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The bestrophin- and TMEM16A-associated Ca^{2+}-activated Cl^- channels in vascular smooth muscles

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Abbreviations: CaCC, Ca^{2+}-activated Cl^- channel; cGMP, cyclic guanosine monophosphate; BK_{Ca}, Ca^{2+}-activated K^+ channel; $I_{Cl(Ca)}$, Ca^{2+}-activated Cl^- current; LTCC, L-type Ca^{2+} channel; PKG, protein kinase G; siRNA, small interference RNA; SR, sarcoplasmic reticulum; VSMC, vascular smooth muscle cell

The presence of Ca^{2+}-activated Cl^- currents ($I_{Cl(Ca)}$) in vascular smooth muscle cells (VSMCs) is well established. $I_{Cl(Ca)}$ are supposedly important for arterial contraction by linking changes in $[Ca^{2+}]_i$ and membrane depolarization. Bestrophins and some members of the TMEM16 protein family were recently associated with $I_{Cl(Ca)}$. Two distinct $I_{Cl(Ca)}$ are characterized in VSMCs; the cGMP-dependent $I_{Cl(Ca)}$ dependent upon bestrophin expression and the 'classical' Ca^{2+}-activated Cl^- current, which is bestrophin-independent. Interestingly, TMEM16A is essential for both the cGMP-dependent and the classical $I_{Cl(Ca)}$. Furthermore, TMEM16A has a role in arterial contraction while bestrophins do not. TMEM16A's role in the contractile response cannot be explained however only by a simple suppression of the depolarization by Cl^- channels. It is suggested that TMEM16A expression modulates voltage-gated Ca^{2+} influx in a voltage-independent manner and recent studies also demonstrate a complex role of TMEM16A in modulating other membrane proteins.

Introduction

For more than two decades, the functional role of Ca^{2+}-activated Cl^- channels (CaCCs) in vascular smooth muscle cells (VSMCs) has been actively investigated.[34,41,42,47,95] This research focus was initiated by the first demonstration in 1987[13] of a Ca^{2+}-activated Cl^- current ($I_{Cl(Ca)}$) in smooth muscle cells, which naturally led to the suggestion that CaCCs are also present in VSMCs. Although $I_{Cl(Ca)}$ has subsequently been well-characterized in patch clamp studies of isolated VSMCs[11,48,49,59] these studies have not linked the $I_{Cl(Ca)}$ to its function in the vascular wall. Since Cl^- is actively accumulated in VSMCs,[1,71] opening of CaCCs at resting membrane potentials will lead to Cl^- efflux and membrane depolarization. These CaCC properties integrate intracellular calcium ($[Ca^{2+}]_i$) increases with membrane potential changes and suggest an amplifying link for agonist-induced contraction[32,40,46,62,87] as well as several other important properties of the vascular wall, e.g., vascular rhythmicity[9,37] and myogenic tone.[12,64] Uncertainties in the mechanistic explanation of this amplifying link are, at least in part, due to the lack of specific pharmacological tools and uncertainty about the molecular structure of CaCCs.[30]

Significant progress in this field has occurred over the last years as putative CaCC proteins have been identified.[14,83,88,97,103] Four members of the bestrophin family can produce $I_{Cl(Ca)}$,[83,97] although their direct association with the endogenous $I_{Cl(Ca)}$ has been questioned.[30] TMEM16A and some other members of the TMEM16 protein family are also shown to be essential for $I_{Cl(Ca)}$.[14,88,103] There is no universal profile for the biophysical and pharmacological properties of $I_{Cl(Ca)}$ reported in VSMCs.[54] The CaCCs responsible for these currents are suggested to vary in their Ca^{2+}-sensitivity and the mechanism of Ca^{2+} activation, voltage- and time-dependency, halide permeability and sensitivities to blockers. These variable characteristics could partially be attributable to differences in the methods of registration, protocols for current activation, solutions and origins of VSMCs. It is, however, most probable that the CaCCs represent a heterologous group of channel proteins unified by their anion sensitivity and Ca^{2+}-dependence.[77] Several subgroups of CaCCs have been suggested based upon their activation mechanism, e.g., the CaCCs activated directly by $[Ca^{2+}]_i$ and those that need additional activators such as CaMKII or cGMP-dependent protein kinase (PKG).[24,25,30] The current, general classification of $I_{Cl(Ca)}$ is based on biophysical and pharmacological characteristics obtained under variable experimental conditions. There is a substantial need to improve this classification based on an understanding of the molecular structures that are associated with the specific characteristics.[54] Evidence for the association between $I_{Cl(Ca)}$ with different properties[55,75,76] and different putative CaCC proteins[16,17,33,52,58,93] is needed. Such associations are beginning to appear, e.g., in the vasculature, where the 'classical' $I_{Cl(Ca)}$ co-exists with another $I_{Cl(Ca)}$, which has an absolute requirement for cyclic GMP for its activation and several other biophysical and pharmacological characteristics distinct from the classical $I_{Cl(Ca)}$.[55,75,76] The cGMP-dependent $I_{Cl(Ca)}$ depends upon the expression of

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bestrophins[21,58] while the classical $I_{Cl(Ca)}$ does not. It is therefore of interest to know whether TMEM16A is important for the classical $I_{Cl(Ca)}$ and what relation this protein has to the cGMP-dependent $I_{Cl(Ca)}$ in VSMCs. Interestingly, TMEM16A is essential for both the classical $I_{Cl(Ca)}$[17,52,93] and the cGMP-dependent $I_{Cl(Ca)}$.[16] This complicates the description of $I_{Cl(Ca)}$ with a certain characteristic to a specific protein or protein family.

Although the association of bestrophins and TMEM16A with CaCCs has been originally suggested in heterologous expression experiments, detailed molecular-based studies of the functional importance of CaCCs in the vascular wall in vitro and in vivo have been made possible by novel approaches to studying vascular tissue; siRNA-induced downregulation of the protein of interest,[58] tissue specific knockdown in mice,[33] molecular cloning and mutagenesis[14] and drug-screening strategies.[68] An siRNA approach suggests that the bestrophin-associated cGMP-dependent $I_{Cl(Ca)}$ has no significance for tonic contractile response in small arteries.[10] These findings are further supported by a comprehensive study suggesting minor or little importance of the Cl^- conductance for arterial contraction in rat mesenteric small arteries.[9,33] However, suppressing TMEM16A expression reduces arterial contraction[12,16,17] and lowers arterial blood pressure.[33] A detailed analysis of the functional consequences of TMEM16A expression changes suggests that TMEM16A is more than a channel-forming protein and likely has several other modulatory and expression-related cellular functions.[16]

In spite of significant progress in our understanding the role CaCC has in the vasculature, several significant incongruities between various reports as well as between working hypotheses and experimental results remain to be resolved. Some of these questions will be discussed in this review.

1) the role of Cl^- conductances for arterial contraction;

2) whether the two CaCCs are represented by a single channel-forming protein family;

3) how TMEM16A and bestrophins interact with each other to produce different Ca^{2+}-activated Cl^- conductances;

4) other putative functions of CaCC-associated proteins in the vascular wall.

Two Functionally Distinct $I_{Cl(Ca)}$ in VSMCs

The existence of a depolarizing conductance that can be activated by cGMP and $[Ca^{2+}]_i$ was previously hypothesized based on the model for generation of vasomotion.[37,72] A depolarizing current dependent on or activated by cGMP is unexpected as cGMP normally leads to hyperpolarization in VSMCs. However, the cGMP-dependent $I_{Cl(Ca)}$ in VSMCs in rat mesenteric small arteries has been reported by two independent laboratories.[55,75,76] The functional characterization of this conductance clearly indicates that it is a unique current in comparison with the well-described classical $I_{Cl(Ca)}$.[30,48] In addition to an absolute cGMP-dependence of only one of these two $I_{Cl(Ca)}$, these conductances differ in their Ca^{2+}- and voltage-dependence as well as in their pharmacological profile.[54] The inhibitors which have been shown to be effective, at least in patch clamp experiments, for the classical $I_{Cl(Ca)}$, e.g., niflumic acid, 5-isothiocyanato-2-[2-(4-isothiocyanato-2-sulfophenyl)ethenyl]benzene-1-sulfonic acid (DIDS) and indanyloxyacetic acid 94 (IAA94),[28] affected the cGMP-dependent $I_{Cl(Ca)}$ only at high concentrations.[55] In contrast, the cGMP-dependent $I_{Cl(Ca)}$ was shown to be sensitive to extracellular Zn^{2+} in concentrations that are without effect on the classical $I_{Cl(Ca)}$.[55,56,76] Differences in blocker sensitivity and distinction in cGMP-activity make it possible to differentiate these two $I_{Cl(Ca)}$ in VSMCs during voltage-clamp experiments.[9,16,17,55,56,58,75,76] Until recently this was the approach to isolate and study the different $I_{Cl(Ca)}$ in VSMCs and pharmacological differentiation was limited in situ due to off-target effects of the blockers.[9,28] When inhibitors are tested in patch clamp experiments, the experimental conditions are created to emphasize the current of interest, e.g., the $I_{Cl(Ca)}$. In contrast, in situ experiments do not often provide such possibility and several conductances can therefore be affected by the same blocker. Thus, a commonly used CaCC inhibitor, niflumic acid, is also known to activate the big conductance Ca^{2+}-activated K^+ channels (BK_{Ca}) and to inhibit L-type Ca^{2+} channel (LTCC).[27,35,56,70,74] These effects would have the same consequences for VSMC membrane potential and vascular tone as inhibition of the $I_{Cl(Ca)}$. This limited specificity of the conventional CaCC inhibitors limits their use for studies of the importance of CaCCs for regulation of vascular tone.[28]

The pharmacological overlap between $I_{Cl(Ca)}$ and cation currents has led to the suggestion of molecular interaction between different groups of channels and even that some molecules may be shared in the molecular complexes important for these currents.[28] The overlapping pharmacological profile does not in itself prove protein-protein interaction nor molecular sharing. Although the observation that CaCC blockers can modulate BK_{Ca} and LTCC, and that the modulation of CaCCs by BK_{Ca} and LTCC can also occur,[28] together with localization of these channels in the same membrane microdomains, does suggest a close physical interaction.

Greenwood and co-authors have addressed this question suggesting the potential co-localization of BK_{Ca} and CaCCs in cholesterol-rich lipid rafts in VSMC membrane.[90] While BK_{Ca} are shown to localize in cholesterol-rich lipid rafts and their properties are modulated by interaction with caveolin[2] and cholesterol depletion,[89] the pharmacological overlap between CaCCs and BK_{Ca} cannot be explained by a simple co-localization of these channels. The putative CaCC TMEM16A is shown to be distributed more evenly in the VSMC plasma membrane having only slight overlap with BK_{Ca} localization.[90] This is consistent with the finding that cholesterol depletion omits the effects of BK_{Ca} blockers on $I_{Cl(Ca)}$ but has no major consequences for the effects of niflumic acid. This suggests that the interaction between BK_{Ca} and CaCCs is more subtle or complex than simple consolidation in a microdomain.[90]

In contrast, a close proximity of CaCCs to voltage-gated Ca^{2+} channels (VGCCs) in the membrane of hippocampal neurons was previously shown.[36] This co-localization is, however, tissue specific as CaCCs were shown to be functionally and structurally separate from VGCCs in other types of neurons: CaCCs were instead shown to interact specifically with ryanodine receptors in

avian sensory neurons[100] and with Ins(1,4,5)P_3 receptors in dorsal root ganglia neurons.[39] This variation in the interacting partner for CaCCs could be the reason for variability of side-effects for different pharmacological agents in different types of cells.

An interesting approach to differentiate $I_{Cl(Ca)}$ in VSMCs was recently used where inhibitory antibodies against two putative CaCC proteins – TMEM16A and bestrophin – were applied to suppress the membrane currents associated with these proteins.[17] Importantly, antibodies raised against bestrophin or TMEM16A inhibited the cGMP-dependent $I_{Cl(Ca)}$ and the classical $I_{Cl(Ca)}$, respectively. Moreover, anti-bestrophin antibody was without effect on the classical $I_{Cl(Ca)}$. While the authors did not test whether the anti-TMEM16A antibody affects the cGMP-dependent $I_{Cl(Ca)}$ it is tempting to suggest that these two Ca^{2+}-activated Cl^- conductances have different molecular identities.[17]

Knowledge of molecular candidates significantly improves the efficiency for drug-screening that has led to the generation of novel, small molecule inhibitors.[98] The recently developed inhibitor $T16A_{inh}$-A01[63] effectively inhibits the TMEM16A-associated $I_{Cl(Ca)}$ without effect on the bestrophin-associated cGMP-dependent $I_{Cl(Ca)}$ in VSMCs.[17] This inhibitor is now widely used for pharmacological identification of TMEM16A-associated $I_{Cl(Ca)}$ and its functions,[23,38,60,69,101] including VSMC contraction.[17] Drug-screening also generated another inhibitor, $CaCC_{inh}$-A01, which has been shown to inhibit TMEM16A-associated $I_{Cl(Ca)}$.[18] This inhibitor has, however, some peculiar properties, since it was shown to affect TMEM16A in cancer cells in two distinct ways: in addition to its inhibitory action on $I_{Cl(Ca)}$, $CaCC_{inh}$-A01 reduces protein level in the membrane by facilitating reticulum-associated, proteasomal turn-over of TMEM16A.[8] The latter action of $CaCC_{inh}$-A01 is complex because after prolonged exposure of a cell culture to $CaCC_{inh}$-A01 a pool of $CaCC_{inh}$-A01 resistant cells developed, where the inhibitor could still block the $I_{Cl(Ca)}$ but TMEM16A remained in the membrane. Importantly, the initial $CaCC_{inh}$-A01-induced retrieval of TMEM16A from the membrane is associated with inhibition of cell proliferation and the authors provided evidence that the anti-proliferative effect could be ascribed to the disappearance of TMEM16A. This interpretation was consistent with observation that $T16A_{inh}$-A01, which only blocks the $I_{Cl(Ca)}$, has no effect on proliferation. These findings strongly suggest a role for TMEM16A in proliferation, which is only partially dependent upon its channel function.[8] This conclusion is further complicated, however, by the lack of direct evidence for binding of $T16A_{inh}$-A01 and $CaCC_{inh}$-A01 to TMEM16A[8] and by the lack of knowledge about the mechanism of their inhibitory action. A structurally different inhibitor, MONNA, was recently shown to have high potency and selectivity for the TMEM16A-associated $I_{Cl(Ca)}$, while it was ineffective toward inhibition of bestrophin-1 associated $I_{Cl(Ca)}$.[68] The fact that these novel small molecular inhibitors can inhibit the TMEM16A-associated $I_{Cl(Ca)}$ without an effect on the bestrophin-associated $I_{Cl(Ca)}$ is supportive evidence for different proteins forming these two conductances.[17,68] Unfortunately, a specific inhibitor for the bestrophin-associated $I_{Cl(Ca)}$ is still lacking. Importantly, although the effects of these inhibitors on cation membrane conductances have not been analyzed in detail, $T16A_{inh}$-A01 has been shown to relax different types of vasculature[17] in accordance with the hypothesized role of CaCCs in agonist-induced arterial contraction.[48,49] An in-depth analysis of the effects of $T16A_{inh}$-A01 on voltage-gated Ca^{2+} currents and K^+ currents in VSMC membrane is, however, necessary to substantiate this conclusion. The other small molecular inhibitors, $CaCC_{inh}$-A01 and MONNA, have not been evaluated on vascular tissue.

Is Bestrophin still a Putative CaCC Protein?

The cGMP-dependent $I_{Cl(Ca)}$ in rat VSMCs is absent after knockdown of both TMEM16A[16] and bestrophin-3[58] proteins. However, expression of bestrophin-3 is required only for the cGMP-dependent $I_{Cl(Ca)}$.[16] Is it possible that bestrophin is a channel subunit that confers the cGMP-dependence? In fact, a cGMP-dependence has previously been shown for human bestrophin-1 but the mechanism behind it has not been analyzed.[20,21]

The data from rat VSMCs are inconsistent with the results from a heterologously expressed mouse heart variant of bestrophin-3, which can produce the $I_{Cl(Ca)}$ of significant amplitude without any presence of cGMP under whole-cell configuration.[66,91] Importantly, bestrophins are known to be expressed in various splice variants, sometimes present in the same cell type,[43,91] and this could explain the inconsistent experimental findings. This possibility is supported by a study showing that expression of the full-length clone of mouse bestrophin-3 does not produce any significant Cl^- current due to autoinhibition by the C-terminus.[29,78,85] This autoinhibitory cytoplasmic C-region has been predicted to contain sites for PKG-dependent phosphorylation[58] as well as for other protein-protein interactions.[61] Moreover, mouse bestrophin-3 with the C-terminus deleted loses its autoinhibitory capacity and produces a large Cl^- current when expressed heterologously.[78,85] These studies indirectly suggest that the cGMP-dependency of bestrophin-3-associated $I_{Cl(Ca)}$ could be a consequence of the splice variant expressed. Although the presence of the autoinhibitory C-terminal domain has been suggested for all members of the bestrophin family,[85] the exact autoinhibitory phosphorylation site remains to be determined. There is no evidence for direct PKG-phosphorylation of the bestrophin protein: this effect might be indirect and involve several additional phosphorylation and dephosphorylation steps. Thus, the C-terminal autoinhibitory domain of mouse bestrophin-3 has been suggested to interact directly with negatively-charged membrane phospholipids and this interaction dynamically regulates the bestrophin-3-associated CaCC activation via the phosphatidyl inositol 3-kinase.[80] However, this modulation is not essential for channel activation and other regulatory sites have been suggested.[29] Indeed, it has been previously shown that $[Ca^{2+}]_i$ might activate human bestrophin-3 without going through a freely diffusible messenger or through protein phosphorylation.[96] This conclusion was, however, reached based upon very slow activation and deactivation kinetics of the CaCC and cannot exclude the involvement of membrane-associated messenger(s) acting on the autoinhibitory C-terminal.[96] Some reports suggest

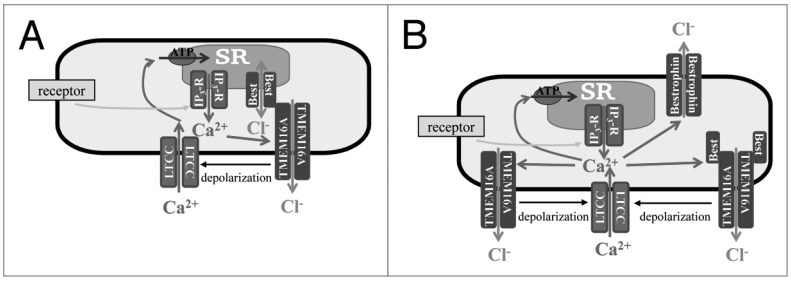

Figure 1. Agonist-induced Ca^{2+}-release activates the $I_{Cl(Ca)}$ which in term depolarises VSMCs leading to influx of Ca^{2+} through voltage-gated L-type Ca^{2+} channels (LTCC) and consequent force development. It has been suggested that TMEM16A is the pore-forming subunit responsible for $I_{Cl(Ca)}$. Bestrophin has been suggested to act as sarcoplasmic reticulum (SR)-located channel that serves as a countercurrent for Ca^{2+} movement (**A**). Other data suggest that bestrophin may be either a subunit of a TMEM16A-formed channel, which modifies the biophysical and pharmacological characteristics of the TMEM16A-associated CaCCs or forms the CaCCs with characteristics distinct from the TMEM16A-associated CaCCs (**B**). Further studies are needed to test for these possibilities.

the presence of potent phosphorylation sites on bestrophin.[6,58,85] Accordingly, bestrophin-1 activation was shown to be dependent on a CaMKII-dependent phosphorylation.[19] This phosphorylation is further regulated by a modulatory action of protein phosphatase 2A, which has been found physically-associated with bestrophin.[53] Finally, the regulation of bestrophin-associated CaCCs could involve the phosphorylation of an accessory subunit, which is as yet unidentified. Very recently, however, novel evidence has been presented to suggest that bestrophins can also form homotetrameric channels and perform highly-regulated CaCC activity independently of other proteins.[7]

The Expression of Bestrophins in Vascular Wall is Modulated by TMEM16A: A Challenge for Structure-Function Analyses

A recent study of siRNA-induced downregulation of TMEM16A demonstrated that both the bestrophin-associated cGMP-dependent $I_{Cl(Ca)}$ and the classical $I_{Cl(Ca)}$ disappeared.[16] Based on these results it is tempting to suggest that TMEM16A might be the channel-forming protein and association with bestrophin generates the cGMP-dependent $I_{Cl(Ca)}$ (**Fig. 1**). This suggestion is, however, complicated by the demonstrated ability of TMEM16A to modulate the expression of other membrane proteins including bestrophins and L-type Ca^{2+} channels.[16] It is therefore not clear how the expression of bestrophins is essential for the cGMP-dependent $I_{Cl(Ca)}$ and whether TMEM16A downregulation affects this current indirectly via reduction in bestrophin expression. Other experimental models, e.g., TMEM16A knockout mice,[33] will be helpful in addressing this question.

The mechanism by which TMEM16A affects expression of other genes and proteins is unknown but might involve protein kinase-dependent signaling pathways. In fact, bestrophin-3 expression in renal epithelial cells has been shown to be modulated by extracellular-signal-regulated kinases (ERK1/2).[50] This ERK1/2 signaling is in turn known as the mechanism involved in TMEM16A-induced cancer cell proliferation.[22] Interestingly, the small-molecular inhibitors ($T16A_{inh}$-A01[22] and $CaCC_{inh}$-A01[8]) have been shown to abrogate the tumorigenic action of TMEM16A, but their mechanism of action is not fully understood, as discussed above. While the effect of $CaCC_{inh}$-A01is independent from CaCC activity,[8] the pore-forming region is necessary for successful inhibition of ERK1/2 signaling by $T16A_{inh}$-A01.[22] The involvement of ERK1/2 signaling for the control of bestrophin expression by TMEM16A remains to be shown; nevertheless, ERK1/2 signaling is known to be active in VSMCs. It is tempting to consider that similar mechanism(s) have a place in regulation of the function and growth of the vascular wall. Moreover, TMEM16A has also been shown to activate nuclear factor–kB (NF-kB), which mediates the expression of numerous genes.[51] Whether bestrophins are also modulated by NF-kB is unknown. Moreover, whether NF-kB signaling in VSMCs is under control of TMEM16A is also unknown.

There is no molecular evidence to date that TMEM16A can work directly as a transcription regulator but it is possible that proteolytically processed part(s) of TMEM16A may act as gene transcription factor. A similar function has previously been suggested for a C-terminal segment of voltage-gated L-type Ca^{2+} channels in neurons and VSMCs.[3,26] Recent evidence from siRNA-mediated downregulation of TMEM16A suggests that TMEM16A can modulate the expression of L-type Ca^{2+} channels.[16] This is inconsistent with a recently reported tamoxifen-induced VSMC-specific TMEM16A knockout where voltage-dependent Ca^{2+} influx was unaffected.[33] However, in this knockout study the $I_{Cl(Ca)}$ was abolished suggesting a defective CaCC function but a truncated TMEM16A protein was still present in VSMCs and this could potentially be sufficient for gene transcription regulation.

Finally, it is possible that the expression-related effect of TMEM16A is indirectly mediated via changes in intracellular spatio-temporal Ca^{2+} signaling, affecting the Ca^{2+}-dependent transcription factors in VSMCs.[57] Although it remains to be found which gene(s) are modulated by TMEM16A and the mechanism behind this modulation, lessons from cancer cell proliferation studies and the recent demonstration of TMEM16A regulation of gene expression in VSMCs[16] clearly indicate that TMEM16A serves as a modulator for gene transcription.

Although the role of TMEM16A in modulation of gene expression is completely unexplored, the importance of this mechanism can be highlighted by the involvement of TMEM16A expression in several physiological and pathophysiological changes in the vascular wall. Thus, it has been shown that hypoxia-induced pulmonary artery hypertrophy and experimental pulmonary hypertension are associated with an increase in TMEM16A-associated $I_{Cl(Ca)}$ and TMEM16A protein expression.[23,92] Whether these changes are primary or secondary to the remodeling-associated changes in gene expression is not known, but TMEM16A has been suggested to be a negative regulator of VSMC proliferation

in hypertension-induced cerebrovascular remodeling.[99] This importance of TMEM16A for arterial structure can vary between different vascular beds in addition to being a stimulus-specific response. Interestingly, no structural changes in peripheral arteries have been reported for the recently published TMEM16A knockout mice.[33] Further studies are needed to clarify the role of TMEM16A for arterial structure.

TMEM16A and Bestrophins can Interact in the Vascular Wall

There are several potential modes of interaction between TMEM16A and bestrophins (**Fig. 1**). One possibility is that bestrophin are intracellular channels in the sarcoplasmic reticulum membrane providing countercurrent for Ca^{2+} release, which in turn stimulates TMEM16A-formed CaCCs.[6,44] However, voltage-clamp studies where bestrophin-associated $I_{Cl(Ca)}$ was measured do not provide experimental support for this model.[4,5,15,19,29,55,58,66,73,78,79,81-85,96,97,102] Indeed, the bestrophin-associated cGMP-dependent $I_{Cl(Ca)}$ was elicited independently from the sarcoplasmic reticulum by elevating $[Ca^{2+}]_i$ via patch pipette solution and via membrane permeabilization for Ca^{2+}.[58] Although it cannot entirely be ruled out that the high cytosolic Ca^{2+} leads to increased release of Ca^{2+} from the sarcoplasmic reticulum[6] these experiments[58] together with single channel recordings,[17] suggest that bestrophin is directly associated with a plasma membrane conductance. Whether this is also the case for all the different bestrophin isoforms is unclear.

There are two possibilities for bestrophin functions in the cell membrane (**Fig. 1**). Bestrophins could be responsible for atypical $I_{Cl(Ca)}$ currents, i.e., the cGMP-dependent $I_{Cl(Ca)}$, while TMEM16A proteins could be responsible for the classical $I_{Cl(Ca)}$. This possibility cannot be tested easily as the expression of TMEM16A modulates bestrophin expression,[16] as discussed above. Moreover, heterologous expression of bestrophins may affect endogenous currents. The bestrophin expression profile has not yet been compared between wild-type and TMEM16A knockout mice.[33] Another possibility is that bestrophin forms a regulatory subunit modifying the biophysical and pharmacological properties of a TMEM16A-formed channel (**Fig. 1**): similar modulation has been shown for the inward-rectifying K^+ channels, which change their electrophysiology and pharmacology substantially after binding to the sulfonylurea receptor subunit to form ATP-dependent K^+ channels.[86] Testing of this possibility for interaction between TMEM16A and bestrophin is difficult due to technical difficulties in independently modulating the expression of TMEM16A and bestrophin proteins. The majority of cells used for heterologous expression studies possess an endogenous CaCC and express either bestrophins or TMEM16A or both.[30,31] A CaCC-free expressional system, such as axolotl (*Ambystoma Mexicanum*) oocytes,[88] would be suitable for addressing this question. Importantly, recent single-molecule subunit analysis suggested that bestrophins are preferentially homotetrameric[7] channels which have little or no interaction with each other and TMEM16A. Similarly, homotypic self-assembly into CaCCs was shown for TMEM16A.[94] Whether these proteins interact with each other remains to be studied.

What is the Role of the Ca^{2+}-Activated Cl^- Conductance for Arterial Contraction?

Since Cl^- is actively pumped into VSMCs and opening of Cl^- channels consequently produces membrane depolarization, the CaCCs have been suggested to participate in the excitation-contraction coupling where they link $[Ca^{2+}]_i$ and membrane potential changes.[48,49] An agonist-induced Ca^{2+} increase leads to CaCC-dependent membrane depolarization, opening of voltage-gated Ca^{2+} channels and further increases in $[Ca^{2+}]_i$. This amplifying mechanism has been suggested to be important for agonist-induced contraction of VSMCs. If the Cl^- conductance is important for contraction, the contraction should depend on the Cl^- gradient across the plasma membrane. Several studies have shown that replacement of extracellular Cl^- with impermeable anions acutely amplifies agonist-induced depolarization and contraction[45,46,64] but this is not always the case. Substitution of extracellular Cl^- was shown to have only mild effect on both resting membrane potential and membrane potential in noradrenaline-stimulated rat mesenteric small arteries[65] and no significant effect on contraction.[9] Nevertheless, a variable significance of Cl^- for VSMC contraction depending on the type of vasculature and stimulation can be suggested.[46] In fact, this is consistent with recent results from TMEM16A knockout mice showing a lack of effect of knockout in mesenteric arteries, while in other blood vessels, e.g., aorta and carotid artery, the contractile response was significantly diminished by TMEM16A knockout.[33]

The Functional Significance of TMEM16A in the Vascular Wall

The lack of the effect of downregulation of bestrophin-associated cGMP-dependent $I_{Cl(Ca)}$ on force development supported the suggestion that Cl^- conductance has little role for agonist-induced contraction of rat mesenteric small arteries.[10] However, when the TMEM16A-associated classical $I_{Cl(Ca)}$ and cGMP-dependent $I_{Cl(Ca)}$ were downregulated, the arteries depolarized significantly less to noradrenaline, and the $[Ca^{2+}]_i$ increase and contraction were reduced[16] as was the response to vasopressin in these arteries. If this effect was due to lower CaCC-dependent depolarization, it should be rescued by depolarizing the arterial wall but this was not the case: KCl-induced depolarization and contraction were significantly reduced in the TMEM16A-downregulated arteries.[16] Furthermore, in the same experimental series, when the membrane potential was similar, $[Ca^{2+}]_i$ was lower in the arteries where TMEM16A was downregulated. Taken together these functional results suggest that TMEM16A may modify voltage-dependent Ca^{2+} influx independently of the membrane potential.[16] Importantly, these results do not exclude

CaCCs from having a role in VSMC depolarization but rather suggest that this role is not exclusive, at least in rat mesenteric arteries.

In contrast, the agonist-induced contractions of other arteries have been shown to be strongly dependent on CaCC depolarizations with no indication of the involvement of voltage-gated Ca^{2+} channels. Thus, in functional studies on mouse thoracic aorta and mesenteric artery as well as human abdominal visceral adipose artery a novel blocker of TMEM16A-associated CaCCs, e.g., $T16A_{inh}$-A01, relaxed preconstricted arteries in vitro.[17] $T16A_{inh}$-A01 was without significant effect on arterial contraction induced by 60 mM KCl.[17] This contrasts with the findings in rat mesenteric arteries discussed above.[16] This could be due to a chronic effect of siRNA-induced downregulation that reduces voltage-gated Ca^{2+} channels vs. an acute pharmacological effect of the blocker. However, siRNA-induced downregulation of TMEM16A in rat cerebral artery organ culture does not support this suggestion.[12] Rat cerebral arteries downregulated for TMEM16A with siRNA demonstrate a reduction in pressure-induced depolarization and myogenic vasoconstriction, but the contraction to 60 mM KCl depolarization was unaffected. However, the authors suggested that Ca^{2+} influx via mechanosensitive nonselective cation channels stimulates TMEM16A-associated channels to induce myogenic vasoconstriction and demonstrated no importance of voltage-dependent Ca^{2+}-influx or Ca^{2+}-release for activation of TMEM16A-associated channels.[12]

The reason for this inconsistency is unclear and could reflect different roles of CaCC in different vascular beds. In fact, it has recently been shown that mouse mesenteric small arteries have a very weak expression of TMEM16A and an $I_{Cl(Ca)}$ of low amplitude.[33] Consistently, an inducible VSMC-specific knockout of TMEM16A in mice was without any effect on mesenteric small artery contractility. In contrast, larger blood vessels, e.g., aorta and carotid artery from TMEM16A knockout mice, had reduced contractility to angiotensin II and U46619 (thromboxane analog) compared with their wild type counterparts.[33] These larger arteries from wild type mice have a large TMEM16A-associated $I_{Cl(Ca)}$. It is of interest to determine whether the responses to adrenergic agonists are also reduced in the larger vessels. Knockout of TMEM16A had no effect on the contraction to 60 mM KCl suggesting that the expression of voltage-gated Ca^{2+} channels was unaffected in this model.[33] However, as mentioned above, although the $I_{Cl(Ca)}$ was gone a significant part of the truncated TMEM16A protein was still present in the VSMCs which could be relevant for function.

Concluding Remarks

The current evidence indicates that TMEM16A is essential for generation of $I_{Cl(Ca)}$ suggesting that this protein is a channel-forming subunit of CaCCs.[12,16,17,23,52,67,93] It remains to be determined whether TMEM16A is associated with different forms of CaCCs and whether other $I_{Cl(Ca)}$-associated proteins, e.g., bestrophins, serve as regulatory proteins that modify the channel's properties. This challenge is hampered by the surprising finding that TMEM16A expression is important for the expression of other genes, such as bestrophins, which are associated with some forms of the $I_{Cl(Ca)}$, and voltage-gated Ca^{2+} channels.[16] These non-contractile functions of TMEM16A protein in VSMCs further complicate the analysis of CaCC function in the vascular wall. It seems that the expression and the function (either membrane depolarization or modulation of voltage-gated Ca^{2+} channels) of TMEM16A vary in different vascular beds and thus may greatly influence experimental results. There is however a general consensus that the TMEM16A-associated $I_{Cl(Ca)}$ is an important component of VSMC function and is important for maintenance of total peripheral resistance and, thus, blood pressure.[12,16,17,33]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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