Symposium Report

Anoctamin 2/TMEM16B: a calcium-activated chloride channel in olfactory transduction

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In vertebrate olfactory transduction, a Ca^{2+}-dependent Cl^- efflux greatly amplifies the odorant response. The binding of odorants to receptors in the cilia of olfactory sensory neurons activates a transduction cascade that involves the opening of cyclic nucleotide-gated channels and the entry of Ca^{2+} into the cilia. The Ca^{2+} activates a Cl^- current that, in the presence of a maintained elevated intracellular Cl^- concentration, produces an efflux of Cl^- ions and amplifies the depolarization. In this review, we summarize evidence supporting the hypothesis that anoctamin 2/TMEM16B is the main, or perhaps the only, constituent of the Ca^{2+}-activated Cl^- channels involved in olfactory transduction. Indeed, studies from several laboratories have shown that anoctamin 2/TMEM16B is expressed in the ciliary layer of the olfactory epithelium, that there are remarkable functional similarities between currents in olfactory sensory neurons and in HEK 293 cells transfected with anoctamin 2/TMEM16B, and that knockout mice for anoctamin 2/TMEM16B did not show any detectable Ca^{2+}-activated Cl^- current. Finally, we discuss the involvement of Ca^{2+}-activated Cl^- channels in the transduction process of vomeronasal sensory neurons and the physiological role of these channels in olfaction.

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The sense of smell and olfactory transduction

The sense of smell allows the organisms to detect chemicals present in the external environment. Vertebrates mainly detect odorants by the main olfactory epithelium located in the nasal cavity (Fig. 1*A*), but rodents and many other mammals also use additional sensory systems, such as the vomeronasal organ, which is mainly involved in pheromone detection, the septal organ and the Grüneberg ganglion (Munger *et al.* 2009; Tirindelli *et al.* 2009).

In the main olfactory epithelium, primary olfactory sensory neurons (OSNs) are responsible for the detection of odorant molecules and the generation of the neural signal that is transmitted to the brain (Fig. 1*B*). At the apical part of these bipolar neurons, the dendritic tip is slightly enlarged into an olfactory knob, from which tens of cilia protrude into the olfactory mucus that covers the surface of the epithelium (Fig. 1*A*–*C*). A single axon projects from the basal part of the neuron directly to the olfactory bulb. The cilia are the site of sensory transduction; indeed, at this level odorant molecules bind to odorant receptors, and this interaction triggers an increase in the intraciliary concentration of cAMP through the activation of the receptor-coupled G protein and adenylyl cyclase (Fig. 1*D*). Cyclic nucleotide-gated (CNG) channels located in the ciliary membrane are directly activated by cAMP, inducing a depolarizing influx of Na^+ and Ca^{2+} ions (reviewed by Schild & Restrepo, 1998; Pifferi *et al.* 2006*a*, 2009*c*; Kleene, 2008). The intracellular increase of Ca^{2+} concentration directly gates Ca^{2+}-activated Cl^- channels (CaCCs). The OSNs maintain an unusually high internal concentration of Cl^- (about 50 mM), which is in the same range as the 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 CaCCs causes an efflux of Cl^- ions from the cilia, corresponding to an inward current that further contributes to the depolarization of OSNs (Kleene & Gesteland, 1991; Kleene, 1993, 1997; Kurahashi & Yau, 1993; Lowe & Gold, 1993; Boccaccio & Menini, 2007; reviewed by Frings *et al.* 2000; Kleene, 2008; Frings, 2009). The depolarization spreads passively to the dendrite and soma of the neuron, triggering action potentials that are

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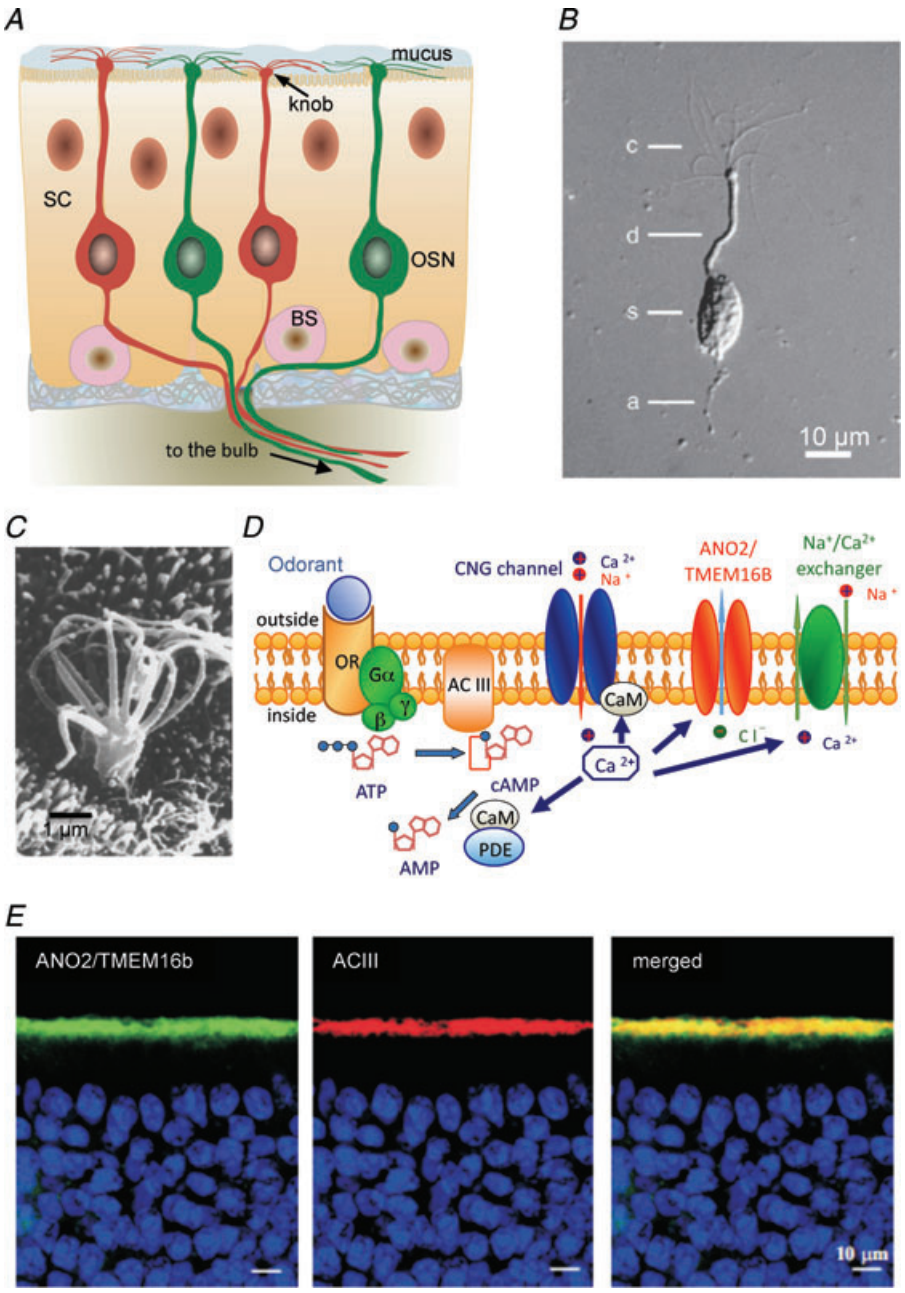

Figure 1. The olfactory epithelium and olfactory transduction
A, schematic diagram showing the various cell types composing the olfactory epithelium: OSN, olfactory sensory neuron; SC, supporting cell; and BS, basal cell. *B*, photograph of an isolated frog OSN under differential interference optics: c, cilia; d, dendrite; s, soma; and a, axon. Reprinted from Kleene & Gesteland (1981), copyright 1981, with permission from Elsevier. *C*, scanning electron micrograph of the knob of a human OSN showing the protrusion of several cilia. Adapted from Morrison & Costanzo (1990), with permission. *D*, schematic representation of the olfactory transduction taking place in the cilia. Abbreviations: ACIII, adenylyl cyclase III; CNG channel, cyclic nucleotide-gated channel; CaM, calmodulin; G, G protein; OR, odorant receptor; and PDE, phosphodiesterase. ANO2/TMEM16B indicates the Ca^{2+}-activated Cl^- channel. Modified from Pifferi *et al*. (2006*a*), with permission. *E*, immunostaining of sections of the mouse olfactory epithelium. Confocal micrographs showing TMEM16B and ACIII expression at the surface of the olfactory epithelium. Cell nuclei were stained by 4′,6-diamidino-2-phenylindole (DAPI). The image on the right was obtained by merging the left and centre images. Reprinted from Sagheddu *et al*. (2010).

conducted along the axon to the olfactory bulb. As OSNs have a high resting membrane resistance (>1 GΩ), a very small depolarizing current is sufficient to trigger action potentials (Lynch & Barry, 1989; Schild & Restrepo, 1998; Pun & Kleene, 2004).

Calcium-activated chloride channels in the cilia of olfactory sensory neurons

The presence of a Ca^{2+}-activated Cl^- current in frog OSNs has been known since the pioneering study of Kleene & Gesteland (1991), which showed that a rise in intraciliary Ca^{2+} concentration directly activates an anion-selective current in the ciliary membrane. Since this first study, the functional properties of olfactory CaCCs have been investigated with several electrophysiological techniques. For example, the field potential recorded at the surface of the olfactory epithelium in response to odorants (the electro-olfactogram) is primarily caused by the depolarizing action of Cl^- current, because more than 80% of the response can be blocked by niflumic acid, a blocker for CaCCs (Nickell *et al*. 2006). The large contribution of the Cl^- conductance to the transduction current was confirmed by experiments in isolated OSNs obtained with the suction pipette or in the whole-cell voltage-clamp configuration (Reisert *et al*. 2005; Boccaccio & Menini, 2007). In a set of experiments, the contribution of CaCCs to the transduction current was estimated by activating CNG channels in the ciliary region by flash photolysis of caged cAMP (Boccaccio & Menini, 2007). Upon flash photolysis, CNG channels are activated by cAMP, allowing the flux of Ca^{2+} ions in the cilia and the subsequent opening of CaCCs (Boccaccio *et al*. 2006; Boccaccio & Menini, 2007). The rising phase of the response at −50 mV in Ringer solution containing 1 mM Ca^{2+} was multiphasic, composed of a primary phase of the response due to Na^+ and Ca^{2+} influx through CNG channels and a secondary phase due to Cl^- efflux through CaCCs. Moreover, the secondary phase of the response was absent with low extracellular Ca^{2+} concentrations or at +50 mV, when the influx of Ca^{2+} through CNG channels is strongly reduced and therefore the contribution of CaCCs is expected to be negligible (Boccaccio & Menini, 2007). These experiments showed that up to 90% of the transduction current is carried by Cl^-.

A more precise characterization of the biophysical properties of olfactory CaCCs was achieved by experiments on excised inside-out membrane patches from the dendritic knob/cilia of mouse OSNs, a technique that allows a control of the concentration of Ca^{2+} at the intracellular side of the channels (Reisert *et al*. 2005; Pifferi *et al*. 2006*b*, 2009*b*). The dose–response relation was well fitted by the Hill equation with half-maximal activation between 2.2 and 4.7 μM Ca^{2+} and Hill coefficient between 2.0 and 2.8. Reisert *et al*. (2003) estimated a CaCC channel density of 62 μm^{-2} compared with only 8 μm^{-2} for CNG channels. Moreover, this conductance showed a Ca^{2+}-dependent inactivation, which was reversible after removal of Ca^{2+} for a few seconds, but also an irreversible run-down, indicating that some modulatory component of the channel may be lost after the excision of the membrane (Reisert *et al*. 2003, 2005). The olfactory CaCC is apparently not affected by Ca^{2+}–calmodulin (Kleene, 1999; Reisert *et al*. 2003) and, at present, no modulators of the channel activity are known.

All these studies clearly showed that CaCCs are present at a high density in the cilia of OSNs and that they contribute to the transduction current. However, it is important to note that, as previously pointed out (Smith *et al*. 2008; Kleene, 2009), it is possible that the secondary Cl^- current may not be required for normal olfactory behaviour. Indeed, given the high resting membrane resistance of OSNs, it is possible that a small primary current through CNG channels is sufficient to trigger action potentials and to allow near-normal olfactory behaviour.

Anoctamin 2/TMEM16B

The molecular identity of the CaCC present in the cilia of OSNs, as well as that of CaCCs in general, has been elusive for a long time. In 2008, three independent studies reported that anoctamin 1/TMEM16A displays many features of native CaCCs (Caputo *et al*. 2008; Schroeder *et al*. 2008; Yang *et al*. 2008). The role of TMEM16A as a CaCC suggested that other members of the family may also act as CaCCs (reviewed by Flores *et al*. 2009; Galietta, 2009; Hartzell *et al*. 2009; Kunzelmann *et al*. 2011). Indeed, when anoctamin 2/TMEM16B (which we will term TMEM16B in this review) was expressed in axolotl oocytes (Schroeder *et al*. 2008) or in HEK 293 cells (Pifferi *et al*. 2009*a*; Sagheddu *et al*. 2010; Stephan *et al*. 2009; Stöhr *et al*. 2009) it also displayed properties resembling those of CaCCs.

In 2005, Yu *et al*. showed by *in situ* hybridization that TMEM16B is expressed in mature sensory neurons of the mouse olfactory epithelium, well before knowing its possible role as a CaCC. Moreover, TMEM16B was found to be a prominent protein in the olfactory ciliary proteome (Mayer *et al*. 2009; Stephan *et al*. 2009), and the relative expression level between TMEM16B and CNG channel subunits (Rasche *et al*. 2010) was in good agreement with the electrophysiological results obtained by Reisert *et al*. (2003). Immunohistochemistry experiments (Fig. 1*E*) showed that TMEM16B is expressed in the ciliary layer of the olfactory epithelium (Hengl *et al*. 2010; Rasche *et al*. 2010; Sagheddu *et al*. 2010; Billig *et al*. 2011) together with the Na^+–K^+–2Cl^- cotransporter NKCC1 (Hengl *et al*.

2010), which mediates Cl^- accumulation into the cilia (Reisert *et al*. 2005).

A side-by-side comparison of the functional properties measured in excised inside-out patches from the native olfactory current and the TMEM16B-induced current in HEK 293 cells showed remarkable similarities (Fig. 2*A* and *B*). Dose–response relations indicate that the half-maximal concentration of Ca^{2+} is very similar: 4.9 μM for TMEM16B and 4.7 μM for native channels at −50 mV (Fig. 2*C*). Moreover, both channels have the same anion selectivity; indeed, they are more permeable to anions larger than Cl^-, and almost impermeable to methanesulfonate (Fig. 2*D*–*F*). TMEM16B and native CaCCs show also a similar sensitivity to Cl^- channel blockers; both are reversibly blocked by niflumic acid but are insensitive to SITS (Fig. 2*G*–*I*). Moreover, TMEM16B

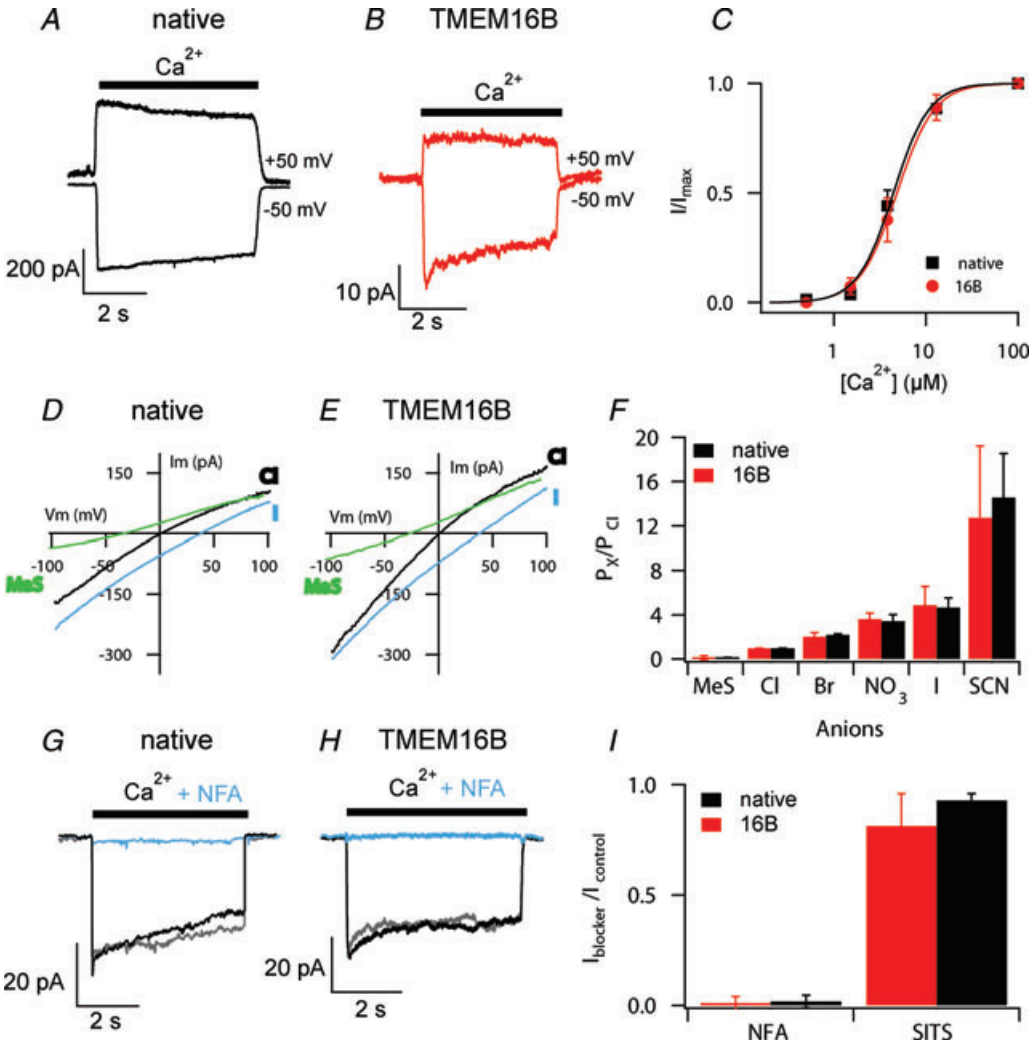

Figure 2. Comparison between the electrophysiological properties of native olfactory Ca^{2+}-activated Cl^- channels and TMEM16B-induced currents in HEK 293 cells
Currents were measured by exposing the cytoplasmic side of membrane patches excised from dendritic knob/cilia of mouse olfactory sensory neurons or from HEK 293 cells expressing TMEM16B. *A* and *B*, currents activated by 100 μM Ca^{2+} at −50 or +50 mV. *C*, normalized currents measured at −50 mV were plotted *versus* Ca^{2+} concentrations and fitted to the Hill equation. The Ca^{2+} concentration producing half-maximal activation was 4.7 μM for olfactory native currents and 4.9 μM for TMEM16B-induced currents. The Hill coefficient was 2.5. *D* and *E*, current–voltage relations from a ramp protocol activated by 100 μM Ca^{2+}. Bath solutions contained 140 mM NaCl, or the sodium salt of iodide (I) or of methanesulfonate (MeS), as indicated. *F*, comparison between the relative permeability ratios (P_x/P_{Cl}) calculated with the Goldman–Hodgkin–Katz relation from measured reversal potentials. *G* and *H*, intracellular blockage by 300 μM niflumic acid (NFA) of the current activated by 100 μM Ca^{2+} at −50 mV. *I*, comparison between the current ratios measured at −50 mV in the presence and in the absence of 300 μM NFA or 5 mM SITS. Modified from Pifferi *et al*. (2006*b*), copyright (2006) National Academy of Sciences, USA, and from Pifferi *et al*. (2009*a*), with permission.

displays a Ca^{2+}-dependent inactivation and an irreversible run-down in a similar manner to native olfactory CaCCs (Reisert *et al.* 2003; Pifferi *et al.* 2009*a*; Stephan *et al.* 2009).

A more recent study also reported a side-by-side comparison obtained in whole-cell recordings with flash photolysis of caged Ca^{2+}, showing that the reversal potential for some external large anions changes with time, both in native olfactory CaCCs and in TMEM16B-induced currents in HEK 293 cells (Sagheddu *et al.* 2010). Further experiments are required to establish the mechanisms of dynamic selectivity. This behaviour was also observed in TMEM16A expressed in axolotl oocytes (Schroeder *et al.* 2008) and in cation channels such as TRPV1 and P2X (Khakh & Lester, 1999; Chung *et al.* 2008).

Recently, Billig *et al.* (2011) succeeded in knocking out TMEM16B in mice and showed that Ca^{2+}-activated Cl^- currents were undetectable in knockout mice. This important result, together with previous data from several laboratories, clearly indicates that TMEM16B is the principal subunit of the ciliary CaCC.

What about the physiological role of TMEM16B in olfaction? Confirming the initial report on *NKCC1* knockout mice (Smith *et al.* 2008), Billig *et al.* (2011) found that disruption of TMEM16B did not reduce mouse performance in some olfactory behavioural tasks, suggesting that CaCC may be dispensable for near-normal olfaction.

Calcium-activated chloride channels in vomeronasal sensory neurons

Some recent results indicated the presence of a Ca^{2+}-activated Cl^- current in vomeronasal sensory neurons also, where it contributes up to 80% of the response to urine (Yang & Delay, 2010; Kim *et al.* 2011). In addition, experiments using flash photolysis of caged Ca^{2+} in the microvilli of isolated mouse vomeronasal sensory neurons showed that a large Cl^- current of more than 300 pA can be activated at −50 mV by Ca^{2+} in symmetrical Cl^- solutions (M. Dibattista & A. Menini, unpublished results). Immunohistochemistry experiments showed that TMEM16B (Rasche *et al.* 2010) and TMEM16A are expressed at the apical surface of the vomeronasal epithelium (Billig *et al.* 2011). Furthermore, Billig *et al.* (2011) measured Ca^{2+}-activated currents in vomeronasal sensory neurons that were not present in knockout mice for TMEM16B.

Conclusions

Current evidence suggests that anoctamin 2/TMEM16B is the major subunit of the Ca^{2+}-activated Cl^- current in the cilia of OSNs, although other subunits may also be expressed. However, the physiological role of this current in olfaction remains unclear, because knockout mice for TMEM16B show near-normal olfactory behaviour. Future experiments will have to establish whether CaCCs are involved in a slight increase of olfactory sensitivity not detected in previous experiments. It is possible that their presence at high density in the olfactory cilia is useful to preserve the detection of odorants also in the presence of modifications that may change extracellular ion concentrations. Furthermore, recent studies indicated that vomeronasal sensory neurons also possess a high density of CaCCs in their microvilli and that both TMEM16A and TMEM16B are expressed at the apical surface of the vomeronasal epithelium. Further experiments will be necessary to clarify the role of CaCCs in the vomeronasal system.

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