TMEM16A channels: molecular physiology and pharmacological regulation

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy (PhD) in the Faculty of Life Science

2012

Aiste Tamuleviciute
List of contents

List of tables......................................................................................................................... 4
List of abbreviations .............................................................................................................. 5
Abstract................................................................................................................................. 7
Declaration............................................................................................................................. 8
Copyright statement............................................................................................................. 8
Author contribution............................................................................................................. 9
Acknowledgements........................................................................................................... 10
Chapter 1. Introduction ........................................................................................................ 11
  1.1. Ion channels ............................................................................................................... 12
  1.2. Calcium-activated chloride channels ........................................................................ 12
  1.3. Regulation of cytosolic Ca\textsuperscript{2+} levels .......................................................... 14
  1.4. Intracellular Ca\textsuperscript{2+} stores ............................................................................. 14
  1.5. Membrane potential and regulation of vascular tone ................................................ 15
  1.6. Active Cl\textsuperscript{-} transport in smooth muscle cells ................................................ 17
  1.7. CaCCs in vascular smooth muscle ............................................................................ 17
  1.8. Molecular candidates for CaCCs ............................................................................... 18
  1.9. Identification of TMEM16 family of CaCCs ............................................................... 19
  1.10. Pharmacology of TMEM16 channels ...................................................................... 20
  1.11. Structure of TMEM16 channels .............................................................................. 22
  1.12. Other TMEM16 family members ............................................................................ 24
  1.13. Aims of the thesis ................................................................................................... 26
Chapter 2. Methods ............................................................................................................. 28
  2.1. RT-PCR ..................................................................................................................... 29
  2.2. Quantitative PCR ..................................................................................................... 29
  2.3. Small interfering RNA ............................................................................................. 30
  2.4. Generation of chimeric DNA constructs .................................................................. 30
  2.5. Generation HA-tagged DNA constructs ................................................................... 31
  2.6. Heterologous expression .......................................................................................... 31
  2.7. Immunocytochemistry .............................................................................................. 32
  2.8. Configurations of the patch-clamp technique ........................................................... 33
  2.9. Electrical properties of the cell membrane ............................................................ 34
Chapter 3. Publication I ....................................................................................................... 37
Chapter 4. Publication II .................................................................................................... 38
Chapter 5. Publication III ................................................................................................... 39
List of tables

Chapter 1
Table 1.1. Intracellular chloride concentration and resting membrane potential in vascular smooth muscle........................................................................................................... 17

Chapter 3
Supplementary Table 1. siRNAs sequences........................................................................ 3
Supplementary Table 2. Oligonucleotide primers used in PCR reactions for detection of rTMEM16A (NM_001107564.1) splicing variants......................................................... 3
Supplementary Table 3. Oligonucleotide primers used in PCR reactions for detection of various TMEM16 family members................................................................. 4

Chapter 4
Table 1. Parameters ($V_{0.5}$ and $z$) obtained from the Boltzmann fit of TMEM16A, TMEM16B, TMEM16A-B and TMEM16B-A conductance versus voltage relationships at various $[Ca^{2+}]_i$........................................................................................................... 17
Table 2. Parameters ($EC_{50}$ and $h$) obtained from the Hill-Langmuir fit of TMEM16A, TMEM16B, TMEM16A-B and TMEM16B-A $[Ca^{2+}]_i$-response relationships............. 17
Supplementary Table 1. Location of the putative pore-loop of various TMEM16 proteins used in this study........................................................................................................... 4

Chapter 5
Table 1. Parameters ($K_i$ and $\gamma$) obtained from Hill fit of $[A9C]_{ext}$ - TMEM16A inhibition relationships at various membrane potentials......................................................... 10
Table 2. Parameters ($V_{0.5}$ and $z$) obtained from the Boltzmann fit of TMEM16A, conductance versus voltage relationships at various $[Ca^{2+}]_i$ and $[A9C]_{ext}$................. 13
Table 3. Parameters ($EC_{50}$ and $h$) obtained from the Hill fit of TMEM16A $[Ca^{2+}]_i$-response relationships in the presence of various $[A9C]_{ext}$ at various potentials........ 14
## List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>[A9C]_{ext}</td>
<td>extracellular A9C concentration</td>
</tr>
<tr>
<td>[Ca^{2+}]_i</td>
<td>intracellular Ca^{2+} concentration</td>
</tr>
<tr>
<td>[Cl^-]_{ext}</td>
<td>extracellular Cl^- concentration</td>
</tr>
<tr>
<td>[Cl^-]_i</td>
<td>intracellular Cl^- concentration</td>
</tr>
<tr>
<td>A9C</td>
<td>anthracene-9-carboxylic acid</td>
</tr>
<tr>
<td>Best-3</td>
<td>bestrophin-3</td>
</tr>
<tr>
<td>CaCCs</td>
<td>Ca^{2+}-activated Cl^- channels</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>CLCA</td>
<td>chloride channel, Ca^{2+}-activated</td>
</tr>
<tr>
<td>DIDS</td>
<td>4,4-diisothiocyanatostilbene-2,2-disulphonic acid</td>
</tr>
<tr>
<td>DM</td>
<td>dissociation medium</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOCA</td>
<td>deoxycorticosterone acetate</td>
</tr>
<tr>
<td>DPC</td>
<td>diphenylamine-2-carboxyl acid</td>
</tr>
<tr>
<td>EC_{50}</td>
<td>half maximal effective Ca^{2+} concentration</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>E_m</td>
<td>membrane potential</td>
</tr>
<tr>
<td>E_X</td>
<td>reversal potential for X ion</td>
</tr>
<tr>
<td>FRET</td>
<td>fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>FRT</td>
<td>Fischer rat thyroid</td>
</tr>
<tr>
<td>G</td>
<td>ionic conductance</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GABA_A receptor</td>
<td>ligand-gated chloride channel</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>h</td>
<td>slope factor (Hill coefficient)</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>HEDTA</td>
<td>2-Hydroxyethylethylenediaminetriacetic acid</td>
</tr>
<tr>
<td>HEK-293T</td>
<td>Human Embryonic Kidney 293T</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>i</td>
<td>single channel current</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>$I_{CaCC}$</td>
<td>Ca$^{2+}$-activated Cl$^-$ current</td>
</tr>
<tr>
<td>ICC</td>
<td>immunocytochemistry</td>
</tr>
<tr>
<td>$I_{max}$</td>
<td>maximal ionic current</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>$I_t$</td>
<td>tail current amplitude</td>
</tr>
<tr>
<td>$K_d$</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>$K_i$</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>$K_{i(0)}$</td>
<td>inhibition constant at 0 mV</td>
</tr>
<tr>
<td>$N$</td>
<td>number of functional channels on the plasma membrane</td>
</tr>
<tr>
<td>NAADP</td>
<td>nicotinic acid adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NMERA receptor</td>
<td>glutamate receptor, ligand-gated non-selective cation channel</td>
</tr>
<tr>
<td>NMERA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NPPB</td>
<td>5-nitro-2-(3-phenylpropylamino)-benzoic acid</td>
</tr>
<tr>
<td>P</td>
<td>ionic selectivity</td>
</tr>
<tr>
<td>PA</td>
<td>pulmonary artery</td>
</tr>
<tr>
<td>PASMC</td>
<td>pulmonary artery smooth muscle cell</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>$P_i$</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>pK$_a$</td>
<td>logarithmic constant of acid dissociation constant</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>$P_o$</td>
<td>channel open probability</td>
</tr>
<tr>
<td>qPCR</td>
<td>real-time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SITS</td>
<td>4-acetamido-4-isothiocyanatostilbene-2,2-disulfonic acid</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>STIC</td>
<td>spontaneous transient inward Cl$^-$ current</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane domain</td>
</tr>
<tr>
<td>TMEM16</td>
<td>transmembrane 16</td>
</tr>
<tr>
<td>$V_{0.5}$</td>
<td>voltage of half-maximal $G$</td>
</tr>
<tr>
<td>VSM</td>
<td>vascular smooth muscle</td>
</tr>
<tr>
<td>$z$</td>
<td>electric charge</td>
</tr>
<tr>
<td>$\delta$</td>
<td>fraction of voltage electric field</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>slope factor (Hill coefficient)</td>
</tr>
<tr>
<td>$\tau_{0.5}$</td>
<td>time of half-maximal ionic current activation/deactivation</td>
</tr>
</tbody>
</table>
Abstract

Calcium-activated chloride channels (CaCCs) are a class of the ligand-gated channels involved in numerous cellular functions. In vascular smooth muscle, these ion channels couple agonist-induced Ca^{2+}-release from the sarcoplasmic reticulum to membrane depolarisation and vasoconstriction. For this reason, CaCCs have been suggested as a potential molecular target to treat a range of vascular disorders. These ion channels, however, have not been yet explored as a drug target because their molecular identity has been elusive and their pharmacology has been restricted to compounds with low potency and poor specificity. The general aims of this work of thesis are: i) to define the molecular identity of CaCCs in vascular smooth muscle, ii) to investigate how the structural features of the identified channel relate to its functional properties and iii) to examine how drug binding modulates CaCC activity. The main findings are the following:

1) By using RNA interference technology and patch-clamp analysis, the *Tmem16A* gene was found to encode for CaCCs in pulmonary artery smooth muscle. Furthermore, *Tmem16A* appeared to be expressed in other vascular smooth muscles suggesting that this ion channel may represent CaCCs in various vascular beds.

2) To understand the physiology and pharmacology of TMEM16A channels it is of a fundamental importance to elucidate the molecular mechanisms by which channel gating and conductance are achieved. TMEM16A comprises eight putative transmembrane domains (TMs) with TM5 and TM6 flanking a putative re-entrant loop, which resembles the pore of other ion channels. Using a chimeric approach the role of this region was investigated. The re-entrant loop of TMEM16A was found to mediate a range of functional roles: it controlled the response of the channel to intracellular Ca^{2+}, the permeation of anions and the expression of channels on the plasma membrane. Specifically, a non-canonical trafficking motif was identified within in a 38 amino acid region within the re-entrant loop.

3) Drugs that modulate the function of TMEM16A channels are currently limited. The generic chloride channel blocker anthracene-9-carboxylic acid (A9C) was found to produce a bimodal effect on TMEM16A currents: low concentrations of A9C activated the channels, while doses higher than ~300 µM produced current inhibition. These two effects were mediated via A9C binding to two separate sites. Binding of A9C into the pore resulted in channel inhibition, while A9C binding to an extracellular site increased the open probability of the channel.

To conclude, this work of thesis has revealed the molecular identity of CaCCs in vascular smooth muscle and elucidated the functional roles of the re-entrant loop of the TMEM16A channel protein. The identification of the activating and inhibiting A9C binding sites may help the development of selective blockers and activators of TMEM16A channels.
Declaration

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

Copyright statement

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the “Copyright”) and s/he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made only in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trade marks and other intellectual property (the “Intellectual Property”) and any reproductions of copyright works in the thesis, for example graphs and tables (“Reproductions”), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IP Policy (see http://www.campus.manchester.ac.uk/medialibrary/policies/intellectualproperty.pdf), in any relevant Thesis restriction declarations deposited in the University Library, The University Library’s regulations (see http://www.manchester.ac.uk/library/aboutus/regulations) and in The University’s policy on presentation of Theses.


Author contribution

This work of thesis is written in an alternative format, which includes three publications arising from my PhD project. The alternative format allows including experiments performed by other members of the research team, which highly enhances the quality and scientific scope of the thesis. The list below specifies my contribution to the following publications:

Publication I
In this collaborative study, I performed an enzymatic isolation and culture of vascular smooth muscle cells, RT-PCR for TMEM16 family members and TMEM16A alternative exons in vascular smooth muscle, RNA interference experiments and a part of a quantitative PCR to test the expression levels of TMEM16 family members. I also did a proofreading of the paper manuscript.

Publication II
I designed molecular biology and immunocytochemistry experiments, performed all the experimental work and analyzed the data. I wrote the paper manuscript together with Dr Paolo Tammaro.

Publication III
I designed a part of electrophysiological patch-clamp experiments, performed all the experimental work, analyzed the data and wrote the manuscript.
Acknowledgements

This PhD project would not have been possible without the invaluable guidance and continuous support from my supervisor Dr Paolo Tammaro. I am grateful for his patience in teaching me how to master the patch-clamping and to present my findings to the scientific society. His daily supervision providing help, constructive criticism and encouragement made my PhD project challenging and enjoyable.

I would also like to thank my advisor Dr Peter Brown and the second supervisor Professor Alison Gurney for their feedback and advice on the direction of my research project. For the friendly and supportive working environment I am grateful to all Dr Tammaro and Professor Gurney’s lab group members. Special thanks go to Iris Egner and Tomas Adomavicius for all the scientific discussions we had and for going together along the PhD progression pathway.

This PhD project has been funded by BBSRC and the Faculty of Life Sciences, University of Manchester.
Chapter 1

Introduction
1.1. Ion channels

Ion channels are membrane-spanning proteins that form pores to permit fluxes of ions across the otherwise impermeable membrane lipid bilayer. Fluxes of ions through ion channels are driven by the electrochemical gradient established across the membrane. Therefore, ion channels represent a mechanism of “passive” ion transport as opposed to “active” transport mediated by transporter proteins, which require energy consumption to transfer ions against their electrochemical gradient. Ion channels are involved in cellular signaling and maintenance of ionic homeostasis. Due to their vital roles ion channels are found in virtually every prokaryotic and eukaryotic cell. Ion channels can be classified according to their selectivity for ions (e.g. Na\(^+\), K\(^+\), Ca\(^{2+}\) and Cl\(^-\) channels), subcellular localization (plasma membrane versus intracellular membranes) and the type of stimuli controlling channel opening and closing (a process termed gating). Channel gating can be energetically coupled to changes in the membrane potentials (voltage-gated channels), binding of a ligand (ligand-gated channels), mechanical tension (stretch-activated channels) or a combination of factors. My thesis centers on Ca\(^{2+}\)-activated Cl\(^-\) channels (CaCCs), which are regulated by both cytosolic Ca\(^{2+}\) and the membrane potential.

1.2. Calcium-activated chloride channels

CaCCs, similarly to other chloride channels, are not perfectly selective for Cl\(^-\) and can conduct different anions, such as I\(^-\) and NO\(_3^-\). Nevertheless, they are termed chloride channels because Cl\(^-\) is the most abundant anion in biological fluids and thereby Cl\(^-\) fluxes are predominant through these anion channels in physiological conditions (Jentsch et al., 2002; Nilius & Droogmans, 2003). Plasma membrane Cl\(^-\) channels perform many diverse cellular functions, including cell volume regulation, maintenance of ionic homeostasis, transepithelial transport and regulation of cell excitability (Jentsch et al., 2002; Nilius & Droogmans, 2003). This range of roles is in part due to the different values of the reversal potential for Cl\(^-\) (\(E_{Cl}\)) in various cell types. For example, \(E_{Cl}\) is highly negative in skeletal muscle (~−85 mV) and hippocampal neurons (~−65 mV). Thus, an activation of Cl\(^-\) channels in these cells is an important repolarizing force during action potentials (Fahlke & Rudel, 1995; Huang et al., 2012). In contrast, \(E_{Cl}\) is far more positive in other cell types, such as dorsal root ganglion neurons (~−25 mV) and smooth muscle cells (~−35 mV). Therefore, in these cells opening of Cl\(^-\) channels leads to Cl\(^-\) efflux and membrane depolarization (Gerstheimer et al., 1987; Large & Wang, 1996; Cho et al., 2012).
CaCCs were first observed in *Xenopus* oocytes, where they cause membrane depolarization after spermatozoid penetration through the cell membrane, which prevents any additional sperm entry (Cross & Elinson, 1980; Barish, 1983). CaCCs subsequently have been found in vertebrates rod photoreceptors (Bader *et al.*, 1982; MacLeish & Nurse, 2007) and olfactory receptor neurons (Kleene & Gesteland, 1991; Kurahashi & Yau, 1994), where they are involved in transduction of photo or olfactory stimuli, respectively. CaCCs expressed in the epithelial cells are responsible for transepithelial fluid secretion (Galietta, 2009; Hartzell *et al.*, 2009). Moreover, CaCCs have been shown to contribute to the electrical activity of neurons (Andre *et al.*, 2003) and cardiac myocytes (Guo *et al.*, 2008) and the regulation of vascular tone in vascular smooth muscle (VSM) (Large & Wang, 1996; Hartzell *et al.*, 2005; Leblanc *et al.*, 2005) (Figure 1.1). This work of thesis focuses on CaCCs in VSM. Therefore, the next five sections will centre on Ca$^{2+}$ homeostasis and regulation of membrane potential in this tissue.

**Figure 1.1.** Physiological roles of CaCCs in various cell types. In epithelial cells, activation of CaCCs by intracellular Ca$^{2+}$ causes Cl$^{-}$ efflux, followed by transepithelial transport of Na$^{+}$ and water. In smooth muscle cells, CaCCs play a role in signal amplification mechanism: CaCCs activated by increase in intracellular Ca$^{2+}$ levels allow Cl$^{-}$ efflux, membrane depolarization and opening of voltage-gated Ca$^{2+}$ channels with the subsequent Ca$^{2+}$ influx leading to the vasoconstriction. Another amplification mechanism takes place in olfactory receptors, where CaCCs are activated by Ca$^{2+}$ influx through cAMP-gated channels. CaCCs also play a role in phototransduction and excitability of neurons.
1.3. Regulation of cytosolic Ca\(^{2+}\) levels

An increase or decrease in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) is the main mechanism causing constriction or relaxation of VSM. At rest [Ca\(^{2+}\)]\(_i\) ranges from 50 to 100 nM, which is around 20,000 times lower concentration compared to [Ca\(^{2+}\)] in the extracellular space (1-2 mM) (Hille, 2001). This difference accounts for the strong driving force leading to Ca\(^{2+}\) influx upon opening of Ca\(^{2+}\) channels in the plasma membrane or in the sarcoplasmic reticulum (SR), which is the main intracellular Ca\(^{2+}\) store with [Ca\(^{2+}\)] approaching values observed for the extracellular space (Hille, 2001). Elevated [Ca\(^{2+}\)]\(_i\) leads to phosphorylation of motor protein myosin, which utilizes the energy obtained from ATP hydrolysis for sliding over the actin filaments ultimately causing contraction (Webb, 2003). To initiate the vasorelaxation, [Ca\(^{2+}\)]\(_i\) needs to be reduced to the resting levels. Ca\(^{2+}\) can be pumped back into the SR by sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\)-ATPase and back to the extracellular space by plasmalemmal Ca\(^{2+}\)-ATPase and Na\(^+\)/Ca\(^{2+}\) exchanger (Webb, 2003; Bruce, 2010). Ca\(^{2+}\)-ATPases use the energy obtained from ATP hydrolysis to transfer Ca\(^{2+}\) ions against their electrochemical gradient. In contrast, Na\(^+\)/Ca\(^{2+}\) exchanger uses the energy stored in the Na\(^+\) electrochemical gradient, which allows the influx of three Na\(^+\) ions down the electrochemical gradient in exchange for the extrusion of one Ca\(^{2+}\) ion (Iwamoto, 2006).

1.4. Intracellular Ca\(^{2+}\) stores

The SR has its own and unique system of Ca\(^{2+}\) homeostasis. Free [Ca\(^{2+}\)] within the SR ranges between 200-800 µM (Case et al., 2007). Ca\(^{2+}\) release from the SR is mediated by several types of channels: inositol 1,4,5-triphosphate (IP\(_3\))-gated IP\(_3\) receptors, Ca\(^{2+}\)-gated ryanodine receptors, and nicotinic acid adenine dinucleotide phosphate (NAADP)-gated receptors (Case et al., 2007). IP\(_3\) receptors are tetrameric channels that are directly activated by the secondary messenger IP\(_3\) (Bezprozvanny, 2005). IP\(_3\) is generated in response to agonist binding to plasmalemmal receptors coupled to phospholipase C (PLC). PLC hydrolyses the membrane lipid phosphatidylinositol-4,5 bisphosphate to IP\(_3\) and diacylglycerol producing two secondary messengers. IP\(_3\) is a soluble molecule that diffuses to the SR and binds to the IP\(_3\) receptor. IP\(_3\)-triggered channel opening causes a rapid increase in [Ca\(^{2+}\)]\(_i\) (Bezprozvanny, 2005). Degradation of IP\(_3\) is mediated mainly by IP\(_3\) phosphorylation by IP\(_3\) 3-kinase and dephosphorylation by inositol polyphosphate 5-phosphatase (Coburn & Baron, 1990). Ryanodine receptors mediate Ca\(^{2+}\) release from intracellular stores when activated by cytosolic Ca\(^{2+}\). Ca\(^{2+}\)-induced Ca\(^{2+}\) release is the
main mechanism of cardiac muscle contraction (Fabia to, 1983) and plays an important role in cellular signaling in many other cell types, including insulin secreting pancreatic beta cells (Islam et al., 1992). In vascular smooth muscle, ryanodine receptors have been suggested to cause local Ca$^{2+}$ sparks triggering opening of Ca$^{2+}$-sensitive channels in the plasma membrane (Kotlikoff et al., 2002). Recently, a new type of Ca$^{2+}$ release channels has been identified. These are two-pore channels localized in the lysosomes rather than SR and are activated by intracellular messenger NAADP (Galion & Ruas, 2005). The [Ca$^{2+}$] is as high as ~600 µM within lysosomes (Shaughnessy et al., 2006), where NAADP-induced Ca$^{2+}$ release represents a novel mechanism for [Ca$^{2+}$], regulation.

1.5. Membrane potential and regulation of vascular tone

Each cell spends metabolic energy to transport ions, accumulating some of them into the cytoplasm and extruding others into the extracellular space. As a result, the concentrations of various ions inside and outside the cell differ. The resulting separation of charge produces a voltage difference or membrane potential. Each ion tends to diffuse across the cell membrane down the concentration gradient until the electrochemical equilibrium ($E$) is reached. At equilibrium, electrical and chemical forces acting on a particular ion are opposite and equal. The $E$ value for an ion can be calculated using Nernst equation:

$$E = \frac{RT}{zF} \ln \frac{C_{\text{out}}}{C_{\text{in}}},$$

where $R$ is the universal gas constant; $T$ the absolute temperature; $[C]_{\text{out}}$ and $[C]_{\text{in}}$ are the concentration of the ion outside and inside the cell, respectively; $z$ is the valence of the ion and $F$ is the Faraday constant (thus $zF$ is the number of electric charges carried by a mole of ions).

The value of the resting membrane potential can be estimated using Goldman equation, which supplements the Nernst equation by taking into consideration the membrane permeability for the various ions:

$$E_{m} = \frac{RT}{F} \ln \left( \frac{\sum P_{i} [K^{+}]_{\text{out}} + P_{Na} [Na^{+}]_{\text{out}} + P_{Cl} [Cl^{-}]_{\text{out}}}{\sum P_{i} [K^{+}]_{\text{in}} + P_{Na} [Na^{+}]_{\text{in}} + P_{Cl} [Cl^{-}]_{\text{in}}} \right)$$

where $E_{m}$ is membrane potential and $P$ is permeability of the noted ion.
For the majority of animal cells the plasma membrane at rest is mainly permeable for K⁺, which drives the membrane potential towards the electrochemical equilibrium for K⁺ ($E_K$) (Hille, 2001; Costanzo, 2006). The resting membrane potential in VSM, however, is more positive than $E_K$ (predicted by Nernst equation to be ~-80 mV) (Table 1.1). This discrepancy suggests that other ionic currents contribute to the resting membrane potential. Further evidence for other than K⁺ conductance at rest is the fact that inhibition of K⁺ channels causes membrane depolarization. Closure of K⁺ channels by itself cannot cause membrane depolarization and there must be another ionic current/s with a more positive reversal potential/s that would produce a shift in membrane potential when K⁺ channels close. Cl⁻ currents with $E_{Cl}$ ranging from -30 and -40 mV could contribute to the resting membrane potential and account for membrane depolarization upon closure of K⁺ channels. In VSM, the permeability for Cl⁻ at rest is relatively small, about 5-10 times smaller than for K⁺. For example, in guinea-pig ileal arteriole and rat arterial smooth muscle $P_{Cl}/P_K$ ratio is 0.09 and 0.19, respectively (Hirst & van Helden, 1982; Brown et al., 1999). This small permeability, however, might affect the resting membrane potential. A replacement of extracellular Cl⁻ with more permeable anion SCN⁻ results in a significant current increase in rabbit pulmonary artery smooth muscle cells (PASMCs), supporting the idea of Cl⁻ conductance involvement in setting the resting membrane potential in VSM (Hogg et al., 1993).

The vascular tone is highly dependent on the membrane potential of smooth muscle cells. A change of only a few milivolts in membrane potential causes a significant change in the diameter of blood vessel (Nelson et al., 1990; Brayden & Nelson, 1992). This steep dependency most likely results from a high voltage-sensitivity of voltage-dependent Ca²⁺ channels in the plasma membrane. For example, a 3 mV increase in the membrane potential can produce a two-fold increase in Ca²⁺ currents ultimately leading to vasoconstriction (Nelson et al., 1990).
### Table 1.1. Intracellular chloride concentration and resting membrane potential in vascular smooth muscle

<table>
<thead>
<tr>
<th></th>
<th>$[Cl^-]$, mM</th>
<th>$E_m$, mV</th>
<th>$E_{Cl}$, mV</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit aorta</td>
<td>33</td>
<td>-60</td>
<td>-38</td>
<td>(Gerstheimer et al., 1987)</td>
</tr>
<tr>
<td>Rabbit pulmonary artery</td>
<td>N/A</td>
<td>-55</td>
<td>N/A</td>
<td>(Clapp &amp; Gurney, 1992)</td>
</tr>
<tr>
<td>Rat femoral artery</td>
<td>44</td>
<td>-62</td>
<td>-33</td>
<td>(Davis, 1992)</td>
</tr>
<tr>
<td>Human umbilical artery</td>
<td>38</td>
<td>-57</td>
<td>-36</td>
<td>(Chien et al., 2000)</td>
</tr>
<tr>
<td>Human placental artery</td>
<td>34</td>
<td>-55</td>
<td>-36</td>
<td>(Chien et al., 2000)</td>
</tr>
</tbody>
</table>

$[Cl^-]$, intracellular Cl$^-$ concentration; $E_m$, resting membrane potential; $E_{Cl}$, Cl$^-$ reversal potential; N/A, data not available.

#### 1.6. Active Cl$^-$ transport in smooth muscle cells

In smooth muscle cells, approximately 40% of the total energy used for secondary active transport is spent for Cl$^-$ accumulation (Chipperfield & Harper, 2000). As a result, these cells contain relatively high intracellular Cl$^-$ concentration ($[Cl^-]$). For instance, in some central nervous system neurons, $[Cl^-]$ is ~1 – 5 mM, whereas $[Cl^-]$ in smooth muscle is in the range of 30 – 50 mM (Table 1.1). This high $[Cl^-]$ is achieved by three active transport systems: Cl$^-$/HCO$_3^-$ exchanger, Na$^+$-K$^+$-Cl$^-$ co-transporter and the third, more elusive system, called “pump III” (O'Donnell & Owen, 1988; Chipperfield et al., 1993; Chien et al., 2000). Cl$^-$/HCO$_3^-$ exchanger transfers intracellular HCO$_3^-$ to the extracellular space down its electrochemical gradient and in exchange uptakes Cl$^-$. Na$^+$-K$^+$-Cl$^-$ co-transporter uptakes Na$^+$, K$^+$ and 2 Cl$^-$ ions by using energy provided by the Na$^+$ electrochemical gradient. Inhibition of Na$^+$-K$^+$-Cl$^-$ co-transporter results in a decrease of $[Cl^-]$, which in turn causes VSM hyperpolarisation and relaxation (Kreye et al., 1981), suggesting that Cl$^-$ uptake is linked to the regulation of vascular tone. The third Cl$^-$ accumulation system called “pump III” has been reported to work in parallel with the Na$^+$-K$^+$-Cl$^-$ co-transporter and contribute to the rise in $[Cl^-]$. Although “pump III” has been found in rat and human smooth muscles, its precise role and tissue distribution is still very poorly understood (Chipperfield et al., 1993; Harper et al., 1997; Chipperfield & Harper, 2000).

#### 1.7. CaCCs in vascular smooth muscle

The major Cl$^-$ current in VSM is the Ca$^{2+}$-activated Cl$^-$ current (I$_{CaCC}$). The first observation of a Cl$^-$ current activated by agent that induced Ca$^{2+}$-release from SR was made in 1987 (Byrne & Large, 1987). It was concluded that these Cl$^-$ currents were directly activated by Ca$^{2+}$, similarly to I$_{CaCCs}$ observed in many other tissues. To date, it
is well established that CaCCs perform a significant role in the regulation of VSM tone. These channels provide a link between agonist stimulation and activation of voltage-gated Ca\(^{2+}\) channels. CaCCs, activated by Ca\(^{2+}\) released from the SR, enables Cl\(^-\) efflux, which causes membrane depolarization. This change in membrane potential leads to the activation of voltage-gated Ca\(^{2+}\) channels (mainly L-type) and Ca\(^{2+}\) influx. The resulting increase in [Ca\(^{2+}\)]\(_i\) ultimately causes vasoconstriction (Large & Wang, 1996; Pollock et al., 1998; Kitamura & Yamazaki, 2001).

I\(_{\text{CaCC}}\)s in VSM have distinctive electrophysiological features: 1) they are activated by elevation in [Ca\(^{2+}\)]\(_i\); 2) at [Ca\(^{2+}\)]\(_i\) lower that 1 µM I\(_{\text{CaCC}}\)s are outwardly-rectifying; 3) at high [Ca\(^{2+}\)]\(_i\) (>1 µM) CaCCs become active at all membrane potentials and the current-voltage relationship becomes linear; 4) CaCCs display slow kinetics of activation (200-500 ms) and deactivation (~100 ms) and their single-channel conductance is in the low picosiemens range (Large & Wang, 1996; Chipperfield & Harper, 2000; Kuruma & Hartzell, 2000; Hartzell et al., 2005; Leblanc et al., 2005).

In addition to classical I\(_{\text{CaCC}}\), which is purely dependent on the intracellular Ca\(^{2+}\), cGMP-dependent I\(_{\text{CaCC}}\) has also been reported in VSM (Piper & Large, 2004a, b; Matchkov et al., 2005). The cGMP-dependent CaCCs require intracellular Ca\(^{2+}\) as well as cGMP for their activation (Piper & Large, 2004b; Matchkov et al., 2005). Also, they exhibit an almost instantaneous response to depolarizing voltages rather than a slow rise in current amplitude observed for classical I\(_{\text{CaCC}}\)s. Classical and cGMP-dependent CaCCs co-exist in the majority of vascular beds. A notable exception is the pulmonary artery smooth muscle, which have been reported to exhibit only the purely Ca\(^{2+}\)-dependent I\(_{\text{CaCC}}\) (Matchkov et al., 2008).

**1.8. Molecular candidates for CaCCs**

Despite the fact that CaCCs have been studied in various cell types for over 30 years, their molecular identity has been elusive (Nilius & Droogmans, 2003; Hartzell et al., 2005). The difficulty in elucidating the molecular candidate for this channel was due to a number of reasons. First, selective pharmacological tools for anion channels are very limited compared to those for cation channels, making it difficult to isolate the currents electrophysiologically. Second, heterologous expression of Cl\(^-\) channel candidates often resulted in upregulation of endogenous anion currents, producing false-positives (Hartzell et al., 2009). Third, one of the most commonly used expression systems,
Xenopus oocytes, was unsuitable for expression cloning due to the fact that endogenous I_{Ca}CCs are abundant in these cells. Finally, homology cloning was not possible, since CaCCs had no sequence similarity with any other ion channel gene. In spite of all these difficulties, over the years a number of candidates for CaCCs have been proposed. CLCA (Cunningham et al., 1995), CIC-3 (Huang et al., 2001), bestrophins (Qu et al., 2003) and Tweety (Suzuki & Mizuno, 2004) have been suggested as candidates for CaCCs. However, these members are absent in some tissues that generate classical I_{Ca}CCs (Papassotiriou et al., 2001; Hartzell et al., 2009). Also, heterologous expression studies have shown that some biophysical properties of these candidates, including the degree of Ca^{2+}-sensitivity and unitary conductance, are highly different compared to those of native CaCCs (Britton et al., 2002; Hartzell et al., 2009). Furthermore, it has recently been suggested that CLCA may in fact be adhesion molecules, which contribute to the activity of chloride channels indirectly rather than forming the ion channels themselves (Loewen & Forsyth, 2005). In contrast to classical CaCCs, the bestrophin family members do not respond to changes in the membrane potential and can be activated by factors other than intracellular Ca^{2+}, such as cGMP and the osmotic cell swelling (Fischmeister & Hartzell, 2005; Matchkov et al., 2005). Also, heterologously expressed bestrophins are not activated by agonist-induced Ca^{2+}-release from the endoplasmic reticulum, which is a distinctive property of the classical CaCCs (Hartzell et al., 2009).

1.9. Identification of TMEM16 family of CaCCs

In 2008, a novel candidate for CaCCs was discovered. Three research groups independently identified TMEM16A (also called Anoctamin 1) as a strong candidate for CaCCs (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008). Heterologous expression of TMEM16A in mammalian cell lines or amphibian oocytes resulted in an I_{Ca}CC with biophysical and pharmacological properties that closely resembled those of native classical I_{Ca}CCs. TMEM16A channels revealed slow gating kinetics and were modulated by changes in the intracellular Ca^{2+} and membrane potential. TMEM16A currents were inhibited by CaCC blockers, such as niflumic acid and 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008). Yang et al. (2008) reported that TMEM16A currents exhibited Ca^{2+}-sensitivity and voltage-dependence similar to those of native CaCCs. The half maximal effective Ca^{2+} concentration (EC_{50}) was 2.4 μM at −60 mV, whereas at the depolarized membrane potential (+60 mV) TMEM16A was more sensitive to Ca^{2+} with a EC_{50} value of 0.4 μM (Yang et al., 2008). Co-expression of TMEM16A and G-protein coupled
Chapter 1

receptor (GPCR), such as endothelin receptor subtype A and angiotensin II receptor subtype 1, generated $I_{\text{CaCC}}$ in response to endothelin and angiotensin II, respectively (Yang et al., 2008). TMEM16A knockout mice had abolished CaCC currents in various cell types providing further evidence that TMEM16A form native CaCCs (Ousingsawat et al., 2009; Romanenko et al., 2010). TMEM16A has been shown to be involved in vasoconstriction (Manoury et al., 2010), transepithelial fluid secretion in airways (Ousingsawat et al., 2009; Rock et al., 2009), gastrointestinal motility (Hwang et al., 2009), and fluid excretion in salivary glands (Romanenko et al., 2010).

Immediately after identification of TMEM16A, another protein from the same family, TMEM16B, was also identified as CaCCs (Pifferi et al., 2009). TMEM16B accounted for $I_{\text{CaCC}}$ in olfactory sensory neurons (Stephan et al., 2009; Billig et al., 2011), photoreceptor terminals (Stohr et al., 2009) and hippocampal neurons (Huang et al., 2012). Despite high sequence similarity (~60%), TMEM16B currents exhibited different properties than TMEM16A. First, TMEM16B was reported to have 10-fold smaller single-channel conductance than that of TMEM16A (0.8 pS versus 8.3 pS) (Yang et al., 2008; Stephan et al., 2009). Second, TMEM16B currents had faster gating kinetics compared to $I_{\text{CaCC}}$ induced by TMEM16A. For example, at depolarized membrane potential (+100 mV) $I_{\text{CaCC}}$ activation time constant was 4 ms for TMEM16B (Pifferi et al., 2009), and hundreds of milliseconds for TMEM16A (Caputo et al., 2008; Schroeder et al., 2008). TMEM16A and TMEM16B also differed in their sensitivity to Ca$^{2+}$. The $EC_{50}$ of Ca$^{2+}$ for TMEM16B was 4.9 µM Ca$^{2+}$ at −50 mV and 3.3 µM Ca$^{2+}$ at +50 mV (Pifferi et al., 2009). In contrast, TMEM16A affinity to Ca$^{2+}$ increased more significantly at positive voltages, i.e. $EC_{50}$ was 2.6 µM Ca$^{2+}$ at −60 mV and 0.4 µM Ca$^{2+}$ at +60 mV (Yang et al., 2008). The permeability sequence for both these CaCCs was: SCN$^-$ > I$^-$ ≥ NO$_3^-$ > Br$^-$ > Cl$^-$ > F$^-$ (Yang et al., 2008; Pifferi et al., 2009; Stephan et al., 2009). In contrast, Schroeder et al. (2008) stated that TMEM16A-induced channels did not have fixed permeability sequence as it varied depending on channel activity. This finding suggested the existence of multiple open states with a different ion selectivity, which was in the agreement with the previously proposed kinetic schemes of native CaCCs gating (Kuruma & Hartzell, 2000; Piper & Large, 2003; Leblanc et al., 2005).

1.10. Pharmacology of TMEM16 channels

At present there is a limited number of pharmacological agents that inhibit or activate TMEM16 channels. The vast majority of classical Cl$^-$ channels blockers are acidic
compounds that in physiological solutions are negatively charged. These include anthracene-9-carboxylic acid (A9C), 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB), 4,4-diisothiocyanostilbene-2,2-disulphonic acid (DIDS), diphenylamine-2-carboxyl acid (DPC) and 4-acetamido-4-isothiocyanostilbene-2,2-disulphonic acid (SITS). All these compounds produce a voltage-dependent block of Cl⁻ channels by binding within the anion permeation pathway (Bosma, 1989; Zhang et al., 2000; Qu & Hartzell, 2001). Other Cl⁻ channels blockers, such as niflumic acid, are also known to block TMEM16 and other Cl⁻ channels (Lerma & Martin del Rio, 1992; Schroeder et al., 2008). Niflumic acid could also influence the activity of TMEM16 channels indirectly by inducing Ca²⁺ release from SR (Cruickshank et al., 2003).

The list of blockers has recently been supplemented by new compounds identified using high-throughput screening of large chemical libraries. CaCCₐₙh-A01 (De La Fuente et al., 2008), tannic acid and related compounds (Namkung et al., 2010) have been described as potent blockers of TMEM16A channels. These compounds, however, are not particularly selective and also block other channels in various cell types. Aminophenylthiazole T16ₐₙh-A01 is another recently identified potent TMEM16A blocker that does not affect Cl⁻ currents mediated by other anion channels, including cystic fibrosis transmembrane conductance regulator (CFTR) (Namkung et al., 2011a).

Activators for TMEM16A channels have been identified just recently. Aroylaminothiazole compounds activate TMEM16A channels in the absence of cytosolic Ca²⁺, while tetrazolylbenzamide agents potentiate the current by increasing TMEM16A sensitivity to Ca²⁺ (Namkung et al., 2011b). Both types of activators bind to the channel directly and produce a large and sustained increase in ionic current. The selectivity of these compounds remains to be tested. It is noteworthy that CaCCs can be activated indirectly by ligands of G-protein coupled receptors leading to Ca²⁺ release from the endoplasmic reticulum. Endothelin-1, ATP, acetylcholine, angiotensin II, carbachol and histamine have been shown to evoke I_CaCCs in smooth muscle cells and in HEK-293 cells expressing TMEM16A or TMEM16B channels (Large & Wang, 1996; Yang et al., 2008; Pifferi et al., 2009). Two compounds, denufosol and duramycin, which activate CaCCs indirectly by increasing intracellular Ca²⁺ levels, are currently in clinical trials to treat cystic fibrosis (Kellerman et al., 2008; Steiner et al., 2008).
1.11. Structure of TMEM16 channels

TMEM16 gene family is composed of 10 members, named TMEM16A-K (Galindo & Vacquier, 2005) (Figure 1.2). The highest sequence similarity (~60%) is observed between TMEM16A and TMEM16B proteins. Their sequence homology with other family members is much lower, about 20-40%. Hydropathy analysis suggests that each member contains eight putative transmembrane domains with cytosolic N- and C-termini. This topology model has been confirmed experimentally for TMEM16G by inserting hemagglutinin (HA) tags at various positions and testing the accessibility of epitopes by extracellular and intracellular antibodies (Das et al., 2007). The fifth (TM5) and the sixth (TM6) transmembrane segments flank the predicted re-entrant loop. Mutations of positively charged amino acids within the re-entrant loop of TMEM16A have resulted in channels with altered ionic selectivity, suggesting that this region might form part of the channel pore (Yang et al., 2008). Cysteine scanning mutagenesis has provided further evidence that the re-entrant loop of TMEM16A constitutes the anion permeation pathway (Yu et al., 2012) (Figure 1.3). The predicted topology model of TMEM16A recently has been questioned based on accessibility of inserted HA epitopes (Yu et al., 2012). An alternative topology model has been proposed suggesting that the re-entrant loop of TMEM16A fully crosses the phospholipid bilayer rather than being partially embedded in it from the extracellular side (Yu et al., 2012).
Figure 1.3. Topology model of TMEM16A. Hydropathy analysis suggests the existence of eight transmembrane domains with intracellular N- and C- termini. $a$ (116 residues), $b$ (22 residues), $c$ (4 residues) and $d$ (26 residues) indicate the alternative segments. Yellow circles highlight residues involved in voltage-dependent gating of TMEM16A: four sequential glutamates (444EEEEE447) in the N-terminal region (Yu et al., 2012), R563 in TM3 and Q757 in TM6 (Caputo et al., 2008). Green circles within the re-entrant loop represent residues R621, K645, and K668 important for anion selectivity (Yang et al., 2008). Circles in red show residues C625, G628, G629, C630, L631, M632, I636 and Q637 that line the anion permeation pathway (Yu et al., 2012).

Fluorescence resonance energy transfer (FRET), chemical cross-linking and co-immunoprecipitation studies have revealed that TMEM16A forms a homodimer (Fallah et al., 2011; Sheridan et al., 2011). TMEM16A subunits associate before being trafficked to the plasma membrane. It is not known, however, whether TMEM16A can form heteromers with other members of TMEM16 family or any other accessory proteins. It is not clear yet if TMEM16A subunits form a channel with a single pore or if each subunit contains independent anion permeation pathway, similar to the double-barreled structure of CIC chloride channels (Miller, 1982; Dutzler et al., 2002).

The voltage sensor and Ca$^{2+}$ binding site/s within TMEM16A and TMEM16B channels remain to be identified. TMEM16 channels lack of the canonical voltage sensing region, such as S4 in voltage-gated potassium or sodium channels (Hille, 2001). Also, neither of these channels possesses canonical motifs to bind Ca$^{2+}$ (e.g. EF-hands) or calmodulin (IQ motifs). Patch-clamp experiments show that I$_{\text{CaCCS}}$ can be generated in the absence of ATP, which is consistent with an idea that TMEM16 channels bind Ca$^{2+}$ directly. If Ca$^{2+}$ is coordinated by carboxyl groups of peptide backbone, an extensive mutagenesis study might be needed to identify its binding pocket/s. So far, two residues E702 and E705 have been suggested to be directly involved in Ca$^{2+}$ binding within TMEM16A (Yu et al., 2012). The complete elucidation of the Ca$^{2+}$ binding site/s will require additional mutagenesis and structural evidence (e.g. X-ray crystallography) to be fully solved.
The sensitivity of TMEM16 channels to Ca\(^{2+}\) and voltage can also be regulated by the alternative splicing. TMEM16A can exist in different isoforms composed of various combinations of four alternative segments, termed \(a\), \(b\), \(c\) and \(d\) (Caputo et al., 2008) (Figure 1.3). TMEM16A with all four alternative segments forms a full-length protein containing 1008 residues. Segment \(a\) codes for a 116 amino acid stretch at the N-terminus and can be skipped if an alternative promoter is induced (Ferrera et al., 2009). Interestingly, TMEM16A lacking of segment \(a\) displays no voltage-dependence. Another alternative exon 6b codes for 22 residues (segment \(b\)) in N-terminus. This region is important for Ca\(^{2+}\) sensitive gating. The exclusion of segment \(b\) gives rise to TMEM16A channels with 4-fold higher affinity to intracellular Ca\(^{2+}\) (Ferrera et al., 2009). The opposite effect is observed upon deletion of the alternative exon 13 (segment \(c\)) coding for four amino acids (\(44qEAVK45l\)) in the first intracellular loop. TMEM16A without segment \(c\) form channels with 50-fold lower affinity to cytosolic Ca\(^{2+}\) (Xiao et al., 2011). Importantly, splice \(c\) is preceded by four glutamic residues, which in total gives five consecutive glutamates (\(44qEEE448\)) in the first intracellular loop of TMEM16A. This acidic region is also present in the first intracellular loop of TMEM16B and closely resembles the “Ca\(^{2+}\) bowl” of large conductance Ca\(^{2+}\)-activated K\(^{+}\) channels (Cui et al., 2009) and a cluster of acidic residues in bestrophin-1 Cl\(^{-}\) channels (Xiao et al., 2008), which are involved in Ca\(^{2+}\) sensing. Interestingly, these glutamate residues have no or very little effect on apparent Ca\(^{2+}\) affinity but instead are involved in sensitivity to voltage of TMEM16A (Xiao et al., 2011) and TMEM16B channels (Cenedese et al., 2012). Finally, an alternative exon 15 (segment \(d\)) codes for 26 amino acids in the first extracellular loop and tends to be skipped if segment \(b\) is included and \textit{vice versa} (Ferrera et al., 2009). The alternative splicing might provide an explanation why CaCCs in various cell types have different biophysical properties (Hartzell et al., 2005).

1.12. Other TMEM16 family members

To date, the physiological role and molecular function of the other TMEM16 family members are elusive. TMEM16A, TMEM16C, TMEM16F and TMEM16E were found to be highly expressed in osteoblasts and chondrocytes (Mizuta et al., 2007; Gritli-Linde et al., 2009). TMEM16E is known to play important roles in musculoskeletal development (Tsutsumi et al., 2004). An alternative name for TMEM16E is GDD1 because mutation in this protein is associated with an autosomal dominant skeletal disease called gnathodiaphyseal dysplasia (Tsutsumi et al., 2004). Mutations of a highly conserved C356 in the first extracellular loop of TMEM16E lead to abnormal bone...
mineralization and bone fragility (Tsutsumi et al., 2004). TMEM16E is also predominantly found in cardiac and skeletal muscles (Mizuta et al., 2007), where the mutations in TMEM16E is associated with some recessive muscular dystrophies (Bolduc et al., 2010; Hicks et al., 2011). One of the symptoms of these diseases is defective skeletal muscle membrane repair, suggesting a possible role of TMEM16E in Cl⁻ conduction as Cl⁻ currents have been shown to be important in membrane repair (Fein & Terasaki, 2005). So far, no studies have demonstrated the involvement of TMEM16E in plasma membrane Cl⁻ fluxes.

**TMEM16F**

The functional role of TMEM16F currently is elusive. This protein was found to be ubiquitously expressed in many tissues (Schreiber et al., 2010). Martins et al. (2011) reported that TMEM16F is a crucial component of outwardly rectifying Cl⁻ channels in airway epithelial cells and Jurkat T lymphocytes. These channels were reported to have a single channel conductance of about 50 pS and play an important role in apoptosis and cell volume regulation (Martins et al., 2011). In contrast, Suzuki et al. (2010) demonstrated that TMEM16F is an essential component of phospholipid scramblase that mediates the transfer of phosphatidylserine between the outer and inner layers of the plasma membrane. In blood platelets TMEM16F was shown to mediate the Ca²⁺-dependent phosphatidylserine exposure on the cell surface triggering the blood clotting. Also, a mutation in TMEM16F gene was found in a patient with Scott syndrome, a rare bleeding disorder caused by impaired blood clotting (Suzuki et al., 2010). Surprisingly, one more function has been assigned to TMEM16F by Lily Yeh Jan’s group. Yang et al. (2011) reported that TMEM16F form Ca²⁺-activated non-selective cation channels with a very small, sub-picosiemens, single-channel conductance. These channels were suggested to permeate divalent anions and to be synergistically gated by cytosolic Ca²⁺ and membrane potential (Yang et al., 2011). Finally, a recent report from Criss Hartzell group suggested that TMEM16F was expressed intracellularly rather than on the plasma membrane (Duran et al., 2011) and apparently would not be able to perform any of the functional roles described above.

**TMEM16G**

TMEM16G, is predominantly expressed in the prostate, where it may play a role in cells association (Das et al., 2007). TMEM16G exists in two isoforms: a long (934 amino acids) plasma membrane protein and a short (179 amino acids) soluble cytosolic protein (Bera et al., 2004). The long isoform has been reported to be an anion channel as
measured by ATP-induced iodide uptake assays (Schreiber et al., 2010). In this study, however, iodide fluxes produced by long isoform of TMEM16G were similar to those generated by the short isoform that is clearly not associated with channel activity. Also, no patch-clamp recordings have been reported to support the idea of TMEM16G involvement in Cl⁻ conductance.

**TMEM16H**
TMEM16H mRNA has been found in embryonic stem cells, fetal brain, and neural tissues (Katoh & Katoh, 2005). In contrast to other TMEM16 proteins, TMEM16H has a largely extended and negatively charged extracellular loop between TM5 and TM6 due to the presence of 20 aspartate and glutamate residues (Galietta, 2009; Kunzelmann et al., 2009). To date, no reports have been published on TMEM16H functional role.

**TMEM16K**
TMEM16K is ubiquitously expressed protein in mammalian tissues (Schreiber et al., 2010). Mutations in TMEM16K have been associated with autosomal recessive cerebellar ataxias (Vermeer et al., 2010). However, the exact role of TMEM16K in physiological conditions and disease states is unknown. *Drosophila* ortholog of TMEM16H/K, named Axs, is a component of microtubules in dividing cells and is important for the normal cell cycle progression (Kramer & Hawley, 2003). It remains to be seen if TMEM16K has a similar function in mammalian cells.

### 1.13. Aims of the thesis

My work of thesis consists of three parts: i) elucidation of the molecular identity of CaCCs in VSM, ii) identification of functional properties of the putative pore-loop of TMEM16 channels and iii) investigation of the mechanisms of action of a TMEM16A modulator.

**Identification of TMEM16A as a CaCC in VSM**
Motivated by the recent discovery of TMEM16 as a new family of CaCCs (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008; Pifferi et al., 2009; Stephan et al., 2009), we hypothesized that TMEM16A or other TMEM16 proteins form CaCCs in VSM. This hypothesis was reinforced by the notion that co-expression of TMEM16A and G-protein coupled receptor ubiquitously found in the vascular system, such as endothelin receptor subtype A and angiotensin II receptor subtype 1, gave rise to $I_{CaCC}$ in response to
endothelin-1 and angiotensin II, respectively (Yang et al., 2008). The specific aims have been set to elucidate the molecular identity of CaCCs in VSM:

a) Establish which members of TMEM16 family are expressed in rat PASMCs;

b) Test whether siRNAs against TMEM16A reduces or abolishes native $I_{\text{CaCC}}$.

These studies were carried out in PASMCs because previous reports indicated that these cells exhibit only the classical $I_{\text{CaCC}}$ (Matchkov et al., 2005; Matchkov et al., 2008). Thus, PASMCs represents an ideal system to evaluate the physiological role of TMEM16A in the absence of the closely related cGMP-dependent $I_{\text{CaCC}}$.

Towards an understanding of the molecular mechanisms of regulation of cloned TMEM16 channels

Elucidation of the fundamental properties of CaCCs gating and conductance is crucial to understand their physiological function in various tissues. Despite high sequence homology between TMEM16A and TMEM16B, these ion channels have different functional properties, including Ca$^{2+}$-sensitivity and voltage-dependence (Schroeder et al., 2008; Yang et al., 2008; Pifferi et al., 2009; Stephan et al., 2009). Recent reports indicate that the re-entrant loop of TMEM16A might be involved in the regulation of ionic selectivity, permeation and sensitivity to Ca$^{2+}$ (Yang et al., 2008; Yu et al., 2012). We, therefore, aimed to isolate the functional role/s of the re-entrant loop of TMEM16A by constructing chimeric channels, in which the re-entrant loop is replaced by analogous regions of other TMEM16 proteins.

Insights into pharmacological properties of TMEM16A channels

The development of selective inhibitors and activators is essential for the investigation of TMEM16A physiological roles in various cell types. Also, selective blockers might be of a therapeutic use to treat a range of disorders, such as hypertension, diarrhea and pain; whereas activators of TMEM16A channels could be used for the treatment of hypotension, cystic fibrosis, gastrointestinal hypomotility disorders and dry mouth syndrome. We aimed to describe the mechanisms of action of a generic open Cl$^-$ channel blocker A9C that produces a bimodal effect on TMEM16A currents. The identification of activating and inhibiting A9C binding sites as well as elucidation of drug physicochemical properties responsible for these two effects might provide a starting point for the development of potent and selective modifiers of TMEM16A channels.
Chapter 2

Methods
The aim of this chapter is to provide the reader with an overview of the principles underlying the methods used in this work of thesis. Notice that this chapter does not include specific details for the various experimental procedures, such as the composition of solutions, sequences of DNA primers, PCR protocols, etc. These details are provided in the methods section of each publication.

### 2.1. RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR) was used to assess the expression of TMEM16 genes in various smooth muscle cell types (Publication I). This molecular biological technique uses RNA strands as a template to synthesize the complementary DNA (cDNA) using the RNA-dependent DNA polymerase.

To purify RNA, cell pellets or tissue samples were lysed and homogenized in a highly denaturing buffer that inactivates RNases. The lysate was applied to columns containing silica-based membranes, which selectively binds RNA molecules longer than 200 bases. Silica membranes were then treated with DNase to catalyse the digestion of genomic DNA that might also bind to the column. RNA was washed and eluted from the columns with RNase-free water. In the presence of random hexonucleotides, RNA-dependent DNA polymerase used purified RNA as a template to synthesize the first-strand cDNA, which then was amplified using the PCR. PCR products were analysed by agarose gel electrophoresis that separated DNA fragments by length. DNA fragments were then visualised by exposing the gel to ultraviolet light (Green & Sambrook, 2012).

### 2.2. Quantitative PCR

The real-time quantitative PCR (qPCR) technique was used to amplify and quantify the expression of TMEM16 family members is VSM (Publication I). The technique is based on a real-time detection of PCR products using a non-specific SYBR Green fluorescent dye that gives a fluorescence signal when intercalates within the double-stranded DNA. The intensity of fluorescence is plotted against the number of PCR cycles on a logarithmic scale. The cycle at which the fluorescence exceeds the set threshold value is termed the cycle threshold (Ct). The relative mRNA levels were calculated using the $2^{-\Delta\Delta\text{Ct}}$ method (Livak & Schmittgen, 2001). According to this method, the Ct of a housekeeping gene was used to correct for the sample-to-sample variation in the quantity of RNA. The housekeeping gene was considered to be expressed at constant levels in all samples and, thus, could be used a reference gene to correct for any loading differences.
Specifically, the Ct value obtained for the housekeeping gene was subtracted from the Ct value for TMEM16 ($\Delta Ct = Ct_{TMEM16} - Ct_{housekeeping}$). Subsequently, the $\Delta \Delta Ct$ value was obtained by subtracting the $\Delta Ct$ value of the samples treated with siRNA from the $\Delta Ct$ value of a calibrator sample (non-treated with siRNA). The fold-difference in mRNA levels was calculated by raising 2 to the power of $-\Delta \Delta Ct$ value.

2.3. Small interfering RNA

Small interfering or silencing RNA (siRNA) is a short (usually 21 nucleotides) double-stranded RNA, which is used for post-transcriptional gene silencing in plants and mammalian cells (Elbashir et al., 2001). The double-stranded siRNA has an overhang of two nucleotides at 3’ end of each strand. The 3’ ends are hydroxylated, while 5’ ends of both strands are phosphorylated. Custom made siRNAs have been introduced into cells via transfection (Publication I). Once internalised within a cell, each double-stranded siRNA is unwound into two single-stranded siRNAs, termed the guide and the passenger strands. The passenger siRNA is degraded, while the guide strand of siRNA is integrated into the RNA-induced silencing complex. The guide strand interacts with a complementary region of a target mRNA and induces cleavage by the catalytic component of the RNA-induced silencing complex. Cleaved mRNA is degraded by ribonucleases preventing the translation of a target protein (Hannon, 2002). This technology of gene silencing has been effectively used to suppress the expression of TMEM16A in PASMCs (Publication I).

2.4. Generation of chimeric DNA constructs

TMEM16 chimeras were constructed using a PCR mutagenesis strategy that allows swapping regions from one gene to another (Kirsch & Joly, 1998). Two PCR reactions were needed to construct each chimera. Figure 2.1 provides a schematic illustration of this method. The region that needs to be transferred (in our case it was the putative pore-loop) is amplified during the first PCR reaction. Importantly, the primers anneal to the template just with their 3’ ends. Overhanging 5’ ends are complementary to the gene, into which the amplified region is going to be inserted. The PCR product then serves as a complementary pair of megaprimers for a second PCR reaction, which results in the generation of chimeric DNA.
2.5. Generation HA-tagged DNA constructs

TMEM16 proteins were tagged with the human influenza hemagglutinin (HA) epitope in the putative extracellular loops using the inverse PCR mutagenesis strategy (Gama & Breitwieser, 1999). Figure 2.2 provides a schematic illustration of this approach. The entire plasmid DNA was amplified using primers, whose 3’ ends annealed on the region where HA insertion was required. 5’ ends of reverse and forward primers coded for the first and second halves of HA epitope, respectively. Inverse PCR reaction resulted in the linear DNA with two halves of HA epitope on both ends. The 5’ ends of DNA strands were phosphorylated and subsequently ligated generating HA-tagged plasmid DNA construct.

2.6. Heterologous expression

Plasmid DNA constructs were heterologously expressed in HEK-293T cells for patch-clamp recordings or immunocytochemistry experiments. Cells were transfected using Escort II Transfection Reagent (Sigma Aldrich, UK) according to the manufacturer’s instructions. This transfection reagent mixed with DNA forms liposomes, which trap DNA inside. After fusion with cell membrane, cargo DNA is released inside the cell. To identify successfully transfected cells for patch-clamping, Tmem16 subcloned into
pcDNA3.1 plasmid was co-transfected with CD8 gene in pCI expression vector. CD8 codes for transmembrane glycoproteins that were visualized by applying polystyrene beads (4.5 µm diameter) coated with a CD8-specific antibody. HEK-293T cells covered in beads were used for patch-clamp recordings.

2.7. Immunocytochemistry

Immunocytochemistry (ICC) was used for to visualize TMEM16 proteins and determine their levels of expression on the plasma membrane (Publication II). Various extracellular regions of TMEM16 proteins were tagged with HA epitopes, which are commonly used for protein detection via antibodies. HA-tagged TMEM16 constructs were transiently transfected into HEK 293T cells. The cells were grown on silicate glass coverslips that acquire a negative surface charge density when immersed in aqueous solutions. The surface of living mammalian cells also has a net negative charge due to negatively charged phospholipids and membrane glycoproteins (Freshney, 2005). To overcome the repellent forces between negative charges and to enhance cell attachment to the glass coverslips, coverslips were coated with a poly-D-lysine, a positively charged synthetic polypeptide that is resistant to enzymatic degradation.

To detect the HA-epitopes, secondary (or indirect) immunofluorescence was used. In contrast to primary (or direct) immunofluorescence, which uses a single antibody chemically linked to a fluorophore, we used two antibodies. The unlabelled primary antibody recognizes the antigen, in our case HA-epitope, while the secondary fluorophore-conjugated antibody specifically binds to the primary antibody. The advantage of the secondary immunofluorescence is that a single primary antibody can bind many fluorophore-conjugated secondary antibodies, this way allowing signal amplification and providing more sensitive antigen detection technique.

The general steps of immunocytochemistry are i) cell fixation, which preserves cell morphology and architecture, ii) permeabilization that enabled the access of antibodies to intracellular epitopes and iii) application of primary and subsequent secondary antibodies, which labels the protein of interest. For cell fixation, we used a cross-linking reagent paraformaldehyde (PFA) that covalently binds to free amino groups forming the network of intermolecular bridges, which inhibits the function and movement of surface proteins. Cells were permeabilized with a mild detergent saponin, which is a natural plant-derived chemical compound that extracts cholesterol from cellular membranes.
enabling the access of antibodies to intracellular epitopes. To probe for the HA-tagged TMEM16 proteins embedded solely in the plasma membrane ICC experimental protocol was modified and performed in non-permeabilizing conditions. To prevent permeabilisation, cells were treated on ice and primary antibodies were applied before the fixation. Another reason of performing ICC on ice was to stop endocytosis, the process by which HA-tagged TMEM16 proteins on the plasma membrane might be internalized. After the application of antibodies, cell nuclei were stained with 4,6-diamidino-2-phenylindole fluorescent dye that binds to A-T rich regions in DNA. ICC images were collected using epifluorescence microscope.

2.8. Configurations of the patch-clamp technique

Electrophysiological patch-clamp technique was used to assess the activity of CaCCs in PASMCs (Publication I) and transfected HEK-293T cells (Publication II and III). This technique allows clamping the membrane potential and measuring the ionic currents conducted by ion channels present in the plasma membrane. The ionic currents are measured using a chlorinated silver electrode placed in the glass micropipette. The bath, or reference, electrode is used to set the zero current level, which allows the voltage to be kept constant while observing changes in current. The tip of the micropipette (~1 µm in diameter) is pressed against the cell membrane and suction is applied to form a high resistance (>1 GΩ) seal. When the gigaohm seal is formed, the membrane patch within the tip of micropipette can be excised by pulling the pipette away from the cell. The extracellular side of the membrane patch remains exposed to the interior of the micropipette, while the cytosolic side becomes exposed to the bath solution. This patch-clamp mode is called the inside-out configuration (Figure 2.3). Alternatively, when the gigaohm seal is formed, suction can then be applied to rupture the membrane patch enclosed within the tip of micropipette. It brings the cytosol into contact with the interior of the pipette and allows measurement of the ionic currents conducted by ion channels present in the plasma membrane of the cell. This patch-clamp mode is called the whole-cell configuration (Figure 2.3).
Chapter 2

Figure 2.3. Diagram illustrating inside-out and whole-cell configurations of the patch-clamp technique.

Native CaCCs in vascular smooth muscle cells (Paper I) and cloned TMEM16 channels heterologously expressed in HEK-293T cells (Paper II and III) were investigated in the presence of physiological $[\text{Ca}^{2+}]_i$, ranging from 17 nM to 1.25 µM. A wider range of $[\text{Ca}^{2+}]_i$ (from 0 to 78 µM) was used to construct $\text{Ca}^{2+}$-sensitivity and voltage-dependence relationships for wild-type and chimeric TMEM16 channels (Paper II and III). For whole-cell experiments, 274 nM $[\text{Ca}^{2+}]_i$ was used to compare the current density of various TMEM16 channels as they all had the same open probability at this $[\text{Ca}^{2+}]_i$ (Paper II). Inhibition of TMEM16A by anthracene-9-carboxylic acid was studied in the presence of 12.5 µM $[\text{Ca}^{2+}]_i$ (Paper III). This concentration was chosen because it fully activates TMEM16A channels enabling the study of blockage mechanisms.

CaCC currents recorded in inside-out or whole-cell configurations were investigated in the presence of various $[\text{Ca}^{2+}]_i$, different permeant anions or various drug concentrations (Paper II and III). The exchange of solutions was achieved by using a local perfusion system consisting of eight tubes of 1.2 mm diameter in which the tip of the patch pipette was inserted. In some cases, an ultra-rapid computer-controlled perfusion system (Warner Instruments) was used to exchange solutions in less than ~5 ms.

2.9. Electrical properties of the cell membrane

The membrane lipid bilayer constitutes an almost impermeable barrier to ions. In electrical terms the membrane can therefore be represented as a resistor. The cell membrane also acts as a capacitor because it is an insulating layer separating two conducting solutions represented by the internal and external cellular environments. Therefore, a complete representation of the cell membrane is an electrical circuit composed of resistor and capacitor elements connected in parallel. In whole-cell configuration, an applied voltage pulse ($V_p$) generates current transients, associated with
the charging of the cell membrane (Figure 2.4). The area of the transient represents the charge difference across the membrane given by \( Q = C_m V_p \). Cell capacitance \( (C_m) \) has an important biological significance because it can be used as a measure of surface area of the cell \( (A) \):

\[
C_m = A \varepsilon \varepsilon_0 / d ,
\]

where \( \varepsilon \) is a dielectric constant of the lipid bilayer, \( \varepsilon_0 \) is a natural constant termed the polarizability of free space and \( d \) is the thickness of the membrane.

It is noteworthy that \( C_m \) is equal to 16.2±1.2 pF \( (n=7) \) and 15.8±0.7 pF \( (n=22) \) for non-transfected and transfected HEK-293T cells, respectively. The decay of the current transient can be well-described by an exponential function (Figure 2.4). The time constant of this decline is given by \( \tau = C_m R_s \), where \( R_s \) is a series resistance. \( R_s \) is a sum of the pipette resistance \( (R_p) \), which depends mainly on the diameter of the tip and the thickness of the glass, and the access resistance \( (R_{access}) \), located between the micropipette and the cell. Low resistance pipettes ensure that \( R_s \) is maintained as low as possible because it introduces two types of errors in patch-clamp recordings (Sherman et al., 1999):

1) Steady state errors. These errors are produced because the amplifier clamps the \( V_p \) rather than the potential of the cell membrane \( (V_m) \). \( V_p \) and \( V_m \) are related by the following equation:

\[
V_m = V_p - I_m R_s ,
\]

where \( I_m \) is a whole-cell current.

2) Dynamic errors. Step changes in \( V_p \) cause changes in \( V_m \) with a delay whose time constant can be estimated by \( \tau \approx R_s C_m \). The increase and decay of macroscopic currents can be delayed by milliseconds, which impair kinetic measurements of fast gated ion channels.

CaCCs, which are studied in this work of thesis, have rather slow gating kinetics with time constants of activation and deactivation ranging from tens to hundreds of milliseconds. Thereby, \( R_s \) has a very little effect on the kinetic measurements. The current magnitude, however, is susceptible to errors due to \( R_s \). The use of low resistance \( (~1.5 \text{ M}\Omega) \) pipettes typically results in \( R_s \) of 4-6 M\Omega. If \( I_m \) is smaller that 1 nA, then
$V_m \approx V_p$. However, in the presence of channel activators or highly permeant ions, $I_m$ increases substantially and $V_m$ deviates from $V_p$. To correct for this voltage error, $R_s$ has to be compensated by continuously calculating $R_s$ and the pipette current ($I_p$). The fraction of $I_p R_s$ value is added to $V_p$ as a correction signal that brings $V_m$ to the desired value. This positive feedback element, built into the patch-clamp amplifier, was used to reduce the effective value of $R_s$ by 70%. Greater compensation was unacceptable because it introduces noise to $I_m$ and becomes prone to oscillations (Sherman et al., 1999). Considering that in most experiments $V_m$ was $\leq 100$ mV and the average current was lower than ~5 nA, the maximal expected voltage error was ~7.5 mV.

Figure 2.4. Current transients recorded in response to a 10 ms pulse at +10 mV in whole-cell configuration of HEK 293T cell. The panel of the right shows the enlarged current transient. The area of the transient represents the charge difference across the membrane bilayer ($Q$) that has been used to calculate the cell capacitance ($C_m$). The blue trace represents the exponential fit of transient current decay with a time constant ($\tau$) representing $C_m$ times the series resistance ($R_s$).
Chapter 3

Publication I

TMEM16A/Anoctamin1 protein mediates calcium-activated chloride currents in pulmonary arterial smooth muscle cells

*Published in The Journal of Physiology*
TMEM16A/Anoctamin1 protein mediates calcium-activated chloride currents in pulmonary arterial smooth muscle cells

Boris Manoury, Aiste Tamuleviciute and Paolo Tammaro

Faculty of Life Sciences, University of Manchester, 46 Grafton Street, Manchester M13 9NT, UK

Calcium-activated chloride channels (CaCCs) play important roles in several physiological processes. In vascular smooth muscle, activation of these ion channels by agonist-induced Ca$^{2+}$ release results in membrane depolarization and vasoconstriction. The molecular identity of vascular CaCCs is not fully defined. Here we present evidence that TMEM16A (or anoctamin 1), a member of the transmembrane 16 (TMEM16) protein family, forms CaCCs in pulmonary artery smooth muscle cells (PASMCs). Patch-clamp analysis in freshly isolated PASMCs revealed strongly outward-rectifying, slowly activating Ca$^{2+}$-activated Cl$^{-}$ currents sharing a high degree of similarity with heterologous TMEM16A currents. TMEM16A mRNA was identified in rat and human pulmonary arteries and various other vascular smooth muscle cell types. Further analyses revealed that several TMEM16A splice variants were detected in rat PASMCs and that TMEM16F and TMEM16K were also expressed in these cells, while TMEM16B, TMEM16D and TMEM16E were all at least 50 times less abundantly expressed and the remaining TMEM16 family members were absent. Downregulation of TMEM16A gene expression in primary cultures of rat PASMCs, with small interfering RNAs, was accompanied by almost total loss of whole-cell CaCC currents. Based on these results, we propose that TMEM16A is the major constituent of the vascular calcium-activated chloride channel in rat pulmonary artery smooth muscle.

(Resubmitted 4 March 2010; accepted after revision 23 April 2010; first published online 10 May 2010)

Corresponding author P. Tammaro: Faculty of Life Sciences, University of Manchester, The Core Technology Facility, 46 Grafton Street, Manchester M13 9NT, UK. Email: paolo.tammaro@manchester.ac.uk

Abbreviations Best-3, bestrophin-3; CaCC, calcium-activated chloride channel; $I_{\text{CaCC}}$, calcium-activated chloride current; PASMC, pulmonary artery smooth muscle cell; qPCR, real-time quantitative polymerase chain reaction; siRNA, small interfering RNA; STIC, spontaneous transient inward chloride currents; VSM, vascular smooth muscle.

Introduction

Calcium-activated chloride channels (CaCCs) play important roles in several cellular functions. They are of key importance in vascular smooth muscle (VSM), where they are activated by a rise in the intracellular Ca$^{2+}$ concentration following agonist-induced Ca$^{2+}$ release from intracellular stores, leading to membrane depolarization and muscle contraction. In addition, CaCCs are activated by Ca$^{2+}$ released from ryanodine receptors located in the sarcoplasmic reticulum and are responsible for spontaneous transient inward Cl$^{-}$ currents (STICs) observed in several VSM cell types (Large & Wang, 1996; Leblanc et al. 2005).

Two types of CaCC currents ($I_{\text{CaCC}}$) have been identified in VSM, a ‘classic’ Ca$^{2+}$-dependent current (Byrne & Large, 1987) and a cGMP-dependent $I_{\text{CaCC}}$ (Matchkov et al. 2004; Piper & Large, 2004a,b). Classic $I_{\text{CaCC}}$ in VSM exhibits distinctive outward rectification, a small (~1–3 pS) single-channel conductance, high thiocyanate permeability and cGMP is not mandatory for channel activation (Large & Wang, 1996).

Although CaCCs have been studied for almost three decades, their molecular identity has been elusive (Nilius & Droogmans, 2003; Hartzell et al. 2009). Elucidating the molecular identity of the classic CaCC is an important goal given its ubiquitous presence in vascular (and non-vascular) smooth muscle and its essential role in regulating smooth muscle tone (Large & Wang, 1996; Leblanc et al. 2005). Several molecular candidates have been proposed for vascular CaCCs, including members of the CLCA (Ca$^{2+}$-activated chloride channel) and bestrophin gene families. Based on RT-PCR analysis, CLCA1 was found to be expressed in mouse portal vein smooth muscle (Britton et al. 2002), and CLCA4 transcripts were detected in many VSMs, including...
aorta and coronary vessels (Elble et al. 2002). However, several of the biophysical properties of native \( I_{CaCC} \), including single-channel conductance, degree of outward rectification and \( Ca^{2+} \) sensitivity, differ from those of heterologously expressed CLCA channels (Britton et al. 2002). Bestrophin-3 (Best-3) mRNA and protein were found in several VSMs, but appeared to be regulated by both \( Ca^{2+} \) and cGMP (Matchkov et al. 2005, 2008). Gene silencing experiments with small interfering RNA (siRNA) indicated that Best-3 represents the cGMP-activated CaCC, but its involvement in the classic \( I_{CaCC} \) is unlikely (Matchkov et al. 2008).

Novel candidates for CaCC have recently been proposed: the TMEM16/anoctamin family (Caputo et al. 2008; Schroeder et al. 2008; Yang et al. 2008). TMEM16A/anoctamin 1 was the first member of this family shown to function as a CaCC. Heterologous expression of TMEM16A, or of the closely related TMEM16B/anoctamin 2 protein, resulted in \( Cl^{-} \) currents sensitive to intracellular \( Ca^{2+} \) and with the degree of outward rectification, ion selectivity and pharmacological profile typical of native \( I_{CaCC} \) observed in many tissues (Caputo et al. 2008; Schroeder et al. 2008; Yang et al. 2008; Galietta, 2009; Hartzell et al. 2009). It is currently unknown whether other members of the TMEM16 family form CaCCs. Several TMEM16A splice variants have been described, which result in channels with different biophysical properties (Caputo et al. 2008; Ferrera et al. 2009). The alternatively spliced exons code for segments of 116 (segment \( a \)) and 22 residues (segment \( b \)) at the N-terminus and segments of four (segment \( c \)) and 26 residues (segment \( d \)) in the first putative intracellular loop.

Motivated by these discoveries, we set out to test the hypothesis that TMEM16A, or other members of the TMEM16 family, are responsible for the classic \( I_{CaCC} \) in VSM. The notion that heterologous coexpression of TMEM16A with receptors ubiquitously found in the vascular system, such as endothelin receptor subtype A or angiotensin II receptor subtype 1, giving rise to endothelin- or angiotensin-induced \( Cl^{-} \) currents, reinforced the hypothesis that TMEM16A may form CaCCs in VSM (Yang et al. 2008). We focused our studies on pulmonary artery smooth muscle cells (PASMCs) because previous findings suggested that this cell type exhibits a pure classic \( I_{CaCC} \), i.e. neither the cGMP-activated current nor the expression of Best-3 have been detected (Matchkov et al. 2005, 2008); thus PASMCs represent a simple system to assess the properties and molecular identity of \( I_{CaCC} \), in the absence of the closely related cGMP-dependent CaCC current.

**Methods**

A detailed Methods section is provided in the online Supplemental material.

**Human samples**

Intralobar human pulmonary arteries were carefully dissected from healthy areas of distal lung sections obtained from two patients undergoing lung resection for lung cancer. Both subjects gave written informed consent. The study was approved by the local research ethics committee (South Manchester Research Ethics Committee).

**Cell isolation and culture**

Male Sprague–Dawley rats (weight 225–300 g) were killed by cervical dislocation in accordance with the UK Home Office guidelines as outlined by Drummond (2009). The intrapulmonary artery was dissected out, cleaned of connective tissue and cut into rings, which were then used for enzymatic cell isolation. Cells were stored at \(+4^\circ C\) and used on the same day or cultured for up to 15 days under standard conditions.

**Composition of solutions**

For whole-cell recordings, the extracellular solution contained (mM): 150 NaCl, 1 CaCl\(_2\), 1 MgCl\(_2\), 10 glucose, 10 mannitol and 10 Hepes; pH was adjusted to 7.4 with NaOH. The pipette solution contained (mM): 130 CsCl, 10 EGTA, 1 MgCl\(_2\), 10 Hepes, 1 MgATP and 1.0, 6.0, 8.0 or 9.5 mM CaCl\(_2\) to obtain \( \sim 17, 225 \) or 600 nM or 1.5 \( \mu \)M free \( Ca^{2+} \), respectively (Caputo et al. 2008); pH was adjusted to 7.3 with NaOH. In some experiments, \( Cl^{-} \) was substituted by gluconate by replacing NaCl with equimolar sodium gluconate, and liquid junction potential was calculated (Neher, 1992) and corrected off-line.

**Small interfering RNA**

Small interfering RNAs (Sigma Aldrich, UK) directed against exon 15 or exon 18 of the rat TMEM16A gene were used in this study (Supplemental Table 1). As a negative control, a scrambled siRNA was used. Primary cultured PASMCs were transfected with 10 or 40 nM siRNA using N-TER (Sigma-Aldrich, Gillingham, Dorset, UK) according to the manufacturer’s instructions. Cells were used 72 h later for quantitative PCR (qPCR) or patch-clamp studies.

**Reverse transcriptase-PCR and RNA quantification**

Reverse transcriptase-PCR was used to assess the expression of the following RNAs: (1) rat and human TMEM16A; (2) all of the rat TMEM16 family members; and (3) rat TMEM16A splice variants. Primers were designed to amplify intron-spanning sequences (Supplemental Tables 2 and 3). The RT-PCR products were sequenced (GATC
biotech, Konstanz, Germany) to confirm the specificity of the reaction.

Real-time quantitative polymerase chain reaction (qPCR) was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and carried out with an ABI Prism® 7500 Sequence Detection System (Applied Biosystems). Relative TMEM16x mRNA levels were calculated using the threshold cycle (Ct) value and the 2−ΔΔCt method (Livak & Schmittgen, 2001).

Electrophysiology

All current recordings were performed with the whole-cell configuration of the patch-clamp technique. Currents were filtered at 2 kHz and sampled at 10 kHz, unless stated otherwise.

Current versus voltage relationships were constructed by measuring the current at the end of 1 s voltage steps from −80 to +100 mV in 10 mV increments, elicited every 5 s. Holding potential was 0 mV. Instantaneous tail current versus voltage relationships were constructed by measuring the tail current amplitude at each potential (from −80 to +130 mV in 10 mV increments; pulse duration 0.7 s) after a 1 s depolarizing step to +100 mV, elicited every 5 s from a holding potential of 0 mV. Tail currents at each potential were fitted with a single exponential function, and the instantaneous tail current amplitude was estimated from extrapolation of the fit to the beginning of the test pulse. The instantaneous tail currents (I(t)) were normalized against the current at the end of the prepulse (I(pp)) and plotted as a function of the test potential.

For non-stationary noise analysis (Heinemann & Conti, 1992) 20–100 identical pulses to a test potential of +100 mV (filtered at 6 kHz and sampled at 20 kHz) were applied, and the mean response, I, was calculated. The variance, σ², was computed from the averaged squared difference of consecutive traces. Background variance at 0 mV was subtracted and the variance-mean plot was fitted by:

$$\sigma^2 = i I - I^2/N$$  \hspace{1cm} (1)

with the single channel current, i, and the number of channels, N, as free parameters.

The maximal channel open probability (Po,max) was determined as:

$$P_{o,max} = I_{max}/Ni$$  \hspace{1cm} (2)

where I_max is the maximal current.

Data analysis

Data were analysed with self-written routines developed in the IGOR Pro (Wavemetrics, Lake Oswego, OR, USA) environment or using custom-written software (Dr M. Pusch, CNR, Genoa, Italy). Data are given as means ± S.E.M. Statistical significance was evaluated using a Student’s two-tailed t test and P < 0.05 taken to indicate a significant difference.

Results

Calcium-activated Cl⁻ currents in freshly isolated rat PASMCs

Pulmonary artery smooth muscle cells exhibit prominent K⁺ currents, including Ca²⁺-activated K⁺ currents. To suppress these endogenous conductances, we replaced K⁺ with Cs⁺ in our intracellular solutions. In the first set of experiments, cells were dialysed with intracellular solutions containing 17 or 600 nM free Ca²⁺ ([Ca²⁺]₀). Figure 1Aa shows that 600 nM [Ca²⁺]₀ activated an outward current that increased and stabilized about 200–260 s after rupturing the cell membrane (whole-cell configuration). The time course of this activation was assessed by measuring the current at the end of 2 s voltage ramps from −100 to +100 mV elicited at various time points (Fig. 1Ab). On average, the current increased by about twofold (from 16 ± 3 to 31 ± 4 pA pF⁻¹, n = 6) after 260 s in the whole-cell configuration. In contrast, whole-cell currents measured in the presence of 17 nM [Ca²⁺]₀ were much smaller, the current averaging 8 ± 1 pA pF⁻¹ (n = 6), and no significant time-dependent increase was observed on establishing the whole-cell configuration. These findings suggest that Ca²⁺ is essential for activation of an outwardly rectifying current.

In the presence of 17 nM [Ca²⁺]₀, very small currents were observed in response to depolarizing voltage pulses, while in the presence of 600 nM [Ca²⁺]₀, distinct outward currents and deactivating inward tail currents were observed in response to depolarizing steps from −80 to +100 mV followed by repolarization to −60 mV (Fig. 1B); similar results were obtained in the presence of an intermediate [Ca²⁺]₀ of 225 nM. The outward currents observed at 225 or 600 nM [Ca²⁺]₀ had two components: a small, instantaneous time-independent component, indicating channels that were open at the holding potential (0 mV), followed by a time-dependent current activation. The time-dependent components at various potentials were well fitted with a single exponential function. The time constant of activation (τ_a) increased mildly (∼twofold) with the voltage, being equal to 234 ± 33 ms (n = 11) at +30 mV versus 491 ± 62 ms (n = 11) at +90 mV in 600 nM [Ca²⁺]₀ (P < 0.05; Fig. 1Bb, inset). The tail currents measured upon repolarization to −60 mV were also well fitted with a single exponential that did not change with voltage over the range of +30 to +90 mV (Fig. 1Bb, inset). The time constant (τ_d) of the deactivating tail current was 108 ± 34 s (n = 11) when preceded by a prepulse to +30 mV and 101 ± 11 s (n = 11) when preceded by a prepulse to +90 mV.
Figure 1. Whole-cell Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents in rat PASMCs

Aa, whole-cell membrane currents recorded from single PASMCs in response to 2 s voltage ramps from −100 to +100 mV. Traces marked from a to e indicate currents recorded at 20, 50, 80, 200 and 260 s from rupturing the membrane (whole-cell configuration), respectively. Calcium ions were added to the intracellular solution as indicated. The dashed line represents the zero-current level. Each recording is representative of five others. Ab, time course of whole-cell current activation at +100 mV in the presence of 17 nM (filled circles, n = 6) or 600 nM [Ca\textsuperscript{2+}]\textsubscript{i} (open circles, n = 6). Current amplitude was obtained from ramp protocols as described in the main text. The continuous line through the open circles is the best fit to a single exponential function with a time constant of 96 s. The continuous line through the filled circles was drawn by eye.

Ba, whole-cell currents recorded in response to 1 s voltage pulses (prepulse) from −80 to +100 mV in 10 mV increments followed by 700 ms pulses to −60 mV, in a single PASMC. The cell was dialysed with a solution containing 600 nM [Ca\textsuperscript{2+}]\textsubscript{i}. The dashed line represents the zero-current level. Bb, mean current–voltage relationship measured at the end of the prepulse for cells dialysed with 17 (open diamonds, n = 9), 225 (filled circles, n = 5) or 600 nM [Ca\textsuperscript{2+}]\textsubscript{i} (open circles, n = 11). Inset shows mean time constants of current activation (filled triangles) and deactivation (open squares) in the presence of 600 nM [Ca\textsuperscript{2+}]\textsubscript{i} (n = 11).

C, mean rectification index determined as the ratio of the current measured at +80 and −80 mV with respect to [Ca\textsuperscript{2+}]\textsubscript{i} concentrations of 225, 600 and 1250 nM.
The current versus voltage relationship measured at the end of 1 s voltage steps from −80 to +100 mV in the presence of 600 nM [Ca\(^{2+}\)], (or 225 nM [Ca\(^{2+}\)]) exhibited a pronounced outward rectification (Fig. 1Bb), while in the presence of higher (1.25 μM) [Ca\(^{2+}\)]\(_{i}\), this relationship was almost linear (not shown). We quantified the degree of current rectification as the ratio between the current measured in response to 1 s voltage pulses to +80 and to −80 mV (rectification index, \(I_{80}/I_{-80}\)). In the presence of 225 or 600 nM [Ca\(^{2+}\)]\(_{i}\) the \(I_{80}/I_{-80}\) index was 4.9 ± 0.8 (\(n = 5\)) and 5.2 ± 1.0 (\(n = 11\)), respectively, but when [Ca\(^{2+}\)]\(_{i}\) was raised to 1.25 μM this changed to 1.5 ± 0.2 (\(n = 5\)) (\(P < 0.05\)), indicating a loss of outward rectification (Fig. 1C).

The reversal potentials of these Ca\(^{2+}\) -activated currents (∼0 mV) were near to the equilibrium potential for Cl\(^−\) in our ionic conditions. To assess the value of the reversal potential more precisely, we determined the instantaneous current versus voltage relationship in the presence of 600 nM [Ca\(^{2+}\)]\(_{i}\), by measuring the amplitude of tail currents (\(I_t\)) at various potentials, as described in the Methods. The instantaneous tail currents in five separate experiments were averaged and plotted as a function of the test potential (Fig. 1D). The fit of the data to the Goldman–Hodgkin–Katz equation yielded a value of −0.3 ± 0.1 mV (\(n = 5\)) for the reversal potential. Lowering the extracellular Cl\(^−\) concentration ([Cl\(^−\)]\(_o\)) (from 154 to 34 mM) by replacing Cl\(^−\) with gluconate resulted in a shift of the reversal potential to 26 ± 2 mV (\(n = 5\)). The reversal potential expected for a perfectly selective Cl\(^−\) channel is 33.7 mV. This small discrepancy may be attributed to a small permeability to gluconate of the CaCC channels in PASMCs. We conclude that these currents are \(I_{CaCC}\) because they are carried by Cl\(^−\), activated by Ca\(^{2+}\), show time-dependent activation and deactivation at depolarizing and hyperpolarizing potentials, respectively, and exhibit a loss of inward rectification in the presence of high [Ca\(^{2+}\)]\(_{i}\), as observed for \(I_{CaCC}\) in other cell types (Kuruma & Hartzell, 2000).

Finally, we used non-stationary noise analysis to determine the single-channel conductance of the CaCC in PASMCs (Supplemental Fig. 1). The average \(i\) (measured at +100 mV) in six separate experiments was 0.35 ± 0.06 pA, and \(P_{o,max}\) was 0.65 ± 0.06.

**TMEM16A is expressed in VSM**

The biophysical characteristics described above are broadly similar to those reported for heterologous TMEM16A currents (Caputo et al. 2008; Schroeder et al. 2008; Yang et al. 2008). We therefore set out to test whether TMEM16A is expressed in PASMCs and various other VSM cell types. Using RT-PCR with gene-specific primers we identified mRNA expression of TMEM16A in rat PASMCs, rat aorta and two VSM cell lines (A7R5 and A10 cells; Fig. 2A). We also assessed the presence of TMEM16A mRNA in human pulmonary arteries from two donors. Figure 2A illustrates that TMEM16A is expressed in human pulmonary arteries.

We used RT-PCR amplification to assess which TMEM16A splicing variants are expressed in rat PASMCs. The single band amplified by primers targeting the a and c segments indicated that these exons are constitutively expressed in rat PASMCs (\(n = 3\)). Primers targeting either b or d exons gave rise to two bands, indicating the presence of TMEM16A transcripts that either contain or lack these alternatively spliced exons in rat PASMCs (Fig. 2B). Thus, a combination of TMEM16A channel types are expected to be expressed in these cells.

We next examined whether other members of the TMEM16 family were expressed in rat PASMCs. Reverse transcriptase-PCR analysis demonstrated the presence of transcripts for TMEM16B, TMEM16F and TMEM16K, while TMEM16D and TMEM16E appeared to be expressed at very low levels and expression of the remaining members was not detected (\(n = 3\); Fig. 2C). We quantified the relative amounts of mRNA for TMEM16A, TMEM16B, TMEM16F and TMEM16K via qPCR but did not attempt a precise quantification of TMEM16D and TMEM16E because qPCR of transcripts expressed at very low level is unreliable due to low signal to background noise ratio. TMEM16B was found to be expressed at very low levels, 49 ± 21 (\(n = 3\)) times less than TMEM16A, while TMEM16A, TMEM16F and TMEM16K were expressed at approximately the same levels (Fig. 2D).

**TMEM16A protein produces \(I_{CaCC}\) in primary cultured PASMCs**

At present, TMEM16A and TMEM16B are the only TMEM family members that have been shown to form CaCCs; but, as described above, TMEM16B is expressed at very low levels in rat PASMCs. We therefore explored the possibility that TMEM16A mediates \(I_{CaCC}\) in these cells by using a siRNA approach. Primary cultured PASMCs were transfected with non-related...
(scrambled) siRNA or with one of two different siRNAs (TMEM16A-siRNAs) directed against exon 15 or exon 18 of the TMEM16A gene. Transfection of primary cultured rat PASMCs with 10 or 40 nM of TMEM16A-siRNA (exon 15) significantly ($P < 0.05$) reduced TMEM16A expression in a dose-dependent manner to $57 \pm 5$ ($n = 4$) and $30 \pm 1\%$ ($n = 7$) of non-transfected control cells, respectively (Fig. 3A). Similarly, TMEM16A-siRNA against exon 18 (40 nM) reduced TMEM16A expression to $16 \pm 9\%$ ($n = 3$) of non-transfected control cells. In contrast, in cells that were exposed to 10 or 40 nM scrambled siRNA the level of TMEM16A mRNA was unchanged ($n = 5$, $P > 0.05$; Fig. 3A).

The downregulation of TMEM16A expression was mirrored by a reduction in $I_{\text{CaCC}}$ (Fig. 3B). In cells treated with 40 nM TMEM16A-siRNA against exon 15 or exon 18 and studied with 600 nM [Ca$^{2+}$]$_i$, $I_{\text{CaCC}}$ at $+100$ mV was $16 \pm 5$ ($n = 10$) and $10.5 \pm 0.8$ pA pF$^{-1}$ ($n = 5$), respectively i.e. $\sim 84$ and $\sim 90\%$ less than the current measured in cells treated with the scrambled siRNA control ($102 \pm 22$ pA pF$^{-1}$, $n = 5$, $P < 0.05$; Fig. 3B).

In contrast, treatment with scrambled siRNA had

---

**Figure 2. TMEM16A is expressed in vascular smooth muscle**

A, RT-PCR analysis of TMEM16A in rat and human pulmonary artery (PA), rat aorta, A7R5 and A10 cells using cDNA samples (+) or the corresponding negative controls (−) as starting material. The expected product size was 305 bp. For rat aorta and PA, similar results were obtained from samples collected from a total of three animals; for human PA, similar results were obtained from samples obtained from a total of two donors; for A7R5 and A10 cells, similar results were obtained from samples obtained from a total of three separate batches. B, topology diagram of the TMEM16A protein illustrating the position of the alternative spliced exons (a, b, c and d; upper panel) and RT-PCR analysis of TMEM16A splice variants in rat PASMCs (lower panel). The expected product sizes are reported in Supplemental Table 2. Similar results were obtained from samples collected from a total of three rats. C, RT-PCR analysis of TMEM16 family members in rat PASMCs using cDNA samples (+) or negative controls as starting materials (−). The expected product size for each family member is reported in Supplemental Table 3. Similar results were obtained from samples obtained from a total of three rats. D, mean TMEM16x mRNA expression, measured via qPCR, relative to the expression of TMEM16A in rat PASMCs ($n = 3$).
no significant effect on $I_{CaCC}$ amplitude. The current density at $+100 \text{ mV}$ averaged $82 \pm 18$ ($n = 5$) and $102 \pm 22 \text{ pA} \text{pF}^{-1}$ ($n = 6$) for non-treated cells and cells exposed to 40 nM scrambled siRNA, respectively (Fig. 3Bb).

**Specificity of TMEM16A-directed siRNAs**

A potential problem associated with gene silencing mediated by siRNAs is the risk of non-specific effects due to knockdown of transcripts other than the intended one, owing to partial sequence complementarity between a transcript and the siRNA molecules used (Jackson & Linsley, 2010). We tested for potential unwanted target effects by using both an *in silico* and an experimental approach. We first searched the available databases for any sequence similarity between the siRNAs and unwanted targets. A search conducted on the Genbank database using the BLAST algorithm revealed that TMEM16A-siRNAs against either exon 15 or exon 18 did not present identity with any other TMEM16 family members. Furthermore, TMEM16A-siRNA against exon 18 did not present sequence identity with the sequence of any other ion channel gene, while the siRNA against exon 15 had 84% identity with the voltage-gated sodium channel $\alpha$-subunit 10a ($Scn10a$) gene and 68% with the transient receptor potential subfamily M, member 6 ($Trpm6$) gene. However, voltage-gated sodium currents are not present in pulmonary artery smooth muscle and Trpm6 is a Mg$^{2+}$-permeable channel that cannot account for the $\text{Cl}^{-}$ current seen in this study.

We then tested experimentally whether exposure of rat PASMCs to 40 nM TMEM16A-siRNAs (exon 15 and exon 18) affected the expression of TMEM16F or TMEM16K, by using qPCR. We detected no change in the level of expression of these TMEM16 family members compared with cells treated with 40 nM scrambled siRNA. However, treatment with 40 nM scrambled siRNA caused an overall ~20% reduction of the expression of these mRNAs (Fig. 3C, $n = 3$).

**Discussion**

The key finding of this paper is the observation that siRNA directed against TMEM16A resulted in a dramatic
reduction in mRNA level and concomitant reduction in $I_{\text{CaCC}}$ in rat PASMCs, while control (scrambled) siRNA had no effect. Since TMEM16A was found to be expressed in both rat and human PASMCs, as well as in several VSM cell types, it can be argued that this channel may be responsible for $I_{\text{CaCC}}$ in the smooth muscle of several vascular beds and across species.

**TMEM16A as the CaCC of rat PASMCs**

Our whole-cell experiments revealed an $I_{\text{CaCC}}$ in rat PASMCs with electrophysiological features similar to those reported for $I_{\text{CaCC}}$ in coronary artery, portal vein and other VSM cells (Leblanc et al. 2005). The time constant of current activation measured at $+70$ mV was 400 ms, compared with a range of 200–300 ms in rabbit pulmonary artery, coronary artery and portal vein (Greenwood et al. 2001). The $I_{\text{CaCC}}$ deactivation time constant was 108 ms, very close to the values measured in rabbit pulmonary artery, coronary artery and portal vein (90–100 ms range; Greenwood et al. 2001). These analogies suggest that common, or similar, ion channel proteins mediate $I_{\text{CaCC}}$ in VSM of different vascular beds.

The overall characteristics of rat PASMC $I_{\text{CaCC}}$ are similar to those of heterologous TMEM16A currents (Caputo et al. 2008; Schroeder et al. 2008; Yang et al. 2008). Both currents were potently activated by 225 or 600 nM $[\text{Ca}^{2+}]$, and exhibited comparable degrees of outward rectification (Caputo et al. 2008). Furthermore, the current kinetic properties are similar, the $I_{\text{CaCC}}$ deactivation time constant being in the 100 ms range in both cases (Caputo et al. 2008). In contrast, heterologous TMEM16B channels display much faster kinetics of current activation (4.4 ms at $+100$ mV) and deactivation (7.1 ms at $-100$ mV; Pifferi et al. 2009). Therefore, the kinetic characteristics of $I_{\text{CaCC}}$ in PASMCs closely resemble those of TMEM16A but not those of TMEM16B. Although rat PASMCs also express other TMEM16 family members, none of them has yet been shown to function as a CaCC.

The unitary conductance of CaCC in rat PASMC measured via noise analysis was $\sim3$ pS. A similar value was obtained from single-channel recordings in rabbit PASMCs and other VSM cell types (Large & Wang, 1996; Leblanc et al. 2005). This value is lower than the single-channel conductance of TMEM16A heterologously expressed in HEK cells ($\sim8$ pS; Yang et al. 2008). It is tempting to speculate that this discrepancy may reflect association of TMEM16A with one or more of the other TMEM16 family members found to be expressed in rat PASMCs. It could also reflect association with accessory subunits or post-translational modifications in native VSM cells. A precise comparison between native and heterologously expressed TMEM16A currents is greatly complicated by the fact that several splicing variants are present in rat PASMCs. Native currents in rat PASMCs could therefore arise from several TMEM16A channel types, possibly expressed at different levels. A recent report showed that the various TMEM16A splicing variants give rise to $I_{\text{CaCC}}$ with substantially different biophysical properties, including the degree of outward rectification, kinetics of activation and $\text{Ca}^{2+}$ sensitivity (Ferrera et al. 2009).

**Rat PASMCs express multiple TMEM16 family members**

We found that three members of the TMEM16 family are predominately expressed in rat PASMCs, namely TMEM16A, TMEM16F and TMEM16K. Furthermore, Gritli-Linde et al. (2009) reported expression of the same TMEM16 proteins along the walls of the dorsal aorta and of other blood vessels in mice. Thus, expression of TMEM16A, TMEM16F and TMEM16K may be ubiquitous in the vasculature. At present, TMEM16A is the only one of them that has been shown to function as a CaCC. The protein sequence of rat TMEM16F presents an overall identity of less than $\sim20\%$ with the sequence of rat TMEM16A. However, the degree of identity between TMEM16A and TMEM16F is much higher ($\sim55\%$) in the region comprising the fifth and sixth putative transmembrane domains and the loop in between them, which were suggested to form part of the pore of the channel (Yang et al. 2008). Furthermore, some of the residues of TMEM16A that are important for regulating channel selectivity (R701 and K725) are conserved in TMEM16F. These considerations may suggest that TMEM16F is capable of forming CaCCs. In contrast, the primary structure of rat TMEM16K presents much less similarity with TMEM16A (less than $20\%$). Furthermore, TMEM16K lacks a part (25 amino acids) of the putative pore-forming loop that includes R701, while lysine 725 is replaced with a serine. These considerations may indicate that TMEM16K has functions other than channel activity.

A recent study showed that tracheal epithelia from mice in which the TMEM16A gene was deleted (knockouts) had greatly reduced (of about 60%) CaCC activity (Rock et al. 2009). In this tissue, TMEM16J, TMEM16F and TMEM16K mRNAs were detected, and it was speculated that these TMEM family members may account for the small residual response to UTP observed in TMEM16A knockout mice. In rat PASMCs, however, we noted that treatment with 40 nM scrambled siRNA caused a $\sim20\%$ reduction of the expression of TMEM16F and TMEM16K but had no effect on $I_{\text{CaCC}}$ amplitude. This suggests that these two TMEM family members do not form CaCCs in rat PASMCs. Moreover, TMEM16F and TMEM16K are ubiquitously found (Galietta, 2009), even in tissues that do
not display CaCC activity. Thus, it is possible to speculate that they play some cellular functions other than CaCC.

Physiological significance

In VSM, [Cl\(^-\)], ranges between 30 and 60 mM (Large & Wang, 1996; Chipperfield & Harper, 2000; Leblanc et al. 2005). Thus, the reversal potential for Cl\(^-\) in VSM varies between \(-20\) and \(-30\) mV. As a consequence, activation of Cl\(^-\) conductances leads to membrane depolarization, increased Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels and ultimately enhanced contraction. It is unclear whether CaCCs contribute to the resting membrane potentials in VSM. The resting levels of [Ca\(^{2+}\)]\(_c\) in the cytoplasm vary between 40 and 140 nm, and only a small fraction of TMEM16A channels are expected to be active in those conditions. However, the observation that replacement of extracellular Cl\(^-\) with SCN\(^-\), an anion which is highly permeable through CaCCs, resulted in a pronounced outward current in rabbit PASMCs is consistent with the idea that CaCCs contribute to the resting membrane potential in this vascular bed (Hogg et al. 1993). The notion that conditions associated with an increase in [Cl\(^-\)]\(_c\), such as rat deoxycorticosterone acetate (DOCA)–salt hypertension, result in a more depolarized resting membrane potential also supports the idea that Cl\(^-\) conductances may contribute to the resting membrane potential in VSM (Davis et al. 1993).

TMEM16A channels are expected to play a role in a variety of pathological conditions that are associated with an increase in [Ca\(^{2+}\)]\(_c\), such as pulmonary hypertension or sepsis. Furthermore, upregulation of 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS)-sensitive chloride currents was associated with PASMC proliferation, which occurs in pulmonary hypertension (Liang et al. 2009). This is in agreement with the observation that TMEM16A is upregulated in cancer and may participate in cell proliferation (Hartzell et al. 2009).

The functional importance of TMEM16A in vivo will be best understood by studying cardiovascular changes in knockout mice. Such mice were generated prior to the discovery that TMEM16A functions as a CaCC (Rock et al. 2008). However, homozygous knockout mice die shortly after birth, probably due to malformation of tracheal cartilage rings (Rock et al. 2008). This short lifespan precludes the use of this model for studying the long-term effects of TMEM16A deletion on VSM function and blood pressure regulation. Thus, generation of tissue-specific knockouts for TMEM16A will be crucial for elucidating the role of TMEM16A channels in the vasculature.

In conclusion, our data suggest that TMEM16A appears to be at least an essential component of CaCCs in rat PASMCs.

References


**Author contributions**

P.T. designed the experiments; all authors performed the experiments and analysed the data; P.T. drafted the manuscript; and all authors critically reviewed and approved the final version. All experiments were carried out at the University of Manchester.

**Acknowledgements**

We thank Drs S. D. Singh and J. Plumb (University of Manchester, Northwest Lung Centre, Wythenshawe Hospital, Manchester) for providing human samples. We are grateful to Professor Alison Gurney, Dr Peter Brown and Professor David Eisner for critical reading of the manuscript. P.T. is a Research Council UK fellow. This work was supported by BBSRC, Wellcome Trust and Royal Society grants to P.T.
SUPPLEMENTAL MATERIAL

ADDITIONS TO THE METHODS

Cell isolation and culture
Artery segments were incubated in dissociation medium (DM*) containing 1.5 mg/mL papain (Sigma Aldrich, UK) for 1 h at 4°C. Dithiothreitol (1 mg) was subsequently added to the medium to catalyse papain activity and a 6 min incubation at 37°C followed. The tissue pieces were then transferred into a new vial containing 1.4 mg/mL collagenase (type IA, Sigma Aldrich, UK) and incubated at 37°C for 5 min. The tissue pieces were subsequently washed twice in fresh DM and gently triturated with a smoothed glass Pasteur pipette.

Cells were cultured under standard conditions in DMEM-F12 (1:1) medium supplemented with 10% foetal calf serum, 2 mM glutamine and 0.05 mg/100 ml gentamicin. Culture media were changed every 3–4 days and cells were split to 1/20 when reaching 80–90% confluence. Cultured cells were used within a maximum of 15 days from cell isolation. Each set of data was obtained from at least three different batches of cells obtained from separate animals. A7R5 cells and A10 cells were obtained from American Tissue Culture Collection (ATCC) and grown according to ATCC specifications.

*Composition of DM was (in mM): NaCl 110, KCl 5, HEPES 10, KH₂PO₄ 0.5, NaH₂PO₄ 0.5, NaHCO₃ 10, taurine 10, EDTA 0.5, D-glucose 10, CaCl₂ 0.16, MgCl₂ 2, phenol red 0.03, pH adjusted to 7.0 with NaOH.

RT-PCR and RNA quantification
The primers (Suppl.Table 2 and 3) were designed to amplify intron-spanning sequences using the Primer 3 v.0.4.0 software (Whitehead Institute and Howard Hughes Medical Institute, http://primer3.sourceforge.net/). When the sequence of a rat TMEM16 member was not available, primers were designed based on the mouse sequence. The PCR products obtained with these primers were sequenced and new primers were designed from the actual rat sequence obtained in this way.

The result of each RT-PCR experiment performed in this study was repeated with at least another different pair of primers and the results confirmed (not shown).

DNAase-treated total RNA was extracted from isolated blood vessels or ~90% confluent primary PASMCs using RNAeasy minikit (Qiagen, UK). First strand cDNA was synthesised using Superscript reverse transcriptase (SuperscriptTM III, Invitrogen, UK) and random hexamers (Invitrogen, UK). For each sample, 250-500 ng of total RNA were used. A reaction mix in which SuperscriptTM III had been omitted was used as negative control.
First-strand cDNA (1.5 µL) or its respective negative control were used as the template in a PCR reaction including forward and reverse primers (0.25 µM each) (MWG Biotech, Germany), 12.5 µL Hotstart Taq polymerase mastermix (Qiagen, UK) and DNase-free water up to a volume of 25 µL. To reveal the expression of TMEM16 family members or of the various TMEM16A splicing variants, a first step of 15 min at 94°C was followed by 35 repetitions of the following sequence: denaturation (94°C, 45 s), annealing (60°C, 45 s) and extension (72°C, 60 s); followed by final elongation stage (72°C, 10 min).

The various PCR products were analysed by electrophoresis with 1% (TMEM16 family members), 2% (for TMEM16A a, b and d variants) and 4% (for TMEM16A c segment) agarose gels containing ethidium bromide (0.01%). DNA was visualised by exposure to untraviolet light, while images were recorded with GelDoc2000 system (Bio-RAD, USA).

For qPCR, each PCR mix contained cDNA (10 µL) or negative control, 12.5 µL Sybr Green PCR Master Mix, forward and reverse primers (0.3 µM each) and DNase-free water up to a volume of 25 µL. An initial denaturation step of 10 min at 95°C was followed by 40 cycles of steps at 95°C (15 s) and 60°C (60 s). Each reaction was performed in triplicate. To assess the purity of the amplicon a melting curve analysis was performed: 95°C for 15 s followed by 60°C for 20 s and measurement from 60°C to 95°C every 0.5°C. These curves confirmed that there was only one PCR product (data not shown). Negative controls did not undergo significant amplification (data not shown).

Average threshold cycle (Ct) values were calculated for each sample in triplicate reactions using the SDS software (Applied Biosystem, USA). To account for any load difference, all samples were normalised for total cDNA input by subtracting Ct value obtained for a reference gene (glyceraldehyde 3-phosphate dehydrogenase (GAPDH)) from the Ct value for TMEM16x (ΔCt=Ct_TMEM16x-Ct_GADPH). Subsequently, ΔΔCt values for all the samples were calculated by subtracting the ΔCt value of each sample from the ΔCt value of a calibrator sample (non-transfected controls). The obtained ΔΔCt values were converted to fold differences by raising 2 to the power of -ΔΔCt value.

**Electrophysiology**

All patch-clamp current recordings were performed using a MultiClamp 700B or an Axopatch 200A amplifier controlled by pCLAMP8.02 (Axon Instruments, CA, USA) or Strathclyde Electrophysiology software, respectively. Pipettes were pulled from borosilicate glass capillaries and had resistances between 1 and 5 MΩ in the working solutions. Series resistance was usually compensated to achieve a maximal effective series resistance generally lower than 5-10 MΩ. To allow for equilibration of the pipette solution with the cell interior, recordings started 5 min after establishing the whole-cell configuration (with exception of the recording shown in Figure 1A, which started immediately on establishing the whole-cell configuration). Experiments were conducted at 20-22°C.
### SUPPLEMENTARY TABLES

**Table 1. siRNAs sequences.**

<table>
<thead>
<tr>
<th>siRNA target</th>
<th>Sequence (5'-'3')</th>
<th>Location on transcript</th>
</tr>
</thead>
<tbody>
<tr>
<td>rTMEM16A</td>
<td>GUCUUAGAGAAGUCACUGA</td>
<td>Exon 15</td>
</tr>
<tr>
<td>rTMEM16A</td>
<td>CCUAUACUCCCAUCUUCUA</td>
<td>Exon 18</td>
</tr>
</tbody>
</table>

**Table 2. Oligonucleotide primers used in PCR reactions for detection of rTMEM16A (NM_001107564.1) splicing variants.**

<table>
<thead>
<tr>
<th>Splicing variant</th>
<th>Primer pairs (5'-'3')</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
</table>
| a                | Fw: CACAAGAGAGCCTCGGTTAG  
Rw: ATCTTCACAAACCGACACC | 267                |
| b                | Fw: CAAAACCGGAGCACAATAG   
Rw: CAGGAGTTTCCTGTGTTGA | 175 or 241          |
| c                | Fw: CTCTGGGCTGCCACCTTC    
Rw: TGGCTTCTAATCTGCTCTGG | 118 or 130          |
| d                | Fw: TCCCAGAGCAGATATGAAGC  
Rw: AGATGAGGAGGAGTTCATGG | 230 or 305          |

F, forward primer; R, reverse primer; bp, base pair.
Table 3. Oligonucleotide primers used in PCR reactions for detection of various TMEM16 family members.

<table>
<thead>
<tr>
<th>gene</th>
<th>Primer pairs (5'-3')</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hTMEM16A</td>
<td>Fw : TCCAAAGACTTCTGGGCTGT Rw : TACTCGTAGCTGGGACTGG</td>
<td>305</td>
</tr>
<tr>
<td>rTMEM16A</td>
<td>Fw : TCCAAAGACTTCTGGGCGGT Rw : TAGGCACTGGCTGGGACTGG</td>
<td>305</td>
</tr>
<tr>
<td>rTMEM16B</td>
<td>Fw : CCAAGGAACAGAGTCTTTTG Rw : TGGTTGTCGAGAAGACAG</td>
<td>297</td>
</tr>
<tr>
<td>rTMEM16C</td>
<td>Fw : CCAAGACTACACTGGCCCT Rw : CAGCGTGCCCAGCGCTCATA</td>
<td>290</td>
</tr>
<tr>
<td>rTMEM16D</td>
<td>Fw : TGGCTTCGAGGGCCAAGGACA Rw : TGAGGCGGGTCGCTAGTC</td>
<td>319</td>
</tr>
<tr>
<td>rTMEM16E</td>
<td>Fw : ACCTGCAAGTACCCACCCCT Rw : ACTGCCCCATCGAGCGGGG</td>
<td>293</td>
</tr>
<tr>
<td>rTMEM16F</td>
<td>Fw : AGCCATCCTGGGGTGGTGA Rw : GCCCGGCGGTGGAGGGG</td>
<td>304</td>
</tr>
<tr>
<td>rTMEM16G</td>
<td>Fw : GCCCGGTGCTCTGGAAGGTGGG Rw : GCCGCGGCGGTGGAGGGG</td>
<td>327</td>
</tr>
<tr>
<td>rTMEM16H</td>
<td>Fw : AGCTCGCTCGCTCGAGGCGG Rw : GCAAGTCCGGTGCTGGCC</td>
<td>302</td>
</tr>
<tr>
<td>rTMEM16K</td>
<td>Fw : GGTACCCCGAGCCAAAATTG Rw : CCCACAGCAAGTGTAGTGGG</td>
<td>280</td>
</tr>
<tr>
<td>rTMEM16L</td>
<td>Fw : AGCTACCCCGAGCCAAAATTG Rw : CACATCGAGCGCGAC</td>
<td>324</td>
</tr>
<tr>
<td>rGAPDH</td>
<td>Fw : CACCAGCTACCCCCCCATTT Rw : CCATCAAGGACCCCCCTTCATT</td>
<td>180</td>
</tr>
</tbody>
</table>

F, forward primer; R, reverse primer; bp, base pair. h, human; r, rat.
Suppl. Figure 1. Non-stationary noise analysis of $I_{\text{CaCC}}$ in rat PASMC.

Current variance, $\sigma^2$, plotted versus the mean current, $I$, fitted with a parabola (smooth line) as described in Methods. The parameters obtained from the fit are: $i = 0.33$ pA, $N = 2676$, $P_{\text{slope}} = 0.60$. Similar results were obtained in a total of 6 patches.
Chapter 4

Publication II

The putative pore of TMEM16/Anoctamin channels controls gating and surface expression

In the process of re-submission
The putative pore of TMEM16/Anoctamin channels controls gating and surface expression

Aiste Tamuleviciute and Paolo Tammaro*

Faculty of Life Sciences, The University of Manchester, 46 Grafton Street, Manchester M13 9NT, United Kingdom.

*Address for correspondence: Dr Paolo Tammaro
Faculty of Life Sciences, University of Manchester
The Core Technology Facility
46 Grafton Street
M13 9NT, Manchester, United Kingdom
Tel. +44 (0)161 2751703
paolo.tammaro@manchester.ac.uk

Running title: Functional analysis of the pore-loop of TMEM16 channels
Subject category: Membranes & Transport, Signal Transduction
Total characters, 51,265; title, 85. Abstract 170 words.
ABSTRACT
The primary structure of the newly identified TMEM16/Anoctamin anion channels diverges substantially from other ion channels, and the structural features controlling anion permeation remain enigmatic. TMEM16A comprises eight putative transmembrane domains (TMs) with TM5-TM6 flanking a putative re-entrant loop resembling the pore of other channels. Using a chimeric approach the role of this region was investigated. Transfer of the pore-loop of non-conducting TMEM16 proteins into TMEM16A resulted in non-conducting channels, consistent with a role in anion permeation. It was also found that the pore-loop controls the magnitude of whole-cell current densities when transplanted between TMEM16 proteins. Unexpectedly, this was achieved via controlling the number of functional channels expressed on the membrane and not by open-channel conductance. A non-canonical trafficking motif within the TMEM16A pore-loop was identified. The pore-loop was, moreover, found to differentially influence the Ca$^{2+}$- and voltage-sensitivities of TMEM16A and TMEM16B channels. The results reveal that the pore of TMEM16 channels is a multifunctional region controlling the recruitment of channels on the cell membrane, as well as channel gating.
INTRODUCTION

Ca\(^{2+}\)-activated Cl\(^{-}\) channels (CaCCs) play key roles in a plethora of cellular functions. Cl\(^{-}\)-currents activated by Ca\(^{2+}\) were observed for the first time about three decades ago in *Xenopus* oocytes (Barish, 1983; Miledi, 1982), in the inner segment of the photoreceptor (Bader et al, 1982) and in lachrymal acinar cells (Marty et al, 1984). Since these early observations, CaCC currents have been detected in several other cell types of both animal and plant species. CaCCs are involved in processes as diverse as epithelial secretion, nociception, fertilisation and regulation of smooth muscle tone (Hartzell et al, 2005; Jentsch et al, 2002; Nilius & Droogmans, 2003). Because CaCCs are regulated by changes both in the intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) and in the membrane potential, they provide a link between Ca\(^{2+}\) signalling and cell electrical activity. Furthermore, expression of functional CaCCs on the plasma membrane, which modulates the whole-cell CaCC conductance, is regulated by chemical factors such as interleukins (Galietta et al, 2002) and cholesterol (Sones et al, 2010). There are also cell mechanisms that result in non-uniform distribution of CaCCs on the cell surface (French et al, 2010).

The genes coding for CaCCs were only recently identified as the *TMEM16/Anoctamin* family (Caputo et al, 2008; Schroeder et al, 2008; Yang et al, 2008). This gene family is composed of ten members. The electrophysiological properties of heterologously expressed TMEM16A/Anoctamin1, or of the closely related TMEM16B/Anoctamin2, directly resemble those of native CaCCs in terms of sensitivity to intracellular Ca\(^{2+}\), extent of outward rectification, ion selectivity and sensitivity to pharmacological agents (Duran & Hartzell, 2011; Flores et al, 2009; Huang et al, 2012a; Kunzelmann et al, 2011b; Scudieri et al, 2011). It is unclear whether other members of the TMEM16/Anoctamin family form CaCCs (Duran & Hartzell, 2011; Galietta, 2009; Kunzelmann et al, 2011b; Scudieri et al, 2011). Furthermore, different TMEM16A splice variants give rise to channels with unique biophysical properties (Caputo et al, 2008; Ferrera et al, 2009). Studies involving knock-out mice or RNA silencing technology have provided further evidence that TMEM16A and TMEM16B are essential components of CaCCs in several cell types (Billig et al, 2011; Manoury et al, 2010; Rock et al, 2009; Thomas-Gatewood et al, 2011).

The membrane topology for TMEM16 proteins predicted from hydropathy analysis involves eight TMs with intracellular N- and C- termini (Caputo et al, 2008;
Importantly, the predicted topology of eight TMs has been experimentally confirmed for TMEM16G/Anoctamin7 (Das et al, 2008). So far, reports on the structure-function relationship of TMEM16 channels are few. The voltage sensing region of some types of voltage-gated channels, such as voltage-gated sodium and potassium channels, was first identified by analysis of their primary structure to be a series of basic residues (arginines or lysines) within the “S4” domain (Hille, 2001). The sequence of TMEM16A and TMEM16B do not present equivalent putative voltage-sensing regions. However, a series of four/five glutamic acids in the first intracellular loop of TMEM16A and TMEM16B appear to contribute to the voltage sensitivity of the channel (Cenedese et al, 2012; Xiao et al, 2011).

The location of the Ca\(^{2+}\) binding site has also not been fully elucidated, but a series of four residues (EAVK) in the first intracellular loop appear to contribute to the Ca\(^{2+}\) sensitivity of TMEM16A (Xiao et al, 2011). Other studies have proposed that the N-terminus may participate in Ca\(^{2+}\) binding, directly or via binding of calmodulin at this site (Ferrera et al, 2009; Tian et al, 2011). Recently, the region between TM5 and TM6 of TMEM16A was proposed to bear a Ca\(^{2+}\) binding site (Yu et al, 2012). Thus, the portions of the channel involved in Ca\(^{2+}\) binding and transduction of Ca\(^{2+}\)-binding into channel opening may involve several residues in different parts of the channel. The involvement of an auxiliary subunit cannot, however, be ruled out. The Ca\(^{2+}\)-sensitivity of TMEM16A channels is dependent on the permeant anion, suggesting that permeation and gating are coupled in TMEM16A channels (Xiao et al, 2011).

A key question is what structural features of TMEM16 channels contribute to anion permeation. The region between the TM5 and TM6 of TMEM16A is predicted to be a re-entrant domain, and re-entrant loops are common features of ion channel pores. Furthermore, mutations in this region (R621E, K645E and K668E) have been reported to alter the ion selectivity of TMEM16A (Yang et al, 2008). Interestingly, a recent study from the Hartzell group challenges the view that the region forms a re-entrant loop from the extracellular side, and proposes an inverted topology instead (Figure 1B in Yu et al., 2012).

In this study, the function of the putative pore-loop was investigated by constructing chimeras involving TMEM16A and other TMEM16 proteins. The chimeric strategy
using homologous, but functionally dissimilar, proteins has been used extensively to identify primary sequence elements associated with particular functions. The TMEM16 proteins used for chimeras in this study were chosen according to the following criteria: i) TMEM16A and TMEM16B because they reportedly function as CaCCs, but have different electrophysiological properties, including Ca\(^{2+}\) sensitivity and kinetics of activation (Duran & Hartzell, 2011; Flores et al, 2009; Huang et al, 2012a; Kunzelmann et al, 2011b; Scudieri et al, 2011); ii) TMEM16 proteins that are unable to conduct Cl\(^{-}\) or do not give rise to prominent whole-cell Cl\(^{-}\) current, such as TMEM16F, TMEM16G and TMEM16K (Duran & Hartzell, 2011; Kunzelmann et al, 2011b; Scudieri et al, 2011), with the rationale that they may not possess conducting pores. All these proteins have different degrees of homology with TMEM16A within the pore-loop: TMEM16B (68%) > TMEM16F (48%) > TMEM16G (41%) > TMEM16K (13%).

The initial aim of this study was to determine the anion conduction properties of the putative pore-loop. Unexpectedly, we found that the “pore” of TMEM16 channels has additional roles. It regulates the expression of these channels on the plasma membrane and participates in their regulation by voltage and intracellular Ca\(^{2+}\). Thus, the putative pore-loop of TMEM16 channels is a functionally critical region that integrates diverse roles of these Ca\(^{2+}\)- and voltage- operated channels.

RESULTS

Comparison of the electrophysiological properties of TMEM16A and TMEM16B channels

A side by side comparison of the TMEM16A and TMEM16B currents has never been reported. This study began by directly comparing the capacity of heterologously expressed TMEM16A and TMEM16B to mediate Ca\(^{2+}\)-activated Cl\(^{-}\) currents, using whole-cell and inside-out patch-clamp of transfected HEK-293T cells.

1) Whole-cell current amplitudes:

Figure 1A shows that in the absence of intracellular Ca\(^{2+}\) the currents recorded from cells transfected with TMEM16A or TMEM16B were indistinguishable from the very small currents observed in non-transfected cells over a wide range of membrane potentials. When cells were dialysed with an intracellular solution containing a physiological [Ca\(^{2+}\)], (274 nM), hyperpolarising or depolarising steps elicited instantaneous, time-independent TMEM16A or TMEM16B currents, followed by time-dependent relaxations towards
new steady state levels. For TMEM16A and TMEM16B the average instantaneous current *versus* voltage relationships were linear, but the relationship between the membrane potential and steady-state currents measured at the end of a 1 s voltage steps was outwardly rectifying (Figure 1B, C). The steady-state currents at negative potentials were smaller while those at positive potentials were larger than the instantaneous currents. In agreement with previous reports (Cenedese et al, 2012; Xiao et al, 2011), the extent of outward rectification of TMEM16A and TMEM16B currents appeared to diminish as [Ca$^{2+}$]$_i$ was raised (Supplementary Figure 1). Although outward rectification was observed for both TMEM16A and TMEM16B, the magnitude of the time-dependent current component at each voltage differed between the two channels. Expressing this phenomenon as the ratio between the steady-state current and the instantaneous current at +100 mV ($I_{ss}/I_{inst}$), gives fraction of 5.4±0.8 (n=7) for TMEM16A and 3.0±0.1 (n=8) for TMEM16B (p<0.05).

An electrophysiological parameter that clearly differed between TMEM16A and TMEM16B was the current density. In the presence of 274 nM [Ca$^{2+}$]$_i$, whole-cell currents generated by TMEM16A at +100 mV were approximately six times larger (739±127 nA/pF; n=7) than currents mediated by TMEM16B (116±14 nA/pF; n=8) (Figures 1B, C).

2) *Kinetics of the currents*:

Figure 1 suggests that the rate of the current activation of TMEM16A and TMEM16B differs. The time-course of the rise in current was quantified as the time required to reach the half maximal current ($\tau_{0.5}$). As the voltage increased, $\tau_{0.5}$ slightly increased, equalling 88±8 ms (n=7) at +40 mV and 120±16 ms (n=7) at +100 mV for TMEM16A channels (Figure 1B, inset) (p<0.05). TMEM16B currents activated ~15 times more rapidly than TMEM16A: $\tau_{0.5}$ was 5.3±0.5 ms (n=8) at +40 mV and 8.4±0.5 ms (n=8) at +100 mV (Figure 1C, inset) (p<0.05). The tail currents measured upon repolarisation to -60 mV were quantified in the same way. For both channels, $\tau_{0.5}$ did not show a significant change with voltage over the range of +40 mV to +100 mV (insets in Figure 1B, C). The $\tau_{0.5}$ for the deactivating tail current was 41±4 ms (n=7, TMEM16A) and 2.5±0.1 ms (n=8, TMEM16B) when preceded by a prepulse to +40 mV and 55±5 (n=7, TMEM16A) and 3.4±0.6 (n=8, TMEM16B) when preceded by a prepulse to +100 mV. Thus, the time necessary to respond to depolarisation or hyperpolarisation differed between TMEM16A and TMEM16B by more than an order of magnitude.
Figure 1 (A) Whole-cell currents recorded from a non-transfected HEK-293T cell or from HEK-293T cells expressing TMEM16A or TMEM16B in the presence of 0 or 274 nM \([Ca^{2+}]_i\), as indicated. Dashed horizontal lines represent the zero-current level. Voltage protocol is shown in the upper left panel. (B) Mean whole-cell current density versus voltage relationships measured at the beginning (Inst.) or at the end (Steady state) of 1 s voltage pulses from -100 to +100 mV in 20 mV increments, for HEK-293T cells expressing TMEM16A in the presence of 274 nM \([Ca^{2+}]_i\) (n=7). Mean whole-cell currents obtained from non-transfected (NT) HEK-293T cells ([Ca^{2+}]_i=274 nM) (n=5), and from transfected cells in 0 [Ca^{2+}]_i (n=5) were measured only at the end of voltage pulses. (C) Mean whole-cell current density versus voltage relationships for TMEM16B (n=8). Experimental conditions were as described in B. Insets in B and C show mean \(\tau_{0.5}\) of current activation (filled symbols) and deactivation (open symbols) for TMEM16A and TMEM16B, measured in the presence of 274 nM \([Ca^{2+}]_i\) at various membrane potentials (n=7-8).

3) Voltage-sensitivity:
The voltage-dependence of TMEM16A and TMEM16B channels was studied in more detail by constructing conductance versus voltage relationships at various \([Ca^{2+}]_i\) (Figure 2A, B). These curves provide a measure of the change in channel open probability \((P_o)\)
as a function of the voltage. Table 1 reports the parameters ($V_{0.5}$ and $z$, as defined in Methods) obtained from the Boltzmann fit of these relationships. $V_{0.5}$ progressively shifted to more negative values as the $[\text{Ca}^{2+}]_i$ was increased from 274 nM to 2.27 µM. For both channels, $V_{0.5}$ values were reduced by 180-200 mV as $[\text{Ca}^{2+}]_i$ was increased to ~1 µM (Figure 2 and Table 1). For both channels, the values of $z$ did not change as $[\text{Ca}^{2+}]_i$ was varied.

4) $\text{Ca}^{2+}$-sensitivity:
To analyse the $\text{Ca}^{2+}$-sensitivity of TMEM16A and TMEM16B channels, $[\text{Ca}^{2+}]_i$-response relationships were constructed by measuring currents at a constant potential while varying $[\text{Ca}^{2+}]_i$ (Figure 2C, D). Table 2 reports the parameters ($EC_{50}$ and $h$, as defined in Methods) obtained from the Hill-Langmuir fit of these relationships. The slope factor $h$ was the same for TMEM16A and TMEM16B channels (~2.7-2.8). In contrast, the $EC_{50}$ differed by ~40%, with TMEM16A being more sensitive to $\text{Ca}^{2+}$ than TMEM16B.
Figure 2 (A) Left panel: tail currents recorded from an inside-out patch excised from a HEK-293T cell expressing TMEM16A, in the presence of 605 nM $[\text{Ca}^{2+}]_i$. The stimulation protocol is shown above. Horizontal dashed line indicates the zero-current level. Right panel: mean normalised TMEM16A conductance $\nu$s voltage relationships obtained in the presence of 274, 605 or 1040 nM $[\text{Ca}^{2+}]_i$, as indicated (n=9).

(B) Left panel: tail currents recorded from an inside-out patch excised from a HEK-293T cell expressing TMEM16B in the presence of 605 nM $[\text{Ca}^{2+}]_i$, in response to the stimulation protocol shown in A. Horizontal dashed line indicates the zero-current level. Right panel: mean normalised TMEM16B conductance versus voltage relationships obtained in the presence of 274, 605, 1040 or 2270 nM $[\text{Ca}^{2+}]_i$, as indicated (n=7).

(C) Currents recorded from inside-out patches excised from HEK-293T cell expressing TMEM16A or TMEM16B in response to various $[\text{Ca}^{2+}]_i$ (µM), as indicated. The voltage was maintained at +70 mV for the whole duration of the recordings.

(D) Mean relationships between $[\text{Ca}^{2+}]_i$ and the current measured at +70 mV and normalised to the maximal response for TMEM16A (n=5) and TMEM16B (n=6). The error bars (mean±SEM) are hidden within the symbols. Statistical analysis is provided in Table 2. The smooth curves are the best fits to the data with eq. 4.

5) Conduction properties:

Non-stationary noise analysis was used to determine the single-channel current ($i$) of TMEM16A and TMEM16B (Figure 3). The average $i$ measured at +70 mV from the
The relationship between the mean current and the variance around the mean in five separate experiments was 0.23±0.01 and 0.27±0.01 pA for TMEM16A and TMEM16B, respectively. This corresponds to a single channel conductance of 3.2±0.2 and 3.8±0.1 pS for TMEM16A and TMEM16B, respectively (p<0.05).

**Figure 3** Non-stationary noise analysis for heterologously expressed TMEM16A and TMEM16B currents. (A) Mean TMEM16A current and variance around the mean obtained from 165 current traces recorded in response to 1.5 s pulses to +70 mV followed by 1 s repolarizations to -60 mV in the presence of 274 nM [Ca$^{2+}$]. Horizontal dashed lines represent the zero-current or zero-variance level, respectively. (B) Current variance plotted against the mean current for the experiment shown in A. The parabolic line is the best fit of eq. 5 to the data. The single-channel current, $i$, calculated from the fit was 0.24 pA. (C) Mean TMEM16B current and variance around the mean obtained from 200 current traces recorded in response to the stimulation protocol described in A. Horizontal dashed lines represent the zero-current or zero-variance level. (D) Current variance plotted against the mean current for the experiment shown in B. The parabolic line is the best fit of eq. 5 to the data. The single-channel current, $i$, calculated from the fit was 0.27 pA.

The relative anion permeability and conductance of TMEM16A and TMEM16B channels were also determined. Figures 4A and 4B show typical current versus voltage...
relationships recorded in the presence of extracellular Cl\(^-\), I\(^-\) or SCN\(^-\). Figure 4C reports the selectivity sequence of TMEM16A and TMEM16B for several anions, while Figure 4D shows the mean chord conductance for the same anions. No significant difference was seen between TMEM16A and TMEM16B in either of these properties. The relative order of selectivity (\(P_x/P_{Cl}\)) for TMEM16A and TMEM16B was SCN\(^-\) (thiocyanate) > ClO\(_4\)\(^-\) (perchlorate) > I\(^-\) (iodide) > NO\(_3\)\(^-\) (nitrate) > N\(_3\)\(^-\) (azide) > Cl\(^-\) (chloride) >> gluconate. The relative conductance (\(G_x/G_{Cl}\)) sequence for both channels was: N\(_3\)\(^-\) > SCN\(^-\) > I\(^-\) > NO\(_3\)\(^-\) > ClO\(_4\)\(^-\) > Cl\(^-\) >> gluconate.

**Figure 4** (A) Whole-cell currents recorded from a HEK-293T cell expressing TMEM16A in the presence of 274 nM [Ca\(_{2+}\)]\(_i\) and different extracellular anions, as indicated. Dashed horizontal lines indicate zero-current levels. For the currents recorded in the presence of Cl\(^-\) only traces every 20 mV are shown for clarity. The stimulation protocol is shown above. (B) Instantaneous currents (obtained from traces in panel A) plotted versus the voltage. (C) Mean relative anion selectivity (\(P_x/P_{Cl}\)) for TMEM16A (n=6-12) and TMEM16B (n=6-9) channels. (D) Mean relative anion conductance (\(G_x/G_{Cl}\)) for TMEM16A (n=6-12) and TMEM16B (n=6-9) channels.

To summarise, the first set of experiments illustrates that TMEM16A and TMEM16B share a range of electrophysiological characteristics including the fact that, under the experimental conditions used: i) Ca\(_{2+}\) is mandatory for TMEM16A and TMEM16B channel activity and ii) both channels display the same degree of permeability to various anions. However, TMEM16A and TMEM16B also differ in a range of properties including: i) the magnitude of the time-dependent current increase observed in response
to depolarising voltage steps; ii) the kinetics of activation and deactivation; iii) the overall \( \text{Ca}^{2+} \)- and voltage-sensitivity and iv) the magnitude of the whole-cell currents (current density).

**Effects of chimeric constructs involving the putative pore-loop of TMEM16A and TMEM16B**

To investigate the role of the putative pore-loop of TMEM16A and TMEM16B channels, chimeric constructs were engineered in which the putative pore-loop of TMEM16A was substituted with that of TMEM16B (TMEM16A-B) and *vice versa* (TMEM16B-A). The exact regions transferred in these chimeras are listed in the Supplementary Table 1. As with wild-type channels, in the absence of \( \text{Ca}^{2+} \) no currents were observed in the -100 to +100 mV range in cells expressing TMEM16A-B or TMEM16B-A (unpublished data). When the \( [\text{Ca}^{2+}]_i \) was raised to 274 nM, prominent outwardly rectifying currents became apparent (Figure 5A). At +100 mV, the current density for TMEM16A-B and TMEM16B-A was 123±27 (n=7) and 734±94 (n=8), respectively. The whole-cell current densities for TMEM16A-B and TMEM16B-A were equal to those observed for TMEM16A and TMEM16B, respectively (Figure 5B).
Whole-cell currents recorded from HEK-293T cells expressing TMEM16A, TMEM16B, TMEM16A-B or TMEM16B-A, as indicated. Currents were elicited by 1 s voltage pulses from -100 to +100 mV in 20 mV increments followed by 0.5 s steps to -60 mV in the presence of 274 nM \([\text{Ca}^{2+}]_i\). Dashed horizontal lines represent the zero-current level. Diagrams above electrophysiological traces are schematic illustrations of the membrane topology of TMEM16 proteins (wild-type, chimeras). TMEM16A and TMEM16B are represented in blue and red, respectively (B) Mean whole-cell current density versus voltage relationships for TMEM16A (n=7), TMEM16B (n=8), TMEM16A-B (n=7) and TMEM16B-A (n=8). Data for TMEM16A and TMEM16B are re-plotted from Figure 1.

Visual inspection of Figure 5A suggested that TMEM16A-B currents increased more slowly in response to depolarising voltage pulses compared to TMEM16B-A currents. We therefore quantitatively compared the electrophysiological properties of wild-type and chimeric whole-cell currents elicited by depolarising pulses to +100 mV followed by a 0.5 s hyperpolarisation to -60 mV (Figure 6A). Parameters measured were \(I_{ss}/I_{inst}\) (Figure 6B), the fraction (in %) of the small residual current at the end of the hyperpolarising (tail) pulse relative to the steady-state current at +100 mV (\(I_{tail}/I_{100}\)) (Figure 6C) and the \(\tau_{0.5}\) of activation at +100 mV (Figure 6D). For TMEM16A-B and TMEM16B-A channels, all three parameters were either indistinguishable from, or very close to the values measured from TMEM16A and TMEM16B, respectively (Figure 6). The \(\tau_{0.5}\) was slightly hastened (by ~44 ms) for TMEM16A-B channels with respect to TMEM16A and slower (by ~16 ms) for TMEM16B-A channels relative to TMEM16B.
(p<0.05). Thus, the putative pore-loop of TMEM16A and TMEM16B appears to control the magnitude of current density and influence the rate of current activation.

The next series of experiments aimed to determine what causes changes in whole-cell current density. A whole-cell macroscopic ionic current ($I$) is the product of:

$$I = iNP_o,$$  \[1\]
where $N$ is the number of functional channels present in the membrane. Thus, differences in macroscopic current densities observed for wild-type and chimeric channels must be due to a change in at least one of the parameters of equation 1.

$i$ – Single channel current

Figure 3 shows that the single-channel current of TMEM16A and TMEM16B channels is almost identical. Thus a change in $i$ cannot underlie the changes in current densities described in Figure 5.

$P_o$ – Sensitivity of chimeric channels to voltage and Ca$^{2+}$

The dependence of the $P_o$ on voltage for chimeric channels was assessed as shown in Figure 2. Interestingly, $z$ and $V_{0.5}$ parameters for TMEM16A-B were the same as those obtained for TMEM16B (Figure 7A, C and Table 1). For TMEM16B-A, $V_{0.5}$ was the same as that of TMEM16A, but the $z$ parameter resembled that of TMEM16B (Figure 7B, C and Table 1). Importantly, there was no change in the fraction of TMEM16A, TMEM16B, TMEM16A-B and TMEM16B-A currents at potentials $\leq$100 mV in the presence of 274 nM [Ca$^{2+}$]. This implies that the different current densities reported in Figure 5 cannot be due to a differential response of wild-type and chimeric channels to voltage.

The putative pore region of TMEM16A has been shown to bear a Ca$^{2+}$ binding site (Yu et al, 2012). We therefore explored whether the chimeric channels display an altered Ca$^{2+}$-sensitivity. Figure 7D indicates that $EC_{50}$ and $h$ of TMEM16A-B channels are identical to those of TMEM16A (see also Table 2). In contrast, TMEM16B-A maintained the $h$ of TMEM16B, but the Ca$^{2+}$-response curve was shifted to the left with $EC_{50}$ being reduced by $\sim$1 µM (Figure 7E and Table 2). Importantly, at 274 nM [Ca$^{2+}$], there was no significant difference between the fraction of current observed for wild-type (TMEM16A and TMEM16B) and chimeric (TMEM16A-B and TMEM16B-A) channels, which was in all cases $\sim$2% of the maximum current observed at [Ca$^{2+}$] $\geq$12.5 µM.

Thus, the differences in TMEM16A, TMEM16B, TMEM16A-B and TMEM16B-A current densities cannot be due changes in $P_o$ because in the presence of 274 nM [Ca$^{2+}$], all these channels display the same degree of sensitivity to both voltage and Ca$^{2+}$.
together with the observation that TMEM16A and TMEM16B share a very similar single-channel conductance, these data imply that the different current densities of TMEM16A, TMEM16B, TMEM16A-B and TMEM16B-A channels are due to a distinct number of functional channels on the plasma membrane (i.e. N).

**Figure 7** (A) Mean normalised TMEM16A-B conductance versus voltage relationships obtained in the presence of 274, 605, 1040 or 2270 nM $[\text{Ca}^{2+}]_i$, as indicated (n=5). (B) Mean normalised TMEM16B-A conductance versus voltage relationships obtained in the presence of 274, 605 or 1040 nM $[\text{Ca}^{2+}]_i$, as indicated (n=5). (C) Mean $V_{0.5}$ and (D) mean $z$ parameters for TMEM16A (n=9), TMEM16B (n=7), TMEM16A-B (n=5) and TMEM16B-A (n=5) assessed in the presence of 1040 nM $[\text{Ca}^{2+}]_i$. Asterisks indicate significant difference (p<0.05). (D) Mean relationships between $[\text{Ca}^{2+}]_i$ and inside-out TMEM16A-B currents normalised to the maximal response measured at +70 mV (n=6). (E) Mean relationships between $[\text{Ca}^{2+}]_i$ and inside-out TMEM16B-A currents normalised to the maximal response measured at +70 mV (n=6). The smooth curves in D and E represent the best fits of the data with eq. 4. Dashed curves in D and E are the fits of the data for TMEM16A and TMEM16B re-plotted from Figure 2.
Table 1. Parameters (V_{0.5} and z) obtained from the Boltzmann fit of TMEM16A, TMEM16B, TMEM16A-B and TMEM16B-A conductance versus voltage relationships at various [Ca^{2+}].

<table>
<thead>
<tr>
<th>[Ca^{2+}], nM</th>
<th>274</th>
<th>605</th>
<th>1040</th>
<th>2270</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMEM16A V_{0.5} (mV)</td>
<td>247±6° (n=7)</td>
<td>131±6° (n=9)</td>
<td>68±6° (n=9)</td>
<td>N/A</td>
</tr>
<tr>
<td>z</td>
<td>1.0±0.1° (n=7)</td>
<td>1.0±0.1° (n=9)</td>
<td>1.1±0.1° (n=9)</td>
<td>N/A</td>
</tr>
<tr>
<td>TMEM16B V_{0.5} (mV)</td>
<td>343±12 (n=7)</td>
<td>229±15 (n=7)</td>
<td>143±12 (n=7)</td>
<td>29±9 (n=7)</td>
</tr>
<tr>
<td>z</td>
<td>1.8±0.1° (n=7)</td>
<td>1.8±0.1° (n=7)</td>
<td>1.6±0.1° (n=7)</td>
<td>1.9±0.1° (n=7)</td>
</tr>
<tr>
<td>TMEM16A-B V_{0.5} (mV)</td>
<td>313±18 (n=5)</td>
<td>196±21 (n=5)</td>
<td>133±15 (n=5)</td>
<td>30±17 (n=5)</td>
</tr>
<tr>
<td>z</td>
<td>1.7±0.2° (n=5)</td>
<td>1.4±0.1° (n=5)</td>
<td>1.6±0.2° (n=5)</td>
<td>1.8±0.2° (n=5)</td>
</tr>
<tr>
<td>TMEM16B-A V_{0.5} (mV)</td>
<td>222±7 (n=5)</td>
<td>129±2 (n=5)</td>
<td>53±7 (n=5)</td>
<td>N/A</td>
</tr>
<tr>
<td>z</td>
<td>1.4±0.2° (n=5)</td>
<td>1.6±0.1° (n=5)</td>
<td>1.7±0.1° (n=5)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

* and ° indicate statistically significant difference from TMEM16A and TMEM16B, respectively.

Table 2. Parameters (EC_{50} and h) obtained from the Hill-Langmuir fit of TMEM16A, TMEM16B, TMEM16A-B and TMEM16B-A [Ca^{2+}]- response relationships

<table>
<thead>
<tr>
<th></th>
<th>TMEM16A</th>
<th>TMEM16A-B</th>
<th>TMEM16B</th>
<th>TMEM16B-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC_{50} (µM)</td>
<td>1302±33 * (n=5)</td>
<td>1304±69 * (n=6)</td>
<td>1860±53 * (n=6)</td>
<td>881±40 * * (n=6)</td>
</tr>
<tr>
<td>h</td>
<td>2.7±0.1 (n=5)</td>
<td>2.7±0.2 (n=6)</td>
<td>2.8±0.1 (n=6)</td>
<td>2.5±0.1 (n=6)</td>
</tr>
</tbody>
</table>

* and ° indicate statistically significant difference from TMEM16A and TMEM16B, respectively.

N – The pore loop of TMEM16A and TMEM16B as a regulator of channel trafficking

The fact that chimeric TMEM16A-B and TMEM16B-A channels give rise to altered current density suggests that the putative pore-loop may contain a motif/s that control channel trafficking. Analysis of the sequence of the putative pore-loop of TMEM16A and TMEM16B indicated that this region contains areas of complete sequence identity, while for two stretches of sequence the percentage of identity is only 32-44% (Figure 8A). These are a region of nine (608-616 in TMEM16A) and 38 (656-693 in TMEM16A) amino acids, respectively. Thus, we constructed chimeras where only the region of nine or 38 amino acids of TMEM16B was inserted into TMEM16A. We termed these chimeric constructs TMEM16A-9-B and TMEM16A-38-B, respectively.
Both constructs gave rise to functional channels when transfected in HEK-293T cells (Figure 8B). Figure 8C shows that in the presence of 274 nM \([\text{Ca}^{2+}]\), TMEM16A-9-B and TMEM16A-38-B were associated with current density identical to that of TMEM16A and TMEM16B, respectively. Thus, a region of 38 amino acids within the putative pore-loop of TMEM16A contains elements that control trafficking of functional channels to the plasma membrane.

**Figure 8 (A)** Sequence alignment of the putative pore-loop of TMEM16A (599-705) and TMEM16B (644-750) proteins. Asterisks indicate residues that are identical for the two channels. Boxes indicate the regions of nine and 38 residues that exhibit substantial difference between TMEM16A and TMEM16B channels. (B) Whole-cell currents recorded from HEK-293T cells expressing TMEM16A-B-9 and TMEM16A-B-38 chimeric channels in response to the voltage protocol shown in Figure 1A and in the presence of 274 nM \([\text{Ca}^{2+}]\). Dashed horizontal lines represent the zero-current level. (C) Mean whole-cell current density versus voltage relationships for TMEM16A (n=7), TMEM16B (n=8), TMEM16A-B-9 (n=5) and TMEM16A-B-38 (n=4), as indicated. Data for TMEM16A and TMEM16B are re-plotted from Figure 1.

**Expression of chimeras involving the putative pore-loop of TMEM16F, TMEM16G or TMEM16K**

In a final series of experiments, the capacity of the putative pore-loop to conduct anions was examined by engineering chimeric constructs in which the putative pore-loop of TMEM16A is substituted with the equivalent region of non-conducting TMEM16 protein (e.g. TMEM16F, TMEM16G and TMEM16K) (Duran & Hartzell, 2011; Kunzelmann et al, 2011b; Scudieri et al, 2011). These constructs were termed TMEM16A-F, TMEM16A-G and TMEM16A-K, respectively. Figure 9A shows that all these chimeric constructs when expressed in HEK-293T cells did not give rise to whole-cell currents in response to voltages as high as +80 mV.
Whole-cell recordings in the presence of 78.1 µM [Ca^{2+}], were also performed to test if the lack of TMEM16A-F, TMEM16A-G and TMEM16A-K currents may be due to impaired sensitivity to Ca^{2+}. In the presence of 78.1 µM [Ca^{2+}], the current density for TMEM16A-F, TMEM16A-G and TMEM16A-K was 3.4±1.0 pA/pF (n=4), 3.4±0.4 pA/pF (n=4) and 4.0±0.7 pA/pF (n=4), respectively (non-significant to current density from non-transfected cells, 3.8±0.7 pA/pF (n=5)) (unpublished data).

Another possible explanation for the lack of whole-cell currents is that these chimeric channels are not trafficked to the plasma membrane. Immunocytochemical assays were performed to assess the surface expression of TMEM16A-F, TMEM16A-G and TMEM16A-K channels compared to TMEM16A channels (Figure 9B). New constructs were engineered in which an HA-tag was inserted in five different positions within the putative extracellular loops of TMEM16A (Supplementary Figure 2). One position was identified that did not affect the current amplitude while being effectively detectable in immunocytochemistry experiments (see Supplementary information). All chimeras were therefore tagged in this position. Figure 9C shows that ~30% of TMEM16A channels in transfected HEK-293T cells were expressed on the plasma membrane. The fraction of TMEM16A-F and TMEM16A-G on the plasma membrane was much smaller (6-8%), while for TMEM16A-K this fraction was below detectable levels. Thus, heterologously expressed TMEM16A-F and TMEM16A-G are trafficked to the plasma membrane but are unable to conduct Cl⁻ ions.
Chapter 4

Figure 9 (A) Mean whole-cell current density recorded from non-transfected HEK-293T cells and cells expressing TMEM16A, TMEM16A-F, TMEM16A-G or TMEM16A-K measured at +80 mV in the presence of 274 nM [Ca$^{2+}$]$_i$ (n=5-7). (B) Epifluorescence images of non-transfected HEK-293T cells or cells expressing HA-tagged TMEM16A, TMEM16A-F, TMEM16A-G or TMEM16A-K, as indicated (see Supplementary information for details). Anti-HA antibodies were visualised with Alexa Fluor 596-labeled secondary antibodies (red) in non-permeabilised (Cell surface) or permeabilised (Total) conditions, as indicated. For each construct images were acquired using identical acquisition settings. (C) Mean cell surface staining expressed as a percentage of the total staining for all HA-tagged constructs (n=35-73). Asterisks indicate significant difference to TMEM16A (p<0.05).

DISCUSSION

The key finding of this paper is the observation that the putative pore-loop of TMEM16A and TMEM16B plays a number of functional roles: 1) it is involved in controlling anion permeability, and unexpectedly 2) regulates the expression of functional channels on the membrane and 3) contributes to the regulation of these channels by Ca$^{2+}$ and voltage.

*TMEM16A and TMEM16B share electrophysiological similarities*

TMEM16A and TMEM16B are the two most closely related members of the TMEM16 family and have an overall sequence identity of ~58% with homology being higher within the putative transmembrane segments. These channels also share some
electrophysiological characteristics. For both channels, the response to depolarising pulses consists of two components: instantaneous currents followed by time-dependent current relaxations. The instantaneous current is mediated by channels that are open at the holding potential (Cenedese et al., 2012; Scudieri et al., 2011). Usually, the conductance of an open channel is almost constant at various voltages, except when the ionic concentrations at the two sides of the membrane are largely asymmetrical or in cases of voltage-dependent block of the ion channel pore (Hille, 2001). Thus, the relationship between the instantaneous TMEM16A and TMEM16B currents and the voltage was linear (this study and Cenedese et al., 2012). The strong outward rectification of the steady-state current versus voltage relationship (Figure 1 and Supplementary Figure 1) that we observed at \([\text{Ca}^{2+}]_i < 1 \mu\text{M}\) is therefore the result of the modulation of the channel (TMEM16A or TMEM16B) \(P_o\) by voltage (Cenedese et al., 2012; Ferrera et al., 2009).

The single-channel conductance of TMEM16A and TMEM16B differed by only \(~0.6\) pS. Both TMEM16A and TMEM16B were permeable to various anions (Pifferi et al., 2009; Schroeder et al., 2008; Yang et al., 2008). The degree of permeability and conductance varied depending on the anion but the \(P_x/P_{Cl}\) and \(G_x/G_{Cl}\) sequences were the same for both channels. The ion permeability is a measure of the ability of an ion to enter into the channel pore, while the conductance is an indication of the energy required for the ion to pass through the whole length of the pore (Halm & Frizzell, 1992; Lauger, 1973; Qu & Hartzell, 2000). Thus, the fact that \(P_x/P_{Cl}\) and \(G_x/G_{Cl}\) sequences do not coincide indicates that the processes of the ion entering the channel pore and passing through it are unequally favourable. This may be the result of ion binding within the pore. We demonstrated that TMEM16A and TMEM16B display identical \(P_x/P_{Cl}\) and \(G_x/G_{Cl}\) sequences. This implies that the regions within the pore-loop that differ between TMEM16A and TMEM16B do not contribute to anion selectivity and conductance.

The putative pore-loop of TMEM16 channels as a regulator of channel trafficking

The trafficking of ion channels is controlled by a variety of factors, including association with auxiliary subunits, ubiquitin ligases, as well as interactions with other membrane receptors (such as G protein coupled receptors) (Schwappach, 2008; Simms & Zamponi, 2012). Several classes of specific amino acid motifs within membrane proteins have been identified that regulate the export/retention from/within the endoplasmic reticulum (Ma & Jan, 2002). For ion channels these motifs are usually present at the N- or C-termini
(Ma & Jan, 2002; Schwappach, 2008; Simms & Zamponi, 2012). Here we show that a region of 38 amino acids within the putative pore-loop of TMEM16A regulates the number of channels expressed on the plasma membrane. A role for the pore of an ion channel in the regulation of trafficking is not unprecedented. For example, pore residues of K\textsuperscript{+} channels participate in channel trafficking (Manganas et al, 2001; Zhu et al, 2005). The sequence of the putative pore region of TMEM16A and TMEM16B does not contain canonical trafficking motifs. The region of 38 amino acids that we have identified in TMEM16A includes recognition sites for protein kinase C and casein kinase 2 that have been implicated in the regulation of ion channel trafficking, including CFTR (Luz et al, 2011), but these sites appear to be conserved in TMEM16B. Thus, they cannot be responsible for the differential current density associated with the two channels. This suggests the presence of a non-canonical trafficking motif/s within the 38 amino acid stretch that we have identified.

**The putative pore-loop of TMEM16A and TMEM16B regulates channel Ca\textsuperscript{2+}- and voltage-sensitivity**

We (this study) and others (Scudieri et al, 2011) have shown that TMEM16A and TMEM16B differ in their sensitivity to Ca\textsuperscript{2+}. A recent study shows that the putative pore-loop of TMEM16A may form a part of the Ca\textsuperscript{2+} binding site (Yu et al, 2012). Specifically, evidence has been provided that two glutamates (702 and 705) are directly involved in Ca\textsuperscript{2+} binding (Yu et al, 2012). It is noteworthy that these glutamates are conserved in TMEM16B. This may indicate that the binding site for Ca\textsuperscript{2+} in TMEM16 channels involves additional residues. The \([\text{Ca}\textsuperscript{2+}]\)-response curves for TMEM16A and TMEM16B are characterised by the same Hill coefficient (equal ~3) but different \(EC_{50}\). \([\text{Ca}\textsuperscript{2+}]\)-response curves for native CaCC also are characterised by a Hill coefficient ~3 (e.g. Kuruma & Hartzell, 2000). A Hill coefficient > 1 is consistent with the idea that more than one Ca\textsuperscript{2+} binds to the channel to produce channel opening. \([\text{Ca}\textsuperscript{2+}]\)-response curves for chimeric channels had this same slope factor. However, unlike TMEM16A-B channels that had Ca\textsuperscript{2+}-sensitivity identical to that of TMEM16A, TMEM16B-A channels were much more sensitive to Ca\textsuperscript{2+} than TMEM16B. This may suggest that the elements involved in Ca\textsuperscript{2+} sensing differ between TMEM16A and TMEM16B and that Ca\textsuperscript{2+} binding site would involve regions outside the putative pore-loop.

TMEM16A and TMEM16B channels differed in their regulation by voltage. The Boltzmann fit of the conductance *versus* voltage relationships revealed that the values of
$z$ for TMEM16A and TMEM16B differed by a factor ~2, but $z$ did not change as $[Ca^{2+}]_i$ was increased. Thus, $Ca^{2+}$ does not alter the coupling between voltage and channel opening in both TMEM16A and TMEM16B. The progressive leftward shift of $V_{0.5}$ as $[Ca^{2+}]_i$ was increased suggests that $Ca^{2+}$ reduces the activation energy required for channel opening to a similar extent for TMEM16A and TMEM16B channels. Transfer of the pore of TMEM16B into TMEM16A resulted in channels with voltage dependent properties identical to those of TMEM16B, while the complementary chimera (TMEM16B-A) preserved the sensitivity to voltage of TMEM16B. The results indicate that the contribution of the putative pore-loop to channel voltage gating is different between the two channels.

*Evidence that the putative pore-loop participates to anion permeation*

We showed that TMEM16A chimeras involving the putative pore-loop of TMEM16 proteins that are unable to conduct $Cl^-$ are non-conductive. This is consistent with the idea that the pore-loop is part of the permeation pathway (Yang et al, 2008; Yu et al, 2012). Experiment involving cysteine accessibility scanning have recently identified residues within the putative pore-loop of TMEM16A that compose part of the permeation pathway (C625, G628, G629, C630, L631, M632, I636, Q637) (Yu et al, 2012). These residues are all conserved in TMEM16B. The capacity of TMEM16 proteins to conduct anions have been examined by various groups (Duran & Hartzell, 2011; Kunzelmann et al, 2011b; Scudieri et al, 2011). TMEM16G exists in two isoforms: a long (934 amino acids) plasma membrane protein and a short (179 amino acids) soluble cytoplasmic protein (Bera et al, 2004). In heterologous expression systems, plasma membrane TMEM16G did not mediate CaCC currents or iodide uptake (Duran et al, 2012; Scudieri et al, 2011). There is some controversy regarding the capacity of TMEM16F to act as CaCC. Indeed, TMEM16F has been shown to i) mediate phospholipids scramblase activity (Suzuki et al, 2010), ii) be a non-selective cation channel (Yang et al, 2011) and iii) act as an outwardly rectifying chloride channel of intermediate single-channel conductance (Martins et al, 2011). In heterologous expression systems, we (unpublished data) and others (Duran et al, 2012; Scudieri et al, 2011) detected no CaCC activity associated with TMEM16F. TMEM16K lacks a part (25 amino acids) of the putative pore-loop and has been reported to be an intracellular protein (Duran et al, 2012). Therefore, TMEM16K may be expected to have functions other than CaCCs. Indeed, we (unpublished data) and other groups (Duran et al, 2012;
Scudieri et al, 2011) found that heterologous expression of TMEM16K did not result in CaCC currents.

Chimeras involving the putative pore region of TMEM16F or TMEM16G spliced into TMEM16A gave rise to proteins that reached the plasma membrane, but did not generate CaCC currents. This was not due to the fact that these chimeras have an impaired sensitivity to Ca\(^{2+}\), because even at very high [Ca\(^{2+}\)], (>70 µM) no CaCC current was detected. Chimeras involving the pore of TMEM16K were not detected on the plasma membrane. This is consistent with the notion that TMEM16K may be an intracellular protein (Duran & Hartzell, 2011; Duran et al, 2012) and that the putative pore-loop of TMEM16 protein is a regulator of channel trafficking.

**Membrane topology of TMEM16A**

We inserted HA epitopes in five different positions in three putative extracellular loops (first, second and last) of TMEM16A. We identified positions for HA-tagging in the second and the last extracellular loops that were accessible to extracellular antibodies. This is consistent with the predicted topology of TM3, TM4, TM7 and TM8. These results, combined with the finding from a recent paper (Yu et al, 2012) that determined the accessibility of extracellular loop 3 and 4, provide experimental confirmation of the predicted membrane topology of TMEM16A between TM3-5 and TM7-8. The same published work suggests that part of the putative pore-loop is exposed to the intracellular side of the membrane (Figure 1B in Yu et al., 2012). Our observation that the pore-loop contributes to Ca\(^{2+}\) sensitivity is consistent with the “inverted topology” model of the pore-loop (Yang et al, 2008; Yu et al, 2012).

**Biological significance**

CaCCs are present in many cell types and their activation may have opposite effects on cell electrical activity, depending on the value of the reversal potential for Cl\(^-\) (E\(_{Cl}\)). Furthermore, E\(_{Cl}\) can be spatially and temporally regulated within cells (Hartzell et al, 2005). For example, in vascular smooth muscle, E\(_{Cl}\) varies between -20 and -30 mV (Chipperfield & Harper, 2000; Large & Wang, 1996). Thus, activation of TMEM16A channels leads to membrane depolarisation, increased Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels and ultimately enhanced contraction. In contrast, in some neurons, such as hippocampal neurons, E\(_{Cl}\) is as negative as ~-70 mV and opening of CaCC channels leads to hyperpolarisation and suppression of cell electrical activity (Huang et al, 2012b).
The amount of Cl\(^{-}\) that leaves or enters the cell through CaCC channels depends on the functional properties of channels and the number of channels expressed on the plasma membrane. Indeed, CaCC expression is altered in pathological conditions (Liang et al, 2009; Wang et al, 2012).

Here we have identified a first structural element that regulates the number of functional TMEM16 channels on the membrane. Future work will be needed to elucidate the precise cellular and molecular mechanisms that regulate CaCC trafficking in various cell types and to determine how alterations in this process lead to human and animal disease.

**MATERIALS AND METHODS**

Details of the cell culture and patch-clamp recordings are provided in the online Supplementary Information.

*Molecular biology and cell transfection*

Mouse TMEM16A (Genbank NM_178642), TMEM16B (NM_153589.2), TMEM16F (NM_175344), TMEM16G (BC116706.1) and TMEM16K (BC099688) all subcloned into pcDNA3.1 vector were used in this study. TMEM16 chimeras were constructed using PCR mutagenesis strategy for sequence swapping between related genes (Kirsch & Joly, 1998). HA-tags were inserted in the putative extracellular loops of TMEM16 proteins using inverse PCR mutagenesis (Gama & Breitwieser, 1999).

*Immunocytochemistry*

To visualise and quantify the expression of the various TMEM16 proteins on the plasma membrane, immunocytochemistry experiments were performed on transiently transfected HEK-293T cells. Permeabilised or non-permeabilised conditions were used to detect either the all cell proteins or only membrane-spanning proteins, respectively. Experiments were conducted 48 hours after transfection at +4°C unless stated otherwise. Cells were washed with PBS and incubated with 2.5 µg/mL anti-HA tag antibody (Abcam; ab9110) in PBS (1 hour). Cells were then fixed with 4% (w/v) paraformaldehyde (15 min) and free aldehydes neutralised with 0.1 M glycine (10 min). Primary antibodies were visualised with 2 µg/mL Alexa 594-conjugated donkey anti-rabbit IgG antibody (Invitrogen; 1 hour, room temperature). For detection of intracellular epitopes, after fixation cells were blocked and permeabilised with 4% donkey serum and 0.1% saponin, respectively (1 hour, room temperature). Cells were incubated with
2.5 µg/mL anti-HA tag antibodies (Abcam; ab9110) with 4% donkey serum and 0.1% saponin in PBS (3 hours, room temperature). Primary antibodies were detected with 2 µg/mL Alexa 594-conjugated donkey anti-rabbit IgG antibody (Invitrogen) in PBS with 4% donkey serum (1 hour, room temperature). Images were collected on the Olympus BX51 upright microscope maintaining identical settings and analysed using ImageJ software. The fraction of TMEM16 proteins expressed on the plasma membrane was expressed as the ratio of the average fluorescence measured from at least 35 cells in non-permeabilised and permeabilised conditions.

Electrophysiology

**Composition of solutions**: The extracellular solution contained (mM): 150 NaCl, 1 CaCl2, 1 MgCl2, 10 glucose, 10 D-mannitol and 10 HEPES; pH was adjusted to 7.4 with NaOH. The intracellular solution contained (mM): 130 CsCl, 10 EGTA, 1 MgCl2, 10 HEPES and 8.0 mM CaCl2 to obtain 274 nM free [Ca2+], pH was adjusted to 7.3 with NaOH. For intracellular solutions with higher [Ca2+]i, EGTA was replaced with equimolar HEDTA and 2.1, 3.1, 4.8, 7.8 or 9 mM CaCl2 were used to obtained in 605, 1040, 2270, 12460 and 78070 nM free [Ca2+], respectively (calculated with Patcher’s Power tool, Dr. Francisco Mendez and Frank Würriehausen, Max-Planck-Institut für biophysikalische Chemie, Göttingen, Germany). In anion selectivity experiments, the extracellular solution was composed of (mM): 150 NaX, 0.1 CaCl2, 10 glucose and 10 HEPES, where X= Cl-, SCN-, NO3-, I-, ClO4-, N3- or gluconate; pH was adjusted to 7.4 with NaOH and osmolarity was adjusted to 320 mOsmol/kg with D-mannitol. Liquid junction potentials were calculated (Barry & Lynch, 1991; Neher, 1992) and corrected off-line.

**Protocols**: Currents were recorded from transfected HEK-293T cells using the whole-cell or inside-out configuration of the patch-clamp technique. Current versus voltage relationships were constructed by measuring the current at the beginning (instantaneous) or at the end (steady state) of 1 s voltage steps from -100 mV to +100 mV in 20 mV increments, elicited every 3 s. Holding potential was 0 mV. Membrane current densities were calculated by dividing the current for the cell capacitance.

The voltage dependencies of TMEM16A and TMEM16B channels were determined by constructing conductance (G) versus voltage relationships in the presence of various [Ca2+]. A 1 s pre-pulse applied to different voltage (from -100 to +180 mV in 40 mV
increments) was followed by a 0.5 s tail pulse to -60 mV. Tail currents at each potential were fit with a single exponential function, and the instantaneous tail current amplitude was estimated from extrapolation of the fit to the beginning of the test pulse and $G$ calculated as $G = I/(V_m - E_{Cl})$. Normalised $G$ ($G/G_{\text{max}}$) was plotted against the voltage of the pre-pulse. Under these conditions, the amplitude of the $G/G_{\text{max}}$ is directly proportional to the channel $P_o$ at the voltage of the pre-pulse (Bezanilla, 2000; Tammaro et al, 2005). The constructed relationships were fit with the Boltzman equation of the form:

$$G/G_{\text{max}} = 1/[1 + \exp\left(\frac{V_m - V_{0.5}}{zF/RT}\right)]$$

where $z$ is the number of gating charges moving through the entire applied field during channel activation, $V_{0.5}$ is the voltage at which the $G$ is half maximal and it is associated with the conformational energy required for the channel to open, $F$ is Faraday's constant, $R$ is the universal gas constant and $T$ is the absolute temperature.

Relative anion permeabilities of TMEM16A and TMEM16B were assessed by determining the shift in the reversal potential ($E_{\text{rev}}$) of the currents when extracellular Cl$^-$ (154 mM) was replaced with an equimolar concentration of other monovalent anions (X). The permeability ratio was estimated using the following equation (Hille, 2001):

$$P_x/P_{Cl} = \exp(\Delta E_{\text{rev}} F/RT)$$

where $\Delta E_{\text{rev}}$ represents the difference of the $E_{\text{rev}}$ for the anion X relative to the $E_{Cl}$. To determine the $E_{\text{rev}}$ in the presence of different anions, tail current versus voltage relationships were constructed by measuring the tail current amplitude ($I_t$) at each voltage (from -60 mV to +60 mV in 10 mV increments; pulse duration 0.5 s) after a 1 s depolarising steps to + 70 mV, elicited every 3 s from a holding potential of 0 mV. Instantaneous $I_s$s at each voltage were determined from single exponential fit of the current as describe above. $I_s$s were plotted as a function of the voltage steps. The relative chord conductance was measured between in the interval of ±25 mV around the $E_{\text{rev}}$. The [Ca$^{2+}$]-response relationships were fit with the Hill-Langmuir equation of the form:
\[ I / I_{\text{max}} = 1 / [1 + \text{[Ca}^{2+}]_i / EC_{50} ]^h \]  \hspace{1cm} [4]

where, \( I \) is the current measured at a given \([\text{Ca}^{2+}]_i\), \( I_{\text{max}} \) is the current measured at the highest \([\text{Ca}^{2+}]_i\), \( EC_{50} \) is the half maximal \([\text{Ca}^{2+}]_i\), and \( h \) is the slope factor (Hill coefficient).

For non-stationary noise analysis (Heinemann & Conti, 1992; Tammaro & Ashcroft, 2007) 50-200 identical pulses to a test potential of +70 mV (filtered at 10 kHz and sampled at 50 kHz) were applied and the mean response, \( I \), was calculated. The variance, \( \sigma^2 \), was computed from the average squared difference of consecutive traces. Background variance at 0 mV was subtracted and the variance-mean plot was fitted by:

\[ \sigma^2 = iI - I^2 / N \]  \hspace{1cm} [5]

with the single channel current, \( i \), and the number of channels, \( N \), as free parameters.

**Data analysis**

Data were analyzed with self-written routines developed in the IgorPro (Wavemetrics, OR, USA) environment or using Ana (http://users.ge.ibf.cnr.it/pusch/programs-mik.htm). Student's two-tailed \( t \) test or ANOVA with Bonferroni's post-test were used for statistical analysis as appropriate and \( p<0.05 \) was considered significant. Data are given as mean±SEM.

**ACKNOWLEDGEMENTS**

We thank Prof Alison Gurney and Dr Peter Brown for helpful comments on this work and Dr Annukka Lehtonen for reading the manuscript. We are grateful to Mr Adam Seed for his assistance in the preliminary experiments of this work and to Mr Keith Smith for outstanding technical assistance. P.T. holds a Research Council (RCUK) Fellowship. This research is supported via a BBSRC PhD studentship to A.T. and a BBSRC New Investigator Award (BB/H000259/1) to P.T.
AUTHOR CONTRIBUTIONS
Author contributions: P.T. initiated the project and designed the research; A.T. and P.T. designed the experiments; A.T. did the experiments; A.T. and P.T. analysed the data and wrote the paper. All experiments were carried out at the University of Manchester.

CONFLICT OF INTEREST
The authors declare that they have no conflict of interest.
REFERENCE LIST


Bezanilla F (2000) The voltage sensor in voltage-dependent ion channels. Physiological reviews 80(2): 555-592


Duran C, Qu Z, Osunkoya AO, Cui Y, Hartzell HC (2012) ANOs 3-7 in the anoctamin/Tmem16 Cl$^{-}$ channel family are intracellular proteins. American journal of physiology 302(3): C482-C493


Huang WC, Xiao S, Huang F, Harfe BD, Jan YN, Jan LY (2012b) Calcium-activated chloride channels (CaCCs) regulate action potential and synaptic response in hippocampal neurons. *Neuron* **74**(1): 179-192


Yang H, Jin T, Cheng T, Jan YN, Jan LY (2011) Scan: a novel small-conductance Ca\textsuperscript{2+}-activated non-selective cation channel encoded by TMEM16F. *Biophysical journal* **100**: 259a


ADDITIONS TO THE METHODS

Cell Culture and Transfection

Human embryonic kidney 293T (HEK-293T) cells were cultured in DMEM-F12 (1:1) supplemented with 10% foetal calf serum, 2 mM L-glutamine and 0.05 mg/100 ml gentamicin at 37°C in a humidified atmosphere with 5% CO₂. Solutions were changed every 3–4 days and cells were split to 1/20 when they reached 80–90% confluence. For patch-clamp experiments, HET-293T cells were plated in 35 mm Petri dishes at density of ~10000 cells/cm² and transfected with 1 µg of TMEM16 and 0.1 µg of CD8 constructs using Fugene HD (Roche) according to manufacturer’s instructions and used 12-48 hours after transfection. Transfected cells were visualized using the anti-CD8 antibody-coated beads method (Jurman et al., 1994). Experiments aimed at comparing the current density (e.g. Figure 1) were conducted 48±3 hours after transfection. For immunofluorescence, HEK-293T cells were plated at a density of ~10000 cells/cm² on poly-D-lysine coated glass coverslips and transfected with 2 µg of HA-tagged TMEM16 constructs.

Electrophysiology

All patch-clamp current recordings were performed using an Axopatch 200B amplifier controlled by GePulse software (http://users.ge.ibf.cnr.it/pusch/programs-mik.htm) with an analog-to-digital and digital-to-analog converter (USB-6221, National Instruments). Pipettes were pulled from borosilicate glass capillaries and had resistances between 1 and 2.5 MΩ in the working solutions. In whole-cell recordings, series resistance was usually compensated by ≥70 % to achieve a maximal effective series resistance generally lower than ~5 MΩ. To allow for equilibration of the pipette solution with the cell interior, recordings started 5 min after establishing the whole-cell configuration. Experiments were conducted at 20-22°C. The bath was grounded through a 3 M KCl agar bridge connected to a Ag–AgCl reference electrode. Rapid exchange of solutions was achieved by using a local perfusion system consisting of eight tubes of 1.2 mm diameter in which the tip of the patch pipette was inserted.
ADDITION TO THE RESULTS

Rectification properties of TMEM16A and TMEM16B currents in the presence of varying intracellular Ca$^{2+}$

Previous studies indicate that native CaCC current (e.g. (Manoury et al., 2010; Qu and Hartzell, 2000) as well as cloned TMEM16A (Yu et al., 2012) and TMEM16B (Cenedese et al., 2012) currents present an almost linear steady-state current versus voltage relationship in the presence of [Ca$^{2+}$]$_i$ > 10 µM. We assessed the relationship between [Ca$^{2+}$]$_i$ and the degree of current rectification by using the inside-out configuration of the patch-clamp technique for both TMEM16A and TMEM16B. The extent of current rectification was quantified as the ratio between the steady-state current measured in response to 1 s voltage pulses to +70 mV and to -70 mV (rectification index: $I_{+70}/I_{-70}$). Supplementary Figure 1 shows that as [Ca$^{2+}$]$_i$ was increased from 0.6 to 78 µM the $I_{+70}/I_{-70}$ index varied from 17.2±1.9 to 0.7±0.1 for TMEM16A (n=5) (p<0.05) and from 6.0±0.3 to 0.7±0.1 for TMEM16B (n=5) (p<0.05), indicating a loss of outward rectification. Supplementary Figure 1 indicates that the rate of TMEM16A and TMEM16B current activation in response to depolarising pulse to +70 mV is hastened as [Ca$^{2+}$]$_i$ is increased and the currents become almost instantaneously activated at [Ca$^{2+}$]$_i$ ≥ 12.5 µM.
Supplementary Figure 1  (A) Currents recorded from inside-out patches excised from HEK-293T cells expressing TMEM16A or TMEM16B in response to 0.7 s voltage steps to +70 mV and -70 mV, as indicated. Holding potential was 0 mV. Records were obtained in the presence of various \([\text{Ca}^{2+}]_i\), as indicated. (B) Mean rectification index \(I_{+70}/I_{-70}\) versus \([\text{Ca}^{2+}]_i\) relationships for TMEM16A (n=5) and TMEM16B (n=5) channels. (C) Mean \(\tau_{0.5}\) of current activation versus \([\text{Ca}^{2+}]_i\) relationships for TMEM16A (n=5) and TMEM16B (n=5) channels. Asterisks indicate significance difference to TMEM16A (p<0.05).

**Determination of the putative pore-loop and extracellular loops in TMEM16 proteins via bioinformatics tools**

The putative topology of TMEM16 proteins was determined using a range of programs: 1) PHDhtm (Rost et al., 1995), 2) TMHMM (Krogh et al., 2001); 3) TopPred (Claros and von Heijne, 1994); 4) DAS (Cserzo et al., 1997); 5) TMPred (Hofmann and Stoffel, 1993) and 6) TOPCONS (Bernsel et al., 2009). A recent report indicates that a bioinformatic analysis of the primary structure of TMEM16A with these and other programs produces somewhat different outputs (Yu et al., 2012). We examined the capacity of each program to predict the topology of TMEM16G, which has been determined experimentally (Das et al., 2008). PHDhtm and TOPCONS predicted that TMEM16G has eight TMs, which is in the agreement with the experimental data. In contrast, TopPred, TMPred and DAS predicted at least 10 TMs and TMHMM suggested the existence of only seven transmembrane segments. Thus, we used PHDhtm and TOPCON to identify i) the location of the putative pore-loop (i.e. the region between...
TM5 and TM6) of TMEM16A, TMEM16B, TMEM16F, TMEM16G, TMEM16K and ii) putative extracellular loops of TMEM16A.

Supplementary Table 1 provides the assigned accession number for the various TMEM16 proteins used in this study and the predicted location of their putative pore-loops. The first, second and last putative extracellular loops for TMEM16A are at positions 355-406, 511-535 and 780-856, respectively.

**Supplementary Table 1. Location of the putative pore-loop of various TMEM16 proteins used in this study.**

<table>
<thead>
<tr>
<th>Protein</th>
<th>TMEM16A</th>
<th>TMEM16B</th>
<th>TMEM16F</th>
<th>TMEM16G</th>
<th>TMEM16K</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accession number</td>
<td>NP_848757.3</td>
<td>NP_705817.2</td>
<td>NP_780553.2</td>
<td>NP_996914.1</td>
<td>NP_598740.1</td>
</tr>
<tr>
<td>Putative pore-loop</td>
<td>599-705</td>
<td>644-750</td>
<td>574-674</td>
<td>552-651</td>
<td>423-504</td>
</tr>
</tbody>
</table>

The location of the putative pore-loop is expressed as the position it occupies in the primary structure.

*Insertion of hemagglutinin (HA) epitopes in the extracellular loops of TMEM16A*

We inserted HA tags in five different positions in predicted extracellular loops (first, second and the last) as well as at the N-terminus of TMEM16A (Supplementary Figure 2A). Each tagged channel was transiently transfected in HEK-293T cells to test for: i) the capacity of each construct to mediate whole-cell CaCC currents and ii) accessibility of HA epitopes to extracellular anti-HA antibodies via immunocytochemistry. These constructs were termed 16A-X-HA, where “X” indicate the position of HA tag insertion. Supplementary Figure 2B shows that two constructs, tagged at position 523 or 822, gave rise to fully functional channels with unaltered whole-cell current density. In contrast, channels HA-tagged at position 380, 393 or 844 were associated with highly reduced whole-cell current density. Only constructs 16A-523-HA and 16A-822-HA coded for channels that were detectable by extracellular anti-HA antibodies (Supplementary Figure 2B). We identified the position 822 as the most suitable location for HA insertion because 16A-822-HA channels were associated with normal whole-cell current density and resulted in a brighter signal in immunocytochemistry assay compared to 16A-523-HA channels.
Supplementary Figure 2 (A) Topology model of TMEM16A. Numbers close to the circles in the diagram indicate the positions at which HA tags were introduced. Blue circles correspond to HA-tagged TMEM16A channels that i) gave rise to functional CaCCs and ii) were detectable on the plasma membrane in immunocytochemistry experiments. Red circles represent positions corresponding to HA-tagged TMEM16A channels that did not give rise to CaCC currents and were not detectable in immunocytochemistry experiments. (B) Immunofluorescent staining of HEK-293T cells expressing TMEM16A channels HA-tagged at different positions, as indicated. The surface expression was detected with anti-HA antibodies that were visualised using Alexa Fluor 596-labeled secondary antibodies (red) in non-permeabilised conditions. TMEM16A HA-tagged at the N-terminus (16A-N-HA) served as a negative control. For each construct, images were acquired using identical acquisition settings. (C) Mean whole-cell current density recorded for the various HA-tagged TMEM16A channels measured at +80 mV in the presence of 274 nM $[\text{Ca}^{2+}]_i$ (n=5-8). Asterisks indicate significance difference to TMEM16A.
REFERENCES


Chapter 5

Publication III

Bimodal effect of anthracene-9-carboxylic acid on TMEM16A channels

Manuscript in preparation
Bimodal effect of anthracene-9-carboxylic acid on TMEM16A channels

Aiste Tamuleviciute and Paolo Tammaro

Faculty of Life Sciences, The University of Manchester, 46 Grafton Street, Manchester
M13 9NT, United Kingdom.

*Address for correspondence: Dr Paolo Tammaro
Faculty of Life Sciences, University of Manchester
The Core Technology Facility
46 Grafton Street
M13 9NT, Manchester, United Kingdom
Tel. +44 (0)161 2751703
paolo.tammaro@manchester.ac.uk
Abstract

TMEM16A channels are ubiquitously expressed and play a manifold of physiological roles, including epithelial secretion, neuronal excitability, phototransduction and regulation of smooth muscle contraction. The effect of a Cl⁻ channel blocker, anthracene-9-carboxylic acid (A9C), on TMEM16A channels heterologously expressed in HEK-293T cells was studied with the patch-clamp technique. We report that A9C has a bimodal effect on TMEM16A currents. At concentrations higher than 300 µM A9C blocked TMEM16A currents, while at lower doses A9C produced current activation. The blocking effect of A9C was studied in the presence of high intracellular free Ca²⁺ concentration (~12.5 µM) to fully activate TMEM16A channels. A9C accessed the inhibiting site from the extracellular side and produced a voltage-dependent block of TMEM16A currents. The inhibition constant at 0 mV, $K_{i(0)}$, was ~350 µM and the electric distance of the A9C binding site was ~0.7, suggesting that the inhibiting A9C binding site is situated within the pore of TMEM16A channel. The activating effect of A9C was observed when the drug was applied extracellularly in the presence of physiological intracellular Ca²⁺ concentration. The extent of TMEM16A current activation by A9C was the same at different membrane potentials, indicating that the activating A9C binding site is located on the extracellular region of the TMEM16A channel outside the membrane electric field. A9C was shown to activate TMEM16A currents by enhancing the sensitivity of the channel to intracellular Ca²⁺ and voltage. This study might provide a starting point for the identification of compounds that could selectively activate or block TMEM16A channels.
**Introduction**

Ca\(^{2+}\)-activated Cl\(^-\) channels (CaCCs) are ubiquitously expressed and play many physiological roles, such as epithelial secretion, neuronal excitability, transduction of olfactory and photo-stimuli and contraction of smooth muscle cells (Verkman & Galietta, 2009; Duran & Hartzell, 2011; Scudieri *et al.*, 2011). The molecular identity of CaCCs has been elusive up until recently when three independent research groups identified TMEM16A (alternative name Anoctamin-1) as a CaCC (Caputo *et al.*, 2008; Schroeder *et al.*, 2008; Yang *et al.*, 2008). TMEM16A expressed in null cells, such as HEK-293, Fischer rat thyroid (FRT) or axolotl oocytes, gave rise to CaCC currents with biophysical properties that closely resembled those of native CaCCs. Studies involving transgenic mice or gene silencing via RNA interference have confirmed that TMEM16A is responsible for CaCC activity in a number of cell types, including biliary and airway epithelium (Rock *et al.*, 2009; Dutta *et al.*, 2010), vascular smooth muscle (Manoury *et al.*, 2010; Thomas-Gatewood *et al.*, 2011), submandibular salivary gland (Romanenko *et al.*, 2010) and nociceptive neurons (Cho *et al.*, 2012). Despite the physiological importance of TMEM16A channels, pharmacological tools that could selectively modulate TMEM16A activity are limited.

Commonly used CaCC inhibitors, such as niflumic acid and 4,4-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS), lack of selectivity. Therefore, they are not fully suitable for the investigation of the functional roles of TMEM16A channels in cells and tissues. For example, DIDS not only inhibits CaCCs but also blocks Na\(^+\)/HCO\(_3^-\) co-transporters (Praetorius *et al.*, 2001), Na\(^+\)-dependent P\(_i\) transporters (Bellocchio *et al.*, 2000) and many Cl\(^-\)/HCO\(_3^-\) exchangers (Nickell *et al.*, 2007). Niflumic acid, in addition to CaCCs inhibition, also modulates other ion channels, such as NMDA and GABA\(_A\) receptors (Lerma & Martin del Rio, 1992). Moreover, niflumic acid is known to induce Ca\(^{2+}\) release from the sarcoplasmic reticulum (Cruickshank *et al.*, 2003). Another CaCC inhibitor usually extracted from cloves, eugenol, has been also reported to block Na\(^+\), K\(^+\) and Ca\(^{2+}\) channels (Yao *et al.*, 2012).

Recently, new inhibitors were identified using high-throughput screening of large chemical libraries. A new small molecule termed CaCC\(_{inh}\)-A01 (De La Fuente *et al.*, 2008), tannic acid and related compounds (Namkung *et al.*, 2010) were described as potent TMEM16A inhibitors. However, these compounds were not perfectly selective for TMEM16A as they also blocked additional types of CaCCs coded by genes other
than Tmem16A (Nakung et al., 2010). Another recently identified compound, aminophenylthiazole T16A\textsubscript{inh}-A01, has been suggested to selectively block TMEM16A without affecting other Cl\textsuperscript{\textprime} channels, including cystic fibrosis transmembrane conductance regulator (CFTR) (Nakung et al., 2011a). The possibility that T16A\textsubscript{inh}-A01 interacts with other types of channels has not yet been tested.

Functional screening of small-molecule chemical libraries has revealed two types of activators: i) aroylaminothiazole compounds that activate TMEM16A channels in the absence on intracellular Ca\textsuperscript{2+} and ii) tetrazolylbenzamide compounds that increase TMEM16A sensitivity to Ca\textsuperscript{2+} (Nakung et al., 2011b). Both types of TMEM16A activators have been suggested to bind to the channel directly, possibly from the intracellular side, to produce a large and sustained increase in ionic current. The selectivity of these compounds remains to be explored.

In this study we examine the effects of a generic Cl\textsuperscript{\textprime} channel blocker anthracene-9-carboxylic acid (A9C) on TMEM16A currents. Unexpectedly, A9C produced a bimodal effect of TMEM16A currents. At concentrations lower than 300 µM, A9C enhanced TMEM16A currents, while higher doses caused current inhibition. We demonstrate that the two effects require binding of two A9C molecules to separate binding sites, both accessible from the extracellular side of the membrane. The inhibitory site appears to be located deep within the pore of TMEM16A, while the activating site is situated on the extracellular part of the channel outside the voltage electric field established across the membrane. Furthermore, we show that A9C activates TMEM16A currents by altering the response of the channel to intracellular Ca\textsuperscript{2+} and voltage.

**Methods**

*Cell Culture and Transfection:* Human embryonic kidney 293T (HEK-293T) cells were cultured in DMEM supplemented with 10% foetal bovine serum and 10 µg/mL gentamicin at 37°C in a humidified atmosphere with 5% CO\textsubscript{2}. For electrophysiology, HEK-293T cells were transfected with 1 µg of mouse TMEM16A (Genbank NM_178642) and 0.1 µg of CD8 constructs subcloned into pcDNA3.1 vector using Fugene HD (Roche). 4 – 48 hours after transfection successfully transfected cells were visualised by applying polystyrene beads coated with a CD8-specific antibodies (Invitrogen).
**Composition of solutions:** The extracellular solution contained (mM): 150 NaCl, 1 CaCl₂, 1 MgCl₂, 10 glucose, 10 D-mannitol and 10 HEPES; pH was adjusted to 7.4 with NaOH. The intracellular solution contained (mM): 130 CsCl, 10 EGTA, 1 MgCl₂, 10 HEPES and 8 mM CaCl₂ to obtain 274 nM free [Ca²⁺] (calculated with Patcher’s Power tool, Dr. Francisco Mendez and Frank Wurriehausen, Max-Planck-Institut für biophysikalische Chemie, Göttingen, Germany); pH was adjusted to 7.3 with NaOH. Higher free Ca²⁺ concentrations were obtained by replacing EGTA with equimolar HEDTA and 2.1, 3.1, 4.8, 7.8 or 9 mM CaCl₂ resulted in 605, 1040, 2270, 12460 and 78070 nM free Ca²⁺, respectively. A9C was dissolved in dimethyl sulfoxide (DMSO) at 0.3 M stock and then applied to working solutions, keeping the concentration of DMSO ≤1%. The exchange of solutions was achieved by using a local perfusion system consisting of eight tubes of 1.2 mm diameter in which the tip of the patch pipette was inserted. In some experiments, ultra-rapid changes (≤5 ms) in A9C concentration (“concentration jumps”) were achieved using computer-controlled perfusion system (Warner Instruments). The bath was grounded through a 3 M KCl agar bridge connected to a Ag-AgCl reference electrode.

**Electrophysiology:** All current recordings were performed with the whole-cell or inside-out configuration of the patch-clamp technique at room temperature (22−24°C). Currents were filtered at 2 kHz and sampled at 10 kHz. Current versus voltage relationships were constructed by measuring steady-state or instantaneous currents at the end of 1 s voltage steps from −100 to +100 mV in 40 mV increments, elicited every 2 s from holding potential of 0 mV. Steady-state currents were measured at the end of test pulses and instantaneous currents were estimated from extrapolation of single exponential fits of the currents to the beginning of test pulses.

The relationships between A9C concentration ([A9C]) and TMEM16A inhibition were obtained by measuring currents at the end of 250 ms steps from +20 to +120 mV in 20 mV increments, elicited every 2 s from holding potential of -70 mV. Currents in the presence of A9C were normalised to currents in the absence of A9C and plotted versus A9C concentration. The obtained concentration-response curves were fitted with the Hill equation of the form:

\[
I_{A9C}/I_0 = 1/[1 + ([A9C]/K_i)^Y],
\]

[1]
where \( I_{A9C} \) and \( I_0 \) are currents measured in the presence or absence of A9C, respectively; \( K_i \) is the A9C dissociation constant from the inhibitory site and \( \gamma \) is the slope factor (Hill coefficient).

The fraction of the voltage field experienced by A9C at the inhibiting site was estimated by fitting the relationship between \( K_i \) and membrane potential (\( V \)) with Woodhull equation (Woodhull, 1973):

\[
K_i = K_{i(0)} \exp\left(-\delta V / RT\right),
\]

where \( K_{i(0)} \) is dissociation constant of A9C from the inhibitory site at 0 mV, \( z \) is the A9C electric charge, \( \delta \) is the fraction of the membrane potential field sensed by A9C at its inhibitory site, \( F \) is Faraday's constant, \( R \) is the universal gas constant and \( T \) is the absolute temperature.

Activation-response curves for A9C were fitted with Langmuir isotherm of the form:

\[
I_{A9C} / I_0 = 1 + (I_{\text{max}} - 1) [A9C]/([A9C] + K_d),
\]

where \( I_{\text{max}} \) is the maximally activated current and \( K_d \) is the dissociation constant of A9C from the activating site.

For voltage sensitivity, tail current \textit{versus} voltage relationships were constructed by measuring the amplitude of instantaneous tail currents at −60 mV after a 1 s step to various potentials ranging from −100 to +180 mV in 40 mV increments, elicited every 2 s from a holding potential of 0 mV. Tail currents were fitted with an exponential function, and the amplitude of instantaneous tail currents was estimated from extrapolation of the fit to the beginning of test pulses. The instantaneous tail currents were plotted \textit{versus} test potentials and normalized against the maximal current value obtained from the sigmoidal fittings. The normalised conductances obtained in this way were plotted \textit{versus} the voltage of test pulses and fitted by the Boltzmann equation:

\[
G / G_{\text{max}} = 1 / \left[1 + \exp\left[z(V_{0.5} - V) / F / RT\right]\right],
\]

where \( G / G_{\text{max}} \) is the normalised conductance, \( z \) is the gating charge associated with channel activation, \( V_{0.5} \) is the voltage producing half-maximal activation.
Ca\(^{2+}\) concentration-response relationships were constructed by exposing an excised patch to various \([\text{Ca}^{2+}]_i\) and measuring the current at the end of 1 s pulses to +70 and -70 mV. The holding potential was 0 mV. The amplitude of steady-state currents was plotted versus \([\text{Ca}^{2+}]_i\) and fitted with the Hill equation of the form:

\[
\frac{I}{I_{\text{max}}} = \frac{1}{1 + \left(\frac{[\text{Ca}^{2+}]_i}{EC_{50}}\right)^h},
\]

where, \(I\) is the current at a given \([\text{Ca}^{2+}]_i\), \(I_{\text{max}}\) is the maximal current, \(EC_{50}\) is \([\text{Ca}^{2+}]_i\) producing the half maximal activation and \(h\) is the slope factor (Hill coefficient).

**Data analysis:** Data were analysed with self-written routines developed in the Igor Pro software (WaveMetrics) environment or using the program Ana (http://users.ge.ibf.cnr.it/pusch/help-ana.htm). Data are given as mean±sem. Statistical significance was evaluated using a Student’s two-tailed \(t\) test and \(p<0.05\) was considered to be significant.

**Results**

The project began by examining the effects of increasing concentrations of A9C applied extracellularly ([A9C]\(_{\text{ext}}\)) on whole-cell TMEM16A currents. Since A9C is known to block Cl\(^-\) channels in the open state (Qu & Hartzell, 2001), the following stimulation protocol was used: TMEM16A channels were first activated by a depolarizing step to +70 mV (pre-pulse) and then the effect of A9C on open channels was examined by stepping to membrane potentials from -100 to +100 mV in 40 mV increments (test pulses). These experiments were carried out in the presence of 274 nM \([\text{Ca}^{2+}]_i\).

Figure 1A shows that in response to the test pulses the instantaneous currents progressively relaxed to new steady-state levels. In the absence of A9C, this relaxation was monotonic. In contrast, in the presence of A9C, current relaxation followed a biphasic time course. This was particularly evident at negative potentials where the current firstly increased reaching the peak amplitude in ~5 ms and then relaxed (Figure 1B). The kinetics of relaxation was measured by calculating the time required to reach the half-maximal current decrease (\(\tau_{0.5}\)). In the presence of 300 µM [A9C]\(_{\text{ext}}\), current decay at -100 mV was 3-fold slower with \(\tau_{0.5}\) being equal to 39±4 ms (n=7) compared to 13±2 ms (n=7) in the absence of A9C.
The magnitude of instantaneous currents was estimated by extrapolating the exponential fit of current decay to the beginning of the test pulses. Figure 1A and C demonstrate that the amplitude of instantaneous currents at negative potentials was progressively increased as $[\text{A9C}]_{\text{ext}}$ was increased. In the presence of 30 µM $[\text{A9C}]_{\text{ext}}$, instantaneous currents were increased nearly ~2-fold, while 1 mM $[\text{A9C}]_{\text{ext}}$ produced ~16-fold increase in current amplitude at -100 mV (Figure 1C).

A9C also affected the amplitude of the steady-state TMEM16A currents. Figure 1D presents the effect of A9C on currents measured at the end of 1 s voltage steps to +100 mV. In the presence of 30 or 100 µM $[\text{A9C}]_{\text{ext}}$, steady-state currents were increased by ~20%. In contrast, 300 µM $[\text{A9C}]_{\text{ext}}$ had no effect on the average current magnitude and 1 mM $[\text{A9C}]_{\text{ext}}$ caused ~40% inhibition of TMEM16A currents.

Figure 1 (A) Whole-cell currents recorded from HEK-293T cells expressing TMEM16A in the presence of 274 nM $[\text{Ca}^{2+}]_{i}$ and various $[\text{A9C}]_{\text{ext}}$ (µM), as indicated. Stimulation protocol is shown above. Dashed horizontal lines represent the zero-current level. (B) Normalized TMEM16A currents, obtained from panel A, recorded at -100 mV in the absence or presence of 300 µM A9C, as indicated. Dashed horizontal lines represent the zero-current level. (C) Mean changes in the instantaneous current amplitude ($\Delta I_{\text{Inst}}$) measured at -100 mV in the presence of 274 nM $[\text{Ca}^{2+}]_{i}$ and various $[\text{A9C}]_{\text{ext}}$ (µM), as indicated. Asterisks indicate significant difference to control conditions. (D) Mean changes in steady-state current amplitude ($\Delta I_{\text{SS}}$) measured at the end of voltage steps to +100 mV in the presence of 274 nM $[\text{Ca}^{2+}]_{i}$ and various $[\text{A9C}]_{\text{ext}}$ (µM), as indicated. Asterisks indicate significant difference to control conditions.

Characterization of the inhibiting effect of A9C on TMEM16A channels

The previous set of the experiment demonstrate that A9C has a bimodal effect of TMEM16A function. Based on the previous reports indicating that A9C is an open Cl’ channel blocker (Qu & Hartzell, 2001; Qu et al., 2003), the possibility that A9C blocks
open TMEM16A channels was examined. The blocking effect was studied by fully activating TMEM16A channels with 12.5 µM \([\text{Ca}^{2+}]_i\).

Consistent with the previous studies (Schroeder et al., 2008; Xiao et al., 2011), in the presence of 12.5 µM \([\text{Ca}^{2+}]_i\), TMEM16A currents elicited at positive potentials were of the same magnitude as those observed at negative potentials (Figure 2). The rectification index, calculated as a ratio between currents measured at -100 and +100 mV (\(I_{-100}/I_{+100}\)), was equal to 1.05±0.03 (n=11). To test from which side of the membrane A9C accesses its inhibiting site, 300 µM [A9C] was applied to inside-out patches intracellularly ([A9C]_{int}) (bath solution) or extracellularly (pipette solution). Extracellular and intracellular A9C had no effect on currents measured at negative potentials. However, currents elicited at positive potentials were highly suppressed by extracellular A9C (\(I_{-100}/I_{+100} = 6.01±0.09, n=6\)) and slightly inhibited by intracellular A9C (\(I_{-100}/I_{+100} = 1.55±0.04, n=4\)), suggesting that A9C reaches its inhibiting site from the extracellular side of the membrane.

![Figure 2](image)

**Figure 2 (A)** TMEM16A currents recorded from inside-out patches in the presence of 12.5 µM \([\text{Ca}^{2+}]_i\), in the absence or presence of 300 µM A9C applied intracellularly or extracellularly, as indicated. Stimulation protocol is shown in the upper left panel. Dashed horizontal lines represent the zero-current level. (B) Steady-state TMEM16A currents normalized to current measured at -100 mV recorded from inside-out patches in the presence of 12.5 µM \([\text{Ca}^{2+}]_i\), in the absence or presence of 300 µM A9C applied intracellularly or extracellularly, as indicated (n=4-11).

The inhibiting effect of extracellular A9C was investigated in more detail by recording macroscopic whole-cell currents in the presence of 12.5 µM \([\text{Ca}^{2+}]_i\), at membrane potentials ranging from +20 to +120 mV. Figure 3A shows that TMEM16A currents
recorded at positive potentials were highly suppressed by A9C. The extent of inhibition was dependent on [A9C]_{ext} and the membrane potential (Figure 3B). The [A9C]_{ext}-current relationships at various potentials were constructed by plotting currents measured at the end of 250 ms voltage steps versus the [A9C]_{ext}. The concentration-response relationships were fit with an eqn. 1 with $\gamma \approx 1$ at all membrane potentials, while $K_i$ decreased as the membrane potentials were increased (Table 1). Figure 3B (panel) shows that the relationship between the membrane potential and $K_i$ fitted with the eqn. 2 with $\delta=0.7\pm0.1$ and $K_i(0)=342\pm38$ µM ($n=6$).

![Figure 3](image)

**Figure 3** (A) Whole-cell currents recorded from HEK-293T cells expressing TMEM16A in the presence of 12.5 µM [Ca$^{2+}$] in the absence or presence of 30 µM [A9C]_{ext}, as indicated. Stimulation protocol is shown in upper right panel. Dashed horizontal lines represent the zero-current level. (B) Mean relationships between the [A9C]_{ext} and whole-cell currents normalised to the currents in the absence of A9C ($I_{A9C}/I_0$) recorded at various membrane potentials, as indicated ($n=4-6$). The smooth curves are the best fits of the data with eqn. 1. The inset shows the relationship between the membrane potential and $K_i$ ($n=4-6$). The smooth curve is the best fit of the data with eqn. 2.

<table>
<thead>
<tr>
<th>$V_m$</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>80</th>
<th>100</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$</td>
<td>196±23</td>
<td>111±7</td>
<td>62±6</td>
<td>37±3</td>
<td>24±2</td>
<td>15±2</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>1.0±0.1</td>
<td>1.0±0.1</td>
<td>0.9±0.1</td>
<td>0.9±0.1</td>
<td>0.8±0.1</td>
<td>0.9±0.1</td>
</tr>
</tbody>
</table>

$V_m$, membrane potential; $K_i$, inhibitory constant; $\gamma$, slope factor.

**Table 1. Parameters ($K_i$ and $\gamma$) obtained from Hill fit of [A9C]_{ext}-TMEM16A inhibition relationships at various membrane potentials.**

Characterization of the activating effect of A9C on TMEM16A channels
Figure 1 showed the existence of two A9C-mediated effects on TMEM16A channels. To separate the activating effect of A9C from the inhibition, the following stimulation protocol was used. In the presence of 274 nM [Ca\textsuperscript{2+}], whole-cell or inside-out patches excised from HEK-293T expressing TMEM16A channels were exposed to various [A9C] using a rapid perfusion system. Rapid [A9C] jumps revealed the magnitude and the time course of activation and inhibition. A9C was applied from extracellular or intracellular side of the membrane to test from which side A9C accesses its activating site. Figure 4A shows TMEM16A currents recorded in the presence of various [A9C] at a constant membrane potential of +100 mV. When applied from the extracellular side, A9C produced a distinct inhibition observed instantly upon application of A9C. Currents then gradually increased reaching a steady-state level in ~1 s. When the drug was removed a large transient increase in current was observed. In contrast, only a modest current increase was observed when A9C was applied intracellularly even at concentrations as high as 1 mM.

The activation was measured by fitting current decay upon A9C washout with a single exponential fit and estimating the peak amplitude by extrapolating the fit to the beginning of A9C washout. Figure 4B shows that at +100 mV, 300 µM [A9C]\text{ext} produced ~10-fold increase in current amplitude, while the same concentration of intracellular A9C activated current by only ~30%. The \( K_d \) for A9C was estimated by fitting the relationship between the amplitude of activated current and [A9C]\text{ext} with eqn. 3. To test if the activating effect of A9C is voltage-dependent, \( K_d \) was determined at different membrane potentials. Figure 4C shows that A9C affinity to its activating binding site did not change with the voltage with \( K_d \) equalling to 415±33 (n=8) and 435±52 µM (n=8) at +40 and +70 mV, respectively.
Figure 4 (A) Whole-cell (upper panel) and inside-out (lower panel) currents recorded from HEK-293T cells expressing TMEM16A channels. Various [A9C] (µM) were applied from extracellular (upper panel) or intracellular (lower panel) side of the membrane using the rapid perfusion system. The bars above current traces indicate the duration of A9C application. The voltage was maintained at +100 mV for the whole duration of the recordings. Dashed horizontal lines represent the zero-current level. (B) Mean changes in the amplitude of current activated by 300 µM [A9C]_ext or [A9C]_int at a membrane potential of +100 mV. Asterisk indicates significant difference between the two groups. (C) Mean $K_d$ of A9C determined at +40 and +70 mV (n=8).

**A9C effects on voltage-dependent gating of TMEM16A channels**

The activity of TMEM16A channels is modulated by intracellular Ca$^{2+}$ and the membrane potential. Thus, we hypothesised that A9C potentiates the current by modifying channel sensitivity to voltage and/or Ca$^{2+}$. The voltage-dependence of TMEM16A channels was studied by exposing inside-out patches to various [Ca$^{2+}$]$_i$ in the presence of fixed [A9C]$_{ext}$ (Figure 5A, B). Instantaneous currents were estimated by extrapolating an exponential fit of the current decay to the beginning of the test pulse. Constructed normalised conductance versus voltage relationships revealed that A9C highly increased TMEM16A sensitivity to voltage measured at various [Ca$^{2+}$]$_i$. $V_{0.5}$ parameters, derived from eqn. 4, showed a progressive shift towards more negative values, when exposed to the increasing [A9C]$_{ext}$ (Table 2).

Figure 5 (A) Tail currents recorded from an inside-out patch excised from HEK-293T cells expressing TMEM16A in the presence of 274 nM [Ca$^{2+}$]$_i$ in the absence or presence of 300 or 1000 µM [A9C]$_{ext}$, as indicated. The stimulation protocol is shown above. Horizontal dashed lines indicate the zero-current level. (B) Mean normalised TMEM16A conductance versus voltage relationships obtained in the presence of various [A9C]$_{ext}$.
and 274, 605 or 1040 nM \([\text{Ca}^{2+}])_i\), as indicated (n=4-14). The smooth curves are the best fits of the data with eqn. 4.

**Table 2. Parameters \((V_{0.5} \text{ and } z)\) obtained from the Boltzmann fit of TMEM16A, conductance versus voltage relationships at various \([\text{Ca}^{2+}])_i\) and \([\text{A9C}]_{\text{ext}}\).**

<table>
<thead>
<tr>
<th>([\text{Ca}^{2+}])_i, \text{nM}</th>
<th>[\text{A9C}]_{\text{ext}}, \mu\text{M}</th>
<th>0</th>
<th>300</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>274</td>
<td>(V_{0.5}, \text{mV})</td>
<td>309±28 (n=10)</td>
<td>124±9 (n=8) *</td>
<td>89±7 (n=14) *</td>
</tr>
<tr>
<td></td>
<td>(z)</td>
<td>1.4±0.3 (n=10)</td>
<td>0.7±0.1 (n=8) *</td>
<td>0.7±0.1 (n=14) *</td>
</tr>
<tr>
<td>605</td>
<td>(V_{0.5}, \text{mV})</td>
<td>130±6 (n=10)</td>
<td>45±4 (n=8) *</td>
<td>18±4 (n=14) *</td>
</tr>
<tr>
<td></td>
<td>(z)</td>
<td>0.9±0.1 (n=10)</td>
<td>0.7±0.1 (n=8) *</td>
<td>0.7±0.1 (n=14) *</td>
</tr>
<tr>
<td>1040</td>
<td>(V_{0.5}, \text{mV})</td>
<td>64±6 (n=10)</td>
<td>-14±8 (n=4) *</td>
<td>-22±7 (n=9) *</td>
</tr>
<tr>
<td></td>
<td>(z)</td>
<td>1.3±0.1 (n=10)</td>
<td>0.9±0.1 (n=4) *</td>
<td>0.9±0.1 (n=9) *</td>
</tr>
</tbody>
</table>

* indicate statistically significant difference from parameters obtained in the absence of A9C.

**A9C effects on TMEM16A sensitivity to \(\text{Ca}^{2+}\)**

The effects of A9C on the sensitivity of TMEM16A channels to intracellular \(\text{Ca}^{2+}\) were tested by recording macroscopic currents from inside-out patches exposed to increasing \([\text{Ca}^{2+}])_i\) in response to voltage steps to +70 and -70 mV (Figure 6A). \([\text{Ca}^{2+}])_i\)-response relationships were constructed by plotting normalised TMEM16A currents measured at the end of 1 s test pulses versus \([\text{Ca}^{2+}])_i\) in the presence of various \([\text{A9C}]_{\text{ext}}\) (Figure 6B). TMEM16A sensitivity to \(\text{Ca}^{2+}\) was enhanced by A9C at positive and negative potentials. In the presence of 1 mM \([\text{A9C}]_{\text{ext}}, EC_{50}\) for \(\text{Ca}^{2+}\) decreased by ~30% and ~60%, when measured at -70 and +70 mV, respectively. \([\text{Ca}^{2+}])_i\)-response relationships were fitted with eqn. 5 with the parameter \(h\) being the same at positive and negative potentials in the presence of various \([\text{A9C}]_{\text{ext}}\) (Table 3).
Figure 6 (A) Inside-out currents recorded from patches excised from a HEK-293T cell expressing TMEM16A in response to various $[\text{Ca}^{2+}]_i$ (nM) in the absence or presence of 1000 µM $[\text{A9C}]_{\text{ext}}$, as indicated. The voltage protocol is shown above. (B) Mean relationships between $[\text{Ca}^{2+}]_i$ and inside-out TMEM16A currents normalised to the maximal response measured at +70 and -70 mV (n=6-10), as indicated. The smooth curves are the best fits of the data with eqn. 5.

Table 3. Parameters ($EC_{50}$ and $h$) obtained from the Hill fit of TMEM16A $[\text{Ca}^{2+}]_i$ -response relationships in the presence of various $[\text{A9C}]_{\text{ext}}$ at various potentials.

<table>
<thead>
<tr>
<th>Vm, mV</th>
<th>$[\text{A9C}]_{\text{ext}}, \mu\text{M}$</th>
<th>0</th>
<th>30</th>
<th>300</th>
<th>1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>-70</td>
<td>$EC_{50}, \mu\text{M}$</td>
<td>2.2±0.1 (n=10)</td>
<td>2.0±0.1 (n=7)</td>
<td>2.3±0.1 (n=6)</td>
<td>1.6±0.1 (n=9) *</td>
</tr>
<tr>
<td></td>
<td>$h$</td>
<td>2.3±0.2 (n=10)</td>
<td>2.3±0.3 (n=7)</td>
<td>2.2±0.2 (n=6)</td>
<td>2.1±0.2 (n=9)</td>
</tr>
<tr>
<td>+70</td>
<td>$EC_{50}, \mu\text{M}$</td>
<td>0.8±0.1 (n=10)</td>
<td>0.6±0.1 (n=7) *</td>
<td>0.5±0.1 (n=6) *</td>
<td>0.3±0.1 (n=9) *</td>
</tr>
<tr>
<td></td>
<td>$h$</td>
<td>2.3±0.2 (n=10)</td>
<td>3.0±0.5 (n=7)</td>
<td>2.6±0.2 (n=6)</td>
<td>2.7±0.5 (n=9)</td>
</tr>
</tbody>
</table>

* indicate statistically significant difference from parameters obtained in the absence of A9C.
Discussion

The main discovery of this study is that A9C produces inhibition and activation of TMEM16A currents and that these effects require binding of A9C to two separate sites on the channel protein. In physiological [Ca^{2+}], (i.e. 274 nM), negative potentials elicited the instantaneous currents followed by an exponential current decay. The exponential TMEM16A current decay at negative potentials reflects the conformational changes leading to the channel closure. In the presence of A9C, however, the instantaneous tail currents were followed by a rapid current increase, presumably representing dissociation of A9C from the inhibiting site. The kinetics of a subsequent current decay was much slower compared to control conditions, suggesting that the activating site of A9C was still occupied and involved in the regulation of channel gating. Another piece of evidence for two binding sites is a distinct voltage-sensitivity for activation and inhibition by A9C. Activating effect showed no voltage-dependence, while inhibition was enhanced with depolarization, suggesting the different localization of two A9C binding sites.

A9C as an open channel blocker

In physiological conditions, A9C is a negatively charged molecule that is known to block the permeation pathway of many Cl⁻ channels, including CaCCs (Qu & Hartzell, 2001), CIC-1 (Estévez et al., 2003), CFTR and swelling-activated Cl⁻ channels (Shuba et al., 2004). Extracellular A9C effectively blocked TMEM16A currents only at positive potentials and the inhibition was enhanced with increasing membrane depolarization. This is consistent with a model according to which the A9C inhibitory binding site is accessible from the extracellular side and is situated within protein regions spanning the voltage electric field. According to this model, A9C applied from the intracellular side is expected to have no or very little effect on TMEM16A currents. This is because hyperpolarized potentials would not favour entry of intracellular A9C into the channel pore. However, intracellularly applied A9C produced a small inhibition at positive potentials. This result suggests that A9C could reach its inhibitory site by crossing the membrane bilayer and then blocking TMEM16A channels from the extracellular side of the membrane. This is plausible considering that A9C contains a hydrophobic region of three aromatic rings, which could enable the drug to cross the membrane lipid bilayer. This finding is consistent with a previous report on native CaCCs, which were blocked by extracellular but not intracellular A9C (Qu & Hartzell, 2001; Qu et al., 2003).
The fact that A9C blocks TMEM16A channels in a voltage-dependent manner is consistent with the idea that the inhibitory site is situated in the anion permeation pathway (Hille, 2001). Indeed, our unpublished data shows that TMEM16A inhibition by the extracellular A9C is enhanced in low [Cl\textsuperscript{-}]\textsubscript{ext} confirming that A9C enters the pore in the competitive manner with permeant anions.

The parameter $\gamma \approx 1$ derived from fitting [A9C]\textsubscript{ext}-response relationships with the Hill equation indicates that one A9C molecule is sufficient to inhibit one TMEM16A channel. The $\delta$ value of $\sim 0.7$ obtained from Woodhull analysis suggests that the A9C molecule penetrates about 70% the length of the pore (voltage field) to reach its inhibitory site. This finding is consistent with a previous report on native CaCCs in *Xenopus* oocytes suggesting that A9C passes the pore from the extracellular side to a distance of $\sim 60\%$ to occupy its blocking position (Qu & Hartzell, 2001). Structural determinants involved in A9C binding have been identified for ClC-1 channels (Estévez *et al.*, 2003). A serine residue situated close to the anion selectivity filter has been described as essential for A9C binding (Estévez *et al.*, 2003). Other residues affecting the coordination of A9C also seem to be located close to the anion binding site based on the crystal structure of bacterial ClC channels (Dutzler *et al.*, 2002). The identification of TMEM16A residues involved in A9C coordination will likely become possible when the crystal structure of this protein or its analog is revealed.

**A9C as an allosteric Cl\textsuperscript{-} channel activator**

In addition to inhibitory effect, A9C also activated TMEM16A channels. A dual effect of A9C has also been reported for native CaCCs in rabbit pulmonary artery smooth muscle cells (Piper & Greenwood, 2003) and CFTR channels in guinea pig ventricular myocytes (Zhou *et al.*, 1997). Different groups did not agree on the mechanism by which A9C activates CFTR channels. Zhou *et al.* suggested that A9C enhances CFTR currents indirectly by inhibiting intracellular Mg\textsuperscript{2+}-sensitive protein phosphatases (Zhou *et al.*, 1997). In contrast, Ai *et al.* reported that A9C binds to CFTR channels directly in a voltage-independent manner and modifies channel gating by increasing the open probability (Ai *et al.*, 2004). The electroneutral A9C analog, anthracene-9-methanol, enhanced CFTR currents similarly to A9C but no blockage was observed suggesting the existence of two separate A9C binding sites for blockage and activation (Ai *et al.*, 2004). Unpublished work from our lab showed that the effect of electroneutral analogs of A9C, anthracene-9-methanol and 9-anthraldehyde, could not be tested as these compounds
were insoluble in our working solutions at room temperature. Therefore, the activating and blocking effects were separated using rapid $[\text{A9C}]_{\text{ext}}$ jumps. The activating effect was isolated and measured upon washout of A9C, which abolished the block immediately revealing the fraction of activated current. The fact that TMEM16A currents were activated instantly and remained steady even in excised inside-out patches suggests that A9C binds TMEM16A directly and not via interactions with intracellular regulatory proteins. TMEM16A currents were activated by extracellular but not intracellular A9C in a voltage-independent manner suggesting that residues coordinating A9C are situated on the extracellular part of the channel. Importantly, when bound to the activating site, A9C acts as a gating modifier that increases the open probability of TMEM16A channels. Mutagenesis of the extracellular loops will be required to identify the exact A9C binding pocket and amino acids involved in drug coordination.

**TMEM16A as a pharmaceutical target**

Selective modulators of TMEM16A could be of a therapeutic use for a number of pathological conditions. Since TMEM16A is abundantly expressed in vascular smooth muscle (Manoury *et al.*, 2010; Thomas-Gatewood *et al.*, 2011), TMEM16A channel openers and blockers could be used to treat hypotension and hypertension, respectively. In pulmonary hypertension, an overexpression of TMEM16A enhances pulmonary vasoreactivity (Sun *et al.*, 2012). Also, the upregulation of Cl$^-$ currents has been implicated in proliferation of pulmonary artery smooth muscle cells (Liang *et al.*, 2009), suggesting that TMEM16A blockers could potentially prevent cell proliferation in pulmonary hypertension. Another potential pharmacological implication of TMEM16A is the treatment of cystic fibrosis. This autosomal recessive disease is caused by mutations in CFTR channel, which impairs its function and/or expression on the apical membrane in airway and other secretory epithelia (Chiaw *et al.*, 2011). TMEM16A channels have been shown to be expressed on the apical membrane in airway epithelium (Ousingsawat *et al.*, 2009). Also, *Tmem16A* knock-out mice resemble cystic fibrosis phenotype by having decreased Cl$^-$ secretion, accumulation of mucus and impaired mucociliary clearance (Ousingsawat *et al.*, 2009; Rock *et al.*, 2009). This suggests that TMEM16A and CFTR have similar physiological implications in airway epithelium secretion. Indeed, two CaCC activators, denufosol and duramycin, already have been in clinical trials as potential drugs to treat cystic fibrosis (Kellerman *et al.*, 2008; Steiner *et al.*, 2008). However, both of them target CaCCs indirectly by inducing only a transient increase in intracellular Ca$^{2+}$ levels and, subsequently, in Cl$^-$ secretion. The activators
that involve direct interaction with TMEM16A channels would potentially lead to more sustained increase in CaCC currents.

A new role of TMEM16A in the development of cancer is emerging. *TMEM16A* gene is situated in the 11q13 human chromosome, where many tumour-related genes are found (Xin *et al.*, 2006; Yang *et al.*, 2008). The overexpression of TMEM16A promotes tumour cell proliferation in head and neck squamous cell carcinoma, while an inhibition of TMEM16A currents with T16A<sub>inh</sub>-A01 abolishes the growth of tumour (Duvvuri *et al.*, 2012). The expression of TMEM16A is also upregulated in gastrointestinal stromal tumour (Espinosa *et al.*, 2008) and metastatic prostate carcinoma, where *TMEM16A* gene interference using small hairpin RNAs significantly suppresses cell proliferation and metastasis (Liu *et al.*, 2012). All together these observations promote TMEM16A as a potential pharmaceutical target for the treatment of cancer and a range of other disorders, including vascular and respiratory diseases.

**Acknowledgements**

P.T. holds a Research Council (RCUK) Fellowship. This research is supported via a BBSRC PhD studentship to A.T. and a BBSRC New Investigator Award (BB/H000259/1) to P.T.

**Author contributions**

Author contributions: P.T. initiated the project; A.T. and P.T. designed the experiments; A.T. did the experiments, analysed the data and wrote the paper. All experiments were carried out at the University of Manchester.

**Conflict of interest**

The authors declare that they have no conflict of interest.
Reference list


Namkung W, Thiagarajah JR, Phuan P-W & Verkman AS. (2010). Inhibition of Ca\(^{2+}\)-activated Cl\(^{-}\) channels by gallotannins as a possible molecular basis for health benefits of red wine and green tea. The FASEB Journal 24, 4178-4186.


Rock JR, O'Neal WK, Gabriel SE, Randell SH, Harfe BD, Boucher RC & Grubb BR. (2009). Transmembrane Protein 16A (TMEM16A) Is a Ca\textsuperscript{2+}-regulated Cl\textsuperscript{-} Secretory Channel in Mouse Airways. *Journal of Biological Chemistry* 284, 14875-14880.


Chapter 6

Discussion
The work described in this thesis had three general aims: i) to investigate the molecular identity of CaCCs in vascular smooth muscle, ii) to begin to elucidate the structure to function relationship of the identified ion channel and iii) to examine how drug binding affects CaCC activity. The main findings of my work can be summarized as follows:

1) Heterologous TMEM16A currents had electrophysiological properties closely resembling those of native $I_{CaCC}$ in rat PASMCs;
2) siRNA directed against TMEM16A resulted in reduction of TMEM16A expression and concomitant decrease of $I_{CaCC}$ in rat PASMCs;
3) Several TMEM16 genes and multiple TMEM16A splice variants were expressed in rat PASMCs;
4) The putative pore-loop of TMEM16A was found to be involved in regulation of channel gating;
5) A non-canonical trafficking motif was identified within a 38 amino acids stretch in the putative pore-loop of TMEM16A;
6) The small molecule compound anthracene-9-carboxylic acid (A9C) was shown to produce a dual effect on TMEM16A currents: inhibition and activation. Channel inhibition involved occlusion of the pore from the extracellular side, while TMEM16A activation required A9C binding to an extracellular site, which allosterically increased the sensitivity of the channel to intracellular $Ca^{2+}$ and voltage.

6.1. Functional properties of heterologous TMEM16A currents versus native $I_{CaCC}$ in PASMCs

$I_{CaCC}$ in PASMCs and heterologous TMEM16A currents exhibited very similar electrophysiological properties, including $Ca^{2+}$-sensitivity, voltage-dependence and kinetics of current activation and deactivation. However, while the kinetic of $I_{CaCC}$ in PASMCs was well-described with a single exponential function, the heterologous TMEM16A currents were better described with a double exponential function (Figure 6.1). This difference in gating kinetics could be explained by a number of reasons: 1) in heterologous expression studies we used mouse TMEM16A containing just a alternative exon, while $b$, $c$, and $d$ segments were absent. In contrast, TMEM16A channels expressed in rat PASMCs were composed of various combinations of alternative exons (Manoury et al., 2010). Ferrera et al. (2009) demonstrated that various TMEM16A isoforms generate $I_{CaCC}$ with distinct biophysical properties, including
degree of outward-rectification, current kinetics and Ca\textsuperscript{2+}- sensitivity; 2) TMEM16A contains potential glycosylation sites on the extracellular side of the protein and multiple phosphorylation sites on the intracellular part (Yang \textit{et al.}, 2008). Therefore, TMEM16A might undergo different posttranslational modifications in various cell types causing alterations in gating kinetics; 3) Also, TMEM16A in native cells might associate with other TMEM16 proteins or regulatory subunits forming heteromeric channels with modified gating kinetics, which cannot be the case in heterologous expression system due to overexpression of TMEM16A alone.

For a channel with a simple gating mechanism, comprising a single closed state and a single open state linked by voltage-dependent transition rates one would expect the macroscopic current to rise in response to membrane depolarization and to decay, in response to membrane hyperpolarization, according to a single exponential function. The fact that TMEM16A have a bimodal kinetics of activation and deactivation suggests a more complex scheme for channel gating, which would include multiple open states. This observation is in agreement with previous studies of native CaCCs, in which the existence of multiple closed and open states was proposed (Kuruma & Hartzell, 2000; Piper & Large, 2003). Furthermore, bimodal kinetics is not unusual for ion channels. For instance, fast and slow inactivation modes have been reported for type III Na\textsuperscript{+} channel (Moorman \textit{et al.}, 1990; Tammaro \textit{et al.}, 2002).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{Comparison of gating kinetics of native CaCCs and heterologous TMEM16A channels. Whole-cell I_{CaCC} recorded from a single rat PASMC or HEK-293T cell expressing TMEM16A in response to a depolarized pulse (1 s) at +100 mV, followed by 0.5 s repolarization to -60 mV. Dashed horizontal lines represent the zero-current level. The current increase and decay recorded from rat PASMCs was well-fitted with a single exponential function (blue line), while TMEM16A current was fitted with a double exponential function (red line) more accurately than with a single exponential function (blue line).}
\end{figure}
6.2. Functional role of other TMEM16 family members

Two other TMEM16 family members, TMEM16F and TMEM16K, were found to be highly expressed in PASMCs. Their contribution to the $I_{CaCC}$, however, was unlikely since in RNA interference study the $I_{CaCC}$ was highly reduced even though the expression of TMEM16F and TMEM16K was not affected. Also, these two members are expressed in tissues that do not show CaCC activity (Galietta, 2009), suggesting that TMEM16F and TMEM16K might perform functional roles other than CaCCs. The cellular functions of TMEM16 proteins might be understood if their subcellular localization is determined. For example, TMEM16G was found preferentially expressed in prostate at cell-cell contact regions (Das et al., 2007). This observation together with a notion that RNA interference of TMEM16G prevented formation of cell aggregates suggested that TMEM16G promotes cell association (Das et al., 2007). The subcellular localization of other TMEM16 family members is currently debatable. Schreiber et al. (2010) reported that all TMEM16E-K proteins were localized on the plasma membrane when heterologously expressed in Fisher Rat Thyroid (FTR) cells. In contrast, Duran et al. (2011) argued that expression of TMEM16C-G and TMEM16K in FRT and other cell lines gave rise to intracellular proteins. Both groups used the confocal microscopy to probe for co-localization of TMEM16 proteins with membrane markers, such as $\beta$-catenin, F-actin (Schreiber et al., 2010) or wheat germ agglutinin (Duran et al., 2011). Despite the fact that some of the images acquired by two groups were surprisingly similar, the conclusions they made were opposite, suggesting that this experimental approach is open to interpretations and might not be the best method to detect protein subcellular localization. More reliable approach to detect and quantify the surface expression of TMEM16 proteins would be labeling of membrane proteins by covalently binding biotin. Biotinylated surface proteins after cell lysis can be separated using streptavidin/avidin and the expression levels of TMEM16 in cell surface and whole-cell fractions can then be quantified via immunoanalytical methods.

6.3. Structure-function studies of TMEM16A channels

The second part of my project aimed to elucidate how TMEM16A channels operate at the molecular level. In order to fully understand the physiology of ion channels it is fundamentally important to understand the molecular mechanisms by which channel gating and conductance are achieved. This is also very important because many diseases associated with ion channels (or channelopathies) are associated with changes in channel
gating and/or conductance. Although channelopathies due to mutations in the \textit{TMEM16A} gene have not yet been found, it is highly likely that they will be discovered. Mutations in almost every currently known class of ion channels have been linked to disease. The studies on the structure to function relationship of TMEM16A are expected to help elucidate the mechanism/s by which genetic mutations in \textit{TMEM16A} may result in altered channel function.

Many questions about TMEM16A biophysics will need to be addressed. One of them is elucidation of the mechanisms of Cl\textsuperscript{−} permeation through these channels. We believe this is a particularly important question since we currently know little about anion permeation through anion channels as opposed to cation permeation through cation channels, which is relatively well understood. Understanding how Cl\textsuperscript{−} permeates through TMEM16A channels may eventually help our understanding of how Cl\textsuperscript{−}/anion permeation through anion channels is achieved in general.

\textbf{6.4. Functional roles of the re-entrant loop of TMEM16 channels}

TMEM16 family proteins show no sequence similarity with other known ion channels. Re-entrant loops, however, are a common feature of ion channel pores (Hille, 2001). Indeed, the re-entrant loop of TMEM16A was suggested to regulate the ionic selectivity (Yang \textit{et al.}, 2008) and permeability (Yu \textit{et al.}, 2012). Also, two glutamate residues in the re-entrant loop of TMEM16A have been suggested to be directly involved in binding of intracellular Ca\textsuperscript{2+} (Yu \textit{et al.}, 2012). Therefore, we hypothesized that the re-entrant loop of TMEM16 channels might regulate anion conduction and sensitivity to cytosolic Ca\textsuperscript{2+}.

To our surprise TMEM16A and TMEM16B channels revealed the same single-channel conductance and selectivity sequence for various anions. These findings contradicted the published data suggesting that a single-channel conductance of TMEM16A is \textasciitilde10-fold higher than that of TMEM16B (8.3 \textit{versus} 0.8 pS) (Yang \textit{et al.}, 2008; Stephan \textit{et al.}, 2009). This discrepancy might be explained by the fact that two groups used different approaches to determine the single-channel conductance. Yang \textit{et al.} (2008) estimated the single-channel conductance of TMEM16A from recordings of single-channel currents, while Stephan \textit{et al.} (2009) used noise analysis to estimate the single-channel conductance of TMEM16B. We believe that side by side comparison of TMEM16A and TMEM16B using identical experimental conditions (e.g. the same methodology,
expression system and \([\mathrm{Ca}^{2+}]_i\) provides a more reliable comparative analysis of channel properties.

Importantly, the re-entrant loop was found to regulate the magnitude of TMEM16 currents. The stretch of 38 amino acids within the re-entrant loop of TMEM16A was found to control the expression of functional channels on the plasma membrane. This region contains recognition sites for protein kinase C and casein kinase 2, which have been implicated in the regulation of CFTR channel trafficking (Luz et al., 2011). These regulatory binding sites, however, are present in both TMEM16A and TMEM16B channels and therefore could not explain the difference in their expression levels. Since no other regulatory segments were found within the region of 38 amino acids, we predicted the existence of a non-canonical trafficking motif. 38 amino acid stretch of TMEM16A, but not of TMEM16B, contains a segment with arginine, serine and proline residues, which closely resembles a recognition site for regulatory protein 14-3-3. This molecule is ubiquitously expressed with at least seven isoforms being found in mammalian cells. The involvement of 14-3-3 in regulation of channel trafficking is not unprecedented. Recent studies demonstrate that 14-3-3 directly interacts with CFTR and ATP-sensitive \(\mathrm{K}^+\) channels promoting their expression on the plasma membrane (Heusser et al., 2006; Liang et al., 2012). It remains to be clarified whether and which 14-3-3 isoform/s promote the cell-surface expression of TMEM16A channels. Specifically, RT-PCR will be employed to reveal the expression of 14-3-3 isoforms in our expression system, HEK-293T cells. Cells then will be treated with siRNA against one 14-3-3 isoform per time before transfecting with TMEM16A. The current density measurements will indicate whether and which 14-3-3 isoform/s regulate the magnitude of TMEM16A currents. A subsequent co-immunoprecipitation study will demonstrate if 14-3-3 physically interacts with TMEM16A channels.

### 6.5. Pharmacology of TMEM16A channels

The third part of my PhD project aimed to examine the pharmacological properties of TMEM16A channels. They were found to contain two separate A9C binding sites for activation and inhibition. A9C inhibited TMEM16A channels by occluding their pore, while the activation was achieved by A9C binding to the extracellular part of the channel and increasing its open probability. The ability to modify channel gating is a desirable property of therapeutic compounds. For example, some antiarrhythmic drugs, such as lidocaine, alter the voltage-dependent gating of cardiac \(\mathrm{Na}^+\) channels (Hanck et al.,
Also, channel modification from the extracellular side can be beneficial for inhaling drugs to treat disorders such as asthma or cystic fibrosis. Since TMEM16A activation and inhibition is mediated via A9C binding to two separate sites, it is likely that different drug properties are responsible for two opposite effects. We intend to use A9C as a starting compound to isolate drug physicochemical properties required for channel activation and inhibition. A9C contains a hydrophobic part consisting of three conjugated six-membered rings and a hydrophilic carboxyl group with $pK_a \approx 3.68$, which means that in our working solutions more than 99% of A9C is the conjugated base. To define the role of hydrophobic and hydrophilic parts of A9C on channel activity we intend to investigate the effect of A9C-related compounds on TMEM16A currents. Molecules with a variable number of conjugated rings with or without the carboxyl or other acidic groups will help understanding which drug properties are required for channel activation and inhibition. These studies will help to elucidate which functional groups of the drug interact with TMEM16A channel, the type of chemical bonds formed and the size of the binding pocket. This information is essential for the rational design of potent and selective modifiers of TMEM16A channels.
Appendix

Muscarinic receptors: electrifying new insights

Journal Club article published in The Journal of Physiology
Proteins embedded within the cell membrane are exposed to an electric field. The function of some of these proteins is regulated by and dependent upon the transmembrane potential. The degree to which voltage influences a protein’s conformation and hence its function varies, with some proteins being strongly voltage dependent while others appear to have little or no voltage dependence at all. G-protein coupled receptors (GPCRs) have been considered to be voltage insensitive because they do not have a transmembrane region rich in charged amino acids as in the case of typical voltage-sensitive proteins. Studies carried out on muscarinic receptors, a subfamily of the rhodopsin-like class of GPCRs, have, however, questioned this view. Ben-Chaim et al. (2003) demonstrated that the sensitivity of muscarinic receptors for the agonist acetylcholine (ACh) was strongly dependent on the membrane potential. Muscarinic-2 receptors (M2Rs) are predominantly expressed in the sino-atrial node, atrial myocytes and some Purkinje fibres, where they play a role in the vagal regulation of heart rate. M2R stimulation by agonists mediates dissociation of G-protein subunits, with the \( \beta \gamma \) subunit directly activating G protein-coupled inwardly rectifying potassium (GIRK) channels. The resulting outward \( K^+ \) current (GIRK current) hyperpolarizes the cardiomyocyte membrane, in this way elongating periods between contractions, an effect known as negative chronotropy (Salazar et al. 2007). Voltage-induced conformational changes within M2Rs were suggested to alter the association of the receptor with the trimeric G-proteins, thus regulating the activation of GIRK channels (Ben-Chaim et al. 2006).

In a recent issue of *The Journal of Physiology*, Navarro-Polanco et al. (2011) shed new light on the relationship between membrane potential and ligand binding to M2Rs. The authors proposed that the membrane potential modifies agonist affinity to M2Rs by inducing conformational changes within the ligand binding pocket rather than altering receptor coupling to G-proteins. This paper therefore offers an alternative mechanism for voltage sensitivity.

The authors noted that the response of M2Rs to two different agonists, ACh and pilocarpine (Pilo), varies with the voltage. The receptor response was assessed by measuring GIRK current in atrial myocytes using the whole-cell patch-clamp technique. The magnitude of currents activated by Pilo, which is a partial agonist, was compared with that elicited by ACh, a reference full agonist, over a wide range of concentrations. Concentration–response curves for the agonists showed that membrane depolarization decreased M2R affinity for ACh, while increasing the affinity and efficacy for Pilo. The authors considered various factors that could interfere with their analysis. The use of blockers for a range of \( K^+ \) and \( Ca^{2+} \) channels, as well as the highly \( Ca^{2+} \)-buffered intracellular solution, eliminated the possibility that channels other than GIRK contributed to the agonist-induced responses. Furthermore, the currents activated by Pilo and ACh had similar characteristics, arguing that both agonists activated the same GIRK currents. Finally, the effects of Pilo and ACh on the currents recorded from HEK-293 cells co-transfected with M2Rs and GIRK channels were voltage dependent and virtually identical to those recorded in atrial myocytes. Based on these results, Navarro-Polanco and colleagues (2011) propose that the agonist-specific voltage dependence of M2Rs is due to voltage-induced conformational changes occurring within the agonist binding pocket.

In order to attain a direct indication of the actual conformational changes within the receptor in response to voltage, the authors measured M2R gating currents and assessed how they were modified by agonists. Gating currents correspond to the net charge moved within the receptor upon changes in membrane potential. To record the minuscule gating currents, the authors favoured the cut-open voltage-clamp technique using oocytes expressing heterologous M2Rs. This technique allows clamping of a large surface of the membrane (about half of the oocytes) thereby including many receptors, while ensuring a fast clamping speed. The charge that moved in response to voltage pulses was calculated as the integral of the gating currents. The resulting sigmoidal charge versus voltage distribution (\( Q–V \) curve) had a half-point of maximal charge movement (\( V_{1/2} \)) at around \(-70 \) mV and the apparent charge moved per receptor, the effective valence, was \( 0.55 e_0 \). This value is relatively small compared to the effective valence of voltage-gated \( K^+ \) channels, which has been reported to be \( \sim 12 e_0 \) (Hille, 2001), indicating that M2Rs undergo rather subtle conformational changes. Importantly, the gating currents occurred at membrane potentials within the physiological range. These gating currents were modulated by agonists: ACh suppressed the gating charge displacement, whereas Pilo increased it in a dose-dependent manner. Interestingly, ACh concentrations that inhibited the M2R gating currents elicited GIRK currents lacking voltage dependence. In contrast, increasing Pilo concentrations enhanced gating charge movement, which was accompanied by pronounced voltage dependence of the ionic currents. This suggests that M2Rs confer voltage sensitivity to GIRK currents. Inspired by the diverse effects of agonists on gating currents, the authors questioned whether mutations within the ligand binding pocket could also affect gating charge displacement. Five conserved amino acids within the ACh binding site were systematically replaced with alanine (W99A, D103A, Y104A, S107A and Y403A). Three types of mutation were identified: those that shifted the Q–V distribution towards hyperpolarized potentials (D103A, Y104A and Y403A), depolarized potentials (W99A) or did not perturb gating charge movement (S107A). None of these mutants showed a decrease in the effective valence indicating that these amino acids do not form part of the primary voltage sensor. The authors also tested how two of the mutants, S107A and W99A, determine the voltage dependence of ACh-activated GIRK currents. As might be expected, both mutants showed significantly decreased...
affinity for ACh; however, the S107A mutant, which did not affect gating charge displacement, mediated GIRK currents with similar voltage dependence to that of wild-type. Interestingly, one of the mutations which altered the gating charge movement (W99A) was associated with GIRK currents lacking voltage dependence. This suggests that the affinity of W99A-M2R for ACh was the same at positive and negative potentials implying that the W99A mutation might have disrupted a link between voltage sensor movement and conformational changes within the ligand binding pocket. It would be interesting to test the voltage dependence of GIRK currents using Pilo and other agonists, as the ligand binding pockets may not be identical for all agonist due to their structural differences. In addition, structural analogues of Pilo and ACh could be employed to determine the structural features of the ligands responsible for their opposing effects. It would also be useful to know whether mutants that shifted the Q–V relationship towards hyperpolarized potentials could maintain the voltage sensitivity of agonist binding. This might help us to understand how the primary voltage sensor is linked to the ligand binding site.

A key question still remains: what is the origin of the voltage sensor? Previously proposed candidates for the primary voltage sensor, D120 and R121 (Ben-Chaim et al. 2006), were excluded in this study as the D120N–R121N mutant generated gating currents with the same effective valence as the wild-type M2R. Mutation of the negatively charged amino acid within the transmembrane electric field (D69A) resulted in a mutant with impaired protein expression. Therefore, the contribution of D69 to voltage sensing has not been determined.

This research provides new important information on how muscarinic receptors operate at the molecular level. The authors introduce a new idea in the field, suggesting that voltage controls M2R function by changing its affinity for specific agonists, independent of their coupling to downstream proteins. The observed correlation between M2R gating charge movement and voltage sensitivity of agonist-induced ionic currents suggests that when gating charge movement is suppressed, the conformation of the ligand binding pocket becomes voltage independent, such that agonist binds to the receptor with the same affinity at positive and negative potentials. In voltage-gated ion channels there is a distinct voltage sensor region, composed of charged amino acids. In contrast, M2Rs have only two charged amino acids in the transmembrane electric field and at least one of them (D103) has been shown not to be a part of the voltage sensor. This implies that dipoles rather than charged residues move in the electric field in response to changes in membrane potential. Voltage-sensing residues are unlikely to be localized within one relatively well-defined domain of the receptor, as in the case of voltage-gated K+ channels, but rather distributed within protein regions that are confined within the membrane electric field. The exact structure of the voltage sensor may therefore be difficult to identify.

In heart, ACh at concentrations occurring in failing hearts. Cardiac GPCRs: GPCR signaling in healthy and failing hearts. Biochim Biophys Acta 1768, 1006–1018.

Acknowledgements

The authors would like to thank Dr Paolo Tammaro and Professor Alison Gurney for their advice during the preparation of this article.
References

Relevant to chapters 1, 2 and 6


Duran C, Qu Z, Osunkoya AO, Cui Y & Hartzell HC. (2011). ANOs 3-7 in the anoctamin/Tmem16 Cl\(^-\) channel family are intracellular proteins. American Journal of Physiology - Cell Physiology 302, C482-C493.


Namkung W, Thiagarajah JR, Phuan P-W & Verkman AS. (2010). Inhibition of Ca$^{2+}$-activated Cl$^{-}$ channels by gallotannins as a possible molecular basis for health benefits of red wine and green tea. The FASEB Journal 24, 4178-4186.


