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Evidence of a novel transduction pathway mediating detection of polyamines by the zebrafish olfactory system

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Summary

To better understand the full extent of the odorant detection capabilities of fish, we investigated the olfactory sensitivity of zebrafish to a monoamine and several polyamines using electrophysiological and activity-dependent labeling techniques. Electro-olfactogram (EOG) recording methods established the relative stimulatory effectiveness of these odorants as: spermine >> spermidine ≈ agmatine > glutamine > putrescine ≥ cadaverine ≥ histamine > artificial freshwater. The detection threshold for the potent polyamines was approximately 1 μmol l^{-1}. Cross-adaptation experiments suggested that multiple receptors are involved in polyamine detection. Three observations indicated that polyamine signaling may involve a transduction cascade distinct from those used by either amino acids or bile salts. Like bile salts and the adenylate cyclase activator forskolin, but unlike amino acid odorants, polyamines failed to stimulate activity-dependent labeling of olfactory sensory neurons with the cation channel permeant probe agmatine, suggesting a signaling pathway different from that used by amino acid stimuli. Also supporting distinct amino acid and polyamine signaling pathways is the finding that altering phospholipase C activity with the inhibitor U-73122 significantly reduced amino acid-evoked responses, but had little effect on polyamine- (or bile salt-) evoked responses. Altering cyclic nucleotide-mediated signaling by adenylate cyclase activation with forskolin, which significantly reduced responses to bile salts, failed to attenuate polyamine responses, suggesting that polyamines and bile salts do not share a common transduction cascade. Collectively, these findings suggest that polyamines are a new class of olfactory stimuli transduced by a receptor-mediated, second messenger signaling pathway that is distinct from those used by amino acids or bile salts.

Key words: electro-olfactogram, activity labeling, olfactory receptor neuron, odorant receptor, zebrafish, *Danio rerio*.

Introduction

Detecting and discriminating odors is the primary function of the vertebrate olfactory system. The nature of the odors detected is determined by the niche occupied. Terrestrial species detect a wide array of volatile odorants, whereas aquatic species primarily detect water-soluble odorants. By some estimates, the number of identified odorant receptors used by terrestrial species exceeds by tenfold or more the 100 predicted odorant receptors for teleosts (Buck and Axel, 1991; Ngai et al., 1993a,b; Naito et al., 1998; Speca et al., 1999; Asano-Miyoshi et al., 2000). For aquatic species, the number of water-soluble compounds potentially serving as odorants is virtually unlimited, but for most fish species, the number of confirmed odorants is well below the predicted number of receptors. Understanding the full odorant detection capabilities of a species requires continued testing of novel odorants that are likely to be of biological significance.

Polyamines are present in all cells and their concentrations in carrion are likely to increase due to bacterial degradation (Morgan 1999; Du et al., 2001). To date, only two polyamines have been tested as odorants in fish, and in both cases they were tested on zebrafish *Danio rerio*. Putrescine failed to stimulate pre-synaptic olfactory sensory neuron activity in the olfactory bulb (Fuss and Korsching, 2001). Agmatine (AGB), a relatively rare guanidium-based diamine synthesized by decarboxylation of L-arginine, was found to elicit large olfactory responses and to enter a small population of microvillar olfactory sensory neurons, which could subsequently be visualized by an anti-AGB antibody (Michel et al., 1999; Lipschitz and Michel, 2002).

Structurally, polyamines are similar to other potent aquatic odorants such as amino acids and biogenic amines. Polyamines are small molecular mass, linear aliphatic molecules that are water-soluble and have positively charged amino groups at physiological pH values, making them organic bases. The fixed spacing of their positive charges gives polyamines unique steric and cationic properties.

Cells closely regulate intracellular polyamine levels through mechanisms controlling biosynthesis, degradation and uptake (Morgan, 1999). Minimal levels of intracellular polyamines are needed for the optimal growth and replication of plant,

bacterial, fungal and animal cells. Polyamines alter the transcriptional and translational stages of protein synthesis, stabilize membranes, modulate ion channel activity, change intracellular free calcium levels and possibly possess important messenger functions (reviewed by Morgan, 1999). Additionally, spermine, spermidine, putrescine (Lynch, 1999; Nevin et al., 2000) and agmatine (Michel et al., 1999) have been shown to block olfactory cyclic nucleotide-gated (CNG) channel activity.

Physiological methods have been used to explore structure–activity relationships of many different classes of odorants in the zebrafish *Danio rerio*, and electrophysiological methods (Michel and Derbidge, 1997), in combination with activity-dependent labeling studies (Lipschitz and Michel, 1999b), identified at least partially independent receptor sites for amino acids, bile salts and guanidine-based substances. These findings were largely confirmed and additional sensitivity to nucleotides, saponins and sex pheromones was identified when presynaptic olfactory sensory neuron (OSN) activity was imaged in the olfactory bulb (Friedrich and Korsching, 1997, 1998; Fuss and Korsching, 2001). In the present investigation, we used electro-olfactogram (EOG) recording methods and activity-dependent labeling techniques to explore polyamine-stimulated signaling in the zebrafish olfactory system.

Materials and methods

Animal maintenance

Zebrafish *Danio rerio* Hamilton-Buchanan were purchased from a commercial supplier (Scientific Hatcheries, Oceanside, CA, USA), housed for up to 4 months in mixed-sex populations in recirculating, charcoal-filtered, 40–80 l aquaria at 28.5°C and fed flake food (Tetramin) daily. Approximately 80% of the water was replaced weekly with fresh, deionized water. The zebrafish were subjected to a light cycle of 12 h:12 h dark:light, which was provided by fluorescent laboratory lighting.

Recording procedures

All experimental procedures have been approved by the University of Utah Animal Care and Use Committee. Before a recording session, each zebrafish was immobilized with an intramuscular injection of Flaxedil (60 mg g^{-1} body mass), secured to a custom silastic-polymer (Sylgard) recording chamber and immediately provided with a continuous flow of artificial fresh water (AFW; see Solutions) to the olfactory epithelium (OE) and a separate flow of approximately 3 ml min^{-1} of AFW containing MS-222, a general anesthetic (20 mg l^{-1} in AFW), over the gills. In order to prevent the loss of afferent sensory activity (Spath and Schweickert, 1977), the fish were anesthetized only after immobilization, thus preventing any contact between the anesthetic and the OE. Each zebrafish was given 10 min for the anesthetic to fully act before the small flap of the epithelium covering the left olfactory organ was surgically removed to expose the olfactory lamellae. Throughout the experiment, zebrafish were monitored for any reflex movements of the gills or eyes and, if noted, provided with additional anesthetic by increasing the gill irrigation flow. Following each experiment, while still anesthetized, each fish was measured (total body length) and weighed, then killed by decapitation. The sex of the zebrafish was then determined by the presence of ovaries or testes in the abdominal cavity as seen under a dissecting microscope.

Electrophysiological methods

The olfactory responses of the zebrafish were measured using EOG recording methods as described previously (Michel and Lubomudrov, 1995; Michel and Derbidge, 1997). A characteristic, negative DC voltage potential shift was recorded in response to odors, reflecting the extracellular ionic flux associated with the summed receptor potentials of the activated olfactory receptor neurons. To record these responses, a reference electrode was placed on the top of the head and a recording electrode was placed in the left olfactory organ, between adjacent olfactory lamellae and near the midline raphe. Both of these electrodes were made from silver/silver chloride wire bridged to the fish by way of 3 mol l^{-1} NaCl/agar-filled (1–3%) glass electrodes with tip diameters of approx. 10–20 μm. A silver/silver chloride wire, placed in the AFW bath directly beneath the body of the fish, served as the ground electrode. The responses to the olfactory stimuli were amplified and filtered at 1–2 kHz by a low-noise, differential, DC amplifier, displayed on an oscilloscope and stored digitally (100 Hz; Digidata 1200 A/D board and Axotape software, Axon Instruments, Union City, CA, USA). Before beginning an experiment, a stable baseline and a response of at least 0.5 mV to 100 μmol l^{-1} L-glutamine were required. The olfactometer and both electrodes were adjusted before deciding to reject a fish on the basis of its small response to glutamine. Fewer than 5% of the zebrafish test subjects were rejected.

Odorant testing

The competitor odorant or AFW solution was selected by a six-way valve from elevated, polyethylene bottles and delivered to the OE at a rate of 3 ml min^{-1}. Teflon or polyethylene tubing and connectors were used in the olfactometer construction to minimize contamination. A piece of 18-gauge stainless steel tubing directed the olfactometer output flow over the OE. A rotary loop injector (Rheodyne, Inc., Rohnert Park, CA, USA) was used to introduce the test odorant (50 μl) into the olfactometer flow. Fluorescent dye calibration determined that the odor solution arrived at the OE in approximately 8 s, achieved peak odor concentration (approximately 84% of stock) at approx. 10 s, and then returned to baseline levels in another 12–15 s. The concentrations reported have not been corrected for this dilution. To minimize adaptation, a period of at least 2 min was allowed between odorant tests, and ascending concentrations were tested during dose–response determinations. The odorants/stimulants used in these experiments were: the amino acid L-glutamine (Gln); the polyamines agmatine (AGB),

putrescine (Put), cadaverine (Cad), spermidine (Spd) and spermine (Spm); the monoamine histamine (His); and, in some experiments, the bile salt taurocholic acid (TCA). The amino acid L-glutamine was used as a positive control to establish the viability of the preparation and to allow comparison with earlier studies. With the exception of TCA (10 μmol l^{-1}), all of the odorants were tested at 100 μmol l^{-1}.

Identification of odorant receptor site types

The protocol used for the cross-adaptation study was similar to the procedures previously used to characterize olfactory receptors in zebrafish and channel catfish (Caprio and Byrd, 1984; Michel and Derbidge, 1997). A standard test series measured the responses to test odor before, during, and after adaptation of the OE with a competitor odorant. The test series was performed in three steps. First, the response to a test odorant was measured with AFW bathing the OE. Then, the response to a test odorant, prepared in the competitor odorant background, was measured while the competitor odorant bathed the OE. During changes between AFW and a competitor odorant, the competitor odorant background bathed the OE for a minimum of 60 s before a test odorant was applied. Finally, the flow across the OE was returned to AFW and the response to the test odorant was verified to ensure that adaptation was reversible. During each phase of testing, each odorant was tested in duplicate. The response to L-glutamine (100 μmol l^{-1}) was checked between test series to assess long-term preparation viability. The order of the odorants tested was randomized for each zebrafish examined, and a minimum of three fish were tested in each of the seven adapting backgrounds. All adapting odorant backgrounds were used at a concentration of 100 μmol l^{-1}.

Transduction cascades involved in polyamine signaling

We have previously shown that adaptation with the adenylate cyclase activator forskolin or the phospholipase C inhibitor neomycin largely eliminates bile salt- and amino acid-evoked responses, respectively (Ma and Michel, 1998; Michel, 1999), suggesting the presence of at least two transduction cascades for odorants in zebrafish. To explore the potential transduction cascade mediating polyamine signaling, the responses to glutamine, taurocholic acid and the polyamines were measured before, during and after adaptation to forskolin (10 μmol l^{-1}) or U-73122 (1 μmol l^{-1}), a more specific PLC inhibitor than neomycin, using the procedure described above for identification of odorant receptor site types.

Activity-dependent labeling

Amino acids, but not the bile salt taurocholic acid (or the adenylate cyclase activator, forskolin), were shown to stimulate transduction pathways linked to ion channels within OSNs permissive to permeation by the guanidinium analog, agmatine (AGB) (Michel et al., 1999; Lipschitz and Michel, 1999b; Lipschitz and Michel, 2002). Methods reported in detail in these earlier studies were used to determine if polyamines were capable of stimulating activity-dependent labeling of OSNs with AGB. Briefly, a fish was immobilized and placed in the recording chamber as described above. The OE was bathed with fish Ringer and once a minute for 10 min a 10 s bolus of odorant and 5 mmol l^{-1} AGB in fish Ringer was introduced into the olfactometer flow to stimulate the OSNs. Presentation of 5 mmol l^{-1} AGB in fish Ringer served as a control. After a brief rinse in fish Ringer, the olfactory rosettes were fixed overnight (see Solutions), embedded in Eponate plastic and sectioned at 500 nm using a diamond knife (Delaware Diamond Knife, Wilmington, DE, USA) and an ultramicrotome (Leica Ultracut UCT, Leica Microsystems, Bannockburn, IL, USA). After deplasticization with 25% sodium ethoxide in ethanol, the sections were incubated in anti-AGB antibody (1:100 in 0.1 mol l^{-1} phosphate buffer, 1% goat serum) overnight and visualized with a goat anti-rabbit, nanogold-conjugated, secondary antibody and silver intensification (Michel et al., 1999). After placing coverslips on top, eight-bit digital bright-field images were captured on an Axioplan 2 microscope equipped with an Axiocam digital camera and Axiovision software (Carl Zeiss Inc., Thornwood, NY, USA).

To quantify the proportion of labeled epithelium in each image, an area of interest (AOI) was drawn to include only sensory epithelium. Labeled pixels within this region were defined as those having intensity values less than 2 standard deviations (S.D.) below the mean pixel intensity value of a second AOI (drawn within the larger AOI) that contained no labeled neurons. The percentage of labeled epithelium was calculated by dividing the number of labeled pixels by the total number of pixels and multiplying by 100. For each olfactory rosette, at least 2–6 areas of interest were examined on each of 3 (or more) planes of section separated by a minimum of 10 μm, to ensure that the same OSNs were not repeatedly sampled.

Data analysis

Relative stimulatory effectiveness and concentration–response relationships were calculated by measuring the peak response (in mV) for replicate odorant presentations for each fish from a minimum of three fish per odorant. One-way analysis of variance (ANOVA; SPSS ver.11, SPSS Inc., Chicago, IL, USA) established overall significance of polyamine specificity and concentration-response functions. A Dunnett's *post-hoc t*-test was used to determine which polyamines (or polyamine concentrations) elicited responses that were significantly greater than the responses to the AFW controls. For cross-adaptation experiments, the average response to a test odorant in the adapting background was normalized to the average response to the same test odorant measured in the AFW background immediately before adaptation. A one-way ANOVA established the overall significance of an adapting background and a Dunnett's *post-hoc t*-test was used to determine responses significantly greater than the self-adapted response. A paired *t*-test was used to determine if forskolin or U-73122 significantly affected the responses to the test odorants. Data are presented as means ± standard error of the mean (S.E.M.) unless otherwise indicated.

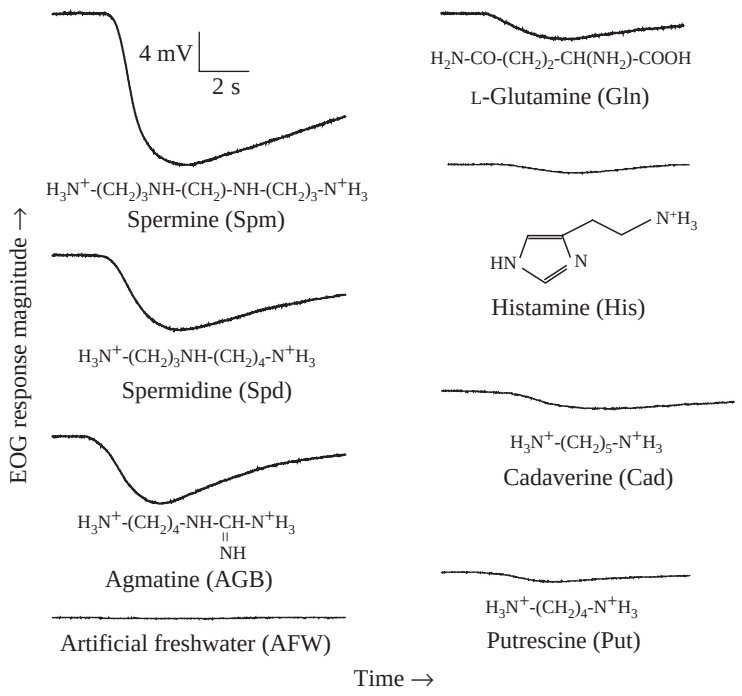

Fig. 1. Typical electro-olfactogram (EOG) responses elicited by the monoamine and polyamine odorants and the amino acid standard L-glutamine. The chemical structure, common name and abbreviation (in parentheses) of each odorant is shown below the response. The peak response was used to quantify response magnitude. All responses were obtained from the same fish. Odorants were tested at the concentration of 100 μmol l^{-1}.

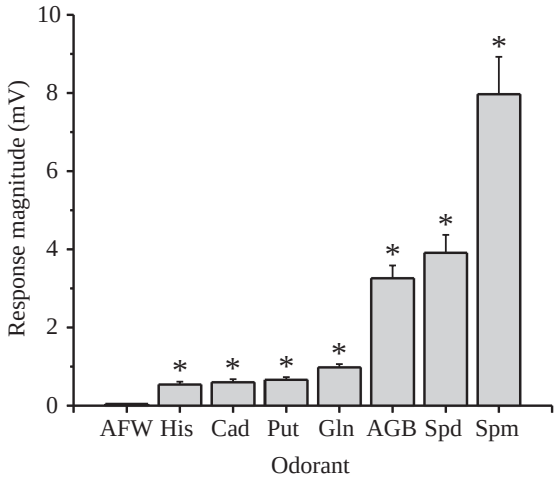

Fig. 2. The stimulatory effectiveness of monoamine and polyamine odorants compared to L-glutamine, the amino acid standard. All odorants were tested at 100 μmol l^{-1}. The average electro-olfactogram (EOG) response (mV) for each odorant was calculated from data obtained from three fish tested with only 100 μmol l^{-1} concentrations of each of the test odorant and from the responses obtained to each stimulus in the pre-adapted state during the cross-adaptation experiments (N=20). Asterisks designate responses significantly greater than the response to AFW (one-way ANOVA, Dunnett's *post hoc* t-test; P<0.05). Values are means ± S.E.M. Abbreviations as in Fig. 1.

Solutions

All of the odorant solutions used were prepared in deionized water with a resistivity of >18 MΩ cm^{-1}. The composition of the AFW was (in mmol l^{-1}): NaCl, 3; KCl, 0.2; $CaCl_2$, 0.2; Hepes, 1; pH 7.2. The composition of fish Ringer was (in mmol l^{-1}): 140 NaCl, 10 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 5 Hepes; pH 7.2. Fixative contained 2.5% glutaraldehyde, 1% paraformaldehyde, 3% sucrose and 0.01% $CaCl_2$ in 0.1 mol l^{-1} phosphate buffer, adjusted to pH 7.4. Odorants were prepared every other week as 10 mmol l^{-1} stocks in AFW (pH 7.00–7.15) and refrigerated at 4°C. The working solutions for each odorant were prepared daily in fresh AFW. For cross-adaptation experiments, test odorants were prepared in AFW and in the competitor odorant for use during background adaptation. All of the odorants were tested at a concentration of 100 μmol l^{-1} except for TCA, which was tested at 10 μmol l^{-1}. These concentrations allowed reproducible responses to be obtained over the lengthy experimental procedure time (often up to 3 h). All chemicals were obtained from Sigma Chemical Co.

Results

Relative stimulatory effectiveness of the polyamines

The odorants tested in this study included histamine, a monoamine, a series of polyamines and the amino acid L-glutamine as a standard for comparison with earlier studies (Michel and Lubomudrov, 1995; Michel and Derbidge, 1997; Lipschitz and Michel, 1999b). The polyamines tested included the 5- and 4-C aliphatic diamines cadaverine and putrescine, the triamine spermidine and the tetra-amine spermine; spermidine and spermine are produced by the sequential addition of amino-propyl groups to putrescine. Agmatine, a diamine precursor to putrescine and previously shown to be an effective olfactory stimulus for zebrafish (Lipschitz and Michel, 1999b) was also tested. Typical EOG responses evoked by these odorants and their structures are shown in Fig. 1.

Glutamine and all of the other odorants elicited responses that were significantly greater than the response to the AFW control (Fig. 2). The average response to the glutamine of 0.98±0.08 mV was similar to that recorded in our earlier studies (Michel and Lubomudrov, 1995). The least potent odorants, histamine, putrescine and cadaverine, elicited responses that were smaller than the glutamine standard. In contrast, responses to spermidine and AGB were nearly fourfold larger than the glutamine standard and spermine elicited the largest olfactory response we have observed in zebrafish (at 100 μmol l^{-1}), nearly eightfold larger than the response to the glutamine standard.

Dose–response relationship

The large responses to spermine, spermidine and AGB might be an indication of heightened olfactory sensitivity to these stimuli. To assess this possibility we determined

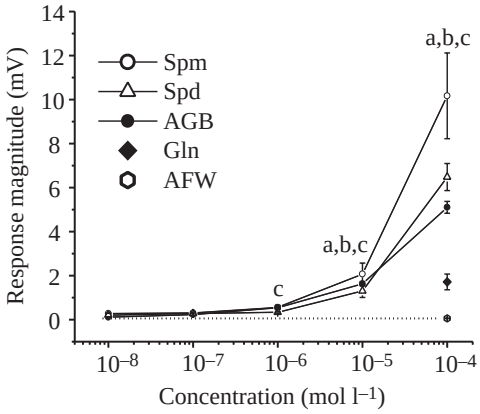

Fig. 3. The concentration–response relationships for spermine, spermidine and agmatine reveal detection thresholds for each of these stimuli in the 1 μmol l^{-1} range. For spermine and spermidine, data from a total of three fish were pooled; for agmatine, data from 20 fish was analyzed. Each fish was used for only one concentration–response series. Odorants were tested as an ascending concentration series. The average responses to the 100 μmol l^{-1} L-glutamine and AFW controls are shown as single points and as a dotted line (AFW only). Values are means ± S.E.M. Responses to spermine, spermidine and agmatine that are significantly greater than the response to the AFW control are designated with a, b and c, respectively (one-way ANOVA; Dunnett's *post hoc t*-test; $P<0.05$). Abbreviations as in Fig. 1.

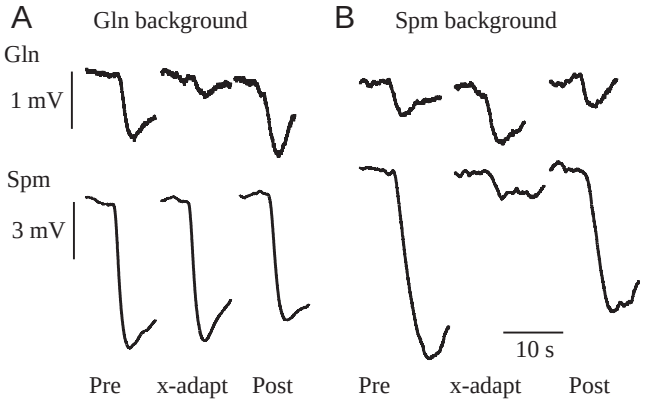

Fig. 4. Examples from two cross-adaptation experiments demonstrating that the amino acid L-glutamine (Gln) and the polyamine spermine (Spm) interact with different odorant receptors. Electro-olfactogram (EOG) responses to 100 μmol l^{-1} glutamine and 100 μmol l^{-1} spermine were selectively attenuated when the background bathing the olfactory epithelium was switched from AFW to (A) 100 μmol l^{-1} glutamine and (B) 100 μmol l^{-1} spermine, respectively. Responses to glutamine (upper traces) or spermine (lower traces) before (Pre), during (x-adapt) and after (Post) exposure to a background odorant are shown for each adapting odorant. Data plotted in A and B were obtained from two different fish.

their threshold concentrations and concentration–response characteristics (Fig. 3). The detection threshold for each of these polyamines was approximately 1 μmol l^{-1} (one way ANOVA; $P<0.05$), similar to the range of detection thresholds previously noted for amino acid and bile salt stimuli in this species (Michel and Lubomudrov, 1995). Olfactory responses to the highest concentrations tested (100 μmol l^{-1}) had not saturated.

Receptor site characterization

To determine if the polyamine odorants bound to unique odorant binding sites, we continuously exposed the OE to a background odorant to desensitize the OE to that odorant, then tested the responsiveness of the desensitized OE to other odors. When the response to a test odorant is abolished in an odorant-adapting background, it was assumed that the test and adapting odorant interact at some level of the transduction cascade, probably at the odorant binding site (the odorant receptor). The response to each of the seven odorants in its own adapting background was eliminated (Figs 4, 5), indicating that self-adaptation was complete. In contrast, the responses to the test odorants were sometimes partially, but never fully, cross-adapted.

Adaptation to putrescine (Fig. 5A) and cadaverine (Fig. 5B) reduced the response to histamine by about 50%, but otherwise had little effect on the responses to polyamines or to glutamine. Histamine adaptation (Fig. 5C) reduced to the greatest extent the responses to putrescine and cadaverine, but responses to all of the test odors remained significantly larger than the self-adapted response to histamine. Adaptation to spermidine (Fig. 5D) and spermine (Fig. 5E) generally reduced the responses to the other test odorants, but affected the responses to each other and to AGB to a greater extent than the responses to the less potent odorants. During spermine adaptation, the responses to the test odorants in different fish were variable. As a result of this variability, the cross-adapted responses to histamine, cadaverine, AGB and spermidine were not significantly greater than the self-adapted response to spermine. Similar variability in test odorant response magnitude was noted when AGB was used as the adapting background (Fig. 5F). In the AGB background, the responses to putrescine and glutamine were largely unaffected, while responses to cadaverine, histamine, spermidine and spermine were not significantly greater than the self-adapted response to AGB. Interestingly, the response to glutamine was not significantly affected by any adapting background and was actually significantly larger than its unadapted response in the spermidine background. During adaptation to glutamine (Fig. 5G), the responses to the test odorant were all significantly larger than the self-adapted response to glutamine.

Signaling cascade mediating polyamine responsiveness

Adenylate cyclase- and phospholipase C-mediated signaling cascades were implicated in the transduction of bile salt (Michel, 1999) and amino acid odorants (Ma and Michel, 1998), respectively. To implicate one of these cascades in polyamine detection, we tested the effects of the adenylate cyclase activator forskolin and the phospholipase C inhibitor U-73122 on polyamine-evoked responses.

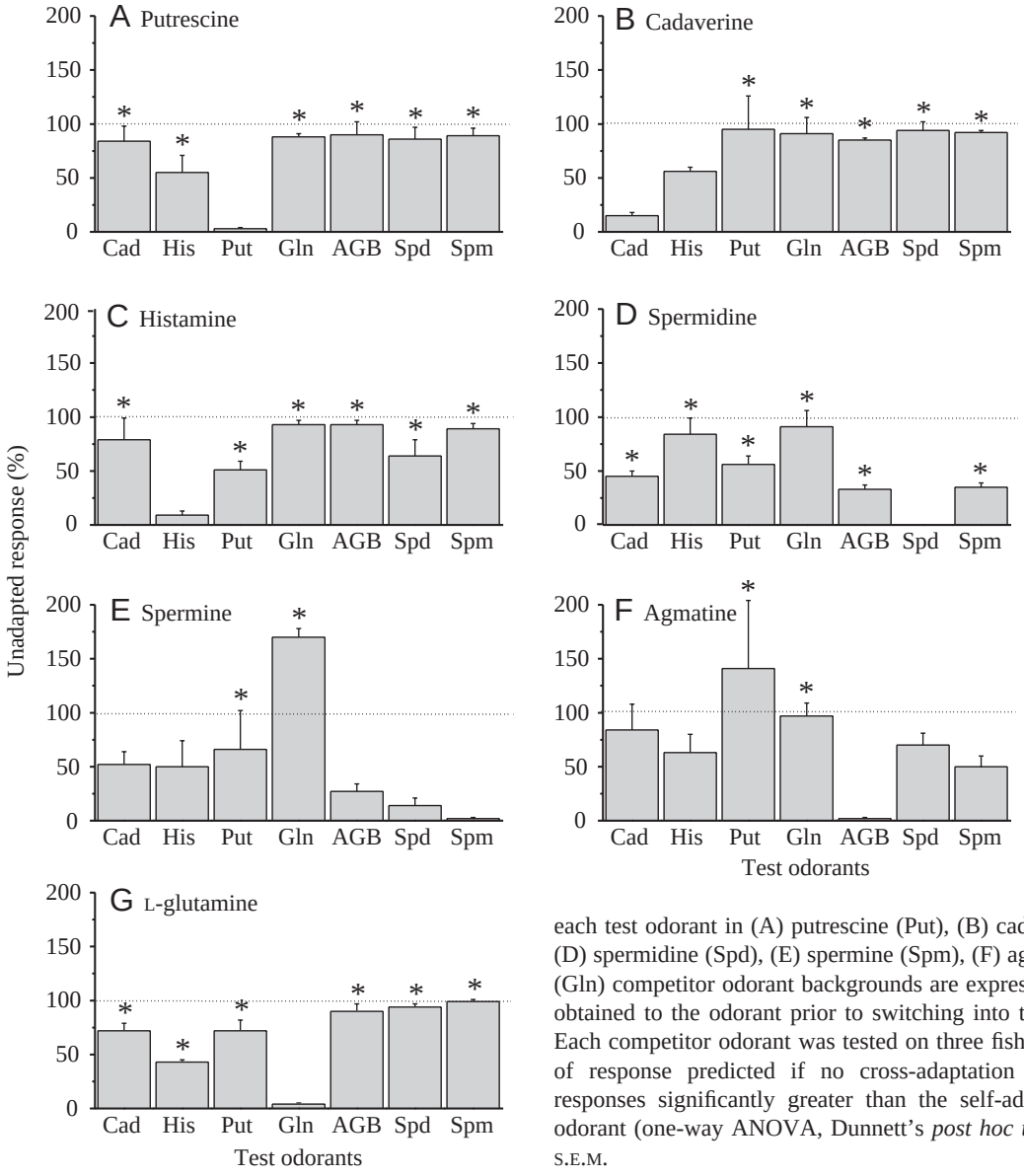

Fig. 5. Cross-adaptation experiments suggest the presence of relatively independent receptor sites for each of the polyamine odorants tested. The electro-olfactogram (EOG) responses to each test odorant in (A) putrescine (Put), (B) cadaverine (Cad), (C) histamine (His), (D) spermidine (Spd), (E) spermine (Spm), (F) agmatine (AGB) and (G) L-glutamine (Gln) competitor odorant backgrounds are expressed as a percentage of the response obtained to the odorant prior to switching into the competitor odorant background. Each competitor odorant was tested on three fish. The dotted line indicates the level of response predicted if no cross-adaptation is occurring. Asterisks designate responses significantly greater than the self-adapted response to the background odorant (one-way ANOVA, Dunnett's *post hoc* *t*-test; $P<0.05$). Values are means ± S.E.M.

A response of 6.0±3.3 mV (N=6) to 10 μmol l^{-1} forskolin confirmed our previous observation that forskolin elicited a large EOG response (Michel, 1999). An inactive forskolin analog, 1,9 dideoxyforskolin (10–100 μmol l^{-1}), used as a negative control for adenylate cyclase activation (Doi et al., 1990), was non-stimulatory (0.09±0.05 mV, N=5). To implicate adenylate cyclase in the polyamine signaling cascade, we compared polyamine-elicited responses obtained in AFW with responses measured when the OE was bathed with 10 μmol l^{-1} forskolin to desensitize OSNs that transduce olfactory input *via* the CNG pathway (Fig. 6). In the forskolin background, the response to forskolin was eliminated, indicating that self-adaptation was complete (not shown). In the forskolin background, the responses to TCA and cadaverine were significantly reduced to about 35–46% of their unadapted levels (paired *t*-test; $P<0.05$). The responses to spermine, spermidine, putrescine, AGB, cadaverine and glutamine were not significantly affected by forskolin adaptation, suggesting that adenylate cyclase activation is not required for transduction of these polyamines.

Application of the phospholipase C inhibitor U-73122 (1 μmol l^{-1}) did not elicit an olfactory response, but the drug did affect the responses elicited by some odorants (Fig. 7). U-73122 significantly reduced responses to amino acid odorants from 53% (glutamine) to 67% (arginine) and significantly reduced the response to spermine by 30% (paired *t*-test; $P<0.05$). Inhibition of PLC had no effect on taurocholic acid or AGB-evoked responses.

Polyamine-stimulated labeling of ORNs

In view of the robust EOG responses obtained to the more potent polyamine stimuli and the failure of the manipulation of either adenylate cyclase or phospholipase C signaling to affect polyamine responses, we were interested in

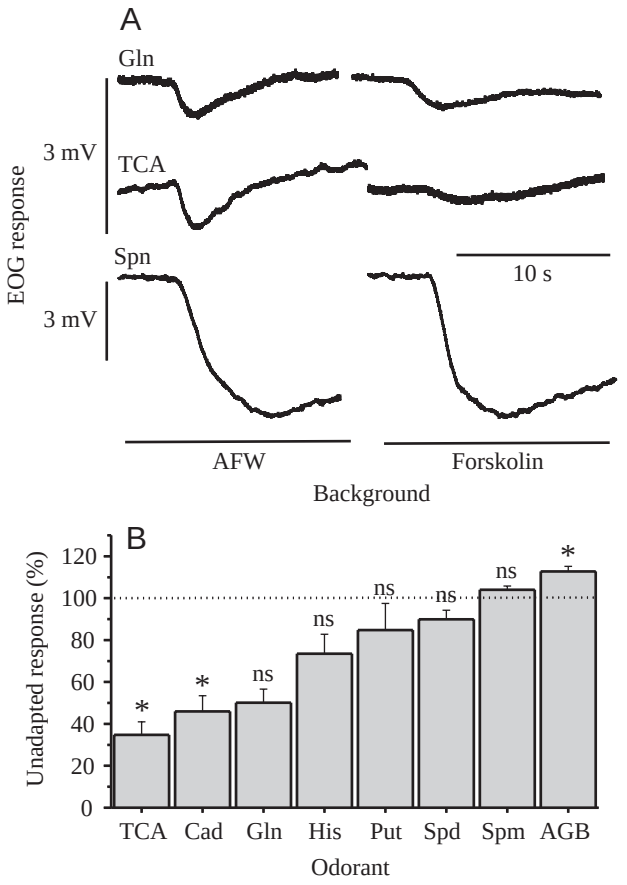

Fig. 6. Application of the adenylate cyclase activator forskolin has little effect on most polyamine-evoked electro-olfactogram (EOG) responses, suggesting that the adenylate cyclase activation is not critical during the initial transduction of a polyamine stimulus. (A) Example responses elicited by glutamine, spermidine and TCA in an AFW background (left traces) or in the presence of 10 μmol l^{-1} forskolin (right traces). (B) Summary changes in odor-evoked responses in the presence of forskolin normalized to the pre-drug treatment level. The magnitude of the taurocholic acid-evoked response was significantly reduced (*t*-test; $P<0.05$) but the L-glutamine-evoked response, while reduced, was not significantly smaller than the pre-forskolin exposure response level (*t*-test; $P>0.05$). Asterisks designate responses significantly reduced compared to the pre-forskolin exposure responses (paired *t*-test; $P<0.05$). Each odorant was tested on three preparations and values are means ± S.E.M. ns, non-significant. Abbreviations as in Fig. 1.

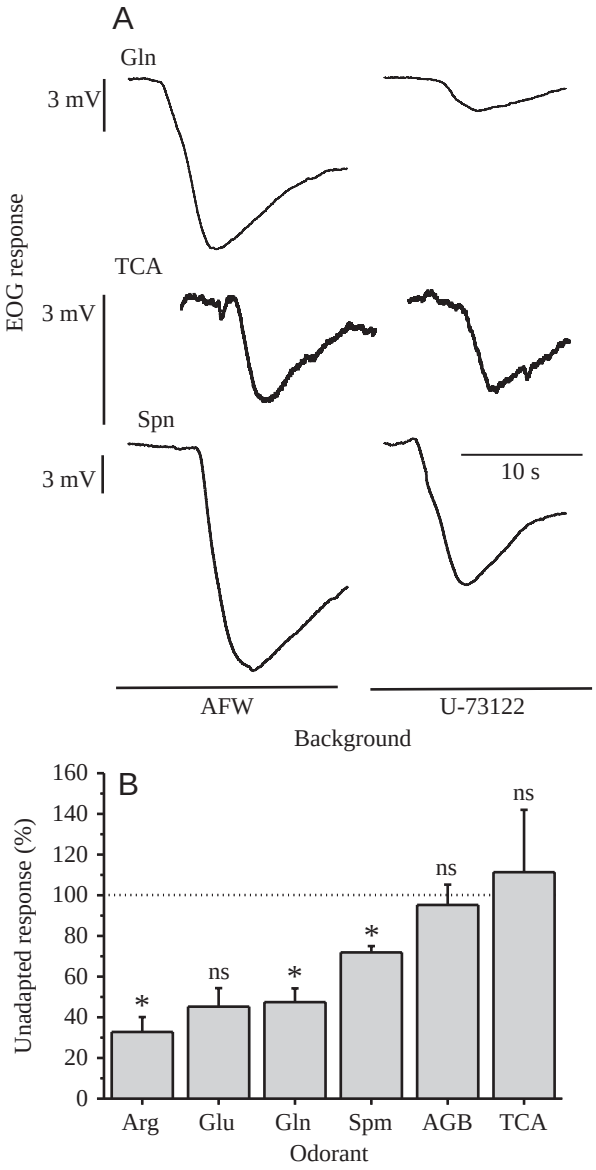

Fig. 7. Application of the phospholipase C inhibitor U-73122 (1 μmol l^{-1}) differentially affected odorant-evoked electro-olfactogram (EOG) responses. (A) Example responses elicited by glutamine, spermidine and TCA in an AFW background (left traces) or in the presence of U-73122 (right traces). (B) Summary changes in odor-evoked responses in the presence of U-73122 normalized to the pre-drug treatment level. U-73122 had little effect on taurocholic acid-, AGB- and spermidine-evoked responses, suggesting that the phospholipase C activation is not critical during the initial transduction of bile salt or polyamine input. Amino acid-evoked responses were significantly reduced to 20–50% of their pre U-73122 levels. Asterisks designate responses significantly reduced compared to the paired pre U-73122 responses (paired *t*-test, $P<0.05$). Values are means ± S.E.M. Abbreviations as in Fig. 1.

determining the cell type involved in polyamine signaling. We previously showed that amino acids, but not bile salts or forskolin, stimulated activity-dependent labeling (Michel et al., 1999; Michel, 1999; Lipschitz and Michel, 1999a) of primarily microvillar OSNs (Lipschitz and Michel, 2002). Using identical procedures, polyamine stimulation failed to significantly increase activity-dependent labeling of the OE, whereas L-glutamine-stimulated preparations resulted in a significantly higher proportion of labeled OE (Figs 8, 9). Stimulation with putrescine resulted in slightly more labeled epithelium than did stimulation with either spermine or spermidine. Qualitatively, the intensity of labeling during spermine stimulation was lighter than in either the AGB control condition or the L-glutamine-stimulated test condition.

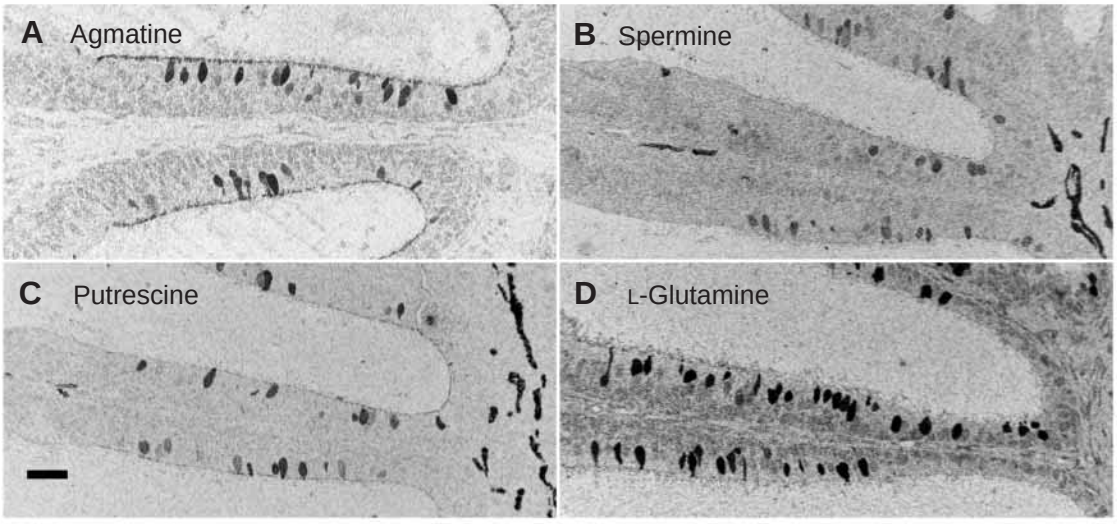

Fig. 8. L-glutamine stimulates a significant increase in activity-dependent labeling of the zebrafish olfactory epithelium compared to agmatine (AGB) control preparations, but the polyamine stimuli do not. The olfactory epithelium was stimulated with (A) 5 mmol l^{-1} AGB, (B) 5 mmol l^{-1} AGB + 100 μmol l^{-1} spermine, (C) 5 mmol l^{-1} AGB + 100 μmol l^{-1} putrescine or (D) 5 mmol l^{-1} AGB + 100 μmol l^{-1} L-glutamine. In all preparations the labeling is largely restricted to cells within the sensory epithelium. In all panels the midline raphe (R) is located on the right and non-sensory epithelium can be seen on the left end of the lamellae (L). Scale bar, 10 μm.

Discussion

Our observation that polyamine stimulation elicits large olfactory responses from zebrafish adds yet another chemical class of olfactory stimuli to an already structurally diverse repertoire of stimuli detected by the fish olfactory system. Of perhaps greater interest is the observation that polyamine signaling is mediated by distinct polyamine receptors that appear to couple to a unique transduction cascade.

Although zebrafish have been subjects for numerous physiological characterizations of olfactory sensitivity (Michel and Lubomudrov, 1995; Friedrich and Korsching, 1997, 1998; Michel and Derbidge, 1997; Lipschitz and Michel, 1999b; Friedrich and Laurent, 2001; Fuss and Korsching, 2001), only two of the odorants investigated in the present study had previously been examined. AGB was shown to be a potent odorant with the unique property of being ion channel-permeant, thus allowing activity-dependent labeling of some odor-stimulated olfactory sensory neurons (Michel et al., 1999; Lipschitz and Michel, 1999b). Putrescine, a weak stimulus in the present investigation, failed to elicit a measurable bulbar response in experiments using optical imaging methods (Fuss and Korsching, 2001). Explanations for this apparent discrepancy include the possibility that putrescine input might be communicated along extrabulbar pathways (Hofmann and Meyer, 1995), or that the optical imaging method employed in the bulb might not have been sufficient to detect a weak stimulus, particularly if the glomerular field activated was not routinely examined.

The potency of the polyamines is correlated with the structure of the molecule. Decarboxylation of the basic amino acids lysine and ornithine produces cadaverine and putrescine, respectively. These 4- and 5-C aliphatic diamines were relatively weak stimuli, as was lysine (Michel and Lubomudrov, 1995). Addition of an amino propyl group to putrescine produces the triamine spermidine, a significantly more potent odorant. Addition of another amino propyl group to spermidine produces the tetra-amine spermine. At 100 μmol l^{-1}, spermine elicited the largest EOG response so far recorded for any olfactory stimulus in the zebrafish. AGB,

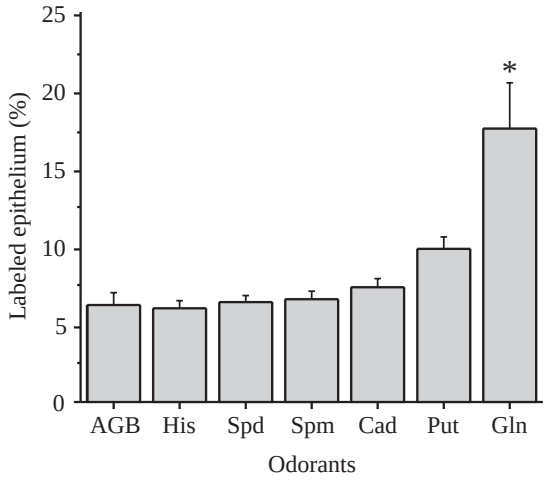

Fig. 9. Polyamine stimulation of the olfactory epithelium resulted in a modest, non-significant, increase in activity-dependent labeling of OSNs compared to the level of labeling noted during control stimulation with AGB alone or the robust stimulation elicited by glutamine. Each odorant was tested on a minimum of two olfactory rosettes from two different fish. A minimum of six areas of interest from three planes of section were sampled for each fish. Asterisks designate significantly greater labeled epithelium than in the AGB control preparations (one way ANOVA, *post hoc t*-test; $P<0.05$). Values are means ± S.E.M. Abbreviations as in Fig. 1.

a structurally distinct diamine produced by the decarboxylation of arginine, was approximately as potent as spermidine. Histamine, the only monoamine tested, was the least potent odorant tested in this study, but it did elicit a response that was significantly greater than the AFW control.

Fish OSNs express odorant receptors, which are either members of the classical odorant receptor (Ngai et al., 1993a,b; Barth et al., 1996) or V2R receptor (Naito et al., 1998; Speca et al., 1999) families. Odor-stimulated activation of these receptors has been shown to activate either cyclic nucleotide-gated or IP_3-mediated signaling pathways through g-protein coupled mechanisms (reviewed by Schild and Restrepo, 1998). Although the molecular structure of ORs activated by polyamines remains to be determined, physiological and pharmacological data from zebrafish suggest that polyamine odorants activate receptors linked to a transduction cascade differing from those used by either amino acid or bile salt odorants. The polyamines putrescine, spermidine and spermine activate a pathway that is seemingly impermeable to AGB uptake and largely unaffected by adenylate cyclase activation or phospholipase C inhibition (present study). The bile salt taurocholic acid activated a pathway that is also impermeable to AGB uptake and unaffected by PLC inhibition, but is significantly affected by adaptation to forskolin (Michel et al., 1999; Michel, 1999), which implicates adenylate cyclase signaling; however inositol phosphate signaling has been implicated in bile salt detection by the Atlantic salmon (Lo et al., 1994). Amino acids activate a pathway in microvillar cells (Lipschitz and Michel, 2002) that is permeable to AGB uptake. Amino acid-evoked responses are partially reduced by either adenylate cyclase activation or phospholipase C inhibition (Michel et al., 1999; Michel, 1999). Adenylate cyclase activation reduced the responses to amino acid odorants to 50–70% of their unadapted response levels (Michel, 1999). Phospholipase C inhibition reduced responses to the same amino acids to 20–50% of their unadapted levels. Thus, both signaling pathways may be involved in amino acid transduction.

In the present study, adenylate cyclase activation reduced the response to cadaverine to less than 50% of its unadapted response, while the responses to histamine, putrescine, agmatine, spermidine and spermine were not significantly affected. These results suggest that cadaverine, a 4-C diamine, may be linked to a different transduction pathway from the other polyamines, perhaps interacting with a receptor for the basic amino acid lysine. Phospholipase C inhibition had little effect on evoked responses to spermine and AGB. The failure of both forskolin and U-73122 to significantly affect evoked responses to AGB and spermine indicates that these odorants are unlikely to use either adenylate cyclase-mediated or phospholipase C-mediated transduction cascades. The signaling cascade activated by polyamines warrants further investigation.

Polyamines reportedly play many roles in cell growth and biosynthesis through interactions with nucleic acids, proteins and membranes (Morgan, 1999). Particularly relevant to the present study is the involvement of polyamines in ion channel regulation. Ion channels known to be affected by polyamines include ionotropic glutamate receptor/channels, potassium channels and the olfactory CNG channel (Williams, 1997). Physiological concentrations of intracellular spermine, spermidine and putrescine rectify the olfactory CNG channels (Lynch, 1999). More importantly, extracellular spermine (0.1–1 mmol l^{-1}) also blocks olfactory CNG channel-mediated currents (Nevin et al., 2000). Our observations of a reduction in response magnitude noted during spermine and spermidine cross-adaptation and a reduction in the intensity of activity-dependent labeling in spermine stimulated preparations may be an indication of an extracellular polyamine-mediated block of ion channels involved in odor transduction.

Spermine and spermidine were originally isolated from seminal fluid and are likely to be present in elevated concentrations in the chromatin-enriched spawning fluids of fish; however, their extracellular concentrations remain to be determined. As their names imply, putrescine and cadaverine are olfactory stimuli originally associated with decaying or rotting tissue (Goldberg et al., 1994; Greenstein et al., 1997) and with the decomposition of food products by bacteria (Morgan, 1999), but were subsequently found to be present in all tissues. Increasing levels of putrescine or cadaverine stimulate rats to bury conspecifics 24–48 h after death and anesthetized rats sprinkled with either putrescine or cadaverine are also buried (Pinel et al., 1981). Although the behavioral responses elicited by polyamines in zebrafish remain to be determined, the behavioral observations of polyamine detection by mammals indicate that this class of stimuli are of general significance to many vertebrates.

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