A cyclic AMP cascade mediates the inhibitory odor response of isolated toad olfactory receptor neurons.

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Key words: olfactory neuron, cAMP, Ca\(^{2+}\)-dependent K\(^+\) channel, odor-transduction, inhibitory response.

Running head: Cyclic AMP mediates inhibitory odor transduction.

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ABSTRACT

Odor stimulation may excite or inhibit olfactory receptor neurons (ORNs). It is well established that the excitatory response involves a cAMP transduction mechanism that activates a non-selective cationic cyclic nucleotide-gated (CNG) conductance, accompanied by the activation of a Ca\(^{2+}\)-dependent Cl\(^-\) conductance, both causing a depolarizing receptor potential. In contrast, odor inhibition is due to a hyperpolarizing receptor potential. It has been proposed that a Ca\(^{2+}\)-dependent K\(^+\) (K\(_{Ca}\)) 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\(^{2+}\) and InsP\(_3\) on isolated toad ORNs. The odor-triggered K\(_{Ca}\) current was reduced upon 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\(^{2+}\) activated a Cl\(^-\) current sensitive to niflumic acid (10 µM) and a K\(^+\) current blockable by charybdotoxin (CTx, 20 nM) and iberiotoxin (IbTx, 20 nM). In contrast, photoreleased Ca\(^{2+}\) 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\(^+\) current in intact ORNs. Photorelease of InsP\(_3\) did not increase the membrane conductance of olfactory neurons, arguing against a direct role of InsP\(_3\) in chemotransduction. We conclude that a cAMP cascade mediates the activation of the ciliary Ca\(^{2+}\)-dependent K\(^+\) current and that the Ca\(^{2+}\) 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 rising cAMP levels within the cilia. This second messenger directly activates non-selective cationic cyclic nucleotide-gated (CNG) channels, allowing the influx of Ca\(^{2+}\) and other cations into the cilia (Nakamura and Gold, 1987; Firestein and Werblin 1989; Kurahashi, 1989). Calcium opens ciliary Ca\(^{2+}\)-dependent Cl\(^{-}\) channels, allowing Cl\(^{-}\) 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 can not only excite, but can also inhibit vertebrate ORNs (Gesteland et al 1965; O’Connel and Mozell, 1969). It was first shown in *Necturus* that odor inhibition was due 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 (Duchamp-Viret et al, 1999; Sanhueza et al, 2000; Delay and Restrepo, 2004).

Morales et al. (1994, 1995) proposed that a Ca$^{2+}$-dependent K$^+$ conductance was the target of the transduction mechanism producing the inhibitory receptor potential in toad. This conductance is present in the cilia (Morales et al, 1995; Delgado et al 2003; Delgado and Bacigalupo, 2004) and is sensitive to charybdotoxin and iberiotoxin (Morales et al, 1995; Sanhueza et al, 2000; Castillo et al, 2005). The mechanism underlying the inhibitory response was unknown. It had been determined that Ca$^{2+}$ ions that activate these K$^+$ channels cross the ciliary membrane from the external milieu (Morales et al. 1997), but their permeability pathway was also unknown. Pun and Kleene (2002) reported the presence of a Ca$^{2+}$-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$^{2+}$ influx, upon which both responses depend. Here we investigated the inhibitory cascade making use of pharmacological agents and caged compounds for cAMP, Ca$^{2+}$ and InsP$_3$. Our results show that the activation of the ciliary Ca$^{2+}$-dependent K$^+$ conductance responsible for the inhibitory response is mediated by a cAMP-cascade, and that this cascade opens Ca$^{2+}$-permeant cyclic nucleotide-gated channels through which 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, sacrificed and pithed, and the olfactory epithelia were removed from their nasal cavity. The tissue was cut into 1 mm² 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 (mM):
Normal Ringer: 115 NaCl, 2.5 KCl, 1 CaCl₂, 0.4 MgCl₂, 10 HEPES, 3 glucose, pH 7.6.
Low-Cl⁻ external solution: 115 NaAc, 5 NaCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, pH 7.6.
Internal solution: 120 KCl, 4 HEPES, 0.1 Na-GTP, 1 Mg-ATP, 1 CaCl₂, 2 EGTA, pH 7.6, pCa 8.0. Low Cl⁻ internal solution: 115 KAc, 5 KCl, 1 MgCl₂, 0.5 EGTA, 10 HEPES, pH 7.6. Caged Ca²⁺ internal solution: 120 KCl, 4 HEPES, 0.1 Na-GTP, 1 Mg-ATP, 2 CaCl₂, 4 DM-Nitrophen (Calbiochem-Novabiochem Corp.), pH 7.6.

Electrical recording and data analysis.

Cells were viewed in an Olympus IX70 inverted microscope, with a 100x DIC objective (Plan, 1.25 N.A.). We used an Axopatch 1D patch-clamp (Axon Instruments) for electrical recording. Capacitance and series resistance (Rₛ) were cancelled; cells with Rₛ > 20 MΩ were discarded. The pClamp 6 software (Axon Instruments) was utilized for data acquisition and analysis. The patch pipettes were made of soft glass capillaries
(Bris, Globe Scientific Inc.) and electrode resistances were 2 - 4 MΩ. Rapid external solution exchange was accomplished with multibarreled pipettes (Sutter Instrument Co.), with tip diameters of ~5-10 µm per barrel, positioned ~30 µm from the cell. In the experiments using odorants, the tip diameters were < 1 µm, the pipette was positioned ~10 µm 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, Inc.) (250 µM, patch pipette concentration), caged Ca²⁺ (DM-Nitrophen, cage for Ca²⁺, Calbiochem-Novabiochem Corp.) (2 mM) and caged InsP₃ (D-myoinositol 1,4,5-triphosphate, P₄(5)-(1-(2-nitrophenyl)ethyl) ester, trisodium salt; NPE-caged Ins 1,4,5-P₃ (Molecular Probes, Inc.)) (150 µM, patch pipette concentration) were used. These messengers were added to the pipette solution and were released from their respective caged compounds using a 75W Xenon lamp as the UV light source. The kinetics of Ca²⁺ release is slower than that of cAMP or InsP₃, 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 Corp., Milton, MA). Normal Ringer was used externally.

In order to test whether InsP₃ was effectively being liberated from its caged compound, we recorded the emission signal generated by the InsP₃-triggered Ca²⁺ release from rat hepatocyte endoplasmic reticulum membrane vesicles; the emitted
signal was collected from a ~50 µm² area, delimited by an adjustable rectangular diaphragm. The vesicles (protein concentration 0.3 mg/ml) were suspended in normal ORN internal solution, supplemented with 20 µM Fluo-3 (Molecular Probes Inc.) and 150 µM caged InsP₃. UV exposures of identical characteristics as those applied to the ORNs generated a large fluorescence signal (not shown). This result indicates that InsP₃ was being successfully photoreleased, since it was inducing the release of Ca²⁺ 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-octadienitrile, kindly provided by D. Restrepo) and citronellal (3,7-dimethyl-6-octenal).

The CNG channel blocker LY83583 (6-(phenylamino)-5,8-quinoliniodine, RBI) was prepared in DMSO and kept as a 20 mM stock solution at −20°C; it was applied at a final concentration of 20 µM. The final DMSO concentration was no higher than 0.1%, which has no side effects on ORNs (Chen et al, 2000). SQ22536 ((9- terahydro-2ˈfuril)adenine, Calbiochem-Novabiochem Corp.) was prepared as a 100 mM stock solution in H₂O and used at a final concentration of 100 µM. Charybdotoxin (CTx) and iberiotoxin (IbTx, Alomone Labs.) were prepared from a 12 µM stock solution and from a 115 µM stock solution, respectively, and kept at −20°C. Both toxins were used at a final concentration of 20 nM. Niflumic acid was used at a final concentration of 10 µM, 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 (Kleene, 1993; Dubin and Dionne, 1994).

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 $K^+$ 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 $+20 \text{ mV}$ and $+50 \text{ mV}$ (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 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 $Ca^{2+}$ responsible for the activation of the ciliary $K^+$ conductance may in principle correspond either to an as yet unidentified ciliary $Ca^{2+}$ conductance or to the $Ca^{2+}$-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 mV, was reversibly and completely abolished when the odorant was applied together with 20 $\mu$M LY83583 (Fig. 1A). The inset shows the cadaverine-induced current, after subtracting the voltage-gated currents. In two other ORNs (of 5 cells responsive to this odorant) the effect of the drug was only partial. These observations suggest that $Ca^{2+}$ 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 ORN responsive to odor mixture F to 100 µM of the adenylyl cyclase inhibitor SQ22536. Figure 1B illustrates one of the five 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 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 three of eight responsive cells, while 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 2A shows that the photorelease of cAMP from its caged compound induced an inward current at –70 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 Cl⁻ solutions, in order to avoid any significant contribution of the excitatory Ca²⁺-activated Cl⁻ current. When released in an ORN held at +50 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, while in the other three cells there it had no effect (Fig. 2B). These results support the notion that a cAMP cascade mediates the activation of the ciliary Ca\(^{2+}\)-dependent K\(^+\) conductance by odors.

\textit{Ca}^{2+} \textit{increases a} K^{+} \textit{and a} Cl^{-} \textit{conductance.}

We tested whether the current activated by the photoreleased Ca\(^{2+}\) consisted of a K\(^+\) and a Cl\(^-\) component, as expected. For this we examined whether blockers of the K\(^+\) and of the Cl\(^-\) conductances, such as CTx or IbTx, and niflumic acid, respectively, affected this current. Figure 3A shows the prominent outward current that developed when an ORN loaded with caged Ca\(^{2+}\) was illuminated with UV light during a voltage pulse that depolarized the cell to 20 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 nM CTx, a strong blocker of the inhibitory current in \textit{Caudiverbera} (Morales et al, 1995; Sanhueza et al, 2000); this toxin has no significant effect on the somatic K\(^+\) currents (Delgado and Labarca, 1993; R Madrid and J Bacigalupo, unpublished observations). In this cell, the toxin blocked over 60 \% of the current induced by the released Ca\(^{2+}\) (Fig. 3B). Similar results were observed in 3 other cells, but CTx had no visible effect on other two ORNs. Another ORN was challenged with 20 nM IbTx, a rather specific blocker of large conductance K\(_{ca}\) channels. IbTx abolished nearly 50 \% of the outward current (Fig. 3C; \(n = 2\)). Finally, 10 \(\mu\)M niflumic acid blocked around 70\% of the outward current in a separate cell (Fig. 3D); a similar result was observed in the four neurons tested. These results are indicative that
released Ca\(^{2+}\) activated a K\(^+\) and a Cl\(^-\) conductance, and are in agreement with the notion that both conductances activate upon odorant-induced increments in luminal Ca\(^{2+}\) in the olfactory cilia. 

*The membrane conductances gated by the photoreleased Ca\(^{2+}\) localize to the olfactory cilia.*

A crucial question of our study was to what extent the currents induced by photoreleased Ca\(^{2+}\) ions flow through the ciliary membrane, specially considering the fact that the somatic plasma membrane of olfactory neurons is known to contain K\(_{Ca}\) channels (Trotier, 1986; Delgado and Labarca, 1993; Madrid et al, 2003). To address this question, we compared the effect of photoreleased Ca\(^{2+}\) in intact ORNs with ORNs that had lost their cilia during the dissociation process. The 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. 4A, right; n = 56) in an intact ORN (Fig 4A, left). Both currents were absent in the control trace, where no UV was given, while their voltage-gated currents seemed completely normal. In contrast, UV illumination had no effect in an ORN lacking its cilia (Fig 4B, left) (Fig 4B, right, n= 8). These results indicate that both conductances activated by Ca\(^{2+}\) under our experimental conditions reside in the chemosensory cilia, and therefore most likely they corresponded to the Ca\(^{2+}\)-dependent transduction conductances.

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

The possible physiological role of the odor-induced 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 (upper 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 on reducing the discharge rate of another cell, which presented a high spontaneous firing rate (Fig. 5, lower 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.

_Inositol trisphosphate does not appear to mediate the activation of ORN membrane conductances._

InsP$_3$ has been proposed as a possible messenger in olfactory transduction (see Schild and Restrepo, 1998). Photoreleasing InsP$_3$ in an ORN loaded with 150 µM caged InsP$_3$ 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$_3$-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, raising ciliary cAMP
levels. This second messenger activates CNG channels, allowing the influx of Ca\(^{2+}\) in the cilium. It is possible that calcium concentration quickly increases within the minute ciliary volume, where it might either activate Ca\(^{2+}\) -dependent Cl\(^-\) channels, originating the excitatory receptor potential, or it activate Ca\(^{2+}\) -dependent K\(^+\) channels, generating the inhibitory receptor potential. Since 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 cyclic nucleotide gated-channel has a key role in excitatory odor transduction, by allowing the influx of Ca$^{2+}$ that gates the Cl$^{-}$ conductance (Kleene and Gesteland, 1991; Kurahashi and Yau, 1993; Lowe and Gold, 1993b). It had been previously reported that intraluminal Ca$^{2+}$ increases occur in the chemosensory cilia of salamander ORNs, via a Ca$^{2+}$ influx from the extracellular milieu through the CNG conductance (Morales et al, 1997; Leinders-Zufall et al. 1997). We found that LY83583, a blocker of the CNG conductance that suppressed the excitatory current (Leiders-Zufall and Zufall, 1995), reversibly abolished the odor-induced ciliary KCa current as well. Since LY83583 has no known effect on K$^{+}$ 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 while 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 alternatively explanation for both situations would be that those cells where the
drugs had only a partial effect posses an additional transduction pathway.

In addition to the pharmacological evidence, the data obtained with caged cAMP
supports 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 $K^+$ currents could be observed in isolation under low $Cl^-$ (Fig. 2B). The
photorelease of cAMP in these conditions induced the $K^+$ current in 51% of the cells
examined. Blockade of this current by CTx is consistent with the notion that it corresponds
to the transduction $K_{Ca}$ 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 two
proteins are also key participants in chemotransduction. Taken together, one may argue on
favor of the notion that a cAMP cascade is central and perhaps the only chemotransduction
pathway in vertebrates.

An 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 Gold, 1999; Chen et al, 2000; Takeuchi and Kurahashi 2003; Takeuchi et al,
2003 and Barry, 2003). We explored the possibility that InsP₃ may open plasma membrane InsP₃ receptor channels during odor stimulation, allowing a Ca²⁺ influx that could subsequently activate Cl⁰ or K⁰ channels. We, however, failed to detect any clear effect of InsP₃ in experiments where this second messenger was released into the cell from a caged compound. There was no response in the eleven ORNs tested at -70 mV. The same occurred in two 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 InsP₃ on transduction. However, we did not explore the possibility of a regulatory role of InsP₃ on the cAMP pathway.

Participation of Ca²⁺ in the inhibitory cascade.
Calcium is involved in excitatory transduction by activating the Cl⁻ conductance (Kleene and Gesteland, 1991). We have proposed that it also participates in inhibitory transduction by activating the ciliary Ca²⁺-dependent K⁺ conductance (Morales et al, 1995; Delgado and Bacigalupo, 2004). Thus, we expected that increases in intracellular Ca²⁺ should result in the activation of both Ca²⁺-dependent transduction conductances, which is exactly what we observed upon photoreleasing it from a caged compound in isolated ORNs. However, one might additionally expect that this raise in Ca²⁺ would cause the activation of the somatic Ca²⁺-dependent K⁺ conductance as well (Firestein and Werblin, 1987; Delgado and Labarca, 1993; Madrid et al, 2003), since the caged compound presumably distributes uniformly in the cell. Since only the voltage-gated currents were activated by the photoreleased Ca²⁺ in an ORN lacking its cilia (Fig. 4), we conclude that the effect of this Ca²⁺ in intact ORNs was exerted exclusively over their transduction conductances, localized to the cilia (Lowe and Gold, 1993a,b; Delgado et al, 2003). A possible
explanation for this observation is that Ca$^{2+}$ influx through somatic voltage-dependent channels during depolarization already increased intracellular Ca$^{2+}$ concentration in the vicinity of the somatic plasma membrane to saturating levels for the somatic $K_{Ca}$ conductance, such that an additional 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 (Lowe and Gold, 1993a; Delgado et al, 2003), and diffusion of Ca$^{2+}$ into the cilia from the dendrite and vice versa is extremely unlikely (Zufall et al, 2000) due to strong buffering and Ca$^{2+}$ extrusion. The UV light would increase ciliary Ca$^{2+}$ concentration significantly, opening the ciliary Ca$^{2+}$-dependent channels. Our results provide strong evidence for a pronounced segregation of the $K_{Ca}$ transduction channels to the ciliary membrane and the voltage-gated channels to the non-ciliary 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 ~ 400-fold lower than 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 (Stern et al, 1982; Johnson and Bacigalupo, 1992; Nasi and Gomez, 1992), 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 figure 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; Wong et al, 2000; Delay and Restrepo, 2004). 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 extracellularly, so that any damage to the cells will result in a significant increment in external K⁺. Potassium concentration values measured with various techniques in the mucus of the olfactory epithelia of different species range from 11 to 77 mM (Joshi et al, 1987; Bronshtein and Leont’ev, 1972; Chiu et al, 1989; Reuter et al, 1998). No measurements are available for Caudiverbera. If the K⁺ concentration were within this range, the opening of a ciliary K⁺ conductance would be depolarizing, but it would be hyperpolarizing at lower K⁺ levels (~10 mM or less) (Delgado et al, 2003).

The model that we propose raises two major questions: How can an ORN give origin to two 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); and how does an ORN manages to generate two 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.
ACKNOWLEDGEMENTS

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

GRANTS

Supported by grants MIDEPLAN ICM P99-031-F and FONDECYT 1020964 (JB), and FONDECYT 4000014 and 2990003 (RM).
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FIGURE LEGENDS

Figure 1. Blockade of the odor-dependent inhibitory K⁺ current by LY83583 and SQ22536. 
A. The inhibitory K⁺ current activated by 50 µM cadaverine was abolished by 20 µM 
LY83583. The inset shows the net current abolished by the drug. B. In a separate cell, 30 
µM mixture F induced the inhibitory and excitatory currents, both of which were abolished 
by 1 minute incubation in 100 µM SQ22536. The inset shows the net current abolished by 
the drug.

Figure 2. Cyclic AMP activates the inhibitory current. A. Inward current induced by 
photoreleasing cAMP (200 ms UV light, Vh = -70 mV). B. Outward current activated by 
cAMP as the membrane was held at +50 mV using low Cl⁻ internal solution and low Cl⁻ 
external solution. The current was abolished by 20 nM CTx.

Figure 3. Ca²⁺ activates a current consisting of a Cl⁻ component and a K⁺ component. A. 
Typical whole cell current induced by photoreleased Ca²⁺. B. 20 nM CTx partially 
abolished the Ca²⁺-induced outward current. C. 20 nM IbTx partially abolished the current 
induced by Ca²⁺, in another ORN. D. 10 µM niflumic acid partially blocked the outward 
current activated by Ca²⁺. In all cases the blockers were supplemented to the normal Ringer 
solution.

Figure 4. The conductances activated by photoreleased Ca²⁺ are confined to the olfactory 
cilia. A. Left. DIC image of an intact ORN, bearing its cilia. Right: Whole cell current in 
response to photoreleased Ca²⁺, superimposed to the control current (evoked by the
Figure 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 (upper panel). Hyperpolarizing currents of identical magnitudes as above reduced the discharge rate in another ORN, that had a high spontaneous firing rate (lower panel). I_h = 0 pA in both cases.

Figure 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 raise in cAMP activates a CNG channel, allowing Ca^{2+} influx. In excitatory transduction, Ca^{2+} activates a Ca^{2+}-dependent Cl^{-} channel (top), whereas in inhibitory transduction, it activates a Ca^{2+}-dependent K^{+} channel (bottom).
Figure 1, Madrid et al.
A.

$V_h = -70$ mV

UV

B.

UV

recovery

CTx

Figure 2, Madrid et al.
Figure 3, Madrid et al.
Figure 4, Madrid et al
Figure 5, Madrid et al
Excitatory transduction

Inhibitory transduction

Figure 6, Madrid et al.