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Activation by odorants of a multistate cation channel from olfactory cilia

(olfaction/chemosensory transduction/planar lipid bilayers/single channels)

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ABSTRACT **Single-channel records were obtained after fusion of ciliary membranes from the olfactory epithelium of *Rana catesbeiana* to planar lipid bilayers, and odorant-activated cation-selective channels were identified. In addition, a 190-pS potassium-selective channel and a 40-pS cation-selective channel were found in a 0.2 M salt-containing buffer. Odorant-sensitive channels were directly and reversibly activated by nanomolar concentrations of the bell pepper odorant 3-isobutyl-2-methoxypyrazine and the citrus odorant 3,7,-dimethyl-2,6-octadienenitrile. These channels display burst kinetics, multiple conductance levels between 35 and 420 pS, and open times in the millisecond range. With increasing concentrations of odorant, the probability of populating the higher conductance levels increases. These results show that direct activation of channels by odorants may mediate excitation of the olfactory receptor cell.**

Initial transduction events in olfaction occur at the membranes of chemosensory cilia, which protrude from the dendritic tips of olfactory receptor neurons (1–3). The binding of an odorant to the ciliary membrane must result directly or indirectly in the activation of ion channels, which in turn culminates in the generation of action potentials.

It has been postulated that signal transduction at the olfactory membrane may be mediated by cAMP and that this second messenger would activate ion channels either directly or via a protein kinase cascade (2, 4). The electroolfactogram, a summated potential recorded from the surface of the olfactory epithelium after application of odorant, is modified by cAMP (5, 6). Olfactory cilia contain a high activity of adenylate cyclase (7). In addition, Nakamura and Gold (8) recently patch-clamped olfactory cilia and observed a conductance that is activated by micromolar concentrations of both cAMP and cGMP. Moreover, the olfactory adenylate cyclase activity can be stimulated by micromolar concentrations of some, but not all, odorants via a regulatory GTP-binding protein (7, 9, 10).

Several laboratories have investigated ion channels in olfactory receptor cells. Patch-clamp studies on the dendrite and soma of isolated olfactory receptor cells revealed several types of K^+ channels (11, 12). Furthermore, Trotier (12) demonstrated the activation of a cationic current by micromolar concentrations of the odorants isoamyl acetate and *n*-butanol with a reversal potential near 0 mV. Finally, Vodyanoy and Murphy (13) added membrane homogenates of rat olfactory epithelium to planar lipid bilayers and observed activation of K^+ channels with a mean open time of about 40 sec by nanomolar concentrations of the odorant diethyl sulfide.

We fused ciliary membrane vesicles from the olfactory epithelium of the bullfrog, *Rana catesbeiana*, to planar lipid bilayers and observed three distinct cation-selective channels. Only one of these, a multistate channel with millisecond open times, is reversibly activated by nanomolar concentrations of two structurally unrelated odorants. Our observations indicate that direct activation of ion channels by odorants may underlie the initial event in olfactory transduction.

MATERIALS AND METHODS

Bullfrogs (*R. catesbeiana*) were obtained from Amphibians of North America (Nashville, TN) and were maintained in a well-ventilated facility in a tank with circulating water. Olfactory cilia were prepared as described (14) and resuspended in a small volume of Ringer's solution containing 2 mM EGTA. The protein concentration of the suspension was measured by the method of Lowry *et al.* (15) with bovine serum albumin as standard.

The bilayer system used was modeled after the description of the apparatus by Alvarez (16). The chambers contained a volume of 2–3 ml each. A push–pull arrangement of syringes was used to perfuse the chambers. In separate experiments it was determined that perfusion with 12 ml of odorant-free buffer reduces the concentration of odorant by a factor of 25. Voltage was applied, and current was measured by using Ag/AgCl electrodes connected to the chambers through 1 M KCl/agar bridges. All records were obtained at room temperature.

Bilayers were formed across 200- to 400-μm-diameter holes in polycarbonate septa from a mixture of 20 mg of phosphatidylethanolamine (from *Escherichia coli*) and 20 mg of phosphatidylserine (from bovine brain) per ml of *n*-decane. Lipids were purchased from Avanti Polar Lipids (Birmingham, AL). After bilayer formation, ciliary membrane vesicles, preequilibrated in 0.5 M sucrose, were added to the cis side of the bilayer to a final protein concentration of 1–5 μg/ml. The vesicles were fused to the bilayer by first adding 15 μl of 1.0 M $CaCl_2$ and subsequently adding, if necessary, 200–300 μl of 4.0 M KCl to the vesicle-containing (cis) side. The voltage was defined with respect to the trans side of the bilayer. Once channels were observed, the cis side was perfused with 12–20 ml of 200 mM potassium acetate/0.5 mM EGTA/5 mM Hepes, pH 7.0, unless indicated otherwise, to prevent further incorporation of channels and to eliminate salt gradients. Channel activity was not observed with olfactory cilia suspended in symmetrical salt solutions or in the absence of olfactory cilia upon the addition of 3-isobutyl-2-methoxypyrazine (IBMP) or 3,7-dimethyl-2,6-octadienenitrile (citralva) to the aqueous solutions. Replacing chloride by acetate eliminates a frequently observed anion conductance. Odorants were added to the trans side of the bilayer chamber from aqueous stock solutions while stirring. It was determined that the trans side of

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Abbreviations: IBMP, 3-isobutyl-2-methoxypyrazine; citralva, 3,7-dimethyl-2,6-octadienenitrile.

the bilayer corresponds to the extracellular side of the ciliary membrane because the 190-pS K^+ channel described in Fig. 1*A* is activated by Ca^{2+} added to the cis side. IBMP was obtained from Pyrazine Specialties (Atlanta). Citralva was donated by International Flavors and Fragrances (Union Beach, NJ). Single-channel current records were low-pass filtered as indicated and stored on magnetic tape and on a Gould chart recorder. Average conductance, conductance histograms, and channel lifetimes were obtained by computer analysis.

RESULTS

After fusion of the chemosensory membranes with the bilayer, two channels were identified in the absence of odorants that are distinct in ion selectivity and conductance (Fig. 1). One channel was K^+ selective and exhibited an average conductance of 190 pS and an average open time of 4 ms at 50 mV (Fig. 1*A*). The selectivity sequence derived from permeability ratios (P_{X^+}/P_{K^+}) as determined from biionic potentials was K^+ (1.0) > Rb^+ (0.6) > NH_4^+ (0.1) >> Na^+,Cs^+ (<0.05). This channel was activated by Ca^{2+} added to the cis side of the bilayer (data not shown). A 40-pS cation-selective channel was also observed (Fig. 1*B*). This channel had an average open time of 20 ms and was about equally permeable to Na^+ and K^+.

In 0.2 M sodium acetate-containing buffer, a third cation-selective channel was observed that was activated reversibly by nanomolar concentrations of odorants (Fig. 2*A*). In Fig. 2*A*, traces 1 and 2 represent the conductance of a bilayer after fusion of ciliary membrane vesicles but prior to the addition of odorant. Addition of IBMP to 40 nM elicited discrete bursts of conductance fluctuations displaying multiple sizes that decayed over time to lower levels of activity (Fig. 2*A*, trace 3). Perfusion of the chamber with odorant-free buffer resulted in virtual disappearance of the conductance fluctuations (Fig. 2*A*, traces 4 and 5). A second addition of IBMP to 40 nM resulted in reactivation of the conductance fluctuations (Fig. 2*A*, trace 6).

The characteristics of the conductance fluctuations induced by IBMP are shown in greater detail in Fig. 2 *B* and *C*. The odorant-induced fluctuations were composed of discrete bursts that contain well-defined conductance levels. Computer analysis of burst activity revealed the occurrence of a conductance at 35 pS as well as multiples of this conductance. A higher resolution picture of burst activity demonstrating the occurrence of discrete conductance levels that are multiples of a 35-pS conductance level is shown in Fig. 2*C*.

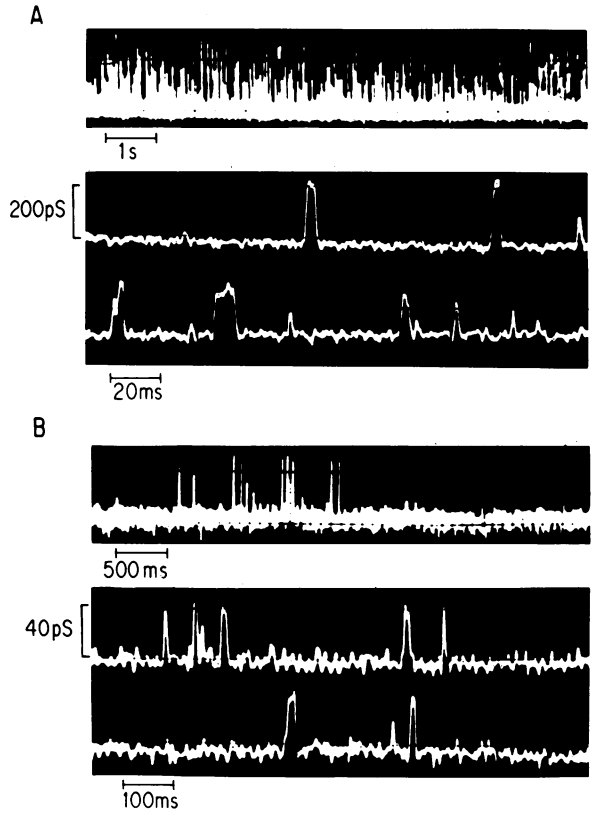

FIG. 1. Ion channels from olfactory cilia recorded in the absence of odorant. (*A*) K^+-selective channel. The record is shown both at slow (upper trace) and fast (lower trace) sweep speeds and was obtained in symmetrical solutions of 0.2 M potassium acetate/5 mM Hepes/0.5 mM EGTA, pH 7.0, at an applied voltage of 50 mV. Upward deflections represent channel openings. The record was filtered at 500 Hz. (*B*) Forty-picosiemen cation-selective channel. The record is shown both at slow (upper trace) and fast (lower trace) sweep speeds and was obtained under the same conditions as in *A*. Upward deflections represent channel openings. The record was filtered at 200 Hz.

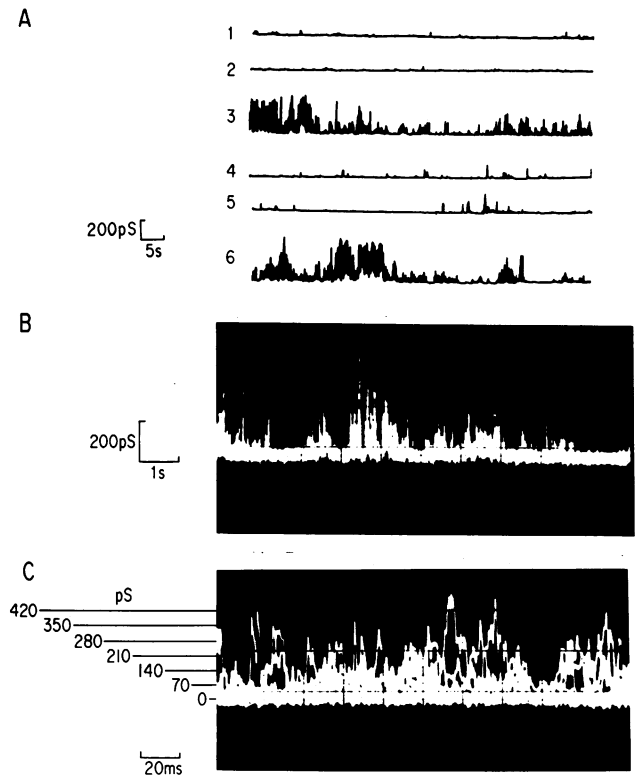

FIG. 2. Activation of the odorant-sensitive channel by IBMP. (*A*) Reversible activation of the channel by IBMP. The records were obtained from a bilayer bathed in symmetric solutions of 0.2 M sodium acetate/5 mM Hepes/0.5 mM EGTA, pH 7.0, at an applied voltage of 50 mV. Traces 1 and 2 show the conductance record after fusion of ciliary membranes to the bilayer but prior to the addition of 40 nM IBMP to the trans side. The third trace was obtained 1 min after the addition of IBMP. The fourth and fifth traces were obtained after perfusion of the chamber with odorant-free buffer. Note the reversal of the activation. The last trace was obtained 1 min after a second addition of 40 nM IBMP. Upward deflections represent channel openings. (*B*) Oscilloscope trace of the odorant-sensitive channel exhibiting bursting behavior. Note that discrete bursts lasting several hundred milliseconds display multiple conductance levels. The record was obtained under the same conditions as in *A*, 1 min after the addition of 30 nM IBMP. Upward deflections represent channel openings. The record was filtered at 500 Hz. (*C*) Superposition of several fast sweeps of the odorant-sensitive channel. Traces like those shown in *B* were superimposed to accentuate the presence of defined conductance levels. The 35-pS level can just be resolved.

Odorant-sensitive channels can also be observed in the presence of the structurally unrelated isoterpenoid odorant, citralva. Activation of channels by 100 nM citralva was similar to that induced by IBMP, although larger concentrations were needed to induce bursts of activity. Furthermore, several minutes after the addition of either odorant, channel fluctuations were observed that have similar average conductances of 90 pS and average open times of 6 ms (at 50 mV) (Fig. 3). The conductance fluctuations induced by the odorant exhibited Nernstian behavior for cations and a $P_{Na^+}/P_{K^+} \approx 1$.

Channel activity increased with increasing odorant concentration and was characterized by an increase in the frequency and duration of the bursts (Fig. 4*A*). Bursts often terminated abruptly and were followed by a diminished activity (Fig. 4*A*). Most importantly, the probability of populating the higher conductance levels increased with increasing odorant concentrations (Fig. 4*B*).

The odorant-sensitive channel, the K^+-selective channel, and the 40-pS channel have all been observed in each of 10 different preparations of olfactory cilia. Neither of the channels shown in Fig. 1 appeared to be affected by the two odorants tested. Channels activated by IBMP or citralva were not observed after fusion of ciliary membranes from the respiratory epithelium of the palate with planar lipid bilayers.

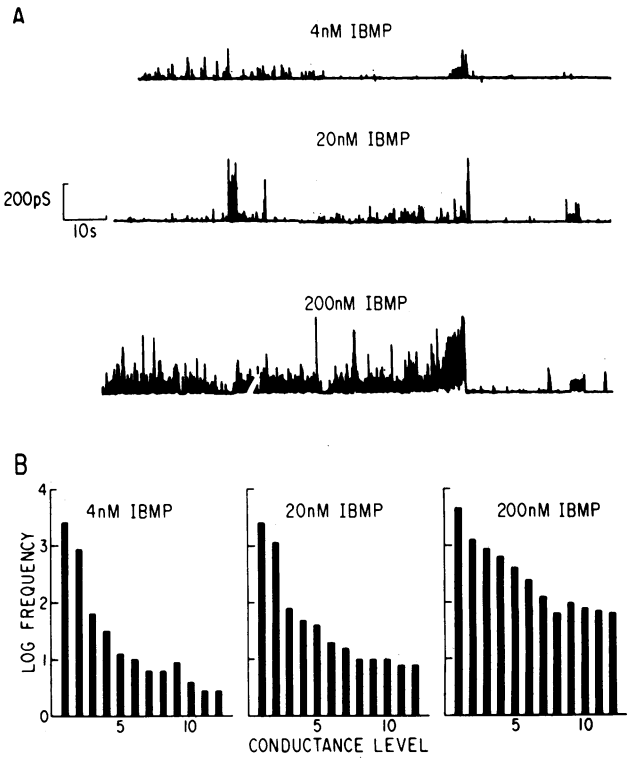

FIG. 4. Effect of odorant concentration on channel activation. (*A*) Channel activation by increasing concentrations of IBMP. The records were obtained from the same membrane 2 min after subsequent additions of 4, 20, and 200 nM IBMP. Conditions were the same as those described in Fig. 2. Upward deflections represent channel openings. The records were filtered at 500 Hz. (*B*) Frequency–conductance histograms of the odorant-activated channel at 4, 20, and 200 nM IBMP. The frequencies of channel opening to the different conductance levels were obtained by computer analysis of records similar to those shown in Fig. 4*A*. Each histogram contains 3 min of data analysis beginning 2 min after the addition of odorant. The indicated conductance levels are integral multiples of the 35-pS unitary conductance.

DISCUSSION

To acquire insights into the channel mechanisms underlying signal transduction in olfaction we detached chemosensory cilia from the olfactory epithelium of the bullfrog and fused the isolated ciliary membrane vesicles to planar lipid bilayers. We measured conductance fluctuations and discovered three cation-selective channels. One of these is K^+-selective and has the same permeability sequence as the Ca^{2+}-activated K^+ channel from T tubules of skeletal muscle (17). This channel resembles the 130 pS calcium-activated potassium channel observed by Maue and Dionne in patch-clamp studies on the dendritic knob and soma of the olfactory receptor cell (11, 18). Neither this channel nor a second 40-pS channel was activated by the two odorants tested. A third cation-selective channel was reversibly activated by nanomolar concentrations of both odorants, suggesting that direct channel activation by odorants may be the initial event in olfactory transduction.

Odorants trigger discrete episodes of channel activity, displaying multiple conductance levels. Further experimen-

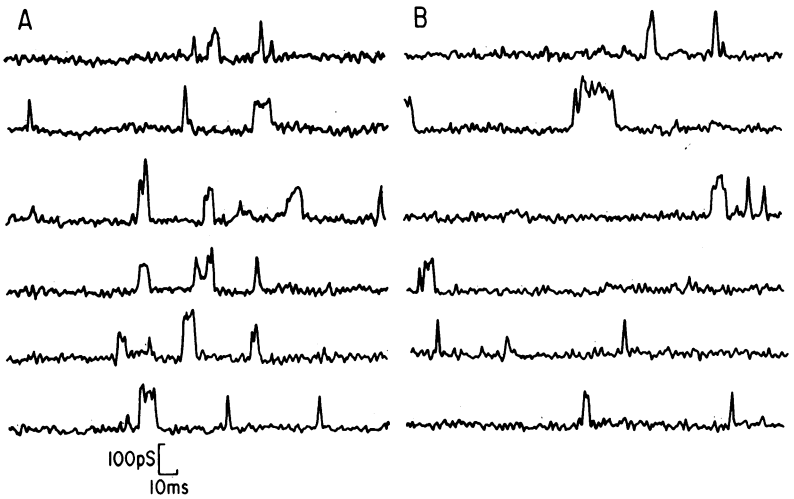

FIG. 3. Odorant-sensitive channels in the presence of IBMP and citralva. (*A*) Records of single channels 5 min after the addition of 30 nM IBMP. (*B*) Records of single channels 5 min after the addition of 100 nM citralva. The records were obtained under the same conditions as in Fig. 2 and were filtered at 500 Hz. Upward deflections represent channel openings. Computer analysis of records such as these yield an average single-channel conductance of 90 pS.

tation and analysis are necessary to determine the exact number of independent conductance levels. The higher conductance levels become more prominent at increasing odorant concentrations (Fig. 4*B*). Channel openings during bursts seem to occur sequentially from lower to higher levels (Fig. 2 *B* and *C*). Likewise, bursts end in a stepwise fashion and channels may remain inactive for long times. Plausible mechanisms that may give rise to such behavior include cooperative opening of discrete units or sequential conformational transitions within a single channel moiety triggered by binding of odorant.

The occurrence of multiple conductance states within the burst raises the question: To what extent may odorants differ in their ability to activate different conductance levels (7). The high membrane impedance of the olfactory receptor neuron would imply that activation of one burst of channel activity to the higher conductance levels would result in excitation of the olfactory neuron (12). Thus, the ability to induce higher conductance states might be related to a lower odorant detection threshold. Furthermore, selectivity for cations of the odorant-activated channel would be consistent with an excitatory conductance in the ciliary membrane.

Presently, it is not clear whether the time-dependent decay of channel activity after odorant application reflects properties inherent to the channel or arises from evaporation of the odorant, absorption of the odorant to the walls of the bilayer chamber, or partitioning of the odorant in the torus. Application of additional odorant results in increased channel activity, which again decays to a lower level of activity. Interestingly, Trotier (12) also reported a decay of odorant-induced currents recorded from patch-clamped receptor cells isolated from the olfactory epithelium of the salamander.

Although neither of the two odorants tested affects the K^+ channel or the 40-pS cation-selective channel, it is conceivable that these channels may be affected by other odorants. Our results suggest that IBMP and citralva may activate the same ion channel, although higher concentrations of citralva are needed.

Although we cannot exclude the presence of contaminating membranes from other components of the olfactory epithelium in our preparation, the use of forskolin-stimulated adenylate cyclase as a marker reveals a 150-fold enrichment of the specific activity of this enzyme in the cilia preparation as compared to membranes prepared from the remainder of the epithelium after detachment of the cilia, indicating a substantial degree of purity (7, 9).

The channels described here are distinct from the diethyl sulfide-sensitive channel from whole rat olfactory epithelium described previously, which is a K^+-selective, two-state channel with mean open times of about 40 sec (13).

Both IBMP and citralva induce bursting behavior, similar conductance levels, and similar average open times at steady state. Activation of odorant-sensitive channels is observed in the absence of ATP, GTP, or cAMP at nanomolar concentrations of odorants. It is, however, possible that limitations on the sensitivity with which stimulation by odorants of the olfactory adenylate cyclase can be detected distort the macroscopic dose–response behavior of this enzyme to apparently higher concentrations. Although the interrelationships between the adenylate cyclase system and the odorant-sensitive channels in olfactory cilia remain to be clarified, our observations suggest that direct activation of these channels by odorants is involved in excitation of the olfactory neuron.

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