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Calcium mediates the activation of the inhibitory current induced by odorants in toad olfactory receptor neurons

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Abstract In toad olfactory neurons, a putrid odorant mixture inducing inhibitory responses increases Ca^{2+}-activated K^+ conductance, developing a hyperpolarizing receptor potential. Removal of extracellular Ca^{2+} or exposure to nifedipine reversibly reduced the inhibitory response, suggesting that odorants induce a Ca^{2+} influx. We show evidence for an odorant-induced Ca^{2+} current. Using confocal microscopy, it is shown that odorants induce a nifedipine-sensitive elevation of Ca^{2+} in the apical end of the cell. These results suggest an inhibitory mechanism in which an apical Ca^{2+} influx causes an increase in internal Ca^{2+}, opening Ca^{2+}-activated K^{2+} channels that lead to membrane hyperpolarization.

Key words: Calcium; Ca^{2+}-activated K^+ conductance; Inhibition; Nifedipine; Olfactory neurons

1. Introduction

Floral and frutal odorants trigger excitatory responses in vertebrate olfactory neurons, activating a cAMP cascade [1–3] that leads to a depolarizing receptor potential [4]. Inhibitory responses to odorants have also been reported in vertebrates [5–7], although in contrast to the excitatory cascade, our knowledge of the inhibitory transduction mechanism is quite limited. Inhibition by odors also occurs in invertebrate olfactory cells; in this case, the underlying mechanism is understood in some detail [8].

Previous work demonstrated that a mixture of putrid odorants induced inhibitory responses in isolated toad olfactory neurons [6], by triggering a hyperpolarizing K^+ current that inhibits action potential firing. Activation of this K^+ current appears to be mediated by a second messenger cascade, as suggested by the prolonged latency (hundreds of ms) preceding the development of the K^+ current. The nature of this cascade is unknown.

Recent evidence indicates that the odorant-induced K^+ conductance is activated by Ca^{2+} [9]. The shape of its I-V curve closely resembles that of other Ca^{2+}-activated K^+ conductances [10]. In addition, this odorant-triggered inhibitory conductance is sensitive to charybdotoxin (CTX), a toxin that blocks Ca^{2+}-activated K^+ channels [11]. In support of this notion, a CTX-sensitive Ca^{2+}-activated K^+ conductance has been recently reported to be present in toad olfactory cilia [12].

We previously showed that extracellular Ca^{2+} is important for the inhibitory current, because it is reversibly abolished upon removal of Ca^{2+} from the Ringer solution [9]. There is evidence for an odorant-induced Ca^{2+} influx in olfactory cells [13]. An elevation of intracellular free Ca^{2+} has been shown to be associated with odorant stimulation in fish [14], frog [15], rat [16,17] and human olfactory neurons [18]. Ca^{2+} enters the cell through the cyclic nucleotide-gated channel (CNG channel) and is involved in excitation [19] and adaptation [20,21]. However, in rat, Ca^{2+} appears to enter the cell by a different pathway [22,17], but its physiological effect has not been defined. Since Ca^{2+} ions have a central role in the inhibitory chemotransduction mechanism [9], it is important to investigate the origin of the calcium that participates in the inhibitory response to odorants.

Here we confirm that the odorant-induced outward current of toad olfactory neurons depends on extracellular Ca^{2+}, and show that odorants inducing inhibition trigger a calcium influx through Ca^{2+}-permeant channels, in association with a localized rise in free Ca^{2+}. This Ca^{2+} influx would determine the opening of the Ca^{2+}-activated K^+ channels that are responsible for the inhibition.

2. Materials and methods

Isolated olfactory neurons were obtained from the Chilean toad *Caudiverbera caudiverbera*, as previously described [6]. After decapitation, the animals were pithed and the olfactory epithelia were dissected out. Olfactory neurons were obtained by mechanical dissociation of small pieces of epithelium with a Pasteur 3 pipette.

Whole-cell recording was achieved with an Axopatch 1-D patch clamp (Axon Instruments, Foster City, CA). Currents were filtered with an 8-pole Bessel filter, set at a 0–10 kHz bandwidth.

Odorant stimulation was achieved with a multibarreled pipette operated with a picospritzer. The chemical stimulus consisted of an equimolar mixture of the odorants isovaleric acid, pyrazine and triethylamine, previously shown to inhibit olfactory neurons from *C. caudiverbera* [6,9]. Odorants were applied focally, 20 μm from of the olfactory cilia. Concentrations at the ciliary level of odorants or other agents delivered from the pipette were, as previously [6], estimated as in Firestein and Werblin [23].

The composition of the solutions (in mM, except when indicated) was as follows: Ringer solution, 115 NaCl, 2.5 KCl, 1 $CaCl_2$, 0.4 $MgCl_2$, 10 HEPES, 3 glucose, pH 7.6. Low Ca^{2+}-Ringer solutions contained either 10^{-4} or 10^{-5} M Ca^{2+}, all other components remained the same. Extracellular Ba^{2+} solution, 10 $BaCl_2$, 84.5 *N*-methyl-D-glucamine, 1.5 $MgCl_2$, 20 tetraethylammonium-HCl (TEA), 10 HEPES, 3 glucose, pH 7.6 (adjusted with HCl). Normal internal solution, 120 KCl, 4 HEPES, 1 $CaCl_2$, 2 EGTA, 0.1 Na_2-GTP, 1 Mg-ATP, pH 7.6, 50 nM free Ca^{2+}. Cs^+-internal solution, 120 CsCl replaced KCl, all other components as in the normal internal solution.

Fluorescence images were obtained with a laser-scanned confocal microscope (Zeiss, model Axiovert 135M), implemented with a 1.3 numerical aperture, 100× oil-immersion objective. Pinhole was kept constant throughout the experiments. Illumination was confined to the periods in which images were acquired. Fluo-3,AM (Molecular Probes, Eugene, OR) was used to monitor intracellular Ca^{2+}. Excitation wavelength, 490 nm; emission wavelength, 530 nm. For these experiments, cells were stuck with polylysine to the bottom of the dish, and incubated for at least 20 min at room temperature in Ringer

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containing 5 μM fluo-3,AM (added from a stock of 1.4 mM fluo 3,AM, made in 20% pluronic acid in dimethylsulfoxide).

All chemicals, including the odorants, were purchased from Sigma Chemical Co. (St. Louis, MO), except when indicated.

3. Results

3.1. Effect of modifying extracellular Ca^{2+} on the I-V relation of odorant-activated conductance

It was previously shown that exposure of the cell to extremely low-Ca^{2+} Ringer (10 nM free Ca^{2+}) abolished the odorant-induced inhibitory outward current [9]. Fig. 1A illustrates the Ca^{2+} dependence of this current for an olfactory neuron exposed to external Ca^{2+} concentrations within a range of 0.01–1 mM. As shown, the outward current decreased from 70 pA to 35 pA when the extracellular Ca^{2+} concentration was lowered from 1 mM to 0.1 mM. On the

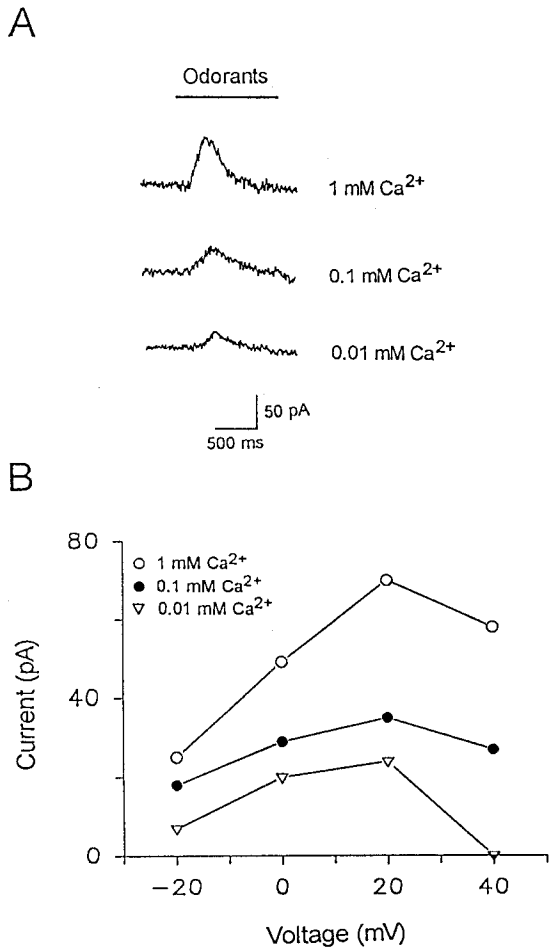

Fig. 1. Odorant-induced inhibitory current depends on extracellular Ca^{2+}. A: The outward current recorded under normal extracellular Ca^{2+} (1 mM, upper trace) decreased as a function of external Ca^{2+} (middle and lower traces). This experiment was done as follows: after recording the odorant-induced outward K^+ current in normal Ringer, the bath solution was replaced by the next Ca^{2+} solution to test. Subsequently, the olfactory neuron was stimulated with odorants in the same Ringer solution of the bath. This allowed a uniform Ca^{2+} level around the cell during focal odorant stimulation. The solutions were tested in the following order: 1, 0.01 and 0.1 mM Ca^{2+}, and then again control (1 mM Ca^{2+}), to check reversibility. The holding voltage, −70 mV, was stepped to +20 mV 700 ms before the onset of odorant stimulation. B: I-V relations for the odorant-induced currents under the Ca^{2+} concentrations tested, for the same cell as in A. Current values correspond to the peaks of the odorant-induced currents.

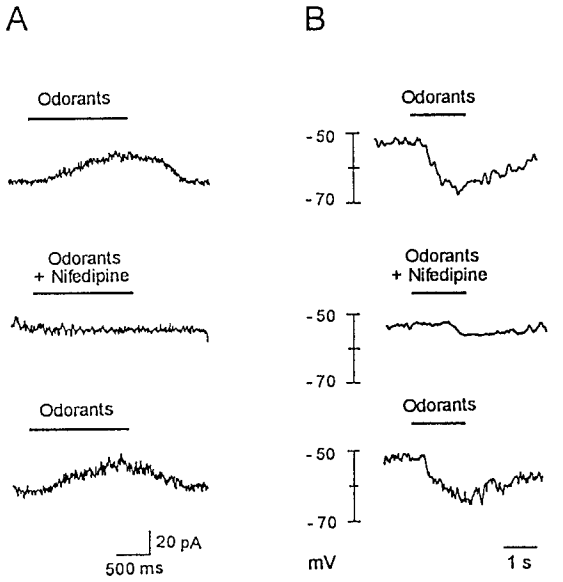

Fig. 2. Nifedipine abolishes odorant-induced outward current and hyperpolarization. A: The odorant-induced outward current (upper trace) did not develop when the stimulus was supplemented with nifedipine (middle trace), but it recovered (bottom trace) when stimulating back with nifedipine-free odorants (V_{hold} = 0 mV). B: Receptor potential recorded under current clamp (I_{hold} = 0 pA) from the same cell as in A (upper trace). When stimulating with odorants plus nifedipine, the receptor potential was partly abolished (middle trace), in a reversible fashion (bottom trace). Odorant concentration was 55 μM and nifedipine was at 25 μM, at the cell level. In these experiments, the bath was perfused with 25 μM nifedipine-Ringer before the application of the odorant/nifedipine stimulus, to ensure uniform exposure of the cell. Subsequently, the bath was perfused back with normal Ringer, to test for reversibility.

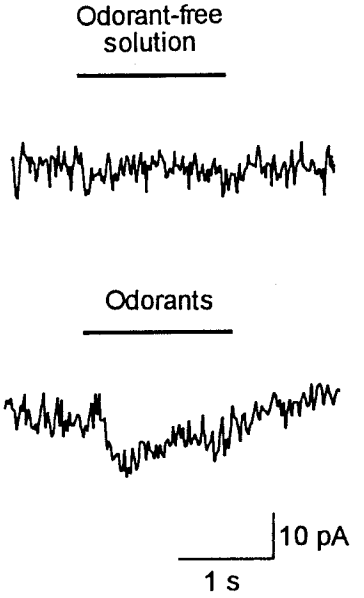

Fig. 3. Odorants of inhibitory effect induce an inward current. An inward current, preceded by a latency of about 250 ms (V_{hold} = −50 mV; bottom trace). Application of odorant-free had no effect (upper trace). The only permeant cation in the Ringer solution was Ba^{2+} (10 mM); in the intracellular solution, Cs^+ replaced K^+.

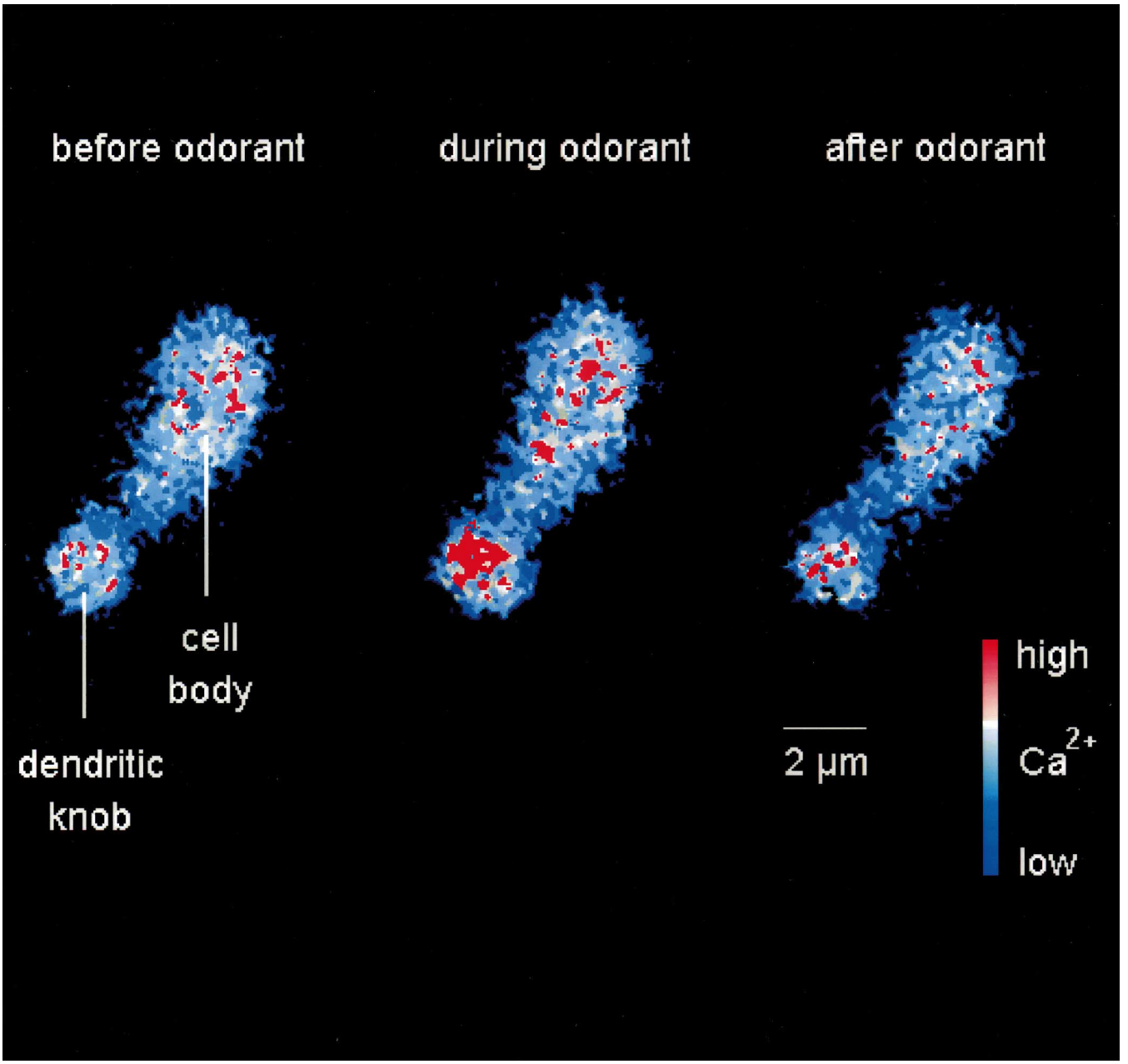

Fig. 4. Inhibitory odorants trigger a transient elevation of intracellular Ca^{2+}. A rise in Ca^{2+} in the apical region of an olfactory neuron was induced by an odorant stimulus (100 μM, 4 s duration) and monitored with a confocal microscope, using the Ca^{2+} indicator fluo-3. Ca^{2+} rise occurs mainly in the apical region of the neuron. The third image was taken 4 s after ending odorant application, and shows that the Ca^{2+} rise was transient.

other hand, a decrease to 24 pA was observed after lowering the Ca^{2+} concentration to 0.01 mM.

We further examined the Ca^{2+} dependence of the odorant-induced conductance by building I-V curves at the same three Ca^{2+} concentrations, for the same cell as in Fig. 1A. The position of the curve along the voltage axis was virtually unaltered upon modification of external Ca^{2+}. Similar observations were made in two other olfactory neurons examined. The average values for the peak outward currents, measured at +20 mV, were 90 ± 35 pA (mean ± S.D.; $n = 7$) at 1 mM Ca^{2+}, 43 ± 20 pA ($n = 4$) at 0.1 mM Ca^{2+} and 26 ± 5 pA at 0.01 external Ca^{2+} ($n = 3$).

These results show that the odor-triggered inhibitory conductance depends on external calcium.

3.2. *Nifedipine reduces the odorant-activated K^+ conductance*

The previous results are consistent with the view that odorant exposure triggers a Ca^{2+} influx through Ca^{2+}-permeant channels, and the resulting increase in Ca^{2+} opens Ca^{2+}-activated K^+ channels, which hyperpolarize the cell.

In order to test this hypothesis, we first examined the effect of nifedipine, a Ca^{2+} channel blocker, on the current induced in olfactory neurons by odorants of inhibitory action. Fig. 2A shows that this drug effectively abolished the outward current

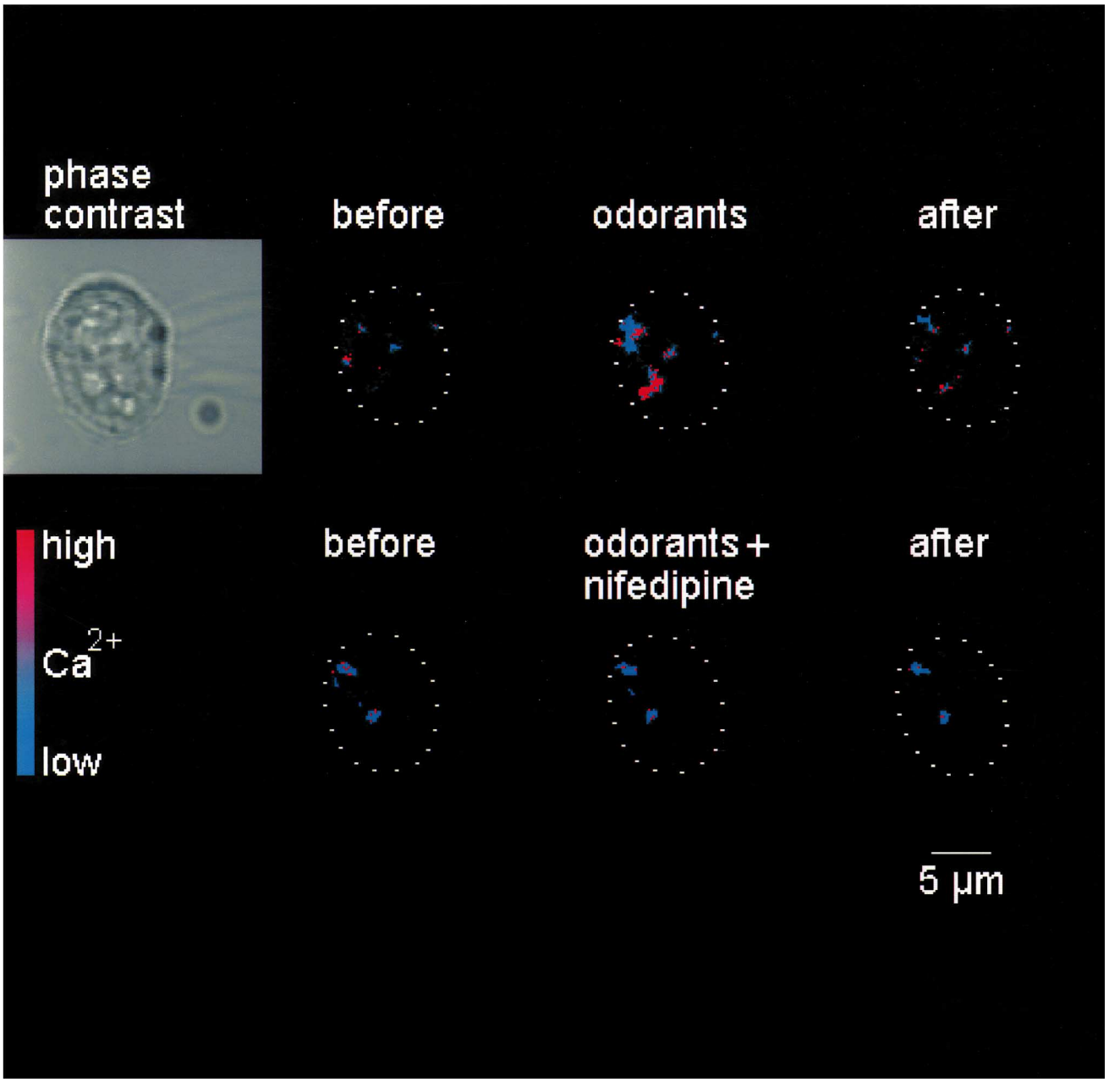

Fig. 5. Nifedipine abolishes odorant-induced elevation of Ca^{2+}. Top, left: Phase-contrast image of the cell. The dendrite and knob are not visible because they are located above the soma in this view of the cell. The cilia can be clearly distinguished to the right of the cell. Top, right: A sequence of three fluorescence images of the same cell is presented, showing that stimulation with odorants transiently elevated free Ca^{2+}. The periphery of the cell is depicted by a broken line. The discrete pseudocolored spots indicate a localized elevation of Ca^{2+} (middle image); they are located in the region where the cilia converge, presumably corresponding to the sites where the cilia insert onto the dendritic knob. Bottom: This sequence of three images was obtained with the same protocol, except that here 20 µM nifedipine was applied with the odorants. Nifedipine abolished the elevation of Ca^{2+} (middle image). Subsequently, the cell was stimulated again with nifedipine-free odorants, and the response partly recovered (not shown), indicating reversibility of the nifedipine effect. The intensity of the signals in this panel is lower than in the top series due to bleaching of the Ca^{2+} indicator.

in a reversible fashion, at a concentration of 25 µM. A similar observation was made on five other receptor neurons.

The high input resistance of olfactory neurons (approximately 5 GΩ [6]) makes it more appropriate to examine the effect of nifedipine under current clamp, since the presence of a small inhibitory current will become more obvious monitoring the membrane potential rather than the whole-cell current. Under current clamp ($I_{hold} = 0$ pA), nifedipine largely (76%), although not completely, suppressed the hyperpolarizing receptor potential in the same cell (Fig. 2B). Similar observations were made on two other olfactory neurons tested in this manner (average suppression: 73 ± 18%, mean ± S.D.; $n = 3$). None of the neurons tested responded to odorant-free nifedipine applications, ruling out the possibility that this drug may be detected as an odorant.

These results support the notion that an influx of Ca^{2+}

through nifedipine-sensitive ion channels at the plasma membrane is part of the chemotransduction mechanism associated with the inhibitory response.

3.3. Odorants with inhibitory effect induce a Ca^{2+} inward current

A direct demonstration for the existence of these putative Ca^{2+} channels would be to record the associated odorant-induced inward current. However, under normal ionic conditions we failed to resolve an inward current that could be attributable to Ca^{2+}, upon stimulation with inhibitory odors. Nevertheless, such a Ca^{2+} current is likely to be rather small, since the amount of Ca^{2+} needed for elevating Ca^{2+} in the cilia (as well as in the dendritic knob) to levels required for the activation of the K^+ channels might be quite minute (see Section 4).

Therefore, we modified the experimental conditions in order to increase the chance of recording such a putative Ca^{2+} inward current. For this aim, we bathed the cells with an extracellular solution in which all permeant cations had been replaced by the non-permeant ion *N*-methyl-D-glucamine and 20 mM TEA, except for Ca^{2+}, which was replaced by Ba^{2+}, a cation that permeates most Ca^{2+} channels better than Ca^{2+} [10]. Furthermore, the Ba^{2+} concentration was 10 mM, 10-fold higher than Ca^{2+} in normal Ringer. Additionally, potassium was replaced by cesium in the internal solution. Under such conditions, an odorant stimulus induced an inward current. In the case illustrated in Fig. 3, this current had a latency of ~250 ms, reached a peak of 7.0 pA and decayed somewhat during odorant exposure. Only 6 of the 42 cells examined this way (14.3%) exhibited detectable inward currents. On average, their peak amplitude was 4.7 ± 2.0 pA (mean ± S.D.) and their mean latency was 258 ± 158 ms (range 100–500 ms). Even though the fraction of olfactory neurons exhibiting the odorant-induced inward current was smaller than expected from the percentage of cells that are responsive to the same odorant mixture (36% [6]), the results suggest that odorants having an inhibitory effect in toad olfactory neurons induce an inward current, most likely carried by Ca^{2+} (see Section 4).

3.4. Odorants of inhibitory effect induce a localized increase of intracellular Ca^{2+} in olfactory receptor neurons

Using confocal microscopy technology, we tested whether odorants of inhibitory effect trigger a rise in intracellular Ca^{2+}. Odorant stimulation of an olfactory neuron loaded with fluo-3,AM induced an increase in Ca^{2+} levels confined to the apical region of the cell (Fig. 4). Samples were taken 2 s before odorant stimulation, 2 s after the onset of a 4 s stimulus and 4 s after the odorant puff, delivered from a pipette positioned 20 μm away from the cilia. A pronounced increase in Ca^{2+} occurred in the apical end of the cell during odorant exposure, as indicated by the red spot of the pseudocolored picture. Similar observations were made in seven other neurons, of a total of 19 examined (37%); the rest of the cells did not respond. This result is in close agreement with the percentage of cells responding to the same odors with an outward current (36% [6]).

3.5. Nifedipine prevents odorant-induced elevation of Ca^{2+}

According to our interpretation that nifedipine is acting against Ca^{2+}-permeant channels at the plasma membrane, opened upon stimulation with odorants that trigger inhibitory responses, it was expected that this drug may also prevent the odorant-triggered elevation in intracellular Ca^{2+}. To test this idea, we stimulated olfactory neurons with odorants supplemented with 20 μM nifedipine. Fig. 5 shows that a cell in which odorant stimulation induced an increase in internal Ca^{2+} (Fig. 5, top, right) failed to produce a Ca^{2+} signal in the presence of nifedipine (Fig. 5, bottom, middle image). The cell was then stimulated with nifedipine-free odorant solution and the response was recovered (not shown). A similar observation was made in two other olfactory neurons tested this way.

These results support the view that the influx of Ca^{2+} is crucial for the elevation of Ca^{2+} in the apical region of the cell during stimulation with inhibitory odorants.

4. Discussion

Previous work demonstrated that toad olfactory neurons may be both excited and inhibited upon chemical stimulation, depending on the odorant [6]. Inhibition, expressed as a transient cessation of spontaneous action potential firing induced by odorants, is due to an increase in a CTX-sensitive, Ca^{2+}-activated K^+-conductance confined to the apical region of the olfactory neuron [9]. The associated K^+-current hyperpolarized the cell, causing the inhibition.

Extracellular Ca^{2+} appeared to be essential for the generation of odorant-triggered inhibitory responses in *C. caudiverbera* olfactory receptor cells, since removing Ca^{2+} from the bathing solution (to 10 nM free Ca^{2+}) completely and reversibly abolished the odorant-induced outward current [9]. Here we show that the I-V relation for the odorant-induced inhibitory current was highly sensitive to modification of extracellular Ca^{2+}, its maximal amplitude being dependent on the level of Ca^{2+}.

The dependence of the odorant-induced K^+ current on extracellular Ca^{2+} is an indication that an influx of Ca^{2+} through a Ca^{2+}-permeant conductance may be crucial for inhibitory chemotransduction. This notion received strong support from the finding that the Ca^{2+} channel blocker nifedipine abolished both the inhibitory response to odorants (Fig. 2) and the associated increase in internal Ca^{2+} (Fig. 5). Since nifedipine was supplemented to normal Ringer, the effect of lowering external Ca^{2+} was due to a reduced Ca^{2+} influx, rather than to an effect of low external Ca^{2+} levels on the membrane.

The CNG channel that participates in the excitatory chemotransduction mechanism is sensitive to micromolar nifedipine, when applied onto the cytoplasmic side of inside-out patches from salamander olfactory neurons [24]. The similar nifedipine sensitivity exhibited by the putative Ca^{2+} conductance involved in the inhibitory response of the toad, although tested from the external side of the membrane, raises the possibility that the CNG channel also plays a role in odorant inhibition. However, the fact that in the same olfactory neuron, odorants causing inhibition do not excite the cell and excitatory odorants do not inhibit it [6] is inconsistent with this possibility, but in contrast it agrees with the notion that both channels are different molecular entities controlled by distinct transduction pathways.

Under physiological ionic conditions, we were unable to resolve a Ca^{2+} current activated by the inhibitory mixture

due to its small magnitude, which is below the limit of resolution of the patch clamp technique. We estimate its value as ~0.1 pA. To obtain this value (which does not consider Ca^{2+} buffering in the ciliary lumen), we calculated how much Ca^{2+} must enter the cilia (a total of 10 cilia, each considered a cylinder of 40 μm long and 0.25 μm in diameter) to reach 46 μM, a concentration at which Ca^{2+}-activated K^+ channels from olfactory cilia incorporated into artificial bilayers open 50% of the time [12]. In order to record this inward current, we removed all other permeant ions from both the internal and external solutions and replaced external Ca^{2+} by 10 mM Ba^{2+}. This way we were able to record a current of an average value of ~5 pA. The latency preceding the development of this inward current (range 100–500 ms) is compatible with the latency of the inhibitory outward current (range 200–500 ms). The percentage of receptor cells presenting a detectable inward current (14.3%) was about half of that in which the odorants induced an outward current under normal conditions (36% [6]), most likely because in the other half of the cells the size of the inward current was too small to be resolved. In spite of the small size of this inward current under physiological conditions, our calculations suggest that it would be sufficient to open enough Ca^{2+}-activated K^+ channels to cause inhibition. Nevertheless, an additional possibility that cannot be ruled out by our results is that this small Ca^{2+} current may be part of a Ca^{2+}-induced Ca^{2+} release mechanism.

Tareilus et al. [17] observed that both odorants that elevate cAMP levels and odorants that induce the production of $InsP_3$ cause an elevation of Ca^{2+} preferentially in the dendritic knob of rat olfactory neurons. Although both odorant types had a similar effect on Ca^{2+} levels, their pharmacological evidence is consistent with two different underlying mechanisms. Ca^{2+} elevation mediated by a cAMP pathway is due to a Ca^{2+} influx through the CNG channel [20]. In contrast, the target for the $InsP_3$ pathway in the rat appears to be an $InsP_3$ receptor at the ciliary membrane [13,25], but it remains unclear what the physiological effect is of this putative pathway in the olfactory neuron. Utilizing the same preparation and two odorant mixtures analogous to those of Tareilus et al. [17], Restrepo et al. [16] reported that both stimuli increased Ca^{2+}, finding no difference between their effects. Using confocal microscopy, we have monitored increases in Ca^{2+} in the apical region of toad olfactory neurons, induced by putrid odorants, in agreement with the notion that Ca^{2+} is involved in the inhibitory mechanism. The fraction of cells that responded to increases in Ca^{2+} upon odorant stimulation (37%) was similar to the fraction of cells that elicited inhibitory responses to the same odorants (36% [6]), as expected if Ca^{2+} increase is an intermediate step of the inhibitory transduction mechanism. It remains to be established how odorants lead to the influx of Ca^{2+} to produce inhibition in the toad.

In a variety of cellular systems, a Ca^{2+} influx triggers the release of Ca^{2+} from intracellular reservoirs. Internal membrane systems are not present in olfactory cilia [26]. However, membrane vesicles exist in the dendritic knob [27,28] and it is conceivable that they may operate as Ca^{2+} reservoirs with the ability to release Ca^{2+} upon odorant stimulation; but there is no evidence for this at present. Although our results do not rule out this possibility, they illustrate that Ca^{2+} influx is essential for mediating odorant-induced inhibition.

The evidence provided here is consistent with the view that Ca^{2+} is an important component of the inhibitory transduction mechanism in toad olfactory receptor neurons. This cation would enter the cell through a nifedipine-sensitive Ca^{2+} conductance and accumulate within the apical end of the cell, activating K^+ channels that hyperpolarize the neuron.

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