University of Nevada, Reno

Modulation of the Pharmacology of Ca$^{2+}$-Activated Cl$^{-}$ Channels of Pulmonary Artery Smooth Muscle Cells and the Molecular Candidate TMEM16A by Phosphorylation

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Cell and Molecular Pharmacology and Physiology

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**DOCTOR OF PHILOSOPHY**

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Abstract

The membrane depolarization associated with the opening of Ca$^{2+}$-activated Cl$^-$ channels (Cl$_{Ca}$) is thought to be an important contributor to the development of vascular smooth muscle tone induced by constricting hormones and neurotransmitters. In arterial smooth muscle cells, Ca$^{2+}$-activated Cl$^-$ currents (I$_{Cl(Ca)}$) are inhibited by phosphorylation. The first two studies presented in this dissertation tested the hypothesis that the state of Cl$_{Ca}$ channel phosphorylation affects their pharmacology. The third study explored whether TMEM16A may be a valid molecular candidate for the native Cl$_{Ca}$ channel of pulmonary artery myocytes and in particular whether this protein is similarly altered by phosphorylation and classical Cl$^-$ channel blockers.

The Ca$^{2+}$-activated Cl$^-$ channel (Cl$_{Ca}$) blocker niflumic acid (NFA) produces a paradoxical dual effect on I$_{Cl(Ca)}$, causing stimulation or inhibition at potentials below or above 0 mV, respectively. We first tested whether the effects of NFA on I$_{Cl(Ca)}$ are modulated by phosphorylation. Eliciting I$_{Cl(Ca)}$ with 500 nM free internal Ca$^{2+}$ in rabbit pulmonary artery myocytes, the channel’s state of phosphorylation was altered by cell dialysis with either 5 mM ATP, or 0 ATP with or without the CaMKII inhibitor KN-93 (10 $\mu$M). We found dephosphorylation to enhance the ability of 100 $\mu$M NFA to inhibit I$_{Cl(Ca)}$, an effect attributable to a large negative shift in the voltage-dependence of block. Channel inhibition was converted to stimulation at potentials < -50 mV, ~70 mV more negative than cells dialyzed with 5 mM ATP. NFA dose-dependently blocked I$_{Cl(Ca)}$ in the range of 100 nM to 250 $\mu$M in cells dialyzed with 0 ATP and KN-93, which contrasted with the significant stimulation induced by 100 nM, which converted to block at concentrations > 1 $\mu$M when cells were
dialyzed with 5 mM ATP. Based on these data and the observations that phosphorylation results in state-dependent block and that NFA preferentially interacts with the open channel, we propose two binding sites for NFA; an inhibitory site residing near or partially in the channel pare, and a stimulatory site removed from the pore.

We then tested the same hypothesis with anthracene-9-carboxylic acid (A9C), a Cl⁻ channel block chemically dissimilar to NFA that like NFA, has a unique dual effect on \( I_{\text{Cl(Ca)}} \), both inhibiting and stimulating the channels depending on voltage and the state of gating. The whole-cell patch clamp technique was used to record \( I_{\text{Cl(Ca)}} \) in rabbit pulmonary artery smooth muscle cells, where currents were generated by 500 nM free internal \( \text{Ca}^{2+} \). Again, the state of phosphorylation was altered by cell dialysis with either 5 mM ATP or an ATP-free pipette solution. Similar to NFA, dephosphorylation enhanced the ability of A9C to inhibit \( I_{\text{Cl(Ca)}} \) in a concentration-dependent manner, with a negative shift in the voltage-dependence of block. Stimulation of \( I_{\text{Cl(Ca)}} \) tail current by 500 µM A9C at -80 mV was enhanced in cells dialyzed with 5 mM ATP. While the tail current of cells dialyzed with 0 ATP was stimulated following depolarization to +40 mV, the stimulation was abolished following steps to +140 mV. These data again suggest the presence of two drug binding sites. It appears that A9C blocks the open channel and that phosphorylation partially occludes access to a blocking site found at the mouth or within the pore. Minimizing phosphorylation resulted in decreased stimulation by A9C of the \( I_{\text{Cl(Ca)}} \) tail current when compared to cells in which phosphorylation was supported, suggesting a phosphorylation-dependent effect on a stimulatory site distinct from the inhibitory site.
The final study presented in this dissertation explored TMEM16A as a possible candidate for the native Cl_{Ca} channel of pulmonary artery myocytes. RT-PCR and immunocytochemistry identified TMEM16A expression in rat and mouse pulmonary artery. HEK293 cells over-expressing TMEM16A displayed large Cl⁻ currents when dialyzed with 500 nM free Ca^{2+}. These currents displayed similar time- and voltage dependence to native I_{Cl(Ca)} of rabbit pulmonary artery smooth muscle cells. Currents ran down, although not to the extent of I_{Cl(Ca)} of the native rabbit pulmonary myocytes. The TMEM16A-dependent currents were sensitive to block by NFA and A9C, but demonstrated stimulation by A9C dissimilar from the native current. A mutation of Threonine 610 to an Alanine – a putative CaMKII phosphorylation site – did not affect rundown, although it did alter TMEM16A pharmacology, increasing block and stimulation by NFA and A9C, respectively. This study indicates that TMEM16A is a strong candidate for the Cl_{Ca} channels of pulmonary artery smooth muscle.

In summary, these studies revealed for the first time that phosphorylation influences the interaction of Cl⁻ channel blockers with Cl_{Ca} channels. Dephosphorylation enhanced the ability of both NFA and A9C to inhibit I_{Cl(Ca)} of rabbit pulmonary artery myocytes. The enhanced block by these drugs was accompanied by a negative shift in the voltage-dependence of block. Promoting phosphorylation on the other hand, resulted in greater stimulation by A9C of the inward tail current upon repolarizing the cell to -80mV. The recently proposed Cl_{Ca} molecular candidate TMEM16A was identified in pulmonary artery of both mouse and rat. Transient transfection of HEK293 cells with a TMEM16A-eGFP fusion protein resulted in large Ca^{2+}-activated chloride currents stimulated by 500 nM free
Ca^{2+}. These currents displayed similar time- and voltage-dependence to native $I_{\text{Cl(Ca)}}$ of rabbit pulmonary artery smooth muscle cells, and while blocked by both NFA and A9C, the pharmacology of TMEM16A-dependent currents differed in some aspects such as magnitude of block and stimulation, as well as voltage-dependence. Overall, TMEM16A is a strong candidate for the Cl$_{Ca}$ channels of pulmonary artery myocytes.
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<tbody>
<tr>
<td>[Ca(^{2+})](_i)</td>
<td>intracellular Ca(^{2+}) concentration</td>
</tr>
<tr>
<td>A9C</td>
<td>anthracene-9-carboxylic acid</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ALK1</td>
<td>activin receptor-like kinase-1</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>adenylyl-imidodiphosphate, tetralithium salt</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>Best</td>
<td>bestrophin</td>
</tr>
<tr>
<td>BK(_{\text{Ca}})</td>
<td>Ca(^{2+})-activated large conductance K(^+) channel</td>
</tr>
<tr>
<td>BMPR2</td>
<td>bone morphogenic protein receptor 2</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Ca(^{2+})/calmodulin-dependent kinase II</td>
</tr>
<tr>
<td>CaN</td>
<td>calcineurin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CFTR</td>
<td>cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CLC</td>
<td>voltage-gated chloride channel family</td>
</tr>
<tr>
<td>Cl(_{\text{Ca}})</td>
<td>Ca(^{2+})-activated Cl(^-) channel</td>
</tr>
<tr>
<td>CLCA</td>
<td>Ca(^{2+})-activated Cl(^-) channel family (molecular candidate)</td>
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<tr>
<td>CPA</td>
<td>cyclopiazonic acid</td>
</tr>
<tr>
<td>CsA</td>
<td>cyclosporine A</td>
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<tr>
<td>DIDS</td>
<td>4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
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</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>E(_{\text{Cl}})</td>
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<tr>
<td>ENG</td>
<td>endoglin</td>
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<td>ER</td>
<td>endoplasmic reticulum</td>
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<td>HEK</td>
<td>human embryonic kidney</td>
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<td>HPV</td>
<td>hypoxic pulmonary vasoconstriction</td>
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<tr>
<td>IAA-94</td>
<td>indanyloxyacetic acid 94</td>
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<td>I(_{\text{Cl(Ca)}})</td>
<td>Ca(^{2+})-activated Cl(^-) current</td>
</tr>
<tr>
<td>IP(_3)</td>
<td>inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>I-V</td>
<td>current-voltage relationship</td>
</tr>
<tr>
<td>KCC</td>
<td>K(^+)-Cl(^-) co-transporter</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo-Dalton</td>
</tr>
<tr>
<td>KN-93</td>
<td>N-[2-{N-(4-Chlorocinnamyl)-N-methylaminomethyl}phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulfonamide phosphate salt</td>
</tr>
<tr>
<td>KO</td>
<td>knock out</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>-----------</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>M-βCD</td>
<td>methyl-β-cyclodextrin</td>
</tr>
<tr>
<td>NFA</td>
<td>niflumic acid</td>
</tr>
<tr>
<td>NKCC</td>
<td>Na⁺-K⁺-Cl⁻ co-transporter</td>
</tr>
<tr>
<td>NO-cGMP</td>
<td>nitric oxide-cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>NPPB</td>
<td>5-nitro-2-(3-phenylpropylamino)benzoic acid</td>
</tr>
<tr>
<td>OA</td>
<td>okadaic acid</td>
</tr>
<tr>
<td>PA</td>
<td>pulmonary artery</td>
</tr>
<tr>
<td>PAH</td>
<td>pulmonary arterial hypertension</td>
</tr>
<tr>
<td>PASM</td>
<td>pulmonary artery smooth muscle</td>
</tr>
<tr>
<td>PASMC</td>
<td>pulmonary artery smooth muscle cell</td>
</tr>
<tr>
<td>PDE-5</td>
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<td>transmembrane protein 16</td>
</tr>
<tr>
<td>TRPC</td>
<td>transient receptor potential</td>
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<tr>
<td>VDCC</td>
<td>voltage-dependent Ca²⁺ channel</td>
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<tr>
<td>VMD</td>
<td>vitelliform macular dystrophy</td>
</tr>
<tr>
<td>VSM</td>
<td>vascular smooth muscle</td>
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<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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<td>WT</td>
<td>wild type</td>
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Chapter 1: Introduction and Literature Review

1.1 Pulmonary Arterial Hypertension

Pulmonary arterial hypertension (PAH) in humans is a progressive and lethal disease. With a prevalence of 30-50 cases per million (1), PAH is rare, although the true burden of PAH is unknown and certainly underestimated (World Health Organization, 2009). It is a disease characterized by elevated pulmonary artery pressures (>25 mm Hg at rest, >30 mm Hg during physical activity (2, 3, 4)), resulting in high right ventricular systolic pressures, eventually progressing to right ventricular failure and death (5). Idiopathic PAH, where there is no known cause, is likely to account for at least 40% of PAH cases (6), and is twice as common in women as in men (7). Remaining cases are either familial, or associated with various conditions such as congenital heart disease, HIV infection, sickle cell anemia, drug- or toxin-induced toxicity, thyroid diseases, some inflammatory diseases, portal vein hypertension, and some tumors (2, 4, 8). In a few of these groups, PAH prevalence is substantially higher than in the general population. For example, in HIV-infected patients the prevalence is 0.5% (9), in patients with sickle cell disease the prevalence is 20-40% (10) and in patients with systemic sclerosis the prevalence has been reported to be up to 16% (11). Prognosis for survival depends largely on when the disease is detected, with patients in functional classes I and II living for 6 years without treatment, while those in classes III and IV live only 2.6 and 0.5 years, respectively (World Health Organization, 2009).

Three factors are commonly thought to cause increased pulmonary vascular resistance in PAH patients: vasoconstriction, remodeling of the pulmonary arteries resulting in reduction of lumen diameter, and \textit{in situ} thrombosis (3). While several signaling pathways
involved in PAH have been identified, the pathogenesis of most forms of the disease remain unknown (12). Historically characterized as a disease of endothelial dysfunction, PAH is thought to result from an imbalance between vasoconstrictors (thromboxane, endothelin, serotonin, angiotensin, others) and vasodilators (prostacyclin, nitric oxide, others) (5). Several experimental models have demonstrated this imbalance between vasoconstrictors and vasodilators in PAH.

1.1.1 Genetic Basis of Pulmonary Arterial Hypertension

In 2000, two groups independently found a link between mutations of the bone morphogenetic protein receptor 2 (Bmpr2) and familial PAH (13, 14). Mutations in this member of the TGF-β superfamily have been reported in patients suffering from familial PAH (up to 60%) and those developing idiopathic PAH (25%) (14, 15). Bone morphogenic proteins have been shown to induce apoptosis of human PASMCs. Several studies involving animal models of PAH suggest that the loss of BMPR2 activity may be involved in the medial hypertrophy and arteriolar narrowing that occur in the disease, likely by reducing TGF-β-induced SMC apoptosis (5). Interestingly, loss of function mutations of Bmpr2 have the opposite effect when it comes to endothelial cells, increasing susceptibility to apoptosis (5). Endothelial cell apoptosis may be associated with initiating experimental PAH either directly by degradation of pre-capillary arterioles, or by leading to the selection of apoptosis-resistant endothelial cells that may contribute to plexiform lesions (5).

Mutations of either the activin receptor-like kinase-1 (ALK1) receptor or the accessory receptor endoglin (ENG) underlie hereditary hemorrhagic telangiectasia (HHT), an
autosomal dominant vascular disorder characterized by the appearance of cutaneous telangiectasias and arteriovenous malformations (16). Of all known ALK-1 mutations, up to 20% are associated with the development of PAH. In a few rare cases, idiopathic and familial PAH appear to be caused by mutations of ALK-1, and present without HHT (16).

1.1.2 Pulmonary Arterial Hypertension Therapies

For some patients, calcium channel blockers are extremely effective at lowering pulmonary artery pressure for periods exceeding 20 years (17). For this reason, it is recommended that all PAH patients be tested for acute vasoreactivity using a short-acting vasodilator. Those showing a positive response may then be considered for long-term oral calcium channel blocker treatment (18, 19). This approach ends up being effective for only 10-20% of patients, necessitating alternative therapeutic approaches for the remaining (>80%) patients (18, 19, 20). These alternatives include prostacyclin analogues (epoprostenol, iloprost, treprostinil, beraprost), endothelin receptor antagonists (bosentan, ambrisentan, sitaxsentan), and phosphodiesterase-5 (PDE-5) inhibitors (sildenafil, tadalafil) (19).

Prostacyclin analogues carry potent vasodilatory activity, and may also inhibit thrombosis, inflammation, and vascular smooth muscle proliferation. The goal with endothelin receptor antagonists is to preferentially block the ET-A receptor, as the vasoconstrictive effects stemming from ET-1 binding to ET-A receptors predominate over the vasodilatory effects of ET-1 binding to ET-B receptors. Finally, inhibition of PDE-5 promotes vasodilation by freeing up the NO-cGMP pathway. Often, several PAH specific
drugs may be employed simultaneously in order to target multiple molecular pathways. Combination therapy is common practice in most expert centers, with evidence of synergistic effects between some treatments. The use of gene therapy in the treatment of PAH is currently being explored, while targeting ion channel expression and function may also lead to additional therapies.

1.1.3 Ion Channels in Pulmonary Arterial Hypertension

Similarly to systemic vessels, ions channels play a key role in regulating pulmonary vascular tone. Indeed, several studies have found Ca\textsuperscript{2+} and K\textsuperscript{+} channels to play a role in PAH. In animal models of PAH and human PAH patients, PASMCs are more depolarized and have a higher [Ca\textsuperscript{2+}]\textsubscript{i} than cells from healthy individuals. This depolarization likely alters responsiveness to endogenous vasoconstrictors (21, 22). A hallmark of human PAH and animal models of PAH is a decrease in voltage dependent K\textsuperscript{+} current (I\textsubscript{K}) due to a decrease in expression of certain K\textsubscript{v} channels (K\textsubscript{v}1.5, K\textsubscript{v}2.1, K\textsubscript{v}4.3, and K\textsubscript{v}9.3) (20, 21, 23, 24, 25, 26, 27, 28, 29, 30). The channel K\textsubscript{v}1.5 is of particular interest, as decreased expression or activity and mutations of K\textsubscript{v}1.5 occur in human (12) and animal models (23, 31) of PAH. The role of K\textsubscript{v}1.5 in PAH is further highlighted in the study by Pozeg et al. (31), where in vivo gene transfer of K\textsubscript{v}1.5 reduces PAH. Prolonged K\textsubscript{v} down-regulation results in reduced apoptosis rates, which manifests as hypertrophy and hyperplasia of SMCs, leading finally to obstructive vascular remodeling (28, 32, 33, 34, 35).

There are both voltage-dependent and –independent Ca\textsuperscript{2+} entry pathways in PASMCs. Many of the effects of reduced I\textsubscript{K} are likely mediated by an increase in [Ca\textsuperscript{2+}] via
voltage-gated L-type Ca\(^{2+}\) channels. The increase in intracellular Ca\(^{2+}\) may then trigger gene transcription leading to the reduced apoptosis rates mentioned above (21, 25, 36, 37, 38, 39). In one animal model for PAH, an up-regulation of L-type Ca\(^{2+}\) channel activity has been observed, accompanied by elevations in protein levels of the \(\alpha_{1C}\) pore forming subunit (40). Evidence also suggests that increased receptor and store-operated Ca\(^{2+}\) entry (SOCE) may play an important role in PAH. Several members of the canonical subfamily of Transient Receptor Potential (TRPC) genes demonstrate increased expression in pulmonary arteries of both human PAH patients and animal models of PAH (41, 42, 43, 44). TRPC channels have also been implicated in PASMC proliferation (45, 46).

Despite the fact that many of the molecular pathways found to be important in the development and progression of PAH affect Cl\(^{-}\) chloride channels either directly or indirectly, few studies have looked into a potential direct role in PAH. In a study involving monocrotaline-induced PAH in rats, expression of the voltage-gated Cl\(^{-}\) channel CIC-3 was increased (47). It was proposed that the up-regulation is an adaptive response, counteracting the harmful reactive oxygen species generated by inflammation. In rat PASMCs, an increase in Cl\(^{-}\) currents was observed in proliferating cultured cells when compared to non-proliferating acutely dissociated cells (48). Investigators propose the increase to be due to an increase in swelling-activated Cl\(^{-}\) currents, which have been demonstrated to be involved in regulating apoptosis, which suggests a possible role in PAH (48, 49). Lastly, the calcium-activated chloride channel (CIC\(_{ca}\)) blocker niflumic acid (NFA) was more effective at attenuating the contraction mediated by \(\alpha_1\)-adrenergic receptor stimulation of PA of monocrotalin-treated versus saline-injected rats (50). As Cl\(^{-}\) channels
are involved either directly or indirectly in many of the pathways associated with PAH, including cell volume regulation, apoptosis, growth, proliferation, and control of vascular tone, a more thorough knowledge of their role in PAH must be gained, and their potential as new therapeutic targets explored.

1.1.4 Hypoxic Pulmonary Vasoconstriction

Hypoxic pulmonary vasoconstriction is an adaptive mechanism that optimizes ventilation by redistributing blood flow from poorly ventilated areas of the lung to better-ventilated areas (51). This is acutely advantageous, as it increases the total area of lung involved in gaseous exchange. However, with global hypoxia resulting from respiratory disease or altitude, HPV leads to a harmful increase in PA resistance. Chronically, HPV may also result in remodeling of the pulmonary vasculature, which in turn leads to the development of pulmonary hypertension (51). While there remains controversy regarding the signaling pathways involved in HPV, like in PAH, there is an established role for ion channels in HPV.

Early studies demonstrated activation of VDCC in HPV. It was shown that hypoxia inhibits K⁺ channels in PASMCs, resulting in depolarization and the subsequent activation of L-type Ca²⁺ channels (52) (Figure 1). Several studies have since demonstrated block of K⁺ channels, specifically the Kv channel family, in PASMCs results in increased [Ca²⁺]i and consequent contraction (53, 54) (Figure 1). The inhibition of K⁺ channels (Kv) by acute hypoxia has also been shown to be specific to PASMC, as Kv channels of systemic artery smooth muscle cells were not inhibited, and hypoxia failed to produce a depolarization
While the mechanism of inhibition of $K_V$ channels by hypoxia remains elusive, chronic hypoxia may alter $K_V$ channel expression, which could affect the resting membrane potential of PASM, resulting in pulmonary vasoconstriction (56, 57, 58, 59). Concerning $Ca^{2+}$ influx, further studies demonstrated HPV under conditions inhibiting both VDCCs and depolarization, suggesting that any increase in $[Ca^{2+}]_i$ must come from either voltage-independent $Ca^{2+}$ entry or intracellular stores. It was then reported that cyclic ADP ribose-mediated $Ca^{2+}$ release from RyRs was critical for HPV. While there is still some debate over the contribution of voltage-dependent vs. –independent $Ca^{2+}$ entry, it is clear that an increase in $[Ca^{2+}]_i$ of PASMCs is a major trigger for pulmonary vasoconstriction (60). Additionally, this rise in $[Ca^{2+}]_i$ stimulates cell migration, proliferation and gene expression, resulting in remodeling of the pulmonary vasculature (60). Since elevated $[Ca^{2+}]_i$ exists in HPV, regardless of the mechanism triggering the rise, it is plausible that $Cl_{Ca}$ channels may contribute to HPV (Figure 1). One recent preliminary study has demonstrated increased $Cl_{Ca}$ channel open times in response to acute hypoxia (61), while another has shown that long term hypoxic stress alters $Cl_{Ca}$ channel functionality in the pulmonary artery (62).

1.2 Chloride Channels

As $Cl^-$ is the most abundant anion and predominant permeating anion species in organisms, anion channels are more generally referred to as “$Cl^-$ channels”. These channels and their resulting currents have been found to play a wide variety of physiological roles, including regulation of cell excitability, secretion, regulating cellular volume, organelle acidification, cell migration, proliferation and differentiation, and apoptosis (63). $Cl^-$ channel
Figure 1. $\text{Ca}^{2+}$ mobilization in HPV: mechanisms that have been implicated in the hypoxia-induced elevation of $[\text{Ca}^{2+}]_i$, their potential signaling pathways, and the ion channels involved. Please refer to text and Ward and McMurty (51) for details. DAG, diacylglycerol; cADPR, cyclic ADP ribose; depol, depolarization; $K_V$, voltage-gated $K^+$ channels; L-type, voltage-gated $\text{Ca}^{2+}$ channels; NCX, $\text{Na}^+-\text{Ca}^{2+}$ exchanger; RyR, ryanodine receptors; SOC, store-operated channels; $\text{Cl}_{\text{Ca}}$, $\text{Ca}^{2+}$-activated $\text{Cl}^-$ channel. Figure adapted from \textit{Ward JPT and McMurty IF. Curr Opin Pharmacol. 2009 June; 9(3): 287–296}.
gating may depend on several factors, including voltage, cell swelling, molecule binding (ligand-gated), ions (anions, H\(^+\) (pH), Ca\(^{2+}\)), and by phosphorylation, either of the pore forming unit itself, or a regulatory subunit (64). Classification thus far has been based on channel location, single-channel conductance, and mechanism of activation or regulation. However, as a channel may lie in both the plasma membrane as well as intracellular organelles, and mechanisms of activation undoubtedly overlap, this method of classification is unlikely to correlate to gene families (64). While the study of Cl\(^-\) channels has historically taken a backseat to works focusing on Na\(^+\), K\(^+\), Ca\(^{2+}\), several anion channels have been well established in the literature; the CLC family, CFTR, and the ligand gated GABA- and glycine-receptors. Others, such as CLIC and CLCA remain controversial, as some investigators do not believe they constitute bona fide channels. More recently, the Bestrophin and TMEM16/Anoctamin families have been suggested to be responsible for the calcium-activated chloride current (I\(_{\text{Cl(Ca)}}\)), with recent data leaning more toward TMEM16/Anoctamin as a better candidate, which will be discussed further in this dissertation.

Given the varied cellular and physiological functions of Cl\(^-\) channels, it would seem plausible that several diseases may be due to Cl\(^-\) channel abnormalities (Cl\(^-\) channelopathies). Indeed, several diseases have been linked to mutations of anion channels; cystic fibrosis, myotonia, epilepsy, hyperekplexia, lysosomal storage disease, deafness, renal salt loss, kidney stones, and osteopetrosis (64). While Best’s vitelliform macular dystrophy is linked to loss-of-function mutations in the Bestrophin family of Cl\(_{\text{Ca}}\) channels, there are doubts as to whether the disease is directly due to a loss of Cl\(^-\) channel...
activity (65). Finally, gnathodiaphyseal dysplasia – associated with bone dysplasia and fragility – has been linked to a mutation in TMEM16E, a member of the newly discovered family of Cl\(_{Ca}\) channels (65).

In addition to the possibility of treating some of the diseases discussed above, understanding the pharmacology of Cl\(^-\) channels is important in the basic research setting. Isolating specific ion channels in a complex background of conductances, distinguishing between gating states, and investigating pore structure are all facilitated by having selective, high-potency pharmacological tools. Unfortunately, such tools are lacking for Cl\(^-\) channels. Those blockers that are available seldom achieve complete block, habitually carry several side-effects, and are display low specificity (64). Despite the classes of compounds known to block Cl\(^-\) channels largely being chemically unrelated, they are similar (with few exceptions) in the fact that they are negatively charged at physiological pH (64). This negative charge is likely related to how the compound interacts with and blocks the channel. Given what is known of cation channels and their negatively charged selectivity filters, it may be postulated that anion channels should have a positively charged selectivity filter. It would be plausible then, that the negatively charged blocker would not pass the charge filter, would not able to translocate across the entire membrane, ending up getting stuck in and occluding the pore. There are other possibilities however, such as a compound binding to a secondary site, resulting in a conformational change of the selectivity filter or pore. This could potentially result in partial or complete block of the channel. Block of Cl\(^-\) channels may be voltage-dependant (blocker binding site lies within the transmembrane
electric field), pH-dependant (charged and uncharged forms of the blocker may bind with different affinities), or concentration-dependant (competitive block).

1.3 Calcium-Activated Chloride Channels

Many cell types from various tissues express Cl\(^-\) channels that are activated by cytosolic Ca\(^{2+}\). These calcium-activated chloride (Cl\(_{\text{Ca}}\)) channels were first described in Xenopus oocytes, where the depolarizing Cl\(_{\text{Ca}}\) current (I\(_{\text{Cl(Ca)}}\)) plays a key role in blocking polyspermy (66, 67). In this system, there is an IP\(_3\)-mediated Ca\(^{2+}\) wave across the cytoplasm upon fertilization, which in turn activates store-operated Ca\(^{2+}\)-entry. This sustained elevation in [Ca\(^{2+}\)]\(_i\) activates Cl\(_{\text{Ca}}\) channels, producing the depolarization that blocks polyspermy. Around the same time, I\(_{\text{Cl(Ca)}}\) was also described in rod inner segments from salamander retinas (68). Within ten years, I\(_{\text{Cl(Ca)}}\) had been described in central and peripheral neuronal cell bodies, developing skeletal muscle, lacrimal gland cells, pituitary cells, cardiac muscle, and smooth muscle (69). In these cell types, along with others more recently identified, they are responsible for important physiological functions. For example, Cl\(_{\text{Ca}}\) channels play a role in olfactory, taste, and photo-transduction, regulate neuronal and cardiac excitability, smooth muscle contraction, and endothelial function, are responsible for Ca\(^{2+}\)-dependent Cl\(^-\) secretion in epithelial cells, may contribute to agonist induced retinal vasoconstriction, and as mentioned above, prevent polyspermy upon fertilization (70).

These channels open in response to [Ca\(^{2+}\)]\(_i\), in the range of 200-500 nM, which may originate from the release of intracellular stores, or by the influx of Ca\(^{2+}\) through channels in the plasma membrane. Activation of Cl\(_{\text{Ca}}\) by Ca\(^{2+}\) often varies by cell type, and may be either
a direct or indirect process (70). Some channels are likely directly gated by Ca\(^{2+}\) via specific Ca\(^{2+}\)-binding domains (70, 71). This has been demonstrated by experiments performed utilizing the inside-out patch-clamp configuration with hepatocytes and *Xenopus* oocytes, where application of Ca\(^{2+}\) in the absence of ATP elicits \(I_{\text{Cl(Ca)}}\) (72, 73). This activation usually results in a depolarization of the plasma membrane, which in some cells increases the open probability (\(P_o\)) of voltage-dependent Ca\(^{2+}\) channels (VDCCs), resulting in additional Ca\(^{2+}\) influx and further depolarization (Figure 2).

### 1.3.1 Reversal Potential for Chloride Ions

There are two main factors determining the direction of Cl\(^-\) flux through Cl\(_{\text{Ca}}\) channels: the membrane potential and the Cl\(^-\) concentration gradient (70). As most cells have a resting potential more negative than \(E_{\text{Cl}}\), activation of Cl\(_{\text{Ca}}\) channels generally results in depolarization. The effect of \(I_{\text{Cl(Ca)}}\) on membrane potential depends on the relative amplitude of the chloride conductance, and \(E_{\text{Cl}}\) (69). With \([\text{Cl}^-]_i\) of most cells being in the range of 4 to 45 mM, \(E_{\text{Cl}}\) is centered in the physiological range of membrane potentials (~-89 to -24 mV at 37\(^\circ\)C). This means that an increase in membrane permeability to Cl\(^-\) could potentially depolarize, hyperpolarize, or stabilize the resting membrane potential (69). In order to set \(E_{\text{Cl}}\), there must be active transport of Cl\(^-\) either into or out of the cell. K\(^+\)-Cl\(^-\) co-transporters (KCC) remove both K\(^+\) and Cl\(^-\) from the cytoplasm, causing a negative shift in \(E_{\text{Cl}}\). KCC activity results in Cl\(^-\) channels being inhibitory, as any increases in Cl\(^-\) conductance will stabilize or hyperpolarize the resting membrane potential (e.g. in developing neurons).
Figure 2. Cartoon representing proposed steps involved in activation of Cl\textsubscript{Ca} channels of vascular smooth muscle during receptor-mediated signaling. Ca\textsubscript{L}, L-type Ca\textsuperscript{2+} channels; CH, constricting hormone; G\textsubscript{q}PCR, G\textsubscript{q} protein coupled receptor; G\textsubscript{q}, G-protein q; PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; SR, sarcoplasmic reticulum. This figure is further described within the text. Adapted from Leblanc et al. Can J Physiol Pharmacol Jul;83(7): 541-56
Alternatively, Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} (NKCC) co-transporters coordinate the entry of Na\textsuperscript{+}, K\textsuperscript{+}, and Cl\textsuperscript{−} into the cytoplasm, resulting in a shift of $E_{\text{Cl}}$ to more positive potentials. An increase in Cl\textsuperscript{−} conductance would then result in depolarization of the membrane potential. The poorly understood ‘pump III’ rounds out the mechanisms of active Cl\textsuperscript{−} transport.

1.3.2 Classes of Calcium-Activated Chloride Channels

There appears to be three functional classes of channels that underlie $I_{\text{Cl(Ca)}}$: Cl\textsuperscript{−} channels activated by Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII), Cl\textsuperscript{−} channels activated by Ca\textsuperscript{2+}/cGMP, and Cl\textsuperscript{−} channels activated by Ca\textsuperscript{2+} without requirement of a secondary activator (termed Cl\textsubscript{Ca} for clarity). While the latter Cl\textsubscript{Ca} channels will be the focus of this dissertation, especially those found in smooth muscle, the other two classes are briefly described below.

1.3.3 CaMKII-Activated Chloride Channels

The CaMKII-activated Cl\textsuperscript{−} channels have been described in the human colonic tumor cell line T84, airway epithelia, T lymphocytes, human macrophages, bilary epithelial cells, neuronal cells, and cystic fibrosis-derived pancreatic epithelial cells (70). The role of CaMKII in activating Cl\textsuperscript{−} currents in these cells come from studies employing inhibitors of calmodulin or CaMKII, or by cell dialysis with purified CaMKII (70). In neurons, CaMKII activates the channel and sensitizes it to increases in $[\text{Ca}^{2+}]_i$ (71). In epithelial tissues, the resulting currents have a medium to large conductance (tens to hundreds of pS). This is in contrast to the Cl\textsubscript{Ca} channels, which are generally small conductance channels (<5 pS).
1.3.4 cGMP-Dependent Calcium-Activated Chloride Channels

In 2001, it was suggested that that vasomotion – the oscillatory contractile activity driven by membrane receptor agonists – was generated by a cGMP-dependent Cl⁻ current $I_{Cl(Ca,cGMP)}$ (74). This current, described in rat mesenteric resistance arteries, seems to combine the characteristics of classical $I_{Cl(Ca)}$ channels and voltage-gated Cl⁻ channels (75). Evoked using either 10 µM cGMP in the intracellular solution or 300 µM 8Br-cGMP (membrane permeable) along with 10 mM caffeine, the resulting currents were characterized by a high sensitivity to inhibition by Zn²⁺, low sensitivity to the classic Cl⁻ channel inhibitors NFA, IAA-94 and DIDS, and did not rectify (75). Additionally, $I_{Cl(Ca,cGMP)}$ is time-independent, has an intermediate conductance (15-55 pS (76)), and a thiocyanate permeability only slightly greater than chloride. This is in stark contrast to the biophysical properties of the ‘classical’ $I_{Cl(Ca)}$, which are described in detail below. Interestingly, both $I_{Cl(Ca)}$ and $I_{Cl(Ca,cGMP)}$ are co-expressed in several smooth muscle types (75, 77). Matchkov et al. (75) exploited the pharmacological profiles of these currents in order to distinguish them, using niflumic acid (NFA) and Zn²⁺ to inhibit $I_{Cl(Ca)}$ and $I_{Cl(Ca,cGMP)}$, respectively. Setting $[Ca^{2+}]_i$ to 900 nM and stepping to potentials between – 60 mV and +60 mV, the investigators observed time-dependent outward relaxations, followed by a slow deactivating tail current upon repolarization (77). Next, NFA was applied (100 µM) in order to block $I_{Cl(Ca)}$, followed by addition of 8Br-cGMP to the bath solution, resulting in the appearance of $I_{Cl(Ca,cGMP)}$. This current was then inhibited with the use of 10 µM ZnCl₂. While these currents appear together in several tissues, including the aorta, tail artery, mesenteric artery, and portal vein, the precise physiological role of $I_{Cl(Ca,cGMP)}$ remains somewhat unclear. Particularly
puzzling is how this excitatory current fits into a cGMP signaling pathway predominated by relaxation. Perhaps the recent discovery of this current’s molecular identity (78) - which will later be discussed - will help shed new light on the physiological role of this current.

1.4 Biophysical Properties of Calcium-Activated (Cl$_{Ca}$) Chloride Channels

Most studies report Cl$_{Ca}$ single channel conductance to be $\leq 5$ pS, which is just at, or below the resolution of single channel recording. There are a few reports of large conductance Cl$_{Ca}$ channels in a few cell types such as human carcinoma cells (79), rat lactotrophs (80), *Xenopus* spinal neurons (81), and *Drosophila* (82), some with conductances $\geq 50$ pS. These reports are rare however, and the predominant Cl$_{Ca}$ channels are those of low conductance.

1.4.1 Ion Selectivity and Permeation

As with many of the biophysical properties of Cl$_{Ca}$ channels, ion selectivity and permeation may differ depending on the cell type. Most Cl$^-$ channels are relatively non-selective, and Cl$_{Ca}$ channels are no exception (70). Cl$_{Ca}$ channels even have trouble selecting between anions and cations. Cl$_{Ca}$ channels of *Xenopus* oocytes and salivary gland acinar cells are among the least selective for Cl$^-$ over cations, with $P_{\text{Cations}}/P_{\text{Cl}}$ being reported as 0.42-0.71 (83, 84) and 0.25 (85), respectively. Slightly more selective are the Cl$_{Ca}$ channels of cone photoreceptor cells ($P_{\text{Cations}}/P_{\text{Cl}} = 0.071$ (86)) and olfactory sensory neurons ($P_{\text{Cations}}/P_{\text{Cl}} = 0.035$ (87)), while Cl$_{Ca}$ channels of neurons and smooth muscle display high selectivity for Cl$^-$ over cations (88, 89, 90, 91). While the selectivity for Cl$^-$ over cations may vary between cell
types, there remains a conserved anion selectivity sequence for Cl\textsubscript{ca} channels. The lyotropic sequence SCN\textsuperscript{−} > I\textsuperscript{−} > Br\textsuperscript{−} ≥ Cl\textsuperscript{−} > F\textsuperscript{−} has been established by many studies employing ion substitution, which results in measurable shifts in the reversal potential. This sequence has been established in many different cell types including smooth muscle myocytes (88, 92, 93), skeletal muscle myotubes (94), parotid secretory cells (95), olfactory receptor neurons (96), olfactory sensory neurons (97), epididymal cells (98), and the T84 (99) and β-TC3 (100) cells lines. Interestingly, many of the Cl\textsuperscript{−} channel families share a similar anion selectivity sequence, including CFTR channels (101), GABA\textsubscript{A} and GABA\textsubscript{C} receptors (102), glycine receptors (103), and volume-activated Cl\textsuperscript{−} channels (104). This similarity among Cl\textsuperscript{−} channels suggests they may also share a common mechanism of ion permeation where a low electric field profile is determined not by the ability of anions to interact with a binding site within the pore, but rather by the dehydration energy profiles of the anions (69).

Permeability ratios, determined by ion substitution experiments, provide an estimate of the difference between the hydration energy in water and the solvation energy of the channel (70). In order to move from the aqueous extracellular space to the channel pore, the energy of stabilization of the ion in bulk water (hydration energy) must be exchanged for the energy of stabilization of the ion by its interaction with the channel (solvation energy) (70). The smaller the difference between hydration and solvation energies, the more easily the ion may enter the channel pore. As larger ions generally carry lower hydration energies, these anions enter the pore relatively easily when compared to smaller anions. However, being able to enter the pore is not synonymous with traversing the channel, meaning the permeability of a channel may not reflect the conductance of that
channel. Indeed, large anions with low hydration energies may enter the pore easily, but are poorly conductive. This is supported by experiments involving the hydrophobic (small hydration energy) anions SCN\(^-\) and C(CN)\(_3\)\(^-\), which block Cl\(^-\) conductance (83). On the other hand, small anions with larger hydration energies are also poorly conductive due to their reduced ability to enter the channel, resulting in a bell-shaped conductance curve (70). By measuring Cl\(_{Ca}\) permeability to organic anions, it has been estimated that the pore is ~6 Å (83), which is in line with reports of pore diameters for several other Cl\(^-\) channels (4.5 to 6 Å). Further investigations involving the voltage-dependence of block by different drugs has led to the proposal that the Cl\(_{Ca}\) pore is an elliptical cone, with the larger opening facing outward (105). This will need to be confirmed by more detailed structural information.

1.4.2 Calcium- and Voltage-Dependence

As their name implies, Cl\(_{Ca}\) channels are activated by elevations in [Ca\(^{2+}\)]\(_i\). Cl\(_{Ca}\) channel activation is also voltage-dependent, but only for a given range of [Ca\(^{2+}\)]\(_i\) (discussed below). However, due to their small single channel conductance preventing the direct measurement of P\(_o\) and a potential range of over 200 mV being required to establish a complete steady state activation curve, precise determination of the Ca\(^{2+}\) and voltage dependence of Cl\(_{Ca}\) channel activation is difficult (69). In order to determine the Ca\(^{2+}\) dependence of activation of Cl\(_{Ca}\), measurements of I\(_{Cl(Ca)}\) are made using the voltage clamp patch clamp configuration, where [Ca\(^{2+}\)]\(_i\) is buffered to known levels by EGTA, BAPTA or HEDTA. A large number of studies have examined the Ca\(^{2+}\)-dependence of activation, giving a range of [Ca\(^{2+}\)]\(_i\) threshold for activation between ~200 and 500 nM. Perhaps best
characterized in several studies involving smooth muscle myocytes, $\text{Cl}_{\text{Ca}}$ channels are outwardly rectifying, but display relatively shallow voltage dependence (106, 107, 108, 109, 110). The shallow voltage dependence of $\text{Cl}_{\text{Ca}}$ channels is evident in the fact that activation from 10% to 90% of maximal activation occurs over a range of over 200 mV. This implies that relatively few charges cross the membrane upon channel opening, which requires the movement of only a small amount of gating charge (69).

Generally, as $[\text{Ca}^{2+}]_i$ increases, there is a negative shift in the voltage dependence of activation. This is readily characterized as a negative shift of the half-maximal activation potential ($E_{0.5}$) (73, 106). However, it has been observed that this shift in $E_{0.5}$ will only occur with $[\text{Ca}^{2+}]_i$ between 0.1 and 1 µM. Beyond 1 µM $[\text{Ca}^{2+}]_i$, it appears that $\text{Cl}_{\text{Ca}}$ channels are activated in a voltage independent manner (73, 106, 111). Interestingly, high $[\text{Ca}^{2+}]_i$ does not activate $\text{Cl}_{\text{Ca}}$ to its maximal theoretical conductance state in smooth muscle myocytes where the intracellular solution contains ATP to promote phosphorylation (106), or at negative potentials in parotid acinar cells (111). When ATP is absent from the pipette solution, high $[\text{Ca}^{2+}]_i$ pushes the conductance towards the theoretical peak (106), and when the $\text{Ca}^{2+}$-dependent decrease in $I_{\text{Cl(Ca)}}$ observed in Xenopus oocytes is minimized, saturating $[\text{Ca}^{2+}]_i$ resulted in a maximal conductance which was independent of voltage (73). These data suggest that $\text{Cl}_{\text{Ca}}$ channel regulation affects the ability of $\text{Ca}^{2+}$ to activate $I_{\text{Cl(Ca)}}$.

1.5 Sources of Calcium

The increase in cytosolic $\text{Ca}^{2+}$ responsible for $\text{Cl}_{\text{Ca}}$ channel activation may occur by entry through VDCCs, by $\text{Ca}^{2+}$ release from intracellular stores through IP$_3$ receptors
activated by the PLC pathway, or by highly localized and transient Ca\textsuperscript{2+} sparks mediated by the opening of ryanodine receptors (112). A close association of RYRs with highly dense clusters of Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels underlies the activation of spontaneous transient inward currents (STICs) by Ca\textsuperscript{2+} sparks in mouse airway smooth muscle (113). Ca\textsuperscript{2+} sparks activate K\textsuperscript{+} and Cl\textsuperscript{-} channels, resulting in spontaneous transient currents in guinea-pig tracheal myocytes (113). In vascular smooth muscle, the focus of this dissertation, membrane depolarization results in activation of L-type calcium channels and Ca\textsuperscript{2+} entry. An elevation in [Ca\textsuperscript{2+}], may then cause activation of Cl\textsubscript{Ca} channels. The enhanced depolarization then recruits further influx through the L-type channels thus establishing a positive feedback loop with the end result of stimulating smooth muscle cell contraction (Figure 2).

Interestingly, the opening of single or clustered L-type channels may occur at hyperpolarized membrane potentials (-70 mV) in arterial myocytes, despite a very low P\textsubscript{o} (114, 115). These openings result in what are termed Ca\textsuperscript{2+} ‘sparklets’, resulting in substantial Ca\textsuperscript{2+} influx and modulate local and global Ca\textsuperscript{2+} in arterial smooth muscle (116). It seems possible that these Ca\textsuperscript{2+} sparklets could activate Cl\textsubscript{Ca} channels. Ca\textsuperscript{2+} sparks on the other hand, where the opening of one or a few ryanodine receptors (RyR) produces a highly localized yet brief Ca\textsuperscript{2+} transient, have been shown to evoke I\textsubscript{Cl(Ca)} in the form of STICs in several types of smooth muscle (113, 117, 118). A recent study has demonstrated a close association of RyRs with Cl\textsubscript{Ca} channels in airway smooth muscle (112). In this study, an image-based approach was applied to measure Ca\textsuperscript{2+} current of the spark while simultaneously employing the whole-cell patch clamp technique to record I\textsubscript{Cl(Ca)}. Ca\textsuperscript{2+} sparks resulted in [Ca\textsuperscript{2+}] of at least 2.4 µM within 600 nm of the spark which activates Cl\textsubscript{Ca}, despite a low Ca\textsuperscript{2+} sensitivity in
these cells (~3.3 µM at -80 mV). This is demonstrated by the observation that Cl<sub>Ca</sub> activation was maintained as long as RyR were open (112). STICs activated by Ca<sup>2+</sup> sparks were blocked by niflumic acid, a commonly used inhibitor of I<sub>Cl(Ca)</sub> (see section discussing Cl<sub>Ca</sub> channel pharmacology). The investigators also proposed that all Cl<sub>Ca</sub> channels localize with Ca<sup>2+</sup> spark sites and concentrate in areas with radii of ~600 nm, with channel densities reaching ~300 channels/µm<sup>2</sup>. This does not seem unreasonable, as Cl<sub>Ca</sub> channels have been suggested to be localized in lipid microdomains or rafts (119). Cl<sub>Ca</sub> channels may be activated following IP<sub>3</sub>-induced Ca<sup>2+</sup> release from the sarcoplasmic reticulum. This type of activation reveals a role for Cl<sub>Ca</sub> channels in a positive feedback loop promoting depolarization and Ca<sup>2+</sup> entry. In this pathway, stimulation of G-protein coupled receptors by agonists such as serotonin or norepinephrine results in activation of PLC, the generation of IP<sub>3</sub>, and finally Ca<sup>2+</sup> release from intracellular stores. Release of Ca<sup>2+</sup> from the SR elevates [Ca<sup>2+</sup>]<sub>i</sub>, activating Cl<sub>Ca</sub> channels and triggering a depolarization due to Cl<sup>-</sup> efflux. L-type Ca<sup>2+</sup> channels are activated by the depolarization, resulting in Ca<sup>2+</sup> influx and contraction (Figure 2) (70, 120, 121). This pathway highlights a role for Cl<sub>Ca</sub> in receptor-mediated vascular tone. A recent interaction between store-operated Ca<sup>2+</sup> entry (SOCE) and Cl<sub>Ca</sub> channels has also been proposed (122). In patch clamp experiments, treatment of rabbit PASMCs with either thapsigargin (TG) or cyclopiazonic acid (CPA) resulted in the activation of a time-independent nonselective cation current (the SOCE current) which activated a NFA-sensitive Cl<sup>-</sup> current (I<sub>Cl(Ca)</sub>). I<sub>Cl(Ca)</sub> increased in proportion to the magnitude of the preceding hyperpolarizing step, which is attributable to larger Ca<sup>2+</sup> influx with more negative potentials (122).
1.6 Regulation of Calcium Activated Chloride Channels

Regulation of $\mathbf{Cl_{Ca}}$ was suspected upon the observation that $\mathbf{I_{Cl(Ca)}}$ in several cell types decreased in amplitude (ran down) following seal rupture or patch excision. As this rundown is observed in the whole-cell patch-camp configuration, it is reasonable to assume that either $[\mathbf{Ca^{2+}}]_i$ somehow declined, or that $\mathbf{Cl_{Ca}}$ channels are being down-regulated. $\mathbf{I_{Cl(Ca)}}$ is often elicited by caffeine or by setting $[\mathbf{Ca^{2+}}]_i$ to elevated levels, and rundown is evident, suggesting that in these studies the rundown is not due to a loss of $[\mathbf{Ca^{2+}}]_i$ but rather $\mathbf{Cl_{Ca}}$ are being down-regulated. The first evidence for kinases regulating $\mathbf{Cl_{Ca}}$ function came in 1997, when $\mathbf{I_{Cl(Ca)}}$ was shown to run down faster than the declining $[\mathbf{Ca^{2+}}]_i$ in airway smooth muscle cells \(^{(123)}\). This study also demonstrated run down of the current when elicited by the $\mathbf{Ca^{2+}}$ ionophore ionomycin, which triggered a maintained elevated $[\mathbf{Ca^{2+}}]_i$. Using the calmodulin antagonist W7, KN-93 (a CaMKII inhibitor), or a specific CaMKII inhibitory peptide, the rate of $\mathbf{I_{Cl(Ca)}}$ rundown was slowed to a level that matched the rate of $[\mathbf{Ca^{2+}}]_i$ decline. Additionally, replacing ATP with the non-hydrolyzable analogue AMP-PNP prolonged the decay of $\mathbf{I_{Cl(Ca)}}$. This provided strong evidence for a role of CaMKII-dependent phosphorylation in the inactivation of $\mathbf{Cl_{Ca}}$ channels of equine tracheal smooth muscle.

While the large multimeric serine/threonine kinase CaMKII is highly expressed in smooth muscle, its effects on $\mathbf{Cl_{Ca}}$ channels may still vary depending on cell type. In both rabbit pulmonary and coronary myocytes, CaMKII was shown to inactivate the channels \(^{(107)}\). Eliciting $\mathbf{I_{Cl(Ca)}}$ with 500 nM $[\mathbf{Ca^{2+}}]_i$ while inhibiting CaMKII activity resulted in a negative shift of the activation curve, suggesting that inhibition by phosphorylation is likely
not due to a simple block of the channel. Interestingly, KN93 increased the amplitude of $I_{Cl(Ca)}$ evoked by a 500 nM Ca$^{2+}$ pipette solution but not 250 nM, suggesting a dependence on [Ca$^{2+}$], for the inhibitory effect of CaMKII. This may be explained by the Ca$^{2+}$ dependence of CaMKII, which has an IC$_{50}$ for activation via calmodulin of ~690 nM (124). Conversely, in the same study $I_{Cl(Ca)}$ of portal vein myocytes displayed positive regulation by CaMKII, suggesting that there are mechanisms downstream of phosphorylation that determine the effects of that phosphorylation on $I_{Cl(Ca)}$ and these are cell and/or tissue specific. There could be several possibilities explaining the cell-specific effects of CaMKII, including a) the channels are made up from different homomeric or heteromeric subunits with distinct phosphorylation profiles, b) it is an associated regulatory subunit which is phosphorylated, and not the pore-forming subunits, or c) CaMKII targets a remote protein, separate from the channels or associated regulatory subunits, that in turn affects the channel (69).

As one might expect, the regulation of Cl$_{Ca}$ channels by the kinase CaMKII is balanced by phosphatase-mediated dephosphorylation. Calcineurin (CaN) was shown to oppose CaMKII activity in rabbit coronary artery myocytes (109). As seen before, inhibition of CaMKII had no effect on $I_{Cl(Ca)}$ amplitude at Ca$^{2+} \leq$ 500 nM, but significantly increased current amplitude at 1 µM [Ca$^{2+}$]. Conversely, inhibition of CaN by cyclosporine A (CsA) had no effect at 1 µM [Ca$^{2+}$], while reducing $I_{Cl(Ca)}$ amplitude at [Ca$^{2+}$] $\leq$ 500 nM. The regulation of Cl$_{Ca}$ channels by CaN was significant at physiological membrane potentials, demonstrated by a 51% reduction in conductance at -50 mV. Another study investigated the role of CaN in pulmonary artery myocyte Cl$_{Ca}$ channel regulation by employing a constitutively active form of the enzyme (108). Including the constitutively active enzyme in the pipette solution
resulted in a considerable enhancement of $I_{\text{Cl(Ca)}}$. This effect was attenuated by dialysis with either the inactivated enzyme or CaN auto-inhibitory peptide. In addition to increasing $I_{\text{Cl(Ca)}}$ amplitude, currents elicited in the presence of the constitutively active form of CaN exhibited faster current development at depolarized potentials, and a slower rate of deactivation upon hyperpolarization. These effects were specific to the α isoform of CaN, even though both α and β isoforms of CaN A are expressed in these myocytes. Finally, as CaN enhanced $I_{\text{Cl(Ca)}}$ in the presence of 500 nM [Ca$^{2+}$], but failed to evoke $I_{\text{Cl(Ca)}}$ when the pipette solution contained only 10 mM BAPTA (essentially Ca$^{2+}$ free), it may be concluded that gating of Cl$_{\text{Ca}}$ channels is not dependent on Ca$^{2+}$-dependent dephosphorylation, but rather gated directly by an increase in [Ca$^{2+}$].

The studies discussed above demonstrate that CaMKII and CaN exert opposite dynamic regulatory effects on $I_{\text{Cl(Ca)}}$. Additionally, their effectiveness at regulating $I_{\text{Cl(Ca)}}$ is Ca$^{2+}$-dependent. It is known that CaN has a higher Ca$^{2+}$ sensitivity than CaMKII (124, 125), making it plausible that CaN might play more of a role in channel regulation during small or moderate elevations in [Ca$^{2+}$], while CaMKII activity would dominate during large elevations in [Ca$^{2+}$], causing $I_{\text{Cl(Ca)}}$ rundown. This would effectively uncouple $I_{\text{Cl(Ca)}}$ from [Ca$^{2+}$], allowing for rapid termination of the depolarizing stimulus.

Ledoux et al (2003) (109) observed that at high [Ca$^{2+}$]$_{i}$ (1 µM), CaN did not enhance $I_{\text{Cl(Ca)}}$ in coronary myocytes. However, inhibiting both CaMKII and CaN with KN93 and CsA, respectively, still led to enhancement of $I_{\text{Cl(Ca)}}$. Dephosphorylation was prevented in equine tracheal myocytes dialyzed with 500 nM Ca$^{2+}$ by thiophosphorylation (ATPγS) or by inhibiting endogenous phosphatase activity with okadaic acid (OA), an inhibitor of protein
phosphatase type 1 and protein phosphatase type 2A (PP1 and PP2A, respectively) (123). Subsequent addition of caffeine resulted in little or no $I_{\text{Cl(Ca)}}$, despite a normal $\text{Ca}^{2+}$ transient. The investigators concluded that the dephosphorylation mediated by the phosphatases was necessary in order to transition $\text{Cl}_{\text{Ca}}$ channels from a phosphorylation-induced inactive state to a closed state, where channels would again be available to open. A recent report has more directly demonstrated a role in $\text{Cl}_{\text{Ca}}$ channel regulation by the $\text{Ca}^{2+}$-independent PP1 and PP2A (126). Several inhibitors, both non-selective (OA, calyculin A, cantharidin) and the PP1 selective inhibitor NIPP-1 antagonized recovery of $I_{\text{Cl(Ca)}}$ when phosphatase activity was promoted (0 ATP in the pipette). Fostriecin, a PP2A-selective antagonist, failed to block recovery of the current. Interestingly, inclusion of a constitutively active form of PP2A in the pipette solution reduced the rundown of the current in the presence of 3 mM ATP, followed by recovery of the current. On the other hand, exogenous PP1 application had no effect on the time course of current rundown. This study also showed that the down-regulation of $I_{\text{Cl(Ca)}}$ by exogenous Calcineurin Aα could be antagonized by the highly selective and potent PP1 antagonist NIPP-1 suggesting that Calcineurin may be exerting its effects on $\text{Cl}_{\text{Ca}}$ by stimulating PP1. While it appears that some sort of involvement in $\text{Cl}_{\text{Ca}}$ channel regulation exists for PP1 and PP2A, the exact role(s) played by these phosphatases remains somewhat unclear, and will require further study.

1.7 Voltage and $\text{Ca}^{2+}$-Dependent Gating Kinetics

In 1996, Arreola et al. (111) proposed a model to describe the kinetics of $\text{Cl}_{\text{Ca}}$ channel activation by voltage and $\text{Ca}^{2+}$ (Figure 3A). In this model, $\text{Ca}^{2+}$ binds to the channel in
Figure 3. Kinetic models describing ClCa channel gating. A) Model proposed by Arreola et al. (111). B) The three open state model proposed by Kuruma and Hartzell (73). Please refer to text for discussion of these two models.
two sequential voltage-dependent steps, resulting in channel opening in a third step that does not involve Ca\(^{2+}\) binding but displays voltage-dependence. The voltage dependence of the transition from the open to closed state was assigned to the rate of channel closing (\(\beta_2\)) based on their data. The shallow voltage-dependence of the equilibrium constant of the step leading to channel opening accounts for saturating Ca\(^{2+}\) failing to activate Cl\(_{ca}\) channels at negative potentials to levels of activation seen with elevated Ca\(^{2+}\) in conjunction with depolarization. In this model, \(P_0\) at saturating [Ca\(^{2+}\)] increases from \(~0.5\) to 0.75 as voltage goes from -50 to +50 mV. While this model is able to simulate Cl\(_{ca}\) channel activation quite well over a wide range of [Ca\(^{2+}\)] and potentials, its ability to simulate deactivation was found to be limited. Kuruma and Hartzell (73) thus proposed a more complex kinetic model, employing three voltage independent Ca\(^{2+}\)-binding steps and three open states (Figure 3B). This model simulates activation well, and is better at simulating deactivation than the model proposed by Arreola et al. Similarly to the Arreola et al. model, the Kuruma and Hartzell model proposes that the voltage dependence of channel gating lies in the closing rate constants (\(\beta(V)\)). Again, equilibrium constants for this model also exhibited shallow voltage dependence. Using this model, \(P_0\) increases from 0.92 to 0.97 in the transition from C\(_3\)Ca\(_3\) to O\(_3\)Ca\(_3\), as voltage goes from -50 to +50. Thus, replacing C\(_3\)Ca\(_3\) with the O\(_3\)Ca\(_3\) state has little effect at physiological potentials. These two models may be reduced to the following conclusions; a) Ca\(^{2+}\) dependence of activation is characterized by voltage dependent Hill coefficients between 1.2 and 3.5, meaning two or more Ca\(^{2+}\) ions bind during channel activation, b) transitions between closed and open states do not involve Ca\(^{2+}\) binding, and c) the time courses of activation and deactivation are Ca\(^{2+}\) and voltage dependent, implying
rate constants are also Ca\(^{2+}\) and voltage dependent. Angermann et al. (106) expanded upon the Kuruma and Hartzell model in order to explain the effects of phosphorylation on Cl\(_{Ca}\) channels. By using either 3 mM ATP or 3 mM AMP-PNP in the pipette solution, phosphorylation was promoted or minimized, respectively. In the presence of 3 mM ATP, \(I_{Cl(Ca)}\) amplitude and the rate of activation were reduced while deactivation was sped up, compared to cells dialyzed with 3 mM AMP-PNP. Interestingly, the \(K_D\) for \(I_{Cl(Ca)}\) activation by Ca\(^{2+}\) was not different between the two groups in the range of potentials allowing for comparison. Instead, dephosphorylation shifted the voltage dependence of activation to more negative potentials resulting in a sustained current that could not be shut off by membrane hyperpolarizations of up to -200 mV. By converting \(O_2\) and \(O_3\) to closed states and increasing the values of voltage dependant rate constants (\(\beta(V)\)), the model was able to simulate \(I_{Cl(Ca)}\) in PASMCs where phosphorylation was promoted. The authors proposed that phosphorylation results in a state-dependent block of Cl\(_{Ca}\) channels, especially at elevated \([Ca^{2+}]\) (106).

1.8 Pharmacology

Having a selection of highly specific and potent pharmacological tools is invaluable in the study of ion channels. Such tools allow investigators to isolate specific channels and selectively suppress particular currents in a complex background, can help distinguish between gating states, and allows for investigations of the pore structure. Unfortunately, there is a general lack of specific antagonists of Cl\(^-\) channels. Compounds employed as Cl\(^-\) channel antagonists often carry side effects such as inhibiting or activating other channels,
Table 1. Effects of various blockers used to inhibit $I_{Cl(Ca)}$ on other ion channels

<table>
<thead>
<tr>
<th>Cl− channel blocker</th>
<th>Ionic current</th>
<th>Effect</th>
<th>Cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Niflumic acid1</td>
<td>$I_{Cl(swell)}$</td>
<td>Inhibit</td>
<td>Portal vein SMC</td>
</tr>
<tr>
<td></td>
<td>$I_{K(BKCa)}$</td>
<td>Stimulate</td>
<td>Lipid bilayer, portal vein SMCs, urethra SMCs</td>
</tr>
<tr>
<td></td>
<td>$I_{f}$</td>
<td>Decrease</td>
<td>Cerebral artery SMCs, urethra SMCs</td>
</tr>
<tr>
<td></td>
<td>$I_{Kv4}$</td>
<td>Decrease</td>
<td>Xenopus oocytes</td>
</tr>
<tr>
<td></td>
<td>$I_{Cl(Ca)}$</td>
<td>Stimulate</td>
<td>VSMCs</td>
</tr>
<tr>
<td>Flufenamic acid1</td>
<td>$I_{Ks}$</td>
<td>Increase</td>
<td>Xenopus oocytes</td>
</tr>
<tr>
<td></td>
<td>$I_{K(BKCa)}$</td>
<td>Stimulate</td>
<td>SMCS, lipid bilayer</td>
</tr>
<tr>
<td>Mefenamic acid1</td>
<td>$I_{K(BKCa)}$</td>
<td>Stimulate</td>
<td>Lipid bilayer, portal vein SMCs</td>
</tr>
<tr>
<td></td>
<td>$I_{Ks}$</td>
<td>Increase</td>
<td>Xenopus oocytes</td>
</tr>
<tr>
<td></td>
<td>$I_{K(dr)}$</td>
<td>Increase</td>
<td>Canine jejunum SMCs</td>
</tr>
<tr>
<td></td>
<td>$I_{K(ATP)}$</td>
<td>Increase</td>
<td>Urethra SMCs</td>
</tr>
<tr>
<td>Meclofenamic acid1</td>
<td>$I_{KCNQ2/3}$</td>
<td>Increase</td>
<td>CHO cells</td>
</tr>
<tr>
<td>NPPB</td>
<td>$I_{Cl(swell)}$</td>
<td>Inhibit</td>
<td>NIH-3T3 cells, 8226 myeloma cells</td>
</tr>
<tr>
<td></td>
<td>$I_{K(BKCa)}$</td>
<td>Inhibit</td>
<td>Glioblastoma cells</td>
</tr>
<tr>
<td></td>
<td>$I_{CFTR}$</td>
<td>Activate</td>
<td>HEK293 cells</td>
</tr>
<tr>
<td>A9C</td>
<td>$I_{K(BKCa)}$</td>
<td>Stimulate</td>
<td>PA SMCs</td>
</tr>
<tr>
<td></td>
<td>$I_{Cl(Ca)}$</td>
<td>Stimulate</td>
<td>Cerebral artery SMCs</td>
</tr>
<tr>
<td>IAA94</td>
<td>$I_{Cl(Ca)}$</td>
<td>Stimulate</td>
<td>Portal vein SMCs</td>
</tr>
<tr>
<td></td>
<td>$I_{K(BKCa)}$</td>
<td>Stimulate</td>
<td>Portal vein SMCs</td>
</tr>
<tr>
<td></td>
<td>$I_{K(ATP)}$</td>
<td>Stimulate</td>
<td>Portal vein SMCs</td>
</tr>
<tr>
<td>Ethacrynic acid</td>
<td>$I_{K(BKCa)}$</td>
<td>Stimulate</td>
<td>Portal vein SMCs</td>
</tr>
<tr>
<td></td>
<td>$I_{K(ATP)}$</td>
<td>Stimulate</td>
<td>Portal vein SMCs</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>$I_{Cl(swell)}$</td>
<td>Inhibit</td>
<td>Portal vein SMCs</td>
</tr>
<tr>
<td></td>
<td>$I_{K(BKCa)}$</td>
<td>Stimulate</td>
<td>Colonic SMCs</td>
</tr>
</tbody>
</table>

All compounds listed have been used as blockers of $I_{Cl(Ca)}$ in various cell types. Abbreviations: $I_{CFTR}$, Cl− current generated by the gene encoding the cystic fibrosis transmembrane regulator CFTR; $I_{Cl(swell)}$, swelling-activated Cl− current; $I_{f}$, hyperpolarization-activated pacemaker current; $I_{K(ATP)}$, ATP-sensitive K⁺ channel current; $I_{K(BKCa)}$, Ca²⁺-activated K⁺ channel current; $I_{K(dr)}$, delayed rectifier K⁺; $I_{K(KCNQ2)}$, K⁺ current generated by the gene encoding the intermediate conductance Ca²⁺-activated K⁺ current generated by the co-association of proteins encoded by KCNQ1 and KCNE1 (mink); $I_{Kv4}$, transient outward K⁺ current belonging to the $K_{o}$ family of voltage-dependent K⁺ channel genes. ¹Structurally similar. Adapted from I.A. Greenwood and N. Leblanc Trends Pharmacol. Sci 2007 Jan;28(1):1-5. Epub 2006 Dec 5.
have poor specificity and potency (effective concentrations in the µM to mM range), and complete block of their target is seldomly achieved (64). Despite coming from a range of unrelated chemical classes, drugs used to block Cl- channels generally carry a negative charge at physiological pH. This likely relates to their mechanism of action, in which the negatively charged blocker lodges in a positively charged anion selectivity filter, occluding the pore (64). This section will discuss the current literature regarding the pharmacology of I_{Cl(Ca)} with particular focus on vascular smooth muscle.

Many different compounds have demonstrated the ability to inhibit I_{Cl(Ca)} with varying degrees of success. As Table 1 demonstrates, these drugs do not specifically target Cl_{Ca} channels. Niflumic acid – a focal point for this section - for example, inhibits I_{Cl(Iswell)} (127), potentiates I_{K(BKCa)} (128, 129) and I_{K(KNa)} (130), decreases current amplitude of VDCC (131, 132), I_{f} (133), I_{Kv4} (134), and as will be discussed below, may also stimulate I_{Cl(Ca)} (120, 135). Many different drugs have been employed to inhibit Cl_{Ca} channels the most common being the fenamates niflumic and flufenamic acid, antracene-9-carboxylic acid (A9C), indanyloxyacetic acid (IAA94) and 4,4'-diisothiocyano-2,2'-stilbene disulphonic acid (DIDS).

1.8.1 Overlapping Pharmacology of Calcium-Activated Chloride and Potassium Channels

Several studies have demonstrated overlapping pharmacology of Ca^{2+}-activated Cl- and K+ channels. In smooth muscle cells, the fenamates, A9C, IAA94 and ethacrynic acid activate BK_{Ca} channels in addition to inhibiting their intended target Cl_{Ca} channels (128, 136). NFA, flufenamic and mfenamic acids have also been shown to potentiate BK_{Ca} in lipid bilayers (129). One study demonstrated the dissimilar 1,3-dihydro-1-[2-hydroxy-5-
(trifluoromethyl)phenyl]-5-trifluoromethly-2H-benzimidazol-2-one (NS1619) and isopimarene to elevate $I_{\text{Cl(Ca)}}$ in vascular myocytes (137). This occurs at concentrations similar to those used to activate $B_{\text{KCa}}$. A follow-up study involving the selective $B_{\text{KCa}}$ channel blocker paxilline significantly inhibited $I_{\text{Cl(Ca)}}$ at a concentration of 1 µM, while the $B_{\text{KCa}}$ blockers penitrem A and iberiotoxin only slightly reduced $I_{\text{Cl(Ca)}}$ amplitude, but did slow channel deactivation (138). Upon depletion of cholesterol with methyl-β-cyclodextrin (M-βCD) the inhibitory action of paxilline on $I_{\text{Cl(Ca)}}$ was abolished, as was the stimulatory effect of NS1619 (119). These results suggest that the effects of the $B_{\text{KCa}}$ channel modulators on $I_{\text{Cl(Ca)}}$ rely on the presence of functional lipid microdomains, and that $B_{\text{KCa}}$ and $\text{ClCa}$ channels may interact in some fashion (119).

1.8.2 Niflumic Acid

Among the various $\text{Cl}^-$ channel blockers, NFA is often considered to be ‘specific’ for $\text{ClCa}$ channels. This is really a misnomer, given some of the non-specific effects discussed above. However, of the known $\text{Cl}^-$ channel blockers it is the most effective inhibitor of $I_{\text{Cl(Ca)}}$ (70). To the best of my knowledge, NFA was first demonstrated to inhibit $I_{\text{Cl(Ca)}}$ of smooth muscle in 1989 (91). Since then, NFA at concentrations between ~ 1-50 µM has been shown to block $I_{\text{Cl(Ca)}}$ evoked in a variety of ways, including by L-type $\text{Ca}^{2+}$ current (139), reverse-mode $\text{Na}^+$/Ca$^{2+}$ exchange (140), constricting agonist or caffeine (141), $\text{Ca}^{2+}$ sparks (STICS) (141), or flash photolysis of caged $\text{Ca}^{2+}$ (92). In this concentration range, NFA has no effect on the magnitude of $\text{Ca}^{2+}$ current (91, 139, 142), SR $\text{Ca}^{2+}$ release evoked by caffeine or norepinephrine (141, 143), or the swelling-activated $\text{Cl}^-$ current (127). Criddle et al. (1996)
(143) demonstrated that NFA blocked norepinephrine-induced contractions in rat aorta, but could not block KCl-induced contraction. These results suggest that NFA blocks agonist-mediated tone not by inhibiting VDCCs, but Cl$_{Ca}$ channels directly (143, 144). Similar results were found in the isolated mesenteric vascular bed of the rat (145), rat pulmonary artery (139), and rabbit mesenteric arterioles (146). The wide range of reported IC$_{50}$ values for NFA suggests its potency linked to the method used to evoke the current. NFA inhibits STICs with an IC$_{50}$ less than 4 µM (Figure 4), whereas the affinity of NFA for blocking I$_{Cl(Ca)}$ evoked by an agonist or caffeine was at least twofold lower (141). Although dose-response relationships were not obtained, NFA appeared less efficacious at inhibiting arterial I$_{Cl(Ca)}$ elicited by inward Ca$^{2+}$ currents than STICs, with 10 µM NFA producing only ~50-80% of the deactivating Cl$^{-}$ tail current recorded after return to the holding potential (139, 142). A further reduction in potency was evident during NFA-induced block of I$_{Cl(Ca)}$ evoked by an elevated sustained intracellular Ca$^{2+}$ concentration (500 nM) in coronary myocytes with an IC$_{50}$ of 159 µM at +50 mV (120). One possible explanation for these observations may relate to the ability of higher [Ca$^{2+}$]$_{i}$, either induced cyclically or maintained, to promote down-regulation of I$_{Cl(Ca)}$ by phosphorylation via CaMKII (106, 107, 108, 109, 123).

As mentioned above, NFA acid has a dual effect, and is also capable of stimulating I$_{Cl(Ca)}$ in vascular smooth muscle cells (Figure 4) (110, 135). Piper et al. (135) first reported this effect in PASMCs, where currents were evoked with 250 or 500 nM [Ca$^{2+}$]$_{i}$ (Figure 4B). Bath application of 100 µM NFA resulted in increased inward currents at negative potentials, but inhibited outward currents at positive potentials. Substituting external NaCl with NaSCN resulted in a shift in $E_{r}$ of about -50 mV. Under these conditions it was found
that NFA increased $I_{\text{Cl}(\text{Ca})}$ at potentials negative to $E_r$ while inhibiting the current at potentials positive to $E_r$. This result suggests that NFA increases the net outward flux of anions while inhibiting the net inward movement of anions. NFA surprisingly increased the holding current when dialyzed with 500 nM $\text{Ca}^{2+}$, increasing the holding current from -26 to -50 pA (Figure 4B). Upon washout of NFA, $I_{\text{Cl}(\text{Ca})}$ was greatly enhanced at all potentials. This increase appears to be due to an increase in conductance, as the instantaneous currents recorded at both +70 and -80 mV were enhanced whereas the current relaxations recorded at these potentials were much smaller. This stimulation upon washout was also observed as an increase in holding current of ~80 pA which peaked after around 30 s before gradually declining to control values after ~2 min. These effects were concluded to be mediated by NFA binding to an external site, likely close to the Cl$^-$ channel pore, as inclusion of NFA in the pipette solution had no effect on $I_{\text{Cl}(\text{Ca})}$.

Ledoux et al. (2005) (110) further investigated this phenomenon in coronary artery myocytes by utilizing fast-flow superfusion system that allowed the investigators to quickly modify the external medium surrounding the patched cell (exchange time < 300 ms). As in the study above, sustained $I_{\text{Cl}(\text{Ca})}$ was evoked by 500 nM $[\text{Ca}^{2+}]_i$. These currents were dose-dependently inhibited by NFA with an IC$_{50}$ of 159 µM. Application of 100 µM NFA quickly blocked both time dependent outward current and the tail current. Quickly washing out NFA resulted in a transient increase in current amplitude of ~2-fold where the time to peak and duration of current enhancement was dose-dependent (Figure 4B). This could be repeated multiple times during the same experiment. NFA washout also appeared to accelerate activation kinetics at +90 mV, while converting the deactivation kinetics at -80
Figure 4. Effects of niflumic acid and anthracene-9-carboxylic acid on Ca$^{2+}$-activated Cl$^{-}$ channels of vascular smooth muscle cells. A) Niflumic acid (NFA) and anthracene-9-carboxylic acid (A9C) reversibly block spontaneous transient inward currents of isolated rabbit portal vein cells. B) NFA both blocks and stimulates $I_{\text{Cl(Ca)}}$. Bi) upper trace: NFA (100 μm) increased the holding current by nearly 20 pA in a rabbit pulmonary artery myocyte dialyzed with 500 nM Ca$^{2+}$. lower trace: In the presence of NFA, the inward current at negative potentials was enhanced, while outward current at positive membrane potentials was inhibited in a second rabbit pulmonary artery myocyte. Bii) Rapid washout of 100 μM NFA stimulates $I_{\text{Cl(Ca)}}$ in rabbit coronary artery myocytes dialyzed with 500 nM Ca$^{2+}$. C) A9C inhibits $I_{\text{Cl(Ca)}}$ evoked by 500 nM Ca$^{2+}$ at +70 mV, while stimulating the tail current at -80 mV. Please refer to text and original manuscripts for greater detail. Figure composed from Hogg RC, Wang Q, and Large WA. Br J Pharmacol. 1994 Apr;111(4):1333-41 (A NFA), Hogg RC, Wang Q, and Large WA. J Physiol. 1993 May;464:15-3 (A A9C), Piper AS, Greenwood IA, and Large WA. J Physiol. 2002 Feb 15;539(Pt 1):119-31 (Bi), Ledoux J, Greenwood IA, and Leblanc N. Mol Pharmacol. 2005 Jan;67(1):163-73. Epub 2004 Oct 1 (Bii), and Piper AS and Greenwood IA. Br J Pharmacol. 2003 Jan;138(1):31-8 (C).
mV from a monophasic time course to a biphasic time course. As investigators observed a partial recovery of the current following rapid block, it may be that the net current recorded in the presence of NFA was the product of a superimposed inhibition and stimulation. Investigators attempted to mathematically model the dual-effect behavior of NFA, using models assuming a single binding site or one inhibitor state and either one or two stimulatory binding sites. The single binding site model could account for block and transient stimulation of $I_{\text{Cl}(\text{Ca})}$ with 10 $\mu$M NFA but it could not account for the biphasic stimulation with either 100 $\mu$M or 1 mM NFA. In the models involving stimulatory sites, block by NFA occludes stimulation unless the drug is washed out quickly. The model that was best able to reproduce the data comprised two NFA binding sites, a high-affinity binding site (10 to 23 $\mu$M) and low-affinity binding site (1.25 mM) (110).

1.8.3 Niflumic Acid – Mechanism of Action

Several groups have postulated that NFA interacts with the hydrophilic channel pore or an extracellular vestibule containing NFA binding site(s) (110, 128, 135, 141). However, until the structure of the underlying protein is resolved, we can only speculate as to how NFA mediates its effects on the channels. It could be that NFA-induced stimulation results from a negative shift of the voltage-dependence of $I_{\text{Cl}(\text{Ca})}$, leading to saturation at elevated $[\text{Ca}^{2+}]$ (110). Or perhaps, as suggested by the fact that the stimulatory effects of NFA were abolished by increasing $[\text{Ca}^{2+}]$, 500 nM to 1 $\mu$M (135), perhaps there is an increase in single-channel conductance or silent channels are being recruited (110). As mentioned above, there appears to be an overlap of the inhibitory and stimulatory effects. Perhaps the
increase in $I_{\text{Cl(Ca)}}$ upon washout is due to a faster reversal of the inhibitory effects, unmasking the stimulatory effects. Block of $I_{\text{Cl(Ca)}}$ by NFA appears to occur quite rapidly, as there was no evidence of developing channel block in PASMCs at +70 mV, with the current immediately settling at the blocked level (135). These findings may be added to previous thoughts on NFA-Cl$_{\text{Ca}}$ channel binding derived from observations of the effects of NFA on STICS. At -80 mV, niflumic acid converted the normal monoexponential STIC decay to a biexponential time course where the fast time constant and the slow time constant were, respectively, faster and slower than the control time constant (147). This effect was concentration dependent, and is consistent with open channel block by NFA where the drug binds relatively slowly to the open channel.

### 1.8.4 Anthracene-9-Carboxylic Acid

A9C is a Cl$^-$ channel blocker chemically unrelated to NFA that inhibits STICs in rabbit portal vein smooth muscle (148, 149). This compound also inhibits $I_{\text{Cl(Ca)}}$ evoked by Ca$^{2+}$ entry through VDCCs in several tissues (132, 150, 151, 152). A9C shares a few characteristics with NFA, such as that in smooth muscle, their inhibitory effects on STICs may be rapidly reversed in a time frame corresponding with the complete changeover of the bath solution (147). This provides evidence that these drugs interact with the channel on the extracellular surface, at a site close to the pore. A9C also appears to block open channels, as STIC amplitude decreased while the time course of decay was lengthened. Block by A9C is highly voltage dependent, with $IC_{50}$ for inhibition of STICs of 300 µM at -50 mV and 90 µM at +90 mV (149). In a follow up to their study examining the dual effects of NFA on $I_{\text{Cl(Ca)}}$ in rabbit
PASMCs, Piper and Greenwood (153) examined the effects of A9C in the same preparation. Their results were equally surprising. Application of 500 µM A9C resulted in a small inhibition of the outward current recorded at +70 mV, but increased the amplitude of the instantaneous inward relaxation at -80 mV by over 300%. Unlike NFA, A9C had no significant effect on the kinetics of current decay. Voltage clamp protocols revealed that this effect of A9C required prior depolarization, as the drug did not simply activate $I_{Cl(Ca)}$ at negative potentials. Interestingly, the increase of the instantaneous inward relaxation was found to be Ca$^{2+}$-independent for concentrations between 100 nM and 1 µM. The effects of A9C were reversible, with current levels returning to control levels with progressive wash out.

NFA was applied in the continued presence of A9C in order to determine whether the two compounds affected $I_{Cl(Ca)}$ through a similar mechanism. Addition of 100 µM NFA rapidly inhibited the A9C-enhanced inward current at -80 mV. Wash out of NFA in the continued presence of A9C saw the current return to pre-NFA levels. There was no stimulation of the current upon wash out, as described previously. Reversing the drug order and applying A9C in the continued presence of NFA failed to alter the effects seen with NFA alone. This suggests that these two drugs may act through a similar mechanism, possibly the same binding site(s), where the effects of NFA overshadow those of A9C (153). More evidence for the two-site model discussed above comes from the observation that lower concentrations of A9C are required for enhancing the instantaneous current at -80 mV compared to those required to produce block at +70 mV. Additionally, the inhibitory site would have a weaker affinity for NFA and A9C than the stimulatory site (153).
1.9 Calcium-Activated Chloride Channels: Molecular Candidates

The fact that Cl⁻ channels seem to lack conserved motifs or shared membrane topology combined with the lack of specific channel blockers has made identification of ClCa difficult. Several studies have identified four families of proteins that may potentially carry the native I Cl(Ca): Tweety, CLCA, bestrophin and TMEM16. These four families and their viability as molecular candidates for the native ClCa channels discussed above will be reviewed below. Representative currents from each family may be found in Figure 5.

1.9.1 Tweety

The human Tweety gene family is comprised of three genes, hTTHY1, hTTHY2 and hTTYH3, which are homologous to a gene in the flightless locus of Drosophila (154). While lacking a distinct voltage sensor, Tweety’s putative protein structure resembles that of BKCa, in that it has 6 predicted trans-membrane domains, a predicted pore region similar to BKCa, as well as a similar glutamate- and aspartate-rich region that may function as a Ca²⁺ bowl. This gene family was first found to carry a chloride conductance when transfected into CHO cells, resulting in a maxi-Cl⁻ conductance with a single channel conductance of ~260 pS (154) (Figure 5). Both hTTHY2 and hTTH3 appear to be activated by Ca²⁺, while hTTHY1 is volume-activated. Tweety channels are blocked by 10 µM DIDS, while 10 µM SITS, 300 µM NFA, 10 µM DTT, or 10 µM ZnCl₂ failed to inhibit the current, which is similar to native maxi-Cl⁻ conductances. Tweety expression has been found in the heart, kidney, skeletal muscle, and brain, but has not been identified in smooth muscle (154, 155, 156). Upregulated in astrocytoma, glioma and several other cancers (156), Tweety proteins are regulated by
Nedd4-2 ubiquitination, which controls cell surface and total cellular protein levels (157). As Cl\textsubscript{Ca} channels of the vasculature are of small conductance, it is unlikely that Tweety is responsible for the native I\textsubscript{Cl(Ca)} (154). Rather, it is more likely to encode the maxi-Cl\textsuperscript{−} channel found in spinal neurons and skeletal muscle (80).

1.9.2 The CLCA Family of Proteins

Since the first isoform was cloned, the CLCA gene family has been a highly contentious molecular candidate for the elusive native I\textsubscript{Cl(Ca)} (70). Cloned from a bovine tracheal cDNA expression library, investigators were pursuing a 140 kDa protein isolated from tracheal epithelial cells that was found to carry a Ca\textsuperscript{2+}/CAMKII-regulated Cl\textsuperscript{−} conductance when reincorporated into an artificial bilayer system (158). Authors were able to clone bovine CLCA1 (bCLCA1) by screening the cDNA expression library using a polyclonal antibody developed against a reduced form (38 kDa) of the protein (158). The underlying native channel was sensitive to Ca\textsuperscript{2+}, with 7.75 \mu M Ca\textsuperscript{2+} increasing the open probability three-fold (159). Additionally, it was found that at a concentration of 0.4 \mu g/ml, CAMKII increased the Ca\textsuperscript{2+} sensitivity to where 1 \mu M Ca\textsuperscript{2+} further increased the open probability (159). Since the cloning of bCLCA1, several additional paralogs and orthologs were discovered, including another bovine isoform (160), four human (161, 162, 163), pig (164), horse (165), rat (166), and six mouse isoforms (167, 168, 169, 170, 171). The various isoforms arise from different genes, alternate splicing, and post translational modification (120).
Originally thought to consist of four 38 kDa subunits held together by disulfide bonds, the first-cloned 140 kDa bCLCA1 was later found to be comprised of a 100 kDa subunit, and a 38 kDa post-translational cleavage product that remains associated (158, 172). With significant homology retained across the family, this two subunit model has since been suggested for several other cloned isoforms (173). Hydropathy data suggests four transmembrane domains for bCLCA1, a homologue of hCLCA3, mCLCA1, mCLCA2 and mCLCA4 (173). Interestingly, hCLCA3 cDNA contains two internal stop codons, producing a NH$_2$ terminal polypeptide that stops short of predicted transmembrane domains, and a second fragment whose amino acid sequence contains the first two potential transmembrane domains of other CLCA proteins (163). The second fragment lacks a signal sequence however, and only the NH$_2$ terminal polypeptide was detected in transfected HEK293 cells. Mouse homologues mCLCA1, mCLA2 and mCLCA4 lack the internal stop codons found in hCLCA3, suggesting significant structural differences.

The 902 amino acid mCLCA1 is post-translationally processed, giving 90 kDa NH$_2$ terminal and 32/38 kDa COOH terminal components (170). The resulting protein is predicted to contain four transmembrane domains, all residing within the NH$_2$ terminus. While little is currently known of mCLCA2 structure, it shares 96% identity with mCLCA1, which might suggest similar structural organization. mCLCA4, at 909 amino acids, is similar to both mCLCA1 and 2, with a conserved cleaved NH$_2$ domain (90 kDa) and second COOH terminal product (30-40 kDa) (168). mCLCA3, which unlike mCLCA1 and -4 has not been found to be expressed in smooth muscle (168, 174), has been found to secreted as a soluble protein (175). The two cleavage products of mCLCA3 (75 kDA NH$_2$ terminal and 35 kDa COOH
terminal products) were found to remain physically associated, as in other CLCA isoforms, but were shown to associate with secretory vesicles. Overall, little is known of the CLCA structure. While homology across species may help in predicting structure, important differences, such as the stop codons of hCLCA3, may drastically affect protein structure.

The CLCA family can be divided into structurally related groups. Cross species orthologs hCLCA1, mCLCA3 and pCLCA1 share a similar distribution within their respective species, concentrated in mucus-producing epithelium in the gastrointestinal and respiratory tracts, where they may be involved in mucin secretion and Cl\(^{-}\) conductance modulation in secretory epithelial tissue (173). hCLCA2 and hCLCA4, along with mCLCA5 and -6, form the second major group, with two subgroups: 1) mCLCA1, -2, and -4, and 2) hCLCA3, bCLCA1, and bCLCA2 (173). The presence of this group has been noted in most tissue where their expression has been investigated (173). Generally, the electrophysiological characteristics are maintained across the CLCA gene family when transfected into HEK293 cells, maintaining anion selectivity, Ca\(^{2+}\) sensitivity, outward rectification, and sensitivity to DIDS, DTT, and sometimes tamoxifen and NFA. When human CLCA1 (hCLCA1) was transiently expressed in the HEK293 cell line, the current increased from 1.57 ± 0.72 pA/pF in control untransfected cells to 11.06 pA/pF in transfected cells. This current was blocked by DIDS, DTT and NFA (161). When activated in the presence of 1 mM Ca\(^{2+}\) and ionomycin, a single channel conductance of 13.4 pS was determined. Current was time-independent and outwardly rectifying, however the Cl\(^{-}\) dependence of the current was not determined. The study did not mention whether there were any endogenous channels that could be responsible for the background of nonselective anion current previously documented in
HEK293 cells (173, 176, 177). Similarly, mCLCA1 expression in HEK293 cells resulted in a Ca\(^{2+}\)-activated current upon application of ionomycin, which increased from 2.05 ± 1.09 to 10.23 pA/pF (170). This current, like that of hCLCA1, was time-independent, outwardly rectifying, and inhibited by DIDS, DTT, and NFA. Transfected *Xenopus* oocytes with mCLCA1 resulted in a Cl\(^{-}\) dependent current that was sensitive to both DIDS and NFA (178). However, Ca\(^{2+}\) dependency was difficult to ascertain due to the large background currents present in *Xenopus* oocytes.

Despite being cloned over 10 years ago, there is still significant debate over whether CLCA proteins are responsible for the native \(I_{Cl(Ca)}\). While activated by Ca\(^{2+}\), transfected CLCA channels often require supraphysiological concentrations of Ca\(^{2+}\) approaching the millimolar range, which is significantly higher than Ca\(^{2+}\) concentrations required to activate the native channels (~ 500 nM) (161, 178). This is clearly demonstrated in a direct comparison of the biophysical properties of whole cell \(I_{Cl(Ca)}\) recorded from freshly isolate murine portal vein SMCs to those generated in HEK293 cells stably transfected with cDNA encoding \(mCLCA1\) (174). The expressed currents demonstrated need for a much higher \([Ca^{2+}]\), for activation (~ 2 µM compared to ~500 mM) and unlike the native current, were time-independent (174). Additionally, while the native \(I_{Cl(Ca)}\) in murine portal vein myocytes was DTT-insensitive, the expression of \(mCLCA1\) in HEK293 cells was significantly inhibited by DTT (Figure 5C). The currents of transfected HEK293 cells did show some similarities to the native \(I_{Cl(Ca)}\) in that they were DIDS- and NFA-sensitive and maintained a similar anion permeability. Another study was then conducted in order to investigate the relative Ca\(^{2+}\)-insensitivity of the expressed mCLCA1 current (179). Investigators found that co-expression of mCLCA1 with
the β1 subunit of the BKCa channel resulted in a Ca\(^{2+}\)-activated Cl\(^{-}\) current with higher Ca\(^{2+}\) sensitivity compared to mCLCA alone, and in some recordings did display time-dependent kinetics. These results suggested that CLCA proteins could interact with accessory subunits, altering their biophysical characteristics. This was confirmed in the case of the BKCa β1 subunit, which was shown to interact with mCLCA1 in a mammalian two-hybrid system (179).

There remains a severe lack of structure-function data for CLCA proteins. It is still unclear due to contradictory data as to which fragment produced by proteolytical processing is responsible for channel activity, and there remains to be seen any site mutation analysis within any putative pore forming region (70). It has also been questioned whether CLCA proteins form Cl\(_{Ca}\) channels at all, with little unifying molecular basis for actions of family members. In addition to the evidence for a carrier of Cl\(^{-}\) conductance, specific isoforms have been implicated in completely different and sometimes opposite processes, such as having a role in tumor cell adhesion (160, 180), metastasis (LuECAM-1) (160, 180, 181, 182), and possessing significant tumor suppressing activity (155, 183). CLCA proteins mCLCA3/hCLCA1 have also been implicated as key element of the mucous production mechanism (163) (184). It is however entirely possible that these capabilities are a result of the ability to carry a Cl\(^{-}\) current. For example, it has been reported that blocking chloride conductance results in cell proliferation and provides protection from apoptotic death (173). As there is usually an influx of Ca\(^{2+}\) during early stages of apoptosis, both K\(^{+}\) and Cl\(^{-}\) channels are activated. With an increasing potassium conductance hyperpolarizing the cell, there would be an increase in driving force for anion conductance through the opened
Cl_{ca} channel, the end result being a significantly lowered intracellular pH, resulting in DNA destruction via activated caspase 3 (173). The hCLCA1, mCLCA3, and mCLCA4 isoforms do not appear to be channels at all, as they do not associate with the plasma membrane, but are secreted soluble proteins with globular domains, more likely to be involved in protein-protein interactions (175, 185, 186). In the case of mCLCA4, the 125 kDa gene product is cleaved to 90 and 40 kDa fragments with the N- and C-terminal fragments being secreted (186). Once outside the cell, both fragments appear to associate with the plasma membrane. hCLCA increased the amplitude of an endogenous I_{Cl(Ca)} in HEK293 cells (187). The protein did not modify the permeability sequence, nor did it appear to enhance Cl_{ca} channel trafficking or plasma membrane insertion, but rather appeared to elevate the single channel conductance of the endogenous HEK293 Cl_{ca} channels. The suggestion that there is an increase in single channel conductance stems from the observation that hCLCA1 decreased the whole cell conductance ratio G_{SCN^-}/G_{Cl^-}, which implies that ion permeability is not increased but rather ion conductance is increased (187). While not addressed in the study, perhaps these modifications are a result of an association between hCLCA1 and Cl_{ca} channels on the extracellular surface, similarly to mCLCA4 discussed above, which associates with the plasma membrane outside of the cell. Finally, native cells expressing I_{Cl(Ca)} have been identified that do not express any isoform of CLCA protein, questioning whether CLCA alone can be responsible for the endogenous current. It has been proposed that CLCA gene products either combine with other proteins to form a multimeric channel complex, or CLCA may modulate an endogenous Cl^- current (70).
1.9.3 The Bestrophin Family of Proteins

Bestrophins have been identified in mammals, birds, bony fish, amphibians, echinoderms, insects, nematodes, and flatworms, with homologs found in fungi, plants and bacteria (188). In 1999, mutations in the Bestrophin gene were found to be responsible for the development of Best vitelliform macular dystrophy (VMD), an autosomal dominant inherited disorder of the eye with juvenile onset (189). The disease results in the accumulation of lipofuscin-like material in the retinal epithelium, and is associated with a decrease in the slow light peak (190). Expression of many different bestrophin proteins in HEK293 cells, including those from human, mouse, *Xenopus*, *C. elegans* and *Drosophila*, result in the appearance of a Cl⁻ current (97, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200). Relatively little is known about the physiological roles of bestrophin proteins, and it is still not even agreed that Best VMD is caused by Cl⁻ channel dysfunction (201). Doubt exists as mBest1 knock-out mice display no ocular pathology, and normal Cl⁻ currents are found in their retinal pigment epithelial cells (RPE) (201). Most if not all of the hBest1 mutations linked with disease produce a dysfunction in the Cl⁻ channel function of expressed hBest1 (197, 202, 203, 204). The debate of whether bestrophin proteins carry the \( I_{\text{Cl(Ca)}} \) in native tissue will be discussed in further detail below.

Bestrophins are quite ubiquitously expressed, although paralogs appear to maintain similar expression across species. Best1 shows high expression in the retina and RPE cells, with significantly lower expression in brain, spinal cord, kidney, colon, trachea, testes and heart. Best2 has a limited distribution, with expression in the colon and testes. There is some evidence that Best2 may be found in olfactory epithelium (205) and the eye, where it
may play a role in determining intraocular pressure (188). Both Best3 and Best4 RNA are widely expressed, although few studies have demonstrated protein expression (188).

As no X-ray crystal structure has been resolved for any of the bestrophin proteins, our knowledge of protein structure comes mainly from theoretical assumptions and mutagenesis studies. Even prior to the identification of Bestrophins as Cl⁻ selective channels, computer simulations involving the causative gene of VMD predicted a membrane bound protein containing four putative transmembrane domains (206). This was corroborated by Milenkovic et al. (207), who suggested that of the six putative trans-membrane domains (TMDs), only TMD1, -2, -5, and -6 crossed the membrane. This model results in the creation of a large cytoplasmic loop. Another, contradictory model for hBest1 topology has also been proposed by Tsunenari et al. (199). In this model, TMD1, -2, -4 and -6 cross the membrane, while TMD5 may form a reentrant loop. While the Milenkovic model results in a cytoplasmic loop, the Tsunenari model predicts a large extracellular loop. Neither model has been confirmed, with both models possessing their own strengths (188). Bestrophins retain high homology between family members at the N-terminus, and as the putative TMDs reside in the N-terminus, the transmembrane topology is also likely conserved across the protein family.

In accord with computer simulations suggesting an integral membrane protein, the localization of bestrophins to the plasma membrane has been confirmed in several studies (190, 192, 195, 199). Expression of xBest2a in HEK293 cells results in two patterns of localization by immunocytochemistry (195). Some cells demonstrated xBest2a concentration at the cell surface, as demonstrated by co-localization with wheat germ
agglutinin, while other cells demonstrated protein localization to early endosomes (195). This dual distribution was also seen by Tsunenari et al. (199), but protein was found in significant quantities at both the plasma membrane as well as in cytosolic vesicles. Bestrophins have also been found to localize to the endoplasmic reticulum (208, 209).

As most known disease-associated mutations are of the missense type and clusters predicted near TMDs, 18 such mutations were investigated to determine what effect mutations may have on the TMDs (207). Three of the investigated mutations (I73N, Y85H, F281del) significantly affect integration of hBest1 into the membrane, presenting a potential disease mechanism (207). Mutagenesis studies have been successful in identifying several residues involved in a putative Bestrophin pore. Qu et al. (194) first mutated S79 of mBest2, resulting in an altered relative permeability and conductance to SCN. As S79 is located in TMD2, the same group investigated the role of other residues in TMD2 in anion permeability of mBest2. Several residues within TMD2, when mutated to a cysteine, decreased permeability of the channel to SCN relative to Cl⁻ three- to fourfold (residues at positions 78, 79, 80, 83, 84, 86, and 87). Additionally, side chains at positions 78 and 80 appear to be situated close to the permeant anion, as demonstrated by mutations at these residues altering the electrostatic charge, which in turn significantly altered permeation (194). Yet another study conducted by the same group confirmed that the mBest2 selectivity filter is formed by amino acids contained in TMD2 (210). By systematically replacing every amino acid in mBest2 between residue 69 and 104 with cysteine, it was discovered that residues forming the selectivity filter are spread out over approximately 20
amino acids (210). These results provide valuable information into how mutations may produce abnormal channels.

Bestrophin proteins may form either tetramers or pentamers when heterologously expressed (197). This was determined by tagging hBest1 with either a c-myc or Rim3F4 tag and co-expressing them in HEK293 cells. By transfecting with different ratios of c-myc- and Rim3F4-tagged hBest1 and employing immunoprecipitation, hBest1 was found to form either tetramers or pentamers. Similar experiments involving hBest1 and hBest2 demonstrated that these two bestrophin paralogs can form heteromers. Another study used hydrodynamic studies propose that pBest1 only forms a dimer, and that the tetrameric/pentameric stoichiometry is an artifact of overexpression (211). Utilizing a sucrose gradient and calculating the mass of the pBest1 complex (taking into account the detergent’s mass), pBest1 was estimated to have a mass of 138 kDa, almost exactly twice the mass of the monomer. However, it is possible that the conditions used in this study (detergent) may disrupt higher order protein structure. While the bestrophin stoichiometry remains unclear, the observation that bestrophin mutants can inhibit the Cl⁻ channel activity of wild-type subunits demonstrates that bestrophin proteins oligomerize.

Bestrophins became a viable molecular candidate for I_{Cl(Ca)} when it was discovered that expressed Bestrophin proteins appeared to form a Cl⁻ selective channel gated by Ca^{2+} at concentrations near 200 nM (97, 192, 193, 194, 195, 197, 198, 199) (Figure 5D & E). Murine and Xenopus Best2 expression in HEK293 cells resulted in Ca^{2+}-activated Cl⁻ currents with a K_d between 210 and 400 nM (97, 194, 195). Isolated from mouse heart, mBest3 had a K_d of ~ 175 nM Ca^{2+}. Using the excised patch configuration, Ca^{2+} appears to directly gate
Figure 5. Representative currents of the various Ca\textsuperscript{2+}-activated chloride channel molecular candidates. A) Current recorded from rabbit pulmonary artery myocyte dialyzed with 500 nM Ca\textsuperscript{2+}. B) Ionophore-elicited current in CHO cells transfected with tweety (hTTYH3). C) Families of currents evoked by pipette solutions containing either 500 nm or 2 mM Ca\textsuperscript{2+} in HEK293 cells stably transfected with mCLCA1. D) Currents recorded from TRex-293 cells transfected with mBest1 and expression induced with tetracycline. Currents were evoked using pipette solutions containing either 250 or 500 nM Ca\textsuperscript{2+}. E) Representative family of currents evoked by a pipette solution containing 500 nM Ca\textsuperscript{2+} from COS-7 cells transiently transfected with mBest3. F) Whole-cell membrane currents recorded in FRT cells stably-transfected with TMEM16A(abc) dialyzed with either 600, 255, or 17 nM Ca\textsuperscript{2+}. G) Whole-cell currents induced by mTMEM16B transfection in HEK 293T cells activated by 1.5 μM Ca\textsuperscript{2+}. Figure composed from Chapter 3 of this dissertation (A), Suzuki M. Exp Physiol. 2006 Jan;91(1):141-7. Epub 2005 Oct 11 (B), Britton FC, Ohya S, Horowitz B, and Greenwood IA. J Physiol. 2002 Feb 15;539(Pt 1):107-17 (C), O’Driscoll KE, Leblanc N, Hatton WJ, and Britton FC. Biochem Biophys Res Commun. 2009 Jul 10;384(4):476-81. Epub 2009 May 6 (D), O’Driscoll KE, Hatton WJ, Burkin HR, Leblanc N, and Britton FC. Am J Physiol Cell Physiol. 2008 Dec;295(6):C1610-24. Epub 2008 Oct 22 (E), Caputo A, Caci E, Ferrera L, Pedemonte N, Barsanti C, Sondo E, Pfeffer U, Ravazzolo R, Zegarra-Moran O, and Galietta L. Science. 2008 Oct 24;322(5901):590-4. Epub 2008 Sep 4 (F), and Pifferi S, Dibattista M, and Menini A. Pflugers Arch. 2009 Oct;458(6):1023-38. Epub 2009 May 28 (G).
bestrophin Cl\(^-\) channels (198, 212). In excised patches, hBest4 had a \(K_0\) of 230 nM, with negligible I\(_{Cl}\) in Ca\(^{2+}\)-free solution (198). Bestrophins have a conserved highly acidic region with a group of five acidic residues, which somewhat resembles the high-affinity ‘Ca\(^{2+}\)-bowl’ of BK\(_{Ca}\) channels. Single channel conductances between \(~0.26\) and 2 pS have been found (97, 212), and the expressed channels also display a similar anion permeability sequence to the native Cl\(_{Ca}\) channels, following the sequence I\(^-\) > Br\(^-\) > Cl\(^-\) > F\(^-\) (192, 193, 194, 195, 197). Expressed bestrophin Cl\(^-\) currents are NFA-, DIDS- and SITS-sensitive (97, 192, 193, 194, 195, 197). RNA\(_i\) against dBest1 and dBest2 but not dBest3 or dBest4 disrupted native I\(_{Cl(Ca)}\) in Drosophila S2 cells, while RNA\(_i\) against hBest1 produced similar effects in human airway epithelial cells (200, 213). Matchkov et al. (78) claim that Best3 is responsible for the cGMP-dependent Cl\(^-\) channel of rat mesenteric artery. This is supported in that the C-terminus of Best3 contains PKG phosphorylation sites (the current has a cGMP-dependence which is mediated via PKG activation), the Best3 expression and appearance of the cGMP-dependent Cl\(^-\) current overlap, and RNA\(_i\) results in the ablation of the current in freshly isolated and cultured myocytes. Additionally, the ‘classic’ I\(_{Cl(Ca)}\) is unaffected by Best3 RNAi. However, it should be noted that the native cGMP-dependent Cl\(^-\) channels carry a unitary conductance of 15 to 55 pS, the SCN\(^-\)/Cl\(^-\) permeability ratio for bestrophins and the cGMP-dependent current differ, and there is no data demonstrating the activation of bestrophin proteins by a rise in cGMP (214). Another study using similar methods has also claimed that Best1 is the Cl\(_{Ca}\) in hippocampal astrocytes (215).

Despite all the evidence suggesting that bestrophins are Cl\(_{Ca}\) channels, there is a similar amount of evidence refuting this claim, or at least that they could not make up the
‘classic’ $I_{\text{Cl}(\text{Ca})}$ of vascular smooth muscle. In general, bestrophins lack both time- and voltage-dependence. Two exceptions include hBest3 and C. elegans Best1 (197). Point mutations of hBest1 and mBest2 can confer voltage-dependence and rectification (188). It is also possible that a regulatory subunit may associate with bestrophins, altering time- and voltage-dependence. Bestrophins have also been shown to have other functions, such as modulating other channels such as the voltage-dependent L-type Ca$^{2+}$ channel (201, 216, 217). This modulation occurs though an association of the hBest1 C-terminus with the Ca$_{\alpha}$β subunit through a src-homology-binding domain (217). This domain and effect is specific to hBest1. Best2 has been shown to mediate bicarbonate transport in goblet cells of mouse colon (218). Bestrophins have also been seen to give rise to volume-sensitive currents (191, 219). HEK293 cells transfected with hBest1 exhibited volume-sensitive Cl$^-$ currents that were about 10-fold larger than untransfected cells (219). Perhaps most interestingly, bestrophin proteins have recently been associated with regulating intracellular Ca$^{2+}$ signaling (208, 209). Localized to the ER of HEK293 cells, hBest1 is seen to interact with stromal interacting molecule 1, an ER Ca$^{2+}$ sensor (208). Best1 KO mice displayed an expansion of ER cisterns and enhanced Ca$^{2+}$ deposits, suggesting Best1 is important in the Ca$^{2+}$ handling in the ER. Indeed, intracellular Ca$^{2+}$ transients elicited by P2Y$_2$ receptor activation in HEK293 cells were significantly augmented by hBest1. The protein kinase Pak2 phosphorylates hBest1 in this system, increasing calcium signaling resulting in the activation of TMEM16A, a new Cl$_{\text{Ca}}$ candidate discussed the next section (208). The authors propose that hBest1 functions as a counterion channel in the ER, balancing transient membrane potential changes occurring during IP$_3$-induced Ca$^{2+}$ release and store refilling. Another study involving mBest1 KO mice
found that the RPE cells of KO mice had higher resting Ca\(^{2+}\) levels than WT mice, and that ATP-induced Ca\(^{2+}\) release rose faster and decayed slower in the KOs (209). This group also found Best1 localized to the ER. This is interesting as suppressed Ca\(^{2+}\) signaling has been observed in a mouse model of Best VMD (59).

It appears the bestrophin story is quite convoluted and complex. While it appears that bestrophins may be good candidates for Cl\(_{\text{Ca}}\) channels in some cell types and tissues, it seems unlikely for others. In the case of VSMCs, there are no studies demonstrating the disruption of Bestrophins resulting in a reduction of native I\(_{\text{Cl(Ca)}}\), nor have there been any single channel studies involving Bestrophins performed in VSMCs. These issues question the viability of the Bestrophin gene family in being singularly responsible for encoding the Cl\(_{\text{Ca}}\) channel of vascular smooth muscle.

1.9.4 The TMEM16 Family

Prior to three independent groups publishing that TMEM16A may function as a Cl\(_{\text{Ca}}\) channel (220, 221, 222), the TMEM16 family had already received some significant attention. A missense mutation of *GDD1* at C356 was reported to be involved in the autosomal dominant disease gnathodiaphyseal dysplasia, characterized by bone fragility, sclerosis of tubular bone, and cemento-osseous lesions of the jaw bone (223). The GDD1 protein was localized to the ER, leading investigators to propose a possible role for GDD1 in calcium homeostasis. An independent group had previously identified a group of genes (FLJ10261, C12orf3, C11orf25 and FLJ34272) that would encode proteins sharing a structural homology of 8 trans-membrane domains (TMDs) with cytosolic NH\(_2\)- and COOH-termini
(224). After consulting with the Human Gene Nomenclature Committee, these closely related genes were named \textit{Tmem16a}, \textit{Tmem16b}, \textit{Tmem16c} and \textit{Tmem16d}. This group later reported four additional genes, \textit{Tmem16e}, \textit{Tmem16f}, \textit{Tmem16g}, and \textit{Tmem16h}, and pointed out that \textit{GDD1} was in fact \textit{Tmem16e} (225, 226, 227). TMEM16E was found to be expressed in the developing mouse embryo, predominantly in differentiating and developing somites where the protein was localized to the membranes of intracellular vesicles (228). Immunohistochemical analysis revealed TMEM16E expression in cardiac and skeletal muscle tissues, as well as in growth-plate chondrocytes and osteoblasts in bone. mRNA analysis revealed complex alternative splicing involving five exons of the \textit{Tmem16e} gene (229). This alternative splicing resulted in lacking conserved amino acids at the N-terminus, as well as changes in transmembrane topology. This is particularly interesting, as several splice variants of the Ca$^{2+}$-activated Cl$^{-}$ current-carrying TMEM16A have been found to modify channel characteristics (discussed later in this section).

A microarray analysis of the zone of polarizing activity (a signaling center required for normal antero-posterior patterning of vertebrate limbs) in developing mice revealed expression of \textit{Tmem16a} (230). Further study by the same group of investigators revealed that TMEM16A is required for normal development of the mouse trachea (231). Mice homozygous for a null allele of \textit{Tmem16a} displayed severe pathology of the trachea, with gaps in the tracheal cartilage rings along the entire length of the trachea. As \textit{Tmem16a} was not found to be expressed at any time in the chondrogenic mesenchym, investigators postulate that the resulting tracheomalacia is secondary to expansion of the embryonic trachea (231). Human \textit{TMEM16A} is found on chromosome 11q13, a region often amplified
in esophageal cancer, bladder tumor, breast cancer (224), and esophageal- (232), oral- (233), and head and neck squamous cell carcinomas (234). TMEM16 family members may be involved in cell proliferation and tumor progression of several cancers, although it does not seem as if mutations of TMEM16A are linked to carcinogenesis (235). *Tmem16a* (also known as *DOG1*) has also been found to be a specific marker for gastrointestinal stromal tumors (236, 237, 238).

### 1.9.5 TMEM16A is a Calcium-Activated Chloride Channel

In 2008, three independent groups reported that TMEM16A, or Anoctamin1 (*Ano1*; this term will not be used for this dissertation) was in fact a Ca^{2+}-activated Cl^{-} channel (220, 221, 222). Fascinatingly, the groups used three distinct approaches in identifying TMEM16A during their studies. Based on the observation that Interleukin-4 (IL-4) increased I_{Cl(Ca)} of murine airway epithelial cells, Caputo et al. (220) employed a microarray analysis approach to identify up-regulated genes of integral membrane proteins. The global gene expression analysis revealed a 7-fold increase of *Tmem16a*. siRNA designed against the murine *Tmem16a* significantly decreased I_{Cl(Ca)} as measured by halide-sensitive fluorescent proteins in monolayers of CFPAC-1 and CFBE41o- cells, both of which express large I_{Cl(Ca)}. Similar reduction in anion flux was noted with purinergic receptor stimulation, as well as with ionomycin. COS-7 and HEK293 cells were transiently transfected with TMEM16A, resulting in the appearance of Ca^{2+}-activated Cl^{-} currents. Currents evoked in stably-transfected FRT cells were inhibited by NFA and NPPB (Figure 5F).
In a search of public domain databases for putative channel genes with more than two TMDs as well as multiple isoforms, TMEM16A was particularly interesting to Yang et al. (222). Their interest was piqued by the protein’s multiple putative membrane-spanning domains and the fact that ten homologs are found in human. *Tmem16a* cDNA was obtained from mouse eye and was coexpressed along with endothelin receptor subtype A (ET-A) in HEK293 cells. Activation of ET-A receptors by 50 nM edothelin evoked robust inward currents in cells held at -60 mV. Replacing extracellular Cl⁻ with gluconate abolished the current. The current was also completely blocked by 10 µM DIDS, NPPB, tamoxifen, and NFA. At -60 mV, the half-maximal Ca²⁺ concentration for activation (EC₅₀) was 2.6 µM, while at +60 the EC₅₀ was significantly lower at 0.4 µM. This shift in apparent binding sensitivity is similar to that seen in PASMCs (106). As the acinar cells of mouse salivary glands have been demonstrated to possess I_{Cl(Ca)} which play a key role in saliva secretion, Yang et al. (2008) (222) utilized siRNA against *Tmem16a* to see whether they could eliminate the current. Four days following siRNA administration, saliva secretion was reduced by ~35%. Isolated submandibular glands from siRNA-injected mice demonstrated a significantly reduced current compared to control and scrambled siRNA-treated mice (222).

The first study to report that TMEM16A carries a Ca²⁺-activated Cl⁻ current (by about a month) employed an expression cloning approach in an attempt to identify a novel Cl_{Ca} channel subunit (221). For this technique, the commonly used *Xenopus* oocyte is not suitable, as it has a large endogenous I_{Cl(Ca)} which functions to block polyspermy. The group found a suitable expression system in the polyspermic *Axolotl* oocyte. A cDNA library prepared from size-fractionated RNA isolated from *Xenopus* oocytes was screened.
xTMEM16A was found to induce $I_{\text{Cl(Ca)}}$. The resulting currents were similar to the native $I_{\text{Cl(Ca)}}$ of *Xenopus* oocytes, displaying outward rectification with lower $[\text{Ca}^{2+}]_i$ and a linear I-V relationship with elevated $[\text{Ca}^{2+}]_i$. The expressed current displayed a similar anion selectivity to *Xenopus* oocytes of $\text{SCN}^- > \text{I}^- > \text{Br}^- > \text{Cl}^-$, as well as a similar pharmacological profile. Functional expression of murine Tmem16a in HEK293 cells resulted in an outwardly rectifying $\text{Cl}_{\text{Ca}}$ current, evoked by 500 nM $[\text{Ca}^{2+}]_i$.

**1.9.6 The TMEM16 Family and Expression**

Mammals, insects, worms, plants, protozoa and yeast all appear to express some type of TMEM16 homolog (239). Mammals have ten gene members, while invertebrates and plants have fewer. This makes TMEM16 the second largest $\text{Cl}^-$ channel family behind the GABA/glycine receptor family (239). TMEM16A is expressed in many of the tissues that express $I_{\text{Cl(Ca)}}$, with expression demonstrated in bronchiolar epithelial cells, pancreatic acinar cells, proximal kidney tubule epithelium, retina, dorsal root ganglion sensory neurons, and the submandibular gland (220, 221, 222). In the submandibular gland, TMEM16A may be found on the apical membrane (222), and localizes to the membrane when expressed in HEK293 cells (221, 222). Interestingly, confocal images of non-transfected HEK293 cells, which do demonstrate some endogenous TMEM16 expressions, do not demonstrate any co-localization of TMEM16A with membrane-localized N-cadherin (240). With the development of specific antibodies, TMEM16A expression was localized to apical membranes of epithelial cells in exocrine glands and trachea, airway smooth muscle, smooth muscle of the reproductive tract, and interstitial cells of Cajal (ICCs) in the
gastrointestinal tract (241, 242). Several transcript variants were reported in ICCs, with all but the $Tmem16g$ paralog being expressed (242). TMEM16A has actually been shown to be a highly specific marker for ICCs, with virtually no cross-labeling of mast cells, unlike the common Kit labeling (237, 242). The Cl⁻ channel blockers NFA and DIDS decreased the frequency and blocked slow wave generation in murine, primate and human small intestine in a concentration-dependent manner (242). Slow waves were absent in the $Tmem16a$ KO mouse. While earlier reports involving cultured ICCs failed to observe Ca²⁺-activated Cl⁻ currents (243), a recent report has linked a Cl$_{Ca}$ conductance in ICCs to slow wave generation and pacemaker activity in the gut (244). It therefore appears that TMEM16A plays a key role in the slow wave activity of gastrointestinal muscle.

1.9.7 TMEM16 Protein Structure

While it has yet to demonstrate the ability to conduct Cl⁻, the topology TMEM16G has been well examined. Termed a ‘prostate-specific gene’, TMEM16G was localized to the apical and lateral surfaces of normal and cancerous prostate cells. The protein appeared at the plasma membrane and concentrated at cell-cell contacts. RNAi application decreased TMEM16G levels, preventing the formation of cell aggregates (245). Hydropathy analysis revealed 8 putative TMDs. This was supported by incorporating epitope tags in all putative extracellular and intracellular regions (246). The accessibility of the epitope tags to antibodies in both intact and permeabilized cells was examined, thus determining TMD orientation. These studies reveal an 8 TMD protein with cytoplasmic NH$_2$- and COOH-termini (Figure 6). Additionally, it appears as though there is a re-entrant loop between
**Figure 6.** Cartoon representing the proposed topology of murine TMEM16A, comprising eight transmembrane domains (TMD). Figure highlights the location of the four alternatively spliced segments (a, b, c, and d), as well as the putative pore position. Denoted is one N-linked glycosylation site, as well as five consensus sites for phosphorylation by CaMKII: T216, S276, S492, T514, and T610. Sites indicated by a red arrow are conserved in human, mouse and rat. *Figure graciously provided by Normand Leblanc.*
TMD5 and TMD6, a common feature of ion channel pores (246). As the predicted TMDs are highly conserved across the TMEM16 family, it is reasonable to assume that they share a basic topology. Mutational analysis of TMEM16A revealed that the reentrant loop mentioned above is important in ion selectivity (222). The Arg669Glu mutation results in a large increase in channel cation permeability, where the $P_{\text{Na}}/P_{\text{Cl}}$ increases from 0.03 to 0.83, while the Lys716Glu mutation yields to a smaller shift in ion selectivity and the Lys693Glu mutation is not functional (222). These data suggest that positively charged amino acids residing in the reentrant loop form at least part of the ion conduction pathway. More evidence that the reentrant group forms part of the conducting pore from MTSET (a membrane impermeant sulfhydryl-reactive reagent), where mutation of Cys673, Cys678 and Cys683 to alanines abolished the inhibition of the channel by MTSET suggesting that these amino acids are accessible to the extracellular space and participate in ion conduction (222). While a few mutations in TMD1 and TMD7 (Lys349Ala, Lys631ala, and Thr830Ala) had no effect, Lys636Ala (TMD5) resulted in reduced currents with slow deactivation kinetics and Gln757Ala (TMD6) altered the voltage-dependence, abolished the time-dependence of the channel, and decreased Cl$^-$ selectivity (220). It is concluded from these experiments that the reentrant loop along with the TMDs that flank them may form the channel pore. It is worth noting that TMEM16H lacks a reentrant loop but contains two 60 amino acid insertions, one of which contains 20 consecutive acidic residues (239). Family members TMEM16J and TMEM16K are poorly conserved in the reentrant loop region. Other than these few members, there is considerable homology around the putative pore-forming region
amongst TMEM16 family members. They also possess several consensus sites for phosphorylation by different kinases, but lack an obvious Ca$^{2+}$ binding domain.

1.9.8 TMEM16 Single Channel Conductance

Ca$^{2+}$-activated Cl$^{-}$ channels may be divided into three groups based on their single channel conductance. Low conductance channels ranging between 1 and 3 pS have been identified in A6 kidney cells (247), endocrine cells from the pituitary gland (248), *Xenopus* oocytes (249), the *Drosophila* S2 cell line (212), cardiac myocytes (250), and arterial smooth muscle (88, 91, 142, 251, 252). Interestingly, rabbit PASMCs have demonstrated three distinct Cl$_{Ca}$ conductances of 1.2, 1.8, and 3.5 pS which were Ca$^{2+}$-dependent, with high [Ca$^{2+}$], (>1 µM) favoring the two lower conductance states, while Cl$_{Ca}$ activation by low [Ca$^{2+}$], (50 nM) showed conductances of 3.5 and 1.8 pS (251). TMEM16B, which has been shown to carry a Ca$^{2+}$-activated Cl$^{-}$ current, falls into this low-conductance group with reported single channel conductances of 1.2 (253) and 0.8 pS (254). Intermediate conductance Cl$_{Ca}$ channels with unitary conductances of ~8 to 15 pS have been identified in endothelial cells (255), hepatocytes (72), as well as several smooth muscle types (75, 77). This intermediate conductance falls in line with TMEM16A, which has a reported single channels conductance of ~8 pS (222). High-conductance Ca$^{2+}$-activated chloride channels have been reported in Jurkat T-cells (256), *Xenopus* spinal neurons (81), and airway epithelial cells (257) with single channel conductances in the range of ~50 pS. As of yet, no TMEM16 protein has demonstrated a similar conductance.
**1.9.9 TMEM16 Calcium- and Voltage-Dependence**

Evoking $I_{\text{Cl}(\text{Ca})}$ in HEK293 cells co-expressing TMEM16A and $\text{ET}_A$ receptor with 50 nM endothelin resulted in an outwardly rectifying current which became linear over time (222). As $[\text{Ca}^{2+}]_i$ in this case would also increase with time, this is consistent with reports for endogenous Cl$_{\text{Ca}}$ channels, where high $[\text{Ca}^{2+}]_i$ results in linear I-V relationships while lower Ca$^{2+}$ concentrations display an outwardly rectifying current (73, 106). Outwardly rectifying currents maintained time-dependent activation and deactivation kinetics while those displaying a linear I-V relationship were time-independent (221). This is again due to high Ca$^{2+}$ concentrations, which will push the channel towards maximal stimulation. Interestingly *Xenopus* TMEM16A expressed in Axolotl oocytes produced currents with multiple components and curiously with different reversal potential (221). This suggests several open states that differ not only in gating kinetics, but also in ion selectivity. Perhaps these different open states have different conductance states, as demonstrated in rabbit PASMCs and discussed above (251). As TMEM16 proteins don’t appear to have any EF hand-like Ca$^{2+}$ binding sites or IQ-domain calmodulin binding sites, it may be that additional subunits associate with TMEM16 proteins in order to confer Ca$^{2+}$ sensitivity. Such a scenario has been suggested for mCLCA1, where the BK$_{\text{Ca}}$ β1 subunit was shown to interact with the protein, significantly increasing its Ca$^{2+}$ sensitivity (179). As expression of TMEM16A in HEK293 cells has resulted in the appearance of a Cl$^-$ current sensitive to Ca$^{2+}$, if a regulatory subunit exists that confers Ca$^{2+}$ sensitivity to TMEM16A, it must be endogenous to these cells. As both NH$_2$- and COOH-termini have several regions rich in acidic amino acids that could bind Ca$^{2+}$, another possibility is that TMEM16 proteins possess a novel Ca$^{2+}$ binding site.
1.9.10 Regulation by Alternative Splicing

Caputo et al. (2008) (220) reported that \textit{Tmem16a} was alternatively spliced, with transcripts likely generated by selection of alternatively spliced exons. Alternative protein segments ‘a’, ‘b’, ‘c’, and ‘d’ can combine to make various TMEM16A isoforms, all of which were predicted to maintain the basic 8 TMD structure (Figure 3). The alternative segments range in size, with ‘a’ containing 116 residues, ‘b’ 22, ‘c’ only 4 and ‘d’ 26 residues (258). The ‘a’ and ‘b’ sequences reside in the NH\textsubscript{2}-terminus while c and d are found within the first intracellular loop. A detailed study was performed in order to determine the biophysical profiles of the various splice variant and to assess whether the alternative protein segments were responsible for specific channel functions (258). As their previous study revealed that the ‘d’ sequence did not appear to affect TMEM16A activity in any specific way (220), Ferrera et al. (258) studied transcripts ‘abc’, ‘ab’, ‘ac’ and (0), where TMEM16A(0) has none of the alternative protein segments. The alternative segments ‘b’, ‘c’, and ‘d’ were found to correspond to exons 6b, 13, and 15 of \textit{Tmem16a}, respectively (258). Skipping of segment ‘a’ is the result of the usage of an alternate promoter, and appears to be very strictly regulated as attempts to amplify this transcript in different human RNA tissues and cell lines were unsuccessful. TMEM16A(abc) demonstrated strong outward rectification, and was essentially identical to previous studies (220, 258). TMEM16A(ab) resulted in a current demonstrating decreased voltage-dependance, and at high depolarizing potentials (+80 and +100 mV) displayed little time-dependence (258). This decrease in voltage-dependence resulted in an increase of instantaneous current upon depolarization from the holding
potential. It appears that segment ‘c’ affects the voltage-dependence of the channel. Being only four amino acids long with a sequence of EAVK, investigators removed either the first two residues (ΔEA) or the last two residues (ΔVK) to examine their effects. TMEM16A(abΔEA) currents were very similar to those of TMEM16A(abc), suggesting that the last two residues of segment ‘c’ (VK) are responsible for much of the voltage-dependence of the channel. Excluding exon 6b resulted in an increase in channel Ca\(^{2+}\) sensitivity. At nearly all voltages, the K\(_D\) for Ca\(^{2+}\) was nearly 4-fold higher for TMEM16A(abc) than TMEM16A(ac), clearly demonstrating a role for segment ‘b’ in Ca\(^{2+}\) sensitivity. Additionally, TMEM16A(ac) carried a larger absolute current than TMEM16A(abc), and demonstrated decreased rectification. This is in agreement with the idea that high Ca\(^{2+}\), or in this case higher Ca\(^{2+}\) sensitivity, will shift the activation by voltage towards more negative potentials (106). While the number of transcripts including exon 6b varies significantly between tissues, the only Tmem16a mRNAs lacking exon 13 were found in brain and skeletal muscle (258). This suggests that while some degree of voltage dependence of Cl\(_{Ca}\) is maintained across tissues (although other channels, post translational modification and regulatory subunits may alter voltage-dependence), their Ca\(^{2+}\) sensitivities may vary significantly. This appears to be the case with native Cl\(_{Ca}\) channels. Alternative splicing of pre-mRNA is important in expanding the proteome diversity, as well as being a mechanism to regulate tissue-specific expression and function. Alternative splicing, as well as the possibility of hetero-oligomerization, may help explain the varying functional characteristics of endogenous Cl\(_{Ca}\) channels.
1.9.11 TMEM16A: Also a Volume-Sensitive Channel?

Similarly to hBest1, TMEM16A has also been suggested to be responsible for a swelling- or volume-activated Cl⁻ current ($I_{Cl(swell)}$) (259). Three cell lines possessing $I_{Cl(swell)}$ activated by hypotonic bath solutions were examined, all of which expressed multiple TMEM16A paralogs. CFPAC, a human pancreatic cell line lacking CFTR and expressing TMEM16A, -F and –H, the human colonic epithelial cell line HT29 expressing TMEM16A, -F, -H, and –J, and HEK293 cells expressing TMEM16A, -F, and –H were used in the study. A RNAi approach revealed that siRNA against Tmem16a decreased $I_{Cl(swell)}$ in each cell line. siRNA against CIC3, CLCA1, or CLCA4 – all of which are endogenously expressed in CFPAC cells – had no effect on $I_{Cl(swell)}$ in these cells. It was not reported whether CFPAC cells expressed any bestrophin proteins, or if bestrophin-targeted siRNAs decreased $I_{Cl(swell)}$. Overexpression of both TMEM16A and –B in HEK293 cells resulted in a significant increase of $I_{Cl(swell)}$, suggesting that several TMEM16 proteins may contribute. This idea is supported by the observation that siRNA targeting endogenous Tmem16a –f and –h all decreased $I_{Cl(swell)}$ in a non-additive manner. As evidenced from experiments carried out in the absence of Ca²⁺ where hypotonic solutions failed to evoke $I_{Cl(swell)}$, Ca²⁺ is required for current activation, although it likely only has a permissive function (259). Physiologically, siRNA targeting endogenous Tmem16a reduced apoptotic volume decrease in HEK293 cells where apoptosis was triggered by staurosporin. This implicates TMEM16A in apoptosis, a process in which Cl⁻ has long been thought to have a role. Cells isolated from various tissues of Tmem16a KO mice also demonstrated either a loss or severe reduction of $I_{Cl(swell)}$ (259).
1.9.12 TMEM16B is Also a Calcium-Activated Chloride Channel

As briefly mentioned above, TMEM16B has recently been shown to carry a Ca\(^{2+}\)-activated Cl\(^{-}\) conductance (253, 254). Expressing murine TMEM16B in HEK293 cells resulted in I\(_{\text{Cl(Ca)}}\) in the whole-cell configuration, as well as inside-out excised patches (253) (Figure 5G). Whole-cell I\(_{\text{Cl(Ca)}}\) could be elicited by having a known [Ca\(^{2+}\)] in the pipette solution, by activation of G-protein coupled receptors, and by photolysis of caged Ca\(^{2+}\). TMEM16B of inside-out excised patches was quickly activated by applying bath solutions of various [Ca\(^{2+}\)], suggesting direct gating by Ca\(^{2+}\). However in the excised patches, an irreversible rundown was observed that was absent in the whole-cell recordings. With the notion that patch excision separated the channel from a modulator/accessory protein, investigators applied ATP, DTT, CaM, PIP\(_{3}\), and cAMP to the bath solution, none of which had any effect. Therefore, there is some unknown factor that is lost upon patch excision. Exposure to 1.5 µM Ca\(^{2+}\) resulted in outwardly rectifying and time-dependent currents, whereas 13 µM Ca\(^{2+}\) evoked time-independent current with a linear I-V relationship. A single channel conductance of 1.2 pS was reported, and the channel had an ion selectivity sequence of SCN\(^{-}\) > I\(^{-}\) > NO\(_{3}\)^{-} > Br\(^{-}\) > Cl\(^{-}\) > MeS. Currents were inhibited by NFA, DIDS, SITS, and NPPB. NFA (300 µM) also blocked I\(_{\text{Cl(Ca)}}\) when applied to the intracellular surface of inside-out excised patches (253). This is contrary to another study where intracellular application of NFA in the whole-cell configuration via inclusion in the pipette solution had no effect on I\(_{\text{Cl(Ca)}}\) (135). A second study involving a proteomic screen for cilial membrane proteins of mouse olfactory sensory neurons (OSN) identified TMEM16B as the Ca\(^{2+}\)-activated Cl\(^{-}\) channel in these tissues (254). The protein was found to be expressed specifically in OSNs of olfactory
epithelium by means of a TMEM16B::EGFP fusion protein expressed in vivo via an adenoviral vector. Expression in HEK293 cells gave a similar current to the native $I_{Cl(Ca)}$. Currents of inside-out excised patches again displayed rundown, but had a slightly smaller single channel conductance of 0.8 pS (254). TMEM16B expression has also been found to be specifically expressed in the olfactory epithelium compared to the more broadly expressed TMEM16A (260). Finally, TMEM16B has been shown to associate with a presynaptic protein complex in mouse photoreceptor terminals, where $I_{Cl(Ca)}$ is believed to regulate synaptic output by local feedback mechanisms (261).

1.9.13 TMEM16 Proteins’ Physiological Role

In the brief time since TMEM16A and TMEM16B have been demonstrated to carry a Cl$^-\$ current, roles for TMEM16 proteins in salivary secretion (222, 262, 263, 264), pacemaker cells of the GI and GI slow wave generation (242, 244), regulatory volume decrease (259, 265), apoptotic volume decrease (259), pain perception (266), and possibly olfactory amplification (254, 260) have been shown. In addition to Yang et al. (222) demonstrating that $Tmem16a$-siRNA reduced saliva secretion follow TMEM16A-siRNA administration and reduced $I_{Cl(Ca)}$ recorded from isolated submandibular glands from siRNA-injected mice, several groups have confirmed a role for TMEM16A in airway fluid secretion. Cl$^-$ secretion in native epithelia normally activated by Ca$^{2+}$-dependent agonists is significantly reduced in $Tmem16a$ KO mice (262, 263). $Tmem16f$ and $Tmem16k$ were still detectable in the TMEM16A KO and could possibly explain the small remaining Ca$^{2+}$-activated Cl$^-$ current, however this reduced current was inadequate for normal airway hydration as seen by the
significant neonatal luminal mucous accumulation (263). Reductions in Cl⁻ secretion were observed in several tissues, including tracheal and colonic epithelium, and hepatocytes and acinar cells of pancreatic and submandibular glands (262). Adding to the suggestion that TMEM16 proteins may also form a volume-sensitive channel when expressed in HEK293 cells, a recent study has demonstrated that erythrocytes isolated from Tmema16a KO mice display significantly impaired crenation and increased lysis when compared to cells from wild-type littermates (265). Another recent report has proposed a mechanism underlying spontaneous pain induced by bradykinin that involves Cl⁰ Ca channel stimulation (266). Through bradykinin-2 receptors and PLC, bradykinin causes release of Ca²⁺ from intracellular stores in small nociceptive neurons from rat dorsal root ganglia (DRG). The increase in cytoplasmic Ca²⁺ inhibits M-type K⁺ channels while opening Cl⁰ Ca channels. These effects result in the depolarization and increase action potential firing induced by bradykinin in DRG neurons. This effect by bradykinin was significantly reduced by local injection of DIDS (50 nmol/injection site) or NPPB (10 nmol/injection site). Using Tmem16a-siRNA, investigators saw significantly reduced inward currents compared to those of small DRG neurons transfected with scrambled oligos, suggesting a role for TMEM16A in inflammatory nociception (266).

Given the data discussed in the above section, it appears as though the TMEM16 family of proteins is a good candidate for the native ICl(Ca) of various tissues, as varying functional properties may be explained by alternative splicing as well as the potential for the formation of hetero-oligomers. The fourth chapter of this dissertation will explore the
possibility that TMEM16A makes up at least part of the $I_{\text{Cl(Ca)}}$ channel of rabbit PASMCs, as well as further exploring the pharmacology of the protein when expressed in HEK293 cells.

1.10 Aims of This Study

Since first described in the early 1980’s, calcium-activated chloride channels have been studied in many different cell types, and have been implicated in many basic physiological functions. These studies have been carried out despite the lack of specific pharmacological tools. Many investigators studying $I_{\text{Cl(Ca)}}$ and the current’s physiological role employ admittedly non-specific $I_{\text{Cl(Ca)}}$ channel blockers (ex: niflumic acid) in order to demonstrate a role for this current in their chosen system. However, as evidenced by the dual effects of both niflumic acid and anthracene-9-carboxylic acid, the effects of $I_{\text{Cl(Ca)}}$ channel blockers on even their ‘main target’ aren’t so cut-and-dry. Since 1997, several studies have demonstrated a role for phosphatase and kinase activity in regulating $I_{\text{Cl(Ca)}}$ channels of vascular smooth muscle cells. It has been proposed that phosphorylation results in a state-dependent block of the channel. The goal of this study was to determine whether the state of phosphorylation of either the pore-forming subunit or an associating regulatory protein of $I_{\text{Cl(Ca)}}$ channels altered the effects of the chemically unrelated $I_{\text{Cl(Ca)}}$ channel ‘blockers’ niflumic acid and anthracene-9-carboxylic acid on $I_{\text{Cl(Ca)}}$ in arterial smooth muscle myocytes. Additionally, given the recent emergence of the TMEM16 family as molecular candidates for the native ‘classic’ $I_{\text{Cl(Ca)}}$, this study explores the pharmacology of expressed TMEM16A. While several studies have made the simple observation that $\text{Cl}^-$ channel blockers inhibit $\text{Ca}^{2+}$-activated $\text{Cl}^-$ currents carried by TMEM16A, this study looks at the
pharmacology of this newly proposed channel in greater detail. Such pharmacological studies may shed light on channel properties such as gating and regulation, as well as provide clues into channel structure.
Chapter 2: Phosphorylation Alters the Effects of Niflumic Acid on Ca\textsuperscript{2+}-Activated Cl- Channels in Rabbit Pulmonary Arterial Smooth Muscle Cells


### 2.1 Summary

**Background and purpose:** Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} currents (I_{Cl(Ca)}) in arterial smooth muscle cells are inhibited by phosphorylation. The Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channel (Cl\textsubscript{Ca}) blocker niflumic acid (NFA) produces a paradoxical dual effect on I_{Cl(Ca)}, causing stimulation or inhibition at potentials below or above 0 mV, respectively. We tested whether the effects of NFA on I_{Cl(Ca)} are modulated by phosphorylation.

**Experimental approach:** I_{Cl(Ca)} was elicited with 500 nM free internal Ca\textsuperscript{2+} in rabbit pulmonary artery myocytes. The state of global phosphorylation was altered by cell dialysis with either 5 mM ATP, or 0 ATP with or without the CaMKII inhibitor KN-93 (10 \(\mu\)M).

**Key results:** Dephosphorylation enhanced the ability of 100 \(\mu\)M NFA to inhibit I_{Cl(Ca)}. This effect was attributed to a large negative shift in the voltage-dependence of block, which was converted to stimulation at potentials < -50 mV, ∼70 mV more negative than cells dialyzed with 5 mM ATP. NFA dose-dependently blocked I_{Cl(Ca)} in the range of 100 nM to 250 \(\mu\)M in cells dialyzed with 0 ATP and KN-93, which contrasted with the significant stimulation induced by 100 nM, which converted to block at concentrations > 1 \(\mu\)M when cells were dialyzed with 5 mM ATP.

**Conclusions and implications:** Our data indicate that the presumed state of phosphorylation of the pore-forming or regulatory subunit of Cl\textsubscript{Ca} channels influences the interaction of NFA in a manner that obstructs an interaction of the drug with an inhibitory binding site.
2.2 Introduction

The membrane depolarization associated with the opening of Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels (Cl\textsubscript{Ca}) is thought to be an important contributor to the development of vascular smooth muscle tone induced by constricting hormones and neurotransmitters (120, 147). Among several classes of agents, the non-steroidal anti-inflammatory agent niflumic acid (NFA) has been extensively used as a “relatively” selective blocker of Cl\textsubscript{Ca} channels in vascular smooth muscle cells (VSMCs) (147). At concentrations in the range of ~ 1-50 μM, NFA blocks Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents (I\textsubscript{Cl(Ca)}) evoked by L-type Ca\textsuperscript{2+} current (139), reverse-mode Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (140), constricting agonists or caffeine (141), spontaneous transient inward Cl\textsuperscript{−} currents (STICs) triggered by spontaneous release of Ca\textsuperscript{2+} from the SR (113, 128, 141, 267), or flash photolysis of caged Ca\textsuperscript{2+} (92). Within the same concentration range, NFA has no effect on the magnitude of Ca\textsuperscript{2+} current (91, 139, 144), swelling-activated Cl\textsuperscript{−} current (127), KCl-induced contractions (139, 143, 144, 145, 146), or SR Ca\textsuperscript{2+} release evoked by caffeine or norepinephrine (141).

The effects of NFA on VSMC Cl\textsubscript{Ca} channels are complex, with widely ranging IC\textsubscript{50} values required for block. A close examination of the studies that have investigated the effects of NFA on I\textsubscript{Cl(Ca)} suggests that its potency appears to be linked to the method used to evoke the current. Indeed, the IC\textsubscript{50} for inhibiting STICs was < 4 μM whereas the affinity of NFA for blocking I\textsubscript{Cl(Ca)} evoked by an agonist or caffeine was at least two-fold lower (141). Although dose-response relationships were not obtained, NFA appeared less efficacious at inhibiting arterial I\textsubscript{Cl(Ca)} elicited by inward Ca\textsuperscript{2+} current than STICs with 10 μM NFA producing ~ 50-80% of the deactivating Cl\textsuperscript{−} tail current recorded after return to the holding potential (139, 142).
A further reduction in potency was evident from the NFA-induced block of \( I_{\text{Cl(Ca)}} \) evoked by elevated sustained intracellular \( \text{Ca}^{2+} \) concentration (500 nM) in coronary myocytes with an \( IC_{50} \) at +50 mV of 159 \( \mu \text{M} \) (110). One possibility to explain these observations may relate to the ability of higher [\( \text{Ca}^{2+} \)]\(_i\), either induced cyclically or maintained, to promote down-regulation of \( I_{\text{Cl(Ca)}} \) by phosphorylation via CaMKII (106, 107, 108, 109, 123). This study was undertaken to test the hypothesis that the presumed state of phosphorylation of \( \text{Cl}_{\text{Ca}} \) channel influences their pharmacology. To this end, we tested the effects of NFA on \( I_{\text{Cl(Ca)}} \) evoked by 500 nM internal \( \text{Ca}^{2+} \) in rabbit pulmonary artery myocytes dialyzed with either 5 mM ATP to induce global phosphorylation, or in the absence of ATP with or without the CaMKII inhibitor KN-93, to promote dephosphorylation of the channel or a regulatory protein (106). At positive potentials, the block displayed steep voltage-dependence that was markedly attenuated in conditions favoring global dephosphorylation. On the other hand, \( \text{Cl}_{\text{Ca}} \) channels suppressed by conditions promoting global phosphorylation exhibited enhanced stimulation by NFA at negative potentials. These results demonstrate that the presumed state phosphorylation of the pore-forming or regulatory subunit of \( \text{Cl}_{\text{Ca}} \) channels has a profound influence on the interaction of NFA with these channels, which may explain the wide range of sensitivities of these channels to NFA reported in many studies.

### 2.3 Materials and Methods

#### 2.3.1 Isolation of Pulmonary Artery Myocytes

A similar method to that previously used by our group was used to isolate smooth muscle cells (106, 108). In brief, cells were prepared from the main and secondary
pulmonary arterial branches dissected from New Zealand white rabbits (2-3 kg) killed by anesthetic overdose in accordance with British and American guidelines for animal care. The animals were allowed free access to food and water and kept on a 12-12 h light/dark cycle until the day of being killed. The University of Nevada Institutional Animal Care and Use Committee approved the experimental protocol. After dissection and removal of connective tissue, the pulmonary arteries were cut into small strips and incubated overnight (~ 16 hours) at 4°C in a low Ca²⁺ physiological salt solution (PSS; see composition below) containing either 10 or 50 μM CaCl₂ and ~ 1 mg ml⁻¹ papain, 0.15 mg ml⁻¹ dithiothreitol and 1 mg ml⁻¹ bovine serum albumin. The next morning, the tissue strips were rinsed three times in low Ca²⁺ PSS and incubated in the same solution for 5 min at 37°C. Cells were released by gentle agitation with a wide bore Pasteur pipette, and then stored at 4°C until used (within 10 h following dispersion).

2.3.2 Whole-Cell Patch Clamp Electrophysiology

Ca²⁺-activated Cl⁻ currents were elicited using the conventional whole-cell configuration of the patch clamp technique with a pipette solution containing either no ATP with KN-93, a specific inhibitor of CaMKII, or 5 mM ATP. The pipette solution also contained 10 mM BAPTA as the Ca²⁺ buffer and free [Ca²⁺] was set to 500 nM by the addition of 7.08 mM CaCl₂. The free [Ca²⁺] was estimated by the calcium chelator program WinMaxC (v. 2.50; http://www.stanford.edu/~cpatton/downloads.htm). Using a Ca²⁺-sensitive electrode and calibrated solutions, the total amounts of CaCl₂ and MgCl₂ calculated by the software were previously shown to yield accurate free Ca²⁺ concentrations with both EGTA and BAPTA as
2.3.4 Experimental Protocols

$I_{\text{Cl(Ca)}}$ was evoked immediately upon rupture of the cell membrane and the voltage-dependent properties were monitored every 5 or 10 s by stepping from a holding potential (HP) of $-50 \text{ mV}$ to $+90 \text{ mV}$ for 1 s, followed by repolarization to $-80 \text{ mV}$ for 1 s. Current-voltage ($I-V$) relationships were constructed by stepping in 10 mV increments from HP to test potentials between $-100 \text{ mV}$ and $+140 \text{ mV}$ for 1 s after 10 min dialysis. For $I-V$ relationships, $I_{\text{Cl(Ca)}}$ was expressed as current density ($\mu\text{A}/\mu\text{F}$) by dividing the amplitude of the current measured at the end of the voltage clamp step by the cell capacitance. For all figure panels showing a time course of $I_{\text{Cl(Ca)}}$ changes, the late current measured at $+90 \text{ mV}$ was normalized to the amplitude of first current elicited at time=0 ($\sim 30 \text{ s}$ after breaking the seal and measuring cell capacitance). After measuring cell capacitance, the constant step protocol described above was started to monitor the changes of $I_{\text{Cl(Ca)}}$ over the course of 10 min after which a control $I-V$ relationship was obtained. While monitoring $I_{\text{Cl(Ca)}}$ elicited by a similar constant step protocol described above, the external solution was switched to one containing NFA at a concentration of 0.1, 1, 10, 100 or 500 \(\mu\text{M}\). During these experiments, cells were stepped from the HP to $+80 \text{ mV}$ before being repolarized to $-80 \text{ mV}$. After a steady-state effect was noticed, another $I-V$ relationship was obtained in the presence of Ca\(^{2+}\) buffers (106, 108, 109). Contamination of $I_{\text{Cl(Ca)}}$ from other types of current was minimized by the use of CsCl and tetraethylammonium chloride (TEA) in the pipette solution, and TEA in the external solution. Data for each group were collected in cells from at least two animals but generally more.
NFA. If the seal was still stable, washout was subsequently initiated and an $I-V$ curve generated after washout. Only one concentration of NFA was tested per cell.

2.3.5 Solutions and Reagents

Single pulmonary artery smooth muscle cells were isolated by incubating pulmonary artery tissue strips in the following low Ca$^{2+}$ (10 or 50 μM) PSS (in mM): NaCl (120), KCl (4.2), NaHCO$_3$ (25; pH 7.4 after equilibration with 95% O$_2$, 5% CO$_2$ gas), KH$_2$PO$_4$ (1.2), MgCl$_2$ (1.2), glucose (11), taurine (25), adenosine (0.01) and CaCl$_2$ (0.01 or 0.05). The K$^+$-free bathing solution used in all patch clamp experiments had the following composition (in mM): NaCl (126), Hepes-NaOH (10, pH 7.35), TEA (8.4), glucose (20), MgCl$_2$ (1.2) and CaCl$_2$ (1.8). The pipette solution had the following composition (in mM): TEA (20), CsCl (106), Hepes-CsOH (10, pH 7.2), BAPTA (10), ATP.Mg (0 or 5), GTP.diNa (0.2), and KN-93 (0 or 0.01). To this solution, the following total amounts of CaCl$_2$ and MgCl$_2$ were added to set free [Mg$^{2+}$] at 0.5 mM and free [Ca$^{2+}$] at various desired levels: 5 mM ATP and 500 nM Ca$^{2+}$ (in mM): CaCl$_2$ (7.08), MgCl$_2$ (3.0); no ATP and 500 nM Ca$^{2+}$: CaCl$_2$ (7.08), MgCl$_2$ (0.545). All enzymes, analytical grade reagents and niflumic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Niflumic acid was initially prepared as 1, 10 or 100 mM stock in dimethyl sulfoxide (DMSO) and an appropriate aliquot was added to the external solution to reach the final desired concentration. The maximal concentration of DMSO never exceeded 0.1%, a concentration that had no effect on $I_{Cl(Ca)}$. 
2.3.6 Statistical Analysis

All data were pooled from $n$ cells taken from at least two different animals with error bars representing the s.e.m. All data were first pooled in Excel and means exported to Origin 7.5 software (OriginLab Corp. Northampton, MA, U.S.A.) for plotting and curve fitting. All graphs and current traces were exported to CorelDraw 12 (Ottawa, Ontario, Canada) for final processing of the figures. Origin 7.5 software (OriginLab Corp. Northampton, MA, U.S.A.) was also used to determine the statistical significance between two groups using a paired or unpaired Student’s $t$ test, or one-way ANOVA test followed by Bonferroni post-hoc multiple range tests in multiple group comparisons. $P < 0.05$ was considered to be statistically significant.

2.4 Results

2.4.1 Impact of Global Phosphorylation Status on Ca$^{2+}$-activated Cl$^-$ current

In all experiments we systematically dialyzed the cells for 10 min with 5 mM ATP, or 0 mM ATP with or without 10 $\mu$M KN-93, a specific inhibitor of calmodulin-dependent protein kinase II (CaMKII). KN-93 was added to facilitate a presumed state of dephosphorylation of the channel or regulatory subunit by endogenous phosphatases. As previously shown by our group (106, 107, 108), these conditions allowed us to first establish conditions favoring completely different states of cellular phosphorylation before examining the effects of niflumic acid (NFA) on Ca$^{2+}$-activated Cl$^-$ current ($I_{\text{Cl(Ca)}}$) evoked by 500 nM free intracellular Ca$^{2+}$ concentration. Figure 1A-a shows current traces recorded at different times after seal rupture from two sample experiments under the conditions described above. $I_{\text{Cl(Ca)}}$ was
Figure 1. Attenuation of rundown of Ca\(^{2+}\)-activated Cl\(^-\) currents in rabbit pulmonary artery myocytes by minimizing phosphorylation. A-a) Representative current traces demonstrate the time-dependent changes of Ca\(^{2+}\)-activated Cl\(^-\) current recorded from pulmonary arterial smooth muscle cells dialyzed with either 5 mM ATP or 0 mM ATP + 10 μM KN-93. Currents depicted were recorded immediately following breaking the seal (0 min), and after 2 and 10 min of cell dialysis. The currents were elicited by repetitive 1 s step depolarizations (every 5 s) to +90 mV from a holding potential (HP) of -50 mV. Each depolarizing pulse was followed by a repolarizing step to -80 mV to enhance the magnitude of the tail current. A-b) This graph depicts the mean time course of \(I_{Cl(Ca)}\) amplitude elicited by 1-s depolarizing pulses to +90 mV, followed by 1-s repolarizing steps to -80 mV, in the presence of 5 mM ATP (filled squares, \(n = 42\)), or in the absence of ATP with 10 μM KN-93 (filled triangles, \(n = 32\)). Currents were normalized to the initial current amplitude at the beginning of the protocol. Each step was applied from HP = -50 mV at a frequency of one pulse every 5 s for 10 min. *** the group dialyzed with 0 ATP and 10 μM KN-93 was significantly different from 5 mM ATP with \(P < 0.001\) (one-way ANOVA). B) This graph shows the mean time course of changes of late \(I_{Cl(Ca)}\) measured at the end of 1-s steps to +90 mV from HP = -50 mV (1 step every 10 s) in cells dialyzed with 0 mM ATP, with (empty circles) or without (filled circles) 10 μM Cyclosporine A to inhibit Calcineurin. ** The group of cells dialyzed with 0 ATP and 10 μM Cyclosporine A was significantly different from that dialyzed with no ATP alone with \(P < 0.01\) (one-way ANOVA).
evoked by 1 s steps to +90 mV from a holding potential (HP) of -50 mV which was followed by a 1 s repolarizing step to -80 mV before returning to the HP. As previously shown by our group (106, 107, 108), step depolarization to +90 mV induced an initial instantaneous membrane current after the capacitative current that was followed by a slow time-dependent current component, both arising from basal and voltage-dependent gating properties of Ca^{2+}-activated Cl^- channels. A slow tail current was apparent upon repolarization to -80 mV and is consistent with channel closure caused by voltage-dependent deactivation. Immediately after seal rupture (0 min), \( I_{\text{Cl(Ca)}} \) evoked in both conditions displayed similar amplitude and kinetics. \( I_{\text{Cl(Ca)}} \) exhibited marked rundown during the first 2 min and remained suppressed throughout the initial 10 min of cell dialysis with 5 mM ATP. Removal of ATP and inclusion of KN-93 to suppress CaMKII activity in the pipette solution also resulted in \( I_{\text{Cl(Ca)}} \) rundown but the magnitude of this process was clearly attenuated after 2 min. This initial rundown most likely reflects a partial state of phosphorylation of an unknown regulatory or pore-forming subunit due to consumption of endogenous levels of ATP before depletion ensues. With continued dialysis, \( I_{\text{Cl(Ca)}} \) progressively recovered and eventually exceeded (see traces at 10 min) the amplitude of the initial current recorded at the onset of dialysis. The graph in panel A-b shows mean data obtained from 32 or 42 cells in each group. For each data set, the amplitude of \( I_{\text{Cl(Ca)}} \) recorded at the end of the step to +90 mV was normalized to that of the initial current recorded at time = 0. Consistent with the experiment described in panel A-a, \( I_{\text{Cl(Ca)}} \) exhibited marked rundown after breaking the seal with 5 mM ATP reaching ~ 15% of the initial level in less than 2 min (filled squares), a level that remained very stable
throughout the rest of the experiment. As in panel A, removing ATP and the addition of KN-93 (filled triangles) from the pipette attenuated the rundown and resulted in a complete recovery that continued to develop beyond the initial level (~30% above the initial level). The global state of phosphorylation also affected deactivation kinetics, where removal of ATP from the pipette solution caused a slowing of the time constant from 70.5 ± 3.4 ms (n=42) to 101.9 ± 6.1 ms (n=32; P < 0.001), with 5 mM ATP and no ATP and 10 μM KN-93, respectively. An opposite trend was observed for the activation kinetics although the differences were just at the limit of significance; τ for activation was 322.9 ± 11.1 ms for 5 mM ATP (n=42) and 300.5 ± 14.1 ms for 0 mM ATP with KN-93 (n=32; P = 0.0576). The effects of different ATP levels on I_{Cl(Ca)} are very similar to those reported by Angermann et al. (2006) (106) who proposed that phosphorylation causes a state-dependent block of the channels. Another series of experiments was carried out to further demonstrate that changing ATP levels influenced an important phosphorylation step regulating I_{Cl(Ca)}. Figure 1B shows that removing ATP alone also attenuated rundown I_{Cl(Ca)} and led to a recovery of the current that still exceeded the initial level after 10 min but to a lesser extent (~10% instead of 30%) than when KN-93 was included in the pipette solution. As shown in panel B including cyclosporine A in the pipette solution, a specific inhibitor of the Ca^{2+}-dependent phosphatase calcineurin (125, 268), obliterated the recovery of I_{Cl(Ca)}. These experiments established that the very different characteristics of I_{Cl(Ca)} before any exposure to NFA was carried out were dictated by diametrically opposite states of global phosphorylation.
2.4.2 Global State of Dephosphorylation Enhances the Potency of NFA-Induced Block of $I_{\text{Cl(Ca)}}$

The next series of experiments were conducted to determine whether NFA exerts a differential effect on $I_{\text{Cl(Ca)}}$ when the state of global phosphorylation is altered. Panel A of Figure 2 depicts typical experiments in which the effects of 100 $\mu$M NFA were tested on $I_{\text{Cl(Ca)}}$ recorded from a cell dialyzed with 5 mM ATP, and 0 ATP and 10 $\mu$M KN-93, respectively. The superimposed traces shown to the right of the graph were obtained immediately before (labeled a) and after steady-state block achieved by NFA (labeled b). The voltage clamp protocol used was similar to that of Figure 1, with changes outlined in the Methods section. The graph shows the two superimposed time courses of normalized $I_{\text{Cl(Ca)}}$ amplitude before and during the application of NFA. In both cases, NFA blocked the instantaneous and time-dependent components of $I_{\text{Cl(Ca)}}$ but the amount of inhibition was notably increased for the cell dialyzed with no ATP and KN-93. Figure 2B shows plots of mean normalized $I_{\text{Cl(Ca)}}$ at +90 mV as a function of time for similar experiments performed with a pipette solution containing 5 mM ATP (filled squares) or 0 ATP and KN-93 (filled triangles). Although the time course of block of $I_{\text{Cl(Ca)}}$ by NFA was similar in both groups, the ATP-free pipette solution containing KN-93 clearly enhanced the potency of the drug. On average NFA blocked $I_{\text{Cl(Ca)}}$ by 42.0 ± 9.9 % with 5 mM ATP and 79.5 ± 3.3 % with no ATP + KN-93. These results demonstrate that inhibition of $I_{\text{Cl(Ca)}}$ mediated by a presumed state channel or regulatory subunit phosphorylation reduces the ability of NFA to inhibit the channels.

We next examined the voltage-dependence of interaction of NFA under both conditions (Figure 3). On the left hand side of each panel, typical families of $I_{\text{Cl(Ca)}}$ currents recorded
Figure 2. Effect of phosphorylation on the ability of NFA to block $I_{\text{Cl(Ca)}}$. A) Representative time courses from pulmonary artery smooth muscle cells dialyzed with pipette solutions containing either 5 mM ATP or an ATP-free solution with 10 μM KN-93. Currents were measured at the end of 1 s depolarizing steps to +90 mV from HP = -50 mV and plotted against time over a 10 min period of dialysis. Following current stabilization, 100 μM NFA was applied to the cells, as indicated by the filled bar. Corresponding to points a) and b) on the time-courses, superimposed sample traces taken before (a) and after (b) the application of NFA are shown to the right of the graph for each experiment. B) Time courses representing mean data collected as described above for cells dialyzed with 5 mM ATP (filled squares, n = 10) or 0 mM ATP + 10 μM KN-93 pipette solution (filled triangles, n = 5). ** both groups with no ATP were significantly different from 5 mM ATP with $P < 0.01$ (one-way ANOVA).
Figure 3. The voltage dependence of \( I_{\text{Cl(Ca)}} \) block by NFA is altered by phosphorylation levels. 

A) On the left, two representative families of currents from a cell dialyzed with 5 mM ATP, before (upper) and after (lower) a 10 min application of 100 \( \mu \)M NFA. Currents were evoked by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV following a 10 min period of dialysis. On the right, mean current-voltage relationships generated from similar families of currents after 10 minute of cell dialysis with 5 mM ATP (filled boxes, \( n = 10 \)), and after 10 min with the addition of 100 \( \mu \)M NFA to the external solution (open circles, \( n = 10 \)). The inset highlights an expanded segment of the current-voltage relationship from -100 mV to -20 mV.

B) was generated in a similar fashion to A) except for the fact that in B), cells were dialyzed with a pipette solution containing 0 mM ATP + 10 \( \mu \)M KN-93 (\( n = 5 \)). Please note in B) the negative shift in voltage where block is converted to stimulation when phosphorylation is minimized (indicated by the arrow in the inset). * \( P < 0.05 \) (Paired \( t \) test).
from the same cell in control (top) and after exposure to 100 μM NFA for 10 min (bottom) are shown. For each panel, the graph on the right hand side reflects the mean I-V relationships of I_{Cl(Ca)} density registered at the end of 1 s steps ranging from -100 to +140 mV from HP = -50 mV and obtained before and after the application of NFA. Consistent with phosphorylation-induced changes in Cl_{Ca} channel gating (106, 107, 108, 109, 120), control currents recorded with 5 mM ATP (Fig. 3A) were considerably smaller (note the different calibration bars), and displayed slower activation and faster deactivation kinetics than those obtained in the absence of ATP (Fig. 3B). For both groups of cells, NFA blocked instantaneous and time-dependent outward I_{Cl(Ca)} (> 0 mV) but the block was greater in the absence of ATP along with KN-93 (Fig. 3B) than with 5 mM ATP (Fig. 3A). NFA was previously shown to enhance I_{Cl(Ca)} in the negative range of membrane potentials (135). The same group also reported marked rebound stimulation of the current in the entire range of membrane potentials examined during washout of the drug and a similar observation was also made for I_{Cl(Ca)} in rabbit coronary myocytes (110). The insets in the graphs displayed in Figure 3 represent an expanded scale in the negative range of voltages of the I-V relationships of I_{Cl(Ca)} density. The results confirm that NFA stimulated this current at negative potentials without inducing any shifts in reversal potential which matched the equilibrium for Cl^− (~ 0 mV). While NFA produced stimulation in cells dialyzed with 5 mM ATP at potentials negative to -20 mV (Fig. 3A), the stimulation was only apparent for potentials negative to -50 mV in cells dialyzed with no ATP and KN-93 (Fig. 3B); in fact NFA blocked I_{Cl(Ca)} at potentials between -40 and -10 mV and this led to the appearance of a crossover point at -50 mV (see arrow). Taken together these results suggested that a
phosphorylation step might be causing a shift in the voltage-dependence of NFA-induced block and stimulation.

To test this hypothesis, % block or stimulation by NFA of steady-state $I_{Cl(Ca)}$ was plotted as a function of voltage (Fig. 4) and the calculated parameters presented in Table 1. In Figure 4, the data in 0 mM ATP alone were omitted for the sake of clarity but fell in between those obtained with 5 mM ATP and 0 mM ATP + KN-93 (see Table 1). All data sets could be well fitted by simple Boltzmann relationships. Figure 4 clearly shows that the interaction of NFA with the $Cl_{Ca}$ channel is voltage-dependent and appears to be linked to channel gating (see Discussion). In the absence of ATP, a significant block of $I_{Cl(Ca)}$ was apparent above -50 mV and saturated around +50 mV. In contrast, steps more positive than +30 mV were required to detect NFA-induced block of $I_{Cl(Ca)}$ with 5 mM ATP. Another interesting feature is the much shallower slope of the relationship obtained with 5 mM versus 0 mM ATP + KN-93.

**Table 1. Voltage-dependent parameters describing the interaction of niflumic acid with Ca$^{2+}$-activated Cl$^{-}$ currents recorded from pulmonary artery myocytes.**

<table>
<thead>
<tr>
<th></th>
<th>$V_{0.5}$ (mV)</th>
<th>Slope (mV)</th>
<th>$V$ where $y = 0$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 mM ATP</td>
<td>+16.0 ± 2.1</td>
<td>59.2 ± 3.9</td>
<td>29.7</td>
</tr>
<tr>
<td>0 ATP</td>
<td>-8.9 ± 3.5</td>
<td>24.2 ± 3.1</td>
<td>-3.3</td>
</tr>
<tr>
<td>0 ATP + 10 µM KN-93</td>
<td>-59.7 ± 0.8</td>
<td>26.1 ± 1.5</td>
<td>-53.7</td>
</tr>
</tbody>
</table>

Half-maximal voltage ($V_{0.5}$) and Slope values were estimated by curve fitting of the data plotted in Figure 4 (except for the data with 0 mM ATP alone which was not shown) to a Boltzmann equation. $V$ where $y = 0$ represents the voltage where the fitted Boltzmann relationships cross the $y$-axis and corresponds to the voltage point where conversion from block to stimulation was observed.
Figure 4. Altering global phosphorylation status shifts the voltage-dependence of the dual effect of NFA on $I_{Cl(Ca)}$. The two data sets were obtained after 10 min of cell dialysis with either 5 mM ATP (filled squares) or ATP-free pipette solution containing 10 μM KN-93 (filled triangles) before and after addition of 100 μM NFA. % Block was determined at each individual voltage step for each cell using the formula \((I_{NFA}/I_{Control})\times 100\)-100, where $I_{NFA}$ and $I_{Control}$ is the current recorded in the presence and absence of NFA, respectively. For $I_{Control}$, the mean of the first five traces was used in the calculation, while for $I_{NFA}$ the mean of the last 5 stable traces was used. Stimulation is manifest as a positive value using the above formula and is graphed as such. Mean values were then plotted at each voltage, and fitted to a Boltzmann function. Parameters estimated from these fits are presented in Table 1.
Figure 5. Effects of various concentrations of NFA on representative $I_{\text{Cl(Ca)}}$ traces recorded from cells under different states of global phosphorylation. Average currents are shown before and after application of NFA in cells dialyzed with 5 mM ATP, or 0 mM ATP + 10 μM KN-93. Current were elicited by 1 s repetitive depolarizing steps from HP = -50 mV to +80 mV, each followed by a 1 s repolarizing step to -80 mV to record tail current. Averaged traces were calculated using Clampfit 9.2 (Molecular Devices). For unmarked control currents, the first five stable traces at the end of 10 min of cell dialysis were averaged for each cell, and subsequently averaged to give a mean current trace for the entire group of cells. Following a 10-minute application of NFA, currents (marked ‘NFA’) were similarly averaged. Note the concentration-dependent effect of NFA on deactivation kinetics by comparing the effect of 10 μM NFA on $I_{\text{Cl(Ca)}}$ in cells dialyzed with 5 mM ATP ($n = 6$), or 0 mM ATP + 10 μM KN-93 ($n = 3$), to the effect of 250 μM NFA on cells dialyzed with 5 mM ATP ($n = 3$), or 0 mM ATP + 10 μM KN-93 ($n = 3$).
(Fig. 4 and Table 1) suggesting a reduced sensitivity to voltage when phosphorylation is promoted. Thus, global phosphorylation has a major impact on the voltage-dependence of interaction of the fenamate compound with the channel favoring inhibition over stimulation, both appearing to be functionally linked.

2.4.3 Concentration-Dependence of NFA Interaction with Cl\textsubscript{Ca} Channels Altered by Phosphorylation

Because of the dual nature of the interaction of NFA with Cl\textsubscript{Ca} channels (110, 135), we postulated that it might be possible to better distinguish the inhibitory and stimulatory effects of NFA on I\textsubscript{Cl(Ca)} recorded under different states of phosphorylation by examining the effects of a wide range of concentrations (100 nM to 250 \(\mu\text{M}\)) of this molecule. Consistent with this possibility, Ledoux et al. (110) showed that NFA blocked and stimulated I\textsubscript{Cl(Ca)} with different affinities. Figure 5 illustrates the effects of NFA on I\textsubscript{Cl(Ca)} elicited by the standard step protocol described in Figure 1 and depicted at the bottom. Each trace is an average of five traces from 4 to 8 cells recorded immediately prior to, or after a steady-state effect of NFA was observed after 10 min. Interestingly 100 nM NFA consistently stimulated the outward current at +80 mV and tail current at -80 mV as well as the steady-state current at the holding potential in cells dialyzed with 5 mM ATP (upper left traces). In contrast, in cells dialyzed with 0 ATP and KN-93 the same concentration of NFA produced inhibition of the outward current at +80 mV, a slight inhibition of the peak inward tail current at -80 mV, and stimulation of steady-state current as evident from the tail current deactivating to a higher negative level (upper right traces). Increasing the concentration of NFA to 10 \(\mu\text{M}\) converted the stimulation to block at +80 mV with 5 mM ATP (middle left traces), little effect on peak
inward tail current at -80 mV but a profound and well characterized slowing of deactivation (141, 269). In the absence of substrate, NFA produced a more potent inhibition of the time-dependent outward current at +80 mV and tail current at -80 mV; as with ATP, NFA markedly slowed deactivation kinetics of I_{Cl(Ca)} which crossed over with the control current (middle right traces). Increasing NFA concentration to 250 μM led to more potent block of the current at +80 mV, and now a significant but small inhibition of the peak inward tail current at -80 mV accompanied by the typical slowing of deactivation kinetics at -80 mV with a detectable cross-over with the control current (lower left traces). With 0 ATP and KN-93, the same concentration of NFA led to marked block of the outward and inward I_{Cl(Ca)} at +80 and -80 mV, respectively; it is apparent that the cross-over described above disappeared due to the marked inhibition of the inward tail current.

Figure 6 reports mean data for all NFA concentrations tested on I_{Cl(Ca)} measured at the end of the step to +80 mV (panel A) and peak inward tail current at -80 mV (panel B). For both graphs, % block or % stimulation is indicated by a negative or positive value, respectively. With 0 ATP and KN-93, NFA dose-dependently blocked the outward and inward current relaxations with an IC_{50} of ~ 46 μM. We were also unable to observe stimulation of I_{Cl(Ca)} when testing even lower concentrations of NFA (100 nM, n=3; 1 μM, n=4; and 10 μM, n=3). Enabling global phosphorylation with 5 mM ATP led to a significant stimulation of the outward current at +80 mV with 100 nM NFA, and inward tail current at -80 mV in the range of 100 nM to 10 μM NFA. 100 and 250 μM NFA led to significant inhibition of both current components but the magnitude of block was considerably reduced relative to cells dialyzed with no ATP and KN-93. Taken together, these results
Figure 6. Concentration-dependence of the dual effect of NFA on $I_{\text{Cl(Ca)}}$.

A) In cells dialyzed with either 5 mM ATP (filled bars), or 0 mM ATP + 10 μM KN-93, NFA was added for 10 min at concentrations of 0.1 μM, 1 μM, 10 μM, 100 and 250 μM. Current was recorded at the end of 1 s depolarizing pulses to +80 mV from HP of -50 mV, and % block for each cell was calculated by the formula ($I_{\text{NFA}}/I_{\text{Control}}$)*100-100, where $I_{\text{NFA}}$ and $I_{\text{Control}}$ are the currents recorded in the presence and absence of NFA, respectively. For $I_{\text{Control}}$, the mean of the first five traces was used in the calculation, while for $I_{\text{NFA}}$ the mean of the last 5 stable traces was used. Stimulation is manifest as a positive value using the above formula, and is graphed as such. Mean calculated values were then determined for each group (5 mM ATP: 0.1 μM NFA, n = 8; 1 μM NFA, n = 5, 10 μM NFA, n = 6; 100 μM NFA, n = 4; 250 μM NFA, n = 4; 0 mM ATP + 10 μM KN-93: 0.1 μM NFA, n = 7; 1 μM NFA, n = 4; 10 μM NFA, n = 3; 100 μM NFA, n = 5; 250 μM NFA, n = 4) and graphed.

Panel B) was generated similarly to A), but the instantaneous tail current was recorded upon repolarizing the cell to -80 mV for 1 s following the depolarizing step described in A). Note that while low concentrations of NFA stimulate $I_{\text{Cl(Ca)}}$ at +80 mV and -80 mV when cells were dialyzed with 5 mM ATP, only block by NFA was detected in cells dialyzed with ATP-free pipette solution containing 10 μM KN-93. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Unpaired Student t tests).
provide convincing evidence for a direct impact of the presumed state of phosphorylation on the ability of NFA to inhibit and stimulate $\text{Cl}_{\text{Ca}}$ channels.

### 2.5 Discussion

This study reports for the first time the impact of a presumed state of channel or regulatory subunit phosphorylation on the interaction of niflumic acid on native $\text{Ca}^{2+}$-activated $\text{Cl}^-$ currents recorded in freshly isolated rabbit pulmonary arterial smooth muscle cells. $I_{\text{Cl(Ca)}}$ elicited by sustained elevated intracellular $\text{Ca}^{2+}$ concentration under conditions favoring either phosphorylation or dephosphorylation led to very marked differences in sensitivity to NFA. NFA produces a paradoxical dual effect on $I_{\text{Cl(Ca)}}$ in pulmonary artery myocytes, stimulating and blocking the channels at negative and positive potentials, respectively (135). Analysis of the voltage-dependence of interaction of NFA with $I_{\text{Cl(Ca)}}$ showed that although the maximal level of block at positive potentials and stimulation at negative potentials were similar, promoting global dephosphorylation shifted the voltage at which block was “apparently” converted to stimulation occurs by more than -70 mV. Examination of the concentration-dependence of the interaction revealed that a very low concentration of NFA (100 nM) led to stimulation of fully activated $I_{\text{Cl(Ca)}}$ at +80 mV and tail current at -80 mV in cells dialyzed with ATP to support global phosphorylation; stimulation was converted to block between 1 and 10 $\mu$M NFA and higher concentrations inhibited the current albeit at a lower extent than for cells dialyzed with no ATP and 10 $\mu$M KN-93. In contrast, $I_{\text{Cl(Ca)}}$ in cells dialyzed with 0 ATP and KN-93 was blocked in a concentration-dependent manner by NFA in the range of 0.1 to 250 $\mu$M. These results demonstrate that
alteration of $\text{Cl}_{\text{Ca}}$ channel gating by an unidentified phosphorylation step profoundly influences the mode of interaction of NFA with the channel.

2.5.1 Conditions Establishing Global States of Phosphorylation

$\text{Ca}^{2+}$-activated $\text{Cl}^-$ channels in airway and arterial myocytes are subject to $\text{Ca}^{2+}$-dependent suppression when intracellular $\text{Ca}^{2+}$ levels are raised and this process involves activation of $\text{Ca}^{2+}$-Calmodulin and CaMKII (107, 123). Phosphorylation by CaMKII of an unknown target on the channel protein or closely associated accessory regulator results in a time-dependent decline of $I_{\text{Cl(Ca)}}$ that is faster than the $\text{Ca}^{2+}$ transient triggering channel opening (123), or current rundown when intracellular $\text{Ca}^{2+}$ levels are clamped above 200 nM (106). It is hypothesized that CaMKII-induced inhibition of $I_{\text{Cl(Ca)}}$ serves an important role in attenuating the strong depolarizing influence of $\text{Cl}_{\text{Ca}}$ channels during excitation (120). This “breaking” or negative feedback regulation by CaMKII is antagonized by calcineurin (CaN) in rabbit coronary (109) and pulmonary artery (108). The massive rundown of $I_{\text{Cl(Ca)}}$ seen in our study when phosphorylation is supported by including 5 mM ATP in the pipette solution, the substantial recovery of the current in myocytes supplied internally with no ATP, with or without KN-93, and the inhibition of the delayed recovery by the CaN inhibitor Cyclosporine A, are consistent with this hypothesis and are similar to a recent report from our group contrasting the effects of cell dialysis with 3 mM ATP or 3 mM AMP-PNP, a non-hydrolysable analogue of ATP (106). Also in accord with this idea, the higher ATP concentration used in the present study (5 vs. 3 mM in Angermann et al., 2006) enhanced the rate and magnitude of $I_{\text{Cl(Ca)}}$ rundown. Angermann et al. (2006) (106) proposed a kinetic model whereby
phosphorylation induces state-dependent block through voltage-dependent gating steps favoring closure at high levels of Ca$^{2+}$ occupancy on the cytoplasmic side of the channel. This model predicts more rapid activation at positive potentials and slower deactivation kinetics at negative potentials at high [Ca$^{2+}$], when the channel or an unknown regulatory element is dephosphorylated by endogenous phosphatases. Currents elicited in cells supplied with no ATP and KN-93 to inhibit CaMKII activated more quickly and deactivated more slowly than those dialyzed with 5 mM ATP. Our results thus firmly establish that the underlying pore-forming or regulatory subunit of Cl$_{Ca}$ channels was either in a strong state of phosphorylation (5 mM ATP) or dephosphorylation (0 ATP with or without KN-93) after 10 min of cell dialysis prior to any application of drug took place.

2.5.2 Global Phosphorylation Attenuates the Block of I$_{Cl(Ca)}$ by NFA

Our first series of experiments were designed to examine the effects of 100 μM NFA, a widely used concentration, on presumed phosphorylated and dephosphorylated Cl$_{Ca}$ channels. The rationale for these experiments was based on the observation that NFA preferentially interacts with opened Cl$_{Ca}$ channels and that the block is voltage-dependent (141, 147). We thus hypothesized that presumed phosphorylation-induced channel closure might reduce the ability of NFA to block I$_{Cl(Ca)}$. Our data confirmed this hypothesis by showing that steady-state block at +90 mV by this compound was significantly greater in cells lacking ATP. Although the maximal levels of achievable inhibition or stimulation were similar, analysis of the voltage-dependence of the interaction of NFA with the channels revealed striking differences. For presumed dephosphorylated channels, the block was
sharply voltage-dependent below 0 mV and stimulation was apparent below -50 mV. In contrast, stimulation was evident at potentials negative to +20 mV in cells dialyzed with 5 mM ATP whereas block was only apparent at potentials more positive than +20 mV. These data suggest that presumed phosphorylation reduces the blocking efficacy of NFA, an effect that may favor the unmasking of the stimulatory effect of NFA. Whether these two opposing effects of NFA are independent as previously suggested by our group for the effects of NFA on \( I_{\text{Cl(Ca)}} \) in rabbit coronary artery myocytes (109) will require more investigation. One possibility to explain these observations is that the NFA binding site responsible for blocking the channel becomes more easily accessible by channel opening, a situation favored by presumed dephosphorylation; consistent with this idea was the observation that cell dialysis with 500 nM \( \text{Ca}^{2+} \) and 3 mM AMP-PNP caused an elevation of basal \( C_{\text{Ca}} \) channel conductance between -100 to -50 mV, and increased progressively from -50 to +130 mV (106). This contrasted with \( C_{\text{Ca}} \) conductance in myocytes dialyzed with 5 mM ATP which was very small from -100 to \( \sim \) 0 mV and increased sharply beyond this voltage. An alternative explanation is that presumed phosphorylation favored NFA-mediated stimulation that counteracted inhibition.

### 2.5.3 Pharmacological Significance

To our knowledge, there is only one other study demonstrating the impact of altered channel gating due to phosphorylation on the pharmacology of an anion channel. Dérand et al. (2003) (270) showed that the activation of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) by benzimidazolone NS004 is influenced by the state of
phosphorylation of the R domain by cAMP-dependent protein kinase (PKA). This compound was shown to activate phosphorylated CFTR with an EC$_{50}$ of 11 μM while an EC$_{50}$ in excess of 100 μM was determined for non-phosphorylated CFTR channels. Dérand et al. (2003) (270) suggested that phosphorylation facilitates binding site accessibility through domain interactions or conformational changes. We propose a similar paradigm, albeit in the opposite direction, for the attenuated ability of NFA to inhibit presumed phosphorylated Cl$_{\text{Ca}}$ channels. CaMKII-induced phosphorylation may occlude the binding site by voltage-dependent steric hindrance, or by reduced availability through a state-dependent mechanism.

The present study offers some interesting clues relating to the relatively wide range of IC$_{50}$ values for NFA to block I$_{\text{Cl(Ca)}}$ in vascular smooth muscle cells. In the first comprehensive description of the mechanism of block of this current in rabbit portal vein smooth muscle cells, Hogg et al. (141) showed that NFA blocked STICs produced by Cl$_{\text{Ca}}$ channels with an EC$_{50}$ of ~ 1-4 μM. 10 μM NFA blocked the slow inward I$_{\text{Cl(Ca)}}$ tail evoked by Ca$^{2+}$ entry through L-type Ca$^{2+}$ channels (I$_{\text{CaL}}$) by 71% in rabbit coronary artery (142), suggesting an EC$_{50}$ value lying below that concentration. In the same preparation, NFA reduced I$_{\text{Cl(Ca)}}$ elicited by sustained 500 nM Ca$^{2+}$ with a much higher EC$_{50}$ (159 μM (110)). One important difference in the methods used to elicit I$_{\text{Cl(Ca)}}$ is that STICs and I$_{\text{CaL}}$-induced I$_{\text{Cl(Ca)}}$ is that they are evoked by transient elevations of [Ca$^{2+}$]. Autophosphorylation of CaMKII may be quite limited; in fact, the phosphorylation balance is likely to be shifted toward a dephosphorylation state as CaN displays ~ 2 orders of magnitude higher affinity for Ca$^{2+}$-CaM than CaMKII and is directly activated by Ca$^{2+}$ via an interaction with the B subunit of CaN (109, 124, 125, 268). These
results suggest being cautious when using NFA as a pharmacological tool to assess the role of $\text{Cl}_{\text{Ca}}$ channels in controlling vascular tone. Large sustained elevations in $[\text{Ca}^{2+}]_i$ caused by high concentrations of constricting agonists is likely to promote a higher state of phosphorylation of $\text{Cl}_{\text{Ca}}$ channels and reduced efficacy of NFA. Indeed, a study by Remillard et al. (2000) (146) showed a progressive attenuation of NFA-induced vasorelaxation of pressurized rabbit mesenteric small arteries when the concentration of the $\alpha_1$-adrenergic receptor agonist phenylephrine was raised from 100 nM to 10 $\mu$M. The recent discoveries of the Bestrophin (120, 188) and TMEM16A/B genes (220, 221, 222) as novel molecular candidates for $\text{Cl}_{\text{Ca}}$ genes will soon bring novel insight into the mechanisms regulating their activation by $\text{Ca}^{2+}$, voltage and phosphorylation, and how the interplay of these factors influence their pharmacology.
Chapter 3: The Anomalous Effects of Anthracene-9-Carboxylic Acid are Altered by Promoting or Minimizing Global Phosphorylation

3.1 Summary

Background and purpose: Ca\(^{2+}\)-activated Cl\(^-\) currents (I\(_{Cl(Ca)}\)) in arterial smooth muscle cells are inhibited by phosphorylation. It was recently found that the state of phosphorylation of the pore-forming or regulatory subunit of Cl\(_{Ca}\) channels influences the interaction of niflumic acid (NFA) with its inhibitory binding site. We tested whether the interaction of the chemically dissimilar Cl\(^-\) blocker anthracene-9-carboxylic acid (A9C) was similarly affected by the state of global phosphorylation. A9C was chosen as it has been found, like NFA, to have a unique dual effect on I\(_{Cl(Ca)}\), both inhibiting and stimulating the channels depending on voltage and the state of gating.

Experimental approach: I\(_{Cl(Ca)}\) was elicited with 500 nM free internal Ca\(^{2+}\) in rabbit pulmonary artery myocytes. The state of global phosphorylation was altered by cell dialysis with either 5 mM ATP, or 0 ATP.

Key results: Dephosphorylation enhanced the ability of A9C to inhibit I\(_{Cl(Ca)}\) in a concentration-dependent manner. Similar to NFA, this was attributed to a negative shift in the voltage-dependence of block. Stimulation of I\(_{Cl(Ca)}\) tail current by 500 µM A9C at -80 mV was enhanced in cells dialyzed with 5 mM ATP. While the tail current of cells dialyzed with 0 ATP were stimulated following depolarization to +40 mV, the stimulation was abolished following steps to +140 mV.

Conclusions and implications: Our data indicate that supporting phosphorylation of the pore-forming or regulatory subunit of Cl\(_{Ca}\) channels influences the interaction of A9C in a
manner that promotes interaction of the drug with a stimulatory binding site while dephosphorylation enhances the ability of the compound to inhibit the channel, most likely due to an increased open state interaction.

3.2 Introduction

Activation of calcium-activated chloride channels (Cl<sub>Ca</sub>) of vascular smooth muscle by constricting hormones and neurotransmitters is thought to be an important contributor to the development of vascular smooth muscle tone (120, 147). Cl<sup>-</sup> is actively accumulated in vascular myocytes which results in an equilibrium potential for this anion of ~ -20 mV, a value that is more positive than the resting membrane potential of the cell (~-50 mV). Thus, activation of Cl<sub>Ca</sub> produces membrane depolarization caused by net Cl<sup>-</sup> efflux, resulting in an increase in the open probability of voltage-dependent Ca<sup>2+</sup> channels, leading to increased Ca<sup>2+</sup> entry and muscle tone (120, 147). In the whole-cell patch clamp configuration, I<sub>Cl(Ca)</sub> in several cell types decreases in amplitude (rundown) following seal rupture or patch excision. As current rundown is evident when I<sub>Cl(Ca)</sub> is elicited by caffeine or even by setting [Ca<sup>2+</sup>], to elevated fixed levels by using a Ca<sup>2+</sup> buffer in the pipette solution, thus making it unlikely that the rundown is due to a decline in [Ca<sup>2+</sup>]. Instead Cl<sub>Ca</sub> channels appear to be down regulated by key signaling molecules including kinases and phosphatases which were suggested to regulate Cl<sub>Ca</sub> channel function by phosphorylation. Blockers of the Ca<sup>2+</sup>-calmodulin–dependent kinase CaMKII were shown to prolong the duration of I<sub>Cl(Ca)</sub> in myocytes isolated from trachea (123) and to enhance I<sub>Cl(Ca)</sub> in pulmonary and coronary artery smooth muscle cells (107). Dialysis of pulmonary artery smooth muscle cells with a
constitutively active form of CaMKII significantly reduced $I_{\text{Cl(Ca)}}$ evoked by 500 nM Ca$_{\text{2+}}$ (107). Omitting ATP from the pipette solution or replacing it with its non-hydrolyzable analog AMP-PNP resulted in impaired rundown, followed by complete recovery of the current (106). It has been proposed that phosphorylation of the Cl$_{\text{Ca}}$ channel or a regulatory subunit influences current generation through one or more voltage dependent steps (106). The inhibitory effect of CaMKII is counteracted by phosphatases, particularly calcineurin (108, 109) (Chapter 2). Inhibition of CaN by cyclosporine A (CsA) in isolated rabbit coronary artery myocytes reduces $I_{\text{Cl(Ca)}}$ amplitude at $[\text{Ca}^{2+}]_{\text{i}} \leq 500$ nM (109), while including a constitutively active form of the enzyme in the pipette solution resulted in a considerable enhancement of $I_{\text{Cl(Ca)}}$ in pulmonary artery myocytes (108). In addition to increasing $I_{\text{Cl(Ca)}}$ amplitude, currents elicited in the presence of the constitutively active form of CaN exhibited faster current development at depolarized potentials, and a slower rate of deactivation upon hyperpolarization (108). These effects were specific to the $\alpha$ isoform of CaN, even though both $\alpha$ and $\beta$ isoforms of CaN A are expressed in these myocytes (108). A recent report has more directly demonstrated a role in Cl$_{\text{Ca}}$ channel regulation by the Ca$_{\text{2+}}$-independent PP1 and PP2A (126). Several inhibitors, both non-selective (OA, calyculin A, cantharidin) and the PP1 selective inhibitor NIPP-1 antagonized recovery of $I_{\text{Cl(Ca)}}$ when phosphatase activity was promoted (0 ATP in the pipette). Fostriecin, a PP2A-selective antagonist, failed to block recovery of the current. Interestingly, inclusion of a constitutively active form of PP2A in the pipette solution reduced the rundown of the current in the presence of 3 mM ATP, followed by recovery of the current. On the other hand, exogenous PP1 application had no effect on the time course of current rundown. This study also
showed that the down-regulation of $I_{\text{Cl(Ca)}}$ by exogenous Calcineurin $A\alpha$ could be antagonized by the highly selective and potent PP1 antagonist NIPP-1 suggesting that Calcineurin may be exerting its effects on $\text{Cl}_{\text{Ca}}$ by stimulating PP1. These data clearly demonstrate a delicate balance between kinase and phosphatase activity in the regulation of $\text{Cl}_{\text{Ca}}$ channels.

We have recently published that the state of phosphorylation of the pore-forming or regulatory subunit of $\text{Cl}_{\text{Ca}}$ channels alters the interaction of niflumic acid (NFA) with an inhibitory binding site (Chapter 2) (271). Dephosphorylation enhanced the ability of 100 µM NFA to inhibit $I_{\text{Cl(Ca)}}$, an affect attributed to a large negative shift in the voltage-dependence of block (271). The present study was conducted to determine whether the pharmacology of a Cl$^-$ channel blocker chemically unrelated to NFA, anthracene-9-carboxylic acid (A9C), is also altered by phosphorylation. A9C has been shown to inhibit $I_{\text{Cl(Ca)}}$ evoked by Ca$^{2+}$ entry through VDCCs in several tissues (132, 150, 151, 152). Block of $I_{\text{Cl(Ca)}}$ by A9C is highly voltage dependent, with $IC_{50}$'s for inhibition of spontaneous transient inward currents (STICs) of 300 µM at -50 mV and 90 µM at +90 mV (149). Inhibition may be rapidly reversed by hyperpolarization and evidence suggests that A9C blocks the channels while in the open state (147). In rabbit pulmonary artery smooth muscle cells (PASMCs), application of 500 µM A9C resulted in a small inhibition of the outward current recorded at +70 mV, but increased the amplitude of the instantaneous inward tail current relaxation at -80 mV by over 300% (153). Stimulation of $I_{\text{Cl(Ca)}}$ upon hyperpolarization requires a preceding depolarization step, as A9C alone does not stimulate $\text{Cl}_{\text{Ca}}$ channels (153).
We tested the effects of A9C on \( I_{\text{Cl(Ca)}} \) elicited by 500 nM \([\text{Ca}^{2+}]_i\) in rabbit PASMCs dialyzed with either 5 mM ATP to support global phosphorylation, or in the absence of ATP to promote dephosphorylation of the \( \text{Cl}_{\text{Ca}} \) channel or a regulatory protein (106). Similarly to previous studies involving NFA, block of \( I_{\text{Cl(Ca)}} \) by A9C at positive potentials was markedly reduced in conditions favoring global phosphorylation. Alternatively, stimulation of the inward tail current at negative potentials following very positive step depolarizations was enhanced by A9C under these conditions. These results provide further evidence that the state of phosphorylation of the pore-forming or regulatory subunit of \( \text{Cl}_{\text{Ca}} \) channels significantly influences their pharmacology.

3.3 Materials and Methods

3.3.1 Isolation of Pulmonary Artery Myocytes

A similar method to that previously used by our group was used to isolate smooth muscle cells (106, 108). In brief, cells were prepared from the main and secondary pulmonary arterial branches dissected from New Zealand white rabbits (2-3 kg) killed by anesthetic overdose in accordance with British and American guidelines for animal care. The animals were allowed free access to food and water and kept on a 12-12 h light/dark cycle until the day of being killed. The University of Nevada Institutional Animal Care and Use Committee approved the experimental protocol. After dissection and removal of connective tissue, the pulmonary arteries were cut into small strips and incubated overnight (~ 16 hours) at 4°C in a low \( \text{Ca}^{2+} \) physiological salt solution (PSS; see composition below) containing either 10 or 50 \( \mu \text{M} \) \( \text{CaCl}_2 \) and ~ 1 mg ml\(^{-1}\) papain, 0.15 mg ml\(^{-1}\) dithiothreitol and
1 mg ml⁻¹ bovine serum albumin. The next morning, the tissue strips were rinsed three times in low Ca²⁺ PSS and incubated in the same solution for 5 min at 37°C. Cells were released by gentle agitation with a wide bore Pasteur pipette, and then stored at 4°C until used (within 10 h following dispersion).

3.3.2 Whole-Cell Patch Clamp Electrophysiology

Ca²⁺-activated Cl⁻ currents were elicited using the conventional whole-cell configuration of the patch clamp technique with a pipette solution containing either no ATP, or 5 mM ATP. The pipette solution also contained 10 mM BAPTA as the Ca²⁺ buffer and free [Ca²⁺] was set to 500 nM by the addition of 7.08 mM CaCl₂. The free [Ca²⁺] was estimated by the calcium chelator program WinMaxC (v. 2.50; http://www.stanford.edu/~cpatton/downloads.htm). Using a Ca²⁺-sensitive electrode and calibrated solutions, the total amounts of CaCl₂ and MgCl₂ calculated by the software were previously shown to yield accurate free Ca²⁺ concentrations with both EGTA and BAPTA as Ca²⁺ buffers (106, 108, 109). Contamination of I_{Cl(Ca)} from other types of current was minimized by the use of CsCl and tetraethylammonium chloride (TEA) in the pipette solution, and TEA in a K⁺-free external solution. Data for each group were collected in cells from at least two animals but generally more.

3.3.3 Experimental Protocols

I_{Cl(Ca)} was evoked immediately upon rupture of the cell membrane and its voltage-dependent properties monitored every 5 or 10 s by stepping from a holding potential (HP)
of –50 mV to +90 mV for 1 s, followed by repolarization to –80 mV for 1 s. Current-voltage (I-V) relationships were constructed after 10 min of cell dialysis by stepping in 10 mV increments from HP to test potentials between –100 mV and +140 mV for 1 s, each step followed by a constant 1 s repolarizing pulse to -80 mV to record the inward tail current. For I-V relationships, \( I_{\text{Cl(Ca)}} \) was expressed as current density (pA/pF) by dividing the amplitude of the current measured at the end of the voltage clamp step or at the beginning of the repolarizing step by the cell capacitance. For all figure panels showing a time course of \( I_{\text{Cl(Ca)}} \) changes, the late current measured at +90 mV was normalized to the amplitude of the first current elicited at time=0 (~ 30 s after breaking the seal and measuring cell capacitance). After measuring the cell capacitance, the constant step protocol described above was started to monitor the changes of \( I_{\text{Cl(Ca)}} \) over the course of 10 min after which a control I-V relationship was obtained. While monitoring \( I_{\text{Cl(Ca)}} \) elicited by a similar constant step protocol described above, the external solution was switched to one containing A9C at a concentration of 1, 10, 100, 250, or 500 \( \mu \)M. During these experiments, cells were stepped from the HP to +80 mV for 1 s before being repolarized for 1 s to -80 mV. After a steady-state effect was noticed, another I-V relationship was obtained in the presence of A9C. If the seal was still stable, washout was subsequently initiated and an I-V curve generated after washout. Only one concentration of A9C was tested per cell.

### 3.3.4 Solutions and Reagents

Single pulmonary artery smooth muscle cells were isolated by incubating pulmonary artery tissue strips in the following low Ca\(^{2+}\) (10 or 50 \( \mu \)M) PSS (in mM): NaCl (120), KCl (4.2),
NaHCO₃ (25; pH 7.4 after equilibration with 95% O₂, 5% CO₂ gas), KH₂PO₄ (1.2), MgCl₂ (1.2), glucose (11), taurine (25), adenosine (0.01) and CaCl₂ (0.01 or 0.05). The K⁺-free bathing solution used in all patch clamp experiments had the following composition (in mM): NaCl (126), HEPES-NaOH (10, pH 7.35), TEA (8.4), glucose (20), MgCl₂ (1.2) and CaCl₂ (1.8). The pipette solution had the following composition (in mM): TEA (20), CsCl (106), HEPES-CsOH (10, pH 7.2), BAPTA (10), ATP·Mg (0 or 5) and GTP·diNa (0.2). To this solution, the following total amounts of CaCl₂ and MgCl₂ were added to set free [Mg²⁺] at 0.5 mM and free [Ca²⁺] at desired levels: 5 mM ATP and 500 nM Ca²⁺ (in mM): CaCl₂ (7.08), MgCl₂ (3.0); no ATP and 500 nM Ca²⁺: CaCl₂ (7.08), MgCl₂ (0.545). All enzymes, analytical grade reagents and A9C were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anthracene-9-carboxylic acid was initially prepared as a 500 mM stock in dimethyl sulfoxide (DMSO) and an appropriate aliquot was added to the external solution to reach the final desired concentration. The maximal concentration of DMSO never exceeded 0.1%, a concentration that had no effect on ICl(Ca).

3.3.5 Statistical Analysis

All data were pooled from n cells taken from at least two animals with error bars representing the s.e.m. All data were first pooled in Excel and means exported to Origin 7.5 software (OriginLab Corp. Northampton, MA, U.S.A.) for plotting and curve fitting. All graphs and current traces were exported to CorelDraw 12 (Ottawa, Ontario, Canada) for final processing of the figures. Origin 7.5 software (OriginLab Corp. Northampton, MA, U.S.A.) was also used to determine the statistical significance between two groups using a paired or
unpaired Student’s t test, or one-way ANOVA test followed by Bonferroni post-hoc multiple range tests in multiple group comparisons. $P < 0.05$ was considered to be statistically significant.

3.4 Results

3.4.1 Impact of General Phosphorylation Status on $I_{\text{Cl(Ca)}}$

Figure 1A shows representative traces of $I_{\text{Cl(Ca)}}$ recorded at different times following seal rupture (black trace recorded at $T = 0s$, blue trace recorded at $T = 600 s$) from two sample experiments. Cells were dialyzed for 10 min with either a 5 mM ATP (left traces) or ATP-free (right traces) pipette solution containing 500 nM free calcium. $I_{\text{Cl(Ca)}}$ was evoked by 1 s depolarizations to +90 mV from a HP of -50 mV, followed by a 1 s repolarizing step to -80 mV before returning to the HP. This protocol resulted in an initial instantaneous membrane current followed by a slow time-dependent component upon depolarization to +90 mV. Repolarizing to -80 mV yielded a slow tail current indicative of channel closure by voltage-dependent deactivation. This is consistent with previous studies by our group (106, 107, 108, 126), as well as with chapter 2 of this dissertation. $I_{\text{Cl(Ca)}}$ exhibited significant rundown during the first ~100 s when dialyzed with 5 mM ATP, with the current remaining suppressed throughout the remainder of the 10 min experiment. The cell dialyzed with ATP-free pipette solution also demonstrated rundown of $I_{\text{Cl(Ca)}}$ but the magnitude of rundown was significantly reduced when compared with 5 mM ATP. $I_{\text{Cl(Ca)}}$ began to recover after the initial rundown. This recovery was complete after 10 min with $I_{\text{Cl(Ca)}}$ amplitude even slightly exceeding the initial current level measured immediately after seal rupture. The initial
Figure 1. Attenuation of rundown of Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} currents in rabbit pulmonary artery myocytes by minimizing phosphorylation. A) Representative current traces demonstrate the time-dependent changes of Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} current recorded from pulmonary arterial smooth muscle cells dialyzed with either 5 mM ATP or 0 mM ATP. Currents depicted were recorded immediately following breaking the seal (0 s, black trace), and after 10 min (600 s, blue trace) of cell dialysis. The currents were elicited by repetitive 1 s step depolarizations (every 5 s) to +90 mV from a holding potential (HP) of -50 mV, as depicted. Each depolarizing pulse was followed by a repolarizing step to -80 mV to enhance the magnitude of the tail current. B) This graph depicts the mean time course of I_{Cl(Ca)} amplitude elicited by 1-s depolarizing pulses to +90 mV, followed by 1-s repolarizing steps to -80 mV, in the presence of 5 mM ATP (filled black squares, n = 27), or in the absence of ATP (filled red circles, n = 28). Currents were normalized to the initial current amplitude at the beginning of the protocol. Each step was applied from HP = -50 mV at a frequency of one pulse every 5 s for 10 min. The group dialyzed with 0 ATP was significantly different from 5 mM ATP with P < 0.001 (one-way ANOVA).
rundown with ATP-free solution is possibly due to a partial state of phosphorylation of the pore-forming subunit or an unknown regulatory protein due to consumption of endogenous ATP at the time of seal rupture. Figure 1B depicts mean data from 27 or 28 cells dialyzed with 5 ATP or ATP-free solution, respectively. The amplitude of \( I_{\text{Cl(Ca)}} \) for each group was recorded at the end of the depolarizing step to +80 mV and was normalized to that of the initial current recorded at time = 0. As with the representative traces described above and shown in Figure 1A, \( I_{\text{Cl(Ca)}} \) displayed significant rundown after seal rupture with 5 mM ATP, declining to ~40% of the initial current level after 100 s. This diminished current level was maintained throughout the remainder of the experiment. Removing ATP from the pipette attenuated the rundown and resulted in a complete recovery of the current. Removal of ATP from the pipette solution slowed the time constant of deactivation from 61.75 ± 3.17 ms (\( n = 27 \)) to 90.93 ± 5.74 ms (\( n = 29; \ P < 0.01 \)), with 5 mM ATP and no ATP, respectively. Meanwhile, activation kinetics tended to be slower when cell were dialyzed with 5 mM ATP (360.41 ± 27.87 ms, \( n = 27 \)) compared to 0 ATP (312.92 ± 24.66, \( n = 29 \)), although this trend was not found to be of statistical significance. These effects of different ATP levels on \( I_{\text{Cl(Ca)}} \) are quite similar to those seen in Chapter 2 of this dissertation, as well as an earlier study by our group, where it was proposed that phosphorylation causes a state-dependent block of the underlying channels (106). These experiments were conducted prior to the addition of A9C in order to establish the characteristics of \( I_{\text{Cl(Ca)}} \) of rabbit PASMCs when phosphorylation is either promoted or minimized.
3.4.2 The State of Phosphorylation Alters Both A9C-induced Block and Stimulation of $I_{\text{Cl(Ca)}}$

In 2003, Piper and Greenwood (153) reported that A9C produced a small inhibition of the outward Cl$^-$ current upon depolarization to +70 mV, but increased the amplitude of the instantaneous inward relaxation at -80 mV. The next series of experiments were carried out in order to determine whether these effects of A9C on $I_{\text{Cl(Ca)}}$ are altered upon varying the global state of phosphorylation. Following the initial 10 min dialysis with either 5 mM ATP or ATP-free pipette solution and recording an $I-V$, $I_{\text{Cl(Ca)}}$ amplitude was monitored over another 10 min during which the effects of A9C were tested. Figure 2 displays plots the time course of changes of mean normalized $I_{\text{Cl(Ca)}}$ recorded at the end of a 1 s depolarization to +80 mV (left column) and $I_{\text{Cl(Ca)}}$ tail upon repolarization to -80 mV (right column). Approximately 60 s into this second test period, the extracellular bath solution was switched to one containing A9C at one of the following concentrations; 1, 10, 100, 250, or 500 µM A9C. Only one concentration of A9C was tested per cell. Application of 1 µM A9C resulted in minimal block in both groups (16.18 ± 2.12 %, $n$ = 4 and 14.10 ± 2.55 %, $n$ = 5 for cells dialyzed with 5 mM ATP and 0 ATP respectively) upon depolarization to +80 mV. Block in both data sets occurred with a similar time course. At this concentration, no stimulation of $I_{\text{Cl(Ca)}}$ was apparent following repolarizing to -80 mV. Block was similar with 10 µM A9C (7.43 ± 8.28 %, $n$ = 5 and 13.59 ± 11.49 %, $n$ = 5 for cells dialyzed with 5 mM ATP and 0 ATP respectively) however unlike 1 µM A9C, 10 µM A9C resulted in stimulation of the tail current in cells dialyzed with both 5 mM ATP and 0 ATP (27.80 ± 7.92 %, $n$ = 5 and 28.89 ± 9.67 %, $n$ = 5, respectively). Both 100 and 250 µM A9C markedly blocked $I_{\text{Cl(Ca)}}$ of cells dialyzed with 0 ATP (43.65 ± 6.79 %, $n$ = 6 and 71.27 ± 4.43 %, $n$ = 4 for 100 and 250 µM, respectively), which
was significantly greater than in cells dialyzed with 5 mM ATP (7.25 ± 7.52 %, \( n = 5 \) and 19.79 ± 13.15 %, \( n = 7 \) for 100 and 250 µM, respectively; \( P < 0.05 \)). Stimulation by these concentrations on the other hand, remained similar between the two groups, any slight differences failing to be statistically significant (100 µM A9C: 113.01 ± 15.60 %, \( n = 5 \) and 89.55 ± 25.94 %, \( n = 6 \) for 5 mM ATP and 0 ATP respectively; 250 µM A9C: 88.54 ± 9.04 %, \( n = 7 \) and 79.27 ± 32.78 %, \( n = 4 \) for 5 mM ATP and 0 ATP respectively). While the time course of stimulation by 250 µM A9C appeared to differ between cells dialyzed with 5 mM ATP vs. 0 ATP, we do not believe this effect to be real as it may have been attributed to variations in flow rate of the A9C-containing bath solution. Increasing the concentration of A9C to 500 µM greatly increased the block of \( I_{\text{Cl(Ca)}} \) in cells dialyzed with 5 mM ATP (33.75 ± 13.21 % block, \( n = 4 \)), although block was still significantly greater with 0 ATP (66.27 ± 3.57 % block, \( n = 5, P < 0.05 \)). The only concentration of A9C to produce a significant difference in stimulation of the tail current at -80 mV was seen with 500 µM A9C (190 ± 46.80 %, \( n = 4 \) and 59.71 ± 11.39 % stimulation, \( n = 5 \) for 5 mM ATP and 0 ATP respectively, \( P < 0.05 \)). Interestingly, stimulation of \( I_{\text{Cl(Ca)}} \) at -80 mV in cells dialyzed with ATP-free solution exhibited a rapid increase to a peak with both 250 and 500 µM A9C (112.23 ± 31.59 %, \( n = 4 \) and 101.13 ± 13.42 % stimulation, \( n = 5 \), respectively) before decreasing and stabilizing at a slightly lower level (79.27 ± 32.78 and 59.71 ± 11.39 % stimulation). The time course of activation was significantly slowed by intermediate concentrations of A9C (10 µM: \( \tau = 292.93 ± 29.69 \) ms to 442.12 ± 56.11 ms, \( n = 5 \); \( P < 0.01 \); 100 µM A9C: 264.38 ± 16.66 ms to 346.68 ± 23.14 ms, \( n = 6 \); \( P < 0.001 \)) in cells dialyzed with 0 ATP, while \( \tau \) of activation was
Step to +80 mV

A9C

1 µM A9C

10 µM A9C

100 µM A9C

250 µM A9C

500 µM A9C

Tail at -80 mV

A9C

5 mM ATP

0 ATP

5 mM ATP

0 ATP

5 mM ATP

0 ATP

5 mM ATP

0 ATP

5 mM ATP

0 ATP

5 mM ATP

0 ATP

5 mM ATP

0 ATP
Figure 2. Effect of phosphorylation on the ability of A9C to both block and stimulate $I_{Cl(Ca)}$. Time courses representing mean data from pulmonary artery smooth muscle cells dialyzed with pipette solutions containing either 5 mM ATP (black squares) or an ATP-free solution (red circles). Currents were measured at the end of 1 s depolarizing steps to +80 mV from HP = -50 mV (left column) and immediately following the capacitance spike of a hyperpolarizing step to -80 mV from +80 mV (right column). Currents were plotted against time over a 10 min period of dialysis. Following current stabilization, A9C was applied to the cells at concentrations of 1 (5mM ATP $n = 5$; 0 ATP $n = 5$), 10 (5mM ATP $n = 5$; 0 ATP $n = 6$), 100 (5mM ATP $n = 6$; 0 ATP $n = 8$), 250 (5mM ATP $n = 8$; 0 ATP $n = 4$), and 500 µM (5mM ATP $n = 5$; 0 ATP $n = 5$), as indicated by the filled bar. * indicates significant difference between 0 ATP and 5 ATP groups, with $P < 0.05$ (one-way ANOVA).
only slowed in cells dialyzed with 5 mM ATP by 100 µM A9C ($\tau = 284.52 \pm 40.24$ ms to $391.04 \pm 42.16$ ms, $n = 5$; $P < 0.05$). Other groups demonstrated a tendency for A9C to increase $\tau$, although the effects were not statistically significant. Taken together, these data suggest that phosphorylation limits the ability of A9C to block $I_{Cl(Ca)}$ at depolarized potentials, while enhancing the ability of the drug to stimulate the current upon repolarizing the cell.

3.4.3 The Voltage-Dependence of Block by A9C is Shifted by Dephosphorylation

The voltage dependence of block by A9C under conditions either promoting or minimizing phosphorylation was then determined, as it has been shown in chapter 2 that the voltage-dependence of block by NFA is altered by the state of global phosphorylation. Figure 3 shows typical families of $I_{Cl(Ca)}$ recorded from the same cell in control (top) and after exposure to 500 µM A9C for 10 min in cells dialyzed with either 5 mM ATP (left) or ATP-free (right) pipette solution. Currents were generated by stepping in 10 mV increments from HP to 1 s test potentials between -100 to +140 mV followed by a 1s repolarizing step to -80 mV before returning to the HP. As observed in previous studies (106, 107, 108, 109, 120) and chapter 2 of this dissertation, control currents (before A9C) recorded with 5 mM ATP (Figure 3 left) were notably smaller (note different calibration bars), and displayed slower activation and faster deactivation kinetics than cells dialyzed with an ATP-free solution (Figure 3 right). This is consistent with phosphorylation-induced changes in $Cl_{Ca}$ channel gating. At a concentration of 500 µM, A9C blocks $I_{Cl(Ca)}$ with both 5 mM ATP and 0 ATP (Figure 3). Although A9C tended to slow the time constant of deactivation this effect was not
significant, suggesting that A9C rapidly dissociates from the channel. Figure 4 reflects the
mean I-V relationships of I_{Cl(Ca)} density recorded at the end of the 1s test pulse before and
after a 10 min exposure to A9C at the noted concentration in cells dialyzed with either 5
mM ATP (black unfilled squares = before A9C; black filled squares = with A9C) or 0 ATP (red
unfilled circles = before A9C; red filled circles = with A9C). A9C blocked time-dependent
outward I_{Cl(Ca)} (>0 mV) in both groups, although the block was greater with 0 ATP. Block in
both groups was voltage-dependent, with dephosphorylation resulting in a negative shift in
the voltage-dependence, as evident from the leftward shift in the potential where block is
first evident. The negative shift in the voltage-dependence of block was also concentration-
dependent, being more pronounced with A9C concentrations. This effect was evident in
both groups.

In order to better demonstrate the shift in the voltage-dependence of A9C-induced
block, the intensity of block (estimated as a % of control) by A9C of steady-state I_{Cl(Ca)} was
plotted as a function of voltage (Figure 5). Data sets were fitted with either a linear fit or by
a simple Boltzmann relationship (see table found in Figure5 for slope or V_{0.5} values). The
negative shift resulting from dephosphorylation was largest with 250 µM A9C, causing a
shift in V_{0.5} of ~ -80 mV. V_{0.5} for cells dialyzed with 0 ATP became less positive as A9C
concentration was increased (10 µM A9C: 101.44 ± 2.41 mV, n = 5; 100 µM A9C: 71.80 ±
1.18 mV, n = 6; 250 µM A9C: 48.45 ± 3.42 mV, n = 4; 500 µM A9C: 47.15 ± 17.04 mV, n = 5),
clearly demonstrating that the negative shift is concentration-dependent. Such a clear trend
was not evident with 5 mM ATP, as V_{0.5} fluctuated from 107.43 ± 19.67 mV with 100 µM
Figure 3. Representative I-V traces demonstrating block and stimulation of $I_{\text{Cl(Ca)}}$ by A9C. Two representative families of currents from a cell dialyzed with 5 mM ATP, before (upper left) and after (lower left) a 10 min application of 500 μM A9C. To the right are currents from a cell dialyzed with 0 ATP before (upper) and after (lower) application of 500 μM A9C. Currents were evoked by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV (see protocol representation) following a 10 min period of dialysis.
Figure 4. The voltage dependence of $I_{Cl(Ca)}$ block by A9C is altered by phosphorylation levels. Mean current-voltage relationships generated from families of currents elicited by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV (see protocol representation) after 10 minutes of cell dialysis with 5 mM ATP before (open black squares) and after 10 min with the addition of 1, 10, 100, 250, or 500 μM A9C to the external solution (filled black squares: 1 μM A9C n = 5; 10 μM A9C n = 5; 100 μM A9C n = 6; 250 μM A9C n = 8; 500 μM A9C n = 5), or after 10 minutes of cell dialysis with 0 ATP before (open red circles) and after 10 min with the addition of 1, 10, 100, 250, or 500 μM A9C to the external solution (filled red circles: 1 μM A9C n = 5; 10 μM A9C n = 6; 100 μM A9C n = 8; 250 μM A9C n = 4; 500 μM A9C n = 5). Please note the negative shift in voltage where block begins to occur when phosphorylation is minimized, particularly in the range of 10 to 250 μM A9C.
(n = 5) to 130.79 ± 2.81 mV with 250 µM A9C (n = 7) before decreasing to 99.09 ± 3.15 mV with 500 µM A9C (n = 4). Phosphorylation clearly alters the voltage-dependence of block by A9C.

### 3.4.4 Dephosphorylation Alters the Stimulation of $I_{\text{Cl(Ca)}}$ Tail Current by A9C

Due to the observation that A9C increases the amplitude of the inward relaxation at -80 mV following depolarization, our next series of experiments examined whether phosphorylation had any effect on this stimulatory effect. Mean I-V relationships of $I_{\text{Cl(Ca)}}$ tail density recorded upon repolarization to -80 mV from the voltage-steps described in Figure 4 were plotted and are presented in Figure 6. It is important to keep in mind that these currents were all measured at -80 mV, and the voltages shown are those of the preceding depolarization. Tail currents in cells dialyzed with ATP-free solution (control: red unfilled circles; with A9C: red filled circles) are larger than those of cells dialyzed with 5 mM ATP (control: black unfilled squares; A9C: black filled squares), which like the larger amplitude of the outward current with 0 ATP, is consistent with state-dependent block of $\text{Cl}_{\text{Ca}}$ channels by phosphorylation. No stimulation of the $I_{\text{Cl(Ca)}}$ tail current was evident in either cell group with either 1 or 10 µM A9C. Beginning with addition 100 µM A9C, stimulation was apparent with both 5 mM ATP and 0 ATP, and displays a slight leftward shift. This trend continued with application of both 250 and 500 µM. However unlike with 0 ATP where the stimulation appeared sigmoidal, with greater stimulation following a depolarization to +60 mV than +140 mV, $I_{\text{Cl(Ca)}}$ tail in cells dialyzed with 5 mM was stimulated progressively following all depolarizations greater than ~ +40 mV. Additionally, while A9C resulted in a consistent leftward shift among all stimulation-inducing A9C concentrations with 0 ATP, this shift was
Figure 5. Altering global phosphorylation status shifts the voltage-dependence of the blocking effect of A9C on $I_{\text{Cl(Ca)}}$. The two data sets for each concentration of A9C were obtained after 10 min of cell dialysis with either 5 mM ATP (black squares) or ATP-free pipette solution (red circles) before and after addition of 500 μM A9C. Currents were elicited by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV and measured at the end of the 1 s test step. % block was determined at each individual voltage step for each cell using the formula 100 - ($I_{\text{A9C}} / I_{\text{Control}}$)*100, where $I_{\text{A9C}}$ and $I_{\text{Control}}$ is the current recorded in the presence and absence of A9C, respectively. For $I_{\text{Control}}$, the mean of the first five traces was used in the calculation, while for $I_{\text{A9C}}$ the mean of the last 5 stable traces was used. Stimulation is manifest as a negative value using the above formula and is graphed as such. Mean values were then plotted at each voltage, and fitted to a Boltzmann function. Parameters estimated from these fits are presented in the table of this figure.
concentration-dependent in cells where phosphorylation was promoted with 5 mM ATP. Similarly to the effect of A9C on the time course of activation, both 10 and 100 µM A9C significantly slowed deactivation kinetics with 0 ATP (10 µM A9C: 83.92 ± 5.60 ms to 103.32 ± 7.30 ms, n = 5, P < 0.05; 100 µM A9C: 79.42 ± 3.51 ms to 95.04 ± 4.76 ms, n = 6, P < 0.01). The time course of deactivation for cells dialyzed with 5 mM ATP, and all other concentrations of A9C in those dialyzed with 0 ATP, remained quite similar. This suggests that in most cases A9C rapidly dissociates from its binding site (see Discussion).

The effect of phosphorylation on the stimulatory effect of A9C is more readily apparent in Figure 7, where the stimulation of $I_{Cl(Ca)}$ tail by A9C upon repolarization (as %) was plotted as a function of voltage. As previously described, 1 and 10 µM had no or little effect on $I_{Cl(Ca)}$ tail, although 10 µM A9C may have slightly stimulated $I_{Cl(Ca)}$ tail in cells dialyzed with 5 mM ATP following large positive depolarizations. As was seen in Figure 6, the state of phosphorylation dramatically altered the stimulatory effect of A9C on $I_{Cl(Ca)}$ tail. Stimulation was greatly reduced when a state of dephosphorylation is supported. Under these conditions, stimulation by A9C reached a peak at intermediate voltage steps (e.g. ~ +60 mV with 100 µM A9C) then declined at more positive potentials until the stimulatory effect disappeared after depolarizing steps to +140 mV. Interestingly, while the magnitude of the stimulation remained relatively constant (~ 60 to 75%), the voltage corresponding to the peak in stimulation appeared to slightly shift towards the right with increasing [A9C]. Unfortunately a Boltzmann function did not fit these data well. Thus a Lorentzian fit was used, which does not provide any important parameters (ex: $V_{0.5}$). When dialyzed with 5 mM ATP, a large stimulation of $I_{Cl(Ca)}$ was apparent with 100, 250, and 500 µM A9C. As for
Figure 6. The voltage dependence of $I_{Cl(Ca)}$ stimulation by A9C is altered by phosphorylation levels. Families of currents were generated by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV, followed by a hyperpolarizing step to -80 mV, where currents were measured immediately following the capacitative spike (see protocol representation). Mean current-voltage relationships were calculated from these families of currents are plotted against time after 10 minutes of cell dialysis with 5 mM ATP before (open black squares and after 10 min with the addition of 500 µM A9C to the external solution (filled black squares: 1 µM A9C n = 5; 10 µM A9C n = 5; 100 µM A9C n = 6; 250 µM A9C n = 8; 500 µM A9C n = 5), or after 10 minutes of cell dialysis with 0 ATP before (open red circles) and after 10 min with the addition of 500 µM A9C to the external solution (filled red circles: 1 µM A9C n = 5; 10 µM A9C n = 6; 100 µM A9C n = 8; 250 µM A9C n = 4; 500 µM A9C n = 5). Please note the negative shift in voltage where block begins to occur when phosphorylation is minimized, particularly in the range of 10 to 250 µM A9C.
Figure 7. Altering global phosphorylation status shifts the voltage-dependence of the stimulatory effect of A9C on $I_{\text{Cl(Ca)}}$. The two data sets for each concentration of A9C were obtained after 10 min of cell dialysis with either 5 mM ATP (black squares) or ATP-free pipette solution (red circles) before and after addition of 500 μM A9C. Currents were elicited by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV, followed by a hyperpolarizing step to -80 mV, where currents were measured immediately following the capacitative spike. % stimulation was determined at each individual voltage step for each cell using the formula $(I_{\text{A9C}}/I_{\text{Control}})*100$-100, where $I_{\text{A9C}}$ and $I_{\text{Control}}$ is the current recorded in the presence and absence of A9C, respectively. For $I_{\text{Control}}$, the mean of the first five traces was used in the calculation, while for $I_{\text{A9C}}$ the mean of the last 5 stable traces was used. Block is manifest as a negative value using the above formula and is graphed as such. Mean values were then plotted at each voltage, and fitted to a Boltzmann function wherever possible, otherwise a Lorentzian fit was used to more easily visualize the data. Parameters estimated from these fits are presented in the table of this figure.
the stimulation in cells dialyzed with 0 ATP, the magnitude of peak stimulation remained quite constant for all three drug concentrations (~ 200 to 250 %), considering the standard error of the measurements. However, phosphorylation resulted in a leftward shift in the voltage-dependence of stimulation. Stimulation by A9C was well fitted by a simple Boltzmann function in cells exposed to 10, 100, 250, and 500 µM, which revealed a shift in the V_{0.5} of stimulation (92.21 ± 8.18 mV, 98.94 ± 1.75 mV, 75.5 ± 1.29 mV, and 64.36 ± 1.26 mV, respectively). These experiments clearly show that global phosphorylation enhances the ability of A9C to stimulate the I_{Cl(Ca)} tail following depolarizing steps, and that this effect is both voltage- and concentration-dependent, where the effect of high concentrations is not to further increase the tail current, but rather to shift the voltage-dependence to more negative potentials.

### 3.4.5 Concentration- and Voltage-Dependence of the Dual Effects of A9C on I_{Cl(Ca)}

While the data described above demonstrate a shift in voltage-dependence and concentration-dependence for both the blocking and stimulatory effects, Figure 8 eases visualization of these changes. Mean % block at +40 mV, +90 mV, and +140 mV (left column) and % stimulation of the tail current following these depolarizing steps (right column) are pictured. Currents were elicited and measurements taken as described in Figures 5 & 7. At +40 mV, no block of I_{Cl(Ca)} was apparent until the concentration of A9C was raised to 250 µM in cells dialyzed with 0 ATP (35.68 ± 13.12 % block, n = 4). Increasing the concentration of drug to 500 µM did not increase the amplitude of block in these cells at +40 mV (35.10 ± 11.77 % block, n = 5), but did result in block of I_{Cl(Ca)} in cells dialyzed with 5 ATP to levels
comparable to those with 0 ATP (31.00 ± 10.55 % block, n = 4). Further depolarization to +90 mV resulted in block of $I_{\text{Cl(Ca)}}$ by all concentrations of A9C tested. Block was significantly greater in cells dialyzed with the ATP-free pipette solution with 100 µM A9C (7.20 ± 7.32 % n = 5, and 58.52 ± 2.94 n = 6 for 5 mM ATP and 0 ATP respectively, $P <0.001$) and 250 µM A9C (18.29 ± 14.22 % n = 7, and 80.04 ± 4.02 % n = 4 for 5 mM ATP and 0 ATP respectively, $P <0.05$). Similar results were found for steps to +140 mV, although block at all concentrations was elevated compared to +90 mV. Again, block of $I_{\text{Cl(Ca)}}$ by 100 and 250 µM was significantly greater when global phosphorylation was minimized.

Stimulation of inward tail currents by A9C recorded following depolarization to +40 mV, +90 mV, or +140 mV showed distinct voltage- and concentration-dependence (Figure 8, right column). Following depolarization to +40 mV, the tail current of cells dialyzed with 0 ATP tended to be more highly stimulated across the range of A9C concentrations tested, although this effect never reached statistical significance. The effect was opposite with depolarization to +90 mV. With concentrations of A9C of 100 µM and higher, stimulation was greatest when phosphorylation was supported. The difference in stimulation magnitude was significant with 500 µM A9C (216.62 ± 62.22 %, n = 4, and 60.90 ± 26.13 %, n = 5, for 5 mM ATP and 0 ATP respectively). Following depolarization to +140, $I_{\text{Cl(Ca)}}$ tail of cells dialyzed with 0 ATP appear to have lost nearly all stimulation, across the A9C concentration range tested. Cells dialyzed with 5 mM ATP on the other hand demonstrated significantly larger stimulation at the three highest drug concentrations (100 µM A9C: 212.88 ± 70.36 %, n = 5 and 1.57 ± 4.55 %, n = 6 $P <0.01$; 250 µM A9C: 187.37 ± 37.17 %, n = 7
Figure 8. Concentration- and voltage-dependence of the dual effect of A9C on I_{Cl(Ca)}. Cells were dialyzed with either 5 mM ATP (black bars), or 0 mM ATP (red bars). A9C was added for 10 min at concentrations of 1, 10, 100, 250, and 500 μM. Current was recorded at the end of 1 s depolarizing pulses to +40, +90, or +140 mV from HP of -50 mV (left column), or immediately following the capacitative spike during a 1 s hyperpolarizing step to -80 mV following the 1 s depolarizing pulses to +40, +90, or +140 mV (right column). % block for each cell was calculated by the formula 100−((I_{A9C}/I_{Control})*100), while % stimulation was calculated by ((I_{A9C}/I_{Control})*100)-100, where I_{A9C} and I_{Control} are the currents recorded in the presence and absence of A9C, respectively. For I_{Control}, the mean of the first five traces was used in the calculation, while for I_{A9C} the mean of the last 5 stable traces was used. Mean calculated values were then determined for each group (5 mM ATP: 1 μM A9C, n = 5, 10 μM A9C, n = 5; 100 μM A9C, 6 = X; 250 μM A9C, n = 8; 500 μM A9C, n = 5; 0 ATP: 1 μM A9C, n = 5; 10 μM A9C, n = 6; 100 μM A9C, n = 8; 250 μM A9C, n = 4, 500 μM A9C, n = 5) and graphed. * P < 0.05; ** P < 0.01; *** P < 0.001 (Unpaired Student t tests).
and 10.93 ± 18.79 %, n = 6 P <0.05; 500 µM A9C: 263.29 ± 110.50 %, n = 4 and 11.55 ± 14.32 %, n = 5 P <0.05).

These data demonstrate the impact of phosphorylation on the voltage- and concentration-dependence of both the inhibitory and stimulatory effects of A9C on I_{Cl(Ca)}.
Dephosphorylation appears to favor an interaction of A9C with an inhibitory binding site, while phosphorylation appears to favor an interaction of the drug with a second stimulatory binding site (see Discussion).

3.5 Discussion

This study presents further evidence that the state of phosphorylation of the Cl_{Ca} channel or a regulatory subunit affects the interaction of known Cl⁻ channel blockers with the channel in freshly isolated rabbit pulmonary arterial smooth muscle cells. As was seen with niflumic acid in Chapter 2 of this dissertation, I_{Cl(Ca)} evoked by dialysis with an elevated fixed intracellular Ca^{2+} concentration under conditions favoring either global phosphorylation or dephosphorylation dramatically altered the interaction of antracene-9-carboxylic acid with the underlying channel. Previous reports have demonstrated A9C (500 µM) to moderately block I_{Cl(Ca)} evoked by pipette solution containing 500 nM Ca^{2+} at +70 mV in rabbit pulmonary artery smooth muscle cells (153). However, upon repolarizing the cell from +70 mV to -80 mV, A9C significantly increased the amplitude of the instantaneous inward current (153). Similarly to NFA, promoting phosphorylation by inclusion of 5 mM ATP in the pipette solution reduced the ability of A9C to inhibit I_{Cl(Ca)} at +90 mV in a dose-dependent manner, whereas cells dialyzed with an ATP-free pipette solution exhibited
increased block that was accompanied by a leftward shift in the voltage-dependence of block. Stimulation of the inward $I_{\text{Cl(Ca)}}$ tail current by A9C upon repolarization of the cell to -80 mV was also impacted by the state of phosphorylation, albeit in a more complex manner. The voltage-dependence of $I_{\text{Cl(Ca)}}$ was shifted leftward by promoting dephosphorylation, but reached a peak amplitude following depolarizing steps from +50 to +70 mV. Stimulation of the inward relaxation then declined steadily following larger depolarizations until stimulation was virtually abolished after repolarization to -80 mV from +140 mV. Supporting phosphorylation resulted in stimulation by A9C reaching greater levels, an effect that was both voltage- and concentration-dependent.

3.5.1 Conditions Establishing Differential States of Phosphorylation

In order to determine the impact of phosphorylation on the interaction of A9C with $I_{\text{Cl(Ca)}}$ channels in rabbit PASMCs, it was necessary to establish that the underlying pore-forming or regulatory subunit was either phosphorylated (5 mM ATP) or dephosphorylated (0 ATP). $I_{\text{Cl(Ca)}}$ channels in airway and arterial myocytes are inactivated by Ca$^{2+}$-calmodulin and CaMKII, and may thus be considered subject to Ca$^{2+}$-dependent suppression (107, 123). Down-regulation of channel activity by CaMKII may then serve to attenuate the depolarizing influence of $I_{\text{Cl(Ca)}}$ channels during excitation (120). In rabbit coronary (109) and pulmonary artery (108), the kinase activity of CaMKII is opposed by the phosphatase calcineurin (CaN). A more recent study from our group postulated that PP1 (and possibly PP2A) is mediating the effects of CaN on $I_{\text{Cl(Ca)}}$, with the former likely acting downstream of CaN (126). Similarly to Chapter 2, significant rundown of $I_{\text{Cl(Ca)}}$ in the present study was apparent when phosphorylation was promoted by including 5 mM ATP in the pipette solution, while removal of ATP from the pipette solution resulted in complete
recovery of the current. These effects are likely due to the dominance of CaMKII activity in the presence of substrate, and a shift to greater CaN activity upon removal of ATP from the pipette. In support of this claim, the recovery of $I_{\text{Cl(Ca)}}$ in PA myocytes observed in the absence of ATP in the pipette solution was recently shown to be abrogated by the CaN inhibitor cyclosporine A (271). Moreover and as reported in Chapter 2, cells demonstrated faster activation at positive potentials and slower deactivation kinetics at negative potentials when phosphorylation was minimized. This is consistent with a recently proposed kinetic model whereby phosphorylation induces state-dependent block by altering voltage-dependent kinetic gating steps favoring channel closure at elevated intracellular Ca$^{2+}$ concentrations (106). Taken together, these data demonstrate that a significantly different global phosphorylation state was achieved in these cells prior to the addition of A9C.

3.5.2 Phosphorylation Inhibits Block of $I_{\text{Cl(Ca)}}$ by A9C at Depolarized Potentials While Potentiating Stimulation Upon Repolarization

A9C, a Cl⁻ channel blocker chemically dissimilar to NFA, inhibits spontaneous transient inward currents and $I_{\text{Cl(Ca)}}$ evoked by Ca$^{2+}$ entry via voltage-dependent Ca$^{2+}$ channels (132, 148, 149, 150, 151, 152). Like NFA, block by A9C is reversible and occurs with a time course similar to that required for a complete changeover of the bath solution, suggesting that A9C interacts on the extracellular side, perhaps near the pore in the vestibular region (147). Block by A9C at $+80 \text{ mV}$ was greater when phosphorylation was minimized by removing ATP from the pipette solution. This effect was concentration-dependent, saturating at 250 µM A9C. While never reaching the levels of block obtained with 0 ATP, A9C also blocked $I_{\text{Cl(Ca)}}$ in cells dialyzed with 5 mM ATP in a concentration-
dependent manner. This suggests that A9C blocks the open channel and that phosphorylation partially occludes access to a blocking site found at the mouth or within the pore. Block of the open channel had previously been proposed based on the observation that STIC amplitude decreased while the time course of decay was lengthened (147). A previous study reported only ~20% block of $I_{\text{Cl(Ca)}}$ by 500 µM A9C in rabbit PASMCs upon depolarization to +70 mV in the presence of 3 mM ATP (153), while we describe ~40% block at +80 mV with 5 mM ATP. Given that phosphorylation of the channel or regulatory subunit is likely to be greater with 5 vs. 3 mM ATP, and thus we might expect to see even less block if phosphorylation does disrupt drug access to an inhibitory binding site. However, block by A9C is highly voltage-dependent, with an IC$_{50}$ for inhibition of STICs of 300 µM at -50 mV and 90 µM at +90 mV (149). In fact in our study, very little block if any, was apparent at potentials below 0 mV (near $E_{\text{Cl}}$), even with 500 µM A9C and irrespective of the presence or absence of intracellular ATP. Therefore, it is possible that the greater depolarizing step employed by the present study may account for at least part of this discrepancy in block by A9C at positive potentials.

As described by Piper and Greenwood (153), A9C increased the amplitude of the $I_{\text{Cl(Ca)}}$ tail at -80 mV following a prior depolarization. Stimulation was concentration-dependent, and similar in cells dialyzed with either 5 mM ATP or 0 ATP, except in the presence of 500 µM A9C. At this concentration, minimizing phosphorylation resulted in decreased stimulation when compared to cells in which phosphorylation was supported, suggesting a phosphorylation-dependent effect on a stimulatory site distinct from the inhibitory site. This two-site model is further supported by the fact that block was
instantaneously relieved upon repolarizing the cell, leading to the stimulation of the inward tail current. It may be speculated that A9C binds to both a pore-associated inhibitory site and a stimulatory site away from the pore simultaneously. The effect of the block masks any stimulation, and the drug is quickly dislodged from the inhibitory site upon repolarization, unmasking stimulation. This theory is supported by the observation that A9C generally slows activation at positive potentials while having little or no effect on deactivation at negative potentials. Alternatively, A9C may increase single channel conductance upon binding to the above-mentioned stimulatory site. Block by A9C of this more conductive pore would still occur at depolarizing potential and upon repolarization A9C would be kicked out of the inhibitory site thus revealing the increased conductive state, essentially revealing an “apparent” inwardly rectifying behavior. Although this possibility could, at least in part, contribute to the enhanced conductance observed at negative potentials, it cannot explain the A9C-induced negative shift in voltage-dependence that is more consistent with an effect on channel gating. Future studies at the single channel level will help delineating the exact mechanism by which A9C stimulates \( I_{\text{Cl(Ca)}} \).

3.5.3 The State of Phosphorylation Alters the Voltage-Dependence of the Interaction of A9C with \( \text{Cl}_{\text{Ca}} \)

As mentioned above, block of STICs is highly voltage-dependent (149). We therefore tested whether the voltage-dependence of either block or stimulation, or both, was impacted by phosphorylation. Similarly to our previous report concerning NFA (Chapter 2), dephosphorylation resulted in a leftward shift in the voltage-dependence of block of \( I_{\text{Cl(Ca)}} \) by
A9C. This suggests that both NFA and A9C inhibit $I_{\text{Ca}}$ via a similar mechanism where CaMKII induced phosphorylation may occlude either a common or distinct inhibitory binding site(s) by voltage-dependent steric hindrance, or by reduced availability of the site through a state-dependent mechanism.

In order to evaluate the voltage-dependence of stimulation, enhancement of the $I_{\text{Cl(Ca)}}$ tail was measured at -80 mV following depolarizing steps to various potentials. It was observed that the degree of stimulation was dependent on the preceding depolarizing step. Dephosphorylation caused a leftward shift in the voltage-dependence of stimulation by A9C, although the shift was much smaller than that observed with block. Interestingly, stimulation under these conditions reached a peak following depolarizations lying between ~ +50 and +70 mV before decreasing until stimulation disappeared after stepping to +140 mV. While A9C would have uninhibited access to a stimulatory site away from the channel pore, more channels would be opened by greater depolarization, which would mean greater access to an inhibitory site within the pore. Thus, stimulation predominates following lesser depolarizations, whereas block remains and counteracts the stimulation following greater depolarization. This theory is supported by the shift in voltage-dependence of block at positive potentials described above.

With phosphorylation supported by inclusion of 5 mM ATP in the pipette solution, the peak in stimulation of the tail current by A9C appeared to be abolished, with stimulation increasing progressively in a voltage-dependent manner. In this case, it appears that that stimulation occurred exclusively. This may be due to poor access to the blocking site resulting from the phosphorylation-mediated occlusion of the inhibitory site previously described, despite large depolarizing steps favoring channel opening. There was a hint with 250 and 500 µM A9C of
peak stimulation being attained following depolarizations $\geq \sim +120 \text{ mV}$, but as it is difficult to maintain a good seal even at $+140 \text{ mV}$, further depolarizations were not attempted in order to determine if block would start to overcome stimulation.

While most studies concerning $I_{\text{Cl(Ca)}}$ typically employ NFA and DIDS, we set out to investigate the interaction of A9C with the underlying channel due to its unique dual effect on the current. As in our previous study of the impact of phosphorylation on the effects of NFA on $I_{\text{Cl(Ca)}}$, phosphorylation profoundly alters A9C interactions with $\text{Cl}_{\text{Ca}}$ channels. The results of these two studies provide further evidence for two drug binding sites, an inhibitory site associated with the channel pore and a stimulatory site removed from the pore, although it is very possible that both sites are unique to each drug especially the stimulatory site which produces very distinct stimulation patterns (110, 135, 153, 271). With the recent discovery that TMEM16A and TMEM16B form $\text{Ca}^{2+}$-activated $\text{Cl}^-$ channels with properties similar to native currents (220, 221, 222, 253, 261), both of which are expressed and play a role in generating $I_{\text{Cl(Ca)}}$ in pulmonary arterial smooth muscle cells (272), more structural data will hopefully become available soon, allowing for the identification of drug binding sites, and new insights into how phosphorylation impacts on these sites.
Chapter 4: The Cl\(_{\text{Ca}}\) Molecular Candidate TMEM16A in Pulmonary Artery

4.1 Summary

*Background and purpose:* The molecular identity of Ca\(^{2+}\)-activated Cl\(^-\) currents (I\(_{\text{Cl(Ca)}}\)) in arterial smooth muscle cells remains unclear. The discovery that TMEM16A carries a Cl\(^-\) current activated by elevations of intracellular Ca\(^{2+}\) has led to studies implicating this protein in I\(_{\text{Cl(Ca)}}\) of epithelial cells, as well as interstitial cells of Cajal in the murine gastrointestinal tract. We investigated the possibility that TMEM16A may form the Ca\(^{2+}\)-activated Cl\(^-\) channel of pulmonary artery smooth muscle cells.

*Experimental approach:* RT-PCR and immunocytochemistry were employed to identify TMEM16A in the pulmonary artery. A TMEM16A-eGFP fusion protein was then transiently transfected into HEK293 cell in order to investigate its biophysical properties, including pharmacology.

*Key results:* TMEM16A was identified in rat and mouse pulmonary artery. HEK293 cells over-expressing TMEM16A displayed large Cl\(^-\) currents when dialyzed with 500 nM free Ca\(^{2+}\). These currents displayed similar time- and voltage-dependence to native I\(_{\text{Cl(Ca)}}\) of rabbit pulmonary artery smooth muscle cells. Currents ran down nominally, although not to the extent of I\(_{\text{Cl(Ca)}}\) of the native rabbit pulmonary myocytes. The TMEM16A-dependent currents were sensitive to block by NFA and A9C, but demonstrated stimulation by A9C dissimilar from the native current. A mutation of threonine at position 610 to an alanine – a putative CaMKII phosphorylation site – did not affect rundown, although it did alter TMEM16A pharmacology; increasing block and stimulation by NFA and A9C, respectively.
Conclusions and implications: Our data indicate that TMEM16A is a strong candidate for the Cl\textsubscript{Ca} channels of pulmonary artery smooth muscle.

4.2 Introduction

Cell types from various tissues express Cl\textsuperscript{−} channels that are activated by cytosolic Ca\textsuperscript{2+} in the range of 200-500 nM, which may originate from the release of intracellular stores, or by the influx of Ca\textsuperscript{2+} through channels in the plasma membrane. These calcium-activated chloride (Cl\textsubscript{Ca}) channels were first described in *Xenopus* oocytes almost thirty years ago, where the depolarizing Cl\textsubscript{Ca} current (I\textsubscript{Cl(Ca)}) plays a key role in blocking polyspermy (66, 67). Since these early studies, I\textsubscript{Cl(Ca)} have been described in central and peripheral neuronal cell bodies, developing skeletal muscle, lacrimal gland cells, pituitary cells, cardiac muscle, and smooth muscle (69). Several important physiological functions have been associated with I\textsubscript{Cl(Ca)}, such as playing a role in olfactory, taste, and photo-transduction, regulating neuronal and cardiac excitability, smooth muscle contraction, and endothelial function, being responsible for Ca\textsuperscript{2+}-dependent Cl\textsuperscript{−} secretion in epithelial cells, contributing to agonist-induced retinal vasoconstriction, and as mentioned above, preventing polyspermy upon fertilization (70).

In vascular smooth muscle (VSM), the classic I\textsubscript{Cl(Ca)} has a small unitary conductance (1-3 pS) and exhibits outward rectification (120, 147). Activation of I\textsubscript{Cl(Ca)} in VSM by agonist-induced Ca\textsuperscript{2+} release from intracellular stores or Ca\textsuperscript{2+} influx across the plasma membrane results in membrane depolarization, contributing to the development of muscle contraction. Spontaneous Ca\textsuperscript{2+} release from ryanodine receptors, known as Ca\textsuperscript{2+} sparks, may
also stimulate activation of VSM $\text{Cl}_{\text{Ca}}$ (120, 147). These highly localized calcium signals result in spontaneous transient inward Cl$^-$ currents (STICs) carried by $\text{Cl}_{\text{Ca}}$ channels, which are thought to contribute to the maintenance of vascular tone.

Despite their importance in many essential physiological functions, the molecular identity of $\text{Cl}_{\text{Ca}}$ channels has remained a contentious issue in the literature. Recently, the TMEM16A family has been proposed as new candidates for $\text{Cl}_{\text{Ca}}$ channels (220, 221, 222). Both TMEM16A and TMEM16B have been found to carry a $\text{Ca}^{2+}$-activated Cl$^-$ current when heterologously expressed (220, 221, 222, 253). Additionally, channels resulting from several TMEM16A splice variants possess varying biophysical properties (220, 258). While segment ‘a’ (116 residues) is often present, the alternatively spliced exons that code for segments ‘b’ (22 residues), ‘c’ (4 residues), and ‘d’ (26 residues) are differentially expressed in various tissues (258). While segment ‘d’ did not appear to alter TMEM16A channel activity in any particular manner (220), segment ‘b’ was found to influence $\text{Ca}^{2+}$ sensitivity and segment ‘c’ altered channel voltage-dependence. Splice variants possessing different biophysical properties may account for some of the variety in $I_{\text{Cl(Ca)}}$ of native tissues.

This study was designed to investigate the possibility that TMEM16A carries the native $I_{\text{Cl(Ca)}}$ in pulmonary artery smooth muscle cells (PASMCs), a cell type extensively studied by our group (106, 109, 110, 126), Chapters 2 and 3 of this dissertation. Using RT-PCR, Western Blot analysis, and immunocytochemistry, TMEM16A was identified in PASMCs. TMEM16A(a) (lacking segments ‘b’, ‘c’, and ‘d’, termed TMEM16A for the remainder of this chapter for simplicity) was then expressed in HEK293 cells so that the properties of the expressed currents could be compared to those of the native currents. In
particular, the pharmacology of the expressed channels and their regulation by phosphorylation were evaluated and compared. As we have previously shown, phosphorylation appears to result in a state-dependent block of PASMC Cl\textsubscript{Ca} (106) and alters the interaction of both niflumic acid (NFA) (Chapter 2) and anthracene-9-carboxylic acid (Chapter 3) with the native channel. The interaction of these two drugs with TMEM16A(a) under conditions favoring or minimizing phosphorylation were studied. Finally, a putative kinase consensus site (T610) was mutated in an attempt to locate the site of kinase activity that seems to cause rundown of I\textsubscript{Cl(Ca)} in PASMCs.

4.3 Materials and Methods

4.3.1 Isolation of Vascular Smooth Muscle Cells

A similar method to that previously used by our group was used to isolate smooth muscle cells (106, 108). In brief, cells were prepared from the main and secondary pulmonary arterial branches dissected from New Zealand white rabbits (2-3 kg), killed by anesthetic overdose in accordance with British and American guidelines for animal care. The animals were allowed free access to food and water and kept on a 12-12 h light/dark cycle until the day of being killed. The University of Nevada Institutional Animal Care and Use Committee approved the experimental protocol. Single smooth muscle cells were isolated as previously described (See Chapters 2 and 3). BALB/c mice (6-8 weeks) were sacrificed either by overdose of pentobarbitone in accordance with schedule 1 of the United Kingdom Animals Act (1986), or sedated with isoflurane prior to cervical dislocation as approved by the local Institutional Animal Care and Use Committee (IACUC) in accordance with the
Animal Welfare Act of the United States. Single smooth muscle cells were isolated from murine portal vein in a similar manner to rabbit pulmonary artery. Both the murine thoracic aorta and murine carotid artery were digested in a physiological salt solution containing 50 μM Ca^{2+}, 1.5 mg ml^{-1} Collagenase XI (Sigma) and 1 mg ml^{-1} Protease XIV (Sigma) for 27 min and triturated gently at 7 min and 20 min. Cells were kept at 4°C before use.

4.3.2 RNA Extraction and cDNA Synthesis

Total RNA was extracted from various murine tissues using the RNeasy Micro Kit (Qiagen) or PureZOL RNA Isolation Reagent (Bio-Rad) according to manufacturer’s instruction and included an on-column DNase treatment step. RNA quality was measured using a Nanodrop Spectrophotometer (Agilent Technologies) and reverse-transcribed with Oligo(dT) (12-18) primers and M-MLV or SuperScript III Reverse Transcriptase (Invitrogen). Negative controls (RT-) were carried out in the absence of reverse transcriptase and used to check for genomic contamination. All samples were stored at -20°C prior to PCR amplification.

4.3.3 Primers

The housekeeping genes β-actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as references to which the gene of interest (GOI) was normalized (QPCR studies). Primers for all genes were designed to allow amplification of exons spanning seven amplicons, thereby allowing for identification of contaminating genomic DNA. Primers were synthesized by Invitrogen or Eurofins MWG Operon (See appendix table 1)
4.3.4 Polymerase Chain Reaction

Non-quantitative or semi-quantitative PCR was performed using Platinum Taq DNA Polymerase (Invitrogen) or Gotaq Green Mastermix (Promega). The two Taq Polymerases have slightly different amplification profiles: initial denaturation step at 94°C followed by 35 cycles of 94 °C for 30 s, annealing temperature specific for each primer set for 30 s and 72 °C for 1 min (Invitrogen) or initial denaturation step at 95°C followed by 35 cycles of 95 °C for 30 s, annealing temperature specific for each primer set for 30 s and 72 °C for 30 s; the reaction was completed with a 5 min extension step. β-Actin or GapDH served as internal controls. PCR product amplification was confirmed with subsequent 2% agarose gel electrophoresis and sequence analysis (University of Dundee Sequencing Facility or Nevada Genomics Center) and checked using the NCBI BLAST program.

4.3.5 TMEM16A Over-Expression

Mouse Tmem16a(a) (lacking alternatively spliced segment ‘b’, ‘c’, and ‘d’) was kindly supplied by Professor Lily Y. Jan, Howard Hughes Medical Institute, University of California San Francisco. The full-length open reading frame was sub-cloned into pcDNA3.1/CT-GFP upstream of a Green Fluorescent Protein (GFP) tag. Endotoxin-free plasmid DNA was prepared using the Qiagen Endo-Free kit (Qiagen, Valencia, CA). HEK293 cells were transfected with 250 ng DNA per cm² surface area using TransIT-LT1, -293 or -2020 reagents (Mirus, Madison, WI). After 18-48 hours these cells were used for immunofluorescence, Western blotting or electrophysiology. A mutation at threonine 610 to alanine was introduced using the QuickChange Site directed Mutagenesis Kit.
(Stratagene). Stratagene’s online primer design program (http://www.stratagene.com/qcprimerdesign) was used to design the mutagenic primers, which were then synthesized by Eurofins MWG Operon. Mutation was verified by sequence analysis (University of Nevada Genomics Center).

4.3.6 Western Blot Analysis

Protein was prepared by homogenisation of tissue in lysis buffer (20 mM Tris Base, 137 mM NaCl, 2 mM EDTA, 1 % NP40, 10 % Glycerol, pH 8, and 10 μL ml⁻¹ protease inhibitor cocktail; Sigma) and subsequent incubation on ice for 15 min prior to centrifugation to remove cell debris. HEK293 cells were lysed by incubation in lysis buffer with two 1 min bursts of vortexing, followed by centrifugation to remove cell debris. H441 cell protein was a kind gift from Dr Baines, SGUL. All supernatants were short-term stored at -20 °C until required. Equal concentration of all proteins were denatured at 95 °C for 5 min in the presence of sample buffer and reducing agent (Invitrogen), followed by separation on SDS-PAGE gels (Invitrogen) and transfer onto PVDF membranes. TMEM16A expression products were immunodetected using a rabbit polyclonal anti-TMEM16A antibody (1:500; ab53213: an undiluted form used previously (8, 18) and visualized with ECL (ThermoFisher Scientific) and hyperfilm (Amersham). No control peptide was supplied for this antibody and the sequence of the epitope targeted by this antibody is not available. Reference 8, did not report quantifiable differences in antibody binding between different TMEM16A isoforms suggesting the epitope for ab53213 does not lie over spliced sites. Protein lysates were interrogated with serum from a non-immunogenic rabbit (negative control for ab53213).
4.3.7 Immunocytochemistry

Enzymatically isolated smooth muscle cells were fixed in 3.7 % formaldehyde solution for 15 min at room temperature, spun down at 1000 rpm for 2 min, re-suspended in PBS and then stored at 4°C until use. Cells were cytospun onto poly-L-lysine-coated slides (10 µg/ml) at 600 rpm for 5 min and allowed to dry for 10 min prior to treatment with 1% bovine serum albumin (BSA) in PBS for 1 hour. Cells were then incubated with primary antibody (ab53213; Abcam) diluted in 1 % BSA for 1 hour in a humidified chamber at room temperature (rat PASMCs) or overnight at 4°C (mouse PASMCs). Control experiments were performed in which the primary antibody was omitted. After washing, cells were incubated with a biotinylated goat anti-rabbit secondary antibody (1:200) along with bis-benzamide (1:500; for detection of nuclei) for 1 hour at room temperature in a humidified chamber. After removal of unbound secondary antibody, slides were incubated in streptavidin (1:1000) for 30 min at room temperature prior to being washed and mounted with coverslips and gelmount anti-fade media (Biomed, CA). Single cells were imaged using an Olympus FV1000 laser scanning confocal microscope with laser excitation set at 488 nm or 345 nm (bis-benzamide). Images for each cell type were gain-matched to ensure accuracy between samples. A cross-section of the cell was selected for display purposes. Transfected HEK293 cells were fixed in 3.7 % formaldehyde and permeabilized with 0.1 % TritonX-100 in PBS for 3 min. Cells were then blocked for 1 hour with 1 % BSA in PBS prior to addition of the rabbit polyclonal Tmem16a antibody (1:50, ab53213 Abcam) for 1 hour at room temperature. Slides were washed and then incubated with Alexa Fluor 594 (1:1000, Invitrogen; excitation= 594 nm) for 1 hour at room temperature in a humidified chamber.
Slides were again washed, and then mounted with anti-fade media. TIRFM images were generated using an Olympus IX71 microscope along with an argon ion laser (488 nm).

4.3.8 Whole-Cell Patch Clamp Electrophysiology

Ca\(^{2+}\)-activated Cl\(^-\) currents were elicited using the conventional whole-cell configuration of the patch clamp technique with a pipette solution containing either no ATP, or 5 mM ATP. The pipette solution also contained 10 mM BAPTA as the Ca\(^{2+}\) buffer and free [Ca\(^{2+}\)] was set to 500 nM by the addition of 7.08 mM CaCl\(_2\). The free [Ca\(^{2+}\)] was estimated by the calcium chelator program WinMaxC (v. 2.50; http://www.stanford.edu/~cpatton/downloads.htm). Using a Ca\(^{2+}\)-sensitive electrode and calibrated solutions, the total amounts of CaCl\(_2\) and MgCl\(_2\) calculated by the software were previously shown to yield accurate free Ca\(^{2+}\) concentrations with both EGTA and BAPTA as Ca\(^{2+}\) buffers (106, 108, 109). Contamination of I\(_{Cl(Ca)}\) from other types of current was minimized by the use of CsCl and tetraethylammonium chloride (TEA) in the pipette solution, and TEA in the external solution. Data for each group were collected in cells from at least two animals but generally more.

4.3.9 Experimental Protocols

I\(_{Cl(Ca)}\) was evoked immediately upon rupture of the cell membrane and its voltage-dependent properties were monitored every 5 s by stepping from a holding potential (HP) of −50 mV to +90 mV for 1 s, followed by repolarization to −80 mV for 1 s. Current-voltage (I-V) relationships were constructed by stepping in 10 mV increments from HP to test
potentials between −100 mV and +140 mV for 1 s, and then repolarized to -80 mV for 1 s after 5 min dialysis. For I-V relationships, $I_{Cl(Ca)}$ was expressed as current density (pA/pF) by dividing the amplitude of the current measured at the end of the voltage clamp step or at the beginning of the repolarizing step by the cell capacitance. For all figure panels showing a time course of $I_{Cl(Ca)}$ changes, the late current measured at +90 mV was usually normalized to the amplitude of first current elicited at time=0 (~30 s after breaking the seal and measuring cell capacitance). After measuring cell capacitance, the constant step protocol described above was started to monitor the changes of $I_{Cl(Ca)}$ over the course of 5 min after which a control I-V relationship was obtained. While monitoring $I_{Cl(Ca)}$ elicited by a similar constant step protocol described above, the external solution was switched to one containing either 100 µM NFA or 500 µM A9C. After a steady-state effect was noticed, another I-V relationship was obtained in the presence of NFA or A9C. If the seal was still stable, washout was subsequently initiated and an I-V curve generated after washout. Only one concentration of NFA or A9C was tested per cell.

4.3.10 Solutions and Reagents

Single pulmonary artery smooth muscle cells were isolated by incubating pulmonary artery tissue strips in the following low Ca$^{2+}$ (10 or 50 µM) PSS (in mM): NaCl (120), KCl (4.2), NaHCO$_3$ (25; pH 7.4 after equilibration with 95% O$_2$, 5% CO$_2$ gas), KH$_2$PO$_4$ (1.2), MgCl$_2$ (1.2), glucose (11), taurine (25), adenosine (0.01) and CaCl$_2$ (0.01 or 0.05). The K$^+$-free bathing solution used in all patch clamp experiments had the following composition (in mM): NaCl (126), Hepes-NaOH (10, pH 7.35), TEA (8.4), glucose (20), MgCl$_2$ (1.2) and CaCl$_2$ (1.8). The
pipette solution had the following composition (in mM): TEA (20), CsCl (106), Hepes-CsOH (10, pH 7.2), BAPTA (10), ATP.Mg (0 or 5) and GTP.diNa. To this solution, the following total amounts of CaCl$_2$ and MgCl$_2$ were added to set free [Mg$^{2+}$] at 0.5 mM and free [Ca$^{2+}$] at various desired levels: 5 mM ATP and 500 nM Ca$^{2+}$ (in mM): CaCl$_2$ (7.08), MgCl$_2$ (3.0); no ATP and 500 nM Ca$^{2+}$: CaCl$_2$ (7.08), MgCl$_2$ (0.545). All enzymes, analytical grade reagents, NFA and A9C were purchased from Sigma-Aldrich (St. Louis, MO, USA). Niflumic acid was initially prepared as a 100 mM stock in dimethyl sulfoxide (DMSO) and an appropriate aliquot was added to the external solution to reach the final desired concentration. Anthracene-9-carboxylic acid was initially prepared as a 500 mM stock in dimethyl sulfoxide (DMSO) and an appropriate aliquot was added to the external solution to reach the final desired concentration. The maximal concentration of DMSO never exceeded 0.1%, a concentration that had no effect on $I_{\text{Cl(Ca)}}$.

4.3.11 Statistical Analysis

All data were pooled from $n$ cells taken from at least two different animals with error bars representing the s.e.m. All data were first pooled in Excel and means exported to Origin 7.5 software (OriginLab Corp. Northampton, MA, U.S.A.) for plotting and curve fitting. All graphs and current traces were exported to CorelDraw 12 (Ottawa, Ontario, Canada) for final processing of the figures. Origin 7.5 software (OriginLab Corp. Northampton, MA, U.S.A.) was also used to determine the statistical significance between two groups using a paired or unpaired Student’s $t$ test, or one-way ANOVA test followed by Bonferroni post-hoc
multiple range tests in multiple group comparisons. \( P < 0.05 \) was considered to be statistically significant.

4.4 Results

4.4.1 TMEM16A is Expressed in Pulmonary Artery Smooth Muscle

Our group has published several studies concerning \( I_{\text{Cl(Ca)}} \) of vascular smooth muscle of portal vein, coronary artery, and pulmonary artery (106, 108, 109, 110, 126). This study was intended to determine whether TMEM16A is expressed in pulmonary smooth muscle and to ascertain whether the current resulting from transiently transfecting HEK293 cells with TMEM16A display similar pharmacology to native \( I_{\text{Cl(Ca)}} \) as presented in chapters 2 and 3 of this dissertation. Thus far, mRNA for TMEM16A has been identified in several different tissues including brain, heart, lung, and kidney (221, 222, 258). While a recent study has identified TMEM16A in human and rat pulmonary artery smooth muscle cells (272), this study was begun prior to the emergence of the report and aimed to determine the viability of TMEM16A as a molecular correlate for native \( I_{\text{Cl(Ca)}} \) channels of this tissue. Figure 1A shows that \( \text{Tmem16a} \) transcripts were identifiable after 35 PCR cycles in rat pulmonary artery, as well as murine portal vein, thoracic aorta, and carotid artery. No studies have subjected native tissues to Western blot analysis. We also examined protein translation of TMEM16A in several vascular tissues. Figure 1B depicts bands (~120 kDa) close to the theoretical molecular weight of TMEM16A (~114 kDa for the mouse TMEM16A protein including all
Figure 1. Expression of Tmem16a in various vascular smooth muscle tissues. A) RT-PCR expression analysis of Tmem16a. RNA was extracted from whole tissues, subjected to reverse-transcription and semi-quantitative PCR performed. After a standard 35 cycles, sequencing verified Tmem16a product was amplified in several tissues. The housekeeping gene β-actin was used as an internal positive control for all PCRs. Representative of > 3 animals. rPA: rat pulmonary artery, mCA: mouse coronary artery, mPV: mouse portal vein, mTA: mouse thoracic artery. B) Western blot analysis of murine protein lysates where Tmema16A protein expression in various murine tissues was determined by SDS-PAGE analysis. H441 cells acted as a negative control for Tmem16a protein expression. C) RT-PCR expression analysis of Tmem16a splice variants in murine pulmonary artery. Lane 1 shows a band consistent with the predicted size of 413 bp and demonstrated presence of ‘a’. Lane 2 contains double bands suggesting splice variants that may be ‘+b’ or ‘-b’, as the primers span the region that contains ‘b’. The presence of exon ‘b’ is confirmed in lane 3, where one of the primers anneals to a region of the exon (predicted band size: 455 bp). Lane 4 depicts a double band (predicted band size: 421 bp) as the primers span exon ‘d’, while the forward primer partially anneals to exon ‘c’. Lane 5 suggests that exon ‘c’ is always present, given the single band (predicted band size: 395 bp) resulting from primers that span exon ‘c’. The primers used in the lane 6 PCR products span exon ‘c’ and give only one band, again suggesting that ‘c’ is always present. Primers were designed to give a smaller product size (188 bp) with the aim that picking up a second band only 12 bp smaller would be made easier. Lane 7 shows double bands at the predicted size (393 bp), suggesting that there are splice variants that may be ‘+d’ or ‘-d’. Lane 8 confirms the presence of exon ‘d’ by using a primer that anneals to a region of the target exon. Figure adapted from Davis AJ, Forrest AS, Jepps TA, Valencik ML, Wiwchar M, Singer CA, Sones WR, Greenwood IA, and Leblanc N. Expression profile and protein translation of TMEM16A in murine smooth muscle. Am J Physiol. In review. 2010.
four alternatively spliced variants) identified in protein lysates from murine brain and lung and HEK293 cells over-expressing GFP-tagged TMEM16A, which were used as positive controls. No bands were detected in cell lysates of H441 cells, a negative control. Mouse carotid artery and thoracic aorta exhibited a band similar to those of the positive controls (~120 kDa), while portal vein protein presented a band of ~80 kDa, with no obvious band at ~120 kDa. This was not due to non-specific binding, as no bands were present when non-immune serum was used.

At least four alternatively spliced exons (termed ‘a’, ‘b’, ‘c’ and ‘d’) were predicted by sequence analysis for human TMEM16A (220, 258). We therefore examined the expression pattern of these putative transcripts in murine pulmonary artery by RT-PCR using sets of primers that either span the target axon, or anneal at one end. Lane 1 of Figure 1C shows that a primer pair designed with one primer annealing to exon 2 (corresponding to splice variant ‘a’) yielded a single band, indicating the presence of this variant. However, we cannot exclude the possibility that transcripts lacking this sequence are also expressed, as it is not possible to design primers that span this exon. Double bands were obtained when using a primer pair spanning the exon encoding variant ‘b’ (lane 2), with sequencing confirming that the 741 bp amplicon corresponded to Tmem16a lacking the ‘b’ variant. We were unable to confirm either by direct sequencing or sub-cloning PCR products that the upper band corresponded to a transcript containing ‘b’, although the molecular weight was consistent with the predicted 66 bp transcript reported by Hwang et al. (242) in mouse colon. Presence of exon ‘b’ was confirmed by a second pair of primers designed such that one of the pair annealed to the exon, producing a single band at the predicted 455 bp (lane
3). Lane 4 demonstrates the presence of the ‘c’ variant in mouse pulmonary artery, as the forward primer partially anneals to exon ‘c’. The double band found in lane 4 may be explained by the fact that the primer pair also spans exon ‘d’. Two set of primers were designed to span exon 14 (variant ‘c’). Lane 5 shows a single band with the predicted product size of 395 bp, while the primers used in lane 6 were designed to amplify a smaller region (200 bp) with the hope of resolving splice variants differing by only 12 bp. As these primers were able to produce a double band in mouse carotid artery (data not shown), this suggests that ‘c’ is always present in mouse pulmonary artery. Alternative splicing of the ‘d’ variant isoform was demonstrated by primer pairs designed to span exon ‘d’ (lane 7), while primers that annealed at one end of the exon showed a single band at the expected size of 298 bp (lane 8). This demonstrates that transcripts with or without exon ‘d’ exist in mouse pulmonary artery.

4.4.2 Transfection of Tmem16a into HEK293 Cells

Figure 2 shows the results of immunocytochemical experiments on single smooth muscle cells isolated from murine (A) and rat pulmonary artery (B). While some Tmem16a-specific fluorescence was detected in the cytoplasm, considerable fluorescence appeared at the plasma membrane. For a positive control, HEK293 cells transiently transfected with GFP-tagged Tmem16a were incubated with the same antibody used on the single smooth muscle cells (ab53213). Overlaying the GFP and Tmem16a fluorescence demonstrated co-localization (Figure 3A). In figure 3B, standard epifluorescence demonstrated Tmem16a-eGFP to be localized at or near the plasma membrane (top) while total internal reflection
Figure 2. *Tmem16a* protein localization in enzymatically isolated pulmonary artery smooth muscle cells. A) Mouse pulmonary artery smooth muscle cells incubated with secondary antibody only (left) or with Tmem16a antibody (ab53213). The Tmem16a-dependent signal was excited at 488 nm and pseudocolored green. Images courtesy of Ramon Ayon. B) Rat pulmonary artery smooth muscle cells incubated with secondary antibody only (left) or with Tmem16a antibody (ab53213). The Tmem16a-dependent signal was excited at 488 nm and pseudocolored red. Images courtesy of Abigail Forrest.
Figure 3. Tmem16a protein expression in transiently transfected HEK293 cells. A) Confocal image of Tmem16a-eGFP transfected HEK293 cell excited at 488 nm (top), where TMEM16A was targeted using antibody ab53213 (middle, excitation=594). The bottom image displays an overlay of the eGFP and Alexa Fluor 594 images. Image courtesy of Abigail Forrest. B) Detection of Tmem16a-eGFP transfected HEK293 cells excited by direct excitation of GFP with a 488 nm argon laser in standard epifluorescence mode to show Tmem16a-eGFP at the periphery of the cell (top, pseudocolored green) or total internal reflection fluorescence mode to demonstrate the construct’s presence at the basal membrane (middle, pseudocolored red). These images were then merged (bottom). Image courtesy of Normand Leblanc. C) Western blot analysis of TMEM16A expression in HEK293 cells transiently transfected with TMEM16A-eGFP. In the blot performed using antibody ab53213, the molecular weight of the principle product seen, most likely corresponding to monomeric protein, is consistent with the predicted size for a Tmem16a-GFP construct (~138 kDa). The fainter band appearing at ~114 is likely to correspond to endogenous Tmem16a. A similar band at ~138 kDa is detected by an anti-GFP antibody (Rockland, 600-101-215; 1:1000). Adapted from Davis AJ, Forrest AS, Jepps TA, Valencik ML, Wiwchar M, Singer CA, Sones WR, Greenwood IA, and Leblanc N. Expression profile and protein translation of TMEM16A in murine smooth muscle. Am J Physiol. In review. 2010.
fluorescence microscopy (TIRFM) showed intense labeling at the plasma membrane of the base of the cell with some areas displaying denser fluorescence suggesting the presence of hot spots (*middle*). These two images are overlaid at the bottom panel of figure 3B, which demonstrates that transfection results in abundant protein expression. Transfection of the HEK293 cells was confirmed by Western blot, where protein lysate from cells transfected with the Tmem16a-eGFP construct displayed a band at ~147 kDa when probed with the TMEM16A antibody ab53213. Another band appearing at ~120 kDa represents endogenous expression of Tmem16a, which was visible in non-transfected cells as well. Interestingly, a band of ~300 kDa was present suggesting that Tmem16a may assemble minimally as a dimer, at least under these artificial conditions. A band of ~147 kDa was found when the cell lysate was probed with a specific anti-GFP antibody (predicted molecular weight is ~ 138 kDa). Cell lysate from non-transfected cells did not display any band when probed with the anti-GFP antibody.

4.4.3 Currents Generated by Transfecting HEK293 Cells With Tmem16a Display Reduced Rundown

The data described above provides strong evidence that Tmem16a is present in pulmonary artery myocytes. Additionally, it is shown that transiently transfecting HEK293 cells with GFP-tagged Tmem16a results in abundant protein expression which may be localized to the plasma membrane. Based on these results, we wanted to determine whether the Ca\(^{2+}\)-activated currents of HEK293 cells expressing Tmem16a behaved similarly to native currents of PASMCs. A previous study by our group has suggested that
phosphorylation of Cl\(_{\text{Ca}}\) channels of PASMCs results in a state-dependent block of the channel (106). This resulted in rundown of I\(_{\text{Cl(Ca)}}\) when cells were dialyzed with 3 mM ATP. Removing ATP from the pipette solution or replacing it with the non-hydrolyzable analogue AMP-PNP resulted in attenuation of the rundown followed by complete recovery over the course of the experiment. Similar results are presented in chapters 2 and 3 of this dissertation. Based on these studies, we subjected the transfected HEK293 cells to similar conditions to determine whether Tmem16a is similarly regulated by phosphorylation. Figure 4Ai displays two representative experiments from HEK293 cells expressing Tmem16a-eGFP dialyzed with either 5 mM ATP (top) or ATP-free (bottom) pipette solution, each containing 500 nM Ca\(^{2+}\). Immediately upon seal rupture, cells in both conditions displayed an instantaneous current when depolarized to +90 mV from a holding potential of -50 mV (traces marked ‘a’). This was followed by the development of a time-dependent outward relaxation over the course of the 1 s depolarizing step. Within 1 min the current of the cell dialyzed with 5 mM ATP had markedly decreased (marked ‘b’, where t = 50 s) to ~85% of the initial current at seal rupture. Over the course of 5 min the current continued to run down to a level ~70% of the initial current (trace marked ‘c’, where t = 300 s). In cells dialyzed with an ATP-free pipette solution current amplitude did not appear to decrease after 50 s of cell dialysis (trace marked ‘b’), although the current did demonstrate some rundown over the course of the 5 min experiment, settling at a level ~85 % of the initial current amplitude (trace ‘c’), similar to the rundown seen with 5 mM ATP at t = 50 s. In both cells, repolarizing to -80 mV from +90 mV for 1 s resulted in an inward tail current with an amplitude proportional to the preceding outward relaxation that is indicative of channel deactivation.
Figure 4. Comparison of $I_{\text{Cl(Ca)}}$ in cells transiently transfected with Tmem16a tagged with eGFP and isolated rabbit pulmonary artery smooth muscle cells. Ai) Representative current traces demonstrate the time dependent changes of Ca$^{2+}$-activated Cl currents recorded from HEK293 cells transiently transfected with Tmem16a-eGFP and dialyzed with either 5 mM ATP (top) or 0 ATP (bottom). Currents depicted were recorded immediately following breaking the seal (0 s, trace ‘a’), 50 s into the experiment (trace ‘b’), and after 5 min (300 s, trace ‘c’) of cell dialysis. The currents were elicited by repetitive 1 s step depolarizations (every 5 s) to +90 mV from a holding potential (HP) of -50 mV. Each depolarizing pulse was followed by a repolarizing step to -80 mV to enhance the magnitude of the tail current. Aii) Pulses to +90 mV, followed by 1-s repolarizing steps to -80 mV, in cells transiently transfected with Tmem16a-eGFP and dialyzed with either 5 mM ATP (black squares, $n = 19$), or 0 ATP (red circles, $n = 11$), and in isolated rabbit pulmonary artery smooth muscle cells under similar conditions (5 mM ATP: dark blue triangle, $n = 27$; 0 ATP: light blue triangle, $n = 28$). Currents were normalized to the initial current amplitude at the beginning of the protocol. Each step was applied from HP = -50 mV at a frequency of one pulse every 5 s for 5 min. Time points marked ‘a’, ‘b’, and ‘c’ correspond to those described above in Ai. B) Mean current-voltage relationships for HEK293 cells transiently transfected with Tmem16a-eGFP (left 5 mM ATP = black squares, $n = 19$; 0 ATP = red circles, $n = 11$), non-transfected HEK293 cells (magenta diamonds, $n = 4$), and transiently transfected with pcDNA3.1/CT-GFP (green stars, $n = 4$). Mean current-voltage relationships for isolated rabbit pulmonary artery smooth muscle cells are shown for comparison (right 5 mM ATP = dark blue triangles, $n = 27$; 0 ATP = light blue triangles, $n = 28$). Currents were elicited by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV after 5 min of cell dialysis. C) Representative families of currents recorded from non-transfected HEK293 cells (left) and HEK293 cells transiently transfected with only pcDNA3.1/CT-GFP. Currents were elicited as described above in B.
Figure 4Aii represents mean data for HEK293 cells expressing Tmem16a-GFP as well as isolated rabbit pulmonary artery smooth muscle cells dialyzed with either 5 mM ATP or 0 ATP to allow for a visual comparison of the rundown of Tmem16a to that seen in native cells. All currents were elicited by a 1 s depolarization to +90 mV from the HP of -50 mV followed by 1 s repolarizing step to -80 mV before returning to the HP. These steps were repeated every 5 s, and current was recorded at the end of the 1 s depolarizing step and normalized to the current amplitude of the first elicited current. While rundown of \( I_{\text{Cl(Ca)}} \) carried by Tmem16a expressed in HEK cells did not exactly follow that of the native current, there are some similarities. Currents of cells where phosphorylation was supported by 5 mM ATP in the pipette solution ran down considerably more than in cells where phosphorylation was minimized (67.34 ± 5.12 and 84.39 ± 6.50 normalized current amplitude at \( t = 300 \) s for 5 mM ATP and 0 ATP respectively, \( P < 0.05 \)), and this rundown occurred more rapidly in the presence of 5 mM ATP. Differences between the Tmem16a-expressing HEK293 cells and the native cells may be attributable to differences in kinase and phosphatase activities in these cell types (see Discussion). It is important to note that neither non-transfected (\( n = 4 \)) cells nor cells transfected with just the eGFP-tagged construct (\( n = 3 \)) displayed little time-dependence (Fig. 4C, right) or modest time-dependent inactivation at positive potentials (Fig. 4C, left), when dialyzed with 500 nM free Ca\(^{2+} \) (Figure 4C). In fact, currents recorded at positive potentials displayed signs of mild inactivation. While these cells do demonstrate the presence of a small current, as the I-V relationships of Figure 4B demonstrate, currents of the non-tranfected and pcDNA3.1/CT-GFP transfected
HEK293 cells are much smaller than those of Tmem16a transfected cells and do not display a similar time-dependence.

Figure 4B presents mean current-voltage relationships of $I_{\text{Cl(Ca)}}$ in HEK293 cells expressing the Tmem16a-eGFP construct (left). Current-voltage relationships were recorded following a 5 min dialysis with either 5 mM ATP (black squares) or 0 ATP (red circles), and were generated by stepping to test potentials between -100 mV and +140 mV in 10 mV increments for 1 s from the HP of -50 mV. I-V relationships of $I_{\text{Cl(Ca)}}$ in rabbit pulmonary artery cells dialyzed with either 5 mM ATP or 0 ATP were generated similarly, and are shown to the right for comparison. As with the time courses in figure 4Aii, it appears that phosphorylation has a slight but significant inhibitory influence on $I_{\text{Cl(Ca)}}$ although the effect is smaller than that observed in rabbit PASMCs.

4.4.4 HEK293 Cells Transfected With Tmem16a Display Similarities in Pharmacology to PASMC $I_{\text{Cl(Ca)}}$

Having established that phosphorylation does affect $I_{\text{Cl(Ca)}}$ of HEK293 cells expressing Tmem16a-eGFP – albeit to a lesser degree than native current of PASMCs – our next set of experiments were carried out in order to determine whether NFA similarly blocked currents in these two cells types. Figure 5A displays representative currents for HEK293 cells transiently transfected with Tmem16a-eGFP and dialyzed with either 5 mM ATP (left) or 0 ATP (right). Families of currents were generated following 5 min of dialysis as described in figure 4B. The bathing solution was then switched to one containing 100 µM NFA (bottom traces), after which a second family of currents was generated for each cell in the continued
Figure 5. Block of $I_{Cl(Ca)}$ by niflumic acid in cells transiently transfected with TMEM16A-eGFP. A) Representative families of currents from cells dialyzed with either 5 mM ATP (left) or 0 ATP (right) both before (upper traces) and after (lower traces) a 5 min application of 100 µM NFA. Currents were evoked by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV following a 5 min period of dialysis. B) Mean current-voltage relationships generated from families of currents elicited as described in A from TMEM16A-eGFP transfected HEK293 cells (left, n = 6) and rabbit pulmonary artery smooth muscle cells (right, n = 10) dialyzed with 5 mM ATP. I-V relationships before and after addition of 100 µM NFA are depicted. C) Mean current-voltage relationships generated from families of currents elicited as described in A from TMEM16A-eGFP transfected HEK293 cells (left, n = 5) and rabbit pulmonary artery smooth muscle cells (right, n = 7) dialyzed with 0 mM ATP. I-V relationships before and after addition of 100 µM NFA are depicted.
presence of NFA. Mean data are displayed in Figures 5B and C. Cells used to generate the I-V displayed in figure 5B were dialyzed with 5 mM ATP in order to support phosphorylation of Tmem16a. I-V relationships were generated as described above both before (black squares) and after (red circles) addition of 100 µM NFA to the bathing solution. Similar $I_{\text{Cl(Ca)}}$ I-V relationships of rabbit PASMCs are shown at left (dark blue triangle = before NFA; light blue triangle = with 100 µM NFA). Currents generated by Tmem16a appeared quite similar to those of rabbit PASMCs, although they seemingly carry a relatively larger inward current at negative potentials. The voltage dependence of block was also shifted towards less depolarized potentials in HEK293 cells expressing Tmem16a-eGFP and was slightly more voltage-dependent (see figure 8), although block at +140 mV was comparable in the two cell types. Additionally, there was evidence for some stimulation of the inward current at negative potentials. Figure 5C is similar to 5B with the exception that these cells were dialyzed with an ATP-free pipette solution rather than 5 mM ATP. As in cells dialyzed with 5 mM ATP, currents carried by Tmem16a displayed greater relative inward current at negative potentials when compared to native $I_{\text{Cl(Ca)}}$ of rabbit PASMCs. However, NFA did not exert as great of block in Tmem16a-eGFP expressing HEK293 cells than that was seen in rabbit PASMCs (see figure 8). As in rabbit PASMCs, NFA was more potent at blocking $I_{\text{Cl(Ca)}}$ of Tmem16a when phosphorylation was minimized. NFA also markedly slowed deactivation kinetics of $I_{\text{Cl(Ca)}}$ in HEK293 cells expressing Tmem16a-eGFP when dialyzed with either 5mM ATP or 0 ATP (see figure 5A), as it did in rabbit PASMCs (Chapter 2). These results suggest that NFA may interact with Tmem16a in a similar fashion to the native ClCa channel of PASMCs.
Given these results, we next sought to determine whether the chemically dissimilar Cl⁻ channel blocker anthracene-9-carboxylic acid (A9C) would affect $I_{\text{Cl(Ca)}}$ of Tmem16a-eGFP expressing HEK293 cells in a comparable manner to the native currents of rabbit PASMCs. Piper and Greenwood (153) demonstrated that while A9C blocks $I_{\text{Cl(Ca)}}$ of rabbit PASMCs at +70 mV, repolarizing the cell to -80 mV in the presence of 500 µM A9C resulted in significant stimulation of the inward tail current. Similar results were found in a study by our group (chapter 3), where we also found that promoting phosphorylation reduced the ability of A9C to block inward current at positive potentials but increased the drugs’ ability to stimulate the inward current upon repolarizing the cell to -80 mV. Figure 6A presents two representative families of currents recorded from HEK293 cells transiently transfected with Tmem16a-eGFP dialyzed or 5 min with either 5 mM ATP (left) or 0 ATP (right). Currents were elicited as described in figure 4B. The bathing solution was then switched to one containing 500 µM A9C. After current reached a steady state, another family of currents was generated in the continued presence of drug (bottom traces). Mean data are presented in figure 6B and C and figure 7A and B, where current density (pA/pF) were plotted against voltage. Figure 6B shows mean current-voltage relationships of $I_{\text{Cl(Ca)}}$ in HEK293 cells expressing Tmem16a-eGFP (left) and rabbit PASMCs (right). While 500 µM A9C blocked $I_{\text{Cl(Ca)}}$ at depolarized potentials in both cell types, A9C blocked currents of transfected HEK293 cells less than those of rabbit PASMCs. Additionally, the depolarized potential where block appeared was shifted to the right in the Tmem16a-eGFP expressing HEK293 cells (~ +30 mV shift). Cells dialyzed with an ATP-free pipette solution demonstrated similar behavior, where A9C’s ability to block $I_{\text{Cl(Ca)}}$ was lesser in transfected HEK293 cells.
Figure 6. Block of $I_{Cl(Ca)}$ by anthracene-9-carboxylic acid in cells transiently transfected with TMEM16A-eGFP. A) Representative families of currents from cells dialyzed with either 5 mM ATP (left) or 0 ATP (right) both before (upper traces) and after (lower traces) a 5 min application of 500 µM A9C. Currents were evoked by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV following a 5 min period of dialysis. B) Mean current-voltage relationships generated from families of currents elicited as described in A from TMEM16A-eGFP transfected HEK293 cells (left, n = 7) and rabbit pulmonary artery smooth muscle cells (right, n = 5) dialyzed with 5 mM ATP. I-V relationships before and after addition of 500 µM A9C are depicted. C) Mean current-voltage relationships generated from families of currents elicited as described in A from TMEM16A-eGFP transfected HEK293 cells (left, n = 6) and rabbit pulmonary artery smooth muscle cells (right, n = 5) dialyzed with 0 mM ATP. I-V relationships before and after addition of 500 µM A9C are depicted.
Interestingly, block in both cell types began to develop at $\sim+20$ mV. Unlike in rabbit PASMCs, phosphorylation did not appear to influence the block of the outward current at positive potentials by 500 µM A9C in HEK293 cells expressing Tmem16a-eGFP (see figure 8). Thus, while A9C still blocked $I_{Cl(Ca)}$ at positive potentials in these cells in a voltage dependent manner similarly to rabbit PASMCs, block was of a lesser magnitude and was not affected by conditions that either support or minimize phosphorylation.

Current-voltage relationships in figure 7 were generated similarly to figure 6 with the exception that current was recorded at the beginning of the 1 s repolarizing step to -80 mV. Thus, graphs depict the tail current at -80 mV following a voltage step to the noted test potential. Figure 7A shows I-V relationships for HEK293 cells transfected with the Tmem16a-eGFP construct (left) and rabbit PASMCs (right) dialyzed with 5 mM ATP. While the I-V relationships prior to A9C exposure appear qualitatively similar, A9C produced a notably reduced amount of tail current stimulation in the transfected HEK293 cell when compared to native cells. When cells were dialyzed with ATP-free pipette solution, the HEK293 cells expressing Tmem16a-eGFP (Figure 7B left) and rabbit PASMCs (Figure 7B right) were similarly affected (qualitatively) by 500 µM A9C.

In order to better demonstrate the difference in block by NFA and A9C at positive potentials and stimulation by A9C at -80 mV, the intensity of block or stimulation (presented as a % of control) by either NFA or A9C was plotted as a function of voltage (Figure 8). Figure 8A displays block of $I_{Cl(Ca)}$ by NFA over a range of voltages between +20 mV and +110 mV in transfected HEK293 cells (left) and rabbit PASMCs (right). It is apparent that the state of phosphorylation of Tmem16a had little effect on the block by NFA, although there was a
tendency for greater block when phosphorylation was minimized by removing substrate from the pipette solution. This effect was not significant at any voltage ($P > 0.05$). Figure 8B shows % block of $I_{\text{Cl}(\text{Ca})}$ by A9C at positive potentials in transfected HEK293 cells (left) and rabbit PASMCs (right). In Tmem16a-eGFP expressing HEK293 cells, % block by 500 µM A9C appeared virtually identical, while A9C was better able to block $I_{\text{Cl}(\text{Ca})}$ of rabbit PASMCs when phosphorylation was minimized. The most dramatic difference in the pharmacology of Tmem16a and native $\text{Cl}_{\text{Ca}}$ channels can be seen by examining the stimulatory effect of A9C on the $I_{\text{Cl}(\text{Ca})}$ tail current. While rabbit PASMCs dialyzed with 5 mM ATP displayed a marked increase in tail current stimulation by 500 µM A9C compared to cells dialyzed with 0 ATP following depolarizations greater than $+40$ mV, Tmem16a tended to follow an opposite trend where stimulation was slightly greater when phosphorylation was minimized. This effect however, was not significant. Interestingly, stimulation of $I_{\text{Cl}(\text{Ca})}$ tail currents of HEK293 cells expressing Tmem16a-eGFP possessed similar voltage-dependence of stimulation by A9C to rabbit PASMCs dialyzed with 0 ATP, where stimulation reached a peak before beginning to decrease. This figure highlights some of the similarities in pharmacology of Tmem16a and native $\text{Cl}_{\text{Ca}}$ channels, while clearly demonstrating several key differences.

4.4.5 The T610A Tmem16a Mutation

In analyzing the mouse Tmem16a protein sequence, which is 91% identical to the human sequence reported by Caputo et al. (220), five consensus sites for phosphorylation by CaMKII were found. All five are speculated to lie on cytoplasmic protein segments, and are located at T216, S276, S492, T514, and T610 (please refer to Figure 6 of Chapter 1,
Figure 7. Stimulation of $I_{\text{cl(Ca)}}$ by anthracene-9-carboxylic acid in cells transiently transfected with Tmem16a-eGFP. A) Current-voltage relationships generated from families of cuurents elicited by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV before repolarizing to -80 mV, where currents were recorded. Mean I-V relationships for HEK293 cells transiently transfected with Tmem16a-eGFP (left, n = 7) and isolated rabbit pulmonary artery smooth muscle cells (right, n = 5) dialyzed with 5 mM ATP are shown both before and after application of 500 µM A9C. B) Current-voltage relationships for HEK293 cells transiently transfected with Tmem16a-eGFP (left, n = 6) and isolated rabbit pulmonary artery smooth muscle cells (right, n = 5) dialyzed with ATP-free pipette solution are shown both before and after application of 500 µM A9C. Currents were generated as described above for panel A.
**Figure 8. The voltage-dependence of interaction of niflumic acid anthracene-9-carboxylic acid with Tmem16a.** A) The two data sets were obtained after 5 min of cell dialysis with either 5 mM ATP or ATP-free pipette solution before and after addition of 100 μM NFA in HEK293 cells transiently transfected with Tmem16a (left, 5 mM ATP: n = 6; 0 ATP: n = 5) and rabbit pulmonary artery smooth muscle cells (right, 5 mM ATP: n = 10; 0 ATP: n = 7). Currents were elicited by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV and measured at the end of the 1 s test step. % Block was determined at each individual voltage step for each cell using the formula 100-((I_{NFA}/I_{Control})*100), where I_{NFA} and I_{Control} is the current recorded in the presence and absence of NFA, respectively. For I_{Control}, the mean of the first five traces was used in the calculation, while for I_{NFA} the mean of the last 5 stable traces was used. Stimulation is manifest as a negative value using the above formula and is graphed as such. Mean values were then plotted at each voltage. B) Generated similarly to panel A, this figure demonstrates the voltage dependence of block of I_{Cl(Ca)} by A9C at positive potentials in Tmem16a-eGFP transfected HEK293 cells (left, 5 mM ATP: n = 7; 0 ATP: n = 6) and rabbit pulmonary artery smooth muscle cells (right, 5 mM ATP: n = 5; 0 ATP: n = 5) dialyzed with either 5 mM ATP or 0 ATP. % Block was determined at each individual voltage step for each cell using the formula 100-((I_{A9C}/I_{Control})*100), where I_{A9C} and I_{Control} is the current recorded in the presence and absence of A9C, respectively. For I_{Control}, the mean of the first five traces was used in the calculation, while for I_{A9C} the mean of the last 5 stable traces was used. Stimulation is manifest as a negative value using the above formula and is graphed as such. Mean values were then plotted at each voltage. C) The two data sets were obtained after 5 min of cell dialysis with either 5 mM ATP or ATP-free pipette solution before and after addition of 500 μM A9C in HEK293 cells transiently transfected with Tmem16a (left, 5 mM ATP: n = 7; 0 ATP: n = 6) and rabbit pulmonary artery smooth muscle cells (right, 5 mM ATP: n = 5; 0 ATP: n = 5). Currents were elicited by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV, followed by a hyperpolarizing step to -80 mV, where currents were measured immediately following the capacitative spike. % stimulation was determined at each individual voltage step for each cell using the formula ((I_{A9C}/I_{Control})*100)-100, where I_{A9C} and I_{Control} is the current recorded in the presence and absence of A9C, respectively. For I_{Control}, the mean of the first five traces was used in the calculation, while for I_{A9C} the mean of the last 5 stable traces was used. Block is manifest as a negative value using the above formula and is graphed as such. Mean values were then plotted at each voltage.
As phosphorylation has been proposed to cause state-dependent block of native $\text{Cl}_{\text{Ca}}$ channels (106), we chose to focus on T610 for this study as it lies near the putative pore-forming region and is conserved in human. We mutated threonine 610 to an alanine in order to determine whether this putative phosphorylation site affects the regulation of Tmem16a as seen by changes in rundown of $I_{\text{Cl(Ca)}}$ when phosphorylation is either promoted or minimized by pipette solutions containing 5 mM ATP or 0 ATP, respectively.

Figure 9 Ai displays two representative experiments recorded in HEK293 cells transfected with either wild-type (WT) Tmem16a, or Tmem16a where threonine 610 has been mutated to an alanine (T610A), each of which are tagged with eGFP for identification of successfully transfected cells. Cells were dialyzed with a pipette solution containing 500 nM free $\text{Ca}^{2+}$ along with 5 mM ATP. Immediately upon seal rupture (traces marked ‘a’), an instantaneous current was observed in HEK293 transfected with either WT or T610A Tmem16a upon stepping from $\text{HP} = -50$ mV to $+90$ mV for 1 s, followed by an outward current that developed with time. The mean time constant of activation of Tmem16a T610A was slightly faster than that of WT Tmem16a, although the difference was not statistically significant ($114.07 \pm 11.67$ ms and $90.07 \pm 14.01$ ms for WT and T610A, respectively). Cells were then repolarized to $-80$ mV for 1 s in order to enhance the magnitude of the tail current. WT and Tmem16a T610A both displayed tail currents that were proportional in magnitude to their preceding outward relaxation, with Tmem16a T610A deactivating slightly quicker ($54.83 \pm 8.22$ ms compared to $70.39 \pm 7.29$ ms for WT TMEM16A, $P > 0.05$). Following 50 s of dialysis (traces marked ‘b’), currents of both WT and T610A Tmem16a ran down to $\sim 85$ to 90% of the initial current. At the end of the 5 min experiment, currents had...
run down even further and stabilized at ~ 67 % of the initial current amplitude (traces marked ‘c’). Figure 9Aii shows mean data for the experiments described in figure 9Ai. This figure clearly demonstrates similar rundown of both WT and T610A Tmem16a (WT Tmem16a = black squares, n = 19; Tmem16a T610A = green triangles, n = 12). The only noticeable difference occurred immediately following seal rupture, where the outward current measured at the end of the 1 s depolarization in HEK193 cells transfected with the T610A mutant increased from 100 (normalized amplitude) to 110.56 ± 8.05 % within 5 s before quickly running back down to levels similar to WT TMEM16A. Time points marked ‘a’, ‘b’, and ‘c’ correspond to the traces found in figure 9Ai.

Figure 9Bi displays representative families of currents recorded from HEK293 cells transfected with either WT Tmem16a (top), or the Tmem16a T610A mutant (bottom). Currents were generated as previously described in figure 4. While the two families of currents are quite similar, the slightly quicker time courses of both activation and deactivation are quite clear in these two cells. Plotting current density against voltage for both WT and mutant Tmem16a, as in figure 9Bii, results in two virtually identical I-V relationships. Taken together, the data presented in figure 9 suggest that T610 of murine Tmem16a is likely not the main site of phosphorylation responsible for the state-dependent block previously observed when promoting phosphorylation (106, 126), Chapter 2, Chapter 3).
4.4.6 The T610A Mutation Alters Tmem16a Pharmacology

While our data suggested that T610 does not appear to be involved in the rundown of \( I_{\text{Cl(Ca)}} \) when phosphorylation is supported, we nevertheless decided to examine the impact of such mutation on NFA and A9C since T610 is speculated to lie the pore of the Tmem16a protein and both agents exert open state block on \( I_{\text{Cl(Ca)}} \) (chapters 2 and 3). These experiments yielded some interesting results. Figure 10A depicts families of currents generated from HEK293 cells expressing the Tmem16a T610A mutant before (left) and after (right) application of 100 µM NFA. Currents were evoked as described in figure 4. NFA significantly slowed both the time constant of activation and deactivation of WT and T610A Tmem16a (see figure 12). Figure 10B displays mean I-V relationships for both the T610A mutant (left) and WT Tmem16a (right). Not only did NFA block \( I_{\text{Cl(Ca)}} \) of HEK293 cells transfected with the TMEM16A T610A mutant considerably more than WT Tmem16a, but the mutation also induced a large leftward shift of the potential where block began to occur (~ -30 mV shift). Interestingly, the stimulation by NFA observed at the extreme negative potentials with WT Tmem16a disappeared with the T610A mutation Plotting the % block of \( I_{\text{Cl(Ca)}} \) as a function of voltage (figure 10C) unmistakably demonstrates the greater block of Tmem16a T610A by NFA, which was significantly greater than WT Tmem16a at +90, +100, and +110 mV (\( P < 0.05 \)). While block at the other potentials was not significantly different, it was often close enough that a few additional experiments would have probably pushed the difference to significance. Another difference between block of WT and T610A Tmem16a at positive potentials is the voltage-dependence of block by 100 µM NFA. Mutating threonine
610 to an alanine resulted in a condition where block by NFA was nearing voltage-
independence, as seen by the much flatter curve of % block.

Representative families of currents recorded both before (left) and after (right) addition of 500 µM A9C to an HEK29 cell expressing Tmem16a T610A are displayed in figure 11A. Currents were elicited as described in figure 4. Note the considerable block of Tmem16a at +140 mV by 500 µM A9C, and the subsequent stimulation upon repolarizing the cell to -80 mV. As few cells maintained a good whole-cell patch long enough to record a current-voltage relationship in the presence of A9C, % block by A9C at +90 mV was determined and graphed in figure 11B (time course protocol consisting of constant 1 s steps from a HP of -50 mV to +90 mV, followed by a 1 s return step to -80 mV before returning to HP, repeated every 5 s). The % block by A9C was determined by using the average of the first five traces (before A9C) and last five traces (after A9C) of a 5 min dialysis. Block of $I_{Cl(Ca)}$ by 500 µM NFA was slightly greater in HEK293 cells expressing the Tmem16a mutant (27.80 ± 13.14 % and 36.57 ± 15.38 % for WT and T610A TMEM16A, respectively), this increase was not significant. The % stimulation of the tail upon repolarization was similarly calculated (figure 11C). While stimulation appears to be much greater with the mutated Tmem16a, this effect again was not statistically significant (80.47 ± 49.03 % and 426.06 ± 224.06 % for WT and T610A Tmem16a, respectively). Interestingly, two cells in each group displayed block of the tail current at -80 mV. In cells transfected with WT Tmem16a ($n = 7$), block in these two cells was 29.67 % and 2.72 %. In cells transfected with the T610A Tmem16a mutant ($n = 5$), block of the two cells by A9C was 56.97 % and 20.35 %. Looking only at the remaining cells displaying stimulation at -80 mV (WT: $n = 5$; T610A: $n = 3$), stimulation was
Figure 9. $I_{\text{Cl(Ca)}}$ of the T610A mutant in HEK293 cells. Ai) Representative current traces demonstrate the time dependent changes of Ca$^{2+}$-activated Cl⁻ currents recorded from HEK293 cells transiently transfected with wild-type TMEM16A-eGFP (top) and the T610A TMEM16A mutant (bottom) dialyzed with 5 mM ATP. Currents depicted were recorded immediately following breaking the seal (0 s, trace ‘a’), 50 s into the experiment (trace ‘b’), and after 5 min (300 s, trace ‘c’) of cell dialysis. The currents were elicited by repetitive 1 s step depolarizations (every 5 s) to +90 mV from a holding potential (HP) of -50 mV. Each depolarizing pulse was followed by a repolarizing step to -80 mV to enhance the magnitude of the tail current. Aii) This graph depicts the mean time course of $I_{\text{Cl(Ca)}}$ amplitude elicited by 1-s depolarizing pulses to +90 mV, followed by 1-s repolarizing steps to -80 mV, in cells transiently transfected with wild-type TMEM16A-eGFP (black squares, n = 19) or the T610A TMEM16A mutant (green triangles, n = 12). Currents were normalized to the initial current amplitude at the beginning of the protocol. Each step was applied from HP = -50 mV at a frequency of one pulse every 5 s for 5 min. Time points marked ‘a’, ‘b’, and ‘c’ correspond to those described above in Ai. Bi) Representative families of currents from cells transfected with either wild-type TMEM16A (top) or the T610A mutant (bottom). All cells were dialyzed with 5 mM ATP. Currents were evoked by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV following a 5 min period of dialysis. Bii) Mean current-voltage relationships generated from families of currents elicited as described in Bi from wild-type TMEM16A-eGFP transfected HEK293 cells (black squares, n = 19) and HEK293 cells transfected with the T610A mutant (green triangles, n = 12).
significantly greater in HEK293 cells expressing T Tmem16a T610A (119.14 ± 61.00 % and 735.87 ± 217.35 % for WT and T610A Tmem16a, respectively. $P < 0.01$). Perhaps simply performing more experiments would push the difference in stimulation between WT and the mutant Tmem16a towards statistical significance. However, the block of $I_{\text{Cl(Ca)}}$ tail by A9C in a minority of cells may prove be a real effect, deserving further investigation.

Figure 12 displays the mean time constant of activation at +80 and the mean time constant of deactivation at -80 mV of HEK293 cells transfected with either WT Tmem16a (closed bars) or the T610A mutant (open bars). Time constants were measured for the first trace following seal rupture (black bars), after 5 min of dialysis with 5 mM ATP pipette solution (red bars), and following application of 100 µM NFA (figure 12A) or 500 µM A9C (figure 12B). Unlike native $I_{\text{Cl(Ca)}}$ of rabbit PASMCs, the time constant of activation of HEK cells expressing either WT or T610A Tmem16a did not slow down during the course of the initial dialysis (figure 12A and B, left), although unlike chapters 2 and 3 where dialysis was allowed to proceed for 10 min, dialysis in this case was only 5 min. As described in figure 9, the mean time constant of activation of Tmem16a T610A was slightly faster than that of WT Tmem16a, although not significantly. The time constant of $I_{\text{Cl(Ca)}}$ deactivation at -80 mV did slow down slightly over the course of the 5 min dialysis in cells expressing WT Tmem16a (by only 9 ms, $P > 0.05$), while remaining unchanged in cells transfected with the T610A mutant. The notable difference between WT and T610A Tmem16a time constants of either activation or deactivation occurred following drug application. NFA application significantly slowed the time constant of activation at +90 mV of WT Tmem16a from 115.68 ± 8.15 ms following 5 min of dialysis to 351.19 ± 108.89 ms ($P < 0.001$), and had a similar effect on the
time constant of activation of the Tmem16a T610A mutant (91.70 ± 10.80 ms after 5 min dialysis and 349.03 ± 40.47 ms with 500 µM A9C, \( P < 0.001 \)). NFA had a similar effect on the time constant of deactivation of \( I_{\text{Cl(Ca)}} \) of HEK293 cells transfected with either WT or T610A TMEM16A at -80 mV. The time constant of deactivation of WT Tmem16a was slowed from 79.96 ± 8.14 ms to 482.62 ± 86.12 ms (\( P < 0.001 \)) by NFA. The time constant of deactivation of Tmem16a T610A was slowed by NFA from 54.92 ± 5.47 ms to 447.93 ± 6.15 ms (\( P < 0.001 \)).

Figure 12B displays time constants of activation (left) and deactivation (right) before and after addition of 500 µM A9C. Drug application following a 5 min dialysis resulted in a significant slowing of the time constant of activation of both WT Tmem16a (115.68 ± 8.15 ms to 270.95 ± 32.73, \( P < 0.001 \)) and Tmem16a T610A (91.70 ± 10.80 ms to 415.64 ± 111.13 ms, \( P < 0.01 \)). These results are similar to those seen in rabbit PASMCs (chapter 3). Also, and as in PASMCs, A9C significantly enhanced the time constant of deactivation of WT Tmem16a from 79.96 ± 8.14 ms to 222.76 ± 46.86 ms (\( P < 0.001 \)). Interestingly, A9C did not affect the time course of deactivation of the T610A mutant, suggesting that T610 may play a role in channel gating (see Discussion).

Taken together, the data involving the Tmem16a T610A mutant suggest that while this putative CaMKII phosphorylation site may not play a major role in down regulation of \( I_{\text{Cl(Ca)}} \), it does appear to impact the interaction of both NFA and A9C with the channel and may participate in channel gating.
4.5 Discussion

The data presented in this study provide strong evidence for Tmem16a being at least a component of Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels of the pulmonary artery. We demonstrate here that TMEM16A mRNA is expressed in rat and mouse pulmonary arteries, which is in agreement with a recent study that demonstrated the presence of Tmem16a mRNA in rat and human pulmonary arteries (272). Our data also convincingly demonstrate that alternative splicing occurs in the mouse pulmonary artery, demonstrating that alternative segments ‘a’ and ‘c’ are always present, while transcripts may either contain or lack the alternatively spliced exons ‘b’ and ‘d. Again, these results mirror those found by Manoury et al. (272), who also identified TMEM16B, TMEM16D, TMEM16E, TMEM16F, and TMEM16K in rat and human pulmonary artery, although TMEM16B, TMEM16D, and TMEM16E were all at least 50 times less abundantly expressed than TMEM16A, TMEM16F, and TMEM16K. Transient transfection of HEK293 cells with eGFP-tagged Tmem16a resulted in the appearance of a robust time- and voltage-dependent current when dialyzed with a pipette solution containing 500 nM free Ca\textsuperscript{2+}, which displayed outward rectification and reversed near 0 mV, which corresponds to the calculated reversal potential of Cl\textsuperscript{-} in these experiments. Comparable currents have been seen by others using similar methods (220, 221, 222, 258, 264). These currents respond to niflumic acid in a similar manner as native \(I_{Cl(Ca)}\) of rabbit pulmonary artery, while their response to anthracene-9-carboxylic acid was quite different. While \(I_{Cl(Ca)}\) of Tmem16a did not run down when phosphorylation was supported to the same extent as it did in rabbit PASMCs, it did run down slightly. Therefore, we mutated a putative CaMKII phosphorylation site (T610) that is conserved in human and
Figure 10. Effect of niflumic acid on $I_{\text{Cl(Ca)}}$ of HEK293 cells transfected with the T610A Tmem16a mutant. A) Representative families of currents from cells dialyzed with 5 mM ATP or 0 ATP before (left) and after (right) a 5 min application of 100 µM NFA. Currents were evoked by stepping in 10 mV increments from $HP = -50$ mV to 1 s test potentials ranging from -100 mV to +140 mV following a 5 min period of dialysis. B) Mean current-voltage relationships generated from families of currents elicited as described in A from wild-type Tmem16a-eGFP transfected HEK293 cells (left, n = 6) and HEK293 cell transfected with the T610A Tmem16a mutant (right, n = 4). All cells were dialyzed with 5 mM ATP. I-V relationships before and after addition of 100 µM NFA are depicted. C) The two data sets were obtained after 5 min of cell dialysis with 5 mM ATP before and after addition of 100 µM NFA in HEK293 cells transiently transfected with either wild-type Tmem16a (black squares, n = 6) or the T610A mutant (light blue triangles, n = 4). Currents were elicited by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV and measured at the end of the 1 s test step. % Block was determined at each individual voltage step for each cell using the formula 100-($I_{\text{NFA}}/I_{\text{Control}}$)*100, where $I_{\text{NFA}}$ and $I_{\text{Control}}$ is the current recorded in the presence and absence of NFA, respectively. For $I_{\text{Control}}$, the mean of the first five traces was used in the calculation, while for $I_{\text{NFA}}$, the mean of the last 5 stable traces was used. Stimulation is manifest as a negative value using the above formula and is graphed as such. Mean values were then plotted at each voltage.
located near the projected pore-forming region in an attempt to identify the main site responsible for phosphorylation-induced rundown. Mutating T610 to an alanine had no effect on the rundown, but rather altered the effects of both NFA and A9C on the current.

4.5.1 Expression of Tmem16a in the Pulmonary Artery

*Tmem16a* is expressed in many of the tissues that express $I_{\text{Cl(Ca)}}$, with expression demonstrated in bronchiolar epithelial cells, pancreatic acinar cells, proximal kidney tubule epithelium, retina, dorsal root ganglion sensory neurons, and the submandibular gland (220, 221, 222). Most recently, *Tmem16a* mRNA has been identified in both rat and human arteries (272). Begun before this recent report of Tmem16a in the pulmonary artery, our study confirms the results of Manoury et al. (272), showing expression in several vascular tissues including mouse carotid and thoracic arteries, portal vein, and rat pulmonary artery. The RT-PCR data is corroborated by Western blot analysis, which demonstrated Tmem16a protein expression in these tissues. Using immunocytochemistry, Tmem16a was identified in both rat and mouse pulmonary artery and appeared to localize near or at the plasma membrane, although the protein was apparent in the cytoplasm as well. Similarly, Tmem16a was found on the apical membrane of the submandibular gland (222), suggesting that Tmem16a is a membrane-bound protein.

As Tmem16a chloride channel properties have been reported to be regulated by alternative splicing (220, 258), we investigated which splice variant(s) were present in the mouse pulmonary artery. We found alternative segments ‘a’ (116 residues) and ‘c’ (4 residues) always to be present while segments ‘b’ (22 residues) and ‘d’ (26 residues) were
Figure 11. Effect of anthracene-9-carboxylic acid on $I_{\text{Cl(Ca)}}$ of HEK293 cells transfected with the T610A TMEM16A mutant. A) Representative families of currents from cells dialyzed with 5 mM ATP or 0 ATP before (left) and after (right) a 5 min application of 100 µM NFA. Currents were evoked by stepping in 10 mV increments from HP = -50 mV to 1 s test potentials ranging from -100 mV to +140 mV following a 5 min period of dialysis. B) Current in HEK293 cells transfected with wild-type TMEM16A (black bar, n= 7) or the T610A mutant (green bar, n = 5) was recorded at the end of 1 s depolarizing pulses to +90 mV from HP of -50 mV, and % block for each cell was calculated by the formula $(1 - (I_{\text{A9C}}/I_{\text{Control}}) \times 100) \times 100$, where $I_{\text{A9C}}$ and $I_{\text{Control}}$ are the currents recorded in the presence and absence of A9C, respectively. For $I_{\text{Control}}$, the mean of the first five traces was used in the calculation, while for $I_{\text{A9C}}$ the mean of the last 5 stable traces was used. C) Current in HEK293 cells transfected with wild-type TMEM16A (black bar, n= 7) or the T610A mutant (green bar, n = 5) was recorded at the start of 1 s repolarizing steps to -80 mV following a 1 s depolarizing pulses to +90 mV from HP of -50 mV, and % stimulation for each cell was calculated by the formula $100 - ((I_{\text{A9C}}/I_{\text{Control}}) \times 100)$, where $I_{\text{A9C}}$ and $I_{\text{Control}}$ are the currents recorded in the presence and absence of A9C, respectively. For $I_{\text{Control}}$, the mean of the first five traces was used in the calculation, while for $I_{\text{A9C}}$ the mean of the last 5 stable traces was used.
found to be either present or lacking, suggesting the exons encoding these segments (exons 6b for ‘b’ and 15 for ‘d’) occurs in this tissue. These results match those of Manoury et al. (272) in the rat pulmonary artery. It should be emphasized that any claim that segment ‘a’ is present in all transcripts must be made with caution, as it is not possible to design primers that span this exon, as it appears to be under the control of an alternate promoter (258). An extensive study has identified the role of these alternatively spliced segments in regulating human Tmem16a (258). The 22 residue segment ‘b’ was found to decrease Ca^{2+} sensitivity of Tmem16a by nearly 4 fold when the corresponding exon 6b was expressed. Segment ‘c’, although only four amino acids, appears to play an important role in regulating the voltage-dependence of Tmem16a. In transcripts lacking segment ‘c’, currents were larger, with a large instantaneous component and small time-dependent component with reduced rectification when compared to transcripts possessing segment ‘c’. Interestingly, skipping of the corresponding exon (13) is quite rare, with brain and skeletal muscle the only tissues investigated in the study that displayed alternative splicing of this exon (our lab does have some evidence – not presented in this dissertation – that exon 13 may be alternatively spliced in the murine carotid artery). A previous study had found no discernable role for segment ‘d’ (220). Alternative splicing of pre-mRNA is important in expanding proteome diversity and regulating tissue-specific expression (258). In the case of the pulmonary artery, alternative splicing could potentially produce heteromers composed of several different transcripts resulting in the specific biophysical properties of I_{Cl(Ca)} recorded in pulmonary artery smooth muscle cells. Other TMEM16 proteins may also be involved in producing a
distinct $\text{Cl}_{\text{Ca}}$ channel, a notion supported by the identification of $\text{Tmem16f}$ and $\text{Tmem16k}$ mRNA in the rat pulmonary artery (272).

### 4.5.2 Tmem16a as a Molecular Candidate for $\text{Ca}^{2+}$-Activated $\text{Cl}^-$ Channels of Pulmonary Artery

In the present study, HEK293 cells were transiently transfected with a Tmem16a-eGFP fusion protein. Overlaying the epifluorescence of the GFP with the Tmem16a-dependent fluorescence revealed considerable co-localization to the plasma membrane. It has been proposed that Tmem16a-dependent fluorescence exhibits clustering when over-expressed, and may localize to lipid microdomains (240). Interestingly, another study demonstrated that the amplitude and pharmacology of native $I_{\text{Cl(Ca)}}$ in vascular smooth muscle is affected by cholesterol depletion, suggesting that native $\text{Cl}_{\text{Ca}}$ channels may occur in plasma membrane microdomains (119). However, no plasma membrane staining was in non-transfected HEK293 cells, which express Tmem16a (240). This may be attributed to the low abundance of this protein in these cells (small or undetectable $I_{\text{Cl(Ca)}}$ in untransfected HEK293 cells) and low affinity of the antibody used in our study. The latter possibility is supported by the much lower intensity of the band detected by the Tmem16a antibody relative to the one targeting GFP in Western blot experiments.

Currents generated by Tmem16a over-expression in HEK293 cells resemble native $I_{\text{Cl(Ca)}}$ of acinar cells, neurons, and smooth muscle cells (71, 106, 222). The role of Tmem16a in $I_{\text{Cl(Ca)}}$ is further supported by data collected from Tmem16a knock-out mice, as well as siRNA experiments in epithelial cells (240, 262, 263), interstitial cells of Cajal of murine stomach and intestine (242, 244), and recently in the rat pulmonary artery (272). In our study, HEK293 cells
expressing Tmem16a -eGFP that lacked alternative segments ‘b’, ‘c’, and ‘d’ demonstrated a Cl− current elicited by 500 nM free Ca2+ with notable similarities to PASMC $I_{Cl(Ca)}$, including strong outward rectification and slow voltage-dependent kinetics of activation and deactivation. Native $I_{Cl(Ca)}$ is activated in voltage dependent manner, with a $V_{0.5}$ at +60 mV of $\sim$ 300 nM (106, 252), which is similar to HEK293 cells over-expressing Tmem16a (222, 258). Tmem16b on the other hand, is considerably less abundant in pulmonary artery smooth muscle cells (unpublished data from our group, reference (272)), and has an IC50 at +50 mV of 3.3 µM (253).

There have been several reports demonstrating that $I_{Cl(Ca)}$ of PASMCs are regulated by a balance between kinase (CaMKII) (106, 107, 123) and phosphatase (CaNA α, PP1, PP2A) (108, 109) activities. This regulation is manifest as current rundown when ATP is included in the pipette solution (3 or 5 mM), or reduced rundown of the current followed by complete recovery back to initial levels when ATP is either removed from the pipette solution or replaced with the non-hydorlyzable ATP analogue AMP-PNP (106, 126), Chapter 2 and 3 of this dissertation). This study demonstrates that while HEK293 cells follow a similar pattern, rundown is considerably less when phosphorylation is supported, and is much slower when phosphorylation is minimized when compared to the native $I_{Cl(Ca)}$ of PASMCs. While unexplored, this may be due to differences in endogenous kinase and phosphatase activities, enzyme distribution, translocation mechanisms and the profile of protein partners in HEK293 cells compared to PASMCs. As of yet, there are no data demonstrating if or how Tmem16a is regulated by CaMKII or phosphatases.
4.5.3 Pharmacology of Tmem16a

Among the various Cl⁻ channel blockers, niflumic acid is often considered to be ‘relatively specific’ for Cl₉ Ca channels. Of the known Cl⁻ channel blockers it is the most effective inhibitor of $I_{Cl(Ca)}$ (70). NFA was first demonstrated to inhibit $I_{Cl(Ca)}$ of smooth muscle in 1989 (91). Since then, NFA at concentrations between ~ 1-50 µM has been shown to block $I_{Cl(Ca)}$ evoked in a variety of ways, that include Ca²⁺ entry through L-type Ca²⁺ channels (139) or reverse-mode Na⁺/Ca²⁺ exchange (140), or by intracellular Ca²⁺ mobilization mediated by various constricting agonists, caffeine (141), spontaneous Ca²⁺ sparks (STICS) (141), or by flash photolysis of caged Ca²⁺ (92). NFA has been shown to have a dual effect, both inhibiting and stimulating $I_{Cl(Ca)}$ in vascular smooth muscle cells (110, 135). Piper et al. (135) first reported this effect in PASMCs, where bath application of 100 µM NFA resulted in increased inward currents at negative potentials, but inhibited outward currents at positive potentials. We demonstrated that Cl⁻ currents generated by Tmem16a expression are blocked in a similar voltage-dependent manner by NFA, although the magnitude of block was considerably less than in PASMCs. The difference in the magnitude of block was also less between Tmem16a-expressing HEK293 cells dialyzed with 5 mM ATP or 0 ATP than in native cells. This decrease may be due to the smaller difference in rundown observed between cells dialyzed with 5 mM ATP vs. 0 ATP in HEK293 cells over-expressing Tmem16a than in rabbit PASMCs. As discussed in chapter 2, phosphorylation results in a state-dependent block of the channel, limiting the ability of NFA to further block the current. Another explanation may be that different splice variants respond to the drug differently, and in a heteromer with either different splice variants or isoforms (or both) of Tmem16 the
Figure 12. Time course of activation and deactivation is altered in the T610A mutant. A) The time course of activation during a 1s depolarization to +90 mV (left) and deactivation during a subsequent repolarization to -80 mV (right) were measured in HEK293 cells transfected with wild-type Tmem16a (filled bars, n = 6) or the T610A mutant (open bars, n = 4). Time courses of activation and deactivation were measured immediately following seal rupture (black bars), after 5 min of dialysis with 5 mM ATP (red bars), and at the end of a second 5 min dialysis in the presence of 100 µM NFA (blue bars). B) Generated similarly to panel A, with the exception that cells were subjected to 500 µM A9C rather than NFA (WT TMEM16A: n = 7; T610A: n = 5).
pharmacology of the channel is altered. NFA significantly slowed the time constants of both activation and deactivation of Tmem16a currents like in rabbit PASMCs, and Tmem16a currents were stimulated by NFA at highly negative potentials (-100 mV to -60 mV), which suggests that even given a decreased magnitude of block by NFA, the drug appears to interact with Tmem16a and native Cl\textsubscript{Ca} channels in a similar manner.

A9C is a Cl\textsuperscript{-} channel blocker chemically unrelated to NFA that inhibits STICs in rabbit portal vein smooth muscle (148, 149). This compound also inhibits I\textsubscript{Cl\textsubscript{Ca}} evoked by Ca\textsuperscript{2+} entry through VDCCs in several tissues (132, 150, 151, 152). Block by A9C is highly voltage-dependent (IC\textsubscript{50} for inhibition of STICs of 300 µM at -50 mV and 90 µM at +90 mV (149)), and appears to interact with open channels on the extracellular surface (147). Unlike native Cl\textsubscript{Ca} channels, block of Tmem16a currents was identical in cells dialyzed with 5 mM ATP or 0 ATP, suggesting that phosphorylation did not affect, at least in our conditions, the ability of A9C to inhibit Tmem16a-induced currents. A9C did still block the current considerably however, to levels comparable to rabbit PASMCs in which phosphorylation was supported by 5 mM ATP. Piper and Greenwood (153) reported that while application of 500 µM A9C resulted in a small inhibition of the outward current recorded at +70 mV, the amplitude of the instantaneous inward relaxation at -80 mV increased by over 300%. Similar results were reported in chapter 3. Stimulation of I\textsubscript{Cl\textsubscript{Ca}} of HEK293 cells transfected with Tmem16a was found to be severely reduced. Chapter 3 documented stimulation of I\textsubscript{Cl\textsubscript{Ca}} in rabbit PASMCs by A9C of over 250% in cells dialyzed with 5 mM ATP pipette solution. In this study, Tmem16a-expressing HEK293 cells dialyzed with 5 mM ATP did not reach 100% stimulation. Cells dialyzed with either 5 mM ATP or 0 ATP followed a trend similar to that of rabbit
PASMCs with 0 ATP, reaching a peak stimulatory effect before decreasing. Stimulation of $I_{\text{Cl(Ca)}}$ by A9C is therefore also present upon repolarizing the Tmem16a-expressing HEK293 cells, but occurs to a much lesser extent than in rabbit PASMCs. As discussed for NFA this may be due to the relative lack of phosphorylation of the channel, resulting in reduced availability of a hypothetical A9C binding stimulatory site.

4.5.4 Effect of Mutating a Putative Phosphorylation Site of Tmem16a Residing Near the Pore-Forming Region

Several studies have performed single-point mutations in order to better understand the Tmem16a channel (reviewed in Hartzell et al. (239)). These studies have provided important information regarding the channel pore, where mutations in the region between transmembrane domains 5 and 6 alter properties such as ion selectivity, gating, voltage-, and time-dependence. Angermann et al. (106) proposed a kinetic model whereby phosphorylation induces state-dependent block through voltage-dependent gating steps favoring channel closure. In an attempt to identify at least one of the phosphorylation sites involved in this process, we mutated threonine 610 to an alanine. This particular phosphorylation consensus site was chosen as it lies near the putative pore-forming region and is conserved in human Tmem16a. This mutation disappointingly did not alter the relatively small rundown observed in Tmem16a over-expressing HEK293 cells, suggesting that this particular phosphorylation consensus site does not seem to be a contributor to the well documented ATP-mediated rundown of rabbit PASMCs (106, 126), chapters 2 and 3. Interestingly, the T610A mutation did alter the pharmacology of the Tmem16a channel. The
mutation resulted in a dramatic shift in the voltage-dependence of block by NFA compared to the wild-type Tmem16a, with block becoming nearly voltage-independent, at least in the positive range of membrane potentials. This finding may suggest that NFA blocks Tmem16a T610A in a largely state-independent manner at positive membrane potentials, although the mutation might have affected the interaction of NFA with the channel in such a way that the voltage-dependence was not apparent at positive potentials, but may well have been apparent and voltage-dependent at negative potentials. This could result from an increase in the apparent affinity of the channel for NFA at positive potentials, although more experiments are required to confirm this hypothesis as voltage-dependence of block could be present at negative potentials. While block by A9C at +90 mV was comparable between wild-type and the T610A Tmem16a mutant, the mutant exhibited considerably more stimulation following repolarization to -80 mV. Additionally, while A9C significantly slowed deactivation of wild-type Tmem16a, the T610A demonstrated no change. As the mutation presumably did not affect phosphorylation, as demonstrated by the lack of change in the rundown during dialysis with 5 mM ATP, the mutation may be indirectly affecting interaction of the drug with the channel or altering channel gating.

4.5.6 Possible Limitations

HEK293 cells were transiently transfected with Tmem16a (a) tagged with eGFP in order to visually identify successfully transfected cells while patch clamping. We did not test however, the possibility that the eGFP tag may have had some effect on the channel. Having
a large globular protein (~28 kDa) attached to the C-terminal end of Tmem16a may affect several factors, including physically blocking phosphorylation of the channel. If this were the case, the present study would greatly underestimate the phosphorylation-induced rundown of the Tmem16a-dependent current, which could in turn mask any differences in pharmacology when phosphorylation is either promoted or minimized. In the future, a bicistronic approach will be employed. A bi-cistronic system would allow for the expression of two proteins under the control of a unique promoter, permitting the expression of Tmem16a and eGFP as separate proteins within the same cell. As the eGFP protein would no longer be physically linked and maintained in close proximity to Tmem16a, it is unlikely that eGFP could have any direct effect on the current.

Another concern in the present study involved the recordings obtained from HEK293 cells transiently transfected with TMEM16A-eGFP. The issue lies in the size of the currents and the difficulty of effectively clamping the currents due to the presence of a significant series resistance between the patch micropipette and the intracellular milieu. Although we tended to patch ‘faint’ cells displaying presumably a lower level of Tmem16a expression, recorded currents often exceeded 10 nA in magnitude, yielding to an undesirable situation of not precisely clamping voltage, especially at the extremes of our voltage step protocol where errors of up to 90 mV may have been encountered (with a measured access resistance of ~ 9 to 10 MΩ in some cases). While series resistance compensation was considered, we decided against employing it in these preliminary studies. First, all cells would be subject to a similar error. As seen by the shape of the I-V relationships presented, no large abnormalities were apparent (e.g. an all or none step due to complete loss of
voltage control), even at the most extreme potentials. Additionally, good stable whole-cell seals were already difficult to come by, and we did not want to further complicate the matter as over-compensation often leads to unbounded oscillations that could have led to seal breakdown and loss of the cell. It must be noted however, that by not employing series resistance compensation we clearly underestimated current amplitude and and the magnitude of phosphorylation-induced current rundown, which we represented on a percentage basis relative to initial current amplitude (normalized). Future studies involving similar recordings from HEK293 cells transiently transfected with \( Tmem16a \) must either: a) decrease the amount of Tmem16a expression thus resulting in smaller currents, where errors due to series resistance would be negligible, or b) effectively employ series resistance compensation.

4.5.7 Conclusion

This study presents indirect evidence that Tmem16a may be at least a part of the native \( Cl_{Ca} \) channel of pulmonary artery smooth muscle cells. We demonstrate that \( Tmem16a \) mRNA is present in rat pulmonary artery, as well as located in or near the plasma membrane of rat and mouse single pulmonary artery myocytes. Additionally, transiently transfecting HEK293 cells with Tmem16a – although lacking alternative segments ‘b’, ‘c’, and ‘d’ – resulted in \( Cl^{-} \) currents that were qualitatively quite similar to the native \( I_{Cl(Ca)} \). These currents were reasonably well blocked by NFA. Currents of the Tmem16a over-expressing HEK293 cells did not display rundown to the same extent as in rabbit PASMCs
however, and the effects of A9C were quite different between the two groups of cells.

These differences may be due to the splice variant tested, or perhaps the native channel is composed of a multimeric complex that exhibits a variable composition of several different splice variants, perhaps along with other Tmem16 isoforms. Further studies examining this possibility are required, and may further uncover the precise identity of pulmonary arterial Cl<sub>ca</sub> channels.
Chapter 5: Summary and Conclusions

The studies described in this dissertation examined the effect of global phosphorylation status on the properties of Ca$^{2+}$-activated Cl$^{-}$ channels of rabbit pulmonary artery smooth muscle cells and how it impacts on their pharmacology utilizing two distinct Cl$^{-}$ channel blockers (niflumic acid and anthracene-9-carboxylic acid). Additionally, we investigated the possibility that the newly proposed Ca$^{2+}$-activated Cl$^{-}$ channel candidate Tmem16a makes up at least a part of the native Cl$_{Ca}$ channel of rabbit PASMCs. We present for the first time that phosphorylation affects the interaction of Cl$^{-}$ channel blockers with Cl$_{Ca}$ channels. Tmem16a, which we demonstrate to be expressed in pulmonary artery smooth muscle cells, demonstrates striking pharmacological similarities to rabbit PASMCs when over-expressed in HEK293 cells.

In chapter 2 we tested the hypothesis that the state of phosphorylation of Cl$_{Ca}$ channels influences their pharmacology. This hypothesis was based upon a key observation. The literature reported a relatively wide range of IC$_{50}$ values for NFA to block I$_{Cl(Ca)}$ in vascular smooth muscle cells. NFA blocked STICs with an IC$_{50}$ of $\sim$1-4 µM (141), between 4 and 10 µM in coronary artery where I$_{Cl(Ca)}$ was evoked by Ca$^{2+}$ entry through L-type Ca$^{2+}$ channels, and 159 µM in coronary artery myocytes where I$_{Cl(Ca)}$ was elicited by sustained 500 nM Ca$^{2+}$. Ca$^{2+}$-activated chloride channels are regulated by at least one phosphorylation step involving the Ca$^{2+}$-Calmodulin-dependent protein kinase CaMKII (107, 123). Phosphorylation by CaMKII of a still undefined protein target is opposed by the phosphatases calcineurin-A$\alpha$ (108, 109), PP1, and PP2A (126). Dialyzing cells with 500 nM free Ca$^{2+}$ along with 5 mM ATP results in significant phosphorylation as manifest by
significant rundown of the current, while removing the substrate from the pipette solution
resulted in attenuated rundown followed by the complete recovery of the current. Autophosphorylation of CaMKII may be quite limited; in fact, the phosphorylation balance is
likely to be shifted toward a dephosphorylation state as CaN displays ~ 2 orders of
magnitude higher affinity for Ca\(^{2+}\)-CaM than CaMKII and is directly activated by Ca\(^{2+}\) via an
interaction with the B subunit of CaN (109, 124, 125, 268). Large sustained elevations in
[Ca\(^{2+}\)]\(_i\) caused by high concentrations of constricting agonists is likely to promote a higher
state of phosphorylation of \(\text{Cl}_{\text{Ca}}\) channels and reduced efficacy of NFA. Inhibition of \(I_{\text{Cl(Ca)}}\) by
NFA was significantly reduced in cells dialyzed with 5 mM ATP compared to those dialyzed
with an ATP-free pipette solution. Minimizing phosphorylation resulted in a large negative
shift in the voltage-dependence of block, which was converted to stimulation at potentials <
-50 mV, ~70 mV more negative than cells dialyzed with 5 mM ATP. NFA acid stimulated \(I_{\text{Cl(Ca)}}\)
of cells dialyzed with 5 mM ATP at a concentration of 0.1 µM, converting to block at
concentrations > 1 µM. The drug blocked \(I_{\text{Cl(Ca)}}\) in cells dialyzed with 0 ATP at all
concentrations tested. Stimulation of \(I_{\text{Cl(Ca)}}\) by NFA has been reported in vascular smooth
muscle; in PASMCs 100 µM NFA stimulated inward currents below \(E_{\text{Cl}}\) but inhibited outward
currents above \(E_{\text{Cl}}\) (135), and in coronary artery myocytes, where a mathematical model
developed based on the data proposed two binding sites where block by NFA binding to an
inhibitory site (high affinity) occludes simulation, a phenomenon revealed when the drug is
quickly washed out and resulting from the drug still occupying a stimulatory site (low
affinity). Based on our observation that phosphorylation reduced the ability of NFA to block
\(I_{\text{Cl(Ca)}}\) due to induced channel closure and previous reports demonstrating that NFA
preferentially interacts with open ClCa channels (141, 147), we propose that the inhibitory site lies near or perhaps close to the channel pore (Figure 1). With a stimulatory site removed from the pore, phosphorylation-induced channel closure would thus favor stimulation of ICi(Ca) (Figure 1).

Chapter 3 continued to test the hypothesis proposed in chapter 2, but utilized the Cl− channel blocker A9C in the place of NFA. A9C has been shown to inhibit ICi(Ca) evoked by Ca2+ entry through VDCCs in several tissues (132, 150, 151, 152). Block of ICi(Ca) by A9C is highly voltage-dependent, with IC50's for inhibition of spontaneous transient inward currents (STICs) of 300 µM at -50 mV and 90 µM at +90 mV (149). Inhibition may be rapidly reversed by hyperpolarization and evidence suggests that A9C blocks the channels while in the open state (147). In rabbit pulmonary artery smooth muscle cells (PASMCs), application of 500 µM A9C resulted in a small inhibition of the outward current recorded at +70 mV, but increased the amplitude of the instantaneous inward tail current relaxation at -80 mV by over 300% (153). Stimulation of ICi(Ca) upon hyperpolarization requires a preceding depolarization step, as A9C alone does not stimulate ClCa channels (153). Dephosphorylation enhanced the ability of A9C to inhibit ICi(Ca) in a concentration-dependent manner. Similar to NFA, this was attributed to a negative shift in the voltage-dependence of block. Stimulation of ICi(Ca) tail current by 500 µM A9C at -80 mV was enhanced in cells dialyzed with 5 mM ATP. While the tail current of cells dialyzed with 0 ATP were stimulated following depolarizations to +40 mV, the enhancement was abolished following steps to +140 mV. These data again suggest the presence of two binding sites: an inhibitory site and a stimulatory site. It appears that A9C blocks the open channel and that phosphorylation partially occludes
access to a blocking site found at the mouth or within the pore, similarly to NFA (Figure 1). Block of the open channel by A9C has previously been proposed based on the observation that STIC amplitude decreased while the time course of decay was lengthened (147). Minimizing phosphorylation resulted in decreased stimulation by A9C of the \( I_{\text{Cl(Ca)}} \) tail current when compared to cells in which phosphorylation was supported, suggesting a phosphorylation-dependent effect on a stimulatory site distinct from the inhibitory site. The two-site model is further supported by the fact that block was instantaneously relieved upon repolarizing the cell, leading to the stimulation of the inward tail current. It may be speculated that A9C binds to both a pore-associated inhibitory site and a stimulatory site away from the pore simultaneously. The effect of the block masks any stimulation, and the drug is quickly dislodged from the inhibitory site upon repolarization, unmasking stimulation (Figure 1). This theory is supported by the observation that A9C generally slows the time course of activation at positive potentials while having little or no effect of the time course of deactivation at negative potentials.

As discussed above, a model involving two binding sites is supported by both the NFA and A9C data. It is unclear though whether the effects of these two channel blockers function through a common mechanism. Piper and Greenwood (153) addressed this question by applying 100 \( \mu \text{M} \) NFA in the continued presence of 500 \( \mu \text{M} \) A9C. The current augmented by A9C at -80 mV was quickly inhibited with a significant slowing of the current decay, effects very similar to those produced by NFA alone. Washing out NFA while maintaining A9C resulted in a return of the current to levels similar to those prior to NFA application. When 500 \( \mu \text{M} \) A9C was added in the continued presence of 100 \( \mu \text{M} \) NFA, no
Figure 1. Cartoon demonstrating the effects of channel phosphorylation on $\text{Cl}_{\text{ca}}$ pharmacology. 
A) Phosphorylation (P) of the channel results in state-dependent inhibition of the channel. B) Phosphorylation of the channel results in diminished access of niflumic acid (NFA) to the inhibitory binding site. C) Dephosphorylated $\text{Cl}_{\text{ca}}$ channels demonstrate higher block but reduced stimulation upon repolarization to -80 mV by A9C compared to phosphorylated channels (D). Please refer to text for further discussions.
significant effect was observed; i.e.: there was no stimulation of the inward tail current at -80 mV. These results suggest that NFA and A9C may act via a similar mechanism, perhaps interacting with a common binding site, with the block by NFA appearing to be dominant. We thus propose a model where phosphorylation of ClCa channels favors drug binding to a high affinity stimulatory binding site removed from the pore, while dephosphorylation favors binding to a lower affinity inhibitory site near or partially within the channel pore.

Chapter 4 of this dissertation explored the possibility that the newly emerging Ca\(^{2+}\)-activated Cl\(^-\) channel candidate Tmem16a may form at least a portion of the native Cl\(_{Ca}\) channel of pulmonary artery smooth muscle cells. We demonstrated expression of Tmem16a mRNA in several vascular tissues including murine carotid and thoracic aortic arteries, portal vein, and pulmonary arteries of both mouse and rat. Tmem16a has been shown to undergo alternative splicing (220, 258). Using primers designed to either span or anneal to the alternatively spliced exons, we found alternative segments ‘a’ (116 residues) and ‘c’ (4 residues) to be always present in murine PA while segments ‘b’ (22 residues) and ‘d’ (26 residues) were found to be either present or lacking, suggesting the exons encoding these segments (exons 6b for ‘b’ and 15 for ‘d’) are alternatively spliced in this tissue. As segment ‘a’ appears to be under the control of an alternative promoter, we were not able to design primers to span mRNA for this sequence, and thus could not determine whether this segment may be alternatively spliced. Our results are in agreement with another recent study investigating whether Tmem16a makes up the Cl\(_{Ca}\) channel of rat PASMCs, which demonstrated comparable alternative splicing (272). Immunocytochemistry confirmed the expression of Tmem16a in both mouse and rat PASMCs. Tmem16a-dependent fluorescence
was evident in both species, with immunofluorescence apparent in the cytosol and more importantly at or near the plasma membrane.

HEK293 cells were then transiently transfected with a Tmem16a-eGFP fusion protein. Overlaying the epifluorescence of the GFP with the Tmem16a-dependent fluorescence revealed considerable co-localization at the plasma membrane. It has been proposed that TMEM16A-dependent fluorescence exhibits clustering when over-expressed, and may localize to lipid microdomains (240). Interestingly, no plasma membrane staining was found with non-transfected HEK293 cells, which do show endogenous Tmem16a expression (240). Chapter 4 further demonstrated a Cl⁻ current elicited by 500 nM free Ca²⁺ in HEK293 cells expressing Tmem16a-eGFP that lacked alternative segments ‘b’, ‘c’, and ‘d’. Currents were notably similar to PASMC I_{Cl(Ca)} possessing slow voltage-dependent kinetics of activation and deactivation. Several other studies have demonstrated HEK293 cells over-expressing Tmem16a to possess Ca²⁺-activated Cl⁻ currents resembling those of acinar cells, neurons, and smooth muscle cells (71, 106, 222). The role of Tmem16a in the native I_{Cl(Ca)} is further supported by data collected from Tmem16a knock-out mice, as well as siRNA experiments in epithelial cells (240, 262, 263), interstitial cells of Cajal of murine stomach and intestine (242, 244), and recently in the rat pulmonary artery (272). While Tmem16a-dependent currents did show signs of phosphorylation-dependent regulation, I_{Cl(Ca)} of HEK293 cells expressing Tmem16a and dialyzed with 5 mM ATP displayed significantly less rundown than PASMCs. And while the TMEM16A over-expressing cells dialyzed with an ATP-free pipette solution demonstrated similar normalized current amplitude to the PASMCs at the 5 min mark, rundown occurred with a much slower time course. In an attempt to identify a potential consensus site for phosphorylation site by CaMKII on Tmem16a, we mutated a site conserved in humans that lies near the putative
pore-forming region. The T610A mutation did not alter current rundown, suggesting that T610 is not the site involved in the regulation of $I_{\text{Cl(Ca)}}$ by CaMKII.

We demonstrated that Cl$^-$ currents carried by Tmem16a were blocked by NFA in a similar voltage-dependent manner, although the magnitude of block was considerably less than in PASMCs. The difference in the magnitude of block was also less between Tmem16a-expressing HEK293 cells dialyzed with 5 mM ATP or 0 ATP than in native cells. This decrease may be due to the smaller difference in rundown between cells dialyzed with 5 mM ATP vs. 0 ATP in HEK293 cells over-expressing Tmem16a than in rabbit PASMCs. As phosphorylation results in a state-dependent block of the channel, dialyzing cells with 5 mM ATP would limit the ability of NFA to further block the current. NFA significantly slowed the time constants of both activation and deactivation of Tmem16a currents as in rabbit PASMCs, and Tmem16a currents were stimulated by NFA at negative potentials (-100 mV to -60 mV), suggesting that even given a decrease in the magnitude of block by NFA, the drug may interact with Tmem16a and native Cl$^-$Ca channels in a similar manner. Block of the Tmem16a-dependent current by A9C differed from PASMCs in that the block was similar in cells dialyzed with 5 mM ATP and 0 ATP. Stimulation of the tail current at -80 mV was also significantly different than isolated PASMCs. Stimulation of $I_{\text{Cl(Ca)}}$ in rabbit PASMCs by A9C reached over 250% in cells dialyzed with 5 mM ATP pipette solution, while the current in Tmem16a-expressing HEK cells dialyzed with 5 mM ATP did not even reach 100% stimulation. Cells dialyzed with either 5 mM ATP or 0 ATP followed a trend similar to that of rabbit PASMCs with 0 ATP; reaching a peak stimulatory effect before decreasing. These differences in pharmacology may be attributable to the reduced rundown observed in
Tmem16a-transfected HEK293 cells, where phosphorylation-dependent block of the channel would affect drug interaction with the channel. These differences in pharmacology may be due to differences in the pharmacology of the Tmem16a splice variant used in this study and those of the native tissue. Alternatively, heteromers composed of several spliced variants and even other Tmem16a family members may form a channel complex with a distinctive pharmacological profile.

Interestingly, while the mutation discussed above had no effect on the rundown of the current as we had hypothesized, it dramatically altered the pharmacology of the Tmem16a channel. The mutation resulted in a marked shift in the voltage-dependence of block by NFA compared to the wild-type, where the mutation may have resulted in an increase in the apparent affinity of the channel for NFA at positive potentials resulting in block that is seemingly state-independent. More experiments are required to confirm this hypothesis however, as voltage-dependence of block could be present at negative potentials.

In summary, the studies described in this dissertation demonstrate for the first time that phosphorylation of Cl\textsubscript{Ca} channels of pulmonary artery smooth muscle cells alters their pharmacology. Minimizing phosphorylation enhanced the ability of 100 μM NFA to inhibit I\textsubscript{Cl(Ca)}. This effect was attributed to a large negative shift in the voltage-dependence of block. NFA dose-dependently blocked I\textsubscript{Cl(Ca)} in the range of 100 nM to 250 μM in cells dialyzed with 0 ATP and KN-93, which contrasted with the significant stimulation induced by 100 nM, which converted to block at concentrations > 1 μM when cells were dialyzed with 5 mM ATP. Dephosphorylation enhanced the ability of A9C to inhibit I\textsubscript{Cl(Ca)} in a concentration-
dependent manner. Similarly to NFA, this was attributed to a negative shift in the voltage-dependence of block. Stimulation of $I_{\text{Cl(Ca)}}$ tail current by 500 µM A9C at -80 mV was enhanced in cells dialyzed with 5 mM ATP. While the tail current of cells dialyzed with 0 ATP were stimulated following depolarization to +40 mV, the stimulation was abolished following steps to +140 mV. This dissertation also demonstrated Tmem16a to be a valid candidate for the native $\text{Cl}_\text{Ca}$ channels of PASMCs. Tmem16a was identified in rat and mouse pulmonary artery smooth muscle cells. HEK293 cells over-expressing TMEM16A displayed large $\text{Cl}^-$ currents when dialyzed with 500 nM free $\text{Ca}^{2+}$. These currents displayed similar time- and voltage dependence to native $I_{\text{Cl(Ca)}}$ of rabbit pulmonary artery smooth muscle cells. Currents ran down, although not to the extent of $I_{\text{Cl(Ca)}}$ of the native rabbit pulmonary myocytes. The Tmem16a-dependent currents were sensitive to block by NFA and A9C, but demonstrated stimulation by A9C somewhat dissimilar from the native current. A mutation of threonine 610 to an alanine – a putative CaMKII phosphorylation site – did not affect rundown, although did alter Tmem16a pharmacology; increasing block and stimulation by NFA and A9C, respectively.
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## Appendix

### Table 1. PCR primer pair sequences

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