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Characteristics of odorant elicited calcium fluxes in acutely-isolated chick olfactory neurons

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Abstract To understand avian olfaction, it is important to characterize the peripheral olfactory system of a representative bird species. This study determined the functional propertiets of olfactory receptor neurons of the chicken olfactory epithelium. Individual neurons were acutely isolated from embryonic day-18 to newborn chicks by dissection and enzymatic dissociation. We tested single olfactory neurons with behaviorally relevant odorant mixtures and measured their responses using ratiometric calcium imaging; techniques used in this study were identical to those used in other studies of olfaction in other vertebrate species. Chick olfactory neurons displayed properties similar to those found in other vertebrates: they responded to odorant stimuli with either decreases or increases in intracellular calcium, calcium increases were mediated by a calcium influx, and responses were reversibly inhibited by 100 μM L–*cis*–diltiazem, 1 mM Neomycin, and 20 μM U73122, which are biochemical inhibitors of second messenger signaling. In addition, some cells showed a complex pattern of responses, with different odorant mixtures eliciting increases or decreases in calcium in the same cell. It appears that there are common features of odorant signaling shared by a variety of vertebrate species, as well as features that may be peculiar to chickens.

Keywords Bird · Calcium imaging · Olfactory receptor neurons · Odorant sensitivity · Domestic chicken

Abbreviations $[Ca^{2+}]_i$: Intracellular calcium concentration · cAMP: Cyclic adenosine monophosphate · IP_3: Inositol 1,4,5–trisphosphate · ORN: Olfactory receptor neuron

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Introduction

Olfaction is a primitive sense that is present in a wide variety of animals. Given the cosmopolitan presence of this sensory system in a variety of phyla, one would expect that olfactory systems would employ a diversity of mechanisms for coding odorant stimuli. Yet recent studies have shown that some physiological features of olfactory receptor neurons (ORNs) are common to many species, including the ability of odorant stimuli to elicit intracellular calcium fluxes in ORNs (Schild and Restrepo 1998). Thus, a study of calcium signaling in ORNs from a variety of species may give researchers valuable insight into commonalities in neural function in animals.

Olfaction in birds has been treated as a peculiarity because of their exceptional visual capabilities, yet every bird studied exhibits a functional sense of smell (Tucker 1965; Wenzel 1971; Wenzel and Sieck 1972; Oley et al. 1975). Birds use their sense of smell to mediate a number of important behaviors such as foraging (Nevitt et al. 1995; Nevitt 1999; 2000; Nevitt and Haberman 2003), feeding (Roper and Jones 1997; Roper 1999) and social interactions (Hagelin et al. 2003). The avian olfactory epithelium is situated on a single spiral turbinate that occupies the nasal cavity (Bang and Wetzel 1985). As in other vertebrates, ORNs are found embedded in the epithelium and directly innervate the olfactory bulb (Mendoza 1980; Bang and Wetzel 1985). Chick ORNs possess a morphology similar to those of other vertebrate species (Breipohl and Fernandez 1977; Matsusaki 1995) and express molecules typical of ORNs of other vertebrates, such as the olfactory marker protein (OMP) (Buiakova et al. 1994). Odor stimulation elicits neuronal responses in avian peripheral nerves (Tucker 1965; Kiyohara and Tucker 1978; Koch et al. 1991) and olfactory bulb (McKeegan and Lippens 2003). Avian olfactory

bulb neurons respond with monotonic increases in firing frequency with increasing stimulus intensity (McKeegan et al. 2002), and different odorants elicit different patterns of activation and inhibition in single bulbar neurons (McKeegan 2002).

Although the sense of smell appears to be universal for avian species, the mechanism of odorant sensitivity in this vertebrate class is poorly understood. Previous studies on other vertebrates have shown that olfactory receptors present on the plasma membrane of the ORN denritic cilia mediate odorant detection (Buck 1996). Stimulation of the ORNs triggers a G-protein-mediated production of the second messenger molecules cyclic adenosine monophosphate (cAMP) and inositol 1,4,5–trisphosphate (IP_3), which open ion channels (for review, see Schild and Restrepo 1998). These cause the ORN to depolarize and trigger the opening of voltage gated Ca^{2+} channels. Ca^{2+} rushes into the cell, increasing the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$, Restrepo et al. 1990). Thus measurements of odorant elicited $[Ca^{2+}]_i$ changes are a useful tool for assessing ORN activation, and were used in this study on bird ORNs.

Understanding the similarities and differences in the odor signaling mechanisms becomes especially important when comparing different species. Such comparisons provide a unique insight into the evolution and behavioral significance of physiological phenomena in the olfactory system. In this study, the functional characteristics of avian olfactory neurons were studied using embryonic and newborn chicks (*Gallus domesticus*). Odorant sensitivity in chicks begins early in the embryo: the chick expresses a limited complement of odorant receptor molecules starting at embryonic day 5 (E5, Leibovici et al. 1996), and embryonic ORNs respond to odorant stimuli by E13 (Lallou et al. 2003). At birth, chicks detect and respond to single odorants and complex odor mixtures in behavioral assays (Roper and Jones 1997; Porter et al. 1999; Roper 1999). Chicks achieve this olfactory behavioral repertoire using a limited repertoire of 12 different olfactory receptors (Nef et al. 1996; Nef and Nef 1997), and single ORNs express only one type of these receptors (Leibovici et al. 1996). This suggests that avian ORNs may play a complex role in processing odorant inputs (Gomez 2000). We tested the physiological properties of chick ORNs using $[Ca^{2+}]_i$ imaging techniques that have been previously applied to a variety of animal models to allow a direct comparison of these properties across different species of vertebrates.

Materials and methods

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Scranton in order to minimize the suffering and the number of animals used. Fertilized chick (*G. domesticus*) eggs were obtained from Charles River Laboratories SPFAS (North Franklin, CT, USA), or from local farmers. Eggs were incubated in a humidified 38°C incubator. Chicks were used between embryonic day-18 (E18) and birth.

Tissue dissection and cell isolation

If unhatched, the embryo was carefully removed from the shell and immediately decapitated. For newborn chicks, the animal was sacrificed by cervical dislocation followed immediately by decapitation. The tip of the upper beak was excised, and a shallow longitudinal cut along the midline (starting form the edge of the upper beak to the first sight of the forebrain), and cuts along each orbital bone at the level of the forebrain, were made. The lateral halves of the head were then spread to reveal single spiral turbinates on either side of the nasal septum (Breipohl and Fernandez 1977). The turbinates and septum were gently removed and placed in a dish containing Ringer's solution (see Materials). The tissue overlying turbinate and septal bones were gently stripped with fine forceps, then transferred to an "isolation solution" (see Materials) supplemented with 2 mM cysteine and 3 U/ml papain. The dissected tissue was minced using iridectomy scissors and transferred into a centrifuge tube to allow the tissue to settle. After a 15-min incubation, most of the isolation solution was removed, and the remaining tissue was triturated using a fire-polished glass pipette until the tissue was broken up into small pieces. Stop solution (see Materials) supplemented with leupeptin and fura–2/AM was then added to the tissue. Cells were situated onto #0 24×60 coverslips (Thomas Scientific Co., Swedesboro, NJ, USA) coated with 5 μg Concanavalin A. The coverslips were placed in a humidified chamber for at least 1 h and the cells were allowed to settle and adhere to the coverslips.

Immunocytochemistry

Immunocytochemical staining was conducted using standard methods (DellaCorte 1995). Briefly, acutely isolated cells were fixed with 4% paraformaldehyde for 10 min. Cells were washed in phosphate-buffered saline (PBS) and incubated for 1 h in PBS supplemented with 0.3% Triton X100 and 10% normal serum. Cells were then incubated overnight at 4°C in PBS with the primary antibody for OMP (1:5,000, gift of F. Margolis, University of Maryland). For controls, we conducted the same procedure but substituted nonimmune serum for the primary antibody. Secondary antibody binding and 3,3′–diaminobenzidine (DAB) staining was conducted using the Vectastain Elite Kit and DAB solutions (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's instructions. Cells were viewed and photographed using a Nikon microscope and Nikon Coolpix digital camera.

Measurement of odorant responses

Odorant responses were measured using standard calcium imaging techniques (Restrepo et al. 1995) and a Nikon TS–2000 inverted microscope. Briefly, olfactory cells were loaded with fura–2 by incubating the cells in the stop solution supplemented with 5 μM fura–2/AM and 20 μg/ml pluronic F127 for at least 1 h. Coverslips with fura–2-loaded cells were then situated in a recording chamber and continuously superfused with Ringer's solution. Stimulus solutions were applied by switching the superfusion; this allowed a complete change of bath solutions within 5–20 s. Cells were illuminated with UV light at two wavelengths (340 and 380 nm) under the computerized control of a Sutter filter wheel. Emitted light from the fura–2 in the cells under 400× magnification was filtered at 510 nm and recorded with an intensified CCD camera (Opelco KS 1380 intensifier and Sanyo CCD camera). Image pairs were acquired every 3–4 s. Images were digitized using a Merlin Imaging Workstation (Perkin Elmer Life Sciences, Bethesda, MD, USA), which performed the imaging ratioing and display of pseudocolor images. Intracellular calcium concentrations ($[Ca^{2+}]_i$) were computed from the ratio of emitted fluorescence intensities from 340 nm to 380 nm excitation based on a two-point calibration of the calcium imaging system as described previously (Restrepo et al. 1995). Cells remained viable in the recording set up for up to 2 h without visible effects of dye bleaching.

$[Ca^{2+}]_i$ was measured as the average concentration in regions of interest placed over the cell body and dendrite. Studies of ciliary Ca^{2+} regulation have shown that although there is a difference in time course between dendritic and somatic $[Ca^{2+}]_i$ changes, increases in somatic Ca^{2+} are causally related to increases in dendritic Ca^{2+} (Leinders-Zufall et al. 1997). Thus it is expected that our average measurements of $[Ca^{2+}]_i$ throughout the cell reflect odorant-induced events in the dendrite. In addition, odor-induced Ca^{2+} changes in the soma are important because they affect ORN function, and therefore play an integral part in signal transduction in ORNs.

Responses were determined as follows: since the resting $[Ca^{2+}]_i$ was intrinsically noisy, we determined the resting $[Ca^{2+}]_i$ based on the average ratio values over ten data points (approx. 30–40 s) immediately prior to the application of a stimulus solution via superfusion. If we noted a distinct change in $[Ca^{2+}]_i$ within 20 s following the solution switch that was at least twice in magnitude as the baseline noise level, and noted a return of $[Ca^{2+}]_i$ *toward* the original resting baseline (even if the original baseline was never reached) within 20 s following odorant solution removal, this $[Ca^{2+}]_i$ change was counted as an odorant-elicited response. To quantify the response, values for the stimulus-elicited change in $[Ca^{2+}]_i$ were measured by determining the maximum or minimum average value across five consecutive data points measured within the response itself (i.e., from the initiation of the $[Ca^{2+}]_i$ change to the start of the return toward the basleine). Values for baseline and the magnitude of stimulus elicited changes in $[Ca^{2+}]_i$ were measured and reported as mean ± SEM. To illustrate an example of a response that just meets the criteria, refer to the first response shown in Fig. 4c. In this data trace, the baseline was 57.18 nM with a noise level of 8 nM; following the stimulus application, the $[Ca^{2+}]_i$ dropped by 16 nM to 41.1 nM.

Materials

Unless otherwise specified, all materials were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Ringer's solution contained (in mM) 145 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 1 Na-pyruvate and 20 Na N-(20 hydroxyethyl) piperzaine-N'-2-ethanesulfonic acid (Na-HEPES). For high K^+ stimulation, 130 mM NaCl was substituted with 130 mM KCl. For Ca^{2+} -free Ringers, $CaCl_2$ was removed and replaced with 2 mM ethleneglycol tetraacetic acid (EGTA). For the isolation solution, $CaCl_2$ and $MgCl_2$ were omitted from the Ringer's and replaced with 2 mM ethylene-diamine tetraacetic acid (EDTA). L–cysteine (1 mM) and papain (3 U/ml) were added immediately prior to use. The stop solution consisted of Ringers supplemented with an additional 1 mM $CaCl_2$ and 5 mM D-glucose. Prior to use, the stop solution was supplemented with 6 mg/ml leupeptin, 5 μM fura–2/AM (Molecular Probes, Eugene, OR, USA), and 20 μg/ml pluronic F127. All solutions were pH 7.4.

Odorants were dissolved in Ringer's solution by several rounds of mixing and sonication. Odorant Mix A contained citralva, citronellal, eugenol, geraniol, hedione, menthone and phenylethylalcohol, and Mix B contained ethyl vanillin, isovaleric acid, lilial, lyral, phenylethylamine, and triethylamine. These two odorant mixes were identical to ones used in previous studies on mammalian ORNs (Restrepo et al. 1993a, b; Rawson et al. 1997; Gomez et al. 2000). Mix AV contained amyl acetate, cineole, ethyl vanillin, eugenol, geraniol, limonene, and octanal and was selected based on previous publications showing that these odorants triggered behavioral or physiological responses in chicks (Table 1). Odorants known to stimulate trigeminal chemoreceptors of birds (such as methyl anthranilate, Mason et al. 1989) were omitted from the mix. The concentration of each individual odorant was 100 μM in Ringers. In addition, 100 μM amyl acetate, an odorant known to be a potent stimulus for chicks and other bird species (Table 1) was used as the representative single odor.

The pharmacological agents 20 μM U73122 (Calbiochem/EMD Biosciences, San Diego, CA, USA), 1 mM Neomycin, and 100 μM L–*cis*–diltiazem were mixed in Ringers immediately prior to each experiment.

Results

Olfactory epithelia from 73 animals (E18 to newborns) were obtained for study. Previous studies have shown

Table 1 Odorant composition of Mix AV

Odorant	Reference for compound's activity in birds
Amyl acetate	Tucker (1965); Tolhurst and Vince (1976)
Cineole	Tolhurst and Vince (1976)
Ethyl vanillin (vanilla extract)	Marples and Roper (1996)
Eugenol (clove oil)	Vallortigara and Andrew (1994); McKeegan (2002)
Geraniol	McKeegan (2002)
Limonene	McKeegan (2002)
Octanal	Hagelin et al. (2003)
Octanol	Koch et al. (1991)

that by E18, mature olfactory neurons are present in the olfactory epithelium (Lalloue et al. 2003). Olfactory neurons were visually identified by their morphology (Fig. 1a): a goblet-shaped cell with a slender dendritic process of varying length that terminates in a knob with cilia or microvilli. Immunocytochemical studies verified that cells that demonstrate this morphology ($n = 47$) express OMP (Fig. 1b), a marker molecule that is expressed by mature olfactory neurons in a variety of animal species (Buiakova et al. 1994). It is known that birds posses both cilated and microvillar receptor cells in their olfactory epithelium (Breipohl and Fernandez 1977; Matsusaki 1995). Although the ORN shown in Figure 1b appears to possess cilia, it cannot be said with certainty that these protrusions from the dendritic knob are not microvilli. Under our microscope, we could not distinguish ciliated from microvillar cells; thus all the cells that were selected for testing possessed this characteristic shape.

We only analyzed data from 299 neurons that loaded with fura–2 and maintained their viability (determined by a stable $[Ca^{2+}]_i$ level) throughout the experiment. These ORNs maintained baseline calcium levels ranging from 17 nM to 223 nM and averaged 87 ± 14 nM. Baseline calcium levels were not significantly different for cells that responded to odorants versus those that did not respond to odorants (ANOVA, $P > 0.05$)

Chick ORNs respond to odorants with increases or decreases in $[Ca^{2+}]_i$

We tested ORNs with a variety of odorant stimuli. For our initial studies, we used Mix A, Mix B, Mix AV, and a single odorant (amyl acetate). We also tested the ORNs' ability to respond to depolarization with a high K^+ Ringer's solution. Figure 2 shows examples of the stimulus elicited $[Ca^{2+}]_i$ responses that were obtained. Figure 2a shows a cell that responded to a single odorant (amyl acetate) with an increase in $[Ca^{2+}]_i$. Odorant elicited increases in $[Ca^{2+}]_i$ constituted 68% of all odorant responses (15% of all cells tested, see Table 2). The average magnitude of an odorant elicited increase was 54 ± 14.8 nM from baseline. Although the timecourse of this particular response appeared to be relatively slow (i.e., it took about 45 s to reach peak value), data from other cells (see subsequent figures) show faster response timecourses. It is unclear whether this slower response timecourse is an artifact of stimulus application or whether it is an inherent property of the cell; it should be noted that previously published studies using identical stimulation and recording techniques have also demonstrated this variety in the timecourse of ORN responses (Gomez et al. 2000). This cell also responded to depolarization with high K^+ with an increase in $[Ca^{2+}]_i$, indicating the involvement of voltage-gated Ca^{2+} channels in generating the $[Ca^{2+}]_i$ change.

Figure 2b shows a different cell that responded to amyl acetate, this time with decreases in $[Ca^{2+}]_i$. This type of response is less common (32% of all responses, Table 2) but has been observed in a variety of species (see Table 3). The average magnitude of an odorant elicited decrease in $[Ca^{2+}]_i$ was 19 ± 5.8 nM from baseline. The response of a cell (increase or decrease in $[Ca^{2+}]_i$) was not related to its resting $[Ca^{2+}]_i$ level (ANOVA, $P > 0.05$). Interestingly, this particular cell also responded to a different odor stimulus (Mix B) with an *increase* in $[Ca^{2+}]_i$. Figure 2c shows a similar phenomenon in a different ORN, wherein the cell responded to Mix A with a decrease in $[Ca^{2+}]_i$ and to Mix AV with

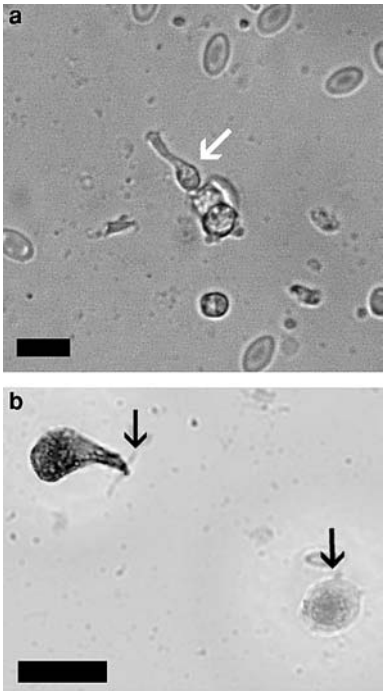

Fig. 1 Photomicrographs (400×) of representative ORNs isolated from the newborn chick olfactory epithelium. *Scale bars* on each figure indicate 20 μm. **a** ORNs (*arrow*) possess a distinct morphology. Physiological data from this cell is seen in Fig. 2c. **b** ORN immunostained for the OMP. Cilia or microvilli were also visible in this cell (see *arrow on the left*). For comparison, a supporting cell (*arrow on the right*) did not stain for OMP

an increase in $[Ca^{2+}]_i$; this cell also responded to high K^+ depolarization with an increase in $[Ca^{2+}]_i$. This unusual pattern was observed in 9% of all cells tested with this stimulus battery ($n = 21$).

The proportion of cells that responded to odorants with increases or decreases in $[Ca^{2+}]_i$ is remarkably similar to those seen in other species (Table 3). In the species which demonstrate odorant-elicited decreases in $[Ca^{2+}]_i$, about one-third of all cell responses are of this type.

Increases in $[Ca^{2+}]_i$ are mediated by a Ca^{2+} influx

To investigate the mechanisms and transduction pathways involved in generating the $[Ca^{2+}]_i$ changes, we focused on manipulations of the Mix AV-generated responses to remove the complications of the complex response patterns (shown in Fig. 2b, c). ORNs can trigger stimulus-elicited $[Ca^{2+}]_i$ increases either by influx of calcium through the plasma membrane or by releasing calcium from intracellular stores (Schild and Restrepo 1998). We tested 31 cells that responded to Mix AV with increases in $[Ca^{2+}]_i$ with the odorant mix delivered in the absence of extracellular Ca^{2+} (Fig. 3). In the absence of extracellular Ca^{2+}, the stimulus elicited $[Ca^{2+}]_i$ increases were reversibly abolished in all cells tested. This indicates that chick ORNs use a calcium influx through the membrane as the primary mechanism for generating stimulus-elicited $[Ca^{2+}]_i$ increases (Restrepo et al. 1990).

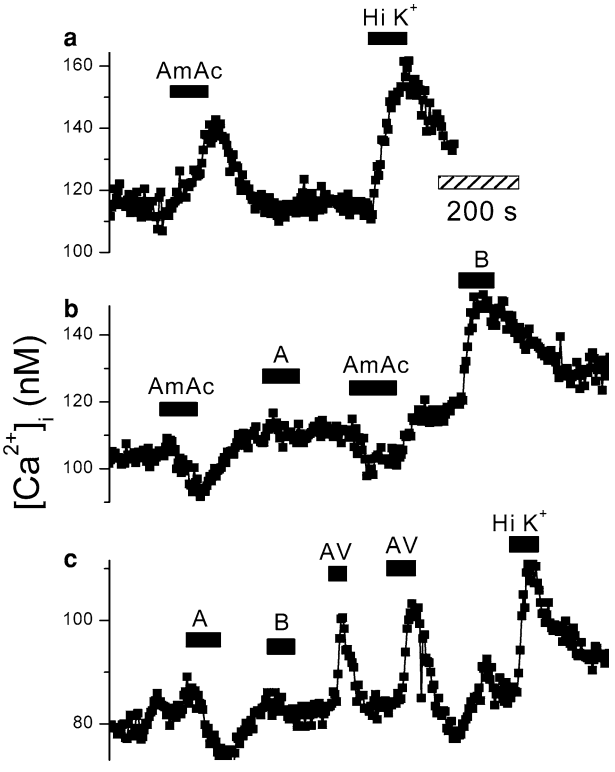

Fig. 2 Samples of the different types of odorant responses recorded from acutely-isolated chick ORNs. Ordinate values show the computed $[Ca^{2+}]_i$ concentrations. In this sample and in all succeeding graphs, the *solid bars* indicate the application of a stimulus, while the *hatched bars* indicate a specified time interval (200 s). **a** Sample of a cell that responded to a single odorant (amyl acetate, *AmAc*) and 135 mM K^+ Ringer's (*Hi* K^+) with increases in $[Ca^{2+}]_i$. **b** A different cell responded to amyl acetate with decreases in $[Ca^{2+}]_i$. This type of response was seen in 32% of all cell responses to odorants. This cell did not respond to Mix A (*A*) but responded to Mix B (*B*) with an increase in $[Ca^{2+}]_i$. Occasionally, cells would display this complex pattern of responses (increase to one odorant and decrease to another); cells from this and other species ordinarily respond with either an increase or a decrease in $[Ca^{2+}]_i$. **c** This particular cell responded to Mix A with a decrease $[Ca^{2+}]_i$ and to Mix AV with increases in $[Ca^{2+}]_i$. This cell also responded to High K^+ with an increase in $[Ca^{2+}]_i$

Table 2 Frequency of odorant-elicited $[Ca^{2+}]_i$ changes observed in isolated chick ORNs

Stimulus ($[Ca^{2+}]_i$ Response Type)	Response frequency (#/# tested)	Percentage of all cells tested	Percentage of cell responses
Hi K^+ (Increase)	40/182	22	85
Hi K^+ (Decrease)	8/182	4	15
Mix AV (Increase[a])	46/299	15	68
Mix AV (Decrease[a])	21/299	7	32
Amyl acetate (Increase)	8/88	9	67
Amyl acetate (Decrease)	4/88	5	33

[a] The numbers reported for Mix AV responses in this table are those that were tested with Mix AV only. These do not include the cells that were tested with Mix A, Mix B, and Mix AV (as seen in Fig. 2)

Pharmacological manipulation of odorant responses

The prevalent theory of olfactory transduction holds that odorants stimulate membrane receptors which activate signal transduction pathways leading to the elevation of intracellular cAMP, which opens cyclic nucleotide gated channels and leads to an influx of calcium (Schild and Restrepo 1998). Other studies have elucidated the role of other signal pathways that may either directly activate olfactory neurons, or modulate the cAMP generated current; these pathways include phospholipase C-mediated production of IP_3 (Schild and Restrepo 1998). Previous studies have tested the involvement of these pathways using biochemical inhibitors to modulate odorant triggered calcium fluxes (Restrepo et al. 1993a; Tarelius et al. 1995; Rawson et al. 1997; Spehr et al. 2002). Studies on mammalian ORNs employed odorant mixtures (Mix A and B, Restrepo et al. 1993a, b; Rawson et al. 1997; Gomez et al. 2000) to stimulate pharmacologically distinct pathways. We did not assume that these mixtures acted similarly in chicks. Thus we focused on using Mix AV as our primary odorant stimulus and tested the effects of number of pharmacological agents. Cyclic nucleotide-gated cation channels are selectively inhibited by L–*cis*–diltiazem (Kolesnikov et al. 1990). Neomycin and U73122 are

Table 3 A comparison of odorant response rate from different vertebrate species, measured using odorant-elicited calcium changes

Species	# Responses/# cells tested	# Odors tested	Response rate	$[Ca^{2+}]_i$ increase (% of responses)	$[Ca^{2+}]_i$ decrease (% of responses)
Chick	67/299	8	22.4	68	32
Cat (newborn to 2 years) [a]	193/657	13	29.4	73.3	27.7
Human (adult) [b]	97/322	13	30	76.7	23.3
Mouse (adult) [c]	53/200	32	26.5	100	0
Mudpuppy [d]	84/ > 330	8	< 25	23	2.4
Rat (adult) [b]	24/72	13	33	100	0

The table shows a comparison of response rates of olfactory neurons from a variety of species. Each of these studies employed calcium imaging methods similar to the one used in this study. The response rates reported in the last two columns are determined from the total number of cells tested. Note that rodent ORNs generally do not respond to odorants with decreases in $[Ca^{2+}]_i$In the mudpuppy study, the numbers were not directly comparable because not all cells were tested with all eight amino acid stimuli. They reported that they tested over 330 neurons; 76 responded with an increase in $[Ca^{2+}]_i$, and eight responded with a decrease in $[Ca^{2+}]_i$

[a]Gomez et al. (submitted); [b]Gomez et al. (2000); [c]Bozza and Kauer (1998); [d]Delay and Dionne (2002)

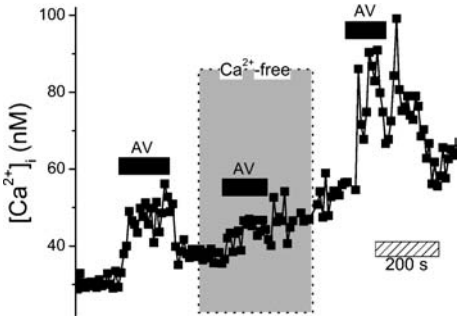

Fig. 3 Odorant elicited $[Ca^{2+}]_i$ increases depend on extracellular Ca^{2+} . Cells that responded with an increase in $[Ca^{2+}]_i$ were tested with the odorants applied in the absence of extracellular Ca^{2+} (*Ca^{2+} free*, *shaded box*). Responses to odorant stimulation in the absence of extracellular Ca^{2+} odorants were reversibly abolished in all cells tested, indicating that odorant elicited increases in $[Ca^{2+}]_i$ were mediated by a Ca^{2+} influx

known to inhibit phospholipase C (Smith et al. 1990; Striggow and Bohnensack 1994), although it has been shown that Neomycin can affect cAMP-dependent cell responses in some species (Ma and Michel 1998).

Figure 4a shows a cell that responded to Mix AV; the odorant responses of this cell were inhibited by the phospholipase C inhibitors Neomycin (1 mM) or U73122 (100 μM). Neomycin was an effective inhibitor of odorant responses: 90% of all odorant elicited $[Ca^{2+}]_i$ increases (Fig. 4a) and decreases (Fig. 4b) in $[Ca^{2+}]_i$ ($n = 50$) were reversibly inhibited by the co-application of Mix AV with 1 mM Neomycin. Upon removal of Neomycin, the odorant responses recovered (Fig. 4b). In contrast, 20 μM U73122 reversibly inhibited odorant responses (increases or decreases) in only 44% of cells tested ($n = 7$); Figure 4a shows an odorant-elicited increase in $[Ca^{2+}]_i$ that was reversibly inhibited by U73122, while Figure 4c shows a cell whose odorant response was not affected by U73122. It is unclear whether the difference in the relative efficiencies of Neomycin and U73122 (90% vs 44%, respectively) were due to their differential pharmacological characteristics, or if Neomycin was acting on multiple signaling pathways (Ma and Michel 1998).

The cyclic nucleotide gated channel inhibitor L–*cis*–diltiazem (100 μM) reversibly inhibited Mix AV elicited responses (Fig. 4d); this effect was observed in only 41% of the cells ($n = 9$).

These results collectively suggest that there are two pharmacologically distinct pathways employed by chick ORNs: one involving cAMP-gated cation channels, and another involving phospholipase C. The fact that U73122 and L–*cis*–diltiazem did not block 100% of the responses suggests that specific odorants may selectively activate one of these two pathways.

Discussion

In this study, we show that chick ORNs appear to possess characteristics that are similar to those found in ORNs of other species. Although embryonic and newborn animals were used for this study, the ORNs from these animals appear to be fully functional, based on both physiological (Lalloue et al. 2003) and behavioral (Tolhurst and Vince 1976; Roper and Jones 1997) studies; we therefore assumed that the characteristics of the ORNs from these animals approximate those found in adult animals.

Previous studies of ORNs described characteristics of odorant elicited $[Ca^{2+}]_i$ changes common to most species. ORNs respond to odorants with increases in $[Ca^{2+}]_i$, initiated in the dendrite and subsequently spreading to the soma (Leinders-Zufall et al. 1997). With a few exceptions (Sato et al. 1991), these $[Ca^{2+}]_i$ increases generally do not occur when odorants are applied in the absence of extracellular Ca^{2+}, indicating that $[Ca^{2+}]_i$ increases are mediated by an influx of extracellular Ca^{2+} (Restrepo et al. 1990). Odorant-elicited $[Ca^{2+}]_i$ changes are reversibly inhibited by biochemical inhibitors of cAMP and/or IP_3 signal transduction, indicating that $[Ca^{2+}]_i$ fluxes are a result of second messenger-mediated activity (Schild and Restrepo 1998). Recently, studies

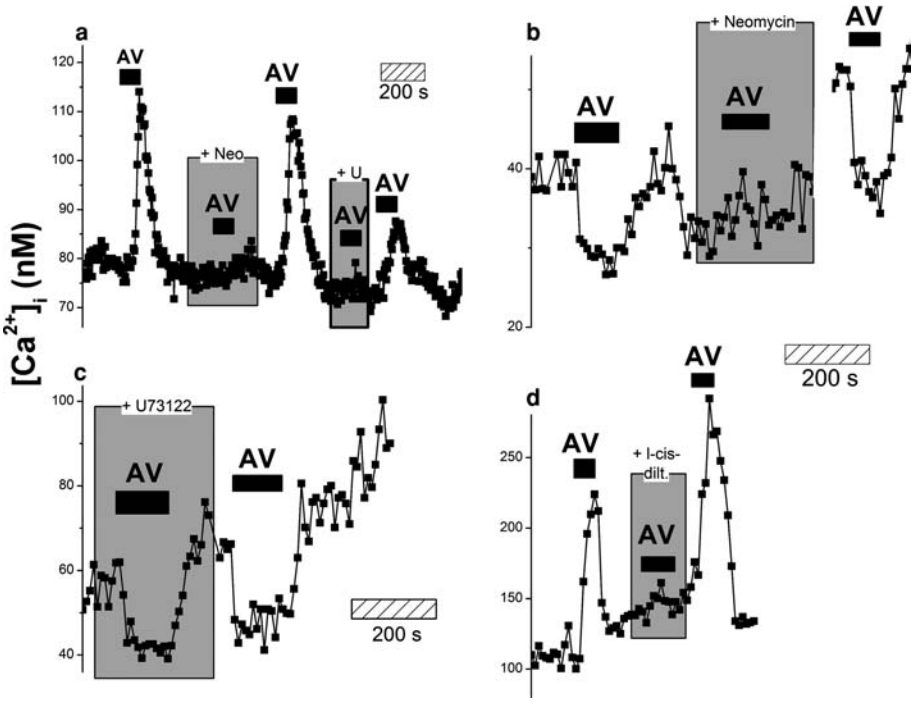

Fig. 4 Pharmacological characterization of odorant signaling was conducted by delivering odorant stimuli in the presence of phospholipase C inhibitors (20 μM U73122 or 1 mM Neomycin) or a cyclic nucleotide gated channel blocker (100 μM L–*cis*–diltiazem). For these figures, the hatched box indicates a time of 200s. **a** This cell responded to Mix AV with increases in $[Ca^{2+}]_i$. These increases were reversibly inhibited by the co-application of Mix AV with either Neomycin (+*Neo*) or U73122 (+*U*). Note that the time scale bar for **a** is different from those of **b**, **c**, and **d**. **b** Odorant elicited $[Ca^{2+}]_i$ changes were reversibly inhibited by Neomycin. This cell responded to AV with an increase in $[Ca^{2+}]_i$. The addition of Neomycin (*shaded box*) resulted in a reversible block of the Mix AV-elicited $[Ca^{2+}]_i$ change in a majority of cells (90%, $n=50$). Following a recovery period, the cell recovered its response. **c** This particular cell responded to Mix AV with a decrease in $[Ca^{2+}]_i$ that was unaffected by U73122 (*shaded box*). Odorant elicited $[Ca^{2+}]_i$ changes were sometimes reversibly inhibited by U73122 (44%, $n=7$). **d** Odorant elicited $[Ca^{2+}]_i$ changes were reversibly inhibited by 100 μM L–*cis*–diltiazem (41%, $n=9$). This cell responded to AV with an increase in $[Ca^{2+}]_i$ that was reversibly blocked by the co-application L–*cis*–diltiazem (+ *l–cis–dilt*, *shaded box*) with Mix AV

have revealed a number of properties that appear to be unique to certain species. In human (Rawson et al. 1997; Gomez et al. 2000) and feline (Gomez et al. in press) ORNs, about a third of the responsive cells respond with decreases in $[Ca^{2+}]_i$; odorant elicited decreases in $[Ca^{2+}]_i$ are not observed in rat ORNs (Gomez et al. 2000) and are occasionally seen in catfish (Restrepo and Boyle 1991) and mudpuppies (Delay and Dionne 2002). In rat biochemical preparations, specific odorants appear to stimulate pharmacologically distinct signaling pathways, related to the differential production of two second messengers. Odorant mixes (Mix A and B, see methods) consisting of odorants that selectively caused the production of cAMP or IP_3 in rat biochemical assays (Breer and Boekhoff 1991) elicit $[Ca^{2+}]_i$ changes that are reversibly inhibited by biochemical inhibitors such as diltiazem or L–*cis*–diltiazem and Neomycin (rat: Tarelius et al. 1995; human: Restrepo et al. 1993a; Rawson et al. 1997). Studies using these odorant mixes show that they selectively stimulate pharmacologically distinct transduction pathways (Rawson et al. 1997; Gomez et al. 2000). While studies on rodents indicate that the primary transduction pathway involves cAMP (Brunet et al. 1996; Belluscio et al. 1998), IP_3 has been shown to participate in signal transduction by modulating cAMP mediated signaling in rat ORNs (Spehr et al. 2002). Mudpuppy ORNs appear to use both of these signaling pathways for odor signal transduction (Delay and Dionne 2002).

Chick ORNs respond to odorant stimulation with increases or decreases in $[Ca^{2+}]_i$ (Fig. 2) that are reversibly inhibited by enzyme or ion channel inhibitors (Fig. 4). These pharmacological manipulations suggest the differential involvement of both IP_3 (via phospholipase C, Fig. 4a, b) and cyclic nucleotide gated channels (Fig. 4d). The similarities of the functional properties of chick ORNs to those found in other species suggests that some features and components of odorant-elicited $[Ca^{2+}]_i$ signaling are remarkably conserved.

Another such conserved mechanism is the odorant-elicited decrease in $[Ca^{2+}]_i$. When present in the organism, this mechanism appears to mediate 25–30% of all odorant responses (Table 3). The exact biochemical components or signaling pathways behind this type of response has yet to be elucidated in olfactory neurons. The diversity of odorants that elicit decreases in the different evolutionarily-distant species (Table 3) suggests that this mechanisms is routinely utilized by ORNs and plays an important role in odorant coding, possibly by contributing to contrast enhancement of an odorant mix (Ache et al. 1998).

A unique feature seen in chick ORNs is that individual cells would occasionally respond to one odorant with an increase in $[Ca^{2+}]_i$ and to another odorant with a decrease in $[Ca^{2+}]_i$ (Fig. 2b, c). This has a number of interesting implications. First, this would imply that individual ORNs process information generated by odor mixtures at the periphery. The prevalent hypothesis of olfactory coding describes a scheme wherein cells that express the same receptor project to specific glomeruli, and the olfactory bulb interprets the pattern of activation generated across these receptors. Studies on rat (Lischka et al. 1999) and toad (Sanhueza et al. 2000) show that odorant-elicited suppressive or inhibitory currents are routinely activated during odorant stimulation, allowing individual ORNs to generate complex patterns of activation and inhibition when given an odorant mix. Thus, these peripheral neurons can act as complex stimulus processors (Gomez 2000). This is especially useful in a species that can detect several different odorants (Jones and Roper 1997) using a relatively narrow repertoire of only 12 different olfactory receptors (Nef and Nef 1997). Studies demonstrate that individual chick ORNs express only one receptor type (Leibovici et al. 1996). Thus, the ability of an ORN to respond with either increase or decreases (Fig 2b, c) suggests that single olfactory receptors are linked to multiple signaling pathways that are differentially activated in an odorant-specific fashion. This phenomenon has been demonstrated and modeled in pharmacological studies of G-protein coupled receptors (Scaramellini and Leff 1998) and may be an adaptation of the chick olfactory system to compensate for its relatively narrow receptor expression profile.

Another interesting finding resulting from this study is the possible involvement of two different signal transduction pathways in the avian olfactory system. The chick olfactory epithelium consists of neurons that are either ciliated or microvillar (Breipohl and Fernandez 1977; Matsuzaki 1995). This arrangement has been observed in fish (Muller and Marc 1984; Hansen et al. 2004), but in mammals and in some amphibians such as axolotls, these cell types are segregated: microvillar cells are restricted to the vomeronasal organ, and the ciliated olfactory neurons are located in the main olfactory cavity (Eisthen 1992; Mendoza 1993). Previous studies of the bird's nasal cavity have not described a distinct vomeronasal organ (Meredith 1991). Studies on rodents have shown that vomeronasal neurons employ phospholipase C-mediated diacylglycerol and IP_3 production as the primary transduction pathway (Kroner et al. 1996; Inamura et al. 1997; Lucas et al. 2003), while olfactory neurons use cAMP as the primary second messenger for excitation (Schild and Restrepo 1998; Gold 1999). Our recording techniques could not resolve the morphological difference between these two cell types in the bird epithelium, thus we could not determine whether these different cell types employed pharmacologically distinct signaling pathways. However, this is an intriguing possibility that merits further investigation.

In summary, odorant-elicited $[Ca^{2+}]_i$ changes in ORNs in this evolutionarily unique group have some features that are identical to those found in other vertebrate species, as well as some properties that are unique to chicks. The study of olfaction in this unique class of vertebrates highlights features that are common to many species that could be important and utilized for olfactory coding.

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