Chemosensory Responses in Isolated Olfactory Receptor Neurons from *Necturus maculosus*

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ABSTRACT Olfactory receptor neurons were isolated without enzymes from the mudpuppy, *Necturus maculosus*, and tested for chemosensitivity. The cells responded to odorants with changes in firing frequency and alterations in excitability that were detected with tight-seal patch electrodes using on-cell and whole-cell recording conditions. Chemosensitive cells exhibited two primary response characteristics: excitation and inhibition. Both types of primary response were observed in different cells stimulated by mixtures of amino acids as well as by the single compound L-alanine, suggesting that there may be more than one transduction pathway for some odorants. Using the normal whole-cell recording method, the chemosensitivity of competent cells washed out rapidly; a resistive whole-cell method was used to record odorant responses under current-clamp conditions. In response to chemical stimulation, excitability appeared to be modulated in several different ways in different cells: odorants induced hyperpolarizing or depolarizing receptor potentials, elicited or inhibited transient, rhythmic generator potentials, and altered excitability without changing the membrane potential or input resistance. These effects suggest that olfactory transduction is mediated through at least three different pathways with effects on four or more components of the membrane conductance. Polychotomous pathways such as these may be important for odor discrimination and for sharpening the "odor image" generated in the olfactory epithelium.

INTRODUCTION

Sensory information about smell is communicated to the brain by patterns of action potentials evoked by odors in peripheral olfactory receptor neurons located in the nasal airways. Mammals have 1 million to 100 million or more olfactory neurons and detect more than 10,000 different odorous compounds; however, each sensory neuron responds only to a subset of the odor spectrum. Odors are detected because they modulate the membrane conductance of sensory neurons, altering membrane excitability and firing patterns. In the central nervous system, regulation of electrical

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activity is achieved by modulating a variety of different types of ion channels; however, it is unclear whether the capacity of the olfactory system to transduce different compounds arises because receptors in different olfactory neurons modulate a single component of the membrane conductance or whether a variety of components are affected. Both cAMP (Pace, Hanski, Salomon, and Lancet, 1985) and inositol trisphosphate (Boekhoff, Tareilus, Strotmann, and Breer, 1990) levels in olfactory tissue are modulated by exposure to odorants, and a cAMP-sensitive conductance (Nakamura and Gold, 1987) has been identified that may be an important mediator of odor action. Recently, several other conductances have also been identified as potential mediators of odor activity (Restrepo, Miyamoto, Bryant, and Teeter, 1990; Kleene and Gesteland, 1991; Schild and Bischofberger, 1991), but the evidence that odors modulate more than a single conductance is not yet compelling.

The enormous variability of chemosensitivity among individual olfactory neurons means that the likelihood of any particular cell being sensitive to a particular odor is low. This presents a significant experimental problem for studying odor transduction at the cellular level. To minimize this problem, I have studied olfactory transduction in the mudpuppy, *Necturus maculosus*, an aquatic salamander, for which the diversity of chemical stimuli may be much smaller than in mammals.

Here I report that odor-elicited responses from isolated olfactory neurons have characteristics resembling those found with extracellular recordings in vivo: olfactory neurons respond to odorants with both excitation and inhibition. Excitatory responses have been detected in vertebrate olfactory neurons before, but this is the first report of inhibitory responses from isolated vertebrate cells. The responses are produced by depolarizing and hyperpolarizing receptor potentials in some cells, by transient, rhythmic generator potentials in other cells, and by conductance changes that alter excitability without changing the resting potential or input resistance. These results suggest that several different conductance mechanisms are modulated during odor transduction, and demonstrate that individual cells may have one or more of these conductances. The distribution of transduction processes among olfactory receptor neurons may be the basis for odor discrimination by the olfactory system.

Some of these results have been reported in abstract form (Dionne, 1990, 1991).

METHODS

This study was conducted on dissociated, visually identified olfactory receptor neurons from the mudpuppy, *Necturus maculosus*. The mudpuppy olfactory organ is a tubular epithelium lined by sensory neurons, support cells, basal cells, and several other cell types. Mudpuppies were purchased from licensed suppliers in Wisconsin from October to May. The animals were housed in the laboratory 3 wk or less to minimize losses in chemosensitivity that were noticed after lengthy periods in captivity.

Potential odorants were tested in vivo. For these experiments the animals were doubly pithed and the olfactory transepithelial potential was measured with a sensitive, low-noise voltage monitor. This signal, termed the electro-olfactogram (EOG) by Ottoson (1956), was measured as the voltage difference between a saline-filled microelectrode on the surface of the olfactory epithelium and a moist metal clip attached to skin. The magnitude of the EOG, typically

$\leq$1,100 μV, was sensitive to the position of the recording electrode. All other studies were conducted on dissociated olfactory neurons.

Olfactory neurons were dissociated from the sensory epithelia of adult animals of either sex using an enzyme-free procedure similar to that described by Frings and Lindemann (1988). Animals were killed by decapitation and the paired olfactory organs were removed, severing the axons of the receptor neurons. The organs were transferred to amphibian saline (APS; see below) and cut open, and the epithelial layer was removed with fine forceps. In fresh APS the epithelial tissue was quickly cut into small pieces (< 1 mm) with iridectomy scissors, then placed in a low-calcium dissociation solution (DS; see below) at pH 10.4 for 30 min. The treated tissue was transferred to APS containing 0.5 μg/ml DNAse (Sigma Chemical Co., St. Louis, MO) to destroy DNA released from broken cells, then either gently stirred or triturated three to six times with a fire-polished Pasteur pipette to mechanically separate the cells. This protocol produced olfactory neurons that remained viable for 2–3 h and retained their morphology.

The dissociated olfactory neurons were studied with patch techniques (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981) using on-cell voltage-clamp and whole-cell current-clamp modes. Recordings were made with an Axopatch amplifier (Axon Instruments Inc., Foster City, CA). The data were digitized on-line with a laboratory computer system (Indec Systems, Inc., Sunnyvale, CA) and stored on magnetic disk for later analysis. The programs for data acquisition and analysis were written in BASIC-23 in this laboratory. Patch electrodes were prepared from Corning 8161 capillary (Corning Glass Inc., Corning, NY) with a Brown-Flaming micropipette puller (Sutter Instrument Co., Novato, CA) and coated with wax to within ~1 mm of the tip to reduce both the capacitance and the wetted outer surface of the pipette. The nominal resistance of the electrodes was 1–3 MΩ in APS.

Whole-cell recordings from odor-sensitive cells were performed with methods that reduced intracellular dialysis. The membrane patch was permeabilized using the antibiotic nystatin (Horn and Marty, 1988) or by applying a few brief pulses of high voltage. Both methods were effective, but the high-voltage breakdown method was preferred because it could be applied quickly. Recordings were made in the presence of a series resistance $\geq$100 MΩ; this large resistance reflected the poor quality of the exchange pathway between the pipette lumen and the cell interior. This recording method, referred to as the resistive whole-cell method, allowed cells to be studied for up to 30 min; the series resistance had only minor effects on the intracellular potentials that were monitored in the current-clamp mode. The input resistance of the neurons was 1–5 GΩ in both the normal and resistive whole-cell recording conditions, in agreement with earlier results (Dionne, 1988). Routine recordings were made with the capacity compensation optimized but without compensation for series resistance since the correction circuit became unstable under these conditions.

All studies were performed in a drop of saline containing dissociated cells that had settled loosely on the glass stage of an upright, compound microscope. The cells were visualized with Nomarski optics at 500× using a water immersion 40× Zeiss objective (Carl Zeiss, Inc., Thornwood, NY). The saline drop (~100 μl in volume) bathing the cells was held between the objective and the glass stage by capillary forces, thus eliminating the need for a conventional recording chamber. To protect against evaporation, gravity-fed inflow and aspirated outflow tubes were suspended from the objective to perfuse the drop (Maue and Dionne, 1987); an inflow rate of 1–2 ml/min could be maintained during recording.

Chemical stimuli were dissolved in APS and applied by ejection from a glass micropipette (a puffer pipette) with a tip diameter of ~10 μm. Air pressure was used to eject the solution; pressure was controlled by an electric solenoid valve that was interfaced with the computer for precise timing during data acquisition. Typically, the puffer pipette tip was placed ~100 μm from the neuron being studied while the bath was constantly perfused with fresh APS. This served to minimize background stimulation from residual odorants or from odorants that

might have leaked from the puffer pipette. Puffer pipettes with small tips such as these were often seen to draw the bath saline into the tip between "puffs," thus rediluting the stimuli. It is important to note that the odorant concentrations specified in the text of this report are those added to the puffer pipette. The combined effects of bath-perfusion, dilution, and redilution reduced the actual concentrations of the chemical stimuli applied to the cell surface below those of the bulk contents of the puffer pipettes. Although the concentrations sensed at the cell surface were not measured, they may have been as much as 10-fold or more below the concentrations in the puffers.

The compositions of the salines used during this study were as follows. APS (amphibian physiological saline) contained (mM) 112 NaCl, 3 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 NaHEPES, 5 glucose, and 5 Na-pyruvate, pH 7.4. DS (low-calcium dissociation saline) contained (mM) 90 NaCl, 4 NaOH, 3 KCl, 1 $CaCl_2$, 10 Na_2CO_3, 10 $NaHCO_3$, 10 Na_3-citrate, and 1% albumin, pH 10.3. IS (low-chloride intracellular saline) contained (mM) 90 K-gluconate, 5 $CaCl_2$, 3 $MgSO_4$, 10 NaHEPES, 10 K_4BAPTA, pH 7.4, and 0.1 μM free Ca^{2+}. Test odorants were dissolved in APS at the concentrations indicated in the text.

RESULTS

Identification of Suitable Olfactory Stimuli

Necturus is a gilled salamander that lives in fresh water. Odors are important stimuli in its environment as judged by its large olfactory epithelia and olfactory bulbs that occupy a major portion of its forebrain. Yet little is known about the natural odors that stimulate *Necturus*. In preparation for the single-cell studies described below, a group of compounds was tested for ability to stimulate electrical activity in the olfactory epithelium in vivo. Five amino acids and five water-soluble volatile compounds were examined; each is a potent odorant to other aquatic or air-breathing salamanders (Artz, Silver, Mason, and Clark, 1986). Stimuli were applied briefly as individual compounds and as mixtures in solutions of APS that flowed steadily through the olfactory organ. The concentrations tested ranged from 5 to 100 μM, and all solutions were applied at near-neutral pH (7.4). The tests were performed on three separate animals.

All five of the amino acids tested and two of the five volatile compounds were effective olfactory stimuli (Table I). The two stimulatory volatile compounds were cineole and cinnamaldehyde, and the most potent amino acids were L-alanine and L-glutamate. EOGs could be elicited repetitively with these compounds, and the responses were dose dependent when tested with two different concentrations. The responses showed a rapid onset to peak and usually declined to a lower value during sustained exposure to a compound. Repeated applications showed smaller responses unless the tissue was washed with clean saline for at least 2–4 min. The L-isomers of alanine and glutamate were more potent than the D-isomers. The results indicate that these compounds are effective olfactory stimuli for the mudpuppy and suggest that other amino acids may be useful also. Isobutyl alcohol, ethyl acetate, and benzaldehyde had little or no effect on the EOG when tested at 100 μM.

Selection of Isolated Cells

Cells were selected for an initial screening of chemosensitivity if they satisfied several morphological criteria: they had to have soma, dendrite, and cilia intact and, for the

majority of cells, had to exhibit ciliary motility. The initial screening was made using a mixture of odorants in most cases, since this increased the likelihood of producing a response. 103 cells were tested for chemosensitivity; responses were observed from 31. Cells that did not respond may have been sensitive to untested compounds or may have been damaged during isolation. Cells with demonstrable chemosensitivity typically exhibited spontaneous or odor-evoked action potentials. Ciliary motility was not necessary for chemosensitivity since cells with nonmotile cilia were sensitive.

TABLE I

Evaluation of Odorants In Vivo by Electro-Olfactogram

Compound	Properties	EOG	Dose dependent	Adapt
L-Alanine	Hydrophobic	+++	Yes	Yes
D-Alanine	Hydrophobic	+	ND	Yes
L-Arginine	Polar (+)	++	ND	Yes
L-Glutamate	Polar (−)	+++	Yes	Yes
D-Glutamate	Polar (−)	++	ND	Yes
L-Proline	Hydrophobic	++	ND	Yes
L-Serine	Polar (0)	+	ND	ND
Benzaldehyde	Volatile	s	ND	ND
Cineole	Volatile	++	Yes	Yes
Cinnamaldehyde	Volatile	+++	Yes	Yes
Ethyl acetate	Volatile	s	ND	ND
Isobutyl alcohol	Volatile	s	ND	ND

All compounds were tested at a nominal 25- or 100-μM concentration; the more potent compounds were also tested at lower concentrations to determine whether their effects were dose dependent. The five volatile compounds were dissolved in APS and sonicated; since they are only slightly soluble in water, it is likely that their actual concentrations were less than stated. The symbols in the table indicate relative responses, with a single + equal to 10–20% of the maximum response evoked by L-alanine or L-glutamate. The symbol s indicates a slight response, < 10%; ND, not determined. The column labeled Adapt indicates whether the EOG response for each compound showed evidence of adaptation during exposure to the compound.

Extracellular Responses

The initial characterization of chemosensitivity in dissociated olfactory neurons was made with on-cell recordings by monitoring extracellular current transients elicited by action potentials (Fig. 1 *A*). Test odorants caused a change in the frequency of action potentials in sensitive cells.

Primary response characteristics. Two qualitatively different types of odor-elicited responses were observed: excitation and inhibition. These responses appeared as an odor-elicited increase or decrease, respectively, in the frequency of action potentials (Fig. 1, *B* and *C*); odors also elicited action potentials from some cells when no spontaneous activity was observed. Of the 31 cells that responded to test odorants, 16 showed excitation and 15 showed inhibition. Three cells that were strongly inhibited by mixtures of amino acids were also weakly stimulated by single compounds. Although this later observation of cells with a mixed response character must be regarded as preliminary, it suggests that olfactory neurons should not be classified simply as "excitatory" or "inhibitory," and I have avoided this terminology.

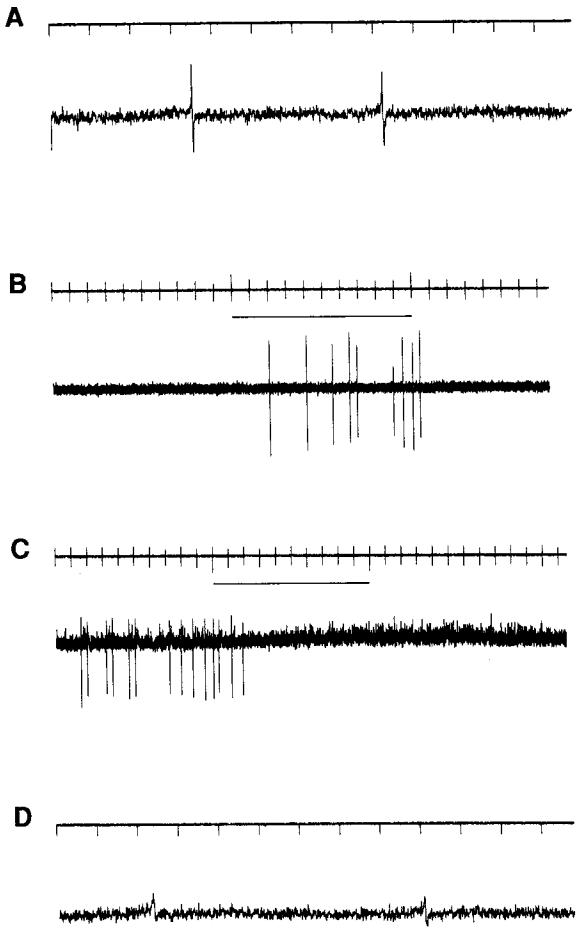

FIGURE 1. Current transients recorded from dissociated mudpuppy olfactory neurons. Biphasic current transients induced by action potentials in isolated olfactory neurons were recorded on-cell with tightly sealed patch electrodes. (*A*) Current transients induced by spontaneous action potentials in the absence of test odorants. The pipette was held at −67 mV; time marks at 100-ms intervals. (*B*) Excitation. A single 27.65-s record showing biphasic currents elicited by 1 mM L-alanine applied during the 10-s interval marked by the horizontal bar; time marks at 1-s intervals. The pipette was held at 0 mV. This cell showed no spontaneous activity (3-505.89). (*C*) Inhibition. A single 32.5-s record from a spontaneously active cell; the biphasic current transients were completely suppressed by a mixture of 8 L-amino acids (R, A, L, E, D, Q, K, C; 500 μM each). The pipette was held at 0 mV. Recovery occurred after the interval shown (4-20011.89). (*D*) Biphasic current transients recorded in the presence of odorants. In some cells, odorants appeared to depress the amplitudes of the current transients. The data in this panel are from the same cell as in *A*, both shown at the same gain and same pipette potential, but here in the presence of a mixture of seven L-amino acids (T, R, A, H, L, E, D; 100 μM each). All data were sampled at 1-ms intervals and filtered at 5 kHz. During on-cell recordings, the pipette potential had no effect on the frequency of spontaneous or evoked action potentials. Examples of cells with spontaneous activity at different pipette voltages are shown in this figure. Amino acids are listed using their single-letter codes: A, ala; C, cys; D, asp; E, glu; F, phe; G, gly; H, his; I, ile; K, lys; L, leu; M, met; N, asn; P, pro; Q, gln; R, arg; S, ser; T, thr; V, val; W, trp; Y, tyr.

The odor-elicited responses were reversible; cells typically recovered their basal activity rate in <30 s in the absence of odorants. In some cells the change in activity induced by odorants declined even in the continued presence of the odorant. Examples of recovery of basal activity after exposure to odors can be seen in Figs. 1 *B*, 2 *A*, 3, 5, and 6.

Inhibition was typified by an odor-induced reduction in frequency of action potentials, but in a few cells this was accompanied by a marked decrease in the magnitude of extracellular current transients (cf. Fig. 1, *A* and *D*). Since this did not occur in all cells, it demonstrates that these are two different manifestations of the cells' responses to odors. Separation of the decline in current transient amplitude from the reduction in frequency of action potentials suggests that odors can modulate two or more different conductance mechanisms during inhibition. A decline in current transient amplitude could be due to a slowing of the action potential or to an effect on a particular component of the membrane conductance. In an effort to detect these possible mechanisms, current transients were averaged and numerically integrated to estimate the time course of the underlying action potentials; however, this approach was not sensitive enough to reveal the source of the amplitude reduction.

Secondary response characteristics. Although the responses of olfactory neurons fell into two primary classes, excitation and inhibition, considerable variation was noted within each class. The more prominent of the variable features were termed secondary response characteristics; these included adaptation, intensity, and the off-response. Similar features have been described in other sensory systems where they affect information processing. In the olfactory system these features may be important for discrimination and chemical coding.

The secondary response characteristics of an individual neuron were identified by examination of the neuron's averaged response to stimulation. The protocol for obtaining these data is illustrated in Fig. 2. The cells were repetitively stimulated by perfusion with odorants for 10 s at intervals of 1–5 min. On-cell recordings were made for nominal periods of 30 s in most cases, beginning 10 s before perfusion with odorants. The time at which action potentials occurred in each record was then marked, the records were aligned with the start of stimulation, and the marks were collected in a single summed record, producing an integrated portrait of the response. From these summed responses the average rate at which action potentials occurred was computed. The illustration of this procedure in Fig. 2 *A* uses data from a cell that was excited by a mixture of amino acids. Fig. 2 *B* shows the result of a similar analysis of a different cell whose firing was inhibited by amino acids. Response latencies such as those seen in these averages after the start and end of an odor puff varied from cell to cell; the most likely cause of variable latency was dilution of odorants in the puffer pipette tip due to back-flow during intervals between stimulation. Since back-flow was not controlled, the latency delays cannot be interpreted as important features of the odor response; however, they do indicate that perfusion per se was not a sufficient stimulus to elicit responses.

Two examples of secondary characteristics, adaptation and the off-response, can be seen in Fig. 2 *A*. Adaptation appears as a decline of the average firing rate during exposure to odorants. As opposed to desensitization, here the cell was still capable of responding to a change in stimulus concentration at the termination of the puff. Five cells showed evidence of adaptation; six others showed a decline in response during the odor application which could have been adaptation or desensitization. These features were detected during both excitation and inhibition. The off-response of the

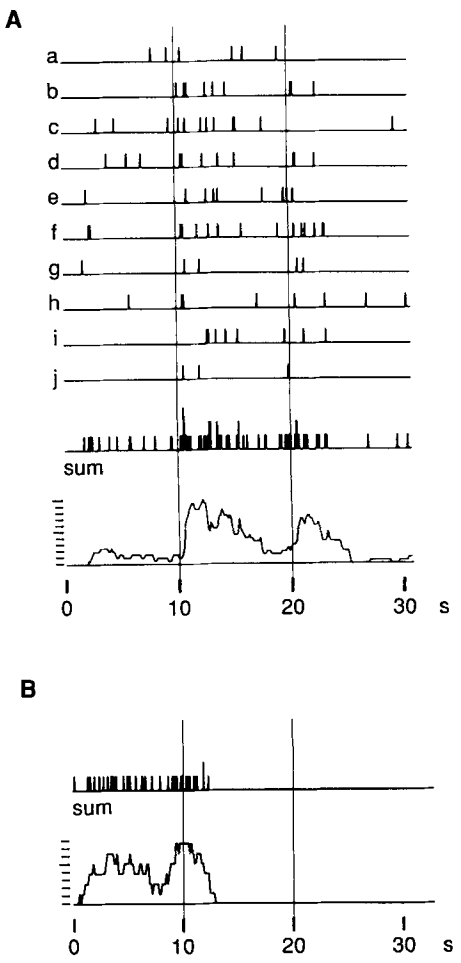

FIGURE 2. Estimating the average cellular response. This figure shows the average responses from two different cells and explains how these were determined. (*A*) Excitation. 10 responses to repeated odor puffs were recorded from a cell and aligned, and the action potentials in each record were marked; the 10 marked records are shown here as traces *a–i*. Each trace is 30.7 s long; chemical stimuli were applied during the 10-s interval between the two vertical lines. The time base of each trace has been divided into 30-ms-wide bins, and action potentials occurring in any bin are represented by a short vertical mark. The marks in all 10 traces were then collected into a single "summed record," shown here as the eleventh trace. In the summed record, bins with more than one action potential are shown by double or triple length marks according to the number represented. The relative rate at which action potentials occurred in the summed record is plotted as the bottom trace in the panel. The rate was computed by scanning the summed record with a 2-s-wide window and plotting the number of action potentials within the window. The calibrations on the ordinate of the rate plot in this panel and in the panel below are in 0.1-Hz increments referred to a single average trace. This cell was excited by application of the odorants and showed both adaptation and an off-response. 10 s after termination of the puff, the rate of firing was lower than that before the stimuli were applied, suggesting that there might have been a late period of inhibition. This cell was exposed to a mixture of seven amino acids (T, R, A, H, L, E, D; 100 μM each) (3-18012.90). (*B*) Inhibition. The summed record and average rate plot are shown for a cell in which activity was inhibited by amino acids. The summed record was derived from four traces during which the cell was exposed to a mixture of eight amino acids (R, A, L, E, D, Q, K, C; 500 μM each). Each trace was 32.5 s long; the mixture was applied during the 10-s interval between the two vertical lines. The mixture suppressed all action potentials with its effect outlasting the portion of the recovery period shown. Firing returned to normal within 30–60 s. All the individual traces showed identical behavior (4-20011.89).

cell in Fig. 2 *A* produced a substantial increase in the firing rate upon termination of the stimulus. Off-responses were seen in four cells.

The other common secondary characteristic of odor responses was a variability in the intensity of the response. Weak excitatory responses showed a two-fold increase in firing rate, while the most robust excitatory responses showed sustained increases of 20-fold and more during exposure to odorants. Inhibitory responses ranged from partial suppression of the firing rate by ~30% (less was difficult to detect) to complete suppression. Although the secondary features were characterized using averaged responses, their essential nature could often be seen in individual data traces, as in Fig. 2 *A*.

Chemosensitivity of apical membrane. Although amino acids are known to be potent olfactory stimuli for aquatic animals (Artz et al., 1986; McClintock and Ache, 1989), they are also important cellular nutrients that are actively transported across the plasma membrane. To evaluate whether the chemical responses induced by amino acids in olfactory neurons were candidates for odor transduction or were nontransduction effects possibly related to uptake, the apical and basolateral membranes were tested for relative chemosensitivity. Odorant detection is expected to be confined to the apical surfaces of the sensory neurons, since this is where odorants are encountered in vivo. The data in Fig. 3 were obtained from a cell positioned so that its basolateral (mostly soma) and apical (mainly cilia and dendritic ending) membrane regions could be perfused separately by the puffer; the perfusate was washed away from the cell by the flowing bath saline. Application of the odor mixture to the ciliated region (Fig. 3 *A*) but not to the soma (Fig. 3 *B*) elicited a substantial increase in the rate at which action potentials were generated. This suggests that the responses described here resulted from transduction processes used by olfactory neurons for odor detection in vivo. Using whole-cell methods to measure membrane current or membrane potential, many cells were examined that were insensitive to the test amino acids. These insensitive cells showed no induced change in current or potential, suggesting that amino acid uptake mechanisms in mudpuppy olfactory neurons are not electrogenic or are low in total turnover, producing negligible net ionic fluxes.

Intracellular Responses

Whole-cell current-clamp recordings were made from 62 olfactory neurons, including 23 of the 31 cells in which chemosensitivity was demonstrated. Odors appeared to modulate membrane excitability by affecting several different components of the membrane conductance.

Under normal as opposed to resistive whole-cell conditions, dissociated neurons were insensitive to odorants, and cells with proven on-cell chemosensitivity became insensitive within seconds. Reducing the number of on-cell test responses elicited before making normal whole-cell measurements had no effect, indicating that loss of chemosensitivity was due to events occurring in whole-cell mode and not to fatigue. The loss of sensitivity in whole-cell mode occurred whether or not the pipette contained 5 mM MgATP and 100 μM GTP. Using the resistive whole-cell recording method, chemosensitivity was preserved and features of the potential changes underlying odor-induced changes in excitability were examined. These results

suggest that dialysis of the intracellular contents with saline from the patch pipette led to disruption of odor transduction.

Sustained receptor potentials. Slow, sustained receptor potentials (Fig. 4) were elicited by odorants from cells that responded with excitation and from other cells that responded with inhibition. However, not all chemosensitive cells showed these

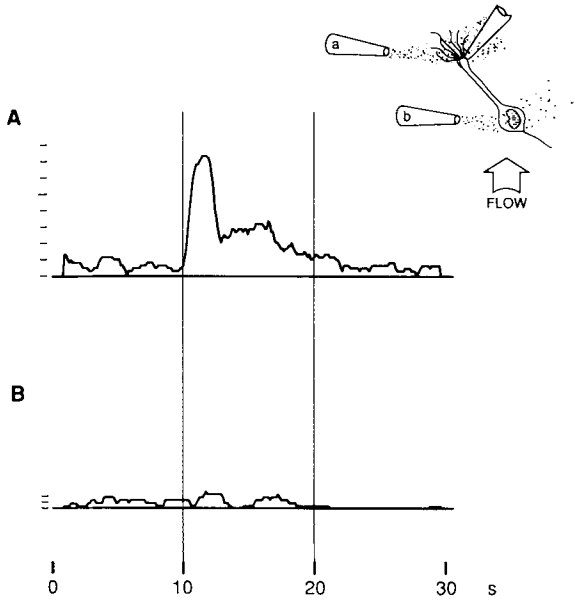

FIGURE 3. Localization of the chemosensitive membrane. These panels show the average rate of action potentials that were recorded when odorant puffs were applied for 10 s during the interval between the vertical lines. The inset shows the relative positions of the olfactory neuron (settled and lightly attached to the glass stage), patch electrode (sealed on the end of the dendrite), and puffer pipette (positions *a* and *b*), as well as the direction of bath flow. The puffer contained a mixture of seven L-amino acids (T, R, A, H, L, E, D; 100 μM each). For purposes of illustration, the approximate distribution of odorants is shown by the stippled area; it was not actually measured. (*A*) The rate of action potentials elicited by amino acids directed at the ciliated end of the dendrite. The average response to application of the odor mixture was an ~10-fold increase in the rate of firing that lasted ~3 s before declining to a level that remained above the background rate. The rate plot was derived from a summed record of 15 traces. (*B*) The rate of action potentials elicited by stimuli directed at the soma. This rate plot, derived from a summed record of six traces, shows no significant change of the rate of firing when the puffer was directed at the soma. The ordinates of both rate plots are marked in 0.1-Hz increments referred to a single trace. The somal and dendritic responses were interspersed in the sequence of data collection, moving the puffer pipette back and forth between the two stimulation positions. Both on-cell and resistive whole-cell recordings were used in each rate plot (see text for explanation of the whole-cell method); responses of each type were similar to the composite. On-cell responses were recorded for the first 10 min, then resistive whole-cell responses for 16 min. Throughout this time the cell remained chemosensitive. The recording condition was finally changed to normal whole-cell mode by applying suction to the pipette; the chemosensitivity disappeared immediately (6-11012.90).

potentials. Of 13 cells in which test stimuli caused excitation, the same odorants elicited sustained depolarizing receptor potentials (10–20 mV) from only six. Similarly, sustained hyperpolarizing receptor potentials (5–20 mV) were seen in only 3 of 11 cells whose primary response to the odorants was inhibition. Odors that caused inhibitory responses in a particular cell never caused depolarizing receptor potentials in that cell, nor were excitatory responses to a particular odor ever paired with

hyperpolarizing receptor potentials in the same cell. The three cells in which hyperpolarizing receptor potentials were observed did not show spontaneous activity in whole-cell mode. Hyperpolarizing receptor potentials had an apparent null voltage near −90 mV (Fig. 4 *C*) in the expected vicinity of the K^+ equilibrium potential, suggesting that they were produced by activation of a K^+ conductance.

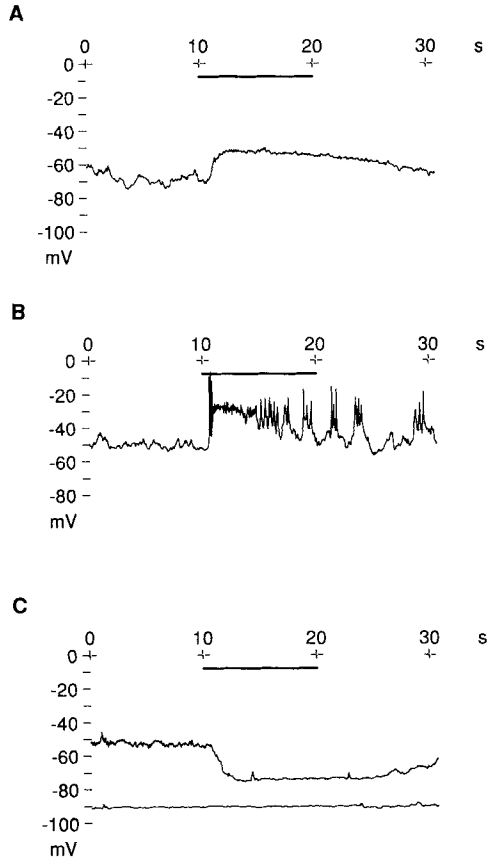

FIGURE 4. Receptor potentials. Receptor potentials, induced by the application of amino acids, were recorded from cells in resistive whole-cell mode. In each of the three panels, a different cell is illustrated; odorants were applied for 10 s during the interval marked with the horizontal bar. (*A*) A depolarizing receptor potential induced by 400 μM L-alanine. The membrane potential did not reach threshold and no action potentials were elicited (6-1103.90). (*B*) A depolarizing receptor potential together with depolarizing generator potentials (see Fig. 5) and action potentials elicited by 500 μM L-alanine. The cell fired briefly when it first depolarized and then less frequently during recovery. After the stimulus was applied, the receptor potential was sustained for ~4 s and then began to decline in the continued presence of alanine. Coincident with this decline was the appearance of the generator potentials, which subsided over a slower time course. The transient generator potentials each induced several nonovershooting action potentials (6-18012.89). (*C*) A hyperpolarizing receptor potential induced by a mixture of four amino acids (100 μM each; A, H, R, T). The two traces in this panel are from the same cell, the lower one recorded at the resting potential and the upper one recorded when the cell was depolarized by a constant 8 pA injected through the recording pipette. The stimulus mixture was applied for 10 s in both cases. This cell was also tested with 100 μM cinnamaldehyde; cinnamaldehyde failed to induce any receptor potential either at the resting potential or when the cell was depolarized to −50 mV with constant current (6-2803.90).

Transient generator potentials. In some olfactory receptor neurons, action potentials were produced by transient generator potentials, rhythmic depolarizations of ~20 mV magnitude that lasted a few hundred milliseconds (Fig. 5). Odorants could modulate the generator potentials to alter the frequency of action potentials. Both suppression and stimulation were observed using the resistive whole-cell

method. Modulation of the generator potentials did not affect the nominal resting potential of the cell. Fig. 5 *A* shows an example in which transient depolarizations were stimulated as a response to application of a mixture of amino acids, while Fig. 5 *B* illustrates a sustained and near complete suppression of generator potentials. In both examples, a return to the unstimulated levels of activity occurred within 30 s after removal of the odorants. In one cell, odorants appeared to stimulate both a sustained receptor potential together with transient generator potentials (Fig. 4 *B*). These data suggest that odor-dependent modulation of transient generator potentials occurs by different mechanisms than does production of sustained receptor potentials.

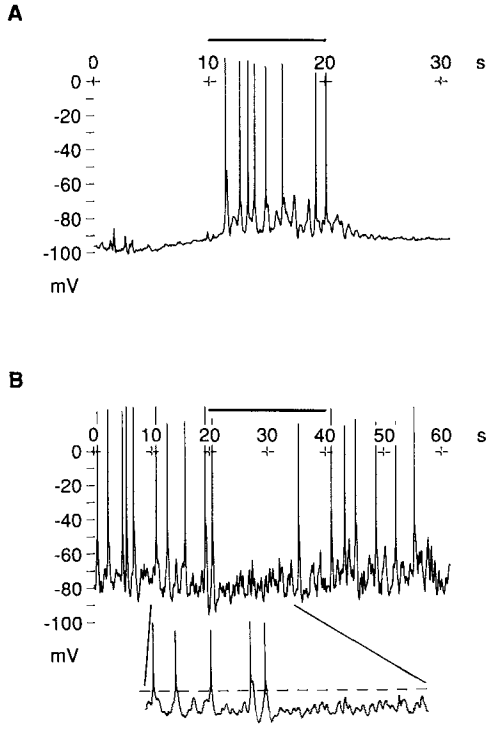

FIGURE 5. Generator potentials. This figure shows odor-induced modulation of rythmic generator potentials from two different cells in response to stimuli applied during the intervals marked by the horizontal bars. (*A*) Excitation. A mixture of seven amino acids (100 μM each; T, R, A, H, L, E, D) activated a train of slow depolarizations. Each depolarization lasted ~0.5 s, and most elicited an overshooting action potential. When application of the stimulus mixture was stopped, the repetitive depolarizations subsided (3-18012.90). (*B*) Inhibition. A mixture of five amino acids (100 μM each; R, E, G, H, S) caused a pronounced inhibition by suppressing the slow, repetitive depolarizations that this cell produced in the absense of the stimulus. The slow depolarizations had approximately the same duration as those in *A* (note that the time base is different), with each bringing the membrane potential to threshold and eliciting one action potential. The inset is an expanded portion of the record showing the triangular shape of the slow depolarizations and their suppression during the first 15 s of application of the odor mixture. The dashed line through the inset was drawn at −55 mV (2-1903.91).

Silent modulation of excitability. Several cells were found that appeared to be chemosensitive during on-cell testing, but that showed no odor-elicited potential under resistive whole-cell recording conditions. This phenomenon might occur if odors affected a conductance that was not reflected as a generator or receptor potential (a silent response), or if the cells were inadvertently rendered unresponsive by the whole-cell recording condition. To evaluate whether the excitability of these cells was affected by the test odorants during resistive whole-cell recordings, step depolarizations were elicited by constant current pulses in the absence and presence

of odorants. In two cells, both of which were inhibited by odorants during on-cell recording, the same chemical stimuli reversibly reduced the number of action potentials that were elicited by the step depolarizations, but did not cause a sustained receptor potential, modulate a generator potential, or affect the input resistance (Fig. 6). Neither the amplitudes of the depolarizing voltage steps nor the resting

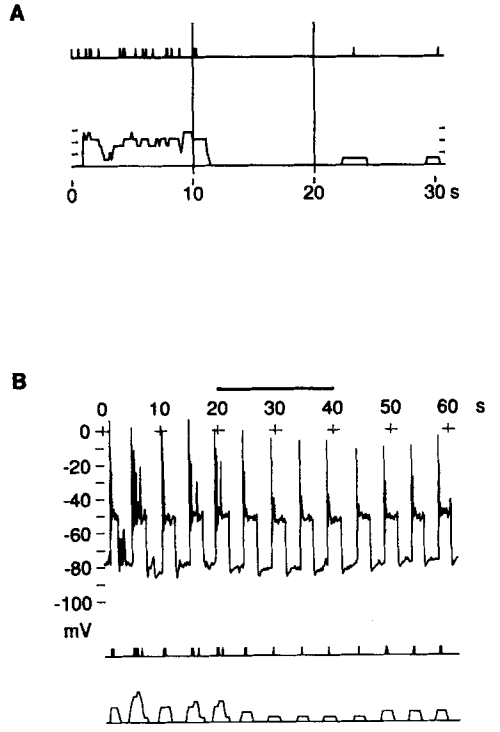

FIGURE 6. Silent modulation of excitability. The data in this figure are from a single cell stimulated with a mixture of amino acids (100 μM each; T, R, A, H, L, E, D). (*A*) Action potentials recorded on-cell were inhibited by the odor mixture. The upper trace in this panel is the summed response from 10 tests. Note the rapid onset and complete suppression of the action potentials after application of the odorants. The lower trace in *A* shows the average rate of action potentials in each trace, estimated using a 2-s window; the calibrations on the ordinate are marked at 0.1-Hz intervals referred to a single average trace. (*B*) Odor-induced reduction of excitability in whole-cell mode. Recording with the resistive whole-cell method from this cell revealed no receptor or generator potentials. Step depolarizations induced by 10-pA current pulses of 1–2 s duration were then applied before, during, and after application of the odorants. Each voltage step depolarized the cell close to its firing threshold and elicited a brief burst of action potentials. The upper trace is a 64-s record of membrane potential during which 13 depolarizing voltage steps were applied. The action potentials induced by each step have been marked on the middle trace in this panel. With application of the odorants (applied for 20 s, horizontal bar) the pattern of action potentials changed with a time course similar to the change seen on-cell (*A*), and the number of action potentials was reduced to one per pulse. Recovery began soon after the odorant application was stopped. The bottom trace is the rate plot of induced action potentials estimated with a 2-s window. The transient decrease in relative peak height corresponds to application of the odorants followed by partial recovery during the final 20 s as the odorants were washed out (4-14012.90).

membrane potential seemed to be significantly altered by the odorants, indicating that the resting membrane conductance was largely unaffected. In one other cell, odorants increased the firing frequency about twofold under similar conditions; however, this cell was excited during initial on-cell recordings, and its first few whole-cell responses resembled those in Fig. 5 *A*. Testing the cell with step depolarizations was done only after these whole-cell responses declined.

Responses to L-Alanine

Recordings were made from cells stimulated with the single compound L-alanine (as opposed to odor mixtures) to test whether one of the two primary odor response characteristics could be uniquely associated with any pure odorant. L-Alanine was selected since it had been identified as a potent odorant in the EOG measurements, and its use might maximize the number of responses seen. Each cell was screened for chemosensitivity with an odor mixture before performing the single odor measurements. Of 15 responsive cells, L-alanine elicited excitatory responses from 9 cells and inhibitory responses from 4 cells. Two other cells were insensitive to L-alanine alone, but were responsive to a mixture of four amino acids that included L-alanine. Both excitatory and inhibitory responses were observed in on-cell and whole-cell recordings, showing that different conductances were activated in the two cases. Chemosensitive cells that were excited by, inhibited by, and indifferent to L-alanine were found in the same preparation from a single animal as well as in different preparations, indicating that neither preparative differences nor animal-to-animal variations account for the results. Thus, a single compound appears capable of eliciting both excitatory and inhibitory responses from different cells as well as eliciting no response from some cells that were shown to be chemosensitive.

DISCUSSION

The olfactory system in the mudpuppy, *Necturus maculosus,* is structurally similar to that found in air-breathing vertebrates, but the mudpuppy is a gilled salamander that lives underwater. I have used it to study the physiology of odor detection because of the advantages of working in aqueous solutions, and because the chemical stimuli that aquatic animals smell may be less numerous than for air-breathing animals. In addition, the olfactory neurons of this polyploid animal are large compared with those of other vertebrates, simplifying the single-cell recording techniques that were used.

Before 1988, few examples of odor-elicited responses from isolated olfactory receptor neurons had been described because the cells seemed to lose their chemosensitivity after dissociation (Anderson and Ache, 1985; Trotier, 1986; Anderson and Hamilton, 1987; Maue and Dionne, 1987). Enzyme-free methods (Frings and Lindemann, 1988; Firestein and Werblin, 1989) have successfully redressed the problem, and mudpuppy olfactory neurons prepared without enzymes retain their chemosensitivity. It was also noticed that housing mudpuppies in the laboratory for more than a few weeks led to diminished olfactory responses, the reasons for which are uncertain. Thus, early difficulties in preparing chemosensitive cells may have resulted from several causes acting simultaneously.

Both excitatory and inhibitory responses were detected in isolated neurons in this study. Excitatory responses have been detected commonly in vertebrate olfactory neurons (Gesteland, Lettvin, and Pitts, 1965; O'Connell and Mozell, 1969; Duchamp, Revial, Holley, and MacLeod, 1974; Getchell, 1977; Getchell and Shepherd, 1978; Revial, Duchamp, and Holley, 1978; Trotier and MacLeod, 1983; Frings and Lindemann, 1988, 1990; Firestein and Werblin, 1989; Kurahashi, 1989; Firestein, Darrow, and Shepherd, 1991), but inhibitory responses, when seen, have been

reported to be infrequent (Gesteland et al., 1965; O'Connell and Mozell, 1969; Duchamp et al., 1974; Getchell and Shepherd, 1978; Revial et al., 1978). Those early observations of inhibition were made in single units using microelectrodes. This, together with a low frequency of detection, led investigators to question whether odors directly elicit inhibitory responses from vertebrate olfactory neurons (Getchell and Shepherd, 1978), and to attribute apparent inhibition to activity-induced local changes in ion concentrations (Gesteland and Adamek, 1987). By comparison, odorants elicit both excitatory and inhibitory responses from lobster olfactory neurons (McClintock and Ache, 1989). The evidence presented here of excitatory and inhibitory responses from isolated mudpuppy olfactory neurons demonstrates that the responses are a primary result of odorant activity. It suggests that much of the complexity detected in odor responses in vivo originates in different receptor cells and not from intercellular or nonreceptor mechanisms.

The low incidence of inhibitory responses in frogs and land-phase salamanders, together with their more common appearance in mudpuppies and lobsters, suggests that inhibition may not be a feature of olfactory transduction in all animals. Possibly only aquatic animals show prominent odor-elicited inhibition of olfactory responses, or possibly only amino acids elicit these responses. It is still not known whether inhibitory responses occur in mammalian olfactory neurons; however, limited indirect evidence from dissociated mouse neurons (Maue and Dionne, 1987) suggests that inhibitory odorant responses may occur in mammals also.

It is important to question whether responses elicited by odor stimuli in vitro represent true olfactory transduction or result from nontransduction effects. This is a special problem for cellular and subcellular studies in olfaction, since odorants may elicit a variety of quite specific membrane effects that may be unrelated to transduction in vivo (Lerner, Reagan, Gyorgyi, and Roby, 1988; Kashiwayanagi, Suenaga, Enomoto, and Kurihara, 1990). Two observations suggest that the responses described here are true transduction. First, they were generated only when the apical membrane of cells was exposed to odorants. Second, only a small percentage of the cells responded to the test odors, and in many cases cells were seen to respond to one chemical stimulus and not to another. The first observation would be expected if the responses were specific to odor transduction since only the apical membrane is exposed to odorants in vivo; a similarly restricted distribution of chemosensitive membrane has been reported in other studies (Kurahashi, 1989; Firestein et al., 1990; Lowe and Gold, 1991). The second observation is consistent with earlier single unit studies using more intact preparations (reviewed in Getchell, 1986): individual olfactory neurons respond to some compounds and not to others. Possibly more surprising in this regard was that the fraction of responsive cells (30% overall) was so high. This may reflect the particular selection of odorants or the possibility that mudpuppies may detect few odorants, or it may indicate a wide distribution of chemosensitivities among olfactory neurons in the mudpuppy. More extensive measurements will be required to evaluate these alternatives.

While extracellular, odor-elicited responses were robust and repeatable, the sensitivity to odorants rapidly (within 5 s) and irreversibly declined completely in all cells examined with normal whole-cell methods. Similar washout, but with a slower onset, has been reported in other studies (Frings and Lindemann, 1988; Kurahashi, 1989;

but see also Firestein et al., 1990). The comparatively rapid washout of chemosensitivity in these studies may reflect the placement of the patch electrode on the dendritic ending as opposed to the soma. This could reduce the length of a critical diffusion pathway. Such a labile chemosensitivity suggests that direct odorant-gated conductance mechanisms may be neither prominent nor critical for odor transduction. Instead, the primary transduction mechanisms are more likely to depend on diffusible cytoplasmic constituents, consistent with a role for second messengers in transduction. These varied features of odor transduction are compatible with the large family of seven-transmembrane domain proteins that have been identified recently as putative olfactory receptors (Buck and Axel, 1991; Nef, Heinemann, and Dionne, 1991*a*; Nef, Hermans-Borgmeyer, Gasic, Heinemann, and Dionne, 1991*b*).

L-Alanine elicited excitation in some cells and inhibition in others. The excitatory responses were associated with depolarizing receptor potentials and initiation of generator potentials in different cells (Fig. 4); in two other cells (data not shown), inhibitory responses were accompanied by a hyperpolarizing receptor potential (one cell) and by suppression of generator potentials (one cell). These data indicate that several different components of the membrane conductance can be modulated by a single odorant, and that the distinct transduction pathways being stimulated diverge at their most distal points, the membrane ion channels. It is not known whether the different transduction pathways for L-alanine involve the same or different receptor proteins and second messengers.

The advantages accruing from more than one transduction pathway for a single odorant are uncertain, but they may provide a means to enhance sensory discrimination among odorants. Possibly L-alanine typifies most odorants in that each may stimulate several different transduction pathways with varying efficacies in different cells. This would produce cells that responded best to a specific subset of odors and less well to other compounds, and that were inhibited by still others. Depending on the pathways represented, a cell could be excited or inhibited to varying degrees by particular odor subsets while being insensitive to many other odorants. Inhibition of electrical activity in a group of sensory neurons could help to sharpen a pattern of excitation produced in other neurons by that odor. Secondary response characteristics could enhance these properties. This diversity of responsiveness was first remarked upon by Gesteland et al. (1965), who characterized it in the extreme as utter chaos. Although chaotic in appearance, the olfactory system resembles other sensory systems (Shepherd, 1988), and the diversity of responses from its receptor cells may underlie its competence in odor detection and discrimination.

The different effects of odorants on receptor potentials, generator potentials, and the firing properties of isolated neurons described here suggest that several different conductances are modulated. Although the specific conductances were not identified in these studies, there are a number of strong candidates. The sustained depolarizing receptor potential could be produced by activation of nonselective cation channels by cAMP (Nakamura and Gold, 1987), while the sustained hyperpolarizing receptor potential appears to arise from activation of a K^+ conductance. Modulation of the transient generator potentials that underlie both excitation and inhibition in some neurons could reflect regulation of intracellular free Ca; the transient potentials might then depend on activation of a Ca-sensitive conductance such as Cl^- (Kleene

and Gesteland, 1991), K^+ (Potter and Dionne, 1986; Maue and Dionne, 1987), Na^+ (Frings and Lindemann, 1988), or one poorly selective for cations (Schild and Bischofberger, 1991). The "silent modulation" illustrated in Fig. 6 could reflect changes in the repolarizing phase of the action potential. Ion channels that affect repolarization in other neurons include Ca-activated and delayed rectifier K^+ channels (e.g., Schwindt, Spain, Foehring, Statstrom, Chubb, and Crill, 1988). Each of these conductances is present in mudpuppy olfactory receptor neurons (Dionne, 1988). The presence of several different ion channels as the final targets of odor transduction may necessitate multiple second messengers. In other olfactory studies, likely roles for cAMP (Pace et al., 1985; Nakamura and Gold, 1987; Frings and Lindemann, 1991), inositol trisphosphate (Boekhoff et al., 1990), and Ca^{2+} (Restrepo et al., 1990) have been described. The functional roles of these agents must still be assessed at the single-cell level.

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