Cell Calcium 84 (2019) 102103

Contents lists available at ScienceDirect

Cell Calcium

journal homepage: www.elsevier.com/locate/ceca

Wasted TMEM16A channels are rescued by phosphatidylinositol 4,5-bisphosphate

Jorge Arreola[a,*], H. Criss Hartzell[b]

[a] Physics Institute, Universidad Autónoma de San Luis Potosí, Ave. Dr. M. Nava #6, San Luis Potosí, SLP 78290, Mexico
[b] Department of Cell Biology, Emory University School of Medicine, Atlanta, GA, 30322, United States

ARTICLE INFO

Keywords:
Phospholipids
Anion channels
Ion channel gating
Calcium
Ion channel regulation

ABSTRACT

Recently there has been a flurry of interest in the regulation of the homo-dimeric calcium-activated chloride channel ANO1 (also known as TMEM16A) by phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$). These recent studies show that upon Ca^{2+} binding, $PI(4,5)P_2$ cooperates to maintain the conductive state of ANO1. $PI(4,5)P_2$ does so by binding to sites or modules on the protein's cytosolic side. These findings add a new function to the $PI(4,5)P_2$ repertoire and a new dimension to ANO1 gating.

1. Introduction

The ANO1 anion channel (also known as TMEM16A) controls a mesmerising number of important physiological functions [1–7]. Elevation of cytosolic Ca^{2+} concentration opens ANO1 to conduct Cl^- ions, but relatively little is known about other pathways that regulate the channel. Recently, considerable attention has become focussed on ANO1 regulation by phosphatidylinositol (4,5)-bisphosphate ($PI(4,5)P_2$) [8–14]. $PI(4,5)P_2$ is a low-abundance phospholipid in the inner leaflet of the plasma membrane that has a remarkable number of cellular functions including regulation of cytoskeletal organization, membrane trafficking, and ion channel gating [15–18]. While the role of $PI(4,5)P_2$ in cation channel regulation is well-known, there is relatively little information about its role in anion channel gating.

Studies aimed at understanding gating and regulation of ANO1 were given a tremendous boost in 2008 by its cloning [19–21] followed in 2017 by cryo-EM structure determinations [22–24]. The structures reveal dimers with each subunit consisting of 10 transmembrane segments with the dimer interface formed in large part by the C-termini of the subunits. Each monomer has a hydrophilic pore surrounded by transmembrane helices TM4-7 (Fig. 1). The large cytoplasmic and extracellular vestibules narrow to form a constricted pore with a radius of ~ 1 Å near the cytoplasmic side of the membrane. Structural analysis, which confirmed earlier mutagenesis and patch clamp studies, revealed the presence of two Ca^{2+} ions coordinated by sidechain oxygens of glutamic and aspartic acid residues located in a hydrophilic pocket situated close to the cytosolic end of the pore. A comparison of apo and Ca^{2+}-bound structures suggested that Ca^{2+} binding induced a major conformational change of the cytoplasmic half of TM6 [22–25]. At the same time, binding of Ca^{2+} reduces the electronegative field within the narrow neck of the pore because the binding pocket is situated in the pore wall [26]. The helix rearrangement sandwiches Ca^{2+} ions between TM6, TM7, and TM8 and at the same time opens the permeation pathway. From this finding, one might fantasize that Ca^{2+} binding stabilizes an open conformation of ANO1, but the mechanisms are certainly more complicated because high Ca^{2+}concentrations also cause open channels rapidly to inactivate. Indeed, the pores of Ca^{2+}-bound cryo-EM structures are too small to conduct Cl^- ions and probably represent inactivated conformations. Surprisingly, application of a water-soluble analog of $PI(4,5)P_2$ (diC8-$PI(4,5)P_2$) to inside-out excised patches nearly abolishes Ca^{2+}-induced inactivation, whereas depletion of $PI(4,5)P_2$ from the plasma membrane using *Danio rerio* voltage-sensitive phosphatase decreases ANO1 activity [11,12]. $PI(4,5)P_2$ could likely be the cofactor necessary to maintain the conductive state of ANO1.

2. $PI(4,5)P_2$ helps ANO1 gating to evade Ca^{2+}-induced inactivation

ANO1 is gated by physiological intracellular Ca^{2+} concentrations (0.1–5 μM) in a voltage-dependent manner. Under this condition, repetitive depolarisations activate a current that reaches a steady plateau without decaying. However, simultaneous activation of the depolarization-activated phosphatase to dephosphorylate $PI(4,5)P_2$ rapidly induces current decay (Fig. 2A). This phenomenon is reversed by shutting off the phosphatase by holding cells at negative voltages,

* Corresponding author.
E-mail addresses: arreola@dec1.ifisica.uaslp.mx (J. Arreola), criss.hartzell@emory.edu (H.C. Hartzell).

https://doi.org/10.1016/j.ceca.2019.102103
Received 8 October 2019; Received in revised form 15 October 2019; Accepted 16 October 2019
Available online 18 October 2019

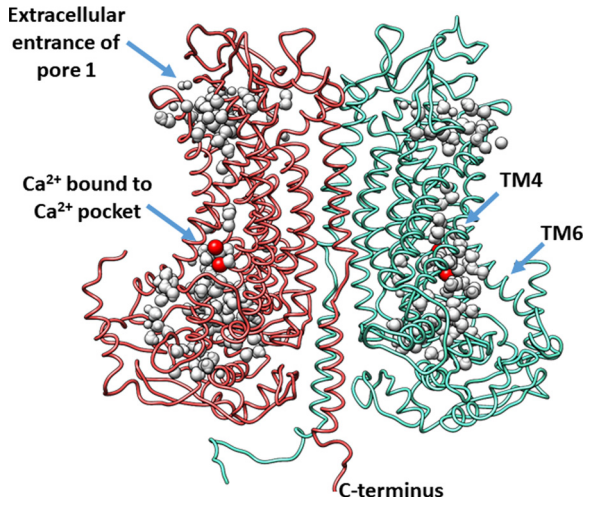

Fig. 1. Homo-dimeric structure of Ca^{2+}-bound ANO1. The structure with two independent subunits (maroon and dark cyan) is shown in the plane of the membrane. The dimer interface formed by the apposition of the two C-terminus is perpendicular to the membrane. Ten transmembrane domains per monomer are depicted. TM6 indicated by light blue arrow rotates from TM4 towards TM8 upon Ca^{2+} binding to reach a straight conformation. The anion permeation pathway with large vestibules at both ends within each monomer is revealed by water molecules (grey). The Ca^{2+} pocket, located towards the intracellular side almost in contact with the permeation pathway, houses two Ca^{2+} ions (red spheres).

providing that ATP is present in the intracellular solution [11,12]. Presumably, under these conditions $PI(4,5)P_2$ is re-synthetized to restore its membrane content. Similarly, when ANO1 is activated in excised inside-out patches exposed to low Ca^{2+}, $PI(4,5)P_2$ fully rescues channels that have previously been stripped of $PI(4,5)P_2$ using polylysine [11,12]. The effect of $PI(4,5)P_2$ is greatest at lower Ca^{2+} concentrations and decreases with depolarization. However, the ability of $PI(4,5)P_2$ to regulate ANO1 at high intracellular Ca^{2+} (i.e. 100 μM) is different. Under this condition, fully active ANO1 channels rapidly inactivate, but inactivation is prevented when $PI(4,5)P_2$ is provided along with high intracellular Ca^{2+} (Fig. 2B). Surprisingly, $PI(4,5)P_2$ is unable to rescue channels previously inactivated by high Ca^{2+} [11,12]. Somehow high Ca^{2+} induces a profound inactivated state, presumably in a process that involves removing $PI(4,5)P_2$.

3. Several $PI(4,5)P_2$ binding sites exist on ANO1

$PI(4,5)P_2$ could facilitate ANO1 gating by electrostatically attracting Ca^{2+} to the channel or by directly altering gating, as has been shown previously for certain potassium channels [15]. Guided by the Ca^{2+}-bound cryo-EM structure of mouse TMEM16A, Huanghe Yang's lab [12] performed an alanine scanning analysis of basic lysine and arginine residues located in the vicinity of the plasma membrane inner leaflet. To gauge $PI(4,5)P_2$ sensitivity, they used the rate of channel inactivation induced by 100 μM Ca^{2+}. They found that residues R451, K461, R482, K567, R575, and K579 (numbering based on mouse ANO1 variant a, lacking the EAVK segment) form a putative $PI(4,5)P_2$ binding site near the cytosolic interface of TMs 3–5. Accordingly, mutations in the $PI(4,5)P_2$ binding site were nearly insensitive to $PI(4,5)P_2$ application and the mutant channels inactivated faster than wild type channels [12]. Ko and co-workers have confirmed that R486 (equivalent to R482 in [12]) in the first intracellular loop is part of a putative $PI(4,5)P_2$ binding site [27].

In addition to this site identified by Le et al, other $PI(4,5)P_2$ binding sites have been found on ANO1. The Hartzell lab performed a glutamine scanning analysis combined with patch clamp and molecular dynamics simulation of lysine and arginine residues located within 10 Å of the cytoplasmic membrane interface, including those residues located in the cytoplasmic N-terminus predicted to interact with $PI(4,5)P_2$ [14]. In contrast to Le et al., they measured the stimulatory effect of 10 μM diC8-$PI(4,5)P_2$ on wild type and mutant ANO1 currents activated by low (270 nM) Ca^{2+} in inside-out patches. This approach yielded three putative binding sites: site A near the dimer interface, site B at the cytoplasmic end of TM6, and site C in the short intracellular loop between TM2 and TM3. Of these sites, site A is close to the site located by Le et al., and near a lipid-filled cavity at the dimer interface according to the cryo-EM structure of TMEM16A. Interestingly, some of the residues in sites B and C identified as being important in regulation channel amplitude in low Ca^{2+} by the Hartzell lab were not found by the Yang lab to be important in regulating inactivation. This difference may be explained if $PI(4,5)P_2$ has different effects on the fully open and partially open conformations of the channel as proposed by Le et al. [12].

It appears that $PI(4,5)P_2$ regulation may be a general feature of the ANO family. There are 10 ANO genes in mammals and while two of them (ANO1 and ANO2) are clearly Ca^{2+}-activated Cl^- channels, others, notably ANO6, are phospholipid scramblases [28]. Previous studies from the Jan lab have shown that ANO6 is regulated by $PI(4,5)P_2$ and they have identified a $PI(4,5)P_2$ binding site in the N-terminus of the protein [29]. The Hartzell lab found amino acids in the N-terminus of ANO1 that alter $PI(4,5)P_2$ effects when they are mutated [14], but the molecular mechanisms by which these residues regulate the channel remain uncertain because the cryo-EM structures do not fully model this region of the protein. However, the multiplicity of $PI(4,5)P_2$ "binding sites" raises interesting questions. Both structural and electrophysiological data suggest that ANO1 apparently can adopt a number of different conformations. Different conformational states likely have different $PI(4,5)P_2$ binding properties. Mutations that stabilize different conformational states (such as K579 [12] or R579 [14]), but do not directly coordinate $PI(4,5)P_2$, could have profound effects on the interpretation of assays that rely on channel function to assess $PI(4,5)P_2$ binding. This emphasizes the importance of melding electrophysiological studies with structural, computational, and biochemical approaches.

4. How can $PI(4,5)P_2$ prevent ANO1 inactivation and exactly how does it participate in ANO1 gating?

While ANO1 gating requires Ca^{2+} binding to the Ca^{2+} pocket, high Ca^{2+} concentrations could trigger depletion of $PI(4,5)P_2$ to induce ANO1 inactivation as has been suggested for ANO6 [29]. Inactivation could be caused by pore collapse as suggested by the Ca^{2+}-bound cryo-EM structures showing a pore with a radius smaller than that of Cl^- ions [22–24]. Indirect evidence from the Yang lab gathered using highly permeant anions, pore blockers, and pore mutants suggest that an occupied pore inactivates slower than an empty pore probably by precluding pore collapse [12]. A direct interaction of $PI(4,5)P_2$ with the gating machinery of ANO1 was suggested by molecular dynamics simulations. The simulation suggests that binding of $PI(4,5)P_2$ to sites A and B/C induces a rotation of the cytoplasmic half of TM6 that dilates the conduction pathway ~30%, but still smaller than a Cl^- ion [14]. Co-incidentally, the Ca^{2+}-bound structure of ANO1 shows that the cytoplasmic end of TM6 adopts a straight conformation and forms one side of the channel pore. The fact that $PI(4,5)P_2$ alters the conformational state of the Ca^{2+} bound structure suggests that $PI(4,5)P_2$ acts by inhibiting Ca^{2+}-induced pore collapse and that the cooperative action of $PI(4,5)P_2$ and Ca^{2+} stabilize the conducting conformation. Further analysis is necessary to determine if pore collapse is prevented by rotation of TM6.

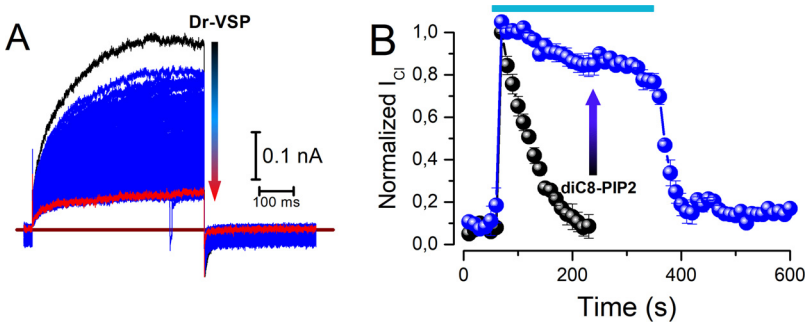

Fig. 2. ANO1 opening requires PI(4,5)P_2. A: Depletion of PI(4,5)P_2 from the plasma membrane induces a progressive decay (from black to red) of the ANO1 Ca^{2+}-activated Cl^- current in a HEK cell also expressing the *Danio rerio* voltage-sensitive phosphatase (Dr-VSP). The cell was dialyzed with 0.2 μM Ca^{2+} and currents were induced by applying a +80 mV step from a holding potential of −30 mV every 2 s during 250 s. B: Ca^{2+}-induced inactivation of ANO1 is dampened by diC8-PIP$_2$, a PI(4,5)P_2 analog. Currents were induced by applying 100 μM Ca^{2+} (top bar) to inside-out patches held at +100 mV in the absence (black) and in the presence of 25 μM diC8-PIP2 (blue).

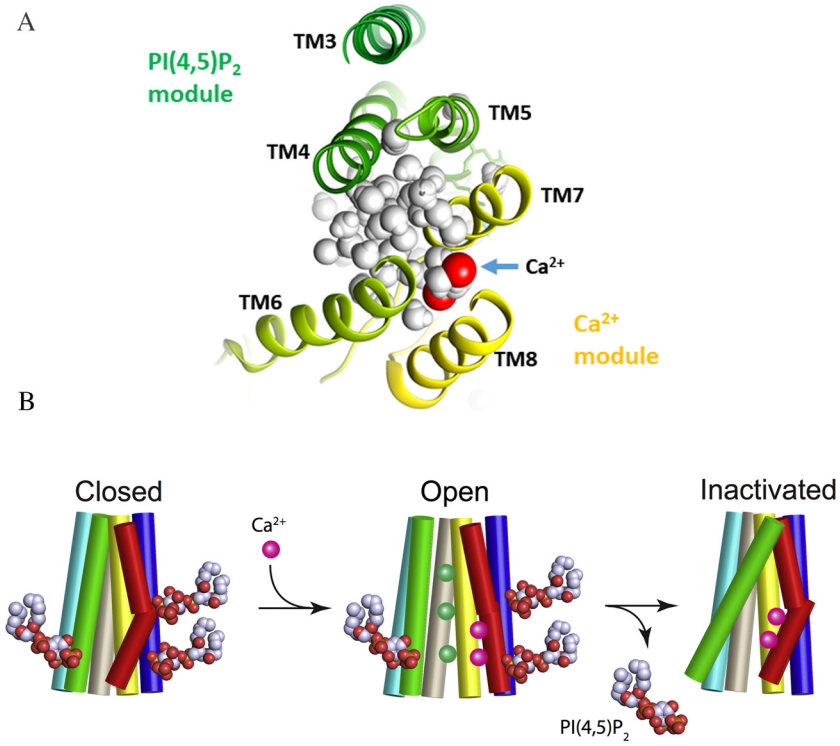

Fig. 3. Modular architecture and cooperative gating of ANO1. A: Modular structure of the ANO1 pore. The scheme shows one pore filled with water molecules (grey) surrounded by the PI(4,5)P_2 regulatory module (TM3-TM5 in green) and by the Ca^{2+} binding module (TM6-TM8 in yellow). B: Hypothetical co-operative ANO1 gating mechanism. (1) When intracellular Ca^{2+} is low, ANO1 is in a closed state with PI(4,5)P_2 bound. In this state, ANO1 can be opened by strong depolarization [30] and removal of PI(4,5)P_2 decreases current amplitude. The current strongly rectifies outwardly because of the strong electronegative field at the intracellular end of the channel due to the acidic residues in the Ca^{2+} binding pocket and bound PI(4,5)P_2 molecules [26]. (2) When Ca^{2+} increases and binds to the Ca^{2+} pocket, the cytoplasmic half of TM6 straightens with the help of PI(4,5)P_2 [14]. This relaxation may be partly a result of neutralization of the negative electric field by Ca^{2+} binding. The open state will be determined by the balance between the electric field and the position of the cytoplasmic end of TM6. The current will be less rectifying as intracellular Ca^{2+} reaches near 5 μM. Dephosphorylation of membrane PI(4,5)P_2 with the voltage-sensitive phosphatase or removal of PI(4,5)P_2 by poly-L-lysine will cause the pore to partially close because PI(4,5)P_2 is not available to adjust the conformation of TM6. But, the pore would not be inactivated (collapsed) because the electric field has not been fully neutralized. Also, in the absence of PI(4,5)P_2, the voltage dependence of open probability has shifted such that a strong depolarization or an increase in Ca^{2+} is necessary to open the pore. (3) Binding of additional Ca^{2+} will fully neutralize the electric field and presumably remove PI(4,5)P_2. This condition will inactivate the channel by collapsing the pore.

5. Paradigm shift of ANO1 structure and gating: modular architecture and cooperative gating

From these studies a new, clearer picture of ANO is starting to emerge: a modular structure of the pore and a gating mechanism that depends on the cooperative action of PI(4,5)P_2 and Ca^{2+}. Le et al found that channels with mutations in the PI(4,5)P_2 binding site displayed rapid inactivation but unchanged apparent Ca^{2+} sensitivity [12]. Surprisingly, mutating residues near the Ca^{2+} binding pocket decreased the apparent Ca^{2+} affinity but did not change inactivation. This result led to the idea that two modules control the anion permeation pathway: a PI(4,5)P_2 binding regulatory module (TM3-TM5) responsible for inactivation and a Ca^{2+} binding gating module (TM6-TM8) responsible for channel activation (Fig. 3A). These modules are arranged around the pore but they obviously do not operate in isolation. Le at al. [12] show that with wild type ANO1, PI(4,5)P_2 alters the Ca^{2+} sensitivity of the channel and that inactivation may be induced by a Ca^{2+}-dependent dissociation of PI(4,5)P_2 from the regulatory module. In this structural scenario, gating would depend on direct binding of PI(4,5)P_2 and Ca^{2+} to their respective modules (Fig. 3B). Under resting Ca^{2+} conditions ANO1 directly interacts with PI(4,5)P_2 via a complex set of binding sites. A sudden Ca^{2+} increase will rapidly activate ANO1 by occupying the Ca^{2+} pocket. Sustained activation will depend on both binding of PI(4,5)P_2 and Ca^{2+} sitting in the hydrophilic pocket. The combined actions of PI(4,5)P_2 and Ca^{2+} will ensure an open state by holding the cytoplasmic end of TM6 from collapsing the pore and by partially neutralizing the electronegative field in the narrowest section of the pore. Structural information about different conformational states of ANO1 together with molecular dynamic simulations will help resolve these remaining questions about ANO1 gating.

Acknowledgements

Thanks to Huanghe Yang, Son Le, and Steven Foltz for helpful comments. Research on ANO 1 channels is supported by grants FC 2016-01-1955 and 219949 from CONACYT, Mexico (JA) and by grants EY0114852, AR067786, and GM132598 from the National Institutes of Health (HCH).

References

[1] N. Pedemonte, L.J. Galietta, Structure and function of TMEM16 proteins (Anoctamins), Physiol. Rev. 94 (2) (2014) 419–459.
[2] Q. Ji, et al., Recent advances in TMEM16A: structure, function, and disease, J. Cell. Physiol. 234 (6) (2019) 7856–7873.
[3] D. Crottes, L.Y. Jan, The multifaceted role of TMEM16A in cancer, Cell Calcium 82 (2019) p. 102050.

[4] J.M. Whitlock, H.C. Hartzell, Anoctamins/TMEM16 proteins: chloride channels flirting with lipids and extracellular vesicles, Annu. Rev. Physiol. 79 (2017) 119–143.
[5] K. Kunzelmann, et al., Modulating Ca(2+) signals: a common theme for TMEM16, Ist2, and TMC, Pflugers Arch. 468 (3) (2016) 475–490.
[6] U. Oh, J. Jung, Cellular functions of TMEM16/anoctamin, Pflugers Arch. 468 (3) (2016) 443–453.
[7] A. Picollo, M. Malvezzi, A. Accardi, TMEM16 proteins: unknown structure and confusing functions, J. Mol. Biol. 427 (1) (2015) 94–105.
[8] Y. Tian, et al., Control of TMEM16A by INO-4995 and other inositolphosphates, Br. J. Pharmacol. 168 (1) (2013) 253–265.
[9] H.A. Pritchard, et al., Inhibitory role of phosphatidylinositol 4,5-bisphosphate on TMEM16A-encoded calcium-activated chloride channels in rat pulmonary artery, Br. J. Pharmacol. 171 (18) (2014) 4311–4321.
[10] C.M. Ta, et al., Contrasting effects of phosphatidylinositol 4,5-bisphosphate on cloned TMEM16A and TMEM16B channels, Br. J. Pharmacol. 174 (18) (2017) 2984–2999.
[11] J.J. De Jesus-Perez, et al., Phosphatidylinositol 4,5-bisphosphate, cholesterol, and fatty acids modulate the calcium-activated chloride channel TMEM16A (ANO1), Biochim. Biophys. Acta 1863 (3) (2017) 299–312.
[12] S.C. Le, et al., Molecular basis of PIP2-dependent regulation of the Ca(2+)-activated chloride channel TMEM16A, Nat. Commun. 10 (1) (2019) 3769.
[13] M. Tembo, et al., Phosphatidylinositol 4,5-bisphosphate (PIP2) and Ca(2+) are both required to open the Cl(-) channel TMEM16A, J. Biol. Chem. (2019).
[14] K. Yu, et al., A network of phosphatidylinositol 4,5-bisphosphate binding sites regulates gating of the Ca(2+)-activated Cl(-) channel ANO1 (TMEM16A), Proc. Natl. Acad. Sci. U. S. A. (2019).
[15] B. Hille, et al., Phosphoinositides regulate ion channels, Biochim. Biophys. Acta 1851 (6) (2015) 844–856.
[16] S.B. Hansen, Lipid agonism: the PIP2 paradigm of ligand-gated ion channels, Biochim. Biophys. Acta 1851 (5) (2015) 620–628.
[17] D.E. Logothetis, et al., Phosphoinositide control of membrane protein function: a frontier led by studies on ion channels, Annu. Rev. Physiol. 77 (2015) 81–104.
[18] B.C. Suh, B. Hille, PIP2 is a necessary cofactor for ion channel function: how and why? Annu. Rev. Biophys. 37 (2008) 175–195.
[19] A. Caputo, et al., TMEM16A, a membrane protein associated with calcium-dependent chloride channel activity, Science 322 (2008) 590–594.
[20] Y.D. Yang, et al., TMEM16A confers receptor-activated calcium-dependent chloride conductance, Nature 455 (2008) 1210–1215.
[21] B.C. Schroeder, et al., Expression cloning of TMEM16A as a calcium-activated chloride channel subunit, Cell 134 (2008) 1019–1029.
[22] C. Paulino, et al., Activation mechanism of the calcium-activated chloride channel TMEM16A revealed by cryo-EM, Nature 552 (7685) (2017) 421–425.
[23] C. Paulino, et al., Structural basis for anion conduction in the calcium-activated chloride channel TMEM16A, Elife 6 (2017).
[24] S. Dang, et al., Cryo-EM structures of the TMEM16A calcium-activated chloride channel, Nature 552 (7685) (2017) 426–429.
[25] C.J. Peters, et al., The sixth transmembrane segment is a major gating component of the TMEM16A calcium-activated chloride channel, Neuron 97 (5) (2018) p. 1063-1077 e4.
[26] A.K. Lam, R. Dutzler, Calcium-dependent electrostatic control of anion access to the pore of the calcium-activated chloride channel TMEM16A, Elife 7 (2018).
[27] W.J. Ko, S.-R, C.-G. Park, J.H. Nam, B. Hille, B.C. Suh, Allosteric modulation of Ca2+-activated Cl− channels TMEM16A by PIP2 and CAMKII, Biophys. J. 114 (2019) 41a.
[28] J. Suzuki, et al., Calcium-dependent phospholipid scrambling by TMEM16F, Nature 468 (7325) (2010) 834–838.
[29] W. Ye, et al., Phosphatidylinositol-(4, 5)-bisphosphate regulates calcium gating of small-conductance cation channel TMEM16F, Proc. Natl. Acad. Sci. U. S. A. 115 (7) (2018) E1667–E1674.
[30] Q. Xiao, et al., Voltage- and calcium-dependent gating of TMEM16A/Ano1 chloride channels are physically coupled by the first intracellular loop, Proc. Natl. Acad. Sci. 108 (21) (2011) 8891–8896.