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Gene identification and evidence for expression of G protein α subunits, phospholipase C, and an inositol 1,4,5-trisphosphate receptor in *Aplysia californica* rhinophore☆

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Abstract

In the marine mollusk *Aplysia californica*, waterborne protein pheromones that are released during egg laying act in concert to stimulate mate attraction. However, molecular information concerning the cellular receptors and signaling mechanisms that may be involved in waterborne peptide and protein pheromonal communication is lacking. As a first step toward examining whether members of the G protein family and phosphoinositide signaling pathway are present in the primary peripheral chemosensory organs (i.e., rhinophores), we isolated five full-length cDNA clones from an *A. californica* central nervous system cDNA library. These clones encoded (1) the G protein α subunits of the G_q, G_i, and G_o families, (2) a protein with homology to phospholipase C (PLC) isoforms, and (3) an inositol 1,4,5-trisphosphate receptor (IP_3R). The expression of these genes was examined using laser capture microdissection/reverse transcription-polymerase chain reaction and in situ hybridization. All of them are expressed in the rhinophore sensory epithelium, suggesting that $G\alpha_q$, $G\alpha_i$, $G\alpha_o$, PLC-like protein, and IP_3R may be involved in waterborne protein pheromone detection in *Aplysia*—possibly via a phosphoinositide signaling mechanism.
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Keywords: *Aplysia*; Pheromonal communication; Signal transduction

Chemical signaling is the most ancient form of communication and is used by mollusks and most, if not all, other organisms [1–8]. For most marine organisms, pheromonal communication is essential for attracting potential mates since vision and auditory signaling are often restricted. To date, few waterborne peptide or protein pheromones have been characterized, although evidence has been accumulating for their role in *Aplysia* mate attraction. Field studies have shown that this habitually solitary animal moves into breeding aggregations, and laboratory studies have found that this is likely caused by stimulation with binary pairs of the protein pheromones attractin, enticin, temptin, or seductin [1,4,6]. The first of these, attractin, was isolated from seawater eluates of *Aplysia* egg cordons and is a 58-residue protein [1,9].

In *Aplysia* and other opisthobranchs, pheromone detection is achieved by olfactory sensory neurons (OSNs) located in specialized anterior sensory organs known as rhinophores. The rhinophores include two tentacle-like organs on the dorsal surface of the head. Rhinophores sense pheromones released during egg laying [10], and, when the rhinophores are removed, *Aplysia* spend significantly less time mating when exposed to pheromones from egg cordons [10]. The neuroanatomical organization of rhinophores has been described [11,12] and includes a rhinophore groove within which most of the sensory cells are concentrated (Fig. 1). For attraction to be initiated, released pheromones must first bind to specific receptor proteins located on the dendrites of rhinophore OSNs in *Aplysia* conspecifics and elicit pheromone signal processing. The three-dimensional nuclear magnetic resonance solution structure of attractin [3] and comparative sequence alignments of six aplysiid attractins indicate that a conserved heptapeptide region (IEECKTS) is critical for cellular receptor binding [2].

The molecular and cellular bases of pheromone detection have been well studied in a variety of vertebrates and inver-

☆ Sequence data from this article have been deposited in the GenBank/EBI Data Libraries under Accession Nos. DQ397515 ($G\alpha_q$), DQ656111 ($G\alpha_i$), DQ656112 ($G\alpha_o$), DQ397516 (phospholipase C protein), and DQ397517 (inositol triphosphate receptor).

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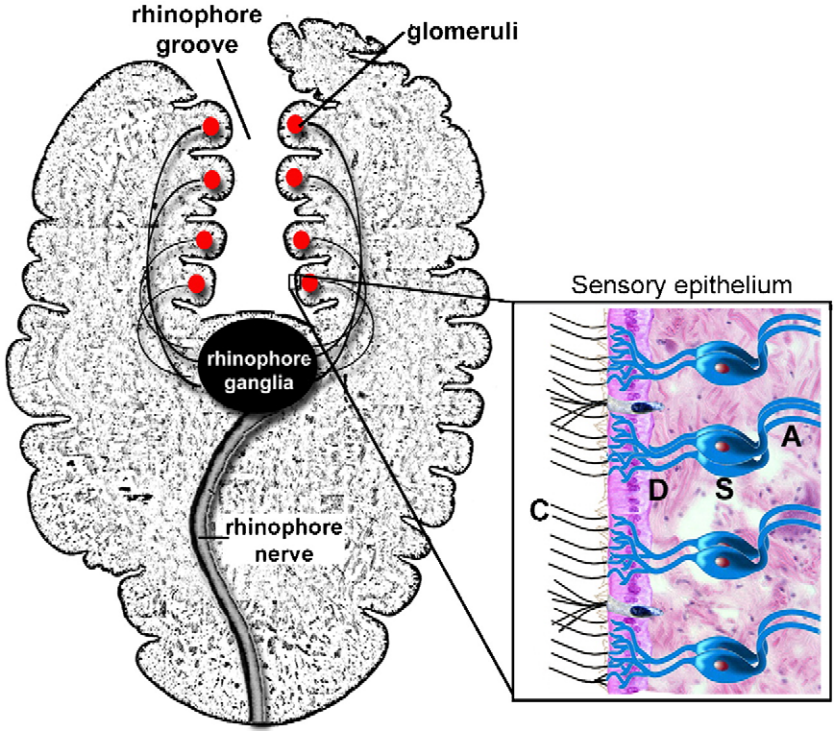

Fig. 1. Schematic diagram of the rhinophore neuroanatomy showing the location of the rhinophore groove, glomeruli underlying the sensory epithelium, rhinophore ganglia, and rhinophore nerve in *Aplysia* (modified from [12]). Enlarged box shows schematic diagram of rhinophore olfactory receptor afferent neurons located beneath the olfactory epithelium. The location of neuron soma (S), dendrites (D), axons (A), and cilia (C) are labeled. Gastropod olfactory neurons send processes to glomeruli, rhinophore ganglia, or directly to the cerebral ganglia.

tebrates [13–18]. However, unlike vertebrates, the molecular mechanisms mediating pheromone signaling in invertebrates has been poorly elucidated. In either case, G protein signaling pathways appear to be primarily responsible for transducing pheromone responses. The evolutionary importance of this system in chemosensory signal transduction is revealed in yeast, which responds to mating pheromones by activating a G protein signaling cascade [19].

Most information concerning pheromonal communication in vertebrates has been derived from rodent research. In rodents, volatile molecules and nonvolatile pheromones are primarily detected by putative pheromone receptors belonging to the heptahelical G-protein-coupled receptor (GPCR) superfamily, present on vomeronasal sensory neurons (VSNs) [20,21]. Signal transduction is driven by activation of trimeric guanine-nucleotide-binding (GTP-binding) proteins (G proteins–$G_{\alpha\beta\gamma}$), specifically $G\alpha_q$, followed by phospholipase-C (PLC)-induced production of inositol 1,4,5-trisphosphate (IP_3) and the subsequent increase in intracellular calcium (Ca^{2+}) concentration. IP_3 plays a key role in membrane depolarization by binding to the IP_3 receptor (IP_3R) located in the endoplasmic reticulum membrane, mobilizing sequestered Ca^{2+} and increasing cytosolic Ca^{2+}.

Several studies have reported that the molecular components and mechanisms of invertebrate pheromone detection are strikingly similar. For example, in insects, pheromones are detected by extremely sensitive sensory neurons localized in specialized sensory organs, the sensilla. Pheromones bind to seven-transmembrane receptors and the message is possibly relayed intracellularly via $G\alpha_q$, which in turn activates a specific PLC, resulting in a rapid and transient increase in IP_3 and intracellular Ca^{2+}. Although the precise mechanism of OSN pheromone signaling in lobsters is unknown, studies have shown that $G\alpha_q$ is expressed in the dendrites [22], and odorant exposure increases IP_3 levels leading to cellular depolarization [23,24]. A PLC and an IP_3R have also been localized to the outer dendrites of lobster OSNs [25–27]. Emerging evidence by Ache and colleagues [28] demonstrates that a calcium-sensitive transient receptor potential (TRP) channel is a downstream target of phosphoinositide signaling in lobster sensory neurons.

In contrast, prior to this study, little was known about olfactory sensory signal transduction in molluscan OSNs, and no IP_3R had been isolated. However, G proteins had been identified in the mantle and gill of the pearl oyster *Pinctada* [29], the cilia of abalone larvae [30], and the central nervous system (CNS) of *Lymnaea* [31]. Furthermore, a single retina-specific PLC isoform had been characterized in squid (*Loligo*) as being similar in structure and organization to vertebrate PLC-β members [32]. Finally, biochemical studies have implicated G-protein-induced IP_3 signaling in oocyte activation [33], and IP_3 is important for *Aplysia* neuroendocrine bag cell depolarization [34]. Taken together, the data suggest that G-protein-mediated IP_3 signaling is also important in molluscan chemosensory and perhaps pheromonal signaling. The identification by Moroz [35] of nitric-oxide-synthase-containing cells

in peripheral sensory organs of *Aplysia* suggests that nitric oxide may also play a role in olfactory processing.

As a first step toward characterizing pheromone transduction pathways that may be used in *Aplysia* waterborne protein pheromone signaling, we isolated cDNAs from an *Aplysia* central nervous system (CNS) cDNA library that encode proteins homologous to $G\alpha_q$, $G\alpha_i$, $G\alpha_o$, PLC, and IP_3R. Multiple alignment analysis with invertebrate and vertebrate homologs showed a high degree of identity, suggesting a conserved function. Consistent with a potential role in *Aplysia* olfactory transduction, mRNAs for all five proteins were expressed in rhinophore sensory epithelium.

Results

Laser capture microdissection (LCM) of rhinophore sensory epithelium

The method of the LCM of rhinophore sensory epithelium and reverse transcription-polymerase chain reaction (RT-PCR) analysis is shown in Fig. 2. The process was monitored by examining the epithelium before (Fig. 2A) and after (Fig. 2B) laser capture and the captured cells that were lifted off of the epithelium section (Fig. 2C) and used for subsequent total RNA isolation and RT-PCR (Fig. 2D). RT-PCR products were fractionated by agarose gel electrophoresis and resulted in a 357-bp cDNA for $G\alpha_q$, a 434-bp cDNA for $G\alpha_i$, a 422-bp cDNA for $G\alpha_o$, a 759-bp cDNA for PLC, and a 282-bp cDNA for the IP_3R. No amplification products were observed in reactions where cDNA was omitted. For comparison, RT-PCR was performed using total RNA extracted from the cerebral ganglia, and the sizes of the RT-PCR products were identical to those obtained from rhinophore sensory epithelium (data not shown).

Identification of Aplysia $G\alpha_q$, $G\alpha_i$, and $G\alpha_o$ and tissue-specific expression

The 357-bp cDNA product amplified by LCM/RT-PCR, using $G\alpha_q$ degenerate oligonucleotide primers, was cloned and sequenced. A comparison of the deduced *Aplysia* amino acid sequence with sequences in the GenBank database indicated that this product was similar to members of the $G\alpha_q$ subunit family. This 357-bp insert was therefore used as a probe to isolate a 1230-bp cDNA clone that encoded a full-length 353-amino-acid predicted protein (Fig. 3A) with a calculated molecular mass of 41.5 kDa. The predicted protein sequence shared a high degree of sequence identity with $G\alpha_q$ proteins from *Lymnaea* (97%), lobster (82%), *Drosophila* (81%), and human (81%) and shared a lower degree of identity with other $G\alpha$ subunit types (Fig. 3A). Similarly, $G\alpha_i$ and $G\alpha_o$ LCM/RT-PCR cDNA products were cloned, sequenced, and used to isolate *Aplysia* CNS cDNA clones. Both encoded full-length 354-amino-acid predicted proteins (Figs. 3B and 3C) with calculated molecular masses of 40.5 kDa; a comparison with GenBank database sequences indicated that the predicted

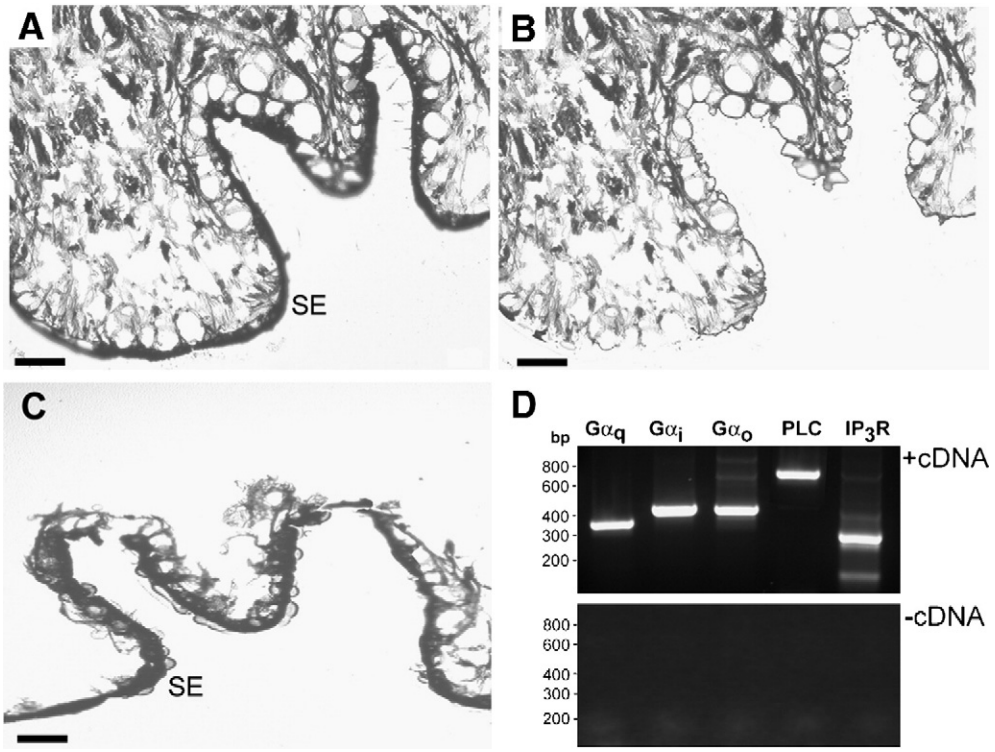

Fig. 2. Laser capture microdissection of *Aplysia californica* rhinophore sensory epithelium. Frozen sections (10 μm) of rhinophore sensory epithelium were made, and total RNA was obtained by LCM and used to amplify $G\alpha_q$, $G\alpha_i$, $G\alpha_o$, PLC, and IP_3R cDNA by RT-PCR. (A) Tissue before LCM. (B) Tissue after LCM. (C) Captured cells. SE, sensory epithelium. Scale bars, 100 μm. (D) RT-PCR and agarose gel electrophoresis of $G\alpha_q$ (357 bp), $G\alpha_i$ (434 bp), $G\alpha_o$ (422 bp), PLC (759 bp), and IP_3R (282 bp) using captured cells cDNA and without cDNA.

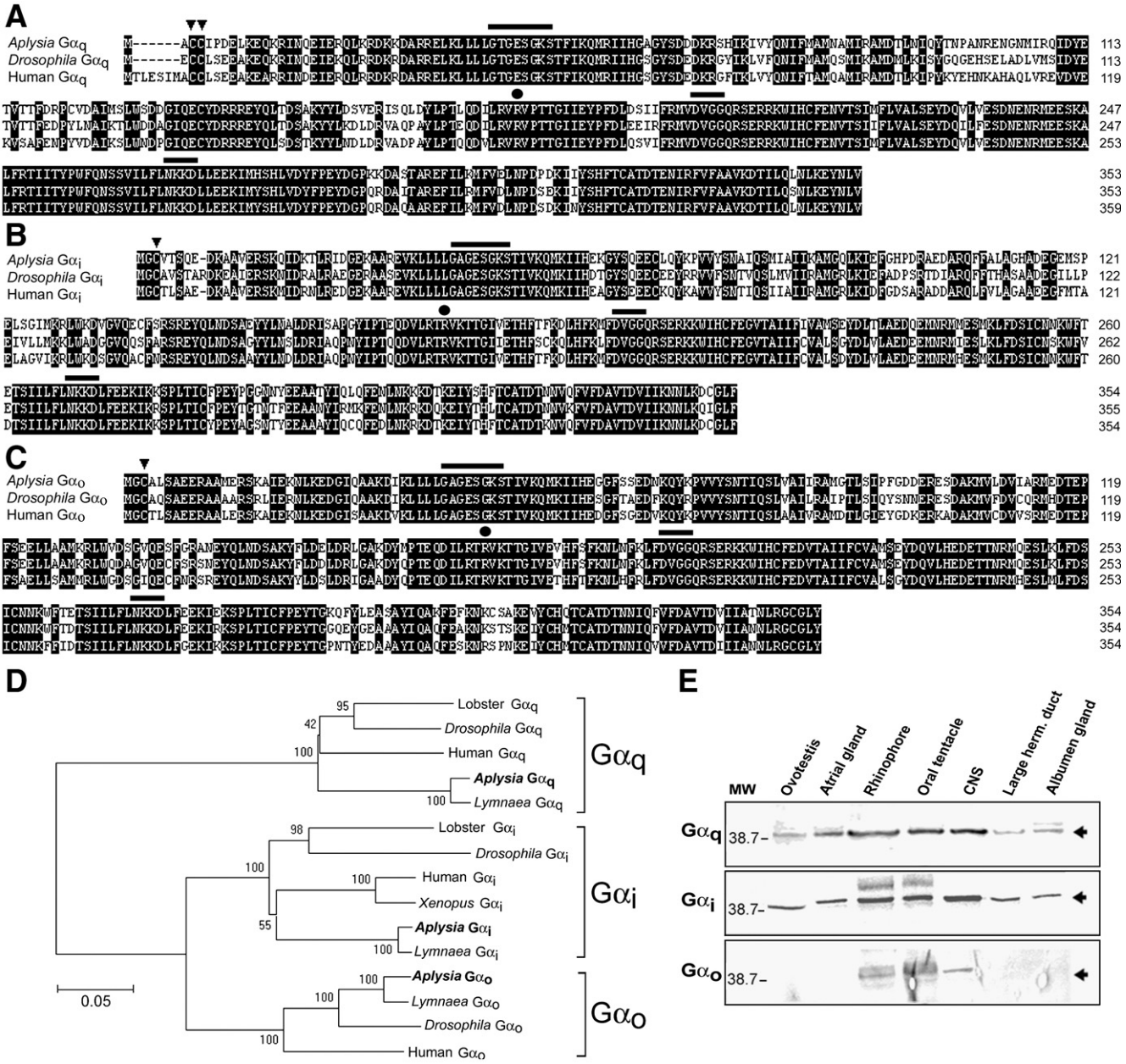

Fig. 3. Identification and phylogenetic analysis of *Aplysia* $G\alpha_q$, $G\alpha_i$, and $G\alpha_o$ proteins. (A–C) Comparison of $G\alpha_q$, $G\alpha_i$, and $G\alpha_o$ sequences from *Aplysia*, *Drosophila*, and human. Identical residues are indicated in black. *Aplysia* contains several motifs indicative of the Gα family, including N-terminal cysteines (▼), GTP-binding sites (overline), and putative cholera toxin ADP-ribosylation site (●). (D) Phylogenetic tree of the G protein family. A tree was constructed from deduced amino acid sequences by using the neighbor-joining method [36]. The number at each branch point represents the bootstrap probability that two lineages join together to form a cluster. Scale bar indicates estimated number of amino acid substitutions per site. GenBank Accession Nos.: lobster $G\alpha_q$ (P91950), *Drosophila* $G\alpha_q$ (AAM49880), human $G\alpha_q$ (AAB64301), *Aplysia* $G\alpha_q$ (ABD62078), *Lymnaea* $G\alpha_q$ (CAA80653), lobster $G\alpha_i$ (P41776), *Drosophila* $G\alpha_i$ (NP_477502), human $G\alpha_i$ (NP_002060), *Xenopus* $G\alpha_i$ (NP989250), *Aplysia* $G\alpha_i$ (DQ656111), *Lymnaea* $G\alpha_i$ (CAA78807), *Aplysia* $G\alpha_o$ (DQ656112), *Lymnaea* $G\alpha_o$ (CAA78806), *Drosophila* $G\alpha_o$ (P16378), and human $G\alpha_o$ (P09471). (E) Protein (40 μg) was isolated from ovotestis, atrial gland, rhinophore, oral tentacle, CNS, large hermaphroditic duct, and albumen glands, and probed by immunoblot analysis using antisera raised against rat, human, or mouse $G\alpha_q$, $G\alpha_i$, and $G\alpha_o$. In each case, *Aplysia* $G\alpha_q$, $G\alpha_i$, and $G\alpha_o$ are indicated by an arrow and are ~41 kDa; the molecular weight marker (MW) is in kilodaltons.

products were similar to members of the $G\alpha_i$ and $G\alpha_o$ subunit families, respectively.

Sequence analysis of *Aplysia* $G\alpha_q$, $G\alpha_i$, and $G\alpha_o$ indicated that they contain one ($G\alpha_i$, $G\alpha_o$) or two ($G\alpha_q$) N-terminal cysteine residues (Cys^3-Cys^4) that are conserved in all Gα proteins and a putative site for palmitoylation (Figs. 3A–3C). The majority of amino acid differences between the *Aplysia*, *Drosophila,* and human Gα subunits are in the N-terminal half of the proteins. The C-terminal half, which contains domains that specify interactions with receptor and effector subtypes, exhibits a high degree of sequence identity with the equivalent regions of *Drosophila* and human Gα proteins; for $G\alpha_q$, only 15 of 159 residues differ in the C-terminal half. All cloned *Aplysia* G protein α subunits contain conserved putative

cholera toxin ADP-ribosylation sites (Arg^{177}; $G\alpha_q$ numbering) and consensus amino acid sequences for GTP-binding that are common to all Gα proteins, the G^{40}XXXSGKS (A domain), D^{199}XXG, and N^{268}KXD sequence [37].

A phylogenetic tree of the Gα family based on amino acid identity is shown in Fig. 3D. The *Aplysia* Gα protein members are most closely related to *Lymnaea* Gα protein homologs. In situ hybridization experiments to localize $G\alpha_q$ mRNA did not reveal any specific labeling, despite positive controls using antisense rRNA riboprobes showing extensive labeling of all cell types (data not shown). This suggested that in situ hybridization may not be sensitive enough to detect G protein mRNA transcripts. Subsequently, immunoblot analyses were performed to determine the tissue expression of $G\alpha_q$, $G\alpha_i$, and $G\alpha_o$ protein in selected *Aplysia* tissues using subunit-specific antibodies raised against the C-terminal region of rat $G\alpha_q$ (Q^{344}LNLKEYNLV), mouse $G\alpha_i$ (K^{345}NNLKDCGLF), and human $G\alpha_o$ (A^{345}NNLRGCGLY). Immunoreactive $G\alpha_q$ and $G\alpha_i$ were present as a single prominent protein band at ~41 kDa in all tissues examined, including the rhinophores and another chemosensory tissue, the oral tentacles (Fig. 3E). By contrast, immunoreactive $G\alpha_o$ (~41 kDa) was restricted to the rhinophores, oral tentacles, and CNS (Fig. 3E). The apparent molecular mass of the immunoreactive *Aplysia* $G\alpha_q$, $G\alpha_i$, and $G\alpha_o$ proteins matched that predicted by the corresponding cDNA (~41 kDa), indicating that the antibody reacts specifically, as expected. No specific antibody binding was identified when primary antiserum was omitted (data not shown).

Identification of an Aplysia phospholipase C and rhinophore expression

A 759-bp LCM/RT-PCR product that was amplified using degenerate PLC-β oligonucleotide primers was cloned and sequenced. A comparison of the deduced *Aplysia* amino acid sequence with sequences in the GenBank database indicated that this product was similar to members of the PLC family. This 759-bp insert was used as probe for CNS library screening and one positive clone was isolated, sequenced, and used to identify the 5′ region by 5′-rapid amplification of cDNA ends (5′-RACE). The *Aplysia* PLC cDNA had an open reading frame of 3367 bp and encoded a predicted 965-amino-acid protein (Fig. 4A). The predicted protein had a calculated molecular mass of 105 kDa and shared the highest degree of sequence identity with PLC-L (PLC-like) proteins from mouse (51%) and human PLC-L (49%) and a lower degree of identity to other PLC types. Analysis shows that the *Aplysia* PLC shares typical PH (residues 109–217) and X and Y domains (residues 370–716) with other classes of PLC (based on Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de/). Most conservation was found within the X and Y catalytic domains. Experimental evidence is needed to verify that the protein has catalytic activity.

A phylogenetic tree of the PLC family (PLC-L, PLC-β, PLC-γ, and PLC-δ), based on amino acid identity, is shown in Fig. 4B. The *Aplysia* PLC protein is most closely related to PLC-L homologs of rodent and human. In situ hybridization experiments demonstrated the expression of *Aplysia* PLC mRNA in the rhinophore sensory epithelium (Fig. 4C); controls using sense mRNA were negative (Fig. 4C inset).

Identification of an Aplysia IP_3R and rhinophore expression

The 282-bp cDNA product amplified by LCM/RT-PCR (with degenerate IP_3R oligonucleotide primers) was cloned and sequenced. A comparison of the deduced *Aplysia* amino acid sequence with sequences in the GenBank database indicated that this product was similar to members of the IP_3R family. The 282-bp insert was used as probe to isolate a 3-kb cDNA clone. Rescreening of the CNS cDNA library (using the 5′-end of the 3-kb clone as a probe) identified an overlapping 4.5-kb cDNA clone. The 5′ region of *Aplysia* IP_3R was identified by sequential 5′ RT-PCR steps and subsequently by 5′-RACE. A 1.4-kb cDNA was amplified by RT-PCR and a 0.4-kb cDNA product was amplified by 5′-RACE to obtain the remaining 5′ sequence.

The *Aplysia* IP_3R cDNA predicted an open reading frame encoding a 2762-residue protein having a calculated molecular mass of 315 kDa. The deduced full-length amino acid sequence shared a high degree of identity with IP_3R proteins from starfish (61%), lobster (55%), and *Drosophila* (54%). The amino acid sequence of the *Aplysia* IP_3R is shown in Fig. 5A. As described in mammalian IP_3Rs, the structure of *Aplysia* IP_3R can be divided into three different domains. The first, the N-terminal ligand-binding domain, contains all three basic sites known to have a crucial role in IP_3 binding and two sites for calcium binding [38]. The second domain, the middle portion of the receptor, is a regulatory region known to be involved in regulation of channel activity. Although experimental determination will be required, this region in *Aplysia* IP_3R contains several residues that are predicted to be subject to posttranslational modification, including consensus sites for N-glycosylation, tyrosine phosphorylation, numerous putative sites for phosphorylation by protein kinase C, and one consensus Gly-rich sequence (G^{2013}XGXXGL) for nucleotide binding. As in the lobster IP_3R, there is a stretch of hydrophilic residues which is not present in other known IP_3Rs (1118-1144). The third domain, the membrane-spanning domain, contains six putative transmembrane regions (TM1–TM6) based on hydropathy analysis, which are predicted to form the channel region. In addition, this region contains four potential N-glycosylation sites, a putative pore helix, and a selectivity filter sequence (G^{2561}IGD) between TM5 and TM6. A conserved region near the C terminus (2634–2650) is known to bind mitochondrial cytochrome *c* and to mediate calcium release during apoptosis [39].

A phylogenetic tree of the IP_3R family based on amino acid identity is shown in Fig. 5B and indicates that the *Aplysia* IP_3R constitutes a separate branch, more closely related to invertebrate IP_3R homologs; in mammals, it is most similar to type I and type II IP_3Rs. Consistent with a potential role in pheromonal sensory transduction in *Aplysia*, IP_3R mRNA was expressed in the rhinophore epithelium, as demonstrated by in

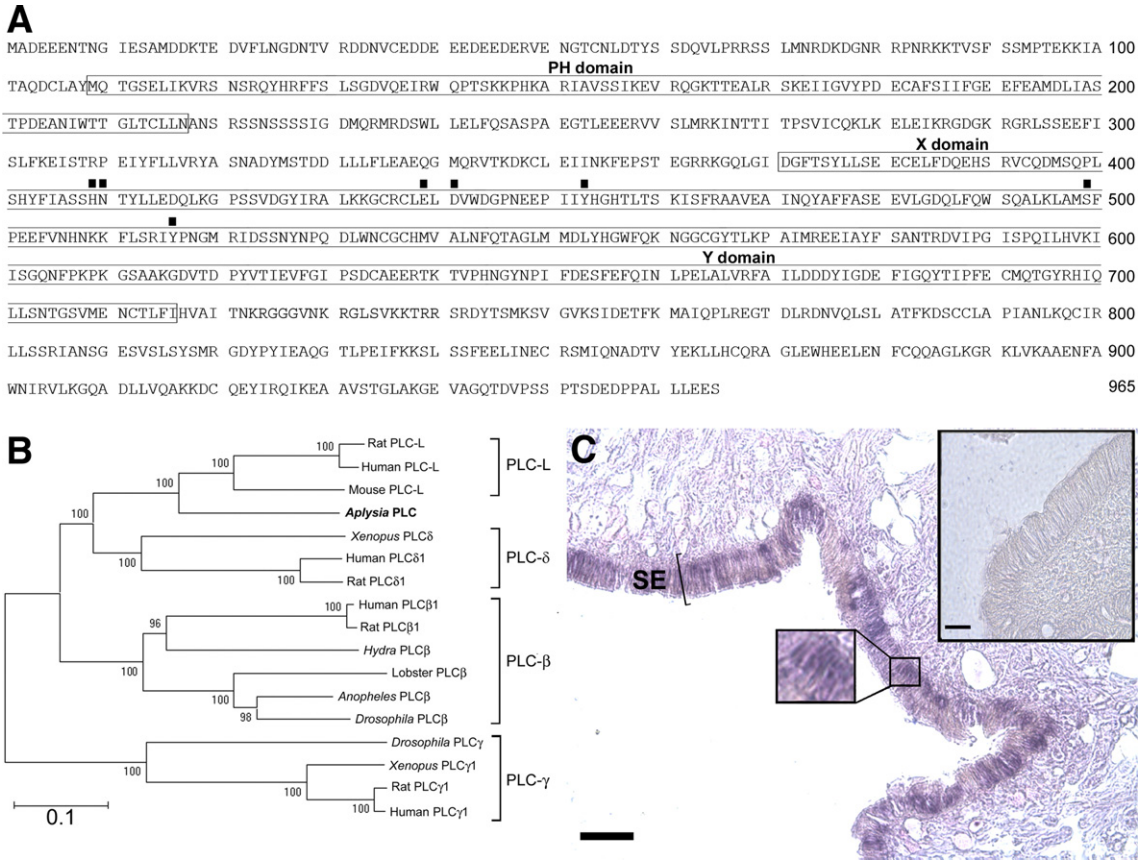

Fig. 4. Identification and analysis of *Aplysia* PLC protein. (A) Predicted amino acid sequence of *Aplysia* PLC protein. Amino acids thought to be essential for PLC activity are indicated (■) and locations of the PH, X, and Y domains are boxed. (B) Phylogenetic tree of PLC-related members. A bootstrap consensus tree was constructed from deduced amino acid sequences by the neighbor-joining method [36]. The number at each branch point represents the bootstrap probability that two lineages join together to form a cluster. Scale bar indicates estimated number of amino acid substitutions per site. GenBank Accession Nos.: rat PLC-L (NP_445908), human PLC-L (AAI01532), mouse PLC-L (BAE24416), *Aplysia* PLC-like protein (ABD62079), *Xenopus* PLCδ (AAI08619), human PLCδ1 (NP_006216), rat PLCδ1 (AAP31521), human PLCβ1 (NP_000924), rat PLCβ1 (NP_077329), *Hydra* PLCβ, lobster PLCβ (AAD32609), *Anopheles* PLCβ (EAA43070), *Drosophila* PLCβ (P13217), *Drosophila* PLCγ (AAN39332), *Xenopus* PLCγ1 (AAD03595), rat PLCγ1 (NP_037319), and human PLCγ1 (ABB84466). (C) Tissue expression of *Aplysia* PLC protein. In situ hybridization showing expression of *Aplysia* PLC mRNA in the rhinophore sensory epithelium. An enlarged region (boxed) shows a typical staining pattern for PLC mRNA; SE, sensory epithelium. Scale bar, 50 μm. Negative control using sense riboprobe is shown in inset. Scale bar, 25 μm.

situ hybridization (Fig. 5C). mRNA labeling was also identified in other cell types within the inner region of the rhinophore. A comparison of the membrane-spanning regions of *Aplysia* IP_3R (2240–2762) and rat IP_3R Type I (2224–2749) is shown in Fig. 6. *Aplysia* IP_3R is 57% identical to the corresponding membrane-spanning region, with most conservation falling in TM5 and TM6.

Discussion

Heterotrimeric G proteins are the most commonly used signal transducers in eukaryotic cells. In the well-studied vertebrate chemosensory systems, it is recognized that pheromone sensory transduction is mediated via $G\alpha_q$, $G\alpha_i$, and $G\alpha_o$ subunits, eventually leading to neuronal activation. To test the hypothesis that G proteins may also be important in sexually mature *Aplysia* chemosensory communication, we isolated sequences by performing LCM on rhinophore sensory epithelia followed by RT-PCR, which were then used to identify full-length cDNAs. We found that *Aplysia* $G\alpha_q$, $G\alpha_i$, and $G\alpha_o$ displayed strong sequence identity with homologous Gα subunit family members, and all of them are expressed in the rhinophore sensory epithelia. Likewise, several studies have demonstrated that G-protein-mediated pheromone signaling systems may also exist in other invertebrates (e.g., *Drosophila*, *Bombyx)*, although further experiments are needed to verify this.

Aplysia Gα protein sequences contain Gly^2 which may be required for myristoylation after removal of the initiation methionine, Cys^3 which may also be needed for palmitoylation, and Cys^{351} which can be ADP-ribosylated by pertussis toxin, thereby inactivating the G protein (see Fig. 3). To directly mediate olfactory transduction, the Gα subunit must be present in the outer dendritic segments of the rhinophore OSNs, where they would be in close association with putative olfactory and pheromone GPCRs. This type of distribution pattern has been described for *Bombyx* $G\alpha_q$ [18] and rodent $G\alpha_o$ [21] subunits. For *Bombyx*, $G\alpha_q$ expression is also not tissue specific but is detected in dendrites of the sensilla trichodea that receive sex pheromones such as bombykol [18]. Similarly, in the rodent

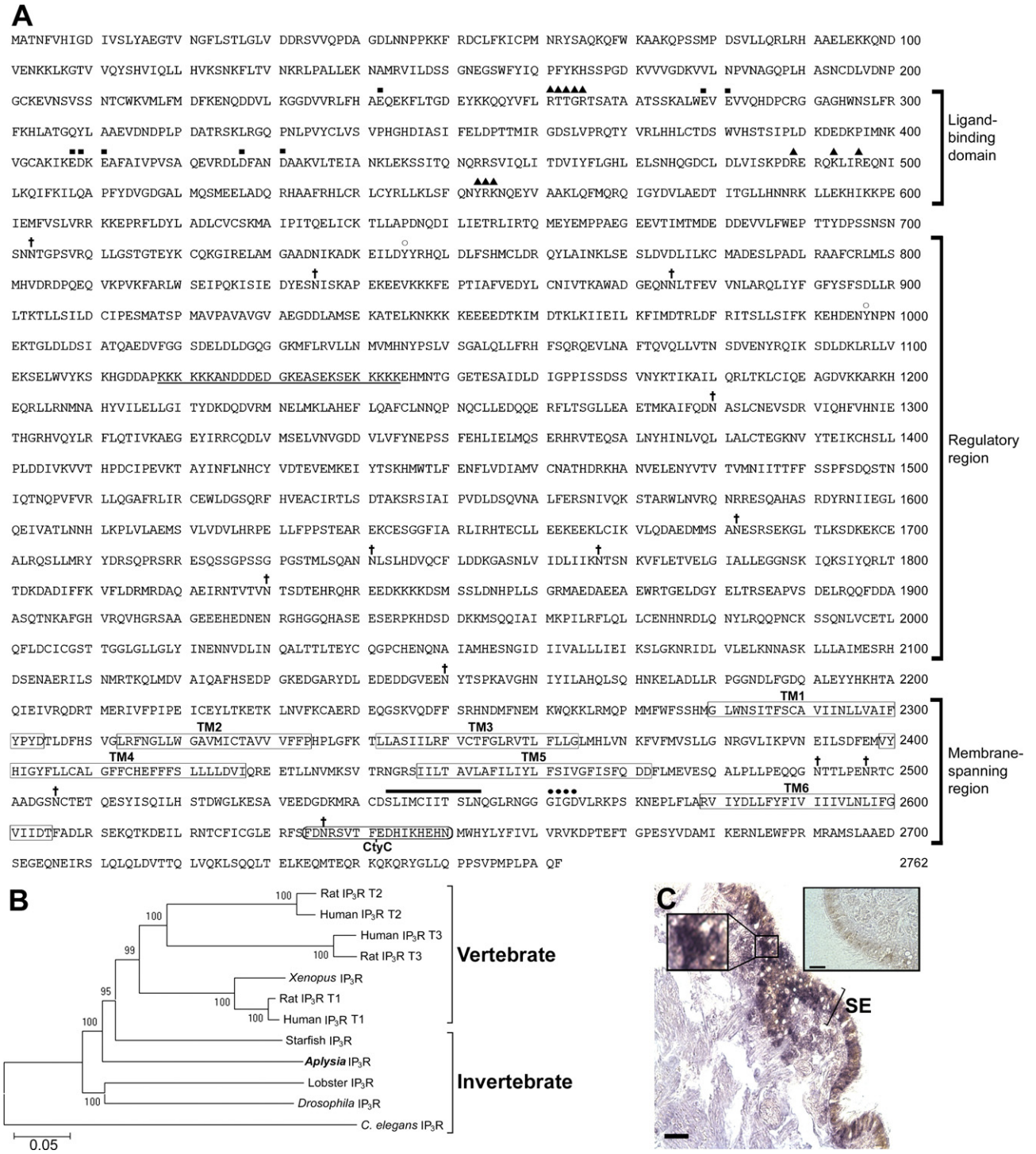

Fig. 5. Identification and phylogenetic analysis of *Aplysia* IP_3R. (A) Predicted amino acid sequence of the *Aplysia* IP_3R. The ligand binding domain, regulatory region, and membrane-spanning regions are indicated, and the six proposed transmembrane (TM) regions are boxed. Potential sites for N-glycosylation (crosses) and tyrosine-kinase-dependent phosphorylation (○) are indicated. Symbols indicate conserved residues important for Ca^{2+} binding (■), IP_3-coordinating residues (▲), the region of the pore helix (overlined), the selectivity filter (●), a stretch of hydrophilic residues (underline), and the site of cytochrome *c* binding (CytC). (B) A phylogenetic tree of IP_3R members. A bootstrap consensus tree was constructed from deduced amino acid sequences by the neighbor-joining method [36]. The number at each branch point represents the bootstrap probability that two lineages join together to form a cluster. Scale bar indicates estimated number of amino acid substitutions per site. GenBank Accession Nos.: rat IP_3R T2 (AAQ82910), human IP_3R T2 (Q14571), human IP_3R T3 (NP_002215), rat IP_3R T3 (NP_037270), *Xenopus* IP_3R (BAA03304), rat IP_3R T1 (NP_001007236), human IP_3R T1 (NP_002213), starfish IP_3R (BAB84088), *Aplysia* IP_3R (ABD62080), lobster IP_3R (AAC61691), *Drosophila* IP_3R (P29993), and *C. elegans* IP_3R (AAK68365). (C) In situ hybridization showing expression of *Aplysia* IP_3R mRNA in the rhinophore sensory epithelium. An enlarged region (boxed) shows a typical staining pattern for IP_3R mRNA; SE, sensory epithelium. Scale bar, 50 μm. Negative control using sense riboprobe is shown in the inset. Scale bar, 25 μm.

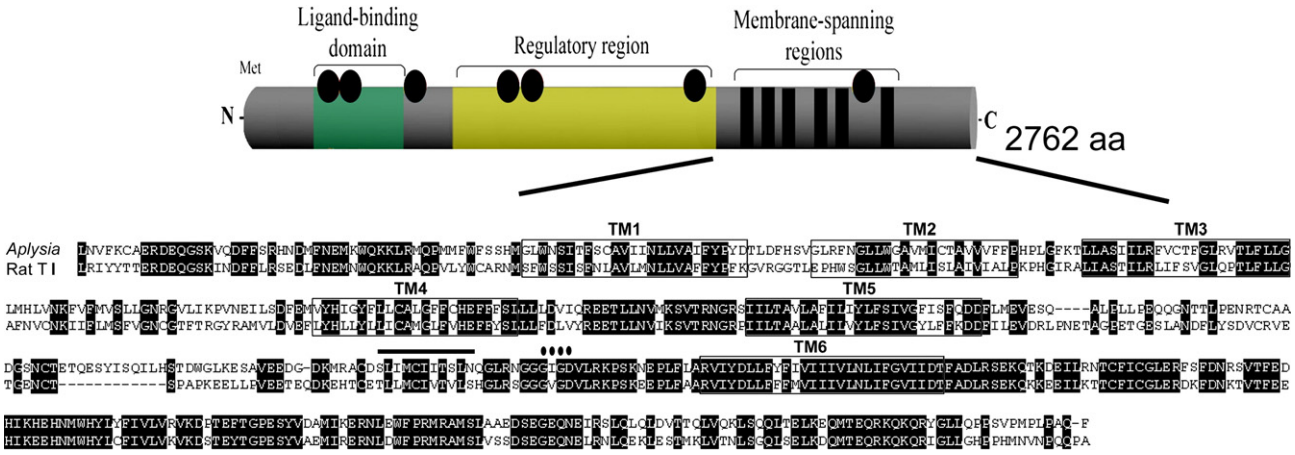

Fig. 6. Schematic diagram of *Aplysia* IP_3R showing ligand binding domain, regulatory region, and membrane-spanning regions. Circles represent potential calcium binding sites. Comparison of putative membrane-spanning amino acid sequences from *Aplysia* and rat IP_3R Type I. Identical residues are indicated in black. Transmembrane domains (boxed), region of the pore helix (overlined), and the selectivity filter (●) are indicated.

vomeronasal organ (VNO), vomeronasal-2-receptor (V2R)-positive sensory cells express the $G\alpha_o$ subunit and are involved in recognition of peptide pheromones. In that pheromone system, male rodents secrete a pheromone peptide (exocrine gland secreting peptide 1; ESP1) from the eyes which is transferred by physical interaction to the female VNO. ESP1 stimulates V2R-expressing sensory neurons and elicits an electrical response following $G\alpha_o$ subunit activation [21]. Subsequently, $G\alpha_o$ then activates PLC leading to IP_3 production. *Aplysia* $G\alpha_q$ and $G\alpha_i$ gene expression appears not to be tissue specific, whereas $G\alpha_o$ expression appears to be primarily restricted to the sensory tissues tested. The presence of immunoreactive $G\alpha_q$, $G\alpha_i$, and $G\alpha_o$ in rhinophore suggests a potential role in *Aplysia* pheromone signal transduction, although further detailed analysis is necessary to precisely localize the identified *Aplysia* G proteins to rhinophore sensory neuron dendrites.

Activation of PLC is an important component to the signal transduction process of many cell types. Four major isoforms of PLC are known, PLC-β, PLC-γ, PLC-δ, and PLC-ε, in addition to the more recently discovered PLC-L form. PLC-β activity is classically involved in the receptor-mediated IP_3 pathway and has been localized to the outer dendrites, including cilia, of mammalian and lobster olfactory tissues [24,40]. In the present study, we describe the isolation, sequence analysis, and expression of an *Aplysia* PLC. We obtained a partial PLC cDNA by performing LCM/RT-PCR, using degenerate primers that were designed within the regions that are highly conserved among all known PLC-β sequences, and screened a CNS library to obtain the full-length cDNA. Detailed sequence analysis, however, suggested that this clone was not a close homolog of PLC-β. Phylogenetic amino acid sequence analysis indicated that it constitutes a separate branch, with the highest homology to PLC-L isoforms. This was also the case when the X and Y domains alone were used in multiple sequence alignments. Unlike other known PLC proteins, *Aplysia* PLC has no linker between X and Y (thought to be the site for interaction with Gβγ). However, *Aplysia* PLC does contain conserved residues and motifs that are known to be critical for catalytic function (H^{409}, N^{410}, E^{439}, D^{441}, Y^{453}, S^{499}, and Y^{516}—based on *Aplysia* numbering). Also, *Aplysia* PLC does not contain a C-terminal extension, typical of PLC-β isoforms. Extensive rescreening of the *Aplysia* CNS library failed to identify PLC-β-like clones. Nevertheless, we cannot preclude the presence of other PLC isoforms, more closely resembling PLC-β, since a squid homolog gene has been isolated and sequenced [32]. The presence of the *Aplysia* PLC mRNA identified here in rhinophore sensory epithelium suggests that this protein may be involved in pheromone signaling.

To determine whether the cloned *Aplysia* IP_3R-like transcript could be assigned to the IP_3R family, we constructed a phylogenetic tree based on a multiple alignment of amino acid sequences representing known invertebrate and vertebrate (including isoforms) IP_3Rs. Given its similarity with other IP_3R sequences (61% starfish; 54% *Drosophila*), and its somewhat distant relationship with ryanodine receptors, we believe this to be the *Aplysia* IP_3R, the first IP_3R to be sequenced in mollusks. Mammalian IP_3Rs are regulated by multiple nucleotide and protein binding molecules. Indeed, the *Aplysia* IP_3R contains many conserved consensus sequences for phosphorylation and binding of ATP, cytochrome *c*, Ca^{2+}, and IP_3; thus, it may also be regulated by these factors. Of particular significance was the conservation within the ligand binding region of multiple charged residues including Arg^{489}, Arg^{493}, and Lys^{496}, known to bind IP_3 [41]. Interestingly, the function of a strong hydrophilic segment (amino acids 1118–1144) present in the regulatory domain remains unknown. It has been suggested by Munger and colleagues [25] that a similar hydrophilic stretch of amino acids found in the lobster IP_3R may either act as a signal sequence to transport the receptor to the plasma membrane or be involved in some other unknown function. The conservation of this segment in crustaceans and mollusks indicates that it is of evolutionary significance and may even be essential to survival in the marine environment.

Significant conservation can also be found in two important regions of the IP_3R putative membrane-spanning domain. First,

the pore helix, which is α helical in shape and slants toward the pore axis (as is predicted in the mammalian homolog [41]), is only 55% identical to rat IP_3R TI but still contains predominantly negatively charged residues. It is thought to be important in concentrating Ca^{2+} ions around the putative pore [42]. Following the pore helix, the motif GXGD (which is highly conserved in all known IP_3R and ryanodine receptors) corresponds to the selectivity filter [43]. In common with other known invertebrate IP_3Rs (*Caenorhabditis elegans*, *Drosophila*, lobster), *Aplysia* IP_3R contains an Ile (GIGD) instead of a Val (GVGD) common to all mammalian IP_3Rs. Studies have shown that mutating Val to Ile in the mammalian IP_3R leads to channels with increased K^+ conductance but the same Ca^{2+} selectivity as the wild-type channels [43]. Taken together, it is likely that the *Aplysia* IP_3R functions similarly to the mammalian homologs.

Three isoforms of IP_3R (Types I, II and III) and multiple splice variants have been characterized in mammals, whereby the type III has been localized to the VNO microvillar layer, possibly within, or in close proximity to, the plasma membrane [44]. In contrast, only one isoform appears to exist in invertebrates. From the multiple (~14) partial sequences that we obtained from screening a *Aplysia* CNS cDNA library, we did not obtain any transcripts that differed from the sequence shown in Fig. 5, except for a single transcript that appeared to be pre-mRNA and contained an intron. In situ hybridization studies established that IP_3R is expressed throughout the rhinophore sensory epithelium. It will be of interest to ultimately localize IP_3R protein within this region and possibly in cilia or microvilli. There is evidence to suggest that lobster IP_3R associates with the plasma membrane of OSNs [25].

Vertebrate and insect olfactory detection is mediated by a large and diverse superfamily of seven-transmembrane domain receptors. To date, members of the putative olfactory, including pheromone receptor family, have not been identified in mollusks. Knowledge of the mechanism of pheromone signal transduction should prove helpful, following the eventual elucidation of putative *Aplysia* pheromone receptors. Olfactory and pheromone receptors are notoriously difficult to express in heterologous systems [45], so knowledge of the signaling system may facilitate the efficient expression of these receptors. Heterologous systems often require exogenous factors, usually Gα proteins, to amplify signal intensity by coupling receptors more effectively to downstream signaling pathways.

The main findings of this study were the identification and localization of Gα proteins and phosphoinositide signaling components in rhinophore sensory epithelium, supporting the notion that waterborne protein pheromones could mediate mate attraction via the G-protein-coupled activation of OSNs leading to IP_3 secondary signaling. Our knowledge of the structure and behavioral actions of four *Aplysia* protein pheromones provides a unique opportunity for studying invertebrate waterborne protein pheromone detection and signal transduction at the molecular level. It will be important to molecularly characterize the pheromone receptors and pattern of activation of specific pheromone-responsive olfactory sensory neurons.

Materials and methods

Animals

Adult *A. californica* individuals (>250 g) were obtained from Marine Research and Educational Products (Escondido, CA). Immediately following arrival, animals were anesthetized by injection of isotonic $MgCl_2$ and relevant tissues were processed by either (1) embedding in optimal cutting temperature (OCT) compound for LCM or (2) snap freezing in liquid nitrogen for RNA and protein isolation.

Laser capture microdissection, RNA isolation, and RT-PCR cloning of Aplysia $G\alpha_q$, $G\alpha_o$, $G\alpha_i$, PLC, and IP_3R cDNAs

The locations of the rhinophore groove, the glomeruli underlying the sensory epithelium, and the rhinophore ganglia in *Aplysia* are shown in Fig. 1. To examine whether *Aplysia* $G\alpha_q$, $G\alpha_i$, $G\alpha_o$, PLC, and IP_3R mRNA are expressed in rhinophore sensory epithelial tissue and to obtain a cDNA probe, a combination of laser capture microdissection, total RNA isolation, and RT-PCR were performed. Rhinophore tissue, which had been embedded in OCT compound, was sectioned (10 μm) onto slides and dehydrated. LCM was performed using a PixCell II laser capture microscope with an infrared diode laser (Arcturus Engineering Inc., Mountain View, CA) and a laser spot size of 15 μm. Cells were marked and captured on CapSure HS caps (Arcturus), and total RNA was isolated using the Picopure Isolation Kit (Arcturus) including DNase I incubation. RNA quality was determined by measurement of absorbance ratio at 260/280 nm.

The sequences of oligonucleotide primers (Sigma–Genosys) used for RT-PCR, 3′-RACE, and 5′-RACE are located in Table 1. For $G\alpha_q$, $G\alpha_i$, and $G\alpha_o$, first-strand cDNA was generated by RT using antisense adaptor primer OL1 and the Superscript Preamplification System for First Strand Synthesis (Invitrogen, Carlsbad, CA). PCR was performed using a degenerate sense primer OL2 corresponding to the common Gα sequence KWIHCFE and a degenerate antisense primer corresponding to either the common $G\alpha_q$ sequence HFTCATDT (OL3), the $G\alpha_i$ sequence KNNLKDCGLF (OL4), or the $G\alpha_o$ sequence ATNLRGCGLY (OL5). Samples were heated at 94 °C for 3 min and amplified for 36 cycles (94 °C, 60 s; 40 °C, 30 s; 72 °C, 60 s), followed by a 7-min extension at 72 °C. RT-PCR products were cloned into the TA vector pGEM-T (Promega, Madison, WI).

To obtain an RT-PCR probe for a PLC, first-strand cDNA was generated by RT using random hexamers and the Superscript Preamplification System for

Table 1
Sequences of oligonucleotide primers

OL1	AAGCAGTGGTATCAACGCAGAGTGAATTCT$_{17}$VN
OL2	AARTGGATHCAYTGYTTYGA
OL3	NGTRTCNGTNGCRCANGTRAARTG
OL4	RAANARNCCRCARTCYTTNARRTTRTTYTT
OL5	RTANARNCCRCANCCNCKNARRTTRTTNGC
OL6	ATYAAYTCVTCYCACAACACBTA
OL7	ATGRTTTTCRAADGAYARRATRACTGG
OL8	GGNGTNATHATHGAYACNTTYGC
OL9	CATNGCNCKCATNCKNGGRAACC
OL10	TARTGCCACATRTTRTGYTC
OL11	TCGCCAACGTCGTAGAAGGGTGC
OL12	CCNCCNAARAARTTYMGNGAYTG
OL13	CATGGACTGTATGGCGTTACTG
OL14	CTTGGTCGTATTCACTCATAGC
OL15	GCTCTAATACGACTCACTATAGG
OL16	GTGAAGTGGGAGTAGATCTCCT
OL17	GTCTACTGTCATCAAACTTGCG
OL18	AAGCAGTGGTATCAACGCAGAGT
OL19	AAGATGATGGAGAAGGCGCACTCG
OL20	TGCCGAGTAGCGGTTCATGGGACAG

N,A/T/C/G; V,A/C/G; R,A/G; H,A/T/C; Y,T/C; K,T/G; B,T/C/G; D,A/T/G; M,A/C.

First Strand Synthesis. PCR was performed using degenerate sense primer OL6 corresponding to the highly conserved catalytic region INSSHNT and degenerate antisense primer OL7 corresponding to the common PLC sequence PVILSFENH. PCR conditions were as described above, except that the PCR was followed by a 10-min extension. To obtain an RT-PCR probe for an IP_3R, first-strand cDNA was generated by RT using random hexamers and the Superscript Preamplification System for First Strand Synthesis.

PCR was performed using degenerate sense primer OL8 corresponding to the sixth transmembrane region GVIIDTFA and degenerate antisense primer OL9 corresponding to the common IP_3R sequence WFPRMRAM. PCR was performed as described above, except that the PCR was followed by a 10-min extension. The specificity of the product was confirmed by nested PCR using sense primer OL8 and nested degenerate antisense primer OL10 corresponding to the common IP_3R sequence EHNMWHY. Following library screening, RT-PCR was again performed to obtain the 5′ cDNA sequence. This was accomplished using the specific antisense primer OL11 corresponding to the *Aplysia* IP_3R sequence APFYDVG and degenerate sense primer OL12 corresponding to the common IP_3R sequence PPKKFRD. The primary PCR products were cloned into pGEM-T vector. Nucleotide sequence analyses verified the identity of the cloned $G\alpha_q$, $G\alpha_i$, $G\alpha_o$, PLC, and IP_3R RT-PCR products.

cDNA library construction and screening

Poly(A)$^+$ RNA from the *A. californica* CNS (pooled cerebral, pleural, buccal, pedal, abdominal ganglia) was selected by oligo(dT)-cellulose chromatography (Poly(A) Pure Kit; Ambion, Austin, TX). Double-stranded cDNA was synthesized with a ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA), cloned into the *Eco*RI/*Xho*I sites of Lambda ZAP II vector, and the cDNA library (complexity 2.2×10^5) was amplified once. *Aplysia* $G\alpha_q$, PLC, and IP_3R RT-PCR inserts were purified and labeled with [α-^{32}P]dCTP by using a Prime-It II Random Primer Labeling kit (Stratagene), and unincorporated nucleotide was removed by chromatography on NucTrap columns (Stratagene); labeled inserts were used as probes for library screening of 1×10^6 plaques essentially as previously described [4,6]. One $G\alpha_q$-positive clone, 1 PLC positive clone, and 16 IP_3R-positive clones were rescreened and sequenced.

5′- and 3′-Rapid amplification of cDNA ends

The SMART RACE cDNA Amplification Kit (Clontech Laboratories, Mountain View, CA) was used to isolate the 5′ region of *Aplysia* $G\alpha_\iota$, $G\alpha_o$, PLC, and IP_3R by 5′-RACE. 3′-RACE was used to isolate the 3′ region of *Aplysia* $G\alpha_i$ and $G\alpha_o$. In both cases, PCR was performed using gene-specific primers and confirmed by DNA sequence analysis (Sequencher Ver. 4.7). For 5′-RACE of $G\alpha_i$ and $G\alpha_o$, antisense primers OL13 and OL14, respectively, were used in combination with the 5′-RACE sense primer OL15. For 3′-RACE of $G\alpha_i$ and $G\alpha_o$, sense primers OL16 and OL17, respectively, were used in combination with the 3′-RACE antisense primer OL18. For PLC, the antisense and sense primers used for 5′-RACE were OL19 and OL15, respectively. For the IP_3R, the antisense and sense primers used for 5′-RACE were OL20 and OL15, respectively.

Phylogenetic analysis

Sequences were aligned using Clustal X, 8.1 [46]. Phylogenetic trees were constructed from respective multiple alignments using MEGA3 software [36]. The unrooted tree was generated with 1000 bootstrap trials using the neighbor-joining method [47] and presented with a cutoff bootstrapping value of 50.

In situ hybridization

In situ hybridization was performed using the protocol described by Braissant and Wahli [48] using 10-μm paraffin sections cut from *A. californica* rhinophores. Digoxigenin (DIG)-labeled riboprobes were produced using T3 and T7 RNA polymerase (DIG RNA-labeling kit, Roche) and recombinant clones containing *A. californica* $G\alpha_q$, PLC, and IP_3R. Postfixation was performed in diethylpyrocarbonate (DEPC)-treated phosphate-buffered saline (PBS, pH 7.4) containing 4% paraformaldehyde (10–30 min), followed by acetylation with DEPC-treated PBS (2× 15 min) containing 0.1% active DEPC. Sections were subsequently incubated in DEPC-treated 5× standard saline citrate (SSC) (15 min), prehybridization buffer (>2 h, 58 °C), and 30 μl hybridization buffer containing 5–10 ng of DIG-labeled riboprobe (overnight, 58 °C). Sections were sequentially washed with 2× SSC (30 min, room temperature), 2× SSC (1 h, 65 °C), and 0.1× SSC (1 h, 65 °C). Detection of probe was initiated by washing sections with buffer 1 (100 mM Tris–HCl, pH. 7.5, 150 mM NaCl) for 5 min, followed by incubation with buffer 1 and anti-DIG/alkaline phosphatase (1:5000) containing 0.5% casein (2 h). Unbound antibody was removed by washing with buffer 1 (2× 15 min) and buffer 2 (Tris–HCl, pH. 9.5, 50 mM $MgCl_2$) (2× 15 min). NBT/BCIP Liquid Substrate (~200 μl; Sigma–Aldrich) was placed over sections and left in the dark until color development was optimal, and the reaction was stopped by incubation with buffer 3 (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). Nonspecific staining was removed by incubation in 95% ethanol (1 h) with gentle agitation. Sections were mounted in aqueous mounting media and viewed under a Zeiss Axioskop MC 80 microscope, and images were captured on a spot-cooled charge-coupled device camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Control slides included using a DIG-labeled sense riboprobe as negative controls.

Immunoblot analysis

Protein was quantified using the BCA Protein Assay Reagent kit (Pierce). Immunoblot analyses were performed essentially as described previously [4,6] using 12% SDS–polyacrylamide gels. Membranes were incubated with antibodies against either rat $G\alpha_q$ residues 350–359 (1:1000 dilution; Calbiochem, San Diego, CA), human $G\alpha_i$ residues 346–355 (1:1000 dilution; Abcam, Cambridge, MA), or mouse $G\alpha_o$ residues 345–354 (1:1000 dilution; Biogenesis, Kingston, NH), which recognize their *Aplysia* homologs due to the high degree of sequence identity. As a control, the primary antiserum was omitted.

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