*g* at 4 °C to separate soluble from membrane bound proteins. GDP-G_q and GTPγS-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 $MgCl_2$ (TEM) to reduce

[2] J. Mitchell, C. Bamsey, and R. Cheung, unpublished data.

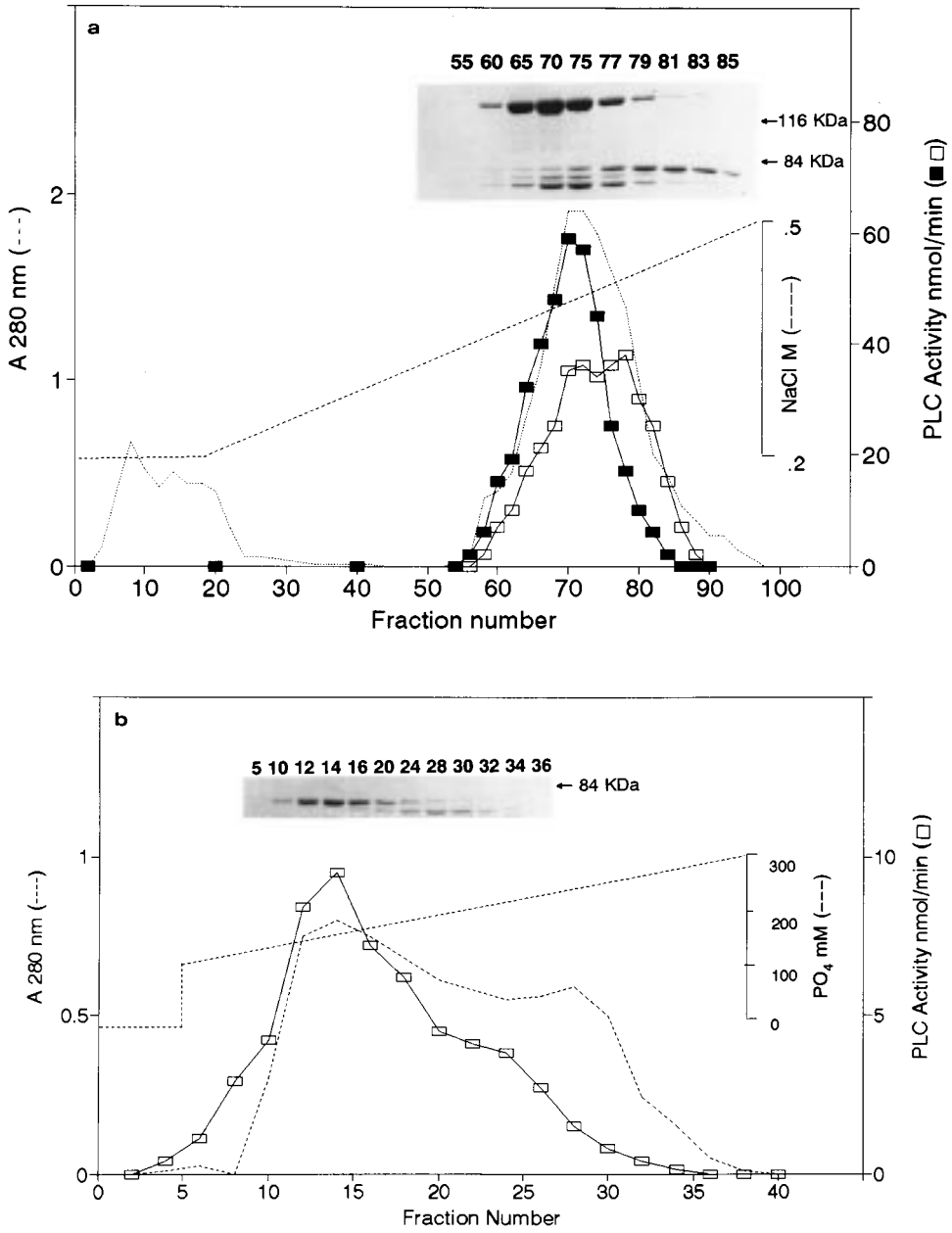

FIGURE 1: Purification of PLC-70 from squid retinal extract by sequential chromatography on heparin−Sepharose (a) and hydroxyapitite (b). PLC activity of each fraction was assayed using PE/PIP_2 mixed phospholipid vesicles in the presence of 1 μM (■), or 100 μM (□) free Ca^{2+} as described under Experimental 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 $G_q\alpha$ was identified by immunoblot using an antibody to mammalian $G_{q/11}\alpha$. G protein samples were pooled and concentrated by filtration using a PM-10 filter.

G_q-PLC Reconstitutions. For measurement of G protein regulated PLC activity, aliquots of GDP- or GTPγS-bound G_q (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_2/PE vesicles (400 μM PIP_2, 8 mM PE containing 25 000 dpm of [^{3}H]PIP_2 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 $MgSO_4$, 2 mM EGTA, 1 μM free Ca^{2+} 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-$G_q\alpha$, 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 $G_q\alpha$ 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[a]

purification step	PLC	protein (mg)	total activity (μmol/min)	specific activity (μmol min^{-1} 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[b]	1.39	7.9	5.1	10.4
hydroxyapitite	−70[b]	0.63	4.7	7.4	15.1

[a] PLC activity in each step was determined using [^{3}H]PIP_2 as a substrate in the presence of 1 μM free Ca^{2+} for total PLC activity and PLC-140. [b] The PLC activity of PLC-70 was measured in the presence of 100 μM free Ca^{2+}.

run on SDS−PAGE gels using six different concentrations of membranes ranging from 0.1 to 10 μg of total protein along with purified PLC enzymes or $G_q\alpha$ 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 Ultroscan XL laser densitometer, and the area of each band was determined using Gel-Scan XL software (Pharmacia). The amount of each PLC enzyme or $G_q\alpha$ 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 μM free Ca^{2+} and PIP_2 in liposomes containing deoxycholate (Figure 1a, closed symbols). When the same samples were assayed in the presence of 100 μM free Ca^{2+} 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^{2+} and pH Dependence of PLC-70 Activity. The Ca^{2+} dependence of hydrolysis of PIP_2 and PI by PLC-70 was examined (Figure 2). PIP_2 hydrolysis by the purified enzyme

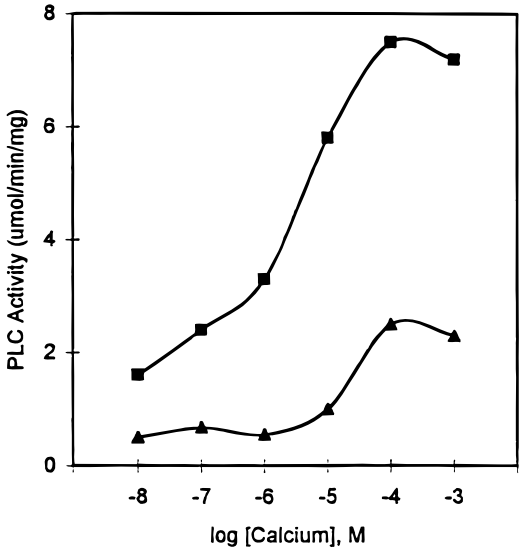

FIGURE 2: Ca^{2+} dependence of phosphoinositide hydrolysis by purified PLC-70. Mixed phospholipid vesicles of PE/PIP_2 (■) or PE/PI (▲) in a molar ratio of 5:1 were used as substrates. Assays were performed using various free Ca^{2+} concentrations at pH 7.0.

increased slowly at low Ca^{2+} concentrations, reached a maximum at 100 μM Ca^{2+}, and then decreased slightly at millimolar concentrations (Figure 2). The effect of Ca^{2+} on the hydrolysis of PI was similar to that seen for PIP_2; 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_2 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. aureaus* 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-

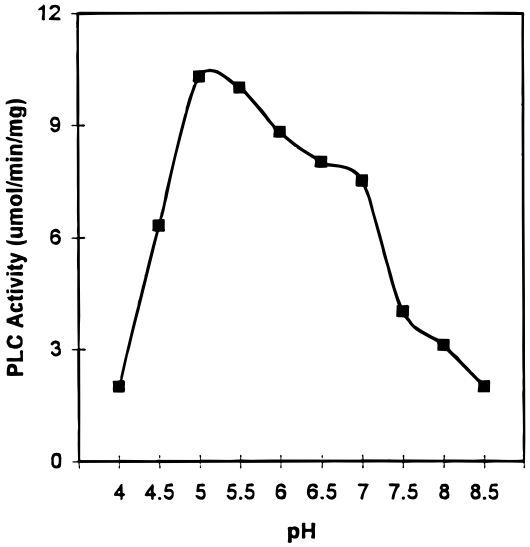

FIGURE 3: Effect of pH on hydrolysis of PIP2 by PLC-70. PLC activity was determined using Bis-Tris propane buffer at various pH values. The reaction mixture contained 100 μM free Ca^{2+} and PE/PIP_2 phospholipid vesicles as substrate and were performed as described under Experimental Procedures.

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 μg 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 μg 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 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.

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

Isozyme	Amino acid sequence[a]
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–β1 747–758	I a a Y E E g g K i l G
PLC–β2 749–760	v a v m E E g n K F i G
PLC–β3 778–789	I a a f E E g g r F v G
PLC–β4 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–β1 733–742	v f K K v V L P s l
PLC–β2 734–743	v f e K I l m P e l
PLC–β3 764–773	d f p K v V L P t l
PLC–β4 759–768	v f r K v i L P D l
PLC–140[b]	v f K K v V L P D l
Peptide 3[c]	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–β1 597–609	i y p K G t r v D S S n y
PLC–β2 601–613	i y p K G t r v D S S n y
PLC–β3 629–641	i y p K G t r v D S S n y
PLC–β4 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–β1 375–391	t h g f T M T t e i s f k e v i e
PLC–β2 372–389	t h g f T M T t d i f f k e a i e
PLC–β3 358–374	t h g f T M T t e V p L r D v l e
PLC–β4 372–389	t h g k a M c t d i l f k D v i q

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

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

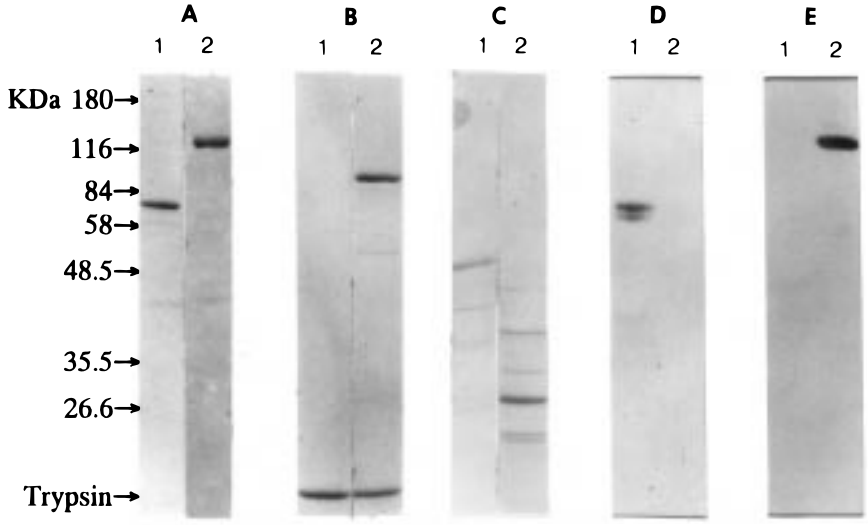

FIGURE 4: Proteolytic digestion and immunological recognition of PLC-70 and PLC-140. Five microgram aliquots of PLC-70 (lanes 1) or PLC-140 (lanes 2) were separated on 11% polyacrylamide gels following incubation without enzymes (A, D, and E) or following digestion with trypsin (B) or *S. aureus* V8 (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.

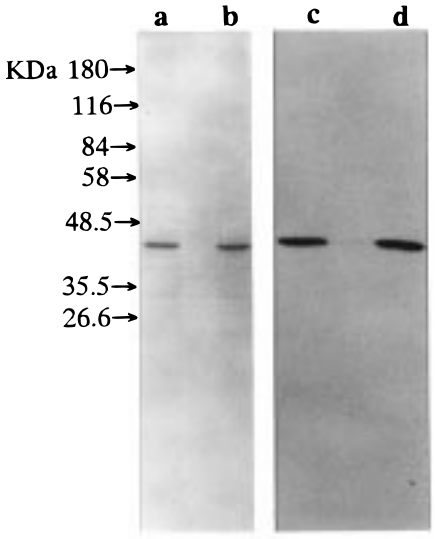

FIGURE 5: SDS−polyacrylamide gel and immunoblot analysis of purified squid retinal $G_q\alpha$. Guanine nucleotides were bound to squid G_q proteins in rhabdomeric membranes prior to extraction in 1 M KCl. Extracts were purified by chromatography on DEAE-Sephacel; fractions containing G_q were pooled 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 $G_{q/11}$ (c and d). GDP-G_q, lanes a and c; GTPγS-G_q, lanes b and d. Numbers at the side of the gel indicate the migration of protein standards.

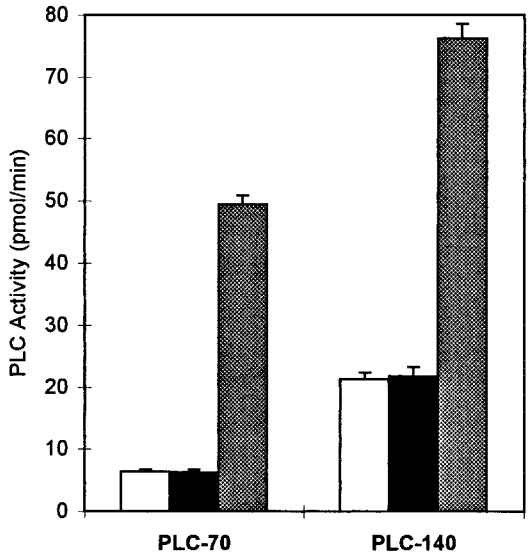

FIGURE 6: Effect of GDP-G_q and GTPγS-G_q on PLC-70 and PLC-140 activity. Purified PLC-70 and PLC-140 (2 ng each) were assayed alone (open bars), with 10 ng of purified GDP-G_q (solid bars), or with 10 ng of purified GTPγS-G_q (stippled bars). Assays were performed in the presence of 1 μM free Ca^{2+} with PE/PIP_2 phospholipid vesicles. Results shown are the means of triplicate determinations ± SE and are representative of three separate preparations of G proteins and PLC enzymes.

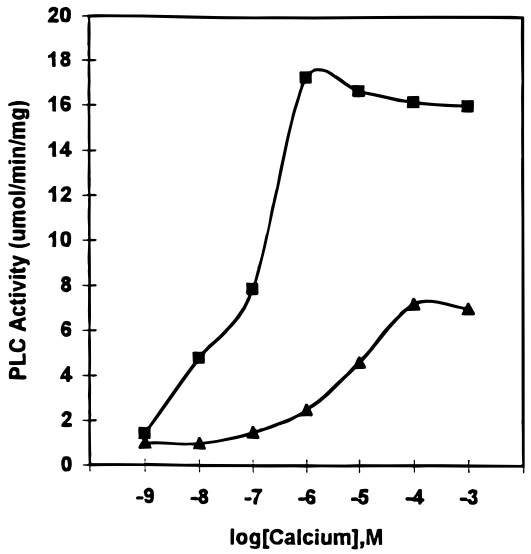

FIGURE 7: Effect of GTPγS-G_q on PLC-70 activity at varying calcium concentrations. Purified PLC-70 (2 ng) was incubated with PE/PIP_2 phospholipid vesicles in the presence (■) or absence (▲) of 10 ng of GTPγS-G_q and the indicated concentrations of free Ca^{2+}. Assays were performed as described under Experimental Procedures. Results shown are the means of triplicate determinations and are representative of two separate preparations of G proteins and PLC enzymes.

identified from other members of the PLC β family of proteins: mammalian PLC-β1−4, *Drosophila* visual norpA, and *Drosophila* neuronal PLC21. PLC-70 sequences 1−3 matched the two *Drosophila* proteins and mammalian PLC-β1 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 (GTPγS-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 GTPγS 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 $G_{q/11}$ proteins and squid G_q (Figure 5). As shown in Figure 6, reconstitution of PLC-70 with a molar excess of GTPγS-bound G_q increased enzymatic activity in the presence of 1 μM 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 AlF^--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 GTPγS-G_q shifted the Ca^{2+} sensitivity of the enzyme such that it was now maximally active in the presence of 1 μM Ca^{2+} (Figure 7).

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

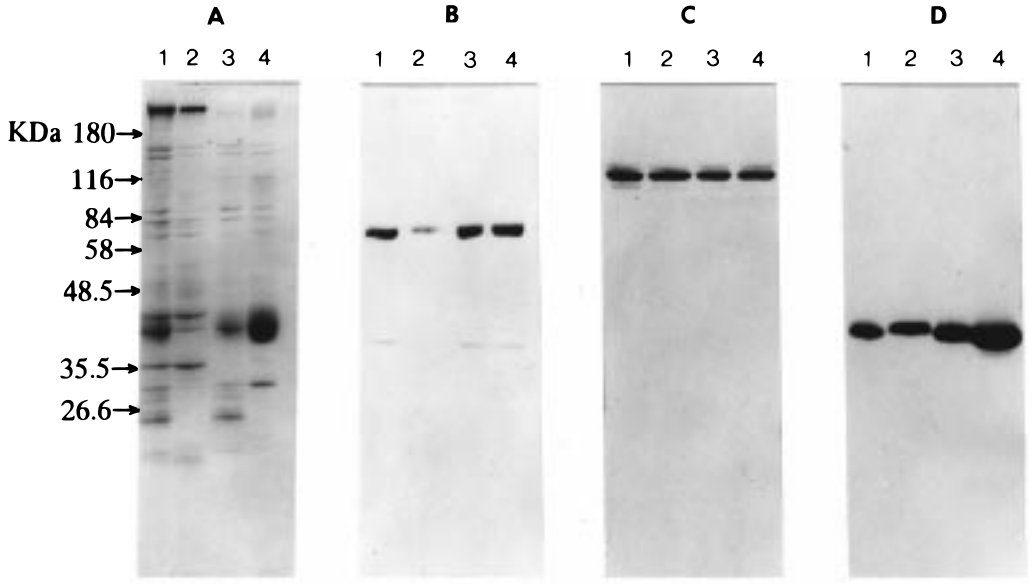

FIGURE 8: SDS−polyacrylamide gel and immunoblot analysis of the distribution of PLC-70, PLC-140, and $G_q\alpha$ in squid retinal fractions. Samples of 10 μg of crude retinal homogenate (lanes 1), soluble proteins (lanes 2), P2 membranes (lanes 3), and rhabdomeral membranes (lanes 4) were separated on 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-β 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 μM Ca^{2+}, similar to the calcium sensitivity of many mammalian PLC isozymes (*38*, *39*), whereas PLC-140 was maximally stimulated with 1 μM 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-β4 (*38*, *40*−*43*), but differs from PLC-140 and mammalian PLC-β1−3 isozymes which are more active at neutral pH (*28*, *43*−*45*). Also in keeping with other PLC-β 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 AlF^--activated G_q (*28*). In this report, $G_q\alpha$ was prepared from photoreceptor membranes in an inactive (GDP-bound) and an active (GTPγS-bound) form by first binding the guanine nucleotides to the G protein in the membrane environment and then purifying the solubilized α subunits. When these were used in reconstitution studies with both enzymes, only the GTPγS-bound form of the G protein activated the PLC enzymes. Activation of PLC-70 by GTPγS-G_q was seen over the entire range of Ca^{2+} concentrations, similar to the effect of AlF^--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-β (*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 μM 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 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. $G_q\alpha$ 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 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 GTPγS-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 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 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 G_q are needed to directly compare the potencies of the two forms of G_q in reconstitution systems with purified PLC enzymes. Nevertheless, the squid G_q protein appears to be unique among the G_q family of proteins in its ability to be solubilized and to activate PLC enzymes in a soluble form as shown here using GTPγS-G_q as well as previously using AlF^--activated G_q. There is an intriguing parallel between the squid G_q and mammalian retinal $G_t\alpha$ which is also soluble in aqueous buffers when activated. Indeed, the solubility of G_t is thought to facilitate the very large amplification of the visual signal from a rhodopsin molecule to hundreds of G_t proteins (*47*). The apparent solubility of visual $G_q\alpha$ 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 Ca^{2+} levels is amplified greater than 10-fold by activated $G_q\alpha$. It remains to be demonstrated what role these two PLC enzymes play in regulating IP_3 levels in vivo, and how this mediates depolarization of the photoreceptor membrane.

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REFERENCES

1. Brown, J. E., Rubin, L. J., Ghalayini, A. J., Tarver, A. L., Irvine, R. F., and Berridge, M. J. (1984) *Nature 311*, 160−163.
2. Fein, A., Payne, R., Corson, D. W., Berridge, M. J., and Irvine, R. F. (1984) *Nature 311*, 157−160.
3. Yoshika, T., Inoue, H., and Hotta, Y. (1985) *J. Biochem. (Tokyo) 97*, 1251−1254.
4. Payne, R. (1986) *Photobiochem. Photobiophys. 13*, 373−397.
5. Fein, A. (1986) *Trends Neurosci. 9*, 110−114.
6. Toyoshima, S., Matsumoto, N., Wang, P., Inoue, H., Yoshioka, T., Hotta, Y., and Osawa, T. (1990) *J. Biol. Chem. 265*, 14842−14848.
7. Zuker, C. S., Cowman, A. F., and Rubin, G. M. (1985) *Cell 40*, 851−858.
8. Hall, M. D., Hoon, M. A., Ryba, N. J. P., Pottinger, J. D. D., Keen, J. N., Saibil, H. R., and Findlay, J. B. C. (1991) *Biochem. J. 274*, 35−40.
9. Lee, Y., Dobbs, M. B., Veradi, M. L., and Hyde, D. R. (1990) *Neuron 5*, 889−898.
10. Pottinger, J. D. D., Ryba, N. J., Keen, L. N., and Findlay, J. B. C. (1991) *Biochem. J. 279*, 323−326.
11. Ryba, N. J. P., Findlay, J. B. C., and Reed, J. D. (1993) *Biochem J. 292*, 333−341.
12. Pang, I.-H., and Sternweis, P. C. (1990) *J. Biol. Chem. 265*, 18707−18712.
13. Strathmann, M. P., and Simon, M. I. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 9113−9117.
14. Smrcka, A. V., Hepler, J. R., Brown, K. O., and Sternweis, P. C. (1991) *Science 252*, 804−807.
15. Taylor, S. J., Chae, H. Z., Rhee, S. G., and Exton, J. H. (1991) *Nature 350*, 516−518.
16. Lee, C. H., Park, D., Wu, D., Rhee, S. G., and Simon, M. I. (1992) *J. Biol. Chem. 267*, 16044−16047.
17. Yarfitz, S., Provost, N. M., and Hurley, J. B. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7134−7138.
18. Ryba, N. J. P., Pottinger, J. D. D., Keen, J. N., and Findlay, J. B. C. (1991) *Biochem. J. 273*, 225−228.
19. Lott, J. S., Ryba, N. J. P., Pottinger, J. D. D., Keen, J. N., Carne, A., and Findlay, J. B. C. (1992) *FEBS Lett. 312*, 241−244.
20. Ray, K., and Ganguly, R. (1992) *J. Biol. Chem. 267*, 6086−6092.
21. Dolph, P. J., Hing, H. M. S., Yarfitz, S., Colley, N. J., Running Deer, J., Spender, M., Hurley, J. B., and Zuker, C. S. (1994) *Nature 370*, 59−61.
22. Yarfitz, S. L., Running Deer, J. L., Froelick, G., Colley, N. J., and Hurley, J. B. (1994) *J. Biol. Chem.* 1251−1254.
23. Running Deer, J. L., Hurley, J. B., and Yarfitz, S. L. (1995) *J. Biol. Chem. 270*, 12623−12628.
24. Inoue, H., Yoshika, T., and Hotta, Y. (1985) *Biochem. Biophys. Res. Commun. 132*, 513−519.
25. Inoue, H., Yoshika, T., and Hotta, Y. (1988) *J. Biochem. (Tokyo) 103*, 91−94.
26. Bloomquist, B. T., Shortridge, R. D., Schneuwly, S., Perdew, M., Montell, C., Steller, H., Rubin, G., and Pak, W. L. (1988) *Cell 54*, 723−733.
27. Scott, K., Becker, A., Sun, Y., Hardy, R., and Zuker, C. (1995) *Neuron 15*, 919−927.
28. Mitchell, J., Goutierrez, J., and Northup, J. K. (1995) *J. Biol. Chem. 270*, 854−859.
29. Suzuki, T., Akihisa, T., Narita, K., Nagai, K., Tsukahara, Y., and Kito, Y. (1995) *FEBS Lett. 377*, 333−337.
30. Harlow, E., and Lane, D., Eds. (1988) *Antibodies: A Laboratory Manual*, pp 92−120, Cold Spring Harbor Laboratory Publications, Cold Spring Harbor, NY.
31. Raafluab, J. (1960) *Methods Biochem. Anal. 3*, 301−325.
32. Kito, Y., Takahara, S., and Higins, F. M. (1982) *Methods Enzymol. 81*, 43−48.
33. LeGendre, N., and Matsudaira, P. (1989) in *A Practical Guide to Protein and Peptide Purification and Microsequencing* (Matsudaira, P., Ed.) pp 49−63, Academic Press Inc., San Diego, CA.
34. Laemmli, U. K. (1970) *Nature 227*, 680−685.

35. Schaffner, W., and Weissmann, C. (1973) *Anal. Biochem. 56*, 502−514.
36. Towbin, H., Staehelin, T., and Gordon, T. (1986) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350−4354.
37. Mitchell, J., and Bansal, A. (1997) *Am. J. Physiol. 273*, E528−E535.
38. Ryu, S. H., Suh, P.-G., Cho, K. S., Lee, K.-Y., and Rhee, S. G. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6649−6653.
39. Jhon, D.-Y., Lee, H.-H., Park, D., Lee, C.-W., Lee, K.-H., Yoo, O. J., and Rhee, S. G. (1993) *J. Biol. Chem. 268*, 6654−6661.
40. Katan, M., and Parker, P. J. (1987) *Eur. J. Biochem. 168*, 413−418.
41. Banno, Y., Yu, A., Nakashima, T., Homma, Y., Takenawa, T., and Nozawa, Y. (1990) *Biochem. Biophys. Res. Commun. 167*, 396−401.
42. Homma, Y., Emori, Y., Shabashaki, F., Suzuki, K., and Takanawa, T. (1990) *Biochem. J. 269*, 13−18.
43. Min, D. S., Kim, D. M., Lee, Y. H., Seo, J., Suh, P.-G., and Rhee, S. G. (1993) *J. Biol. Chem. 268*, 12207−12212.
44. Homma, Y., Imaki, J., Nakanishi, O., and Takenawa, T. (1988) *J. Biol. Chem. 263*, 6592−6598.
45. Baldassare, J. J., Henderson, P. A., and Fisher, G. J. (1989) *Biochemistry 28*, 56−60.
46. Nagy, K. (1991) *Q. Rev. Biophys. 24*, 165−226.
47. Fung, B. K.-K., and Stryer, L. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2500−2504.

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