Odorant-regulated Ca^{2+} Gradients in Rat Olfactory Neurons

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ABSTRACT   Olfactory neurons respond to odors with a change in conductance that mediates an influx of cations including Ca^{2+}. The concomitant increase in $[Ca_i]$ has been postulated to play a role in the adaptation to maintained odorant stimulation (Kurahashi, T., and T. Shibuya. 1990. *Brain Research.* 515:261–268. Kramer, R. H., and S. A. Siegelbaum. 1992. *Neuron.* 9:897–906. Zufall, F., G. M. Shepherd, and S. Firestein. 1991. *Proceedings of the Royal Society of London, B.* 246:225–230.) We have imaged the distribution of $[Ca_i]$ in rat olfactory neurons (RON) using the Ca^{2+} indicator fura-2. A large percentage of the RON (42%, $n = 35$) responded to odorants with an increase in $[Ca_i]$. About half of the responding neurons displayed an increase in $[Ca_i]$ at the apical end of the cell, but not at the soma. Moreover, in those cells that responded to odors with a standing $[Ca_i]$ gradient, the gradient could be maintained for long periods of time (minutes) provided that the cells were continuously stimulated. In contrast, K^+-induced depolarization elicited a more homogeneous increase in $[Ca_i]$. The spatially inhomogeneous increase in $[Ca_i]$ elicited by odorants in some cells has important implications for the role of Ca^{2+} in adaptation because channels and enzymes regulated by Ca^{2+} will be affected differently depending on their location.

INTRODUCTION

Olfactory neurons are asymmetric bipolar cells with a round cell body and a single thin dendrite that ends in a protuberance called the olfactory knob (Fig. 1) (Getchell, 1986; Ronnett and Snyder, 1992). Cilia that extend from the olfactory knob are believed to contain the molecular machinery necessary to initiate the olfactory response (Lowe and Gold, 1991). Stimulation of olfactory neurons with odorants leads to a second messenger (cAMP or IP_3)-mediated increase in membrane conductance (Ronnett and Snyder, 1992), and an influx of cations including Ca^{2+}, which results in an increase in intracellular calcium concentration ($[Ca_i]$) (Inoue, Yamashita, Harada, and Kumazawa, 1992; Restrepo, Miyamoto, Bryant, and Teeter, 1990; Restrepo and Boyle, 1991; Restrepo, Okoda, Teeter, Lowry, Cowart, and Brand, 1993; Sato, Hirono, Tonoike, and Taketayashi, 1991; and Schild and Lischka, 1992).

The odorant-induced increase in $[Ca_i]$ is believed to play an important role in the modulation of the olfactory response because Ca^{2+} is known to affect several different components of the transduction pathway. For example, in olfactory neurons Ca^{2+} blocks the cAMP-gated channel (Zufall, Shepherd, and Firestein, 1991; Kramer and Siegelbaum, 1992) and activates Ca^{2+}-dependent K^+-channels (Maue and Dionne, 1987), both of which would contribute to membrane repolarization, and it opens Ca^{2+}-dependent Cl^--channels (Kleene and Gesteland, 1991), which has been postulated to cause membrane depolarization (Kurahashi and Yau, 1993). In addition, Ca^{2+} may exert indirect effects via modulation of enzymes in the second messenger pathway, e.g., calmodulin-mediated stimulation of adenylyl cyclase (Anholt and Rivers, 1990) or cAMP phosphodiesterase (Borisy, Ronnett, Cunningham, Juilfs, Beavo, and Snyder, 1992). Therefore, the overall effect of Ca^{2+} on the output of an olfactory neuron is a complex summation of several effects.

Some of the Ca^{2+}-regulated conductances present in the olfactory neuron are thought to be localized in the olfactory cilia at the apical end of the cell (e.g., cAMP-gated channels, IP_3-gated channels, Ca^{2+}-dependent Cl^- conductance),
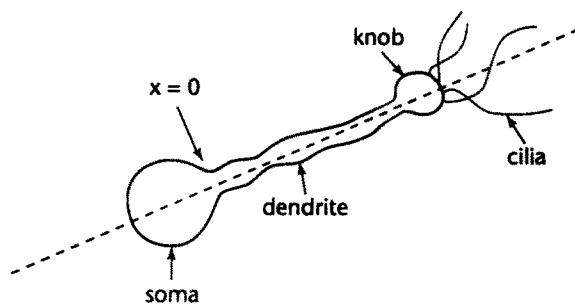
FIGURE 1. Olfactory neurons are bipolar cells with a round soma and a single dendrite, which ends in a protuberance called the olfactory knob. Thin ciliary processes that attach to the olfactory knob are believed to contain most of the biochemical machinery necessary for the initiation of the olfactory response. The principal axis of the cell (dashed line), and the point where the dendrite attaches to the soma (arrow, $x = 0$) were used in the quantification of the standing gradients of $[Ca_i]$ (see Materials and Methods).

whereas others are believed to be located in the soma membrane (e.g., Ca^{2+}-dependent K^+ channels). Because of this, the magnitude and nature (hyperpolarizing vs depolarizing) of the effect of Ca^{2+} on the response of olfactory neurons to odorants depends on the spatial distribution of intracellular Ca^{2+} concentration. We find that sustained odorant stimulation leads to formation of a steady state standing gradient of intracellular Ca^{2+} in some, but not all olfactory neurons. Olfactory neurons can maintain these standing gradients for periods lasting several minutes. This has important implications for the modulation of the olfactory response by Ca^{2+}.

MATERIALS AND METHODS

Solutions

Mammalian Ringer contained (in mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 20 Na-HEPES, pH 7.2. K-Ringer was the same as mammalian Ringer except that sodium was replaced by potassium. Divalent cation-free Ringer contained (in mM): 145 NaCl, 5 KCl, 1 EDTA, 20

NaHEPES, pH 7.2. The stop solution contained (in mM): 145 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 20 NaHEPES, 1 Napyruvate, 10 μg/ml leupeptin and 100 μg/ml gentamicin. Unless otherwise specified, all reagents were from Sigma Chemical Co. (St. Louis, MO). The acetoxymethyl ester of fura 2 (fura 2/AM) and pluronic F127 were obtained from Molecular Probes, Inc. (Eugene, OR). Fura 2/AM was dissolved in DMSO at a concentration of 1 mM. Odorants were either from International Flavors and Fragrances (New York, NY), or from Firmenich (Geneva, Switzerland). Two mixtures of odorants were used. Mixture *A* (100 μM each of hedione, geraniol, phenylethylalcohol, citralva, citronellal, eugenol and menthone) contained odorants that have been shown to stimulate cAMP formation in isolated rat olfactory neurons, whereas the odorants in mixture *B* (100 μM each of lyral, lilial, triethylamine, ethyl vanillin, isovaleric acid, and phenylethyl amine) have been shown to stimulate IP₃ formation (Breer and Boekhoff, 1991).

Olfactory Neuron Isolation

Sprague Dawley rats ranging in weight from 200 to 300 gm were used for this study. Immediately after killing, the olfactory epithelium was excised and minced with scissors in 3 ml of divalent cation-free Ringer supplemented with 15 U/ml activated papain and 5 mM L-cysteine. After a 10 min incubation half of the solution was decanted, and the cells were detached from the tissue by pipetting vigorously through small bore capillaries (0.1–1 mm diam). After dissociation, the cell suspension was filtered through a 300 μm nylon mesh, and 3 ml of stop solution containing 8 μM fura2/AM and 80 μg/ml pluronic F127 were added.

Measurement of Intracellular Ca²⁺

Rat olfactory neurons were loaded with the Ca²⁺-sensitive dye fura-2 (Tsien and Harootunian, 1990) by preincubation for 1 h in stop solution (see above) supplemented with 8 μM of the acetoxymethyl ester of fura-2. Cells were allowed to attach to a coverslip coated with concanavalin A, and were continuously perfused with Ringer or Ringer containing odorant mixtures *A* or *B* during the experiments. Exchange of solutions took place within <5 s. The apparatus used to image [Ca$_i$] was a setup previously used by us to measure [Ca$_i$] in single catfish olfactory neurons (Restrepo and Boyle, 1991) modified to allow imaging of [Ca$_i$] (Tsien and Harootunian, 1990). Modifications to the previous system were as follows: light emitted by the Xenon lamp was alternately filtered with narrow bandpass filters at 340 (Ca²⁺ sensitive) or 360 nm (Ca²⁺ insensitive). A shutter was used to minimize exposure of cells to UV light to prevent bleaching of the dye and cell damage. Filter switching and shutter operation were computer-controlled. Fluorescent light emitted by the cells under 40× magnification (Nikon Fluor 40× 1.3 NA) was imaged by an OPELCO KS-1380 image intensifier coupled with a Sanyo CCD camera. Images were digitized and averaged 16 times by a Quantimet 570 image analysis workstation (Leica, Inc., Deerfield, IL). The Quantimet performs image ratioing and produces a pseudocolor image of the distribution of [Ca$_i$]. Calculation of [Ca$_i$] was as described in Restrepo and Teeter (1990). To quantify standing calcium gradients in isolated olfactory neurons we determined the average intracellular calcium concentration as a function of distance from the point where the dendrite attaches to the cell body (*arrow*, x = 0 in Fig. 1). This distance was measured along the principal axis of the cell (*dashed line*, Fig. 1). To calculate average [Ca$_i$] the olfactory neuron was sliced into segments of equal length by cutting with equidistant lines perpendicular to the principal axis of the cell.

RESULTS

Rat Olfactory Neurons Maintain a Standing [Ca$_i$] Gradient

We measured the distribution of [Ca$_i$] in 43 isolated rat olfactory neurons. Fig. 2, *A* and *B*, show histograms for the average [Ca$_i$] values at the soma and olfactory knob.

The average [Ca$_i$] was 83 ± 57 nM, mean ± SD, n = 43 in the soma and 77 ± 71 nM in the olfactory knob. As shown in the figure, [Ca$_i$] levels varied greatly from cell to cell. The olfactory neurons that displayed higher [Ca$_i$] levels could have been damaged during the dissociation procedure. However, some of these cells were fully responsive upon stimulation with odorants (see below), and all maintained steady Ca^{2+} levels for 10 to 20 min. This suggests that a small percentage of the olfactory
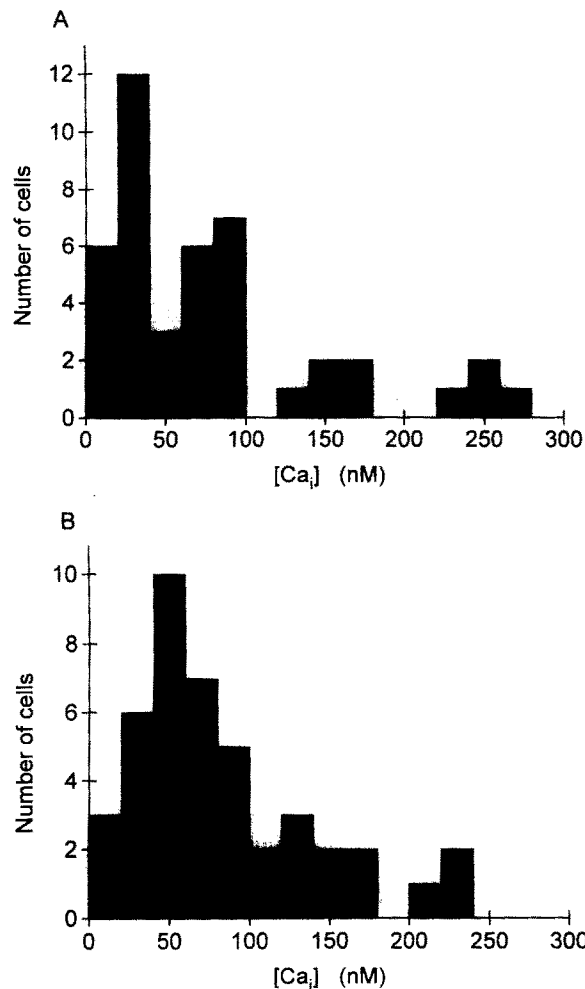

FIGURE 2. (A and B) Basal [Ca$_i$] maintained by isolated rat olfactory neurons. Histograms display the distribution of average [Ca$_i$] values in the olfactory knob (A) and soma (B) (n = 43). Width of the bar graphs represents bin width.

neurons maintain high intracellular [Ca$_i$] levels compared to the average of the population of cells. This could have functional implications for the role of Ca^{2+} in olfactory transduction in this sub-population of neurons.

The average intracellular Ca^{2+} levels at the soma (83 ± 57 nM, n = 43) and the olfactory knob (77 ± 71 nM) were not significantly different for the entire population of neurons. However, as shown in Fig. 3 A, some olfactory neurons clearly maintained

FIGURE 3. Standing gradients of Ca^{2+} maintained by some olfactory neurons at rest. (*A*) Average [Ca_i] plotted as a function of distance from the point where the dendrite attaches to the soma shown for three different olfactory neurons. To calculate the average [Ca_i] in each segment, the olfactory neuron was sliced into segments of equal length by cutting with equidistant lines perpendicular to the principal axis of the cell (*dashed line* in Fig. 1). Data are presented as mean ± SEM. The number of images used in computing each average was 5 for triangles, 5 for circles, and 6 for squares. (*B*) Traces displaying the change in average [Ca_i] induced by addition of 5 μM ionomycin to an isolated olfactory neuron. (*Squares*) [Ca_i] at the olfactory knob. (*Circles*) [Ca_i] at the soma. (*C*) Histogram displaying the distribution of the values of the asymmetry factor α (defined by Eq. 1) in 43 isolated olfactory neurons. A positive value indicates that the average Ca^{2+} concentration at the olfactory knob is higher than the Ca^{2+} concentration at the soma.

a standing gradient of $[Ca_i]$. In some cells $[Ca_i]$ was higher at the knob (Fig. 3 *A*, *squares*), whereas in others, the soma displayed higher $[Ca_i]$ (Fig. 3 *A*, *triangles*). In addition, some cells displayed a uniform distribution for $[Ca_i]$ (Fig. 3 *A*, *circles*). The standing gradient of $[Ca_i]$ was rapidly dissipated by addition of 5 μM of the Ca^{2+} ionophore ionomycin (Fig. 3 *B*), indicating that the measured standing gradients were not an artifact arising from the measurement of $[Ca_i]$.

To quantitate the standing gradient of $[Ca_i]$, we computed the asymmetry factor α defined as the logarithm of the ratio of the calcium concentration in the knob $([Ca_i]_k)$ to the calcium concentration in the soma $([Ca_i]_s)$:

$$\alpha = \log ([Ca_i]_k / [Ca_i]_s) \tag{1}$$

α is positive when $[Ca_i]$ is higher at the knob, it is zero when there is no standing gradient, and it is negative when $[Ca_i]$ is larger at the soma. As shown in Fig. 3 *C*, α ranged from -1.3 to 0.55 with a mean value of -0.1 ± 0.32 (mean ± SD, $n = 43$).

TABLE I

Percent Responses Elicited by Various Stimuli

Stimulus and effect	Responding	n
	%	
Responding to either A or B with an increase in $[Ca_i]$	43	35
Responding to A only	11	35
Responding to B only	17	35
Responding to A and B	14	35
Responding to 2 μM forskolin with an increase in $[Ca_i]$	50	6
Responding to K^+ with an increase in $[Ca_i]$	92	26
Responding to K^+ with a change in shape	46	26

Sustained Odorant Stimulation Elicits Spatially Heterogeneous Increases in $[Ca_i]$

We stimulated olfactory neurons by prolonged exposure to two different odorant mixtures. Mixture A contained odorants that elicit cAMP formation in biochemical assays with isolated rat olfactory cilia, whereas mixture B contained odorants that trigger IP_3 formation (Breer and Boekhoff, 1991). Stimulation of the cells with mixtures A and B elicited an increase in $[Ca_i]$ in 43% (15) of the cells (see Table I). Odorant-induced changes in $[Ca_i]$ could be observed with repeated stimulation. In 6 out of 15 cells the response was primarily localized to the anterior end of the cell (knob and dendrite), with little (<10%) or no change in $[Ca_i]$ at the soma. An extreme example in which the odorant-induced elevation in $[Ca_i]$ occurred only at the knob is shown in Fig. 4, *A–C*. In contrast, in 3 out of 15 cells the response was spatially homogeneous (an example is shown in Fig. 4, *D–F*). Furthermore, in the other six cells, the response occurred at both the olfactory knob and the cell body, and the percent increase at the knob was larger than at the cell body (not shown).

As shown in Fig. 4, prolonged stimulation with odorants modified the steady state distribution of $[Ca_i]$. To document more completely the spatial characteristics of the response of isolated rat olfactory neurons to odorants, we plotted the fractional

increase in [Ca$_i$] at the knob as a function of the fractional increase in [Ca$_i$] at the soma (Fig. 5 *A*). Because we did not find differences in the spatial distribution of the changes in [Ca$_i$] elicited by odorant mixture *A* compared with mixture *B* we did not make a distinction between responses to either mixture in this analysis. Fig. 5 *A* shows that the fractional increase in [Ca$_i$] at the olfactory knob was larger than or equal to the fractional increase at the soma. On the average the increase in [Ca$_i$] at the olfactory knob was 1.8-fold, compared to an average 1.3-fold increase at the soma. In addition, none of the cells responded with an increase in [Ca$_i$] at the soma only. Furthermore, as shown in Fig. 5 *B*, stimulation of the olfactory neurons with odorants either did not modify the standing gradient of Ca^{2+} (quantitated using the asymmetry factor α), or increased the knob to soma gradient.

Forskolin Induces an Increase in [Ca$_i$]

Stimulation of olfactory neurons with mixtures of hydrophobic odorants with diverse chemical structures could cause a nonspecific increase in [Ca$_i$] due to a direct effect on the Ca^{2+} permeability of the cell membrane. While it is unlikely that nonspecific effects would cause a localized increase in [Ca$_i$], such as the response shown in Fig. 4 *A*, it is conceivable that odorant mixtures *A* and/or *B* could have induced nonspecifically a spatially homogeneous response such as the increase in [Ca$_i$] shown in Fig. 4 *D*. This raises the question of whether a direct (nonreceptor mediated) increase in cellular second-messenger concentration induces both localized and spatially homogeneous increases in [Ca$_i$]. We induced formation of cAMP by direct stimulation of the adenylyl cyclase with forskolin, and compared the spatial distribution of the forskolin-induced and the odorant-induced changes in [Ca$_i$]. Forskolin (2 μM) induced an increase in [Ca$_i$] in three out of six olfactory neurons tested (Fig. 6). The response was sustained, and in contrast with the odorant-induced response, it could only be elicited once. In one olfactory neuron the response was spatially localized to the apical portion of the cell (Fig. 6, *A* and *C*), whereas in the other two cells the response was evenly distributed throughout the cell (Fig. 6, *B* and *D*). These experiments indicate that, like the odorant-induced response, an increase in cAMP alone elicits both localized and spatially homogeneous increases in [Ca$_i$] in isolated olfactory neurons. If the increase in cAMP had caused spatially inhomogeneous increases only, then the homogeneous increases in [Ca$_i$] caused by odorants would have been suspect of being a nonspecific effect. A similar experiment could not be performed to test the effect of increases in cellular IP$_3$, because a compound that would specifically stimulate phospholipase C is not available.

The Odorant-induced Increase in [Ca$_i$] Is Likely to be Mediated by Influx of Ca^{2+}

An increase in intracellular Ca^{2+} concentration can be mediated by influx of Ca^{2+} or by release of Ca^{2+} from internal stores. Fig. 7 *A* shows that removal of extracellular Ca^{2+} prevented the increase in [Ca$_i$] elicited by odorant mixture *A*. Similar experiments indicated the same was true for odorant mixture *B* (not shown). In addition, as shown in Fig. 7 *B*, we found that the increase in [Ca$_i$] elicited by odorant mixture *A*
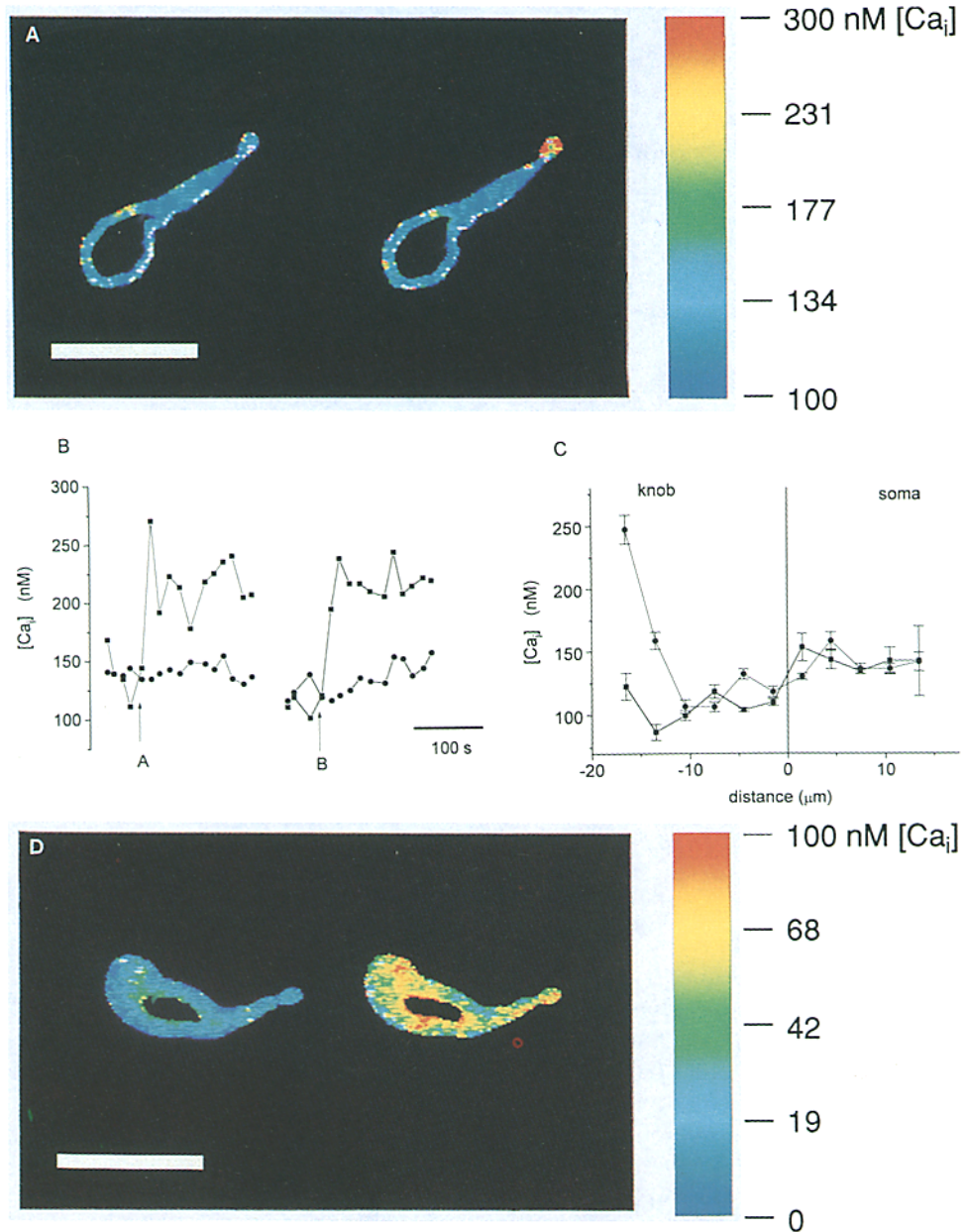
FIGURE 4.

was inhibited by 1-*cis*-diltiazem, an inhibitor of the olfactory cAMP-gated channel, which is peremeable to Ca^{2+} (Kolesnikov, Zhainazarov, and Kosolapov, 1990). These experiments suggest that the odorant-induced increase in $[Ca_i]$ is mediated by influx of extracellular Ca^{2+}, which could possibly be mediated by the second messenger-gated channel(s).

Depolarization-induced Increases in [Caᵢ] Cause a Dissipation of the Standing Ca²⁺ Gradient

Stimulation with odorants causes olfactory neuron depolarization. This odorant-induced depolarization is likely to trigger opening of voltage-gated Ca^{2+} channels leading to an influx of Ca^{2+}, which could participate in the odorant-induced elevation of [Caᵢ]. To determine how the standing [Caᵢ] gradient is affected by opening of voltage-gated Ca^{2+} channels we depolarized rat olfactory neurons by addition of extracellular K^+. We replaced all extracellular Na^+ with K^+. This maneuver is expected to depolarize the cell near 0 mV ensuring that both high and
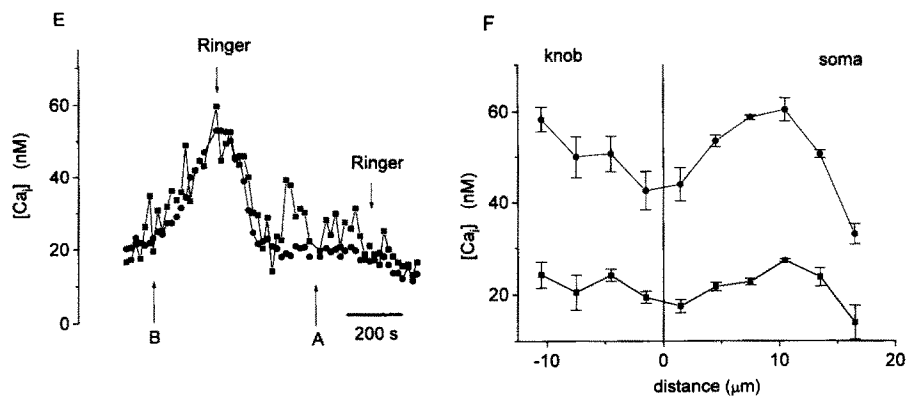
FIGURE 4. Two examples of the changes in [Caᵢ] elicited by maintained stimulation of rat olfactory neurons with odorant mixtures *A* and/or *B*. One cell (*A–C*) responded to stimulation with either mixture with an increase in [Caᵢ] at the olfactory knob, whereas the other neuron (*D–F*) responded to mixture *B* with a spatially homogeneous increase in [Caᵢ]. *A* and *D* show pseudocolor representations of the spatial distribution of [Caᵢ] in the olfactory neurons before (*left*) and during (*right*) stimulation with odorant mixture *A;* the bar shown has a length of 20 μm; *B* and *E* display the time courses for changes in average [Caᵢ] at the knob (*squares*) and the cell body (*circles*); and *C* and *F* display the average [Caᵢ] for the response to odorant mixture *A* as a function of the distance from the point where the dendrite attaches to the cell body, measured along the principal axis of the cell (*dashed line* in Fig. 1). The squares represent the average [Caᵢ] before the response, whereas the circles are the average [Caᵢ] during the response to odorant mixture *A*. Data are mean ± SEM with *n*'s of 5 for *C* (*squares*), 9 for *C* (*circles*), 5 for *F* (*squares*), and 5 for *F* (*circles*).

low threshold voltage-gated Ca^{2+} channels are activated. As shown in Fig. 8 and Table I, 92% of the olfactory neurons tested responded to K^+ with an increase in [Caᵢ]. The rate of increase of [Caᵢ] varied by several orders of magnitude from cell to cell (Fig. 8, *A* and *B*), suggesting that the magnitude of the influx of Ca^{2+} caused by cell depolarization varied greatly from cell to cell. In addition, and as expected for depolarization-induced influx of Ca^{2+}, the increase in [Caᵢ] caused by addition of extracellular K^+ was abolished by removal of extracellular Ca^{2+} (not shown).

In approximately half of the cells, the K^+-induced response was accompanied by retraction of the dendrite resulting in a change in cell shape (Table I). In those cells
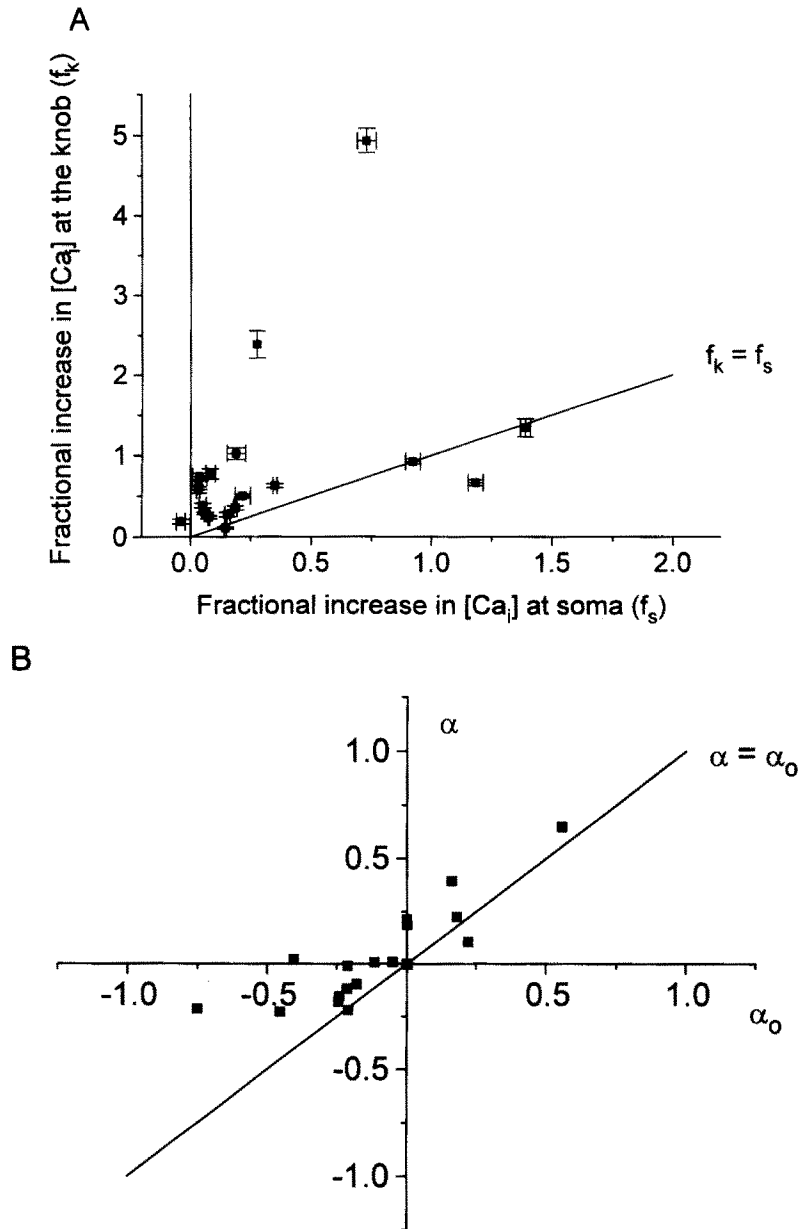
FIGURE 5. A. Spatial heterogeneity in the increase in [Ca$_i$] elicited by stimulation of isolated rat olfactory neurons with odorant mixtures *A* and *B*. For each response, the fractional increase in [Ca$_i$] at the olfactory knob is plotted as a function of the fractional increase in [Ca$_i$] at the cell body. The solid line has a slope of one. Data are mean ± SEM. When measured at the olfactory knob all responses were found to be significantly different from zero at $P < 0.02$ (*t*-test). The average intracellular Ca^{2+} concentrations before and during stimulation, respectively, were 93 ± 14 and 111 ± 15 nM at the cell body and 77 ± 12 and 118 ± 16 nM at the olfactory knob (mean ± SEM, $n = 20$ responses). (*B*) The odorant-elicited change in the [Ca$_i$] gradient

that did not change shape we were able to follow the change in the standing [Ca$_i$] gradient. As illustrated by the example shown in Fig. 8 A, we found that K$^+$-induced depolarization had a tendency to reduce the knob to soma [Ca$_i$] gradient. This is more thoroughly documented by a plot of the asymmetry factor (α) during stimulation as a function of the asymmetry factor before stimulation (α_o) (Fig. 8 C), which shows that stimulation of the olfactory neurons with K$^+$ always resulted in a decrease in the magnitude of the knob to soma [Ca$_i$] gradient (i.e., a reduction in the magnitude of α). This contrasts with odorant-induced responses, which tended to increase the asymmetry factor α (Fig. 5 B). These results suggest that voltage-gated Ca^{2+} channels are more evenly distributed throughout the membrane of the olfactory neuron (dendrite and soma) than the odorant-stimulated conductances.

DISCUSSION

Our experiments clearly show that rat olfactory neurons respond to maintained stimulation with odorants with a tonic increase in intracellular Ca^{2+}, and that in some olfactory neurons this response is spatially localized to the apical end of the cell (Figs. 4, A–C, and 5, A and C). As stated in the results, the magnitude of the odorant-induced increase in [Ca$_i$] at the olfactory knob was on the average 1.8-fold over a mean resting [Ca$_i$] of 77 nM. Such an increase in [Ca$_i$] would be expected to cause a significant increase in the mean open probability for Ca^{2+}-activated K$^+$ channels in olfactory neurons (Maue and Dionne, 1987). Indeed, addition of 5 μM ionomycin, which causes an increase in [Ca$_i$] similar in magnitude to the odorant-induced response, causes an increase in outward current in rat and human olfactory neurons (Restrepo et al., 1993; Okada, Teeter and Restrepo, manuscript in preparation), and a decrease in extracellularly recorded spike rate in frog olfactory neurons (Frings, 1993), as would be expected if Ca^{2+} causes opening of Ca^{2+}-activated K$^+$-channels.

The diversity of the spatial distributions of the odorant-induced changes in [Ca$_i$] in different olfactory neurons has important implications for the role of Ca^{2+} in olfactory transduction because Ca^{2+}-regulated enzymes and channels are affected differently depending on their location within the cell. It has been proposed that Ca^{2+} plays an important role in adaptation to sustained odor stimulation (Kramer and Siegelbaum, 1992; Kurahashi and Shibuya, 1990; Zufall et al., 1991). Ca^{2+} could mediate adaptation through a variety of mechanisms including blockage of the cAMP-gated channel (Kramer and Siegelbaum, 1992; Zufall et al., 1991) and opening of Ca^{2+} activated K$^+$ channels (Maue and Dionne, 1987). An apically localized increase in Ca^{2+} would affect the cAMP-gated conductance, and the Ca^{2+}-activated Cl$^-$-conductance, which are primarily located in the cilia (Nakamura and Gold, 1987, Kleene and Gesteland, 1991), but is unlikely to open significant numbers of Ca^{2+}-activated K$^+$ channels which are found on the soma membrane (Maue and

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maintained by the isolated rat olfactory neurons is illustrated by plotting the value of the asymmetry factor (α, defined by Eq. 1) during the response as a function of the value of the asymmetry factor before stimulation (α_o). Odorants elicited either no change or an increase in α, indicating that the response to odorants had a tendency to increase the knob to soma [Ca$_i$] gradient.

Dionne, 1987). On the other hand, a more spatially homogeneous increase in [Ca$_i$] is likely to have a larger effect on the Ca^{2+}-activated K$^+$ channels. Therefore, the difference in spatial distribution of the [Ca$_i$] response to odorants may underlie differences between individual olfactory neurons in the time course and magnitude of adaptation, as well as in the time course of recovery from adaptation.
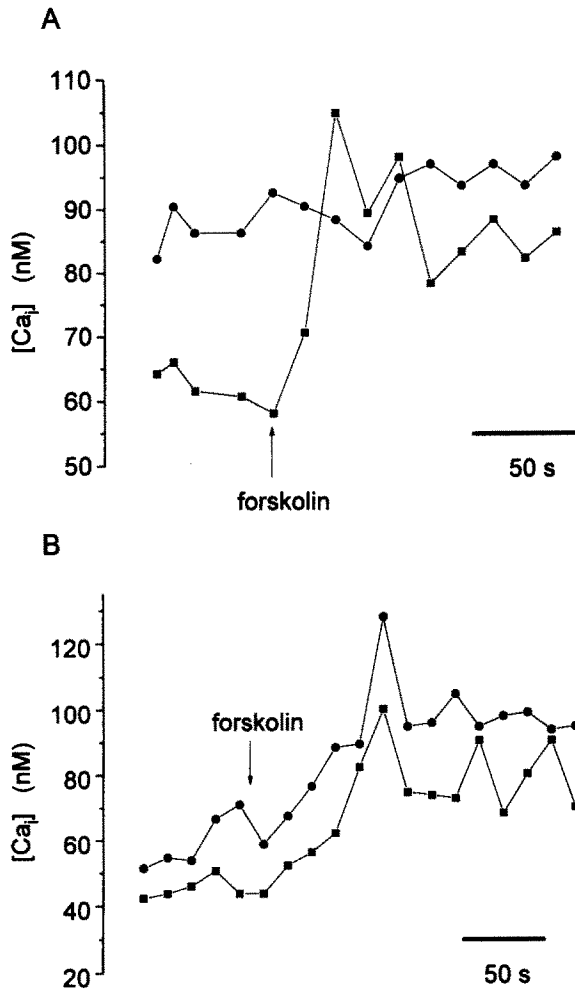
A

B

FIGURE 6. Forskolin (2 μM) induces a spatially heterogeneous increase in [Ca$_i$] in some isolated rat olfactory neurons. Shown is the response to addition of 2 μM forskolin in two isolated olfactory neurons. (*A* and *B*) Traces showing the average [Ca$_i$] at the olfactory knob (*squares*) and the soma (*circles*). *C* and *D* display the average [Ca$_i$] in the cells in *A* and *B*, respectively, as a function of the distance from the point where the dendrite attaches to the cell body, measured along the principal axis of the cell (*dashed line* in Fig. 1). The squares represent the average [Ca$_i$] before the response, while the circles are the average [Ca$_i$] during the response to forskolin. Data are mean ± SEM with *n*'s of 6 for *C* (*squares*), 5 for *C* (*circles*), 5 for *D* (*squares*), and 7 for *D* (*circles*).

To maintain a steady state gradient of Ca^{2+}, olfactory neurons must have an asymmetric distribution of transport proteins or channels involved in net Ca^{2+} influx and efflux. It is known that the cAMP-gated conductance, which is permeable to Ca^{2+} (Kurahashi and Shibuya, 1990; Kolesnikov et al., 1990), is primarily localized in the cilia (Nakamura and Gold, 1987), and it is likely that the IP$_3$-gated conductance (also permeable to Ca^{2+}) is also primarily localized to the cilia (Fadool and Ache, 1992; Restrepo et al., 1990; Restrepo et al., 1992). The reason why some cells display an

increase in [Ca$_i$] at the apical end could be that the cAMP and IP$_3$ gated conductance(s) act as a spatially localized source of Ca^{2+}.

During prolonged exposure to odorants the odorant-activated conductance carries a current of ~4 pA (at a potential of −35 mV, see Kurahashi and Shibuya, 1990). Because the permeability for Ca^{2+} is 6.5 times the permeability for Na$^+$ in the cAMP-gated channel (Kurahashi and Shibuya, 1990), it is possible to estimate using
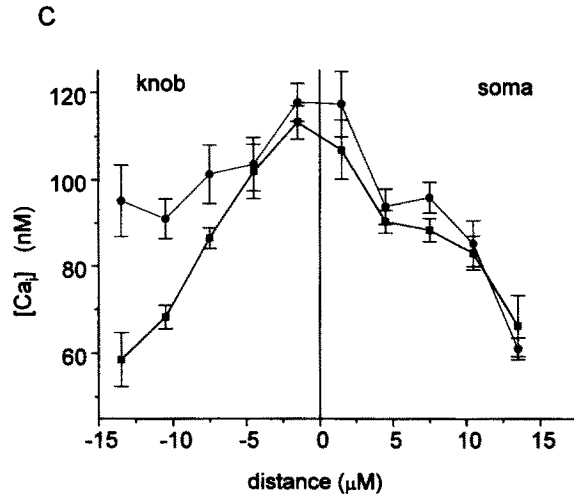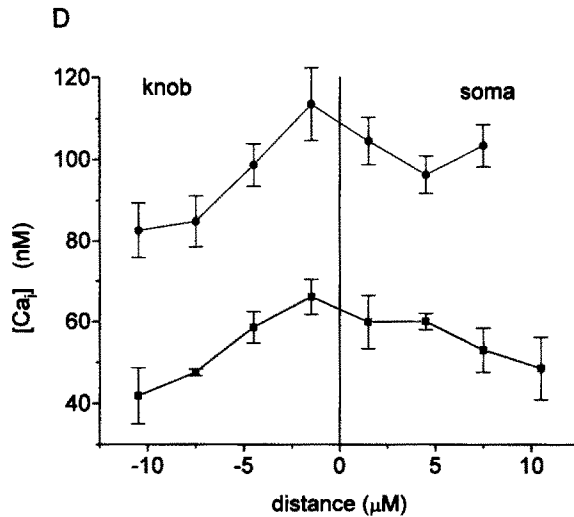

FIGURE 6.

the Goldman flux equation that ~7% of the odorant-stimulated current (or 0.26 pA) is carried by Ca^{2+} ions (see Appendix). To maintain a steady'state Ca^{2+} gradient, this current must be of the same magnitude as the diffusional flow of Ca^{2+} ions along the dendrite. If we take as an example the response of the cell in Fig. 4 C (*circles*), it is possible to calculate this diffusional Ca^{2+} flow for the standing gradient of [Ca$_i$] that is maintained during odorant stimulation between the olfactory knob and the
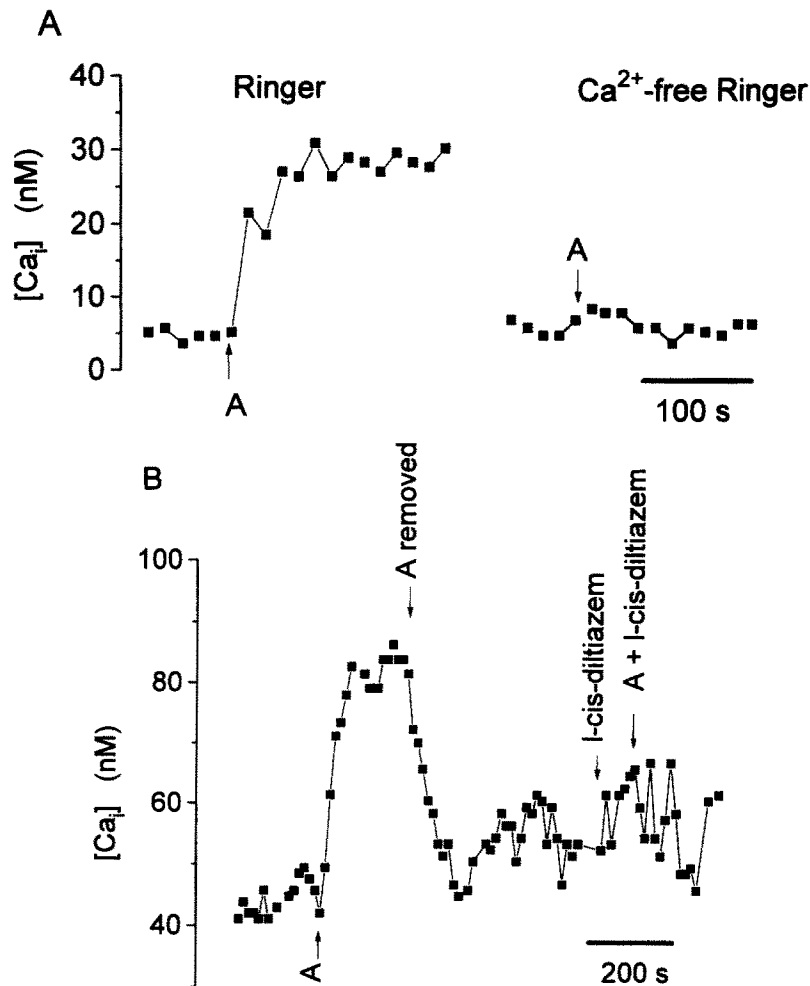
FIGURE 7.   (A) Stimulation of an isolated olfactory neuron with odorant mixture A leads to an increase in [Ca$_i$] only in the presence of extracellular Ca^{2+}. Removal of Ca^{2+} was accomplished by removal of CaCl$_2$ from and addition of 1 mM EGTA to the Ringer solution. The trace is representative of three experiments with mixture A and one with mixture B. This cell responded primarily at the olfactory knob. Traces display average [Ca$_i$] at the olfactory knob. (B) The response to odorant mixture A was inhibited by addition of 20 μM of the cAMP-channel blocker 1-cis-diltiazem. This cell responded with a homogeneous increase in [Ca$_i$]. The trace shown is for the average [Ca$_i$] in the whole cell.

dendrite using Fick's law (Macey, 1987):

$$J = D\, d[\mathrm{Ca_i}]/dx \tag{2}$$

where D is the diffusion coefficient for Ca^{2+}. In the example in Fig. 4 C (circles), the concentration of intracellular Ca^{2+} decreases along the longitudinal axis of the cell at a rate $d[\mathrm{Ca_i}]/dx$ of -28 nM/μm. Using Fick's law and assuming a dendritic radius of

A

B

C
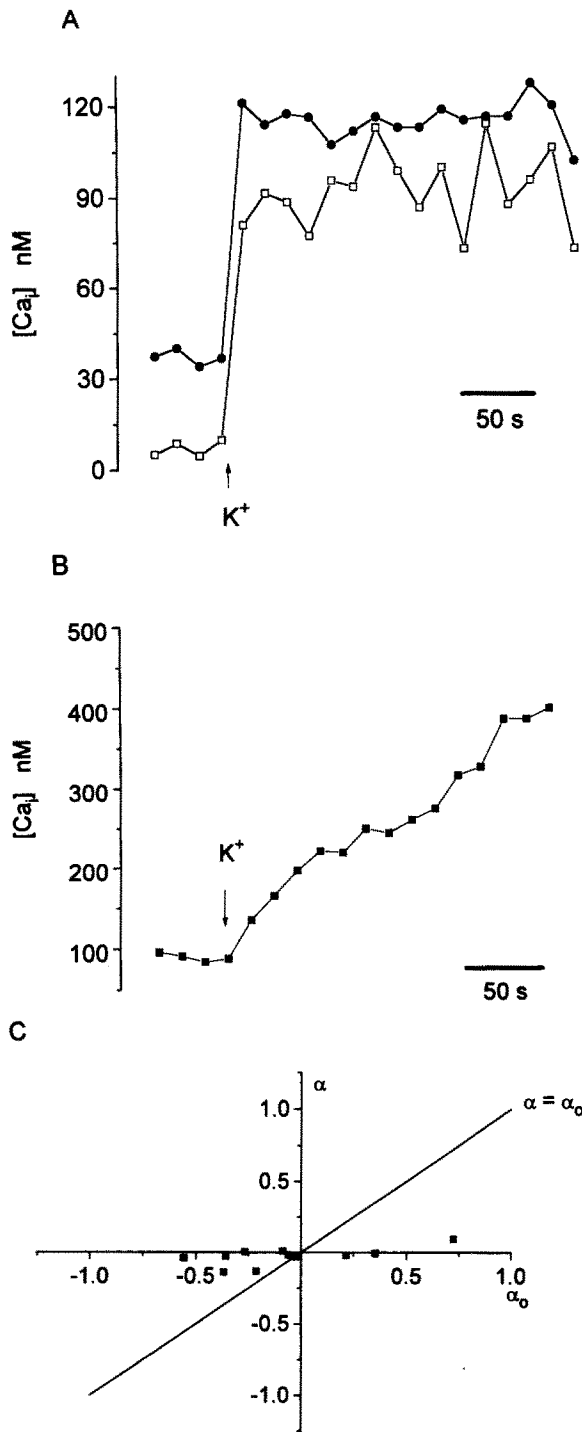

FIGURE 8. K⁺-induced depolarization causes an increase in [Ca$_i$] and a decrease in the Ca²⁺ gradient maintained by isolated rat olfactory neurons. (*A*) Increase in [Ca$_i$] elicited by depolarization of a rat olfactory neuron by replacement of extracellular Na⁺ with K⁺. Circles represent the average [Ca$_i$] in the soma, whereas the squares represent the average [Ca$_i$] at the olfactory knob. (*B*) K⁺-induced change in [Ca$_i$] in a different olfactory neuron. It was not possible to follow the change in the olfactory knob in this neuron, because the cell underwent a change in shape. (*C*) The K⁺-induced change in the magnitude of the [Ca$_i$] gradient maintained by the isolated rat olfactory neurons is illustrated by plotting the value of the asymmetry factor (α, defined by Eq. 1) during the response as a function of the value of α before stimulation (α_o). K⁺ elicited a decrease in the absolute value of α, indicating that the response to K⁺ had a tendency to dissipate the knob to soma [Ca$_i$] gradient.

1 μm and a cytosolic diffusion coefficient for Ca^{2+} of 13 $\mu m^2/s$ (Allbritton, Meyer, and Stryer, 1992) it is possible to calculate the magnitude for the diffusional flux of Ca^{2+} along the dendrite as -0.2 pA, which is of the same order of magnitude as the Ca^{2+} current carried by the odorant-stimulated conductance (0.25 pA). Although these numbers are only rough estimates, the calculation shows that it is not unreasonable to expect that a current of the magnitude of the odorant-activated current can give rise to the standing Ca^{2+} gradients found in this study.

The shape of the standing gradients for Ca^{2+} that are elicited by maintained odorant stimulation in some cells (e.g., Fig. 4 A–C) has implications for the spatial localization of Ca^{2+} extrusion mechanism(s) (presumably the Ca^{2+} ATPase) in olfactory neurons. The reason for this is that there are abrupt changes in the rate at which the calcium concentration changes as a function of distance along the dendrite. An example of this can be seen in Fig. 4 C where the Ca^{2+} concentration decreases steadily as a function of distance at the olfactory knob, while it is nearly constant as a function of distance along the rest of the dendrite. At the point where the magnitude of the slope of the [Ca_i] vs distance plot changes abruptly, the diffusional flux of Ca^{2+} along the dendrite changes from roughly -0.2 pA to nearly zero. Because the cell is at steady state (i.e., [Ca_i] is not changing with time) the net diffusional flux of Ca^{2+} must be balanced by net extrusion of Ca^{2+} across the plasma membrane. However, a 0.2 pA Ca^{2+} current in this very small region constitutes a large Ca^{2+} current per unit of membrane area. As a comparison peak Ca^{2+} current through voltage-gated Ca^{2+} channels, which would be expected to be distributed evenly throughout the total membrane area, can be estimated to be between 1 and 4 pA at 1 mM extracellular Ca^{2+} (Miyamoto, Restrepo, and Teeter, 1992; Schild, 1989; Trotier, 1986). These observations suggest that those olfactory neurons that can maintain large apical [Ca_i] gradients have a high density of Ca^{2+} ATPase at the apical end of the cell.

APPENDIX

Estimation of the Ca^{2+} Current that Flows Through the Odorant-stimulated Conductance

The fraction of the current carried by Ca^{2+} can be estimated using the Goldman flux equation. For simplicity it is assumed that only two cations are present in the extracellular medium, a monovalent cation (M^+) and calcium (Ca^{2+}). The unidirectional influx of these two ions (J_M and J_{Ca}) is assumed to follow the Goldman flux equation (Macey, 1987):

$$J_M = P_M[M]x/(e^x - 1) \tag{A1}$$

$$J_{Ca} = 2P_{Ca}[Ca]x/(e^{2x} - 1) \tag{A2}$$

where P_M and P_{Ca} are the permeability coefficients for M^+ and Ca^{2+} respectively, [M] and [Ca] the concentrations of the two ions, and $x = (V_M F/RT)$, where V_m is the membrane potential and RT/F is a constant equal to 25.2 mV at room temperature.

From Eqs. A1 and A2 we find that the ratio of the flux of calcium to the flux of the monovalent cation can be expressed as:

$$(J_{Ca}/J_M) = [2(P_{Ca}/P_M)([Ca]/[M])((e^x - 1)]/[e^{2x} - 1] \tag{A3}$$

In the case of the response of olfactory neurons to odorants appropriate parameters are

(P_{Ca}/P_{Na}) = 6.5 and V_m = −35 mV (Kurahashi and Shibuya, 1990). In addition, the concentration of monovalent cations in Ringer ([M]) is 150 mM, and the concentration of calcium ([Ca]) is 1 mM. Using these parameters, we find that, under these conditions J_{Ca}/J_M is equal to 0.07, which means that, if the magnitude of the odorant-induced current is 4 pA, the portion of this current which is carried by Ca^{2+} is ~0.26 pA.

REFERENCES

Allbritton, N. L., T. M. Meyer, and L. Stryer. 1992. Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate. *Science.* 258:1812–1815.

Anholt, R. R. H., and A. M. Rivers. 1990. Olfactory transduction: cross-talk between second-messenger systems. *Biochemistry.* 29:4049–4054.

Borisy, F. F., G. V. Ronnett, A. M. Cunningham, D. Juilfs, J. Beavo, and S. H. Snyder. 1992. Calcium/calmodulin-activated phosphodiesterase expressed in olfactory receptor neurons. *Journal of Neuroscience.* 12:915–923.

Breer, H., and I. Boekhoff. 1991. Odorants of the same odor class activate different second messenger pathways. *Chemical Senses.* 16:19–29.

Fadool, D. A., and B. W. Ache. 1992. Plasma membrane inositol 1,4,5-trisphosphate-activated channels mediate signal transduction in lobster olfactory receptor neurons. *Neuron.* 9:1–20.

Frings, S. 1993. Protein kinase C sensitizes olfactory adenylate cyclase. *Journal of General Physiology.* 101:183–205.

Getchell, T. V. 1986. Functional properties of vertebrate olfactory receptor neurons. *Physiological Reviews.* 66:772–818.

Inoue, T., T. Yamashita, N. Harada, and T. Kumazawa. 1992. Responses of the solitary olfactory receptor cell of the guinea pig. *Acta Otolaryngologica (Stockholm).* 112:717–725.

Kleene, S. J., and R. C. Gesteland. 1991. Calcium-activated chloride conductance in frog olfactory cilia. *Journal of Neuroscience.* 11:3624–3629.

Kolesnikov, S. S., A. B. Zhainazarov, and A. V. Kosolapov. 1990. Cyclic nucleotide-activated channels in the frog olfactory receptor plasma membrane. *FEBS Letters.* 266:96–98.

Kramer, R. H., and S. A. Siegelbaum. 1992. Intracellular Ca^{2+} regulates the sensitivity of cyclic nucleotide-gated channels in olfactory receptor neurons. *Neuron.* 9:897–906.

Kurahashi, T., and T. Shibuya. 1990. Ca^{2+}-dependent adaptive properties in the solitary olfactory receptor cell of the newt. *Brain Research.* 515:261–268.

Kurahashi, T., and K-W. Yau. 1993. Co-existence of cationic and chloride components in odorant-induced currents of vertebrate olfactory receptor cells. *Nature.* 363:71–74.

Lowe, G., and G. H. Gold. 1991. The spatial distributions of odorant sensitivity and odorant-induced currents in salamander olfactory receptor cells. *Journal of Physiology.* 442:147–168.

Lowe, G., T. Nakamura, and G. H. Gold. 1989. Adenylate cyclase mediates olfactory transduction for a variety of odorants. *Proceedings of the National Academy of Sciences, USA.* 86:5641–5645.

Macey, R. I. 1987. Mathematical models of membrane transport processes. *In* Membrane Physiology. T. E. Andreoli, J. F. Hoffman, D. D. Fanestil, and S. G. Schultz, editors. Plenum Publishing Corp., New York. 111–130.

Maue, R. A., and V. E. Dionne. 1987. Patch-clamp studies of isolated mouse olfactory receptor neurons. *Journal of General Physiology.* 90:95–125.

Miyamoto, T., D. Restrepo, and J. H. Teeter. 1992. Voltage-dependent and odorant-regulated currents in isolated olfactory receptor neurons of the channel catfish. *Journal of General Physiology.* 99:505–530.

Nakamura, T., and G. H. Gold. 1987. A cyclic nucleotide-gated conductance in olfactory receptor cilia. *Nature.* 325:442–444.

Restrepo, D., and A. G. Boyle. 1991. Stimulation of olfactory receptors alters regulation of [Ca$_i$] in olfactory neurons of the catfish (*Ictalurus punctatus*). *Journal of Membrane Biology.* 120:223–232.

Restrepo, D., T. Miyamoto, B. P. Bryant, and J. H. Teeter. 1990. Odor stimuli trigger influx of calcium into olfactory neurons of the channel catfish. *Science.* 249:1166–1168.

Restrepo, D., Y. Okada, J. H. Teeter, L. D. Lowry, B. Cowart, and J. G. Brand. 1993. Human olfactory neurons respond to odor stimuli with an increase in cytoplasmic Ca^{2+}. *Biophysical Journal.* 64:1961–1966.

Restrepo, D., and J. H. Teeter. 1990. Olfactory neurons exhibit heterogeneity in depolarization-induced calcium change. *American Journal of Physiology (Cell Physiology).* 258:C1051–C1061.

Ronnett, G. V., and S. H. Snyder. 1992. Molecular messengers of olfaction. *Trends in Neuroscience.* 15:508–513.

Sato, T., J. Hirono, M. Tonoike, and M. Takebayashi. 1991. Two types of increases in free Ca^{2+} evoked by odor in isolated frog olfactory receptor neurons. *Neuroreport.* 2:229–232.

Schild, D. 1989. Whole-cell currents in olfactory receptor cells of *Xenopus laevis*. *Experimental Brain Research.* 78:223–232.

Schild, D., and F. W. Lischka. 1992. High time resolution recordings of stimulus dependent calcium increases in *Xenopus* olfactory neurones measured with a laser scanning microscope. *Chemical Senses.* 17:879.

Trotier, D. 1986. A patch-clamp analysis of membrane currents in salamander olfactory receptor cells. *Pflugers Achives.* 407:589–595.

Tsien, R. Y., and A. T. Harootunian. 1990. Practical design criteria for dynamic ratio imaging system. *Cell Calcium.* 11:93–109.

Zufall, F., G. M. Shepherd, and S. Firestein. 1991. Inhibition of the olfactory cyclic nucleotide gated ion channel by intracellular calcium. *Proceedings of the Royal Society of London B.* 246:225–230.