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Molecular identification of candidate chemoreceptor genes and signal transduction components in the sensory epithelium of *Aplysia*

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SUMMARY

An ability to sense and respond to environmental cues is essential to the survival of most marine animals. How water-borne chemical cues are detected at the molecular level and processed by molluscs is currently unknown. In this study, we cloned two genes from the marine mollusk *Aplysia dactylomela* which encode multi-transmembrane proteins. We have performed *in situ* hybridization that reveals expression and spatial distribution within the long-distance chemosensory organs, the rhinophores. This finding suggests that they could be receptors involved in binding water-borne chemicals and coupling to an intracellular signal pathway. In support of this, we found expression of a phospholipase C and an inositol trisphosphate receptor in the rhinophore sensory epithelia and possibly distributed within outer dendrites of olfactory sensory neurons. In *Aplysia*, mate attraction and subsequent reproduction is initiated by responding to a cocktail of water-borne protein pheromones released by animal conspecifics. We show that the rhinophore contraction in response to pheromone stimulants is significantly altered following phospholipase C inhibition. Overall, these data provide insight into the molecular components of chemosensory detection in a mollusk. An important next step will be the elucidation of how these coordinate the detection of chemical cues present in the marine environment and activation of sensory neurons.

Key words: *Aplysia*, receptor, pheromones, phospholipase C, inositol trisphosphate receptor.

INTRODUCTION

Humans view smell as a luxury, yet for most animals smell is the primal sense, one they rely on for survival. Incredibly, it is still a mystery as to how many animals in the ocean smell and respond to chemical cues despite the essential role of water-borne signals for all aspects of survival, for example reproduction (Painter et al., 2004), feeding (Stief and Hölker, 2006), settlement (Dreanno et al., 2006) and predator avoidance (Ferrari and Targett, 2003).

The molecular components and mechanism of chemical detection in some animal phyla have been well studied. Vertebrate and insect chemoreceptors seemingly evolved independently and show no similarity besides containing seven-transmembrane (7TM) domains. In mammals the odorant ligand(s)–receptor interactions cause an activation of downstream effectors *via* G proteins, which can regulate a variety of effectors such as cAMP and inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3], whereas the functional insect olfactory receptor consists of a heteromeric complex forming a ligand-gated ion channel (Sato et al., 2008). Although relatively little is currently known about the molecular mechanism of chemical detection in molluscs, including the identity of chemoreceptors, recent olfactory studies in squid have shown that in olfactory receptor neurons, both phospholipase c (PLC) and cAMP-mediated pathways may be involved in signal processing (Mobley et al., 2007). In our studies of the marine opisthobranch mollusk *Aplysia californica*, we isolated a full-length cDNA from a central nervous system (CNS) cDNA library that encodes a protein homologous to the G protein alpha subunit G_q (DQ397515), as well as a phospholipase C (PLC; DQ397516) and an inositol 1,4,5-trisphosphate receptor (Ins(1,4,5)P_3R; DQ397517) (Cummins et al., 2007). The *Aplysia* Gα_q gene encodes a protein of 353 amino acids, and shares conservation of myristoylation, palmitoylation and ADP-ribosylated sites with known G protein homologs. *Aplysia* PLC cDNA encodes a predicted 965-amino-acid protein that shares the highest degree of sequence identity with PLC-L (PLC-like) proteins. Finally, *Aplysia* Ins(1,4,5)P_3R cDNA encodes a 2762-residue protein with an estimated molecular mass of 315 kDa that shares a high degree of identity with invertebrate Ins(1,4,5)P_3R homologs. Consistent with a potential role in *Aplysia* chemical transduction, recent observations using laser capture microdissection of rhinophore sensory epithelium followed by G protein-, PLC- or Ins(1,4,5)P_3R-specific RT-PCR, indicate that these mRNA are expressed in rhinophore sensory epithelium (Cummins et al., 2007).

For marine organisms, pheromone communication is important for attracting potential mates since vision and auditory signaling may be absent or restricted. Pheromones are molecules released from one animal and detected by conspecifics, resulting in hormonal and behavioral changes. The molecular nature of pheromone-induced behavior on mate attraction has been thoroughly studied in *Aplysia*. In this animal, egg laying results in the release of a cocktail of water-borne attraction pheromones, and among these the small protein ‘attractin’ appears to play a key role. In fact, behavioral T-maze assays have shown that binary pheromone blends of attractin with either enticin, temptin or seductin is sufficient to attract potential mates (Cummins et al., 2004; Cummins et al., 2005a; Cummins et al., 2005b). All four protein pheromones are synthesized in a specialized exocrine gland, the albumen gland, the other primary function of which is to secrete materials that package eggs into capsules and then into cordons. At the anatomical level, long-distance chemoreception is achieved by specialized anterior sensory organs known as rhinophores, which are bilaterally paired, retractile

organs on the dorsal surface of the head. Their ultrastructure and neuroanatomical organization have been described, and includes a rhinophore groove where a specialized epithelium contains the majority of sensory neurons. It has been reported that the sensory neurons have axons that project back to either glomeruli or rhinophore ganglia, and dendrites that end in surface-exposed cilia (Emery and Audesirk, 1978; Wertz et al., 2006). Tentacle retraction (called a twitch) is a mechanism by which odor molecules are removed from the olfactory epithelium, thus providing improved temporal resolution for olfactory perception (Lemaire and Chase, 1998).

The presence of $G\alpha_q$ protein transcripts in the sensory epithelium suggests that *Aplysia* water-borne pheromone detection may involve a transmembrane receptor coupled to a downstream PLC, or other pathway, to provide signal amplification and neuronal excitation. To address this issue, we identified a gene encoding a multi-transmembrane candidate chemosensory receptor found to be expressed within the *Aplysia* rhinophore sensory neuron region that could potentially couple to $G\alpha_q$. Antisera directed against conserved regions of *Aplysia* PLC and Ins(1,4,5)P_3R were used to identify expression in pheromone sensory tissue, and to locate these proteins at the site of olfactory transduction, the outer sensory epithelium of the rhinophores. We provide functional evidence that inactivating PLC activity in rhinophores leads to significant changes in pheromone sampling compared with control tissue.

MATERIALS AND METHODS

Animals

Adult *A. californica* Cooper 1863 individuals (>250 g) were obtained from Marine Research and Educational Products (MREP, Escondido, CA, USA). Immediately following arrival, animals were anesthetized by injection of isotonic $MgCl_2$ (337 mmol l^{-1}) equivalent to 50% of their mass and relevant tissues were processed by either (1) embedding in optimal cutting temperature (OCT) compound for sectioning, (2) snap freezing in liquid nitrogen or (3) placing in filtered artificial seawater (ASW; 425 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 22 mmol l^{-1} $MgCl_2$, 10.2 mmol l^{-1} $CaCl_2$, 2.8 mmol l^{-1} $NaHCO_3$, 26.2 mmol l^{-1} $MgSO_4$, 1 g l^{-1} glucose) for primary culture or organ bath experiments. Adult *A. dactylomela* Rang 1828 (>150 g) were collected from Caloundra (Queensland, Australia). Animals were anesthetized by injection of isotonic $MgCl_2$ and relevant tissues were immediately processed by (1) fixing in fresh 4% paraformaldehyde/0.1 mol l^{-1} phosphate buffer (PB), pH 7.4, for 24 h at 4°C, then embedding in OCT compound for sectioning, or (2) snap freezing in liquid nitrogen for RNA isolation.

Candidate chemoreceptor identification, probe synthesis and *in situ* hybridization

Total RNA was extracted from rhinophore tissue of *A. dactylomela* using Tripure Isolation Reagent (Roche, Applied Science, Castle Hill, NSW, Australia), and any contaminating genomic DNA was removed by treatment with DNase I (Invitrogen, Carlsbad, CA, USA). First strand cDNA synthesis was performed using 1 µg of total RNA in a 20 µl reverse transcription mixture containing oligo$(dT)_{12–18}$ and 200 units Superscript™ III RNase H^- reverse transcriptase (Invitrogen), following the manufacturer's instructions. Degenerate primers (Sigma-Genosys, Sigma-Aldrich Pty. Ltd., Sydney, NSW, Australia) were designed to regions of *A. californica* candidate chemosensory receptors (GenBank accession numbers EU808013 and EU935862). OL1 sense: 5′-Y(C/T)CATCAG-CTTY(C/T)TTTGGCATTGTGGC-3′ (corresponding to ISFFGIV), OL1 antisense: 5′-R(A/G)W(A/T)AY(C/T)TTB(C/G/T)GAR-(A/G)CTCATN(A/T/G/C)TW(A/T)R(A/G)TAATA (corresponding to YYNMSSKY)-3′; OL2 sense: 5′-ATGGATAAGY(C/T)-ATM(A/C)TGCV(A/C/G)W(A/T)GAACCCG (corresponding to WISICVNP)-3′, OL2 antisense: 5′-TCGR(A/G)TATTTY(C/T)-GAACTCATTTTCAG-3′ (corresponding to LKMSSKYR).

Samples were heated at 94°C for 2 min and amplified for 36 cycles (94°C, 60 s; 45°C, 30 s; 72°C, 60 s), followed by a 7-min extension at 72°C. Amplicons of expected size were cloned into the transcription vector pGEM-T (Promega, Madison, WI, USA) and sequenced. Computer analyses of sequences were performed using BLAST and CLUSTALW for nucleotide alignment. Transmembrane helix domain and topology of predicted receptors was performed using HMMTOP version 2.0 program; http://www.enzim.hu/hmmtop/index.html.

After plasmid isolation of the OL2 recombinant and amplification of template DNA using M13 forward and reverse primers, *in vitro* transcription reactions were carried out in the presence of digoxigenin-UTP (DIG RNA Labeling Kit; Roche), with SP6 polymerase for antisense probes and T7 polymerase for sense riboprobes. The template was degraded with RNase-free DNase (Roche) and DIG-labeled riboprobes were purified by ethanol and lithium chloride precipitation and stored at –80°C in RNase-free water until *in situ* hybridization. *In situ* hybridization was performed using DIG-labeled riboprobes according to the method of Hinman et al. (Hinman et al., 2000), with modifications as follows. Rhinophores were sectioned (12 µm) onto slides and incubated in phosphate-buffered saline (PBS) with 0.1% Tween 20 for 10 min. Specimens were pre-hybridized for 5 h in 50% formamide, 5× SSC, 5 mmol l^{-1} EDTA, 1% Denhardt's solution (100 µg ml^{-1} heparin, 100 µg ml^{-1} tRNA, 0.1% Tween 20) at 50°C. Hybridization was performed using the same solution and by adding 200 ng ml^{-1} DIG-labeled riboprobe overnight at 50°C. Specimens were subsequently washed at 55°C twice in 50% formamide, 4× SSC, 0.1% Tween 20, then twice in 50% formamide, 2× SSC 0.1% Tween 20, then twice in 50% formamide, 1× SSC, 0.1% Tween 20, for 15 min each, and then stepped into 0.1 mol l^{-1} maleic acid, pH 7.5, 0.15 mol l^{-1} NaCl, 0.1% Tween 20. Antibody incubation for detection was also performed overnight, followed by several washing steps (Shain and Zuber, 1996). Staining reactions were done by using Nitro Blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma) in glycerol. For analysis, specimens were dehydrated by stepwise ethanol and xylene changes, and mounted in DePeX (Sigma).

Antiserum production

A glutathione S-transferase (GST)–*Aplysia* Ins(1,4,5)P_3R fusion protein (GST–Ins(1,4,5)P_3R) was used to immunize one New Zealand white rabbit (Antibodies Inc., Davis, CA, USA); C-terminal residues 2697-2762 of *Aplysia* Ins(1,4,5)P_3R were included in the fusion protein. GST-Ins(1,4,5)P_3R antibodies were affinity purified using the AminoLink Plus Immobilization Kit (Pierce Biotechnology, Rockford, IL, USA), and antibodies to GST were removed by preincubation with recombinant GST (20 µg ml^{-1}). To generate an antiserum specific to the *Aplysia* PLC, a peptide corresponding to residues 723–740 of *Aplysia* PLC (*cys*-acetyl-aminohexanoate-KRGGGVNKRGLSVKKTRRamide) was synthesized, conjugated to keyhole limpet hemocyanin, and the conjugate used to immunize one New Zealand white rabbit (Antibodies Inc.). The synthetic peptide was coupled to an affinity column, and antiserum to the PLC protein was affinity purified using the AminoLink Plus Immobilization Kit, according to the manufacturer's instructions.

Immunoblot analysis of Ins(1,4,5)P_3R

The rhinophores and CNS (pooled cerebral, buccal, pleural, pedal, and abdominal ganglia) were removed from sexually mature *A. californica*. Organs were extracted at 4°C in 0.1% heptafluorobutyric acid (Sigma) using a Polytron homogenizer (Brinkmann Instruments, Mississauga, ON, USA) and sonicated. Total protein was lyophilized, resuspended in PBS, and then quantified using the BCA Protein Assay Reagent (Pierce). Immunoblot analyses were performed essentially as described previously (Akalal et al., 2003) using 12% SDS-polyacrylamide gels. Membranes were incubated with antibodies against *Aplysia* Ins(1,4,5)P_3R antiserum (1:1000 dilution; 1 mg of affinity-purified mg^{-1} of antibody). The antigen–antibody complexes were detected by enhanced chemiluminescence (SuperSignal; Pierce). As a control, the primary antiserum was replaced with preimmune serum.

Immunohistochemical localization of *Aplysia* PLC and Ins(1,4,5)P_3R

The essential details of the immunohistochemical protocol used here have been described previously (Cummins et al., 2004; Cummins et al., 2005a). Briefly, immunofluorescence staining was performed using cryostat sections of rhinophores of sexually mature *A. californica*. Serial sections (8 μm) were cut, mounted on gelatin-coated slides and placed in running water for 5 min. Blocking was performed in 4% bovine serum albumin for 30 min. Sections were rinsed in PBS (×3), incubated overnight at 4°C in either *Aplysia* PLC antiserum (1:500 dilution; 1 mg of affinity-purified antibody ml^{-1}) or *Aplysia* Ins(1,4,5)P_3R antiserum (1:1000 dilution), rinsed in PBS (×3), incubated in fluorescein-isothiocyanate (FITC)-conjugated goat anti-rabbit Ig (Sigma-Aldrich) for 1 h at 22°C, rinsed in PBS (×3), and then mounted in FITC mounting medium (90% glycerol, 4% *n*-propylgallate in 50 mmol l^{-1} PBS, pH 8.2). Preparations were examined using an Olympus FluoView confocal microscope (Leeds Precision Instruments, Minneapolis, MN, USA), and the images captured on a spot-cooled CCD camera. To aid in visualization of cell nuclei, some sections were counterstained with propidium iodide (PI, 0.05 mg ml^{-1} in PBS; Sigma). As a control, the primary antiserum was replaced with preimmune serum or no primary antibody.

Immunohistochemical localization of Ins(1,4,5)P_3R in dissociated rhinophore cells

A. californica rhinophores were treated with collagenase (1 mg ml^{-1}; Sigma) in ASW for 1 h, followed by a 5 min rinse in filtered ASW. Under a dissecting microscope, epithelial tissue was separated using fine forceps. The underlying olfactory sensory cells were separated from muscle cells by mechanical dissociation and subsequently filtered through a sterile cell strainer (70 μm; Fisher Scientific, Pittsburgh, PA, USA). Dissociated cells were plated onto concanavalin A (Con A)-coated (10 mg ml^{-1}) glass coverslips, allowed to settle for 20 min before rinsing in filtered ASW, and incubated for 24 h at room temperature in filtered ASW. Cells were subsequently fixed in methanol for 20 min at –20°C, and permeabilized in 0.1% Triton X-100 for 15 min. Cells were processed either using Mayer's hematoxylin (Sigma) staining, PI nuclear staining (0.1 mg ml^{-1} in PBS) or reacted with anti-Ins(1,4,5)P_3 as described above.

Purification of egg eluate proteins

A. californica (100–500 g) were induced to lay eggs by egg-laying hormone injection. One hour after injection, and at 15-min intervals thereafter, egg cordons were removed, transferred to 100 ml of fresh ASW for elution, and gently shaken for 15 min. Seawater eluates were acidified to a final concentration of 0.1% trifluoroacetic acid (TFA), filtered (0.45 μm), purified on C_{18} Sep-Pak Vac cartridges (5 g; Waters Corp., Rydalmere, NSW, Australia), and the sample eluted with 60% acetonitrile/0.1% TFA and lyophilized. Based on previous RP-HPLC and amino acid microsequence analysis (Cummins et al., 2004; Cummins et al., 2005a), attractin, enticin and temptin represent a significant fraction of purified egg eluate protein.

Rhinophore bath assay

Physiological experiments were performed on rhinophores from sexually mature *A. californica*. Following anesthetization, the rhinophores were dissected away from the head and pinned to the bottom of a chamber containing 100 ml aerated ASW. A stainless steel S-hook was inserted through the tip of individual rhinophores (approximately 10–20 mm in length). The hook was connected to a force transducer (Grass-FT03) via a cotton thread, and this was linked to a PowerLab™ data collection system, which recorded data for further analysis. An initial resting tension was adjusted until no activity was recorded (10–20 min), by which time tension had reduced to 0 g. Test solutions containing 30 μg purified egg eluate protein were dissolved in 20 μl ASW, gently added to the chamber in close proximity to the rhinophore and the amplitude and number of contractions were measured over a 5 min period. This egg eluate protein concentration has been shown previously to elicit a predictable rhinophore twitch response (Cummins et al., 2008). After each response, rhinophores were incubated for an additional 10 min, or until no additional contractions were observed. In experiments with ASW containing 10^{-6} mol l^{-1} PLC inhibitor (U73122; Calbiochem, Gibbstown, NJ, USA) or 10^{-6} mol l^{-1} PLC inactive inhibitor (U73343; Calbiochem), rhinophores were preincubated for at least 15 min prior to application of 30 μg egg eluate protein. All experiments were performed at room temperature. Responses, including number of contractions and maximum amplitude of twitch contraction, were obtained directly following addition of stimulus. Data are expressed as means ± one standard error (s.e.m.) of five or more experiments. Statistical differences between the values were determined using Student's *t*-test for paired comparisons and significance was assumed at a $P \leq 0.05$.

RESULTS

Identification and distribution of a candidate chemoreceptor mRNAs

PCR amplification of *A. dactylomela* rhinophore cDNA using degenerate primers, which were selective for *A. californica* candidate chemosensory receptor genes, generated amplification products of 862 bp (using OL1 primer set, *A.dac1*) and 739 bp (using OL2 primer set, *A.dac2*). The amplicons were successfully cloned and sequenced. The predicted partial-length cDNAs encode candidate chemosensory receptors for *A. dactylomela*, sharing 41% (*A.dac1*) and 79% (*A.dac2*) identity with *A. californica* (Fig. 1). Kyte–Doolittle hydropathy profiles of the deduced amino acid sequences show that they contain six hydrophobic transmembrane segments, probably representing transmembrane domains 2–7. An antisense riboprobe was generated from the *A.dac2* amplicon to examine the precise distribution of mRNA in sections of mature *A. dactylomela* rhinophores (Fig. 2A–C). At low stringency, mRNA transcripts were detected central to rhinophore sections and within the vicinity of the sensory epithelium. No distinct signal was observed when sections were incubated with a DIG-labeled sense riboprobe (Fig. 2D).

Distribution of *Aplysia* phospholipase C

For the purpose of this study, we focused on PLC as a potential secondary signaling olfactory component because of recent

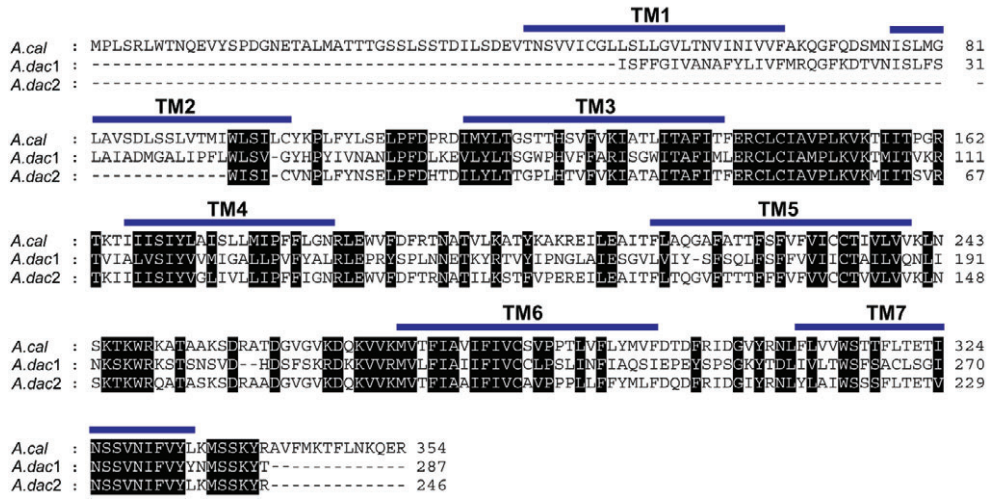

Fig. 1. Alignment of predicted amino acid sequences of three candidate chemosensory receptors cloned from *Aplysia*. (A) In *Aplysia dactylomela*, two gene amplicons were obtained by RT-PCR of rhinophore RNA, encoding multi-transmembrane proteins. An alignment of *A. dactylomela* clones *A.dac1* and *A.dac2* with *A. californica* (*A.cal*; GenBank: EU935862) is shown, including regions encoding putative transmembrane domains (TM) 1–7 deduced from hydropathy analysis. Black shading indicates identical or similar amino acids. The sequence data obtained from this study have been submitted to the GenBank database under accession numbers FJ794819 (*A.dac1*) and FJ159694 (*A.dac2*).

identification of an *Aplysia californica* PLC homolog (Cummins et al., 2007). Fig. 3A shows that the *Aplysia* PLC shares typical pleckstrin homology (PH; residues 109–217) and X and Y catalytic domains (residues 370–716) with other PLC classes (based on Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de/). The location of the peptide sequence that was used for antibody generation is also shown. To examine the expression of PLC within pheromone sensory epithelia, *A. californica* rhinophore sections were reacted with *Aplysia* PLC antibody. Fig. 3B shows that the majority of the expression is in the surface region of sensory epithelium, possibly within cilia. A few additional immunoreactive fibers were located distal to the primary layer of

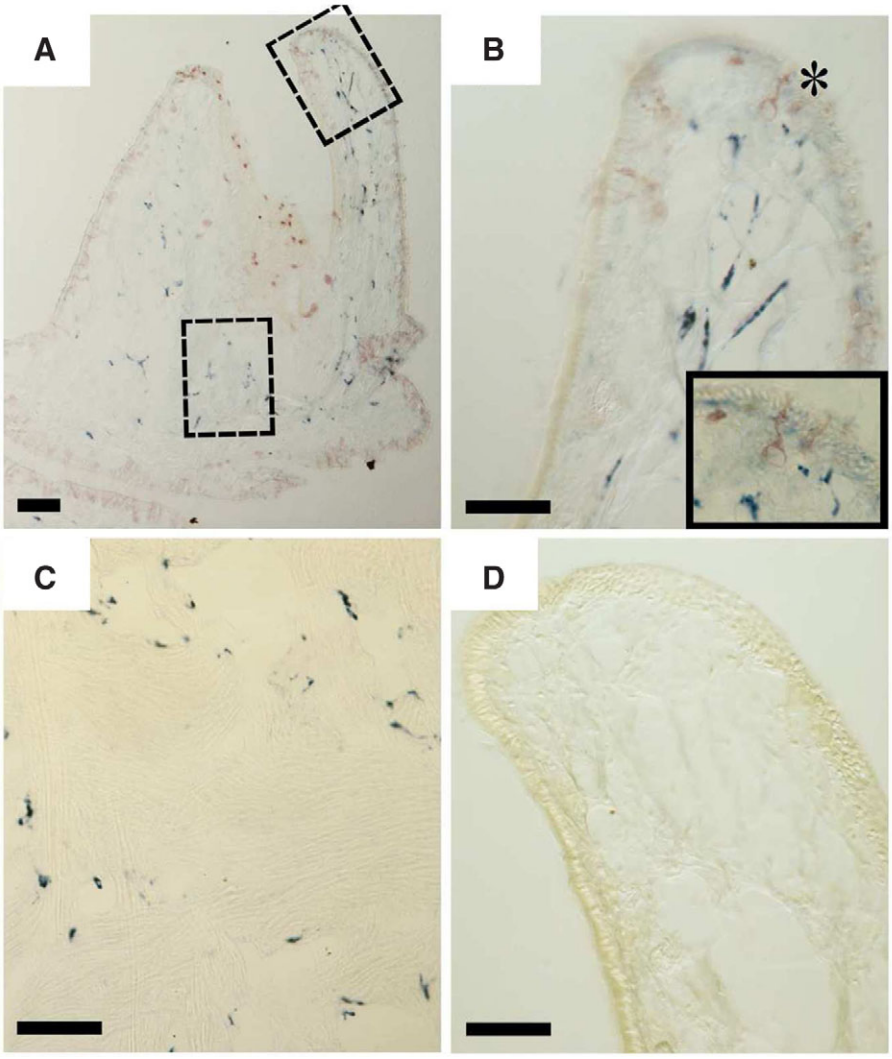

Fig. 2. Spatial expression pattern of a candidate chemosensory receptor in *Aplysia* rhinophore tissue. The *A.dac2* cDNA clone was used to generate a DIG-labeled antisense and sense riboprobe to hybridize to corresponding mRNA. (A) *In situ* hybridization on a transverse section of a rhinophore showing staining (blue) within central and peripheral cells, using the antisense riboprobe. (B,C) Higher magnifications of the sensory epithelium and central regions, respectively (boxed in A). The inset in B shows a digitally enhanced micrograph of the area indicated by the asterisk. (D) Control section using sense riboprobe, showing no signal. Scale bars, 50 μm.

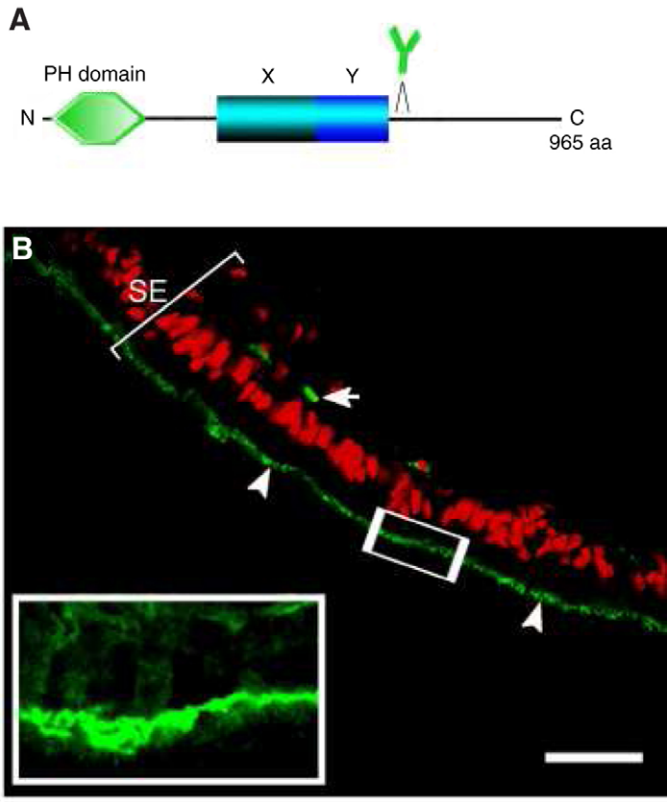

Fig. 3. Analysis of an *Aplysia* PLC. (A) Schematic diagram showing the location of the pleckstrin homology domain (PH) and bipartite catalytic domains (X,Y). An antibody was generated against a hydrophilic region of the *Aplysia* PLC protein as indicated. (B) Immunofluorescence localization of PLC protein in the rhinophore sensory epithelium (SE) neuronal fibers (arrow), and sensory surface (arrowheads). Sections were counterstained with propidium iodide. A region of the sensory surface is enlarged to show expression within the outer surface, potentially cilia. Scale bar, 50 μm.

cells. For negative controls in which primary antibody was replaced with preimmune serum, all immunoreactivity was absent (data not shown), confirming the specificity of the signal for PLC in the tissue section.

Distribution of *Aplysia* Ins(1,4,5)P_3R

A schematic diagram of the *Aplysia californica* Ins(1,4,5)P_3R is shown in Fig. 4A. Extensive screening of a CNS cDNA library suggests that only one *Aplysia* Ins(1,4,5)P_3R exists (Cummins et al., 2007). Immunoblot and immunohistochemical analyses were performed using affinity-purified *Aplysia* Ins(1,4,5)P_3R antiserum. SDS-PAGE followed by western blotting detected immunoreactive Ins(1,4,5)P_3R protein in *A. californica* rhinophore and CNS protein extracts (Fig. 4B). The apparent molecular mass was consistent with its predicted size of 315 kDa. Immunofluorescence studies demonstrated prominent expression of immunoreactive Ins(1,4,5)P_3R in cross sections of rhinophores, within presumptive chemosensory neurons concentrated within the rhinophore epithelium (Fig. 4C,D). We performed negative controls in which the primary antibody was omitted and primary antibody was replaced with preimmune serum. In each case, the signal was absent, confirming the specificity of the signal for *Aplysia* Ins(1,4,5)P_3R in the tissue section (data not shown).

The chemosensory epithelium of the rhinophores was dissociated into individual cells and they were plated onto Con A-coated coverslips and cultured for 1 day. Four cell types could be routinely identified based on size and morphology: (1) large and columnar; (2) large and granulated; (3) small and round; and (4) mid-size, ovoid and ciliated (Fig. 5A). To determine whether Ins(1,4,5)P_3R was still present in this *in vitro* culture system, anti-Ins(1,4,5)P_3R was used to identify immunoreactivity in dissociated culture cells. Fig. 5B shows that Ins(1,4,5)P_3R is present in three of the four cell types.

Changes in pheromone-stimulated rhinophore contraction in the presence of a PLC inhibitor

Aplysia rhinophore organ bath bioassays were used to study changes in rhinophore activity following pheromone stimulation with and without the PLC inhibitor U73122. A typical representation of the rhinophore twitch response to 30 μg purified egg eluate protein (containing attractin, enticin, temptin and seductin) in normal ASW, and in the presence of U73122, is shown in Fig. 6A,B, respectively. Under normal conditions, egg eluate protein initially caused a relatively large twitch response within 10 s of exposure to rhinophore preparations. This was generally followed by one or two smaller twitches. In all preparations, no twitch response was observed when only ASW was applied, indicating that mechanical stimulation was not a factor. In the presence of the PLC inhibitor U73122, egg eluate protein caused a 55% increase in the number of rhinophore twitches (4.8±0.5, N=20; P<0.03) compared with the control (3.1±0.4, N=15; Fig. 6C). Frequently, after the initial contraction, a twitch relaxation was delayed by an additional twitch. In the presence of U73343, an inactive analog of U73122, there was no significant change in rhinophore twitch activity when compared with the control (N=5; Fig. 6C). There was no significant difference in the maximum amplitude of rhinophore twitch contraction between cells treated with the PLC inhibitor U73122 (N=20) or the inactive PLC inhibitor (N=5; data not shown) and the controls.

DISCUSSION

The molecular basis of mammalian and insect chemoreception is not related but is a remarkable case of convergent evolution. In mammals, odorant chemosensory detection is achieved from binding of molecules to one or more of a large family of G-protein coupled receptors that are expressed on the surface of sensory neurons. The vomeronasal organ contains two large receptor families (e.g. 350 receptors in mouse) which detect pheromones and induce hormonal and behavioral responses (Matsunami and Buck, 1997). Meanwhile, insect olfactory and pheromone systems respond to a relatively smaller range of odorants and their genomes in turn encode a smaller family of receptors (de Bruyne and Warr, 2006), typically housed in antenna sensillae. In this study, we asked whether novel multi-transmembrane receptor proteins similar to those used by other Metazoa, could also be present in *Aplysia* rhinophores, which are highly evolved organs that recognize chemosensory information in their marine environment. Indeed, we identified two partial length transcripts in *Aplysia dactylomela* by degenerate RT-PCR and used one transcript to help define spatial and cellular distribution within the epithelium of rhinophore sections. Based on this distribution pattern, these probably encode chemosensory receptors. To discriminate the diverse array of environmental stimuli, it is probable that there exist a large family of similarly novel receptors that are all expressed at any one time. If this is correct then it is probable that the *in situ* probe used in this study could have non-specifically bound to similar transcripts representing closely related subfamily members and therefore the actual number of cells expressing this exact receptor type may be lower than that shown.

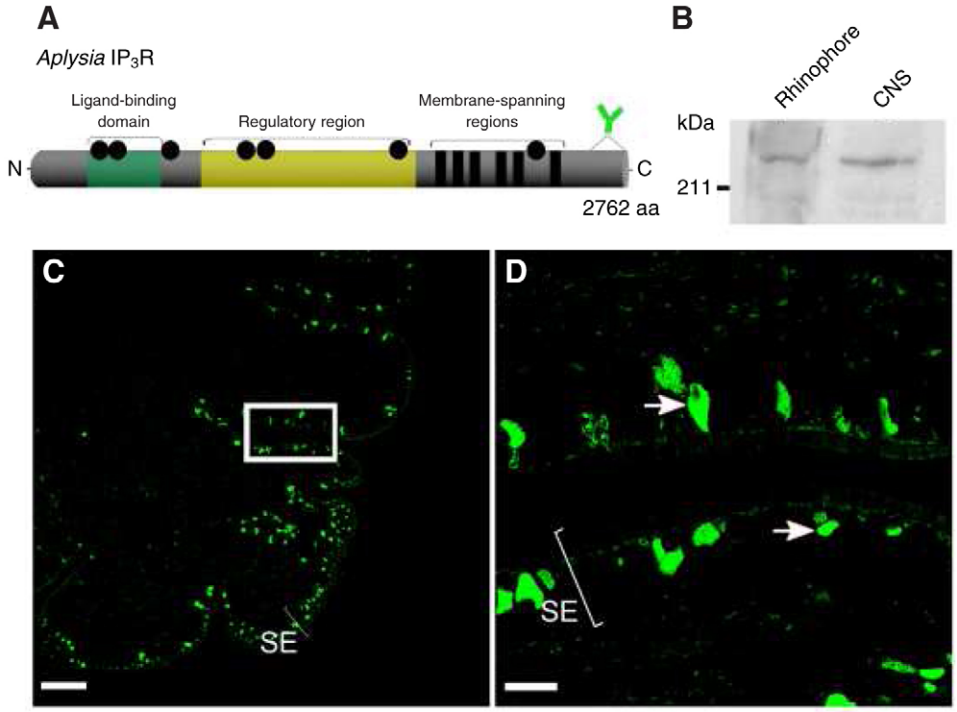

Fig. 4. Analysis of an *Aplysia* Ins(1,4,5)P_3R. (A) Schematic diagram of *Aplysia* Ins(1,4,5)P_3R showing the ligand-binding domain, regulatory region and membrane-spanning regions. Black circles represent potential calcium-binding sites. An antibody was generated against the C-terminus of *Aplysia* Ins(1,4,5)P_3R as indicated. (B) Protein was isolated from rhinophores and CNS, and probed by immunoblot analysis using antisera raised against *Aplysia* Ins(1,4,5)P_3R. In each case, *Aplysia* Ins(1,4,5)P_3R is identified as an immunoreactive band of above 211 kDa. (C) Immunofluorescence localization of *Aplysia* Ins(1,4,5)P_3R in the rhinophore sensory epithelium (SE). Scale bar, 200 μm. The boxed area is enlarged in D, and shows localization within cell bodies (arrows) at the rhinophore primary epithelium. Scale bar, 25 μm.

Chemoreceptor genes, such as those characterized from mammals and insects, generally show localized expression within sensory tissues. For instance, the olfactory chemoreceptor mRNA is found almost exclusively in the olfactory or vomeronasal epithelium of rodents (Buck and Axel, 1991; Saito et al., 1998). Also within these regions, receptor mRNA appears to be expressed in a cell-specific manner. Similarly, members of insect chemosensory receptor gene families are expressed in topographically defined subpopulations of sensory neurons in sensilla (Grosse-Wilde et al., 2007; Vosshall et al., 1999). The availability of the identified *Aplysia* candidate chemosensory receptors may now be used as molecular probes for *in situ* hybridization that will enable us to further explore the precise tissue and cellular spatial distribution of these types of receptors in sensory and non-sensory tissues. Final validation of chemoreceptor function will involve *in vitro* cell-based assays.

In some sensory systems, G-protein activation of PLC is the molecular trigger for intracellular chemosensory transduction and neuronal activation. For example, female opossum urine contains a pheromone that stimulates vomeronasal neurons through activation of PLC by G-protein-coupled mechanisms (Zhang et al., 2007). The canonical model predicts that PLC-induced release of Ins(1,4,5)P_3 leads to an increase in cytoplasmic calcium from internal endoplasmic reticulum stores *via* an Ins(1,4,5)P_3R-gated mechanism. PLC-mediated cell activation has been reported in molluscs using biochemical studies that have implicated G-protein-induced Ins(1,4,5)P_3 signaling in such activities as bivalve oocyte

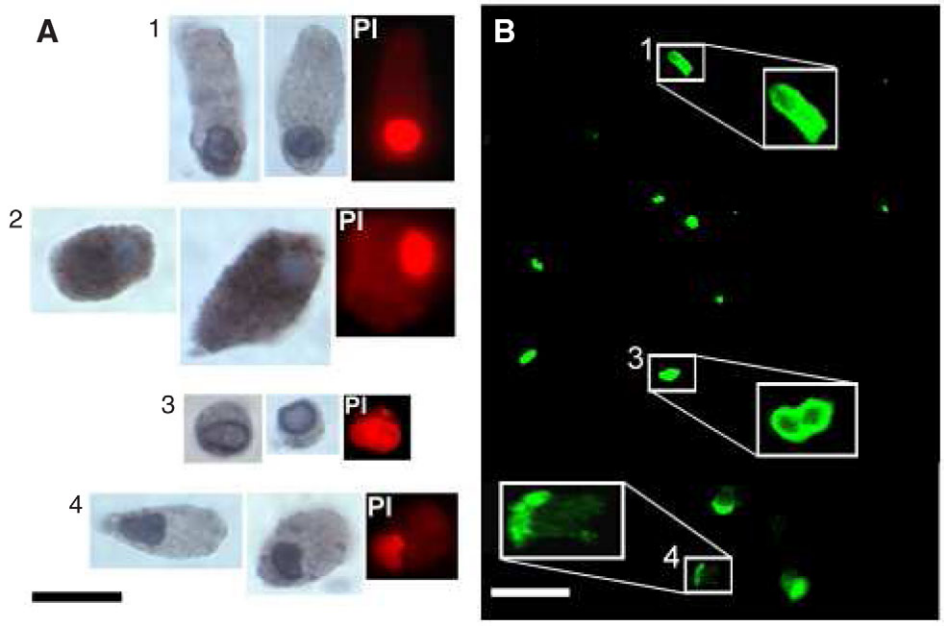

Fig. 5. Dissociation of *Aplysia* rhinophore cells and Ins(1,4,5)P_3R immunolocalization. (A) Four cell types were routinely identified (hematoxylin stain) from the dissociation preparation: 1, large and columnar; 2, large and granulated; 3, small and round; and 4, mid-size, ovoid and ciliated. PI staining clearly shows the nuclei. Scale bar, 25 μm. (B) Cells were attached to Con A-coated coverslips and reacted with anti-Ins(1,4,5)P_3R. Photomicrograph shows immunoreactivity typically observed only within cell types 1, 3 and 4. Scale bar, 100 μm.

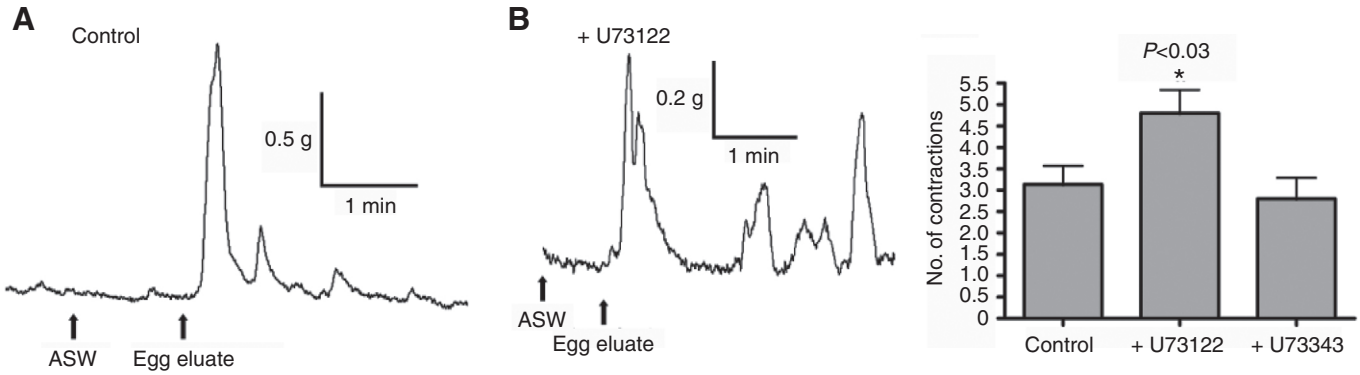

Fig. 6. Effect of PLC inhibitor on pheromone-induced rhinophore twitching. (A,B) Representative tracings showing the contractile effect of egg eluate protein on rhinophores (control; A), and in the presence of 10^{-6} mol l^{-1} of the PLC inhibitor U73122 (B). Arrows indicate the addition of ASW and 30 μg purified egg eluate protein. (C) Mean responses (number of twitches) of rhinophores following stimulation with egg eluate protein without PLC inhibitor (control), and in the presence of 10^{-6} mol l^{-1} U73122 or U73343 (PLC inactive inhibitor). Compared with controls, the number of rhinophore twitches increased significantly (*P<0.03) when rhinophores were preincubated with U73122, but not U73343.

activation (Deguchi et al., 1996) and opisthobranch neuroendocrine bag cell depolarization (Fink et al., 1988). More specifically, within chemosensory cell types, the squid olfactory receptor neurons are known to co-express $G\alpha_q$ and PLC140, suggesting a potential mechanism for odor transduction. Our data, showing the presence of a PLC and Ins(1,4,5)P_3R in cell bodies lining sensory epithelia in *Aplysia* support their potential role in chemosensory signal transduction in molluscs. This observation was further confirmed in dissociated rhinophore sensory neurons where three out of the four cell types identified appeared to contain an Ins(1,4,5)P_3R. The isolation of individual cells described here provides an opportunity for studying chemosensory signaling at the single cell level. The fact that we already know the pheromones involved in *Aplysia* attraction suggests that calcium imaging techniques could be suitably applied to cultured rhinophore cells to enable identification of pheromone-responsive cell type(s). Indeed, this experimental approach has proven successful in other studies (Chamero et al., 2007).

Collective morphological and electrophysiological data indicate that gastropod rhinophores serve a different chemosensory function to the oral tentacles (Boudko et al., 1999). That is, the rhinophores function in long-distance chemoreception whereas the oral tentacles probably serve in contact or short-distance chemoreception. So it is the rhinophores that play a key role in detecting distant water-borne pheromones that regulate mate attraction. In *A. fasciata*, for example, ablating the rhinophores causes a decrease in the time spent mating, as well as a decrease in feeding in the presence of a conspecific; it also blocks respiratory pumping in response to mating and egg cordons (Levy et al., 1997). In this study, we took advantage of our existing knowledge of *Aplysia* water-borne protein pheromones and the rhinophore twitch response to pheromonal stimuli. Rhinophore organ bath bioassays were used to study changes in rhinophore activity following pheromone stimulation with and without the PLC inhibitor U73122. For these experiments, we assumed that although U73122 is membrane permeable, it would be unlikely for it to rapidly penetrate to the muscle cells involved in contraction. It is more probable that the effect of U73122 is specific to sensory epithelial cells, including chemosensory receptor neurons. The proposed mechanism of U73122 action is at the level of $G\alpha_q$ regulation, indicating that U73122 preferentially inhibits PLC-β (Thompson et al., 1991). We anticipated that if a similar PLC-β were used by rhinophore sensory neurons to transduce the pheromonal response in *Aplysia*, we would expect to see a decrease or even elimination of the rhinophore contraction response following U73122 exposure. Instead, however, we observed a significant

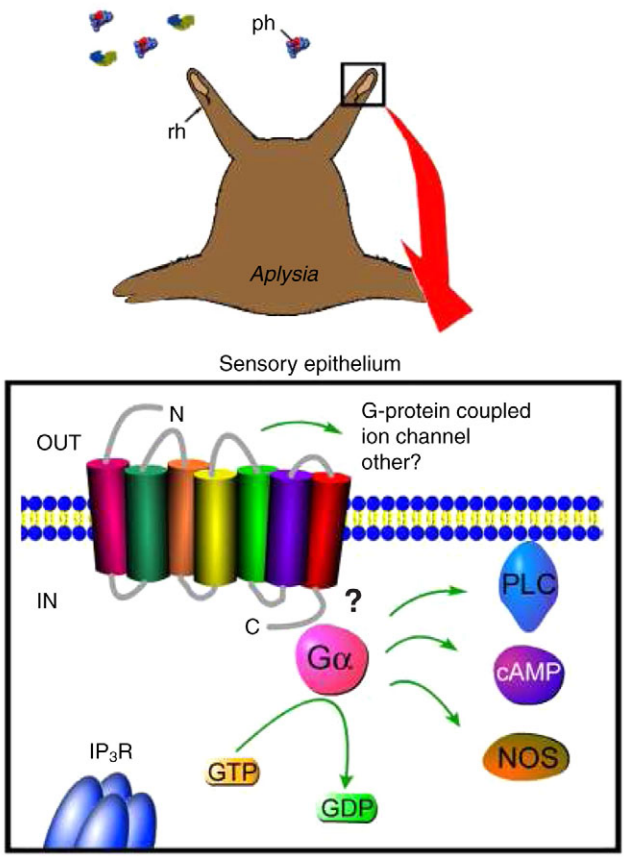

Fig. 7. Hypothetical summary of water-borne chemical detection and signal transduction pathways in *Aplysia*. Pheromones (ph) are detected by the rhinophores (rh). At the level of the sensory epithelium, pheromones may bind to a G-protein coupled ion channel or other multi-transmembrane chemoreceptors and be transduced internally *via* a variety of transduction systems including G proteins, PLC, cAMP and nitric oxide synthase (NOS). We have demonstrated that candidate chemosensory receptors, G proteins, PLC and Ins(1,4,5)P_3R are present in *Aplysia* sensory epithelia. OUT, cell exterior; IN, cell interior.

increase (55%) in the rhinophore twitch response, raising further questions as to the exact role of a PLC in sensory transduction. However, our finding confirms that it does modulate the sensing the pheromone cocktail by an unknown mechanism, or perhaps may underlie transduction of just some of the pheromones in the cocktail, and therefore causes a somewhat different response than to the full cocktail.

In summary, structural and behavioral evidence for pheromone communication in molluscs is most complete in *Aplysia* species, however, we are only beginning to understand the molecular basis of chemosensory detection. In this study, we established, using *in situ* hybridization, immunohistochemical analysis and rhinophore bioassay methods, that multi-transmembrane proteins (probably chemoreceptors), a PLC, and an Ins(1,4,5)P_3R in the rhinophore sensory epithelium may function in chemical detection in *Aplysia* (summarized in Fig. 7). Future work is essential to define their precise role in chemical binding, signal amplification and modulation.

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