STRUCTURE AND FUNCTION OF TMEM16 PROTEINS (ANOCTAMINS)

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TMEM16 proteins, also known as anoctamins, are involved in a variety of functions that include ion transport, phospholipid scrambling, and regulation of other membrane proteins. The first two members of the family, TMEM16A (anoctamin-1, ANO1) and TMEM16B (anoctamin-2, ANO2), function as Ca2+-activated Cl− channels (CaCCs), a type of ion channel that plays important functions such as transepithelial ion transport, smooth muscle contraction, olfaction, phototransduction, nociception, and control of neuronal excitability. Genetic ablation of TMEM16A in mice causes impairment of epithelial Cl− secretion, tracheal abnormalities, and block of gastrointestinal peristalsis. TMEM16A is directly regulated by cytosolic Ca2+ as well as indirectly by its interaction with calmodulin. Other members of the anoctamin family, such as TMEM16C, TMEM16D, TMEM16F, TMEM16G, and TMEM16J, may work as phospholipid scramblases and/or ion channels. In particular, TMEM16F (ANO6) is a major contributor to the process of phosphatidylserine translocation from the inner to the outer leaflet of the plasma membrane. Intriguingly, TMEM16F is also associated with the appearance of anion/cation channels activated by very high Ca2+ concentrations. Furthermore, a TMEM16 protein expressed in Aspergillus fumigatus displays both ion channel and lipid scramble activity. This finding suggests that dual function is an ancestral characteristic of TMEM16 proteins and that some members, such as TMEM16A and TMEM16B, have evolved to a pure channel function. Mutations in anoctamin genes (ANO3, ANO5, ANO6, and ANO10) cause various genetic diseases. These diseases suggest the involvement of anoctamins in a variety of cell functions whose link with ion transport and/or lipid scrambling needs to be clarified.

I. TMEM16A AND TMEM16B AS Ca2+-ACTIVATED Cl− CHANNELS

A. Identification

Of the more than 20,000 genes contained in the human genome, a significant fraction codes for membrane proteins with unknown function (4, 57). In 2008, three independent research teams found that one of these orphan proteins, TMEM16A, is a component of the Ca2+-activated Cl− channel, CaCC (29, 195, 256). This type of Cl− channel had been known at the functional level for more than 25 years, but there was no clear understanding of its molecular identity (48, 80). Typical features of this channel are as follows: 1) outward rectification of the steady-state current-voltage relationship due to activation and deactivation at positive and negative membrane potentials, respectively; and 2) activation by cytosolic Ca2+ with an apparent affinity that is increased by a change of the membrane potential to positive values. CaCC activity may be detected with a series of techniques including patch-clamp, short-circuit current recordings, and fluorescent measurements of I−/Cl− fluxes (FIGURE 1). As discussed later, TMEM16 proteins can also be studied after purification and reconstitution in artificial membranes (135, 219).

Many studies previously proposed a panel of possible molecular candidates for CaCCs, including CLCA, ClC-3, and bestrophins (48, 80, 116). However, the identification of these proteins as components of CaCCs was not totally convincing.

Yang et al. (256) found TMEM16A as a CaCC by searching among unknown proteins with multiple transmembrane segments. Expression of TMEM16A in heterologous expression systems generated Cl− currents activated by receptor-mediated intracellular Ca2+ mobilization. Coexpression of TMEM16A with endothelin, angiotensin, muscarinic, histamine, or purinergic receptors, followed by acute
stimulation with the corresponding receptor agonist, resulted in appearance of large CaCC currents. TMEM16A-dependent currents could also be activated by direct Ca$^{2+}$ application to the cytosolic side of excised membrane patches. The properties of such currents resembled very closely those of native CaCCs in terms of Ca$^{2+}$ sensitivity, voltage dependence, and response to pharmacological inhibitors. In particular, TMEM16A channels showed a typical feature of native CaCCs: the apparent Ca$^{2+}$ affinity was increased by membrane depolarization (EC$_{50}$ of 2.6 and 0.4 µM at −60 and +60 mV, respectively). The single-channel conductance of TMEM16A channels (8.3 pS) appeared larger than that reported for native CaCCs (1.8–3.5 pS) (174). However, in a more recent study, the single-channel conductance of TMEM16A, calculated by noise analysis, was found to be 3.5 pS (2). With regard to expression in native tissues, TMEM16A was found particularly expressed in pancreatic acinar cells, retinal cell layers, proximal renal tubules, sensory neurons of dorsal root ganglia, submandibular glands, and Leydig cells (256). Knockdown in vivo of TMEM16A by injected siRNA resulted in reduced fluid secretion by salivary glands.
To identify the CaCC protein, Schroeder et al. (195) decided to adopt an expression cloning approach after realizing that the oocytes of Axolotl salamander (Ambystoma mexicanum), unlike those of Xenopus laevis frogs, do not have endogenous activity of CaCCs. Interestingly, this difference is due to an important physiological reason. CaCC currents are used in Xenopus oocytes to generate the fertilization potential that prevents polyspermy. In contrast, CaCC currents are not needed in the oocytes of Axolotl salamanders, which are physiologically polyspermic. Injection of Axolotl oocytes with mRNA extracted from Xenopus oocytes generated robust CaCC currents. To identify the cDNA underlying CaCC activity, Schroeder et al. (195) generated a cDNA library. Axolotl oocytes were then utilized to express pools of cDNA clones (195). After cycles of pool fractionation and functional evaluation, a single active cDNA clone containing the coding sequence of TMEM16A was identified. Expression of TMEM16A generated Cl− currents with voltage and Ca2+ dependence resembling those of Xenopus CaCC (195). The TMEM16A-dependent channels were sensitive to classical CaCC blockers such as DIDS, niflumic acid, and NPPB. Similar currents were also found when TMEM16A was expressed in HEK-293, a cell line devoid of endogenous CaCC activity. Interestingly, the relative permeability of TMEM16A channels to various anions was not fixed but changed dynamically with the status of channel activation. Schroeder et al. (195) also investigated the activity of TMEM1B protein, a close paralog of TMEM16A. TMEM16B was also endowed with CaCC activity (195).

Caputo et al. (29) identified TMEM16A using a third type of approach. These authors and others had previously found that human bronchial epithelial cells respond to prolonged (24 h) treatment with interleukin-4 (IL-4) or with interleukin-13 (IL-13) by increasing the activity of CaCCs (40, 67), an effect that appeared to be mediated by upregulation of the corresponding gene(s). Therefore, a functional genomics strategy was adopted to identify the molecular identity of CaCCs. Gene expression microarrays were used to generate a short list of genes upregulated by IL-4 and coding for membrane proteins with unknown function (29). These candidates were then screened using functional assays combined with siRNA-based gene silencing. TMEM16A was the only gene whose downregulation resulted in a significant CaCC inhibition measured by a series of functional assays (iodide influx, short-circuit current, patch clamp) in different cell systems, including bronchial CFBE41o- cells, pancreatic CFPAC-1 cells, and primary cultures of human bronchial epithelial cells (29). Importantly, the effect of TMEM16A knockdown on CaCC activity was not affected by the type of activation (purinergic receptor stimulation versus Ca2+ ionophore). Furthermore, TMEM16A knockdown did not alter the increase in Ca2+ concentration caused by Ca2+ mobilizing agents. These results indicated that TMEM16A is not an indirect regulator of CaCCs (29).

TMEM16A belongs to a protein family whose members (10 proteins, labeled with letters from A to K, excluding I) have a length between 800 and 1,000 amino acid residues (106). TMEM16 proteins have a similar putative structure consisting of eight transmembrane domains and the NH2 and COOH terminals protruding into the cytosol (FIGURE 2). This structure is not only based on computer programs predicting number and localization of transmembrane domains but is also supported by experiments done on TMEM16G, also known as NGEP (41). Insertion of epitope tags at different positions of TMEM16G sequence was used to define the protein topology. This study also proposed that the region between the fifth and sixth transmembrane domain partially crosses the membrane similarly to the reentrant loop that forms the pore of other types of ion channels. However, as discussed later and shown in FIGURE 2A, this topology has been recently questioned (259).

Alignment of amino acid sequence of TMEM16 proteins reveals a high identity in the predicted transmembrane segments. In particular, a relatively high level of sequence conservation is present within a region in the COOH-terminal half that was labeled as domain of unknown function 590 (DUF590). This region is now called Anoctamin domain (http://pfam.sanger.ac.uk/family/Anoctamin).

“Blasting” of the protein sequences available in databases shows that anoctamin family is relatively ancient, with several homologs detected in animal species outside the phylum Vertebrata. For example, there are proteins in insects (Drosophila genus, Aedes aegypti, Tribolium castaneum, Bombus terrestris) with more than 40% amino acid identity to human TMEM16A. A similar degree of identity is also found in proteins from sea anemones (Nematostella vectensis), in nematodes (Brugia malayi), and in flatworms (Schistosoma mansoni). In Caenorhabditis elegans, two anoctamins, ANOH-1 and ANOH-2, were recently identified (240). ANOH-1 appears to be expressed in sensory neurons with chemoreceptor function and in intestinal cells. Inhibition of ANOH-1 function by RNA interference reduced the ability of worms to avoid high osmolarity but not the response to other stimuli, either mechanical or chemical. ANOH-2 was expressed in mechanoreceptors, motor neurons, and spermatheca, but its silencing did not generate a clear phenotype (240). Interestingly, there is also a distant TMEM16 homolog in yeast, Ist2p, that is involved in tolerance to high extracellular salt concentration (109). Recently, Ist2 was found as an important tethering protein between the endoplasmic reticulum (ER) and the plasma membrane (136, 247). Ist2 is localized in the ER, but comes into contact with the plasma membrane by means of a highly basic COOH terminus. Deletion of Ist2 in yeast de-
lays growth, alters intracellular pH, changes ER organization, and triggers the unfolded protein response.

Analysis of amino acid sequence similarity within TMEM16 proteins allows the generation of a phylogenetic tree (150, 240). Given their high amino acid identity (62%), TMEM16A and TMEM16B appear as closely related members belonging to the same subfamily. TMEM16C/D/J, and TMEM16E/F may form two separate subgroups, probably confined to chordates (240). TMEM16G/H/K are instead distant paralogs of TMEM16A and as ancient as Eumetazoa (240).
As expected from the relatively high sequence identity shared with TMEM16A, TMEM16B was also found associated with CaCC activity. This result was initially found by Schroeder et al. (195) and subsequently confirmed by three other teams (172, 208, 209). As discussed later, TMEM16B channels have biophysical characteristics different from those of TMEM16A. Because of the eight putative transmembrane segments and the anion channel function, TMEM16 proteins were also named anoctamins (ANO1–10). Although anion currents have been found to be associated with other anoctamins, it is not sure that all TMEM16 proteins have an ion channel function (77, 139, 194, 202, 223).

So far, there has been an increasing amount of evidence supporting the conclusion that TMEM16A is a CaCC-forming protein. First, in all cells devoid of endogenous CaCC activity, such as HEK-293 or FRT cells, transfection with TMEM16A is enough to generate Ca\(^{2+}\)-activated Cl\(^{-}\) currents with biophysical properties (e.g., voltage dependence) similar to those of native CaCCs (29, 195, 256). This may imply that TMEM16A protein alone is sufficient to form a whole channel or that other subunits of CaCC are already constitutively expressed in all cells. Second, TMEM16A knockdown with siRNAs causes CaCC inhibition (29, 50, 128, 137, 256). Inhibition is never complete, but this partial effect may be explained by the suboptimal efficacy of siRNA transfection methods. Also, site-specific mutagenesis of highly conserved amino acid residues in the TMEM16A sequence leads to specific changes in CaCC properties, such as ion selectivity and voltage dependence. For example, replacement of arginine and lysine residues with glutamate in the putative pore-forming loop between transmembrane segments 5 and 6 altered ion selectivity (256). In addition, mutations of conserved residues in transmembrane segments altered channel gating kinetics (29).

Finally, in a very recent study, TMEM16A protein was purified and reconstituted into proteoliposomes (219). Ion transport activity was determined by measuring the efflux of Cl\(^{-}\) from the liposomes. The presence of TMEM16A was associated with a significant Cl\(^{-}\) transport controlled by Ca\(^{2+}\) and transmembrane voltage in a way that resembled that reported for TMEM16A in cells (219). For example, the half effective concentration of Ca\(^{2+}\) at a given membrane potential was 210 nM, a value close to that measured in cells. Furthermore, the TMEM16A-dependent Cl\(^{-}\) transport was sensitive to classical CaCC blockers (219). All these experiments strongly indicate that TMEM16A is indeed a CaCC-forming protein but do not obviously rule out that other proteins are involved in the composition and regulation of the native channel.

B. Oligomeric Organization and Interactome of TMEM16A

Two studies indicated that TMEM16A protein is structured as a homodimer. In one study, TMEM16A protein was tagged with two different fluorescent proteins, mCherry and eGFP (201). Coexpression of the green and red TMEM16A within the same cell demonstrated a significant level of fluorescence resonance energy transfer (FRET), which is indicative of a close physical interaction. Chemical cross-linking and coimmunoprecipitation was also used to demonstrate dimer formation. In the second study, blue native polyacrylamide gel electrophoresis (BN-PAGE) was applied to demonstrate the presence of high-molecular-weight complexes of TMEM16A protein consistent with homodimer formation (58). Interestingly, the dimers are not abolished by reducing agents and not altered by intracellular Ca\(^{2+}\) concentration changes (58, 201). It is not clear yet whether two TMEM16A subunits form a single- or a double-pore channel.

In a more recent study, the formation of TMEM16A dimers was found to involve a region of the protein localized at the NH\(_2\) terminus (225). By analyzing a panel of truncation and deletion mutants, the authors of this study identified a small segment of 19 amino acids (position 161–179) essential for dimerization (by homotypic interaction) and channel function (FIGURE 2B). The authors also introduced a mutation, A169P, which was predicted to alter the \(\alpha\)-helix structure of the dimerization domain. The mutation abolished dimerization, protein trafficking to the plasma membrane, and channel activity (225). Interestingly, TMEM16A coimmunoprecipitated with TMEM16B but not with TMEM16F in heterologous expression systems. These results suggest that TMEM16A and TMEM16B heterodimers may form in cells coexpressing both genes. However, the real existence of these hybrid channels in native cells and their biophysical properties remain to be studied.

FIGURE 2. Structure-function of TMEM16A protein. A: topology of TMEM16A protein. The figure shows two different topologies proposed for TMEM16A protein. In the first model (left), the region between transmembrane domains 5 and 6 constitutes a reentrant loop that faces the extracellular environment and is important for the formation of the ion pore (258). In the second model, this region crosses completely the membrane (forming the sixth transmembrane domain) and then generates a cytosolic loop important for Ca\(^{2+}\) extracellular environment and is important for the formation of the ion pore (258). Another regulatory calmodulin binding site (RCBM) was also identified (237). In the third intracellular loop, two glutamic acid residues (702 and 705) appear to be the major Ca\(^{2+}\) binding sites that mediate channel activation (259). In the first intracellular loop, the sequence EEEEEAVK is important in the coupling of voltage and Ca\(^{2+}\)-dependent gating mechanisms (250). A small region in the NH\(_2\) terminus, the dimerization domain, is important for the homotypic interaction between TMEM16A proteins.

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The interactome of TMEM16A has been recently investigated using a proteomics approach (171). HEK-293 cells were stably transfected with TMEM16A labeled with a triple FLAG epitope. To stabilize the interaction between TMEM16A and its possible partners, Perez-Cornejo et al. (171) used a membrane permeable bifunctional cross-linker, DSP, which has a 12 Å spacer. After cell lysis, the cross-linked macromolecular complexes were immunoprecipitated with an anti-FLAG antibody and analyzed by mass spectrometry. Among the hundreds of proteins identified, 73 proteins appeared to be enriched more than three-fold in cells transfected with TMEM16A. The most enriched proteins were ezrin, munc18c, radixin, and moesin. Bioinformatic analysis of the results identified two major protein networks for TMEM16A interactome: SNARE proteins, which control vesicle trafficking, and the ezrin-radixin-moesin (ERM) scaffolding complex, which links plasma-membrane associated proteins with the cytoskeleton. The authors focused on the latter group of proteins. The interaction with TMEM16A/ANO1 was confirmed by coimmunoprecipitation in lysates of mouse salivary glands and by bimolecular fluorescence complementation in transfected cells (171). Furthermore, immunofluorescence experiments demonstrated colocalization of ANO1 with ERM proteins in the apical membrane of cells in the acini and ducts of salivary glands. Interestingly, knockdown of moesin expression with shRNA caused inhibition of TMEM16A currents by more than 50% without altering TMEM16A protein localization in the plasma membrane. This finding may indicate a role of ERM proteins in the regulation of TMEM16A activity. In this respect, it is interesting to note that, in another study, modulators of actin polymerization state such as cytochalasin D and phalloidin inhibited TMEM16A activity (222). As discussed by Perez-Cornejo et al. (171), the mass spectrometry analysis did not identify other integral membrane proteins besides ANO1, nor known Ca\(^{2+}\)-binding proteins such as calmodulin. This may suggest that the native CaCC is not composed of other subunits providing Ca\(^{2+}\) sensitivity or forming the channel pore. However, as described in subsequent sections of this review, calmodulin was actually found, with nonproteomics approaches, to contribute to TMEM16A regulation (101, 222, 237). More information about the TMEM16A/ANO1 interactome will certainly derive from future experiments using cross-linkers with different reactive groups and spacer arms, and by using cells with endogenous expression of CaCCs.

Recently, the inositol 1,4,5-triphosphate receptor 1 (IP\(_3\)R1) was discovered as another important interactor of TMEM16A, at least in nociceptive sensory neurons (99). This finding arose from the observation that TMEM16A can be activated in small neurons of dorsal root ganglia by Ca\(^{2+}\) signals dependent on G protein-coupled receptors but not by those deriving from voltage-dependent Ca\(^{2+}\) channels. Indeed, Jin et al. (99) found that TMEM16A is activated by bradykinin receptor 2 and protease-activated receptor 2 through release of Ca\(^{2+}\) from intracellular stores. In contrast, TMEM16A was insensitive to global intracellular Ca\(^{2+}\) elevation caused by membrane depolarization and opening of Ca\(^{2+}\) channels. This particular behavior is probably due to a localization of TMEM16A in regions of the cell where the plasma membrane and the endoplasmic reticulum are very close to each other. The close apposition of the two membranes creates a compartmentalized microenvironment that isolates TMEM16A from Ca\(^{2+}\) influx. As suggested by coimmunoprecipitation and proximity ligation assays, this arrangement may be favored by a direct interaction between IP\(_3\)R1 and two regions of TMEM16A: the first intracellular loop and the COOH terminus (99). Compartmentalized Ca\(^{2+}\) signaling activating TMEM16A was also observed in other studies (15, 118). In particular, bestrophin, by representing a pathway for counter ion transport, was an important factor contributing to Ca\(^{2+}\) release from the intracellular store.

C. Alternative Splicing

Another level of complexity in TMEM16A, and possibly in other anoctamins, arises from alternative splicing. There are multiple isoforms of TMEM16A proteins generated by inclusion/skipping of alternative exons and/or by the use of an alternative promoter (29, 60, 144, 205). TMEM16A was initially reported to have at least four alternative segments, which were simply labeled as a, b, c, and d. Inclusion/skipping of segments b (22 amino acids), c (4 amino acids), and d (26 amino acids) is the result of alternative splicing of exons 6b, 13, and 15, respectively (60). Instead, skipping of segment a, which corresponds to the first 116 amino acids of TMEM16A protein, is dependent on the activation of an alternative promoter. In this case, the sequence corresponding to the first 36 codons is replaced with a sequence lacking an ATG (205). It was initially believed that translation would then start at the next available ATG at position 117, thus entirely skipping segment a (29). It has been now found that noncanonical start codons, possibly CTG, allow translation of a large part of segment a in the absence of the first codon (205).

Expression of TMEM16A isoforms is tissue dependent. While inclusion of microexon 13 appears to occur in most transcripts, the splicing of exons 6b and 15 shows a variety of situations. Interestingly, preferential inclusion of exon 6b in some tissues seems associated with exclusion of exon 15, and vice versa (60, 228). In a recent study, an altered expression of TMEM16A isoforms was found in the stomach of diabetic patients affected by gastroparesis (144). These individuals showed an increased abundance of transcripts coding for TMEM16A proteins with shortened NH\(_2\) terminus (144).

More recently, another study reported additional exons undergoing alternative splicing in the heart (161). Some tran-
scripts were found to miss exon 10, which resulted in deletion of 45 amino acids that include the first putative transmembrane domain. Since this TMEM16A variant would have an altered transmembrane topology, it is not expected to be functional. Another variant, without exon 14, would lack 24 amino acids in the first intracellular loop possibly containing a phosphorylation site for protein kinase C (161). The same intracellular loop could also be affected by inclusion of the alternative exon 13b causing the insertion of another 40 amino acids.

Expression of different isoforms of TMEM16A protein has functional implications. Inclusion of exon 6b, resulting in a protein with additional 22 amino acids (segment b) in the region preceding the first transmembrane domain, was found associated with Cl− channels having a decreased sensitivity to cytosolic Ca2+ (60). This phenomenon was also observed in another study (223). At the cytosolic Ca2+ concentration of 100 nM, the isoform (ac) is significantly active in contrast to the isoform (abc) (60, 223). However, the isoform (ac) is also more prone to inactivation at high Ca2+ concentrations (223). The molecular basis for the observed influence of segment b is not clear. However, future experiments will need to consider that this domain is highly basic (8 arginine/lysines out of 22 amino acid residues), it has potential phosphorylation sites, and has a noncanonical calmodulin-binding site. As discussed later, binding of calmodulin to this domain modulates channel activity (222). Skipping of microexon 13, an event that appears to be rare in human tissues (60), also produces channels with altered characteristics. In a first study, deletion of the four amino acids (Glu-Ala-Val-Lys, EAVK in the single letter code) corresponding to segment c resulted in channels with reduced activation at positive membrane potentials (60). More recently, this TMEM16A variant was found to have significantly reduced Ca2+ sensitivity (250). Normally, the effect of cytosolic Ca2+ on CaCC channels is a shift of the relationship between normalized conductance and membrane potential towards more negative values. Xiao et al. (250) found that an increase in the Ca2+ concentration from 1 to 2 μM dramatically shifted the voltage dependence of wild-type TMEM16A channels from +64 to −145 mV (250). In contrast, the ΔEAVK mutant showed a significant impairment of sensitivity to Ca2+: a concentration of 25 μM was required to obtain an activity comparable to that recorded at 1 μM for the wild-type protein (250). By measuring the kinetics of activation and deactivation of the channel upon fast Ca2+ concentration changes, Xiao et al. (250) concluded that ΔEAVK increases the dissociation rate of Ca2+ from the channel (250). The differences between the two studies may stem from different experimental conditions and/or the use of different TMEM16A backbones, with and without segment b (60, 250). Indeed, the binding of calmodulin at segment b (222) may influence the net response of the channel to cytosolic Ca2+. Despite the different conclusions, these studies point to the EAVK segment, and to the first intracellular loop in general, as an important domain for the regulation of TMEM16A channel activity.

Another isoform of TMEM16A stems from the combined absence of segments a, b, c, and d (29). This isoform, defined as TMEM16A(0) and mostly expressed in testis (205), has altered voltage dependence and ion selectivity (61). Recently, it has been found that skipping of segment a is not complete (205). Actually, the absence of the first ATG does not result in translation from the second ATG as previously thought. The presence of non-canonical start codons allow a large part of segment a to be included in TMEM16A(0) (205).

So far, there have been no results on the role of other alternative regions in TMEM16A (161). However, it may be speculated in general that the expression of different TMEM16A isoforms, even within the same cell and with the formation of heterooligomers, enhances tremendously the complexity of CaCC at the molecular level. This variety of CaCC channels may have different biophysical properties, regulatory mechanisms, and subcellular localization.

The information available so far suggests that alternative splicing may be a common process within the anoctamin family. In particular, alternative splicing of TMEM16B transcripts generates multiple variants. TMEM16B transcripts missing exon 14 (formerly annotated as exon 13) were found in the murine olfactory epithelium but not in the retina (208). Importantly, the four amino acids (ERSQ), that in TMEM16B correspond to segment c of TMEM16A (EAVK), prevent channel inactivation at high Ca2+ concentrations (237). Noninactivating TMEM16B channels containing this sequence were expressed in retina and pineal gland. Inactivating channels, devoid of segment c, were instead expressed in the olfactory epithelium and in most regions of the central nervous system (237).

Other variants of TMEM16B were generated by alternative splicing of exon 4 and by the use of another starting exon that resulted in a shortened NH2 terminus (175). Expression of TMEM16B proteins missing the region corresponding to exon 4 resulted in inactive channels. However, coexpression of proteins with and without exon 4 generated functional channels but with altered properties, particularly regarding the inactivation process and channel rundown (175). Interestingly, exon 4 codes for a region that is homologous to that containing the dimerization domain identified in TMEM16A (225).

Inspection of sequences deposited in databases reveals that multiple isoforms also exist for all TMEM16 proteins. In TMEM16F, the inclusion of an alternative exon coding for
21 amino acids within the NH2-terminal region increased protein function (200).

D. Topology

Predictive computer programs propose for TMEM16A and other anoctamins a structure consisting of eight transmembrane domains with both the NH2 and COOH termini exposed to the cytosolic environment (29, 195, 256). Most algorithms predicting transmembrane domains agree in identifying the position of transmembrane helices 1–4 and 7–8. Such regions have an amino acid composition that is canonical for a protein segment entirely spanning the plasma membrane. In contrast, the topology of the region between transmembrane helix 4 and 7 appears more uncertain. It was proposed that a reentrant loop may exist between transmembrane segments 5 and 6 (FIGURE 2A). In this region, the protein would not cross entirely the membrane but would exit again on the extracellular side forming a hairpin. This model was based on a topology study performed on TMEM16G (ANO-7) by inserting an epitope at different regions of the protein and then looking at the accessibility of the epitope from the extracellular or intracellular side of the membrane (41). The reentrant loop, corresponding to a sequence of 103 amino acids, was proposed as a region constituting the channel pore of TMEM16A and therefore important for ion selectivity (256). Indeed, mutagenesis to glutamate in this region of three highly conserved basic amino acids (R621, K645, and K668) resulted in a significant alteration in ion permeability. In particular, the mutant R621E showed a dramatic loss of selectivity because the relative permeability for Na+/K+ was 0.93 instead of the 0.03 value of the wild-type protein (256). The orientation of this region was also investigated by exploiting three cysteines localized at positions 625, 630, and 635. A cysteine-modifying reagent, MTSET, which is unable to cross the membrane, blocked the activity of wild-type TMEM16A channels when applied from the extracellular side. There was no block when MTSET was applied to TMEM16A channels in which the three cysteines were mutated to alanine. These results indicated that the amino acids in the 620–635 segment are part of the channel pore and are accessible to the aqueous solution on the extracellular side (256).

The reentrant loop model has been recently questioned in another study (259). These authors considered the possibility that part of the putative reentrant loop actually crosses totally the membrane forming the sixth transmembrane domain (FIGURE 2A). The authors introduced hemaglutinin (HA) epitopes at different sites between residues 570 and 824. The epitope was accessible from the outside at positions 614 and 824 but not at positions 672 and 700. These results indicated that the region 650–706 is actually intracellular and not extracellular. As discussed in the subsequent section, this finding represented the basis for the discovery of a novel Ca2+-binding site. Yu et al. (259) also explored the issue of ion selectivity by studying the mutant R621E. In contrast to previous results (256), they found no change in ion selectivity for this mutant. They also adopted a scanning mutagenesis approach by replacing each amino acid between F620 and Q646 with cysteine and then testing the resulting mutants with extracellular MTSET. Changes in ion selectivity were not detected. The authors concluded that amino acid residues beyond position 635 are not accessible from the extracellular side (FIGURE 2A).

In conclusion, determination of TMEM16A structure in the region between transmembrane segments 4 and 7 appears particularly difficult and requires further studies. However, elucidation of this issue is essential to understand the function and regulation of the different anoctamins.

II. REGULATORY MECHANISMS OF TMEM16A/B CHANNELS

A. Calcium/Calmodulin and Membrane Potential

Native CaCCs in many cell types are activated by cytosolic Ca2+ in the 100–600 nM range, although cases with lower (micromolar) sensitivity have also been reported (7, 22, 71, 80, 111, 177). However, Ca2+ is not alone in activating CaCCs since, in most cases, these channels are also influenced by membrane potential (6, 7, 22, 177). More precisely, membrane depolarization enhances the Ca2+ sensitivity of the channels (7, 120). This behavior may be explained by a Ca2+-binding site that is localized within the transmembrane region of the channel and that is therefore sensitive to transmembrane potential (7). Similar characteristics have also been reported for the Cl- currents elicited by TMEM16A and TMEM16B expression. Both proteins are associated with voltage- and Ca2+-dependent Cl- channels. The half effective Ca2+ concentration required for TMEM16A channels is 0.4–0.6 μM at positive membrane potentials (60, 256). Such values increase significantly as the membrane potential becomes more negative, although the extent of change may differ among studies (60, 256). In contrast, TMEM16B channels are less Ca2+ sensitive, with the half effective Ca2+ concentration being around 1–3 μM at positive membrane potentials (172, 208). Furthermore, the kinetics of activation and deactivation of TMEM16B channels is much faster than that of TMEM16A, with time constants differing by more than 10-fold (29, 172, 208, 256). There are also significant differences regarding single-channel conductance: 8 pS for TMEM16A (256) and nearly 1 pS for TMEM16B (172, 208). TMEM16A and TMEM16B channels are also activated, with various potency and efficacy, by other divalent cations, such as Ba2+, Sr2+, and Ni2+ (208, 250, 260). Zn2+ has instead an inhibitory effect (260).
The molecular mechanisms underlying the channel gating by Ca\(^{2+}\) and voltage are unknown. It has been reported that some native CaCCs and TMEM16A/B channels can be directly activated by a Ca\(^{2+}\) increase on the cytosolic side of isolated membrane patches (250, 256). These findings may be interpreted as evidence that soluble cytosolic components are dispensable for channel activation. However, a slow and irreversible rundown of channel activity has also been reported in another study (222). Therefore, it is probable that unknown proteins with a loose binding to the plasma membrane control TMEM16A and TMEM16B activity. For example, a significant fraction of TMEM16A activity in excised patches was restored by adding ATP and calmodulin to the cytosolic solution (222).

Inspection of TMEM16A and TMEM16B primary structure does not reveal the presence of canonical binding sites for Ca\(^{2+}\) (e.g., EF-hands) or calmodulin, which could be expected for a Ca\(^{2+}\)-gated channel. However, there are multiple regions with negatively charged amino acid residues that may represent potential Ca\(^{2+}\)-binding sites. In particular, there is a cluster of five glutamic acid residues (EEEEE) in the first intracellular loop that is highly conserved among orthologs and close paralogs of TMEM16A (FIGURE 2A). This negatively charged cluster may be a binding pocket for Ca\(^{2+}\), as proposed for the “Ca\(^{2+}\) bowl” of Ca\(^{2+}\)-dependent K\(^{+}\) channels (12, 261). Interestingly, the fifth glutamate of the EEEE cluster is actually dependent on the inclusion of segment c (EAVK) in the TMEM16A transcript (in the absence of segment c, the fifth glutamic acid is replaced by an aspartic acid residue from exon 14). If the EEEE cluster is indeed involved in Ca\(^{2+}\) binding, removal of the EAVK alternative segment could directly affect the Ca\(^{2+}\) sensitivity of the TMEM16A protein, as shown by Xiao et al. (250). However, deletion of EEEE abolished the intrinsic voltage dependence without altering Ca\(^{2+}\) sensitivity (250). Therefore, the EEEVEAK domain may have an allosteric role in modulating Ca\(^{2+}\) sensitivity and not a direct participation in Ca\(^{2+}\) binding. Other regions with negatively charged residues could be more directly involved.

Recently, an important candidate site for direct Ca\(^{2+}\) binding has been identified in the TMEM16A sequence (259). As discussed in a previous section of the review, these authors found that the region between amino acids 650 and 706 is actually intracellular and not extracellular (FIGURE 2A). This protein region contains two highly conserved glutamic acid residues, E702 and E705, which were hypothesized to interact with Ca\(^{2+}\) (FIGURE 2B). Mutagenesis of both residues to glutamine shifted the apparent Ca\(^{2+}\) sensitivity of TMEM16A/AN01 channels from the submicromolar to the millimolar range (259). The authors also mutagenized the glutamic acid 702 to cysteine and tested the effect of the cysteine-modifying reagents MTSET and MTSES\(^{-}\) applied to the cytosolic side of the membrane. The two reagents had the opposite effect, consisting of a decrease and an increase of apparent Ca\(^{2+}\) affinity, respectively, relative to the unmodified E702C mutant. These results support the role of E702/E705 in direct binding to Ca\(^{2+}\) for the opening of the TMEM16A channel (259). However, additional studies are needed to demonstrate direct Ca\(^{2+}\) binding. Indeed, an alternative interpretation of results by Yu et al. (259) is that mutagenesis of E702/E705 alters Ca\(^{2+}\) binding at another site by an allosteric mechanism.

It is important to note that E702 and E705 are conserved in the TMEM16B protein despite a nearly 10-fold lower Ca\(^{2+}\) sensitivity relative to TMEM16A. In a recent study, replacement of the third intracellular loop of TMEM16A, which contains E702 and E705, with that of TMEM16B lowered Ca\(^{2+}\) sensitivity (199). The resulting chimeric channel behaved in part like the wild-type TMEM16B. Therefore, other amino acids in the third intracellular loop are also important in determining the Ca\(^{2+}\) sensitivity. Such amino acids may influence the position and orientation of the glutamates 702 and 705 and therefore their binding to Ca\(^{2+}\). Recently, this region was also found important for TMEM16A trafficking to the plasma membrane (2).

In addition to the third intracellular loop and the EEEAVK sequence, other TMEM16A/TMEM16B domains may participate in direct Ca\(^{2+}\) binding or in the allosteric modulation of Ca\(^{2+}\) sensitivity. In the search for these sites, we must consider that, in addition to glutamic and aspartic acid, other amino acid residues with oxygen in the side chain, or even the carboxyls of the peptidic backbone, could participate in Ca\(^{2+}\) coordination. Furthermore, Ca\(^{2+}\)-binding site(s) may include noncontiguous amino acid residues. Indeed, these sites could be generated in the folded TMEM16A protein by residues that reside in different regions of the primary structure. Finally, it is also theoretically possible that Ca\(^{2+}\)-binding sites are created at the interface of TMEM16A subunits during the formation of dimers.

Calmodulin may be another factor contributing to the Ca\(^{2+}\) dependence of TMEM16A channels, as also suggested by studies on native CaCCs. For example, a study carried out in an olfactory cell line concluded that calmodulin was absolutely required for endogenous CaCC activity. Transfection of calmodulin mutants having a dominant negative effect inhibited CaCC currents (102). As stated before, TMEM16A protein has a noncanonical binding site in segment b (coded by exon 6b). In a recent report, segment b of TMEM16A was found to be important for binding to calmodulin (222). Calmodulin coimmunoprecipitated with the TMEM16A(abc) but not with the TMEM16A(ac) variant. Furthermore, TMEM16A currents were strongly reduced by calmodulin inhibitors ( trifluoperazine and J-8) or by inhibitory peptides (222). However, the TMEM16A(ac)
isoform (unable to bind calmodulin according to immunoprecipitation results) was still activated by Ca\(^{2+}\). Therefore, multiple Ca\(^{2+}\)-dependent mechanisms, including calmodulin binding, may be involved in the gating of TMEM16A channels (Figure 2B).

Different results were found in another study (237). These authors identified a different site for calmodulin binding (termed RCBM, i.e., regulatory calmodulin-binding motif), localized in the NH\(_2\)-terminal region, before the first transmembrane domain, and more than 30 amino acid residues after the insertion of segment \(b\) (Figure 2B). The RCBM is present in TMEM16A and TMEM16B, but it is much less conserved in other anoctamins. Vocke et al. (237) showed evidence of direct interaction between calmodulin and peptides containing the RCBM sequence. Mutagenesis of specific residues within the RCBM revealed the existence of two regions or lobes. Both regions were important for binding of TMEM16A RCBM to calmodulin and for the Ca\(^{2+}\)-dependent channel activity of TMEM16A in cells. It is important to note that mutagenesis of RCBM also reduced the trafficking of TMEM16A protein to the plasma membrane. However, activation of currents at +200 mV suggested that a fraction of the mutant protein could reach membrane and be activated by extreme voltages but not by Ca\(^{2+}\), presumably because of impaired binding to calmodulin (237).

The first part of RCBM was also important for the activation of TMEM16B by Ca\(^{2+}\) (237). However, in this protein, the second half of RCBM had another role. When this segment was mutated, TMEM16B was activated normally by Ca\(^{2+}\) but did not inactivate. In contrast, the wild-type TMEM16B initially studied by Vocke et al., cloned from rat olfactory epithelium, showed a process of slow inactivation that, after the peak of activity triggered by Ca\(^{2+}\), closed the channels within 30–40 s (237). Interestingly, these authors found another TMEM16B variant, derived from the retina, that did not inactivate. When the sequences of the two variants were compared, Vocke et al. (237) found that both TMEM16B channels contained the RCBM, but the retinal variant contained four additional amino acids, ERSQ, which correspond to segment \(c\) of TMEM16A. This finding suggests that segment \(c\) controls the inactivation of TMEM16B channels.

Another role for calmodulin in the modulation of TMEM16A channel function was recently reported (101). It was found that the bicarbonate permeability of TMEM16A channels expressed in HEK-293 cells is strongly increased in a calmodulin-dependent way by high cytosolic Ca\(^{2+}\) concentrations. With a submaximal Ca\(^{2+}\) concentration (0.4 \(\mu\)M), the relative permeability to bicarbonate, expressed as \(P_{\text{HCO}_3}/P_{\text{Cl}}\) was 0.35. At a maximal concentration (3 \(\mu\)M), this value became 1.04. The high Ca\(^{2+}\) concentration affects permeability also to other anions so that the pore appears less selective. The authors found that this phenomenon was not observed in outside-out membrane patches, which indicates the involvement of a diffusible factor that is lost during excision of the membrane. This factor was identified as calmodulin because 1) the increase in bicarbonate permeability was blocked by a pharmacological inhibitor of calmodulin binding (J-8), 2) inhibited by transfection of anti-calmodulin siRNAs, and 3) restored in excised patches by adding exogenous calmodulin (101). Direct interaction was demonstrated in pull-down experiments: precipitation of calmodulin fused to GST resulted in coprecipitation of TMEM16A protein. The authors identified two candidate domains for calmodulin binding. The first one, named CBM1, was at the NH\(_2\) terminus right before transmembrane domain 1 (Figure 2B). This domain partially overlaps the RCBM identified by Vocke et al. (237). The second one, CBM2, was localized before transmembrane domain 7. Two critical isoleucines, at positions 317 and 762, were mutagenized to impair calmodulin binding at CBM1 and CBM2, respectively. The single mutants and, more markedly, the double mutant displayed a significantly reduced bicarbonate permeability at 3 \(\mu\)M Ca\(^{2+}\) and decreased interaction between calmodulin and TMEM16A in pull-down experiments (101). The role of Ca\(^{2+}\) concentration in the regulation of TMEM16A ion permeability was confirmed in salivary gland cells. Importantly, the study by Jung et al. (101) was done with the (ac) isoform of TMEM16A. Therefore, this isoform lacks the segment \(b\) shown by Tian et al. (222) to be a site of interaction with calmodulin. This may explain, at least in part, why, in the study by Jung et al. (101), calmodulin inhibition by J-8 or siRNA resulted in alteration of permeability to bicarbonate but not in a change in channel activity, as instead shown by Tian et al. for the isoform (abc) (222).

To sum up, the role of calmodulin in the regulation of TMEM16A/B channels appears complex and requires further investigation. By binding to segment \(b\), calmodulin may influence positively channel activity (222). Vocke et al. (237) found instead that calmodulin binds to a close but separate region, RCBM. According to these authors, calmodulin binding to RCBM is essential for TMEM16A activation and, in TMEM16B, for inactivation. In the third study, by binding to CBM1 and CBM2 (Figure 2B), calmodulin transduces changes in Ca\(^{2+}\) concentrations to changes in bicarbonate permeability (101), a modification that has a high physiological importance. As discussed by Jung et al. (101), high micromolar cytosolic Ca\(^{2+}\) concentrations can be reached in nanodomains at the apical membrane of secretory cells in salivary glands and in pancreas during stimulation. This condition can switch CaCCs to a state highly permeable to bicarbonate, a characteristic that may support the secretion of a bicarbonate-rich fluid (101). It is important to note that other investigators have also reported a dynamic ion permeability of TMEM16A and TMEM16B (189, 195). In these studies, the ion selectivity...
was not constant during the recordings but changed according to the levels of cytosolic Ca\(^{2+}\) concentration.

Ca\(^{2+}\) may also have inhibitory activity on TMEM16A channels (61, 223, 256). High Ca\(^{2+}\) concentrations, particularly in the micromolar range, can lead to channel inhibition. The mechanism is unknown but may involve phosphorylation by Ca\(^{2+}\)/calmodulin kinase II (CaMKII) since the phenomenon is attenuated by the inhibitor KN-62 (223). As discussed in a previous paragraph, in a specific TMEM16B variant, the relatively slow rundown caused by Ca\(^{2+}\) increase seems to be mediated by direct binding to calmodulin (237).

Recently, results obtained with purified TMEM16A in proteoliposomes suggest that calmodulin is not needed for TMEM16A activity (219). In purification steps, calmodulin did not co-elute with TMEM16A, and addition of calmodulin to liposomes did not change Cl\(^{-}\) transport. However, as discussed by the authors of this study, experimental conditions (e.g., the use of detergents) could affect binding of calmodulin. Therefore, it can be concluded that TMEM16A is by itself able to transport Cl\(^{-}\) and to be activated by Ca\(^{2+}\) and voltage. However, additional proteins, such as calmodulin, may modulate channel activity in a native system.

In addition to Ca\(^{2+}\) and calmodulin, TMEM16A channel activity is also regulated by temperature (34). The role of TMEM16A and TMEM16B as “heat sensors” is discussed in the section on nociceptive neurons.

### B. Phosphorylation

Another possible mechanism involved in the regulation/gating of TMEM16A/B channels is phosphorylation. In fact, some native CaCCs, particularly in epithelial cells, appear to be activated by Ca\(^{2+}\)/calmodulin-dependent kinases (CaMKs) (85, 251). Such results contrast with those obtained for CaCCs in other tissues in which phosphorylation by CaMK has actually an inhibitory effect. For example, CaMK in smooth muscle causes a desensitization of CaCCs so that they close in spite of a prolonged intracellular Ca\(^{2+}\) increase (6, 73, 241). In contrast, the Ca\(^{2+}\)-dependent phosphatase activity of calcineurin has a stimulatory effect (74). So far, the molecular basis explaining these different behaviors of native CaCCs has not been elucidated. However, it is important to note that Cl\(^{-}\) channels activated by CaMK are voltage independent (251), whereas channels inhibited by CaMK show activation at depolarizing membrane potentials that is typical of classical CaCCs (73). It is expected that the discovery of TMEM16A and TMEM16B will help to resolve this issue.

There are multiple putative phosphorylation sites in the primary structure of these proteins as indicated by prediction programs such as GPS 2.1, NetPhosK 1.0, Disphos 1.3, Prosite, and pkpS (20, 95, 158, 203, 253). Analysis of TMEM16A amino acid sequence identifies a number of potential phosphorylation sites ranging between 8 and 48 depending on the program. However, there are five sites [S74, S94, T216, S280, T530; residues numbered according to the TMEM16A(abcd) isofrom] that have been recognized as putative PKC sites by at least three prediction programs. The phosphorylation sites in TMEM16A and the corresponding kinase(s) may have a distinct regulatory effect on channel activity. However, experiments are needed to demonstrate that these sites are indeed phosphorylated and that this results in a significant effect on protein function. In a recent study, the role of TMEM16A phosphorylation was investigated using various kinase inhibitors (222). None of these compounds decreased TMEM16A-dependent Cl\(^{-}\) currents. Actually, inhibition of CaMKII resulted in increased activation of CaCC currents by ATP (but not by ionomycin). The issue of TMEM16A phosphorylation was further investigated by mutagenesis in the COOH terminus of two serines that are putative phosphorylation sites for the Erk1,2 kinase. The TMEM16A mutant showed a decreased channel activity when cells were stimulated with extracellular ATP, therefore through purinergic receptors, but not with the Ca\(^{2+}\) ionophore ionomycin (222). The partial or null effect of maneuvers aimed at inhibiting phosphorylation (kinase inhibitors or mutagenesis of putative phosphorylation sites) contrasts with the strong CaCC current inhibition observed when experiments on TMEM16A-expressing cells were carried out in the absence of intracellular ATP (222). This may suggest the involvement of specific kinases that are insensitive to the inhibitors used in the study or the need for ATP to keep TMEM16A active through mechanisms other than phosphorylation.

Another mechanism of regulation of native CaCCs involves changes in the metabolism of inositol polyphosphates. d-Myo-inositol 3,4,5,6-tetrakisphosphate [Ins(3,4,5,6)P\(_4\)] uncouples Ca\(^{2+}\)-dependent Cl\(^{-}\) secretion and CaCC activity from intracellular Ca\(^{2+}\) elevation (30, 86, 229). As well established in many studies, stimulation of cells with phospholipase C-based stimuli (e.g., stimulation with purinergic agonists) causes the production of Ins(1,4,5)P\(_3\), which triggers the release of Ca\(^{2+}\) from intracellular stores. However, this CaCC-activating stimulus is followed by a more delayed increase in Ins(3,4,5,6)P\(_4\), which acts on CaCC as a feedback inhibitory signal (30, 86, 229). The inhibitory effect of Ins(3,4,5,6)P\(_4\), which may involve an okadaic acid-sensitive phosphatase (252), can be antagonized by high intracellular Ca\(^{2+}\) concentrations (85). Therefore, the specific effect of this inositol polyphosphate could be the reduction of the Ca\(^{2+}\) sensitivity of the channel. In a recent study, a synthetic membrane-permeable inositol polysphosphate analog, INO-4995, was used on human epithelial cells (224). Incubation with INO-4995 increased...
TMEM16A-dependent Cl⁻ transport without altering intracellular Ca²⁺ levels. This result reveals the possibility to modulate TMEM16A pharmacologically by intervening on regulatory mechanisms.

III. PHYSIOLOGICAL ROLE OF TMEM16A AND TMEM16B

A. Epithelial Cells

Ca²⁺-activated Cl⁻ currents are a common finding in electrophysiological studies carried out in epithelial cells. Short-circuit current recordings in different types of polarized epithelia reveal the presence of Ca²⁺-activated Cl⁻ secretion that may be triggered by Ca²⁺ mobilizing agents such as purinergic receptor agonists (ATP, UTP) or Ca²⁺ ionophores (ionomycin, A23187) (67, 107, 140). The mechanism of Cl⁻ secretion requires the coordinated activity of various channels and transporters localized in the apical and basolateral membranes of the epithelial cells. In particular, the Na⁺ gradient across the basolateral membrane, maintained by the activity of the Na⁺-K⁺-ATPase, is utilized by the Na⁺/K⁺/2Cl⁻ cotransporter to generate an intracellular accumulation of Cl⁻ (75, 76). This condition favors the exit of the anion through apical channels activated by different second messengers, including cytosolic cAMP or Ca²⁺.

There are at least two main lines of evidence proving that cAMP- and Ca²⁺-dependent Cl⁻ secretion is mediated by separate apical Cl⁻ channels, namely, CFTR and CaCC, at least in the airway epithelium. First, selective pharmacological inhibitors allow the separation of the two components. For example, the thiazolidineone CFTRinh-172 blocks CFTR Cl⁻ channels with high selectivity (235). In the presence of this compound, Ca²⁺-elevating agents are still able to induce Cl⁻ secretion, whereas the cAMP-dependent Cl⁻ secretion is totally inhibited (29). The second important proof derives from studies on epithelial cells from cystic fibrosis (CF) patients. CF is a genetic disease caused by the loss of function of the CFTR protein (181). Transepithelial ion transport recordings on CF airway epithelia show a severe reduction of cAMP-dependent but not of Ca²⁺-dependent Cl⁻ secretion, thus demonstrating that the latter is dependent on another type of protein different from CFTR (5, 66). The CF defect is particularly important in the airways where the deficit in Cl⁻ secretion causes dehydration of the epithelial surface and impairment of mucoiliary transport (143). This favors the colonization of the airways by pathogenic bacteria. In CF epithelia, the presence of a second type of Cl⁻ channel that is not altered by CFTR gene mutations is interesting for the development of therapeutic strategies. Actually, in vitro activation of CaCC activity in CF epithelia increases the thickness of the airway surface fluid, thus compensating for the CFTR defect (218).

In 2008, the discovery of TMEM16A as a Cl⁻ channel protein provided a molecular basis for epithelial CaCCs (29, 195, 256). In fact, TMEM16A was found by searching the genes upregulated by IL-4 in human bronchial epithelial cells (29). Knockdown of TMEM16A expression by transfection with siRNAs caused a significant inhibition of Ca²⁺-dependent Cl⁻ secretion. Silencing was effective not only in cells treated with IL-4 but also in untreated cells (29). These results indicate that TMEM16A is an important component of CaCC in airway epithelia under resting conditions and that stimulation with IL-4 or IL-13 (40, 67), which simulates asthma-like inflammatory conditions in vivo, further enhances the epithelium ability to secrete Cl⁻ by increasing TMEM16A expression. The physiological role of the upregulation of TMEM16A by cytokines is unknown. However, two recent studies have provided a possible answer (89, 198). TMEM16A was found specifically expressed in mucus-producing goblet cells with little expression in ciliated cells (FIGURE 3). More precisely, there was a uniform low expression in the airway epithelium under resting conditions, even in goblet cells. However, induction of goblet cell hyperplasia by treatment of human bronchial epithelial cells with Th-2 cytokines or by sensitization of mice with ovalbumin resulted in an increased number of cells coexpressing at high levels TMEM16A and the mucin 5AC (MUC5AC, a marker of goblet cells). Importantly, specific coexpression of TMEM16A and MUC5AC was also confirmed in the airways of asthmatic patients (89). By analyzing the time course of TMEM16A and MUC5AC expression during treatment with IL-4, Scudieri et al. (198) found that, in the first 24 h, TMEM16A appeared mainly in cells that were nonciliated and devoid of mucin expression. However, after 72 h of treatment, the percentage of cells coexpressing TMEM16A and MUC5AC had largely increased. These results suggest that, during the process of goblet cell metaplasia induced by Th-2 cytokines, TMEM16A overexpression is an early event of differentiation to goblet cells.

IL-4 and IL-13 are potent inducers of mucus secretion and goblet cell metaplasia in the airways (121). TMEM16A overexpression in goblet cells could be a mechanism favoring the secretion and fluidity of mucus (FIGURE 3). This mechanism could be related to the recent discovery that TMEM16A is highly permeable to bicarbonate, particularly under conditions of high Ca²⁺ mobilization (101). It has been shown that HCO₃⁻ is particularly important in allowing maximal expansion of mucus granules upon secretion (69). Therefore, physiological activation of TMEM16A in goblet cells during mucin release could be a way to provide the required concentration of bicarbonate. In this respect, it should be noted that, in goblet cells, the secretory vesicles contain high levels of ATP in addition to mucin (114). Exocytic mucin release would then occur simultaneously with autocrine stimulation of puri-
nergic receptors. The resulting Ca\textsuperscript{2+} increase would activate TMEM16A/CaCC, thus generating a pulse of anion secretion synchronized with mucin release (FIGURE 3). It is also possible that TMEM16A is directly linked to the mechanism of mucin release itself. Actually, Huang et al. (89) have tested the effect of TMEM16A inhibitors, and these compounds caused a significant reduction of mucin release.

Interestingly, IL-4 not only affects TMEM16A but also strongly upregulates the expression of pendrin (SLC26A4), a transporter for Cl\textsuperscript{−}, I\textsuperscript{−}, SCN\textsuperscript{−}, and bicarbonate (70, 170). This effect suggests that inflammatory conditions and mucus hypersecretion in the airways are generally associated with a boost in anion transport capacity.

The elucidation of the role of TMEM16A in the airways needs further studies. Its association with goblet cell hyperplasia/metaplasia is particularly important in the pathogenesis and treatment of several diseases characterized by mucus hypersecretion, including asthma, cystic fibrosis, and chronic obstructive pulmonary disease (185). With regard to cystic fibrosis, it is important to remark that TMEM16A overexpression occurs in goblet cells, whereas CFTR is localized in ciliated cells (198). It is not clear if this finding implies that these two channels have actually different physiological roles. It is also important to assess TMEM16A expression in cystic fibrosis airways. In primary cultures, cells from normal and cystic fibrosis individuals have similar TMEM16A expression (198). However, the severe inflammatory response in the airways, triggered by bacterial colonization, and characterized by massive neutrophil infiltration and mucus hypersecretion, could affect TMEM16A expression in a positive or a negative way. This issue requires further studies to assess the suitability of TMEM16A as a drug target in cystic fibrosis.
Recently, three studies have added further interest on TMEM16A function in airway epithelial cells. It has been shown that TMEM16A affects cell proliferation and influences repair of the bronchial epithelium in cystic fibrosis (187). In another study, it was found that TMEM16A-dependent Cl\(^-\) transport inhibits, with an unknown mechanism, the release of the pro-inflammatory cytokine IL-8 (233). Therefore, activation of TMEM16A could have anti-inflammatory effects. Finally, it has been found that a TMEM16 protein in *Drosophila melanogaster* is involved in host defense activity against the pathogenic bacterium *Serratia marcescens* (248). It is tempting to speculate that a similar role could be played by TMEM16A in the airways.

The relationship between TMEM16A and CaCCs has also been demonstrated in other epithelia outside the airways. For example, TMEM16A protein is expressed in the apical membrane of rodent cholangiocytes, where it is probably involved in the Ca\(^{2+}\)-dependent fluid secretion of biliary ducts, possibly mediated by mechano-sensitive ATP release (186). The properties of CaCCs in cultured cholangiocytes resembled those of TMEM16A-dependent channels and silencing of TMEM16A expression in such cells inhibited Ca\(^{2+}\)-dependent Cl\(^-\) transport (50). In another study, the endogenous Ca\(^{2+}\)-activated Cl\(^-\) currents of salivary gland cells were compared with the currents arising from heterologous expression in HEK-293 cells of TMEM16A or bestrophin-2 (186). The currents were all strikingly similar in terms of Ca\(^{2+}\) sensitivity and time/voltage dependence. However, Ca\(^{2+}\)-dependent Cl\(^-\) transport and fluid secretion were normal in Bestrophin-2 knockout mice. In contrast, Ca\(^{2+}\)-dependent Cl\(^-\) transport was significantly reduced in TMEM16A\(^{-/-}\) animals (186).

A large TMEM16A expression was also revealed in serous and mucous cells of airway submucosal glands (62). Actually, the TMEM16A mRNA was 10 and 100 times more abundant than CFTR mRNA in serous and mucous cells, respectively. The high TMEM16A expression was in agreement with a large size of the Ca\(^{2+}\)-dependent Cl\(^-\) currents measured in whole cell patch-clamp recordings. Also, measurement of Cl\(^-\) secretion by the short-circuit technique revealed a large Ca\(^{2+}\)-dependent component (62).

Despite strong lines of evidence in favor of TMEM16A as an epithelial Cl\(^-\) channel, there are indications that other types of CaCCs may also exist. In a recent study, a selective TMEM16A channel inhibitor (T16A\(_{inh\cdot}\)A01) was used in different epithelial cell types (155). In differentiated bronchial epithelia, the inhibitor caused little effect on the Cl\(^-\) secretion induced by Ca\(^{2+}\)-elevating agents. A similar negative finding was also obtained in intestinal epithelia. In contrast, T16A\(_{inh\cdot}\)A01 abolished Cl\(^-\) secretion in salivary glands. The conclusion of the study was that CaCCs are heterogeneous and TMEM16A is a minor contributor to Ca\(^{2+}\)-dependent Cl\(^-\) secretion in airway and intestinal epithelia (155). This conclusion was also supported by the low amounts of TMEM16A protein detected by western blots (although a large TMEM16A protein increase was observed upon treatment with IL-4). The issue of TMEM16A role in the airway epithelium needs further consideration. In fact, other types of studies, based on gene silencing with siRNA and on gene knockout animals, are in favor of a more significant involvement of TMEM16A in airway Cl\(^-\) secretion (29, 184, 167). It is possible that TMEM16A is indeed expressed at low levels in the airway epithelium under resting conditions and that another type of CaCC exists. In this respect, it is important to note that a previous study had reported that CaCCs in polarized and nonpolarized airway epithelial cells have different properties (5). Nonpolarized cells show Ca\(^{2+}\)-dependent Cl\(^-\) currents with the typical voltage dependence of classical CaCCs. In contrast, the currents of polarized cells are less voltage dependent. If another CaCC protein exists, the role of TMEM16A may be more important under inflammatory conditions when an increase in the ability to secrete Cl\(^-\) is needed. Interestingly, TMEM16A is upregulated by IL-4 also in biliary duct epithelial cells (50), which suggests that stimulation of Cl\(^-\) secretion by proinflammatory stimuli is a general phenomenon in various organs.

The lack of TMEM16A function in the intestine resulting from the T16\(_{inh\cdot}\)A01 inhibitor study (155) deals with a quite controversial issue regarding the presence of one or more types of Cl\(^-\) channels in the apical membrane of intestinal epithelial cells. In many studies, CFTR appears as the most important Cl\(^-\) conductive pathway in the apical membrane, responsible for both cAMP- and Ca\(^{2+}\)-activated Cl\(^-\) secretion (5, 134, 211). The conclusion of these studies is that AMP-dependent Cl\(^-\) secretion is directly due to CFTR activation, whereas the response to Ca\(^{2+}\) would be more indirect. Indeed, Ca\(^{2+}\) activates basolateral K\(^+\) channels and the resulting hyperpolarization of the membrane potential would favor the exit of Cl\(^-\) through open CFTR Cl\(^-\) channels. On the other hand, there are also studies indicating that a separate Ca\(^{2+}\)-activated Cl\(^-\) conductance exists in the apical membrane of intestinal epithelial cells (146, 148). Furthermore, the carbachol-induced Cl\(^-\) secretion was severely reduced in the colon of TMEM16A\(^{-/-}\) mice (167). It is possible that expression of a CaCC conductance in the intestine is not constitutive, such as CFTR, but influenced by age or other physiological and pathological conditions. For example, TMEM16A expression in the intestinal epithelium seems regulated by the epidermal growth factor (152). Another factor influencing the contribution of TMEM16A/CaCC to intestinal Cl\(^-\) secretion is its subcellular localization. Actually, in a recent study, TMEM16A protein was detected by immunofluorescence in the lateral membrane of colonic epithelial cells (81). In agreement with this localization, CaCC inhibitors increased Cl\(^-\) secretion.
Interestingly, expression and function of TMEM16A/CaCC in the apical membrane of intestinal epithelial cells could be the basis for secretory diarrhea induced by rotaviral infection (113, 168). Indeed, the rotaviral toxin NSP4 induces Cl⁻ secretion through activation of CaCCs. Therefore, pharmacological inhibition of CaCCs could represent a useful antidiarrheal approach (113, 168).

Another site of expression of TMEM16A is the kidney. A recent study (26) showed expression of TMEM16A in MDCK, a canine cell line resembling the principal cells of the collecting duct, that is used as an in vitro model to study the growth of kidney cysts. TMEM16A was also expressed in the epithelium lining the cysts of autosomal polycystic kidney disease patients (26). Silencing of TMEM16A, but not of TMEM16F, with siRNAs inhibited Ca²⁺-dependent Cl⁻ secretion in MDCK cells. Similar inhibition was obtained with TMEM16A blockers. Cyst growth was measured in two ways: with MDCK cells suspended in a collagen gel and with an embryonic kidney cultured model. When TMEM16A activity was antagonized with pharmacological agents or with siRNAs, cyst growth was significantly inhibited (26). These results suggest that TMEM16A is an important protein contributing to the progression of polycystic kidney disease.

**B. Smooth Muscle**

Smooth muscle cells from different organs, particularly from blood vessels, show Ca²⁺-activated Cl⁻ currents (124). Actually, there seems to be two different CaCCs: a “classical” one, with voltage-dependent gating (6, 73, 74), and a second one that is instead modulated by intracellular cGMP and has a different sensitivity to niflumic acid and zinc (141, 142). The two types of channels have a different pattern of distribution in the smooth muscle cells from blood vessels and colon (142).

CaCCs in smooth muscle are believed to stimulate contraction. This occurs because Cl⁻ is accumulated intracellularly through the activity of cotransporters like NKCC1. Indeed, NKCC1 knockout mice have reduced blood pressure and vascular smooth muscle tone (149). Because of the favorable electrochemical gradient, the opening of CaCCs results in Cl⁻ exit and membrane depolarization, that in turn activates voltage-dependent Ca²⁺ channels. There may be different sources for Ca²⁺ increase leading to CaCC activation. Stimulation of G protein-coupled receptors for norepinephrine, acetylcholine, and histamine causes Ca²⁺ release from intracellular stores (27, 98, 169, 239). CaCC activation may also derive from a positive feedback loop involving Ca²⁺ entry through voltage-dependent Ca²⁺ channels and Ca²⁺-induced Ca²⁺ release through ryanodine receptors (124, 191).

CaCC inhibitors like niflumic acid have been widely used to support the role of CaCC in smooth muscle. CaCC inhibition with such compounds causes smooth muscle relaxation (36, 37, 100, 122, 191, 262). However, the classical CaCC inhibitors used in many studies, like niflumic acid or NPPB, have a series of different activities on channel and non-channel proteins (124). For example, they may block CFTR (197, 264) or Ca²⁺ channels (11), activate K⁺ channels (165), or cause emptying of Ca²⁺ stores (38, 176). The development of novel and more specific CaCC inhibitors may help to elucidate the role of CaCCs in smooth muscle (43).

The link between CaCCs and TMEM16A protein in smooth muscle cells is currently under investigation in many laboratories. So far, it is still unclear whether TMEM16A is expressed and associated with CaCCs in all smooth muscle cell types. With the use of specific antibodies on murine tissues, TMEM16A protein appears highly expressed in the smooth muscle of the airways and reproductive tract but, intriguingly, not at all in the smooth muscle of blood vessels (87). In contrast, other studies have detected mRNA and protein TMEM16A in freshly isolated vascular smooth muscle cells from rodents (42, 137, 206, 238). Silencing of TMEM16A by siRNA resulted in CaCC inhibition (137, 238). Analysis of TMEM16A mRNA splicing showed the coexpression in these cells of multiple variants mostly due to alternative splicing of segments b and d (42, 137, 238).

Interestingly, TMEM16A expression and function in smooth muscle cells are altered under pathological conditions. In one study, performed on smooth muscle cells isolated from the basilar arteries of hypertensive rats, TMEM16A protein expression was downregulated compared with that of normal rats (238). In particular, TMEM16A expression appeared to correlate inversely with cell proliferation by regulating the levels of cyclin D1 and cyclin E (238). Therefore, downregulation of TMEM16A appears to favor remodeling of the wall of the basal artery, which supplies blood to the brain.

In another study, TMEM16A was involved in the hyperreactivity of airway smooth muscle cells (ASMCs) under asthma-like conditions (263). Mouse sensitized with ovalbumin to induce a chronic inflammation showed increased contractility along with increased TMEM16A expression. Contractility of ASMCs isolated from TMEM16A knockout mice was significantly impaired compared with wild-type animals (263). These results indicate that TMEM16A may be a drug target to prevent bronchoconstriction in asthma. This conclusion also arises from the results of another study in which CaCC inhibitors had a relaxant activity on ASMCs (89).

Chronic hypoxia was found to upregulate TMEM16A expression and function. In particular, rats were exposed for 3–4 wk to low oxygen levels. In these animals, pulmonary arterial smooth muscle cells (PASMCs) showed larger...
CaCC currents, increased TMEM16A protein and mRNA, and increased contractile responses (213).

TMEM16A-dependent CaCC activity is also upregulated in pulmonary hypertension (64). A rat model of pulmonary hypertension was generated by injection of monocrotaline. PASMCs from these animals showed an increase in the expression of TMEM16A at the mRNA and protein levels, and a parallel increase in Ca$^{2+}$-activated Cl$^{-}$ currents. Contractility of PASMCs was antagonized by CaCC inhibitors, including niflumic acid and the TMEM16A-selective T16Ainh-A01 (64). Interestingly, the PASMCs from animals with pulmonary hypertension also showed an altered pattern of TMEM16A splicing; the transcripts with inclusion of segments b were more abundant than in control animals.

It would be interesting to assess if there are changes in the pattern of splicing also in other physiological or pathological conditions. There is evidence that the alternative splicing of many smooth muscle genes is affected by the phenotypic state of the cells (e.g., phasic vs. tonic smooth muscle; reviewed in Ref. 63). Since alterations in the splicing machinery of a cell have pleiotropic effects on multiple genes, we may also expect a change in the relative abundance of TMEM16A isoforms.

Another source of complexity influencing the role of TMEM16A in smooth muscle cells is phosphorylation. As mentioned before, native CaCCs in smooth muscle cells are regulated by a complex mechanism involving Ca$^{2+}$-dependent kinases and phosphatases such as CaMKII and calcineurin, respectively (6, 8, 74, 126, 241). The efficacy of kinase and phosphatase inhibitors is dependent on the cytosolic free Ca$^{2+}$ concentration (126). For example, cyclosporin A, a calcineurin inhibitor, decreases CaCC currents at 500 nM but not at 1 µM Ca$^{2+}$. Therefore, the net balance between activation and inactivation of CaCC is probably dependent on the amplitude and kinetics of intracellular Ca$^{2+}$ transients. It is still not clear whether the activity of kinases and phosphatases on CaCCs directly involves phosphorylation of TMEM16A or of a regulatory subunit.

**C. Interstitial Cells of Cajal**

In the gastrointestinal tract, the contraction of smooth muscle layers is controlled by the electrical activity of a specialized cell type, the interstitial cells of Cajal, ICCs (92, 242). ICCs are interposed between the terminations of enteric neurons and smooth muscle cells, thus mediating the transmission of motor neurotransmitters such as acetylcholine. Rhythmic depolarizations of ICCs are transferred electrically to smooth muscle cells, by gap junctions, triggering contraction. Interestingly, TMEM16A, known as DOG1, was found as a strong marker for gastrointestinal stromal tumors, GISTs (55), and also useful to identify ICCs in the gastrointestinal tract because of its high and specific expression in these cells (72). A microarray analysis had actually identified TMEM16A as a gene highly expressed in ICCs before its identification as a CaCC-forming protein (33). The reason for expression of TMEM16A/DOG1/ANO1 in GISTs may lie in the possible development of these tumors from ICCs.

For years, the ionic basis of membrane depolarization in ICCs was unclear. The discovery of TMEM16A/ANO1/DOG1 as a Cl$^{-}$ channel protein and its high expression in ICCs (72, 87) strongly suggested that a Cl$^{-}$ conductance could be an important component of ICC electrical activity. Indeed, the slow wave depolarization of ICCs appeared to be caused by activation of niflumic acid-sensitive CaCCs (266). To reach this conclusion, Zhu et al. (266) used mice in which ICCs were labeled by specific expression of GFP. This characteristic allowed the identification of ICCs within the mixed cell population isolated from the intestine. Electrophysiological recordings demonstrated the presence of a Ca$^{2+}$-activated Cl$^{-}$ conductance that was responsible for the spontaneous depolarizations shown by the isolated ICCs, as demonstrated by its sensitivity to niflumic acid. The relationship between TMEM16A and the slow wave depolarizations in ICCs was further demonstrated in knockout animals. TMEM16A knockout animals displayed a drastic decrease in the electrical activity and contractility in the intestine and in the stomach (87, 94). Importantly, the impairment of gastrointestinal motility was not due to a loss of ICCs (since these cells were normally distributed, as demonstrated by staining for the C-kit marker), but to a loss of TMEM16A expression (87). Indeed, slow waves were totally absent in the gastric and intestinal muscles of TMEM16A knockout mice (94). These findings strongly demonstrate the importance of TMEM16A in the peristalsis of the gastrointestinal tract and suggest that alterations in the function and expression of TMEM16A protein may be a feature of gastrointestinal motility disorders. In this respect, as previously discussed, the pattern of TMEM16A splicing appeared altered in the stomach of patients with diabetic gastroparesis (144). The resulting TMEM16A variants, with shortened NH$_2$ terminus, showed a change in CaCC gating kinetics. In another study, reduction in immunostaining for TMEM16A revealed a depletion of ICCs as a possible cause of slow transit constipation (103).

ICCs are not important only in the gastrointestinal tract. Slow electrical waves are also present in the oviduct of female mice to control the transport of oocytes. These rhythmic changes in membrane potential and the resulting contraction of myosalpinx were absent in Tmem16a knockout mice (46).

It is interesting to note that the urethra appears as an exception to the rule of specific expression of TMEM16A in ICCs. In urethras from sheep, mouse, and rat, TMEM16A...
was highly expressed in smooth muscle cells but not in ICCs (192).

D. TMEM16B in Olfactory Neurons

Ca\(^{2+}\)-activated Cl\(^{-}\) currents have been described in many types of neurons where they may be involved in the triggering of action potentials and in the modulation of excitability. CaCCs have been particularly studied as mediators of olfactory stimuli. The cilia of receptor neurons in the nasal olfactory epithelium respond to odorants by increasing intracellular cAMP (193). This messenger opens in turn cyclic nucleotide-gated channels (CNGCs) that are permeable to Na\(^{+}\) and Ca\(^{2+}\) (154). The cation influx causes per se membrane depolarization, but Ca\(^{2+}\) also activates CaCCs that mediate Cl\(^{-}\) efflux (21, 110, 119, 130). As in epithelial cells, the efflux of Cl\(^{-}\) is favored by the activity of the bumetanide-sensitive Na\(^{+}\)/K\(^{+}\)/2Cl\(^{-}\) cotransporter, which generates the outwardly directed gradient (82, 159, 180). The depolarization arising from Cl\(^{-}\) efflux through CaCCs represents a mechanism of electrical amplification of the initial olfactory stimulus. This mechanism is favored by the specific compartmentalization of odorant receptors, CNGCs, and CaCCs on the membrane of olfactory cilia (65, 179). This special organization allows the intracellular messengers generated by olfactory stimuli (cAMP, Ca\(^{2+}\)) to reach high concentrations within the small volume of cilia and to reach rapidly the downstream actuators (CNGCs and CaCCs).

In 2009, TMEM16B (ANO2) protein was proposed as the olfactory CaCC. TMEM16B was found as an abundant membrane protein in the proteome of mouse olfactory cilia (208). This finding was confirmed in another study in which quantitative analysis detected TMEM16B protein expression at levels higher than those of CNGC subunits (178). The link between olfactory CaCCs and TMEM16B was also supported by functional studies. Transfection of TMEM16B in HEK-293 cells generated Cl\(^{-}\) currents whose properties resembled those of native olfactory CaCCs (172, 208).

CaCCs have been identified also in vomeronasal sensory neurons (VSNs). The vomeronasal organ is a specialized structure devoted to the detection of pheromones. In VSNs, the mechanism of transduction has evolved differently from that of the main olfactory epithelium neurons. G protein-coupled odorant receptors in VSNs do not increase cAMP but trigger the activation of phospholipase C and therefore the production of diacylglycerol. This molecule stimulates TRPC2 causing Ca\(^{2+}\) influx and CaCC activation (108, 254). As evidenced by various studies, TMEM16B/ANO2 may be the main component of CaCC also in VSNs (19, 254).

Recently, a study has seriously questioned the role of CaCCs in olfactory stimulus transduction (19). A knockout mouse was generated to investigate the relationship of TMEM16B/ANO2 with olfaction. This animal showed no CaCC currents in olfactory neurons, thus demonstrating that TMEM16B protein is indeed the main CaCC-forming protein in these cells. However, electro-olfactograms showed that TMEM16B\(^{-/-}\) animals had only a partial (40%) reduction of the electrical response to odorants. Furthermore, olfactory tests showed that TMEM16B\(^{-/-}\) mice, in contrast to CNGA2\(^{-/-}\) animals, showed no impairment in the ability to detect odorants (19). These intriguing results contradict the common belief, supported by many studies, that CaCCs are indeed required in olfaction. It is not clear at the moment whether CaCCs are completely dispensable for olfaction or if they may serve under particular circumstances. In this respect, it should be noted that the wide use of nonspecific Cl\(^{-}\) channels inhibitors, such as niflumic acid, may have led to an overestimation of the actual role of CaCCs in olfaction. The development of more selective TMEM16B pharmacological inhibitors could be important to elucidate the physiological role of CaCCs in olfactory receptors as well as in other cells.

E. TMEM16B in Photoreceptors

In vertebrate photoreceptors (cones and rods), CaCCs are believed to regulate the release of glutamate at the synaptic terminal by local feedback mechanisms. In the dark, there is a continuous influx of Na\(^{+}\) through cGMP-gated cation channels that depolarize the membrane potential. This depolarization keeps the voltage-dependent Ca\(^{2+}\) channels at the synaptic terminal in the open state causing an influx of Ca\(^{2+}\) and hence a steady release of glutamate through fusion of vesicles. The influx of Ca\(^{2+}\) also activates CaCCs with a consequent effect on the electrical properties of the photoreceptor membrane (13, 14, 35, 133). In cones, which have an equilibrium potential for Cl\(^{-}\) close to the resting membrane potential in the dark (approximately –36 mV), opening of CaCCs tends to stabilize the membrane potential (220). In rods, where the Cl\(^{-}\) equilibrium potential (–20 mV) is more positive than the resting potential, CaCC opening causes Cl\(^{-}\) efflux. This may have a feedback regulatory role on voltage-dependent Ca\(^{2+}\) channels through an anion-binding site (10, 221).

TMEM16B was found highly expressed in the photoreceptor synaptic terminals of mouse retina (209). Stöhr et al. (209) found that the TMEM16B COOH terminus has a PDZ class I binding motif through which it forms a macromolecular complex with PSD95, VELI3, and MPP4 adaptor proteins. This interaction appeared functionally important since TMEM16B was lost from the photoreceptor membrane in mice defective for MPP4 protein. When expressed in HEK-293 cells, TMEM16B generated Cl\(^{-}\) currents very similar to those of native CaCCs previously described in photoreceptors. Subcellular localization and functional properties suggested that TMEM16B is a main CaCC-forming protein of photoreceptors (209).
In another study, carried out on salamander retina, Mercer et al. (147) found evidence for TMEM16A expression and function at the photoreceptor ribbon synapse. Interestingly, these authors analyzed the distribution of voltage-dependent Ca\(^{2+}\) channels (VDCCs), CaCCs, and transmitter release sites by comparing the response to depolarizing voltage steps in the presence of BAPTA or EGTA, two chelating agents with different kinetics for Ca\(^{2+}\) binding. Synaptic release of transmitter was not blocked when the intracellular solution in the photoreceptor contained the slow EGTA agent. Furthermore, the release was only partially reduced by using the fast BAPTA agent. These results indicate that the distance between VDCCs and the site of transmitter release is very short, below 100 nm. In contrast, CaCCs appeared more distant from VDCCs since their activation by a depolarizing pulse was markedly reduced by EGTA in a concentration-dependent manner (147). The estimated average distance between CaCCs and VDCCs was 300–600 nm in cones and 200–450 nm in rods. This more diffuse distribution, estimated by functional measurements, appeared consistent with the results obtained by immunostaining of the Ca\(_{v}1.4\) Ca\(^{2+}\) channel subunit and of TMEM16A (147).

Future studies are needed to clarify the relative contribution of TMEM16A and TMEM16B to photoreceptor function and whether they may coexist in the same cells. This may require the development of conditional knockout mice with selective gene ablation in retinal cells.

F. TMEM16A in Nociceptive Neurons

The role of CaCCs in nociceptive neurons was elucidated by Liu et al. (128) by investigating the mechanism of action of bradykinin, an inflammatory mediator and a potent inducer of pain sensation (FIGURE 4). In small nociceptive neurons of dorsal root ganglia, bradykinin elicited phospholipase C-dependent inhibition of M-type K\(^{+}\) channels and activation of a Ca\(^{2+}\)-dependent Cl\(^{-}\) conductance (128). The resulting membrane depolarization triggered firing of action potentials by neurons. These neurons were found to express TMEM16A protein, as also shown previously by Yang et al. (256), and knockdown of TMEM16A expression by transfection of siRNA decreased the amplitude of CaCC currents (128). Recently, TMEM16A was found to directly interact with the inositol 1,4,5-triphosphate receptor 1 (99). This interaction may be important in nociceptive neurons for the selective activation of TMEM16A by bradykinin and not by voltage-dependent Ca\(^{2+}\) channels (see also paragraph on TMEM16A interactome).

Recently, another important mechanism of activation of TMEM16A, highly relevant to its expression in sensory neurons, was discovered (34). TMEM16A/ANO1 and TMEM16B/ANO2 were found to be highly sensitive to temperature when expressed in HEK-293 cells (FIGURE 4).

In particular, temperatures above 44°C activated TMEM16A Cl\(^{-}\) currents even in the absence of Ca\(^{2+}\) (high BAPTA concentration on the cytosolic side of the membrane). At more physiological temperatures, heat and Ca\(^{2+}\) appeared to act in a synergistic way on channel activity: increases in cytosolic free Ca\(^{2+}\) concentrations decreased the temperature threshold required for TMEM16A activation (34). The role of TMEM16A as a heat sensor was also demonstrated in sensory neurons of dorsal root ganglia (DRG). Actually, DRG neurons were already known to express other heat sensors such as the TRPV1 channel, the capsaicin receptor (16). However, the presence of other temperature-sensitive mechanisms in these cells was expected because Trpv1 knockout mice keep a response to high temperature (249). Cho et al. (34) demonstrated that DRG neurons respond to high temperature with activation of Cl\(^{-}\) currents and that such currents are preserved in cells from Trpv1 knockout mice. The temperature-sensitive Cl\(^{-}\) currents were dependent on the expression of TMEM16A because 1) they were inhibited in neurons isolated from mice injected with anti-TMEM16A siRNA, and 2) they were strongly reduced in neurons derived from a conditional knockout mouse (TMEM16A deleted only in sensory neurons). When TMEM16A was downregulated in vivo by siRNA silencing or by gene knockout, mice showed a decreased response to noxious high temperature (>50°C). The mechanism of action of TMEM16A is based on neuron membrane depolarization, which in turn triggers firing of action potentials. Indeed, the relatively high intracellular Cl\(^{-}\) concentration in sensory neurons (>30 mM) causes Cl\(^{-}\) efflux and, hence, depolarization when TMEM16A channels open. Cho et al. (34) demonstrated that both TRPV1 and TMEM16A contribute to the heat sensitivity of neurons because a combination of capsazepine (TRPV1 inhibitor) and mefloquine (CaCC inhibitor) was required to fully block the depolarization induced by high temperature. In conclusion, the expression and function of TMEM16A in nociceptive neurons indicate this protein as an important target for analgesic therapies. However, the role of TMEM16A in the transduction of painful stimuli also implies that pharmacological activators of this channel, developed for the treatment of other diseases (e.g., cystic fibrosis), may cause serious pain as a side effect.

G. TMEM16B in Hippocampal Neurons

A recent study has revealed the importance of CaCCs in general, and of TMEM16B in particular, in controlling action potential firing and frequency in the somatodendritic region of hippocampal neurons (90; FIGURE 4). The authors found that the application of depolarizing stimuli in murine cells triggered a conductance activated by the voltage-dependent influx of Ca\(^{2+}\). Indeed, this conductance was removed by blocking Ca\(^{2+}\) channels with Cd\(^{2+}\) or by chelating intracellular Ca\(^{2+}\) with BAPTA. The Ca\(^{2+}\)-dependent conductance was identified as a CaCC component because...
of the following lines of evidence: 1) it was abolished by CaCC inhibitors like niflumic acid or NPPB and 2) its reversal potential was dependent on the Cl\(^{-}\) concentration in experimental solutions. To understand the molecular identity of CaCCs in hippocampal neurons, the authors analyzed the expression of TMEM16A and TMEM16B. Only TMEM16B was expressed at mRNA and protein levels. Knockdown of TMEM16B by shRNA significantly inhibited the neuronal CaCC current. Furthermore, this current was unaltered in neurons from TMEM16A knockout mice (90).

Interestingly, CaCC inhibitors caused a broadening of action potentials in CA1 and CA3 pyramidal neurons (by nearly 30% with 100 \( \mu \)M niflumic acid). This finding reveals that the role of TMEM16B is to act as a brake on neuronal excitability. The increase in spike width caused by niflumic acid persisted even when the intracellular solution...
contained EGTA instead of BAPTA (90). Since EGTA is a slow chelating agent, this result indicates that TMEM16B channels are close to the site of Ca\(^{2+}\) entry (i.e., voltage-dependent Ca\(^{2+}\) channels).

The excitatory synaptic response was studied by recording from CA1 neurons while stimulating the axons of CA3 neurons (90). It was found that CaCCs act in the somatodendritic region of CA1 neurons by raising the threshold of action potentials and contrasting the summation of excitatory postsynaptic potentials. Obviously, the inhibitory role of CaCC on action potential firing is dependent on the normal low Cl\(^{-}\) concentration (5–10 mM) in mature neurons. As discussed by Huang et al. (90), increases in Cl\(^{-}\) concentration, as those that may occur during prolonged neuronal activity or under pathological conditions, are expected to change the influence of CaCCs from inhibitory to excitatory.

H. TMEM16A Role in Cell Volume Regulation

All cells are able to regulate their own volume by means of a complex array of channels and transporters that mediate the efflux or uptake of electrolytes and organic osmolytes. For example, when cells are exposed to a hypotonic environment, they initially swell because of osmotic uptake of water. However, they subsequently shrink, returning to their initial volume, by activating in parallel K\(^{+}\) and Cl\(^{-}\) channels (212). The resulting net efflux of KCl drives water outside the cells, thus causing the so-called “regulatory volume decrease” (RVD). RVD is also favored by the characteristic permeability of volume-sensitive Cl\(^{-}\) channels to organic osmolytes, such as taurine and myo-inositol, that are also important in determining the cell shrinkage (96). Cell volume decrease by activation of K\(^{+}\) and Cl\(^{-}\) channels may also occur in isotonic conditions. Isotonic shrinkage can be the mechanism underlying the apoptotic cell volume decrease (123). Volume decrease, particularly if localized to a region of the cell, can also be important in cell shape changes, a process associated with cell migration. Furthermore, cell volume regulation may be continuously needed to compensate for changes in intracellular osmolarity due to uptake and metabolism of nutrients and macromolecules (123).

The anion channel that is most frequently associated with cell volume changes, particularly with activation by hypotonic shock, is the volume-regulated anion channel (VRAC), also known as volume-sensitive organic osmolyte-anion channel (VSOAC). This channel has biophysical characteristics differing from those of CaCCs. It has an intrinsic outwardly rectifying current-voltage relationship (i.e., the channel has a higher conductance at positive membrane potentials) and a typical time-dependent inactivation when the membrane is depolarized (97, 113, 163). These characteristics indicate that VRAC and CaCCs are due to different molecular entities. Actually, the protein(s) constituting VRAC are still unknown. However, this does not exclude TMEM16A/CaCC and other anoctamins as other important physiological determinants of cell volume regulation. In cells expressing TMEM16A, mobilization of intracellular Ca\(^{2+}\) could induce anion efflux, thus contributing to cell volume decrease. This mechanism is supported by experimental data: knockdown of TMEM16A by siRNA in pancreatic CFPAC-1 and intestinal HT-29 cells decreased the amplitude of Cl\(^{-}\) currents evoked by a hypotonic shock (3). Furthermore, RVD was impaired in cells treated with siRNAs against TMEM16A and in epithelial cells from the TMEM16A knockout mouse. Intriguingly, the currents activated by cell swelling and RVD appear to depend also on other anoctamins, particularly TMEM16F, TMEM16H, and TMEM16J (3).

In future studies, it will be important to determine the relationship between anoctamins and VRAC and how the activity of different Cl\(^{-}\) channel proteins are controlled during regulation of cell volume.

IV. TMEM16F AND OTHER ANOCTAMINS

A. TMEM16F as a Multifunctional Membrane Protein

Among anoctamins, TMEM16F (ANO6) is one of the most highly expressed in different tissues and cell types (194, 202). In mouse, TMEM16F, like TMEM16H and TMEM16K, shows a high expression in several tissues, a characteristic that may imply important housekeeping roles (194). In contrast, TMEM16B, TMEM16C, and TMEM16D appear mainly expressed in neuronal tissues. In particular, while TMEM16B is highly expressed in the eye, TMEM16C and TMEM16D are expressed in spinal cord, brain stem, and cerebellum. Instead, TMEM16E is specifically localized in skeletal muscle and thyroid (194).

In terms of phylogenetic analysis, TMEM16F is a close relative of TMEM16E/ANO5 and a distant relative of TMEM16A and TMEM16B (68). In 2010, Suzuki et al. (215) reported the unexpected association of TMEM16F with phospholipid scramblase activity.

The plasma membrane is characterized by an asymmetric distribution of lipids in the bilayer, being the negatively charged phosphatidylserine (PS) much more abundant in the inner leaflet (18). This asymmetrical distribution is maintained by the activity of a protein called “flippase” (aminophospholipid translocase, a P-type ATPase). However, under physiological and pathological conditions, activation of a Ca\(^{2+}\)-dependent “scramblase” dissipates the asymmetry by catalyzing the rapid externalization of PS on
the cell surface (18). This process has a variety of important roles in different cells. In platelets, exposed PS triggers the coagulation cascade. PS externalization is also a general feature of cells undergoing apoptosis. Indeed, exposed PS is a signal for the removal of dying cells by phagocytes (18). Furthermore, transient and localized PS scrambling may be a general signaling phenomenon associated with various cell processes including exocytosis, membrane fusion, and synaptic transmission (1, 93, 105, 210, 231, 258).

The molecular identity of scramblases was unclear. There are four proteins belonging to the same family, which still have the official name of phospholipid scramblases: PLSCR1–4 (18). However, the direct role of these proteins in the translocation of phospholipids is uncertain. Upregulation or knockdown of PLSCR proteins does not change the rate of phospholipid scrambling (1, 18, 56, 267). It appears now that PLSCR proteins are signaling molecules with a possible function in the nucleus as gene activators (190).

Suzuki et al. (215) used an expression cloning approach to look for proteins directly involved in phospholipid scrambling. The authors used a mouse B-cell line, Ba/F3, with endogenous Ca$^{2+}$-dependent scramblase activity, as detected by binding of fluorescent annexin V to the cell surface (FIGURE 5). Cycles of fluorescence-activated cell sorting allowed the isolation of a cell clone characterized by large scramblase activity. A cDNA library generated from these cells was transfected in native Ba/F3 cells to identify the coding sequence inducing scramblase activity (215).
ment of the aspartic acid at position 409 with a glycine, in the first intracellular loop (215). The results indicated that TMEM16F is associated with scramblase activity and that the D409G mutant, spontaneously arisen in Ba/F3 cells, increased the sensitivity of the scramblase to intracellular Ca\(^{2+}\), so that PS was externalized even under resting conditions. Of note, no scramblase activity was found for TMEM16A. Suzuki et al. (215) also found a loss-of-function mutation in the TMEM16F gene of a patient with Scott syndrome. This is a genetic disease in which platelets have a defect in PS scrambling leading to a coagulation deficit and bleeding. The patient had a G-to-T homozygous mutation at the splice-acceptor site in intron 12 causing skipping of exon 13 with sequence frameshift and generation of truncated TMEM16F protein (215).

The results obtained by Suzuki et al. (215) showed that cells with constitutive exposure of PS on the cell surface, as those expressing the mutant TMEM16F-D409G, were viable. This finding was intriguing because PS externalization was considered an important signal of apoptosis. In a subsequent study, the same research group, by further screening of the original cDNA library, identified another version of TMEM16F with a gain of function (200). This TMEM16F version, named D430G-L, corresponded to a splice variant having 21 additional amino acids at position 24 plus the previously identified aspartic-to-glycine mutation. Interestingly, D430G-L protein was even more active than the D409G mutant. Cells expressing D430G-L showed a high and constitutive level of PS on the cell surface, were viable, and grew in response to IL-3 with the same rate as parental cells. Also, there was no difference in terms of apoptosis induced by FasL (200). Finally, cells with high PS on the surface were not engulfed by macrophages in vitro and in vivo. These results indicate that PS externalization is not sufficient per se as a signal to trigger phagocytosis and further emphasize the information that PS scrambling may occur in nonapoptotic cells.

Interestingly, TMEM16F is also endowed with ion channel activity, although the biophysical properties of TMEM16F-dependent channels differ among studies. In the first report on this topic (139), TMEM16F appeared as an essential component of the outwardly rectifying Cl\(^{-}\) channel (ORCC), a type of anion channel characterized by intermediate single-channel conductance (~50 pS) and nonlinear current-voltage relationship. ORCCs are typically activated in different types of cells when excised membrane patches are treated with strong and prolonged membrane depolarization (117). They are also activated in intact Jurkat lymphocytes during apoptosis induced by FasL (216). Martins et al. (139) found that knockdown of TMEM16F/ANO6 (but not of TMEM16E/ANO5 nor of TMEM16H/ANO8) by siRNA strongly inhibited the FasL-induced activation of ORCC chloride currents. A similar inhibitory effect of TMEM16F gene silencing was found when ORCCs were induced by other apoptotic stimuli such as ceramide and staurosporine. Silencing of TMEM16F downregulated staurosporine-induced ORCC also in epithelial cell lines (A549 and 9HTe-). Overexpression of TMEM16F in A549 cells resulted in ORCC Cl\(^{-}\) currents even in the absence of staurosporine (139). The direct role of TMEM16F in forming the ORCC channel was supported by the finding of an altered halide permeability caused by mutations (Y405F, K553A, and K834A) introduced in the putative pore-forming region (139).

It has been previously reported that ORCC activity is modulated by CFTR (196). Martins et al. (139) found that CFTR expression increased the activity of ORCC triggered by staurosporine and that this effect was inhibited by anti-TMEM16F siRNAs (139). Interestingly, the TMEM16F-dependent ORCC activity was not Ca\(^{2+}\)-dependent, since it persisted in the presence of the BAPTA chelating agent. Martins et al. (139) also found that the activity of TMEM16F as an ORCC facilitates apoptosis. This effect may be due to the efflux of Cl\(^{-}\) favoring cell shrinkage, a typical phenomenon occurring during apoptosis.

Activity of TMEM16F/ANO6 as a Cl\(^{-}\) channel has also been recently detected in dendritic cells (217). In contrast to epithelial and Jurkat cells, TMEM16F in dendritic cells appears to be Ca\(^{2+}\)-dependent. Indeed, elevation of intracellular Ca\(^{2+}\) by ionomycin or IP\(_3\) triggered the activation of outwardly rectifying Cl\(^{-}\) currents. Such currents were also activated, in a Ca\(^{2+}\)-dependent way, by CCL21, a physiological agonist of the chemokine receptor CCR7 that triggers chemotactic activity in dendritic cells. Knockdown of TMEM16F by siRNA resulted in reduction of Ca\(^{2+}\)-activated Cl\(^{-}\) currents and, importantly, in inhibition of dendritic cell migration (217).

Two recent studies have also established an association between TMEM16F and Ca\(^{2+}\)-activated Cl\(^{-}\) channels (77, 202). In both studies, intracellular Ca\(^{2+}\) elevation elicited outwardly rectifying Cl\(^{-}\) currents, although after a delay of several minutes. In contrast to TMEM16A, the strong rectification of currents was maintained even at the highest Ca\(^{2+}\) concentrations. However, the two studies reported different values of Ca\(^{2+}\) sensitivity, with an estimated EC\(_{50}\) of 9.6 \(\mu M\) in one study (202) and of nearly 100 \(\mu M\) in the other (77). As discussed by Grubb et al. (77), this discrepancy may be related to experimental conditions. The use of EGTA, a buffer that is not appropriate to control Ca\(^{2+}\) in the micromolar range, may be an important factor. The two studies on TMEM16F also reported differences in ion selectivity. Shimizu et al. (202) found that TMEM16F currents are carried by Cl\(^{-}\) with no significant contribution of cations, as demonstrated by the lack of effect following replacement of Na\(^{+}\) with the large cation NMDG\(^{+}\). In contrast, Grubb et al. (77) found that TMEM16F expression generated Ca\(^{2+}\)-dependent Cl\(^{-}\) channels that were also per-
measurable to Na$. In particular, the relative permeability $P_{Na}/P_{Cl}$ was 0.3 (77). Shimizu et al. (202) also found that the expression of TMEM16F was not associated with the appearance of Cl$^{-}$ channels activated by staurosporine, as instead shown by others (139). They also showed no relationship between TMEM16F and the volume-sensitive Cl$^{-}$ channel (202).

The identification of TMEM16F as a Cl$^{-}$ channel contrasts with the results of another recent study in which TMEM16F was actually found to mediate a cation conductance (255). The authors generated a TMEM16F knockout mouse. The platelets, erythrocytes, and B-cells from these animals showed reduced Ca$^{2+}$-dependent PS exposure, in agreement with the function of TMEM16F as a scramblase component. Importantly, TMEM16F +/- mice showed impaired coagulation, as measured by thrombin generation, and prolonged bleeding, thus resembling the phenotype of Scott syndrome patients. The knockout animals were also markedly less prone to induced carotid artery thrombosis compared with wild-type mice. Megakaryocytes, the precursors of platelets, were studied with patch-clamp recordings. Cells from wild-type mice showed a Ca$^{2+}$-dependent outwardly rectifying conductance that was absent from the cells of TMEM16F +/- animals. TMEM16F channel properties were also studied by its expression in Axolotl oocytes and HEK-293 cells (255). Unlike TMEM16A, the conductance generated by TMEM16F expression did not become linear at high Ca$^{2+}$ concentrations but remained outwardly rectifying. Surprisingly, the TMEM16F currents were found to carry cations and not Cl$^{-}$ (255). Changes in the ion composition of the experimental solutions during recordings caused shifts in reversal potential of the currents consistent with $P_{Na}/P_{Cl}$ of ~7. In the same type of experiments, the $P_{Na}/P_{Cl}$ of TMEM16A was ~0.14. The cation selectivity of TMEM16F channels was confirmed in megakaryocytes of wild-type mice.

TMEM16F channels were almost equally permeable to Na$, K$, Li$, Rb$, and Cs$. Importantly, TMEM16F were more permeable to Ca$^{2+}$ and Ba$^{2+}$ than to monovalent cations (255). Measurements of single-channel conductance gave a very small value of nearly 0.5 pS. Because of these characteristics, TMEM16F channels were called SCAN channels (for “small-conductance Ca$^{2+}$-activated cation channels”). The SCAN acronym was chosen to distinguish the new type of channels from the better known CAC channels. Importantly, TMEM16F or TMEM16F with the D409G mutation identified by Suzuki et al. (215) did not cause any increase in Ca$^{2+}$-induced PS exposure.

Surprisingly, expression in HEK-293 cells of wild-type TMEM16F or TMEM16F with the D409G mutation identified by Suzuki et al. (215) did not cause any increase in Ca$^{2+}$-induced PS exposure.

The results by Yang et al. (255) are intriguing because they suggest that anion and cation channels may coexist within the anoctamin family. However, the discrepancy between these results and those obtained by other authors (77, 139, 202), who identified TMEM16F as an ORCC and/or a CaCC, remains to be clarified.

Another puzzling result is the finding that overexpression of TMEM16F (wild-type or D409G mutant) is able to induce PS scrambling in Ba/F3 cells (215) but not in HEK-293 cells (255). These results would indicate that TMEM16F is required but not sufficient for scramblase activity.

Yang et al. (255) propose three alternative hypotheses to explain TMEM16F involvement in scramblase activity (FIGURE 8). According to the first hypothesis, Ca$^{2+}$ influx through TMEM16F may activate the real (still unknown) scramblase. However, this hypothesis implies that TMEM16F-dependent Ca$^{2+}$ influx could be bypassed by directly increasing cytosolic Ca$^{2+}$ with ionomycin. This contrasts with the results obtained by Kmit et al. (112). Ca$^{2+}$ elevation by ionomycin did not induce PS scrambling in lymphocytes obtained from two patients with Scott syndrome. In the second hypothesis, TMEM16F may work as a Ca$^{2+}$ sensor of the scramblase. Finally, the third hypothesis postulates that the same TMEM16F protein mediates both cation transport and phospholipid scrambling. In one possible model, Ca$^{2+}$ binding to TMEM16F changes its conformation leading to formation of a cleft through which

that is critical for ion selectivity. They noted that TMEM16F has a glutamine residue (Q559) in a position that is instead occupied by a lysine in TMEM16A and TMEM16B. Mutagenesis of Q559 to lysine transformed TMEM16F in a channel more permeable to Cl$^{-}$ ($P_{Na}/P_{Cl}$ = 2.2), whereas mutagenesis of K584 to glutamine converted TMEM16A channels to a less anion-permeable state ($P_{Cl}/P_{Na} = 4.1$ instead of 7.0).
the hydrophilic headgroups of phospholipids may translocate from one side to the other of the membrane. This change in conformation could also create a pore for cations (or anions).

B. Activity of Other Anoctamins as Phospholipid Scramblases

TMEM16F as a scramblase is not alone within the anoctamin family. In a recent study, Suzuki et al. (214) generated a murine cell line devoid of TMEM16F expression. These cells were used as a recipient for the expression of anoctamins. Scramblase activity was detected using fluorescently labeled lipids. Suzuki et al. (214) found that TMEM16D, TMEM16F, TMEM16G, and TMEM16J mediate Ca\(^{2+}\)-dependent scrambling of phosphatidylserine (PS), phosphatidylycholine (PC), and galactosylceramide (GalCer) (TABLE 1). Instead, TMEM16C expression elicited scrambling of PC and GalCer but not of PS. Interestingly, TMEM16D-expressing cells showed a significant level of scramblase activity under resting conditions, in the absence of intracellular Ca\(^{2+}\) elevation (214).

Suzuki et al. (214) also looked for ion channel activity. Only TMEM16A and TMEM16B generated CaCC currents (214). In particular, in contrast to other studies (77, 139, 202), TMEM16F did not appear to function as a channel (TABLE 1). Suzuki et al. (214) also noted that some anoctamins, namely, TMEM16E, TMEM16H, and TMEM16K, had neither scramblase nor channel activity. The authors proposed that the lack of function may be due to a prevalent intracellular localization of these proteins. The subcellular localization of some anoctamins is actually a matter of controversy. In one study, various anoctamins including TMEM16F were found to be intracellular and to lack any ion channel activity (47). This finding is in contrast to the activity of TMEM16F as an anion/cation channel and/or a scramblase component (77, 139, 202, 215, 255). Furthermore, other anoctamins also showed a detectable plasma membrane localization and ion transport activity (194, 223), although TMEM16H-K (AN08–10) were more highly localized in intracellular compartments. The discrepancy among different studies may be due to experimental conditions. It is important to note that TMEM16F and other anoctamins appear to require high micromolar Ca\(^{2+}\) concentrations (77, 202, 216, 255). Therefore, concentrations normally used to activate TMEM16A and TMEM16B may not allow detection of the activity of the other anoctamins. Other factors, possibly related to a cell type-dependent regulation of subcellular localization, may also be involved. It is possible that some anoctamins traffic from intracellular compartments to plasma membrane and back on the basis of regulatory signals that are still to be defined.
Another source of variability may arise from different regulatory mechanisms responsible for anoctamin activation. Some anoctamins, such as TMEM16A and TMEM16B, may be more directly activated by Ca\(^{2+}\), whereas others may require more indirect mechanisms based on Ca\(^{2+}\)-dependent phosphorylation or interaction with other regulatory subunits (194).

It is also interesting to consider the possibility that some anoctamins form heterooligomers. For example, coexpression of TMEM16A and TMEM16J elicited a level of anion transport activity that was smaller than that elicited by TMEM16A alone (194). This inhibition may arise from a direct interaction between the two types of proteins. Such a possibility indicates that the results obtained from heterologous expression of TMEM16A and TMEM16J elicited a level of anion transport activity that was smaller than that elicited by TMEM16A alone (194). This inhibition may arise from a direct interaction between the two types of proteins. Such a possibility indicates that the results obtained from heterologous expression of TMEM16A in some null systems such as HEK-293 or FRT cells may be significantly affected by the endogenous expression of other anoctamins, particularly TMEM16F, TMEM16H, and TMEM16K (194).

**Table 1. Channel/scramblase activity of anoctamins**

<table>
<thead>
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<th></th>
<th>16A</th>
<th>16B</th>
<th>16C</th>
<th>16D</th>
<th>16E</th>
<th>16F</th>
<th>16G</th>
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<tbody>
<tr>
<td>Scramblase</td>
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<td>GaCer</td>
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<td>Channel activity</td>
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</table>

Table 1 reports the ability of the various anoctamins to perform the scrambling of phosphatidylserine (PS), phosphatidylcholine (PC), and galactosylceramide (GalCer) as determined by Suzuki et al. (214). Table 1 also reports channel activity for TMEM16A, TMEM16B, and TMEM16F as determined in multiple studies (29, 77, 139, 195, 202, 255, 256). However, channel activity of TMEM16F has not been confirmed in other studies (47, 214). Other reports suggest Cl\(^{-}\) channel function also for other anoctamins.

**D. TMEM16C, a K\(^{+}\) Channel Regulator**

TMEM16C/ANO3 is an anoctamin with a particular expression in the central and peripheral nervous systems of human, mouse, and rat (32, 88, 194). Immunostaining demonstrated a high TMEM16C expression in a subset of nociceptive neurons in dorsal root ganglia (88). To assess the physiological role of TMEM16C, Huang et al. (88) generated TMEM16C knockout rats. Behavioral tests revealed that animals without TMEM16C were hypersensitive to high temperatures (≥50°C). Electrophysiological recordings from DRG neurons of mutant rats demonstrated a broadened shape of action potentials and a decreased threshold for action potential firing (88). When the electrical properties of neurons were studied in detail under voltage-clamp conditions, Huang et al. (88) found, unexpectedly, that a Na\(^{+}\)-activated K\(^{+}\) current was strongly reduced in knockout animals. Interestingly, TMEM16C was found to colocalize and directly interact with Slack, the protein forming the Na\(^{+}\)-activated K\(^{+}\) channel. In a heterologous expression system, HEK-293 cells, TMEM16C did not form ion channels by itself but enhanced the activity of Slack when both proteins were expressed together (88). In particular, the presence of TMEM16C enhanced the Na\(^{+}\) sensitivity and single-channel conductance of Slack. This finding may explain how TMEM16C regulates the excitability of nociceptive neurons. Prolonged trains of action potentials caused by pain stimuli increase intracellular Na\(^{+}\) concentration. In the presence of TMEM16C, Na\(^{+}\) increase...
upregulates Slack function, thus causing hyperpolarization of the cell membrane.

V. LESSONS FROM ANIMAL MODELS AND HUMAN DISEASES

A. Animal Models

Despite the physiological importance of CaCCs in various tissues, until now there was no evidence of human pathologies caused by defective Ca$^{2+}$-dependent Cl$^{-}$ transport. However, information deriving from knockout mice indicates that a deficit in CaCC activity/expression could have serious consequences in various organs. At the time of the identification of TMEM16A as a channel protein, the effects of the gene knockout were already known. Indeed, Rock et al. (182) published in 2008 an article reporting the importance of TMEM16A protein in murine trachea development. Actually, these authors had initially identified TMEM16A in a screen for genes expressed in the zone of polarizing activity (ZPA) of the developing limb (183). Interestingly, mice homozygous for a null allele of TMEM16A did not show any alteration of limb development but died within 1 mo of birth (182). The most apparent abnormality was the presence of ventral gaps in the cartilage rings of the trachea and an abnormal trachealis muscle, which caused the trachea to collapse, thus mimicking human tracheomalacia (182). The authors also suggested that the cartilage defect is actually secondary to defects in the epithelium or trachealis muscle, where TMEM16A is normally expressed (182). Interestingly, similar congenital cartilaginous defects can also be observed in CFTR knockout mice (24), suggesting the presence of a common defect associated with Cl$^{-}$ transport and mediated by separate Cl$^{-}$ channels, TMEM16A and CFTR (184). TMEM16A$^{-/-}$ animals also show an accumulation of mucus in the airways (184) and an impairment of mucociliary transport (167) that mimic the CF phenotype. In addition to the trachea, TMEM16A plays a fundamental role also in the pacemaker activity (slow waves) generated by interstitial cells of Cajal (ICC) in gastrointestinal smooth muscles (87, 94). Indeed, slow waves fail to develop in muscles of TMEM16A$^{-/-}$ mice, although ICC networks develop normally (87, 94). It is highly probable that the lack of gastrointestinal peristalsis also contributes to the lethal phenotype of TMEM16A$^{-/-}$ animals. Given the very severe and complex phenotype of conventional knockout mice, a more refined analysis of the role of TMEM16A in each organ requires the development of conditional knockout animals. For

![Detection of afTMEM16 as a dual function protein.](http://physrev.physiology.org/)

**FIGURE 7.** Detection of afTMEM16 as a dual function protein. Purification and reconstitution of Aspergillus fumigatus TMEM16 protein (afTMEM16) in liposomes elicits Ca$^{2+}$-dependent ion transport, as detected with an ion-sensitive electrode (135). Liposomes with afTMEM16 also show scramblase activity. This was measured with fluorescent lipids that are transported by afTMEM16 from the inner to the outer leaflet of liposome membrane. The fluorescence of the externalized lipid is quenched by dithionite. The rate of fluorescence quenching is proportional to scramblase activity.
example, the use of mice with selective TMEM16A ablation in nociceptive neurons demonstrated TMEM16A involvement in the detection of thermal stimuli (34).

As discussed before, another important animal model was created by knocking out the TMEM16B gene (19). The results from this mouse confirmed the identification of TMEM16B as the ciliary CaCC (208). In the mouse, TMEM16B protein is expressed on the apical surface of the main olfactory epithelium (where it colocalizes with the ciliary marker acetylated tubulin), in vomeronasal sensory neurons (colocalized with TMEM16A), and on the axons of the olfactory sensory neurons (19). TMEM16B −/− mice lack CaCC currents in the main olfactory epithelium and in the vomeronasal organ and show a 40% reduction in the fluid-phase electroolfactogram responses. However, as stated previously, they have no detectable impairment of olfaction (19). Future studies will need to address this issue by generating conditional knockout mice for the TMEM16B gene. In this way, by knocking out the gene at the desired location and at a specific time, it will be possible to rule out possible compensatory mechanisms that may intervene in the conventional knockout mouse by minimizing the effect of TMEM16B gene loss.

Recently, two knockout mice defective for TMEM16F have been also generated. One of these animal models (255) has been discussed in the previous paragraph. This animal shows a significant impairment in the coagulation process and in the PS scramblase activity of platelets, erythrocytes, and B-cells. The authors reported no other obvious phenotype stating that the knockout mice were viable and fertile (255). In a subsequent study, another group of investigators reported instead a severe defect in mineral deposition in the bone of TMEM16F animals resulting in reduced skeleton size and skeletal deformities (53). This defect was consistent with a particular expression of TMEM16F/ANO6 in ossifying tissues of endochondral and intramembranous bones. In particular, TMEM16F appeared constantly expressed in osteoblast and, less markedly, in osteocytes. Ehlen et al. (53) reported a high (60%) mortality of TMEM16F-deficient mice at birth. This may be caused by asphyxiation since the mutant animals showed a narrow ribcage due to deformed ribs. Abnormalities at birth also involved the limb and the skull bones. Analysis of the embryos demonstrated no alteration of osteoblast differentiation but a strongly reduced bone mineralization ability. This defect was confirmed in vitro on osteoblast cultures from wild-type and mutant animals. Osteoblasts from TMEM16F −/− mice showed a 50–70% reduction in mineral deposition but no alteration of proliferation or apoptosis (53). Interestingly, osteoblasts of knockout mice also showed a markedly reduced PS scramblase activity as detected by annexin V binding following treatment of cells with the Ca2+ ionophore ionomycin. The authors investigated whether TMEM16F-dependent scramblase activity in osteoblasts is related to apoptosis. Therefore, they triggered apoptosis with staurosporine. This treatment resulted in equal PS scrambling in both wild-type and TMEM16F-defective cells (53). These results suggest the presence of two types of scramblase activity: one type activated by intracellular Ca2+ elevation and mediated by TMEM16F/ANO6, and the other type triggered by apoptotic stimuli and due to another unknown protein. Regarding the role of TMEM16F in bone development, Ehlen et al. (53) commented that PS scrambling is important for bone mineralization. TMEM16F may be important for the release of PS-rich vesicles. These vesicles, carrying high concentrations of Ca2+ and phosphate, initiate the mineralization process.

Comparison of the two studies done on TMEM16F knockout mice (53, 255) evokes intriguing questions. What is the reason for the skeletal abnormalities and the high mortality in one study and not in the other? In this respect, it has to be noted that Scott syndrome patients show no obvious skeletal defects. On the other hand, Ehlen et al. (53) did not observe a marked defect in coagulation in their animals, although they did not perform specific tests. It is possible to hypothesize that other anoctamins may differently compensate for the loss of TMEM16F in humans compared with mice. Furthermore, differences in the strategy for the generation of the knockout animals may also explain the different phenotypes (53, 255).

B. Human Genetic Diseases

Precious information on the role of anoctamins may also derive from human genetic diseases (TABLE 2). Among the members of the anoctamin gene family, TMEM16E/ANO5 was the first to be associated with human diseases. In 2004, Tsutsumi et al. (227) proposed that gnathodiaphyseal dysplasia (GDD), a rare dominant skeletal syndrome, could be due to dominant mutations occurring in the so-called GDD1 gene, that corresponds to TMEM16E. A linkage analysis of two independent families allowed the authors to find two different missense mutations, both at codon 356, which corresponds to an evolutionarily conserved cysteine in human, mouse, zebrafish, fruit fly, and mosquito (227).

Recently, a novel mutation, T513I, was found in another family with GDD (138).

Topology prediction performed using the Tmpred software suggested that GDD1 contains eight transmembrane domains, with cytosolic NH2 and COOH termini (227). Immunolocalization studies demonstrated that wild-type GDD1 protein is localized in the ER and that overexpression of the mutant proteins decreased cell adhesion and changed cell morphology (227). Because of protein localization and topology, the authors hypothesized that GDD1 might function as an intracellular channel of the ER membrane involved in the Ca2+-dependent signaling pathway, and that mutations induce excess Ca2+ release from the ER.
Table 2. Anoctamins in physiology and pathology

<table>
<thead>
<tr>
<th>Relationship With Human Genetic and Nongenetic Diseases</th>
<th>Phenotype in Knockout Mouse</th>
<th>Physiological Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMEM16A/ANO1</td>
<td>Upregulation in gastrointestinal stromal tumors (GISTs), in breast cancer, and in head and neck squamous cell carcinomas (HNSCCs); upregulated in asthma</td>
<td>Tracheal malformation, impaired mucociliary clearance, block of gastrointestinal peristalsis</td>
</tr>
<tr>
<td>TMEM16B/ANO2</td>
<td>Absence of CaCCs of olfactory receptors but no impairment of olfaction</td>
<td>Modulation of olfactory transduction, phototransduction, and neuronal excitability</td>
</tr>
<tr>
<td>TMEM16C/ANO3</td>
<td>Mutated in cervical dystonia</td>
<td>Increased sensitivity to heat</td>
</tr>
<tr>
<td>TMEM16E/ANO5</td>
<td>Mutated in gnathodyaphyseal dysplasia (GDD), limb-girdle muscular dystrophy (LGMD2L), and Miyoshi myopathy (MM3)</td>
<td></td>
</tr>
<tr>
<td>TMEM16F/ANO6</td>
<td>Mutated in Scott syndrome</td>
<td>Impaired coagulation and skeleton abnormalities (in 2 different models)</td>
</tr>
<tr>
<td>TMEM16G/ANO7</td>
<td>Upregulation in prostate cancers</td>
<td></td>
</tr>
<tr>
<td>TMEM16K/ANO10</td>
<td>Mutated in cerebellar ataxia</td>
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</tbody>
</table>

(227). In 2007, the same authors demonstrated that, in the adult mouse, GDD1 protein is highly expressed in cardiac and skeletal muscle tissues, and in growth plate chondrocytes and osteoblasts in bone, suggesting diverse cellular roles of GDD1 in the musculoskeletal system (151). In this respect, recessive mutations in TMEM16E/ANO5 were associated with two different muscular dystrophies, proximal limb-girdle muscular dystrophy (LGMD2L) and distal Miyoshi myopathy (MM3), with characteristics that resemble dysferlinopathies caused by mutations in the dysferlin gene (23, 84). Dysferlin is a component of the sarcolemmal repair machinery, predicted to function as a fusogen in the formation of the patch membrane required for membrane resealing (79). Therefore, it was proposed that TMEM16E may play a role in the dysferlin-dependent muscle membrane repair pathway (23). In this respect, Cl⁻ currents were recorded after membrane wounding in sea urchin embryos (59), suggesting that TMEM16E may be responsible for a chloride current needed during membrane repair in muscle (23). Loss-of-function TMEM16E mutations may cause skeletal muscle demise because of defective membrane repair (23).

A second anoctamin gene causing a human genetic disease is TMEM16K. Mutations in TMEM16K/ANO10 were found to cause autosomal-recessive cerebellar ataxia (236). TMEM16K has the highest expression in the adult brain, particularly in the cerebral cortex and in the cerebellum, consistently with the relatively late onset of ataxia (236). Although it has not been elucidated yet whether TMEM16K codes for a CaCC, this hypothesis is suggestive, because these channels participate in the regulation of neuronal excitability. Indeed, one of the most important mechanisms causing cerebellar ataxia is deranged Ca²⁺ signaling in Purkinje cells (104), which is the consequence of mutations of several known ataxia-associated genes, like Ca²⁺ pumps or voltage-gated Na⁺ or K⁺ channels (226, 230, 243). If TMEM16K protein is a (or a subunit of a) CaCC, it might play a role in influencing Ca²⁺ signaling in Purkinje cells, and an abnormal function of TMEM16K could lead to cerebellar ataxia through this mechanism (236).

Recently, a study reported the identification of a third disease-causing gene in the anoctamin family. Linkage analysis combined with whole-exome sequencing identified mutations in the TMEM16C/ANO3 gene that cause a dominant form of cranio cervical dystonia (32). Five missense mutations were identified in different families: T54I, W490C, R494W, S685G, and K862N. A sixth mutation was a C to T base change in the 5′-untranslated region of the gene. The mechanism of action of mutations is unknown since the function of TMEM16C is not well defined. However, TMEM16C seems particularly expressed in striatum and, as discussed previously, appears to regulate a neuronal K⁺ channel (88). Therefore, it is possible that alterations in TMEM16C function caused by the dominant mutations perturb the excitability of striatal neurons. It would be interesting to assess if the causal relationship between TMEM16C and cranio cervical dystonia in humans is related with the regulatory role of TMEM16C on Na⁺-dependent K⁺ channels discovered in rats (88).

As discussed in previous sections of this review, another anoctamin gene, TMEM16F, was found to be mutated in Scott syndrome (215). Scott syndrome is a rare congenital bleeding disorder characterized by a deficiency in platelet procoagulant activity that is not associated with decreased levels of coagulation factors (244, 268). More precisely, Scott syndrome is due to an aberrant phospholipid composition of the outer membrane leaflet of platelets, namely, a defective surface exposure of PS upon Ca²⁺-dependent activation by injured blood vessels (267). Defective function of the Ca²⁺-dependent scramblase protein is the cause of Scott syndrome (127, 267). Direct sequencing of
TMEM16F gene of a subject affected by Scott syndrome revealed that the patient carried a homozygous mutation at the splice-acceptor site in intron 12, which caused skipping of exon 13 with a frameshift resulting in the premature termination of the TMEM16F protein in exon 14, at the third transmembrane segment (215). More recently, Castoldi et al. (31) identified two novel mutations in the TMEM16F gene in a patient with Scott syndrome: a transition at the first nucleotide of intron 6 (IVS6 +1G→A), disrupting the donor splice site consensus sequence of intron 6, and a single-nucleotide insertion in exon 11 (c.1219insT), predicting a frameshift and premature termination of translation at codon 411.

In a study carried out on the platelets of a Scott syndrome patient and from control individuals, the results provided evidence for the existence of at least two mechanisms of phospholipid scrambling (232). Stimulation of platelets with the Ca\(^{2+}\)-mobilizing agonists convulxin and thrombin generated a high level of phosphatidylserine externalization in normal platelets. This process was totally absent in the platelets of the patient, which indicated the total dependence on a functional TMEM16F protein. In contrast, the PS exposure induced by a pro-apoptotic stimulus was only partially inhibited in the platelets of the Scott syndrome patient (232). If TMEM16F is a Ca\(^{2+}\)-dependent scramblase, these results would indicate the existence of a second protein involved in the externalization of PS. Alternatively, it can be hypothesized that TMEM16F is one of the multiple regulators (e.g., the Ca\(^{2+}\) sensor) of a still unknown scramblase.

C. TMEM16 Proteins and Cancer

Even before the discovery of TMEM16A and TMEM16B as possible Cl\(^{-}\) channels, it was found that many anoctamins are highly expressed in human cancers (TABLE 2). TMEM16A, named DOG1 (discovered on gastrointestinal stromal tumor 1), was highly expressed in gastrointestinal stromal tumors (245). Known also as TAOS2 (tumor amplicon 2), TMEM16A gene was amplified and overexpressed in oral squamous cell carcinomas (91). TMEM16G, also termed NGEP, is particularly expressed in prostate cancers (17). Furthermore, the pattern of TMEM16F RNA splicing is associated with the metastatic potential of mammary cancers in mouse and with the malignancy of breast cancers in humans (49).

The reason for the high expression of some anoctamins, particularly TMEM16A, in tumors is unclear. However, various studies have recently started to investigate the relationship between TMEM16A and cancer.

It appears that TMEM16A/ANO1 overexpression is not a general phenomenon but is related to specific types of tumors. In a recent study, 4,000 samples belonging to 80 types of human cancers were analyzed (188). TMEM16A was mostly expressed in head and neck squamous cell carcinomas (HNSCCs) and in gastrointestinal stromal tumors (GISTs). In another study, TMEM16A/ANO1 was found to be overexpressed in breast cancers (25). TMEM16A overexpression is in many cases due to amplification of the corresponding chromosomal region in 11q13 (9, 188). Interestingly, ANO1 protein expression is associated with reduced survival and increased metastatic potential (9, 52, 188). In particular, individuals with cancers having TMEM16A expression were characterized by a median survival time of 23 mo compared with 56 mo for individuals without TMEM16A expression (188).

In some in vitro studies, done on cultured cancer cells, TMEM16A expression correlated with the rate of cell proliferation. Indeed, TMEM16A knockdown by RNA interference (25, 52, 129) or inhibition with CaCC inhibitors (25, 145, 207) resulted in reduced cell growth in vitro. TMEM16A was also found to influence cell migration, as determined by wound healing assays. The rate of cell migration was significantly reduced by TMEM16A downregulation (9, 129, 188). In some studies, TMEM16A appeared to affect migration but not proliferation (9, 188). In another study, TMEM16A influenced GIST cell growth in vivo but not in vitro (204). TMEM16A role in tumor growth in vivo was also demonstrated for HNSCC, prostate carcinoma, and breast cancer (25, 52, 129).

The differences among studies, particularly regarding the effect of TMEM16A in vitro, may imply cell type-dependent mechanisms. For example, Ubby et al. (228) expressed various TMEM16A isoforms in HEK-293 cells using an inducible system. TMEM16A expression did not correlate with cell migration or proliferation in vitro. Therefore, the role of TMEM16A in cell proliferation and migration is not universal but dependent on the particular intracellular and extracellular environment of cancer cells.

The link between TMEM16A/ANO1 and cancer cell growth is still unclear. However, it may be related to altered cell signaling. For example, downregulation of TMEM16A expression in GIST cells resulted in strong upregulation of insulin-like growth factor-binding protein 5 (IGFBP5), a potent antiangiogenic factor (204). In breast cancer cells, TMEM16A expression/function instead correlated with signaling through the EGF receptor and the calmodulin-dependent kinase (25). Furthermore, it was found that TMEM16A overexpression increases the phosphorylation of ERK1/2 and the levels of cyclin D1 (52). In this study, overexpression of TMEM16A with a mutation (K610A) that abolishes Cl\(^{-}\) transport failed to induce phosphorylated ERK1/2 (52). This finding indicates that Cl\(^{-}\) transport is important for the effect on cell cycle and proliferation, a mechanism that may involve a change in intracellular Cl\(^{-}\) concentration. It is important to remark that, in non-cancer
cells (basilar artery smooth muscle cells), TMEM16A expression appears to have an inverse relationship with proliferation (238). Wang et al. (238) found that TMEM16A downregulation induced by angiotensin II stimulation was associated with increased cell proliferation and increased levels of cyclins D1 and E.

Another mechanism that may explain the role of TMEM16A in cell proliferation and, particularly, in cell migration is cell volume regulation. Coordinated efflux of Cl⁻ and K⁺ causes cell shrinkage. This may favor the migration of cancer cells through tissues. Indeed, swelling and shrinkage occur at the front end and rear end of a migrating cell, respectively. The role of TMEM16A in cell volume regulation was demonstrated in an overexpressing cancer cell line (BHY). Application of a hypotonic shock did not cause the expected cell swelling (188). Actually, cell swelling was observed only when TMEM16A was silenced with siRNA. This result may be explained by a fast activation of TMEM16A, in nonsilenced cells, that rapidly shrinks the cells, thus preventing the detection of the transient cell swelling.

In summary, several studies indicate an important role of TMEM16A in some types of human cancers. TMEM16A is therefore an important diagnostic and prognostic marker and may represent an important drug target.

The other anocytotamins may have additional and even more important roles in cancer cell biology. For example, TMEM16F, with its involvement in cell migration, cell volume regulation, and apoptosis (3, 139, 217), could have a particular impact on the growth and metastatization of human cancers.

**VI. DRUGGABILITY OF CACCs**

For many years, the lack of selective inhibitors has limited the studies on CACCs. Selective and potent blockers are valuable research tools to identify ion channels, to analyze tissue distribution and contribution to physiological processes, and also to explore the properties of the channel pore. Available CACC inhibitors were neither selective nor potent, requiring high concentrations to completely block Cl⁻ currents with undesirable side effects. Niflumic acid (FIGURE 8), often considered a specific blocker and even used to identify membrane currents as CACCs in different tissues, can also affect other channels or cause emptying of intracellular Ca²⁺ stores (11, 38, 80, 165, 176, 197, 264). In addition to being valuable research tools, more selective CACC inhibitors could also have important applications as therapeutic agents (234, 235).

When TMEM16A was discovered as a CACC component, it was found that the anion transport associated with its expression is sensitive to a large panel of general Cl⁻ channel inhibitors (FIGURE 8), including niflumic acid, NPPB, DIDS, furosemide, and fluoxetine in the 5–20 μM range (29, 195, 256). DPC, another nonspecific Cl⁻ channel inhibitor, required millimolar concentrations to be effective (195). CFTRinh-172, a compound widely used as a selective CFTR inhibitor (28, 132), was ineffective on TMEM16A currents (29). In contrast, GlyH-101, which is also used to block CFTR (153), showed a significant activity at 20–50 μM (29).

Other CACC/TMEM16A inhibitors have been identified by screening chemical libraries using a high-throughput functional assay. In one of the first studies, the molecular identity of CACCs was still unknown. Therefore, the authors used an intestinal epithelial cell line, HT-29, with endogenous activity of CACC, to perform a high-throughput screening of a library of 50,000 compounds (44). The authors reported the identification of six new classes of CACC inhibitors, two of which (classes A and B) considered particularly interesting, on the basis of multiple criteria, including potency, water solubility, drug-like properties, identification of active analogs, chemical stability, and CACC targeting (44). Among these inhibitors, the compound labeled CaCCinh-A01 (FIGURE 8) is one of the most used to test the contribution of CACCs to physiological processes (81, 166).

After the identification of TMEM16A as a CACC, Verkman et al. (155) performed a second high-throughput screening aimed at the discovery of new classes of small molecule TMEM16A inhibitors, using a rat epithelial cell line, FRT, with heterologous expression of TMEM16A, and a library of 110,000 compounds. The authors found four novel classes of inhibitors that seem to target directly TMEM16A protein, rather than upstream processes, such as extracellular agonist binding or Ca²⁺ signaling. In addition, they compared the effect of one of the newly discovered TMEM16A inhibitors, T16inh-A01 (FIGURE 8), with that of CaCCinh-A01 (44) in various tissues, concluding that, while in salivary gland epithelium TMEM16A is responsible for all CACC activity, in airway and intestinal epithelia it is only a minor contributor to total CACC current (155). T16inh-A01 has also been used by different research groups as a pharmacological probe to demonstrate the involvement of TMEM16A channel in a series of physiological and pathological processes including transepithelial ion transport, smooth muscle function, and cancer (43, 52, 152, 213). T16inh-A01 was also found to inhibit acrosomal reaction in human spermatozoa, thus revealing a possible role of TMEM16A in the fertilization process (164).

In another study, the screening of a relatively small chemical library (2,000 compounds) identified benzbromarone (FIGURE 8), dichlorophen, and hexachlorophen as TMEM16A inhibitors (89). These compounds inhibited airway smooth muscle contraction and mucin release from goblet cells,
which underlines the potential of TMEM16A as a drug target in asthma.

Natural compounds appear as another interesting source of TMEM16A/CaCC inhibitors. In one study, by screening a chemical library with a high representation of natural compounds, the authors found that tannic acid (FIGURE 8) is an effective inhibitor of TMEM16A Cl\(^{-}\)/H\(^{+}\) currents (156). Tannic acids and other related compounds also had an inhibitory effect on the contractility of arterial smooth muscle. The authors concluded that the gallotannin content of red wine and green tea could be the molecular basis for their beneficial effect on the cardiovascular system (156). In another study, the antidiarrheal effect of a Thai herbal preparation was investigated (257). Fractionation of the preparation by HPLC led to the identification of eugenol as a TMEM16A inhibitor, although with potency in the high micromolar range.

In a recent study, the evaluation of a selected panel of anthranilic acid derivatives allowed the identification of a potent TMEM16A inhibitor, termed MONNA (162). This compound acted with an IC\(_{50}\) of 0.08 and 1.3 \(\mu\)M on Xenopus and human TMEM16A, respectively. MONNA appeared to be quite selective regarding other anion channels, since it did not block CFTR, CIC2, and bestrophin 1 (162). As for other TMEM16A inhibitors, it is not clear yet if MONNA acts directly on the channel or indirectly through interaction with a regulatory protein.

Verkman et al. (157) have also looked for activators of TMEM16A, which could be useful as research tools and as potential drugs for the treatment of salivary gland dysfunction, cystic fibrosis, dry eye syndrome, intestinal hypomotility, and other disorders associated with Cl\(^{-}\) channel dysfunction. The chosen strategy was once again the screening of a library of 110,000 compounds using FRT cells with

**FIGURE 8.** Pharmacological modulators of CaCCs. Structure of classical CaCC blockers (niflumic acid, NPPB, DIDS), of new TMEM16A inhibitors (T16inh-A01, CaCCinh-A01, tannic acid, eugenol, benz bromarone), and of a TMEM16A activator (Eact).
heterologous expression of TMEM16A (157). The authors reported the identification of six classes of TMEM16A activators, two of which (classes E and F) considered particularly interesting, because they do not cause elevation of cytoplasmic Ca\(^{2+}\) and produce maximal TMEM16A activation, although class E activator (FIGURE 8) can activate TMEM16B as well (157). Looking at a panel of analogs, the authors also realized that minor chemical structural changes can convert an activator into an inhibitor, since they found compounds belonging to classes B and E that fully inhibited TMEM16A Cl\(^{-}\) conductance, which supported the conclusion that these compounds target TMEM16A directly (157). Interestingly, a similar behavior, i.e., compounds having both activator and inhibitor properties, had also been previously reported for native CaCCs (125, 173). In this respect, it should be noted that the properties of CaCC pharmacological modulators may be affected by phosphorylation (246).

Pharmacological sensitivity of TMEM16A channels may also be affected by cell environment and extent of expression. Indeed, it was found that, in heterologous expression systems, but not in native cells, TMEM16A channels are stimulated by openers of Ca\(^{2+}\)-activated K\(^{+}\) channels such as 1-EBIO (223, 224).

In summary, a large panel of pharmacological inhibitors and, to a lesser extent, of activators of the TMEM16A channel is now available. In contrast, the pharmacology of the other anoctamins is still in its infancy. TMEM16A pharmacological modulators may have broad therapeutic applications. By antagonizing the contractility of smooth muscle cells (43), TMEM16A inhibitors may be useful in hypertension, asthma, and as spasmolitic agents. The inhibition of mucin release shown by some TMEM16A inhibitors (89) suggests that they may be beneficial in asthma not only as bronchodilators but also to prevent mucus hypersecretion. However, it is not clear whether inhibition of mucin release from goblet cells is indeed a good thing for asthmatic patients. Actually, activators of TMEM16A were considered useful in cystic fibrosis and other chronic respiratory disease to promote mucociliary clearance (157). Therefore, the precise role of TMEM16A in the airway epithelium needs to be clarified. Recently, a study done on a transgenic mouse has shown that hypersecretion of MUC5AC in the airways actually has protective role (54). The implications of this study are that the abundant mucin secretion alone is not the cause of airway obstruction in some respiratory diseases. Inadequate support of electrolytes and water, in parallel with mucin release, may be a critical factor.

TMEM16A activators have the potential ability to generate important side effects (FIGURE 9). If delivered to the airways by inhalation, they could potentially generate bronchoconstriction by acting on airway smooth muscle. If delivered systemically, they could induce pain, at least theoretically, by acting on the DRG cells where TMEM16A works as a heat sensor (34). Furthermore, it is not clear whether activators may also generate problems (e.g., hypertension, spasms, colics) in the cardiovascular system, gastrointestinal tract, urinary system, and other organs where the activity of TMEM16A favors smooth muscle contraction. TMEM16A inhibitors may also generate side effects systematically. For example, block of TMEM16A could produce block of gastrointestinal peristalsis. A better understanding of TMEM16A function in different tissues, its interaction with regulatory networks, and the role of splicing variants will be important for the rational development of selective drugs.

VII. CONCLUSIONS

The identification of TMEM16A/ANO1 and TMEM16B/ANO2 apparently ended a search that lasted for several years: the long sought CaCCs had finally a molecular identity (29, 195, 256). In particular, it appeared that the finding of two different proteins working as CaCCs, each one available in multiple variants, was able to explain the variety of biophysical characteristics previously reported for CaCCs in different tissues (48, 80). TMEM16A channels were similar to native CaCCs expressed in epithelial and smooth muscle cells, whereas TMEM16B channels were more representative of CaCCs observed in olfactory receptors and other neurons. Furthermore, the existence of TMEM16A and TMEM16B paralogs in the anoctamin family suggested that other types of Cl\(^{-}\) channels could be discovered. Indeed, TMEM16F/ANO6 expression was found associated with the activity of outwardly rectifying Cl\(^{-}\) channels, ORCCs (139), or with CaCCs having “non-canonical” characteristics (77, 202). However, the intriguing and unexpected discovery of TMEM16F as a membrane protein linked to the scrambling of membrane phospholipids (215) revealed the possibility that some anoctamins have additional or alternative functions.

At the moment, it is difficult to reconcile all results obtained with TMEM16F. One possibility is that TMEM16F is essentially a scramblase that in turn regulates ion channels, possibly by modification of the local lipid environment (FIGURE 6). In this respect, there are several examples of channels or receptors whose activity is influenced by electrostatic interaction with the charged head groups of phospholipids or by changes in the thickness/composition of the lipid bilayer (39, 83, 131). If TMEM16F regulates other channels, this could explain the discrepant results obtained in different studies. Indeed, TMEM16F has been found to associate not only with Cl\(^{-}\) channels (CaCC or ORCC) but also with cation channels (255). To explain these conflicting results, it can be hypothesized that TMEM16F regulates different types of cation and anion channels and that the expression of these channels is cell type dependent or affected by other types of experimental conditions. Other anoctamins may
also behave in this way. For example, TMEM16C/ANO3, although apparently devoid of channel activity, is able to regulate the activity of the Slack K⁺/H¹⁺¹¹ channel (88). The underlying mechanism is unknown, although there is evidence of a direct physical interaction between the two proteins (88). If TMEM16C is a scramblase, as recently proposed (214), the translocation of lipids in close proximity of the Slack channel could be the basis of the regulatory mechanism.

Another alternative hypothesis is that TMEM16F is actually a channel that instead regulates a phospholipid scramblase (FIGURE 6). This scheme would be consistent with the finding of TMEM16F as a cation channel with a particular permeability to divalent cations (255). Accordingly, the TMEM16F-dependent Ca²⁺ influx could be the mechanism through which a separate scramblase is activated. The role of TMEM16F as a channel and not a scramblase would also be consistent with the lack of significant phospholipid scrambling when TMEM16F is overexpressed in HEK-293 cells (255).

At the moment, it is not clear which hypothesis is correct. However, the recent study on the fungal TMEM16 (afTMEM16) reveals that ion transport and lipid scrambling may be indeed carried out by the same anoctamin (135). Similar studies, involving purification and reconstitution in an artificial lipid bilayer, need to be replicated on other anoctamins, particularly on mammalian TMEM16F, to assess their functions in a cell-free system. If TMEM16F is indeed a dual activity protein, working also as an ion channel, it remains to be explained why there are contrasting results about its ion selectivity. Another intriguing aspect of channels associated with TMEM16F is the relatively long delay (minutes) between cytosolic Ca²⁺ elevation and channel activation (77, 202). This behavior may imply an indirect mechanism of activation by Ca²⁺ or a Ca²⁺-induced traffic of the protein from intracellular compartments to the plasma membrane. Actually, there are discrepant results about the subcellular localization of TMEM16F and other anoctamins (47, 77, 202). The contrasting results may derive from different experimental conditions that have an effect on the localization of TMEM16F and other anoctamins.

In contrast to TMEM16F and other close paralogs, TMEM16A and TMEM16B expression is not associated with scramblase activity (214). This could mean, as sug-
gested by results obtained with afTMEM16 (135), that the dual function, particularly the scramblase activity, is an ancestral characteristic. Conversely, CaCC activity is a function that was later acquired by a branch of the anoctamin family represented by TMEM16A and TMEM16B.

Despite the variety of functions, a common characteristic of most, if not all, anoctamins is the regulation by cytosolic Ca\(^{2+}\). The activation seems mediated by direct interaction of Ca\(^{2+}\) with a pair of highly conserved glutamic acid residues (255, 259). Mutagenesis of these acidic residues lowered the Ca\(^{2+}\) sensitivity of channels associated with TMEM16A, TMEM16F, and afTMEM16 (135, 255, 259). However, since mutagenesis of acidic residues can alter Ca\(^{2+}\)-dependent activation in an allosteric way, other Ca\(^{2+}\)-binding sites cannot be excluded.

It is also probable that anoctamins, particularly TMEM16A and TMEM16B, are additionally controlled by calmodulin. Nevertheless, in this case, the situation is more complex. Different calmodulin-binding sites have been identified, each one with a different role on channel activity. For example, one study showed that calmodulin binding essentially affects the ion selectivity of TMEM16A, particularly its permeability to bicarbonate, but not its gating (101). Conversely, in other studies calmodulin binding to TMEM16A influenced its channel activity (222, 237). Understanding the precise role of calmodulin requires further investigation. However, it is interesting to note that the different studies have in common the focus on a region localized in the NH\(_2\) terminus before the first transmembrane segment. Interestingly, calmodulin seems also to have a role on channel inactivation, a process that, at least for TMEM16B, could desensitize the channel during long-lasting cytosolic Ca\(^{2+}\) increases (237).

In summary, the study of anoctamins has started to reveal a variety of functions, from ion transport to membrane lipid dynamics. While TMEM16A and TMEM16B may be considered as “pure” channels, full understanding of the main role played by the other anoctamins needs further investigation. The discovery of TMEM16 proteins also paves the way for the search of novel pharmacological agents that will be potentially useful for the treatment of a variety of human diseases. TMEM16A, as discussed in previous sections of this review, is a potential target for the treatment of asthma, cystic fibrosis, hypertension, gastrointestinal motility disorders, and cancer. Instead, TMEM16F, because of its particular role in blood coagulation, could be a target of antithrombotic therapies (160). However, the expression of TMEM16A, TMEM16F, and other anoctamins in multiple tissues implies a careful design of drugs to avoid important side effects. This requires a better comprehension of the structure-function relationship, physiological role, and regulation of each single anoctamin, a goal that will require a combined effort based on multiple approaches including gene silencing in native cells, overexpression in null systems, and conditional knockout in mice.

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