European Journal of Neuroscience, Vol. 26, pp. 2524–2531, 2007 doi:10.1111/j.1460-9568.2007.05863.x

Plasma membrane Ca^{2+}-ATPase in the cilia of olfactory receptor neurons: possible role in Ca^{2+} clearance

Karen Castillo, Ricardo Delgado and Juan Bacigalupo
Department of Biology, Faculty of Sciences and Millennium Institute for Cell Dynamics and Biotechnology, University of Chile, Santiago, Chile

Keywords: Ca^{2+}-dependent Cl^- current, Na^+/Ca^{2+} exchanger, odour adaptation, olfactory cilia, olfactory transduction

Abstract

Olfactory sensory neurons respond to odorants increasing Ca^{2+} concentrations in their chemosensory cilia. Calcium enters the cilia through cAMP-gated channels, activating Ca^{2+}-dependent chloride or potassium channels. Calcium also has a fundamental role in odour adaptation, regulating cAMP turnover rate and the affinity of the cyclic nucleotide-gated channels for cAMP. It has been shown that a Na^+/Ca^{2+} exchanger (NCX) extrudes Ca^{2+} from the cilia. Here we confirm previous evidence that olfactory cilia also express plasma membrane Ca^{2+}-ATPase (PMCA), and show the first evidence supporting a role in Ca^{2+} removal. Both transporters were detected by immunoblot of purified olfactory cilia membranes. The pump was also revealed by immunocytochemistry and immunohistochemistry. Inside-out cilia membrane vesicles transported Ca^{2+} in an ATP-dependent fashion. PMCA activity was potentiated by luminal Ca^{2+} ($K_{0.5} = 670$ nM) and enhanced by calmodulin (CaM; $K_{0.5} = 31$ nM). Both carboxyeosin (CE) and calmidazolium reduced Ca^{2+} transport, as expected for a CaM-modulated PMCA. The relaxation time constant (τ) of the Ca^{2+}-dependent Cl^- current (272 ± 78 ms), indicative of luminal Ca^{2+} decline, was increased by CE (2181 ± 437 ms), by omitting ATP (666 ± 49 ms) and by raising pH (725 ± 65 ms), suggesting a role of the pump on Ca^{2+} clearance. Replacement of external Na^+ by Li^+ had a similar effect ($\tau = 442 \pm 8$ ms), confirming the NCX involvement in Ca^{2+} extrusion. The evidence suggests that both Ca^{2+} transporters contribute to re-establish resting Ca^{2+} levels in the cilia following olfactory responses.

Introduction

Olfactory transduction begins with odour binding to receptors (ORs) of the cilia of olfactory sensory neurons (OSNs). ORs activate type III adenylyl cyclase (ACIII) through a GTP-binding protein (G_{olf}), raising cAMP levels. Cyclic AMP opens cyclic nucleotide-gated (CNG) channels, allowing Na^+ and Ca^{2+} influx into the cilia, thus depolarizing the cell (Firestein *et al*., 1990; Firestein & Zufall, 1994). Ca^{2+} has a main role in olfactory transduction (Schild & Restrepo, 1998), opening Ca^{2+}-activated Cl^- channels (Cl_{Ca}) that allow Cl^- efflux from the cilia, further depolarizing the cell. This generates an excitatory response, characterized by an increase in action potential frequency (Kleene, 1993; Kurahashi & Yau, 1993; Lowe & Gold, 1993b). Ca^{2+} may also activate Ca^{2+}-dependent K^+ (K_{Ca}) channels in the cilia, which hyperpolarize the cell, generating inhibitory responses consisting of a reduction in discharge rate (Morales *et al*., 1994; Vogler & Schild, 1999; Madrid *et al*., 2005). Calcium also has a fundamental role in odour adaptation (Chen & Yau, 1994; Liu *et al*., 1994; Kurahashi & Menini, 1997), by reducing CNG channel affinity for cAMP and by decreasing cAMP levels through phosphodiesterase activation (Borisy *et al*., 1992; Yan *et al*., 1995) and ACIII inhibition (Wayman *et al*., 1995; Leinders-Zufall *et al*., 1999). The multiple regulatory actions of Ca^{2+} in olfactory transduction indicate that its concentration in the cilia is critical and the Ca^{2+} availability in the cilia needs to be finely controlled, implying that Ca^{2+} gained as a result of the olfactory response must be efficiently removed. It is noteworthy that chemosensory cilia are devoid of internal stores that could capture this calcium.

Correspondence: Dr J. Bacigalupo, as above.
E-mail: bacigalu@uchile.cl

Received 13 June 2007, revised 8 August 2007, accepted 3 September 2007

Fluorescence measurements of ciliary led to the conclusion that ciliary Ca^{2+} may reach nearly 1 μM during an olfactory response (Leinders-Zufall *et al*., 1998). However, single K_{Ca} recordings in membrane patches excised from olfactory cilia showed that in the vicinity of the plasma membrane Ca^{2+} may reach much higher values, close to 100 μM (Delgado & Bacigalupo, 2004). Functional evidence indicates that a Na^+/Ca^{2+} exchanger (NCX) localizes to the cilia, extruding Ca^{2+} after an odour stimulus (Reisert & Matthews, 1998; Danaceau & Lucero, 2000; Lucero *et al*., 2000).

The need for Ca^{2+} removal and the unfavourable voltage dependence of NCX suggest that an additional transport mechanism might be operating in the cilia, most likely a Ca^{2-}-ATPase (PMCA). It has been suggested that a Ca^{2+}-ATPase may be important in maintaining resting Ca^{2+} levels in olfactory sensory neurons (OSNs; Lischka & Schild, 1993). The presence of PMCA in the cilia and cell body of ORNs was recently documented by immunocytochemistry (Weeraratne *et al*., 2006). Here we show the olfactory cilia express PMCA with properties that are highly consistent with a role in ciliary Ca^{2+} clearance. It has been reported that PMCA exhibits a low transport activity at resting Ca^{2+} concentration, but when Ca^{2+} concentration rises, it is vigorously enhanced (Carafoli & Brini, 2000). Our evidence indicates that both NCX and PMCA may contribute to remove Ca^{2+} gained by the cilia during the olfactory response.

Materials and methods

OSNs were obtained from rat (Sprague–Dawley) and toad (*Caudiverbera caudiverbera*). Rat OSNs were used for transport and immunohistochemical experiments, whereas toad OSNs were utilized

for immunocytochemistry and electrical recordings on isolated cells. Animals were handled and killed according to the guidelines of the Animal Ethics Committee of the University of Chile, in full agreement with the NIH guidelines for experimental procedures with animals. Rats were killed by decapitation after being deeply anaesthetized with sodium pentobarbital (100 mg/kg). Toads were anaesthesized by cooling in ice, killed and pithed.

Purified ciliary membrane fraction from rat olfactory epithelium

Ciliary membranes were detached from the epithelium by mechanical agitation (Washburn *et al*., 2002), followed by a purification step in a sucrose gradient (Castillo *et al*., 2005). The purity of the ciliary membrane preparation was previously established by testing for the absence of voltage-dependent Na^+ channels, which are confined to the non-ciliary plasma membrane fraction of OSNs (Madrid *et al*., 2005), whereas the enrichment of the preparation in cilia olfactory membrane was tested by expression of CNG, ACIII and G_{olf}. The sodium channel was absent in the ciliary fraction, but was detected in the whole epithelium and brain membrane fractions, used as positive controls. In addition, single-channel recordings in planar lipid bilayers doped with this preparation showed the presence of Ca^{2+}-dependent K^+ channels but not of voltage-gated K^+ channels (Castillo *et al*., 2005), which have been shown to be confined to the cell body and dendrite of OSNs (Lowe & Gold, 1993a), further supporting the purity of the ciliary membrane fraction.

The deciliated epithelium membrane fraction was obtained as previously described (Washburn *et al*., 2002). Brain, olfactory bulb and whole epithelium were obtained by homogenizing the respective tissues in sucrose buffer (0.2 mM EDTA, 10 mM MOPS, 0.3 M sucrose, 0.02% NaN_3, pH 7.4). The suspensions were centrifuged at 15 000 ***g*** for 10 min. The recovered supernatant was centrifuged for 1 h at 100 000 ***g*** and the pellet obtained was resuspended in equilibrium buffer. The brain soluble fraction was generated after centrifuging the supernatant brain tissue at 100 000 ***g*** for 1 h. All steps of these procedures were carried out at 4 °C and all solutions were supplemented with a protease inhibitor cocktail (Sigma-Aldrich). Membrane fractions were frozen in liquid nitrogen and stored at −80 °C until use. Total proteins were determined by the Hartree method (Hartree, 1972).

Western blots analysis

Proteins were separated in 9% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) for PMCA and NCX detection, and 15% SDS–PAGE gel for calmodulin (CaM). Anti-PMCA (PAN) and anti-CaM antibodies were purchased from Sigma-Aldrich, anti-NCX (PAN) from Research Diagnostics (Concord, MA, USA). Both PAN antibodies recognize all PMCA and NCX isoforms, respectively. Seventy micrograms total proteins was loaded per track. The primary antibodies were used at 1 : 600 dilutions. Membranes were washed three times with phosphate-buffered saline (PBS)-T, 10 min each, and incubated for 1 h at room temperature with the appropriate secondary antibodies coupled to peroxidase: anti-rabbit for NCX; anti-mouse for PMCA and CaM (Sigma-Aldrich). Membranes were developed with an ECL chemoluminescence assay (Amersham Biosciences. Fairfield, CT, USA).

Immunocytochemistry

To detect the expression of PMCA in olfactory cilia, small pieces of olfactory epithelia were dissociated by gently passing them several times through a fired-polished Pasteur pipette, allowing them to settle on pegotin (BiosChile, Nunoa Santiago, Chile)-coated slides and fixing in 4% paraformaldehyde (PFA). The cells were incubated overnight at 4 °C with anti-PMCA antibody (1 : 100), washed three times with PBS and then incubated with fluorescent anti-mouse Alexa 468 secondary antibody (1 : 300; Molecular Probes). Images were obtained by means of a Carl Zeiss OX10 VERT 200M epifluorescence inverted microscope, with a 63 × objective (Plan apochromatic, 1.4 NA, oil immersion) and equipped with an AX10 CAM HRM CCD camera.

Immunohistochemistry

To reveal the presence of PMCA in the ciliary layer of olfactory epithelia, rat olfactory epithelia were extracted from the septum of the nasal cavity of killed animals, fixed in 4% PFA in PBS, and kept in 30% sucrose overnight. Treated tissue was frozen in tissue-freezing medium (Electron Microscopy Sciences, Hatfield, PA, USA) and cut into ∼10 μm-thick pieces in a Cryo Cut II cryostat (American Optics, Buffalo, NY, USA). Sections were mounted on pegotin-coated glass slides. Primary antibody (1 : 100) was added to the slides containing the sections and left overnight at room temperature in a humidified chamber. Sections were washed with PBS, the secondary antibody (1 : 2000) added, and the sample incubated overnight at room temperature, washed and then incubated for 4 h with ABC kit solution (Vecstatin kit, Vector Laboratories, Burlingame, CA, USA). Tissue sections were treated with diaminobenzidine, dehydrated and mounted with Entellan (Merck), and viewed with the microscope using a 40 × objective (LD acroplan, 0.6 NA, PH2, KORR).

Inversion of ciliary membrane vesicles

To measure Ca^{2+} accumulated by the ciliary vesicles, they had to be inverted in order to expose their luminal membrane leaflet, where the catalytic and regulatory sites of the pump are located. Ciliary membrane vesicles obtained with the described procedure were mainly right-side out, as revealed by their low Ca^{2+} transport activity, about ∼10% of that of inverted vesicles. Vesicle inversion was accomplished by the method of Fraser *et al*. (1985), which involves the osmotic rupture of the vesicles. Two-hundred microlitres of vesicle suspension were added to an Eppendorf vial containing inversion solution (50 mM Tris-acetate, 10% glycerol and 250 mM sucrose), to a final volume of 2 mL. The inversion was performed in the presence of 2 mM ouabain (Sigma-Aldrich), to prevent possible ATP hydrolysis by the Na^+/K^+-ATPase during the transport experiments. For the Ca^{2+} transport experiments using a fluorescent probe, the vesicle inversion was performed in the presence of 0.5 μM of the cell-impermeant Ca^{2+} fluorophore Fluo 5N (Molecular Probes), in order to load the vesicles with this probe. The suspension was diluted 10 times in equilibrium buffer (in mM: KCl, 140; $MgCl_2$, 1; glucose, 10; EGTA, 1; HEPES/Tris, 5; pH 7.2). This suspension was centrifuged twice at 100 000 ***g*** for 1 h. The pellet containing resealed vesicles was resuspended and homogenized in 200 μL of equilibrium buffer, and its total protein content determined.

It is likely that a fraction of the vesicles did not revert. Nonetheless, the presence of right-side-orientated vesicles should not interfere with our measurements.

Calcium transport experiments

In order to test the functional properties of the ciliary PMCA, we carried out Ca^{2+} transport experiments by two separate approaches: One involving $^{45}Ca^{2+}$ uptake and the other, Ca^{2+} fluorescence.

PMCA activity was measured as the uptake of $^{45}Ca^{2+}$ by the inverted vesicles at different Ca^{2+} concentrations. The vesicles were added to a final concentration of 0.5 μg/μL to 200 μL of reaction buffer (in mM: sucrose, 500; HEPES-BTP, 2.5; KCl, 20; EGTA, 0.5; FCCP, 1 nM; pH 7.0; Ca^{2+} was added as $CaCl_2$ to the desired free concentration). The reaction was started by adding 2 mM ATP-Mg^{2+}. The specificity of pump activity was determined measuring Ca^{2+} uptake in the absence of ATP. Carboxyeosin diacetate (CE; Molecular Probes), a PMCA inhibitor (Gatto & Milanick, 1993), calmidazolium (CMZ; Sigma-Aldrich), a CaM inhibitor (Fischer *et al*., 1987), and the Ca^{2+} ionophore A23187 (Martina *et al*., 1994; Sigma-Aldrich) were added, when appropriate. At 90 s the reaction was filtered (Millipore filters, 0.45 μm. Bellerica, MA, USA) and immediately stopped with 4 mL stop solution (in mM: sucrose, 250; HEPES-BTP, 2.5; $CaCl_2$, 0.2; pH 7.0). Filters were let dry, put into 1.5-mL Eppendorf vials with scintillation liquid and read in a liquid scintillation analyser (TRI-CARB 2100 TR, Packard Instruments, Downers Grove, IL, USA).

CaM modulation was determined as increases in Fluo 5N fluorescence, as an indication of Ca^{2+} accumulation by the ciliary vesicles. Inverted vesicles were added to equilibrium buffer in ELISA plates at 1 μg/μL final concentration. Wells contained different Ca^{2+} concentrations and, when appropriate, CaM, CE or CMZ. The experiments were started by adding 2 mM ATP and measurements were carried out at 3 min, in the equilibrium phase of transport. Unspecific activity was determined in 5 μM Ca^{2+}, in the absence of ATP.

The probe was calibrated for Ca^{2+} concentration between 100 nM and 200 μM. The minimal fluorescence level (F_{min}) was determined in chelating solution with 1 mM EGTA, and maximal fluorescence (F_{max}) by permeabilizing the vesicles with the Ca^{2+} ionophore A23187 in equilibrium buffer.

Experiments were carried out at room temperature (20 ± 2 °C) in Na^+-free solutions, to avoid possible NCX contribution to Ca^{2+} transport. Free Ca^{2+} concentrations at given total Ca^{2+}, Mg^{2+}, ATP and EGTA concentrations were calculated with the WinMaxc v2.05 program (C. Patton, Stanford University).

Electrical recordings

To determine the participation of PMCA in Ca^{2+} removal from the cilia, we measured the transduction Ca^{2+}-dependent Cl^- current relaxation time constant (τ) after photoreleasing cAMP from 4,5-dimethoxy-2-nitrobenzyl adenosine 3',5' cyclic monophosphate-caged cAMP (Molecular Probes) by a UV light flash (∼15 kW, 1 ms), generated by a Xenon flash-lamp system (JML-C2, Rapp OptoElectronic. Gehlenkamp, PA, Germany). The whole-cell current was measured by means of a patch-clamp amplifier (Axopatch 1D, Axon Instruments) from isolated OSNs. Cells were dissociated with fine tweezers from small olfactory epithelia pieces of the Chilean toad *Caudiverbera caudiverbera*, obtained as described above, and resuspended in Ringer's solution (in mM: NaCl, 115; KCl, 2.5; $CaCl_2$, 1; $MgCl_2$, 1.5; HEPES, 10; sucrose, 10; pH 7.6). Cells were added to glass coverslips and allowed to settle. OSNs were viewed with an inverted microscope (Olympus IX70, Center Valley, PH, USA) equipped with a 40X phase contrast objective (Plan N, 0.65 NA). Only cells with visible cilia were used. The transduction cascade was triggered at a late stage, by photoreleasing cAMP. The current evoked by cAMP largely corresponded Ca^{2+}-dependent Cl^- current through the ciliary membrane, where the CNG channels also reside (Lowe & Gold, 1993a). Caged cAMP was added to the pipette solution (100 μM) and allowed to dialyse into the OSN for at least 5 min previous to starting the measurements. The role of PMCA on ciliary Ca^{2+} clearance was studied by recording whole-cell currents in OSNs in three different conditions: dialysed with internal solution containing CE; with ATP-free internal solution; and by raising extracellular pH to 9.4. NCX was abolished by Li^+, which replaced Na^+ equimolarly in the Ringer solution. Intracellular solution (in mM): KCl, 120; NaCl, 5; $CaCl_2$, 1; $MgCl_2$, 1; EGTA, 2; HEPES, 4; Mg-ATP, 2; pH 7.6. Patch pipettes were made of borosilicate glass capillaries, drawn in a P97 Sutter Instruments puller (Novato, CA, USA). Seal resistances were typically 3–5 GΩ. Their tip resistances were 2–4 MΩ.

Results

Expression of PMCA and NCX in olfactory cilia

To investigate whether PMCA was present in the olfactory cilia, we conducted immunochemical determinations: Immunoblot, immunocytochemistry and immunohistochemistry. Western blots of olfactory cilia membranes, deciliated epithelium, olfactory bulb, whole olfactory epithelium and brain membrane preparations were treated with an anti-PMCA antibody (Fig. 1A). The gels revealed that olfactory cilia contain a plasma membrane Ca^{2+} pump. Control without the primary antibody did not show any band. Additionally, NCX was observed in cilia, deciliated epithelium and brain membranes (Fig. 1B). In contrast, endoplasmic reticulum membranes, used as negative control, were not labelled by the anti-NCX antibody. Estimated molecular weights are ∼130 kDa for PMCA and ∼135 kDa for NCX.

The expression of PMCA in chemosensory cilia was confirmed by immunocytochemistry (Fig. 1C). The cilia of dissociated toad olfactory neurons displayed intense fluorescence, revealing the presence of the pump in these organelles. OSNs also exhibited fluorescence in their soma and dendrite. Control without primary antibody did not show any fluorescence (not shown). Additionally, we performed immunohistochemistry on cryosections of rat olfactory epithelium, confirming the presence of this calcium transport protein in the ciliary layer. In these sections, the labelling of the ciliary layer with the anti-PMCA antibody was evident. The control without primary antibody was much less reactive (Fig. 1D).

This evidence indicates that both Ca^{2+} transport proteins, PMCA and NCX, are found in the olfactory cilia, suggesting their involvement in Ca^{2+} homeostasis in the chemosensory organelles of OSNs.

Calcium transport in inverted rat olfactory cilia vesicles

In order to establish whether the olfactory cilia possess an ATP-driven Ca^{2+} pump and display Ca^{2+}-dependent transport activity, we conducted $^{45}Ca^{2+}$ uptake experiments on inverted ciliary membrane vesicles. Ca^{2+} dependence was determined at the initial velocity phase of the activity vs time curves (90 s), at different Ca^{2+}concentrations. The Ca^{2+} uptake vs pCa relation gave a $K_{0.5}$ of 670 nM (Fig. 2A). Ca^{2+} uptake was drastically reduced in the absence of ATP, as well as in the presence of CE or CMZ. When the Ca^{2+} ionophore A23187 was added to vesicles loaded with Ca^{2+}, their calcium content dropped substantially (Fig. 2B). The Ca^{2+} levels retained by the vesicles after the ionophore treatment may be due to binding of the cation to proteins attached to the membranes (Ordenes *et al*., 2002). Calcium fluorescence determinations indicated that maximum pump activity occurs at ∼5 μM Ca^{2+}, and confirmed that the transport rate decreases in the absence of Ca^{2+} and ATP, and on the addition of CE or CMZ to the medium (not shown).

The results indicated that olfactory cilia possess a CE-sensitive, ATP-dependent Ca^{2+} transport, strongly suggesting the participation of

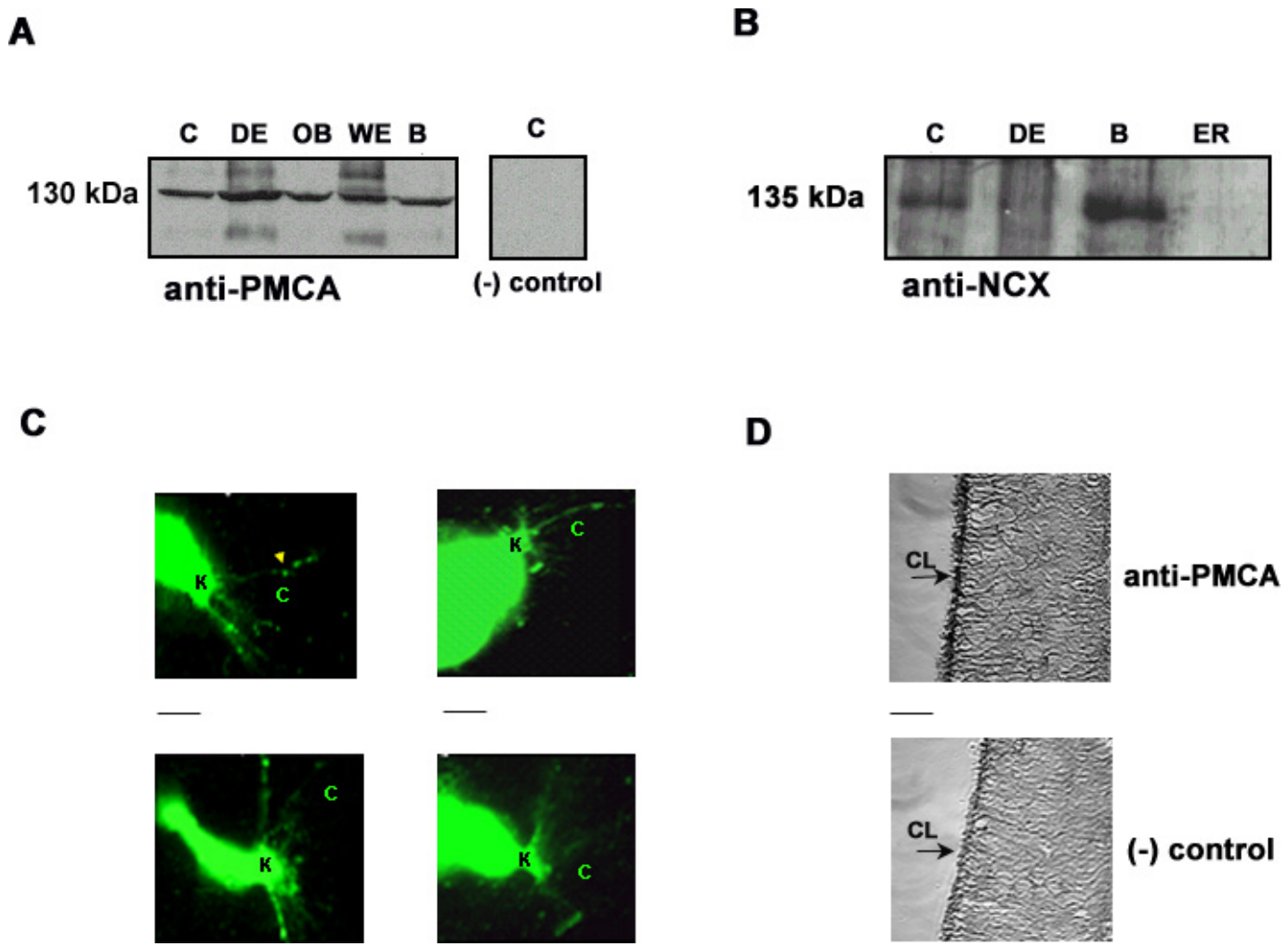

FIG. 1. Olfactory cilia express a plasma membrane calcium ATPase (PMCA). (A) Western blot of rat olfactory ciliary membranes (C), showing PMCA localization to these organelles. B, brain membranes; DE, deciliated epithelia; OB, olfactory bulb; WE, whole olfactory epithelium. Western blot without primary antibody was used as control. (B) Sodium/calcium exchanger (NCX) detected in olfactory cilia, deciliated epithelia and brain, but absent from endoplasmic reticulum (ER) membranes (negative control). (C) Immunocytochemistry of dissociated toad OSNs showing PMCA in olfactory cilia; K, dendritic knob. Arrowhead indicates an example of the bead-like distribution of PMCA in the cilia. (D) Immunohistochemistry of rat olfactory epithelium showing PMCA expression in the ciliary layer (CL). In the absence of the primary antibody, no band is distinguished. Scale bars: 20 μm.

a PMCA on the removal of Ca^{2+} from olfactory cilia. The evidence also shows that Ca^{2+} transport is enhanced by luminal Ca^{2+}, and it appears to be modulated by endogenous CaM that stayed bound to the ciliary membranes, as the Ca^{2+} load was lower in the presence of CMZ.

CaM modulation of ciliary PMCA

In order to confirm that CaM was associated with the ciliary membranes, we carried out Western blots on purified cilium membranes with anti-CaM antibody. CaM was detected in the ciliary membrane fraction, indicating that the protein stays bound to them through the procedure to obtain the inverted ciliary vesicles. Control without the primary antibody did not display mark for CaM (Fig. 3A).

We also tested the effect of CaM on Ca^{2+} transport. To this purpose, we measured Ca^{2+} accumulation by the inverted ciliary vesicles as a function of CaM concentration under 1 μM free Ca^{2+}, using vesicles preloaded with Fluo 5N (Fig. 3B). The maximal activity of the pump, measured by Ca^{2+} fluorescence and $^{45}Ca^{2+}$ uptake, are in the same range of Ca^{2+} concentration (1–10 μM; not shown). There was a basal activity of the pump without added CaM (35 μmol/min/mg protein), consistent with the notion that the vesicles retained bound CaM through the preparation procedure. Nevertheless, adding CaM enhanced Ca^{2+} transport by the vesicles ($K_{0.5}$ = 31 nM), indicating that ciliary PMCA is modulated by Ca^{2+}/CaM.

Altogether, these results indicate that the olfactory cilia express PMCA and that this pump is potentiated by Ca^{2+} in a Ca^{2+}/CaM-dependent manner.

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

The participation of PMCA in Ca^{2+} removal was explored by measuring the relaxation time constant of the current generated upon uncaging cAMP in dissociated OSNs, as the membrane potential was held at −70 mV. Because the transduction Cl^- current directly depends on luminal Ca^{2+}, it closely reflects the dynamics of free Ca^{2+} concentration within the cilia, in the vicinity of the membrane.

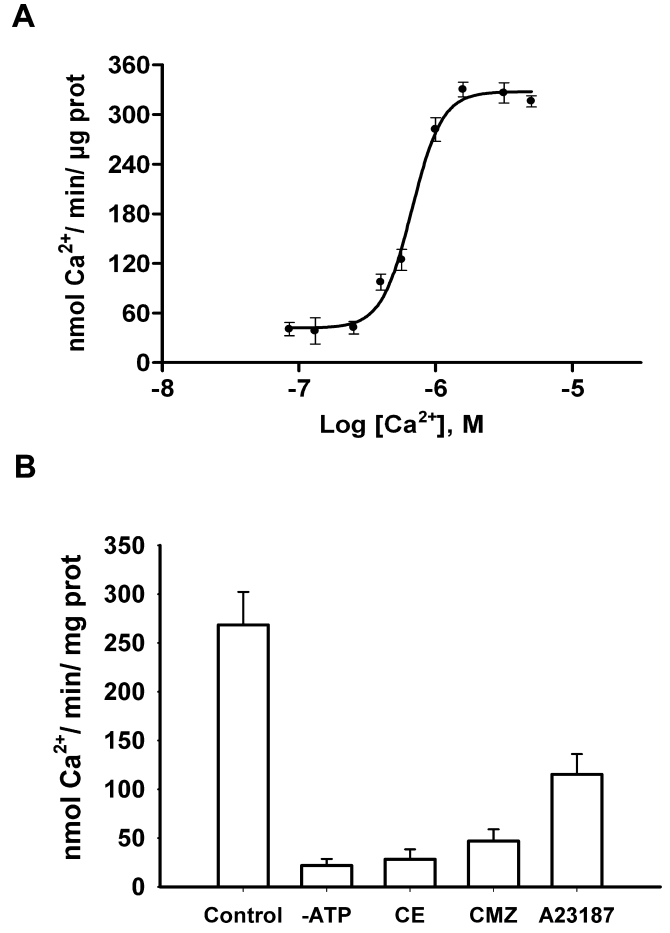

FIG. 2. Ca^{2+}-dependence of PMCA Ca^{2+} transport. Ca^{2+}-dependent Ca^{2+} uptake was measured with $^{45}Ca^{2+}$. (A) The transport assay was run at different Ca^{2+} concentrations. It was started by adding ATP and stopped at 90 s with Stop Solution (see Materials and methods). The experimental points were fitted by a sigmoidal dose–response (variable slope) Hill function; $K_{0.5} = 670$ nM. Measurements done at 45 s gave virtually identical results (not shown). (B) Ca^{2+} transport activity measured under 1 µM Ca^{2+} and 2 mM ATP (control), in the absence of ATP, in the presence of 50 µM carboxyeosin (CE), 10 µM calmidazolium (CMZ), and after adding 10 µM of Ca^{2+} ionophore A23178 to inverted cilia vesicles pretreated identically as the control. Each experimental point corresponds to the average value of three independent experiments, every one of them in triplicate. Values are mean ± SEM.

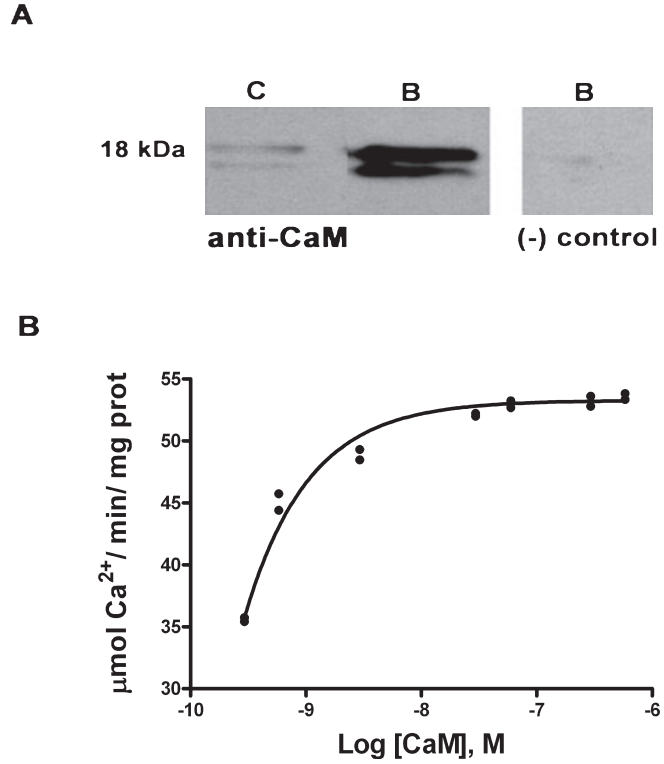

FIG. 3. Calmodulin (CaM)-modulation of PMCA Ca^{2+} transport. (A) Western blot showing the presence of CaM in the ciliary membrane fraction. A soluble brain fraction was used as positive control. Two bands are labelled; the one migrating faster likely corresponds to degradation products of CaM (Sigma data sheet). (B) Ca^{2+} transport vs [CaM] relation exhibited a $K_{0.5}$ of 31 nM; experimental points were fitted to a sigmoidal dose–response (variable slope) Hill function, $n = 2$. Experiments were carried out in 2 mM ATP, 1 µM free Ca^{2+}, Na^{+}-free solutions.

Therefore the current represents an adequate monitor of the ciliary Ca^{2+} transporters activities. The current declined with a $\tau = 272 \pm 78$ ms under control conditions, but the recovery was significantly slowed down by 100 µM CE ($\tau = 2181 \pm 437$ ms), consistent with the participation of PMCA in the extrusion of Ca^{2+} from the cilia. Omitting ATP from the internal solution also increased the relaxation time constant ($\tau = 666 \pm 49$ ms). Extracellular alkalinization (pH 9.4) had a similar effect ($\tau = 725 \pm 65$ ms; Fig. 4A and B). These results, obtained with three different strategies to abolish the pump, support the notion that an ATP-dependent mechanism for Ca^{2+} extrusion, namely a 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 also incremented ($\tau = 442 \pm 8$ ms; Fig. 4A and B), 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 result of the activation of the olfactory transduction cascade.

Discussion

In this work we show that PMCA is expressed in the chemosensory cilia of ORNs, and provide functional evidence that this pump is likely to have a significant role in terminating the olfactory response by extruding Ca^{2+} from these chemosensory organelles, in addition to NCX.

Electrophysiological studies have documented that frog and rat ORNs possess a NCX that extrudes Ca^{2+} from the cilia after odour stimuli (Reisert & Matthews, 1998). Also, NCX mRNA was detected both in rat and mice olfactory epithelia by reverse transcription-polymerase chain reaction and *in situ* hybridization, and different NCX isoforms expression in OSNs have been recently reported (Schulze *et al.*, 2002; Pyrski *et al.*, 2007). However, such findings are not indicative of the expression and functionality of NCX in the cilia. Here we confirmed previous evidence for the presence of NCX in olfactory cilia by means of immunochemistry and electrophysiological recordings.

Measurements of intraluminal Ca^{2+} in the cilia have been difficult to obtain due to their minute dimensions: ~0.2 µm diameter and 5–250 µm long, depending on species. Nonetheless, odour-induced Ca^{2+} increases have been recorded in amphibian olfactory cilia using fluorescence Ca^{2+} indicators (Leinders-Zufall *et al.*, 1998; Reisert & Matthews, 2001). Reisert and Matthews attributed Ca^{2+} removal to NCX, and provided strong evidence supporting NCX involvement in Ca^{2+} efflux from the OSN. Because NCX is driven by the Na^{+} gradient and is electrogenic (exchanging 3 Na^{+} : 1 Ca^{2+}), we

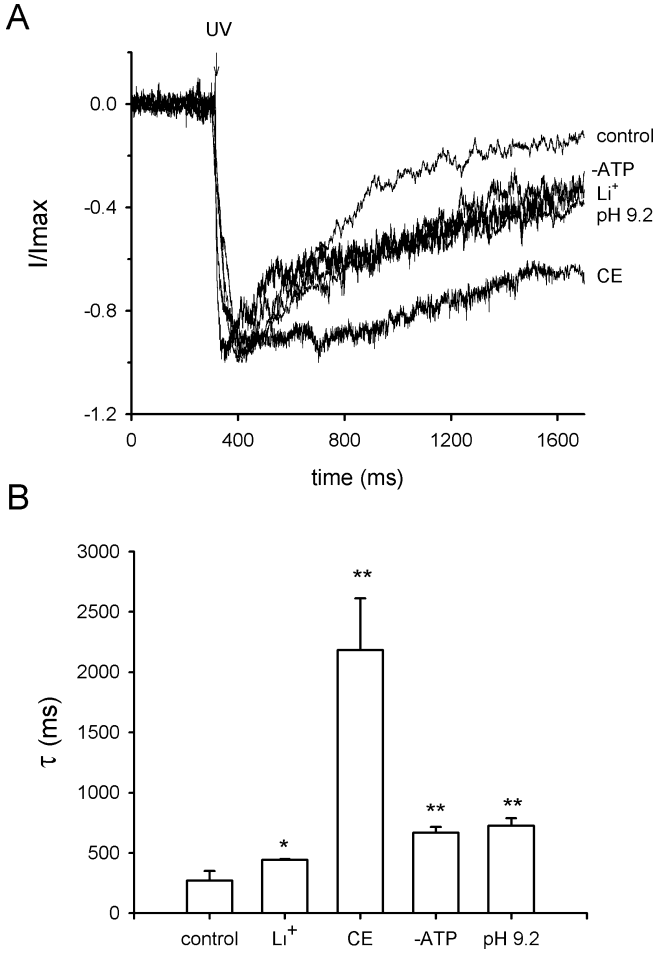

FIG. 4. Role of PMCA in Ca^{2+} clearance in olfactory cilia. (A) Normalized representative whole-cell currents recorded from OSNs after uncaging cAMP with a UV flash (arrow) are shown superposed. The time constants of the currents relaxations were determined in control conditions ($\tau = 272 \pm 78$ ms, $n = 4$), when NCX was abolished by replacing external Na^+ for Li^+ ($\tau = 442 \pm 8$ ms, $n = 3$), blocking PMCA with 50 μM carboxyeosin (CE; $\tau = 2181 \pm 437$ ms, $n = 5$), omitting ATP ($\tau = 666 \pm 49$ ms, $n = 5$) and exposition to high (9.4) extracellular pH ($\tau = 725 \pm 65$ ms, $n = 3$). $V_h = -70$ mV. (B) Histogram of the τ-values obtained in these experiments. (Student's *t*-test, $*P < 0.01$, $**P < 0.005$).

reasoned that the efficiency of NCX might drop as the cell depolarizes during an odour response. Removal of Ca^{2+} from the cilia being absolutely critical for recovering the resting condition, it seemed reasonable to think that the cell might possess a supplementary Ca^{2+} transport system. Added to this is the absence of membrane compartments that could sequester Ca^{2+}, plus the unlikely possibility that Ca^{2+} diffusion to the dendrite may contribute effectively to re-establish ciliary Ca^{2+} levels.

The immunofluorescence images of PMCA suggest that the pump is not homogenously distributed in the cilia, as it is possible to discern discrete spots of fluorescence along them (Fig. 1C), in agreement with Weeraratne *et al*. (2006) and similar to the bead-like distribution of Ca^{2+} fluorescence previously seen in olfactory cilia (Leinders-Zufall *et al*., 1998). This observation may be reflecting a discrete distribution of the constituents of the transduction machinery, as in other systems where the transduction constituents are assembled into macromolecular complexes that permit and ensure high sensitivity, fast activation and deactivation kinetics, and fine feedback regulation mediated by intracellular changes in Ca^{2+} concentration. In addition, such complexes may ensure the specificity of signalling processing, as has been previously proposed (Tsunoda & Zuker, 1999; Zylinska & Soszynski, 2000; Van den Bergh *et al*., 2006).

The ciliary PMCA exhibited a Ca^{2+}-dependent activity, sharply rising by about 5-fold, with $K_{0.5} = 670$ nM. Such Ca^{2+} concentrations are within the physiological range attained in the cilia, supporting the notion that the pump has a relevant participation in the extrusion of Ca^{2+}, as this cation approaches micromolar levels during an odour response. The Ca^{2+} affinity of PMCA is thought to be ≤ 0.5 μM when complexed to CaM, whereas that of NCX is 10-fold lower; however, NCX Ca^{2+} transfer rate is 10-fold higher than the pump (Blaustein & Lederer, 1999). Other properties involved on the function of both transport proteins in the cilia are the voltage dependence of the exchanger, which makes its efficiency decline with depolarization, and the strengthening of the Ca^{2+} pump as intracellular Ca^{2+} increases. An additional factor that may influence the operation of the pump is its stoichiometry, which is still unclear. The pump appears to exchange 1 Ca^{2+} for 1 or 2 H^+, being either electrogenic or electroneutral, respectively. Interestingly, if it were electrogenic, a depolarization would favour the exchange, opposite to NCX. Therefore, the pump bears properties remarkably suitable to satisfy the demand for Ca^{2+} clearance imposed by odour responses.

CaM potentiation of the pump suggests that it may have the effect of shortening the duration of Ca^{2+} transients induced by odorants in olfactory cilia. CaM is involved in the regulation of several transduction proteins in the cilia, being fundamental for the orchestration of their Ca^{2+}-dependent activities during odour responses. CaM has been previously demonstrated to be permanently bound to the olfactory CNG channel (Bradley *et al*., 2004), a feature that is essential to maximize the negative feedback effect of Ca^{2+} over this channel, the major factor responsible for odour adaptation. In the case of the PMCA, its activity would be potentiated by Ca^{2+}/CaM, allowing a fast and efficient control of sudden Ca^{2+} changes within the cilia occurring during odour stimulation.

Resting Ca^{2+} concentration in salamander olfactory cilia was estimated to be ~40 nM, based on fluorescence measurements of the bulk Ca^{2+} levels within the cilia (Leinders-Zufall *et al*., 1997). It is likely that locally at the vicinity of the CNG channels, the Ca^{2+} concentrations during odour responses may be much higher than in the bulk of the ciliary lumen. The tested Ca^{2+} concentration at which PMCA activity was highest (1–10 μM, as determined by fluorescence measurements and experiments with isotopes) is compatible with that presumably attained in the olfactory cilia during the responses to physiological odour levels. In excised frog olfactory cilia the Cl^- transduction current begins to rise as Ca^{2+} reaches ~1 μM, with a $K_{0.5}$ of 5 μM (Kleene & Gesteland, 1991). This value of Ca^{2+} is in agreement with estimates in the salamander (Leinders-Zufall *et al*., 1998), where $K_{0.5}$ is ~1 μM. Single Ca^{2+}-dependent Cl^- channel recordings from membrane patches excised from toad olfactory cilia showed that the channel open probability reached a maximal value at ~1 μM Ca^{2+}, with $K_{0.5} = \sim 0.4$ μM (Delgado and Bacigalupo, unpublished results). Estimates of ciliary Ca^{2+} concentrations based on unitary ciliary Ca^{2+}-dependent K^+ channel recordings indicated a $K_{0.5}$ of ~20–60 μM, depending on the K_{Ca} channel type (Delgado *et al*., 2003; Castillo *et al*., 2005). Thus, calcium concentrations within the cilia during odour exposures likely reach levels in the range in which the pump is fully active.

The current induced by the photorelease of cAMP is carried principally by the Cl^- ions exiting the cilia through the Cl_{Ca} transduction channels, as the cationic current through the CNG channels is only a minor fraction of the depolarizing transduction

current (Kurahashi & Yau, 1993; Lowe & Gold, 1993a), and the K_{Ca} current is negligible at the holding potential used (−70 mV). Therefore, the shape of the transduction current can be considered as indicative of the time course of the Ca^{2+} concentration profile within the chemosensory organelles. The transduction current declined with a time constant of 272 ms under control conditions. The slower decline of the current after replacing external Na^+ by Li^+ ($\tau = 442$ ms) confirmed the participation of NCX in Ca^{2+} removal (Reisert & Matthews, 1998, 2001). The increase in the transduction current relaxation time constant caused by the PMCA blocker CE ($\tau = 2180$ ms) suggests that the pump also participates in this process. The similar effect of the lack of ATP in the internal solution ($\tau = 666$ ms) supports the participation of PMCA in Ca^{2+} clearance. Additional evidence is provided by the effect of high pH on the relaxation time constant of the current ($\tau = 725$ ms). The rise time of the current evoked by the photorelease of cAMP was virtually identical, indicating that neither of the treatments affected the gating kinetics of the CNG channels by the photoreleased cAMP nor that of the subsequent Cl^- conductance activation. Rather, it was the time course of OSN recovery of resting conditions that was affected by inhibiting the cilia Ca^{2+} transporters.

Recently Weeraratne *et al*. (2006) reported the presence of the four PMCA isoforms in mouse OSNs, three of which (1, 2 and 4) would be present in the cilia. Nevertheless, our aim was to examine whether ciliary PMCA exhibited properties compatible with a role in re-establishing resting Ca^{2+} levels and if its activity could be detected in isolated OSNs. The Ca^{2+}-dependent activity of ciliary PMCA that we measured is similar to that of PMCA isoforms 1 and 4, both of which had maximal activities at ∼10 μM Ca^{2+} in the presence of CaM and CaM dependence with $K_{0.5}$ of 28 and 35 nM, respectively (Guerini *et al*., 2003). The different PMCA isoforms have similar maximum transport rates for Ca^{2+}, but isoforms 2 and 3 exhibit a maximum activity at ∼1 μM Ca^{2+} (Caride *et al*., 2001). Isoform 2 has ∼100 times higher sensitivity to CaM ($K_{0.5} = 0.2$ nM) than the other isoforms, and isoform 3 is weakly stimulated by CaM and appears to be constitutively active (Filoteo *et al*., 2000). Thus, the PMCA studied here does not seem to correspond to isoforms 2 or 3; rather, both its CaM and Ca^{2+} dependences match the properties of PMCA isoforms 1 and 4.

Our evidence demonstrates that both PMCA and NCX are expressed in the olfactory cilia, and that PMCA is likely to have a relevant participation in the extrusion of Ca^{2+} occurring as a consequence of odour stimulation.

Acknowledgements

We are indebted to Carolina González, Susana Cabello, Claudio Elgueta and Romina Vargas for help in the preparation of membrane fractions. We thank Juan Reyes and Oliver Schmachtenberg for critical reading of an early version of the manuscript. This work was supported by FONDECYT 1050124, Proyecto Anillo en Ciencia y Tecnología ACT-45, Programa Bicentenario CONICYT; MIDEPLAN ICM-P05-001-F (J.B.) and a CONICYT doctoral fellowship (K.C.). J.B. holds a John S. Guggenheim Memorial Foundation Fellowship.

Abbreviations

ACIII, type III adenylyl cyclase; CaM, calmodulin; CE, carboxyeosin; CMZ, calmidazolium; CNG channel, cyclic nucleotide-gated channel; NCX, sodium/calcium exchanger; OR, olfactory receptor; OSN, olfactory sensory neuron; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PMCA, plasma membrane calcium ATPase; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

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