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INVITED REVIEW

Cl^- channels in smooth muscle cells

Simon Bulley · Jonathan H. Jaggar

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Abstract In smooth muscle cells (SMCs), the intracellular chloride ion (Cl^-) concentration is high due to accumulation by Cl^-/HCO_3^- exchange and Na^+–K^+–Cl^- cotransportation. The equilibrium potential for Cl^- (E_{Cl}) is more positive than physiological membrane potentials (E_m), with Cl^- efflux inducing membrane depolarization. Early studies used electrophysiology and nonspecific antagonists to study the physiological relevance of Cl^- channels in SMCs. More recent reports have incorporated molecular biological approaches to identify and determine the functional significance of several different Cl^- channels. Both "classic" and cGMP-dependent calcium (Ca^{2+})-activated (Cl_{Ca}) channels and volume-sensitive Cl^- channels are present, with TMEM16A/ANO1, bestrophins, and ClC-3, respectively, proposed as molecular candidates for these channels. The cystic fibrosis transmembrane conductance regulator (CFTR) has also been described in SMCs. This review will focus on discussing recent progress made in identifying each of these Cl^- channels in SMCs, their physiological functions, and contribution to diseases that modify contraction, apoptosis, and cell proliferation.

Keywords Chloride channel · Smooth muscle · TMEM16A/Ano1 · Bestrophins · ClC-3 · CFTR

Introduction

Chloride (Cl^-) is the predominant extracellular and intracellular anion with intracellular concentration $[Cl^-]_i$ varying widely between different cell types. In many cells, such as frog skeletal muscle, $[Cl^-]_i$ is similar to that predicted by passive distribution determined by the Donnan equilibrium [47]. In contrast, in vascular smooth muscle cells (SMCs), $[Cl^-]_i$ is much higher than would be expected [14]. $[Cl^-]_i$ ranging from ~30 to ~50 mM has been recorded in SMCs using a variety of techniques, including radioisotopes, fluorescent dyes, and ion-selective electrodes (see [57]). High $[Cl^-]_i$ is maintained by active accumulation through Cl^-/HCO_3^- anion exchange and Na^+–K^+–Cl^- cotransportation [1, 90]. The estimated equilibrium potential for Cl^- (E_{Cl}^-) is between −30 and −20 mV in SMCs [57, 66]. Physiological membrane potential (E_m) in vascular and nonvascular SMCs ranges between ~−60 and ~−40 mV [6, 44, 83, 85, 86, 118]. Cl^- channel activation would result in Cl^- efflux, leading to membrane depolarization, voltage-dependent calcium (Ca^{2+}) channel activation, an elevation in $[Ca^{2+}]_i$, and contraction [19, 46, 65]. In addition to modulation of membrane potential and contractility, intracellular Cl^- has also been proposed to regulate intracellular pH and cell volume in SMCs [14].

Cl^- channels are subdivided into five families: transmembrane protein 16 (TMEM16)/anoctamin (ANO), bestrophins, voltage-gated Cl^- channels (CLCs), cystic fibrosis (CF) transmembrane conductance regulator (CFTR), and ligand-gated Cl^- channels, including glycine and γ-aminobutyric acid (GABA) receptors [30]. This review will summarize knowledge of TMEM16A/ANO, bestrophins, CLCs, and CFTR due to limited evidence for other Cl^- channel members in SMCs. The predicted membrane topologies for each of these Cl^- channels are illustrated in Fig. 1. Ligand-gated Cl^- channels have been described in airway SMCs, where both $GABA_A$ and GlyR1 channels are expressed and functional [81, 143]. A distinct type of Cl^- current ($I_{Cl,acid}$) activated by acidic extracellular pH has also been reported in aortic SMCs that may be generated by CLC-3 [71, 76].

Functional significance of SMC Cl^- currents

Several early studies demonstrated Cl^- flux in a variety of different vascular SMC types [11, 108, 124, 137]. Noradrenaline (NE) stimulated $^{36}Cl^-$ efflux in rat aorta, portal vein, and rabbit pulmonary arteries [11, 108, 124]. Subsequent findings showed that NE-induced depolarization of rat anococcygeus muscle cells was Cl^- current-dependent; endothelin (ET) activated Cl^- currents in porcine coronary artery, human mesenteric artery SMCs, and cultured aortic SMCs; and histamine

S. Bulley · J. H. Jaggar (✉)
Department of Physiology, University of Tennessee Health Science Center, Memphis, TN 38163, USA
e-mail: jjaggar@uthsc.edu

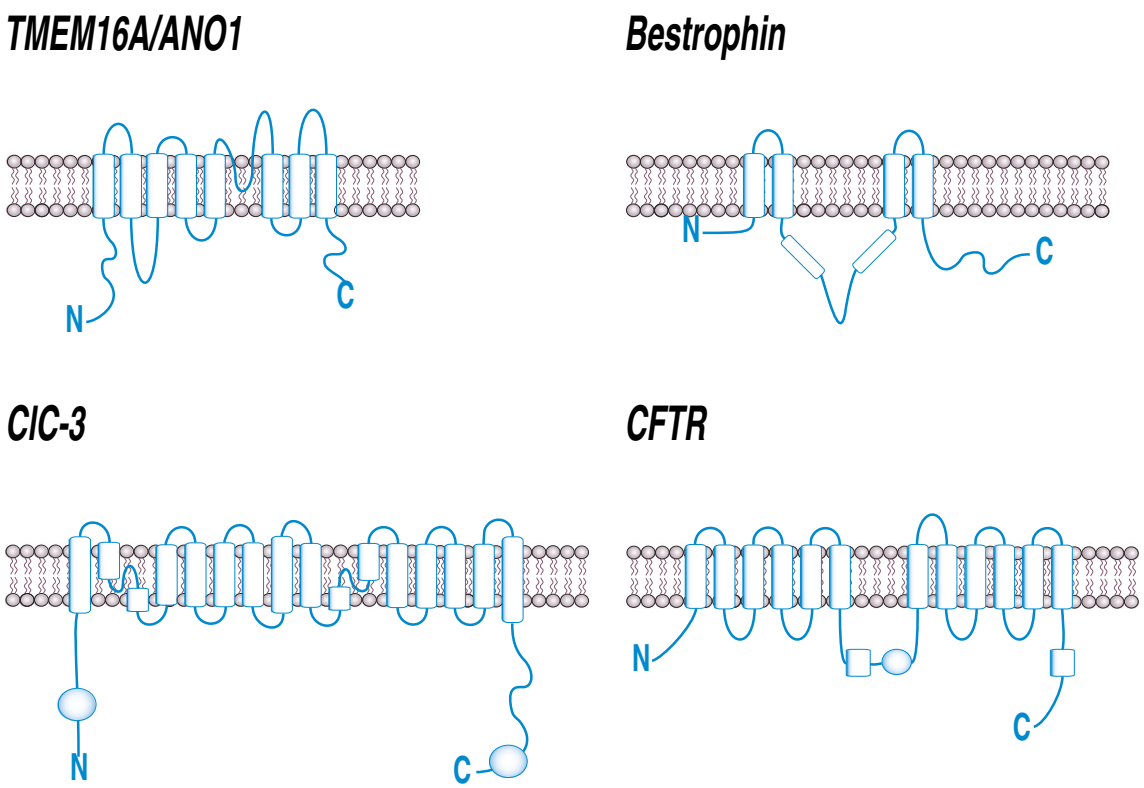

Fig. 1 Predicted membrane topologies of Cl^- channels described in vascular SMCs. TMEM16A/ANO1 was adapted from [144], although an alternative membrane topology has been suggested [144]. Bestrophin modified from ref. [79], ClC-3 from ref. [27], and CFTR from ref. [152]

activated Cl^- currents in rabbit pulmonary artery SMCs [59, 121, 123].

Research using a variety of nonselective Cl^- channel antagonists further supported the concept that Cl^- flux contributes to vasoconstriction. 4,4′-Diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and indaryloxyacetic acid (IAA-94), but not niflumic acid (NFA), hyperpolarized and relaxed pressurized rat cerebral arteries [84]. NFA reduced NE- but not K^+-induced contractions in rat aorta and mesenteric arteries [16, 17, 62]. Histamine-induced depolarization and contraction were also attenuated by NFA in rabbit middle cerebral and basilar arteries, respectively [37, 120]. IAA-94 inhibited ET-induced vasoconstriction in cultured vascular SMCs [114]. Anion replacement has also been utilized to strengthen functional evidence obtained using nonspecific Cl^- channel inhibitors. Substitution of extracellular Cl^- with methanesulfonate potentiated NE-, serotonin-, endothelin-1-, and histamine-induced, but not K^+-induced contractions in rabbit basilar arteries and rat aorta [19, 20, 62]. Lowering extracellular Cl^- potentiated pressure-induced constriction and inhibited histamine-induced contraction in rat cerebral arteries [84, 120]. Substitution with Br^- and NO_3^-, which are more permeant anions than Cl^-, increased contraction to NE in rat portal vein [125].

In addition to modulating SMC contractility, both volume-sensitive Cl^- channels and Ca^{2+}-activated Cl^- channels (Cl_{Ca}) channels have been proposed to control SMC proliferation [12, 138, 142]. DIDS, but not IAA-94 or 5-nitro-2,2″-dicarboxylic acid (NPPB), another nonselective Cl^- channel blocker, suppressed ET-1-induced proliferation in cultured aortic SMCs [138]. In contrast, NPPB and IAA-94, but not DIDS, inhibited insulin-like growth factor (IGF)-induced proliferation in porcine coronary artery SMCs [12]. Under chronic hypoxic conditions, NFA and IAA-94 also inhibited proliferation of rat pulmonary artery SMCs [142].

In summary, studies measuring ion flux and those using nonselective Cl^- channel blockers and extracellular anion replacement suggested that Cl^- currents regulate SMC function. More recent studies have identified some of the proteins that generate and regulate these Cl^- currents and investigated their physiological functions and pathological alterations.

Molecular identification of Cl^- channels in SMCs

Classic Ca^{2+}-activated Cl^- (Cl_{Ca}) channels

Cl_{Ca} currents have been described in a variety of SMC types, including those from human mesenteric, rabbit ear, pulmonary and coronary arteries, rat portal vein and cultured cells from rat pulmonary, and cultured pig aorta [2, 26, 58, 64, 91, 130, 145]. Nonspecific Cl^- channel blockers previously shown to

modulate SMC functions were demonstrated to inhibit whole-cell Cl_{Ca} currents, supporting relevance [64]. Cl^- channel blockers also inhibited spontaneous transient inward currents (STICs) in rabbit portal vein SMCs [48]. STICS occur due to the simultaneous activation of multiple Cl^- channels by a Ca^{2+} spark, a local intracellular Ca^{2+} transient that occurs due to ryanodine (RyR)-mediated sarcoplasmic reticulum Ca^{2+} release [53]. In some SMC types, including those from airways, Ca^{2+} sparks activate both STICs and spontaneous transient outward currents (STOCs), which occur due to the simultaneous activation of multiple large conductance Ca^{2+}-activated potassium (BK_{Ca}) channels. A single Ca^{2+} spark can activate both Cl_{Ca} and BK_{Ca} channel, eliciting a STOC followed by a STIC [151]. STICs induce depolarization, whereas STOCs hyperpolarize the membrane potential. Thus, bimodal regulation of Cl_{Ca} and BK_{Ca} channels by Ca^{2+} sparks permits fine tuning of membrane potential [150].

Cl_{Ca} currents exhibit a distinct phenotype. The IV relationship is outwardly rectifying at low intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) [45]. Elevating $[Ca^{2+}]_i$ linearizes the Cl_{Ca} IV relationship [65]. The relative permeability of SMC Cl_{Ca} currents is $SCN^- > I^- > Br^- > Cl^-$ > aspartate [41]. IP_3R- or RyR-mediated SR Ca^{2+} release, Ca^{2+} entry through voltage-dependent Ca^{2+} channels (VDCC), and local Ca^{2+} influx through transient receptor potential (TRP) channels have all been demonstrated to activate Cl_{Ca} currents in SMCs [9, 64–66]. Some of these regulatory mechanisms appear to be cell type-specific, as blockers of nonselective cation channels but not VDCCs inhibited Cl_{Ca} currents in cerebral artery SMCs [9]. In contrast, Ca^{2+} entry through VDCCs activated Cl_{Ca} currents in rat portal vein and rabbit coronary artery SMCs [64, 92]. Extracellular Ca^{2+} removal had no immediate effect on Cl_{Ca} currents in pig aorta and rabbit ear artery and portal vein SMCs, suggesting that external Ca^{2+} was not a primary direct source for activation [2, 26, 129].

Studies illustrating that Ca^{2+} sparks activate STICs in rabbit portal vein, rat coronary artery, and tracheal SMCs provide direct evidence that intracellular Ca^{2+} release can activate Cl_{Ca} channels, at least in some SMC types [40, 51, 128, 151]. However, STICs do not occur in many SMC types, including those that generate Ca^{2+} sparks and express Cl_{Ca} channels. These findings indicate that some SMC types locate Cl_{Ca} channels in close proximity to sites of intracellular Ca^{2+} release and, more specifically, nearby RyR channels that generate Ca^{2+} sparks [53]. Such organization permits local control of Cl_{Ca} channel activity. In contrast, other SMC types appear to position Cl_{Ca} channels away from Ca^{2+} spark sites, eliminating this regulatory mechanism.

Bestrophins, CLCs, CLCAs, and a *tweety-3* homolog have been proposed to generate Cl_{Ca} currents[66]. *Tweety* appeared to be an unlikely candidate due to its relatively high conductance [113]. Similarly, recombinant CLCA channels generate currents that were kinetically distinct from Cl_{Ca} currents in SMCs [66]. The voltage dependence of recombinant bestrophins or CLCs was also dissimilar to those of SMC Cl_{Ca} currents [45, 87, 111]. Recently discovered TMEM16A/ANO1 channels displayed properties similar to native Cl_{Ca} channels [10, 105, 141]. TMEM16A/ANO1 channel message and protein have been described in rat cerebral, pulmonary and carotid artery, murine portal vein, and cultured rat pulmonary artery SMCs [21, 72, 117]. Evidence supporting the contribution of TMEM16A/ANO1 channels to Cl_{Ca} currents includes the fact that recombinant channels and native SMC Cl_{Ca} currents exhibit similar Ca^{2+} dependence and IV linearization by an elevation in $[Ca^{2+}]_i$ (Fig. 2) [10, 72, 82, 106, 117]. TMEM16A/ANO1 knockdown reduced Cl_{Ca} current density in rat cerebral artery and cultured pulmonary artery SMCs [72, 117]. Cell swelling and membrane stretch activated TMEM16A/ANO1 currents in cerebral artery SMCs [9]. Selective TMEM16A/ANO1 knockdown attenuated intravascular pressure-induced cerebral artery depolarization and vasoconstriction [9]. $T16A_{inh}$-A01, a TMEM16A/ANO1 inhibitor, relaxed methoxamine-contracted murine and human blood vessels, suggesting that agonists can activate these ion channels to induce contraction [22]. These studies provide strong evidence that TMEM16A/ANO1 channels generate classic Cl_{Ca} currents in SMCs.

TMEM16A/ANO1 channels also appear to generate functional Cl_{Ca} currents in nonvascular SMCs. TMEM16A/ANO1 is expressed in sheep, rat, and mice urethral SMCs [103]. Electronic field stimulation (EFS)- and NE-induced uterine contractions were inhibited by NFA and exposure to Cl^--free Krebs solution [103]. The authors suggested that TMEM16A/ANO1 regulates the development and maintenance of excitatory contractile responses in urethral SMCs [103]. TMEM16A/ANO1 is expressed in airway SMCs, and activation contributes to methacholine-induced contraction [146]. Benzbromarone, a TMEM16A/ANO1 blocker, inhibited methacholine-induced contraction of mouse and human airway SMCs [50]. TMEM16A/ANO1 is also expressed in interstitial cells of Cajal (ICC), which control SMC contraction and induce rhythmic slow waves in the gastrointestinal tract [38, 49, 52, 104]. In TMEM16A knockout mice, rhythmic contractions are reduced or absent in gastric and small intestine SMCs [49, 52].

Recent studies suggest that alterations in TMEM16A/ANO1 function contribute to cardiovascular pathology. Cl_{Ca} currents were elevated in pulmonary artery SMCs of rats exposed to hypoxia for 7 days [70]. TMEM16A/ANO1 mRNA/protein and Cl_{Ca} currents were elevated in pulmonary artery SMCs of rats with chronic hypoxic pulmonary hypertension (CHPH) [112]. Cl_{Ca} currents and TMEM16A/ANO1 expression were also increased in conduit and intralobar pulmonary artery SMCs from monocrotaline (MCT)-treated rats, another pulmonary hypertension model [32]. NFA and $T16A_{inh}$-A01 both attenuated an elevation in serotonin-induced vasocontraction in pulmonary

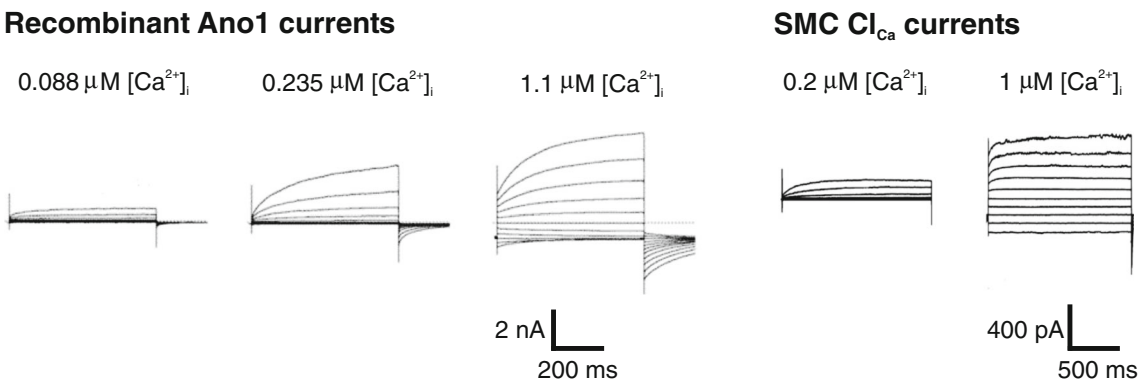

Fig. 2 Original electrophysiological recordings of recombinant TMEM16A/ANO1 and SMC Cl_{Ca} currents. Whole-cell currents of TMEM16A-expressing HEK-293 cells in different free $[Ca^{2+}]_i$ [106]. Reproduced with permission from [106]. © the Biochemical Society. Whole-cell recordings of Cl^- currents in cerebral artery SMCs with 200 nM and 1 μM free $[Ca^{2+}]_i$ (adapted from ref. [117])

arteries from both CHPH and MCT rats [32, 112]. In contrast, TMEM16A/ANO1 protein and Cl_{Ca} currents were both lower in basilar artery SMCs isolated from 2-kidney, 2-clip renohypertensive (2k2c)-rats [133]. The authors concluded that TMEM16A/ANO1 is a negative regulator of cell proliferation and may be important in hypertension-induced cerebrovascular remodeling.

In an ovalbumin (OVA)-sensitized mouse model of chronic asthma, TMEM16A/ANO1 expression was higher, suggesting contribution to airway hyperresponsiveness [146]. NFA and benzbromarone prevented airway hyperresponsiveness and augmented airway SMC contraction. Agonist-mediated contraction was also attenuated in airway SMCs of TMEM16A/ANO1$^{-/-}$ mice [146]. An increase in TMEM16A protein expression and Cl_{Ca} channel activity was observed in asthmatic mouse models and human asthmatic patients, although this increase in protein was primarily observed in epithelial, not smooth muscle, cells [50].

In summary, studies suggest that TMEM16A/ANO1 channels generate Cl_{Ca} currents, and activation leads to membrane depolarization and constriction in both vascular and nonvascular SMCs. Diseases are associated with altered TMEM16A/ANO1 expression and functionality, with differential changes described that may depend on multiple factors, including the pathology involved.

cGMP-dependent Cl_{Ca} channels

A Cl_{Ca} current distinct from classic Cl_{Ca} that requires cGMP for Ca^{2+} activation was initially discovered in rat mesenteric artery SMCs [93]. Subsequently, this current has been described in multiple vascular and colonic SMCs [55, 73, 74]. cGMP-dependent Cl_{Ca} currents are voltage-independent and require lower $[Ca^{2+}]_i$ for activation than classic Cl_{Ca} currents [74, 94]. Halide permeability is also different to classic Cl_{Ca} currents, at $Br^- > I^- > Cl^-$ [74, 94]. cGMP-dependent Cl_{Ca} currents are highly sensitive to Zn^{2+} and relatively insensitive to both NFA and DIDS, effective classic Cl_{Ca} blockers [73]. cGMP-dependent and classic Cl_{Ca} current densities are approximately equal in SMCs from many vascular beds, although deviations from this stereotype have been described [74].

cGMP-dependent Cl_{Ca} currents should induce membrane depolarization and vasoconstriction. Such an effect is counterintuitive to the recognized actions of cGMP-mediated PKG activation, which activates several K^+ channels, including BK_{Ca}, leading to membrane hyperpolarization and relaxation [73, 116]. Conceivably, cGMP-dependent Cl_{Ca} currents act as a break to oppose the cGMP-mediated vasodilation, permitting an additional level of fine tuning of membrane potential and contractility.

The molecular identity of cGMP-dependent Cl^- channels is unclear, but bestrophins, a family of four proteins (1 through 4), can control this current. Cl^- currents generated by recombinant bestrophins are Ca^{2+}-activated, but do not resemble those of classical Cl_{Ca} (Fig. 3) [4, 13, 97, 111]. Bestrophin-3 mRNA and protein are present in rat mesenteric arteries, rat aorta, and cultured A7r5 cells [75]. In contrast, bestrophin-1 and bestrophin-2 are weakly expressed in these tissues [75]. In line with these observations, studies have focused primarily on identifying physiological functions of bestrophin-3 in SMCs [8]. Bestrophin-3 is found in rabbit, but not rat, pulmonary arteries suggesting species-specific expression [66]. The presence of bestrophin protein has been described to match that of cGMP-dependent Cl_{Ca} currents in SMCs. Bestrophin-3 knockdown reduced cGMP-dependent Cl_{Ca} currents in cultured A7r5 cells and rat mesenteric artery SMCs, but did not alter classic Cl_{Ca} currents [75]. Vasomotion in rat mesenteric arteries was reported to have a strong Cl^- dependency that required cGMP [5, 93]. Replacement of extracellular Cl^- with less permeable aspartate inhibited vasomotion in rat mesenteric arteries [5]. Consistent with a role for bestrophins, bestrophin-3 knockdown reduced synchronized vasomotion, but not tonic contractility, in rat mesenteric arteries [8]. Cl_{Ca} current has not been uniformly observed after bestrophin-3 expression in heterologous expression systems; therefore, it is unclear whether the protein forms a prototypical ion channel or is an accessory subunit [88, 96].

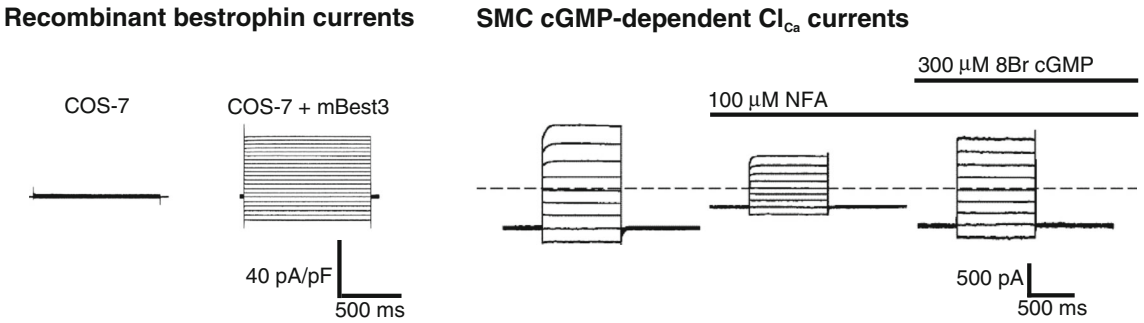

Fig. 3 Recombinant bestrophin-3 and SMC cGMP-dependent Cl_{Ca} currents. Whole-cell mBest3 currents expressed in COS-7 cells at a $[Ca^{2+}]_i$ of 500 nM [88]. Adapted with permission from ref. [88]. © the American Physiological Society (APS). Whole-cell niflumic acid (NFA)-insensitive cGMP-dependent Cl_{Ca} current recorded in a mesenteric artery SMC [5]. Adapted with kind permission from Springer Science+Business Media: (ref. [5], Fig. 7)

In addition to regulating vasomotion, bestrophin-3 has been demonstrated to inhibit H_2O_2-induced apoptosis in basilar artery SMCs [55]. Bestrophin-3 knockdown reduced cell viability, whereas bestrophin-3 overexpression prevented apoptosis. Supporting a protective role, bestrophin-3 overexpression reduced ER stress-induced cell death in cultured renal epithelial cells [67].

In summary, both cGMP-dependent and cGMP-independent Cl_{Ca} currents have been observed in vascular SMCs [74]. Data indicate that two distinct Cl_{Ca} channels generate these currents, including that bestrophin-3 tissue distribution closely matches that of cGMP-dependent Cl_{Ca} currents [75]. The majority of research on bestrophins in SMCs has been in mesenteric arteries. Future studies should investigate bestrophin functions in other vascular beds and whether bestrophins form a prototypical ion channel or an accessory subunit to another ion channel protein. Although bestrophin-3 locates near the cell surface in mesenteric artery SMCs, other bestrophin family members (bestrophin-1 and bestrophin-2) are intracellular proteins when expressed in heterologous expression systems [8, 61, 97]. Conceivably, in SMCs of different vascular beds, other bestrophin proteins may be expressed and perform additional physiological functions.

Volume-sensitive Cl^- channels

In many cell types, cell swelling stimulates compensatory K^+, Cl^-, and H_2O efflux as a mechanism to reestablish cell volume [31]. Volume-sensitive Cl^- channels are expressed in many cell types, including vascular SMCs, and appear to contribute to this process [42]. Although controversy exists as to whether Cl^- channel-3 (ClC-3), a member of the ClCn gene family, operates as a prototypical ion channel, this protein has been proposed to act as a volume-sensitive Cl^- channel (Fig. 4) [54]. Currently, ClC-3 is the only molecular candidate for a volume-sensitive Cl^- channel in SMCs. Therefore, evidence supporting ClC-3 will be summarized in this section.

ClC-3 message was detected in canine pulmonary and renal artery SMCs [140]. Hypotonic solution activated an outwardly rectifying Cl^- conductance with a similar phenotype to cardiac myocyte ClC-3, including anion permeability and inhibition by DIDS and extracellular ATP [28, 140]. Similar data were obtained when studying cultured human aortic and coronary artery vascular SMCs and isolated canine pulmonary artery and colonic SMCs [25, 29, 63]. ClC-3 overexpression elevates volume-regulated Cl^- currents in aortic SMCs [76]. Intracellular dialysis of ClC-3 antibodies abolished volume-activated Cl^- currents in canine pulmonary artery SMCs [127]. ClC-3 knockdown inhibited volume-sensitive Cl^- currents in A10 vascular SMCs [126, 149]. PKC activators differentially regulate swelling-activated Cl^- currents in rabbit portal vein versus canine pulmonary artery SMCs and cardiac myocytes, an effect that may be attributed to differences in intracellular signaling pathways involved [148]. ClC-3 expression and volume-sensitive Cl^- currents were larger in femoral artery than vein SMCs, perhaps due to differences in venous and arterial blood pressures to which these vessels are exposed [56].

Other evidence questions whether ClC-3 acts as a volume-sensitive Cl^- channel in SMCs. ClC-3 expression in *Xenopus* oocytes and HEK-293 cells did not produce volume-sensitive Cl^- currents, suggesting that results may be cell type-dependent [33, 76, 109]. When expressed in immortalized cell lines, ClC-3 was an intracellular channel that was not volume-regulated [69, 89, 135]. There is also variability in the contribution of ClC-3 to Cl_{Ca} currents in different cell types. For example, ClC-3 knockout reduced Cl_{Ca} currents in aortic SMCs, but had no effect in parotid acinar cells [3, 36]. Cell-specific differences may arise due to variability in CaMKII activation, as ClC-3 regulation is CaMKII-dependent in aortic SMCs [36]. Further uncertainty derives from data indicating that volume-sensitive Cl^- currents in pulmonary artery SMCs and other cell types, including cardiac myocytes, are unaltered in ClC-3 knockout ($Clcn3^{-/-}$) mice [3, 39, 110, 132, 139]. One explanation for this finding may be that ClC-3 knockout leads

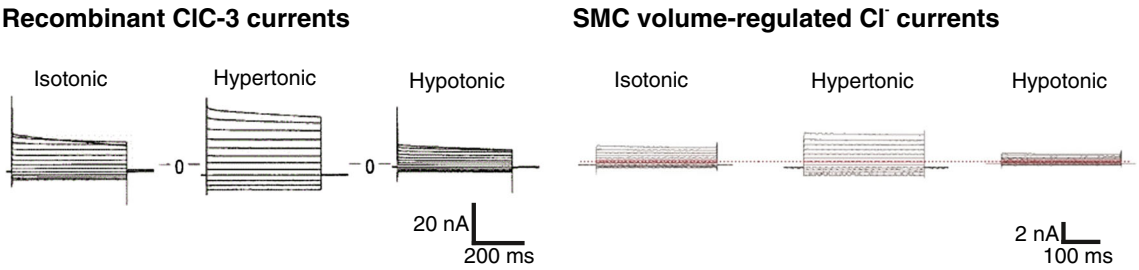

Fig. 4 Recombinant ClC-3 and SMC volume-regulated Cl^- currents. Osmotic regulation of whole-cell currents recorded from gpClC3-transfected NIH/3 T3 cells under isotonic, hypotonic, and hypertonic conditions [28]. Adapted by permission from Macmillan Publishers Ltd: ref. [28]. Volume regulation of whole-cell currents recorded from A10 vascular SMCs under similar conditions [149]. Reproduced with permission from ref. [149]. © 2008 The American Society for Biochemistry and Molecular Biology. All rights reserved

to compensatory upregulation of other volume-regulated ion channels [139]. Consistent with this concept, mRNA for ClC-1 and ClC-2, but not ClC-4 or ClC-5, is elevated in $Clcn3^{-/-}$ mice atrial myocytes [139].

Volume-regulated Cl^- channels may depend on an association between ClC-3 and NADPH oxidase(Nox)-dependent reactive oxygen species (ROS) signaling in SMCs [76]. ClC-3 locates to membrane of organelles, including endosomes, where it regulates Nox1-mediated ROS generation [43, 80]. ClC-3 acts as a Cl^-/H^+ exchanger that neutralizes electron flow generated by Nox1 [80]. SMCs from ClC-$3^{-/-}$ mice did not generate endosomal ROS or activate transcription factor nuclear factor (NF)-κB in response to tumor necrosis factor (TNF)-α and interleukin (IL)-1β [80]. As a result, volume-regulated Cl^- current was not activated by TNF-α and IL-1β in ClC-$3^{-/-}$ mice [76].

Evidence has been provided that ClC channels control SMC function. In pig artery SMCs, ClC-2 knockdown suppressed IGF-1-induced proliferation [12]. ClC-3 knockdown inhibited endothelin-1 (ET-1)-induced aortic SMC proliferation by arresting the cell cycle [115, 131]. Aortic SMCs from $Clcn3^{-/-}$ mice proliferated more slowly than those from wild-type controls [80]. TNF-α and carotid artery injury both stimulated ClC-3 expression with injury-induced carotid artery neointima formation reduced in $Clcn3^{-/-}$ mice [15]. ClC-3 overexpression inhibited apoptosis in pulmonary artery SMCs [18].

ClC-3 is associated with changes in SMC function during disease. A hypotonicity-induced decrease in $[Cl^-]_i$ and an increase in rat basilar artery SMC size correlated with hypertension in 2k2c rats, suggesting that volume-sensitive Cl^- channels are more active and may be involved in vascular remodeling [107]. ClC-3 mRNA and protein were both elevated in pulmonary artery SMCs of rats with experimentally induced pulmonary hypertension [18]. Static pressure stimulated ClC-3 expression, volume-sensitive Cl^- currents, and proliferation in aortic SMCs, and these changes were attenuated by Cl^- channel blockers and ClC-3 knockdown [95]. Ca^{2+}-independent Cl^- currents, but not Cl_{Ca} currents, were larger in proliferating pulmonary artery SMCs from rats exposed to hypoxia, suggesting that antagonists of this current may be useful in the treatment of pulmonary hypertension [70]. Volume-sensitive Cl^- currents increased as femoral artery SMCs switched from a contractile to a proliferative state during vascular remodeling [56]. ClC-3 mRNA and protein were higher in aortic SMCs of diabetic rats than controls, suggesting that the channel may be associated with pathology [34]. Although the contribution of ClC-3 to volume-regulated Cl^- currents is controversial and requires additional study, ClC-3 may represent a therapeutic target in SMC-associated diseases, including during proliferative vascular disease.

In summary, whether ClC-3 generates volume-sensitive Cl^- channels in vascular SMCs is controversial. It is unclear whether ClC-3 is located primarily intracellular or in the plasma membrane. This uncertainty arises, in part, due to the presence of swelling-activated Cl^- currents in the cells of $Clcn3^{-/-}$ mice [3, 76]. However, ClC-3 is expressed in SMCs and both knockdown and knockout result in physiological changes [140]. ClC-3 expression levels are also altered in disease states. Further studies are required to determine SMC CLC-3 cellular localization and whether ClC-3 is a Cl^- channel or an accessory protein.

CFTR

The cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated ATP-gated anion channel, has primarily been studied in epithelial cells, where it was originally identified [98]. CFTR channels have subsequently been found in a number of other cell types, including neurons, cardiac myocytes, and endothelial cells [35, 119, 136]. CFTR functions in SMCs were initially proposed from experiments using highly nonspecific pharmacological modulators [23, 68, 147]. Subsequent studies using immunofluorescence and Western blotting demonstrated CFTR expression in rat thoracic aorta and intrapulmonary artery [101, 102]. cAMP pathway and CFTR activators both activated iodide efflux in cultured vascular SMCs and relaxed precontracted, depolarized endothelium-denuded aortic and intrapulmonary artery rings via a mechanism sensitive to $CFTR_{inh}$-172, a more selective CFTR blocker [100, 102]. cAMP pathway agonists

and pharmacological CFTR activators stimulated iodide efflux in depolarized cultured aortic SMCs of wild-type mice, but not in cells of CFTR$^{-/-}$ mice [100]. Vasoconstrictors also contracted aortic rings from CFTR$^{-/-}$ mice more than those from CFTR$^{+/+}$ mice [100]. These studies suggested that stimulation of the cAMP pathway and CFTR activation were functional when the SMC membrane potential was more positive than the E_{Cl}. Under this condition, CFTR channel activation appears to oppose vasoconstriction. A study demonstrating that myogenic tone is enhanced in both CFTR$^{-/-}$ cerebral and mesenteric arteries supports the concept that CFTR activation hyperpolarizes membrane potential [77].

CFTR is also expressed in nonvascular SMCs [78, 122]. cAMP pathway agonists and CFTR activators stimulated iodide efflux and induced CFTR$_{inh}$-172-sensitive relaxation of tracheal SMCs [122]. CFTR knockdown attenuated histamine-induced intracellular Ca^{2+} release in airway SMCs [78]. CFTR$^{-/-}$ mice also exhibit ileal SMC phenotypes that vary when studied on different mouse strains [99]. Furthermore, CFTR channel knockout results in small intestine circular smooth muscle dysfunction 7 days postnatal in mice [24].

SMC dysfunction, including bronchoconstriction, airway hyperresponsiveness, gastric dysmotility, and intestinal obstruction, may contribute to the cystic fibrosis disease phenotype [78]. Thus, CFTR modulators may have therapeutic benefit by acting on airway SMCs. Conceivably, CFTR activators may also have antihypertensive actions, although many questions still remain regarding function in SMCs. CFTR knockout may induce many different compensatory mechanisms that could modify contractility. Conceivably, CFTR may regulate other Cl^- channels in vascular SMCs. CFTR expression inhibits both volume-sensitive Cl^- and Cl_{Ca} current in bovine pulmonary artery endothelial cells, and upregulation of its expression results in a corresponding downregulation in both channels in recombinant cells [60, 123, 134]. Whether similar regulating mechanisms exist in SMCs is unclear, but possible.

Importantly, CFTR channels have not been directly measured in SMCs using electrophysiological techniques, including patch-clamp electrophysiology. Similarly, SMC-specific inducible CFTR$^{-/-}$ knockout mice should be studied and systemic blood pressure measurements performed. Such data

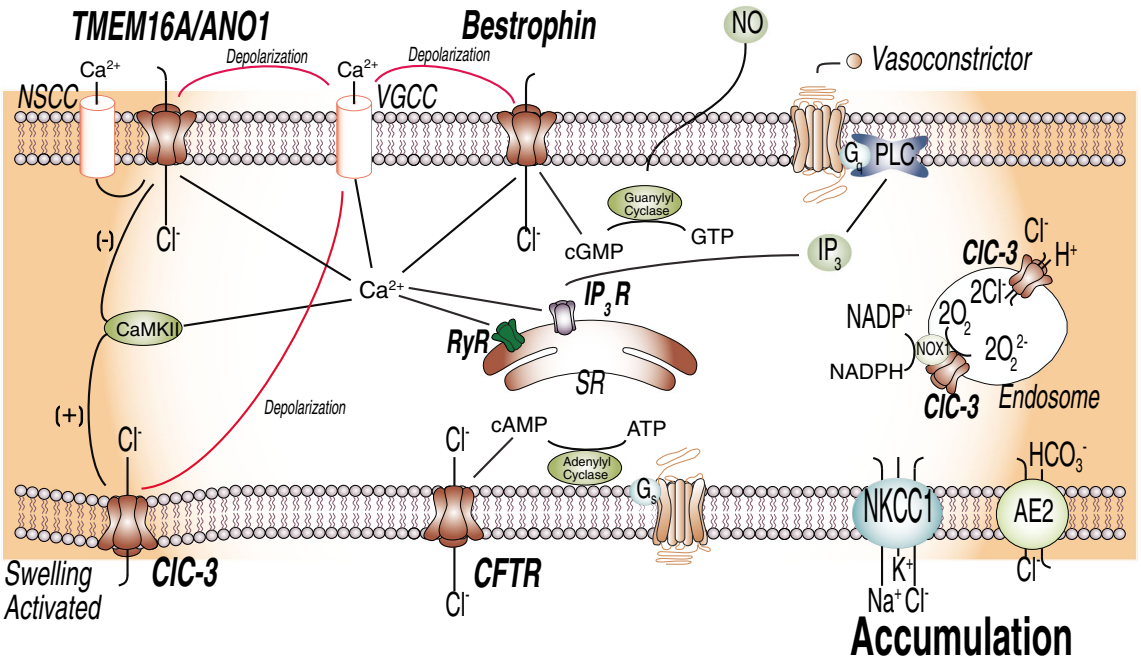

Fig. 5 Cl^- channels present in vascular SMCs. Cl^- accumulates in SMCs due to the Na^+–K^+–Cl^- cotransporter (NKCC1) and the Cl^--HO_3^- exchanger-2 (AE2). cGMP-dependent and independent Cl_{Ca} channels, a volume-sensitive Cl^- channel, and the cystic fibrosis transmembrane conductance regulator (CFTR) have been identified. The molecular identity of the first three channels has been proposed to be bestrophin, TMEM16A/Ano1, and ClC-3, respectively. Numerous mechanisms of Ca^{2+} activation of Cl_{Ca} channels in vascular SMCs have been suggested, including IP_3R- or RyR-mediated SR Ca^{2+} release, Ca^{2+} entry through voltage-dependent Ca^{2+} channels (VDCC), and local Ca^{2+} influx through nonselective cation channels (NSCC). Activation of these channels leads to Cl^- efflux and subsequent depolarization of the cell membrane that activates voltage-dependent Ca^{2+} channels (VDCC). ClC-3 channels have been proposed to be activated by membrane swelling. ClC-3 is present in the plasma membrane and in intracellular compartments, including endosomes. Endosomal ClC-3 channels may regulate volume-regulated Cl^- channels via ROS production. CaMKII inhibits TMEM16A and activates ClC-3 channels. CFTR is a cAMP-activated ATP-gated anion channel that appears to be functional when the SMC membrane potential becomes more positive than the Cl^- equilibrium potential. Under this condition, CFTR channel activation would lead to Cl^- influx and oppose vasoconstriction

would provide stronger support for physiological functions of vascular SMC CFTR.

Conclusions

Research has focused primarily on discovering the molecular identity, physiological functions, and pathological significance of cation channels expressed in SMCs. In contrast, little is known of anion channels, specifically Cl^- channels that are expressed in SMCs. This knowledge gap has arisen, in part, due to a lack of specific Cl^- channel modulators and uncertain molecular identity of the proteins present. Recent discoveries of TMEM16A/ANO1, bestrophin, ClC-3, and CFTR expression in SMCs have provided new insights (Fig. 5). Identification of these proteins has permitted the use of molecular biology techniques to inhibit Cl^- channel expression and study the effects on SMC function. Evidence suggests that multiple Cl^- channel types are expressed in SMCs. These channels can control physiological functions, including contractility and proliferation, and can contribute to SMC pathologies.

Future directions

Future studies should aim to identify intracellular signaling pathways that regulate different Cl^- channels in SMCs and downstream functional effects of such modulation. Many ion channels have one or more auxiliary and regulatory subunits, and these proteins can, in some cases, exhibit SMC-specific expression (e.g., K_{Ca} channel β1 subunits [7]). It is possible that Cl^- channels have auxiliary subunits, although this remains to be determined. Similarly, whether some proteins identified are pore-forming Cl^- channels or accessory subunits is unclear, including some bestrophins and ClC proteins. Similarly, different Cl^- channels may interact and regulate each other directly, for example through heteromultimer formation, and indirectly, via signaling networks. Many of these research directions will benefit from the discovery of specific Cl^- channel modulators and animals with inducible, SMC-specific genetic alterations of the proteins under investigation. The next decade should see a significant increase in knowledge of Cl^- channel signaling, physiology, and pathology in SMCs.

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