Evidence for Multiple Calcium Response Mechanisms in Mammalian Olfactory Receptor Neurons

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Abstract

Olfactory receptor neurons employ a diversity of signaling mechanisms for transducing and encoding odorant information. The simultaneous activation of subsets of receptor neurons provides a complex pattern of activation in the olfactory bulb that allows for the rapid discrimination of odorant mixtures. While some transduction elements are conserved among many species, some species-specificity occurs in certain features that may relate to their particular physiology and ecological niche. However, studies of olfactory transduction have been limited to a relatively small number of vertebrate and invertebrate species. To better understand the diversity and evolution of olfactory transduction mechanisms, we studied stimulus-elicited calcium fluxes in olfactory neurons from a previously unstudied mammalian species, the domestic cat. Isolated cells from cat olfactory epithelium were stimulated with odorant mixtures and biochemical agents, and cell responses were measured with calcium imaging techniques. Odorants elicited either increases or decreases in intracellular calcium; odorant-induced calcium increases were mediated either by calcium fluxes through the cell membrane or by mobilization of intracellular stores. Individual cells could employ multiple signaling mechanisms to mediate responses to different odorants. The physiological features of these olfactory neurons suggest greater complexity than previously recognized in the role of peripheral neurons in encoding complex odor stimuli. The investigation of novel and unstudied species is important for understanding the mechanisms of odorant signaling that apply to the olfactory system in general and suggests both broadly conserved and species-specific evolutionary adaptations.

Key words: calcium imaging, domestic cat, olfaction, signal transduction

Introduction

Mammals can detect and discriminate thousands of different odors under a variety of environmental conditions and use this information to guide essential activities such as feeding, establishing territorial boundaries and in social interactions (e.g. mate selection). Different mammalian species are challenged to detect unique odorants from a rich olfactory environment and extract pertinent information to suit their specific feeding and social activities. Understanding the complexity of odorant signaling involves rigorous investigation of the properties of each component of the system—from the genes encoding the receptor proteins to the cellular transduction mechanisms to neural transmission to central coding by the olfactory bulb through to behavioral output.

The initial steps of odorant detection occur when odorants bind to the receptors found in the olfactory receptor neurons (ORNs) that line the nasal mucosa. This binding triggers a biochemical cascade that eventually results in a change in membrane voltage of the ORN and a change in intracellular calcium ($[Ca^{2+}]_i$). Recent investigations have employed fluorescence imaging techniques to measure stimulus-elicited calcium changes from ORNs as an indicator of odorant sensitivity (Noé and Breer, 1998; Malnic *et al.*, 1999). Since imaging approaches allow the non-invasive study of a relatively large number of cells, a number of these studies have been conducted on a variety of vertebrate species using similar approaches, allowing the direct comparison of odor signaling mechanisms among different species.

Odorant elicited $[Ca^{2+}]_i$ changes in ORNs exhibit characteristics common to most species (for review see Schild and Restrepo, 1998). Either single odorants or odorant mixtures are capable of eliciting $[Ca^{2+}]_i$ changes. ORNs generally respond to these odorant stimuli with increases in $[Ca^{2+}]_i$, although odorant-elicited decreases have been described in catfish (Restrepo and Boyle, 1991), humans (Rawson

et al., 1997) and salamanders (Delay and Dionne, 2002). With a few exceptions (Sato *et al.*, 1991), $[Ca^{2+}]_i$ increases generally do not occur when odorants are applied in the absence of extracellular Ca^{2+}, indicating that $[Ca^{2+}]_i$ increases are due to an influx of extracellular Ca^{2+}. Odorant-elicited $[Ca^{2+}]_i$ changes are reversibly inhibited by ion channel blockers (Restrepo *et al.*, 1993b; Leinders-Zufall *et al.*, 1997) or biochemical inhibitors of signal transduction (Striggow and Bohnensack, 1994; Rawson *et al.*, 1997), indicating that $[Ca^{2+}]_i$ fluxes are a result of second messenger-mediated activity. However, ORNs from different species also exhibit characteristics that are species-specific (Rawson *et al.*, 1997; Delay and Dionne, 2002). An understanding of the nature of such differences can provide unique insight into the evolution and behavioral significance of physiological phenomena in the olfactory system.

In this study, we investigated the properties of odorant elicited $[Ca^{2+}]_i$ changes in feline ORNs (the domestic cat, *Felis felis*). As a naturally solitary and nocturnal animal, the cat depends extensively on its olfactory senses in its social interactions (Bradshaw, 1992). Recent genetic studies place the cat in a superclade that has been largely ignored in studies of chemosensation and is distinct from that of rodents and primates (Sun *et al.*, 2001). As a mammalian species phylogenetically distant from rodents and an obligate carnivore with a remarkably wide range of habitats (Bradshaw, 1992), we hypothesized that feline ORNs might exhibit unusual features reflective of this divergence. Consistent with this hypothesis, we show that ORNs from this species possess an unusually diverse constellation of odorant signaling pathways not reported in studies of other species.

Materials and methods

Olfactory cell dissociation procedures

Experiments described in this work conformed to the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications 80-23, revised 1978), and efforts were made to minimize the number of animals used. Males and females aged 2 weeks–adult were used. Animals were housed with *ad libitum* food and water, 12 h light cycles, at 21°C, with 12–15 air changes/h. Feline olfactory neurons were obtained at autopsy from animals housed and euthanized for other research purposes at the University of Pennsylvania School of Veterinary Medicine in accordance with protocols approved by their Institutional Animal Care and Use Committee and the guidelines of the American Veterinary Medical Association. The turbinates and septum were excised and put in ice-cold Ringer's solution for subsequent transport to the laboratory. Tissue was dissected to remove the underlying septal and turbinate bones/cartilage. Olfactory neurons were dissociated by mincing the tissue with dissecting scissors, then incubating the tissue in an 'isolation solution' supplemented with 6 U/ml pa-

pain and 2 mM L-cysteine for 20 min. Following cell dissociation, tissue was triturated in fire-polished glass pipettes to further break up large pieces. The cells were then filtered through a nylon mesh and transferred to a 'stop solution' containing 10 µg/ml leupeptin and fura 2/AM (see below). Cells were then spread over glass coverslips (22 × 60 cm, No. 0, Thomas Scientific Co.) coated with 8 µg/ml concanavalin A for subsequent testing.

Measurement of odorant responses

Odorant responses were measured using calcium imaging techniques (Restrepo *et al.*, 1995). Olfactory cells were loaded with fura-2 by incubating the cells in the stop solution supplemented with 8 µM fura-2/AM (Molecular Probes Inc., Eugene, OR) and 80 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 a Ringer's solution. Stimulus solutions were applied by switching the superfusion; this allowed a complete change of bath solutions within 5–20 s, based on preliminary experiments with fluorescent dye for each setup and recording chamber. Durations of stimulus exposures are shown in the figures.

Calcium imaging recordings were performed using standard imaging techniques (Restrepo *et al.*, 1995). Cells were illuminated with UV light at two wavelengths (340/360 or 340/380 nm, depending on the setup). Emitted light from the fura-2 in the cells under 400× magnification was filtered at 510 nm and recorded with a cooled CCD camera (Olympix, Perkin Elmer Life Sciences, Bethesda MD). Images were digitized using a Merlin Imaging Workstation (Perkin Elmer Life Sciences, Bethesda MD), which produced the image ratio and display of pseudocolor images. $[Ca^{+2}]_i$ was computed from the ratio of emitted light from the 340/360 or 340/380 nm wavelength. Excitation light was shuttered between acquisitions and exposure times minimized (typically 50–100 ms). Images were acquired every 2–5 s. Using these conditions, cells remained viable in the recording setup for over two hours without significant effects of dye bleaching or baseline shifts. The use of ratiometric imaging reduces the problems associated with fluorescence quenching that can generate artifacts using single wavelength dyes (Restrepo *et al.*, 1995).

This technique allowed measurement of average cellular $[Ca^{2+}]_i$ over the cell body and dendrite. $[Ca^{2+}]_i$ changes in the ORN dendrite and soma are important because they affect ORN function, and therefore play an crucial role in signal transduction in ORNs (for review see Schild and Restrepo, 1998). Although ciliary $[Ca^{2+}]_i$ could not be measured in our experimental setup, studies of odorant-induced ciliary Ca^{2+} fluxes have shown that ciliary and dendritic $[Ca^{2+}]_i$ changes are causally related (Leinders-Zufall *et al.*, 1997). While the odorant-elicited ciliary $[Ca^{2+}]_i$ changes occur at a much faster time course, measurements of dendritic

and cell body $[Ca^{2+}]_i$ that occur over a much longer time course (>60 s) reflect odor-induced changes in ciliary $[Ca^{2+}]_i$ (Restrepo et al., 1993a). In addition, studies have shown that the time course of the change in somatic $[Ca^{2+}]_i$ is much slower (~1 min) than in the dendrites, even when the stimulus is delivered over a short time period (Leinders-Zufall et al., 1997).

Responses were determined as described previously (Gomez et al., 2000). Briefly, the resting $[Ca^{+2}]_i$ was based on the mean $[Ca^{+2}]_i$ over four data points (~20 s) immediately prior to the application of a stimulus solution. If we noted a distinct and sustained change in $[Ca^{+2}]_i$ immediately following odorant stimulation (within 50 s) and a return of $[Ca^{+2}]_i$ toward the original baseline immediately following odorant solution removal (within 50 s), we counted this as an odorant response. Calcium concentrations were estimated from calibrations performed for each experiment as described (Restrepo et al., 1995).

Materials

Mammalian Ringer's solution contained (in mM) 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 1 sodium pyruvate and 20 sodium N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (Na HEPES). For high K^+ stimulation, 130 mM of NaCl was replaced with KCl. For Ca^{2+}-free Ringer's, $CaCl_2$ was removed and replaced with 1 mM ethyleneglycol tetraacetic acid (EGTA). For the isolation solution, $CaCl_2$ and $MgCl_2$ were omitted from the mammalian Ringer's and replaced with 2 mM ethylenediamine tetraacetic acid (EDTA). The stop solution contained (in mM) 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 D-glucose, 1 sodium pyruvate and 20 Na HEPES. All compounds were obtained from Sigma Chemical Co. (St Louis, MO).

Odorants were obtained from Firmenich (Geneva, Switzerland) and from the Shiseido and Takasago Corporations (Tokyo, Japan). Mix A [cAMP-producing odors in rat biochemical assays (Breer and Boekhoff, 1991)] contained citralva, citronellal, eugenol, geraniol, hedione, menthone and phenethylalcohol, while Mix B [IP_3-producing odors in rat biochemical assays (Breer and Boekhoff, 1991)] contained ethyl vanillin, isovaleric acid, lilial, lyral, phenylethylamine and triethylamine. In some experiments, subsets of these mixtures were also tested (see Results). The concentration of each odorant was 100 μM to make the study compatible with previous studies on ORNs from other mammalian species using similar techniques, which demonstrated that these particular odorants activated pharmacologically distinct odorant signaling pathways (Restrepo et al., 1993b; Tarelius et al., 1995; Rawson et al., 1997). Odorants were dissolved directly in standard or Ca^{2+}-free Ringer's by several rounds of vigorous vortexing and sonication.

Pharmacological agents were obtained as follows: Neomycin (1 mM) and 3-isobutyl-1-methylxanthine (IBMX, 1 mM) were from Sigma-Aldrich, Inc. (St Louis, MO); thapsigargin (200 nM), LY83583 (20 μM) and forskolin (100 μM) were from Calbiochem (San Diego, CA). Agents were dissolved in appropriate buffer and used at the concentrations indicated. Pilot tests were run to establish effective concentrations based on data from rat and human olfactory neurons.

Results

Olfactory epithelia from 79 animals were obtained for study (41 males and 38 females). These animals ranged in age from 2 weeks to 6 years (average = 1.4 years, median = 1 month). Although there was a large age range, our preliminary tests did not suggest an age-related change in the functional properties of the cell signal transduction pathways. In addition, no differences were observed between cells from males versus females. Thus, data from all animals were combined.

Olfactory neurons were visually identified by their distinct morphology: a goblet-shaped cell with a slender dendritic process that terminates in a ciliated knob (Figure 1). We recorded from a total of 672 neurons that loaded with the fluorescent dye and maintained their viability, defined as a stable shape and calcium level throughout the experimental protocol. Baseline calcium levels ranged from 5 to 255 nM and averaged 86 ± 3.4 nM (mean ± SEM). Baseline calcium values were not significantly different for cells responding to odorants versus those nonresponsive or for cells responding to depolarization with high K^+ versus those nonresponsive to this stimulus.
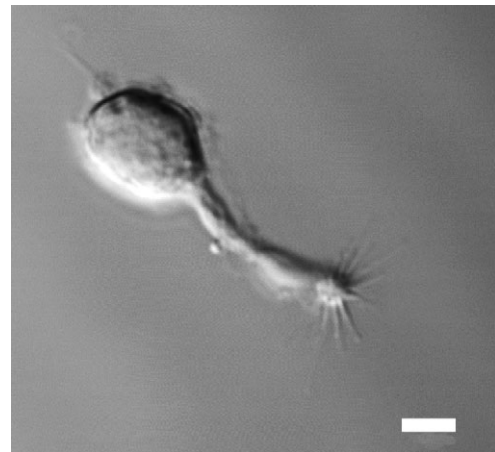
Figure 1 Image of a freshly dissociated feline olfactory neuron captured in transmission mode with the Leica TCS SP2 Spectral Confocal Microscope (Leica Microsystems Inc., Mannheim, Germany) using the Argon laser. Cells were viewed under a HCX APO L U-V-1 63X (0.9 W) water immersion objective. An excitation wavelength of 488 nm was used and emitted light was split by a Wollaston prism to create a transmission image. The image was scanned unidirectionally at a 1024 × 1024 pixel format. Neurons were identified under visual observation; only neurons exhibiting a soma, a dendrite (which may be short or long) and a ciliated terminal knob were tested for odorant sensitivity. Bar = 4 μm.

Odorants elicited increases or decreases in intracellular calcium

Feline olfactory neurons responded to odorant stimulation with changes in $[Ca^{2+}]_i$. Of 657 cells exposed to odorants, 193 responded to at least one of the two odorant mixes (see Table 1). Using the same set of odorants at these concentrations (Mix A and Mix B, a total of 13 individual odorants at 100 μM each), the response frequency of cat ORNs (29%) is similar to those found in rat (33%) and human (30%) ORNs. An example of an ORN's response to odorant stimulation is shown in Figure 2. This cell did not respond to Mix A but did respond to Mix B with an increase in $[Ca^{2+}]_i$ and to depolarization with high K^+ solution with a larger increase in $[Ca^{2+}]_i$. Although the time course of the responses of this cat ORN appears to be slow (for example, the first response to Mix B takes 45 s to attain its maximum magnitude), these kinetic properties are quite similar to those seen in our previous studies on rat and human ORNs (Gomez *et al.*, 2000) using the same stimulation and recording set-ups. In addition, the ability of a cell to respond to an odorant with a decrease in $[Ca^{2+}]_i$ was not related to its resting Ca^{2+} baseline.

Table 1 Summary of odorant elicited calcium changes in cats and in other mammalian species

| Response type	Cat ORN (no./no. tested)	(%)	Rat ORN[a] (%)	Human ORN[b] (%)	Mouse ORN[c] (%)
Mix A ↑	101/652	15	22	11	37
Mix A ↓	21/652	3	0	4	0
Mix B ↑	88/643	14	25	12	19
Mix B ↓	25/643	4	0	3	0
Mix A only	78/638	12	8	15	29
Mix B only	69/638	11	11	15	10
Mix A and B	42/638	7	14	0	9
Mix A or B	193/657	29	33	30	47
High K^+	414/532	78	NR	NR[d]	100
IBMX	108/314	34	NR	NR[d]	NR

[a]From Gomez *et al.* (2000). NR, not reported.
[b]From Rawson *et al.* (1997) and Gomez *et al.* (2000). These ORNs were obtained from healthy young adult volunteers.
[c]From Bozza and Kauer (1998). In this manuscript, seven different odorant mixes were used. The mixes that most closely resembled our Mix A and B were called Mix E (equivalent to our Mix A, except acetophenone was substituted for hedione) and Mix F (equivalent to our Mix B except triethylamine and lilial were omitted). These data were summarized from Figure 4 in their manuscript. In addition, their selection of cells was based on the ability to respond to High K^+ depolarization; hence they report a 100% response rate for this category.
[d]Although these values were not reported in these manuscripts, personal experience has shown that human ORNs from this subject population generally do not respond to high K^+ depolarization or to IBMX with changes in $[Ca^{2+}]_i$.

Like most other vertebrate species, a majority of the feline ORNs tested responded to high K^+ depolarization with increases in $[Ca^{2+}]_i$ (Figure 2, Table 1). This indicates that these ORNs possess functional voltage-activated calcium channels, which allow Ca^{2+} influx into the cell and thus help to amplify and transmit the odorant response to the soma (Restrepo and Teeter, 1990; Schild and Restrepo, 1998). Some researchers have used this response as a selection criterion for inclusion in their analyses. However, we observed a significant number of cells that failed to respond to high K^+ but were odor and/or IBMX (see below) responsive (32/129), and prestimulus calcium levels did not differ among cells responsive and those nonresponsive to depolarization, so all cells were included in our summary statistics.

Single compounds have been shown to be effective odorant stimuli in several species (Firestein *et al.*, 1991; Sato *et al.*, 1994; Ma and Shepherd, 2000, Spehr *et al.*, 2002), and single ORNs have been shown to respond to one or more single odorants (Malnic *et al.*, 1999). To further assess the odorant selectivity of feline ORNs, a subset of cells responsive to the complete mixtures were tested with subsets of Mix A and B. Figure 3 shows samples of two different ORNs that responded to Mix B with an increase (Figure 3A) or a decrease (Figure 3B) in $[Ca^{2+}]_i$. In Figure 3A, this cell responded to the mixture subset B1 (which consists of lyral, lilial and triethylamine) but not to B2 (isovaleric acid and phenylethylamine) or B3 (ethyl vanillin only). Figure 3B shows a different ORN that also responded to Mix B and to subsets B2 and B3, but not to B1. Of 11 cells responsive to one or both mixtures that were also tested with subsets of the effective mixture(s), none responded to all three of the relevant subsets, four responded to two subsets and seven to only one.
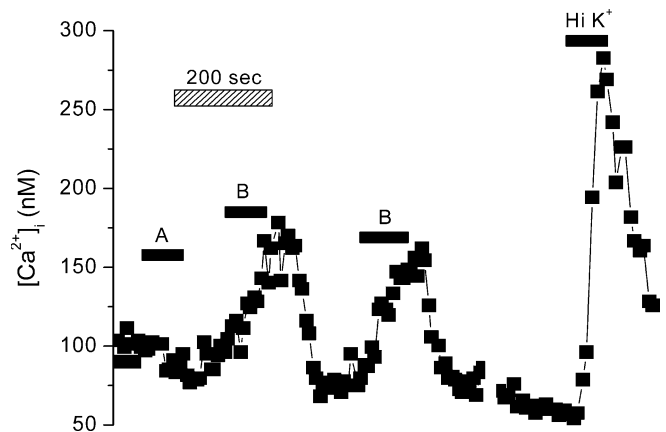
Figure 2 Data trace showing an example of odorant-elicited $[Ca^{2+}]_i$ changes in a feline ORN. Isolated cat ORNs were tested with odorants and other stimuli as described in the methods section. For this and the succeeding figures, the application of a stimulus (Mix A, Mix B or High K^+) is denoted by the solid bars; the hatched bar indicates a time calibration bar (here, 200 s). Many cells also responded to depolarization with high K^+ with increases in $[Ca^{2+}]_i$ (see also Table 1).
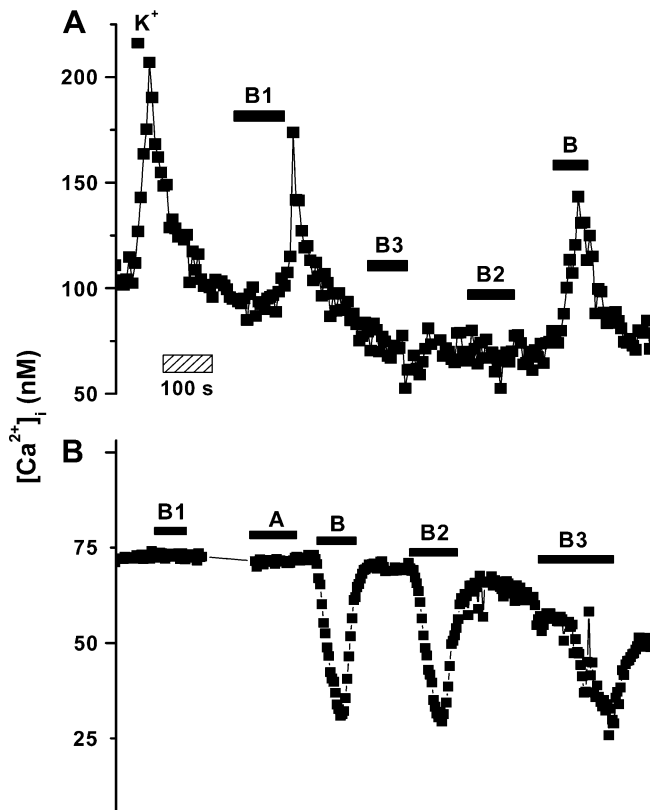
Figure 3 ORNs responded to odorant mix subsets and single odorants. The graphs show two different cells that responded to Mix B with either an increase **(A)** or a decrease **(B)** in $[Ca^{2+}]_i$. When Mix B was delivered in smaller subsets of one to three odorants, the ORNs only responded to some of the subsets. In (A), the cell responded to subset B1 (lyral, lilial and triethylamine), but not to B2 (isovaleric acid and phenylethylamine) or B3 (ethyl vanillin); this cell also responded to depolarization with high K^+ with an increase in $[Ca^{2+}]_i$. In the bottom panel (B), a different cell responded to B2 and B3 with decreases in $[Ca^{2+}]_i$, while neither B1 nor Mix A elicited a response.

These data indicate that feline ORNs are similar to those of rats in their comparatively broad response profile at the concentrations of odorants tested, but also similar to those of human in their ability to respond to odorant stimuli with either increases or decreases in intracellular calcium (Figures 2 and 3, Table 1).

## Multiple mechanisms for odorant-stimulated calcium responses

The selection of the components of Mix A and Mix B were based on biochemical preparations of rat olfactory cilia (Breer and Boekhoff, 1991), showing that components of Mix A and Mix B selectively triggered cAMP and IP₃ formation, respectively. In previous experiments with rat (Tarelius *et al.*, 1995) and human ORNs (Restrepo *et al.*, 1993b; Rawson *et al.*, 1997), these two odorant mixes stimulated pharmacologically distinct pathways: Mix A was selectively inhibited by l-*cis*-diltiazem or LY83583 (blockers of the cyclic nucleotide gated channel (Kolesnikov *et al.*, 1990;

Pawlyk *et al.*, 1991; Leinders-Zufall and Zufall, 1995) and Mix B, by neomycin (a phospholipase C inhibitor, Striggow and Bohnensack, 1994). The contribution of a phospholipase C linked pathway to odorant signaling remains controversial. A number of studies have demonstrated IP₃-dependent currents (Okada *et al.*, 1994; Honda *et al.*, 1995; Kaur *et al.*, 2001) and receptors (Smutzer *et al.*, 1997) in the plasma membrane of rodent ORNs. Recent evidence supports involvement of such a pathway in a modulatory role for some odorants (Spehr *et al.*, 2002). Our selection of the odorant stimuli does not directly imply that these two signaling pathways are functional and distinct in cat ORNs. Rather, we selected these stimuli to facilitate comparison with earlier studies in other species and to investigate whether the pharmacological distinction of responses to these odorant mixes was also present in cat ORNs.

Figure 4 shows an example of an ORN that responded to both Mix A and Mix B (Figure 4A) with increases in $[Ca^{2+}]_i$. When Mix B was applied together with neomycin, the response was selectively and reversibly attenuated. Neomycin did not affect the Mix A response in this cell (Figure 4B). Neomycin eliminated the response in a majority of Mix B responsive cells tested (17 of 21 tested), but was less likely to affect Mix A responses (4/13; $P < 0.005$, χ^2). This suggests a neomycin-sensitive mechanism specific for some odorants. Other studies have employed inhibitors of the cyclic nucleotide-gated channel such as l-*cis*-diltiazem or LY83583 (Leinders-Zufall and Zufall, 1995) to demonstrate the role of this channel in mediating olfactory transduction. Unfortunately, neither of these compounds successfully inhibited responses to forskolin, IBMX or odorants in feline ORNs (data not shown), suggesting a species difference in the cyclic nucleotide gated channel. In view of such species differences in sensitivity to pharmacological agents, we cannot conclude that the effect of neomycin was specific to the phospholipase C pathway, although it has been shown in other studies that neomycin effectively inhibits phospholipase C in cat tissue (Shin *et al.*, 2002). Nonetheless, we can conclude that these odorant stimuli can activate multiple pathways for calcium elevation in cat ORNs, and that individual cells can employ either or both pathways to respond to different odorant stimuli.

Involvement of intracellular Ca^{2+} stores in the stimulus elicited $[Ca^{2+}]_i$ change

The data in Figure 4 also demonstrate differential involvement of intracellular Ca^{2+} stores in individual ORNs. ORNs generate a stimulus-elicited $[Ca^{2+}]_i$ increase by triggering a Ca^{2+}-influx through plasma membrane ion channels or ion pumps. Thus, when odorants are applied in the absence of extracellular Ca^{2+}, the $[Ca^{2+}]_i$ increase mediated by the classical transduction pathway does not occur (Restrepo *et al.*, 1990). In contrast, feline ORNs appeared to use two distinct mechanisms for generating $[Ca^{2+}]_i$ increases.
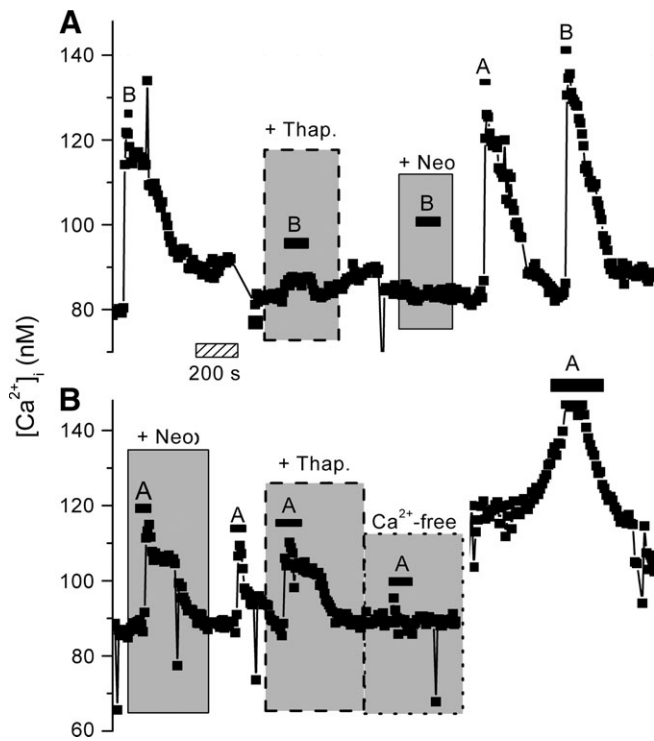
Figure 4 Feline ORNs employ pharmacologically distinct pathways for signaling odorants, similar to ORNs from rats and humans. **(A)** This ORN responded to Mix A and Mix B with an increase in $[Ca^{2+}]_i$. When Mix B was applied in the presence of thapsigargin or neomycin, no response was evoked. Following washout, the cell again responded to Mix B. **(B)** In this same ORN, the response to Mix A was unaffected by either neomycin or thapsigargin, but was eliminated in the absence of external calcium. Upon restoration of normal extracellular calcium, the cell recovered its response to odorants (although the baseline calcium level was elevated). This complex figure demonstrates two phenomena: first, odorant-elicited $[Ca^{2+}]_i$ changes are reversibly inhibited by inhibitors of signal transduction (neomycin); and second, ORNs appear to employ two distinct mechanisms of calcium signaling.

We triggered $[Ca^{2+}]_i$ increases in ORNs using three mechanisms: odorant stimulation, high K^+ depolarization, or by eliciting an increase in intracellular cAMP by inhibiting phosphodiesterase with IBMX (Pawlyk et al., 1991; Lowe and Gold, 1995). IBMX inhibits phosphodiesterase leading to an elevation in cAMP and gating of the cyclic nucleotide channel (Beavo and Reifsnyder, 1990), which causes ORNs to depolarize (Lowe and Gold, 1995) and results in an increase in $[Ca^{2+}]_i$. If a $[Ca^{2+}]_i$ response was observed, the stimulus was applied in the absence of extracellular Ca^{2+} to determine the contribution of the Ca^{2+} influx to the total $[Ca^{2+}]_i$ response.

The ORNs shown in Figure 4 demonstrated differential odorant-dependent responses to 200 nM thapsigargin, an agent known to block Ca^{2+}-ATPase dependent reloading of the intracellular stores (Takemura et al., 1989) and known to be effective in cat tissue (An et al., 2002). In Figure 4A, the addition of thapsigargin in the presence of extracellular Ca^{2+} reduced the magnitude of the $[Ca^{2+}]_i$ changed elicited by Mix

B. In contrast, Figure 4B shows this cell's response to Mix A was unaffected by thapsigargin but was reversibly abolished by the removal of extracellular Ca^{2+}. This suggests that this cell relied mostly on intracellular Ca^{2+} stores to mediate the odorant elicited $[Ca^{2+}]_i$ response to Mix B but not to Mix A. Figure 5A shows a different cell responsive to both odor mixtures. Upon removal of extracellular Ca^{2+}, the response to Mix A persisted. Subsequent stimulation in the absence of extracellular Ca^{2+} resulted in the loss of the odorant response, reflecting a depletion of intracellular stores, it is uncertain whether the lack of a Mix B response was due to its dependence on a Ca^{2+} influx or to depletion of intracellular stores. The Mix A response returned upon restoration of extracellular Ca^{2+} (Figure 5B).

Figure 6 shows another case in which the response to Mix A was dependent on extracellular calcium. This cell responded to both Mix A and Mix B (Figure 6A). The response to Mix B, but not to Mix A, was present in the absence of extracellular calcium (Figure 6B). As in Figure 4, thapsigargin eliminated this response to Mix B. When cells were returned to normal calcium solution, calcium levels tended to rise and remain elevated making recovery of the affected responses difficult to demonstrate. However, delayed and/or smaller recovery responses could be observed (see e.g. Figure 4B).

Although baseline calcium levels in the cells shown here were not elevated by thapsigargin treatment alone as is often the case, it is likely that this treatment was acting as predicted. First, the finding that results with thapsigargin and removal of external calcium were consistent in each case: responses insensitive to removal of external calcium were eliminated by thapsigargin (e.g. Figure 4) while those eliminated by removing external calcium were unaltered by thapsigargin (data not shown). Second, some cells tested with thapsigargin alone did exhibit an increase in baseline calcium, which varied in magnitude (data not shown), supporting the efficacy of the drug on feline olfactory neurons. The impact of thapsigargin on resting cytosolic calcium may be influenced by the activity of other calcium homeostatic mechanisms such as extrusion or binding to calcium-binding proteins. Thus, it is possible that simply preventing reuptake into intracellular stores may elicit only a nominal or very transient effect on baseline calcium that may not be detected. Numerous studies in the literature have established the efficacy of thapsigargin in both neuronal and non-neuronal feline cells (e.g. Lahiri et al., 1996; An et al., 2002). Taken together, we conclude that our thapsigargin treatments were operating as predicted and that in some instances, odor stimulation elicits a release of calcium from intracellular stores.

These data indicate that multiple mechanisms underlie the calcium responses to these odorant mixes and that both pathways can be activated by different odorants in the same ORN. Data from 55 odorant-responsive cells tested similarly indicated that either mixture could elicit release from intracellular stores in a subset of cells. Of 25 cells responsive to
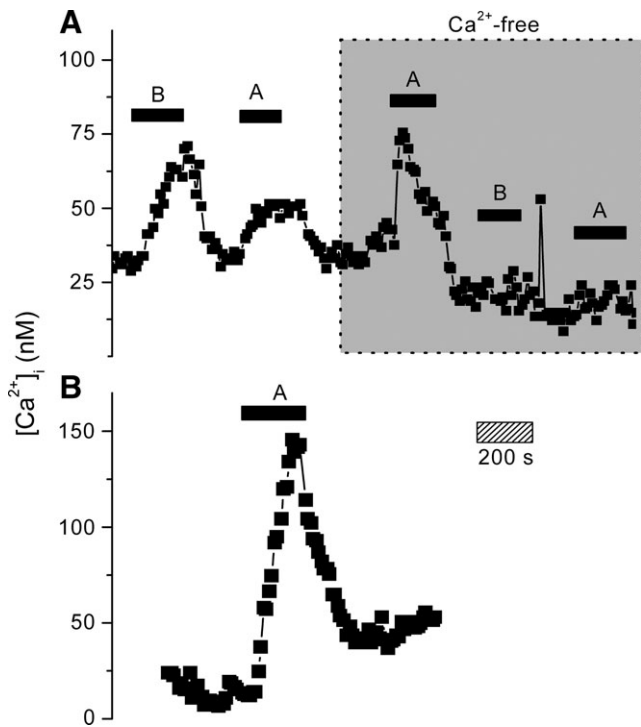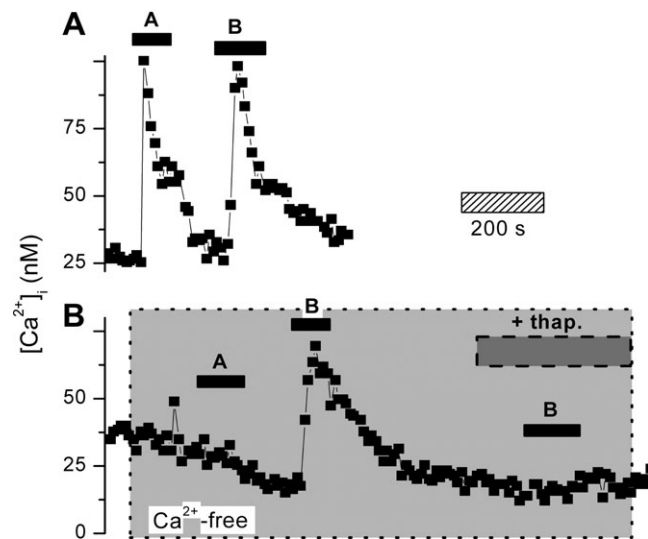
Figure 5 Odorants may activate different calcium response mechanisms in different cells. **(A)** Like the cell in Figure 4, this cell responded to both Mix A and Mix B with an increase in $[Ca^{2+}]_i$. However, in this case, the cell responded to Mix A, but not to Mix B, in the absence of external calcium. The Mix A response in calcium-free Ringer's did not persist upon repeated application, perhaps due to depletion of intracellular stores. **(B)** The response to Mix A recovered following return to normal Ringer's.

Figure 6 The traces in this figure demonstrate an example of another single cell that was responsive to both odorant mixes **(A)**. In this cell, the response to Mix A was dependent on extracellular calcium, while that to Mix B was independent of extracellular calcium but was dependent on the availability of thapsigargin-sensitive internal stores **(B)**. Although baseline calcium levels rose following return to normal Ringer's, a smaller but discernible response to Mix B was observed **(C)**, indicating the recovery of the odorant response.

Mix A, six (24%) responded in the absence of extracellular calcium, while 30% (9 of 30) Mix B-responsive cells responded in the absence of external calcium. These proportions (24% versus 30%, respectively) are not significantly different ($P > 0.5$, χ^2), suggesting that components of each mixture are able to trigger each pathway.

The involvement of intracellular stores appears to be specific to odorant-elicited responses (which involve signal transduction cascades) and are not related to the identity of the odorant mixture. When tested with high K$^+$ depolarization, 80% of the ORNs responded with an increase in $[Ca^{2+}]_i$ (Figure 2; see also Table 1) that did not involve signal transduction cascades. In the absence of extracellular Ca^{2+}, the $[Ca^{2+}]_i$ changes in response to high K$^+$ were completely abolished in all cells tested ($n = 14$; data not shown), indicating that these $[Ca^{2+}]_i$ changes were mediated exclusively by an influx through voltage-sensitive Ca^{2+} channels as seen in other studies (Restrepo and Teeter, 1990).

To further investigate the upstream events triggering release of calcium from intracellular stores, we applied IBMX or odorants in the absence of extracellular Ca^{2+} (Figure 7). In the canonical paradigm for ORN activation, the calcium response to IBMX results from elevation of intracellular cAMP and opening of the cyclic nucleotide gated channel

(Schild and Restrepo, 1998). Consistent with this, IBMX elicited an elevation of intracellular calcium that could be repeated many times without attenuation and in most cases, was dependent on extracellular Ca^{2+} (Figure 7A). This pattern was observed in 69% ($n = 32$) of the cells tested in this fashion. However, in a subset of cells (Figure 7B) the odorant- and IBMX-elicited $[Ca^{2+}]_i$ increases still occurred in the absence of extracellular Ca^{2+} (31% of responsive cells). Thus, increases in $[Ca^{2+}]_i$ were generated from alternate sources, presumably intracellular Ca^{2+} stores (Usachev and Verkhratsky, 1995). Repeated stimulation of this cell in the absence of external Ca^{2+} resulted in a gradual attenuation of the $[Ca^{2+}]_i$ increase, possibly reflecting the depletion of the Ca^{2+} stores (Figure 7B). Thus, the activation of elements of second messenger signaling triggered an increase in $[Ca^{2+}]_i$ via two mechanisms: one that is dependent on Ca^{2+} influx and a second that is independent of external calcium and may involve mobilization of intracellular Ca^{2+} stores, although release from binding proteins or suppression of calcium efflux cannot be ruled out. In addition, the dependence of high K$^+$-induced, but not IBMX- or odorant-induced, $[Ca^{2+}]_i$ changes on extracellular Ca^{2+} implies that this calcium response pathway required the activation of components of a signal transduction cascade, and that odorant-elicited $[Ca^{2+}]_i$ changes did not necessarily require the activation of voltage-gated Ca^{2+} channels.

In summary, feline ORNs used mechanisms that are similar to those seen in other vertebrate species (e.g. Figures 3 and 4). Odorant-stimulated release of calcium from intracellular stores has also been reported in bullfrogs (Sato *et al.*,
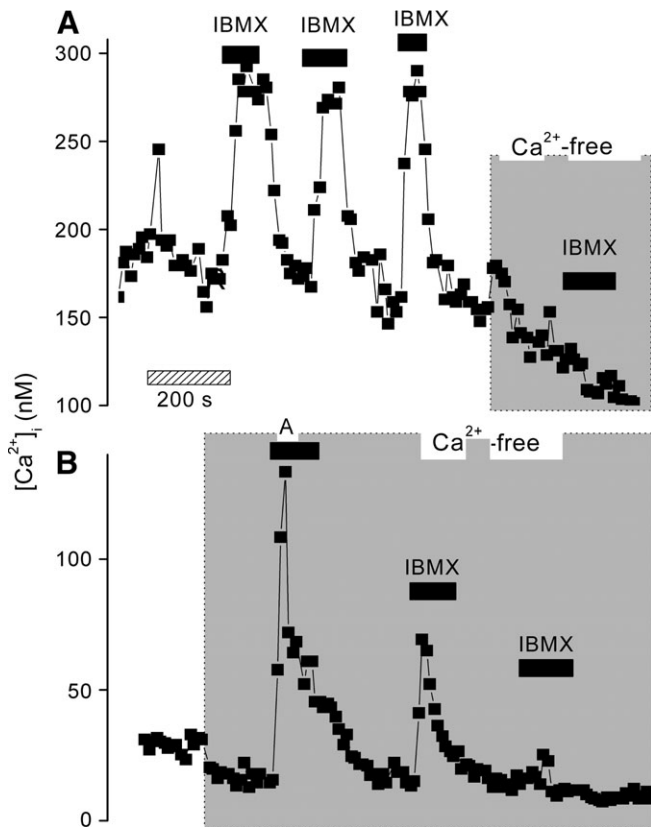
Figure 7 Calcium responses to phosphodiesterase inhibition by IBMX also involved multiple mechanisms. Cells were stimulated with IBMX (1 mM) or odorants in the presence, then absence (Ca²⁺-free, shaded box) of extracellular Ca²⁺. **(A)** Repeatable responses to IBMX were abolished when IBMX was applied in the absence of extracellular Ca²⁺. **(B)** A different cell responded to both Mix A and to IBMX in the presence of extracellular calcium (not shown) and continued to do so even when extracellular Ca²⁺ was removed. The magnitude of the responses in the absence of extracellular Ca²⁺ steadily declined, possibly reflecting the depletion of intracellular Ca²⁺ stores. For this graph, the 200 s time scale bar was the same for A and B.

1991) and salamander (Zufall *et al.*, 2000), but has not been observed in previous studies of rat (Restrepo *et al.*, 1993a; Tareilus *et al.*, 1995) and human ORNs (Restrepo *et al.*, 1993b; Rawson *et al.*, 1997) using similar odorant stimuli and recording methods. Like those of the human, but unlike those of the rat, a subset of feline ORNs also exhibited decreases in $[Ca^{2+}]_i$ in response to odorant stimuli (Table 1).

Discussion

The results of this study show that peripheral olfactory neurons possess complex mechanisms for signaling odorant information. Feline ORNs responded to multiple odorants with dual types of $[Ca^{2+}]_i$ fluxes (increases or decreases) that may or may not involve the mobilization of internal Ca²⁺ stores. This complexity highlights the importance of mechanisms in the periphery that may contribute to modulating and shaping the inputs into the olfactory bulb.

Although the significance of mobilization from Ca²⁺ stores on odorant signaling versus calcium influx via plasma membrane cation channels is unclear, it is interesting to note that cat ORNs employ the mechanisms in a stimulus-selective manner. In many cellular systems, mobilization of Ca²⁺ stores is achieved by the production of IP₃. Although there is no direct relationship between what have been termed 'IP₃-producing odors' (Mix B) and mobilization of the stores, it is possible that upstream activation of two distinct signaling pathways might lead to the downstream activation of shared signaling components such as protein kinases, presumably via pathways that cross-talk (Gomez *et al.*, 2000; Vogl *et al.*, 2000).

Due to the long time course of the responses, the $[Ca^{2+}]_i$ changes measured in this study are likely to affect long-term cellular signaling processes such as adaptation (Boekhoff *et al.*, 1996; Zufall and Leinders-Zufall, 2000). Utilizing two distinct mechanisms for eliciting $[Ca^{2+}]_i$ changes (one that employs intracellular stores and one that does not) may reflect the ability of single ORNs to enhance contrast via a temporal mechanism that relies on differential rates of adaptation to encode odorant stimuli. In vertebrates, olfaction is often associated with respiration, which generates repeated odorant stimulation (Desmukh and Bhalla, 2003). Repeated stimulation triggering distinct calcium responses might result in differential rates of adaptation by two separate odorants in a given cell (Zufall and Leinders-Zufall, 2000). A similar pathway has been demonstrated in salamander ORNs (Zufall *et al.*, 2000) and shown to amplify the calcium response which might have functional significance for both coding and adaptation processes. If release from internal stores occurs independently of influx, the rise in calcium could prevent or delay activation of the cyclic nucleotide-gated channel (Zufall *et al.*, 1991; Kleene, 1999) without necessarily eliciting depolarization. In this scenario, the existence and use of distinct downstream components for odorant-elicited $[Ca^{2+}]_i$ signaling would be a mechanism for ORNs to create temporal contrast between different individual odorants that are applied simultaneously (Jinks *et al.*, 2001). Given the strong evidence for a single receptor type being expressed in each receptor cell in mammalian ORNs (Malnic *et al.*, 1999; Mombaerts, 2004), this would necessitate alternative modes of stimulus–receptor interaction that result in changes in intracellular calcium via multiple pathways (Scaramellini and Leff, 1998). This type of versatility has been recently demonstrated for the human serotonin receptor (Noda *et al.*, 2004).

It is important to note that different species utilize a diversity of calcium signaling mechanisms to transduce odorant information. However, some features that are often treated as peculiarities appear to be more common than previously thought. For example, decreases in $[Ca^{2+}]_i$ are not routinely seen in rodent or salamander ORNs but are seen in humans (Rawson *et al.*, 1997; Gomez *et al.*, 2000), mudpuppies (Delay and Dionne, 2002) and cats (Figure 3). It is difficult to attribute these observations to artifacts or to differences

in experimental protocol or setups because different investigators using a variety of preparations or setups have observed them, and the frequency and characteristics of these responses is not what one would expect from fluorescence quenching or other stimulus-related artifacts (Figure 2, Table 1; see also Materials and methods). In addition, baseline calcium levels for cells exhibiting decreases were not significantly different from those exhibiting increases in calcium in response to odorant stimuli, suggesting that these results are not simply a function of baseline state. The cellular signaling mechanism for odorant-elicited $[Ca^{2+}]_i$ decreases has not yet been elucidated. While IP_3 has been implicated in mediating an inhibitory response in rat ORNs (Spehr et al., 2002), the diversity of odorants that elicit decreases in human (Rawson et al., 1997) and cat (Table 1) ORNs suggests that these mechanisms are routinely activated during odorant stimulation. It is possible that the response is secondary to an increase that is either below the temporal or spatial limit of resolution of our imaging system, or it may be a primary, inhibitory response. The mechanisms behind odorant-elicited $[Ca^{2+}]_i$ decreases merit a more detailed investigation to understand odor signaling in its totality.

While studies in semi-intact preparations (Ma and Shepherd, 2000; Manzini and Schild, 2004) would be helpful to establish whether these findings reflect in vivo phenomenon entirely, existing data suggest that diverse calcium response types may be part of the natural process of odorant signaling, which may be employed to differing degrees and in different ways from species to species (Ache et al., 1998). The evolutionary distance and ecological diversity of species that appear to utilize this feature leads one to assume that these mechanisms are significant and useful for odorant coding in a variety of environments.

In summary, feline ORNs share features of odorant-elicited $[Ca^{2+}]_i$ signaling that are common to most species studied, and display characteristics that appear to be unique to selected species. Understanding the physiological features of ORNs in a unstudied species is important for discovering the mechanisms of odorant signaling that apply to the olfactory system in general. Such studies will also lead us to greater insight into how sensory systems may adapt throughout evolution to meet the needs of animals surviving in a diversity of ecological niches.

Acknowledgements

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