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Transmembrane Currents in Frog Olfactory Cilia

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Summary. We have measured transmembrane currents in intact single cilia from frog olfactory receptor neurons. A single cilium on a neuron was sucked into a patch pipette, and a high-resistance seal was formed near the base of the cilium. Action potentials could be induced by applying suction or a voltage ramp to the ciliary membrane. A transient current was seen in some cells on stimulation with odorants. After excision from the cell, most of the cilia showed increased conductance in a bath containing cAMP, indicating that the cytoplasmic face of the ciliary membrane was accessible to the bath. The estimated resistance of a single cilium was surprisingly low.

Key Words olfaction · receptor · electrophysiology

Introduction

When odorants are applied to the olfactory epithelium, the mucosal surface develops a transient negative potential. Ottoson [20] concluded that this is a result of a receptor current which flows into the olfactory neurons. Intracellular studies of single olfactory neurons have confirmed the existence of an inward current when the neurons are stimulated with odorants [2, 3, 6, 8, 10, 14, 17, 25, 26, 28]. In some cases the resulting depolarization is sufficient to induce action potentials [2, 3, 8, 10, 25, 28].

In the frog, the transepithelial potential is almost entirely due to odorant-induced changes in the cilia of the receptor neurons [1]. Although the odorant-induced current has been demonstrated in isolated neurons, it has not yet been possible to independently measure the ciliary contributions to this current. It has been shown that odorants are most effective when delivered to the apical end of the cell [5, 14, 16], where the cilia reside. A cAMP-induced conductance change has been found in inside-out membrane patches excised from toad olfactory cilia [19] and in artificial bilayers derived from rat olfactory epithelium [31]. Many odorants activate an adenylate cyclase in membranes prepared from olfactory cilia [21, 23, 24], and it is presumed that the cAMP formed acts to increase the ciliary conductance and produce the receptor current (for a recent review *see* ref. [27]). Channels directly gated by odorants have also been found in planar lipid bilayers derived from rat olfactory epithelial homogenate [30] or bullfrog olfactory cilia [15].

Recently loose-patch recording of olfactory cilia was used to measure action potentials in olfactory epithelia [9]. We report the first tight-seal patch-clamp recordings of olfactory cilia. Recording from a cilium attached to an isolated neuron, we measured a receptor current in response to odorants. Injection of current through the ciliary membrane evoked action potentials. After excision from the cell, the cilia showed the cAMP-induced conductance originally reported in patches of ciliary membrane [19]. The resting resistance of the excised cilia was lower than expected. The ciliary patch procedure we describe should allow a complete description of the ciliary conductance properties both at rest and during excitation by odorants.

A preliminary report of this work has appeared [13].

Materials and Methods

Cell Suspensions

Northern grass frogs (*Rana pipiens*) were decapitated and pithed. The dorsal and ventral olfactory mucosae, attached to the adjacent cartilage, were removed and placed in extracellular medium (115 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM NaHEPES, pH 7.2). One of the mucosae was peeled away from the cartilage, and loose debris on the mucosal surface was removed. In a well containing 0.3 ml of extracellular medium, the mucosa was stretched and torn with two pairs of forceps and then placed under a 12-mm diameter cover glass. The cover glass was gently pressed onto the tissue several times to promote dissociation. The resulting suspension was transferred once by Pasteur pipette to a test tube, where medium was added to a final volume of 1 ml. The cell suspension was stored on ice and used for up to 8 hr.

Ciliary Patch Technique

Micropipettes were pulled in two stages on a Kopf 700D vertical puller from Fisher blue-tipped microhematocrit capillaries (soda-lime glass, o.d. 1.5 mm, i.d. 1.1 mm). Pipettes were used without coating or fire polishing. Those used to transfer cells were pulled to a tip diameter of 50 μm, those for stimulus delivery to 1 μm, and those used to isolate the cilia to about 0.5 μm. (In the last case, this is an estimate based on a bubble number of 3.4 [18].) Pipettes were filled with the extracellular medium. All solutions were filtered through Nuclepore polycarbonate membranes of 0.2-μm pore size.

Manipulations were done under phase-contrast microscopy at 600× magnification using a Nikon Diaphot inverted microscope and Narishige MO-103M hydraulic micromanipulators. A neuron or small cluster of cells was sucked into a transfer micropipette (tip diameter 50-μm) along with the smallest possible volume of medium. The pipette was moved through the air into the recording chamber, which contained 200 μl of extracellular medium. The cell was ejected into the chamber by positive pressure. A recording micropipette (tip diameter about 0.5 μm, resistance 8–10 MΩ) was brought near one of the moving cilia and suction applied until the end of the cilium entered the pipette. Suction was continued until the olfactory vesicle touched the tip of the pipette and a high-resistance seal formed. The patch procedure was videotaped, and ciliary lengths were estimated from the video images. The odorant mixture used to stimulate the patched neurons contained the following odorants at 0.1 mM each in extracellular medium: 2-heptanone, (S)(+)-carvone, *iso*amyl acetate, anisole, pyridine, benzaldehyde, *n*-hexanoic acid, cineole, *n*-butanol, and ethyl *n*-butyrate.

To excise the cilium from the cell, the pipette was raised into the air. It was then quickly reimmersed in a new bath containing pseudointracellular medium (110 mM KCl, 5 mM NaCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM tetrapotassium BAPTA, and 5 mM NaHEPES, pH 7.2). BAPTA [1,2-*bis*(2-aminophenoxy)ethane N,N,N′,N′-tetraacetic acid] is a highly specific calcium chelator [29]; in our pseudointracellular medium, free calcium was buffered to 10^{-7} M. The pipette containing the cilium could usually be transferred through the air to new baths several times before the seal would rupture.

For stimulus delivery, a micropipette (tip diameter 1-μm) containing the stimulus was brought within 5 μm of the cell or the tip of a pipette containing an excised cilium. The stimulus was ejected under the control of a General Valve Picospritzer II. All other pressure manipulations were controlled by mouth. All operations were done at room temperature. The cover glass at the bottom of the chamber was rinsed with bovine serum albumin, 10 mg/ml, as necessary to prevent cell adhesion to the glass. Odorants were from Aldrich; cAMP, N^6,2′-O-dibutyryl cAMP, and BAPTA were from Sigma.

Current Recordings

The recording pipette and bath were coupled to a List L/M-EPC7 patch-clamp amplifier via Ag/AgCl electrodes. In all experiments the pipette was voltage clamped relative to the bath. Current recordings were filtered at 10 kHz, digitized by a Medical Systems PCM-1 recording adapter, and stored on videotape. Subsequent digital filtration was as indicated in the figure legends. In all figures, an upward deflection represents increasing positive current from the cilium into the pipette. Voltage ramps (ramp rate 0.2 mV/msec) were generated by pCLAMP software (Axon Instruments). Input resistances were estimated from current measured at pipette potentials of 0 and +50 mV. Results of repeated experiments are reported as mean ± SD. Reported potentials have been corrected for the liquid junction potential.

Results

Ciliary Patch Method

We have been able to suck one cilium from an isolated frog olfactory receptor neuron into a patch pipette and establish a high-resistance seal near the base of the cilium (Fig. 1). This allowed measurement of ciliary transmembrane currents.

In the suspension of mechanically dissociated epithelial cells, the neurons were easily identified by their 6–8 motile cilia, each up to 200 μm long [22]. *In situ*, each olfactory neuron is elongated and has a well-defined dendritic process [11]. An enlargement of the dentrite called the olfactory vesicle lies at the epithelial surface, and the several cilia protrude from this vesicle into the mucus. In suspension, the cells appeared round, and only rarely could an axonal segment or dendrite be distinguished. Neurons were easy to distinguish from respiratory epithelial cells. Each respiratory cell bore several dozen cilia up to 12 μm long. These beat synchronously, while the neuronal cilia moved independently of one another. No ciliary breakage was apparent, since the cilia of a given neuron were usually of a single length.

Because fine debris in the crude suspension could clog the tip of the recording pipette, single neurons or small cell clusters containing neurons were isolated from the suspension and placed in a clean recording chamber. There a cilium was easily sucked into the 0.5-μm tip of a recording micropipette, and in 47% of 547 attempts, a high-resistance seal formed. Although the cells became round in suspension, the olfactory vesicle was usually visible against the tip of the pipette. The average length of the patched cilia, estimated from video images, was 32 μm (n = 209, range 14 to 80 μm).

With the cell attached to the pipette, we were able to measure ciliary currents after a stimulus was delivered to the soma and to those cilia outside the pipette. It was also possible to remove the cell, leaving the single excised cilium sealed into the recording micropipette.

Cell-Attached Configuration

With one cilium inside the recording micropipette and the cell attached, the input resistance averaged 2.2 ± 0.9 GΩ (n = 256, range 0.6 to 6.7 GΩ). The

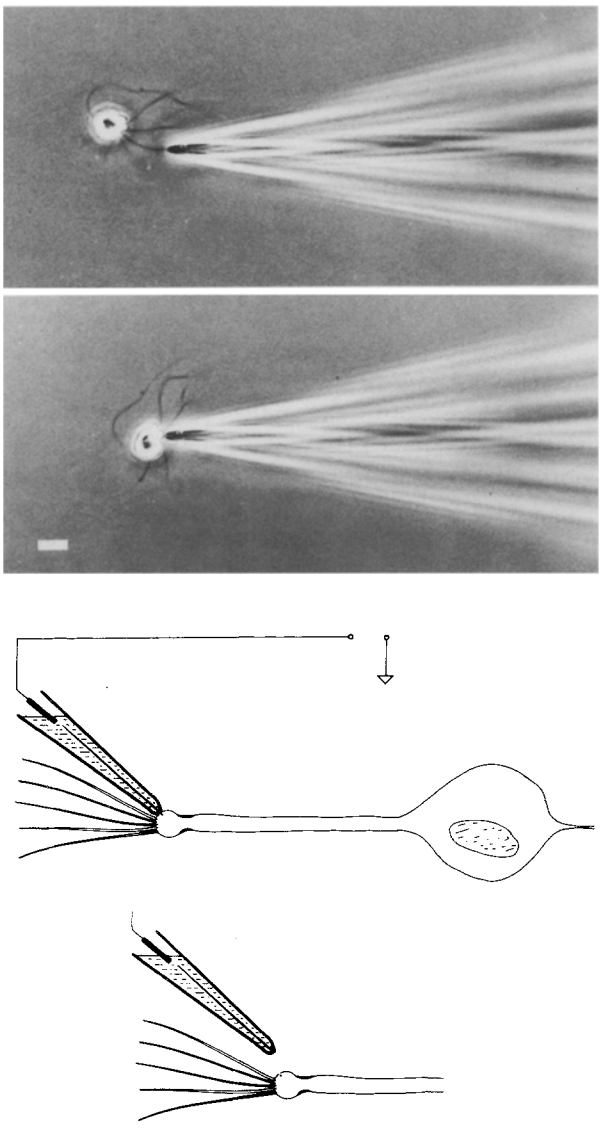

Fig. 1. Ciliary patch procedure. Photomicrographs: One cilium of a single olfactory receptor neuron was sucked into the end of a patch pipette (top) until the pipette contacted the cell near the base of the cilium (bottom). For these photographs only, 0.1% glutaraldehyde was added to the bath to stop ciliary motion. Bar = 10 μm. Schematic diagrams: recording from a single cilium still attached to the cell (top) and after excision from the cell (bottom). The drawings are not to scale, and the dendrite and axonal segment were not usually identifiable in the isolated neurons

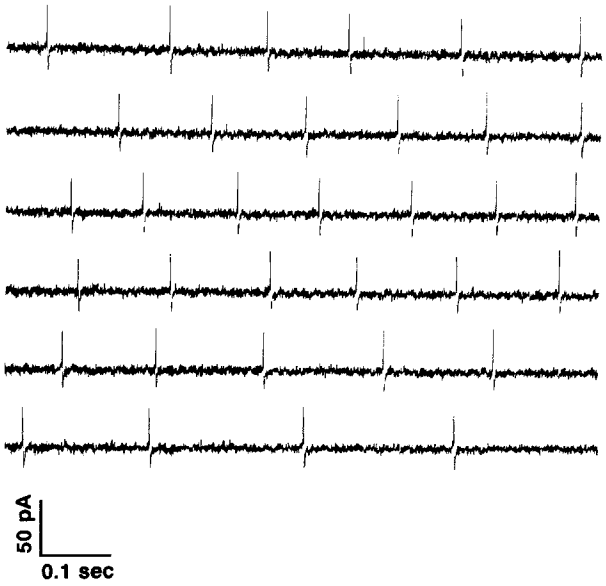

Fig. 2. Currents due to action potentials, detected through the ciliary membrane. With one cilium of a cell inside the recording pipette, suction was applied to the pipette. The record was digitally filtered at 5 kHz

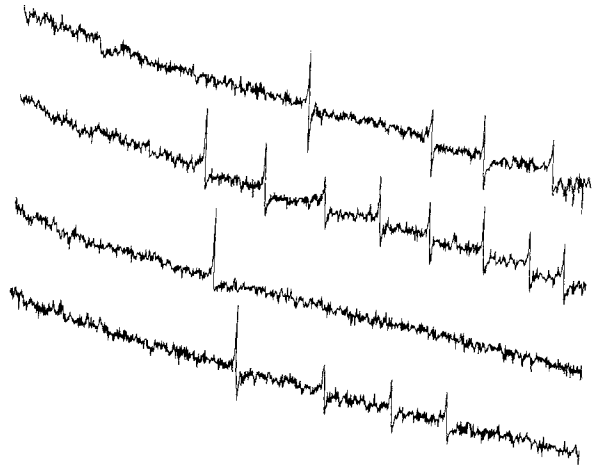

Fig. 3. Currents due to action potentials measured through a patched cilium. With the cell attached to the recording pipette by a cilium (input resistance 5.6 GΩ), voltage ramps (pipette potential −100 to +100 mV, 1 sec per ramp, 1 sec between ramps) were delivered through the pipette. In eight such ramps (four of which are shown), the initial current spikes appeared at an average pipette potential of −25 mV (range −36 to −2 mV). The average amplitude of these initial spikes was 17 pA (range 15 to 18 pA). No action potentials were induced when the direction of the ramp was reversed. The record was digitally filtered at 1 kHz

conductance measured in this configuration was not voltage dependent, and the current reversed near 0 mV (*not shown*).

In some neurons, application of suction to the cilium during or after the patch procedure induced repetitive firing of action potentials (Fig. 2). After seal formation, action potentials could be induced in these cells by applying a voltage ramp to the cilium (Fig. 3). Spontaneous action potentials were extremely rare before or after seal formation. Most neurons (88%) gave no action potentials under any conditions tested.

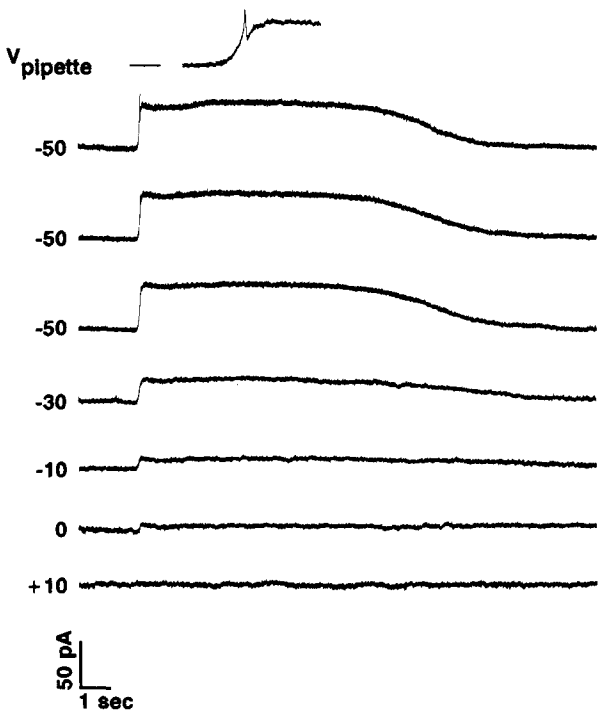

Fig. 4. Response of an isolated olfactory neuron to odorants. One cilium of an isolated olfactory neuron was sucked into a pipette. Input resistance was 4 GΩ. Every 20 sec, a 1-sec pulse of the 10-odorant mixture was delivered to the cells. Pipette holding potential was varied as indicated. Stimulus delivery is shown by the bar above the top trace. The short record at the top is a portion of the first response expanded 10-fold along the time axis to clearly show the action potential. This short record was digitally filtered at 6.7 kHz and the others at 1.4 kHz

Some of the attached cells responded to odorous stimuli. An aqueous mixture of 10 odorants was delivered to the soma and to those cilia outside the pipette. In responding cells, the odorants evoked a transient current from the cilium into the pipette (Fig. 4). The response was repeatable, and the current was greater when the pipette potential was held negative relative to the bath (Fig. 4). Our results at positive pipette potentials were inconclusive; in some cases this seemed to irreversibly inactive the cell. The peak amplitude also increased as the stimulus duration was varied from 50 msec to 3 sec (*not shown*). Often a single action potential was detectable during the response measured at the cilium (first trace, Fig. 4). No response was detectable when bath solution replaced the odorants as the stimulus (*not shown*).

Some cells failed to give a second response when tested 5 min after the first stimulus. Other cells showed no diminution of the response when stimulated at 1-min intervals over a 10-min period. Fourteen of 91 cells tested gave odorant responses of at least 10 pA. With the pipette potential held at −80 mV, the largest peak current was 160 pA; the average was 42 ± 37 pA (n = 14).

Excised Single Cilium

When a pipette with cell attached by a cilium was raised into the air, the cell was pulled off at the air-bath interface. When the pipette was quickly reimmersed in a bath of pseudointracellular solution, the input resistance was still high in 81% of 191 trials. The average input resistance was 1.8 ± 0.9 GΩ (n = 191, range 0.2 to 5.0 GΩ). We believe that most of the resistance was due to a cilium which remained sealed inside the pipette tip (Fig. 1, lower schematic). In our preliminary experiments [13], both sides of the excised cilium were exposed to 5 mM Ca^{2+} to stabilize the membrane. The effect of calcium was to reduce the input resistance of the excised cilium to 0.90 ± 0.76 GΩ (n = 194, range 0.13 to 5.0 GΩ).

Most excised cilia responded to cAMP. With the pipette potential negative relative to the bath, a 50-msec puff of 1 mM cAMP directed at the tip of the pipette induced a current from the bath into the pipette (Fig. 5, top). This current flowed in the opposite direction at positive pipette potentials (*not shown*). No response was detectable when bath solution replaced cAMP as the stimulus (*not shown*). Some cilia gave responses to repeated cAMP stimulation for up to 30 min, although the response amplitude attenuated with successive stimuli. There was no response when a cell attached to the pipette by a cilium was stimulated with 1 mM cAMP (*not shown*).

Stimulation was also possible by transferring a pipette containing an excised cilium through the air to a pseudointracellular bath to which 10 μM cAMP had been added. This increased the ciliary membrane conductance (Fig. 5, bottom, curve *B*). When the cilium was returned to the original bath, which lacked cAMP, its conductance returned to near the prestimulus level (Fig. 5, bottom, curve *C*). The cAMP-induced current reversed at −12.8 ± 8.2 mV (membrane potential, cytoplasmic relative to external, n = 10, range −23.1 to −0.5 mV). Similar results were obtained when the bath contained 10 μM cGMP instead of cAMP (n = 4). Eleven of 45 cilia tested showed no conductance increase in 10 μM cAMP. These cilia also showed no response in 10 μM N^6,2′-O-dibutyryl cAMP, a membrane-permeant analogue of cAMP.

Current fluctuations characteristic of single channels were common in the excised cilia. We have not yet characterized these channels.

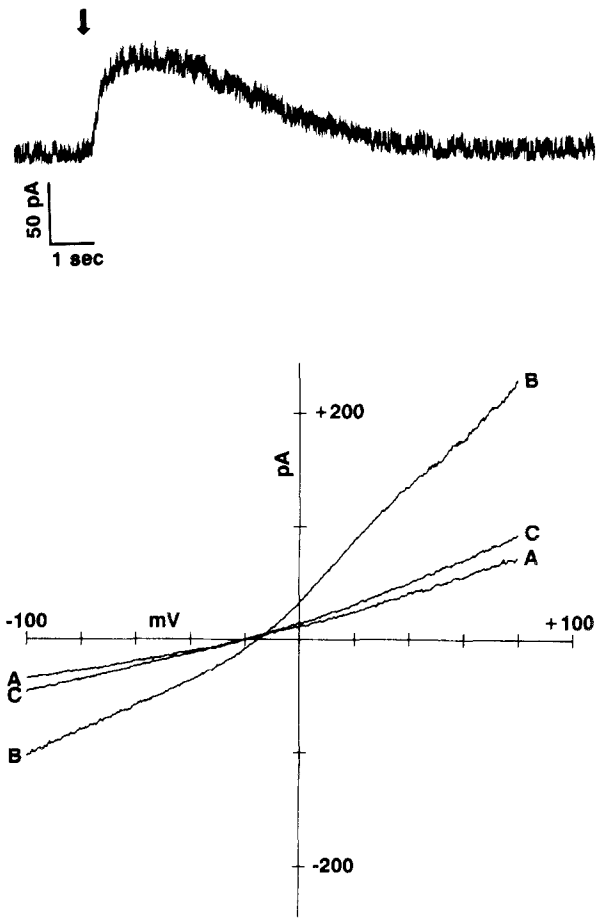

Fig. 5. Responses of excised single cilia to cAMP. Top: One of the cilia of an isolated olfactory neuron was sealed into a patch pipette. The cell was pulled off at the air-bath interface, leaving the cilium inside the pipette. Input resistance was 3.1 GΩ. A 50-msec pulse of 1 mM cAMP was delivered to the tip of the pipette as indicated by the arrow above the trace. Pipette potential was clamped at −40 mV. The record was digitally filtered at 2 kHz. Bottom: Current-voltage relationships of excised single cilia. *A*, in pseudointracellular bath; *B*, in pseudointracellular bath plus 10 μM cAMP; *C*, after return to original pseudointracellular bath. Voltage ramps were delivered 15 sec after immersion of the pipette in each bath. Changing the direction of the voltage ramp did not alter the results (*not shown*). We have plotted bath potential relative to pipette potential. The data shown are the averages of 10 experiments, in each of which the *I-V* relationships were measured in all three conditions. To determine the reversal potential of the cAMP-induced current, a difference curve (*B-A*) was prepared. A 20-mV region near the apparent reversal of the difference curve was fit to a straight line, and the voltage-intercept of this line was taken as the reversal potential. The records were acquired by sampling at 500 Hz

Discussion

We have measured transmembrane currents in intact single cilia of frog olfactory receptor neurons. One of the several cilia of a neuron was pulled inside a recording micropipette. The pipette made a high-resistance seal near the base of the cilium, probably against the olfactory vesicle.

Response to Odorants

Fifteen percent of the cells tested responded to an odorant mixture. We detected the response as a transient increase in current from the cilium into the pipette. The amplitude of this current increased with stimulus duration and with increasingly negative pipette potential. A corresponding odorant-induced current in olfactory neurons has been seen when recording from the soma, either with intracellular micropipettes [10, 25, 28] or the whole-cell patch-clamp technique [2, 3, 6, 8, 14, 17, 26]. One would not expect the cilium inside the pipette to be directly accessible to the odorants. It is more likely that the odorants acted on those cilia outside the pipette, causing receptor current to flow from the bath into those cilia and to depolarize the neuron. Some of this current would flow from the cell interior through the patched cilium and into the pipette.

The odorant response was measured in the cell-attached recording configuration [12]. A patch of membrane, here consisting of a single cilium, provided a resistance to current flow between the pipette and the cell interior. However, we detected a depolarization only when pipette potential was clamped negative relative to the bath (Fig. 4). Apparently the resistance of the ciliary membrane was low enough that the pipette potential influenced the intracellular potential [7]. No odorant-induced current was seen when pipette potential was held at +10 mV (Fig. 4). This is near the reversal potential for the odorant-induced current, as measured intracellularly [2, 3, 6, 14, 26, 28].

Excised Single Cilium

When the pipette with cell attached was raised briefly through the air, the cell was pulled off at the air-bath interface, but the input resistance remained high. We believe that the single cilium remained inside the pipette tip, with the seal between the base of the cilium and the tip of the pipette intact. Such a preparation would expose the cytoplasmic surface of the ciliary membrane to the bath. In most of these preparations, the ciliary conductance was reversibly increased by stimulation with cAMP. Nakamura and Gold [19] have shown that a cAMP-induced conductance is present in olfactory ciliary membrane. In agreement with them, we found that the induced conductance had a reversal potential near 0 mV. A

minority of the cilia responded to neither cAMP nor its membrane-permeant analog dibutyryl-cAMP. These cases could represent recording pipettes clogged by cellular debris or a subset of olfactory cilia that lack the cAMP-induced current.

Ciliary Resistance

We estimated the resistance of an average single cilium to be 1.8 GΩ. The resistivity, normalized for a typical ciliary surface area,[1] is 360 Ω cm^2, a surprisingly low value. For a cell with six cilia, the combined parallel resistances of the cilia would be 0.3 GΩ. The somal and dendritic resistances would reduce the whole-cell resistance even more. However, a low ciliary resistance is not apparent when recording from the intact neuron. When a cell is patched by one cilium, the electrode measures a resistance of 2.2 GΩ. Whole-cell patch experiments at the soma in frog receptor neurons measure an average input resistance of 1.0 GΩ.[2] In neither case are the low ciliary resistances detected. The input resistance we measured with an excised cilium in the pipette was a parallel combination of the membrane-pipette seal resistance and the membrane resistance itself [4, 7]. Thus a high ciliary resistance could be obscured by consistently low seal resistances. Membrane patches excised from chromaffin cells also had unexpectedly low resistances, and it was speculated that the patch procedure itself may have been responsible [4].

Future Applications

The ciliary patch method should allow an exhaustive description of the functional biophysics of the ciliary membrane. Characterizations of the resting ionic conductances and single channels are in progress. Methods are also becoming available for delivering stimuli within the recording pipette. With such methods, it will be possible to apply odorants to the outer face of the membrane of a single cilium and determine how its properties change.

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[1] We have estimated that each cilium is a cylinder 32 μm in length (the mean length of the cilia we patched) and 0.2 μm in diameter. The resulting surface area is 20 μm^2.

[2] R. Pun (*personal communication*).

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