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Olfactory Signal Modulation by Molluscan Cardioexcitatory Tetrapeptide (FMRFamide) in Axolotls (*Ambystoma mexicanum*)

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Abstract

The terminal nerve, which innervates the nasal epithelia of most jawed vertebrates, is believed to release neuropeptides that modulate activity of sensory receptor neurons. The terminal nerve usually contains gonadotropin-releasing hormone as well as at least one other peptide that has not been characterized, but which bears some structural similarity to molluscan cardioexcitatory tetrapeptide (FMRFamide) and neuropeptide tyrosine (NPY). We investigated the effects of FMRFamide on both voltage-gated currents and odorant responses in the olfactory epithelium of axolotls (*Ambystoma mexicanum*), using whole-cell patch clamp and electro-olfactogram (EOG) recording techniques. In the presence of FMRFamide, the magnitude of a voltage-gated inward current was dramatically increased, reaching an average of 136% of the initial (pre-exposure) magnitude in neurons that showed a response to the peptide. This increase is detectable within ~1–2 min of exposure to FMRFamide and is sustained for at least 10 min. In EOG experiments, odorant responses are not affected during FMRFamide application, but are sometimes increased or decreased during the subsequent wash period. On average, the largest single EOG response in each trial was detected ~25 min after initial FMRFamide application, and ranged from 110 to 147% of baseline. These results suggest that a compound similar to FMRFamide, if released from the terminal nerve, may function in peripheral olfactory signal modulation.

Key words: salamander, terminal nerve, whole-cell patch clamp, electro-olfactogram (EOG), neuropeptide, neuromodulation

Introduction

Studies of the peripheral processing of odorant information generally focus on mechanisms involving single olfactory receptor neurons. The role of multicellular interactions, such as those involved in neuromodulation, receives relatively little attention. In the retina and cochlea of vertebrates, transduction of sensory stimuli into neural signals is generally modulated both locally and by centrifugal inputs (Akopian, 2000; Ashmore *et al.*, 2000; Weiler *et al.*, 2000). A complete understanding of peripheral processing of odorant stimuli must include a thorough exploration of the role of neuromodulators.

The terminal nerve is an anterior cranial nerve present in most jawed vertebrates. The fibers of these bipolar neurons extend rostrally to the nasal cavity and caudally to the hypothalamic/preoptic area (Wirsig-Wiechmann *et al.*, 2002). The cells and fibers of the terminal nerve contain several potentially modulatory compounds, including gonadotropin-releasing hormone (GnRH) (Oka, 1992), chemicals that display immunoreactivity to neuropeptide tyrosine (NPY) or FMRFamide (Phe-Met-Arg-Phe-NH_2) (Wirsig-Wiechmann, 1990; Eisthen and Northcutt, 1996; Chiba, 2000) and to tyrosine hydroxylase (White and Meredith, 1995), and acetylcholinesterase (Wirsig and Leonard, 1986; Wirsig-Wiechmann, 1990). These compounds appear to be released into the nasal epithelia or nasal cavity (Wirsig-Wiechmann and Jennes, 1993).

The terminal nerve seems to play a role in reproductive behavior, although the nature of this role has not been clearly established (Wirsig and Leonard, 1987; Yamamoto *et al.*, 1997). In dwarf gouramis (*Colisa lalia*), lesions of terminal nerve cells inhibit initial nest-building behaviors (Yamamoto *et al.*, 1997). In male hamsters, lesions of the terminal nerve decrease mating frequency, and reduce behavioral responses to female vaginal odors (Wirsig and Leonard, 1987). The terminal nerve may play a modulatory role in peripheral sensory systems (Walker and Stell, 1986; Oka, 1992), and this modulation may underlie its behavioral effects.

The only terminal nerve peptide that has been positively identified is GnRH, and its effects on peripheral olfactory systems have recently been examined. Recordings from slices of olfactory epithelium of salamanders suggest that GnRH

increases the excitability of olfactory receptor neurons (Eisthen *et al.*, 2000). Specifically, GnRH applied to olfactory neurons of mudpuppies (*Necturus maculosus*) increases the magnitude of a tetrodotoxin (TTX)-sensitive sodium current, and also alters outward currents. In another study, Park and Eisthen (2003) found that electro-olfactogram (EOG) responses to L-amino acids in axolotls (*Ambystoma mexicanum*) were reduced during the application of GnRH, but then recovered and were sometimes enhanced above the baseline magnitude during the subsequent wash period. These studies demonstrate that GnRH released from the terminal nerve into the nasal cavity can modulate peripheral processing of olfactory signals.

In addition to GnRH, the terminal nerve of most jawed vertebrates contains a second neuropeptide that has not been identified, but which can be labeled using antisera directed against FMRFamide or NPY (Chiba, 1997, 2000). These peptides are evolutionarily unrelated and differ in length, but share a C-terminus that contains the motif -RFamide or -RYamide (Greenberg and Price, 1992; Larhammar, 1996). FMRFamide was first isolated and characterized from the nervous system of molluscs (Price and Greenberg, 1977); since then, FMRFamide and other FMRFamide-like peptides (FLPs) have been isolated in many invertebrates and vertebrates, including amphibians (Koda *et al.*, 2002). FLPs have been shown to serve neuroendocrine functions in vertebrates and neuromodulatory functions in invertebrates, but neuromodulatory effects of FLPs have not been explored in detail in vertebrates (Henry *et al.*, 1999; Lange and Cheung, 1999; Loi and Tublitz, 2000; Tsutsui *et al.*, 2000; Satake *et al.*, 2001; Koda *et al.*, 2002). In cultured spinal neurons from mice, FMRFamide alters ion conductances in different ways in different neurons (McCarthy and Cottrell, 1984). FMRFamide depolarizes horizontal cells from the retinae of white perch, *Morone americana* (Umino and Dowling, 1991), and induces excitatory, inhibitory or biphasic inhibitory–excitatory responses in retinal ganglion cells from goldfish, *Carassius auratus* (Walker and Stell, 1986). FMRFamide-like immunoreactivity has been described in the olfactory epithelium of more than a dozen vertebrate species, including frogs, salmon, shrews, and chickens (Muske and Moore, 1988; Wright and Demski, 1996; Rastogi *et al.*, 2001; Malz and Kuhn, 2002), but the role of FLPs in peripheral olfactory signal modulation has not yet been investigated.

In the present study, we examined the effects of FMRFamide on both voltage-gated currents and odorant responses in the olfactory epithelium of axolotls, using whole-cell patch clamp and EOG recording techniques. We find that FMRFamide increases the magnitude of a voltage-gated inward current in some olfactory neurons, and modulates odorant responses in the olfactory epithelium of some individuals.

Methods

Subjects

Adult axolotls obtained from the Indiana University Axolotl Colony were kept in aquaria (80 × 40 × 50 cm) containing 100% Holtfreter's solution, a standard medium for maintaining amphibians (Mattison, 1982). All aquaria were equipped with a recirculating filter system in which water from groups of tanks passed through mechanical and biological filters and an ultraviolet sterilizer before being returned to tanks.

To minimize stress, no more than six same-sex individuals were housed in each tank. Axolotls were fed commercial salmon chow (Rangen Inc., Buhl, ID) twice weekly. The temperature of the tanks ranged between 18 and 22°C, and the photoperiod was adjusted monthly to match that of the animals' native habitat in Mexico City.

The original research reported in this paper was conducted according to animal care and use guidelines established by the Society for Neuroscience and the US Public Health Service.

Whole-cell recordings

Olfactory epithelial slices were prepared using a protocol described previously (Eisthen *et al.*, 2000). Axolotls were immersed in ice water for 20 min and then decapitated. The nasal sac was dissected out, opened flat, and attached to a support with cyanoacrylate glue. Slices 200–250 μm thick were cut with a vibrating blade and stored in amphibian physiological saline containing (in mM): 120 NaCl, 10 HEPES, 8 $CaCl_2$, 5 glucose, 5 pyruvate, 2.5 KCl, and 1 $MgCl_2$. The pH was adjusted to 7.6 using 1 N HCl or 1 M NaOH. Slices of olfactory epithelium prepared in this manner remained viable for recording at room temperature for at least 4 h, and when stored at 4°C could be used for 48 h.

Epithelial slices were mounted in a recording chamber and viewed with a 40× water immersion objective on a Zeiss Axioskop FS microscope. Patch electrodes of borosilicate glass were pulled on a Flaming-Brown programmable micropipette puller (Sutter Instruments, Novato, CA). For whole-cell voltage-clamp recordings, pipette resistance was generally 3–5 MΩ. All recordings were conducted at room temperature using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) with a low-pass Bessel filter set at 5–10 kHz. Raw data were collected and leak current subtracted before analysis using AxoGraph software (Axon Instruments, Foster City, CA).

For some recordings, we did not use ionic substitutions in the intracellular solution; in these experiments, the recording pipette was filled with a solution containing (in mM): 105 K gluconate, 25 KCl, 10 HEPES, 5 ATP, 3 $MgSO_4$, 1 EGTA, 0.5 GTP and 0.5 $CaCl_2$. Our preliminary experiments suggested that the effects of FMRFamide

on outward currents were variable but that effects on inward currents were more consistent and more reliably obtained, so we chose to study the latter. In most experiments, then, cesium was substituted for potassium to block large outward potassium currents that might mask FMRFamide-sensitive inward currents; calcium-dependent outward currents were blocked through the substitution of $BaCl_2$ for $CaCl_2$ in the amphibian physiological saline described above. In such experiments, the intracellular solution contained (in mM): 135 CsCl, 10 HEPES, 5 ATP, 3 $MgSO_4$, 1 K_4BAPTA, 0.5 GTP and 0.085 $CaCl_2$.

FMRFamide (Phe-Met-Arg-Phe-NH_2; Bachem, Torrance, CA) was dissolved in amphibian physiological solution at a concentration of 10 μM and bath-applied to slice preparations during recording; the dose was selected based on a study using an endogenous amphibian FLP (Koda *et al*., 2002). We added a dye, fast green, to this solution to allow us to verify the timing of FMRFamide application and wash. In previous studies, we have found that fast green does not function as an odorant or alter activity of voltage-gated ion channels in salamander olfactory epithelia (Eisthen *et al*., 2000; Park and Eisthen, 2003).

Solutions were introduced into the recording chamber using a gravity-feed system. Our observations indicate that the amphibian physiological solution covering a slice was completely replaced ~60 s after solution sources were changed. In some experiments, a bath solution containing fast green but not FMRFamide was used as a control for the effects of changing solutions. In the remainder of the paper, we refer to this solution as the 'control solution'; note that the control solution is different from the 'wash', which consisted of plain physiological saline without fast green.

For each recording, we followed a standard protocol to ensure that comparable data were collected from all cells. Once a seal of 1–5 GΩ was attained, the membrane under the electrode was ruptured, and a holding potential between –50 and –90 mV was applied. We recorded responses to a series of 15 ms voltage pulses ranging from –100 mV to 100 mV in 10 mV steps. We then initiated the flow of amphibian physiological solution over the slice and recorded responses to a similar set of pulses 1 min later. Recordings were made every 2 min in a flowing bath for 3–6 min until the seal and series resistance stabilized; usually two or three sets of recordings were made in the flowing bath before FMRFamide was applied. Recordings were made 1 min after the flow of FMRFamide was initiated, then again at 2 min intervals for 10–12 min. While washing off the FMRFamide with amphibian physiological saline, we recorded responses at 2 min intervals for 6–16 min. Once a slice had been exposed to FMRFamide, the slice was discarded.

In some experiments, the effect of FMRFamide appeared to depend on the holding potential applied. To test this possibility directly, we performed some recordings using a protocol similar to that described above, except that the holding potential was adjusted from –50 mV to –80 mV in a cycle of 10 mV steps once per minute. At least two sets of recordings at the four different holding potentials were made before FMRFamide was applied. Beginning 1 min after FMRFamide application, another four or five sets of recordings at each holding potential were made. We recorded responses to at least three sets of pulses at each holding potential during the washing off of the FMRFamide. In this experiment, recording pipettes were filled with cesium-substituted intracellular solution and the amphibian physiological saline contained $BaCl_2$ instead of $CaCl_2$. This experiment was completed in five cells.

We analyzed data for all cells for which we obtained responses to at least four sets of pulses during the 10 min FMRFamide application, provided the seal was >1 GΩ and the series resistance did not increase more than ~10% throughout the recording. Using these criteria, we report data obtained from a total of 39 cells from 20 axolotls.

EOG recordings

Before surgery, axolotls were anesthetized with pH-corrected 0.1% MS 222 (tricaine methanesulfonate, pH 7.5, Sigma Chemical, St Louis, MO) in Holtfreter's solution, and immobilized with an intramuscular injection of gallamine triethiodide dissolved in amphibian Ringer's solution (Flaxedil, Sigma Chemical; 0.1–0.3 mg/100 g body weight, pH 7.6). Supplemental doses of MS 222 were delivered to the gills and additional Flaxedil was injected intramuscularly as necessary throughout the experiment.

The main olfactory epithelium was exposed by removing the tissue dorsal to the nasal capsule. To record electrical field potentials, a glass capillary electrode (100–200 μm tip diameter) was filled with 1% agar in Ringer's solution bridged to a chloride-coated silver wire. An Ag–AgCl reference electrode was placed under the skin of the head (Park *et al*., 2001). Electrodes were coupled to a differential amplifier (DP-301, Warner Instruments, Hamden, CT). Signals were digitized via an ITC-18 interface (Instrutech Co., Great Neck, NY), and then displayed, recorded, and analyzed on a Macintosh computer using AxoGraph software (Axon Instruments, Foster City, CA). The magnitude of the EOG response was measured as the maximal height of phasic displacement from the baseline level. Absolute response values in millivolts were obtained by comparison with the deflection elicited by a known calibration voltage.

During each trial, a continuous flow (3.5–4 ml/min) of Holtfreter's solution bathed the olfactory mucosa. Holtfreter's solution contains (in mM): 60 NaCl, 2.4 $NaHCO_3$, 0.67 KCl, 0.81 $MgSO_4$ and 0.68 $CaCl_2$ in distilled water (pH 7.5–7.6, adjusted by the addition of 1 N HCl or 1 M Tris base). For each EOG recording, ~50 μl of 1 mM L-methionine dissolved in the Holtfreter's solution at room temperature (23–25°C) was injected from a 1 ml syringe connected to a pressure injector (Picospritzer II, General Valve, Fairchild, NJ) into the flow of the Holtfreter's solution. We selected L-methionine (Sigma Chemical) as an

odorant because it is a potent stimulus for EOG responses in axolotls (Park and Eisthen, 2003). To ensure that identical amounts of odorant were delivered throughout EOG recordings, the settings on the Picospritzer were not adjusted during a trial. The time of arrival of the injected stimulus at the olfactory mucosa was measured by adding fast green to the odorant solution on some trials; using this method, we found that the odorant solution arrived at the epithelium ~10 s after injection into the carrier stream and remained on the epithelium for ~2–3 s.

To determine whether FMRFamide affects EOG responses, we recorded EOG responses to 50 µl of 1 mM L-methionine before, during and after FMRFamide application. To establish a baseline response level, we recorded two to four EOG responses to the stimulus odorant before FMRFamide application. The interval between consecutive odorant presentations was 4 min, and did not produce any sign of odorant adaptation during baseline recordings of EOG responses. Once the EOG responses were relatively consistent (less than ~10% difference in EOG magnitude), 5 µM FMRFamide prepared in Holtfreter's solution was delivered to the olfactory epithelium continuously for 12 min. The dose of FMRFamide was based on a previous study using endogenous FLPs in bullfrogs (Koda *et al.*, 2002). Three EOG responses were recorded at 4 min intervals during FMRFamide application. During the period after FMRFamide was applied ('wash'), we recorded another nine EOG responses while bathing the olfactory epithelium in running Holtfreter's solution.

To investigate the effects of consecutive exposures to FMRFamide, we repeated this procedure for another two trials for each subject, with a 60–80 min interval between consecutive trials. To optimize the signal, the recording electrode was sometimes relocated at the beginning of a trial. Fourteen animals were used in these experiments.

In control experiments with five animals, we repeated the procedures described above, except that we substituted Holtfreter's solution for FMRFamide solution. As in experiments with FMRFamide, three trials, separated by a 60 to 80 min interval, were conducted with each animal.

Results

Whole-cell recordings

FMRFamide did not affect the magnitude of the inward current in all olfactory receptor neurons examined; thus, we categorized cells as 'responders' and 'non-responders' for further analysis. A cell was considered a 'responder' if the magnitude of the inward current was elevated above baseline for at least four consecutive data points while exposed to FMRFamide. A cell for which the inward current was steady was categorized as a 'non-responder'. Cells that displayed a variable inward current were categorized as uninterpretable and excluded from further analyses; we did not observe any cases in which the inward current was

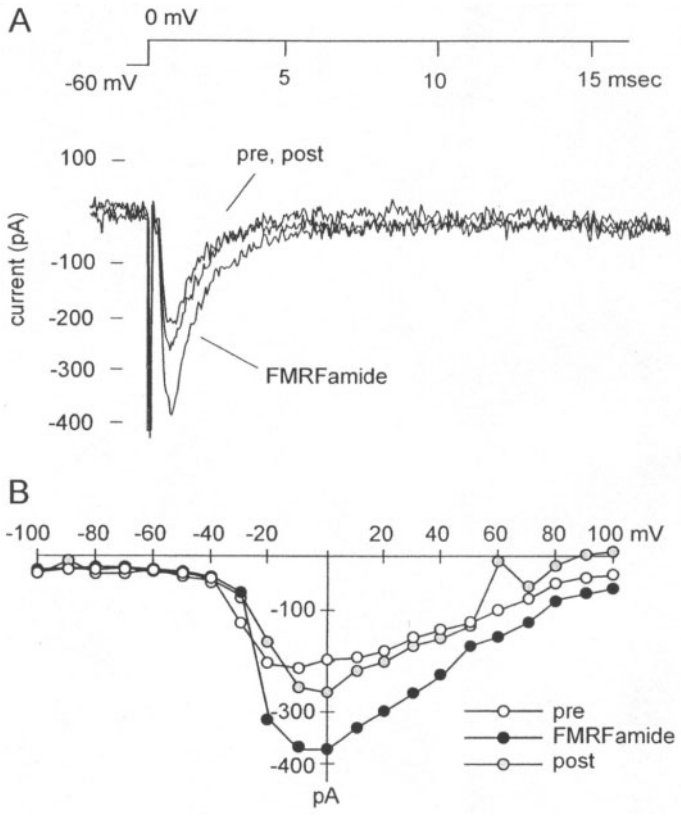

Figure 1 Whole-cell recordings from an olfactory receptor cell, illustrating an effect of bath application of 10 µM FMRFamide. Outward currents have been reduced by substituting Cs^+ for K^+ in the intracellular solution and calcium-dependent currents have been reduced by substituting $BaCl_2$ for $CaCl_2$ in the extracellular solution. V_{hold} = –60 mV. **(A)** Inward currents elicited by a voltage pulse of 0 mV, showing the magnitude of the inward current before application of FMRFamide ('pre'), after 9 min exposure to FMRFamide ('FMRFamide'), and after washing FMRFamide off the slice for 7 min ('post'). **(B)** Relationship between peak current and voltage for the same cell, at the same time points. Currents were elicited by application of a series of voltage pulses ranging from –100 to 100 mV, in 10 mV increments.

decreased relative to baseline for 4 or more consecutive readings. Using these criteria, 10 of 39 olfactory receptor neurons (25.6%) were classified as 'responders'. An example of data from a 'responder' is illustrated in Figure 1. Twenty-three additional cells (59.0%) were classified as 'non-responders', and the responses of the remaining six cells were uninterpretable. Data from the 10 cells that responded to FMRFamide are described below.

As illustrated in Figure 2, the inward current was increased relative to baseline (the average magnitude of the inward current on the last two recordings before FMRFamide application), beginning ~1–2 min after FMRFamide application began. This increase was sustained throughout the FMRFamide exposure period and recovered to the baseline level within 5–10 min after washing FMRFamide off.

To calculate the latency to and magnitude of the largest inward current, we used four cells for which we were able to

complete our recording protocol, including the full wash period. In these cells, the largest inward currents were detected ~10 min (9.71 ± 0.34 min) after initial FMRFamide application, and ranged from 119.5 to 146.2% (136.1 ± 6.02%) of the baseline magnitude.

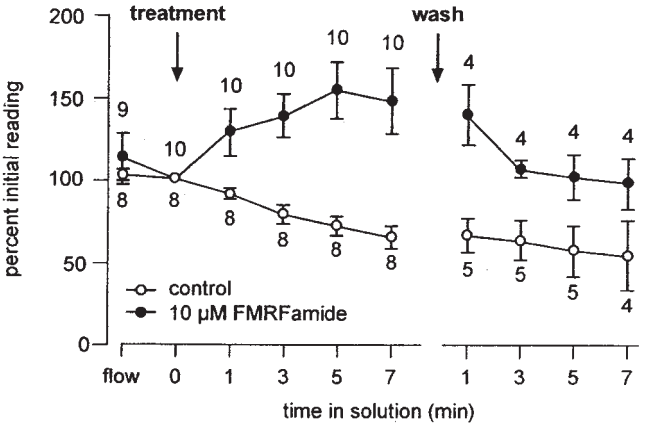

Figure 2 Change, over time, in the magnitude of the inward current. To facilitate comparison, data were normalized such that the average magnitude of two recordings of the inward current immediately before application of the test solution was designated 100%, and the magnitude of the current at other times is expressed as a percentage of this magnitude. The arrow labeled 'treatment' indicates the time at which 10 μM FMRFamide or the control solution was washed onto the slice; the solution was then washed off ('wash'). Means and standard errors are shown for cells that responded to FMRFamide (filled circles) or were exposed to a control solution containing an indicator dye but no FMRFamide (open circles). After 1 min in FMRFamide, the magnitude of the inward current was significantly larger in cells responding to FMRFamide than in cells exposed to the control solution; within 5 min in wash, the magnitude of the inward current had recovered to baseline. The numbers above and below data points indicate the number of cells included in the analysis for each group at each time point. Note that the sample size decreases as cells are lost, e.g. as the wash solution is applied; nevertheless, the small standard errors indicate that the sample sizes are adequate for analysis.

We investigated the possibility that the holding potential interacts with FMRFamide effects. In three of the five cells examined, the inward current was increased after FMRFamide application at all holding potentials (–50, –60, –70 and –80 mV). In two cells the inward current was only increased at a specific holding potential: in one case, –50 mV, and in the other, –80 mV. In these two cells, the inward current was unchanged at other holding potentials during FMRFamide application.

EOG recordings

As illustrated in Figure 3A, EOG responses in control experiments varied within ~5% of the baseline magnitude throughout the experimental protocol. Overall, EOG responses did not differ significantly among the three trials in control experiments (Kruskal–Wallis test, $P = 0.99$), nor did they vary significantly within any of the three trials (Kruskal–Wallis test, $P = 0.39$ for the first trial, 0.73 for the second trial, and 0.57 for the third trial). Thus, we pooled data within and across the three control trials for presentation in Figure 4.

As in our control experiment, 4 of 14 experimental animals displayed less than 5% change in EOG magnitude during FMRFamide application in any of the three trials. These animals were categorized as 'non-responders' and excluded from further analysis. We also excluded from analysis 3 of 30 trials from the remaining 10 animals in which technical problems occurred, such as changes in the subject's level of anesthesia during the trials. The percent of analyzable data obtained did not differ among trials (χ^2, $P = 0.94$). Data from these 27 trials in the 10 animals that responded to FMRFamide are described below.

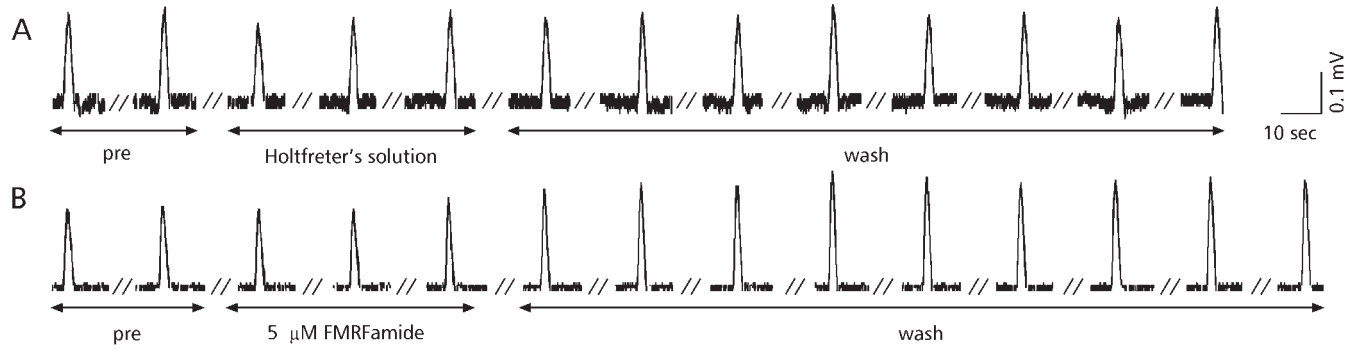

Figure 3 Representative examples of control and experimental EOG responses, taken from the second trial for two different subjects. EOG responses were evoked by delivery of ~50 μl of 1 mM L-methionine. The interval between successive odorant presentations was 4 min. We recorded two to four baseline EOG responses before FMRFamide application ('pre'), three EOG responses during the application of FMRFamide, and nine EOG responses during the period after FMRFamide was applied ('wash'). **(A)** In control experiments, a similar protocol was used, except that Holtfreter's solution was washed into the nasal cavity instead of FMRFamide. Control EOG responses recorded during the second trial of a control experiment varied in magnitude by ~5% relative to baseline. The maximum magnitude of EOG responses in this trial was 101.3% of the baseline magnitude, and was recorded during the wash period. **(B)** FMRFamide generally did not affect odorant responses during application, but EOG responses were often enhanced during the wash period. For this subject, the average magnitude of the EOG responses during FMRFamide application in the second trial was 103.2%. The maximum magnitude was 139.7% of the baseline magnitude, and occurred during the wash period.

Figure 3B illustrates EOG responses during an experiment in which 5 μM FMRFamide was applied to the olfactory epithelium. In such experiments, EOG responses were generally unaffected during the period of FMRFamide application, ranging from 93.6% to 103.8% of the baseline magnitude. Nevertheless, the magnitude of the EOG response was often increased or decreased relative to baseline during the subsequent wash period.

For statistical analysis and data display, we designated the mean magnitude of the two to four EOG responses recorded before the FMRFamide application as 100%, and normalized all other data collected in each trial relative to this mean. EOG responses from each trial were grouped into three categories, as follows: *baseline* EOG responses, the average magnitude of two to four EOG responses recorded before FMRFamide exposure; *FMRFamide* EOG responses, the average magnitude of three consecutive EOG responses recorded during FMRFamide exposure; and *wash* EOG responses, the average magnitude of nine consecutive EOG responses recorded during the wash period. The same analysis was performed for each trial, using the baseline magnitude from that trial as the standard for comparison. The values obtained for each treatment within a trial were averaged across animals, but we did not average the data across trials.

Statistical tests indicated that some of the data were not distributed normally; thus, we used nonparametric statistics for most analyses. To determine whether odorant responses differ before, during and after FMRFamide application within a trial, we used the Kruskal–Wallis test, a nonparametric analog of the one-way ANOVA test. A significant result in the Kruskal–Wallis test indicates that the EOG responses are significantly different between treatments, but does not indicate which particular treatments differ. Therefore, when the Kruskal–Wallis test indicated significant differences among treatments, we performed additional nonparametric *post hoc* tests with an α level of 0.05, as described in Siegel and Castellan (Siegel and Castellan, 1988) for two-point comparisons. In these cases, we performed pairwise comparisons among all data points.

Using this method to compare results *within* trials, we found that EOG responses before, during, and after FMRFamide application varied significantly in the second ($H_{30/2}$ = 19.99, $P < 0.001$) and third trials ($H_{24/2}$ = 6.86, $P = 0.032$), but not in the first trial ($P = 0.86$). *Post hoc* tests indicate that the magnitude of EOG responses obtained during FMRFamide application did not differ from baseline (both $P > 0.05$). Curiously, EOG responses during the wash period were enhanced relative to baseline in the second trial, but were depressed in the third trial (both $P < 0.05$). The results of these analyses are illustrated in Figure 4.

We determined whether the effects of FMRFamide differed *among* the three trials using the Kruskal–Wallis test followed by nonparametric *post hoc* tests for two-point comparisons (Siegel and Castellan, 1988), as described above. The magnitude of the baseline EOG response, calculated from raw data, did not differ among trials ($H_{28/2}$ = 0.124, $P = 0.94$). We found that the effect of FMRFamide on EOG responses differed significantly across the three trials ($H_{81/2}$ = 12.70, $P < 0.002$). Specifically, the magnitude of the EOG responses during the wash period differed significantly across trials (Figure 4: $H_{27/2}$ = 11.64, $P = 0.003$), but those during FMRFamide application did not (Figure 4: $P = 0.62$). *Post hoc* tests indicate that the EOG responses during the wash period in the second trial were larger than those in the third trial ($P < 0.05$), but other comparisons did not indicate significant differences ($P > 0.05$, for all cases).

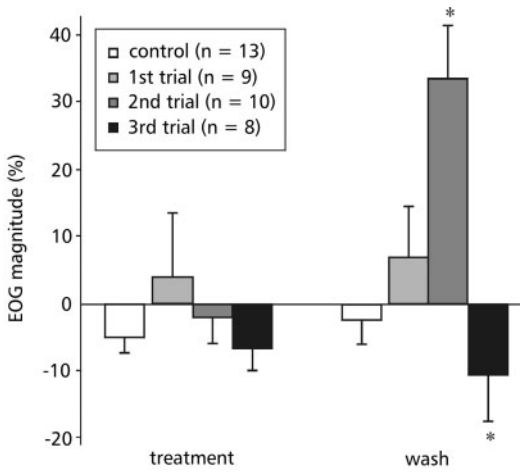

Figure 4 FMRFamide affects EOG responses to L-methionine. All data are normalized such that the mean magnitude of the baseline EOG is designated 100%, and all subsequent EOG response magnitudes are expressed as a percentage of the baseline magnitude. Bar height indicates mean percent deviation from baseline, and vertical lines indicate the standard error of the mean. 'Treatment' indicates period during which FMRFamide or the control solution was applied to the olfactory epithelium; the solution was then washed off ('wash'). Data shown for controls represent the mean magnitude of all EOG responses recorded during all three trials (open bars). Data from the three trials for FMRFamide-treated animals are shown separately (shaded bars). During the period of FMRFamide application, the average magnitude of the three EOG responses did not differ across trials ($P = 0.62$). During the wash period, the average magnitude of the nine EOG responses differed significantly across trials ($P = 0.003$); specifically, the magnitude during the second trial is significantly larger than during the third ($P < 0.05$). In addition, the responses in the second trial were significantly larger than baseline, and those during the third trial were significantly smaller than baseline. Asterisks (*) represent comparisons with the baseline magnitude, and indicate that $P < 0.05$.

Because EOG responses during the wash period were significantly different among three consecutive trials, we also measured and analyzed (i) the magnitude of the largest single EOG response elicited during the wash period for each trial, expressed as a percent of the mean baseline EOG magnitude, and (ii) the duration of the time interval between the initial FMRFamide application and the largest EOG response, or the 'latency' to the largest EOG response within each trial. The data concerning the magnitude of and

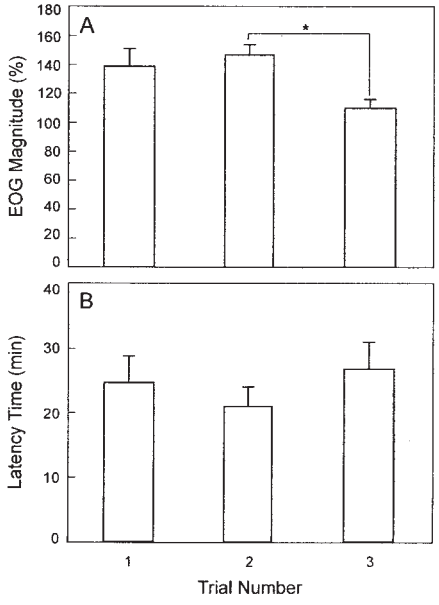

Figure 5 The largest EOG response was generally recorded during the wash period, after FMRFamide had been applied and washed off. **(A)** The magnitude of the largest single EOG response, normalized to the baseline magnitude and averaged across animals, differed significantly among the three trials ($P = 0.029$): the magnitude of the largest EOG response in the second trial was greater than that in the third trial. Asterisk (*) indicates a significant difference between trials ($P < 0.05$). **(B)** The latency to the largest EOG response did not differ among trials ($P = 0.50$). Vertical bars depict the standard error of mean.

latency to the largest EOG responses were normally distributed (Shapiro–Wilk test, $P = 0.27$ and 0.54, respectively), so we analyzed the data using one-way ANOVA, followed by Tukey's *post hoc* test (Sokal and Rohlf, 1981).

In accord with the results of across-trial analyses, we found that the magnitude of the largest EOG response elicited during the wash period differed significantly among the three trials (Figure 5A: $F_{24/2} = 4.10$, $P = 0.029$). The magnitude of the largest EOG response in the second trial (mean ± SEM, 146.54 ± 7.67%; $n = 10$) was larger than that in the third trial (mean ± SEM, 110.14 ± 6.30%; $n = 8$), and this difference was significant (Tukey's test, $P = 0.028$). The magnitude of the largest EOG response obtained during the first trial did not differ significantly from those obtained during other trials. In addition, the latency to the largest EOG response did not differ among trials, ranging from 21.3 to 27.5 min across trials (Figure 5B: one-way ANOVA, $P = 0.50$).

Discussion

To investigate whether FMRFamide affects voltage-dependent currents in single olfactory neurons and odorant responses from the olfactory epithelium of axolotls, we used whole-cell patch clamp and EOG recording techniques. We found that FMRFamide increased voltage-dependent inward currents and altered EOG responses to L-methionine.

Given the magnitude, rate of onset and inactivation, and reversal potential of the current (~60 mV), the inward current that was increased by the application of FMRFamide appears to be carried by sodium. In separate experiments, we have found that a current with the same characteristics can be reversibly blocked by application of 1 μM TTX, indicating that the current that is enhanced in the presence of FMRFamide is a voltage-dependent sodium current (data not shown). The voltage-gated inward current that was affected by FMRFamide was robust after replacing $CaCl_2$ with $BaCl_2$ in the extracellular solution, indicating that the current is not dependent on external calcium, but we cannot rule out the possibility that the altered current is partially carried by calcium (or, in some experiments, barium).

It is possible that FMRFamide is directly gating amiloride-sensitive (Cottrell, 1997; Cottrell *et al.*, 1984) or acid-evoked ion channels that are members of the degenerin/epithelial Na^+ channel (DEG/ENaC) family (Askwith *et al.*, 2000), although our data suggest that this is not the case. The inward current that we found to be altered by FMRFamide is voltage-activated, whereas DEG/ENaC channels are not (Cottrell, 1997; Askwith *et al.*, 2000). In addition, our extracellular solutions contained relatively high concentrations of divalent cations (8 mM Ca^{2+} or Ba^{2+}), which may be sufficient to block amiloride-sensitive channels (Green and Cottrell, 2002). The pH of our extracellular solution was 7.6, too high for FMRFamide to potentiate acid-evoked channels (Askwith *et al.*, 2000). Thus, although we cannot rule out the possibility that the effects we observed were due to activation of one or both of these types of channels in axolotl olfactory receptor neurons, this scenario seems unlikely.

In invertebrates, FMRFamide has been shown to alter the conductance of sodium currents, resulting in modulation of neuronal activity. For example, in peptidergic neurons of the central nervous system that are involved in egg laying in the mollusc *Lymnaea stagnalis*, FMRFamide reduces the magnitude of a TTX-sensitive, voltage-gated Na^+ current (Brussaard *et al.*, 1990). Such a change results in an increased threshold for action potential generation, and arrests ongoing firing activity of the cells. In addition, FMRFamide applied to the abdominal ganglion cells of *Aplysia californica* induces a slow inward current by opening voltage-dependent Na^+ channels, resulting in depolarized cells (Chiba *et al.*, 1992). Furthermore, in LFS neurons in the neural circuit underlying the siphon withdrawal reflex of *Aplysia*, FMRFamide application typically produces a biphasic response, involving a fast excitatory response followed by a prolonged inhibitory response (Belkin and Abrams, 1998). Activation of a TTX-insensitive Na^+ conductance by FMRFamide plays a key role in the early transient depolarization of the neurons (Belkin and Abrams, 1998). These results lend support to our suggestion that FMRFamide may modulate the excitability of olfactory receptor cells by altering voltage-activated sodium currents.

FMRFamide alone does not appear to elicit an odorant response. In our patch-clamping experiments, we found that FMRFamide altered the magnitude of an inward (probably sodium) current, but observed no responses similar to those elicited by odorants. Similar results have been obtained in response to GnRH application in mudpuppy olfactory receptor neurons (Eisthen *et al*., 2000). The results of our EOG experiments also support our contention that FMRFamide does not evoke odorant responses. We conducted a preliminary experiment with one animal, in which FMRFamide solution alone was applied to the olfactory epithelium, and we were not able to record an EOG response to this stimulus (data not shown). In addition, in the data presented here, we found that EOG responses to an odorant solution were not altered during FMRFamide application, but only during the wash period. Taken together, these observations indicate that FMRFamide does not directly induce odorant responses, but instead is functioning to modulate activity of olfactory receptor neurons.

FMRFamide appears to affect odorant responses in the olfactory epithelium. In our EOG study, we found that odorant responses were not affected during the period of FMRFamide application, but were enhanced during the wash period in the second trial. Although the mechanism underlying this phenomenon is unknown, similar effects have been described in other animals, using different compounds. For example, in frog (*Rana esculenta/ridibunda*) olfactory receptor neurons, enhanced responses to odorants that generate adenosine 3:5-cyclic monophosphate (cAMP) were observed after the application of compounds such as carbachol and serotonin (Frings, 1993). Protein kinase C (PKC) activated by intracellular Ca^{2+} serves as a key factor in determining the responsiveness of adenylyl cyclase (AC) in response to odorant stimulation (Anholt and Rivers, 1990; Frings, 1993). In studies of invertebrates, FMRFamide has been shown to alter cAMP and AC concentrations: for example, FMRFamide applied to molluscan heart cells increases both cAMP and AC concentrations (Higgins *et al*., 1978; Willoughby *et al*., 1999), and markedly increases AC concentrations in salivary glands of the freshwater snail, *Planorbarius corneus* (Ferretti *et al*., 1996). These results suggest that FMRFamide might modulate odorant responses in peripheral olfactory systems by interacting with one or more biochemical pathways involved in signal processing.

In our EOG study, we found that the effect of consecutive FMRFamide exposures was significantly different across three trials: the effect of FMRFamide on odorant responses was enhanced in the second trial, and was significantly decreased in the third trial. This difference cannot be explained by deterioration of the EOG responses or changes in the subjects' state, for we found in control recordings that EOG responses were similar in all three trials, and in FMRFamide experiments we found no cross-trial difference in the magnitude of the baseline EOG response. A similar differential response induced by consecutive FMRFamide applications has been reported in a study of retinal ganglion cells in goldfish (*Carassius auratus*). In several such cells, the excitatory response caused by the first application of FMRFamide was much larger than those evoked by subsequent applications (Walker and Stell, 1986). Thus, although we do not understand the mechanisms involved, our data suggest that FMRFamide produces relatively long-term changes in the functioning of the olfactory epithelium.

Given that we have recorded only from semi-intact preparations of olfactory epithelium, we do not know whether FMRFamide induces responses directly by binding to receptors on olfactory receptor neurons, or indirectly by affecting other types of cells in the olfactory epithelium. In addition, we cannot determine whether the effects we observed are due to binding of FMRFamide to FLP receptors, or whether we are activating receptors for other peptides, such as NPY. Finally, we do not yet know whether the terminal nerve of axolotls produces an endogenous FMRFamide-like peptide that is released into the nasal epithelia. Nevertheless, FMRFamide-like peptides have been isolated from several vertebrate species, including an amphibian (Tsutsui *et al*., 2000; Satake *et al*., 2001; Koda *et al*., 2002), and the brain, terminal nerve and olfactory epithelium of amphibians show FMRFamide-like immunoreactivity (Muske and Moore, 1988; Rastogi *et al*., 2001).

In both our patch-clamping and EOG experiments, we found that not all cells and subjects respond to FMRFamide. Similar results have been obtained in previous studies using GnRH, in which we found that some cells and subjects were affected by GnRH and others were not (Eisthen *et al*., 2000; Park and Eisthen, 2003). We have demonstrated that the proportion of cells responding to GnRH varies across the breeding season, suggesting that endogenous factors regulate GnRH responsivity (Eisthen *et al*., 2000). Given that the terminal nerve seems to play a role in reproductive behavior, perhaps the activity of the terminal nerve or responsivity to its peptides generally varies with reproductive condition. If so, we would expect to find that any terminal nerve-derived peptide only affects a subset of cells and subjects.

Several different compounds have now been demonstrated to modulate activity of olfactory receptor neurons. The effects of GnRH and adrenaline have been examined using both whole-cell patch clamp and EOG recording techniques in amphibians. The results from these studies are broadly comparable to the data we present here, although the species examined, odorant stimuli, and concentrations of modulatory compounds vary across studies. We find that only some cells and animals respond to FMRFamide; similar results have been obtained in studies of the modulatory effects of GnRH (Eisthen *et al*., 2000; Park and Eisthen, 2003), but information concerning the proportion of cells and animals that respond to adrenaline is not available. In the present study, we found that FMRFamide increased the

magnitude of the inward current by as much as 136% relative to baseline. GnRH has been shown to increase the magnitude of the Na^+ current ~120% (Eisthen *et al.*, 2000), and adrenaline increases the magnitude of the Na^+ current ~118% (Kawai *et al.*, 1999). The time interval between application of the compound and the maximal effect varies considerably, ranging from ~4 min for adrenaline (Kawai *et al.*, 1999) to ~10 min for FMRFamide and ~15 min for GnRH (Eisthen *et al.*, 2000). In EOG studies, the magnitude of the enhanced responses is similar: 120–150% of the baseline for adrenaline (Arechiga and Alcocer-Cuaron, 1969), 116–157% for GnRH (Park and Eisthen, 2003), and 110–147% for FMRFamide. The time interval between application of the compound and the maximal EOG response is similar for FMRFamide and GnRH: 21–27 min for FMRFamide and 25–40 min for GnRH (Park and Eisthen, 2003). In contrast, Arechiga and Alcocer-Cuaron (Arechiga and Alcocer-Cuaron, 1969) report that adrenaline increases EOG responses to a maximal level within 5–10 min. These results suggest that different compounds may modulate activity of the olfactory epithelium over different time scales. With all three compounds, studies using patch-clamping and EOG recordings consistently reveal discrepant results, but it is not clear whether these discrepancies reflect real differences in the processes and mechanisms measured using the two recording techniques or are due in part to disruptions of physiological processes caused by the different recording preparations.

In conclusion, our data demonstrate that terminal-nerve-derived compounds may be involved in peripheral olfactory signal modulation. Given that the terminal nerve may contain additional compounds, such as NPY, substance P and acetylcholine, a complete characterization of these compounds and their effects on the olfactory epithelium could greatly enhance our understanding of odorant processing in the periphery.

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