TMEM16 chloride channels are two-faced

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Elucidating the oligomeric structure of ion channels is central to understanding their function (Marsh and Teichmann, 2015). The vast majority of ion channels are formed by multiple subunits organized as an annulus surrounding a solitary, centrally located ion-conducting pore. For example, all the potassium channels, Cys-loop receptors like nicotinic acetylcholine receptors and GABA receptors, glutamate receptors, acid-sensing ion channels and epithelial sodium channels, cyclic nucleotide-gated and transient receptor potential channels, orai channels, ryanodine receptors and IP_3 receptors, connexins, and bestrophins are formed by three to six subunits bordering a single aperture. Similarly, voltage-gated Ca^{2+} and Na^{2+} channels have a pore surrounded by four subunits that fused into a single polypeptide during evolution in eukaryotes. However, a small fraction of channels are oligomers, with each subunit forming its own pore. The CLC chloride channels (dimers), aquaporins (tetramers), voltage-gated proton channels (dimers), bacterial porins (trimers), and two-pore Ca^{2+} channels (dimers of a two-repeat fusion protein) fall into this multiple pore/multiple subunit category. Two papers in this issue of *The Journal of General Physiology* by Lim et al. and Jeng et al. add another protein to this minority, namely the Ca^{2+}-activated Cl^- channel (CaCC) TMEM16A (also known as ANO1). The findings in these papers raise fundamental questions about the structural principles underlying ion channel pores by suggesting that the Cl^- ion conduction pathways are located, not in the center of the protein, as we have come to expect, but rather on the surface of the protein in contact with the membrane bilayer.

The anoctamin proteins have recently attracted a great deal of attention because the discovery of their founding members, *TMEM16A* and *TMEM16B*, ended a decade-long search for CaCC genes (Pedemonte and Galietta, 2014; Whitlock and Hartzell, 2017). CaCCs are activated by increases in cytosolic Ca^{2+} and are crucial for many cellular functions including epithelial secretion, regulation of smooth muscle tone, gut motility, neuronal excitability, and nociception. Biochemical and fluorescence resonance energy transfer (FRET) studies have demonstrated that TMEM16A exists as a homodimer, with each subunit containing a Ca^{2+}-binding site that is crucial for channel opening (Fallah et al., 2011; Sheridan et al., 2011). However, answering the fundamental question of whether the TMEM16A channel has a single pore at the dimer interface, or each subunit has its own pore, has been less straightforward than expected.

The two papers in this issue take similar attacks on this question. The investigators create concatemers comprised of two copies of TMEM16A covalently connected in a head-to-tail arrangement by a 31–amino acid flexible linker. By introducing a mutation into one subunit that alters channel function in a way that can be measured electrophysiologically, the contribution of each subunit to the ionic current can be assessed. These investigators first study mutations that alter the ability of Ca^{2+} to open the channel. The amino acids responsible for Ca^{2+} binding to TMEM16A had previously been identified by mutagenesis (Yu et al., 2012; Brunner et al., 2014; Tien et al., 2014). Ca^{2+} is stabilized in its binding site by oxygen atoms contributed by the side chains of four acidic residues in transmembrane domains (TMDs) 7 and 8 (E702, E705, E734, and D738) and an additional glutamic acid (E654) in TMD6. It should be noted that the amino acid numbering in this Commentary has been chosen to coincide with the a,c isoform used by Lim et al. (2016) (four should be added to find the equivalent residue in the Jeng et al. [2016] paper). The TMEM16A splice variants used by the two groups differ by the presence or absence of exon 6, which encodes 4 amino acids. This exon affects the Ca^{2+} sensitivity of the channel quantitatively, but otherwise, the behavior of the two isoforms is very similar (Ferrera et al., 2009; Xiao et al., 2011). Both groups design a concatemer composed of one WT subunit and one subunit containing a mutation of E702 that significantly reduces the Ca^{2+} sensitivity of the channel. The Cl^- current encoded by constructs containing E702 mutations in one subunit activates in a biphasic manner as Ca^{2+} concentration is increased. The Ca^{2+} dose–response curves can be fitted with two EC_{50} values consistent with the independent activation of each subunit (Jeng et al., 2016; Lim et al., 2016).

Although these data provide convincing demonstrations that each subunit can be activated independently,

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they unfortunately do not prove that each subunit has its own distinct pore. One could argue that the dimer has only one pore but that activation of one subunit produces a partial opening and that activation of both subunits dilates the pore further. This idea seems possible because previously published observations showed that TMEM16A becomes less anion selective as Ca^{2+} concentration increases, as if the anatomy of the pore changes with Ca^{2+} concentration (Schroeder et al., 2008; Xiao et al., 2011). However, the two papers in this issue present evidence to the contrary: they conclude that the channel may be more anion selective than previously recognized and that selectivity does not change with Ca^{2+} concentration. Whereas previous studies measured anion/cation selectivity using a NaCl gradient with sucrose or mannitol to compensate osmolarity, these investigators replace NaCl with impermeant $NMDG_2(SO_4)$ and subtract background currents in the absence of activating Ca^{2+}. Under these conditions, TMEM16A appears to be highly Cl^- selective at all Ca^{2+} concentrations. This observation weakens a single-pore model that invokes pore dilation.

Nevertheless, to answer the one- versus two-barrel question rigorously, mutants that alter the biophysical properties of the pore itself are required. Years ago, when the ion channel field was faced with a similar question regarding the functional oligomerization of CLC channels, the problem was solved using concatemers of subunits, each having a different single channel conductance (γ). Single channel recordings of these constructs exhibited two distinct current levels, each contributed by one subunit controlled by its own gate. In the case of TMEM16A, this approach was not feasible. First, single channel recordings are extremely challenging because the estimated γ is so small (1–3 pS). Second, there are no known mutations that clearly alter single channel conductance or anion/cation selectivity, despite considerable effort and disagreement. Although the R621E mutation (Yang et al., 2008) was reported to alter anion/cation selectivity, this finding has been disputed (Yu et al., 2012). The K588Q mutation was also reported to alter selectivity (Yang et al., 2012), but Jeng et al. (2016) could not reproduce these data.

Although the K588Q mutant did not have the altered ion selectivity that Jeng et al. (2016) expected, it did have stronger outward rectification than WT. When testing concatemers composed of one WT and one mutant subunit, Jeng et al. (2016) found that rectification of the current activated by 20 µM Ca^{2+} was significantly greater for the WT + K588Q concatemer than for the WT + (K588Q/E702C) concatemer. Because E702C is expected to reduce the Ca^{2+} sensitivity of the subunit harboring the K588Q mutation, this subunit would not be significantly activated by 20 µM Ca^{2+}, and thus less outward rectification would be apparent. Although these experiments are consistent with the double barrel hypothesis, skeptics might argue that the interpretation is complicated by the observation that the rectification of the WT channel is strongly Ca^{2+} dependent (Xiao et al., 2011). However, the Ca^{2+} dependence of WT TMEM16A probably involves voltage-dependent changes in channel open probability that do not occur at the high Ca^{2+} concentrations used here. Nevertheless, if rectification is an allosteric property of the channel enacted at some site remote from the pore, rectification might be a poor reporter for the pore itself.

Lim et al. (2016) take a slightly different approach. They make a more drastic mutation in K588 (K588E) in the hope of augmenting the ionic selectivity change, but the mutants remain highly anion selective. Nevertheless, the current amplitudes are very small, suggesting that the K588E single channel conductance has been reduced. They confirm this by nonstationary noise analysis and show that K588E channels have an estimated γ (1.05 pS) less than half that of WT (2.63 pS). However, the data are less compelling than the authors would have hoped because a reliable estimate of γ from nonstationary noise analysis requires that the plot of variance versus current amplitude describes a parabola, but the K588E data do not fulfil this requirement. Nevertheless, the data from the concatemer (one WT subunit and one K588E subunit with one or the other subunit having reduced Ca^{2+} sensitivity owing to the E702Q mutation) are consistent with the double-barreled model.

Despite these minor caveats, these authors together make a convincing case that TMEM16A (and by analogy TMEM16B) are double-barreled channels. Unfortunately, the annoying properties of the TMEM16 channels (small single channel conductance, lack of suitable mutations, and biophysical properties that may change with Ca^{2+} concentration) conspire to scuttle experiments like the single channel studies that demonstrated the double-barreled nature of the CLCs.

One important potential experimental artifact that both groups effectively rule out is the possibility that concatemers do not necessarily ensure the stoichiometry of the assembled channel. For example, concatemers have the potential to assemble into higher-order oligomers that function differently than the native dimer (McCormack et al., 1992). Lim et al. (2016) address this question biochemically by showing that the concatemers migrate as dimers both in denaturing SDS gels and in native gel filtration. Further, they show that the concatemers exhibit the same functional properties (channel gating, Ca^{2+} sensitivity, and single channel conductance) as WT subunits, which dimerize biologically rather than chemically. In addition, Jeng et al. (2016) compare FRET between subunits and between concatemers tagged with acceptor and donor fluorophores on the C termini. Although the subunits show measureable FRET, the concatemers do not show FRET,

suggesting that concatemers are not oligomerizing with one another to form higher-order oligomers.

A major advance in our understanding of the TMEM16 family came in 2014, when Brunner et al. (2014) solved the atomic structure of a fungal TMEM16 homologue from *Nectria haematococca* called nhTMEM16. This structure validated previous studies on vertebrate TMEM16A: the nhTMEM16 protein is a homodimer, and the Ca^{2+}-binding site is highly conserved. But nhTMEM16 is not a Cl^- channel. Like another fungal TMEM16 isolated from *Aspergillus fumigatus* (afTMEM16, in the same phylum; Malvezzi et al., 2013), nhTMEM16 is a phospholipid scramblase when purified and reconstituted into liposomes (Brunner et al., 2014). It turns out that the TMEM16 family has a split personality: to date, only vertebrate TMEM16A and TMEM16B have been shown unambiguously to be Cl^- channels. Despite the high sequence similarity of TMEM16A to the other TMEM16s (mouse TMEM16A is 51–59% identical to TMEM16C-16G, excluding the variable cytoplasmic N and C termini), 6 of the 10 mammalian TMEM16 proteins as well as the fungal TMEM16s are Ca^{2+}-dependent phospholipid scramblases (Whitlock and Hartzell, 2017). The structure of the nhTMEM16 dimer reveals the pathway that is potentially used for scrambling phospholipids, one located on the surface of each subunit. The finding that the Cl^- channel TMEM16A also has two pathways is consistent with the idea that lipid scramblases and Cl^- channels use an evolutionarily related structure for these two functions (Whitlock and Hartzell, 2016).

What exactly is phospholipid scrambling? Phospholipid scramblases facilitate the translocation (scrambling) of phospholipids between leaflets of the membrane bilayer (Bevers and Williamson, 2016). Typically, membrane phospholipids are organized as two mono-molecular leaflets with their hydrophilic head groups facing outward toward the aqueous medium and their hydrophobic hydrocarbon chains pointing inward toward the central core. The composition of the two leaflets in the plasma membrane is different—the outer leaflet is enriched in phosphatidylcholine and sphingomyelin and the inner leaflet is enriched in phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine. This lipid asymmetry is generated by ATP-dependent flippases and floppases that actively transport lipids between leaflets. The translocation of phospholipids down their concentration gradients is very slow because there is a large energy barrier for the hydrophilic head groups to flip spontaneously through the hydrophobic core of the membrane (15–50 kcal/mol). Phospholipid scramblases serve to assist with downhill translocation by forming channels that provide a hydrophilic pathway between leaflets and to lower the energy barrier for the passive movement of hydrophilic lipid headgroups from one leaflet to the other. Phospholipid scrambling has two major consequences: phospholipids that are normally sequestered in the cytoplasmic leaflet (mainly phosphatidylserine and phosphatidylethanolamine) become exposed on the cell surface. These externalized phospholipids are recognized by receptors that initiate various signaling cascades. In addition, phospholipid scrambling changes the physical properties of the membrane in ways that favor membrane fusion or fission events, alter the function of integral membrane proteins, and recruit new proteins to the membrane (Whitlock and Hartzell, 2017).

nhTMEM16 is a dimer with each subunit containing 10 membrane helices (Fig. 1 and Video 1; Brunner et al., 2014). A notable feature of each subunit is a hydrophilic furrow, lined by helices 4–6, which is located on the opposite surface to the dimer interface and connects the cytoplasmic and extracellular sides of the membrane. This furrow is thought to function like an open aqueduct to provide a hydrophilic pathway for the phospholipid head groups to translocate between leaflets while the hydrocarbon tails remain in the hydrophobic phase of the bilayer. Thus, nhTMEM16 can be viewed as a sort of "channel" for lipid head groups. However, the "channel" is not enclosed on all sides by protein as it is in ion channels, but rather it is a furrow enclosed by protein only on one side. The nhTMEM16 structure does not reveal lipids within the furrow, but several groups have now produced molecular dynamics simulations depicting the passage of lipid headgroups along the nhTMEM16 aqueducts (Whitlock and Hartzell, 2016). The dimensions of the aqueduct are sufficient to accommodate phospholipid headgroups and are consistent with the broad range of lipids that have been shown to be translocated by afTMEM16, nhTMEM16, TMEM16F, and other Ca^{2+}-activated lipid scramblases (Suzuki et al., 2010; Malvezzi et al., 2013; Brunner et al., 2014; Bevers and Williamson, 2016).

The mammalian TMEM16s, including TMEM16A, are likely to have a similar structure to nhTMEM16 because human TMEM16s have ∼25% sequence identity in their TMDs to nhTMEM16, they are dimers, their transmembrane topology closely matches that predicted for nhTMEM16, and the location of the amino acids that coordinate Ca^{2+} are highly conserved (Fig. 1 A). One side of the nhTMEM16 aqueduct is lined by the scramblase domain formed by TMDs 4–5 (recently identified in TMEM16F; Yu et al., 2015). Because nhTMEM16 and the mammalian TMEM16 proteins are predicted to have similar transmembrane topology and structure (Whitlock and Hartzell, 2016), the question arises whether Cl^- ions in TMEM16A take the same furrowed pathway as lipids in nhTMEM16. One strong argument that ions and lipids do take a structurally conserved path has been provided by mutagenesis studies of TMEM16A, which have identified amino acids that are important in ion transport to be

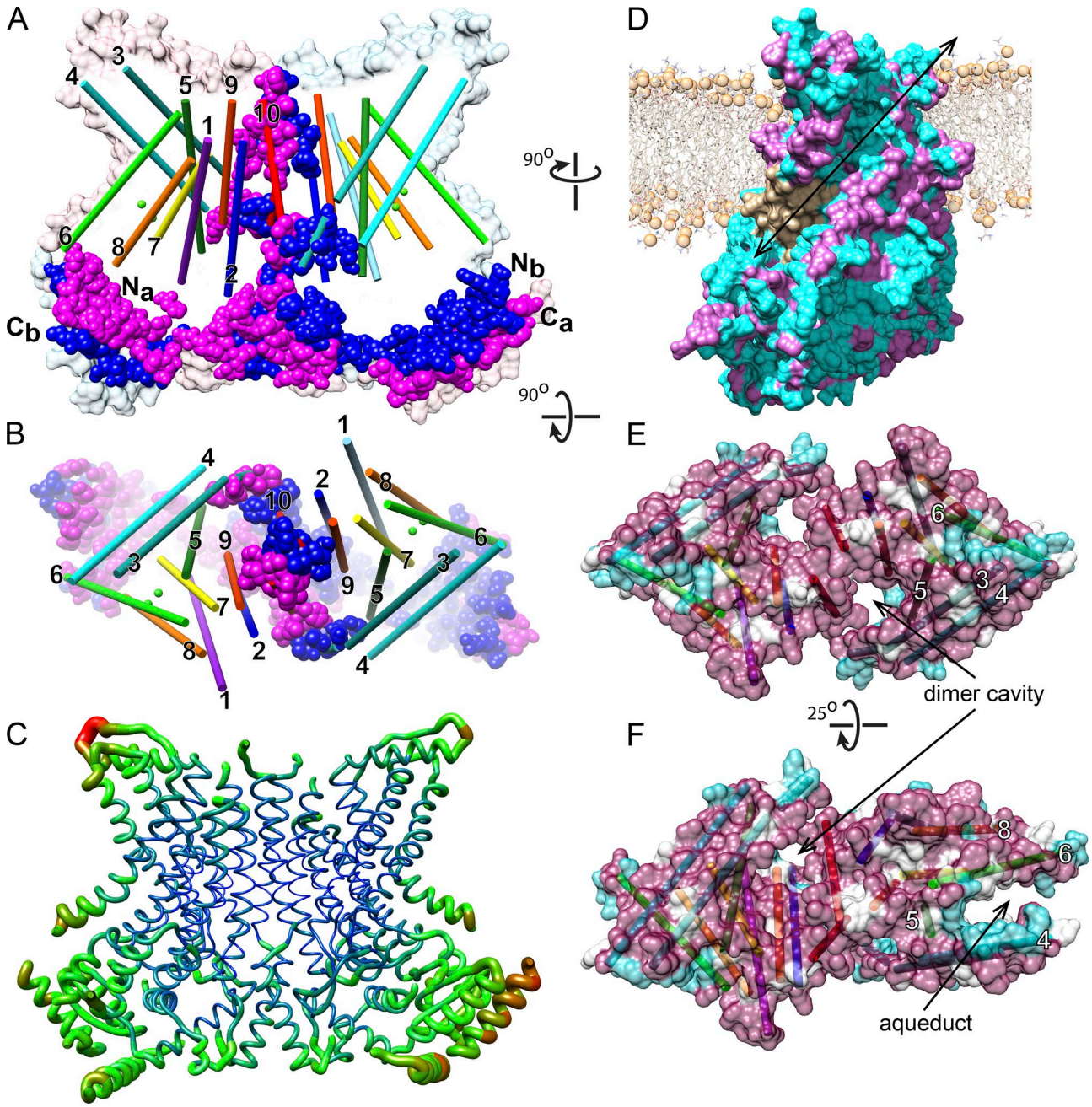

Figure 1. **The structure of nhTMEM16.** (A) Side view from the plane of the membrane. The cutaway surface of subunit *a* is colored pink, and the cutaway surface of subunit *b* is light blue. Within the cutaway surfaces, transmembrane helices are represented as cylinders colored violet (TMD1) to red (TMD10). The TMDs are numbered in subunit *a* but unlabeled in subunit *b*. Interfacial residues (http://www.ebi.ac.uk/pdbe/pisa/) between subunits *a* and *b* are shown as spheres (subunit *a*, magenta; subunit *b*, blue). The C terminus of each subunit (C_a and C_b of subunits *a* and *b*, respectively) extends to wrap around the N terminus (N_a and N_b) of the other. Green spheres are activating Ca^{2+} ions. (B) View from the extracellular space showing the interface between subunits along TMD10 and the cytoplasmic end of TMD3. (C) nhTMEM16 B-factors. B-factors are represented as the color and thickness of the worms (from blue = 62 to red = 288). The cytosolic N and C termini show considerable disorder compared with the TMDs. (D) Surface representation of the hydrophilic aqueduct. The structure in A was rotated in the plane of the membrane 90°. Hydrophilic residues are cyan; hydrophobic residues are magenta. Amino acids corresponding to the scrambling domain identified in TMEM16F are colored tan. (E) Dimer cavities. The same view as in B. The surface is translucent to reveal underlying transmembrane helices shown as cylinders (TMD3–6 are numbered). The surface was constructed using only amino acids in transmembrane helices and is colored cyan for hydrophilic and magenta for hydrophobic. (F) The surface in E was rotated 25° to reveal the aqueduct.

clustered around the aqueduct (Yu et al., 2012; Peters et al., 2015; Lim et al., 2016; Whitlock and Hartzell, 2016). However, although the aqueduct structure is perfectly suited for flipping an amphipathic phospholipid from one leaflet to the other, its structure seems antithetical to an ion channel because the permeant ion in the aqueduct would be exposed on one side to the hydrophobic core of the membrane.

When the nhTMEM16 structure first appeared, Brunner et al. (2014) proposed a solution to the puzzle of how the open aqueduct structure could be reconfigured to transport ions in the Cl^- channel members of the family. They suggested that the oligomeric organization of the TMEM16 Cl^- channels may be different from the TMEM16 scramblases (Fig. 2). The nhTMEM16 subunits are arranged back to back (Figs. 1 A and 2 A) with an extensive dimer interface that buries ~13% of the total molecular surface. More than half of the interface occurs between the cytoplasmic domains of the N and C termini that wrap around one another, whereas the remainder involves hydrophobic and ionic interactions between TMDs 10 and between TMD10 and TMD3 of the partner subunit (Fig. 1 A). Brunner et al. (2014) speculated that, although the TMEM16 scramblases are likely organized like nhTMEM16 with dimers arranged back to back and each aqueduct located on the periphery of the protein opposite the dimer interface, the subunits in TMEM16 Cl^- channels might be turned 180° and dimerize front to front to bring the aqueducts of each subunit together at the dimer interface to form a single, enclosed central ion conduction pathway (Fig. 2 C). The papers by Lim et al. (2016) and Jeng et al. (2016) call this model into question by showing that the channel is double-barreled.

However, the question of whether nhTMEM16 and TMEM16A dimerize differently is worthy of additional consideration. Amino acids 161–179 in the N terminus of TMEM16A are essential and sufficient for dimerization (Tien et al., 2013), and the C terminus does not seem to be involved. Peptides containing amino acids 161–179 coimmunoprecipitate with full-length TMEM16A and with themselves. Mutation of this dimerization domain, or overexpression of peptides containing the dimerization domain, significantly reduces TMEM16A currents. These results suggest that TMEM16A dimerization involves homotypic interaction of the N termini of the two subunits, but this model jars with the structure of nhTMEM16. In nhTMEM16, the N termini of adjacent subunits do not come within 20 Å of one another (Brunner et al., 2014). Furthermore, the observation that deletion of the C terminus of TMEM16A has no effect on channel function (Scudieri et al., 2013) suggests that the C terminus is not necessary for TMEM16A dimerization or that dimerization is not necessary for TMEM16A function/trafficking. Another possibility that should be considered is that the interaction of the N and C termini in the nhTMEM16 structure is an artifact of crystallization. The C terminus is rather disordered: the B-factors are relatively large (Fig. 1 C), and there are several stretches of amino acids that are not modeled. These flexible cytoplasmic domains may normally interact with other proteins in the cell and might fold on one another adventitiously in the absence of binding partners. In any case, it seems likely that the mechanisms of dimerization of TMEM16A and nhTMEM16

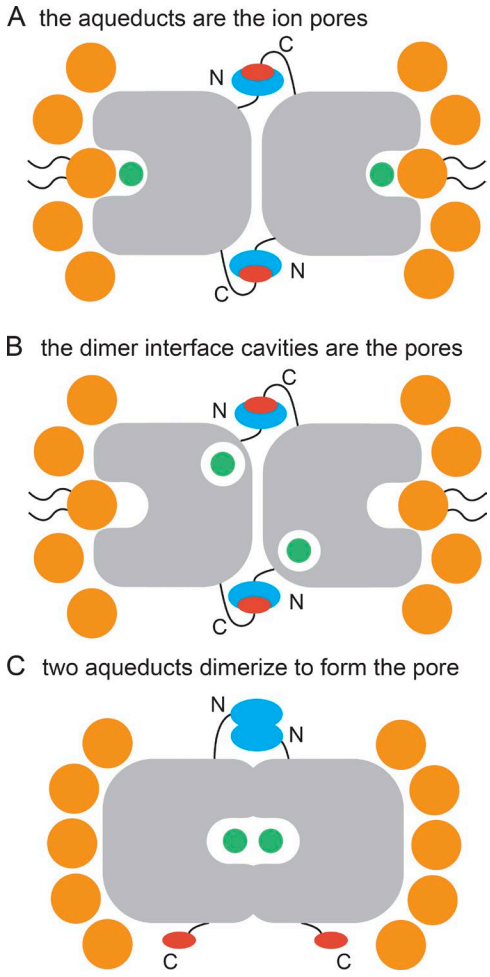

Figure 2. **Potential oligomeric organizations of TMEM16 Cl^- channels.** View from the extracellular space looking down on the membrane. (A) TMEM16A is represented as two gray subunits that dimerize back to back like nhTMEM16 via interaction between extended N and C termini and between TMDs at the interface. Each subunit adopts an open conformation when Ca^{2+} ions (not depicted) bind. The pore for Cl^- ions (green spheres) is formed in the space between the head groups of phospholipids (orange spheres) and the grooved aqueduct on the outer surface of each subunit. (B) Alternatively, cavities (white circles) at the dimer interface similar to those in the nhTMEM16 structure may form the Cl^- permeation pathway. (C) TMEM16A may dimerize differently than nhTMEM16, aqueduct to aqueduct, creating a central, protein-lined ion pore. Dimerization occurs via homotypic interaction between N termini. Two Cl^- ions are shown in the pore to suggest that this pore has dual pore characteristics.

are at least partly similar, because the sequence around the N-terminal dimerization domain of TMEM16A is reasonably well conserved in nhTMEM16.

Another way to explain how TMEM16A transports Cl^- is to propose that the ion-conducting pathway is structurally distinct from the aqueduct (Fig. 2 B). There are two substantial cavities at the dimer interface of nhTMEM16 bounded by TMD10 of one subunit and TMD3, TMD9, and TMD10 of the other subunit (Fig. 1). Each of these dimer cavities is ∼15 Å wide at the extracellular side, and they merge into a single vestibule at the intracellular side of the membrane. However, this is very unlikely to conduct ions because the surface of this cavity is lined almost exclusively by hydrophobic and aromatic amino acids, and most of the cavity is open to the membrane on one side. There are no other obvious pathways for ion conduction through the nhTMEM16 protein.

However, there is a caveat to the conclusion that TMEM16A shares the same structural pathway that is used for lipid translocation in nhTMEM16. nhTMEM16 has not been shown to support ionic currents (Brunner et al., 2014). In contrast, the closely related fungal afTMEM16 (65% similar to nhTMEM16) mediates both ion transport and phospholipid scrambling, but curiously, its ion transport is dependent on the composition of the lipid in which it is reconstituted. In membranes composed of 3:1 POPE/POPG, afTMEM16 ion transport is strongly suppressed (Malvezzi et al., 2013). However, because the published experiments showing that nhTMEM16 has no ion channel activity were performed in this lipid mixture (Brunner et al., 2014), it is very likely that nhTMEM16 supports ion transport.

If the Cl^- permeation pathway in TMEM16A is formed by the open aqueduct on the surface of the protein, how could Cl^- transport occur when the ion will be exposed to lipid along one side of the aqueduct? A clue may be provided by the observation that TMEM16F conducts not only lipids, but also ions that we believe move through a leak between the lipids and protein (Yu et al., 2015). We have suggested that TMEM16 Cl^- channels might have evolved from scramblases by loss of lipid scrambling activity while retaining the ionic leak pathway (Whitlock and Hartzell, 2016). One possible mechanism is that lipid head groups form part of the ion conduction pathway. For example, TMEM16A could stabilize a nonbilayer phase in the membrane so that the two leaflets are continuous where they interact with the aqueduct. If the aqueduct is partially obstructed, disallowing the lipids to move, the lipid head groups would then provide a hydrophilic environment around the open half of the pore and ions could move across the membrane in the "channel" formed between the protein and the lipid head groups (Fig. 2 A). Just as ionic currents flowing during lipid scrambling in TMEM16F likely represent leak of ions around the lipid–protein interface, the ions would flow through TMEM16A in the analogous space with the lipids playing a structural role. This unconventional pore structure can explain several unusual features of the TMEM16A currents (Whitlock and Hartzell, 2016).

The papers by Lim et al. (2016) and Jeng et al. (2016) raise several additional questions. One of the most interesting regards the mechanisms of TMEM16 dimerization. Do TMEM16 channels and scramblases use homologous domains for dimerizing? If TMEM16A dimerizes the same way that nhTMEM16 does, by interactions between the N and C termini, why does TMEM16A with its C terminus deleted form functional channels? What is the role of the TMD10 salt bridges observed in nhTMEM16 that are highly conserved in TMEM16 scramblases but are divergent in the TMEM16 channels? Why can TMEM16A heterodimerize with TMEM16B but not with TMEM16F? Finally, even if TMEM16A has two pores, are we certain where they are located? Do the hydrophilic aqueducts face outward toward the lipid environment of the membrane as these papers imply, or do they face inward, toward the cognate subunit, forming a dual pore in TMEM16A? After all, if the aqueducts face outward, they define a novel structural feature of ion channel pores that has not previously been recognized (Whitlock and Hartzell, 2016). An outward-facing aqueduct would require the membrane lipids to form part of the TMEM16 ion conduction pathway, denoting a novel ionic route through the membrane. This suggestion may also explain why the ionic conductance of afTMEM16 is dependent on the composition of the lipids in which it is reconstituted (Malvezzi et al., 2013).

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