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TMEM16A Protein: A New Identity for Ca^{2+}-Dependent Cl^- Channels

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Ca^+-dependent Cl^- channels (CaCCs) play a variety of physiological roles in different organs and tissues, including transepithelial Cl^- secretion, smooth muscle contraction, regulation of neuronal excitability, and transduction of sensory stimuli. The recent identification of TMEM16A protein as an important component of CaCCs should allow a better understanding of their physiological role, structure-function relationship, and regulatory mechanisms.

Chloride channels have been neglected for a long time and considered little more than a background conductance that passively follows cation transport. This lack of interest was the result of various factors. In general, the physiological role of Cl^- conductance and its regulation in many organs and tissues were not understood. Furthermore, there were no selective pharmacological inhibitors that could help in assessing the contribution of Cl^- channels to specific physiological processes. Finally, the molecular identity of most Cl^- channels has been for a long time an uncertain and controversial topic. Actually, this uncertainty has often led to the wrong conclusions about the type of channels associated with a particular physiological process or disease.

The readers may refer to previous excellent reviews that describe the properties of known Cl^- channels such as CFTR, proteins of the ClC family, and ionotropic receptors for GABA and glycine (11, 18, 42, 47, 61). Here, we will discuss Ca^{2+}-activated Cl^- channels (CaCCs) and their relationship with the family of recently discovered TMEM16 proteins, also known as anoctamins.

Molecular Basis of CACCs

Electrophysiological studies have identified CaCCs in many cell types (18, 35). These channels are activated by increases in cytosolic free Ca^{2+} concentrations due to release from intracellular stores or influx through plasma membrane channels (FIGURE 1). The most common characteristics of CaCCs are I^- and SCN^- permeabilities larger than that for chloride, activation by cytosolic free Ca^{2+} concentrations in the 0.2–1.0 μM range, and modulation of channel activity by membrane potential (25, 35). Usually, CaCCs slowly activate when the membrane is depolarized to positive membrane potentials and deactivate with comparable kinetics when the membrane returns to resting conditions (5, 9, 25, 35, 44) (FIGURE 1). However, activation by membrane potential is markedly dependent on Ca^{2+}. At very low nanomolar Ca^{2+} concentrations, depolarization by itself cannot activate the channel. At high micromolar Ca^{2+} concentrations, the channel is almost fully activated at all membrane potentials.

Several attempts have been made to identify the proteins forming CaCCs. The first candidate, CLCA, was a protein isolated from bovine trachea (15). CLCA proteins have been also called "asthma" channels because of their upregulation in allergic airway disease (21). However, subsequent studies demonstrated that CLCA proteins are cell adhesion molecules anchored to the cell surface or even secreted in the extracellular space (30). This conclusion is in agreement with the initial finding of a CLCA protein as a factor important for attachment of metastatic melanoma cells to lung endothelium (20).

CLC-3 is another CaCC candidate. However, the currents evoked by CLC-3 expression lack voltage dependence and are activated by Ca^{2+}-/calmodulin-dependent phosphorylation (39, 62), whereas in many cases CaCCs seem to be directly activated by Ca^{2+} (35) and even inhibited by phoshorylation (3, 76). CLC-3 has been also found to work as an electrogenic H^+/Cl^- antiporter (50). Similar to other proteins of the same family, such as ClC-4, ClC-5, and ClC-7, ClC-3 may be essentially involved in the acidification of intracellular organelles (34, 42). ClC-3 has been also associated with the activity of cell swelling-activated Cl^- channels, but CLC-3 knockout mice show normal Ca^{2+}- and swelling-activated Cl^- conductances (4). Therefore, the relationship of CLC-3 with CaCCs and other plasma membrane Cl^- channels is unclear.

Bestrophins, initially discovered as proteins involved in vitelline macular dystrophy (Best's disease), represent another candidate for CaCC (59, 72). If compared with "classical" CaCCs, Cl^- currents associated with bestrophin expression have different Ca^{2+} affinity, voltage dependence, and sensitivity to pharmacological inhibitors. Bestrophins have been associated with multiple cell functions, such as regulation of voltage-dependent

Ca^{2+} channels (67), bicarbonate transport in intestinal epithelial cells (79), and Cl^- transport in the endoplasmic reticulum (7). According to this last finding, bestrophins would be important as a shunt conductance required to neutralize the electrical charge of Ca^{2+} moving across the endoplasmic reticulum membrane. In this way, bestrophins would affect CaCC activity indirectly by modulating the shape and amplitude of regulatory Ca^{2+} signals.

In 2008, three teams of investigators postulated TMEM16A as a component of CaCCs (12, 68, 78). Interestingly, this conclusion was obtained independently using different strategies, including expression cloning and functional genomics. Silencing of TMEM16A gene expression in vitro and in vivo caused inhibition of endogenous CaCC activity. On the other hand, heterologous expression of TMEM16A in null cell systems caused the appearance of Ca^{2+}-activated Cl^- channels with the biophysical and pharmacological properties expected for a "canonical" CaCC. For example, the anion permeability sequence found for TMEM16A ($NO_3^- > I^- > Br^- > Cl^- > F^-$; Refs. 68, 78) is similar to that reported for native CaCCs (18, 35). Furthermore, the Cl^- currents generated by TMEM16A expression (12, 78) are inhibited by niflumic acid, NPPB, and DIDS with potency comparable to that reported for CaCCs (18, 35). Subsequent studies on TMEM16A by other research groups have confirmed its association with Cl^- channel activity (46, 56, 66). This very high level of concordant results supports the conclusion that TMEM16A forms a CaCC by itself or in combination with other, yet to be discovered, proteins.

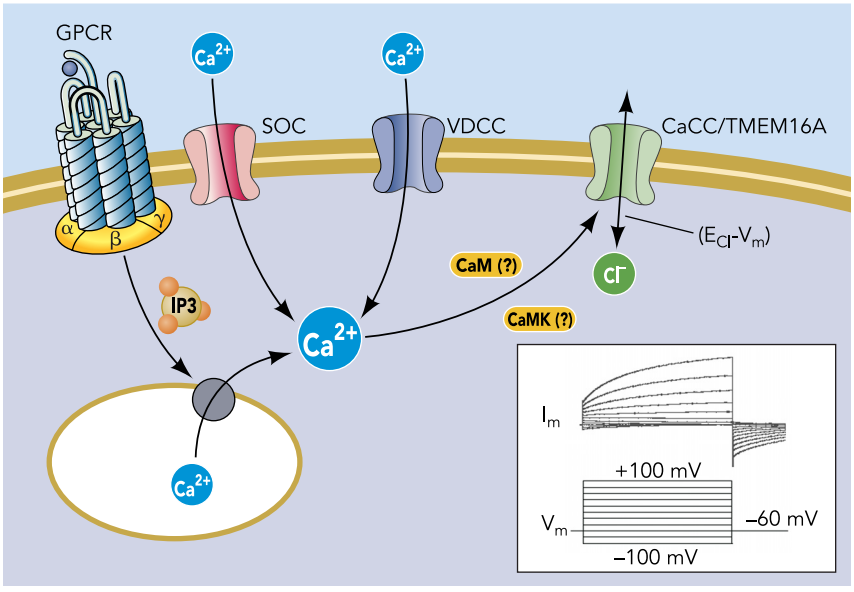

FIGURE 1. **Activation of Ca^{2+}-activated Cl^- channels (CaCCs)**
CaCCs are activated by cytosolic Ca^{2+} increases deriving from release from intracellular stores [triggered by stimulation of a G-protein-coupled receptor (GPCR) and phospholipase C-dependent inositol triphosphate generation] or by influx through the plasma membrane. Ca^{2+} influx may occur through store-operated Ca^{2+} channels (SOCs) or through voltage-dependent Ca^{2+} channels (VDCCs). Opening of CaCCs causes a net efflux or influx of Cl^- depending on the difference between the Cl^- equilibrium potential and the resting membrane potential (E_{Cl}-V_m). *Inset*: representative CaCC currents (*top*) elicited at different membrane potentials (*bottom*). Channel activity increases following membrane depolarization and decreases when the membrane potential is returned to negative values.

Structure-Function Relationship of TMEM16A

The primary structure of the TMEM16A protein has no similarity with other proteins having known function and, in particular, with other ion channels. Examination of TMEM16A amino acid sequence with programs predicting structure and topology evidences at least eight putative transmembrane segments, with both NH_2 and COOH termini protruding into the intracellular medium. Because of the eight transmembrane segments and the anion selectivity, TMEM16A has been also named anoctamin-1 (ANO1). Based on mutagenesis experiments that result in altered ion selectivity, it has been proposed that the region between the fifth and the sixth transmembrane segment forms a reentrant loop that inserts into the plasma membrane and contributes to the formation of the channel pore (78). Intriguingly, TMEM16A sequence does not contain canonical calcium- or calmodulin-binding domains. If TMEM16A directly binds Ca^{2+}, it may occur through a novel type of domain. A possible Ca^{2+}-binding region is a cluster of four contiguous glutamic acid residues localized in the first intracellular loop. This region may be similar to the "calcium bowl" of Ca^{2+}-dependent K^+ channels (6). However, there may be multiple calcium binding sites in TMEM16A, as suggested by the steep relationship between CaCC activity and free Ca^{2+} concentration in many studies (25, 35). Identification of such sites may result in difficulty since each site may include residues residing distantly from each other in the primary sequence. Furthermore, the binding site may be a combination of amino acid side chains and carbonyls of protein backbone. An alternative hypothesis is that the Ca^{2+}-sensing mechanism of CaCCs is not intrinsic to the TMEM16A protein but is provided by an ancillary subunit, possibly calmodulin or another Ca^{2+}-binding protein.

Interestingly, there is not a single version of the TMEM16A protein (FIGURE 2). Indeed, the mechanism of alternative splicing is responsible for the generation of various TMEM16A isoforms (12, 23). This process involves the skipping/inclusion of at least three alternative segments, called *b*, *c*, and *d*, corresponding to *exons 6b*, *13*, and *15*, and being 22, 4, and 26 amino acids long, respectively. Analysis of TMEM16A splicing among different human organs and tissues showed a variety of patterns.

Some tissues co-express multiple isoforms having variable levels of *exons 6b* or *15* skipping (23). Others show a preferential pattern of one isoform only. Interestingly, tissues appearing to preferentially skip *exon 6b* tend to include *exon 15* and vice versa. This coordinated pattern of splicing may suggest that segments *b* and *d* have mutually exclusive functional roles. In contrast, *microexon 13* is always included, with a small degree of skipping in brain and skeletal muscle. The NH_2 terminus of TMEM16A includes a region (*segment a*) that may be skipped when an alternative promoter is used (23). The resulting protein lacks the initial 116 amino acids. We found that the transcript lacking *segment a* was also devoid of *segments b*, *c*, and *d*. The corresponding isoform, called TMEM16A(*0*), is only 840 amino acids long compared with the longest one, TMEM16A(*abcd*), which has 1,008 amino acid residues (12).

Patch-clamp experiments have revealed that TMEM16A alternative splicing has a functional meaning (FIGURE 2). In particular, inclusion of segment *b* reduces the apparent affinity for Ca^{2+} of TMEM16A-dependent channels. Accordingly, the Ca^{2+} sensitivity of isoforms TMEM16A(*abc*) and TMEM16A(*ac*) differ by nearly fourfold (23). On the other hand, the splicing of the four amino acids (Glu-Ala-Val-Lys) corresponding to *segment c* (*exon 13*) alters the voltage dependence. Interestingly, inclusion of *segment c* occurs after the stretch of four glutamic residues discussed above as a possible Ca^{2+}-binding site. Heterologous expression of TMEM16A(*0*) variant generates Cl^- currents that are Ca^{2+} dependent but are unaffected by membrane potential. The physiological relevance of this isoform is unclear.

Summarizing, alternative splicing appears as an important mechanism regulating the CaCC channel properties, such as voltage dependence and Ca^{2+} sensitivity. Alternative splicing may also explain the variety of characteristics reported for CaCCs in different cell types (35).

TMEM16A/CaCC: Role in Epithelia

One of the major sites for CaCC expression and function is represented by epithelial cells. CaCCs constitute a route for Cl^- secretion across the apical membrane of epithelial cells of the airways, intestine, and exocrine glands (35, 43). Elevation of intracellular free Ca^{2+} concentration, triggered by paracrine and autocrine mechanisms, leads to transient CaCC activation. In many epithelial cells, intracellular Cl^- is accumulated by the coordinated activity of basolateral channels and transporters above the electrochemical equilibrium. Therefore, activation of CaCCs generates an efflux of Cl^- in the apical membrane that is followed by Na^+ through the paracellular pathway. The net secretion of NaCl drives transepithelial water transport. In the airways, local activation of CaCCs, through autocrine release of ATP and binding to purinergic receptors, may be a mechanism to increase water supply and hence mucociliary clearance (73). CaCCs probably have additional functions. CaCC-mediated bicarbonate transport may be essential for the expansion of mucins, as described for CFTR (27). In addition, CaCCs are highly permeable to SCN^- (thiocyanate). This pseudohalyde is used by lactoperoxidases on the airways and in the lumen of salivary and mammary glands to generate hypothiocyanite, a molecule with antimicrobial activity (28, 41, 55). Interestingly, the other substrate of lactoperoxidase is hydrogen peroxide, which is produced by plasma membrane dual oxidases (DUOX) in a Ca^{2+}-dependent way (24, 57). Therefore, increases in cytosolic Ca^{2+} may trigger a localized innate defense mechanism based on CaCC and DUOX activation.

In agreement with the important role of CaCCs in epithelial cells, expression of TMEM16A protein or mRNA has been demonstrated in the airway surface epithelium and in the acinar cells of pancreas, salivary glands, and bronchial submucosal glands (38, 45, 56, 63, 66, 68, 78). TMEM16A is also expressed in the mammary gland and in renal tubules (68, 78). Demonstration of TMEM16A expression in other types of epithelial cells such as those of the intestine requires further studies. Indeed, there are controversial results regarding the contribution of CaCCs to intestinal Cl^- secretion (43).

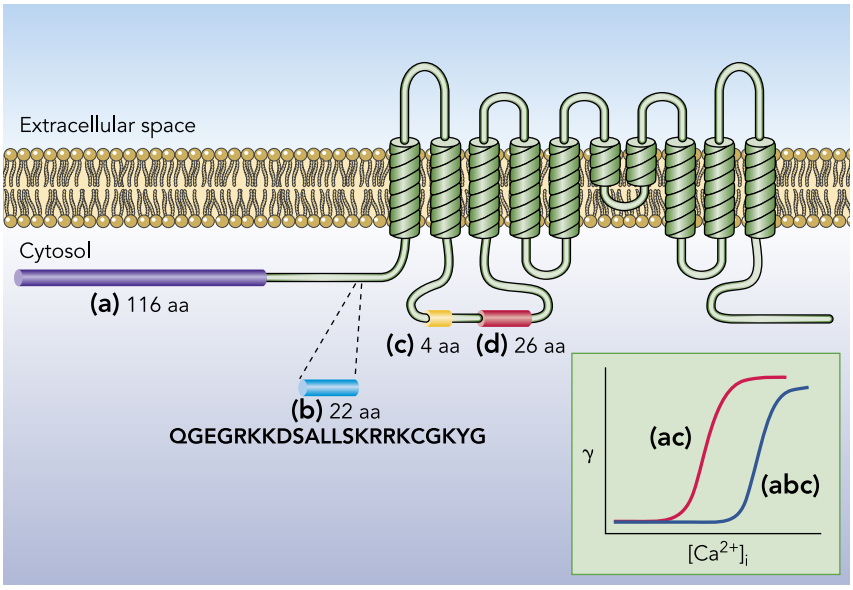

FIGURE 2. **Regulation of CaCC by alternative splicing of TMEM16A**
Predicted topology of TMEM16A protein showing eight putative transmembrane domains with a reentrant loop between the fifth and the sixth domain. The figure also shows the position and size of the four alternative segments: *a*, *b*, *c*, and *d*. Inclusion/skipping of *segment b* (22 amino acid residues) modulates the Ca^{2+} sensitivity of the TMEM16A Cl^- conductance (γ). The Ca^{2+} sensitivity of TMEM16A(*abc*) is nearly fourfold lower than that of TMEM16A(*ac*).

It has been reported that CaCCs are active only in nonpolarized intestinal epithelial cells (2) and not in native epithelium (71). In this case, in polarized epithelia, Ca^{2+}-based signals would induce Cl^- secretion essentialy through cAMP-activated Cl^- channels, i.e., CFTR (16, 48, 71), if such channels are activated constitutively or by concomitant stimuli. Indeed, the intracellular Ca^{2+} increase should act by activating basolateral K^+ channels and therefore increasing the driving force for Cl^- efflux. On the other hand, different studies have shown separate CFTR and CaCC conductances in polarized intestinal epithelia (51, 52). Such discrepant results may derive from a dependence of CaCC expression on unknown regulatory factors. For example, the presence of CaCC-dependent Cl^- secretion, stimulated by the NSP4 rotaviral enterotoxin, was found to depend on the age of mice (54). Another important issue to clarify is the subcellular localization of TMEM16A protein in the various epithelial cells. Indeed, TMEM16A expression would support Cl^- secretion only if the channels are apically localized.

In vitro and in vivo functional studies have demonstrated that TMEM16A is indeed needed for Ca^{2+}-dependent Cl^- secretion. Silencing of TMEM16A by siRNA has been found to inhibit the Ca^{2+}-dependent stimulation of Cl^- secretion in polarized cultures of human bronchial epithelial cells (12) and the fluid secretion by salivary glands (78). Other data supporting the relationship between TMEM16A and CaCCs originate from knockout mice. These animals show a decreased Ca^{2+}-dependent Cl^- transport in tracheal and intestinal epithelium, salivary glands, and hepatocytes (56, 65, 66). The results obtained from the intestine of TMEM16A(−/−) animals suggest that CaCCs are indeed important for intestinal Cl^- secretion (56). As a consequence of defective Cl^- secretion in the airways, there is also a defective mucociliary clearance (56) that may generate accumulation of mucus (65). Intriguingly, TMEM16A knockout mice have a very severe phenotype mainly characterized by incomplete development of tracheal cartilage rings (63). This defect is probably the cause of early death of the animals by suffocation. The mechanism leading to altered cartilage development is unclear but may be a consequence of a defect in the tracheal surface epithelium since expression in the cartilage was not detected (63).

TMEM16A in Smooth Muscle Cells

CaCCs have been repeatedly identified by functional studies in smooth muscle cells (SMCs), particularly in blood vessels (32, 33). Their role is considered essential in the mechanism of signal amplification leading to cell contraction (35). Indeed, cytosolic free Ca^{2+} elevation by paracrine mechanisms triggers CaCC activation. The resulting Cl^- efflux causes membrane depolarization, opening of voltage-dependent Ca^{2+} channels, and hence further Ca^{2+} elevation. As in epithelial cells, the CaCC-dependent depolarization depends on a relatively high intracellular Cl^- concentration (14).

The identification of TMEM16A now allows investigation of the expression and role of CaCCs in different types of SMCs. Immunocytochemistry studies suggest that TMEM16A protein is not uniformly present in SMCs. According to these results, TMEM16A is markedly expressed in SMCs of the airways and of some parts of the reproductive system (i.e., oviduct and epididymis) but not in SMCs of blood vessels (38). However, recent reports indicate that TMEM16A is indeed expressed in such cells and that silencing of TMEM16A causes inhibition of endogenous Ca^{2+}-activated Cl^- currents (16, 49). In the gastrointestinal tract, TMEM16A is strongly expressed in the interstitial cells of Cajal (ICCs), which represent pacemaker cells controlling the contraction of the smooth muscle layers (31, 38, 40). The importance of TMEM16A in gastrointestinal motility is demonstrated by studies on knockout mice (38, 40). These animals are devoid of slow waves, the rhythmic changes in membrane potential controlling contraction. Slow waves are also inhibited by niflumic acid, a CaCC inhibitor, although with different potency in gastric antrum compared with intestine (40).

It is interesting to note that the mechanism of ICC depolarization was previously proposed to depend on a Ca^{2+}-inhibited nonselective cation channel (22), although some studies indicated a possible role of Cl^- channels (37). The identification of TMEM16A (ANO1) and its high expression in ICCs has led to reconsideration of this issue. In a recent study on ICC cells identified by cell-specific GFP expression, the Ca^{2+}-activated Cl^- conductance was indeed found to mediate the membrane depolarization underlying slow waves (80).

TMEM16A/CaCCs in Nervous System and Sensory Receptors

CaCCs also control the excitability of various types of neurons including olfactory sensory neurons, somatosensory neurons, photoreceptors, and spinal cord neurons (25). The opening of CaCCs generates membrane potential depolarization or hyperpolarization depending on whether the Cl^- equilibrium potential is more positive or more negative than the resting potential, respectively.

Somatosensory neurons, whose bodies constitute the root dorsal ganglia (DRG), transduce different stimuli such as skin temperature, pain, and touch. A subpopulation of DRG neurons show CaCC activity, thus indicating that these channels are involved in the transduction of specific sensory pathways (25).

Recently, it has been demonstrated that CaCC currents in small-size DRG neurons mediate the acute nociceptive stimulus in reponse to bradykinin (46). Bradykinin, through B2 receptors and phospholipase C cascade, triggers intracellular Ca^{2+} increase and CaCC activation. The resulting depolarization increases the action potential firing rate. Gene silencing experiments demonstrated that CaCC activity in small DRG neurons depends on TMEM16A expression (46).

CaCCs also play a role in the mechanism of olfactory signal transduction. In the cilia of olfactory sensory neurons, odorous substances trigger cAMP elevation and hence activation of cyclic nucleotide-gated cation channels. The corresponding influx of Na^+ and Ca^{2+} opens CaCCs and causes Cl^- efflux. This event produces an additional membrane depolarization that acts as an amplification step (25, 36). It has been shown that CaCC currents in olfactory neurons are mediated by TMEM16B (alias ANO2), a close homolog of TMEM16A (36, 60, 69).

TMEM16B has been also identified in the synaptic terminals of the mouse retina (70). This finding is consistent with a regulatory role of Ca^{2+}-dependent Cl^- conductance on photoreceptor function (25).

Other TMEM16 Proteins

TMEM16A and TMEM16B are members of a protein family containing another eight members, from TMEM16C to TMEM16K (26). All TMEM16 proteins (anoctamins) have a comparable predicted topology. The amino acid sequence of TMEM16A is ~60% identical to that of TMEM16B. Not surprisingly, TMEM16B appears to work as a CaCC. However, there are some interesting differences. Compared with TMEM16A, TMEM16B-dependent channels have nearly 10-fold smaller unitary conductance, lower Ca^{2+} sensitivity, and much faster activation kinetics (58, 69, 78). These differences may guide in the identification of critical protein domains involved in channel gating and Cl^- transport.

The overall homology between TMEM16A and other anoctamins is much lower, with TMEM16F, G, H, J, and K being only 20–30% identical. However, specific regions in anoctamins, particularly in the putative transmembrane domains, show a much higher level of sequence conservation. These more distant anoctamins may represent different types of anion channels or transporters. TMEM16F and TMEM16K show high and ubiquitous expression in many cells and tissues (12, 64). In contrast, TMEM16C and TMEM16G seem particularly expressed in the nervous system and in prostate, respectively (8, 64). Interestingly, TMEM16E/ANO5 (also known as GDD1) is the only anoctamin found so far to be mutated in human genetic diseases. Indeed, mutations in the TMEM16E cause gnathodiaphyseal dysplasia, a dominant autosomic syndrome associated with fibro-osseous jawbone lesions and long-bone bowing (74). More recently, two recessive diseases, proximal limb-girdle muscular dystrophy and distal non-dysferlin Miyoshi myopathy, were also found to be caused by TMEM16E mutations (10). The physiological role of TMEM16E protein is unknown, but it has been shown to have an intracellular localization (53). Other anoctamins may also have an intracellular localization and function.

It has been reported that expression of many TMEM16 proteins, including TMEM16A, generates Cl^- currents that are activated by cell swelling (1). However, the biophysical properties of the Cl^- channels associated with anoctamin expression appear different from those of volume-sensitive Cl^- channels, also known as VSOAC (75). Therefore, anoctamins and VSOACs may represent different types of channels involved in cell volume regulation.

TMEM16 Proteins and Cancer

One of the intriguing characteristics of TMEM16A is its overexpression in some human cancers such as gastrointestinal stromal tumors (GISTs) and head and neck squamous cell carcinomas (13, 77). Because of this relationship, TMEM16A protein is also known as DOG1 (discovered on gastrointestinal stromal tumor 1), TAOS2 (tumor amplified and overexpressed sequence 2), and ORAOV2 (oral cancer overexpressed 2). The overexpression of TMEM16A may imply that it is important for cancer development and metastasis. However, other hypotheses are also possible. For example, TMEM16A upregulation may be a consequence of amplification of the genomic region (11q13) containing other genes with more relevance to cancer such as cyclin D1 and FADD (29). Alternatively, high TMEM16A expression may be a feature of the cells from which the tumor derived. For example, GISTs probably originate from or have a progenitor in common with ICCs.

Other TMEM16 proteins also have a relationship with cancer. TMEM16G, also known as NGEP, is particularly expressed in prostate cancers (8). The pattern of TMEM16F splicing affects the metastatic capability of mammary cancers in mouse and is associated with poor prognosis of human patients with breast cancer (19).

Concluding Remarks

The identification of TMEM16A/ANO1 as a CaCC protein will keep investigators in the field busy for the next several years. Several questions remain unresolved, including the structure-function relationship, the interactome, the regulatory mechanisms,

and the physiological meaning of the different isoforms.

TMEM16A and other members of the same family may represent novel drug targets for the treatment of various human diseases such as cystic fibrosis, hypertension, gastrointestinal motility disorders, asthma, and cancer. However, the participation of CaCCs in a variety of physiological processes requires the development of tissue-specific pharmacological modulators. ■

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