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Requirement for a phospholipase C in odor response: Overlap between olfaction and vision in *Drosophila*

(electrophysiology/*norpA* gene/signal transduction)

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ABSTRACT **A central problem in sensory system biology is the identification of the signal transduction pathways used in different sensory modalities. Genetic analysis of transduction mutants provides a means of studying *in vivo* the contributions of different pathways. This report shows that odorant response in one olfactory organ of *Drosophila melanogaster* depends on the *norpA* phospholipase C (EC 3.1.4.3) gene, providing evidence for use of the inositol 1,4,5-trisphosphate (IP_3) signal transduction pathway. Since the *norpA* gene is also essential to phototransduction, this work demonstrates overlap in the genetic and molecular underpinnings of vision and olfaction. Genetic and molecular data also indicate that some olfactory information flows through a pathway which does not depend on *norpA*.**

Vertebrate olfactory transduction is believed to be mediated, at least in part, through the adenosine 3′,5′-cyclic monophosphate (cAMP) second messenger pathway (1). There is also evidence for a role for the inositol 1,4,5-trisphosphate (IP_3) second messenger pathway (1). For example, biochemical analysis of a preparation of rat olfactory cilia provided evidence that while some odorants induce formation of cAMP but not IP_3, other odorants induce formation of IP_3 but not cAMP (2). In another study, using a primary culture system of rat olfactory receptor neurons, odorants stimulated both second messenger systems, but with different potencies (3).

In invertebrates, biochemical measurements taken from cockroach antennal homogenates showed that a pheromone stimulated elevation of IP_3 but not cAMP levels (4). IP_3 has also been shown to evoke an inward (excitatory) current in cultured lobster olfactory receptor neurons (ORNs) (5). In some lobster ORNs, cAMP was found to mediate an odorant-induced inhibitory current (6). There is also evidence for a role for cGMP in olfactory transduction in the silkworm moth: cGMP was found to stimulate a sex pheromone-dependent ion channel (7).

Photoreception in invertebrates is thought to be mediated, at least in part, by the IP_3 pathway (8). Important support for this conclusion comes from genetic analysis: strong alleles of the *Drosophila melanogaster* gene *norpA* (no receptor potential A) eliminate the light-evoked responses of photoreceptors (9, 10). The *norpA* gene was found to encode a phospholipase C (PLC; EC 3.1.4.3), providing strong evidence that phototransduction is dependent upon the IP_3 pathway (11, 12).

To examine *in vivo* the possible role of the IP_3 pathway in olfactory transduction in *Drosophila*, we have examined the odorant response of *norpA* mutants. We show that odorant response in one olfactory organ requires *norpA* activity, consistent with a role for the IP_3 signal transduction pathway in olfactory function. The results also provide evidence that some olfactory information is transmitted through a pathway which does not rely on *norpA*. Since *norpA* is also required for phototransduction, the results indicate overlap in the molecular components underlying vision and olfaction.

MATERIALS AND METHODS

Drosophila Stocks and Culture. $norpA^{EE5}$ was obtained from T. Tanimura (Kyushu University). All other *norpA* alleles were from William Pak (Purdue University). Flies were cultured at 22°C in bottles containing yeast-inoculated cornmeal–molasses–agar medium.

Electrophysiology. For maxillary palp recordings, electrodes were glass micropipets (with tips ≤1 μm in diameter) filled with *Drosophila* Ringer's solution. The electrodes made electrical contact with a high-impedance 10X dc amplifier via silver chloride/silver wires. The reference electrode was inserted into the head capsule. The recording electrode was brought into contact with the distal tip of the maxillary palp and advanced until stable electrical contact was just established. Throughout the recording session, the fly was in a constant airstream, 2 liters/min in some experiments and 3 liters/min in others. The airstream was directed at the fly through a polystyrene tube (inner diameter 6 mm), whose end was 1 cm from the fly. The tube contained a small hole for injection of odorant puffs into the airstream. Odorant puffs were created by quickly expelling 3 cm^3 of air from a 5-cm^3 syringe over a filter disk, which was saturated with 200 μl of odorant diluted in paraffin oil. The disk was fitted into the large end of a Pasteur pipet; the small end of the pipet was inserted into the hole in the airflow tube. Antennal recordings were made in analogous fashion, from the dorsomedial portion of the anterior face of the antenna.

Dosages are indicated as dilution factors of the odorants in paraffin oil. The number of molecules which evaporated from the surface of the filter disk (during the injection), made contact with the maxillary palp, and entered the lumen of the olfactory sensilla has not been determined.

To determine whether electrical conduction properties were affected in the maxillary palp of $norpA^{P24}$ flies, a tungsten stimulating electrode was placed in the head capsule at the base of the proboscis, near the maxillary palp, and voltages of 20, 50, 100, and 200 mV were applied for 20 msec each. Direct current was applied with a Grass stimulator. We recorded the potential difference between a recording electrode and a reference electrode, placed in the same positions on the external surface of the maxillary palp and in the head capsule as for electropalpogram recordings. The potential differences recorded (≈15, ≈35, ≈80, and ≈160 mV, respectively, for the different stimulating voltages applied) did not differ between mutant and wild type (n = 5 flies for each genotype).

***In Situ* Hybridization and Immunocytochemistry.** The antisense *norpA* probe used for *in situ* hybridization was made from a genomic clone (the 3.3-kb *Bgl* II fragment of λC1-D,

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Abbreviations: PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate.

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obtained from C. Montell, Johns Hopkins University, Baltimore). Hybridization was as described elsewhere (13).

Immunocytochemistry of tissue sections was as described elsewhere (13). For the whole mounts shown in Fig. 5, the maxillary palps and proboscis were separated from the rest of the head by a single cut made through the head, slightly dorsal to the maxillary palps. This cut divided the head into two portions; the portion which contained the maxillary palps and the proboscis was then fixed in 2% paraformaldehyde at room temperature for 2 h and incubated 4 h at room temperature with a 1:1000 dilution of affinity-purified anti-norpA antibody (14), a generous gift of R. Shortridge. The tissue was then incubated with secondary antibody, using the Vectastain Elite ABC kit (Vector Laboratories) for 2 h at room temperature. All washings, after fixation and after incubation with antibodies, were for 2 h at room temperature with several changes.

This procedure yielded a substantial number of $norpA^{P24}$ maxillary palps which showed at least some staining; we interpret this staining as background, since control experiments showed some staining of palps in the absence of the first-stage (anti-norpA) antibody. In an effort to reduce background staining, we performed a separate experiment in which fixation, incubations, and washings were at 4°C. The fixation and second-stage incubations were for 4 h, the first stage incubation was overnight, and all washings were for 3–4 h. While this procedure did not eliminate the staining that we interpret as background, mutant and wild-type maxillary palps were distinguishable in the great majority of cases, indicating that there was little overlap in staining levels between mutant and wild-type. Specifically, in a blind experiment, the maxillary palps of 17 animals, including both mutant and wild-type, were stained with anti-norpA antibodies in parallel, and then scored by three individuals, independently. These individuals were able to identify $norpA^{P24}$ vs. wild-type maxillary palps correctly in 17/17, 16/17, and 16/17 cases. In a second independent experiment, $norpA^{P24}$ and wild-type were identified correctly in 19/21, 18/21, and 18/21 cases.

RESULTS

The head of *Drosophila* contains two pairs of olfactory organs, the third antennal segments and the maxillary palps (Fig. 1) (15–17). These organs are covered with sensory hairs, and each hair is innervated by up to four neurons (18, 19). The antenna and the maxillary palp both send projections to the antennal lobe of the brain (19, 20), both develop from the eye–antennal imaginal disc (21), and both respond to a wide variety of odorants (17).

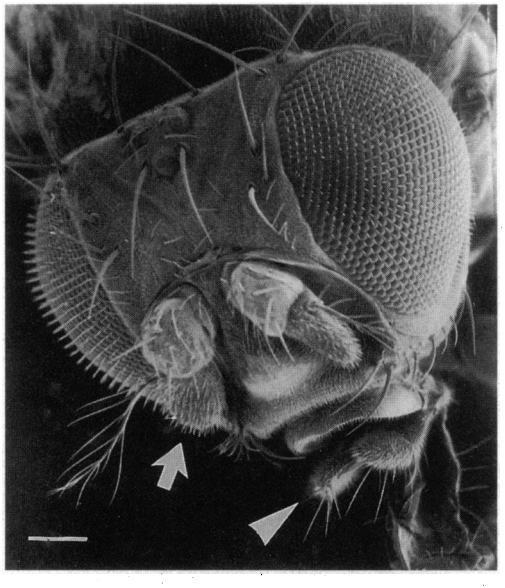

FIG. 1. Scanning electron micrograph of *Drosophila* head, showing third antennal segment (arrow) and maxillary palp (arrowhead). (Scale bar = 100 μm.)

We initially tested odorant response in the $norpA^{P24}$ mutant. This mutation reduces the amplitude of visual response to zero (22), as measured in electroretinogram recordings. The mutant contains no detectable norpA product in the head, as determined with anti-norpA antibodies (14, 23), and it has also been reported to lack norpA product in the thorax, abdomen, and legs (14). Moreover, Zhu *et al.* (14) reported that no *norpA* RNA was detectable in the mutant, as determined by hybridization with a *norpA* probe. We measured odorant response in the maxillary palp by using extracellular recordings, analogous to electroretinograms, that are believed to measure the summed receptor potentials of olfactory receptor neurons in the vicinity of the recording electrode (17).

The amplitude of the response to vapors of all odorants tested in the maxillary palp was found to be significantly reduced (Fig. 2*A*). The odorants included an aldehyde (benzaldehyde, which is the odor of almond), three alcohols (4-methylcyclohexanol, 3-octanol, and 1-butanol), an acetate ester (ethyl acetate), and an organic acid (propionic acid). Dose–response curves were generated for two of these odorants, benzaldehyde and ethyl acetate (Fig. 2 *B* and *C*). The response amplitude of the mutant was 10–55% of the wild-type value for all dilutions of benzaldehyde and ethyl acetate except the very highest concentration of ethyl acetate, where it was 73% of the wild-type value.

Interestingly, antennal response, measured analogously with electroantennograms (17, 24), was not significantly affected in the mutant for any tested odorant (Fig. 3*A*) or at any concentration of benzaldehyde or ethyl acetate (Fig. 3 *B* and *C*).

Six other alleles of *norpA* were also found to show reduced odorant response in the maxillary palp, in a separate experiment (Fig. 4*A*). In this experiment, $norpA^{P24}$ and six of nine other *norpA* alleles all showed reduced ($P < 0.05$) amplitudes of responses to ethyl acetate. These 10 alleles were all induced in an Oregon-R genetic background. Maxillary palp response in an additional allele, $norpA^{EE5}$, was not significantly different from that of its Canton-S genetic background control (data not shown).

Three of the mutants which showed reduced odorant response were chosen for more extensive analysis ($norpA^{P39}$, $norpA^{P40}$, and $norpA^{P55}$). Dose–response curves were generated for ethyl acetate, and responses were found to be reduced across a broad range of concentrations for all three alleles in the maxillary palp (Fig. 4*B*). All of the 11 *norpA* alleles showed normal response in the antenna to vapor of a 10^{-3} dilution of ethyl acetate (data not shown).

Flies carrying a chromosome lacking the *norpA* gene also have a reduced maxillary palp response (Fig. 4*C*). This chromosome, *Df(1)RC40,* has undergone a deletion extending from 4B1 to 4F1 on the X chromosome; the *norpA* locus lies in the 4B6–C1 interval. The $+/Df$ heterozygote shows a reduced response to ethyl acetate, compared with sibling $+/+$ controls. These results provide further evidence that the olfactory defects of the various *norpA* mutants in fact map to the *norpA* gene; they also indicate that the deletion mutation is at least partially dominant for this phenotype.

Fig. 4*C* also shows that the response amplitude of $+/norpA^{P24}$ is equal to that of $+/Df$ and that $norpA^{P24}/norpA^{P24}$ gives a response equal to that of $Df/norpA^{P24}$. In this experiment, then, $norpA^{P24}$ cannot be distinguished from a deletion of the *norpA* locus, indicating that $norpA^{P24}$ is a null allele with respect to this phenotype. [The $norpA^{P24}$ allele was also indistinguishable from the deletion in terms of response to 4-methylcyclohexanol, benzaldehyde, and acetone; for propionic acid, the response of $+/norpA^{P24}$ was indistinguishable from that of $+/Df$, although $norpA^{P24}/norpA^{P24}$ gave a re-

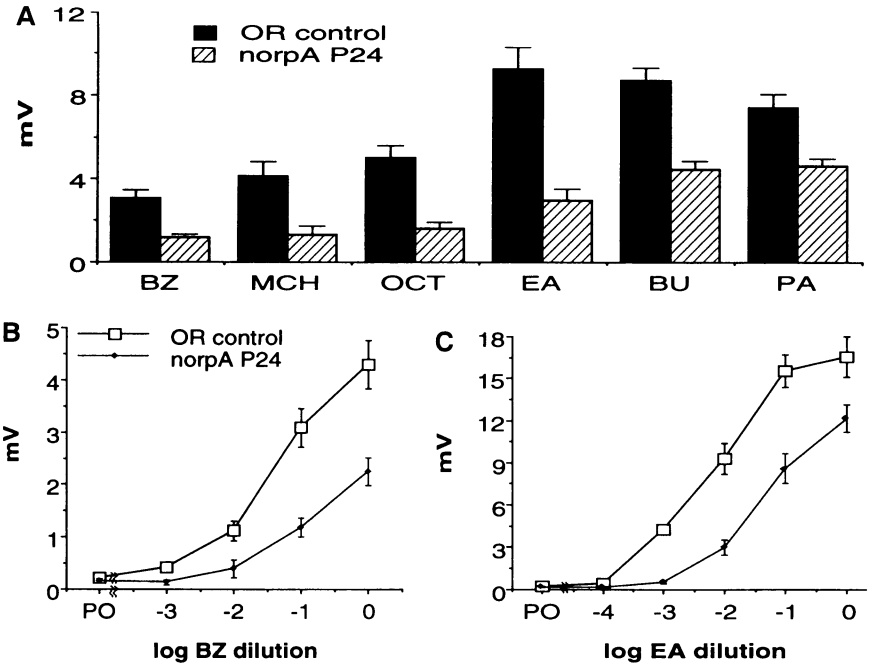

FIG. 2. Olfactory physiology is defective in the maxillary palp of *norpA*^{P24} flies. OR indicates wild-type Oregon-R flies. Values indicate response amplitude ± SEM. Error bars are too small to be seen in some cases. (*A*) Responses to vapors of benzaldehyde (BZ; diluted 10^{-1}; see *Electrophysiology* for explanation of dosage), 4-methylcyclohexanol (MCH; diluted 10^{-1}), 3-octanol (OCT; neat), ethyl acetate (EA; diluted 10^{-2}), 1-butanol (BU; diluted 10^{-1}), and propionic acid (PA; diluted 10^{-1}). $n = 17$. Differences are significant for all odorants ($P < 0.05$; ANOVA). (*B*) Dose–response curves for benzaldehyde. PO, paraffin oil diluent alone. $n = 17$. Differences are significant at all concentrations ($P < 0.05$; ANOVA). (*C*) Dose–response curves for ethyl acetate. Symbol designations are as in *B*. $n = 17$ except $n = 8$ for 10^{-1} and 10^{0} dilutions. Differences are significant at all concentrations except 10^{0} ($P < 0.05$; ANOVA).

sponse which was greater than that of *Df/norpA*^{P24} (data not shown).]

Consistent with our demonstration of a role for the *norpA* gene in the maxillary palp, but not the antenna, we have found evidence for *norpA* gene expression in the maxillary palp, but not the antenna. *In situ* hybridization to RNA in tissue sections with a *norpA* probe showed labeling in the maxillary palp (Fig. 5*A*). No hybridization was observed to a comparable number of maxillary palps examined from the *norpA*^{P24} mutant. We observed no staining in wild-type antennae examined in the same experiment (≈100 antennal sections made from 12 antennae; data not shown). A caveat in interpreting these

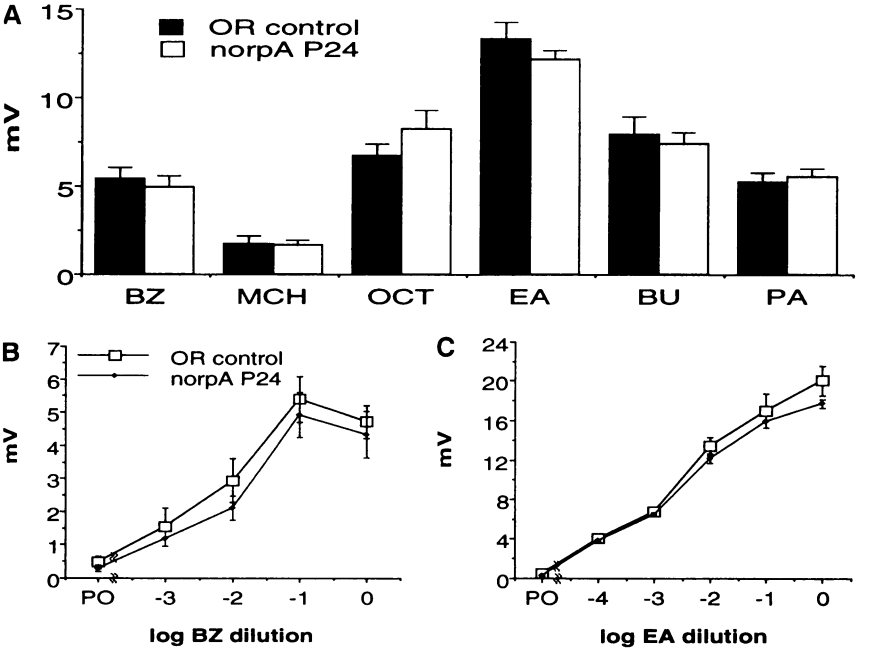

FIG. 3. Olfactory physiology is normal in the antenna of *norpA*^{P24} flies. Values indicate response amplitude (±SEM); $n = 10$ for all values. (*A*) Responses to vapors of benzaldehyde (BZ; diluted 10^{-1}), 4-methylcyclohexanol (MCH; diluted 10^{-1}), 3-octanol (OCT; neat), ethyl acetate (EA; diluted 10^{-2}), 1-butanol (BU; diluted 10^{-1}), and propionic acid (PA; diluted 10^{-1}). (*B*) Dose–response curves for benzaldehyde. PO, paraffin oil diluent alone. (*C*) Dose–response curves for ethyl acetate. Symbol designations are as in *B*.

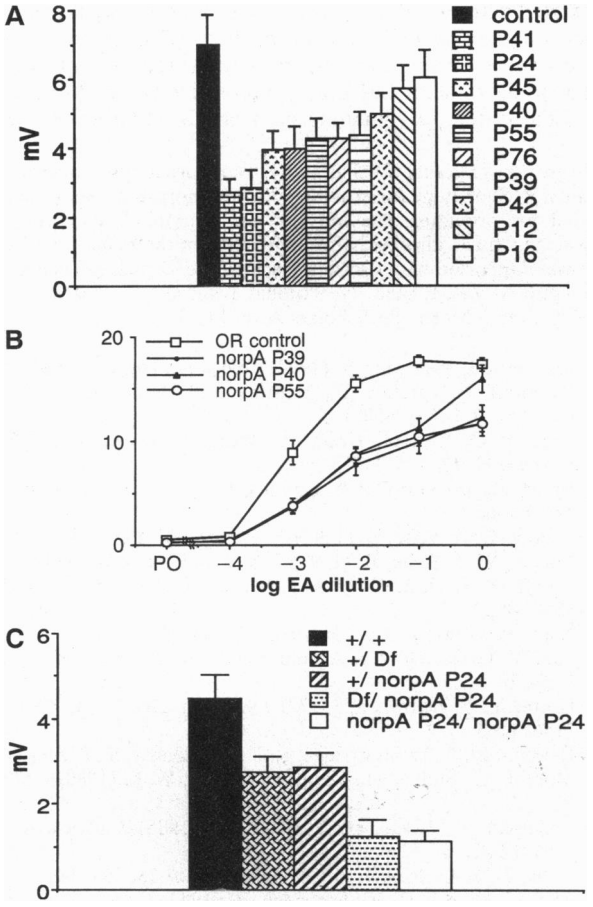

FIG. 4. Genetics of the *norpA* olfactory defect. All panels show response amplitude (±SEM) of the maxillary palp. [Recordings in *A* and *B* were made by using a different odorant delivery system (with a different flow velocity) than that used in *C* and in Fig. 2, which presumably accounts for the somewhat larger amplitudes.] (*A*) Response of a series of *norpA* alleles and their parental background control, Oregon-R, to vapors of a 10^{-3} dilution of ethyl acetate; $n = 10$. The alleles showing significantly reduced response were $norpA^{P41}$, $norpA^{P24}$, $norpA^{P45}$, $norpA^{P40}$, $norpA^{P55}$, $norpA^{P76}$, and $norpA^{P39}$. Significance ($P < 0.05$) was as determined by ANOVA, followed by a post hoc comparison using Dunnett's test. (*B*) Dose–response curves for three *norpA* alleles and their parental Oregon-R wild-type control. PO, paraffin oil diluent alone. $n = 6$. The values for $norpA^{P39}$ and $norpA^{P55}$ are significantly different from the control value at all concentrations tested (the error bars are too small to be seen for the 10^{-4} dilution), and the $norpA^{P40}$ value is significantly different from the control value at 10^{-3}, 10^{-2}, and 10^{-1} dilutions. Significance ($P < 0.05$) was determined as in *A*. (*C*) Comparison of *Df(1)RC40* and $norpA^{P24}$. The stimulus was vapor of a 10^{-3} dilution of ethyl acetate. When the paraffin oil diluent was tested alone, the mean amplitudes were 90% (±3%) smaller than for the ethyl acetate stimulus, in the case of each genotype. The +/+ flies were *OR/FM7c*, the +/*Df* flies were *OR/Df*, and the +/$norpA^{P24}$ flies were *FM7c*/$norpA^{P24}$. *OR* refers to the Oregon-R parental wild type, and *FM7c* is a balancer chromosome, expected to be $norpA^{+}$. $n = 9$ for each genotype. In a separate control experiment, the response amplitudes of *OR/OR* and *OR/FM7c* were found to be indistinguishable: 8.8 ± 0.4 mV for *OR/OR* vs. 8.8 ± 0.7 mV for *OR/FM7c*. $n = 7$ for each genotype.

experiments, however, is that the fraction of wild-type maxillary palp sections showing hybridization comparable to that shown in Fig. 5*A* was low, and therefore *norpA* expression was investigated further in the maxillary palp at the protein level.

Immunocytochemistry with an affinity-purified anti-norpA antibody (14) also revealed expression of the *norpA* product in the maxillary palp (Fig. 5*B*) but not in the antenna (data not shown). The staining is localized along the lateral surface of the maxillary palp, in a region that contains a high density of

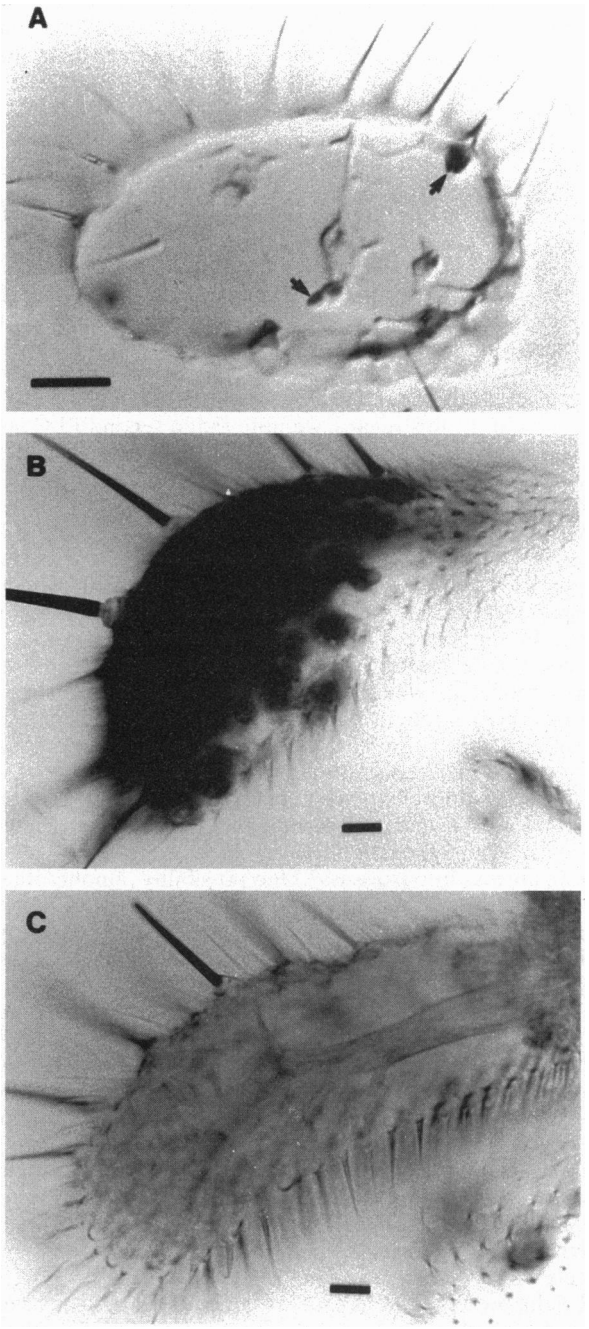

FIG. 5. Expression of the *norpA* gene in the maxillary palp. (Scale bars = 10 μm.) (*A*) *In situ* hybridization to RNA in a maxillary palp section, using antisense *norpA* probe. The entire maxillary palp contains ≈60 olfactory hairs (19); a 7-μm section like that shown is expected to contain cell bodies of only a small number of them. Hybridization was as described elsewhere (13). (*B*) Whole mount staining of a maxillary palp of Oregon-R wild type with the affinity-purified anti-norpA antibody abRN (14). This photograph shows an example of a maxillary palp exhibiting very strong staining. (*C*) $norpA^{P24}$ maxillary palp stained with antibody as in *B*. This photograph shows an example of a maxillary palp exhibiting little if any staining.

olfactory hairs. Staining occurs not only in cell bodies but also in axons; labeling of the maxillary nerve can be clearly seen in planes of focus deeper than that shown in Fig. 5*B*. In the $norpA^{P24}$ mutant, maxillary palp staining is either absent (Fig. 5*C*) or reduced (see *Materials and Methods*).

Scanning electron microscopy revealed no obvious morphological defects in the maxillary palps of $norpA^{P24}$ mutants (data not shown). Examination of sectioned maxillary palps by light

microscopy also revealed no gross anatomical defects. Finally, the electrical conduction properties of the *norpA*^{P24} maxillary palps were normal, as determined by stimulating the interior of the head capsule and recording from the external surface of the maxillary palp (see *Materials and Methods* for details).

DISCUSSION

Results in this paper provide genetic evidence that the IP_3 pathway is used in invertebrate olfactory transduction. The physiological measurements, made *in vivo,* indicate that normal olfactory response in the maxillary palp requires the PLC encoded by the *norpA* gene. By contrast, we have found neither genetic nor molecular evidence that response in the antenna depends on this enzyme. It is possible that the antenna also depends on a PLC-mediated pathway, but that a distinct PLC gene is used. In this regard, we note that a second PLC gene has been isolated in *Drosophila* (25); however, mutations of it have not been described, and antennal expression has not been reported.

We have found that maxillary palp response is not abolished in *norpA*^{P24} (Fig. 2), or in *Df/norpA*^{P24} mutants (Fig. 4*C*), even though *norpA*^{P24} is a null mutant by genetic criteria. One interpretation of these results is that there is genetic redundancy, in the sense that another PLC gene may also be expressed in the maxillary palp. Our results are also consistent with a model in which odorant response in *Drosophila* is elicited in part through a PLC-mediated transduction pathway and in part through a PLC-independent pathway, such as a cyclic nucleotide-mediated pathway.

We have shown that most, but not all, alleles of *norpA* produce defects which are sufficiently severe as to be detectable in our physiological tests. Why do some *norpA* alleles appear normal in our assays? One possibility, among others, concerns the facts that the *norpA* gene consists of at least 13 exons (26) and that the gene gives rise to multiple transcripts of different sizes (14). Perhaps some mutations affect an exon which is expressed in the eye, but not the maxillary palp.

The simplest interpretation of our results is that *norpA* plays a direct role in olfactory transduction in the maxillary palp. We detected no morphological defects in the *norpA* maxillary palp, and it appeared normal in a test of its conduction properties. We cannot exclude the formal possibility that *norpA* mutations affect maxillary palp response wholly or in part through an indirect effect, such as a subtle morphological abnormality. However, speculation concerning such indirect effects is constrained by the observation that olfactory response in the antenna appears normal; many types of general, systemic defects would likely affect both olfactory organs.

Results presented here provide genetic and molecular evidence for overlap between the olfactory and visual pathways. This evidence is consistent with our earlier finding that the *rdgB* (retinal degeneration B) gene is required for normal physiology in both visual and olfactory systems (27, 28). Unlike *norpA, rdgB* function is required for normal olfactory physiology in both the antenna and the maxillary palp, and expression of rdgB protein has been demonstrated in both organs by immunocytochemistry (28, 29). Like *norpA, rdgB* may be associated with the IP_3 signal transduction cascade: it encodes a phosphatidylinositol transfer protein (29). It will be of interest to determine how much of the olfactory pathway is unique to the olfactory system, as opposed to being shared with the visual system or signalling pathways in other cell types.

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