

# Cyclic AMP Cascade Mediates the Inhibitory Odor Response of Isolated Toad Olfactory Receptor Neurons

Rodolfo Madrid,<sup>1</sup> Ricardo Delgado,<sup>1,2</sup> and Juan Bacigalupo<sup>1,2</sup>

<sup>1</sup>Department of Biology, Faculty of Sciences and <sup>2</sup>Cell Dynamics Biotechnology Research Center, University of Chile, Santiago, Chile

**Madrid, Rodolfo, Ricardo Delgado, and Juan Bacigalupo.** Cyclic AMP cascade mediates the inhibitory odor response of isolated toad olfactory receptor neurons. *J Neurophysiol* 94: 1781–1788, 2005. First published April 7, 2005; doi:10.1152/jn.01253.2004. Odor stimulation may excite or inhibit olfactory receptor neurons (ORNs). It is well established that the excitatory response involves a cyclic AMP (cAMP) transduction mechanism that activates a nonselective cationic cyclic nucleotide-gated (CNG) conductance, accompanied by the activation of a Ca<sup>2+</sup>-dependent Cl<sup>-</sup> conductance, both causing a depolarizing receptor potential. In contrast, odor inhibition is attributed to a hyperpolarizing receptor potential. It has been proposed that a Ca<sup>2+</sup>-dependent K<sup>+</sup> (K<sub>Ca</sub>) conductance plays a key role in odor inhibition, both in toad and rat isolated olfactory neurons. The mechanism underlying odor inhibition has remained elusive. We assessed its study using various pharmacological agents and caged compounds for cAMP, Ca<sup>2+</sup>, and inositol 1,4,5-triphosphate (InsP<sub>3</sub>) on isolated toad ORNs. The odor-triggered K<sub>Ca</sub> current was reduced on exposing the cell either to the CNG channel blocker LY83583 (20 μM) or to the adenylyl cyclase inhibitor SQ22536 (100 μM). Photorelease of caged Ca<sup>2+</sup> activated a Cl<sup>-</sup> current sensitive to niflumic acid (10 μM) and a K<sup>+</sup> current blockable by charybdotoxin (20 nM) and iberiotoxin (20 nM). In contrast, photoreleased Ca<sup>2+</sup> had no effect on cells missing their cilia, indicating that these conductances are confined to the cilia. Photorelease of cAMP induced a charybdotoxin-sensitive K<sup>+</sup> current in intact ORNs. Photorelease of InsP<sub>3</sub> did not increase the membrane conductance of olfactory neurons, arguing against a direct role of InsP<sub>3</sub> in chemotransduction. We conclude that a cAMP cascade mediates the activation of the ciliary Ca<sup>2+</sup>-dependent K<sup>+</sup> current and that the Ca<sup>2+</sup> ions that activate the inhibitory current enter the cilia through CNG channels.

## INTRODUCTION

Olfactory receptor neurons (ORNs) exhibit spontaneous action potential firing, at a rate that varies according to species. Odor stimulation may increase the discharge rate by inducing a depolarizing receptor potential. Such an excitatory response is triggered when odorants bind to G-protein-coupled receptors present in the chemosensory cilia of these sensory receptor neurons. A G-protein mediates the activation of adenylyl cyclase, locally increasing cyclic adenosine monophosphate (cAMP) levels within the cilia. This second messenger directly activates nonselective cationic cyclic nucleotide-gated (CNG) channels, allowing the influx of Ca<sup>2+</sup> and other cations into the cilia (Firestein and Werblin 1989; Kurahashi 1989; Nakamura and Gold 1987). Calcium opens ciliary Ca<sup>2+</sup>-dependent Cl<sup>-</sup> channels, allowing Cl<sup>-</sup> efflux from the cilia (Kleene and Gesteland 1991; Kurahashi

and Yau 1993; Lowe and Gold 1993b). Both inward current components are responsible for the depolarizing receptor potential.

Electrophysiological studies based on single-unit recordings from the olfactory epithelium revealed that odorants not only can excite but also can inhibit vertebrate ORNs (Gesteland et al. 1965; O'Connell and Mozell 1969). It was first shown in *Necturus* that odor inhibition was attributed to a hyperpolarizing receptor potential (Dionne 1992). Morales et al. (1994) confirmed such a result in *Caudiverbera* and, furthermore, demonstrated that an olfactory neuron can generate both excitatory and inhibitory odorant responses to different odorants. Kang and Caprio (1995) provided evidence supporting the presence of both response types to amino acids in fish. Similar observations were subsequently made in *Xenopus* tadpoles (Vogler and Schild 1999). Inhibitory responses have been also described in mammalian ORNs (Delay and Restrepo 2004; Duchamp-Viret et al. 1999; Sanhueza et al. 2000).

Morales et al. (1994, 1995) proposed that a Ca<sup>2+</sup>-dependent K<sup>+</sup> conductance was the target of the transduction mechanism producing the inhibitory receptor potential in toad. This conductance is present in the cilia (Delgado and Bacigalupo 2004; Delgado et al. 2003; Morales et al. 1995) and is sensitive to charybdotoxin and iberiotoxin (Castillo et al. 2005; Morales et al. 1995; Sanhueza et al. 2000). The mechanism underlying the inhibitory response was unknown. It had been determined that Ca<sup>2+</sup> ions that activate these K<sup>+</sup> channels cross the ciliary membrane from the external milieu (Morales et al. 1997), although their permeability pathway was also unknown. Pun and Kleene (2002) reported the presence of a Ca<sup>2+</sup>-dependent outward current evoked by odorants in *Rana pipiens*, whose activation seemed to be mediated by cAMP. This current increased with hyperpolarization. However, the authors did not identify the nature of that current. Recently, Delay and Restrepo (2004) reported that both excitatory and inhibitory transduction currents were abolished by the CNG channel blocker *L-cis*-diltiazem and were absent in transgenic mice lacking the CNG channel, suggesting that this channel is required for both types of odorant responses (Brunet et al. 1996), most likely by allowing the Ca<sup>2+</sup> influx, on which both responses depend. Here we investigated the inhibitory cascade making use of pharmacological agents and caged compounds for cAMP, Ca<sup>2+</sup>, and inositol 1,4,5-triphosphate (InsP<sub>3</sub>). Our results show that the activation of the ciliary Ca<sup>2+</sup>-dependent K<sup>+</sup> conductance responsible for the inhibitory response is mediated by a cAMP cascade, and that this cascade opens

Address for reprint requests and other correspondence: J. Bacigalupo, Department of Biology, Faculty of Sciences, University of Chile, P.O. Box 653, Santiago, Chile (E-mail: bacigalu@uchile.cl).

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$\text{Ca}^{2+}$ -permeant CNG channels through which  $\text{Ca}^{2+}$  influx occurs.

## METHODS

### *Preparation, solutions, and solution changes*

Isolated ORNs were obtained by mechanical dissociation of the olfactory epithelium from the Chilean toad *Caudiverbera caudiverbera*. The animals were anesthetized in ice, killed, and pithed, and the olfactory epithelia were removed from their nasal cavity. The tissue was cut into 1-mm<sup>2</sup> pieces, which were maintained in hypertonic Ringer solution supplemented with amino acids, antibiotics, and albumin (Morales et al. 1994).

The composition of the solutions used in this study was as follows (in mM): Normal Ringer: 115 NaCl, 2.5 KCl, 1 CaCl<sub>2</sub>, 0.4 MgCl<sub>2</sub>, 10 HEPES, 3 glucose, pH 7.6. Low-Cl<sup>-</sup> external solution: 115 NaAc, 5 NaCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, pH 7.6. Internal solution: 120 KCl, 4 HEPES, 0.1 Na-GTP, 1 Mg-ATP, 1 CaCl<sub>2</sub>, 2 EGTA, pH 7.6, pCa 8.0. Low-Cl<sup>-</sup> internal solution: 115 KAc, 5 KCl, 1 MgCl<sub>2</sub>, 0.5 EGTA, 10 HEPES, pH 7.6. Caged  $\text{Ca}^{2+}$  internal solution: 120 KCl, 4 HEPES, 0.1 Na-GTP, 1 Mg-ATP, 2 CaCl<sub>2</sub>, 4 DM-Nitrophen (Calbiochem-Novabiochem), pH 7.6.

### *Electrical recording and data analysis*

Cells were viewed in an Olympus IX70 inverted microscope, with a 100 × DIC objective (Plan, 1.25 N.A.). We used an Axopatch 1D patch clamp (Axon Instruments) for electrical recording. Capacitance and series resistance ( $R_s$ ) were cancelled; cells with  $R_s > 20 \text{ M}\Omega$  were discarded. The pClamp 6 software (Axon Instruments) was used for data acquisition and analysis. The patch pipettes were made of soft glass capillaries (Bris, Globe Scientific) and electrode resistances were 2–4 M $\Omega$ . Rapid external solution exchange was accomplished with multibarreled pipettes (Sutter Instrument), with tip diameters of approximately 5–10  $\mu\text{m}$  per barrel, positioned about 30  $\mu\text{m}$  from the cell. In the experiments using odorants, the tip diameters were <1  $\mu\text{m}$  and the pipette was positioned about 10  $\mu\text{m}$  from the cilia. The solution flow from the barrels was controlled by a custom-made computer-operated picospritzer.

### *Caged compounds*

Caged cAMP (4,5-dimethoxy-2-nitrobenzyl adenosine 3',5'-cyclic-monophosphate; DMNB-caged cAMP, Molecular Probes) (250  $\mu\text{M}$ , patch pipette concentration), caged  $\text{Ca}^{2+}$  (DM-Nitrophen, cage for  $\text{Ca}^{2+}$ , Calbiochem-Novabiochem) (2 mM), and caged  $\text{InsP}_3$  [D-myoinositol 1,4,5-triphosphate,  $P_{4(5)}$ -(1-(2-nitrophenyl)ethyl) ester, trisodium salt; NPE-caged  $\text{InsP}_3$  (Molecular Probes)] (150  $\mu\text{M}$ , patch pipette concentration) were used. These messengers were added to the pipette solution and were released from their respective caged compounds using a 75-W xenon lamp as the UV-light source. The kinetics of  $\text{Ca}^{2+}$  release is slower than that of cAMP or  $\text{InsP}_3$ , but this is a characteristic of this particular caged compound (Ellis-Davies et al. 1996). UV-light pulses were controlled and the emitted signals were acquired with the IonWizard 4.2 software, using a Fluorescence System Interface (IonOptix, Milton, MA). Normal Ringer was used externally.

To test whether  $\text{InsP}_3$  was effectively being liberated from its cage compound, we recorded the emission signal generated by the  $\text{InsP}_3$ -triggered  $\text{Ca}^{2+}$  release from rat hepatocyte endoplasmic reticulum membrane vesicles; the emitted signal was collected from an area of about 50  $\mu\text{m}^2$ , delimited by an adjustable rectangular diaphragm. The vesicles (protein concentration 0.3 mg/ml) were suspended in normal ORN internal solution, supplemented with 20  $\mu\text{M}$  Fluo-3 (Molecular Probes) and 150  $\mu\text{M}$  caged  $\text{InsP}_3$ . UV exposures of identical characteristics as those applied to the ORNs generated a large fluorescence

signal (not shown). This result indicates that  $\text{InsP}_3$  was being successfully photoreleased because it was inducing the release of  $\text{Ca}^{2+}$  from the vesicles.

### *Odorants and blockers*

The odorants used in this work were the following: Cadaverine (1,5-diaminopentane). Mixture F: geraniol (3,7-dimethyl-2,6-octadien-1-ol), citralva (3,7-dimethyl-2,6-octadienenitrile, kindly provided by D. Restrepo), and citronellal (3,7-dimethyl-6-octenal).

The CNG channel blocker LY83583 [6-(phenylamino)-5,8-quinolinodine, RBI] was prepared in DMSO and kept as a 20 mM stock solution at  $-20^\circ\text{C}$ ; it was applied at a final concentration of 20  $\mu\text{M}$ . The final DMSO concentration was no higher than 0.1%, which has no side effects on ORNs (Chen et al. 2000). SQ22536 [(9-terahydro-2'furyl)adenine, Calbiochem-Novabiochem] was prepared as a 100 mM stock solution in H<sub>2</sub>O and used at a final concentration of 100  $\mu\text{M}$ . Charybdotoxin (CTx) and iberiotoxin (IbTx, Alomone Labs) were prepared from a 12  $\mu\text{M}$  stock solution and from a 115  $\mu\text{M}$  stock solution, respectively, and kept at  $-20^\circ\text{C}$ . Both toxins were used at a final concentration of 20 nM. Niflumic acid was used at a final concentration of 10  $\mu\text{M}$ , prepared in Ringer from a 20 mM stock solution in ethanol. We have no indication that the ethanol concentration used in the present work had any effect on the ORNs. Higher concentrations have been previously used on ORNs and no effects have been reported either (Dubin and Dionne 1994; Kleene 1993).

All chemicals were purchased from Sigma–Aldrich, unless otherwise indicated.

## RESULTS

### *Inhibitory current is sensitive to drugs affecting the cAMP-dependent pathway*

The odor-triggered inhibitory  $\text{K}^+$  current of olfactory neurons can be best observed as the odor stimulus is applied during depolarizing voltage steps. This current reaches its maximal value between +20 and +50 mV (Morales et al. 1994; Sanhueza et al. 2000). The excitatory odor-dependent current reverses near 0 mV, being outward at positive potentials and inward at negative potentials. This current is also expressed as an inward tail current when the holding potential returns from a depolarized value back to  $-70 \text{ mV}$  (Sanhueza et al. 2000). In the present study we made use of this strategy to determine what transduction currents were activated by the odor stimulus in a given experiment.

The ionic conductance that allows the influx of the  $\text{Ca}^{2+}$  responsible for the activation of the ciliary  $\text{K}^+$  conductance may in principle correspond either to an as yet unidentified ciliary  $\text{Ca}^{2+}$  conductance or to the  $\text{Ca}^{2+}$ -permeant CNG conductance. To distinguish between these possibilities, we tested whether the CNG channel blocker LY83583 affected the inhibitory outward current. The outward current induced by a puff of the odorant cadaverine, delivered during a depolarizing step to  $-10 \text{ mV}$ , was reversibly and completely abolished when the odorant was applied together with 20  $\mu\text{M}$  LY83583 (Fig. 1A). The *inset* shows the cadaverine-induced current, after subtracting the voltage-gated currents. In 2 other ORNs (of 5 cells responsive to this odorant) the effect of the drug was only partial. These observations suggest that  $\text{Ca}^{2+}$  mediating the inhibitory response enters the cell through the CNG conductance.

This result implies that the cAMP cascade may participate in the inhibitory response. To test this hypothesis we exposed an

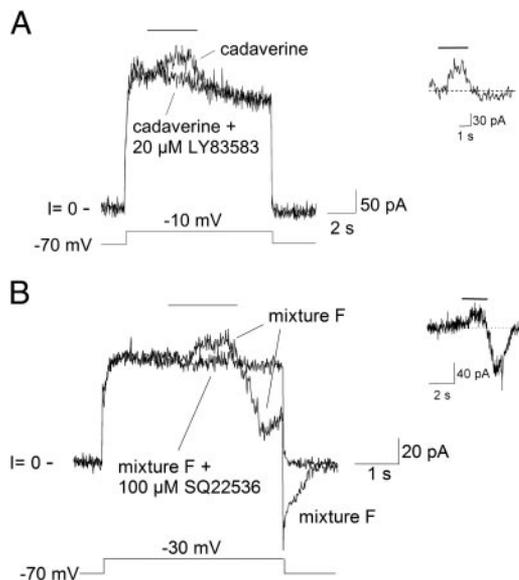


FIG. 1. Blockade of the odor-dependent inhibitory  $K^+$  current by LY83583 and SQ22536. *A*: inhibitory  $K^+$  current activated by  $50 \mu\text{M}$  cadaverine was abolished by  $20 \mu\text{M}$  LY83583. *Inset*: net current abolished by the drug. *B*: in a separate cell,  $30 \mu\text{M}$  mixture F induced the inhibitory and excitatory currents, both of which were abolished by 1 min incubation in  $100 \mu\text{M}$  SQ22536. *Inset*: net current abolished by the drug.

ORN responsive to odor mixture F to  $100 \mu\text{M}$  of the adenylyl cyclase inhibitor SQ22536. Figure 1*B* illustrates one of the 5 ORNs in which we have observed so far that the same odor stimulus activated both the inhibitory outward current and the excitatory inward current (Sanhueza et al. 2000); the inward current followed the outward current during the step to  $-30 \text{ mV}$ . The excitatory current was also expressed as an inward tail current after returning back to the holding potential. The *inset* shows both odor-dependent currents during the depolarizing pulse, after subtracting the voltage-gated currents. Both odorant-dependent currents were abolished by SQ22536, in agreement with the notion that adenylyl cyclase is a key component of excitatory and inhibitory transduction. We observed a complete blockage effect by SQ22536 on the inhibitory current in 3 of 8 responsive cells, whereas in the rest of them its effect was only partial.

#### Cyclic AMP activates the inhibitory current

Our results predict that an increase in cAMP concentration in an ORN should produce the activation of both the excitatory and the inhibitory transduction currents. Figure 2*A* shows that the photorelease of cAMP from its caged compound induced an inward current at  $-70 \text{ mV}$ , confirming previous observations in other species (Kurahashi 1990; Kurahashi and Menini 1997; Takeuchi and Kurahashi 2002). To test whether cAMP activated the  $K^+$  conductance we performed this experiment under external/internal low  $\text{Cl}^-$  solutions, to avoid any significant contribution of the excitatory  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current. When released in an ORN held at  $+50 \text{ mV}$ , cAMP induced an outward current, an observation repeated in 25 out of 49 cells (51%). This current was reversibly blocked by CTx in 4 of the 7 cells tested, whereas in the other 3 cells it had no effect (Fig. 2*B*). These results support the notion that a cAMP cascade mediates the activation of the ciliary  $\text{Ca}^{2+}$ -dependent  $K^+$  conductance by odors.

#### $\text{Ca}^{2+}$ increases a $K^+$ and a $\text{Cl}^-$ conductance

We tested whether the current activated by the photoreleased  $\text{Ca}^{2+}$  consisted of a  $K^+$  and a  $\text{Cl}^-$  component, as expected. For this we examined whether blockers of the  $K^+$  and of the  $\text{Cl}^-$  conductances, such as CTx or IbTx, and niflumic acid, respectively, affected this current. Figure 3*A* shows the prominent outward current that developed when an ORN loaded with caged  $\text{Ca}^{2+}$  was illuminated with UV light during a voltage pulse that depolarized the cell to  $20 \text{ mV}$ . Repolarization back to the holding potential was accompanied by an inward tail current that was absent in the control, where no UV light was applied. An identical protocol was used on a different ORN, in the absence and presence of  $20 \text{ nM}$  CTx, a strong blocker of the inhibitory current in *Caudiverbera* (Morales et al. 1995; Sanhueza et al. 2000); this toxin has no significant effect on the somatic  $K^+$  currents (Delgado and Labarca 1993; Madrid and Bacigalupo, unpublished observations). In this cell, the toxin blocked over 60% of the current induced by the released  $\text{Ca}^{2+}$  (Fig. 3*B*). Similar results were observed in 3 other cells, although CTx had no visible effect on other 2 ORNs. Another ORN was challenged with  $20 \text{ nM}$  IbTx, a rather specific blocker of large-conductance  $\text{K}_{\text{Ca}}$  channels. IbTx abolished nearly 50% of the outward current (Fig. 3*C*;  $n = 2$ ). Finally,  $10 \mu\text{M}$  niflumic acid blocked around 70% of the outward current in a separate cell (Fig. 3*D*); a similar result was observed in the 4 neurons tested. These results are indicative that released  $\text{Ca}^{2+}$  activated a  $K^+$  and a  $\text{Cl}^-$  conductance, and are in agreement with the notion that both conductances activate on

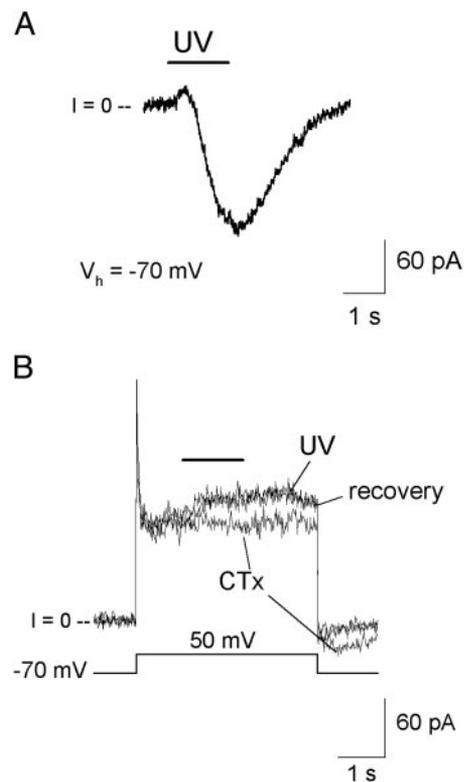


FIG. 2. Cyclic adenosine monophosphate (cAMP) activates the inhibitory current. *A*: inward current induced by photoreleasing cAMP ( $V_h = -70 \text{ mV}$ ). *B*: outward current activated by cAMP as the membrane was held at  $+50 \text{ mV}$  using low  $\text{Cl}^-$  internal solution and low  $\text{Cl}^-$  external solution. Current was abolished by  $20 \text{ nM}$  charybdotoxin (CTx).

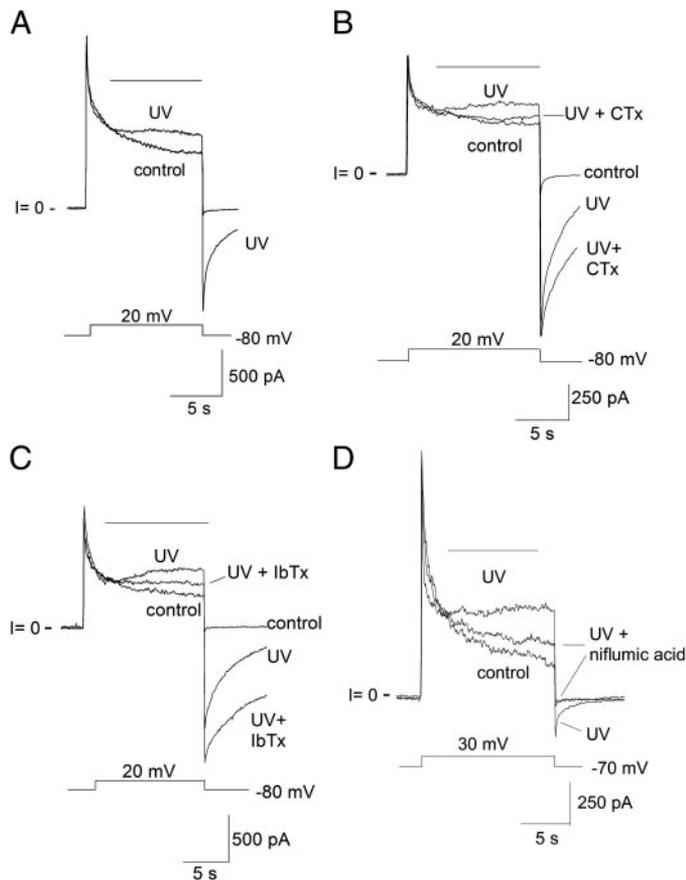


FIG. 3.  $\text{Ca}^{2+}$  activates a current consisting of a  $\text{Cl}^-$  component and a  $\text{K}^+$  component. *A*: typical whole cell current induced by photoreleased  $\text{Ca}^{2+}$ . *B*: 20 nM CTx partially abolished the  $\text{Ca}^{2+}$ -induced outward current. *C*: 20 nM iberiotoxin (IbTx) partially abolished the current induced by  $\text{Ca}^{2+}$ , in another olfactory receptor neuron (ORN). *D*: 10  $\mu\text{M}$  niflumic acid partially blocked the outward current activated by  $\text{Ca}^{2+}$ . In all cases the blockers were supplemented to the normal Ringer solution.

odorant-induced increments in luminal  $\text{Ca}^{2+}$  in the olfactory cilia.

#### *The membrane conductances gated by the photoreleased $\text{Ca}^{2+}$ localize to the olfactory cilia*

A crucial question of our study was to what extent the currents induced by photoreleased  $\text{Ca}^{2+}$  ions flow through the ciliary membrane, especially considering the fact that the somatic plasma membrane of olfactory neurons is known to contain  $\text{K}_{\text{Ca}}$  channels (Delgado and Labarca 1993; Madrid et al. 2003; Trotier 1986). To address this question, we compared the effect of photoreleased  $\text{Ca}^{2+}$  in intact ORNs with ORNs that had lost their cilia during the dissociation process. The  $\text{Ca}^{2+}$  increase triggered an outward current on top of the voltage-gated outward current, that was followed by an inward tail current as the depolarizing voltage pulse to 20 mV returned to  $-80$  mV (Fig. 4*A*, right;  $n = 56$ ) in an intact ORN (Fig. 4*A*, left). Both currents were absent in the control trace, where no UV was given, whereas their voltage-gated currents seemed completely normal. In contrast, UV illumination had no effect in an ORN lacking its cilia (Fig. 4*B*, left; Fig. 4*B*, right,  $n = 8$ ). These results indicate that both conductances activated by  $\text{Ca}^{2+}$  under our experimental conditions reside in the chemo-

sensory cilia and thus most likely they corresponded to the  $\text{Ca}^{2+}$ -dependent transduction conductances.

#### *Effects of small injected currents on action potential firing in ORNs*

The possible physiological role of the odor-induced  $\text{K}^+$  current may seem hard to appreciate, considering its negligibly small magnitude at membrane voltages near the resting potential (Morales et al. 1994; Sanhueza et al. 2000). To assess this, we examined the effect of small current injections on the membrane potential of current-clamped ORNs. Figure 5 (*top panel*) shows that injections of 2- and 3-pA depolarizing currents induced spiking in an ORN exhibiting a low spontaneous activity (Madrid et al. 2003). Likewise, hyperpolarizing currents of identical magnitudes were effective in reducing the discharge rate of another cell, which presented a high spontaneous firing rate (Fig. 5, *bottom panel*). This observation, repeated in 5 ORNs, demonstrates that very small currents (in the low picoampere level) can significantly alter the firing rate of these neurons, supporting a physiological function of a small odor-dependent hyperpolarizing current.

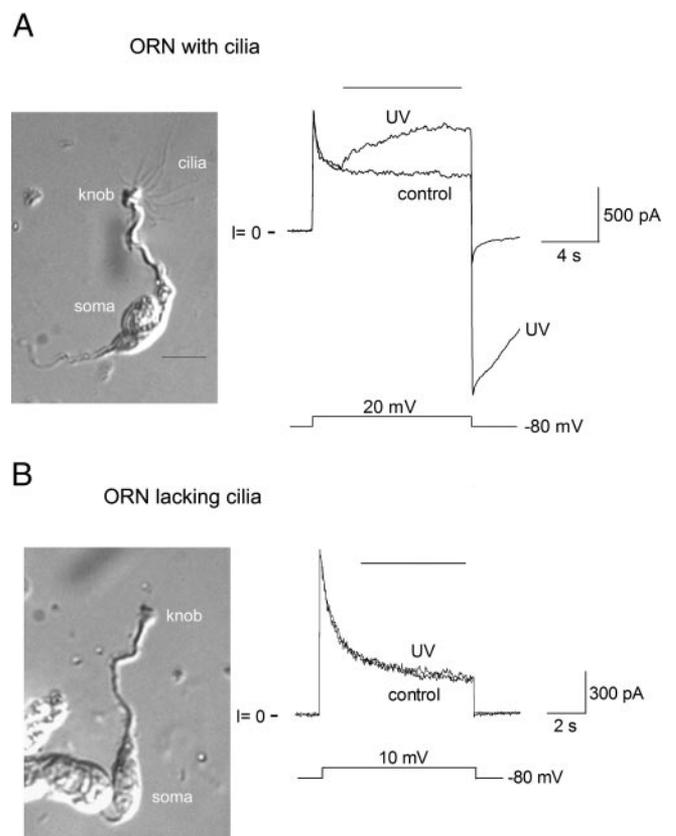


FIG. 4. Conductances activated by photoreleased  $\text{Ca}^{2+}$  are confined to the olfactory cilia. *A*, left: differential interference contrast (DIC) image of an intact ORN, bearing its cilia. Right: whole cell current in response to photoreleased  $\text{Ca}^{2+}$ , superimposed to the control current (evoked by the depolarizing step in the absence of UV light). *B*, left: ORN missing its olfactory cilia. Right: current recorded in response to a voltage step is superimposed to the current obtained after applying the same step in combination with an ultraviolet (UV) stimulus. Currents were recorded under normal Ringer solution. Bar: 5  $\mu\text{m}$ .

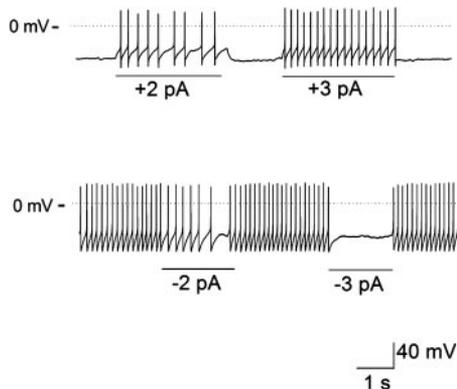


FIG. 5. Effect of current injections on action potential firing. Depolarizing current pulses of 2 and 3 pA induced firing in an ORN presenting a low spontaneous firing activity (*top panel*). Hyperpolarizing currents of identical magnitudes as above reduced the discharge rate in another ORN that had a high spontaneous firing rate (*bottom panel*).  $I_h = 0$  pA in both cases.

#### *Inositol trisphosphate does not appear to mediate the activation of ORN membrane conductances*

InsP<sub>3</sub> has been proposed as a possible messenger in olfactory transduction (see Schild and Restrepo 1998). Photoreleasing InsP<sub>3</sub> in an ORN loaded with 150  $\mu$ M caged InsP<sub>3</sub> did not induce any membrane current when clamped either at  $-70$  ( $n = 11$ ) or at  $0$  mV ( $n = 2$ ) (not shown). These results do not support the involvement of an InsP<sub>3</sub>-dependent pathway in odor transduction.

#### *A model for chemotransduction*

Based on our previous and present results, and those of others on the excitatory transduction mechanism (see Schild and Restrepo 1998), we propose an integrative model for the transduction events taking place in the chemosensory cilia (Fig. 6). Odorant binding enables G-protein-coupled odor receptor to trigger a cAMP cascade, increasing ciliary cAMP levels. This second messenger activates CNG channels, allowing the influx of Ca<sup>2+</sup> in the cilium. It is possible that calcium concentration quickly increases within the minute ciliary volume, where it might activate either Ca<sup>2+</sup>-dependent Cl<sup>-</sup> channels, originating the excitatory receptor potential, or Ca<sup>2+</sup>-dependent K<sup>+</sup> channels, generating the inhibitory receptor potential. Because odors activate either one or the other response in an ORN, we hypothesize that the transduction components are somehow segregated in the cilia.

#### DISCUSSION

The evidence presented here suggests that, similarly to odor excitation, odor inhibition is also mediated by a cAMP cascade, with both mechanisms differing on their particular electrophysiological targets.

#### *Participation of cAMP in the inhibitory cascade*

The CNG channel has a key role in excitatory odor transduction, by allowing the influx of Ca<sup>2+</sup> that gates the Cl<sup>-</sup> conductance (Kleene and Gesteland 1991; Kurahashi and Yau 1993; Lowe and Gold 1993b). It was previously reported that intraluminal Ca<sup>2+</sup> increases occur in the chemosensory cilia of salamander ORNs, by a Ca<sup>2+</sup> influx from the extracellular

milieu through the CNG conductance (Leinders-Zufall et al. 1997; Morales et al. 1997). We found that LY83583, a blocker of the CNG conductance that suppressed the excitatory current (Leinders-Zufall and Zufall 1995), reversibly abolished the odor-induced ciliary K<sub>Ca</sub> current as well. Because LY83583 has no known effect on K<sup>+</sup> channels, this result suggests that the CNG conductance is involved in the inhibitory mechanism and, furthermore, that the cAMP pathway may participate in both excitatory and inhibitory odor transduction. Further support for this notion was provided by the observation that the cyclase inhibitor SQ22536 also abolished this current. Both drugs caused a complete suppression of the inhibitory current in some neurons, whereas in other cells they had only a partial effect. We think that in some cells the CNG channel blocker was less effective simply because the drug concentration around the cilia might have not reached the desired level during stimulation (1–2 s). In the case of SQ22536, there is an additional complication because this compound has to permeate the membrane to reach its target. An alternative explanation for both situations would be that those cells where the drugs had only a partial effect possess an additional transduction pathway.

In addition to the pharmacological evidence, the data obtained with caged cAMP support the role of this second messenger in the inhibitory pathway, indicating that an increase in cAMP mediates the activation of both the excitatory and the inhibitory currents. The odor-induced K<sup>+</sup> currents

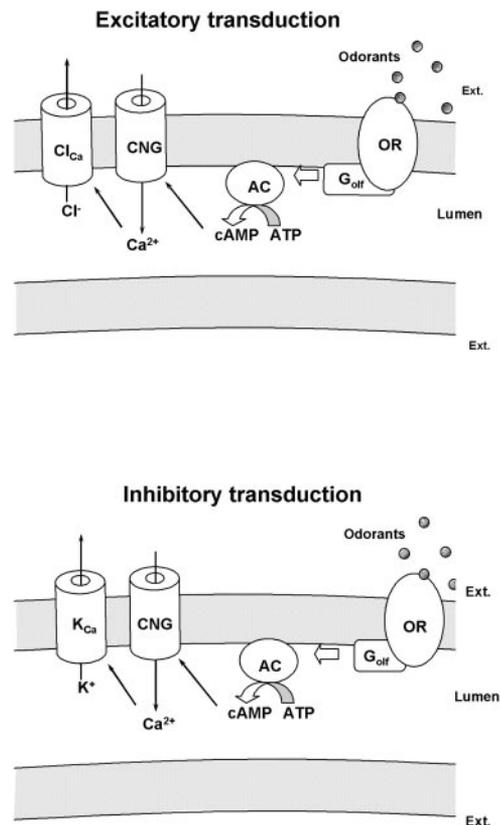


FIG. 6. Model for chemotransduction in an olfactory cilium. Odorants bind to its receptor protein, which activates adenylate cyclase-mediated by a G protein ( $G_{olf}$ ); the resulting increase in cAMP activates a cyclic nucleotide-gated (CNG) channel, allowing Ca<sup>2+</sup> influx. In excitatory transduction, Ca<sup>2+</sup> activates a Ca<sup>2+</sup>-dependent Cl<sup>-</sup> channel (*top*), whereas in inhibitory transduction, it activates a Ca<sup>2+</sup>-dependent K<sup>+</sup> channel (*bottom*).

could be observed in isolation under low  $\text{Cl}^-$  (Fig. 2B). The photorelease of cAMP in these conditions induced the  $\text{K}^+$  current in 51% of the cells examined. Blockade of this current by CTx is consistent with the notion that it corresponds to the transduction  $\text{K}_{\text{Ca}}$  current (Morales et al. 1995). Our results are in agreement with recent work that reports blockade of the CNG channel with *L-cis*-diltiazem abolishes both excitatory and inhibitory responses in mice ORNs (Delay and Restrepo 2004). Our observations are supported by the evidence that CNG-null mice are anosmic (Brunet et al. 1996). Altogether, the experimental evidence favors a crucial role of this channel in odor transduction. Similar phenotypes were also observed in adenylyl cyclase III (Wong et al. 2000) and olfactory G-protein-null mice (Belluscio et al. 1998), indicating that these 2 proteins are also key participants in chemotransduction. Taken together, one may argue in favor of the notion that a cAMP cascade is central and perhaps the only chemotransduction pathway in vertebrates.

An  $\text{InsP}_3$  transduction pathway had been proposed as a parallel transduction pathway in vertebrates, based on biochemical measurements on olfactory cilia membrane preparations (Boekhoff et al. 1990), on electrophysiological recordings (Restrepo et al. 1990; Schild et al. 1995), and on immunohistochemical evidence (Cunningham et al. 1993; see Schild and Restrepo 1998). However, conflicting results have put this idea into question (see Barry 2003; Chen et al. 2000; Gold 1999; Takeuchi and Kurahashi 2003; Takeuchi et al. 2003). We explored the possibility that  $\text{InsP}_3$  may open plasma membrane  $\text{InsP}_3$  receptor channels during odor stimulation, allowing a  $\text{Ca}^{2+}$  influx that could subsequently activate  $\text{Cl}_{\text{Ca}}$  or  $\text{K}_{\text{Ca}}$  channels. However, we failed to detect any clear effect of  $\text{InsP}_3$  in experiments where this second messenger was released into the cell from a caged compound. There was no response in the 11 ORNs tested at  $-70$  mV. The same occurred in 2 of those cells that were also tested at 0 mV. Our results are in agreement with those of Gold and colleagues (Belluscio et al. 1998; see Gold 1999; Wong et al. 2000) and argue against a direct participation of  $\text{InsP}_3$  on transduction. However, we did not explore the possibility of a regulatory role of  $\text{InsP}_3$  on the cAMP pathway.

#### *Participation of $\text{Ca}^{2+}$ in the inhibitory cascade*

Calcium is involved in excitatory transduction by activating the  $\text{Cl}^-$  conductance (Kleene and Gesteland 1991). We have proposed that it also participates in inhibitory transduction by activating the ciliary  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  conductance (Delgado and Bacigalupo 2004; Morales et al. 1995). Thus we expected that increases in intracellular  $\text{Ca}^{2+}$  should result in the activation of both  $\text{Ca}^{2+}$ -dependent transduction conductances, which is exactly what we observed on photoreleasing it from a caged compound in isolated ORNs. However, one might additionally expect that this increase in  $\text{Ca}^{2+}$  would cause the activation of the somatic  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  conductance as well (Delgado and Labarca 1993; Firestein and Werblin 1987; Madrid et al. 2003) because the caged compound presumably is uniformly distributed in the cell. Because only the voltage-gated currents were activated during the photorelease of  $\text{Ca}^{2+}$  in an ORN lacking its cilia (Fig. 4), we conclude that the effect of this  $\text{Ca}^{2+}$  in intact ORNs was exerted exclusively over their transduction conductances, lo-

calized to the cilia (Delgado et al. 2003; Lowe and Gold 1993a,b). A possible explanation for this observation is that  $\text{Ca}^{2+}$  influx through somatic voltage-dependent channels during depolarization already increased intracellular  $\text{Ca}^{2+}$  concentration in the vicinity of the somatic plasma membrane to saturating levels for the somatic  $\text{K}_{\text{Ca}}$  conductance, such that an additional  $\text{Ca}^{2+}$  increment by photorelease would have no further effect on this somatic conductance. The situation in the cilia would be entirely different because they are devoid of voltage-gated channels (Delgado et al. 2003; Lowe and Gold 1993a), and diffusion of  $\text{Ca}^{2+}$  into the cilia from the dendrite and vice versa is extremely unlikely (Zufall et al. 2000) because of strong buffering and  $\text{Ca}^{2+}$  extrusion. The UV light would substantially increase ciliary  $\text{Ca}^{2+}$  concentration, opening the ciliary  $\text{Ca}^{2+}$ -dependent channels. Our results provide strong evidence for a pronounced segregation of the  $\text{K}_{\text{Ca}}$  transduction channels to the ciliary membrane and the voltage-gated channels to the nonciliary membrane in olfactory receptor neurons. Cell-attached patch-clamp recordings have shown the presence of the CNG channel in the dendritic knob (Zufall et al. 1991) and the cell body, but at a density nearly 400-fold lower than that in the cilia of toad ORNs (Kurahashi and Kaneko 1991; Lowe and Gold 1993a). The segregation of the transduction channels to a specialized membrane is common to most sensory cells, such as vertebrate photoreceptors (Fesenko et al. 1985; see Yau and Baylor 1989), invertebrate photoreceptors (Johnson and Bacigalupo 1992; Nasi and Gomez 1992; Stern et al. 1982), and mechanosensory hair cells (Jaramillo and Hudspeth 1991), and olfactory neurons are no exception.

#### *Chemotransduction events in olfactory cilia*

Taking together our own data and those of others, we propose the model illustrated in Fig. 6. According to this model, a single cascade mediates excitatory and inhibitory transduction. Abolishment of any of the individual proteins of the cascade should eliminate both responses, which is what is found either by the application of specific inhibitors (Fig. 1) or by gene-targeted deletion (Brunet et al. 1996; Delay and Restrepo 2004; Wong et al. 2000). Our studies results were obtained in isolated olfactory neurons under well-defined ionic conditions and were interpreted accordingly. In vivo the situation is rather uncertain because the ionic concentrations surrounding the cilia are not entirely clear and might depend on the species and might vary with the environment for a particular animal. The measurements of ion concentrations in the proximity of the cilia are technically difficult, with the additional complication, in the case of potassium, that its intracellular concentration is much higher than its extracellular concentration, so that any damage to the cells will result in a significant increment in external  $\text{K}^+$ . Potassium concentration values measured with various techniques in the mucus of the olfactory epithelia of different species range from 11 to 77 mM (Bronshtein and Leont'ev 1972; Chiu et al. 1989; Joshi et al. 1987; Reuter et al. 1998). No measurements are available for *Caudiverbera*. If the  $\text{K}^+$  concentration were within this range, the opening of a ciliary  $\text{K}^+$  conductance would be depolarizing, but it would be hyperpolarizing at lower  $\text{K}^+$  levels ( $\leq 10$  mM) (Delgado et al. 2003).

The model that we propose raises 2 major questions: 1) How can an ORN give origin to 2 opposite responses to different

odors if every ORN is thought to express only one of the hundreds of odor receptor genes of the genome? (Malnic et al. 1999; Serizawa et al. 2003; see Mombaerts 2004). 2) How does an ORN manage to generate 2 opposite responses independently of each other if they share a common transduction pathway? These are crucial questions that will have to be addressed in future studies. It is tempting to speculate that the transduction proteins that participate in each response type form complexes somehow segregated from each other within the cilia, allowing them to operate as independent functional units.

## ACKNOWLEDGMENTS

We thank Dr. Cecilia Vergara for thoughtful comments on the manuscript. Present address of R. Madrid: Instituto de Neurociencias, Universidad Miguel Hernández, 03550 Alicante, Spain.

## GRANTS

This work was supported by grants Ministerio de Planificación Nacional de Chile Iniciativa Científica Milenio P99-031-F and Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) 1020964 to J. Bacigalupo, and FONDECYT 4000014 and 2990003 to R. Madrid.

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