Molecular Components Involved in the Inhibitory Responses and Ca^{2+} Clearance from the Cilia of Olfactory Receptor Neurons

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Zusammenfassung

Olfaktorische Rezeptorneurone (ORNs) reagieren auf Geruchsstoffe mit Änderungen des Membranpotentials, was in der Folge die Aktionspotenzialfrequenz moduliert. Erregende Rezeptorantworten werden durch eine cAMP Kaskade vermittelt, welche zur Öffnung von nicht-selektiven kationischen zyklischen Nukleotid-aktivierten (CNG) Ionenkanälen und einer darauffolgenden Öffnung von Kalzium-abhängigen Chloridkanälen führt. Beides depolarisiert die Zellmembran und erhöht die Aktionspotenzialfrequenz. Dieser Prozess findet in den olfaktorischen Zilien statt, die alle Proteine dieser Signalkaskade enthalten.

Ausserdem konnte gezeigt werden, dass ORNs von verschiedenen Wirbeltierarten inhibitorische Geruchsantworten produzieren, bei denen die Aktionspotenzialfrequenz sich verringert. Es wurde postuliert, dass eine Kalzium-aktivierte Kaliumkonduktanz (K_{Ca}) eine Schlüsselrolle in diesem Prozess spielt, aber die Expression dieser Art von Kaliumkanälen in den olfaktorischen Zilien und der unterliegende Transduktionsprozess wurden bis jetzt nicht nachgewiesen. In unserem Labor wurde mit Hilfe eines spezifischen Antikörpers ein 116 kDa Protein in Western Blots von purifizierten olfaktorischen Zilien nachgewiesen, welches dem Molekulargewicht von KCa mit hoher Leitfähigkeit entspricht. Derselbe Antikörper markierte ORN Zilien in Gefrierschnitten des olfaktorischen Epithels und bei isolierten Rezeptorneuronen von Ratte und Kröte (*Caudiverbera caudiverbera*).

Hier zeige ich, mit Einzelkanal-Messungen an exzisierten inside-out Membranpatches von chemosensorischen Zilien der Kröte, das Vorhandensein von drei Typen von Kaliumkanälen, mit Leitfähigkeiten von 12, 60 und 210 pS, hoher K^+-Selektivität und Ca^{2+}-Sensitivitäten im unteren Mikromolarbereich. Ausserdem zeige ich, mit Hilfe von on-cell Patches auf chemosensorischen Zilien, die Aktivierung ziliärer Kaliumkanäle durch olfaktorische Stimuli. Dieselben Kanäle konnten nach Exzisierung der Membranpatches durch mikromolares Calcium, aber nicht durch zyklisches AMP aktiviert werden. Mit Hilfe von Photoaktivierung von caged cAMP“ konnte ausserdem ein auswärtsgerichteter K_{Ca}-Strom ausgelöst werden, welcher durch Charybdotoxin und Iberiotoxin (20 nM) blockiert wurde. Die Beobachtung, dass diese Konduktanz nicht in Zellen ohne Zilien beobachtbar war, deutet auf ihre Lokalisierung in ebendiesen Organellen hin. Photoaktivierung von, caged $InsP_3$“ bewirkte im Gegensatz zu cAMP keinerlei Membranstrom, was gegen eine Rolle von InsP3 in der olfaktorischen Transduktionskaskade spricht.

Ein zweites Thema dieser Arbeit war der Mechanismus der Ca^{2+} Eliminierung in olfaktorischen Rezeptorneuronen. Die Ca^{2+} Konzentration steigt im ziliären Lumen während der olfaktorischen Transduktion an, weil Ca^{2+} durch die geöffneten CNG Kanäle einströmt. Ca^{2+} spielt eine fundamentale Rolle in der Geruchsadaptation, indem es die cAMP Stoffwechselrate und die Aktivierung des CNG Kanals durch cAMP reguliert. Aus diesem Grunde muss die Ca^{2+} Konzentration fein geregelt werden. Es konnte gezeigt werden, dass ein Na^+/Ca^{2+} Austauscher eine entscheidende Rolle bei diesem Prozess spielt. Dieser Mechanismus wird vom Na^+-Gradienten angetrieben, welcher während der olfaktorischen Antwort reduziert ist. Mit Hilfe von Immunoblot,

Immunzytochemie und Immunhistochemie konnte in unserem Labor gezeigt werden, dass sowohl NCX als auch Plasma Membran ATPasen in purifizierter Zilienmembran vorhanden sind. Ausserdem wurde an inside-out Membranvesikeln von Zilien gezeigt, dass diese Ca^{2+} in ATP-abhängiger Weise akkumulieren. Dieses deutet auf einen komplementären Ca^{2+}-Eliminierungsmechanismus in olfaktorischen Zilien hin. Hier zeige ich, an whole-cell patch clamp Experimenten, dass eine Ca^{2+}ATPase zur Ca^{2+}-Eliminierung beiträgt. Die Entspannungszeitkonstante des Transduktionsstroms (272 ± 78 ms) wurde durch Carboxyeosin erhöht (2181 ± 437 ms), wie auch durch ATP (824 ± 161 ms) oder eine Erhöhung des pH-Werts von 7.6 auf 9.4 (1205 ± 286 ms). Das Ersetzen von extrazellulärem Na^+ durch Li^+ hatte einen ähnlichen Effekt (442 ± 8 ms), was die Rolle des Na^+/Ca^{2+}-Austauschers bestätigt. Diese Daten suggerieren, dass beide Ca^{2+} Transporter dazu beitragen, die Ca^{2+}-Basalkonzentration in den olfaktorischen Zilien wiederherzustellen.

Stichworte

Olfaktorisches Rezeptorneuron
Erregende Rezeptorantwort
Inhibierende Rezeptorantwort
Patch clamp
Whole cell recording
CNGC
cAMP
caged-cAMP
Ca^{2+}-aktivierte Cl^- Kanäle
Ca^{2+}-aktivierte K^+ Kanäle
NCX
PMCA

Abstract

Olfactory receptor neurons (ORNs) respond to odorants with changes in the membrane potential which leads to changes in the action potential firing rate. Excitatory responses are mediated by a cAMP (adenosine 3',5'-cyclic monophosphate) cascade that leads to the activation of cationic non-selective cyclic nucleotide-gated (CNG) channels and a subsequent opening of Ca^{2+}-dependent Cl^- (Cl_{Ca}) channels, both causing a depolarizing receptor potential that increases action potential firing frequency. This process takes place in the olfactory cilia, where all protein components of this cascade are confined. In addition, it has been shown that ORNs from various vertebrate species generate inhibitory odor responses, expressed as decrease in action potential discharges. It has been proposed, both in toad and rat isolated olfactory neurons, that a Ca^{2+}-dependent K^+ (K_{Ca}) conductance plays a key role in odor inhibition, but the presence of this kind of K^+ channels has not been demonstrated in the olfactory ciliary membrane and the underlying mechanism remains unclear. If these channels are involved in odor-transduction, they are expected to be present in the olfactory cilia. In our laboratory, using a specific antibody against large conductance K_{Ca}, it was possible to recognize in Western blots of purified rat olfactory ciliary membranes, a ~116 kDa protein that matches with the large conductance K_{Ca} molecular weight. This conductance was also found that K_{Ca} antibody labeled ORN cilia in cryosections of the olfactory epithelium as well as in isolated ORNs from rat and toad (*Caudiverbera caudiverbera*). Here I show evidence, obtained by means of single-channel recordings from excised inside-out membrane patches from toad chemosensory cilia, for the presence of three different types of K_{Ca} channels, with unitary conductances of 12, 60 and 210 pS, high K^+-selectivity and Ca^{2+} sensitivities in the low micromolar range. Moreover, through single-channel patch clamp recordings from toad 'on cilium' membrane patches, I was able to show activation of ciliary K^+ channel in response to odorant stimulation, which could be subsequently opened dose-dependently by micromolar Ca^{2+} after excision of the membrane patch, but not by cyclic AMP. In addition, in a separated series of whole cell experiments on toad ORNs, I was able to evoke, by photorelease of caged cAMP, an outward K_{Ca} current that was charybdotoxin (CTx, 20 nM) and iberiotoxin (IbTx, 20 nM) sensitive, but could not be observed in cells missing their cilia, indicating that these conductances are activated through the cAMP cascade and are confined to these organelles. Furthermore, $InsP_3$ photorelease did not increase the membrane conductance of olfactory neurons, arguing against a direct role of $InsP_3$ in chemotransduction.

Ca^{2+}concentration increases within the ciliary lumen by means of the Ca^{2+} influxes through the CNG channel, during olfactory transduction. Ca^{2+} ions have a fundamental role in odour adaptation, regulating cAMP turnover rate and the affinity of the CNG channels for cAMP, therefore the Ca^{2+} concentration must be finely controlled in the ciliary lumen. It has been shown that a Na^+/Ca^{2+} exchanger has a key role in Ca^{2+} clearance from the cilia. This Ca^{2+} extrusion mechanism is driven by the Na^+ gradient; however, this gradient declines during the olfactory response, making unfavourable conditions for this Ca^{2+} clearance mechanism. Recently, it was documented in our

laboratory by immunoblot, immunocytochemistry and immunohistochemistry, that both NCX and plasma membrane Ca^{2+}-ATPase are present on highly purified rat olfactory cilia membranes preparation. Moreover, it was observed in inside-out cilia membrane vesicles, that they accumulate Ca^{2+} in an ATP-dependent fashion. Both evidence support that a complementary Ca^{2+} clearance mechanism operates in ORN cilia. In the present work I show functional evidence, obtained from ORN whole cell patch clamp experiments, that the Ca^{2+}-ATPase contributes to Ca^{2+} removal. The relaxation time constant (τ) of the transduction current (272 ± 78 ms) was increased, by the PMCA blocker carboxyeosin (2181 ± 437 ms), by omitting ATP in the internal solution (824 ± 161 ms) or by raising extracellular pH from 7.6 to 9.4 (1205 ± 286 ms). Replacement of external Na^+ by Li^+ had a similar effect (442 ± 8 ms), confirming the Na^+/Ca^{2+} exchanger involvement in Ca^{2+} extrusion. The evidence suggests that both Ca^{2+} transporters contribute to restore resting Ca^{2+} levels in the cilia following olfactory response.

Keywords

Olfactory receptor neurons
Excitatory response
Inhibitory response
Patch clamp
Whole cell recording
CNG
cAMP
caged-cAMP
Ca^{2+}-dependent Cl^- channels
Ca^{2+}-dependent K^+ channels
NCX
PMCA

INCLUDED PUBLICATONS

The results presented in this thesis have been published in the following research articles:

Delgado Ricardo, Saavedra V., Schmachtenberg O., Sierralta J., and Bacigalupo J. 2003. Presence of Ca^{2+}-dependent K^{+} Channels in Chemosensory Cilia, supporting a role in Odor Transduction. *J Neurophysiol*, 90: 2022-2028. http://www.ncbi.nlm.nih.gov/pubmed/12801890

Delgado R., and Bacigalupo J. 2004. Cilium-attached and excised patch-clamp recordings of odourant-activated Ca-dependent K^{+} channels from chemosensory cilia of olfactory receptor neurons. *Eur J Neurosci*. 2004 Dec; 20 (11): 2975-80. http://www.ncbi.nlm.nih.gov/pubmed/15579151

Madrid R., Delgado R., Bacigalupo J. 2005. Cyclic AMP cascade mediates the inhibitory odor response of isolated toad olfactory receptor neurons. *J Neurophysiol*. 94:1781-1788 http://www.ncbi.nlm.nih.gov/pubmed/15817646

Castillo K., Delgado R., and Bacigalupo J. 2007. Plasma Membrane Ca^{2+}-ATPase in the Cilia of Olfactory Receptor Neurons: Possible role on Ca^{2+} clearance. *Eur J Neurosci*. 26 (9):2524-31. http://www.ncbi.nlm.nih.gov/pubmed/17970729

The text of these articles is not included in the thesis.

Please, use the original articles.

ABBREVIATIONS

Chemicals

Channel blockers

LY83583:	6-(phenylamino)-5,8-quiniolinodine, (RBI)
SQ22536:	9-terahydro-2´furil)-adenine, (Calbiochem-Novabiochem Corp)
CTx	Charybdotoxin , (Alomone Labs).
IbTx	Iberiotoxin, (Alomone Labs)
Niflumic Ac.	2-((3-(trifluoromethyl)phenyl)amino)-3-pyridinecarboxylic acid, (Sigma-Aldrich)

L-cis-diltiazem. L-(-)-*cis*-Diltiazem hydrochloride. (Biomol International, Enzo Life Science. Inc.)

Buffers

HEPES	N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (Sigma-Aldrich)
EGTA	ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid (Sigma-Aldrich)

Odor molecules

citronellal	3,7-dimethyl-6-octenal. (Sigma-Aldrich)
geraniol	3,7-dimethyl-2,6-octadien-1-ol (Sigma-Aldrich)
citralva	3,7-dimethyl-2,6-octadienenitrile. (Sigma-Aldrich)
cadaverine	1,5-diaminopentane. (Sigma-Aldrich)
isovaleric acid	3-Methylbutanoic acid. (Sigma-Aldrich)
pirazyne	1,4-Diazabenzene. (Sigma-Aldrich)

Olfactory response activators

cAMP	adenosine 3',5'-cyclicmonophosphate. (Sigma-Aldrich)
caged-cAMP	4,5-dimethoxy-2-nitrobenzyladenosine ',5'cyclicmonophosphate; DMNB-caged cAMP, (Molecular Probes, Inc).

1. INTRODUCTION

1.1. Olfactory receptor neurons.

Olfactory receptor neurons (ORNs) are located in the olfactory epithelia, within the nasal cavity. These sensory neurons project their axon to the olfactory bulb and a single dendrite toward the mucosal surface, where it terminates in a structure called "*dendritic knob*". The chemosensory cilia, approximately 0.2 µm wide and a few to tens of micrometers long, depending on the species, emanate from this structure, Figure 1. The cilia contain all the molecules involved in chemosensory transduction (Pace et al. 1985; Buck and Axel, 1991; Jones and Reed, 1989; Menco et al. 1992).

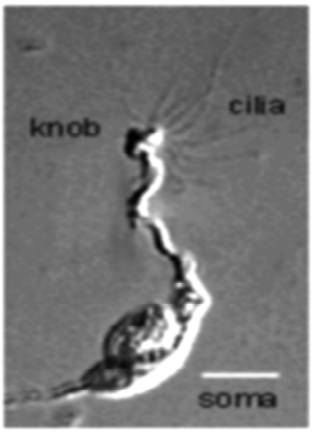

Figure 1. ***Dissociated toad olfactory receptor neuron, (ORN).*** *DIC 60X microscopic ORN image, showing the characteristic morphology. Bar calibration is 5 µm.*

1.2. Olfactory transduction

It is widely accepted that olfactory transduction begins when an odour molecule binds to a specific odour receptor, and activates, by means of a GTP-binding protein ($G_{olf,}$), a type III adenylyl cyclase (ACIII), raising the intraciliary cAMP concentration. cAMP gates the cyclic nucleotide-gated (CNG) channels, allowing Na^+ and Ca^{2+} influx into the ciliary lumen, depolarizing the cell (Firestein *et al.*, 1990; Firestein & Zufall, 1994). Ca^{2+} inside the cilia opens a Ca^{2+}-activated Cl^- channel (Cl_{Ca}) that allow Cl^- efflux from the cilia, (Kleene, 1993; Kurahashi & Yau, 1993; Lowe & Gold, 1993b), amplifying the olfactory response, (Figure 2A). Thus, in the ORNs, the depolarizing receptor potential is generated by positive and negative ions flowing in opposite direction through the ciliary membrane.

1.3 Excitatory response

ORNs exhibit spontaneous action potential firing. CNGCs and the $Cl_{Ca,}$ channels open as a consequence of odor binding to a receptor, depolarize de cilia and generate an excitatory response, (Nakamura and Gold, 1987; Firestein et al. 1991; Kleene and Gesteland, 1991; Lowe and Gold, 1993; Kurahashi and Yau, 1993), characterized by an increase in action potential frequency, Figure 2B.

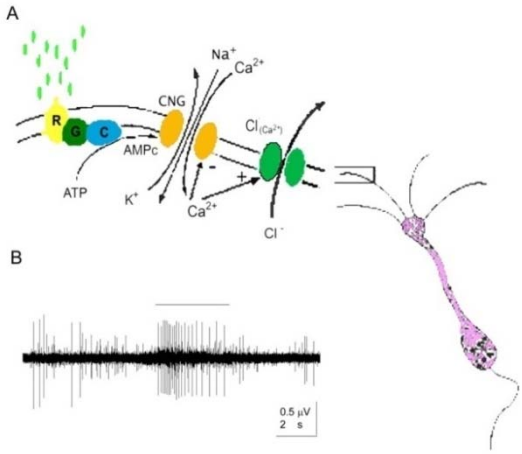

Figure 2: ***Excitatory olfactory transduction mechanisms****.* ***A.*** *Schematic representation of molecules, involved in olfactory transduction. R: olfactory receptor, G: G-protein, C: adenylyl cyclase, CNG: cyclic nucleotide gated channel,* $Cl_{(Ca2+)}$*:* Ca^{2+} *activated chloride channel. (Modified from Schild and Restrepo, 1998).* ***B****. Extracellular record of spontaneous action potential firing, from frog olfactory epithelium, showing an excitatory odor response. Odor mix application: cintronelal, geraniol, 10 µM each, indicated by the bar upper voltage record.*

1.4 Inhibitory odor response

Odorants can also evoke inhibitory responses in ORNs, expressed as a decrease in the spiking frequency, Figure 3. Inhibitory responses have been observed in animal species whose ORNs exhibit a relatively high rate of spontaneous action potentials (Maue and Dionne, 1987; Dionne, 1992; Morales et al. 1994; Kang and Caprio, 1995; see Getchell, 1986; Bacigalupo et al. 1997).

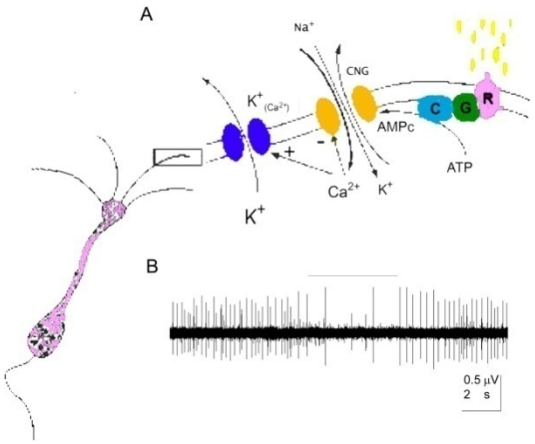

Figure 3. ***Proposed inhibitory olfactory mechanism.*** ***A.*** *Such as in the excitatory olfactory response, we postulate that inhibitory response is caused by the activation of* $K^+(_{Ca}{}^{2+})$ *channel, by* Ca^{2+} *entering through CNG channel.* ***B.*** *Extracellular recording of spontaneous action potential firing from toad olfactory epithelium in response to an odor mix application, composed of isovaleric acid and pyrazine, 100 µM each.*

Inhibitory chemotransduction has been proposed to involve a K_{Ca} conductance, since a charybdotoxin-sensitive odor-induced K_{Ca} current was found both in toad (*Caudiverbera caudiverbera*) and rat ORNs, in whole cell voltage-clamp experiments (Morales et al. 1994, 1995; Sanhueza et al. 2000). This K^+ current generates a hyperpolarizing receptor potential, causing the spiking rate to decrease. Focal odorant stimulation provided indirect evidence supporting the notion that these putative transduction K^+ channels are located in the chemosensory cilia (Morales et al. 1997). A study of the ion channels contained in a purified toad olfactory cilia preparation incorporated into planar lipid bilayers gave additional support to the presence of K_{Ca} channels in this compartment (Jorquera et al. 1995). However, this technique does not rule out the possibility that the K^+ channels recorded in lipid bilayers were not from the cilia. It had been determined that Ca^{2+} ions which activate these K^+ channels flow into the ciliary membrane from the external milieu (Morales et al. 1997). Ca^{2+}-dependent outward currents

evoked by odorants, which increase with hyperpolarization, have also been reported in *Rana pipiens*, and their activation seems to be mediated by cAMP, (Pun and Kleene, 2002), however the authors did not identify the nature of that current. In addition, it has been reported that both excitatory and inhibitory transduction currents were abolished by the CNG channel blocker L-cis-diltiazem (Delay and Restrepo, 2004) and were absent in transgenic mice lacking the CNG channel, (Brunet et al, 1996) suggesting that the CNG channel is required for both types of odorant responses, likely by allowing the Ca^{2+} influx, on which both responses depend.

1.5 Adaptation of olfactory transduction.

Within the olfactory transduction process, Ca^{2+} has a fundamental role in odour adaptation, (Chen & Yau, 1994; Liu *et al.*, 1994; Kurahashi & Menini, 1997). This ion reduces the cAMP affinity of the CNG channel and decreases cAMP levels through phosphodiesterase activation (Borisy *et al.*, 1992; Yan *et al.*, 1995) as well as by ACIII inhibition, (Wayman *et al.*, 1995; Leinders-Zufall *et al.*, 1999). The multiple regulatory actions of Ca^{2+} in olfactory transduction suggest that its concentration needs to be finely controlled. Chemosensory cilia are devoid of internal stores that could capture calcium ions, therefore Ca^{2+} accumulated into the cilia, during the olfactory response needs to be efficiently removed. Fluorescence measurements of ciliary Ca^{2+} concentrations led to the estimation that Ca^{2+} may reach nearly 1 μM during an olfactory response in this sensory organelle (Leinders-Zufall *et al.*, 1998). Functional evidence indicates that a Na^{+}/Ca^{2+} exchanger (NCX) present in the cilia extrudes Ca^{2+} after an odour stimulus (Reisert & Matthews, 1998). However, because the cell depolarizes, during the olfactory response the Na^{+} driving force declines, creating unfavourable conditions for NCX-dependentCa^{2+} removal, suggesting that an additional Ca^{2+} transport mechanism operates in the cilia, most likely a plasma membrane calcium ATPase (PMCA). PMCA can be pharmacologically distinguished from the sarco-endoplasmic reticulum calcium-ATPase (SERCA), by their differential sensitivity to carboxyeosin (Gatto C, and Milanik MA. 1993) and thapsigargin (Inesi and Sagara, 1994) It has been reported that PMCA exhibits a low transport activity at resting Ca^{2+} concentration, but when the Ca^{2+} concentration rises, it is vigorously enhanced (Carafoli & Brini, 2000).

1.6 Hypothesis

The hypotheses proposed in the present works are:

1. K_{Ca} channels are present in the chemosensory ciliary membrane.
2. K_{Ca} participates in the inhibitory odor response,
3. K_{Ca} generates a hyperpolarizing odor induced current which is activated by a cAMP-Ca^{2+} pathway.
4. Calcium is extruded from the ciliary lumen by NCX and a plasma membrane calcium ATPase (PMCA),

1.7 Methodological approach

In order to test these hypotheses, I used the following electrophysiological approaches:

- To examine the presence of K_{Ca} in olfactory cilia, I recorded single-channel currents from ciliary membrane inside-out patches under appropriate ionic condition.
- To test whether the $_{KCa}$ channels participate in odor transduction, I recorded unitary K^+ current from ciliary membrane patches in response to odours.
- In order to establish the nature of cAMP induced Ca^{2+} activated current, I conducted a pharmacological study, taking advantage of caged-cAMP, to activate the cascade
- To investigate the participation of a PMCA on ciliary Ca^{2+} clearance mechanism I measured the Cl_{Ca} transduction current relaxation (τ) after photorelease of cAMP, considering that a major fraction of this current is a Ca^{2+}-dependent Cl^- component. Based on the differential pharmacology and ionic requirements of NCX and PMCA, I established that both Ca^{2+} transporters, NCX and PMCA, contributed to ciliary Ca^{2+} clearance.

2. METHODS

2.1 ORNs dissociation

Isolated ORNs were obtained by mechanical dissociation of the olfactory epithelium, from the Chilean toad *Caudiverbera caudiverbera,* obtained from an authorized local distributor. The animals were anesthetized in ice, sacrificed and pithed, and the olfactory epithelia were removed from their nasal cavity. The tissue was placed into a 30 mm Petri dish containing normal Ringer solution, cut in approximately 1 mm^2 pieces and gently passed through a fire polished Pasteur pipette. 0.5 ml of the cell suspension were transferred to the experimental chamber and let to settle.

2.2 Single channels studies of K_{Ca}

Olfactory receptor neurons were identified morphologically through an Olympus IX70 inverted microscope, endowed with a 60x DIC objective (Plan, 1.25 N.A.) or Plan 100x /1.25 Oil Ph3. Electrical recordings were obtained from inside-out patches by the patch-clamp technique using an Axopatch 1D amplifier (Axon Instruments, Inc., Union City, CA). Recording pipettes were made from borosilicate glass capillaries with filament (Hilgenberg-GmbH, Postfach, Germany) and pulled with a P-97 horizontal puller (Sutter Instruments Co., Novato, CA). The tip resistances for this kind of recording were 40 - 50 MΩ. Recording pipette electrodes were filled with low Cl^- internal solution and the bath contained low Cl^- external solution. To adjust the pCa to 6.0 or higher, EGTA and $CaCl_2$ were added to attain a concentration calculated with the Winmaxc-2 software *(http://www.stanford.edu/~cpatton).* At low pCa in the solutions (≤ 6.0), EGTA was omitted and Ca^{2+} was added to the desired final concentration. The bath solution was exchanged by perfuse the whole experimental chamber (6 chamber volumes in ~30 s)

2.3. Whole cell K_{Ca} current studies.

The recording patch pipette electrodes were built using soft glass capillaries (Bris, Globe Scientific Inc.) or Borosilicate Glass melting point capillaries, (Kimble-Chasse, Thomas Scientific Cat # 6418K07). Patch-electrode had resistances about 2 - 4 MΩ. Rapid external solution exchange was accomplished with multi-barreled pipettes (Sutter Instrument Co.), with tip diameters ~ 5-10 μm per barrel, which were positioned ~ 30 μm from the cell. For odorant stimulation experiments, the stimulus pipettes were built such as the tip diameter were < 1 μm and were positioned ~10 μm from the cilia; the solution flow was controlled by a custom built computer-operated picospritzer.

2.4. PMCA activity on deactivation of ORNs olfactory responses.

To determine the participation of PMCA in Ca^{2+} removal from the cilia, the relaxation time constant (τ) of Ca^{2+}-dependent Cl^- currents activated as a consequence of ORN olfactory transduction were studied. For these experiments, Ca^{2+}-dependent Cl^- currents were evoked by photorelease of cAMP from DMNB-caged cAMP (Molecular probes) through an UV light flash (~15 kW, 1 ms), delivered by a

Xenon flash-lamp system (JML-C2, Rapp OptoElectronic. Gehlenkamp, PA, Germany). Caged cAMP was added to the recording pipette solution to final concentration of 100 μM and allowed to dialyze into the ORNs for at least 5 minutes previous to starting the electrical measurements. The whole cell currents were measured as described above. Only cells with visible cilia were used. The role of PMCA on ciliary Ca^{2+} clearance was studied in three different conditions: dialyzing the cell with internal solution containing carboxyeosin, with an ATP-free internal solution or by raising the extracellular solution pH form 7.6 to 9.4. NCX was abolished by Na^+ replacing Na^+ by Li^+, at equimolar concentration in the Ringer solution.

2.5. Data analysis.

Voltage protocols for K_{Ca} channels and whole cell current studies, as well as data acquisition to PC memory and data analysis were carried out with pClamp 6.0 software, (Axon Instruments, Inc., Union City, CA) installed on a Pentium III, 1000 MHz PC. Figure designs were made with Sigmaplot 10.

2.6. Solutions composition, expressed:

- Normal Ringer (mM): 115 NaCl, 2.5 KCl, 1 $CaCl_2$, 0.4 $MgCl_2$, 10 HEPES, 3 glucose, pH 7.6.
- Normal internal solution (mM): 120 KCl, 4 HEPES, 1 Mg-ATP, 1 $CaCl_2$, 2 EGTA, pH 7.6, pCa 8.0.
- Low-Cl^- external solution (mM): 115 NaAc, 5 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.6.
- Low Cl^- internal solution (mM): 115 KAc, 5 KCl, 1 $MgCl_2$, 2 EGTA, 1 $CaCl_2$, 10 HEPES, pH 7.6., pCa 8.0
- Caged cAMP internal solution (mM): 120 KCI, 4 HEPES, 1 Mg-ATP, 2 EGTA, 1 $CaCl_2$, 4 DMNB-caged cAMP, pH 7.6.

3. RESULTS

3.1 Single-channel Ca2+-dependent K+ current recordings from olfactory cilia.

In order to directly determine the presence of K_{Ca} in chemosensory cilia, inside-out excised membrane patches were exposed to different Ca^{2+} concentrations. The picture in figure 4A, shows the image of the pipette sealed on a cilium previous to excision. Unitary channel electrical recordings from the ciliary membrane patch, under various Ca^{2+} concentrations are shown, (Figure 4B). The ciliary membrane electrical recordings remained silent when the patch was exposed to 0.1 μM free Ca^{2+} solution, while elevation of Ca^{2+} to 50 μM induced K_{Ca} channel activation. Further activation was observed upon increasing Ca^{2+} to 100 μM, and the channel closed upon returning back to the 0.1 μM Ca^{2+} solution. The channel open probability (P_o) as a function of [Ca^{2+}] is displayed in figure 4C. The fit of the Hill function to the experimental data determines a $K_{0.5}$ of ~70 μM. The ciliary channel is strongly voltage dependent, as depicted in the recordings of figure 3D, where P_o increased from 0.03 to 0.38 as the transmembrane potential was raised from –10 to +40 mV, under 50 μM Ca^{2+}. The I-V relation yields a

slope conductance of ~210 pS (obtained under 50 µM Ca^{2+}, figure 3E), in agreement with large conductance Ca^{2+}-dependent K^+ channels (Vergara et al. 1998). Substituting Na^+ for K^+ in the bath caused a shift of the I-V curve that indicates a K^+-selective channel.

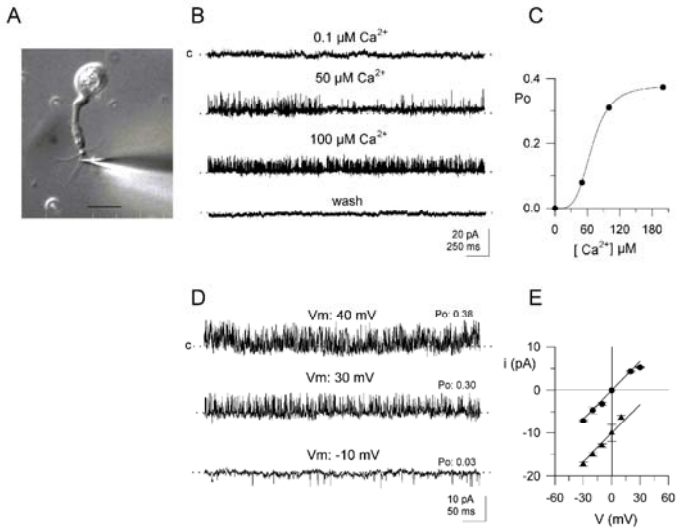

Figure 4. ***Single 210 pS Ca^{2+}-dependent K^+ channel currents activity from toad olfactory cilia A****. An ORN with a patch pipette sealed onto a cilium prior to excision, viewed through Olympus IX70 DIC optics, 40x objective; Bar: 5 µm.* ***B****. Single Ca^{2+}-dependent K^+ channel currents activity from toad olfactory cilia, obtained from an inside-out patch at three different [Ca^{2+}]; 0.1, 50 and 100 µM, 10 mV transmembrane potential.* ***C****. P_o vs. [Ca^{2+}] plot for the data in B. The curve corresponds to a Hill function fit; Hill coefficient (n) = 4. $K_{0.5}$ = 69 µM.* ***D****. Single-channel activity of the same patch as in B, at three different transmembrane potentials, recorded under 50 µM Ca^{2+} in the bath. P_o for each condition is indicated in the figure.* ***E****. I-V relations, under symmetrical K^+ solutions (⊗) as in D, and after replacing Na^+ for K^+ in the bath (Δ). Slope conductance: 210 pS; each data point represents 20 currents transition measurements. Error bars correspond to S.E.M. (n =2).*

In addition to the 210 pS channel, I observed two other K_{Ca} channel types. One of them was exposed to 0.1, 10 and 50 µM Ca^{2+} (at -40 mV), displaying P_o values of 0.0, 0.1 and 0.55, respectively (figure 5A). $K_{0.5}$ for Ca^{2+}, determined from the Hill function fitting to the P_o values , is in the low micromolar range and its unitary conductance is ~ 62 pS (60 and 64 pS, from 2 patches), as determined from the slope of the I-V curve (figure. 5B, circles). Replacement of Na^+ for K^+ in the bath solution shifted the curve by ~ 80 mV in the predicted direction for a K^+-selective channel (Figure. 5B, triangles). The third K_{Ca} channel species found in the olfactory cilia is presented in Figure 5C, under conditions in which the pipette contained Na^+-solution and the bath K^+-solution. The channel is shown under 0.1, 40 and 100 µM Ca^{2+}, at -20 mV transmembrane potential. The unit conductance of this channel is ~12 pS (10 and 14 pS, from 2 patches; figure 5E) and its $K_{0.5}$ for Ca^{2+} is ~ 53 µM (figure. 5D). In all three cases, the recording ionic conditions indicate that the only ion that could account for the observed single-channel currents was potassium.

The electrophysiological data from ciliary membrane patches are in agreement with immunochemical results from our laboratory and confirm the presence of K_{Ca} channels in the

ciliary membrane. Altogether, the evidence presented is consistent with a role of such channels in olfactory transduction.

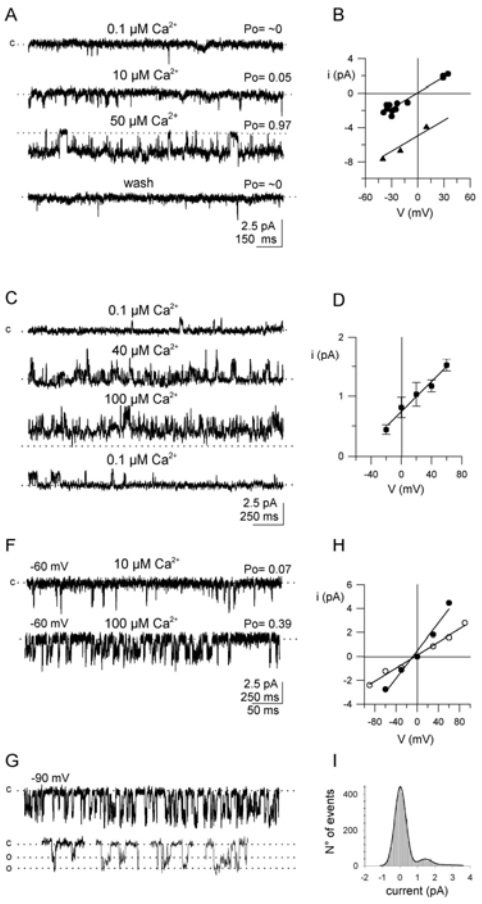

Figure 5. ***Single 60, and 14 pS, and 27, 58 pS Ca^{2+}-dependent K^+ channels from toad olfactory cilia.*** *A. Inside-out patch exposed to three different [Ca^{2+}]: 0.1, 10 and 50 μM. V = -40 mV. B. I-V relation before (●) and after (▲) replacing Na^+ for K^+ in the bath; slope conductance: 60 pS. (n= 2). C. Another different, inside-out patch under various [Ca^{2+}]: 0.1, 40 and 100 μM, under Na^+ solution in the pipette and K^+ solution in the bath. V_m = 20 mV. D D. I-V relation under the same ionic conditions as in C; slope conductance: 14 pS; Each data point represents measurements of 20 individual events at each potential in this patch; the error bars represent S.E.M. E. Po vs [Ca^{2+}] plot. Data fitted to a Hill equation, with n = 4; $K_{0.5}$ = 53 μM. F. Two recordings from another K^+ channel at 10 and 100 μM Ca^{2+}; voltage: -60 mV. G. Recordings from the same patch at -90 mV, under 100 μM Ca^{2+}. Assorted events exhibiting transitions between the two open states are shown in the bottom; the dotted line indicates the close and both open levels (bandwidth: 0-2000 Hz). H. I-V plot from the data at 100 μM Ca^{2+}; slope conductance: 29 and 58 pS. I. Amplitude histogram built from all the events that*

3.2 On-cilia odor activation of single Ca^{2+}-dependent K^+ channels.

Previously it was shown by electrical recording from inside-out patches excised from olfactory cilia, as well as by means of a variety of other methods in our laboratory, that the chemotransduction organelles contained Ca^{2+}-dependent K^+ channels. However, evidence indicating that such channels could be activated during odor stimulation is necessary to support their involvement in odor transduction. Therefore we conducted experiments on cilium-attached membrane patches in order to demonstrate the K_{Ca} channel activation by odor stimuli. Figure 6 shows an on-cilium patch-clamp experiment, in which the recording pipette was sealed onto a cilium and the odor stimulus puffer pipette was located ~10 μm away from the recording pipette

(figure 6A). The bath contained low Cl^- internal solution, to simplify the interpretation of the recordings. The pipette potential was set to 30 mV, determining a driving force for K^+ that favored a K^+ influx across the membrane patch. As shown in figure 6B, there was very little channel activity prior to odorant exposure, but an inward current event developed shortly after the stimulus onset, which gradually decayed back to basal level after the end of odor stimulation. The stimulus was repeated, with similar results (not shown). No channel activity increase was detected upon depolarizing the patch in the absence of odorant (not shown), indicating that channel activation was triggered by the chemical stimulus rather than being a result of an odor-induced cell depolarization. According to the experimental ionic conditions, the only ion that could account for the observed single-channel currents was K^+.

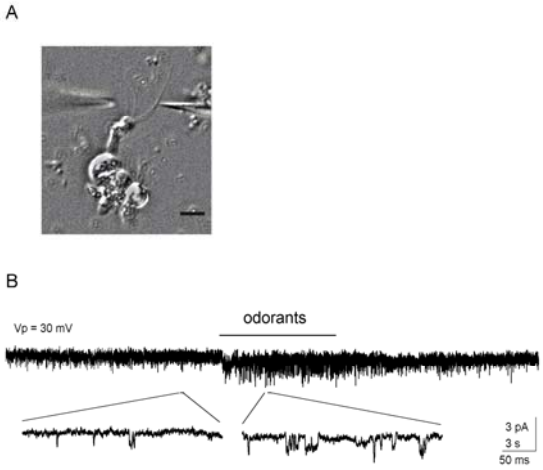

Figure 6. ***Single-channel activity induced by odor stimulation on a chemosensory cilium. A****. DIC 60X ORN microphotography showing the recording pipette patched on a cilium, and the stimulus puffer pipette.* ***B****. Current through the ciliary membrane patch before, during and after the application of an odor stimulus (Bandwidth: 1 kHz), Vm = 30 mV. The line above the record indicates the timing of the stimulus. The short traces correspond to expanded segments. The neuron was bathed in low Cl^- internal solution by both side of membrane patch.*

Figure 7 depicts the experiment of figure 6 in further detail. The top traces were recorded on-cilium, in the absence of any chemical stimulation (figure. 7A). The probability of the channel being open (Po) was 0.01. During odorant application, the channel activity greatly increased, reaching a Po of 0.26 (Figure. 7B). The patch was subsequently excised in the inside-out configuration and its intracellular face was exposed to internal solution. We tested three Ca^{2+} concentrations, 0.5, 10 and 100 μM, at which the channel exhibited P_o's of 0.01, 0.06 and 0.20, respectively (Fig 7C-E), indicating that the channel is Ca^{2+}-dependent

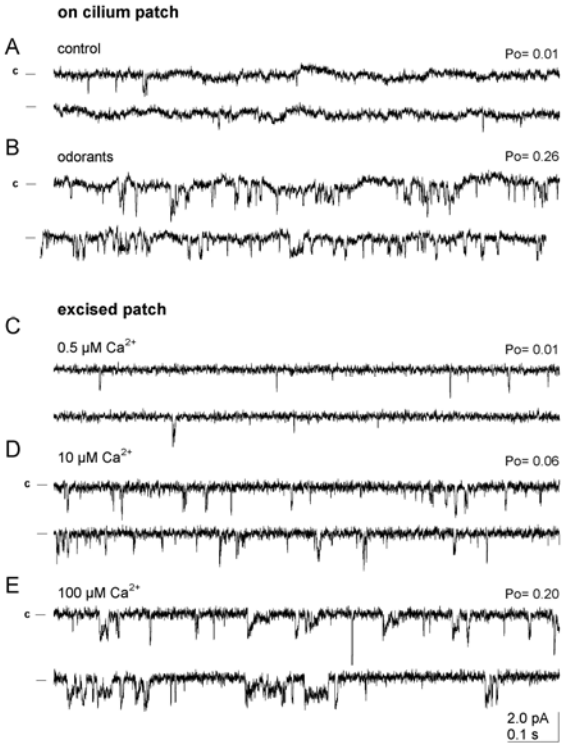

Figure 7. ***On-cilium odor-induced channel activity was activated by Ca^{2+} after patch excision****. **A**. On-cilium recording under odorant-free conditions. **B**. Channel activity during odorant stimulation. **C-E**. Channel activity after the patch was excised (inside-out) and exposed to internal solution with 0.5 µM, 10 µM and 100 µM Ca^{2+} free Ca^{2+}. $V_{pipette}$ = 30 mV.*

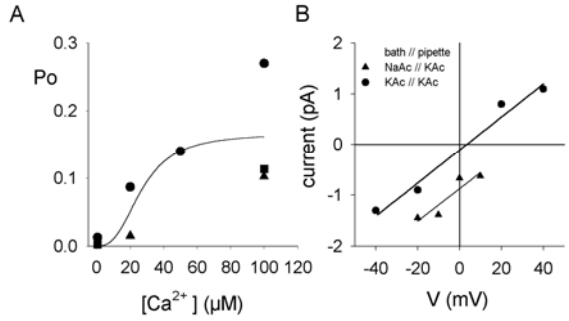

Figure 8. ***Ca^{2+}-dependence, and K^+ vs Na^+ selectivity, of the odorant-dependent ciliary channel****. **A**. Po vs $[Ca^{2+}]$ plot containing the data from 3 different patches (each indicated by a different* symbol). The line represents the fit to the *Hill equation $(A/(1+(K_{0.5}/[Ca^{2+}])^n)$, where $K_{0.5}$= ~25 µM and n= 2.7. **B**. I-V relation for the channel under K^+ (circles) and Na^+ (triangles) intracellular solutions in the bath.*

The Ca^{2+}-dependence of the K^+ channel for three separate patches is depicted in figure, 8A. The data were fitted to a Hill equation. The curve determined a channel $K_{0.5}$ for $[Ca^{2+}]$ of ~25 µM and a Hill coefficient (n) of 2.7. The I-V relation for this channel was previously reported to have two substates, of unitary values of ~27 and ~58 pS. Figure 8B shows the I-V relation for the low conductance level of the channel under symmetrical K^+. The I-V curve shifted ~28 mV to the right after K^+ was replaced by Na^+, indicating that the channel strongly selects for K^+ over Na^+. We explored a range of voltages (-40 to 40 mV) and could not determine any obvious difference of Po with respect to the membrane potential. The channel´s Po typically fluctuated spontaneously

over long time periods under steady conditions, suggesting an apparent change in the channel gating mode (not shown). A similar feature had been previously noticed for the large conductance Ca^{2+}-dependent K^+ channel in skeletal muscle cells (McManus and Magleby, 1988).

3.3 Cyclic AMP activates the inhibitory current.

In the case of excitatory odor response, it is well established that Ca^{2+} entry through CNG channels activates the Cl_{Ca} excitatory channels however the origin of Ca^{2+} for K_{Ca} channels activation was unclear. Considering that there is no other Ca^{2+} source described in the olfactory cilia, and that the CNG blocker L-cis-diltiazem (Delay and Restrepo, 2004) as well as the CNG mutant mouse (Brunet et al, 1996) affect the inhibitory response, we predict that the elevation of cAMP concentration in an ORN, may also produce activation of K_{Ca} channels underlying the inhibitory transduction currents. To test whether cAMP activated the K^+ conductance in ORNs, we performed whole cell experiment under external / internal low Cl^- solutions, in order to avoid any significant contribution of the excitatory Ca^{2+}-activated Cl^- current. Figure 9A shows that the photorelease of cAMP from its caged compound induced an inward current in at –70 mV, confirming previous observations in other species (Kurahashi, 1990; Kurahashi and Menini, 1997; Takeuchi and Kurahashi, 2002. In addition, 51%, (25 out of 49 cells) shows an outward current after photorelease of cAMP, when ORN were held at +50 mV, which was reversibly blocked by CTx in 4 of the 7 cells tested, while in the other 3 cells, CTx had no effect (Figure, 9B).

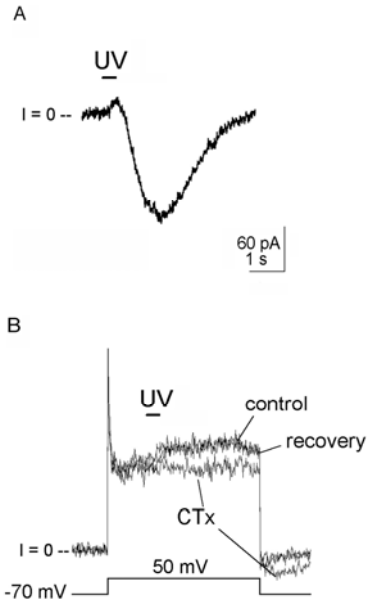

Figure 9. ***Intracellular cyclic AMP elevation activates the inhibitory transduction current.***

A*. Inward current induced by cAMP photoreleasing, by exposure the cells to 200 ms of UV light, recorded under ORN clamped to Vh= -70 mV. The cells were maintained in low* Cl^- *, external and internal solution.*

B*. Outward current activated by cAMP in a ORNs held at +50 mV and dialyzed with low* Cl^- *internal solution and bathed by low* Cl^- *external solution, was abolished by 20 nM CTx*

These results, together with the previously evidence, support the notion that a cAMP cascade mediates the activation of the ciliary Ca^{2+}-dependent K^+ conductance, which would account for the hyperpolarization which decreases the ORN spiking observed in response to some odor substances.

3.4 PMCA participates in Ca^{2+} clearance from the cilia

I addition, I also investigated the possible involvement of a PMCA in Ca^{2+} removal from the ORNs ciliary lumen. To this purpose, I studied the Ca^{2+} clearance from these organelles by measuring the relaxation time constant of the current triggered upon uncaging cAMP in dissociated ORNs, at -70 mV. At this potential the current evoked by cAMP mainly corresponds to the Cl^- component through the Cl_{Ca} channels, plus a minor component of cationic current through the CNG channels (Lowe & Gold, 1993b), while the K^+ current contribution is negligible. Therefore, since the transduction current directly depends on luminal Ca^{2+}, its kinetics closely reflects the dynamics of free Ca^{2+} concentration in the vicinity of the membrane within the cilia. Thus, the cAMP induced current represents an adequate monitor of the ciliary Ca^{2+} transporter activities. Under control conditions the cAMP activated current declined with a τ = 272 ± 78 ms (*n = 4)*, but the decay was significantly slowed down by exposing the cilia to 100 µM carboxyeosin (τ = 2181 ± 437 ms, *n = 5*), consistent with the participation of PMCA in the Ca^{2+} extrusion from this sensory organelle. Similar observations were made by omitting ATP from the internal solution, and the relaxation time constant also increased (τ = 824 ± 161 ms), as well as by extracellular alkalinisation to pH 9.4, (τ = 1205 ± 286 ms, *n = 3*) (Figure, 10). These results, obtained with those three different and independent strategies to abolish the Ca^{2+} pump, support the notion that an ATP-dependent mechanism for Ca^{2+} extrusion, (PMCA), is involved in extruding Ca^{2+} from the cilia. On the other hand, when external Na^+ was replaced by Li^+, the current relaxation time constant incremented as well (τ = 442 ± 8 ms, *n = 3*) (Figure, 10B), confirming the involvement of NCX on Ca^{2+} removal (Reisert & Matthews, 1998). These observations indicate that both PMCA and NCX effectively take part in the clearance of Ca^{2+} gained as a consequence of an increase in cAMP concentration supporting, a role of both transporters in Ca^{2+} removal following the activation of the olfactory transduction cascade.

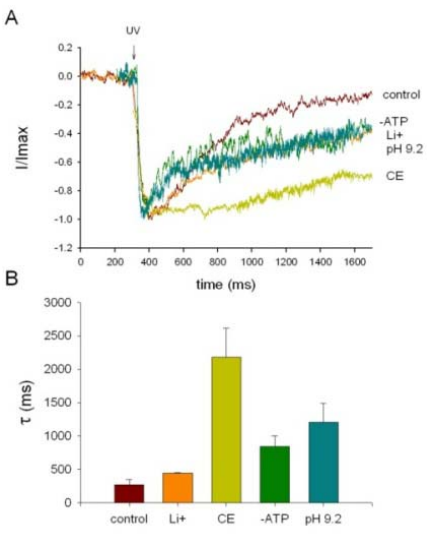

Figure 10. ***PMCA is involved in Ca^{2+} clearance from toad olfactory cilia.*** ***A****. Normalized superimposed representative whole cell currents recorded from ORNs evoke by UV-uncaging cAMP (arrow). The induced cAMP current relaxation time constants were determined in control conditions (τ = 272 ± 78 ms, n= 4), and under PMCA blockage treatment by Ringer solution with 50 µM carboxyeosin (CE) (τ= 2181 ± 437 ms, n= 5), and by interfering the H^+/Ca^{2+} pump, by increasing the pH from 7.6 to 9.2. (τ= 1205 ± 286 ms, n= 3) or by omitting ATP from the intracellular solution (τ= 824 ± 161 ms, n= 5). Current relaxation also was*

*determined after interfering the NCX by replacing external Na^+ for Li^+ (τ= 442 ± 8 ms, n= 3). V_h= -70 mV. **B.** τ values histogram summarizing the results indicated in A.*

4. DISCUSSION

4.1 K_{Ca} in the chemosensory transduction

The participation of a K_{Ca} conductance in the inhibitory odor response in the chemosensory cilia of olfactory receptor neurons (ORNs) was determined from whole cell recordings of isolated ORNs of the Chilean toad *Caudiververa caudiververa*. My studies showed that this conductance is responsible for ORN hyperpolarization and consequently reducing their firing rate, associated with the inhibitiory odor response (Morales et al. 1994, 1995). This conductance is sensitive to charybdotoxin (CTx) (Morales et al. 1995; Sanhueza et al. 2000) and iberiotoxin, (IbTx) (Madrid et al., 2005), both toxins are blocker of the large conductance Ca^{2+}dependent-K^+ channels (Vergara et al, 1998). Odor induced-inhibitory currents have also been reported in *Necturus* (Dionne, 1992) and *Xenopus* (Vogler and Schild, 1999), although their conductance, pharmacological properties and ion selectivity were not studied. The presence of the K_{Ca} channel in ORN olfactory cilia was confirmed by western blot and immunocytochemistry (Delgado et al., 2003). The BK_{Ca} channels are also present in the cell body, where they contribute to the voltage-induced whole cell current (Delgado and Labarca, 1993; Schild and Restrepo 1998; Madrid et al 2003).

Reconstitution experiments of purified toad ciliary membrane preparation into planar lipid bilayer first revealed the presence of a BK_{Ca} (Jorquera et al., 1995). The characterized potassium channel from this study shows a conductance of 240 pS, under symmetric conditions of 200 mM KCl, with a maximal $P_0 \sim 1$, and was sensitive to CTx. More recently, a detailed reconstitution study of a rat ciliary membrane preparation combined with immunoblotting confirmed such findings and, in addition to the presence of BK_{Ca}, revealed the presence of an apamine-sensitive small conductance K_{Ca} (SK_{Ca}), as well as two intermediate conductance K_{Ca} channels, (Castillo et al., 2005). The 210 pS channel reported in this thesis presents the same conductance as the channel blocked by ChTx and IbTx of rat ciliary membrane studies, but as the reconstituted toad BK_{Ca}, the ciliary rat BK_{Ca} exhibited a maximum open probability (P_0~ 1. 0), which was higher than the ciliary $BK_{Ca,}$ (P_0~ 0.38) recorded directly in inside-out patches. It has been shown that the presence of a complementary channel protein can modified the open probability of the BK_{Ca}, (Orio et al., 2002), as well as of the voltage gated Ca^{2+} channel, (Dzhura I. and Neely A. 2003). Therefore, the differences in the maximal open probability measured with both different techniques, may be due to the presence of some regulatory co-factor in the excised patches that was lost during the biochemistry procedure used to obtain the ciliary membrane preparation for the reconstitution experiments. Alternatively, the lipid composition of the artificial membrane might affect channel behavior (Vergara, 1998).

The $K_{0.5}$ for Ca^{2+}, for the three ciliary K_{Ca} channels characterized in this thesis, ranged from 1 to 69 µM Ca^{2+}. Although this value is greater than the submicromolar range of $K_{0.5}$ for Ca^{2+}

dependence reported for small conductance K_{Ca} channels (Vergara et al., 1998) and somewhat higher than that reported for intermediate conductance K_{Ca}, which is in the low micromolar range (Simoes et al. 2002), K^+ channels sensitivity by calcium varies widely, from submicromolar up to hundred μM for BK_{Ca} (Latorre et al., 2000). Fluorescence measurements suggested that resting Ca^{2+} concentration in the cilia is ~ 40 nM, and it might reach hundreds of nM during odor stimulation (Leinders-Zufall et al., 1998). On the other hand, based on the $K_{0.5}$ for Ca^{2+} dependence of frog ciliary Ca^{2+}- dependent Cl^- channels, the Ca^{2+} concentration would reach 5 to 26 μM (Kleene and Gesteland, 1991; Hallani et to the. 1998). Our excised ciliary membrane patch measurements suggest that the Ca^{2+} concentration would be between 10 to 100 μM in the vicinity of ciliary K^+ channels. It has been proposed that the Ca^{2+} concentration may reach such values in the intracellular mouth of Ca^{2+}-permeant channels, and drop sharply with the distance due to the presence of Ca^{2+}-binding proteins and other factors (McCarron JG. et al. 2006, Naraghi N. and Neher e. 1997). This notion may apply to the cilia, specifically around the CNG channels, establishing Ca^{2+} microdomains. Since there are two Ca^{2+}-dependent channels which produce opposite effects, Cl_{Ca} and KCa, in the cilia it is possible that these channels localize to separate microdomains.

It is worth considering that the extraciliary K^+ concentration is not known, and its value would be crucial for determining the true role of the ciliary K_{Ca} conductance, especially because it does not take much to surpass the equilibrium potential for this ion, in which case the K^+ current would become depolarizing. Measurement of extraciliary K^+ is particularly difficult because any damage of the epithelial cell would generate a leak of this ion from the epithelial cells, raising its external concentration significantly. In this thesis it was assumed that the extraciliary K^+ concentration is similar to a regular extracellular fluid, which is what we used in our experiments with dissociated olfactory neurons. Under this condition, the K^+ currents had a hyperpolarizing effect.

Cilium-attached patch clamp recordings were carried out using a pipette solution containing high K^+, no Na^+ and low Cl^-, to prevent an effective contribution of this anion to the recorded currents. These ionic conditions assured us that the channel activity induced by odor stimulation would be due to K^+ channels. After excising such patches they were exposed to various Ca^{2+} concentrations to build a Po vs. $[Ca^{2+}]$ plot that could be used to determine the actual $[Ca^{2+}]$ that induced the observed odor-evoked K^+ channel activity. The channel exhibited a high K^+ vs Na^+ selectivity. Both observations argue against the possibility that the recorded currents were through the CNG, which is cationic and unselective (Nakamura y Gold, 1987) and blocked by divalent cations (Zufall y Firestein, 1993).

The $K_{0.5}$ for Ca^{2+}, 25 μM, determined for the odor activated K^+ channel is similar to the $K_{0.5}$ for Ca^{2+} of BK_{Ca} and SK_{Ca} channels measured in excised ciliary membrane patches, (Delgado et al., 2003) and is close to the $K_{0.5}$ estimated for the Ca^{2+}-dependent Cl^- transduction channel (5-26 μM), (Hallani et al, 1998 (25); Kleene and Gesteland, 1991). These data would indicate that

[Ca^{2+}] may reach concentrations close to 100 μM in the vicinity of the CNG channels under high levels of odor stimulation.

4.2 The inhibitory response is mediated by a cyclic AMP cascade.

The evidence provided above supports a physiological function for K_{Ca} in odor transduction. The question that follows is what underlying mechanisms controls this conductance. The cilia are devoid of an internal membrane system, as well as of any other Ca^{2+} permeate channels other than CNG, which is the only Ca^{2+} entry source in olfactory cilia (Firestein et al., 1990; Firestein & Zufall, 1994). There have been reports suggesting the presence of an IP_3 pathway in olfactory cilia (Boekhoff et al., 1990; Cunningham et al., 1993; see Shild and Restrepo 1998), but the evidence is controversial and is missing convincing electrophysiological support. The evidence presented above, in addition to the fact that the CNG channel blocker L-cis-diltiazem affects the inhibitory response (Schild and Restrepo, 2004), added to the evidence that the CNG knockout mouse mutant shows an impaired inhibitory response (Brunet et al., 1996), and our own evidence, showing that photorelease of cAMP induced the K_{Ca} current, strongly supports the idea that the cAMP pathway may be involved in the inhibitory response.

Further supports to the notion that cAMP activates the K_{Ca} current comes from the observation that LY83583, a blocker of the CNG channel which inhibits the excitatory current (Leiders-Zufall, Zufall, 1995), also inhibits the inhibitory odor responses (Madrid et al, 2005). It was also shown that the adenyl cyclase inhibitor SQ22536, also affected the inhibitory current (Madrid et al., 2005). Although both drugs cause a complete abolition of the inhibitory response in some neurons and they had only a partial effect in other cells, these results shows a participation of the cAMP cascade in inhibitory response. Furthermore, this result suggest that ORN might have a population possessing an additional inhibitory transduction pathway.

4.3 Participation of the PMCA in Ca^{2+} removal in odor transduction.

As mentioned, the activations of the mechanisms underlying the olfactory response result in Ca^{2+} accumulation within the cilia and therefore to terminate it, Ca^{2+} removal is crucial. Electrophysiological studies have documented, both in frog and rat olfactory receptor neuron, the presence of NCX, which extruded Ca^{2+} from the cilia after odor stimulation (Reisert & Matthews, 1998; Reisert et al., 2003). Furthermore, by microscopy and molecular biology techniques, the presence of a PMCA in mouse olfactory neurons was revealed (Weeraratne et al., 2006), but no evidence for Ca^{2+} removal was provided. More Recently, we combined immunoblotting, immunocytochemistry and Ca^{2+} transport measurements and electrophysiological recordings, providing evidence for the presence of a PMCA in olfactory cilia and for its capacity for removing intraciliary Ca^{2+} (Castillo, el al., 2007).

In the present work I present electrophysiological evidence which reveals a participation of PMCA in odor transduction. In this study I used the relaxation kinetics of the whole cell currents induced by photolysis of caged-cAMP as a monitor of ciliary Ca^{2+} levels, since under our

experimental conditions this current was principally carried by Cl^-. The relaxation time course of this current,incremented upon abolishing either PMCA or NCX, supporting the involvement of both proteins on ciliary Ca^{2+} clearance.

Recent reports support our finding (Ponissery Santos et al., 2009; Kwon HJ. et al., 2009). Using fluorescence techniques it was shown that blockade of PMCA prolonged the decay of the Ca^{2+} fluorescence signal. Altogether, current evidence supports the notion that PMCA and NCX participate in Ca^{2+} extrusion from the cilia following an odor response.

5. SUMMARY

In the present work, I provide electrophysiological evidence for the presence of K_{Ca} channels in the cilia of ORNs. The activation of the ciliary K_{Ca} underlies the outward current evoked in response to intracellular cAMP elevation. I also provide electrophysiological evidence supporting a role of the PMCA in Ca^{2+} clearance from the cilia of ORN.

6. REFERENCES

1. Bacigalupo J, Labarca P, Morales B, Ugarte G, and Madrid R. (1997). Inhibitory responses to odorants in vertebrate olfactory neurons. In: From ion channels to cell-to-cell conversations, edited by Latorre R and Saez JC, New York, NY: Plenum Press. Chapter 16, p. 269-284.
2. Bacigalupo J., and Lisman, J.E. (1983). Single-channel currents activated by light in Limulus ventral photoreceptors Nature 304, 268-270.
3. Barry P. (2003). The relative contributions of cAMP and InsP3 pathways to olfactory responses in vertebrate olfactory receptor neurons and the specificity of odorants for both pathways. J Gen Physiol 22:247-50.
4. Belluscio L, Gold GH, Nemes A, and Axel R (1998). Mice deficient in $G_{(olf)}$ are anosmic. Neuron 20: 69-81.
5. Boekoff I, Tareilus E, Strotmann J, and Breer H. (1990). Rapid activation of alternative second messenger pathways in olfactory cilia from rats by different odorants. EMBO J. 9: 2453 2458.
6. Borisy FF, Ronnett GV, Cunningham AM, Juilfs D, Beavo J, Snyder SH. (1992). Calcium/calmodulin-activated phosphodiesterase expressed in olfactory receptor neurons. J Neurosci 12(3):915-23.
7. Brunet LJ, Geoffrey HG and Ngai J. (1996). General anosmia caused by a targeted disruption of the mouse olfactory cyclic nucleotide-gated cation channel. Neuron 17: 681-693.
8. Buck LB, and Axel R. (1991). .A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell 65: 175-187,
9. Carafoli E, Brini M. (2000) Calcium pumps: structural basis for and mechanism of calcium transmembrane transport. Curr Opin Chem Biol. 4(2):152-61.
10. Castillo K, Bacigalupo J and Wolff D. (2005). Ca^{2+}-dependent K^+ channels from rat olfactory cilia characterized in planar lipid bilayers. FEBS Lett, 14: 579(7):1675-82.
11. Cunningham AM, Ryugo DK, Sharp AH, Reed RR, Snyder SH, and Ronnett GV. (1993). Neuronal inositol 1,4,5-trisphosphate receptor localized to the plasma membrane of olfactory cilia. Neuroscience 57:339-52.
12. Chen & Yau. (1994). Direct modulation by Ca^{2+}-calmodulin of cyclic nucleotide-activated channel of rat olfactory receptor neurons. Nature. Apr 7; 368(6471):545-8.
13. Chen S, Lane AP, Bock R, Leinders-Zufall T, and Zufall F. (2000).Blocking adenylyl cyclase inhibits olfactory generator currents induced by "$IP_{(3)}$-odors". J Neurophysiol 84:575-80.
14. Delay R, and Restrepo D. (2004). Odorant responses of dual polarity are mediated by cAMP in mouse olfactory sensory neurons. J Neurophysiol. 92(3): 1312-9.

15. Delgado R, and Labarca P. (1993). Properties of whole cell currents in isolated olfactory neurons from the Chilean toad *Caudiverbera caudiverbera*. Am. J. Physiol. Cell Physiol. 264: C1418-1427.

16. Delgado R, Saavedra MV, Schmachtenberg O, Sierralta J and Bacigalupo J. (2003). Presence of Ca^{2+}-dependent K^+ channels in chemosensory cilia support a role in odor transduction. J Neurophysiol 90:2022-8.

17. Dionne VE. (1992). Chemosensory responses in isolated olfactory receptor neurons from *Necturus maculosus*. J. Gen. Physiol 99: 415-433.

18. Dzhura I, and Neely A. 2003. Differential modulation of cardiac Ca_{2+} channel gating by b-subunits. Biophys. J. 85: 274–289

19. Firestein, S., Shepherd, G.M. & Werblin, F.S. (1990) Time course of the membrane current underlying sensory transduction in salamander olfactory receptor neurones. J Physiol, 430, 135-158.

20. Firestein S, Zufall F, and Shepherd GM. (1991). Single odor-sensitive channels in olfactory receptor neurons are also gated by cyclic nucleotides. J. Neurosci. 11: 3565-3572.

21. Firestein, S. & Zufall, F. (1994). The cyclic nucleotide gated channel of olfactory receptor neurons. Semin Cell Biol, 5, 39-46.

22. Gatto C, Milanik MA. (1993) Inhibition of red blood cell calcium pump by eosin and other fluorescein analogues. *Am J of Physiology*, **264**, C1577–158

23. Getchell, TV. (1986). Functional properties of vertebrate olfactory receptor neurons. Physiol. Reviews 66: 772-818.

24. Gold GH. (1999). Controver**sia**l issues in vertebrate olfactory transduction. Annu. Rev. Physiol. 61:857-871.

25. Gomez, M.P. & Nasi, E. (1994). The light-sensitive conductance of hyperpolarizing invertebrate photoreceptors: a patch-clamp study. J Gen. Physiol. 103, 939-956.

26. Haab, J.E., Vergara, C., Bacigalupo, J. & O'Day, P.M. (2000) Coordinated gating of TRP-dependent channels in rhabdomeral membranes from Drosophila retinas J. Neurosci. 20, 7193-7198.

27. Hallani M, Lynch JW, and Barry, PH. (1998). Characterization of the calcium-activated chloride channels in patches excised from the dendritic knob of mammalian olfactory receptor neurones. J. Membr. Biol. 161: 163-171.

28. Haynes, L., Kay, A.R. & Yau, K.-W. (1986). Single cyclic GMP-activated channel activity in excised patches of rod outer segment membrane. Nature 321, 66-70.

29. Inesi G, Sagara Y. (1994). Specific inhibitors of intracellular Ca^{2+} transport ATPases. J Membr Biol. 141(1):1-6.

30. Jensen BS, Strøbaek D, Olesen SP, and Christophersen P. (2001). The Ca^{2+}-activated K^+ channel of intermediate conductance: a molecular target for novel treatments. Current Drug Targets 2: 401-422.

31. Jones D.T., and Reed R., R. (1989). Golf: an olfactory neuron specific G protein involved in odorant signal transduction. Science 244: 790–795.

32. Jorquera O, Latorre R, and Labarca P. (1995). Ion channel classes in purified olfactory cilia membranes: planar lipid bilayer studies. Am. J. Physiol. Cell. Physiol. 269: C1235-C1244.

33. Kang J. and Caprio J. (1995). .In vivo responses of single olfactory receptor neurons in the channel catfish, Ictalurus punctatus. J. Neurophysiol. 73: 172-177.

34. Kleene SJ, and Gasteland RC. (1991). Calcium-activated chloride conductance in frog olfactory cilia. J. Neurosci. 11: 3624-3629.

35. Kleene SJ. (1993). Origin of the chloride current in olfactory transduction. Neuron 11:123-132.

36. Kramer, R.H. & Siegelbaum, S.A. (1992). Potassium channel inactivation peptide blocks cyclic nucleotide-gated channels by binding to the conserved pore domain Neuron 9, 897-906.

37. Kurahashi T. (1990). The response induced by intracellular cyclic AMP in isolated olfactory receptor cells in the newt. J. Physiol. (London) 430:355-371.

38. Kurahashi T and Yau KW. (1993). Co-existence of cationic and chloride components of odorant-induced current of vertebrate olfactory receptor neurons. Nature 363:71-74,.

39. Kurahashi T and Menini A. (1997). Mechanism of odorant adaptation in the olfactory receptor cell. Nature 385:725-729.

40. Kwon H.J., Koo J.H., Zufall F., Leinders-Zufall T., Frank and Margolis L. (2009). Ca^{2+} extrusion by NCX is compromised in olfactory sensory neurons of $OMP^{-/-}$ mice. PLOS ONE 4, 1, e4260

41. Latorre R, Vergara C, Alvarez O, Stefani E, and Toro L. (2000). Voltage-gated calcium-modulated potassium channels of large unitary conductance: structure, diversity and pharmacology. In: Handbook of Experimental Pharmacology Pharmacology of Ion Channel Functions: Activators and Inhibitors, edited by M Endo, Heidelberg, Springer-Verlag., Vol 147, chapt. 8, p. 197-223.

42. Leinders-Zufall T and Zufall F. (1995). Block of cyclic nucleotide-gated channels in salamander olfactory receptor neurons by the guanylyl cyclase inhibitor LY83583. J Neurophysiol. 74: 2759-2762.

43. Leinders-Zufall T, Rand M, Shepherd GM, Greer CA and Zufall F. (1997). Calcium entry through cyclic nucleotide-gated channels in individual cilia of olfactory receptor cells: spatiotemporal dynamics. J. Neurosci 17: 4136-48.

44. Leinders-Zufall T, Greer CA, Shepherd GM, and Zufall FF. (1998). Imaging odor-induced calcium transients in single olfactory cilia: specificity of activation and role in transduction. J. Neurosci. 18: 5630-5639,.

45. Leinders-Zufall, T., Ma, M. & Zufall, F. (1999) Impaired odor adaptation in olfactory receptor neurons after inhibition of Ca2+/calmodulin kinase II. J Neurosci, 19, RC19.

46. Liu, M., Chen, T.Y., Ahamed, B., Li, J. & Yau, K.W. (1994) Calcium-calmodulin modulation of the olfactory cyclic nucleotide-gated cation channel. Science, 266, 1348-1354.

47. Lowe G and Gold GH. (1993a). Contribution of the ciliary cyclic nucleotide-gated conductance to olfactory transduction in the salamander. J Physiol (London) 462:175-96,

48. Lowe G and Gold GH. (1993b). Nonlinear amplification by calcium-dependent chloride channels in olfactory receptor cells. Nature 366:283-6.

49. Madrid, M., Sanhueza, M., Alvarez, O., and Bacigalupo J. (2003) Tonic and Phasic Receptor Neurons in the Vertebrate Olfactory Epithelium. Biophys J. June; 84(6): 4167–4181.

50. Madrid, R., Delgado, R. & Bacigalupo, J. (2005) Cyclic AMP cascade mediates the inhibitory odor response of isolated toad olfactory receptor neurons. J Neurophysiol, 94, 1781-1788.

51. Malnic B, Hirono J, Sato T and Buck LB. (1999)..Combinatorial receptor codes for odors. Cell 96:713-723.

52. Mathews, G. (1987) Proc. Natl. Acad. Sci. USA 84, 299-302.McManus, O.B. & Magleby, K.L. (1988) J. Physiol. 402, 79-120.

53. Maue RA, and Dionne VE. (1987). Patch-clamp studies of isolated mouse olfactory receptor neurons. J. Gen. Physiol. 90: 95-125.McManus, O.B. & Magleby, K.L. (1988) Kinetic states and modes of single large-conductance calcium-activated potassium channels in cultured rat skeletal muscle. J. Physiol. 402, 79-120.

54. McCarron JG, Chalmers S, Bradley KN, MacMillan D, Muir. TC (2006) Ca^{2+} microdomains in smooth muscle. Cell Calcium 40:461–493

55. Menco BP. (1992). Lectins bind differentially to cilia and microvilli of major and minor cell populations in olfactory and nasal respiratory epithelia. Microsc Res Tech. Oct 15; 23(2):181-99.

56. Morales B, Labarca P, and Bacigalupo J. (1995). A ciliary K^+ conductance sensitive to charybdotoxin underlies inhibitory responses in toad olfactory receptor neurons. FEBS Lett. 359: 41-44.

57. Morales B, Madrid R and Bacigalupo J. (1997).Calcium mediate the activation of the inhibitory current induced by odorants in toad olfactory receptor neurons. FEBS Lett. 402: 259-264.

58. Morales B, Ugarte G, Labarca P, and Bacigalupo (1987). J. Inhibitory K^+ current activated by odorants in toad olfactory neurons. Proc. R. Soc. Lond. B Biol. Sci. 257: 235-242, 1994.

59. Naraghi N. and Neher E. (1997). Linearized buffered Ca^{2+} diffusion in microdomains and its implications for calculation of $[Ca^{2+}]$ at the mouth of a malcium Channel. J. of Neurosci. 17(18):6961–6973

60. Nakamura T and Gold GH. (1989). A cyclic nucleotide gated conductance in olfactory receptor cilia. Nature 325: 442 444.

61. Nasi E and Gomez MP. (1992). Light-activated ion channels in solitary photoreceptors of the scallop *Pecten irradians*. J Gen Physiol 99:747-769.

62. Orio P, Rojas P, Ferreira G, and Latorre R, 2002. New disguises and Old Channel: MaxiK channel β-subunits. News Physiol Sci 17: 156-161

63. Pace U, Hanski E, Salomon Y, and Lancet D. (1985). Odorant-sensitive adenylate cyclase may mediate olfactory reception. Nature 316: 255-258.

64. Ponissery S.S., Weeraratne S.D., Valentine1 M., Delay R. and Van Houten J.L. (2009). Role of plasma membrane calcium ATPases in calcium clearance from olfactory sensory neurons. Chem. Senses 34: 349–358.

65. Pun R. and Kleene S. (2002). .Outward currents in olfactory receptor neurons activated by odorants and by elevation of cyclic AMP. Cell Biochem Biophys. 37(1):15-26.

66. Reisert, J. & Matthews, H.R. (1998) Na^{+}-dependent Ca^{2+} extrusion governs response recovery in frog olfactory receptor cells. J Gen Physiol, 112, 529-535.

67. Reisert, J. & Matthews, H.R. (2001) Simultaneous recording of receptor current and intraciliary Ca^{2+} concentration in salamander olfactory receptor cells. J Physiol, 535, 637-645.

68. Reisert, J., Bauer, P.J., Yau, K.W. & Frings, S. (2003) The Ca^{2+}-activated Cal^{-} channel and its control in rat olfactory receptor neurons. J Gen Physiol, 122, 349-363.

69. Restrepo D, Miyamoto T, Bryant BP and Teeter JH. (1990). Odor stimuli trigger influx of calcium into olfactory neurons of the channel catfish. Science 249:1166-1168.

70. Sanhueza M, Schmachtenberg O and Bacigalupo J. (2000) Excitation, inhibition, and suppression by odors in isolated toad and rat olfactory receptor neurons. Am. J. Physiol. (Cell Physiol) 279:31-39.

71. Schild D and Restrepo (1998) D. Transduction mechanisms in vertebrate olfactory receptor cells. Physiol. Rev. 78:429-466.

72. Schild DF, Lischka W and Restrepo D. (1995). InsP3 causes an increase in apical $[Ca^{2+}]i$ by activating two distinct components in vertebrate olfactory receptor cells. J. Neurophysiol. 89: 862–866.

73. Schild, D. & Restrepo, D. (1998) Transduction mechanisms in vertebrate olfactory receptor cells. Physiol Rev, 78, 429-466.

74. Simoes M, Garneau L, Klein H, Banderali U, Hobeila, F, and Roux B. (2002). Cysteine mutagenesis and computer modelling of the S6 region of an intermediate conductance IK_{Ca} channel. J. Gen Physiol. 120: 99-116.

75. Takeuchi H and Kurahashi T. (2003) Identification of second messenger mediating signal transduction in the olfactory receptor cell. J Gen Physiol. 122:557-567.

76. Takeuchi H and Kurahashi T. (2002). Photolysis of caged cyclic AMP in the ciliary cytoplasm of the newt olfactory receptor cell. J Physiol. 541:825-33.

77. Vergara C, Latorre R, Marrion NV, and Adelman JP. (1998) Calcium-activated potassium channels. Curr. Opin. Neurobiol. 8: 321-329.

78. Vogler C and Schild D. (1999). Inhibitory and excitatory responses of olfactory receptor neurons of Xenopus to stimulation with amino acids. J. Exp. Biol. 202: 997-1003.

79. Wayman, G.A., Impey, S. & Storm, D.R. (1995). Ca^{2+} inhibition of type III adenylyl cyclase in vivo. J Biol Chem, 270, 21480-21486.

80. Weeraratne, S.D., Valentine, M., Cusick, M., Delay, R. & Van Houten, J.L. (2006) Plasma membrane calcium pumps in mouse olfactory sensory neurons. Chem Senses, 31, 725-730.

81. Wong S.T., Trinh K, Hacker B, Chan GC, Lowe G, Gaggar A, Xia Z, Gold G. H. and Storm D. R. (2000). Disruption of the type III adenylyl cyclase gene leads to peripheral and behavioural anosmia in transgenic mice. Neuron 27:487-497.

82. Yan, C., Zhao, A.Z., Bentley, J.K., Loughney, K., Ferguson, K. & Beavo, J.A. (1995) Molecular cloning and characterization of a calmodulin-dependent phosphodiesterase enriched in olfactory sensory neurons. Proc Natl Acad Sci U S A, 92, 9677-9681.

83. Zimmermann, A. & Baylor, D.A. (1986) Cyclic GMP-sensitive conductance of retinal rods consists of aqueous pores. Nature 321, 70-72.

84. Zufall F, Firestein S. (1993) Divalent cations block the cyclic nucleotide-gated channel of olfactory receptor neurons. J Neurophysiol. 69(5): 1758-68.