

# Calcium-activated chloride channels in the apical region of mouse vomeronasal sensory neurons

Michele Dibattista,<sup>1</sup> Asma Amjad,<sup>1</sup> Devendra Kumar Maurya,<sup>1</sup> Claudia Sagheddu,<sup>1</sup> Giorgia Montani,<sup>2</sup> Roberto Tirindelli,<sup>2</sup> and Anna Menini<sup>1</sup>

<sup>1</sup>Neurobiology Sector and Italian Institute of Technology Unit, Scuola Internazionale Superiore di Studi Avanzati (SISSA), 34136 Trieste, Italy

<sup>2</sup>Department of Neuroscience, University of Parma, and Brain Center for Social and Motor Cognition, Italian Institute of Technology, 43121 Parma, Italy

The rodent vomeronasal organ plays a crucial role in several social behaviors. Detection of pheromones or other emitted signaling molecules occurs in the dendritic microvilli of vomeronasal sensory neurons, where the binding of molecules to vomeronasal receptors leads to the influx of sodium and calcium ions mainly through the transient receptor potential canonical 2 (TRPC2) channel. To investigate the physiological role played by the increase in intracellular calcium concentration in the apical region of these neurons, we produced localized, rapid, and reproducible increases in calcium concentration with flash photolysis of caged calcium and measured calcium-activated currents with the whole cell voltage-clamp technique. On average, a large inward calcium-activated current of  $-261$  pA was measured at  $-50$  mV, rising with a time constant of 13 ms. Ion substitution experiments showed that this current is anion selective. Moreover, the chloride channel blockers niflumic acid and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid partially inhibited the calcium-activated current. These results directly demonstrate that a large chloride current can be activated by calcium in the apical region of mouse vomeronasal sensory neurons. Furthermore, we showed by immunohistochemistry that the calcium-activated chloride channels TMEM16A/anoctamin1 and TMEM16B/anoctamin2 are present in the apical layer of the vomeronasal epithelium, where they largely colocalize with the TRPC2 transduction channel. Immunocytochemistry on isolated vomeronasal sensory neurons showed that TMEM16A and TMEM16B coexpress in the neuronal microvilli. Therefore, we conclude that microvilli of mouse vomeronasal sensory neurons have a high density of calcium-activated chloride channels that may play an important role in vomeronasal transduction.

## INTRODUCTION

Many social behaviors in animals are triggered by molecules with various chemical structures. In mammals, several chemosensory organs, such as the main olfactory epithelium, the vomeronasal organ (VNO), the septal organ, and the Grüneberg ganglion, are involved in chemical detection (Brennan and Zufall, 2006; Zufall and Leinders-Zufall, 2007; Brennan, 2009; Ma, 2009; Munger et al., 2009; Tirindelli et al., 2009; Touhara and Vosshall, 2009). Among these, the two main systems are represented by the main olfactory epithelium and the VNO. In both sensory systems, signal transduction occurs in bipolar sensory neurons and leads to membrane depolarization, although different transduction cascades are involved.

In most olfactory sensory neurons of the main olfactory epithelium, signal transduction occurs in the cilia

protruding from the neurons' apical surface. The binding of molecules to odorant receptors leads to cAMP production and to the opening of CNG channels in the ciliary membrane.  $\text{Na}^+$  and  $\text{Ca}^{2+}$  influx through CNG channels produces a depolarization of the neuron, and the increase in cytoplasmic  $\text{Ca}^{2+}$  concentration in the cilia has several effects, including a role in adaptation and the activation of  $\text{Cl}^-$  channels (Schild and Restrepo, 1998; Pifferi et al., 2006, 2009b; Kleene, 2008; Frings, 2009a,b; Reisert and Zhao, 2011).

In most vomeronasal sensory neurons, signal transduction occurs in microvilli that are present at the neurons' apical surface. The binding of molecules to vomeronasal receptors activates a phospholipase C signaling cascade, leading to the opening of ion channels that allow  $\text{Na}^+$  and  $\text{Ca}^{2+}$  influx. The transient receptor potential canonical 2 (TRPC2) channel is expressed in the neurons' microvilli (Liman et al., 1999) and is mainly responsible for such cation influx (Zufall et al., 2005;

Correspondence to Anna Menini: [menini@sissa.it](mailto:menini@sissa.it)

M. Dibattista's present address is Monell Chemical Senses Center, Philadelphia, PA 19104.

Abbreviations used in this paper: DAPI, 4'-6-diamidino-2-phenylindole; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid;  $\text{MeS}^-$ , methanesulfonate; NFA, niflumic acid; PDE4A, phosphodiesterase 4A;  $\text{SCN}^-$ , isothiocyanate; TRPC2, transient receptor potential canonical 2; VNO, vomeronasal organ.

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Munger et al., 2009). Several studies demonstrated that vomeronasal sensory neurons respond to stimuli with the generation of action potentials and an increase in intracellular  $\text{Ca}^{2+}$  concentration (Holy et al., 2000; Leinders-Zufall et al., 2000, 2004, 2009; Spehr et al., 2002; Chamero et al., 2007). However, the role played by cytoplasmic  $\text{Ca}^{2+}$  elevation in the microvilli is still largely unknown. Spehr et al. (2009) have recently shown that  $\text{Ca}^{2+}$  in combination with calmodulin is responsible for sensory adaptation. In addition, other studies suggested that intracellular  $\text{Ca}^{2+}$  might also activate ion channels involved in the transduction process, although it is still a matter of debate whether these channels are cation or anion selective. Indeed,  $\text{Ca}^{2+}$ -activated nonselective cation currents have been measured in hamster (Liman, 2003) or mouse vomeronasal sensory neurons (Spehr et al., 2009). In the whole cell configuration, currents of about  $-177$  pA at  $-80$  mV were activated by dialysis of 0.5 or 2 mM  $\text{Ca}^{2+}$  (Liman, 2003). In excised inside-out patches, the dose–response relation indicated that half-activation of the channels occurred at 0.5 mM  $\text{Ca}^{2+}$  at  $-80$  mV (Liman, 2003). It has been suggested that this  $\text{Ca}^{2+}$ -activated nonselective cation channel could directly mediate vomeronasal sensory transduction or amplify the primary sensory response (Liman, 2003), but at present its role and its molecular identity are still unknown. Other studies suggested that a significant portion of the response to urine in mouse vomeronasal sensory neurons is carried by  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels (Yang and Delay, 2010; Kim et al., 2011). However, these studies used indirect ways to activate channels, as the increase in cytoplasmic  $\text{Ca}^{2+}$  concentration was a secondary effect of urine stimulation.

Thus, at present, it is still unclear whether nonselective cation and/or  $\text{Cl}^-$  channels activated by  $\text{Ca}^{2+}$  are expressed in the apical region of vomeronasal sensory neurons and may be involved in vomeronasal transduction. To contribute to the resolution of this debate, we directly recorded and characterized currents by producing rapid and repeatable increases in intracellular  $\text{Ca}^{2+}$  concentration using flash photolysis of caged  $\text{Ca}^{2+}$ , while recording the induced current in the whole cell voltage-clamp configuration (Boccaccio et al., 2011). The use of photolysis of caged  $\text{Ca}^{2+}$  to produce an increase in  $\text{Ca}^{2+}$  concentration, instead of dialysis of  $\text{Ca}^{2+}$  into the neuron or the production of a secondary  $\text{Ca}^{2+}$  increase, allowed us to release  $\text{Ca}^{2+}$  in a temporally and spatially defined manner into an intact neuron because we could precisely deliver a flash of UV light at the apical region of a vomeronasal sensory neuron. We measured an average inward  $\text{Ca}^{2+}$ -activated current of  $-261$  pA at the holding potential of  $-50$  mV and showed that this current is anion selective. Furthermore, both niflumic acid (NFA) and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), two very well known  $\text{Cl}^-$  channel blockers (Frings et al., 2000; Hartzell et al., 2005), partially

blocked the  $\text{Ca}^{2+}$ -activated current in vomeronasal sensory neurons.

To the best of our knowledge, these are the first recordings providing a direct demonstration that a large  $\text{Cl}^-$  current can be directly activated by  $\text{Ca}^{2+}$  in the apical region of mouse vomeronasal sensory neurons, as this demonstration can only be obtained by using a method that provides a temporal and spatial control of  $\text{Ca}^{2+}$  release, such as photolysis of caged  $\text{Ca}^{2+}$ .

Recent studies indicated that at least two members of the TMEM16/anoctamin family, TMEM16A/anoctamin1 and TMEM16B/anoctamin2, are  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008; Pifferi et al., 2009a, 2012; Stephan et al., 2009; Stöhr et al., 2009; Scudieri et al., 2012). We studied the expression of members of this family in the VNO and found that both TMEM16A and TMEM16B are expressed in the apical region of the VNO, in agreement with recent studies (Rasche et al., 2010; Billig et al., 2011; Dauner et al., 2012). However, microvilli both from vomeronasal sensory neurons and from supporting cells are present at the apical surface of the vomeronasal epithelium, and they are not clearly distinguishable. We therefore investigated the localization of TMEM16A and TMEM16B in isolated vomeronasal sensory neurons and found that these channels are expressed in neurons' microvilli. Because microvilli are the site where transduction events take place, the presence of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels indicates that they may be involved in signal transduction in the VNO. A complete understanding of conductances present in vomeronasal sensory neurons will help to elucidate the molecular mechanisms involved in the generation of the vomeronasal transduction current and the production of action potentials.

## MATERIALS AND METHODS

### Preparation of isolated vomeronasal sensory neurons

All animals were handled in accordance with the Italian Guidelines for the Use of Laboratory Animals (Decreto Legislativo 27/01/1992, no. 116) and European Union guidelines on animal research (no. 86/609/EEC). For experiments, 2-mo-old mice were anaesthetized by  $\text{CO}_2$  inhalation and decapitated before VNO removal. The vomer capsule containing the VNO was removed as described previously (Liman and Corey, 1996; Dean et al., 2004; Shimazaki et al., 2006; Arnson et al., 2010), and vomeronasal sensory neurons were dissociated from the VNO with a standard enzymatic–mechanical dissociation protocol (Di Battista et al., 2008). In brief, the removed vomer capsule was rapidly transferred to a Petri dish containing divalent-free PBS (Sigma-Aldrich) solution where the VNO was extracted. The tissue was cut into small pieces with tiny scissors, transferred to divalent-free PBS containing 1 mg/ml collagenase (type A), incubated at  $37^\circ\text{C}$  for 10 min, cut into small pieces with tiny scissors, and reincubated for 10 min at  $37^\circ\text{C}$ . After a 2-min centrifugation at 1,700 rpm, the tissue was gently triturated with a fire-polished Pasteur pipette. Cells were resuspended in 1 ml of fresh Ringier's solution and plated on a glass coverslip (World Precision

Instruments) coated with poly-L-lysine and concanavalin A (type V; Sigma-Aldrich). Cells were stored at 4°C for up to 7 h before experiments.

### Patch-clamp recordings

Vomer nasal sensory neurons were observed using an inverted microscope (IX 70; Olympus) with an oil immersion  $\times 100$  objective (Carl Zeiss). Currents in whole cell voltage-clamp configuration were recorded using an Axopatch 200B patch-clamp amplifier controlled by Clampex 8 connected with a Digidata 1322A (Molecular Devices). Patch pipettes were made using borosilicate capillaries (World Precision Instruments) and were pulled by a two-stage vertical puller (PP-83; Narishige). Pipette resistance was around 3–6 M $\Omega$ . Currents were low-pass filtered at 1 kHz and acquired at 2 kHz. All the experiments were performed at room temperature (20–24°C).

### Ionic solutions, photolysis of caged Ca<sup>2+</sup>, and perfusion system

The extracellular mammalian Ringer's solution contained (in mM): 140 NaCl, 5 KCl, 1 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES, 10 glucose, and 1 sodium pyruvate, pH 7.4. For flash photolysis of caged compounds, we used a xenon flash-lamp system (JML-C2; Rapp OptoElectronic) coupled with the epifluorescence port of the inverted microscope with a quartz light guide, as described previously (Boccaccio et al., 2006, 2011; Boccaccio and Menini, 2007). The light spot had a diameter of  $\sim 15$   $\mu$ m and was focused on the microvilli and dendritic knob of isolated vomeronasal sensory neurons. The flash duration was  $< 1.5$  ms and was kept constant for each experiment. The interval between flashes was at least 2 min. At the beginning of each experiment, the stability of the response was checked by applying repetitive flashes at intervals of 2 min. Neurons that did not reach a stable response to at least two consecutive flashes were discarded.

The intracellular recording solution for the photorelease of caged Ca<sup>2+</sup> contained (in mM): 3 DMNP-EDTA, 1.5 CaCl<sub>2</sub>, 140 CsCl, and 10 HEPES, pH 7.4. DMNP-EDTA was purchased from Invitrogen, and CaCl<sub>2</sub> was adjusted with a 0.1-M standard solution from Fluka. Aliquots were stored at  $-20^{\circ}\text{C}$  and kept refrigerated in the dark during the experiment.

For ionic selectivity experiments, NaCl was replaced with equimolar NMDG-Cl, or Cl<sup>-</sup> was substituted with other anions, such as methanesulfonate (MeS<sup>-</sup>) or isothiocyanate (SCN<sup>-</sup>), by replacing NaCl on an equimolar basis with NaX, where X is the substituted anion.

The bath was grounded through a 1-M KCl agar bridge connected to an Ag-AgCl reference electrode. Liquid junction potentials were calculated using the Clampex's Junction Potential Calculator (Molecular Devices), based on the JPCalc program developed by Barry (1994), and applied membrane potentials were corrected offline for the calculated liquid junction potentials, as described previously (Saghehdu et al., 2010).

NFA was prepared in DMSO as stock solutions at 200 mM and diluted to the final concentration of 300  $\mu$ M. DMSO alone did not modify the currents. DIDS was directly dissolved in the bathing solution to 1 mM.

Bathing solutions were changed by using a gravity-fed perfusion system with a slow perfusion rate, adjusted in such a way that the position of the neuron was not perturbed. A complete solution change was obtained in  $\sim 10$  s. To measure blocker effects, current recordings were obtained before blocker application until a stable response was obtained (control), 2 min after delivery of the solution with the blocker, and 2–5 min after perfusion with Ringer's solution without the blocker (wash).

Chemicals, unless otherwise stated, were purchased from Sigma-Aldrich.

### Analysis of electrophysiological data

Data analysis and figures were made using Clampfit and IGOR software (WaveMetrics). Current recordings at each holding potential were plotted by subtracting the value of the baseline measured before photorelease of caged Ca<sup>2+</sup>. Data are given as mean  $\pm$  SEM and the total number of neurons (*n*). Statistical significance was tested with a Student's *t* test.  $P < 0.05$  was considered statistically significant.

### RNA extraction and RT-PCR

RNA was extracted from the VNO of FVB mice. Methods and primers for the amplification of TMEM16/anoctamins cDNA were the same as described previously (Saghehdu et al., 2010). All amplicons were gel extracted, subcloned, and sequenced for confirmation.

### Immunofluorescence

HEK 293T cells were grown on coverslips and cotransfected with plasmids containing the cDNA sequence of TMEM16A or TMEM16B (RZPD) and enhanced GFP (Takara Bio Inc.) for fluorescent identification of transfected cells, as described previously (Pifferi et al., 2009a). Transfected cells were fixed in 4% paraformaldehyde for 15 min at room temperature and sequentially washed. Next, they were incubated with a quenching solution (0.1 M glycine) for 10 min and then treated with 0.05% SDS for antigen retrieval for 10 min. Cells were subsequently incubated for 15 min in blocking solution (2% [vol/vol] FBS and 0.2% [vol/vol] Triton X-100 in PBS), followed by incubation with the primary antibody for 3 h at 4°C. After washing with PBS-T (0.1% Tween 20 in PBS), cells were incubated for 45 min with the secondary antibody, prepared in PBS-T. Finally, cells were incubated with 0.1  $\mu$ g/ml 4'-6-diamidino-2-phenylindole (DAPI) for 15 min, and coverslips were mounted with Vectashield (Vector Laboratories).

For immunohistochemistry on tissue sections, the mouse nasal regions or the VNOs extracted from the nasal cavity were fixed in 4% paraformaldehyde (for 4 h at 4°C), then decalcified in 0.5 M EDTA for 12 h at 4°C, and subsequently equilibrated overnight (4°C) in 30% (wt/vol) sucrose for cryoprotection. 14- $\mu$ m coronal sections were cut with a cryostat and stored ( $-80^{\circ}\text{C}$ ) for further use. For antigen retrieval, sections were treated with SDS 0.5% (wt/vol) in PBS for 15 min. Sections were incubated in a blocking solution (2% [vol/vol] FBS and 0.2% [vol/vol] Triton X-100 in PBS) for 2 h, and then with the primary antibody (diluted in the blocking solution) overnight at 4°C. Sections were then rinsed with 0.1% (vol/vol) Tween 20 in PBS (PBS-T) and incubated with the fluorophore-conjugated secondary antibody (diluted in PBS-T) for 2 h at room temperature. After washing with PBS-T, sections were treated with 0.1  $\mu$ g/ml DAPI for 30 min, washed with PBS-T, and mounted with Vectashield (Vector Laboratories).

Immunocytochemistry on isolated vomeronasal sensory neurons was performed as described previously (Fieni et al., 2003). Dissociated vomeronasal sensory neurons were prepared as for electrophysiological experiments. Glass-attached vomeronasal neurons were gently perfused with 4% paraformaldehyde and then washed in PBS. Cells were blocked in 1% albumin and 0.3% Triton X-100 in PBS for 20 min and incubated overnight with the primary antibody. Cells were then washed in PBS and further incubated with the secondary antibody, prepared in PBS. In some experiments, cells were also incubated with 0.1  $\mu$ g/ml DAPI for 15 min. Coverslips were mounted with Vectashield (Vector Laboratories).

Wild-type C57BL6 or genetically modified mice that express GFP in all mature olfactory and vomeronasal sensory neurons (OMP-GFP mice; provided by P. Mombaerts, Max Planck Institute of Biophysics, Frankfurt, Germany) were used.

The following primary antibodies were used: rabbit anti-TMEM16A (1:50; Abcam), rabbit anti-TMEM16B (1:100; Santa Cruz Biotechnology, Inc.), guinea pig anti-TMEM16A and guinea

pig anti-TMEM16B (provided by S. Frings, Heidelberg University, Heidelberg, Germany; Dauner et al., 2012), goat anti-TRPC2 (1:50; Santa Cruz Biotechnology, Inc.), rabbit anti-G $\alpha$ o (1:100; Santa Cruz Biotechnology, Inc.), and rabbit anti-phosphodiesterase 4A (PDE4A; 1:50; Abcam). The following secondary antibodies, obtained from Invitrogen, were used: goat anti-guinea pig Alexa Fluor 594 (1:500), goat anti-rabbit Alexa Fluor 488 (1:500), goat anti-rabbit Alexa Fluor 594 (1:500), goat anti-rabbit Alexa Fluor 405 (1:500), chicken anti-rabbit Alexa Fluor 594 (1:500), and chicken anti-goat Alexa Fluor 488 (1:500).

Immunoreactivity was visualized with a confocal microscope (TCS SP2; Leica). Images were acquired using Leica software (at 1,024  $\times$  1,024-pixel resolution) and were not modified other than to balance brightness and contrast. Control experiments without the primary antibodies gave no signal.

## RESULTS

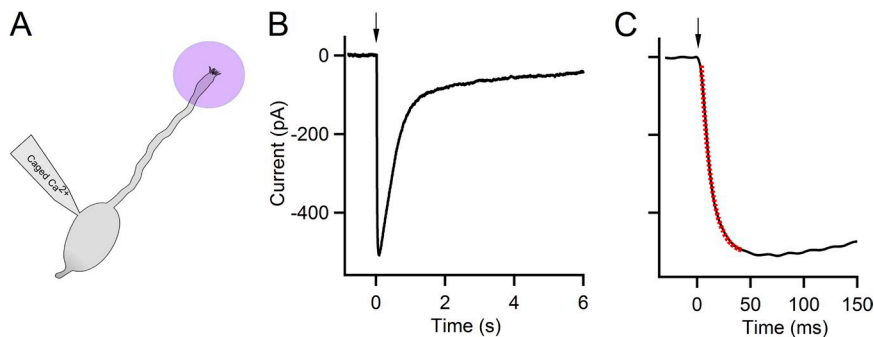
### Whole cell currents activated by photolysis of caged Ca<sup>2+</sup> at the apical region of mouse vomeronasal sensory neurons

We obtained whole cell voltage-clamp recordings from isolated mouse vomeronasal sensory neurons and directly measured Ca<sup>2+</sup>-activated currents by rapidly elevating the Ca<sup>2+</sup> concentration in the apical region (dendritic knob and microvilli) by localized photorelease of caged Ca<sup>2+</sup> (Fig. 1 A). Fig. 1 B shows a typical current response at the holding potential of  $-50$  mV. An inward current rapidly developed upon the flash release, reaching a peak amplitude of  $-508$  pA, and then slowly returned to baseline. The rising phase of the Ca<sup>2+</sup>-activated current was well described by a single-exponential function with a time constant of 9.5 ms (Fig. 1 C). Similar results were obtained from a total of 59 neurons with an average time constant value of  $12.6 \pm 6.0$  ms ( $n = 59$ ; range of 3–26 ms). We observed a large variability in Ca<sup>2+</sup>-activated current amplitudes in different neurons with absolute values ranging between 50 pA and 1 nA at  $-50$  mV, and a mean amplitude of  $-261 \pm 37$  pA ( $n = 59$ ). Such variability has also been observed in recording Ca<sup>2+</sup>-activated currents in the ciliary region of olfactory sensory neurons, where absolute values ranging from 50 pA up to  $\sim 1$  nA were also recorded (Boccaccio et al., 2006; Boccaccio and Menini, 2007). The amplitude variability

can originate both from the illumination conditions in the neuron that may differ between experiments as well as from various numbers or densities of channels that may vary in different neurons. Similarly to olfactory sensory neurons, we also found a large variability in the time necessary for the current to return to baseline in vomeronasal sensory neurons (Figs. 1 B and 3, A and B). This is likely to be the result of differences in the time necessary for the decrease in Ca<sup>2+</sup> concentration by Ca<sup>2+</sup> extrusion and/or by diffusion to other neuronal compartments.

To investigate the ionic nature of the Ca<sup>2+</sup>-activated currents, we measured the reversal potential in various ionic conditions. Currents were recorded at various holding potentials with different ionic compositions in the extracellular solution. In a first set of experiments, we used our standard intracellular and extracellular solutions, in which Cs<sup>+</sup> was chosen as the intracellular monovalent cation to avoid contributions from Ca<sup>2+</sup>-activated K<sup>+</sup> currents, and Na<sup>+</sup> was the main extracellular monovalent cation. Moreover, the intracellular and extracellular Cl<sup>-</sup> concentrations were almost symmetrical. Fig. 2 A shows the responses induced by photorelease of Ca<sup>2+</sup> at the indicated holding potentials. The reversal potential calculated from the current-voltage relation was  $+0.5$  mV, with an average value of  $+0.3 \pm 3.5$  mV ( $n = 6$ ; Fig. 2 E). Because the cation concentrations were [Na<sup>+</sup>]<sub>o</sub> = 149 mM and [Cs<sup>+</sup>]<sub>i</sub> = 143 mM, and the Cl<sup>-</sup> concentrations were [Cl<sup>-</sup>]<sub>o</sub> = 149 mM and [Cl<sup>-</sup>]<sub>i</sub> = 143 mM, a reversal potential value close to 0 mV is consistent both with a nonselective cation current and with an anion-selective current.

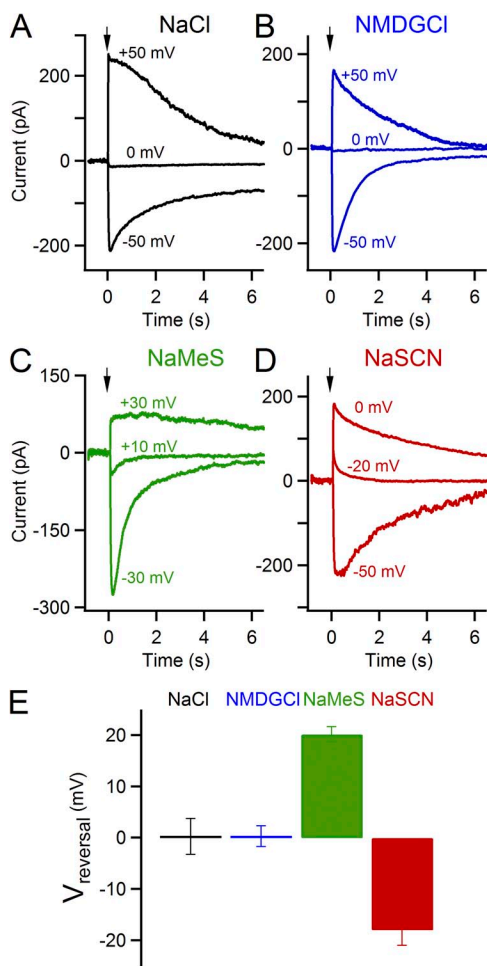
To distinguish between the two types of currents, we performed a first set of experiments by replacing Na<sup>+</sup> in the extracellular solution with NMDG<sup>+</sup>, a large organic monovalent cation largely impermeant in cation channels. If the measured Ca<sup>2+</sup>-activated currents were carried by cations, the replacement of Na<sup>+</sup> by NMDG<sup>+</sup> should produce a shift of the reversal potential toward negative values. Fig. 2 B shows that the reversal potential in the presence of 140 mM NMDG-Cl was  $+0.3$  mV, with an average value of  $+0.5 \pm 3.8$  mV ( $n = 3$ ; Fig. 2 E), indicating that the Ca<sup>2+</sup>-activated current was not carried by cations.



**Figure 1.** Current responses induced by photorelease of Ca<sup>2+</sup> in the apical region of mouse vomeronasal sensory neurons. (A) Schematic drawing of a vomeronasal sensory neuron showing the location of application of the UV flash to photorelease Ca<sup>2+</sup>. (B) Whole cell current induced by photorelease of Ca<sup>2+</sup> at the holding potential of  $-50$  mV. A flash was applied at time  $t = 0$  (indicated by an arrow). (C) Expanded timescale shows the rapid increase in the current upon Ca<sup>2+</sup> photorelease. The current rising phase was well fitted by a single exponential (red dotted line) with a  $\tau$  value of 9.5 ms.



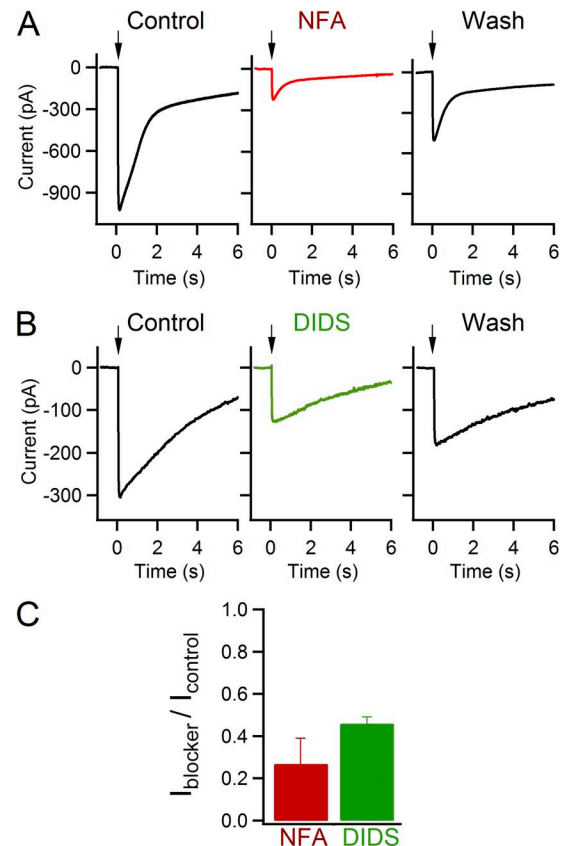
In a second set of experiments, we tested whether the  $\text{Ca}^{2+}$ -activated current was carried by  $\text{Cl}^-$  by substituting most of the extracellular  $\text{Cl}^-$  with  $\text{MeS}^-$ , an anion known to be almost impermeant in  $\text{Cl}^-$  channels. Fig. 2 C shows that, when we replaced 140 NaCl with NaMeS, the reversal potential was +18 mV, with an average value of  $+20.2 \pm 1.5$  mV ( $n = 3$ ; Fig. 2 E). The average reversal potential in the low extracellular  $\text{Cl}^-$  solution was shifted toward more positive values, as expected if  $\text{MeS}^-$  is much less permeant than  $\text{Cl}^-$ . Furthermore, because most  $\text{Cl}^-$  channels are more permeable to  $\text{SCN}^-$  than to  $\text{Cl}^-$ , we measured the reversal potential after replacing 140 mM NaCl with NaSCN: the reversal potential shifted toward more negative values, -20 mV (Fig. 2 D), with an average value of  $-18 \pm 3$  mV ( $n = 8$ ; Fig. 2 E). These



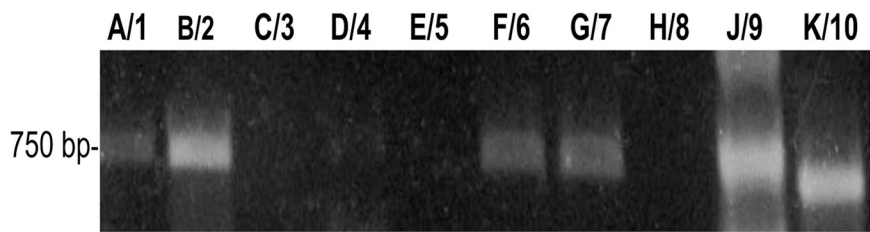
**Figure 2.** Ion selectivity of the  $\text{Ca}^{2+}$ -activated current. Whole cell currents from vomeronasal sensory neurons induced by photorelease of  $\text{Ca}^{2+}$  into the apical region recorded at the indicated holding potentials. A UV flash was applied at the time  $t = 0$  (indicated by an arrow). Recordings in the presence of extracellular Ringer's solution containing 140 mM: (A) NaCl, (B) NMDG-Cl, (C) NaMeS, and (D) NaSCN, each from a different neuron. (E) Average reversal potentials measured in the presence of the indicated ionic solutions: NaCl ( $n = 6$ ), NMDG-Cl ( $n = 3$ ), NaMeS ( $n = 3$ ), and NaSCN ( $n = 8$ ).

results demonstrate that the  $\text{Ca}^{2+}$ -activated current is an anion current and that these ion channels have higher permeability for  $\text{SCN}^-$  over  $\text{Cl}^-$ , as in most  $\text{Cl}^-$  channels (Hartzell et al., 2005).

To further characterize these channels, we measured the extracellular blockage by NFA and DIDS, two compounds commonly used to partially block  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  currents in various tissues (Frings et al., 2000). Fig. 3 A shows the blocking effect by 300  $\mu\text{M}$  NFA of the current elicited by photolysis of caged  $\text{Ca}^{2+}$  at -50 mV. The maximal inward current decreased from -1,022 to -221 pA upon NFA application, corresponding to 22% of its value before blocker application. The blocking effect was partially reversible after perfusion with Ringer's solution without NFA, as the current amplitude recovered to -505 pA, 50% of the control value. On average, the current amplitude in the presence of 300  $\mu\text{M}$  NFA at -50 mV was 27% ( $n = 5$ ) of the value before blocker application (Fig. 3 C). After perfusion with Ringer's



**Figure 3.** Blockage of the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current. Whole cell currents induced by photorelease of  $\text{Ca}^{2+}$  into the apical region of vomeronasal sensory neurons. The holding potential was -50 mV. Current recordings were obtained before blocker application (control), 2 min after application of the indicated blockers, and 2 min after the removal of blockers (wash). The following blockers were used: (A) 300  $\mu\text{M}$  NFA and (B) 1 mM DIDS. (C) Average values of the current in the presence of 300  $\mu\text{M}$  NFA ( $n = 5$ ) or 1 mM DIDS ( $n = 3$ ) normalized to the current in control conditions ( $P < 0.05$ ).



**Figure 4.** Expression of TMEM16s/anoctamins in mouse VNO. TMEM16/anoctamin isoforms A–J (1–10) were amplified from VNO cDNA by RT-PCR. A/1, B/2, F/6, G/7, J/9, and K/10 are expressed in the VNO.

solution without the blocker, the current recovered on average to 68% of its control value.

Fig. 3 B shows recordings from a vomeronasal sensory neuron in which the extracellular addition of 1 mM DIDS produced a block to 42% of its control value. After washout with Ringer’s solution, the current amplitude reached 60% of the value before blocker application. On average, the current amplitude in the presence of 1 mM DIDS was 46% ( $n = 3$ ) of the control value (Fig. 3 C). After perfusion with Ringer’s solution without DIDS, the current was on average 60% of its control value.

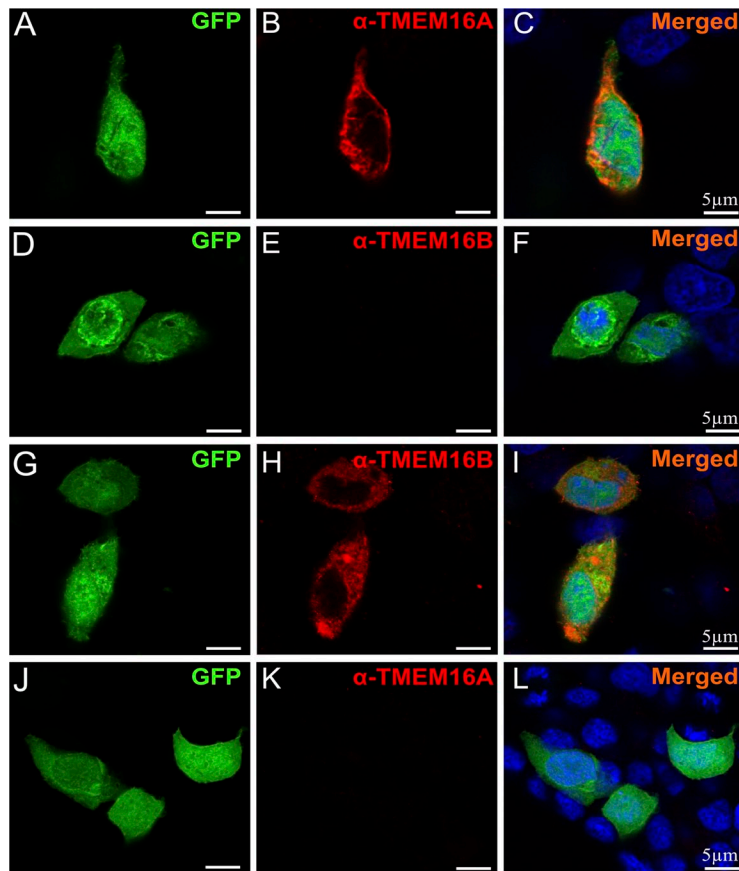
Collectively, these results show that a sudden increase in the intracellular  $Ca^{2+}$  concentration can rapidly activate a large anion current, demonstrating the expression of a high density of  $Ca^{2+}$ -activated  $Cl^{-}$  channels in the apical region of vomeronasal sensory neurons.

#### Expression of TMEM16s/anoctamins in mouse VNOs

Having measured  $Ca^{2+}$ -activated  $Cl^{-}$  currents in the apical region of mouse vomeronasal sensory neurons, we sought to investigate which members of the TMEM16/anoctamin family that are known to function as  $Ca^{2+}$ -activated  $Cl^{-}$  channels are expressed in the VNO.

To analyze the expression of each TMEM16/anoctamin, we performed RT-PCR on cDNA obtained from mouse VNOs. mRNAs of TMEM16A/anoctamin1, B/2, F/6, G/7, J/9, and K/10 were found to be significantly expressed in the VNO (Fig. 4).

Because antibodies against TMEM16A and TMEM16B are commercially available, we first tested their specificity on HEK 293T cells transiently transfected with plasmids containing the cDNA sequence of TMEM16A or TMEM16B and GFP. Fig. 5 shows that cells transfected with TMEM16A (Fig. 5, A–F) or TMEM16B (Fig. 5, G–L)



**Figure 5.** Specificity of rabbit anti-TMEM16A and anti-TMEM16B antibodies in HEK 293T cells expressing TMEM16A or TMEM16B. (A–F) Fluorescence images of the staining with anti-TMEM16A or anti-TMEM16B antibodies (indicated with the prefix  $\alpha$ ) of HEK 293T cells transiently cotransfected with TMEM16A and GFP cDNA. Specific staining was observed only with anti-TMEM16A (B), whereas no immunoreactivity was detected with anti-TMEM16B (E) antibody. (G–L) Fluorescence images of the staining with anti-TMEM16B or anti-TMEM16A antibodies of HEK 293T cells transiently cotransfected with TMEM16B and GFP cDNA. Specific staining was observed only with anti-TMEM16B (H), whereas no immunoreactivity was detected with anti-TMEM16A (K) antibody. Cell nuclei were stained by DAPI (blue). Bars, 5  $\mu$ m.

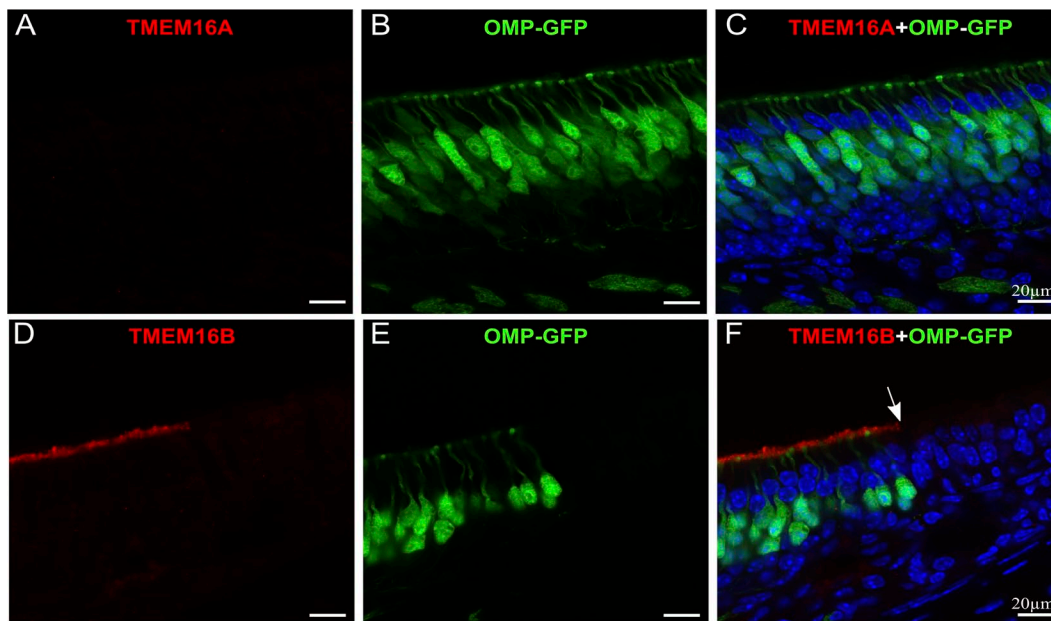
cDNA produced a strong and specific immunoreactivity exclusively to their respective antibody.

We further investigated the specificity of the two antibodies on the olfactory epithelium. Indeed, it is well established that TMEM16B is expressed in the apical layer of the olfactory epithelium but not in the respiratory epithelium (Hengl et al., 2010; Rasche et al., 2010; Billig et al., 2011). We used genetically modified OMP-GFP mice that express GFP in all mature olfactory and vomeronasal sensory neurons (Potter et al., 2001) and confirmed that TMEM16B was expressed at the apical surface of the olfactory epithelium but not in the respiratory epithelium (Fig. 6, D–F), and that TMEM16A immunoreactivity was absent (Fig. 6, A–C). Collectively, these experiments indicate that these antibodies specifically recognized their epitopes and did not show cross-reactivity.

We therefore used the same antibodies to examine the localization of TMEM16A and TMEM16B in the vomeronasal epithelium of OMP-GFP mice and detected immunoreactivity at the luminal surface of the sensory epithelium (Fig. 7, A–F). High magnification images show that both anti-TMEM16A (Fig. 7 G) and anti-TMEM16B (Fig. 7 H) staining lies above the knobs of the vomeronasal sensory neurons, where microvilli are located. We also costained VNO sections with an antibody against the cation channel TRPC2, which is involved in vomeronasal transduction, and observed staining in the microvillar region (Fig. 8, B and E) that largely colocalized with TMEM16A and TMEM16B

immunoreactivity (Fig. 8, A, C, D, and F), indicating that cation (TRPC2) and anion channels (TMEM16A and TMEM16B) coexpress. However, the microvillar region is composed of microvilli of both sensory neurons and supporting cells (Höfer et al., 2000; Dauner et al., 2012), and they cannot be distinguished in immunosignals from VNO coronal sections.

To unequivocally establish whether TMEM16A and/or TMEM16B are expressed in the microvilli of sensory neurons, the compartment responsible for signal transduction, we performed immunocytochemistry on isolated vomeronasal sensory neurons using combinations of the commercially available antibodies raised in rabbit and of those raised in guinea pigs (provided by S. Frings; Dauner et al., 2012). Figs. 9 and 10 illustrate that TMEM16A and TMEM16B are both expressed in the microvilli of vomeronasal sensory neurons. Moreover, Fig. 9 clearly shows the coexpression of TMEM16A and TMEM16B in the microvilli of the same neuron from an OMP-GFP mouse. After dissociation and immunocytochemistry procedures, we obtained 34 intact isolated vomeronasal sensory neurons from four OMP-GFP mice, and 62 intact neurons from 10 wild-type mice. Every intact vomeronasal sensory neuron, both from OMP-GFP and wild-type mice, showed coexpression of TMEM16A and TMEM16B in the microvilli. For coexpression, both combinations of rabbit anti-TMEM16A with guinea pig anti-TMEM16B and guinea pig anti-TMEM16A with rabbit anti-TMEM16B produced the same result. Unfortunately, in our preparation of dissociated



**Figure 6.** TMEM16A and TMEM16B immunoreactivity in the olfactory epithelium. (A–C) Confocal images of coronal sections of the olfactory epithelium from an OMP-GFP mouse showing the absence of TMEM16A immunoreactivity. (D–F) Confocal images of the transition region between the olfactory and the respiratory epithelium (absence of OMP-GFP-expressing neurons). The arrow indicates the transition between the two epithelia. TMEM16B is expressed at the apical surface of the olfactory but not of the respiratory epithelium. Cell nuclei were stained by DAPI (blue). Bars, 20 μm.



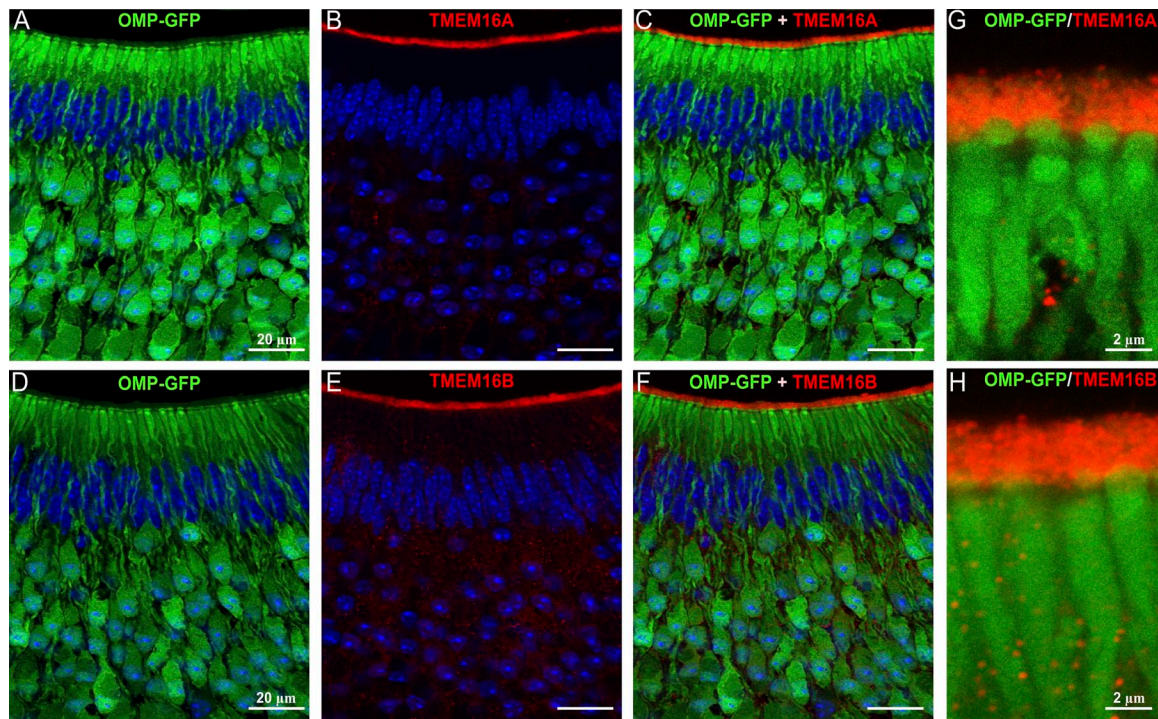
vomeronal cells, we were not able to clearly identify supporting cells; therefore, we cannot exclude the possibility that TMEM16A and/or TMEM16B are also expressed in microvilli of these cells.

The rodent VNO has two major neuronal populations, apical and basal neurons, characterized by their location in the VNO and by the expression of specific proteins and receptors (Berghard and Buck, 1996; Jia and Halpern, 1996; Ryba and Tirindelli, 1997; Lau and Cherry, 2000; Leinders-Zufall et al., 2004; Liberles et al., 2009; Rivière et al., 2009). Apical neurons are located near the lumen of the VNO and express the G protein  $\alpha$  subunit  $G\alpha i2$ , the PDE4A, and receptors of the V1R or formyl peptide receptor family. Basal neurons are close to the basal lamina and express the G protein  $\alpha$  subunit  $G\alpha o$  and receptors of the V2R or formyl peptide receptor family. The finding that TMEM16A and TMEM16B are expressed in the microvilli of neurons raises the question of whether the expression of these proteins is restricted to any of these two neuronal subsets. We used specific markers to identify whether neurons expressing TMEM16A or TMEM16B are basal or apical. We performed immunocytochemistry on isolated vomeronasal sensory neurons using rabbit anti-PDE4A antibody, a marker for apical neurons, or rabbit anti- $G\alpha o$  antibody, a marker for basal neurons

(Lau and Cherry, 2000; Leinders-Zufall et al., 2004), together with the guinea pig anti-TMEM16A or anti-TMEM16B. Fig. 10 shows that TMEM16A and TMEM16B are localized in the microvilli of both apical (Fig. 10, A–C and G–I) and basal (Fig. 10, D–F and J–L) vomeronasal sensory neurons from wild-type mice. These results show that TMEM16A and TMEM16B are expressed in microvilli of both the apical and basal neuronal populations.

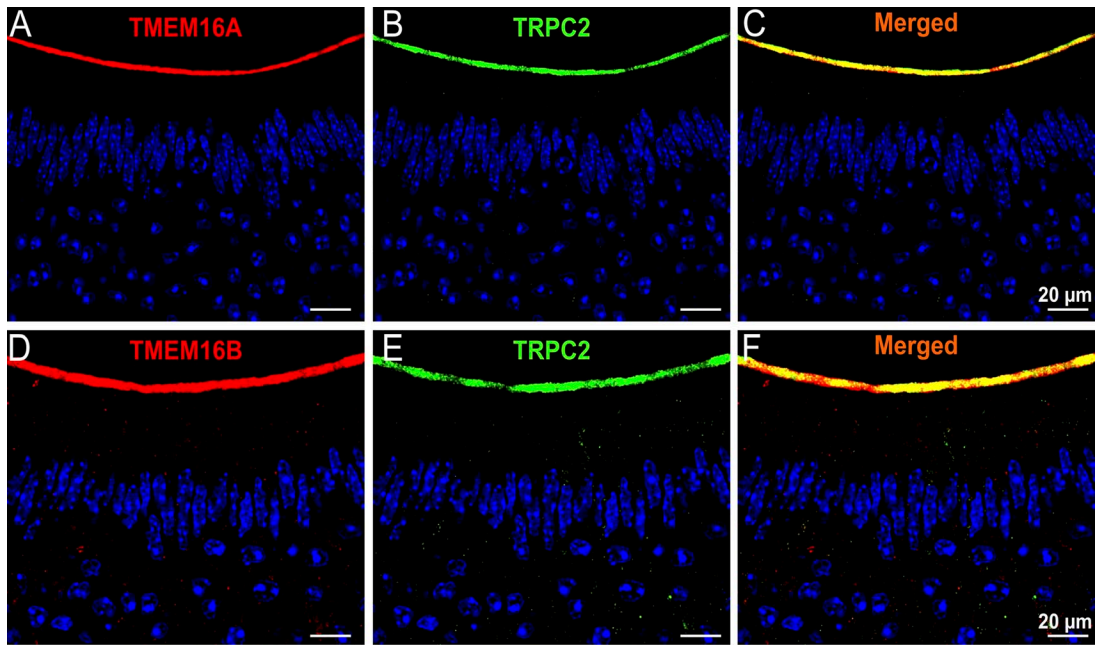
## DISCUSSION

In this study, we have provided the first direct demonstration that a large  $Cl^-$  current can be directly activated by  $Ca^{2+}$  in the apical region of mouse vomeronasal sensory neurons. Indeed, photolysis of caged  $Ca^{2+}$  allowed us to obtain a precise temporal and spatial control of cytoplasmic  $Ca^{2+}$  elevation while recording the current in the whole cell voltage-clamp mode. Moreover, we have demonstrated that TMEM16A and TMEM16B largely colocalize with TRPC2 at the apical surface of the vomeronasal epithelium, and that TMEM16A and TMEM16B are coexpressed in microvilli of both apical and basal isolated vomeronasal sensory neurons, therefore suggesting that these two anion channels are likely to be involved in vomeronasal transduction.



**Figure 7.** TMEM16A and TMEM16B are expressed at the apical surface of the vomeronasal epithelium. (A–H) Immunostaining of sections of VNO from an OMP-GFP mouse. (A and D) Endogenous GFP fluorescence of mature vomeronasal sensory neurons. TMEM16A (B) and TMEM16B (E) are expressed at the luminal surface of the vomeronasal sensory epithelium. (G and H) High magnification image of the apical portion of the VNO showing that TMEM16A and TMEM16B are expressed at the apical surface. Cell nuclei were stained by DAPI (blue). Bars: (A–F) 20  $\mu m$ ; (G and H) 2  $\mu m$ .





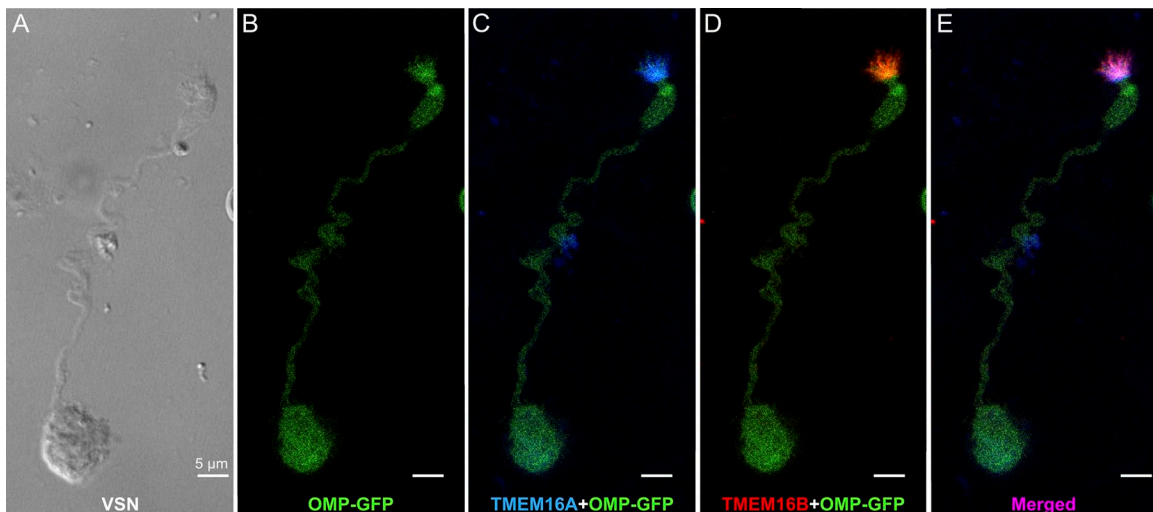
**Figure 8.** TMEM16A and TMEM16B are coexpressed with TRPC2. Double-label immunohistochemistry in tissue sections of VNO from a wild-type mouse showing the coexpression of TRPC2 with TMEM16A (A–C) and TMEM16B (D–F) at the luminal surface of the vomeronasal sensory epithelium. Cell nuclei were stained by DAPI (blue). Bars, 20  $\mu\text{m}$ .

#### Expression of TMEM16A and TMEM16B in the vomeronasal epithelium

Two recent reports (Billig et al., 2011; Dauner et al., 2012) have shown that both TMEM16A and TMEM16B are expressed in the microvillar surface of the VNO. However, as pointed out by Dauner et al. (2012), the VNO apical surface layer contains the microvilli of both sensory neurons and supporting cells (Höfer et al., 2000). Thus, immunohistochemistry on coronal sections of the VNO does not allow for the distinction between

these two microvillar subsets. By performing high resolution confocal imaging on isolated vomeronasal sensory neurons, we were able to detect both TMEM16A and TMEM16B in microvilli of the same neuron, thus providing the first unequivocal evidence of coexpression of these two anion channels in the same cell.

**Ca<sup>2+</sup>-activated currents in vomeronasal sensory neurons**  
 Previous reports showed the presence of Ca<sup>2+</sup>-activated nonselective cation currents in vomeronasal sensory



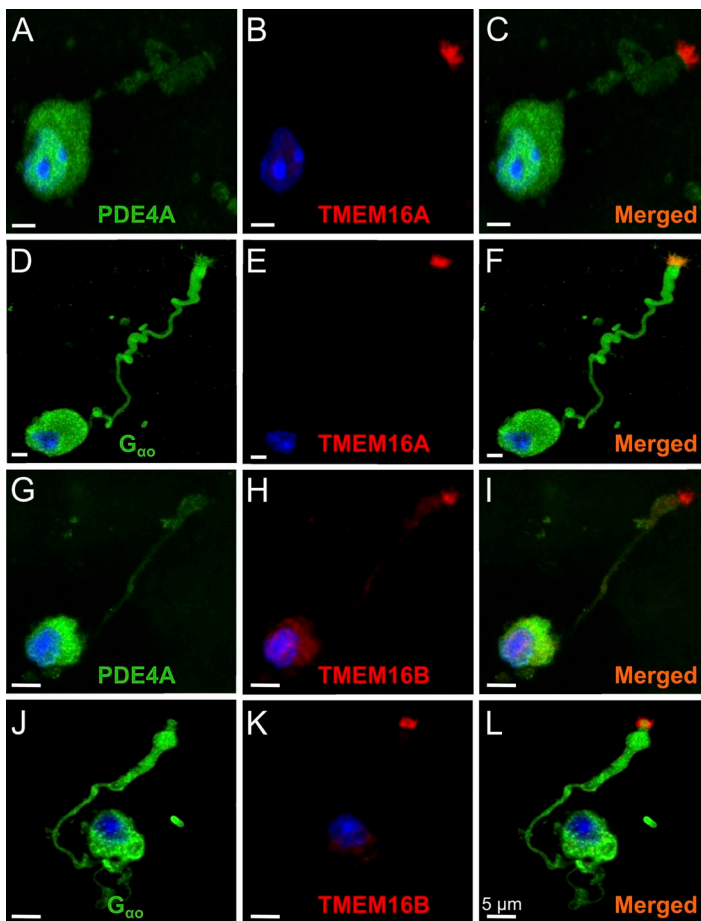
**Figure 9.** TMEM16A and TMEM16B are coexpressed in the microvilli of vomeronasal sensory neurons. (A) Bright field image of a vomeronasal sensory neuron isolated from an OMP-GFP mouse (B). The same neuron was stained with (C) rabbit anti-TMEM16A and (D) guinea pig anti-TMEM16B, showing the coexpression of the two anion channels in the microvilli (E). Cell nuclei were stained by DAPI (blue). Bars, 5  $\mu\text{m}$ .

neurons (Liman, 2003; Spehr et al., 2009). In our experiments, with intracellular  $\text{Cs}^+$  and extracellular  $\text{Na}^+$  as the main monovalent cations, we did not detect any cation current. However, it must be pointed out that the anion and cation channels are likely to be activated in two very different ranges of  $\text{Ca}^{2+}$  concentration. Liman (2003) reported that half-activation of the nonselective cation channel occurred at a very high  $\text{Ca}^{2+}$  concentration: 0.5 mM at  $-80$  mV. The  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels TMEM16A and TMEM16B, located in the microvilli of vomeronasal sensory neurons, are half-activated at  $<5$   $\mu\text{M}$  (Pifferi et al., 2009a; Stephan et al., 2009; Xiao et al., 2011), a  $\text{Ca}^{2+}$  concentration that is 100-fold lower than that required to activate the nonselective cation currents. Moreover, although we did not directly measure the  $\text{Ca}^{2+}$  concentration photoreleased in the microvilli, estimates from our previous studies in olfactory sensory neurons obtained by comparing the normalized currents at various flash intensities (Boccaccio et al., 2006) with the dose-response relation for the native olfactory  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels (Pifferi et al., 2009b) indicate that the maximal photoreleased concentration of  $\text{Ca}^{2+}$  is likely to be  $\sim 10$ – $20$   $\mu\text{M}$ . Thus, a  $\text{Ca}^{2+}$  concentration range lower than that necessary to activate the nonselective cation

channels may explain the lack of detectable cation currents in our experiments.

Liman (2003) also stimulated inside-out patches from dendritic knobs of hamster vomeronasal sensory neurons with 2 mM  $\text{Ca}^{2+}$  and showed that the activated current was almost entirely cationic. Similar results were obtained by Spehr et al. (2009) by activating patches from mouse VNO neurons with 50  $\mu\text{M}$   $\text{Ca}^{2+}$ . It is likely that the explanation of the absence of the chloride component in these inside-out experiments is related to a rapid decrease of the current. Indeed, it has been shown that  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels have a fast run-down in activity after patch excision from the dendritic knob of rat olfactory sensory neurons (Reisert et al., 2003) and that a detectable chloride current is present only in 75% of the patches from mouse olfactory sensory neurons (Fig. 6 C of Pifferi et al., 2009b).

A few recent studies have also provided some evidence for the presence of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels in vomeronasal sensory neurons. Yang and Delay (2010) used the perforated patch-clamp recordings with gramicidin, a technique that does not modify the intracellular  $\text{Cl}^-$  concentration, and demonstrated that 80% of the urine-induced current was carried by  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels. These authors also showed that  $\text{Ca}^{2+}$  influx is



**Figure 10.** TMEM16A and TMEM16B are expressed in the microvilli of both apical and basal vomeronasal sensory neurons. TMEM16A (guinea pig antibody) is expressed in the microvilli of both apical neurons, as shown by PDE4A (A–C) immunoreactivity, and basal neurons, labeled with rabbit anti- $\text{G}\alpha\text{o}$  antibody (D–F). TMEM16B (guinea pig antibody) is also located in the microvilli of both apical (G–I) and basal (J–L) neurons. Cell nuclei were stained by DAPI (blue). Bars, 5  $\mu\text{m}$ .

necessary to activate the  $\text{Cl}^-$  channels. In another study, Kim et al. (2011) confirmed the previous study of Yang and Delay (2010) showing that a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current contributes to urine response, but they also suggested that this current can be activated both by  $\text{Ca}^{2+}$  influx through the TRPC2 channel and by  $\text{Ca}^{2+}$  release from intracellular stores. Indeed, they also showed that knockout mice for TRPC2 still have a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  component in the response to urine that is blocked by compounds that inhibit intracellular  $\text{Ca}^{2+}$  release, thus suggesting that a TRPC2-independent signaling pathway may be operating in the VNO and that the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current may play a relevant role in transduction.

Billig et al. (2011) compared whole cell recordings in vomeronasal sensory neurons from wild-type ( $n = 7$ ) or knockout mice for TMEM16B ( $\text{Ano2}^{-/-}$ ;  $n = 6$ ) obtained with  $1.5 \mu\text{M}$   $\text{Ca}^{2+}$  or  $0 \text{Ca}^{2+}$  in the pipette and reported that “currents of most  $\text{Ano2}^{-/-}$  VSNs were indistinguishable from those we observed without  $\text{Ca}^{2+}$  (Fig. 5n), but a few cells showed currents up to twofold larger. Averaged current/voltage curves revealed that  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  currents of VSNs depend predominantly on  $\text{Ano2}$  (Fig. 5l). Although  $\text{Ano1}$  is expressed in the VNO (Fig. 3a), its contribution to VSN currents seems minor.” Thus, although most of the  $\text{Ca}^{2+}$ -activated current was abolished in knockout mice for TMEM16B, a residual current was still present in some neurons. It is likely that the residual current is carried by TMEM16A channels. Indeed, both TMEM16A and TMEM16B are known to independently function as  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels (Scudieri et al., 2012), and we have demonstrated that TMEM16A and TMEM16B coexpress in microvilli of vomeronasal sensory neurons. Further experiments are necessary to unequivocally establish the origin of the residual current and to determine whether TMEM16A and TMEM16B form heteromeric channels and, if so, what the functional properties of the heteromeric channels are.

#### Physiological role of $\text{Ca}^{2+}$ -activated $\text{Cl}^-$ channels in vomeronasal and olfactory transduction

What is, then, the physiological role of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels in vomeronasal sensory neurons? Depending on the  $\text{Cl}^-$  equilibrium potential, these channels may contribute to the neuron depolarization or hyperpolarization. In vomeronasal sensory neurons, estimates of the  $\text{Cl}^-$  concentration inside the neurons and in the fluid filling its lumen are not available yet. However, experiments with the perforated patch-clamp recordings with gramicidin showed that the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current acts to further amplify a primary inward depolarizing current (Yang and Delay, 2010), indicating that the intracellular  $\text{Cl}^-$  concentration was relatively high. In addition, the same authors showed that bumetanide, a specific blocker for the sodium potassium–chloride cotransporter NKCC1, significantly decreased the urine-induced

inward current, indicating that NKCC1 is involved in chloride accumulation.

In olfactory sensory neurons, direct measurements of  $\text{Cl}^-$  concentrations showed that these neurons maintain an unusually high internal concentration of  $\text{Cl}^-$  of  $\sim 50 \text{mM}$ , which is similar to the  $\text{Cl}^-$  concentration present in the mucus at the external side of the ciliary membrane (Reuter et al., 1998; Kaneko et al., 2001, 2004). Therefore, in physiological conditions, the opening of  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels causes an efflux of  $\text{Cl}^-$  ions from the cilia, which contributes to neuron depolarization. Up to 80% of the transduction current can be carried by  $\text{Cl}^-$ . Furthermore, studies from several laboratories indicated that TMEM16B is the main constituent of the  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels involved in olfactory transduction. Indeed, it has been shown that TMEM16B is expressed in the ciliary layer of the olfactory epithelium, that currents in olfactory sensory neurons and in HEK 293T cells transfected with TMEM16B have very similar characteristics, and that knockout mice for TMEM16B did not show any detectable  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current (Pifferi et al., 2012). However, Billig et al. (2011) found that disruption of TMEM16B did not reduce mouse performance in some classical olfactory behavioral tasks, suggesting that  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels may be dispensable for near-normal olfaction. Future experiments will have to establish whether  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels are involved in more subtle aspects of olfactory sensing not detected in previous experiments.

It is of interest to note that, despite their differences in the molecular mechanisms of transduction, both olfactory and vomeronasal neurons express members of the TMEM16 family at the site of transduction, indicating that these channels are likely to play a physiological role in sensory transduction.

#### Conclusions

Our data contribute to the present understanding of the molecular mechanisms of vomeronasal transduction by providing the first direct evidence of the presence of a large  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current in the apical region of mouse vomeronasal sensory neurons and of the coexpression of the two  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels TMEM16A and TMEM16B in the microvilli of the same sensory neurons. These observations suggest that TMEM16A and TMEM16B are likely to be responsible for the  $\text{Cl}^-$  current reported in this work.

In conclusion, collectively with previous studies indicating the presence of a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  component in urine response (Yang and Delay, 2010; Kim et al., 2011), our results contribute to the indication that  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  channels could play a role in vomeronasal transduction. Future studies in mice in which the TMEM16A and/or TMEM16B gene are deleted will increase our understanding of the role of intracellular  $\text{Ca}^{2+}$  elevation in vomeronasal transduction.



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