A Lobster Phospholipase C- β That Associates with G-Proteins in Response to Odorants

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A cDNA clone encoding a protein of 1116 amino acids with significant homology to β -isoforms of phospholipase C was isolated from lobster olfactory organ cDNA libraries and named lobPLC β . This cDNA hybridized predominantly to a 9 kb transcript in RNA from olfactory organ, pereopod, brain, and eye-eyestalk and to several smaller minor transcripts only in eye-eyestalk. An antiserum raised to the C terminus of lobPLC β detected immunoreactivity in a single 130 kDa band in olfactory aesthetasc hairs, olfactory organ, pereopod, dactyl, and brain. In eye-eyestalk this 130 kDa band was abundant, and minor bands of 100, 79, and 57 kDa also were detected. In cross sections of the aesthetasc hairs, immunoreactivity was de-

tected in the outer dendritic segments of the olfactory receptor neurons, the site of olfactory transduction. A complex odorant caused lobPLC β immunoreactivity to increase in membrane fractions and decrease in soluble fractions of homogenates of aesthetasc hairs. The odorant also increased the amount of lobPLC β in immunoprecipitates of G $_{\alpha q}$ and G $_{\beta}$ from homogenates of aesthetasc hairs. These results support the conclusion that lobPLC β mediates olfactory transduction.

Key words: olfaction; vision; sensory transduction; phospholipase C; inositol phospholipids; inositol 1,4,5-trisphosphate; GTP binding protein; G-protein; Crustacea; Arthropoda

In arthropods the inositol 1,4,5-trisphosphate (IP $_3$) pathway is the primary mechanism of olfactory transduction (Ache, 1994; Hildebrand and Shepherd, 1997). The phospholipase C- β (PLC- β) that mediates olfactory transduction in most arthropod olfactory receptor neurons has yet to be identified. However, the *Drosophila norpA* gene product, a PLC- β that mediates phototransduction, is also necessary for olfactory transduction in a subset of olfactory receptor neurons in the *Drosophila* maxillary palp (Pak et al., 1970; Deland and Pak, 1973; Pak, 1995; Riesgo-Escovar et al., 1995; Zuker, 1996). In the main olfactory organ on the first antennae of lobsters, evidence suggests that a similar PLC- β is the central enzyme in the IP $_3$ olfactory transduction pathway. (1) Odor-activated depolarization is GTP-dependent and can be blocked by antisera specific for G $_{\alpha q}$ (Fadool et al., 1995). (2) Odorants stimulate IP $_3$ production in homogenates containing olfactory dendrites (Boekhoff et al., 1994). (3) Ion channels gated by IP $_3$ are present in the dendrites of the olfactory receptor neurons (Hatt and Ache, 1994). In ~50% of lobster olfactory receptor neurons this IP $_3$ pathway coexists with a cAMP pathway (Michel and Ache, 1992), which results in a hyperpolarizing receptor potential that can sum with depolarization during stimulation with odorant mixtures (McClintock and Ache, 1989; Michel et al., 1991; Michel and Ache, 1992). The role of the cAMP pathway appears to be a modification of the primary, excitatory signal mediated by PLC- β .

Because it is the central enzyme in the primary transduction pathway and a likely site for feedback regulation from both

transduction pathways, we attempted to identify a PLC from the lobster olfactory organ that would respond to odorants. In mammals there are four PLC- β , two PLC- γ , and four PLC- δ isoforms (Rhee and Bae, 1997). The evidence that lobster olfactory transduction involves heterotrimeric G-proteins implicates PLC- β isoforms because they are activated by G $_{\alpha q}$ and G $_{\beta\gamma}$ subunits of G-proteins (Smrcka et al., 1991; Taylor et al., 1991; Jhon et al., 1993; Jiang et al., 1994). PLC- β isoforms have a modular structure that includes a pleckstrin homology (PH) domain that is a possible site for interaction with G $_{\beta\gamma}$, a bipartite catalytic domain separated by a G $_{\beta\gamma}$ interaction site, and a C-terminal domain that contains a G $_{\alpha q}$ interaction site (Ellis et al., 1993; Wu et al., 1993; Essen et al., 1996, 1997; Kim et al., 1996; Kuang et al., 1996; Shaw, 1996; James and Downes, 1997; Rhee and Bae, 1997). In this report we describe the isolation and characterization of a cDNA encoding a lobster PLC- β that is shared by the olfactory and visual systems. Odorants caused this PLC- β to associate with membranes, G $_{\alpha q}$, and G $_{\beta}$.

MATERIALS AND METHODS

Lobsters. American lobsters, *Homarus americanus*, were purchased from Falmouth Fish Market (Falmouth, MA) and held in artificial seawater at 4°C for no more than 1 week.

Recombinant DNA. Lobster olfactory organ λ ZapII cDNA libraries, RNA extraction, and methods for homology cloning that use PCR were described previously (McClintock et al., 1992, 1997; Xu et al., 1997). Degenerate primers were designed from two highly conserved regions of PLC sequences: TG(T/C)GTIGA(A/G)(C/T)TIGA(T/C)TGITGG (residues 359–365 of bovine PLC- β_1) and TT(A/G)TT(T/C)TTIATIA(A/G)IAT(T/C)TT (residues 461–466 of bovine PLC- β_1). PCR was performed in a 50 μ l total volume containing first-strand cDNA prepared from 0.25 μ g poly(A) $^+$ RNA, 50 pmol of each primer, a 200 μ M concentration of each dNTP, 1.5 U of *Taq* polymerase, and 5 μ l of 10 $\times$ buffer (Promega, Madison, WI). The reaction conditions included the following: 94°C/60 sec, 45°C/60 sec, and 72°C/120 sec for 35 cycles. PCR products were purified, subcloned into pCR2.1 (Invitrogen, San Diego, CA), and sequenced. Random-primed cDNA libraries were plated at 20,000 plaques/plate, transferred to nitrocellulose filter lifts, and screened by hybridization with cDNA probes labeled with [α 32 P]dCTP.

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Plasmids (pBluescript) containing positive clones were rescued according to the manufacturer's protocol (Stratagene, La Jolla, CA). The dideoxynucleotide chain termination method of sequencing double-strand DNA was used on cDNA subclones generated by the Erase-a-Base system (Promega). Northern blotting was done as previously described (McClintock et al., 1992, 1997; Xu et al., 1997).

Generation and purification of an antiserum. An *XhoI* fragment of lobPLC β cDNA encoding residues 824–1116 (named PLC293ct) was ligated into pET-28a(+) and grown in B834 cells (Novagen, Madison, WI). PLC293ct was purified from lysed bacteria, using Ni-NTA agarose per the supplier's instructions (protocol 14; Qiagen, Valencia, CA), and was used for the commercial production of a rabbit antiserum named P293 (BioWorld, Dublin, OH). For preabsorption of the P293 antiserum with PLC293ct, the N-terminal His-tag was removed from PLC293ct by cleavage with 1 U of thrombin/mg protein for 2 hr at 20°C in thrombin cleavage buffer, as described in the pET System manual (Novagen). Thrombin was inactivated by incubation at 95°C for 10 min, and the His-tag cleavage product was removed from the solution by adding an excess of the Ni-NTA agarose, incubating for 1 hr with agitation at ambient temperature, and using column filtration to separate the soluble fraction from the resin.

The immunoreactivity of P293 against PLC293ct was confirmed by ELISA (Harlow and Lane, 1988). For affinity purification of P293, PLC293ct was coupled to activated agarose beads in 100 mM phosphate buffer, pH 10, using the manufacturer's procedure (Bio-Rad, Hercules, CA). The column was washed with 20 column volumes of 100 mM phosphate buffer with decreasing pH values (10, 8, 6, and 4) and 100 mM glycine, pH 3.0; then it was equilibrated with PBS, pH 7.4. The antiserum was centrifuged at 20,000 $\times$ g for 10 min to remove insoluble particles. The pH of the supernatant was adjusted to 8.0 by adding 1/10 volume of 1 M Tris buffer, pH 8.0. This solution was added to the column, and the eluate was collected and reloaded twice. The column was washed sequentially with 10 column volumes of 100 mM Tris, pH 8.0, 10 column volumes of 10 mM Tris, pH 8.0, and five column volumes of 10 mM acetic acid buffer, pH 5.5. Then the retained antibodies were eluted from the column with 20 mM glycine, pH 3.0, and frozen at -80°C in aliquots.

Western blotting. Aesthetasc hairs, brains, pereopods, dactyls, eye-stalks, and olfactory organs were dissected from live lobsters chilled on ice. The preparation of tissue homogenates and membrane proteins, SDS-PAGE, and the blotting of proteins were performed as described previously (McClintock et al., 1997). Blots were washed for 5 min with Tris-buffered saline plus Tween 20 (TBST) before immunostaining. Nonspecific binding was blocked by incubation in blocking solution (0.5 M glycine, 2.5% dried milk powder, and 0.5% bovine serum albumin) for 1 hr. A 1:200 (v/v) dilution of purified antiserum P293 was added to the blocking solution for 1 hr. The blots were washed with TBST five times for 5 min, incubated in an anti-rabbit IgG conjugated to horseradish peroxidase for 1 hr, and washed as above. The antigen–antibody complex was detected via enhanced chemiluminescence (SuperSignal, Pierce, Rockford, IL). Relative quantitation of immunoreactivity was done by densitometry (National Institutes of Health Image software). The mean density of bands in digital scans from x-ray films was calculated by measuring the mean density of each band and subtracting the mean density of the background on the film.

Immunocytochemistry. Cross sections of aesthetasc hairs were prepared by making 10 μm cryosections approximately parallel to the ventral surface of groups of 3 mm cylinders dissected from the lateral filament of the first antennae. The preparation of this tissue and the methods for immunostaining were done as described previously (Xu et al., 1998), except that the blocking solution included 0.3% Triton X-100, 2.5% nonfat powdered milk, 0.5% bovine serum albumin, and 5% normal goat serum. Affinity-purified P293 was used at a 100-fold dilution. Preabsorption of the P293 antiserum was done by incubation with 10 $\mu\text{g}/\text{ml}$ of PLC293ct for 1 hr at ambient temperature.

Immunoprecipitation. Aesthetasc hairs were removed from 20 to 30 frozen olfactory organs (stored at -80°C) with a razor blade, transferred to CT buffer [(in mM) 120 NaCl, 5 KCl, 1.6 K_2PO_4 , 1.2 MgSO_4 , 25 NaHCO_3 , 7.5 glucose, and 2 EGTA plus 3 $\mu\text{g}/\text{ml}$ Pefabloc (Boehringer Mannheim, Indianapolis, IN), 1 $\mu\text{g}/\text{ml}$ pepstatin, 1 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ benzamidine, pH 7.4] in a 1.5 ml microcentrifuge tube on ice, and homogenized with eight strokes of a plastic pestle in ice water. The supernatant from a 300 $\times$ g spin for 5 min at 4°C then was diluted to 0.5–2 $\mu\text{g}/\text{ml}$ protein and brought to 1 $\times$ stimulation buffer [(in mM) 200 NaCl, 50 MOPS, 2.5 MgCl_2 , 1 DTT, 10 EGTA, 0.04 ATP, 0.01 GTP, 6.4 Ca^{2+} , and 0.1% Lubrol, pH 7.4] on ice. Eye-eyestalk homogenates were pre-

pared by Polytron homogenization for 15 sec in CT buffer, centrifugation at 300 $\times$ g for 5 min at 4°C to remove insoluble material, and storage at -80°C in aliquots. Just before experiments, aliquots of homogenates were thawed on ice and diluted with water and 3 $\times$ stimulation buffer to a protein concentration of 0.5–2 $\mu\text{g}/\text{ml}$. Homogenates of eye-eyestalk tissue were prepared in a darkened room.

Aesthetasc hair homogenates were stimulated at room temperature for 1 min with 3 μM GTP- γ -S or with a 50-fold dilution of an extract of TetraMarin (TetraWerke, Melle, Germany) prepared in stimulation buffer according to McClintock and Ache (1989). Eye-eyestalk preparations were stimulated by exposure to room light for 1 min at room temperature. Antisera were added to a final concentration of 20 $\mu\text{g}/\text{ml}$ to the samples and incubated for 20 min at room temperature. After the addition of 10 μl of a 50% suspension of protein A-Sepharose (Pharmacia Biotech, Piscataway, NJ), the samples were shaken vigorously for 20 min. The antigen–antibody protein A–agarose complexes were collected by brief centrifugation and washed with the suspension buffer. The complexes were dissociated by boiling for 5 min in loading buffer before SDS-PAGE separation and Western blotting. Antisera to G_β (catalog number T-20) and $\text{G}_{\alpha q}$ (catalog number C-19) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical analyses. Means are reported with their SEs. Means were compared by Student's *t* test or a test of meaningfully paired comparisons (Steel and Torrie, 1980).

RESULTS

Molecular cloning of lobPLC β

A pair of degenerate primers homologous to PLC- β isoforms was used to amplify a PCR product from olfactory organ cDNA. Cloning and sequencing of this product revealed a sequence that was homologous to known PLC- β isoforms. This PCR product was used to screen an olfactory organ cDNA library, and a 1.8 kb cDNA clone (PLC-1) was obtained. The insert contained a putative translational initiation site that was consistent with the consensus initiation site of crustaceans (Mankad et al., 1998). Within 100 bp upstream of this site were two in-frame stop codons. Screening another olfactory organ cDNA library by using the 3'-end of PLC-1 identified another 1.8 kb cDNA clone (PLC-2). Further screening with the 3'-end of PLC-2 yielded a third cDNA clone (PLC-3) that had 500 bp overlapping with PLC-2 and contained the end of the open reading frame plus 350 bp of 3'-untranslated sequence. A clone containing the complete open reading frame was constructed in two steps. A 1328 bp fragment from PLC-2 was obtained by partial digestion with *EcoRV* and *XhoI* and directionally ligated into the *EcoRV* and *XhoI* sites of pBS-PLC1, resulting in a 2646 bp cDNA insert, pBS-PLC-12. An *XhoI*/blunt fragment of PLC-3 then was added to the *XhoI* end of pBS-PLC-12, and this complete cDNA was named lobPLC β . The deduced protein sequence of lobPLC β (Fig. 1) was used to search for homologous sequences in protein databases (pBLAST; Altschul et al., 1997). LobPLC β had significant homology only to the PLC- β subfamily and contained all of the structural modules common to this subfamily (Fig. 2). It had 57% amino acid identity with the NorpA protein (Bloomquist et al., 1988), 50–53% identity with rat and bovine PLC- β_4 sequences (Lee et al., 1993; Ferreira and Pak, 1994), 35% identity with rat PLC- β_1 , 33% with human PLC- β_2 and PLC- β_3 , and only 28% identity with *Drosophila* PLC21 (Shortridge et al., 1991). A truncated PLC isoform from a brine shrimp (Su et al., 1994) had 54% identity with residues 420–960 of lobPLC β , but because this region consists primarily of the highly conserved catalytic domain, this percentage reflects only moderate homology. LobPLC β was therefore most similar to isoforms of PLC- β that function in phototransduction and olfactory transduction.

1PLC β	MIKYDFVWKEVDSRLQEGAVIDRW-TEDKES--VDYERNCTFKVDEYGFVWKSDFREGNVAELSOVSDIRGTLPRDCKLLDKL---LNKFGQDVE	94
r β 4	.A.PYE.N.Q...P.F...F.Y.E...FVF...L...F...LT...K...Q.L.C.LINS..LAAT.K...T.AA.-ESVG.SEN.L.	94
α NORPA	...KYE.D.IIP.PPE.TT.C.F...FENE..TKEN.F.RDAL...LY...D.D.T..C...A.GP.K...I...VIKKGINGINPELD	100
1PLC β	DKMLAICSGLDVSYINIVCKTKDEADAWLEGLRNIFHNIRANNICPMCLKKHAMRLGMVDSGGKIPVKHIVRTFASGKTEKMYSCLEEMGLPSG	194
r β 4	GRI.CV...T.L.N.GFTYM.AENPETT.Q.V...S.I..F...VS..T...K.AFLTNT...RS.T...VIFQA.K.L...	194
α NORPA	KRS.T...NT..IN.T.HHVT.PDAAT..S.QKN..L.T..N..T.V..RWN.M...SYC.EK...TLAK...L..T..KDA...DD	200
1PLC β	KGDSIEKDAFTFEKFKYKLYHTICPRIDIDELYSV--TKGEHVSQAQLVNFNNEKQRPDSLNELLYPLVDEKRCMQIKAHEPNEENIENKRFKSKDGLLA	292
r β 4	...E..PA...Y...E..TQ...ED.FKK.NGD.TDYLITVD...S.L..H...R...F.F..A..A...EMV..D..LKKKGLI..S..FCR	294
α NORPA	...AATM.EQ...D...A...V...N..E..F...TKG.QDFI..E.FIQ...D...RM...E...TE..NDY.LD..KKK.VQM.L..FKR	300
1PLC β	YLMSDENDPVFLRLDIYQIMDQPLSHYVINSSTNTYLSGRQFGGKSTAEYRQITLLAGCRCVELDCWDGKGEDEEPIITHGKAMCTDILFKEAIIAIFD	392
r β 4	...E..EL...H...F.S...T...SV...V...Q...DV.Q..KE	394
α NORPA	...E..F.M...I...SV...N...V...H.Y..E...DC.Q..A	400
1PLC β	CAFTVSDYFVILSPFNHOCKKQYKLAQVDEYFGDLLSKEALPDRPLVPGLHPSNQLKKILIKRKLKPEVEKQELFLVW---GQ-----LYLV	483
r β 4	T...A...S.Y...QMS...EDL...Q..ESH..E..RL...D...KQ..ALKSMEEA..SAAPAST.E	494
α NORPA	...S.A...NRA...DF...P...D..LP..P.CK...M...V...WL...K	489
1PLC β	DDDDAREDAQANPQKPEKNSCITTTTTP--VSQPSLED--DKPLTA--EERAFL--YYOYTGAITHHPVLSSVYNNQFQVNFQGFDAKEKNVHHNMS	576
r β 4	...NEEETESAADQEEEAH.EYKFGN.LS.DDFSHKE.VANSVK...VIVED.Q.WMAS.K.V...NI..Y..T..I..A..K...H..E.R...Y...	594
α NORPA	T...PE...AGK.PEAAAAPAP.AA.--AAG.A.G--GG.AE.--AA.--AN.S.S..N..W...A..IK...K.I...A...	578
1PLC β	SFSETMGLGYLTKQATEFVNMNKRQMSKITYPKGARVDSSNMHVFVWAGQMVSLNFQTSDLPMQINQKFEYNGNGYLLKEDFMRRITDKTFDPFAES	676
r β 4	...N.SV...H...R...G...QI...Y..P..A...S...P.R...S.T	694
α NORPA	...A.SA.MN...QSS.D...R...T.A...Q...S...G...A..D...DA	678
1PLC β	PVDGVIAAQVSVIAGQFLSKKVGIVVEVIMVGLPTDITIRKEFFRTYDSLEMAIPISIMNLPPLFCVCLTWLCCELVCFMKTEKWKAEFLAMVC	776
r β 4	...TC..Q..S...I...M-VMNGLNFPVNEESFVRKVLFDLAVLRIA..EDNN.LIGQRI-LPLDG	792
α NORPA	...C..K...F...S.VK...L-VANGLNFPVNEEDPYVRKWLFDLAVIRFG..EESG.IIGQRI-LPLDG	776
1PLC β	MQAGYRHISLRTEGNFFMSLMLFCNIEKITYVDGLDGLMDALSDPRAPFLSAQEKREEMKAMGTEASDID---TKALKGG-GGKAGGAKPAAGAKAGA	872
r β 4	L...N...K.L...TI...V..T...IV...KK...IT...AD.LR...T...ADVPSDTS.NDKK...NP..ANVTQSSS	892
α NORPA	L...V...A...V...FE.F.AM...G.AG.AKQON...L...EQS-----GG.ARDA-----EE-----	859
1PLC β	CGKPGACA---GGKKKKFFVEYEP-INLEITLRSQKNFLKATKQKQKELESMMKKHMERLSTQKACSAATEKL---AKGK---KVMDDPKIKSLVSE-	960
r β 4	ELR.TTT.ALGS.QEA..GI.LI.Q.RI.D.KOM.AY..HL...N..K...A..HSTM..LH.TQVD.IVAQVD.E..THEK.LEK.MK.KGG.NC	992
α NORPA	-----E..EPPLVF...T..S..QE.G.Q.VG...I...DT...A...T.V..T.NA..D...-I...-KD..RN..N.NSIND-	939
1PLC β	-CMKWSE-----MMEKHKKEEWEMLEKHLK-----SQE-DILKLMEEBQAGQIKKMEAKHEQDMKMKQAQAKAAMETAKIV	1032
r β 4	LEI.KE..IKIQITLSD.KSKVK.IVA..T.EWSEMINTHSAEEQETDHLH..QCEL.R..LINAHEQ.TQOLKLS.DRES.E.R.H...IA..NS.AI	1092
α NORPA	..T...D-----IAR...D..R..VQDSQAM-----A..LTV..A...QL.DR.AR.I..LN.K...MSAD...E	1011
1PLC β	NQDKTLTKADRRRLKERNQNNIKRFIEERKISAMKQKQKEKLVKHDOQMTELTKDQMAIQFYENTEAEYKMNKMEFFC	1116
r β 4	S...SI.N..E.E.V..L.SS...K.L...RL...S.E.DQ...QLEHL.F.E.QNEQ.KE.QQMVKL.AE.DRRPATVV	1176
α NORPA	QN...NEK...R...V...M..K.QIGV...RA...LA.SK.I..FST.V.KLMD..KIE.EA..TQG.T..CA	1095

Figure 1. Comparison of the predicted amino acid sequence of lobPLC β (GenBank accession number AF128539) with that of rat PLC- β 4 and *Drosophila* NorpA (GenBank accession numbers 435757 and J03138). *Consensus PKC phosphorylation site; γ consensus PKA phosphorylation site; #consensus PKA and PKC phosphorylation site. *lPLC β* , lobPLC β ; (.), residues identical to lobPLC β ; (---), spaces introduced to improve alignment.

Figure 2. General features of the lobPLC β modular structure. PH, Pleckstrin homology domain; EF, EF hand homology domain; X, Y, the bipartite catalytic domain; C2, C2 homology domain; P, P box; G, G-box required for functional interaction with G α_q . Underlined are the locations of polypeptide PLC293ct and of the conserved site of stimulatory interaction with G α_q . aa, Amino acids.

Within the identified domains of PLC- β isoforms there are residues and motifs that are known to be critical for function (Fig. 2). These were highly conserved in lobPLC β . All 10 residues in the catalytic domain that are known to be important for ligand discrimination, enzyme activity, or calcium sensitivity (Ellis et al., 1998) were present in lobPLC β . The 62 amino acid region of PLC- β 2 that contains the site required for interaction with G α_q

(Kuang et al., 1996; Yan and Gautam, 1997) shared 74% identity and 90% similarity with residues 588–649 lobPLC β . The C terminus of PLC- β isoforms, which are rich in basic amino acids that contribute to the association of PLC- β with particulate fractions of cells, is predicted to form helical structures (Kim et al., 1996). The C-terminal region of lobPLC β (residues 850–1116) is also highly basic, with an isoelectric point of 10.4. A sequence analysis program (Geneworks, Intelligenetics, Mountain View, CA) predicted the secondary structure of this region to be an α -helix, with residues R⁹⁰⁰, K⁹⁰³, K⁹⁰⁷, K⁹¹⁰, K⁹¹¹, K⁹¹⁴, E⁹¹⁷, R⁹²⁰, K⁹²¹, K⁹²², and K⁹²⁵ forming a positively charged surface on the helix. Several of these basic residues, R(K)⁹⁰⁰, K⁹⁰⁷, and K⁹¹⁰, are critical for the activation of PLC by G α_q (Kim et al., 1996).

LobPLC β mRNA is expressed in many tissues

Northern blotting of mRNA was performed to characterize the expression of lobPLC β . A 1033 bp fragment from the coding region of lobPLC β was used to probe mRNA from several tissues (Fig. 3). A predominant transcript of 9 kb was detected in all of the tissues tested, and in eye-eyestalk there were minor bands of 7, 5, 4, and 3 kb.

Figure 3. Northern blot detection of lobPLC β transcripts. Arrows mark bands at 9, 7, 5, 4, and 3 kb. Each lane contains 2 μ g of poly (A)⁺ RNA isolated from the indicated lobster tissue. E, Eye-eyestalk; L, pereopod; B, brain; N, olfactory organ. Size markers are given in kilobases.

Figure 4. Western blot of lobPLC β , using antisera P293. Each lane contains 20 μ g of membrane protein, except for eye-eyestalk, which contains 5 μ g. The right panel shows a separate comparison of lobPLC β immunoreactivity in lanes containing equal amounts of brain and aesthetasc hair membrane protein. A, Aesthetasc hair; B, brain; D, dactyl; E, eye-eyestalk; L, pereopod; N, olfactory organ. E(s), Short exposure (2 sec) of the eye-eyestalk lane. Size markers are given in kilodaltons.

LobPLC β protein is present in many tissues, including the aesthetasc hairs

Western blotting that used antisera P293 revealed that all of the tissues tested contained an immunoreactive band of 130 kDa, in agreement with the calculated 128 kDa molecular weight (Fig. 4). Consistent with the Northern blots, we observed an abundance of lobPLC β immunoreactivity in eye-eyestalk lanes of Western blots. Reduction of the amount of membrane protein loaded in the eye-eyestalk lane and brief exposure to film revealed that the 130 kDa band was the predominant immunoreactive band in this tissue (Fig. 4). The sizes of the minor bands of in eye-eyestalk membranes were 100, 79, and 57 kDa. These smaller bands were absent in other tissues, except that the 100 kDa band was detected rarely in preparations of aesthetasc hairs and brain.

LobPLC β is expressed in the outer dendritic segments of olfactory receptor neurons

For lobPLC β to mediate olfactory transduction, it must be present in the outer dendritic segments of the olfactory receptor neurons. The distal 80% of the 700 μ m length of aesthetasc hairs contains only these outer dendritic segments (Olesco-Szuts and Atema, 1977; Grünert and Ache, 1988). In cross sections from the distal 500 μ m of these hairs we detected immunoreactivity for lobPLC β (Fig. 5A). This immunoreactivity was absent if the P293

Figure 5. Immunoreactivity for lobPLC β in the outer dendritic segments of olfactory receptor neurons in cross sections from the outer 500 μ m of aesthetasc hairs. A, Staining with P293 antiserum. B, Staining with P293 antiserum preabsorbed with its antigen. C, Omission of P293 from the staining procedure. Scale bar, 20 μ m.

antiserum was preabsorbed with antigen (Fig. 5B). In digital images the tissue inside hairs in sections stained with the P293 antiserum had a mean gray scale value of 143 ± 12 ($n = 16$), compared with 161 ± 11 ($n = 13$) for the preabsorption control and 163 ± 9 ($n = 10$) for sections in which P293 was omitted. The sections stained with P293 differed significantly from the two controls (Student's t test; $p < 0.0005$; $df = 27$ and 24 , respectively).

Membrane association of lobPLC β is tissue- and stimulation-dependent

The majority of lobPLC β was in the soluble fraction of homogenates of aesthetasc hairs. Stimulation with TetraMarin extract or GTP- γ -S caused the distribution of lobPLC β immunoreactivity to shift in favor of the membrane fraction (Fig. 6A,B). This shift involved <30% of the lobPLC β immunoreactivity, but it was repeatable and approached statistical significance for both stimuli after only one replication (tests of meaningfully paired compar-

Figure 6. The membrane association of lobPLC β immunoreactivity increased after odorant stimulation. *A*, Western blot of membrane and supernatant fractions of an aesthetasc hair homogenate. *B*, Densitometry of replicate experiments shown in *A* ($n = 2$). Error bars indicate SEMs. *C*, Western blot for lobPLC β in fractions of an eye-eyestalk homogenate. *d*, Darkness; *l*, light (room illumination); *c*, vehicle control; *g*, 3 μ M GTP- γ -S; *t*, TetraMarin extract; *sup*, supernatant fraction.

isons; $p < 0.1$; $df = 1$). In contrast, densitometry of Western blots of soluble and membrane fractions from eye-eyestalk homogenates showed that 85–90% of lobPLC β was associated with the membrane pellet (Fig. 6C). Stimulation with light shifted even more of lobPLC β from the soluble fraction to the membrane pellet.

Odorants stimulate association of lobPLC β protein with $G_{\alpha q}$ and G_{β}

The similarity of lobPLC β with *norpA* and the detection of lobPLC β immunoreactivity in the aesthetasc hairs indicated that lobPLC β could be part of the olfactory transduction pathway. If so, lobPLC β must be activated by either $G_{\alpha q}$ or $G_{\beta\gamma}$ in this tissue. Because activation of PLC- β isoforms by G-proteins is known to be by a direct interaction with $G_{\alpha q}$ or $G_{\beta\gamma}$, we tested whether the application of odorants or GTP- γ -S would increase the association of lobPLC β with $G_{\alpha q}$ and G_{β} . Immunoprecipitation with antisera to $G_{\alpha q}$ and G_{β} resulted in the precipitation of lobPLC β immunoreactivity. This coprecipitation was increased by stimulation with the TetraMarin extract or with GTP- γ -S (Fig. 7A). The odorant increased lobPLC β immunoreactivity 2.1-fold in

Figure 7. Stimulation increased the association of lobPLC β with $G_{\alpha q}$ and G_{β} . *A*, LobPLC β immunoreactivity in G_{β} and $G_{\alpha q}$ immunoprecipitates from aesthetasc hair homogenates. *B*, Densitometry of replicate experiments shown in *A* ($n = 3$ for all treatments, except $n = 2$ in lane *t* in G_{β} immunoprecipitate). Error bars indicate SEMs. *C*, LobPLC β immunoreactivity in G_{β} and $G_{\alpha q}$ immunoprecipitates from eye-eyestalk homogenates. *c*, Vehicle control; *t*, TetraMarin extract; *g*, 3 μ M GTP- γ -S; *d*, darkness; *l*, light (room illumination). *Significant difference from control by test of meaningfully paired comparisons; $p < 0.05$; $df = 2$).

$G_{\alpha q}$ immunoprecipitates and 2.2-fold in G_{β} immunoprecipitates (Fig. 7B). GTP- γ -S caused even larger increases in the lobPLC β immunoreactivity in the $G_{\alpha q}$ and G_{β} immunoprecipitates, with 3.6- and 3.1-fold increases, respectively (Fig. 7B). For comparison, we also tested eye-eyestalk homogenates, where the level of expression of lobPLC β and its close homology with *norpA* made it very likely to be responsive to light. As expected, increases in association of lobPLC β with $G_{\alpha q}$ and G_{β} were observed in eye-eyestalk homogenates stimulated with light (Fig. 7C). On average, the coprecipitation of the 130 kDa band with G_{β} and $G_{\alpha q}$ was increased 3.5- and 1.6-fold, respectively. Similarly, the coprecipitation of the 100 kDa band with G_{β} and $G_{\alpha q}$ was increased 4.3- and 2.2-fold, respectively. In their specific receptive tissues, odorants and light therefore stimulated the association of lobPLC β with G-protein subunits to similar extents.

DISCUSSION

A cDNA clone that encodes a lobster PLC- β was isolated. LobPLC β mRNA and protein were expressed in all of the tissues that were examined but were especially abundant in the eye-eyestalk. In addition to the predictable role of a NorpA homolog in phototransduction, this broad tissue distribution suggests that lobPLC β is involved in a variety of signaling pathways. Our results provide evidence that lobPLC β mediates olfactory transduction in the lobster.

This conclusion is supported by several lines of evidence. LobPLC β immunoreactivity was present in the aesthetasc hairs where olfactory transduction occurs (Boekhoff et al., 1994; Hatt and Ache, 1994). This immunoreactivity was found in cross sections from the outer 500 μ m of the hairs, where the only cellular material is the outer dendrites of the olfactory receptor neurons (Olesco-Szuts and Atema, 1977; Grünert and Ache, 1988). These outer dendrites are believed to be the site of olfactory transduction. A complex odorant stimulated the translocation of lobPLC β to membranes in homogenates of aesthetasc hairs. Most importantly, the odorant increased the association of lobPLC β with $G_{\alpha q}$ and G_{β} . These interactions are involved in activating PLC- β isoforms (Gutowski et al., 1991; Blank et al., 1992; Boyer et al., 1992; Camps et al., 1992; Jhon et al., 1993; Park et al., 1993; Smrcka and Sternweis, 1993; Lee et al., 1994; Lee and Rhee, 1995; Hamm and Gilchrist, 1996), and they are consistent with the conservation in lobPLC β of residues known to be necessary for stimulatory interaction with $G_{\alpha q}$ and $G_{\beta\gamma}$ (Kim et al., 1996; Kuang et al., 1996; Yan and Gautam, 1997). All PLC- β isoforms are stimulated by $G_{\alpha q}$, and only mammalian PLC- β_4 is not stimulated by $G_{\beta\gamma}$ (Jiang et al., 1994; Lee et al., 1994). Even if the association of lobPLC β with $G_{\beta\gamma}$ is not stimulatory, it still could play a significant role by contributing to the membrane association of lobPLC β . Previous studies in a spiny lobster species show that the primary olfactory transduction mechanism depends on the activation of a G_q heterotrimer and results in the production of IP $_3$ (Fadool and Ache, 1992; Ache, 1994; Boekhoff et al., 1994; Hatt and Ache, 1994; Fadool et al., 1995). Evidence that a $G_{\alpha q}$ expressed by lobster olfactory receptor neurons and a G_{β} cDNA cloned from the lobster olfactory organ also are expressed in the outer dendritic segments (McClintock et al., 1997; Xu et al., 1998; T. Landers and T. McClintock, unpublished results) further supports our conclusion that lobPLC β mediates olfactory transduction. These two G-protein subunits and lobPLC β appear to be components of the primary olfactory transduction pathway in lobsters.

As would be expected of a homolog of the *norpA* gene, lobPLC β appears also to mediate phototransduction in lobsters. It is highly abundant in the eye-eyestalk and associates with $G_{\alpha q}$ and G_{β} in response to light. Also like *norpA*, lobPLC β cDNA hybridizes to multiple bands in eye-eyestalk lanes on Northern blots (Bloomquist et al., 1988). At least some of the *Drosophila* bands are alternatively spliced transcripts of *norpA* (Kim et al., 1995), and we hypothesize that this will prove to be true for lobPLC β as well. In addition, multiple species of immunoreactive protein were observed specifically in the eye-eyestalk lanes on our Western blots, raising the possibility that alternatively spliced transcripts, or highly homologous genes, give rise to these proteins. In *Drosophila* the strong association of NorpA protein with retinal membranes (McKay et al., 1994) is attributable to interaction with a scaffold protein, InaD, which also interacts with the principal light-activated ion channel (Trp) and an eye-specific protein

kinase C (Shieh et al., 1997; Tsunoda et al., 1997). The association of InaD and NorpA are essential for the controlled activation and deactivation of phototransduction. Our results predict that lobster photoreceptor cells express an anchoring protein, perhaps an InaD homolog, that interacts with lobPLC β . This hypothesis is supported by the observation that the residues of NorpA that are critical for interaction with InaD, Phe¹⁰⁹³-Cys¹⁰⁹⁴-Ala¹⁰⁹⁵-COOH (Shieh et al., 1997), are similar to the C terminus of lobPLC β , Phe¹¹¹⁴-Phe¹¹¹⁵-Cys¹¹¹⁶-COOH. NorpA mutations at Phe¹⁰⁹³ or Cys¹⁰⁹⁴ fail to bind to InaD, and the photoreceptors of transgenic *Drosophila* carrying the *norpA*^{C1094S} mutation demonstrate delayed activation and slow repolarization. This evidence that the two common C-terminal residues, Phe and Cys, are critical for NorpA interaction with InaD and for phototransduction is consistent with the prediction that lobPLC β is anchored to retinal membranes by an InaD homolog. In contrast, the dendrites of lobster olfactory receptor neurons appear to lack an anchoring protein because most of lobPLC β was in the soluble fraction of aesthetasc hair homogenates. This difference is consistent with the hypothesis that the olfactory system sacrifices response speed in favor of using subcellular compartmentalization of lobPLC β to regulate transduction.

Another mechanism of regulation of lobPLC β may be phosphorylation. LobPLC β has nine consensus phosphorylation sites, suggesting that this enzyme could be regulated by PKA and PKC. Mammalian isoforms of PLC- β are phosphorylated by these kinases, and there are functional consequences of this phosphorylation for the interaction between $G_{\beta\gamma}$ and PLC- β (Ryu et al., 1990; Liu and Simon, 1996; Litosch, 1997). This is of particular relevance to lobster olfaction, in which at least one-half of the receptor neurons contain two olfactory transduction pathways, one mediated by IP $_3$ and the other by cAMP (Ache, 1994). The two transduction pathways terminate in receptor potentials of opposite polarity, leading to electrical integration of their signals (McClintock and Ache, 1989; Michel et al., 1991; Ache, 1994). Whether biochemical interaction between the two pathways also exists has yet to be determined, but the presence of PKA consensus sites in lobPLC β suggests a possible mechanism for such an interaction.

Previously, the molecular characterization of PLC- β isoforms in the olfactory system was limited to the identification of the role of the *norpA* gene in olfactory receptor neurons of the *Drosophila* maxillary palp (Ache, 1994; Riesgo-Escovar et al., 1995; Bruch, 1996). Although it is possible that lobPLC β might mediate olfactory transduction for only a subset of olfactory receptor neurons, similar to *norpA* (Riesgo-Escovar et al., 1995), we think it is more likely that lobPLC β mediates olfactory transduction for all lobster olfactory receptor neurons. The relative abundance of lobPLC β in the eye-eyestalk as compared with the aesthetasc hairs does not necessarily correlate with the fraction of receptor cells that use lobPLC β for sensory transduction. The Western blot shown in Figure 4 exacerbated the difference between the sensory organs by using membrane preparations, which recovered almost all of the lobPLC β from the eye-eyestalk but only a third of the lobPLC β from the aesthetasc hairs. In addition, recent evidence from other systems indicates that effector enzymes may be less abundant than other components of transduction pathways, a mechanism of regulating the signaling capacity of G-protein-dependent signaling pathways (Gao et al., 1998). We conclude that lobPLC β is an inositol phospholipid phospholipase that mediates olfactory transduction in lobsters.

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