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Phosphatidylinositol 4,5-bisphosphate, Cholesterol, and Fatty Acids Modulate The Calcium-Activated Chloride Channel TMEM16A (ANO1)

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Abstract

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COMPETING INTERESTS

None of the authors have competing interests.

AUTHORS CONTRIBUTIONS

Electrophysiological experiments were performed at JA lab

Inside out patches experiments were performed at HCH lab

ELISA and western blot experiments were performed at PPC lab

Super resolution microscopy experiments were performed at AMT lab

JJDeJP: acquisition, analysis, and interpretation of data, drafting the work

SCR: acquisition, analysis, and interpretation of data, drafting the work

AEES: acquisition, analysis, and interpretation of data, drafting the work

NECF: acquisition, analysis, and interpretation of data, drafting the work

ZQ: acquisition, analysis, and interpretation of data, drafting the work

PPC: conception or design of the work, drafting the work or revising it critically for important intellectual content.

HCH: conception or design of the work, drafting the work or revising it critically for important intellectual content.

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The TMEM16A-mediated Ca^{2+} -activated Cl^- current drives several important physiological functions. Membrane lipids regulate ion channels and transporters but their influence on members of the TMEM16 family is poorly understood. Here we have studied the regulation of TMEM16A by phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂), cholesterol, and fatty acids using patch clamp, biochemistry and fluorescence microscopy. We found that depletion of membrane PI(4,5)P₂ causes a decline in TMEM16A current that is independent of cytoskeleton, but is partially prevented by removing intracellular Ca^{2+} . On the other hand, supplying PI(4,5)P₂ to inside-out patches attenuated channel rundown and/or partially rescued activity after channel rundown. Also, depletion (with methyl- β -cyclodextrin M- β CD) or restoration (with M- β CD + cholesterol) of membrane cholesterol slows down the current decay observed after reduction of PI(4,5)P₂. Neither depletion nor restoration of cholesterol change PI(4,5)P₂ content. However, M- β CD alone transiently increases TMEM16A activity and dampens rundown whereas M- β CD + cholesterol increases channel rundown. Thus, PI(4,5)P₂ is required for TMEM16A function while cholesterol directly and indirectly via a PI(4,5)P₂-independent mechanism regulate channel function. Stearic, arachidonic, oleic, docosahexaenoic, and eicosapentaenoic fatty acids as well as methyl stearate inhibit TMEM16A in a dose- and voltage-dependent manner. Phosphatidylserine, a phospholipid whose hydrocarbon tails contain stearic and oleic acids also inhibits TMEM16A. Finally, we show that TMEM16A remains in the plasma membrane after treatment with M- β CD, M- β CD + cholesterol, oleic, or docosahexaenoic acids. Thus, we propose that lipids and fatty acids regulate TMEM16A channels through a membrane-delimited protein-lipid interaction.

Keywords

Regulation; TMEM16A; Anoctamin; Lipids; Poly-unsaturated fatty acids; Patch clamp; Fluorescence microscopy

INTRODUCTION

The Ca^{2+} -activated Cl^- channel TMEM16A is responsible for Cl^- transport that helps regulate vascular tone, neuronal activity, salivary secretion, gastrointestinal tract motility, insulin secretion, tumorigenesis and cancer progression, among other processes (Frings et al., 2000; Hartzell et al., 2005; Duran et al., 2010; Hwang et al., 2009; Catalán et al., 2015; Duvvuri et al., 2012; Pedemonte and Galletta, 2014; Oh and Jung, 2016). TMEM16A and TMEM16B are homo-dimeric channels that belong to the TMEM16 (Anoctamin) membrane protein family (Sheridan et al., 2011; Pedemonte and Galletta, 2014; Whitlock and Hartzell, 2017). Intracellular Ca^{2+} activates TMEM16A and TMEM16B by binding directly to the channel.

In addition to its regulation by Ca^{2+} , TMEM16A is also regulated by permeant anions that facilitate TMEM16A channel gating (Arreola et al., 1996; Kuruma and Hartzell, 2000; Arreola et al., 2010; Xiao et al., 2011; Terashima et al., 2013; Betto et al., 2014; Contreras-Vite et al., 2016) because ion permeation and gating are coupled (Perez-Cornejo et al., 2004; Betto et al., 2014). Also, protons exert a strong regulatory effect on channel activity. Increasing the intracellular proton concentration decreases TMEM16A activity because protons compete with intracellular Ca^{2+} for binding to glutamic acids E702 and E705 at the

calcium binding pocket (Arreola et al., 1996; Chun et al., 2015). In contrast, extracellular protons increase the open probability of TMEM16A by titrating the outward-facing E623 residue without altering the apparent affinity for intracellular Ca^{2+} (Qu and Hartzell, 2000; Cruz-Rangel et al., 2017).

Membrane lipids also regulate many ion channels. The inner leaflet phospholipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) is a potent regulator of several ion channels and transporters (Suh and Hille, 2008; Huang, 2007). Voltage-gated, inwardly rectifying and two-pore K^+ channels, voltage-gated Ca^{2+} channels, Ca^{2+} release channels, TRP channels, and epithelial ENaC channels are all regulated by PI(4,5)P2. Recent evidence shows that TMEM16 proteins are also regulated by PI(4,5)P2. For example, the TMEM16F phospholipid scramblase is regulated by PI(4,5)P2 in intestinal epithelia stimulated with the accessory cholera enterotoxin (Aoun et al., 2016). This might be because TMEM16F and also TMEM16C, -D, -G, and -J are phospholipid scramblases that transport membrane phospholipids between leaflets of the membrane bilayer (Suzuki et al., 2010; Suzuki et al., 2013). It is thought that during translocation the hydrophilic head groups of the phospholipids move through a hydrophilic cleft on the surface of the scramblase that is in contact with the lipid bilayer (Brunner et al., 2014). While TMEM16A has no scramblase activity, the structure of the Cl^- selective pore of TMEM16A is homologous to the hydrophilic cleft present in the scramblases (Whitlock and Hartzell, 2016; Paulino et al., 2017; Fisher and Hartzell, 2017). This suggests that membrane lipids could be in intimate contact with the pore of TMEM16A. In addition, in secretory epithelial cells, TMEM16A is activated after stimulation of phospholipase C by muscarinic receptors (Melvin et al., 2002). Activation of phospholipase C causes hydrolysis of PI(4,5)P2 to generate inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 in turn triggers release of Ca^{2+} from internal stores and activates the TMEM16A channel (Giovannucci et al., 2002). Thus, in addition to the activating effect exerted by released Ca^{2+} , changes in the PI(4,5)P2 content in the inner leaflet of the plasma membrane by itself could regulate TMEM16A. Indeed two recent reports indicate that TMEM16A activity is under control of PI(4,5)P2; however, these reports show opposite results. One of them demonstrates that exogenous PI(4,5)P2 down regulates the Ca^{2+} -dependent Cl^- current ($I_{\text{Ca,Cl}}$) in inside-out patches from pulmonary artery cells (Pritchard et al., 2014). But the other shows that exogenous PI(4,5)P2 increases TMEM16A activity in inside out patches isolated from HEK 293 cells and that PI(4,5)P2 dephosphorylation induces the opposite effect (Ta et al., 2017).

Regulation of membrane proteins by PI(4,5)P2 could be further complicated because PI(4,5)P2 appears to be concentrated in low-density, caveolin- and cholesterol-rich membrane fractions in A431 cells (Pike et al., 1996; Pike and Miller, 1998; Golub and Caroni, 2005). Interestingly, epidermal growth factor or bradykinin decreased PI(4,5)P2 from caveolae whereas cholesterol depletion redistributed ^3H -PI(4,5)P2 from those domains without changing the total amount of polyphosphoinositides; added cholesterol had the opposite effect (Pike and Miller, 1998). Based on these observations it has been suggested that cholesterol and PI(4,5)P2 interact to regulate ion channels (Rosenhouse-Dantsker et al., 2014). In addition, cholesterol by itself regulates the activity of inwardly rectifying K^+ channels, voltage-gated K^+ channels, Ca^{2+} -sensitive K^+ channels, voltage-gated Na^+ channels, N-type voltage-gated Ca^{2+} channels, volume-regulated anion channels, and

endogenous Ca^{2+} -activated Cl^- channels (Levitan and Barrantes, 2012; Levitan et al., 2010; Sones et al., 2010). Thus, a cholesterol/PI(4,5)P2 interaction could bring about a complex regulation of ionic channels including TMEM16A, which appears to be located in cholesterol rich domains in pulmonary artery cells (Sones et al., 2010).

Recently, the role of mono- and poly-unsaturated (ω -3, ω -6, and ω -9) fatty acids or PUFAs on ion channels has attracted intense interest due in part to their promising effects on cardiac-, brain-, and muscle-related disorders. For example, the mono-unsaturated oleic acid may act as a blood pressure regulator (Teres et al., 2008). Docosahexaenoic acid (DHA) is a major fatty acid in the brain and retina and impaired learning is associated with low concentrations of DHA (Hashimoto et al., 2016; Janssen and Kiliaan, 2014). Arachidonic acid is a second messenger that regulates proteins such as PLCs and PKCs, and, for example, acts as a vasodilator (Proctor et al., 1987). Several ion channels are modulated by fatty acids, including voltage-gated Na^+ , K^+ , Ca^{2+} , H^+ , Ca^{2+} -activated potassium channels and transient receptor potential (TRP) channels (see a recent review by Elinder and Liin, 2017). DHA also modulates desensitization of excitatory nicotinic acetylcholine and inhibitory GABA receptors (Hamano et al., 1996; Bouzat and Barrantes, 1993). In addition, some of these fatty acids form the hydrocarbon tails of PI(4,5)P2 and other phospholipids including phosphatidylserine. Despite this wealth of information about the effects of fatty acids on ion channels and blood pressure regulation, we know nothing about their effects on TMEM16A, a channel that is key in controlling blood pressure (Wang et al., 2015; Hübner et al., 2015) among other roles.

In this work, we analysed the effect of PI(4,5)P2 dephosphorylation, M- β CD-induced cholesterol depletion, and exposure to extracellular fatty acids on TMEM16A channel activity. We found that PI(4,5)P2 dephosphorylation inhibits TMEM16A activity, that M- β CD dampens current run-down, and that fatty acids inhibit TMEM16A in a voltage-dependent manner. The effect of M- β CD and fatty acids was independent of TMEM16A redistribution. Our data demonstrate that the lipid environment regulates the activity of TMEM16A due to the interaction of the channel with specific lipids. These effects might be physiologically relevant in instances like the presence of hormones and drugs that mediate changes in PI(4,5)P2 metabolism.

METHODS

Cell culture and protein expression

HEK 293 cells were grown in DMEM (Dulbecco's modified Eagles's medium) supplemented with 10% fetal bovine serum, 2 mM l-glutamine and 1 mg ml^{-1} geneticin at 37°C in a 95% O_2 /5% CO_2 atmosphere. The mouse TMEM16A (ac) variant cloned into a bicistronic vector for mammalian expression (pIRES2-EGFP, Clontech, Mountain View, CA, USA) was stably transfected into HEK 293 cells as previously described (Contreras-Vite et al., 2016). To study the regulation of TMEM16A by PI(4,5)P2, we transiently co-transfected HEK 293 cells with mouse TMEM16A-3xFlag along with *Danio rerio* voltage-sensitive phosphatase (Dr-VSP) cloned into the pIRES2-EGFP vector. Dr-VSP dephosphorylates PI(4,5)P2 at the 5' position of the inositol ring with a time constant of 300 ms, is inactive at negative voltages and is fully activated by depolarizing voltages (Hossain et al., 2008;

Falkenburger et al., 2010). We also altered PI(4,5)P₂ via the human muscarinic receptor type 1 (M1R) that induces PI(4,5)P₂ hydrolysis via phospholipase C activation. HEK 293 cells were co-transfected with 1 µg/µL of mTMEM16A and 0.25 µg/µL of M1R cDNA. To localize membrane TMEM16A with super-resolution microscopy, ECFP-Mem, a membrane protein marker, was transiently co-transfected with TMEM16A-EGFP. 1 µg cDNA and the Polyfect transfection reagent (Qiagen, Valencia, CA, USA) were added to the media according to the manufacturer's instructions. 12 h post-transfection the cells were re-plated onto 5 mm glass coverslips and allowed to attach for at least 4 h before the electrophysiological recordings were carried out. Dr-VSP was cloned in pIRES2-EGFP vector and cells positive for Dr-VSP expression were identified by their EGFP fluorescence. For microscopy assays, TMEM16A was cloned into the expression vector pEGFP-N1. The TMEM16A-EGFP fusion protein was co-transfected in glass-bottom Petri dishes with the plasma membrane marker pECFP-Mem (both from Clontech).

Electrophysiological recordings

Transfected cells were identified by EGFP fluorescence (488 nm excitation). Whole-cell recordings were carried out at room temperature (21–23°C) using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA). Ca²⁺-activated Cl[−] current (I_{Ca,Cl}) was recorded using voltage steps from −100 to +120 mV delivered in 20 mV increments every 0.5 s, from a holding voltage of −60 mV. Tail currents were recorded by repolarizing to −100 mV. Alternatively, time courses were obtained by recording I_{Cl} at +100 (every 1.5 s) or +120 mV (every 5 s and then every 5 min).

Inside-out excised patches were studied as previously described (Xiao et al., 2011). Briefly, HEK 293 cells were transiently transfected with TMEM16A-EGFP using Fugene-6 (Roche) and patches were excised 24–72 h after transfection. Patches were switched between zero-Ca²⁺ and high 100 µM Ca²⁺ solutions by positioning the patch at the opening of a double-barrelled theta tubing (1.5 mm o.d.; Sutter Instruments) with a tip diameter of ~50 µm attached to a piezobimorph on a micromanipulator. When dioctanoyl phosphatidylinositol 4,5-bisphosphate (C8-PIP₂) or dioctanoyl glycerol pyrophosphate (C8-DGPP) was added to the solution, 25 µM Ca²⁺ was also added to compensate for Ca²⁺ binding to the phospholipid. Data acquisition and generation of voltage protocols were performed using a Digidata 1550 interface (Molecular Devices, Sunnyvale, CA, USA). Currents were filtered at 5 kHz and digitized at 10 kHz using the pClamp10 software (Molecular Devices, Sunnyvale, CA, USA). Patch pipettes were pulled from Corning 8161 glass capillary tubes (Warner Instruments, LLC, Hamden, CT, USA) using a PP830 electrode puller (Narishige, Tokyo, Japan). Pipette resistance ranged from 3–6 mΩ for whole cell recordings. A 3M KCl agar-bridge was used to ground the recording chamber.

We assay the role of actin filaments by treating HEK 293 cells co-expressing TMEM16A-3xFlag and Dr-VSP for 1 h with 25 µM CK-666 or 5 µM latrunculin A. CK-666 binds to Arp2/3 complex to stabilize the inactive state of the complex, which inhibits nucleation of branched actin filaments with an EC₅₀ of 4 µM (Hetrick et al., 2013) whereas latrunculin A inhibits actin polymerization with a dissociation constant of about 0.2 µM (Coué et al., 1987). We also determine the regulation of TMEM16A by PI(4,5)P₂

dephosphorylation in the absence of intracellular Ca^{2+} in cells dialyzed with 25 mM EGTA and bathed in 140 mM SCN^- to increase the current magnitude (Perez-Cornejo et al., 2004; Qu and Hartzell, 2000). Under this experimental condition, we activate M1R with 1 mM carbachol and determine its effect on TMEM16A. The effects of these manipulations on TMEM16A were determined from I_{Cl} time courses.

Solutions

Intracellular (pipette) solution with $[\text{Ca}^{2+}] = 0.2 \mu\text{M}$ contained (in mM): 30 TEA-Cl, CaCl_2 5.2, HEPES 20, 25.2 EGTA, and 85 D-mannitol. The free $[\text{Ca}^{2+}]$ was calculated using the MAXCHELATOR program (maxchelator.stanford.edu). An intracellular solution with 0 Ca^{2+} was prepared by omitting CaCl_2 . We used a 0 Ca^{2+} solution supplemented with 1 mM Mg^{2+} , 0.1 mM GTP- γ -S and 3 mM ATP-Tris to study TMEM16A regulation by muscarinic receptor activation. The pH of both solutions was adjusted to 7.3 with TEA-OH. The osmolality, 290–300 mOsm/kg, was measured using the vapour pressure point method (VAPRO, Wescor Inc., South Logan, UT, USA). Cells were bathed in a standard external solution that contained (in mM): 139 TEA-Cl, 0.5 CaCl_2 , 20 HEPES, and 110 D-mannitol, pH adjusted to 7.3 with TEA-OH. Alternatively, when we recorded TMEM16A in the absence of intracellular Ca^{2+} we used an external solution containing 140 mM SCN^- and 0 Cl^- . To prevent activation of endogenous volume-sensitive Cl^- currents, the external solution was made hypertonic (Hernández-Carballo et al. 2010) by adding D-mannitol. For PI(4,5)P2 recovery experiments the internal solution was supplemented with 1 mM MgCl_2 and freshly made 5 mM ATP. External and standard zero- Ca^{2+} internal solutions for inside-out patches contained (in mM): 150 NaCl, 1 MgCl_2 , 10 glucose, and 10 HEPES (pH 7.3), adjusted with N-methyl-D-glucamine, and 146 CsCl, 5 EGTA, 10 sucrose, and 8 HEPES (pH 7.3), respectively. An internal solution with 100 μM Ca^{2+} was prepared with 146 mM CsCl, 100 μM CaCl_2 , 8 mM HEPES (pH 7.3).

External solutions containing methyl- β cyclodextrin (M- β CD), methyl- β cyclodextrin + cholesterol (M- β CD + chol) or fatty acids (stearic acid, methyl-stearate —Sigma-Aldrich, St. Louis, MO, USA—, oleic acid, arachidonic acid, docosahexaenoic acid, and eicosapentaenoic acid (Tocris Bioscience, Avonmouth, Bristol, UK), or bovine brain phosphatidylserine (PS) (1,2-diacyl-*sn*-glycero-3-phospho-L-serine from Sigma-Aldrich, St. Louis, MO, USA) were prepared as follows. M- β CD (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in the external solution at a concentration of 2.3 mM (3 mg/ml). This concentration induces a mild reduction in the cholesterol content (Zidovetzki and Levitan, 2007). M- β CD + chol (1:1) mixture was prepared by mixing 2.3 mM of cholesterol in 10 ml of 2.3 mM M- β CD. The resulting mix was dissolved by vortexing and sonication for 20 s, 3 times. The final mix was filtered through a 4 μm filter to remove undissolved cholesterol crystals. These solutions were freshly prepared before every experiment. Fatty acids were dissolved in ethanol, thawed and stored at -20°C . On the day of the experiment, the desired fatty acid concentration was prepared by adding the appropriate volume of the stock to the standard external solution. The ethanol concentration was $<0.06\%$. PS was dissolved in chloroform:methanol (95:5). External solutions were gravity-perfused into the recording chamber at a flow rate of $\sim 4 \text{ ml min}^{-1}$.

Super-resolution imaging

Co-localisation of ECFP-Mem and TMEM16A-EGFP at the plasma membrane was determined 24 h post-transfection and then at 0 and 30 min after adding 2.3 mM M- β CD, M- β CD+Chol (1:1), 10 μ M OA or 10 μ M EPA. We used an ELYRA PS.1 super-resolution structured illumination microscopy system (SR-SIM, ZEISS). Fluorescent proteins were excited with the laser line at 488 nm for EGFP and 405 nm for ECFP and observed using a 63 $\times$ oil immersion objective. Images were collected using the Zeiss Zen software and analysed off line.

Lipid-protein interaction assay

To study the interaction between TMEM16A and PI(4,5)P2 we have used a modified protein lipid overlay assay (Dowler et al., 2002). For this assay a recombinant protein must be purified and then incubated with several dilutions of the lipid of interest (1–500 pmol), previously spotted into a nitrocellulose membrane. The bound protein is detected with the help of an antibody. Here we use the mouse TMEM16A-3xFlag protein, which was immunopurified by adding the cell extracts from HEK 293 cells transiently transfected to magnetic beads conjugated to an antibody directed against the tag on the fusion protein as described before (Perez-Cornejo et al., 2012). Briefly, 30 μ L of Dynabeads M-280 sheep antimouse IgG (11201D; Invitrogen) were conjugated to 0.2 μ g mouse anti-Flag M2 antibody (F3165 Sigma-Aldrich, St. Louis, MO). The conjugated beads were incubated with lysates containing 500 μ g total protein for 2 hrs at 4 °C using a tube rotator (C400110Q Labquake, Thermo Scientific). After this incubation, the beads were washed and the bound proteins were eluted using 20 μ L of 2 $\times$ Laemmli buffer.

The lipid strips used in our experiments were purchased from Avanti as ELISA Snoopers strips for PI(4,5)P2 (330633; Avanti, Alabaster, AL). The strips consist of 8 wells pre-coated with 500 pmol of PI(4,5)P2 per well spotted on a nitrocellulose membrane. The strips were blocked for 1 h with 5% skim milk in TBS-T and rinsed 4 times with PBS-T prior to adding either cell lysates (400–500 ng total protein) or isolated TMEM16A (20 μ L). The eluted proteins were allowed to bind to the membrane for 2 h, then the membranes were rinsed five times. 200 μ L of primary antibody (dil 1:5,000) were added, incubated for 2 h and rinsed five times before adding 200 μ L of secondary antibody (dil 1:20,000; Santa Cruz Biotechnology, Santa Cruz, CA) for an additional 2 h incubation. All incubations were performed at room temperature. Five washes were performed before adding 50 μ L of the colorimetric TMB substrate (T0440; Sigma-Aldrich, St. Louis, MO). After a 20 min incubation, the reaction was terminated using a Stop Solution (895032; R&D Systems, Minneapolis, MN). The degree of binding was estimated by absorbance at 450nm (iMark, Bio-Rad). PBS without primary or secondary antibodies was used as blank. Total protein from HEK 293 cells that do not express TMEM16A (untransfected cell lysates) was used to get a reading of unspecific binding, due to PI(4,5)P2 interaction with proteins other than the channel.

Western blots

Untransfected HEK 293 cells were incubated at room temperature with vehicle, 2.3 mM M- β CD or M- β CD+Cholesterol 1:1 during 30 min. After the treatment the cells were placed on ice, washed twice with PBS and lysed using a buffer containing (in mM): 150 NaCl, 10

HEPES, 1 EGTA, and 0.1 MgCl₂, pH 7.4 supplemented with 0.5% Triton X-100 and a protease cocktail (11245200; Roche). Immediately after the cells were scraped and transferred to an Eppendorf tube for incubation on ice during 30 min. Supernatant was recovered by centrifugation at 14,800 g for 10 min and total protein was quantified by BCA assay (Sigma-Aldrich, St. Louis, MO). Proteins were separated by SDS-PAGE on 10% gels, followed by protein transfer to PVDF membranes using a semi-dry transfer apparatus (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked with 5% Carnation non-fat dry milk in TBS-T and probed overnight at 4°C. PI(4,5)P₂ levels were detected using 1:1000 dilution of a mouse monoclonal antibody from Santa Cruz Biotechnology (sc-53412). Blots were visualized by chemiluminescence using the ChemiDoc XRS+ System with Image Lab Software (Bio-Rad Laboratories, Hercules, CA). Band intensities were determined by volume analysis and then plotted against the amount of total protein loaded in each lane.

Data analysis

Electrophysiological data were analysed using pClamp10 (Molecular Devices) and Origin 10 (Origin Lab, Northampton, MA). I_{Cl} vs V_m curves were constructed using the I_{Cl} values at the end of each depolarizing pulse normalized to the I_{Cl} magnitude recorded at +120 mV in the same cell. Time course plots were constructed using normalized currents ($I/I_{t=0}$). $I/I_{t=0}$ was calculated by dividing the current magnitude induced by a depolarizing voltage at any given time by the current magnitude measured before a given treatment. Time constants were calculated from single exponential fits (Equation 1)

$$y_0 * e^{-t/\tau} \quad \text{Equation 1}$$

In which y_0 is the value of the variable at infinite time and τ is the time constant. Doseresponse curve was analysed using a Hill equation (Equation 2)

$$\frac{1}{1 + \frac{EC_{50}}{[EPA]}} \quad \text{Equation 2}$$

here EC_{50} is the [EPA] to obtain 50% of the maximum response. Images were analysed using Fiji software (Schindelin et al., 2012). The voltage dependence of TMEM16A inhibition was quantified as the unblock fraction by dividing the remaining current after exposure to fatty acid by the control current. This ratio was then plotted against voltage. To determine whether or not cholesterol alteration or exposure to fatty acids induced protein relocation, we measured the fluorescence emitted by EGFP-tagged TMEM16A. Fluorescence was measured at $t = 0$ and $t = 30$ min from rectangular sections across the cells and from 4 rectangles of 6×20 pixels drawn at different locations on the membrane. Fluorescence magnitude at $t = 30$ min was divided by the fluorescence measured in the same cell at $t = 0$. Fluorescence ratios obtained from different cells were averaged and plotted.

Statistically significant differences were evaluated using the Student's *t* test with a level of significance of 0.01. Experimental data are presented as mean $\pm$ SEM and *n* represent the number of independent experiments.

RESULTS

PI(4,5)P2 regulates TMEM16A activity

Two reports showed opposite effects of PI(4,5)P2 on TMEM16A; PI(4,5)P2 inhibited the Ca^{2+} -activated Cl^- current ($I_{\text{Ca,Cl}}$) in pulmonary artery cells (Pritchard et al., 2014) but increased TMEM16A $I_{\text{Ca,Cl}}$ in HEK 293 cells (Ta et al., 2017). Here we study the regulation of TMEM16A $I_{\text{Ca,Cl}}$ by PI(4,5)P2 using the *Danio rerio* voltage-sensitive phosphatase (Dr-VSP). Cells expressing Dr-VSP and TMEM16A were identified by EGFP fluorescence and channel activity in cells dialyzed with a solution containing 0.2 μM free Ca^{2+} . We activated TMEM16A and Dr-VSP using depolarisations to +80 mV that lasted 30 s (Figure 1Aa). In cells without ATP or dialyzed with 5 mM ATP and expressing only TMEM16A we recorded an I_{Cl} that slowly increased (Figure 1Ab and 1Ac) as we previously reported (Cruz-Rangel et al 2015). In sharp contrast, cells co-expressing TMEM16A and Dr-VSP displayed an I_{Cl} that peaked around 2 s and then decayed in a bi-exponential manner regardless of the presence of intracellular ATP (Figures 1Ad and Ae). Time constants of inhibition were 2.47 ± 0.43 s and 1.05 ± 0.28 s. These values are close to the time constant ($\tau = 2$ s) we determined for Kir2.1 inhibition by Dr-VSP (data not shown). To measure recovery of TMEM16A channels, we applied three depolarisation steps to +80 mV (P_2 , P_3 , and P_4) lasting 4 s each. These pulses were 4, 10 and 20 s apart while the cells were held at -60 mV (Figure 1Aa). In the absence of MgCl_2 and ATP, we did not observe recovery (Figure 1Ad). On the contrary, the amplitude of the current decayed continuously. However, in cells dialysed with 1 mM MgCl_2 and 5 mM ATP, we observed a partial recovery after 15 s (Figure 1Ae). This compares to 11 s time constant of recovery of PI(4,5)P2 after its dephosphorylation by Dr-VSP (Falkenburger et al., 2010). Figure 1B summarizes this finding and shows ATP was critical for channel recovery. Our findings revealed that the PI(4,5)P2 dephosphorylation caused by Dr-VSP decreased TMEM16A activity in agreement with a recent report showing that exogenous PI(4,5)P2 increases TMEM16A activity whereas PI(4,5)P2 depletion has the opposite effect (Ta et al., 2017).

Because PI(4)P is produced by PI(4,5)P2 dephosphorylation, it is possible that PI(4)P regulates TMEM16A. To test this possibility we co-expressed human muscarinic receptor (M1R) along with TMEM16A and measured the effect of 1 mM carbachol (CCh). Cells were dialyzed with 0 Ca^{2+} and bathed in a SCN^- -containing solution. M1R activation stimulates phospholipase C which generates IP3 and diacyl glycerol from PI(4,5)P2 hydrolysis while reducing PI(4)P (Falkenburger et al., 2010). In addition, PI(4,5)P2 is resynthesized slowly after M1R activation, with a time constant of 130 s (Falkenburger et al., 2010). Although our experimental conditions allow IP3 to release Ca^{2+} from the endoplasmic reticulum, intracellular Ca^{2+} is chelated so that Ca^{2+} -dependent TMEM16A activation does not occur. Figure 1C shows two examples of I_{SCN} recorded at +100 mV in the absence (left) and 5 min after addition of 1 mM CCh (right). In the absence of CCh, I_{SCN} decayed but this decay was more pronounced in cells treated with CCh (Figure 1D). I_{SCN}

was inhibited by CCh with a time constant of 114.2 ± 15.0 s. Because M1R activation reduces PI(4,5)P2 without producing PI(4)P, it is unlikely that PI(4)P regulates TMEM16A.

Patch excision leads to a gradual loss of PI(4,5)P2 and subsequent rundown of Kir channel but Kir activity can be rescued by applying exogenous PI(4,5)P2 (Huang et al., 1998). Rundown of the TMEM16A current is Ca^{2+} -dependent and cannot be rescued by calmodulin (Yu et al., 2014). Our data showing that PI(4,5)P2 depletion impairs TMEM16A function suggested that application of exogenous PI(4,5)P2 might prevent channel rundown. We corroborated this idea by exposing the intracellular side of inside-out patches to C8-PIP2, a soluble analogue of PI(4,5)P2. Figure 2A shows the recording from patches excised from cells overexpressing TMEM16A. The recording was initiated with a zero Ca^{2+} solution on the cytoplasmic side. As indicated by the grey bar, the cytosolic side of the patch was then exposed to a solution containing 100 μM free Ca^{2+} . I_{Cl} rapidly increased and then ran down over the next 2 min with a time constant of 76.5 s. In Figure 2B, 25 μM C8-PIP2 was added to the 100 μM Ca^{2+} solution. In this case I_{Cl} remained stable (time constant > 90 s) until PI(4,5)P2 was removed, then the current quickly ran down. After rundown, PI(4,5)P2 application in the presence of zero Ca^{2+} for 3 min caused partial recovery of I_{Cl} . Figure 2C shows that I_{Cl} ran down quickly in 100 μM Ca^{2+} . The patch was then returned to the zero Ca^{2+} solution and the recording stopped for 3 min. I_{Cl} amplitude in response to a second exposure to Ca^{2+} was unchanged indicating that there was no current recovery. The patch was then returned to zero Ca^{2+} plus 25 μM C8-PIP2 for 3 min. The initial I_{Cl} amplitude in response to the third exposure to Ca^{2+} was significantly increased, but because PI(4,5)P2 was no longer present, I_{Cl} ran down. The patch was then returned to zero Ca^{2+} plus 25 μM C8-PIP2 for 3 min. I_{Cl} amplitude in response to 100 μM Ca^{2+} + C8-PIP2 was significantly increased and persisted as long as PI(4,5)P2 was present. Figure 2D shows the quantification of experiments like the one shown in Figure 2C. The patch was first exposed to 100 μM Ca^{2+} for ~5 min until I_{Cl} had run down to a stable level. The patch was then exposed for 3 min to a solution with zero Ca^{2+} in the absence or presence of the inactive C8-diacylglycerol pyrophosphate (+DGPP) or C8-PIP2 (+C8-PIP2). The bar graph shows that C8-PIP2, but not C8-DGPP, produced an 8-fold recovery of I_{Cl} .

Regulation of TMEM16A by PI(4,5)P2 was independent of actin filaments and subsided after removal of intracellular Ca^{2+}

PI(4,5)P2 could regulate TMEM16A activity by several possible mechanisms. PI(4,5)P2 regulates cortical actin (Zhang et al., 2012) that directly interacts with ion channels (Sasaki et al., 2014) and TMEM16A requires PI(4,5)P2-dependent ERM proteins for proper function (Fehon et al., 2010; Perez-Cornejo et al., 2012). Therefore, actin filaments could mediate the down-regulation of TMEM16A observed after PI(4,5)P2 dephosphorylation. Alternatively, PI(4,5)P2 could electrostatically alter the $[\text{Ca}^{2+}]$ near the channel as shown for KCNQ K^+ channel and its regulation by Mg^{2+} (Suh and Hille, 2007). Finally, PI(4,5)P2 could directly interact with TMEM16A to regulate its function as demonstrated for the Kir2.1 channel (Huang, 1998; Hansen et al, 2011). Thus, we assayed the role of actin filaments by exposing cells to 25 μM CK-666 or 5 μM latrunculin A for 1 h in cells bathed in 140 mM SCN^- (Perez-Cornejo et al., 2004; Qu and Hartzell, 2000). In addition, to evaluate whether PI(4,5)P2 and TMEM16A interact we performed a modified protein lipid

overlay assay using ELISA Snoopers strips for PI(4,5)P2. Figure 3A shows whole-cell traces recorded at +100 mV from two different untreated cells expressing TMEM16A-3xFlag (left) or TMEM16A-3xFlag and Dr-VSP (right). Traces were collected at $t = 0$ (black) and $t = 300$ s (blue). Without Dr-VSP the I_{Cl} magnitude at both time points was nearly identical. However, cells expressing Dr-VSP displayed a sharp reduction in I_{Cl} after 300 s. Experiments like this were repeated in cells treated with CK-666, latrunculin A, or dialyzed with a 0 Ca^{2+} /25 mM EGTA solution. Figure 3B summarizes our findings in a time course graph. In the absence of Dr-VSP, I_{Cl} was nearly the same during the entire 300 s recordings (black). However, when the phosphatase was present in untreated cells I_{Cl} decays with a time constant of 83.1 ± 8.5 s (blue) and after 300 s the I_{Cl} fraction remaining is only 0.26 ± 0.05 . Surprisingly, the I_{Cl} time course in cells treated with CK-666 (Figure 3B, grey) or latrunculin A (Figure 3B, red) was the same as that obtained from untreated cells, indicating that these drugs had no effect on the I_{Cl} decay induced by PI(4,5)P2 dephosphorylation. Similarly, we found that removal of intracellular Ca^{2+} decreased, but did not abolish, the PI(4,5)P2 dephosphorylation-induced I_{SCN} decay (Figure 3B, cherry). Our ELISA assay shows that binding of TMEM16A to PI(4,5)P2-coated nitrocellulose membranes was 1.18 ± 0.21 versus 0.54 ± 0.06 (arbitrary units; $n=4$) for unspecific binding. Thus, our data suggest that PI(4,5)P2 might bind TMEM16A to regulate channel activity independently of actin filaments but partially dependent on intracellular Ca^{2+} .

Changes in cholesterol content altered rundown and slowed the effect of Dr-VSP on TMEM16A

It has been suggested that PI(4,5)P2 and TMEM16A concentrate in low-density membrane fractions, which are characteristic of cholesterol-rich membrane domains (Pike et al., 1996; Sones et al., 2010). In addition, cholesterol depletion with methyl- β -cyclodextrin (M- β CD) down-regulates the PI(4,5)P2 content in HEK 293 cells (Chun et al., 2010). These observations suggest that the response of TMEM16A to PI(4,5)P2 dephosphorylation would be altered in cells with altered cholesterol content. To test this idea, we first evaluated the effects of decreasing (with M- β CD) or increasing (with M- β CD + chol) cholesterol in the membrane, and then we evaluated the effect of Dr-VSP in cells with altered levels of cholesterol. Extended treatments (>2 h) with 2.3 mM M- β CD may decrease the overall cholesterol content (Zidovetzki and Levitan, 2007), while short treatments (5 min) as used by Sones et al. (2010) may produce a small change in cholesterol content (Zidovetzki and Levitan, 2007). In our experiments, we chose a condition that is likely to deplete cholesterol from both rafts and non-raft domains in HEK 293 cells (Omstrom and Insel, 2004). We treated the cells with 2.3 mM M- β CD or with M- β CD + chol (1:1) for 30 min. Figure 4A shows that a 5 min treatment with M- β CD slightly increased I_{Cl} . This effect, summarized in the I_{Cl} vs V_m curve (Figure 4B), reproduces a report showing up-regulation of endogenous Ca^{2+} -activated Cl^- channels from pulmonary artery after M- β CD treatment (Sones et al., 2010). However, the time course of the effect of 2.3 mM M- β CD revealed two additional effects. First, activation and deactivation time constants of I_{Cl} induced by a depolarization to +120 mV (Figure 4C) were changed after 30 min treatment. M- β CD slowed activation and deactivation of TMEM16A while M- β CD + chol speeded up activation but slowed deactivation (Table 1). Similarly, at -60 mV, M- β CD increased TMEM16A activity 1.97-fold whereas M- β CD + chol decreased it 2.08-fold. Second, application of M- β CD or M-

β CD + chol induced an early increase in I_{Cl} above control condition, however, at later times I_{Cl} rundown was nearly abolished by M- β CD while M- β CD + chol enhanced the rundown (Figure 4D). These data indicate that removal of cholesterol with long treatments stabilize TMEM16A from rundown but does not increase its activity. Next, we recorded the I_{Cl} time course of the decay induced by PI(4,5)P2 dephosphorylation in cells incubated for 30 min with either M- β CD or M- β CD + chol (Figure 5A). Activation of Dr-VSP induced I_{Cl} decay with a time constant of 83.1 ± 8.5 s whereas pre-treatment with M- β CD or M- β CD + chol decelerated the time course of the effect of PI(4,5)P2 dephosphorylation on TMEM16A to 182.2 ± 22.5 s and 180.9 ± 26.8 s, respectively. However, after 300 s the magnitude of I_{Cl} inhibition was similar between the three groups. Thus, cholesterol content decelerates the current decay induced by PI(4,5)P2 dephosphorylation.

To test whether or not M- β CD or M- β CD + chol change PI(4,5)P2 content, we quantified PI(4,5)P2 levels using a quantitative western blot assay. First the intensity of the signal was measured for protein isolated from untreated cells (upper membrane in inset in Figure 5B) to generate the standard curve shown in Figure 5B (black squares). The intensity of the signal generated by the PI(4,5)P2 antibody (1:1000 dil) is proportional to the amount of protein loaded onto the membrane. This standard curve was fit by linear regression with a correlation index (R^2) of 0.94. Next, the intensity of the signal for 10 and 20 μ g of protein isolated from cells incubated during 30 min with M- β CD or M- β CD + chol (lower membrane in inset in Figure 5B) was measured and compared to the standard curve. The intensity of the signals are shown as cherry and grey dots in Figure 5B. The data show that PI(4,5)P2 level does not change with these treatments; hence the effect cholesterol on TMEM16A regulation by PI(4,5)P2 dephosphorylation is not due to variations in PI(4,5)P2 content.

Fatty acids and phosphatidylserine regulate TMEM16A activity

In sensory neurons, the fatty acid-regulated TRPV1 channel interacts with and provides the Ca^{2+} that activates TMEM16A to enhance pain sensation (Takayama et al., 2015). Importantly, ablation of TMEM16A or exposure to poly-unsaturated fatty acids causes a decrease in blood pressure (Hübner et al., 2015; Calder, 2012). To determine whether fatty acids directly regulate TMEM16A, we tested the effects of unsaturated stearic acid (SA, 18:0), uncharged methylstearate (Me-S, 18:0), mono-unsaturated oleic acid (OA, 18:1, cis-9), poly-unsaturated arachidonic acid (AA, 20:4, cis-5,8,11,14), poly-unsaturated eicosapentaenoic acid (EPA, 20:5, cis-5,8,11,14,17), and poly-unsaturated docosahexaenoic acid (DHA, 22:6, cis-4,7,10,13,16,19) (see structures in Figures 6 and 7). I_{Cl} was recorded at +120 mV before the start and at the end of a 30 min exposure to fatty acids. Figure 6 shows the effects of OA, SA, and Me-S. Panel A depicts I_{Cl} traces recorded from different cells in control condition (Ctrl), and in the presence of 10 μ M OA, 10 μ M SA, and 5 μ M Me-S. Positive currents were strongly inhibited whilst the tail currents recorded at -100 mV were less affected. See for example that the tail currents were nearly the same in the presence of OA and SA and yet OA produced a more pronounced inhibition of the positive current than SA. Panel B displays the time courses. I_{Cl} displayed a mono-exponential rundown in control conditions (black). Application of SA, Me-S, and OA decreased the amplitude of I_{Cl} with nearly the same time constant. The time constants of current inhibition were (in min)

2.14 ± 0.35 (Ctrl), 0.95 ± 0.13 (10 μM OA), 1.47 ± 0.26 (10 μM SA), and 1.20 ± 0.19 (5 μM Me-S). Of these three 18-carbon fatty acids, OA produced the largest effect. Mono-unsaturated SA and uncharged Me-S were also able to induce channel inhibition albeit to a lesser extent than OA.

Then we tested the effects of 10 μM PUFAs on TMEM16A. Remarkably, AA, EPA, and DHA slowed activation with only slight changes in the tail current and effectively decreased the amplitude of I_{Cl} induced by depolarization (Figure 7A). Figure 7B shows that EPA inhibited TMEM16A I_{Cl} in a dose-dependent manner and inhibition was faster at higher [EPA]. Time courses were fitted with an exponential function (Eq. 1) to calculate the time constant of inhibition. Figure 7C shows that as the [EPA] increased, the rate of I_{Cl} inhibition rose until it saturated at 50 μM; the EC_{50} calculated was 5.4 μM. Analogous time courses were determined for 10 μM of DHA and AA (Figure 7D). These PUFAs inhibited TMEM16A with time constants of 1.39 ± 0.21 and 0.64 ± 0.08 min, respectively. Table 1 summarizes the time constants of activation and deactivation of I_{Cl} in the absence and in the presence of different fatty acids. OA, AA and EPA fatty acids slowed whereas DHA accelerate channel activation during the depolarizing pulse with little or no effect on the deactivation kinetics.

Phosphatidylserine (PS) hydrocarbon tails are most commonly composed of stearic and oleic acids, which we show inhibit TMEM16A. In addition, PS is transported by the TMEM16F scramblase and a single mutation converting V543 to T or S can turn the TMEM16A channel into a scramblase (Jiang et al., 2017). Thus, we investigated whether PS could regulate TMEM16A. Figure 7E shows that 10 μM PS decreased I_{Cl} , albeit this effect does not reach statistical significance. However, increasing PS to 63 μM increased the inhibition of I_{Cl} (Figure 7E) at +120 mV but much less at -100 mV (Figures 8A). This current reduction was statistically significant. Activation was accelerated by increasing the PS concentration while deactivation kinetics were unchanged (Table 1). Figure 7F summarizes this finding as time course plots. The time constant of I_{Cl} inhibition by 63 μM PS was 1.40 ± 0.17 min.

Remarkably, except for DHA, the inhibition of TMEM16A by fatty acids and PS was voltage-dependent. The unblocked current fraction at +120 and -100 mV after the treatment with 10 μM SA, OA, AA, EPA, DHA, 63 μM PS or with 5 μM Me-S is plot in Figure 8A. Inhibition at negative voltages was nearly absent with all fatty acids except for DHA. In contrast, fatty acids inhibited TMEM16A at positive voltages. We found that within the 18-carbon fatty acids, the mono-unsaturated OA induced the most pronounced inhibition whereas the uncharged Me-S and saturated SA produced less inhibition at +120 mV. We performed a more detailed analysis of the voltage-dependence of inhibition induced by 10 μM OA. Figure 8B shows representative I_{Cl} traces recorded between -100 and +140 mV from the same cell before (black) and after addition of 10 μM OA (cherry). Within 5 min OA sharply reduced I_{Cl} at all voltages (Figure 8C). To quantify the voltage dependence of the OA inhibition, we calculated the unblocked I_{Cl} fraction at each voltage. Figure 8D shows the resulting curve. As the membrane potential became positive the effectiveness of OA to inhibit channel activity intensified. Taken together, these data demonstrate that TMEM16A

is down regulated by PUFAs since channel activity is inhibited in a dose- and voltage-dependent manner.

Treatment with M- β CD, M- β CD + cholesterol or fatty acids did not change the plasma membrane distribution of TMEM16A

Membrane cholesterol modulates lipid micro domain segregation, the rate of internalization of clathrin-coated pits, lipid raft-associated signalling molecules, and distribution of plasma membrane proteins including Kv1.5 K⁺ channel (Subtil et al., 1999; Hao et al., 2001; Foster et al., 2003; Abi-Char et al., 2007; Abu-Arish et al., 2015). Thus, to rule out redistribution and/or internalization of TMEM16A in cells treated with M- β CD, M- β CD + chol or fatty acids as the mechanism for down regulation, we determined the fluorescence of GFP-tagged TMEM16A at the plasma membrane using super-resolution microscopy. Figure 9A shows a control cell transfected with GFP-tagged TMEM16A and the CFP-Mem plasma membrane marker. Both green fluorescence (left) and blue fluorescence (middle) were observed at the edge of the cell and localized together in the merged image (right) indicating that GFP-tagged channels are confined to the plasma membrane. This fluorescence pattern was well maintained during the 30 min observation period (upper right panel in Figure 9A). Several intracellular vesicles were also labelled with EGFP, probably corresponding to newly synthesized protein trafficking towards the plasma membrane. We quantify the fluorescence in a rectangle drawn around the cell's equator (Figure 9B) at 0 (olive green) and 30 (red) min in the same cell (Figure 9C). Alternatively, four 6 $\times$ 20 pixel boxes were drawn on top of the membrane (shown as white boxes, upper row of Figure 9B, labelled Ctrl) and the fluorescence was measured at 0 (olive green) and 30 (red) min in the same cell (Figure 9D). This analysis was performed for control cells and cells treated with 2.3 mM M- β CD, 1:1 M- β CD + chol, 10 μ M of OA, or 10 μ M EPA. Figures 9B and 9C show examples of different cells before (0 min) and after treatment (30 min) and their corresponding fluorescence around the cell's equator, respectively. The average relative fluorescence measured from the white boxes from 4 different cells is shown in Figure 9D. None of the treatments altered the fluorescence intensity, indicating that TMEM16A was neither redistributed within the plasma membrane nor lost during the treatment.

DISCUSSION

The present work demonstrates that TMEM16A is regulated by PI(4,5)P₂, cholesterol, and fatty acids. Our data indicate that PI(4,5)P₂ is required for the function of TMEM16A. PI(4,5)P₂ stabilizes the current by preventing rundown but if PI(4,5)P₂ is dephosphorylated then TMEM16A activity decreases. Our results are in agreement with those of Ta and co-workers showing that exogenous PI(4,5)P₂ increases the activity of TMEM16A, but not TMEM16B in a Ca²⁺-dependent manner and activation of the Dr-VSP phosphatase inhibited TMEM16A (Ta et al., 2017). A previous report showed that adding diC8-PIP₂ decreased the current through Ca²⁺-dependent Cl⁻ channels activated by 500 nM Ca²⁺ in inside-out patches excised from rat pulmonary artery cells (Pritchard et al., 2014); however, we do not have an explanation for this discrepancy. In these cells the Ca²⁺-activated Cl⁻ current is generated by activation of TMEM16A (Manoury et al., 2010) and adding PI(4,5)P₂ should favour activation, however, we realize that PI(4,5)P₂ could regulate TMEM16A by

mechanisms that diverge in different cells (see below) but more experiments are necessary to clarify this issue. Collectively, the report by Ta and co-workers and our results demonstrate that in HEK 293 cells the TMEM16A channels require PI(4,5)P₂ for proper function.

PI(4,5)P₂ could regulate TMEM16A indirectly by binding to a protein associated with the channel, by electrostatically altering the Ca²⁺ concentration near the channel as shown for the KCNQ K⁺ channel (Suh and Hille, 2007), or by direct lipid-protein interaction like in the inwardly rectifying Kir2.1 potassium channel (Huang, 1998; Hansen et al, 2011). A dephosphorylated PI(4,5)P₂ could inactivate ERM proteins that subsequently down regulate TMEM16A or impair actin-channel interaction (Perez-Cornejo et al., 2012; Fehon et al., 2010; Zhang et al., 2012; Sasaki et al., 2014) or become less effective at attracting Ca²⁺ ions to bind TMEM16A. However, PI(4,5)P₂ can activate and prevent rundown of TMEM16A in inside out patches that lack cytoskeleton (Ta et al., 2017; present data). In addition, our data show that the channel's activity decreases after PI(4,5)P₂ dephosphorylation in cells treated with latrunculin A that disrupt actin filaments or with CK-666 that inhibits nucleation of branching actin filaments (Hetrick et al., 2013). Also, if PI(4,5)P₂ acted by increasing the Ca²⁺ concentration near the channel then PI(4,5)P₂ dephosphorylation or PI(4,5)P₂ addition would have similar effects on TMEM16A and TMEM16B. This is not the case; PI(4,5)P₂ increases TMEM16A activity but decreased TMEM16B activity (Ta et al., 2017). The effects of Dr-VSP are likely due to PI(4,5)P₂ dephosphorylation and not to PI(4)P production because the M1R activation induces PI(4,5)P₂ metabolism without producing PI(4)P and decreases TMEM16A. Additionally, we show that removing intracellular Ca²⁺ decreases the inhibition induced by PI(4,5)P₂ dephosphorylation. Hence, we conclude that actin filaments do not play a role in the regulation of TMEM16A by PI(4,5)P₂ but intracellular Ca²⁺ is partially needed. We think that the regulation of TMEM16A activity is caused by direct interaction between PI(4,5)P₂ and the channel within the plasma membrane. The following observations support this idea: a) the current decays within seconds after activation of Dr-VSP, b) TMEM16A binds to strips pre-coated with PI(4,5)P₂, c) adding PI(4,5)P₂ to inside out patches nearly abolishes rundown and partially rescues channels after rundown, d) TMEM16A is activated by PI(4,5)P₂ while TMEM16B is inhibited (Ta et al., 2017), and e) activation of muscarinic receptor M1 in the absence of intracellular Ca²⁺ also inhibited TMEM16A. We reason that regulation of TMEM16A by PI(4,5)P₂ would be of physiological relevance. For example, TMEM16A is activated by an IP₃-dependent increase in intracellular Ca²⁺ due to GPCR activation, which also causes PI(4,5)P₂ metabolism. Thus the net result of GPCR activation will be a balance between TMEM16A activation by Ca²⁺ and inhibition by consumption of PI(4,5)P₂. Thus, PI(4,5)P₂ metabolism will dynamically limit the cell responses that are dependent on a Cl⁻ efflux mediated by TMEM16A activation.

It has been suggested that TMEM16A and PI(4,5)P₂ localize to low density membrane fractions that are rich in cholesterol (Pike and Casey, 1996; Pike and Miller, 1998; Sones et al., 2010). Because of this, alterations in cholesterol content would alter protein regulation by PI(4,5)P₂. Interestingly, cholesterol enrichment using M-βCD + cholesterol for 1–2 h only produces a slight down-regulation of the ³²P-PI(4,5)P₂ content in HEK 293 cells (Chun et al., 2010). In contrast, cholesterol depletion delayed the decrease in Kir2.1 and Kir2.3 currents induced by PI(4,5)P₂ depletion with neomycin (Rosenhouse-Dantsker et al., 2014).

Similarly, the activity of the PI(4,5)P₂ sensitive channel HCN4 (Suh and Hille, 2008) is decreased in the presence of M- β CD but not M- β CD + cholesterol and yet both treatments produced a significant positive shift on the voltage dependence of activation (Füerst and D'Avanzo, 2015). Furthermore, the idea that PI(4,5)P₂ is clustered in cholesterol rich membrane domains has been recently challenged since it has been shown that Triton X-100 causes clusters formation (van Rheenen et al., 2005). Thus, most likely PI(4,5)P₂ is homogeneously distributed along the plasma membrane in HEK 293 cells. Treatment with M- β CD appears not to alter this distribution as shown by FRET experiments that measure the interaction between GFP-PH-PH and RFP-PH-PH (van Rheenen et al., 2005). These observations suggest that varying cholesterol content in the membrane may have little effect on the ability of PI(4,5)P₂ to regulate TMEM16A channel in HEK 293 cells. Our data indicate that the amount of PI(4,5)P₂ was not significantly changed after treating untransfected HEK cells with M- β CD or M- β CD + cholesterol during 30 min. However, both depletion and enrichment of membrane cholesterol slows down the inhibition produced by the Dr-VSP phosphatase, which suggest that cholesterol and PI(4,5)P₂ could interact to regulate TMEM16A. Alternatively, M- β CD may extract not only cholesterol from the membrane, but other lipid(s) involved in TMEM16A regulation (Zidovetzki & Levitan, 2007). Our data also suggest that a direct lipid-protein interaction contributes to the regulation of TMEM16A by cholesterol. Our finding that M- β CD stabilizes TMEM16A activity while M- β CD + chol produced the opposite effect supports this idea. A sequence analysis of the primary structure of TMEM16A suggests the presence of 14 putative cholesterol-binding motifs. Still, a docking assay, using a homology model of TMEM16A (Cruz-Rangel et al., 2015), suggests that only the motif located in TM5, near the extracellular side, may actually bind cholesterol. A mutagenesis analysis will define whether these putative sites participate in cholesterol sensing and regulation of TMEM16A channels.

Although the mechanism of ion channel regulation by fatty acids has not been fully established (Bruno et al., 2007), a direct interaction is likely to play a role. In agreement with this idea, a binding site for docosahexaenoic acid formed by an arginine residue in H⁺-gated GLIC channels has been identified using X-ray methods (Basak et al., 2017). In TRPV1 channels, PUFAs can interact with the channel gate or voltage sensor to open the channel or PUFAs could compete with the ligand to shut down the channel (Matta et al., 2007; Morales-Lázaro et al., 2016). In addition, oleic acid induces a pronounced rightward shift (> +100 mV) of the voltage activation of TRPV1 (Morales-Lázaro et al., 2016). In our hands, fatty acids decrease TMEM16A activity in a voltage-dependent manner. Noteworthy, the dose dependence of inhibition by EPA (EC₅₀ 5.4 μ M) is comparable to the most selective inhibitors of TMEM16A (Namkung et al., 2011). All these data support the idea that fatty acids directly interact with ionic channels to regulate its function. This idea is further supported by the observation that PS, a phospholipid with hydrocarbon tails containing stearic and oleic acids, inhibited TMEM16A. PS inhibited TMEM16A in a voltage-dependent manner indicative of an interaction within the membrane electric field. We think that PS is interacting directly with the pore of TMEM16A. In agreement with this idea, it has been shown that TMEM16A channel can be converted into a scramblase able to transport PS (Jiang et al., 2017). Thus, the channel might be able to bind PS but not to translocate it, unless is mutated (Whitlock & Hartzell, 2016).

The voltage dependence of inhibition by fatty acids suggested that the carboxyl group might be involved as was shown in K^+ channels (Börjesson et al., 2008). However, this group plays a modest role since methyl stearate, an uncharged saturated fatty acid of 18 carbons, also inhibited TMEM16A albeit to a lesser extent than oleic acid. Saturated stearic acid, on the other hand, produced effects that were similar to those observed under control conditions. Thus, because oleic acid with one double bond, and PUFAs with 4, 5 and 6 double bonds inhibited TMEM16A in a voltage dependent manner we think the double bonds are important for fatty acids actions on TMEM16A.

PUFAs are beneficial to our health (Calder, 2012) because they regulate blood pressure, heart rate, and inflammation, improve insulin sensitivity, pain sensing, and visual signalling (Calder, 2012). Also, blood pressure (Hübner et al., 2015), pain sensing (Deba and Bessac, 2015), fluid secretion (Catalan et al., 2015), insulin secretion (Edlund et al., 2014) and gastrointestinal movements (Hwang et al., 2009) are controlled by Cl^- efflux via TMEM16A channels. Therefore, it is tempting to speculate that some of those physiological processes that are influenced by PI(4,5)P₂ or PUFAs are in part mediated by the regulation here described. For instance, co-activation of TMEM16A and TRPV1 would enhance pain sensation by inducing neuronal depolarization (Liu et al., 2010) but fatty acids and/or PI(4,5)P₂ hydrolysis could ameliorate pain or blood pressure because of their inhibitory effects on both channels.

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Highlights

1. The Ca^{2+} -dependent Cl^- channel TMEM16A is controlled by phospholipids and cholesterol.
2. TMEM16A is down regulated by muscarinic receptor 1 in the absence of intracellular Ca^{2+} .
3. Fatty acids regulate TMEM16A in a dose- and voltage-dependent manner.
4. Regulation by phospholipids partially required intracellular Ca^{2+} but not actin filaments.
5. Cholesterol, phospholipids and fatty acids could regulate the physiological role of TMEM16A in cells.

Figure 1. PI(4,5)P2 regulates TMEM16A activity

A, I_{Cl} records obtained from three different cells using the voltage protocol shown in **a**. Trace in **b** is from a cell expressing mTMEM16A and dialyzed with $0.2 \mu M Ca^{2+}$ ($n = 4$). Trace in **c** is from a cell expressing mTMEM16A and dialyzed with $0.2 \mu M Ca^{2+} + 5 mM ATP + 1 mM Mg^{2+}$ ($n = 7$). Record in **d** was obtained from a cell co-transfected with mTMEM16A + Dr-VSP that was dialyzed with $0.2 \mu M Ca^{2+}$ ($n = 5$). Record in **e** was obtained from a cell with mTMEM16A + Dr-VSP and dialyzed with $0.2 \mu M Ca^{2+} + 5 mM ATP + 1 mM Mg^{2+}$ ($n = 6$). Single exponential fits are shown in red with time constants of $2.47 \pm 0.43 s$ (middle trace) and $1.05 \pm 0.28 s$ (bottom trace). **B**, Recovery of I_{Cl} . Current magnitudes at pulse P2, P3, and P4 were measured from traces shown **Ad** and **Ae** and normalized to the current generated by pulse P1. Data obtained from cells dialyzed with $0.2 \mu M Ca^{2+}$ are shown in blue whereas cells dialyzed with $0.2 \mu M Ca^{2+} + 1 mM Mg^{2+} + 5 mM ATP$ are shown as grey bars. **C**, Representative I_{SCN} showing that activation of muscarinic receptor type 1 inhibited TMEM16A. I_{SCN} were recorded before (black) and after 300 s in the presence of 1 mM carbachol (blue CCh). For these experiments HEK 293 cells were co-transfected with mTMEM16A-3xFlag and human M1 receptor. Cells were dialyzed with 25 mM EGTA and 0 Ca^{2+} and I_{SCN} was recorded at +100 mV every 1.5 s. **D**, Normalized I_{SCN} as a function of time. Black symbols = control ($n=5$); blue symbols = 1 mM CCh ($n=4$).

Arrow indicates the time of CCh application. Red line: fit using a single exponential function (Equation 1) with time constant of 114.2 ± 15.0 s.

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Figure 2. mTMEM16A rundown was either prevented or partially rescued by application of exogenous C8-PIP2 in inside-out patches

A, I_{Cl} rapidly increased and then ran down ($n = 6$) in inside-out patches excised into a zero Ca^{2+} and subsequently exposed to $100 \mu\text{M}$ free Ca^{2+} (grey bar). **B**, Rundown of I_{Cl} is decelerated in patches exposed to $25 \mu\text{M}$ C8-PIP2 and $100 \mu\text{M Ca}^{2+}$ solution ($n = 5$). Red lines in A and B are mono-exponential fits to estimate the time constant of I_{Cl} decay. **C**, C8-PIP2 application in the presence of zero Ca^{2+} for 3 min causes partial recovery of I_{Cl} . Solutions with $100 \mu\text{M Ca}^{2+}$ were applied in 4 occasions (grey bars) intercalated by 3 min exposures to zero Ca^{2+} solutions. Pink bars indicate application of $25 \mu\text{M}$ C8-PIP2. The first Ca^{2+} application quickly activates the channel and induced rundown, no recovery was observed during the second Ca^{2+} exposure. Exposing the patch to 0- Ca^{2+} plus $25 \mu\text{M}$ C8-PIP2 for 3 min, followed by a third Ca^{2+} exposure caused a transient increase in I_{Cl} . The fourth Ca^{2+} exposure along with $25 \mu\text{M}$ C8-PIP2 caused a sustained increase of I_{Cl} after exposing the patch to 0- Ca^{2+} plus $25 \mu\text{M}$ C8-PIP2 for 3 min. **D**, Quantification of TMEM16A recovery from experiments like those in C. I_{Cl} remaining after rundown (I_{rundown}) was quantified after exposing the patches to $100 \mu\text{M Ca}^{2+}$ for ~5 min (shown as a grey asterisk in C). The rescued current (I_{test}) after the patches were exposed for 3 min to 0- Ca^{2+} solution with no additions (0 Ca), or to C8-diacylglycerol pyrophosphate (+DGPP) or

C8-PIP2 (+PI(4,5)P2) is indicated by black and pink asterisks in C. TMEM16A recovery was quantified as $I_{\text{test}} / I_{\text{rundown}}$. All current recordings were carried out at +100 mV.

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Figure 3. Disruption of actin filaments have no effect on TMEM16A regulation by PI(4,5)P2 dephosphorylation whereas removal of intracellular Ca^{2+} partially blunted it

A, representative I_{Cl} ($t = 0$, black, and $t = 300$ s, blue) from HEK 293 cells transfected with mTMEM16A-3xFlag (left) or co-transfected with mTMEM16A-3xFlag and Dr-VSP (right) 1:1. I_{Cl} were activated using the voltage protocol shown in the upper left corner applied every 1.5 s. **B**, Normalized current as a function of time. Black = mTMEM16A ($n = 7$), blue = mTMEM16A + Dr-VSP ($n = 5$), cherry = mTMEM16A + Dr-VSP, $[\text{Ca}^{2+}]_i = 0$, $[\text{SCN}^-]_e = 140$, ($n = 6$), grey = mTMEM16A + Dr-VSP, 20 μM CK-666 during 1 h ($n = 4$), red = mTMEM16A + Dr-VSP, 5 μM latrunculin A during 1 h ($n = 4$). Red line: fit of a single exponential function (Equation 1) with time constant of 94 s.

Figure 4. Cholesterol regulates TMEM16A

A, representative I_{Cl} recorded from the same cell stably transfected with TMEM16A, dialysed with $0.2 \mu\text{M Ca}^{2+}$ and 40 mM Cl^- , bathed in a solution with 140 mM Cl^- (control condition; black) and after 5 min of treatment with $2.3 \text{ mM M-}\beta\text{CD}$ (cherry). I_{Cl} was generated using the voltage protocol shown in the upper left corner. **B**, I_{Cl} - V_m relationships in control condition (black) and after 5 min of treatment with $2.3 \text{ mM M-}\beta\text{CD}$ (cherry) ($n = 6$). I_{Cl} values in the absence and presence of $\text{M-}\beta\text{CD}$ were normalized to control current value obtained at $+120 \text{ mV}$. **C**, Current traces recorded at 32 min in control (black), $2.3 \text{ mM M-}\beta\text{CD}$ (cherry) or $2.3 \text{ mM M-}\beta\text{CD} + \text{chol}$ (1:1) (grey); the panel show an average of 4–5 traces collected from different cells. For clarity we amplified the currents at -60 mV to show that I_{Cl} was also affected at this voltage. **D**, Normalized I_{Cl} as a function of time. Black arrow indicates the time when the extracellular control solution was changed to control solution (black; $n = 5$), $2.3 \text{ mM M-}\beta\text{CD}$ (cherry; $n = 4$) or $2.3 \text{ mM M-}\beta\text{CD} + \text{chol}$ (1:1) (grey; $n = 4$). Asterisks (*) indicate significantly different ($P < 0.01$) from control I_{Cl} .

Figure 5. M-βCD or M-βCD + Chol decelerate regulation of TMEM16A by PI(4,5)P2 dephosphorylation without changing levels of PI(4,5)P2

A, Time courses of normalized I_{CI} at +100 mV. Cells co-expressing mTMEM16A + Dr-VSP were pre-incubated during 30 min with 2.3 mM M-βCD (cherry, $n = 4-8$), 2.3 mM M-βCD + 2.3 mM Chol (light grey, $n = 7$), or without treatment (black, $n = 5$). Single exponential fits with time constants of 83.1 ± 8.5 s (control), 182.2 ± 22.5 s (M-βCD), and 180.9 ± 26.8 s (M-βCD + chol) are shown as red lines. **B**, The amount of total protein is proportional to the band intensity on a quantitative western blot and shows that the levels of PI(4,5)P2 present in the cells do not change after treatments. Amount of total protein loaded in the control gel was 0, 5, 10, 20 and 30 μg (image shown in top row of inset). 10 or 20 μg of

protein isolated from cells treated with M- β CD (cherry, n = 3) or M- β CD + Chol (light grey, n = 3) during 30 min were loaded on the membrane shown on the lower row of inset. Inset: image from two experiments. Dilution of anti-PI(4,5)P2 antibody was 1:1000.

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Figure 6. Modulation of TMEM16A activity by 18-carbon fatty acids

A, Average of five I_{Cl} traces taken from different cells after a 32 min exposure to 10 μ M OA, 10 μ M SA, or 5 μ M Me-S (structures shown on top). Currents were measured at +120 mV and normalized to the current measured before each treatment. **B**, Normalized I_{Cl} as a function of time in presence of 0 (black, $n = 5$), 10 μ M of stearic acid (SA; blue; $n = 5$), 5 μ M of methyl stearate (Me-S; red; $n = 5$), or 10 μ M oleic acid (OA; light orange; $n = 6$). Continuous lines are single exponential fits with Equation 1 to calculate the time constants.

Figure 7. Modulation of TMEM16A activity by PUFAs and by phosphatidylserine

A, Average of five I_{Cl} traces taken from different cells after a 32 min exposure to $10\ \mu\text{M}$ of the indicated poly-unsaturated fatty acid (structures shown in the upper left corner). **B**, normalized I_{Cl} currents as a function of time in the presence of 0 ($n = 5$), $10\ \mu\text{M}$ (magenta; $n = 5$), $50\ \mu\text{M}$ (dark orange; $n = 6$), and $140\ \mu\text{M}$ (blue; $n = 5$) eicosapentaenoic acid (EPA). Continuous lines are single exponential fits used to calculate time constants. **C**, Time constants plotted as a function of [EPA]. The curve was fit with a Hill equation (Equation 2) and the calculated EC_{50} was $5.4\ \mu\text{M}$. **D**, normalized I_{Cl} currents as a function of time in the presence of $10\ \mu\text{M}$ of docosahexaenoic acid (DHA; cherry; $n = 6$), arachidonic acid (AA; $n = 6$).

blue royal; $n = 5$). Continuous lines are single exponential fits to calculate time constants. Black line is the fit of control data shown in panel B. **E**, I_{Cl} traces recorded from three cells after a 32 min exposure to extracellular solution (Ctrl) or 10 or 63 μM phosphatidylserine (PS). I_{Cl} s were measured and normalized as in A. **F**, Normalized I_{Cl} as a function of time in the presence of 0 ($n = 4$), 10 μM PS ($n=8$) and 63 μM PS ($n=5$). Continuous lines are single exponential fits with time constants of 1.56 ± 0.32 min in control condition and 1.29 ± 0.13 min in the presence of PS.

Figure 8. Fatty acids inhibit TMEM16A in a voltage-dependent manner

A, Normalized I_{Cl} recorded at +120 and -100 mV under conditions indicated on the x-axis. I_{Cl} recorded in standard external solution (Ctrl, n = 5), standard external solution plus 0.03 % ethanol (Ctrl-EtOH, n = 9), standard external solution plus 0.57% chloroform and 0.03 % methanol (Ctrl-CHL-MeOH, n = 6), standard external solution plus 10 μ M SA (n = 5), 10 μ M OA (n = 7), 10 μ M AA (n = 5), 10 μ M EPA (n = 5), 10 μ M DHA (n = 6), 5 μ M methylstearate (Me-S; n = 5), or 63 μ M PS (n = 5). Asterisk indicate significantly different at $p < 0.01$. **B**, Inhibition of TMEM16A by 10 μ M oleic acid. Representative I_{Cl} recorded from the same cell before (black) and after 5 min in the presence of oleic acid (cherry). Currents were generated by steps voltages that varied the membrane potential between -100 to +140

mV in 20 mV increments. **C**, I_{Cl} vs V_m relationships ($n = 5$). Black symbols are control condition while cherry symbols correspond to 5 min treatment with 10 μ M OA. The same cell was used as its own control and I_{Cl} values were normalized to I_{Cl} measured at +140 mV before applying OA. **D**, Unblocked current fraction as a function of voltage. In each cell, we measure the remaining current after 5 min exposure to OA and then divided it by the corresponding value prior to treatment. The data was pooled ($n = 7$) and plotted as a function of voltage as filled circles. Open symbols are control obtained in the absence of OA ($n = 5$). Asterisk indicate significant different at $p < 0.01$.

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Figure 9. TMEM16A remains in the membrane regardless of cholesterol content or PUFA application

A, Super-resolution images of 1 μm thick taken from a control HEK 293 cell co-transfected with TMEM16A-GFP (green) and ECFP-Mem (blue) at $t = 0$ and $t = 30$ min. Merged images show that the distribution of TMEM16A is not altered after 30 min in control conditions. **B**, Images taken at $t = 0$ (left lane) and $t = 30$ min (right lane) from control, 2.3 mM M- β CD, 1:1 M- β CD + cholesterol, 10 μM of OA, or 10 μM EPA treated cells (from top to bottom). Cells were co-transfected with TMEM16A-GFP and ECFP-Mem; only EGFP fluorescence is shown. **C**, Normalized pixel intensities correspond to GFP fluorescence

along the rectangles drawn across the cells in B. Distribution of fluorescence is shown at $t = 0$ (olive green) and $t = 30$ min (red). **D**, Relative fluorescence before and after the 30 min treatment (control, 2.3 mM M- β CD, 1:1 M- β CD + cholesterol, 10 μ M of OA, or 10 μ M EPA from top to bottom), no statistical differences were observed in any case. Relative fluorescence was calculated as fluorescence after 30 min divided by fluorescence at $t = 0$. Fluorescence intensity was measured at 4 different spots on the membrane of the same cell. The spots chosen for control conditions are indicated by the little white rectangles of 6×20 pixels in the upper panel in B. Scale bar = 5 μ m in all cases.

Table 1

Time constants of activation (τ_{on}) and deactivation (τ_{off}) of TMEM16A currents at 32 min without (CONTROL) or after addition of the compounds listed. CONTROL, CONTROL-EtOH (ethanol added), and CONTROL-CHL-MeOH (chloroform and methanol added) are conditions used to dissolve M- β CD, fatty acids and PS, respectively. The time constants were calculated from currents recorded at +120 mV and at -100 mV using Equation 1.

Treatment	τ_{on} (ms)	τ_{off} (ms)	n
CONTROL	237.1 $\pm$ 9.4	36.4 $\pm$ 3.7	5
2.3 mM M- β CD	289.5 $\pm$ 4.9	51.8 $\pm$ 4.9	4
1:1 M- β CD + Chol	209.2 $\pm$ 3.4	63.0 $\pm$ 9.4	4
CONTROL- EtOH	397.0 $\pm$ 42.3	49.37 $\pm$ 4.94	9
10 μ M SA	353.0 $\pm$ 20.16	43.9 $\pm$ 2.9	5
10 μ M OA	449.5 $\pm$ 63.9	34.7 $\pm$ 3.7	6
5 μ M Me-S	374.7 $\pm$ 35.5	39.2 $\pm$ 1.3	5
10 μ M AA	477.2 $\pm$ 70.2	33.9 $\pm$ 1.8	5
10 μ M EPA	413.9 $\pm$ 116.2	43.8 $\pm$ 3.0	5
10 μ M DHA	313.0 $\pm$ 14.7	39.5 $\pm$ 8.1	6
CONTROL-CHL-MeOH	303.8 $\pm$ 52.0	39.5 $\pm$ 4.8	6
10 μ M PS	295.8 $\pm$ 7.9	33.5 $\pm$ 1.3	8
63 μ M PS	274.5 $\pm$ 24.3	31.6 $\pm$ 3.4	5