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Evidence for Multiple Signaling Pathways in Single Squid Olfactory Receptor Neurons

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ABSTRACT

At least two different G-protein-mediated transduction cascades, the adenylate cyclase and phospholipase C (PLC) pathway, process chemosensory stimuli for various species. In squid olfactory receptor neurons (ORNs), physiological studies indicate that both pathways may be present; however, confirmation of the transduction molecules at the protein level is absent. Here we provide evidence that the G-proteins involved in both adenylate cyclase and PLC pathways are present in squid ORNs (*Lolliguncula brevis*). We used immunoblotting to show that $G\alpha_{olf}$, $G\alpha_q$, and a downstream effector, enzyme PLC140, are present in the squid olfactory epithelium (OE). To localize these proteins to one or more of the five morphological cell types described for squid OE, paraformaldehyde-fixed olfactory organs were cryosectioned (10 μm), double-labeled for $G\alpha_{olf}$, $G\alpha_q$, or PLC140, and imaged. Analysis of serial sections from entire olfactory organs for epithelial area and patterns of immunofluorescence revealed a region of highest immunoreactivity at the anterior half of the organ. At the cellular level, type 1 cells could not be distinguished morphologically and were not included in the analysis. The three labeling patterns observed in type 2 cells were $G\alpha_q$ alone, PLC140 alone, and colocalization of $G\alpha_q$ and PLC140. Subsets of cell types 3, 4, and 5 showed colocalization of $G\alpha_{olf}$ with $G\alpha_q$ but not with PLC140. These data suggest that the PLC pathway predominates in type 2 cells; however, coexpression of $G\alpha_{olf}$ with $G\alpha_q$ in cell types 3, 4, and 5 suggests that both pathways may participate in olfactory transduction in non-type 2 squid ORNs. J. Comp. Neurol. 501:231–242, 2007.

Indexing terms: olfaction; cephalopods; G proteins; PLC; adenylate cyclase

Olfactory transduction pathways

Multiple transduction pathways have been described in olfactory systems of both vertebrates and invertebrates. In vertebrates, the adenylate cyclase pathway has been implicated as the major olfactory transduction pathway based on studies utilizing transgenic animals with olfactory-specific deletions (Brunet et al., 1996; Belluscio et al., 1998; Wong et al., 2000). However, the phospholipase C (PLC) (Lin et al., 2004; Elsaesser et al., 2005), PI3 kinase (Spehr et al., 2002), and cGMP (Juilfs et al., 1997) pathways are also functional in olfactory receptor neurons (ORNs) and may either modulate or directly transduce certain classes of odorants. As in their vertebrate counterparts, olfactory chemoreception in invertebrates is transduced by at least two different enzyme pathways: adenylate cyclase and PLC (Pottinger et al., 1991; Fadool and Ache, 1992; Boekhoff et al., 1994; Ache, 1994; Hildebrand and Shepherd, 1997; Gomez-Diaz et al., 2004; Doolin and Ache, 2005). In both vertebrates and invertebrates, olfactory transduction begins when an odorant binds to a receptor and activates its associated G-protein. The alpha subunit of the olfactory specific stimulatory G-protein, $G\alpha_{olf}$, activates adenylate cyclase, which generates cAMP from ATP. The alpha subunit of $G\alpha_q$ activates PLC, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) to form inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). The rapid increase in concentration of cAMP or IP_3 generates a receptor potential in the ORN by opening second

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messenger gated ion channels in the cilia (Hildebrand and Shepherd, 1997).

Invertebrate olfactory transduction

Several studies have identified adenylate cyclase and the more generic form of $G\alpha_{olf}$, named $G\alpha_s$, in invertebrate chemosensory systems. The adenylate cyclase pathway is present in *Drosophila* (Gomez-Diaz et al., 2004) and lobster ORNs (Hatt and Ache, 1994). $G\alpha_s$ is found in lobster ORNs (Xu et al., 1997) and the inner and outer dendritic segments of the putative ORNs in the silkmoth (Miura et al., 2005). In cephalopod neurons, an enzyme with adenylate cyclase-like activity (Capasso et al., 1991), and a $G\alpha_s$-like G-protein (Chin et al., 1994) have been identified. Collectively, these and other studies suggest that the $G\alpha_{olf}$–adenylate cyclase pathway is important in invertebrate chemoreception and that the pathway exists in the cephalopod nervous system.

The PLC pathway is present in the olfactory system of several invertebrates, including the cockroach (Breer et al., 1990; Boekhoff et al., 1990), moth (Maida et al., 2000), and lobster (Fadool and Ache, 1992; Krieger and Breer, 1999). A 140-kDa protein with PLC-like properties (PLC140) was isolated from the retina of squid and has close homology to mammalian PLCβ, *Drosophila* neuronal PLC21, and *Drosophila* visual norpA (the gene that encodes PLC involved in *Drosophila* phototransduction; Mitchell et al., 1995). PLC is activated by $G\alpha_q$ to generate IP_3 and DAG (Jiang et al., 1994). $G\alpha_q$ has been cloned from lobster ORNs (Juilfs et al., 1997) and lepidoptera antenna (Jacquin-Joly et al., 2002). Although $G\alpha_q$ has not previously been identified in cephalopod ORNs, it is present in the squid retina (Pottinger et al., 1991; Ryba et al., 1993), where it has two different structural and functional forms (Narita et al., 1999). These data suggest that the $G\alpha_q$-PLC pathway may also be present in cephalopod olfactory neurons.

Squid olfactory transduction

Behavioral studies in squid have shown that the bilaterally paired olfactory organs located ventral and posterior to each eye are the sites of highest chemosensitivity (Fig. 1A) (Gilly and Lucero, 1992). The olfactory organs are ideally situated to detect water-borne odorants entering the mantle cavity, and are comprised of a pseudostratified olfactory epithelium (OE), which is 50–75 μm thick and ≈1 mm in diameter. A lamina propria beneath the OE consists of connective tissue containing smooth muscle, axon bundles, and blood vessels. The olfactory epithelium consists of five types of morphologically distinct ORNs and one or more classes of supporting cells (Emery, 1975). Three of the five types of ORNs (3, 4, and 5) have large invaginations filled with cilia, which form pockets that retain access to the extracellular environment (Figs. 1B, S1). Cilia pockets are the subcellular region containing the signal transduction machinery (Lucero et al., 2000; Danaceau and Lucero, 2000). Similarly shaped cells named "crypt cells" have recently been described in vertebrate OE (Hansen and Zeiske, 1998; Hansen and Finger, 2000; Hansen et al., 2004).

Type 2 and type 4 squid ORNs respond to odorants with changes in action potential firing rates (Lucero et al., 1992) and elevations in intracellular Ca^{2+} (Piper and Lucero, 1999). In isolated type 4 squid ORNs, inclusion of either IP_3 or cyclic nucleotides in the patch pipette evokes opposing electrical responses, suggesting that both the adenylate cyclase and PLC pathways are functional (Lucero and Piper, 1994). Likewise, structurally distinct odorants (glutamate and betaine) activated opposite current responses in single type 4 ORNs (Danaceau and Lucero, 2000). Interestingly, responses to mixtures of glutamate and betaine were suppressed compared to either alone, indicating cross-talk between transduction pathways. Collectively, these data suggest that single squid ORNs may have more than one signal transduction pathway, and that simultaneous activation results in a unique output that is not a simple summation of two individual odorant responses. In the present work, we used immunocytochemical techniques to determine whether squid ORNs express both G proteins associated with PLC and adenylate cyclase pathways in the same cell, and examined the distribution of $G\alpha_{olf}$ and $G\alpha_q$ across the five cell types in squid olfactory organs. We find that, in agreement with physiological studies, subsets of type 4 cells as well as the physiologically uncharacterized type 3 and 5 cells coexpress $G\alpha_{olf}$ and $G\alpha_q$. This is the first report of the distribution of $G\alpha_{olf}$, $G\alpha_q$, and PLC140 in the squid OE other than in abstract form (Mobley and Lucero, 2005).

MATERIALS AND METHODS

Animal procedures were approved by the University of Utah Institutional Animal Care and Use Committee. Live juvenile *Lolliguncula brevis* (≈10 cm length) were caught in the wild by the National Resource Center for Cephalopods (University of Texas Marine Biomedical Center, Galveston, TX), held for 3–5 days, and shipped overnight to Utah. Experiments were performed immediately upon arrival.

Adult Swiss-Webster mice (30 g, Charles River Laboratory, Wilmington, MA) used for positive controls were deeply anesthetized with 150 μg/kg ketamine and killed by decapitation. All experiments were performed in triplicate.

Protein extraction

Mouse olfactory epithelia (n = 4) were freshly dissected on ice while being nebulized with O_2 saturated Ringer's solution (in mM: NaCl, 140; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; HEPES, 10; glucose, 10). Squid olfactory organs, optic lobes, and retinas (n = 9) were dissected in sterile filtered sea water. Tissue was homogenized in 3 mL of homogenization buffer on ice (in mM: 10 imidazole, 4 EDTA, 1 EGTA, 1-2 phenylmethylsulphonyl fluoride [PMSF], 1× protease cocktail inhibitor, 1% sodium orthovanadate), and then centrifuged for 10 minutes at 4°C at 14,000*g*. Twice, the supernatant was collected and spun at 10,000*g* for 10 minutes. The pooled supernatant was then spun at 60,000 rpm for 1 hour at 4°C to pellet the membranes. The pellet was solubilized in resuspension buffer (In mM: 15 Tris HCl, pH 7.5, 5 EDTA, 2.5 EGTA, 1 PMSF, 1× protease cocktail, and 1% SDS). All chemicals were from Sigma (St. Louis, MO) unless stated otherwise.

Western blotting

We used Western blotting techniques to verify the presence of each signal transduction protein in squid OE, optic lobe, retina, and mouse olfactory epithelium. Protein samples were boiled in Laemelli buffer (0.1 M Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 2% β-mercaptoethanol, 0.2%

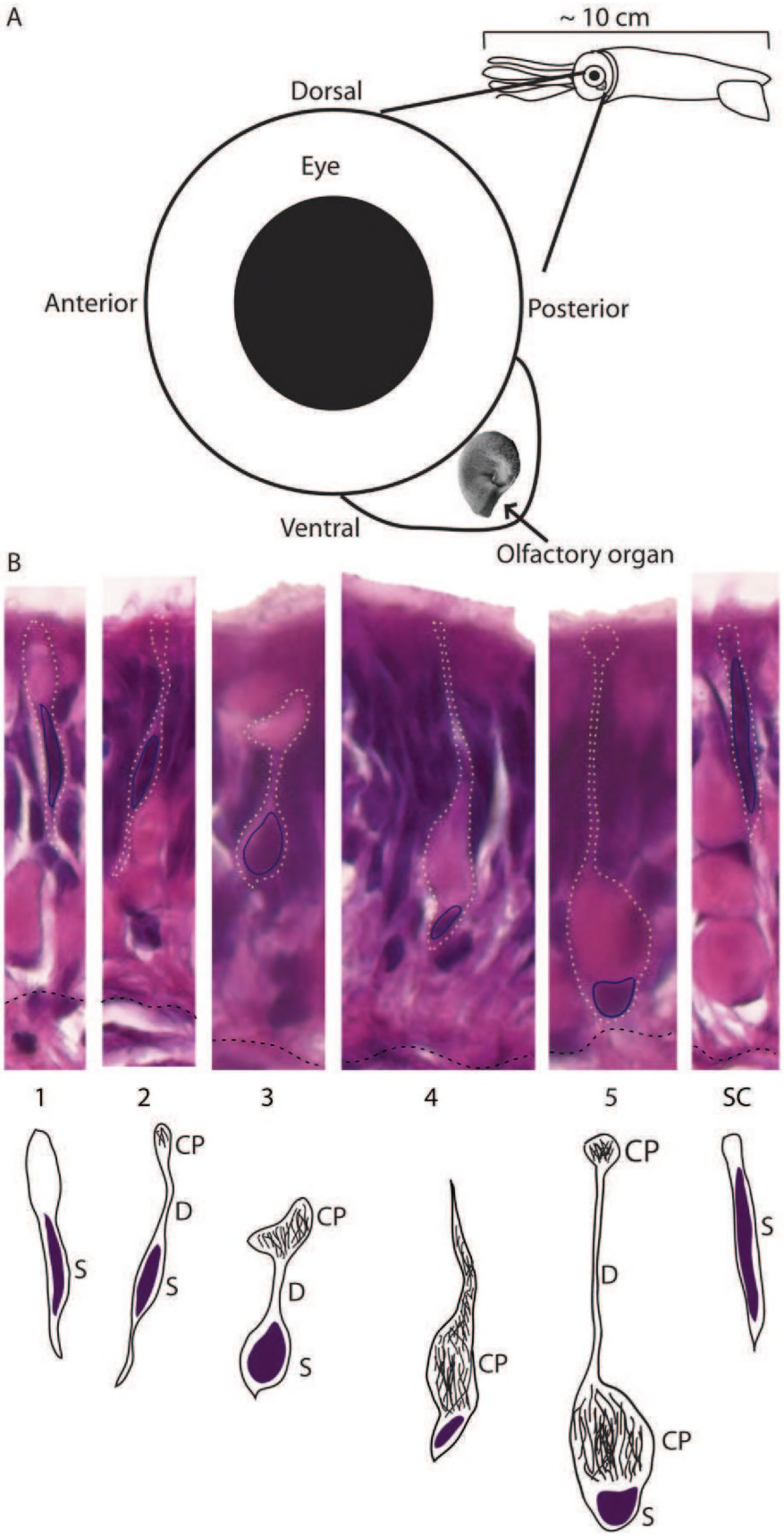

Fig. 1. Anatomy of the squid olfactory organ from *L. brevis*. **A:** The olfactory organ sits upon a muscular ridge of tissue ventral and posterior to each eye, anterior to the mantle cavity. **B:** The five morphological types of olfactory neurons have unique cilia pockets as shown in H&E images (top) and as illustrated (bottom). White dashed lines outline the cells (see supplemental data for image without lines). Blue lines outline the nucleus. Black dashed lines indicate the basement membrane of the OE. CP, cilia pocket; D, dendrite; S, soma, SC, support cell. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE 1. Antibodies and Peptides Used for Western Blotting and Immunohistochemistry

	Supplier	Cat. No.	Lot No.	Sequence
$G\alpha_{s/olf}$	Santa Cruz Biotech.	SC-383	C1104	Rat 377-394
$G\alpha_{q/11}$	Santa Cruz Biotech.	SC-393	G1904	Mouse 13-29
Peptide $G\alpha_{s/olf}$	Santa Cruz Biotech.	SC-383P	K1203	Rat 377-394
Peptide $G\alpha_{q/11}$	Santa Cruz Biotech.	SC-393P	E162	Mouse 13-29
PLC140	Dr. Jane Mitchell	n/a	n/a	Squid 1-140

bromophenol blue) at 80°C for 8 minutes, resolved on 10% SDS-PAGE, and transferred onto PVDF membranes (Bio-Rad, Hercules, CA). PVDF membranes were incubated at room temperature for 1 hour in a blocking buffer containing 5% nonfat powdered milk in TTBS (In mM: 50 Tris, pH 7.5, 150 sodium chloride, 0.1% SDS, 0.1% Tween-20), followed by incubation with indicated antibodies in blocking buffer and kept at 4°C overnight. Polyclonal rabbit anti-$G\alpha_{s/olf}$ does not differentiate between $G\alpha_s$ and $G\alpha_{olf}$, but will be referred to as the $G\alpha_{olf}$ antibody. Polyclonal rabbit anti-$G\alpha_{q/11}$ does not differentiate between $G\alpha_q$ and $G\alpha_{11}$, but will be referred to as the $G\alpha_q$ antibody. $G\alpha_{olf}$ and $G\alpha_q$ antibodies were used at 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-PLC140 was used at 1:10,000 (kind gift from Dr. Jane Mitchell, Univ. of Toronto). Complete source and sequence details for each antibody and peptide are given in Table 1. After washing three times for 10 minutes each with TTBS, the membranes were incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:10,000, Santa Cruz Biotechnology). Immunoreactive bands were detected by Chemiluminescence (BioRad). Molecular weights were determined using SeeBlue Plus2 Pre-stained Standard (Invitrogen, Carlsbad, CA). As a control, each primary antibody was incubated with its original ligand (blocking peptide; BP; 119 nM) at room temperature for 2 hours. The preabsorbed antibody was applied the same as primary antibodies.

Serial section immunohistochemistry

Freshly dissected olfactory organs from *L. brevis* were pinned onto sylgard-coated coverslips and immersed in ice-cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) containing 20% sucrose and 0.2% picric acid overnight at 4°C. After cryoprotecting in 15% and 30% sucrose, the tissue was removed from the coverslips, oriented for cutting, embedded in Tissue-Tek O.C.T. Compound (Sakura, Torrance, CA), and then flash-frozen with liquid nitrogen. Serial sections (10 μm thick) of the entire organ were collected on Superfrost/Plus slides (VWR, West Chester, PA). Sections were rehydrated in wash buffer (0.1 M PBS, 0.1% bovine serum albumin [BSA], 0.2% Triton X-100) for 30 minutes, then blocked in 5% donkey serum wash buffer for 30 minutes. Slides were rinsed three times in wash buffer and incubated in primary antibodies (polyclonal rabbit anti-$G\alpha_s$ 1:500, rabbit anti-$G\alpha_q$ 1:100, Santa Cruz Biotechnology; rabbit anti-PLC140 1:5,000, kind gift from Dr. Jane Mitchell, Univ. of Toronto) at 4°C overnight. The slides were rinsed three times in wash buffer to remove the primary antibodies, then incubated with Fluorescein (FITC)-conjugated AffiniPure Fab Fragment Donkey Anti-Rabbit IgG (Fab; 1:500; Jackson ImmunoResearch, West Grove, PA) at room temperature for 6 hours. Fab fragments allow the use of two primary antibodies made in the same host species (Negoescu et al., 1994; Heilbronn et al., 2003). Slides were rinsed in wash buffer, blocked again in 5% donkey serum, and incubated in the second primary antibody at 4°C overnight. Slides were rinsed in wash buffer followed by incubation in rhodamine (TRITC) conjugated AffiniPure donkey anti-rabbit IgG secondary antibody (1:100; Jackson ImmunoResearch) for 30 minutes at room temperature. Slides were rinsed three times in wash buffer and incubated in 4′, 6-diamidino-2-phenylindole (DAPI: 1 μL / 47.7 mL PBS) for 3 minutes, rinsed three times in wash buffer, and coverslipped with Vectashield Mounting Medium for Fluorescence (Vector Laboratories, Burlingame, CA).

In control experiments the TRITC antirabbit secondary antibody was applied for 30 minutes immediately after washing off the anti-rabbit FITC-conjugated Fab fragments to test whether the TRITC secondary antibody had access to the primary antibody following Fab treatment. We found that Fab fragments completely blocked access of the TRITC secondary antibody to the first primary antibody (data not shown). As a control, the primary antibodies (polyclonal rabbit anti-$G\alpha_{s/olf}$ and rabbit anti-$G\alpha_{q/11}$) were incubated with a 5-fold concentration of its original ligand (119 nM; blocking peptide, purchased from Santa Cruz Biotechnology) at room temperature for 2 hours. The preabsorbed antibody was applied the same as primary antibodies. Additional controls included zero primary antibodies and zero secondary antibodies.

The hematoxylin and eosin (H&E) staining procedure was from http://science.peru.edu/gregarina/html/harris.html (accessed June 16, 2006). Briefly, frozen sections were hydrated in 50% ethanol (EtOH), then ddH_2O. Slides were stained with Harris Hematoxylin-Modified (Sigma) rinsed with ddH_2O and destained with 1% acid EtOH. Slides were exposed to ammonia water until sections turned bright blue, then rinsed with ddH_2O. Slides were dehydrated in an EtOH series, cleared in a graded xylene/EtOH series as follows: 50% xylene/EtOH, 100% xylene, Zelmer's Eosin-xylol, 100% xylene. Slides were coverslipped using Vectamount (Vector Laboratories).

Confocal microscopy

Serial sections were imaged either on a Zeiss upright Axioplan2 scope with an Axiocam CCD camera or an Olympus IX81 confocal microscope. Serial section 3D reconstruction was done using Volocity High Performance 3D imaging software (http://www.improvision.com/products/Volocity/). Photo-merging was performed using Adobe Elements 2.0 (San Jose, CA). Masking and thresholding the OE and all other image processing were performed in Adobe Photoshop 6.0. To obtain an average pixel intensity (PI) of fluorescent antibody labeling across the olfactory organ, six tissue sections collected at ≈150-μm intervals throughout each olfactory organ were imaged. For each image, all tissue was masked off except for the OE. A PI histogram for each image generated a value for the mean PI of OE staining. The mean PI plus three standard deviations (99th percentile value) for all six images from one olfactory organ was calculated and averaged to determine a threshold value for PI. The threshold value was subtracted from every serial section to visualize the fluorescent staining that was significantly above the mean PI. The pixel area of the OE was measured in Adobe Photoshop 6.0 using the OE mask.

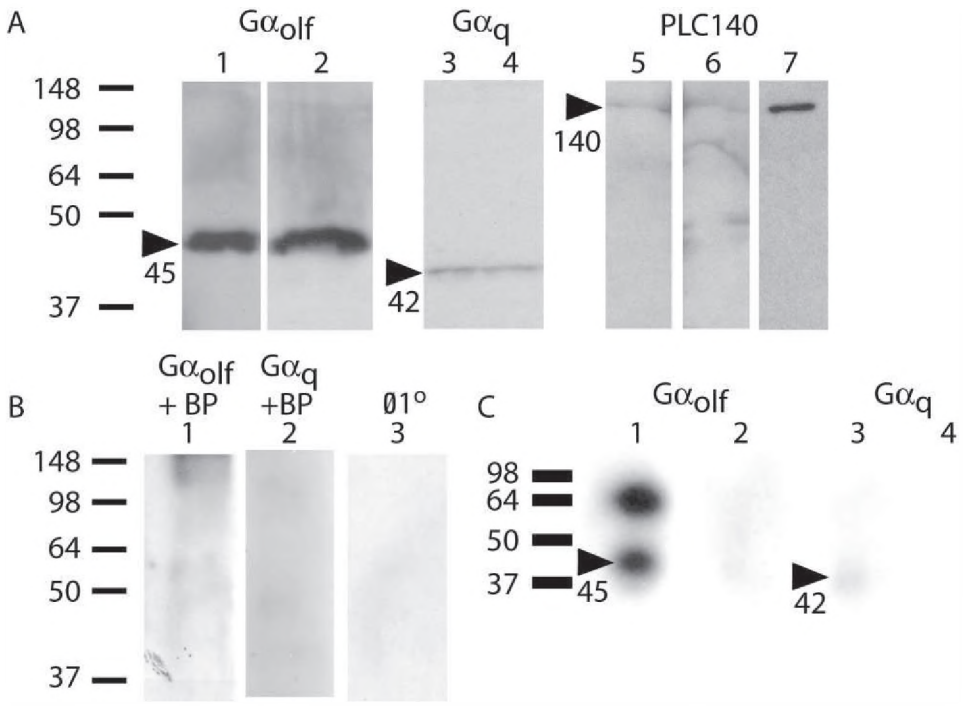

Fig. 2. Expression of $G\alpha_{olf}$, $G\alpha_q$, and PLC140 in squid tissue and mouse OE. **A:** Western blots immunostained for $G\alpha_{olf}$, $G\alpha_q$, and PLC140 in the squid OE (lanes 1, 3, 5), optic lobe (lanes 2, 4, 6), and retina (lane 7). **B:** Preabsorption control Western blots using blocking peptide (BP) for $G\alpha_{olf}$ and $G\alpha_q$ and a zero-primary (Ø 1°) control using squid OE. **C:** $G\alpha_{olf}$ and $G\alpha_q$ expression in mouse OE (lanes 1 and 3); BP controls (lanes 2 and 4).

RESULTS

Western blot analysis for G-proteins and PLC

To confirm the presence of adenylate cyclase and PLC pathways in squid OE, we performed Western blot analysis on squid and mouse tissues. Protein bands with the same molecular weight as $G\alpha_{olf}$ and $G\alpha_q$ in mouse OE were specifically recognized by antibodies against $G\alpha_{olf}$ and $G\alpha_q$ in squid OE (Fig. 2A). $G\alpha_{olf}$ antibody recognized a single band in squid OE and squid optic lobe (lanes 1–2) at 45 kDa, which is the previously determined molecular weight of mammalian $G\alpha_{olf}$ (Pace and Lancet, 1986) (Fig. 2C, lane 1). The antibody to $G\alpha_q$ recognized protein bands at 42 kDa in both squid OE, optic lobe (Fig. 2A, lanes 3–4) and mouse OE (Fig. 2C, lane 3). There was no nonspecific immunoreactivity from the secondary antibody when the primary antibody was omitted, and the $G\alpha_{olf}$ and $G\alpha_q$ band was eliminated by including blocking peptide in the primary antibody (Fig. 2B). These data indicate that proteins of similar molecular weight and immunoreactivity to vertebrate $G\alpha_{olf}$ and $G\alpha_q$ are present in squid OE.

Beta-type phospholipase C (PLCβ) is an enzyme activated by $G\alpha_q$, which catalyzes the breakdown of PIP_2 into IP_3 and DAG. Fragment analysis of PLC140 protein purified from squid retina revealed four fragments that shared homologous sequences with PLCs from *Drosophila* neuronal PLC21, *Drosophila* visual norpA, and a mammalian PLC β rat1 (Mitchell et al., 1995). PLC-like activity of squid PLC140 was demonstrated by hydrolysis of phosphatidylinositol and G-protein regulation. We tested the PLC140 antibody against tissue from the squid OE, squid optic lobe, and squid retina. The PLC140 antibody recognized a single band at 140 kD in the squid OE, optic lobe, and retina (Fig. 2A, lanes 5–7), thus confirming the presence of PLC140 in squid OE.

Immunocytochemistry for G proteins and PLC

To visualize the distribution of the proteins in the adenylate cyclase and PLC pathway across the OE, we double-labeled cryostat serial sections (10 μm thickness) of the entire squid olfactory organ with antibodies against $G\alpha_{olf}$ and $G\alpha_q$, or $G\alpha_q$ and PLC140 (Fig. 3). We found $G\alpha_{olf}$-like immunoreactivity (IR) in cilia pockets of neuron subtypes 3, 4, and 5 (arrowheads) and in the cilia at the apical surface of the epithelium (Fig. 3C,D). We allow for the possibility of $G\alpha_{olf}$ in the sustentacular cells, as there is immunoreactivity in the apical cell layer, below the cilia. We are unable to definitively identify this layer as sustentacular with our current markers. $G\alpha_q$-like IR labeled a subset of types 2, 3, 4, and 5 ORNs (Fig. 3E,F). In ORN, types 3, 4, and 5 $G\alpha_q$-like IR labeled the cilia pockets (arrowheads) and the cilia at the apical surface. PLC140-like IR was found in a subset of type 2 ORNs with long dendritic extensions and discrete soma labeling (arrowheads), minus the nucleus (Fig. 3G,H).

Double-labeled sections were assayed for colocalization of $G\alpha_{olf}$- and $G\alpha_q$-like IR or $G\alpha_q$- and PLC140-like IR. We found that $G\alpha_{olf}$-like IR colocalized with $G\alpha_q$-like IR in types 3, 4, and 5 ORNs (Fig. 4). Type 3 neurons have a large cilia pocket atop a narrowed extension separating the cilia pocket from the soma (Figs. 1B, 4A′ arrow). We

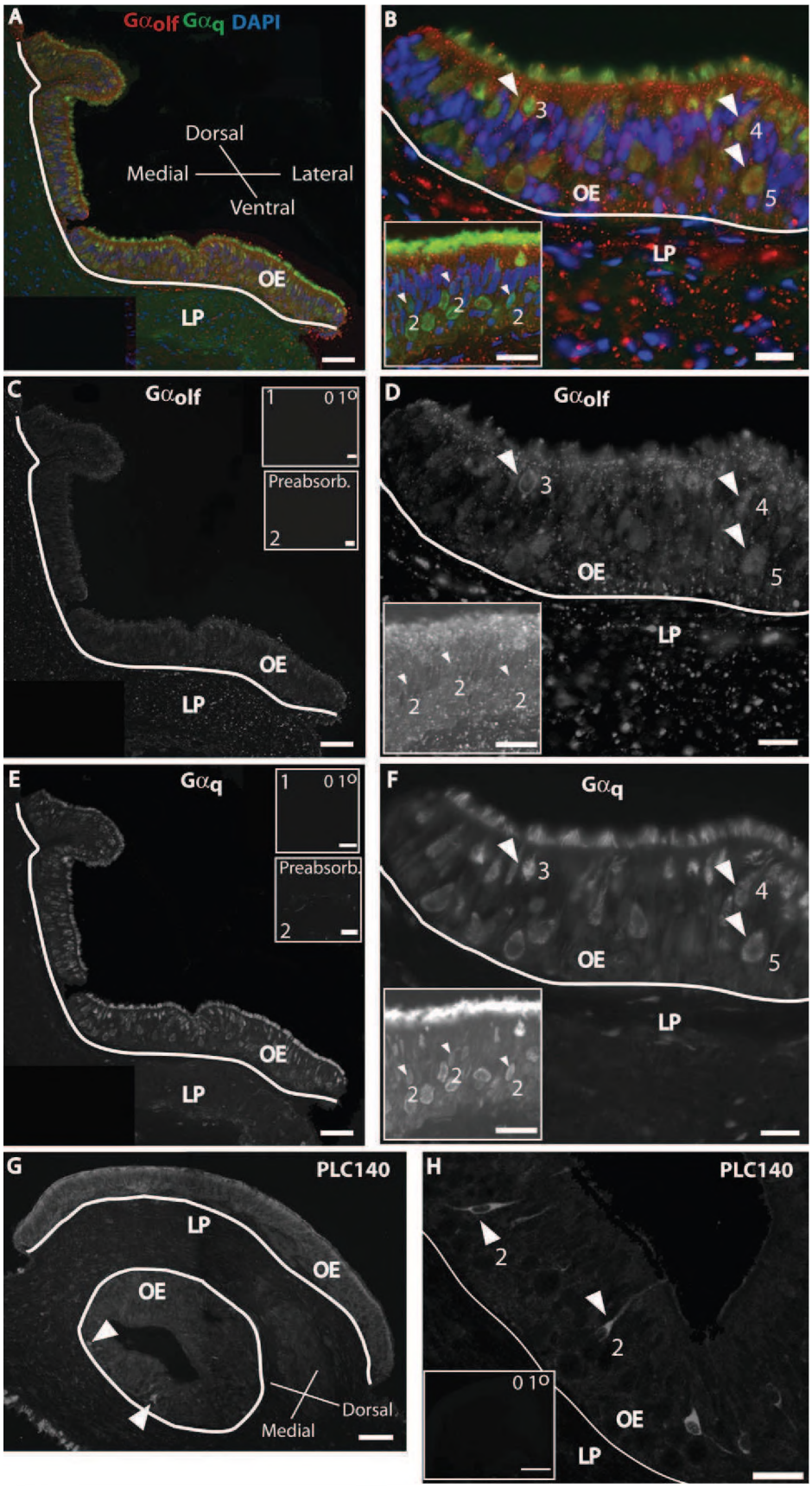

Fig. 3. Fluorescent images of squid olfactory epithelium. **A,B:** RGB images of $G\alpha_{olf}$ (red), $G\alpha_{q}$ (green), and DAPI (blue). **B,D,F,inset:** A different section showing the presence of $G\alpha_{q}$ in type 2 ORNs (B,F) and the absence of $G\alpha_{olf}$ (D). **C,D:** $G\alpha_{olf}$-like IR stains cilia pockets of types 3–5. **C,E,H,inset:** 1, zero primary antibody and 2, preabsorbed primary antibody controls. **E-F:** $G\alpha_{q}$-like IR stains types 2-5. **G,H:** PLC140-like IR only stains a subset of type 2 cells. A,C,E,G: Montaged images, scale bars = 50 μm; B,D,F,H: scale bars = 25 μm; C,E,H insets: scale bars = 20 μm; B,D,F insets: scale bars = 50 μm. LP = lamina propria. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

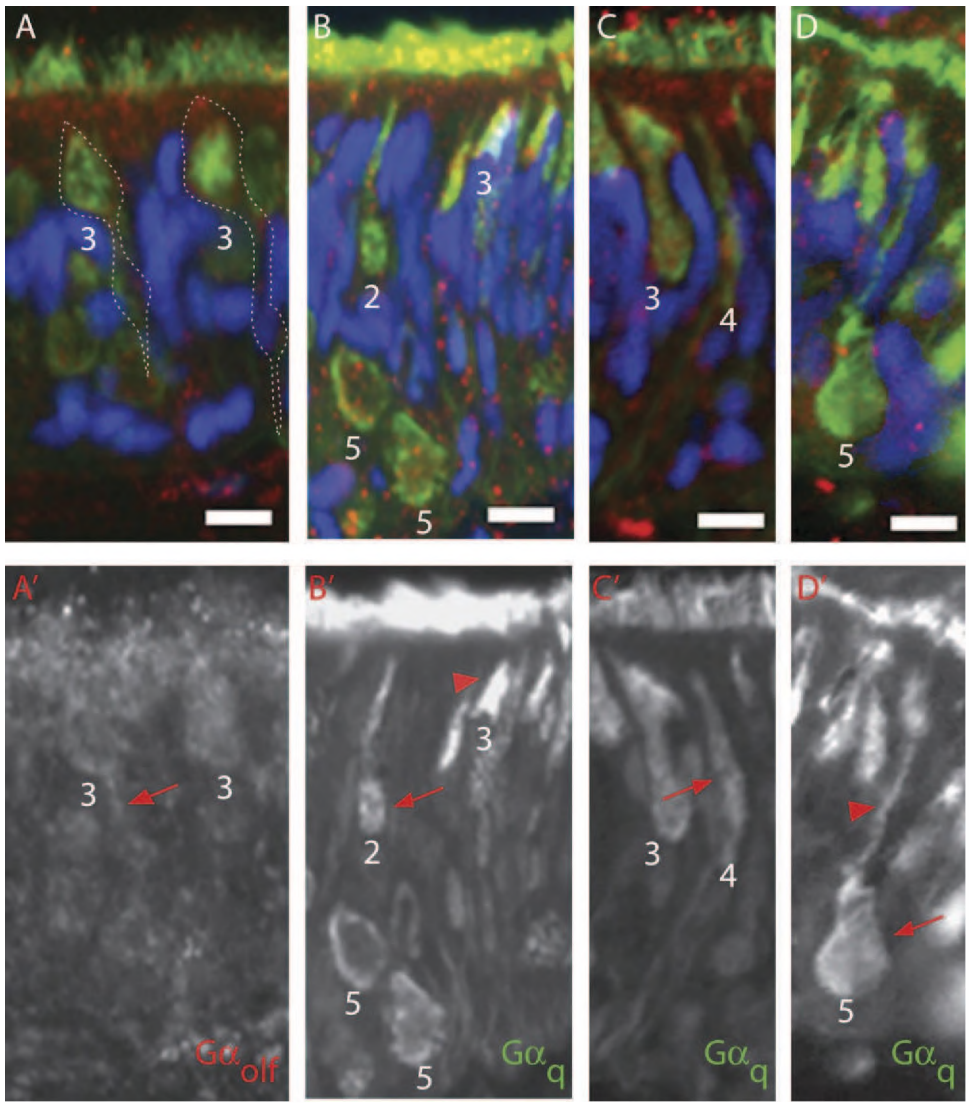

Fig. 4. Colocalization of $G\alpha_{olf}$ and $G\alpha_q$-like IR in squid ORNs. **A–D:** Fluorescent RGB images of $G\alpha_{olf}$ (red), $G\alpha_q$ (green), and DAPI (blue). **A′–D′:** Grayscale images from A–D. A: Two type 3 ORNs clearly show $G\alpha_q$ (green) in cilia pockets. A′: The type 3 neuron on the left has $G\alpha_{olf}$ IR in the outer membrane; its narrowed extension is visible (arrow). The type 3 neuron on the right has $G\alpha_{olf}$ IR in the cilia pocket. B: Two type 5 cilia pockets and a type 3 cilia pocket show $G\alpha_{olf}$-like IR. B′: The arrow indicates the $G\alpha_q$-like IR in type 2 soma and dendrite, the arrowhead indicates a type 3 cilia pocket. C: A type 3 and type 4 neuron both showing $G\alpha_{olf}$ and $G\alpha_q$. C′: The arrow indicates the type 4 cilia pocket. D: A type 5 neuron has $G\alpha_q$ throughout and little or no $G\alpha_{olf}$. D′: The arrow indicates the cilia pocket, the arrowhead indicates the dendritic extension. Scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

observed two types of $G\alpha_{olf}$- and $G\alpha_q$-like IR in the type 3 cilia pockets. In some type 3 ORNs both $G\alpha_{olf}$- and $G\alpha_q$-like IR labeled virtually all the cilia within the pocket. In other type 3 ORNs $G\alpha_q$-like IR labeled the cilia pocket; however, $G\alpha_{olf}$-like IR only labeled the outer membrane (Fig. 4A,A′). The type 2 cilia pocket is a shallow invagination near the apical surface (Emery, 1975). Therefore, it is simple to differentiate type 2 and 3 cells, as type 3 cilia pockets are much larger and slightly below the epithelial surface (Fig. 4B′, arrowhead). Type 4 neurons have large cilia pockets that narrow near the apical surface and are more apical in the OE than type 5 cilia pockets (Figs. 1B, 4C). Both $G\alpha_{olf}$- and $G\alpha_q$-like IR labeled the type 4 cilia pockets. In most type 5 neurons, $G\alpha_q$-like IR only labeled the outer membrane of the cilia pocket, while $G\alpha_{olf}$-like IR labeled the cilia (Fig. 4B). The type 5 cilia pocket is round and located at or near the base of the OE, and has a thin dendritic process connecting it to the apical surface that is rarely seen in sectioned tissue (Figs. 1B, 4D).

Type 2 PLC140-positive neurons could be divided into those with $G\alpha_q$-like IR colocalization, and those without $G\alpha_q$-like IR colocalization (Fig. 5). Colocalization occurred in the small apical cilia pocket, dendrite, and soma in a subset of type 2 cells. This segregation was apparently not related to their location in the olfactory organ because we saw PLC140-positive neurons with and without $G\alpha_q$-like

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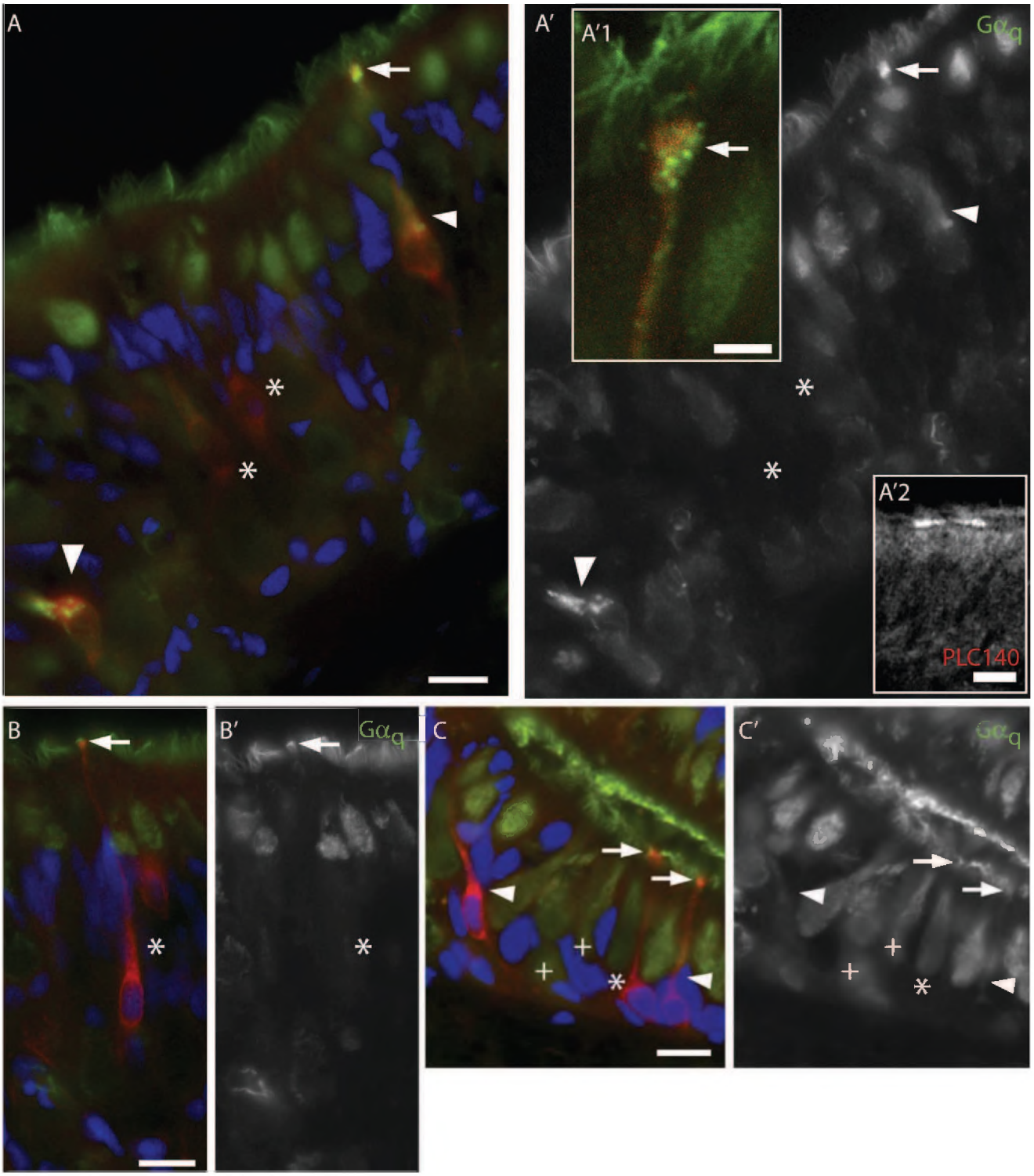

Fig. 5. Colocalization of PLC140- and $G\alpha_q$-like IR in a subset of type 2 neurons. **A–C:** Fluorescent RGB images of $G\alpha_q$ (green), PLC140 (red), and DAPI (blue) from confocal projection of three z-stack images. **A′–C′:** Grayscale images from A–C. A,A′: Examples of $G\alpha_q$ IR-positive and -negative type 2 ORNs that are PLC140 IR-positive. Note colocalization in apical cilia pocket (arrows). A′1: Single plane confocal image of PLC140 and $G\alpha_q$ in an apical cilia pocket. A′2: Isolated PLC140-like IR just below the surface consistent with type 2 apical cilia pockets. B-B′: PLC140-positive type 2 ORN with $G\alpha_q$ IR only in the apical cilia pocket (arrow). C,C′: Example of $G\alpha_q$-only positive type 2 neurons. In all panels arrows indicate type 2 neurons with $G\alpha_q$ and PLC140 colocalization in apical cilia; arrowheads indicate type 2 neurons with $G\alpha_q$ and PLC140 colocalization in soma and dendrite; asterisks indicate PLC140-only positive type 2 neurons; plus signs indicate $G\alpha_q$-only positive type 2 neurons. Scale bars = 20 μm in A,A′,C,C′; 5 μm in A′1; 10 μm in A′2,B,B′. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE 2. Summary of Observed Labeling in Each Cell Type

PLC140 only	PLC140 and $G\alpha_q$	$G\alpha_q$ only	$G\alpha_q$ and $G\alpha_{olf}$	$G\alpha_{olf}$ only
Type 2	Type 2	Type 2		
		Type 3	Type 3	Type 3
		Type 4	Type 4	Type 5
		Type 5	Type 5	Type 5

The columns are organized by positive immunostaining for the antibodies listed in the top row. Positive labeling for a particular antibody was found in all three subcellular regions (cilia pocket, dendrite, soma) of each cell type; however, all subcellular regions were not necessarily labeled within the same cell. Empty boxes indicate absence of label.

IR in the same section (Fig. 5B,C). Punctate PLC140 IR was also observed just below the cilia surface, consistent with type 2 cilia pockets (Fig. 5A′1-2). A third subset of type 2 neurons was characterized as $G\alpha_q$-positive and PLC140-negative IR (Fig. 5C-C′ (+)). Type 1 neurons are the rarest and most difficult to identify morphologically and histochemically. Although we did not see obvious labeling of the type 1 ORNs with any of our antibodies, we cannot be certain that they were not labeled. Table 2 summarizes the labeling patterns for each type of ORN.

Distribution of G proteins and PLC140 across the olfactory organ

Finally, we looked at the distribution of the proteins across the olfactory organ. We used pixel intensity thresholding on six images spaced at ≈150-μm intervals throughout each olfactory organ (Fig. 6A), so that only the 99th percentile of labeled cells was included in the analysis as the total labeled area (see Materials and Methods). The area of pixels with intensities above the threshold in each image was measured and divided by the area of the OE to determine the percentage of immunoreactivity per section (Fig. 6B–D). We observed a differential distribution of immunoreactivity for each antibody that was replicated in olfactory organs from three different squids (Fig. 6E–G). Olfactory epithelia labeled with anti-$G\alpha_{olf}$ showed a majority of labeling within the first 300 μm of the anterior side and in the last 250 μm of the posterior side (Fig. 6E). $G\alpha_q$-like IR was highest at the anterior side and decreased toward the posterior side (Fig. 6F). PLC140 had a similar but smaller pattern of immunoreactivity compared to $G\alpha_q$-like IR (Fig. 6G). The unique but overlapping immunoreactivity expression pattern for each antibody suggests regional colocalization. The total OE area was similar in all preparations; however, by comparing the percent IR/section we found that the relative amount of $G\alpha_q$-IR > $G\alpha_{olf}$-IR > PLC140-IR (Fig. 6E–G).

DISCUSSION

Our present study of G proteins and PLC in squid ORNs confirms previous physiological studies suggesting that multiple transduction pathways are present in single ORNs (Lucero and Piper, 1994; Danaceau and Lucero, 2000). We also extend our understanding of transduction mechanisms in squid ORNs to cell types not previously characterized in physiological experiments. We find that $G\alpha_{olf}$, $G\alpha_q$, and PLC140 are differentially expressed among four of the five types of ORNs. $G\alpha_q$ appears to be the dominant signaling molecule in squid ORNs. It is expressed in the cilia of cell types 2–5 and has the highest relative amount of immunoreactivity across the olfactory organ (Fig. 6). $G\alpha_{olf}$-like IR is found in cilia of cell types 3–5, and PLC140-like IR identifies a subset of type 2 ORNs. In addition to ciliary staining, each antibody showed specific labeling in the dendrites and soma of squid ORNs. Our colocalization studies using triple labeling revealed that with the exception of type 2 cells, squid ORNS of the same cell type express one or both G proteins. Furthermore, the colocalization of G proteins within cells varied such that some cells showed colocalization only in the cilia (Fig. 4A) while others showed colocalization throughout the soma and dendrite (Fig. 4C). We were unable to morphologically identify type 1 ORNs and support cells, although the $G\alpha_{olf}$ IR in the apical and basal regions of the OE may have been labeling either or both cell types.

Immunoreactivity distribution patterns for $G\alpha_{olf}$, $G\alpha_q$, and PLC140 across serially sectioned olfactory organs (Fig. 6) confirm previous studies suggesting that there is a heterogeneous distribution of the ORN subtypes (Lucero et al., 2000). This differential distribution of olfactory transduction proteins across cell types has also been observed in fish olfactory organs (Hansen et al., 2003, 2004) and suggests regional specificity of function. The high immunoreactivity of all three proteins along the anterior edge of the OE (Fig. 6E–G) fits well with the direction of odorant flow relative to the intact olfactory organ in that the anterior edge is where odorants would first contact odorant receptors (ORs; Fig. 1).

Multiple G proteins in single ORNs

A subset of the $G\alpha_q$-positive neurons show colocalization with $G\alpha_{olf}$ IR in the large cilia pockets of cell types 3, 4, and 5. Colocalization of G proteins in cilia compartments support physiological data showing odorant mixture suppression (Danaceau and Lucero, 2000). The present work does not tell us whether different ORs are coupling to different G proteins, or whether a single OR couples to multiple G proteins (Boekhoff et al., 1994). In tadpole, single ORNs can respond to multiple odor stimuli (Manzini and Schild, 2004). In *Drosophila* it has been demonstrated that single neurons coexpress two functional ORs (Goldman et al., 2005) with the potential of integrating responses at the ORN level (Hallem et al., 2004). Interestingly, both the ciliated goldfish ORNs and morphologically similar squid type 2 neurons only express a single G protein, while goldfish crypt cells and morphologically similar types 3–5 squid ORNs express two G proteins. The similarity may end there, however, because dual G protein expression in crypt cells segregates between cilia and soma (Hansen, 2004), which is different from the colocalization in the same cilia compartment that we see in subsets of squid ORNs (Fig. 4). Lobster ORNs represent the other end of the colocalization spectrum by expressing both $G\alpha_q$ and $G\alpha_{olf}$ in most ORNs (McClintock et al., 1997).

Although we found $G\alpha_q$ in type 3–5 ORNs, the absence of PLC140 suggests that a different isoform of PLC may be present in squid ORNs with large cilia pockets. A subset of type 2 ORNs did show colocalized staining for $G\alpha_q$ and PLC140; however, the majority of type 2 neurons were PLC140-negative. These observations suggest that at least one or more additional isoforms of PLC couple to $G\alpha_q$ in squid ORNs. It is notable that PLC140 was also found in a subset of type 2 ORNs that were $G\alpha_q$-negative. PLC140 is a protein that was isolated from the squid

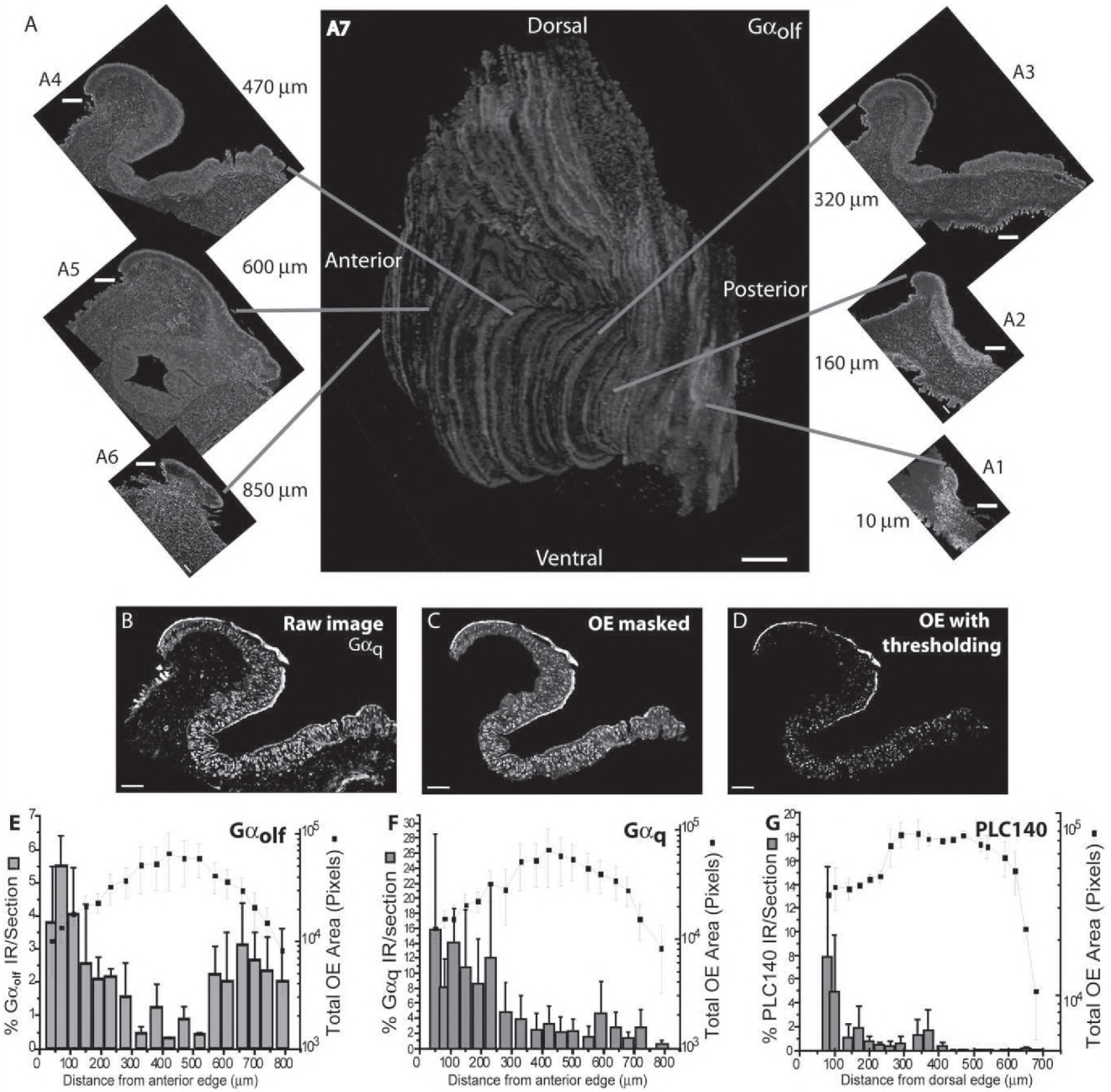

Fig. 6. The distribution of G proteins in the squid olfactory organ. **A:** Three dimensional reconstruction of the anti-$G\alpha_{olf}$-labeled squid olfactory organ (center) made from 85 images at 10-μm increments. A1-A6: Six images used to measure the total OE pixel area and to obtain the threshold value. Gray lines indicate their location within the reconstructed organ (A7), the distance from the posterior edge of the organ is given in microns. **B:** Raw image of a $G\alpha_q$-stained section. **C:** The same image as B with nonolfactory tissue masked off. **D:** OE after the threshold value was subtracted. **E–G:** Total OE area (solid square = log scale) is similar in all preparations. The percent suprathreshold immunoreactivity (IR) area/total OE area is indicated by the gray bars. Error bars indicate SEM; n = 3 for each antibody. E: The largest percentage of $G\alpha_{olf}$-like IR is found at anterior and posterior margins. F: The largest percentage of $G\alpha_q$-like IR is found at the anterior margin. G: PLC140-like IR falls within the area of $G\alpha_q$ labeling. Each olfactory organ was oriented in a similar manner for sectioning. Scale bars = 100 μm.

retina and characterized as having phospholipase C activity, with a substrate preference similar to the mammalian PLC β1 subtype (Mitchell et al., 1995). Although PLC140 was stimulated by GTP (Mayeenuddin et al., 2001), it may be activated by proteins other than $G\alpha_q$. Beta isoforms of PLC are widely recognized as effector enzymes of the $G\alpha_q$ family of G proteins; however, $G\alpha_q$ is not the only binding partner for PLC (Hubbard and Hepler, 2006). Indeed, PLCγ isozymes are activated by polypeptide growth factors, other G proteins, nonreceptor protein tyrosine ki-

nases, and lipid-derived messengers, such as phosphatidic acid. The present work identifies three unique classes within the type 2 ORNs based on the presence and absence of PLC140 and $G\alpha_q$ (Table 2).

Antibody specificity

In 1993, Ryba et al. found 77% amino acid identity of squid visual $G\alpha_q$ with mouse and *Drosophila* $G\alpha_q$. The present study provides additional evidence that $G\alpha_q$ is a conserved protein in both vertebrates and invertebrates. The mammalian rabbit anti-$G\alpha_q$ that we used on squid olfactory epithelium was previously characterized in squid retina (Mitchell and Mayeenuddin, 1998; Mayeenuddin et al., 2001). Western blot data and control experiments (zero primary and preabsorption with $G\alpha_q$ antigen) support the specificity of the antibody for $G\alpha_q$ in squid tissue. $G\alpha_{olf}$-like ($G\alpha_s$) activity has been previously shown in squid retina (Chin et al., 1994) and octopus optic lobe (Capasso et al., 1991). The mammalian rabbit anti-$G\alpha_{olf}$ that we used in this study was not previously characterized in squid tissue; however, Western blot data support antibody specificity. Thus, in the present study we showed that the mammalian rabbit anti-$G\alpha_{olf}$ and $G\alpha_q$ reacted specifically with proteins from squid OE and optic lobe.

Physiological significance and conclusions

When attacked, squids release copious amounts of ink that contains high levels of dopamine (Lucero et al., 1994). Both the type 2 and type 4 squid ORNs respond to dopamine with membrane hyperpolarization and increases in intracellular Ca^{2+} (Lucero et al., 1992; Piper and Lucero, 1999). Our findings that $G\alpha_q$ is the G protein common to both type 2 and type 4 cells suggest that $G\alpha_q$ is mediating dopamine responses. Type 4 cells also respond to glutamate and betaine and we propose that the heterogeneity of G-protein expression allows the observed multiplicity of current responses (Danaceau and Lucero, 2000). Multiple transduction pathways allow ORNs to respond to more than one odor profile or integrate mixture responses (Hallem et al., 2004). Understanding the distribution of G proteins in morphologically distinct cell types will aid in sorting out specificity of odorant responses.

We conclude that $G\alpha_{olf}$, $G\alpha_q$, and PLC140 are present in the squid olfactory organ (Western blots) and are differentially distributed among neuronal subtypes (IR). The immunoreactivity of these proteins includes cellular regions that have been shown to contain signal transduction machinery involved in odor transduction (Lucero et al., 2000; Danaceau and Lucero, 2000). The immunoreactivity distribution of $G\alpha_{olf}$, $G\alpha_q$, and PLC140 suggests that some squid ORNs utilize multiple olfactory transduction pathways. Additional studies are required to tease out the precise signaling properties and odorant response profiles of the ORN subtypes.

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