brief communication

Human olfactory neurons respond to odor stimuli with an increase in cytoplasmic Ca^{2+}

Diego Restrepo,*‡ Yukio Okada,*** John H. Teeter,*‡ Louis D. Lowry,¶ Beverly Cowart,*¶ and Joseph G. Brand*§||

*Monell Chemical Senses Center, Philadelphia, PA 19104, USA; ‡Department of Physiology, School of Medicine, §Department of Biochemistry, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA; ||Veterans Affairs Medical Center, Philadelphia, PA 19104, USA; and ¶Department of Otolaryngology, Thomas Jefferson University, Philadelphia, PA 19107, USA; **Department of Physiology, Nagasaki University, Nagasaki 852, Japan

ABSTRACT The sense of smell allows terrestrial animals to collect information about the chemical nature of their environment through the detection of airborne molecules (7). In humans smell is believed to play an important role in protecting the organism from environmental hazards such as fire, gas leaks and spoiled food, in determining the flavor of foods, and perhaps in infant-parent bonding (8). In addition, the study of human olfaction is relevant to a number of medical problems that result in olfactory dysfunction, which can affect nutritional state, and to the study of the etiology of neurodegenerative diseases which manifest themselves in the olfactory epithelium (8, 26). Although much is known about behavioral aspects of human olfaction (8), little is understood about the underlying cellular mechanisms in humans. Here we report that viable human olfactory neurons (HON) can be isolated from olfactory tissue biopsies, and we find that HON respond to odorants with an increase in intracellular calcium concentration ($[Ca_i]$).

INTRODUCTION

Animal model studies have shown that stimulation of olfactory receptors in the plasma membrane of cilia that extend from the apical dendrite of receptor neurons results in G-protein-mediated activation of second messenger systems (adenosine 3′5′-monophosphate, cAMP and inositol-1,4,5-trisphosphate, IP_3), leading to direct second messenger-gating of ciliary nonselective cation channels (1). Opening of the second messenger-gated channels causes depolarization of the plasma membrane of the olfactory neuron, resulting in an increased frequency of firing of action potentials in the receptor cell axon. However, relatively little is known about the mechanisms of olfactory transduction at the cellular level in humans.

The human olfactory epithelium is accessible for biopsy (14), and olfactory tissue biopsies have been used for morphological studies of olfactory dysfunction in humans (25). To demonstrate the suitability of biopsy material for cellular studies of olfaction in humans, we obtained samples of olfactory epithelium from patients who were already undergoing tissue removal in the course of elective sinus surgery. We isolated viable human olfactory neurons from these biopsies and showed that the olfactory neurons respond to stimulation with odorants with an increase in intracellular Ca^{2+} which appears to be due to influx of Ca^{2+} through the plasma membrane. This isolated human olfactory neuron preparation is suitable for the study of human olfaction at the cellular and molecular levels.

RESULTS AND DISCUSSION

To obtain isolated human olfactory neurons (HON) small biopsies measuring approximately 1 mm^3 were taken from the septum opposite the high middle turbinate (see Experimental Procedures). The tissue samples were treated with papain in divalent cation-free mammalian Ringer solution and cells were dissociated by vigorous pipetting through a thin capillary. This procedure resulted in dissociation of 20–200 human olfactory neurons per biopsy. The receptor neurons were readily distinguished by their characteristic morphology (17), with a round cell body and thin dendrite ending in a round protuberance called the olfactory knob. In some of the cells, cilia could be seen extending from the olfactory knob.

Voltage-dependent conductances were studied in the HON using the whole-cell patch clamp technique (9). Cells maintained an average resting potential of -50 ± 12 mV (mean ± SD, $n = 11$), and the input resistance was 3.4 ± 1.7 GΩ, attesting to the integrity of the cell membrane. The average membrane capacitance was 3.9 ± 1 pF. HON displayed time- and voltage-dependent membrane currents in response to depolarizing voltage steps (Fig. 1, *A* and *B*). One of the 11 HON displayed transient inward currents that activated between −50 and −70 mV, reached a peak at about −20 mV and inactivated rapidly (Fig. 1 *A* and *B*). These are presumably transient Na^+ currents, although further studies are necessary to substantiate this tentative identification. The absence of transient Na^+ currents in many cells may be due to the axotomy that takes place during the isolation procedure. In contrast, all cells displayed sustained outward currents that activated at −40 mV (Fig. 1, *A* and *B*). These currents probably represent outward K^+ currents. None of these cells displayed inactivating outward currents, nor did we observe a decrease in outward current at positive potentials in the IV curve suggesting Ca^{2+}-activated K^+ currents (Fig. 1 *A*). However, when treated with 5 μM ionomycin, which causes an increase

Address correspondence to Diego Restrepo, Monell Chemical Senses Center, 3500 Market St., Philadelphia, PA 19104 USA.

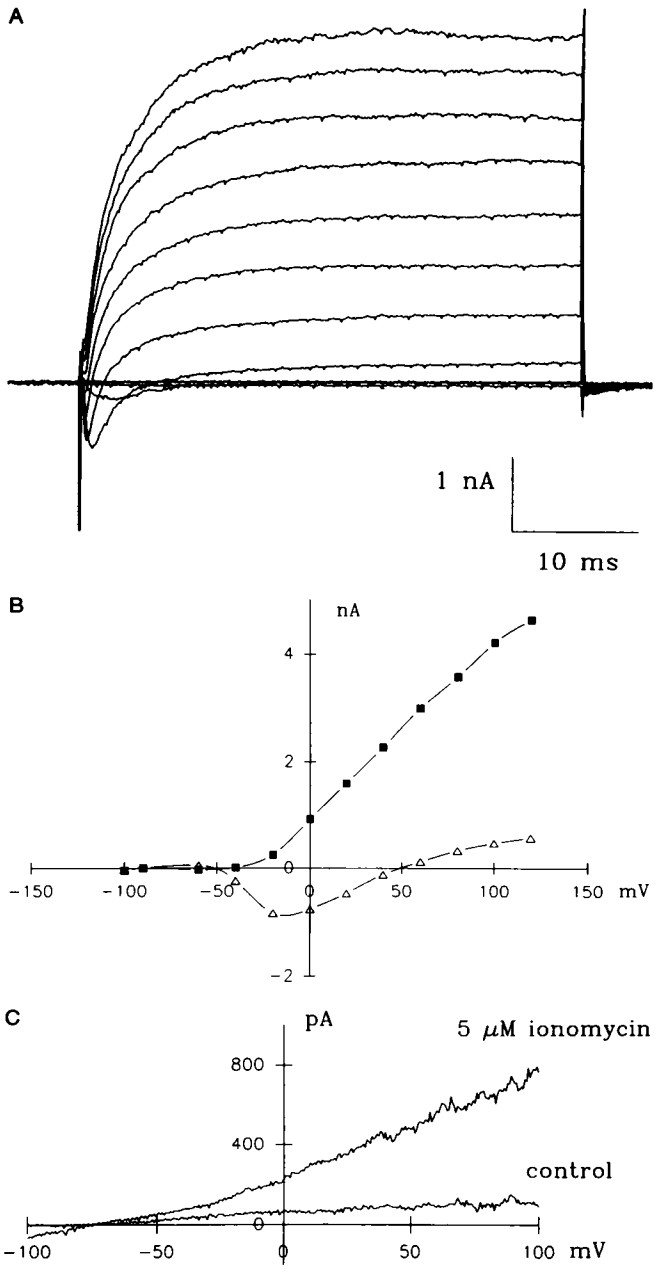

FIGURE 1 Voltage-dependent currents recorded from isolated HON using the whole-cell patch clamp technique (9). The cells were bathed in mammalian Ringer's solution and the pipette contained pseudo intracellular solution (see below for composition). (*A*) Currents elicited by voltage steps from −100 mV to +120 mV from a holding potential of −80 mV. (*B*) Current-voltage relationship for peak inward (triangles) and steady state outward (squares) currents elicited by voltage steps in the records in *A*. (*C*) The pseudo-steady state current-voltage curve for an olfactory neuron was recorded by measuring transmembrane current elicited by a voltage ramp from −100 to 100 mV (166 mV/s). Addition of 5 μM ionomycin induced the development of an outward current that reversed at approximately −70 mV.

in $[Ca_i]$, the current-clamped receptor cells hyperpolarized, and under voltage clamp an outward current developed that reversed at negative potentials (Fig. 1C). This Ca^{2+}-activated current could play an important role in modulating the generator potential induced by stimulation with odorants.

Changes in $[Ca_i]$ have been implicated in the responses of olfactory neurons from various animal species to odorants (10, 20–24). We studied $[Ca_i]$ homeostasis in HON with the Ca^{2+}-sensitive dye fura-2 and dual excitation fluorescence microscopy (reference 27 and Experimental Procedures). HON maintained low $[Ca_i]$ in the range from 1 to 90 nM (26.5 ± 8 nM mean ± SEM, n = 12). Addition of 5 μM of the Ca^{2+} ionophore ionomycin produced a rapid increase in $[Ca_i]$ from 14 ± 9 to 136 ± 6 nM (mean ± SEM, n = 6) (not shown). The functional state of the cells was tested by stimulation with a mixture of odorants (mixture A, containing 100 μM of each of the following odorants: hedione, geraniol, phenylethylalcohol, citralva, citronellal, eugenol and menthone). Half of the cells (6 of 12) responded with an increase in $[Ca_i]$ (Figs. 2, *A*, *B* and *C*). $[Ca_i]$ changed from a mean basal value of 13.5 ± 6 nM to 87 ± 30 nM, and recovered to 15.6 ± 7 nM upon removal of the stimulus (mean ± SEM, n = 6). The HON could be repeatedly stimulated, and stable recordings were sustained for 30 min to 2 h. When the HON were stimulated twice, the ratio of the increase in $[Ca_i]$ during the second response divided by the increase in $[Ca_i]$ during the first response was 1.17 ± 0.08 (mean ± SEM, n = 6). Although the distribution of $[Ca_i]$ was spatially inhomogeneous (Fig. 2 *A*), the odorant-induced increase in $[Ca_i]$ took place throughout the cell at both the distal and proximal ends (Fig. 2 *A*). This contrasts with results in rat olfactory neurons which in some cases display larger odorant-stimulated increases in $[Ca_i]$ at the apical end (22). Further work will be necessary to find the reason for this difference in $[Ca_i]$ distribution between human and rat olfactory neurons. However, it is important to state that odorants could elicit an increase in $[Ca_i]$ throughout the neuron, both at the knob and the soma, even if the source of Ca^{2+} is localized to the apical end, provided that either diffusion of Ca^{2+} in the cytoplasm is relatively fast, and/or that opening of voltage-gated Ca^{2+} channels contributes to the response. In addition, two HON (n = 12) responded to stimulation with odorant mixture A with a decrease in $[Ca_i]$ (Fig. 2 *D*). A similar decrease in $[Ca_i]$ was detected previously in a small number of cells when catfish olfactory neurons were stimulated with amino acid odorants (21).

An increase in $[Ca_i]$ has been postulated to contribute to adaptation of olfactory neurons to maintained stimulation through a variety of mechanisms, including direct blockage of cAMP-gated channels (5, 13, 18), stimulation of phosphodiesterase activity (13), and opening of Ca^{2+}-dependent K^+ or Cl^- channels (11, 16). In this respect it is important that the time course for both odorant-stimulated increases in $[Ca_i]$, and for recovery from stimulation, varied widely in HON. In some cells the half times ($t_{1/2}$) for the increase in $[Ca_i]$ and for recovery were within the time resolution of our apparatus (<10 s)

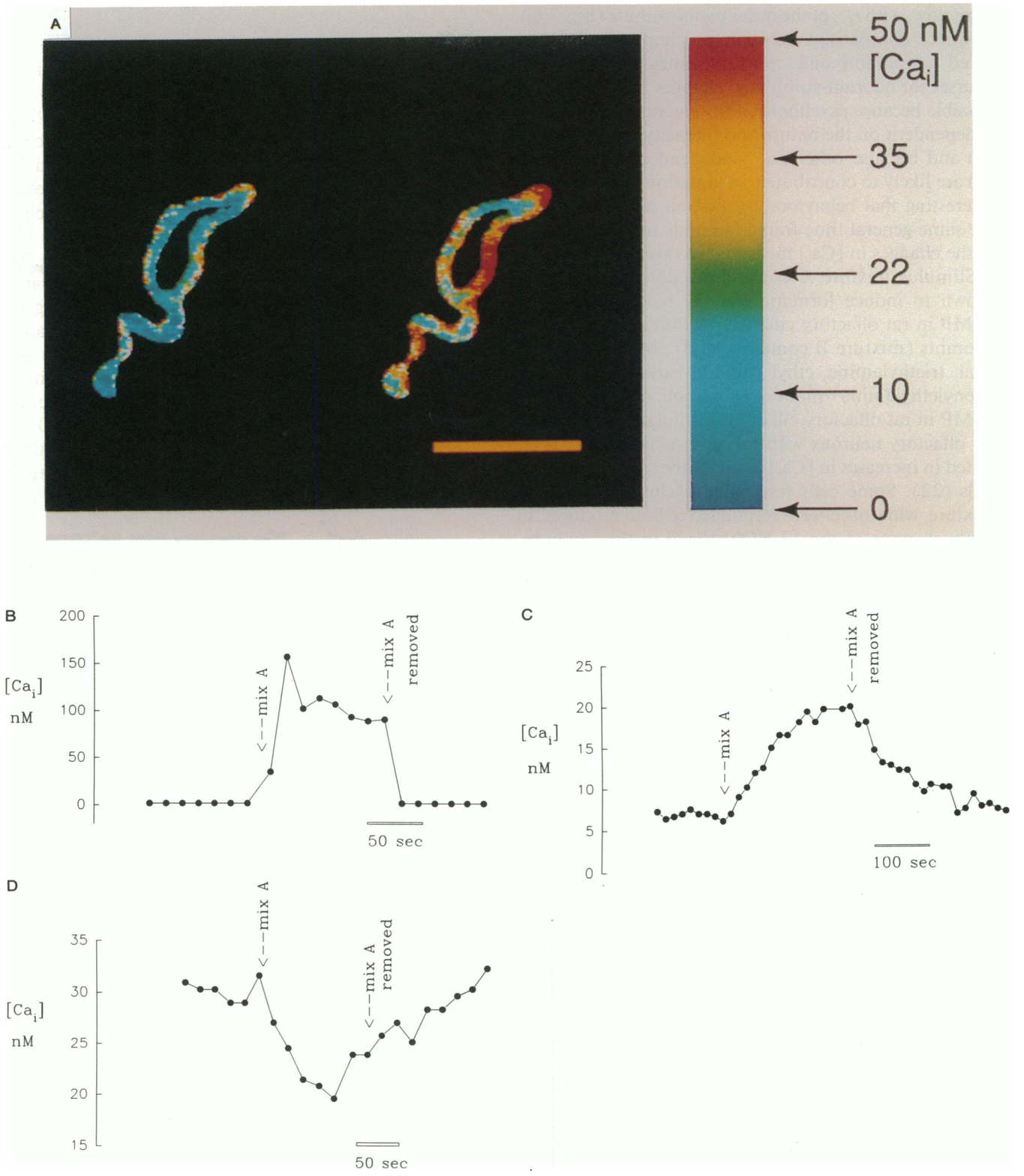

FIGURE 2 Stimulation of HON with a mixture of odorants elicits changes in $[Ca_i]$. (*A*) Pseudocolor image displaying the distribution of $[Ca_i]$ within a human olfactory neuron before (left) and after (right) stimulation with odorant mixture A. The center of the cell body was overexposed on purpose to attain high sensitivity for the measurements of $[Ca_i]$ in the dendrite, which was less fluorescent. Because of this, the central area of the cell body is not shown in pseudocolor but is shown in black instead. The solid bar is 20 μm in length. (*B* and *C*) Average $[Ca_i]$ in receptor cells that responded to stimulation with odorant mixture A with rapid (*B*) and slow (*C*) increases in Ca^{2+} levels. (*C*) Example of a cell that responded to stimulation with odorant mixture A with a decrease in $[Ca_i]$.

(Fig. 2 *B*), whereas in others the increase and recovery were slow, with $t_{1/2}$ of the order of one minute (Fig. 2 *C*). A direct comparison between psychophysically measured adaptation and recovery times and the time courses for odorant-stimulated changes in $[Ca_i]$ are not possible because psychophysically measured adaptation is dependent on the nature and intensity of the odorant (4) and because other Ca^{2+}-independent mechanisms (2) are likely to contribute to adaptation. However, it is interesting that behavioral adaptation takes place over the same general time frame (seconds to minutes) (4), as the changes in $[Ca_i]$ measured in this study.

Stimulus mixture A consists of odorants previously shown to induce formation of the second messenger cAMP in rat olfactory cilia (3). A different mixture of odorants (mixture B containing 100 μM each of lyral, lilial, triethylamine, ethyl vanillin, isovaleric acid and phenylethylamine) induces formation of IP_3, but not cAMP in rat olfactory cilia (3). Stimulation of isolated rat olfactory neurons with mixture A or mixture B resulted in increases in $[Ca_i]$ in about the same number of cells (22). Some cells responded exclusively to either mixture, while other cells responded to both mixtures. In contrast, none of the 12 HON tested in this study responded to stimulation with odorant mixture B (IP_3-generating in rat), while 50% responded to stimulation with odorant mixture A (cAMP-generating in rat). These experiments raise the interesting possibility that the olfactory neurons located in the biopsied area (on the septum at the level of the high middle turbinate) may possess receptors for the odorants in mixture A (cAMP-generating), but not for those in mixture B (IP_3-generating).

To investigate whether the odorant-stimulated increase in $[Ca_i]$ results from an influx of Ca^{2+} through the plasma membrane, or from release of Ca^{2+} from internal stores (IS), the cells were stimulated with odorant mixture A in the absence of extracellular Ca^{2+} ($[Ca_o]$). There was no increase in $[Ca_i]$ under these conditions (Fig. 3 *A*). In contrast, addition of 5 μM ionomycin in the absence of $[Ca_o]$ induced an increase in $[Ca_i]$ due to release of Ca^{2+} from IS (not shown). These experiments suggest that the odorant-induced increase in $[Ca_i]$ is due to an influx of Ca^{2+}. Although it is possible that odorants cause release of Ca^{2+} from internal stores that are depleted very rapidly upon removal of $[Ca_o]$, addition of 20 μM 1-cis-diltiazem, a blocker of the olfactory cAMP-gated channel in rat and frog (6, 12), abolished the response to odorant mixture A (Fig. 3 *B*). Together these experiments (Figs. 3, *A* and *B*) strongly suggest that the increase in $[Ca_i]$ elicited by odorants results from Ca^{2+} influx. While part of this influx may be mediated by influx of Ca^{2+} through the cAMP-gated channels, which are known to be permeable to Ca^{2+} ions (12, 13), it is also likely that some portion of the increase in $[Ca_i]$ is due to influx of Ca^{2+} through voltage-gated Ca^{2+} channels that open in response to the depolarization caused by opening of the cAMP-gated channels. As shown in Fig. 3 *C*, depolarization of the HON leads to an increase in $[Ca_i]$, and this depolarization-induced increase in $[Ca_i]$ did not take place in the absence of $[Ca_o]$.

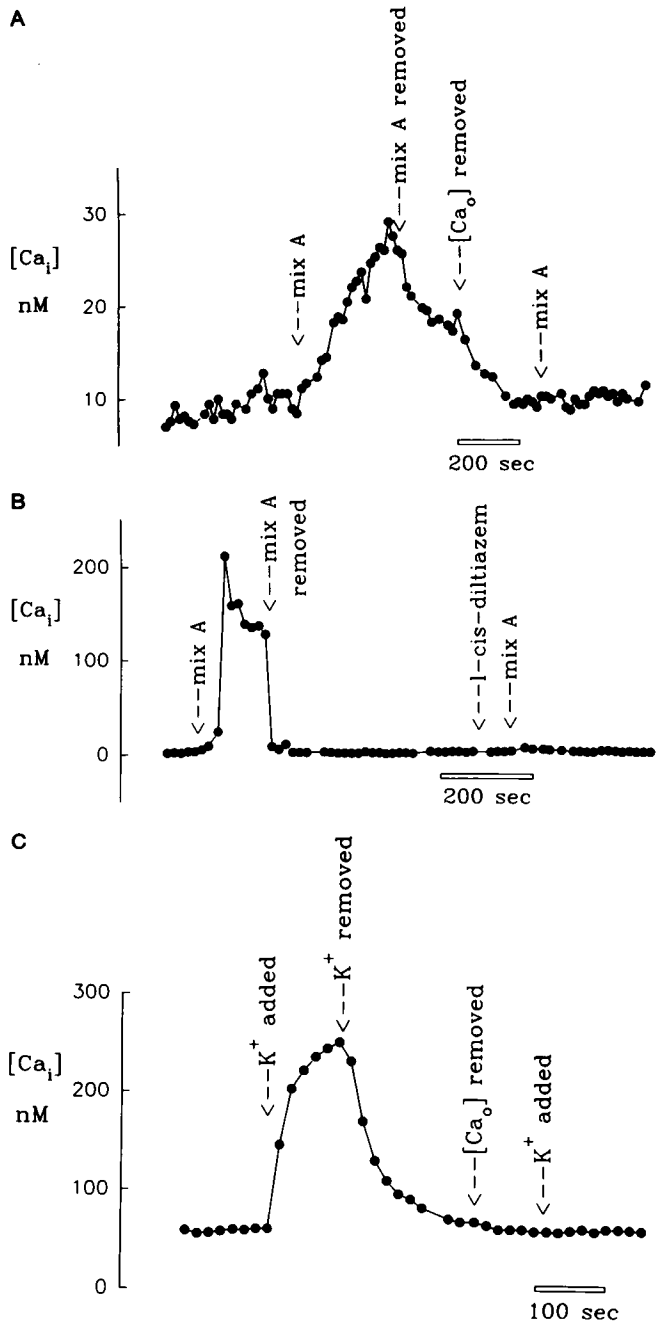

FIGURE 3 (*A*) Removal of extracellular Ca^{2+} ($[Ca_o]$) abolishes the increase in $[Ca_i]$ elicited by stimulation with odorant mixture A. Removal of $[Ca_o]$ was attained by perfusing the HON with Ca^{2+}-free mammalian Ringer with 1 mM EGTA. This record is representative of measurements from four HON. (*B*) Addition of 20 μM 1-cis-diltiazem abolishes the response to odorant mixture A. Record is representative of results in two HON. (*C*) Depolarization of HON, attained by replacing extracellular Na^+ isoosmotically with K^+, results in a rapid increase in $[Ca_i]$. This response to addition of extracellular K^+ does not take place in the absence of $[Ca_o]$.

In conclusion, we have shown that it is feasible to isolate viable HON from olfactory tissue biopsies. Using this preparation we have studied for the first time the cellular mechanisms of olfactory transduction in humans. We find that olfactory stimuli known to induce an increase in cAMP production in rat olfactory neurons elicit an increase in $[Ca_i]$ in HON that is probably mediated by Ca^{2+} influx. The isolated HON should be a useful model for studies of olfactory dysfunction that occurs in normal aging and in a variety of disease states, including Alzheimer's disease (26).

EXPERIMENTAL PROCEDURES

Olfactory tissue biopsies and cell dissociation. Olfactory tissue biopsies were obtained from patients during the course of elective endoscopic sinus surgery. Informed consent was obtained after the nature and possible consequences of the procedure were explained. Biopsies of approximately 1 mm^3 were sampled from the olfactory epithelium lining the septum opposite the high middle turbinate. A total of nine subjects ranging in age from 22 to 69 years (mean 43 years) participated in the study. HON were dissociated from the tissue biopsies by treatment for 10 min with divalent cation-free mammalian Ringer's supplemented with 15 U/ml activated papain as reported previously for catfish olfactory neurons (19). HON were identified by the presence of a round cell body, with one long dendrite terminating in a swelling called the olfactory knob. Occasionally cilia and/or an axon were also visible. Cells were considered to be viable if they maintained a stable level of $[Ca_i]$. In experiments with rat olfactory neurons we have determined that a steady increase in $[Ca_i]$ is correlated with loss of responsivity to odorants.

Current measurements. Whole cell current measurements were performed using the whole-cell patch clamp technique as described in detail in reference 15. The cells were perfused continuously with mammalian Ringer solution containing (in mM): 145 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 20 Hepes (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid) and 1 Na pyruvate, pH 7.2. The pipette contained a pseudointracellular solution with (in mM): 135 KCl, 0.1 $CaCl_2$, 1 EGTA (ethylene glycol-bis(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid), 10 Hepes, 1 $MgCl_2$, pH 7.1 with a calculated free Ca^{2+} concentration of 26 nM.

Measurement of intracellular Ca^{2+}. HON were loaded with the Ca^{2+}-sensitive dye fura-2 (27) by preincubation for 1 h in mammalian Ringer (see composition in legend to Fig. 1) 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 during the experiments. Exchange of solutions took place within less than five seconds. The apparatus used to image $[Ca_i]$ was a setup previously used by us to measure $[Ca_i]$ in single catfish olfactory neurons (21) modified to allow imaging of $[Ca_i]$ (27). Modifications to the previous system were as follows: Light emitted by the Xenon lamp was alternately filtered with narrow bandpass filters at 340 (Ca^{2+} sensitive) or 360 nm (Ca^{2+} 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 n.a.) 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). 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.

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