Contributions of TRPM4 and Rho Kinase to Myogenic Tone Development in Cerebral Parenchymal Arterioles

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CONTRIBUTIONS OF TRPM4 AND RHO KINASE TO MYOGENIC TONE DEVELOPMENT IN CEREBRAL PARENCHYMAL ARTERIOLES

A Dissertation Presented

by

Yao Li

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Specializing in Pharmacology

January, 2016

Defense Date: October 9, 2015
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Cerebral parenchymal arterioles (PAs) play a critical role in assuring appropriate blood flow and perfusion pressure within the brain. PAs are unique in contrast to upstream pial arteries, as defined by their critical roles in neurovascular coupling, distinct sensitivities to vasoconstrictors, and enhanced myogenic responsiveness. Dysfunction of these blood vessels is implicated in numerous cardiovascular diseases. However, treatments are limited due to incomplete understanding of the fundamental control mechanisms at this level of the circulation. One of the key elements within most vascular networks, including the cerebral circulation, is the presence of myogenic tone, an intrinsic process whereby resistance arteries constrict and reduce their diameter in response to elevated arterial pressure. This process is centrally involved in the ability of the brain to maintain nearly constant blood flow over a broad range of systemic blood pressures. The overall goal of this dissertation was to investigate the unique mechanisms of myogenic tone regulation in the cerebral microcirculation. To reveal the contributions of various signaling factors in this process, measurements of diameter, intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), membrane potential and ion channel activity were performed. Initial work determined that two purinergic G protein-coupled receptors, P2Y4 and P2Y6 receptors, play a unique role in mediating pressure-induced vasoconstriction of PAs in a ligand-independent manner. Moreover, a particular transient receptor potential (TRP) channel in the melastatin subfamily, i.e. TRPM4, was also identified as a mediator of PA myogenic responses. Notably, the observations that inhibiting TRPM4 channels substantially reduces P2Y receptor-mediated depolarization and vasoconstriction, and that P2Y receptor ligands markedly activate TRPM4 currents provide definitive evidence that this ion channel functions as an important link between mechano-sensitive P2Y receptor activation and the myogenic response in PAs. Next, the signaling cascades that mediate stretch-induced TRPM4 activation in PA myocytes were explored. Interestingly, these experiments determined that the RhoA/Rho kinase signaling pathway is involved in this mechanism by facilitating pressure-induced, P2Y receptor-mediated stimulation of TRPM4 channels, leading to subsequent smooth muscle depolarization, [Ca\(^{2+}\)]\(_i\) increase and contraction. Since Rho kinase is generally accepted as a “Ca\(^{2+}\)-sensitization” mediator, the present, contrasting observations point to an underappreciated role of RhoA/Rho kinase signaling in the excitation-contraction mechanisms within the cerebral microcirculation. Overall, this dissertation provides evidence that myogenic regulation of cerebral PAs is mediated by mechano-sensitive P2Y receptors, which initiate the RhoA/Rho kinase signaling pathway, subsequent TRPM4 channel opening, and concomitant depolarization and contraction of arteriolar smooth muscle cells. Revealing the unique mechanochemical coupling mechanisms in the cerebral microcirculation may lead to development of innovative therapeutic strategies for prevention and treatment of microvascular pathologies in the brain.
CITATIONS

Material from this dissertation has been published in the following form:


Material from this dissertation has been accepted for publication in the following form:

ACKNOWLEDGEMENTS

First of foremost, I would like to thank my advisor, Dr. Joseph Brayden. I am extremely fortunate to have worked with you for the past four and a half years. You have truly been a great mentor from day one. I would like to thank you for everything, your advice on study design and trouble-shooting, your help with writing proposals and manuscripts, your patience when my research was stuck for a long time, your encouragement when my proposal got rejected, and so on and so forth. The invaluable guidance you gave helped me achieve numerous things, not just knowledge, grants or publications, but scientific passion and self-confidence.

I am very fortunate to have a dissertation committee consisting of experts in their respective fields. I would like to thank every member on this committee: Drs. Margaret Vizzard, Gary Mawe, Mark Nelson, George Wellman. Thank you for your time and efforts in guiding my research and helping me obtain the ability of critical thinking.

I am incredibly lucky to spend the past few years with wonderful lab members. During every lab meeting, my lab family offered me not only great advice on the project but also encouragement that motivated me constantly. I thank Dr. Rachael Baylie for introducing me to the world of patch clamp. I thank Dr. Fabrice Dabertrand for enormous technical and emotional support. I thank Matt Tavares for
assisting me with my research. Though he has left us, I will never forget he was a helpful, kind-hearted and talented fellow.

To my best friend, Arsalan Syed: Thanks for always being so understanding, generous and encouraging. The Ph.D. life was full of ups and downs. It is so wonderful to have you by my side for all the late nights of preparing for exams and all the anxious moments when I did not know what to do.

To my boyfriend, Xirun Chen: Thanks for showing up in my life. After every frustrating day at work, you threw your arms around me and told me everything would be fine. Thanks for all the sacrifices you made to support my career. Thanks for switching your major to life science so that we can have endless topics to talk about in the future.

To my mama and baba: Thanks for being the greatest parents I could ever hope to have. You work so hard to create the best environment for me. You encourage me to build my own dream and to pursue it with all my heart. You support me in every possible way even when it meant separating from you. Thanks for being so supportive of me being myself and doing what I am enthusiastic about. Thanks for teaching me the importance of independence, determination and perseverance.
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CHAPTER 1: LITERATURE REVIEW

Introduction

While the regulation of vascular tone in cerebral pial arteries has been intensely investigated, mechanistic studies of the intracerebral vessels branching from the surface arteries, i.e. the cerebral parenchymal arterioles (PAs), are uncommon, despite the fact that the PAs have unique functional roles and properties. PAs contribute up to 40% of cerebrovascular resistance and play a key role in regulating local blood flow within the brain. By maintaining appropriate local perfusion pressure, PAs serve to protect downstream capillaries from the potential damaging effects of high intravascular pressure. When these small arterioles branch from pial arteries to perfuse the cerebral cortex, they enter a unique environment where surrounding astrocytes and neurons modulate vascular diameter and blood flow through a process called neurovascular coupling. Compared to pial arteries, PAs display distinct sensitivities to vasoconstrictors, including Angiotensin II, norepinephrine, suggesting that PAs may have a unique receptor profile and cellular signaling pathways. The shift of intracellular Ca$^{2+}$ events from Ca$^{2+}$ sparks in pial arterial myocytes to Ca$^{2+}$ waves in PA smooth muscle and concomitantly altered K$^+$ channel activity further point to the existence of different mechanisms for vascular reactivity regulation in PAs. In addition, PA smooth muscle exhibits substantially enhanced myogenic depolarization and vasoconstriction. It is therefore likely that some of the fundamental contractile mechanisms in PA smooth muscle are unique as well. Structurally, in contrast to the robust circulating networks created by pial vessels and capillaries, PA organization creates a bottleneck effect in the
cerebral circulation as they form a fragile, one-dimensional network with limited collateral blood supply. Dysfunction of the cerebral microcirculation is therefore not uncommon, and it is implicated in numerous cerebrovascular diseases, including cerebral small vessel diseases\textsuperscript{8, 9}, subarachnoid hemorrhage\textsuperscript{10}, ischemic stroke\textsuperscript{11}, vascular dementia\textsuperscript{12} and hypertension\textsuperscript{13}. A better understanding of vascular tone regulation in PAs may reveal potential therapeutic targets for treatment of cerebral microvascular diseases.

As one of the essential processes that modulate vascular tone, myogenic reactivity represents the ability of small arteries and arterioles to constrict and reduce their diameter in response to increased intravascular pressure. Myogenic regulation ensures that blood flow remains relatively constant despite moment-to-moment fluctuations in arterial pressure. In the brain, myogenic control plays a key role in cerebral autoregulation. Mechanisms of myogenic tone development have been extensively studied and it is widely accepted that vascular smooth muscle cells respond to increased intraluminal pressure via membrane depolarization\textsuperscript{14}, calcium entry through voltage-dependent calcium channels and vasoconstriction\textsuperscript{15}. However, the signaling pathways accounting for the translation of mechanical stretch into membrane depolarization have not been fully elucidated.

Prior research in our laboratory has demonstrated the contribution of G protein-coupled receptors (GPCRs), specifically P2Y4 and P2Y6 purinergic receptors, to myogenic responsiveness of PAs. P2Y receptors in mesenteric arteries have been previously reported to mediate myogenic response in an autocrine/paracrine fashion whereby pyrimidines released from local sources by mechano-stimulation act on P2Y receptors in smooth muscle, leading to myogenic vasoconstriction\textsuperscript{16, 17}. Interestingly, these
receptors appear to be directly mechano-activated in cerebral microvasculature, leading to subsequent vascular smooth muscle contraction\textsuperscript{4}, similar to a proposed role for angiotensin II receptors in pial arteries\textsuperscript{18}.

Another important finding from our laboratory indicates a critical role of transient receptor potential (TRP) channels, specifically TRPM4, in this pathway involving monovalent cation influx (mainly Na\textsuperscript{+}) and membrane depolarization in the smooth muscle cells of cerebral pial arteries\textsuperscript{19}. Further studies of this channel revealed that it is regulated by protein kinase C (PKC) and Ca\textsuperscript{2+} release from the sarcoplasmic reticulum\textsuperscript{20, 21}. However, neither the significance nor the regulation of TRPM4 channels has been investigated in the PAs.

Emerging evidence points to the dynamic role of the RhoA/Rho-associated protein kinase (ROCK) pathway in regulating cerebrovascular function, including facilitating the myogenic response\textsuperscript{22-24}. It is generally accepted that ROCK promotes contraction by inactivating myosin light chain phosphatase, i.e. via a “Ca\textsuperscript{2+} sensitization” mechanism. Nevertheless, recent findings have revealed that Rho signaling also regulates ion channel activity, including epithelial sodium channels\textsuperscript{25}, inward rectifying potassium channels\textsuperscript{26, 27} and delayed rectifying potassium channels\textsuperscript{28, 29}. Moreover, the observation that ROCK also participates in smooth muscle membrane depolarization in cerebral arterial smooth muscle\textsuperscript{28, 29} further supports the hypothesis that in addition to increasing Ca\textsuperscript{2+} sensitivity of the contractile apparatus, ROCK may also modulate the Ca\textsuperscript{2+}-dependent pathways.

Following from these important findings, the major goals of this dissertation project are 1) to elucidate the contributions of TRPM4 channels to myogenic regulation
in cerebral PAs, 2) to explore the possible coupling between TRPM4 channels and P2Y receptor in mediating myogenic constriction of PAs, 3) to test the hypothesis that RhoA/ROCK signaling mediates pressure-induced activation of TRPM4 channels in the brain microcirculation, and 4) to decipher the mechanisms underlying TRPM4 channel activation by ROCK stimulation in PA smooth muscle cells.

This literature review will provide an overview of regulation of cerebrovascular tone with a major emphasis on myogenic control. Part one will discuss the mechanisms and regulation of pressure-induced depolarization and vasoconstriction. Part two will focus on the key contributors to myogenic tone development in cerebral microvasculature with an emphasis on TRPM4 channels and Rho signaling.

**Part I: Cerebrovascular Physiology**

**1.1 General Anatomy of the Cerebral Arterial Blood Supply**

The brain is highly perfused to ensure constant supply of oxygen and nutrients. In order to meet the demands of energy consumption, blood supply to the brain consists of two pairs of large arteries, the left and right internal carotid arteries, which are essential for blood supply to the cerebrum, and the left and right vertebral arteries, which join distally to form the basilar artery on the base of cerebellum\(^2\). Branches coming off the vertebral and basilar arteries are responsible for perfusing cerebellum and brain stem\(^2\). On the proximal side of the base of brain, a complete anastomotic ring of arteries is formed by the basilar artery joining with the left and right internal carotid arteries, which is known as the Circle of Willis. The unique structure of the Circle of Willis plays a fundamental role in providing collateral and redundant blood supply to the entire brain.
surface. It is particularly important under pathological conditions; occlusion of a cerebral artery or arteriole can be quickly compensated by blood flow coming from other vessels to preserve adequate cerebral perfusion.\textsuperscript{30}

The Circle of Willis gives rise to three pairs of main pial arteries, the anterior cerebral arteries (ACAs), middle cerebral arteries (MCAs) and posterior cerebral arteries (PCAs). The ACAs extend upward and forward from the internal carotid artery on the Circle of Willis and supply the most midline portions of the frontal lobes and superior medial parietal lobes, the part of the brain that control logical thought, personality, and voluntary movement, especially the legs.\textsuperscript{31} The MCAs supply a portion of the frontal lobe and the lateral surface of the temporal and parietal lobes, including the primary motor and sensory areas of the face, throat, hand and arm, and in the dominant hemisphere, the areas for speech.\textsuperscript{31} These arteries have also been found to be the most often occluded vessels in stroke.\textsuperscript{31} The PCAs originate from the basilar arteries for most individuals, but sometimes originate from the ipsilateral internal carotid artery. The PCAs supply the temporal and occipital lobes of the left and right cerebral hemisphere.\textsuperscript{31} All these cerebral arteries are surrounded by cerebrospinal fluid (CSF), which occupies the subarachnoid space and the ventricle system, providing protection to the brain from basic mechanical insults.\textsuperscript{5}

Cerebral pial arteries give rise to smaller arteries that eventually penetrate into the cerebral cortex. These penetrating arterioles lie within the Virchow-Robin space, which is a continuation of the subarachnoid space, and become parenchymal arterioles (PAs) once they penetrate deeper into the brain cortex.\textsuperscript{2} In contrast to the robust and anastomotic pial and capillary networks, PAs form a fragile, one-dimensional organization with limited collateral supply.\textsuperscript{32} They create a bottleneck effect that makes the brain parenchyma
especially vulnerable to any insult that affects flow through PAs. Therefore, it is not surprising to find that dysfunction of PAs is implicated in numerous cardiovascular diseases, including hypertension, subarachnoid hemorrhage, ischemic stroke, cerebral small vessel diseases and vascular dementia.

Fragile as they are, PAs have unique functional roles and properties in the cerebral circulation. Firstly, they contribute as much as 40% to total cerebrovascular resistance, thus regulating PA reactivity is a key process for adjusting blood perfusion within the brain. Next, by maintaining appropriate perfusion pressure, PAs serve to protect downstream capillaries from the potential detrimental effects from high intraluminal pressure. Thirdly, when penetrating into the cerebral cortex, PAs enter a unique cellular and molecular environment where they are completely encased by surrounding astrocytes and neurons that modulate local smooth muscle contractility and blood flow through a process called neurovascular coupling. In other words, PAs are “intrinsically” innervated as compared to pial arteries that are under great influence of sympathetic and parasympathetic nerve activity, i.e. extrinsic innervation. Indeed, PAs are essentially unresponsive to several neurotransmitters, including norepinephrine and serotonin. Additionally, that PAs respond differently to vasoconstrictors, like angiotensin II, suggests PAs may exhibit different receptor profile and subsequent intracellular signaling mechanisms. Furthermore, Ca$^{2+}$ signaling patterns in smooth muscle cells shift from Ca$^{2+}$ sparks in pial arteries to Ca$^{2+}$ waves in PAs, resulting in lack of contribution from large conductance Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$) channels to membrane potential regulation under physiological conditions. More importantly, PAs have considerably more depolarized membrane potentials and greater myogenic tone.
This is physiologically crucial as it sets a contractile background in order for tight regulation of vascular tone, particularly by vasodilatory influences from neurons and endothelium.

1.2 Cerebral Autoregulation and Myogenic Tone

Cerebral autoregulation is a physiological process for resistance arteries in the brain to maintain adequate and stable blood flow. Though most systems in the body exhibit some degree of autoregulation, the brain is extremely sensitive to changes in blood flow. Either under- or overperfusion is potentially detrimental to brain functions. The autoregulatory control of blood flow ensures that adequate and relatively constant oxygen and nutrient supply is provided, and carbon dioxide and waste is disposed. The reasons why the brain is particularly sensitive to changes in blood flow reside in both high level of metabolic activity and lack of fuel storage. Being the most perfused organ in the body, the brain receives up to 15% of the cardiac output, and consumes up to 20% of oxygen and glucose, even though it represents about 2% of body weight.

Though not completely understood, it is widely accepted that a fundamental physiological process, such as cerebral autoregulation, is multifactorial and relies on numerous factors and signaling mechanisms. To date, both extrinsic (neuronal, hormonal) and intrinsic (myogenic, metabolic) mechanisms have been identified. Extrinsic innervation involving sympathetic and parasympathetic nerves plays an important role in regulating vascular tone. Neurotransmitters, like norepinephrine, serotonin and nucleotides, are released from nerve endings, interact with corresponding receptors on vascular smooth muscle and endothelium, and initiate primarily G protein-coupled
signaling pathways to modulate vascular contractility. However, it was found that sympathetically and parasympathetically denervated animals still display cerebral autoregulation, suggesting that rather than playing an exclusive role, neurogenic factors serve only as one of the mechanisms underlaying autoregulatory functions. In addition to extrinsic innervation, cerebral penetrating arterioles are also influenced by intrinsic perivascular innervation (note this is different from the intrinsic mechanisms for cerebral autoregulation), whereby increased activity of surrounding neurons is coupled to arteriolar dilation. It is mediated by signaling pathways in the astrocytes, which completely encase parenchymal arterioles, i.e., neurovascular coupling. Relaxation of downstream arterioles would further lead to dilation of upstream feed arteries to prevent a drop in intravascular pressure. Therefore, neurovascular coupling is also involved in blood flow autoregulation. Furthermore, metabolic byproducts have been proposed to contribute to cerebral vascular tone regulation. For example, H+ and CO2, normally accumulated in underperfused tissue, induce vasodilation as a function to increase blood flow and perfusion.

One fundamental mechanism responsible for autoregulation is myogenic control. This is a process by which resistance arteries constrict and reduce their diameter in response to increased intravascular pressure. It was first observed and described by Bayliss in 1902, hence the name Bayliss effect. Ever since then, elucidating the mechanisms of myogenic response has been a sustained area of research in numerous laboratories. Though endothelium is significant for regulation of myogenic tone, this process is determined by the vascular smooth muscle contractile status based on the observation that the myogenic response is preserved or even enhanced in endothelium-
denuded arteries\textsuperscript{42}. Part of its mechanisms has been well established. Elevation in intraluminal pressure leads to membrane depolarization\textsuperscript{14} and Ca\textsuperscript{2+} entry through activated L-type voltage-dependent Ca\textsuperscript{2+} channels\textsuperscript{15}. Increase in intracellular Ca\textsuperscript{2+} concentration leads to greater Ca\textsuperscript{2+}-calmodulin interaction, and concomitant myosin light chain kinase activation. This kinase in turn phosphorylates and activates myosin light chain. Simultaneously, pressure-dependent, PKC or Rho kinase-mediated inhibition on myosin light chain phosphatase activity further augments myosin light chain activation, and promotes myosin-actin interaction, and thus vasoconstriction\textsuperscript{46}. As implied by this mechanism, pressure-induced depolarization plays a central role. Nevertheless, the nature of the ion channels and intracellular signaling pathways that translate the mechanical force into a biological depolarizing responses remains an open question. The following section will focus on various ion channels, primarily K\textsuperscript{+} channels and TRP channels, which have been shown to be centrally involved in regulation of myogenic depolarization and vasoconstriction.

1.3 **Regulation of smooth muscle membrane potential**

Membrane potential is the difference in electric potential of the interior of plasma membrane in reference to the exterior of a biological cell. It is established by asymmetric concentration gradients of electrolytes maintained by cellular ion transporters and pumps\textsuperscript{47}. The resting membrane potential of arterial smooth muscle is about -60 mV. In pressurized arteries, membrane potential can elevate to about -40 mV\textsuperscript{48}. This depolarization subsequently increases intracellular Ca\textsuperscript{2+} concentration and initiates smooth muscle contraction\textsuperscript{15}. The relationship between membrane potential and arterial
tone is very steep. Numerous studies have demonstrated that even membrane potential changes of a few millivolts cause significant changes in blood vessel diameter\textsuperscript{15, 49, 50}. Therefore, membrane potential is a major determinant of vascular smooth muscle contractility.

The resting membrane potential is modeled by the Goldman-Hodgkin-Katz equation:

\[ E_m = \frac{RT}{F} \ln \left( \frac{P_K [K^+]_{out} + P_{Na} [Na^+]_{out} + P_{Cl} [Cl^-]_{in}}{P_K [K^+]_{in} + P_{Na} [Na^+]_{in} + P_{Cl} [Cl^-]_{out}} \right) \]

\( E_m \) = the membrane potential

\( P_{ion} \) = the permeability for that ion

\( R \) = the ideal gas constant

\( T \) = the temperature in degrees Kelvin

\( F \) = Faraday’s constant

It is clearly indicated by this equation that membrane potential is dictated not only by the concentrations of different ions inside and outside the cell, but also by the permeability of plasma membrane to each ion. Therefore, the activity of various ion channels and pumps plays a key role in modulating membrane potential. In this section, regulators of smooth muscle membrane potential in cerebral arteries will be discussed, including potassium channels, TRP channels, chloride channels and epithelial sodium channels.
1.3.1 $K^+$ channels

Given that the intracellular and extracellular $K^+$ concentrations of cerebrovascular smooth muscle cells are $\approx 140$ mmol/L and 3 mmol/L, respectively, the equilibrium potential of $K^+$ is $\approx -100$ mV calculated with the Nernst Equation:

$$ E_K = \frac{RT}{F} \ln \left( \frac{[K^+]_{\text{out}}}{[K^+]_{\text{in}}} \right) $$

$E_K$ = potassium equilibrium potential

$R$ = the ideal gas constant

$T$ = the temperature in degrees Kelvin

$F$ = Faraday’s constant

The equilibrium potential is a voltage where net movement of $K^+$ is zero since electrical gradient and concentration gradient are balanced. However, the $Na^+$ reversal potential is $\approx +89$ mV (given that $[Na^+]_{\text{out}} \approx 140$ mmol/L and $[Na^+]_{\text{in}} \approx 5$ mmol/L). Though the membrane potential is considerably more depolarized than $E_K$, it is still largely dominated by the $K^+$ conductance due to high permeability to $K^+$. An increase in permeability to $K^+$ would potentiate $K^+$ efflux and bring the membrane potential closer to $E_K$, i.e. cause hyperpolarization; a decrease in $K^+$ conductance would cause depolarization. Therefore, potassium channels contribute essentially to regulating arterial smooth muscle membrane potential and arterial contractility. There are primarily four types of $K^+$ channels present in vascular smooth muscle cells: Voltage-dependent $K^+$ ($K_v$) channels, large-conductance $Ca^{2+}$-activated $K^+$ ($BK_{Ca}$) channels, inward rectifying $K^+$ ($K_{ir}$) channels and ATP-sensitive $K^+$ ($K_{ATP}$) channels. This section briefly reviews the involvement of these channels in modulation of smooth muscle membrane potential.
Voltage-dependent $K^+$ ($K_v$) channels. Several subtypes of $K_v$ channels have been identified in arterial myocytes$^{51}$. Among them, heteromultimeric $K_v$ composed of $K_v1.2$ and $K_v1.5$ have been shown to participate in control of vascular tone$^{52, 53}$. Plan et al. showed that $K_v$ currents were attenuated by pharmacological inhibitors (4-AP and correolide), which enhanced myogenic constriction in rat mesenteric arteries, indicating that $K_v$ channels are active during myogenic regulation$^{54}$. To further differentiate the relative contributions of these subtypes of $K_v$ channels, Chen et al. utilized a dominant negative cDNA construct to suppress $K_v1$ channel activity in rat cerebral arteries$^{53}$. They found that myogenic tone was potentiated in arteries overexpressing a $K_v1.5$ dominant negative construct, whereas myogenic constriction was reduced in arteries overexpressing wild-type $K_v1.5$ channels, pointing to important involvement of $K_v1.5$ in regulating tone development$^{53}$. $K_v$ channels are steeply activated by membrane depolarization, resulting in $K^+$ efflux and membrane hyperpolarization. Together with $B_{KCa}$, these channels initiate an important feedback mechanism to counteract myogenic constriction and stabilize tone. In this regard, $K_v$ channels can be highly significant for membrane potential regulation in the cerebral microcirculation, where $B_{KCa}$ channels are not functionally active under physiological conditions$^6$. In addition to myogenic response, $K_v$ channels are also involved in various vasodilator-induced membrane hyperpolarization, including responses to statins$^{55}$, adenosine$^{56}$, prostacyclin$^{57}$, $H_2O_2$$^{58}$ and endothelial-derived nitric oxide$^{59}$. Furthermore, several vasoconstrictors depolarize smooth muscle by inhibiting $K_v$ channels through either cAMP/PKC or RhoA/Rho kinase pathways$^{28, 60}$. For example, Luykenaar and colleagues have shown UTP inhibits 4-AP-sensitive $K_v$ currents, which is rescued by Rho kinase inhibition, demonstrating that UTP activates
Rho kinase, which subsequently inhibits Kv and facilitates depolarization and constriction\textsuperscript{28}.

\textit{Large-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK\textsubscript{Ca}) channels.} BK\textsubscript{Ca} channels are activated by both elevation of intracellular Ca\textsuperscript{2+} and membrane depolarization\textsuperscript{61, 62}. As both of these activating signals for BK\textsubscript{Ca} channels result from myogenic stimulation, the critical role of BK\textsubscript{Ca} channels in myogenic tone has been intensely investigated. Activation of BK\textsubscript{Ca} channels increases K\textsuperscript{+} efflux and hyperpolarizes plasma membrane. Hence blocking BK\textsubscript{Ca} would cause membrane depolarization and constriction, which was demonstrated in various vascular beds. Brayden et al. showed that BK\textsubscript{Ca} channel blockers tetraethylammonium ion (TEA\textsuperscript{+}) and charybdotoxin depolarized and constricted pressurized rat cerebral arteries with myogenic tone\textsuperscript{50}. In uterine arteries, chronic hypoxia inhibited upregulation of BK\textsubscript{Ca} channels during pregnancy and suppressed BK\textsubscript{Ca} channel current density\textsuperscript{63}. Reduced BK\textsubscript{Ca} channel expression resulting in enhanced myogenic constriction was observed in the uterine arteries in pregnant animals acclimatized to chronic hypoxia\textsuperscript{63}. This work illustrate that BK\textsubscript{Ca} channels play a dynamic role in the control of arterial membrane potential by functioning as a negative feedback pathway to reduce membrane excitability regulated by pressure or even vasoactive agents\textsuperscript{62, 64, 65}. BK\textsubscript{Ca} channels are activated by high levels of intracellular Ca\textsuperscript{2+}, which come from Ca\textsuperscript{2+} release events of the ryanodine receptors (RyRs) in the sarcoplasmic reticulum, i.e. Ca\textsuperscript{2+} sparks\textsuperscript{66}. Ca\textsuperscript{2+} spark sites are in close proximity to BK\textsubscript{Ca} channels, and each spark can increase local [Ca\textsuperscript{2+}] to 10~100 µmol/L and activate 20~100 BK\textsubscript{Ca} channels in the plasma membrane\textsuperscript{66}. This high level of interaction ensures that smooth muscle membrane potential is tightly controlled. Interestingly, this does not seem to be the case the in the
cerebral penetrating arterioles under physiological conditions. Dabertrand et al. reported that in parenchymal arteriolar myocytes, Ca\(^{2+}\) spark activity is extremely low, resulting in very little BK\(_{Ca}\) channel contribution to vascular tone\(^6\). BK\(_{Ca}\) channel blocker paxilline-induced constrictions in parenchymal arterioles that were much smaller than those in cerebral pial arteries\(^6\). These observations imply that in PAs, the role of BK\(_{Ca}\) channels in negative feedback mechanisms that limit myogenic depolarization is largely replaced by K\(_v\) channels.

**ATP-sensitive K\(^+\) (K\(_{ATP}\)) channels.** K\(_{ATP}\) channels are inhibited by intracellular ATP, hence the name ATP-sensitive channels. After they were initially identified in cardiac muscle\(^{67}\), they were later found in skeletal muscle, pancreatic β-cells, and smooth muscle\(^{62, 68}\). K\(_{ATP}\) channel blockers (glibenclamide and tolbuamide) are well-characterized antidiabetic drugs. By inhibiting K\(_{ATP}\) channels and K\(^+\) efflux, glibenclamide causes β-cell membrane depolarization, Ca\(^{2+}\) entry and insulin release. Since this compound is relatively selective for K\(_{ATP}\) channels, it is widely used as a pharmacological tool to study the functional importance of K\(_{ATP}\) channels in other systems. However, recent evidence has shown that high concentrations of glibenclamide (100 µmol/L) also block TRPM4 channels\(^{69}\), since similar to K\(_{ATP}\) channels, TRPM4 channels are also co-expressed with the sulfonylurea receptor 1, which is the target of glibenclamide\(^{70}\). In vascular smooth muscle, through mediating hyperpolarization, K\(_{ATP}\) channels participate in various physiological functions, primarily conducting vasodilator effects and regulating metabolic blood flow. Several vasodilators have been shown to induce glibenclamide-sensitive arterial hyperpolarization and dilation, including calcitonin gene-related peptide (CGRP)\(^{71, 72}\), adenosine\(^{73}\), prostacyclin\(^{74, 75}\), vasoactive
intestinal peptide, nitric oxide, pituitary adenylate cyclase activating polypeptide (PACAP), etc. Moreover, $K_{ATP}$ channels have been reported to mediate metabolic regulation of vascular reactivity. Hypoxia causes vasodilation and increases blood flow in many vascular beds, presumably as a means to elevate blood supply to the hypoxic regions. It has been observed in various vascular beds (cerebral arteries, coronary arteries and renal arteries) that hypoxia-induced vasodilation can be inhibited by glibenclamide, illustrating an essential involvement of $K_{ATP}$ channels in this mechanism. More definitive evidence was also reported that hypoxia activates $K_{ATP}$ currents in isolated myocytes from porcine coronary arteries. Additionally, like other potassium channels, a potential role of $K_{ATP}$ channels in myogenic regulation has been studied. Interestingly, this channel seems to play diverse roles in various vascular beds. For instance, in coronary and mesenteric arteries, application of glibenclamide effectively decreases basal blood flow in vivo, and depolarizes and constricts the arteries in vitro, whereas in cerebral and renal arteries, glibenclamide has little effect on vascular contractility under physiological conditions. However, during hypoxia, glibenclamide is able to restore myogenic vasoconstriction, indicating that $K_{ATP}$ channel activity is differentially regulated based on different tissue type and cellular environment.

*Inward rectifying $K^+$ ($K_{ir}$) channels.* $K_{ir}$ or inward rectifying were named based on the observation that when membrane potential is controlled, inward currents through these $K^+$ channels ($K^+$ influx) are larger than the outward currents ($K^+$ efflux). In addition, $K_{ir}$ channels are activated by membrane hyperpolarization, as opposed to $K_v$ and $BK_{Ca}$ that are stimulated by membrane depolarization. Therefore, when membrane potential is negative to $E_K$, $K_{ir}$ channels readily conduct $K^+$ ions flowing into cells. However, in
vascular smooth muscle cells, resting membrane potential (-60 ~ -40 mV) is always positive to $E_K$ ($\approx -85$ mV); $K_{ir}$ channels therefore normally conduct small outward hyperpolarizing currents\textsuperscript{49}. In cerebral arteries, both $K_{ir}$ channel mRNA transcripts and $K_{ir}$ currents were identified in arterial smooth muscle cells\textsuperscript{87}, suggesting $K_{ir}$ channels may play a role in regulation of cerebral arterial tone. The activity of $K_{ir}$ channels is regulated by both membrane potential and the extracellular $K^+$ concentration\textsuperscript{88, 89}. Quayle et al. discovered that elevation in external [K$^+$] would significantly shift the zero current potential to more positive potentials, and increase the inward currents at membrane potentials negative to the new $E_K$, while the outward currents remained small\textsuperscript{89}. Interestingly, elevating external K$^+$ from 5 mmol/L to 10 mmol/L causes significant membrane hyperpolarization and vasodilation in pressurized arteries\textsuperscript{90}. These effects could be prevented by < 10 µmol/L Ba$^{2+}$, indicative of an essential role of $K_{ir}$ channels in K$^+$-induced dilation in coronary arteries, and cerebral pial and parenchymal arteries\textsuperscript{9, 90}. The mechanism of this K$^+$-induced dilation is that when external [K$^+$] is increased from 5 to 10 mmol/L, $E_K$ shifts from -79.5 to -66.3 mV. The membrane potential of pressurized arteries is still positive to $E_K$ (around -50 ~ -40 mV). Due to the N-shaped current-voltage relationship of $K_{ir}$ channels, an increase in external [K$^+$] shifts the N-shaped curve to the right. So over a certain range of membrane potential, the outward currents through $K_{ir}$ channels increase and hence the membrane hyperpolarizes\textsuperscript{49}. In contrast to $K_v$ and $BK_{Ca}$ channels, this K$^+$-dependent feature of $K_{ir}$ channels accounts largely for its critical importance in mediating K$^+$-induced hyperpolarization and dilation. For instance, increase in neuronal activity can dilate local parenchymal arterioles through activation of $BK_{Ca}$ channels on astrocytes, release of K$^+$, and activation of vascular smooth muscle $K_{ir}$.
channels. Another very important example of $\text{K}^+$-induced dilation comes from endothelium-derived hyperpolarizing factor (EDHF). Edwards et al. have shown that $\text{K}^+$ release from endothelial cells through intermediate and small conductance $\text{Ca}^{2+}$-activated $\text{K}^+$ (IK$_{Ca}$ and SK$_{Ca}$) channels hyperpolarizes and relaxes adjacent myocytes by activating $\text{Ba}^{2+}$-sensitive K$_{ir}$ channels. These studies indicate that K$_{ir}$ channels play a pivotal role in integrating the interactions between smooth muscle and the surrounding environment.

### 1.3.2 TRP channels

As previously mentioned, the equilibrium potential for $\text{K}^+$ in cerebral myocytes is $\approx -100 \text{ mV}$, whereas the resting membrane potential is always positive to $E_K$ in the range of $-60 \sim -40 \text{ mV}$. This indicates that there is considerable number of depolarizing ion channels on the plasma membrane that conduct $\text{Na}^+$ and $\text{Ca}^{2+}$ influx, whereby membrane potential is brought more positive compared to $E_K$. The prime examples are transient receptor potential (TRP) channels, which are non-selective cation channels that are permeable to $\text{Na}^+$ and $\text{Ca}^{2+}$, and participate in regulation of membrane potential and intracellular $[\text{Ca}^{2+}]$ in the smooth muscle of different vascular beds. Many TRP channels have been revealed to be functionally important in vascular smooth muscle, with their contributions ranging from mechano-sensing, GPCR-associated signaling pathways to cell proliferation and migration. The following section will briefly discuss the TRP channels that are implicated in the depolarization-contraction mechanism of smooth muscle cells.

**TRPM4.** TRPM4 channels are monovalent-selective cation channels that conduct only to $\text{Na}^+$, $\text{K}^+$, $\text{Li}^+$ and other monovalents, but are impermeable to divalent cations,
including Ca^{2+} and Mg^{2+}. TRPM4 mRNA transcripts and currents are detected in the cerebral artery and arterioles. Contributions of TRPM4 channels to the coupling between membrane stretch and myogenic depolarization of arterial smooth muscle were first reported by Earley et al. in cerebral arteries. It was observed that arteries treated with TRPM4 antisense oligodeoxynucleotides (ODNs) were significantly less depolarized compared to sense-treated vessels when they were subject to same level of intravascular pressures (80 mmHg). Later, along with the discovery and widespread use of a relatively selective TRPM4 blocker 9-phenanthrol, this channel was further confirmed to participate in pressure-induced membrane depolarization as 9-phenanthrol (30 µmol/L) effectively hyperpolarized pressurized arteries from -40 mV to -73 mV. Although it was later revealed that part of this significant hyperpolarizing effect comes from activation of endothelial K^+ channels, the contribution of TRPM4 to the depolarizing mechanism is still prominent. In accord with this notion, we found that in the penetrating arterioles within the brain, 9-phenanthrol (10 µmol/L) significantly hyperpolarized the smooth muscle of either pressurized arterioles (endothelium-denuded) or arteries exposed to purinergic stimulation, demonstrating that TRPM4 is responsible for mediating mechano-activated and GPCR-mediated membrane depolarization. Moreover, a similar depolarizing mechanism involving TRPM4 has also been strongly suggested in urinary bladder smooth muscle, where 9-phenanthrol attenuated both spontaneous inward TRPM4 currents, intracellular Ca^{2+} levels, and decreased spontaneous as well as nerve-evoked contractions in the rat detrusor smooth muscle. Though membrane potential was not measured directly in this study, TRPM4-mediated depolarization appears to be an important step in the mechanism of detrusor smooth muscle excitability and contractility.
Because TRPM4 is the focal point of this thesis work, a much more detailed elaboration of TRPM4 channels regarding their roles in myogenic regulation as well as other physiological processes will be provided later in this chapter.

**TRPC6 channels.** TRPC6 expression has also been detected in native arterial myocytes from portal veins\(^9^6\), mesenteric arteries\(^9^7\) and cerebral arteries\(^9^8\). TRPC6 conducts both Na\(^+\) and Ca\(^{2+}\) with a higher permeability to Na\(^+\). It is rather well-established that TRPC6 channels are stimulated by the phospholipase C (PLC)-diacylglycerol (DAG) pathway but they are PKC independent, suggesting they are activated by PLC pathway-associated receptors, i.e. G\(_q\)-coupled receptors\(^9^9\). Demonstration of the coupling between TRPC6 and G\(_q\)-coupled receptors in native vascular smooth muscle was first reported by Inoue et al.\(^1^0^0\). They found high levels of TRPC6 mRNA expression in both murine and rabbit portal vein smooth muscle. Moreover, downregulation of the channel with antisense ODNs not only abolished the expression of TRPC6 protein, but also largely decreased \(\alpha\)-adrenoceptor-mediated Ca\(^{2+}\) entry through TRPC6. Later, more TRPC6-coupled GPCRs were revealed in vascular myocytes. For example, in A7r5 aortic smooth muscle-derived cells, nonselective cation channels with TRPC6 channel biophysical properties and pharmacological profile were identified\(^1^0^1\). TRPC6 siRNA substantially reduced vasopressin-induced Ca\(^{2+}\) currents, whereas it had no effect on Thapsigargin-induced store-operated Ca\(^{2+}\) entry\(^1^0^1\). In freshly isolated rabbit mesenteric myocytes, angiotensin II (Ang II) activated nonselective cation currents that were sensitive to TRPC6 antibodies, and were activated by the PLC-DAG pathway in a PKC-independent manner\(^9^7\), strongly indicating these were TRPC6 currents.

In addition to its role in receptor-mediated responses, TRPC6 has also been shown to
facilitate myogenic depolarization and contraction. Welsh et al. reported that antisense ODNs targeted against TRPC6 channels selectively reduced TRPC6 mRNA expression and effectively decreased TRPC6 whole-cell currents. Functionally, it did not affect membrane potential at low intravascular pressure, but significantly hyperpolarized cerebral arteries when vessels were pressurized to 80 mmHg. Consequently, TRPC6 antisense also inhibited myogenic tone at 60-100 mmHg\textsuperscript{98}, indicating an essential contribution of this channel to the regulation of membrane potential and vascular myogenic tone.

However, the exact mechanism by which TRPC6 channels are activated by an increase in intravascular pressure is still a matter of controversy. TRPC6 channels can be activated by cell swelling or suction on the membrane, but whether the channel is directly mechanosensitive remains unclear. To investigate the mechanosensitivity of TRPC6, Mederos y Schnitzler et al. used HEK293 cells overexpressing the channel. They found that pressure application on the inside-out patch failed to activate currents. As a control experiment, TRPC6 channel activity was markedly enhanced by subsequent administration of DAG analogue\textsuperscript{18}. On the other hand, Gonzales et al. utilized on-cell patch clamp configuration to show that the open probability of TRPC6 channels was substantially augmented by application of negative pressures on the membrane of HEK cells expressing TRPC6 channels, suggesting the channel can be directly activated by mechanical stretch\textsuperscript{102}. This controversy may result from the different patch configurations used in the studies. It is possible that on-cell patch configuration maintains cell integrity and allows for stimulation of the channel by cellular factors, which are however dialyzed by membrane excision that occurs with inside-out patch configuration. This implies that
TRPC6 might not be directly activated by pressure, but rather that it is stimulated by upstream stretch-activated signaling pathways. In agreement with this proposal, observations shown by Gottlieb and colleagues regarding the mechanosensitivity of TRPC6 failed to confirm a significant role for this channel as a stretch-activated protein. They found that transient expression of TRPC6 in green monkey kidney (COS) or Chinese hamster ovary (CHO) cells did not alter the amplitude of stretch-activated currents as compared with mock-transfected cells, yet intracellular administration of OAG (DAG analogue) consistently increased channel activity in TRPC6-transfected cells\(^{103}\). Unfortunately, at this point, more detailed molecular and biophysical investigation is required to elucidate the mechanosensitivity of this TRP channel.

Whether or not this channel is directly activated by membrane stretch does not downgrade its functional significance in mediating myogenic depolarization and constriction. Mederos y Schnitzler et al. found that GPCRs are mechanosensors that are activated by membrane stretch in an agonist-independent manner, and subsequently initiate PLC-mediated TRPC6 activation\(^{18}\). Since both TRPC6 and TRPM4 channels seem to participate in the same myogenic excitation-contraction mechanism, the coupling between these two proteins and their relationship with upstream GPCRs and PLC were systemically investigated by Gonzales et al. They demonstrated that Ca\(^{2+}\) entry through TRPC6 channels either by PLC-\(\gamma_1\)-dependent GPCR stimulation or direct mechanical signal reinforces IP\(_3\) receptor-mediated Ca\(^{2+}\) release from the SR, which then activates nearby TRPM4 channels, with subsequent depolarization, Ca\(^{2+}\) influx through VDCCs and vasoconstriction\(^{102}\). Surprisingly, TRPC6 knockout mice exhibited enhanced membrane depolarization and vasoconstriction from either agonists or increased
pressure\textsuperscript{104}, which may be explained by a compensatory upregulation of constitutively active TRPC3 channels\textsuperscript{105}.

**TRPP1 and TRPP2.** The TRPP subfamily consists of TRPP1 (PKD2), TRPP2 (PKD1) and TRPP3 (PKD2L2). TRPP1 is a Ca\textsuperscript{2+}-permeable, non-selective cation channel with a quite large unitary conductance (135~175 pS). TRPP2 is structurally distinct from other TRPP proteins and does not form an ion channel. Both TRPPs are expressed in vascular smooth muscle cells\textsuperscript{106, 107} and contribute to pressure sensing as well as myogenic reactivity. The coupling between intraluminal pressure and TRPP proteins in vascular smooth muscle was first established by Sharif-Naeini et al.\textsuperscript{107}. They found that knocking down TRPP1 channel caused a substantial decrease in stretch-activated cation (SAC) channel activity. Moreover, downregulation of TRPP1 markedly decreased myogenic tone developed by isolated mouse mesenteric arteries, but did not influence KCl or phenylephrine-induced vasoconstriction, indicating the essential contribution of TRPP1 in pressure-initiated tone. Interestingly, TRPP2 transcript knockdown resulted in increased SAC activity in smooth muscle cells from TRPP1 knockdown animals, and also restored myogenic vasoconstriction of mesenteric arteries. These observations demonstrate that it is the TRPP1/TRPP2 ratio that regulates the activity of native SACs in vascular smooth muscle cells and myogenic response of mesenteric arteries; TRPP2 inhibits SAC mechanosensitivity while TRPP1 reverses this inhibition\textsuperscript{107}. However, whether the TRPP1/TRPP2 ratio also modulates myogenic depolarization was not explored by this study. To the contrary, studies of TRPP channels in cerebral arteries reveal a substantially different mechanism, providing compelling evidence that cerebral and peripheral circulation may comprise different pressure-sensing factors and be
differentially regulated. Specifically, Narayanan et al. reported that in cerebral arteries from rats and humans, TRPP2 is the major TRPP isoform expressed\textsuperscript{106}. Suppressing TRPP2 with a selective shRNA strongly decreased cell swelling-activated cation currents. Moreover, TRPP2 shRNA also decreased pressure-induced but not depolarization (KCl)-induced vasoconstriction of cerebral arteries, suggesting that TRPP2 is an important player in myogenic regulation of cerebral vascular tone\textsuperscript{106}, potentially via membrane depolarizing mechanisms, in contrast to its inhibitory role in the mesenteric arteries. The reasons for these apparently distinct physiological functions of TRPP2 protein in mesenteric and cerebral arteries are unclear, and require further interrogation.

1.3.3 Cl\textsuperscript{-} channels

Cl\textsuperscript{-} channels also contribute to regulation of vascular smooth muscle membrane potential. Cl\textsuperscript{-} channels have relatively high conductance with an equilibrium potential of \(-30\) to \(-20\) mV\textsuperscript{49, 108}, therefore like TRP channels, they contribute to bringing membrane potential to more positive values than \(E_K\). Therefore, it was postulated that activation of Cl\textsuperscript{-} channels facilitates pressure-induced membrane depolarization. In support of this proposal, Nelson et al. found that pharmacological chloride channel blockers significantly hyperpolarized and dilated pressurized cerebral arteries, whereas a blocker of Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels had no effect on arterial contractility\textsuperscript{109}. The observation that reducing extracellular Cl\textsuperscript{-} concentration from 140 mmol/L to 60 mmol/L significantly enhanced pressure-induced vasoconstriction strongly points to the role of Cl\textsuperscript{-} channels in myogenic regulation\textsuperscript{109}. Consistent with this study, Doughty et al. reported that myogenic tone development of rat cerebral arteries coincided with a pressure- and temperature-
dependent Cl\(^-\) efflux, which is sensitive to Cl\(^-\) channel blocker, corroborating the contribution of outward Cl\(^-\) currents to smooth muscle depolarization associated with myogenic contraction\(^{110}\). However, controversy exists in terms of the involvement of Ca\(^{2+}\)-activated Cl\(^-\) channels in myogenic tone development. It was recently argued by Bulley et al. that TMEM16A channels, Ca\(^{2+}\)-activated Cl\(^-\) channels identified in cerebral vascular smooth muscle cells, are one component of the mechanosensitive mechanisms that mediate myogenic vasoconstriction\(^ {111}\). These authors provided convincing evidence that TMEM16A channels in arterial myocytes were activated by cell swelling. Knocking down the channels in arterial smooth muscle with selective siRNA, reduced pressure-induced depolarization and vasoconstriction, but not KCl-induced responses, indicating the Ca\(^{2+}\)-activated Cl\(^-\) channels are implicated in the myogenic excitation-contraction mechanisms\(^ {111}\).

### 1.3.4 Epithelial Na\(^{+}\) channels (ENaC)

Lately, new players in the myogenic depolarizing mechanism have been identified. A prime example is ENaC. ENaC channels have a well-established role in Na\(^{+}\) reabsorption in the distal tubules in kidneys\(^ {112}\). Recent studies by Jernigan and Drummond focused on the mechanosensitivity of ENaC and its potential role in myogenic responses of isolated intrarenal arteries. This study showed that ENaC inhibition with amiloride and benzamil abolished pressure-induced constriction and increases in cytosolic Ca\(^{2+}\) as well as Na\(^{+}\) of renal arteries\(^ {113}\). This poses an important physiological function of ENaC on renal blood flow regulation. In support of this notion, mice with reduced levels of βENaC expression exhibited reduced myogenic tone in renal
afferent arterioles, reduced myogenic regulation of \textit{in vivo} renal blood flow and elevated blood pressure\textsuperscript{114}. Similar observations were obtained by Kim et al., who also presented evidence that cerebral arterial myogenic constriction and cytosolic [Ca\textsuperscript{2+}] increases were inhibited by amiloride and benzamil\textsuperscript{115}. Moreover, they found that siRNA specifically targeted against βENaC and γENaC significantly reduced the ability of isolated arteries to develop myogenic tone, presumably through inhibition of ENaC-mediated Na\textsuperscript{+} and/or Ca\textsuperscript{2+} influx and reduced membrane depolarization\textsuperscript{115}. Further investigation into the mechanosensitivity of ENaC by the same group revealed that ENaC and TRPM4, but not ENaC and TRPC6, appeared to be clustered in similar subcellular locations in cerebral arterial myocytes\textsuperscript{116}. Further, co-treatment of ENaC and TRPM4 inhibitors did not have additional inhibitory effects on myogenic tone compared to individual treatment with either inhibitor, suggesting there might be an interaction between ENaC and TRPM4 channels\textsuperscript{116}. However, this study is limited only to functional studies of isolated vessels. In order to corroborate the interactions between these channels, extensive electrophysiological studies are required to investigate their relationship. For example, to test the coupling between ENaC and TRPM4 (or ENaC and TRPC6), TRPM4 and/or TRPC6 channel activity ought to be compared before and after application of an ENaC blocker. In addition, the effects of ENaC siRNA on TRPM4/TRPC6 activity should also be investigated. Proper control experiments, particularly concerning the potential direct inhibitory effect of ENaC blocker on TRPM4/TRPC6 channel activity, should be conducted early on.
1.4 Transient Receptor Potential Channels and Vascular Function

Transient receptor potential (TRP) channels were initially discovered in *Drosophila* in 1969\textsuperscript{17}. Since then, a large number of studies have been dedicated to the identification, characterization and cellular functions of these ion channels. Currently, twenty-eight TRP channels have been described. They are widely distributed to numerous animal cell types, mediating diverse sensations, including pressure, osmolarity, temperature, pain, taste, etc., and facilitating intracellular signal transduction. Notably, numerous TRP channels have also been found in the heart and various vascular beds, contributing essentially to diverse aspects of cardiovascular functions as well as pathophysiology, including regulation of vascular tone, angiogenesis, pace-making, cardiac conduction, cardiac and vascular hypertrophy and hypertension.

According to amino acid sequence homology, the twenty-eight TRP channels are grouped into six subfamilies: canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), ankyrin (TRPA), mucolipin (TRPML) and polycystin (TRPP)\textsuperscript{47}. All TRP channels are polypeptides with 553-2022 amino acids containing six transmembrane domains and intracellular NH\textsubscript{2} and COOH termini. A TRP domain, a conserved sequence with about 25 amino acids, was identified in TRPC, TRPM and TRPV subfamilies\textsuperscript{118}. This sequence is located immediately C-terminal to the 6\textsuperscript{th} transmembrane domain and contains a highly conserved 6-amino acid TRP box\textsuperscript{118}. Though still not fully understood, in several TRP channels, the TRP box region is involved in phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) binding and interactions\textsuperscript{119}. Additionally, transcriptional splice variants exist for almost all TRP subunits, some of which showed very different functional properties\textsuperscript{120}. One TRP channel is formed from the assembly of four subunits. The 5\textsuperscript{th} and 6\textsuperscript{th} transmembrane
domains form the pore region to conduct cations. Both homomeric and heteromultimeric 
TRP channels were reported in native tissue. However, the heteromeric channels exhibit 
distinct conductance properties compared to homomeric subunits\textsuperscript{47}, leaving 
characterization of channel properties much more complicated.

TRP channels are also referred to as “non-selective cation channels”, however, 
that is imprecise since different TRP channels show distinct permeability to monovalent 
and divalent cations. For example, TRPM4 and TRPM5 channels in the melastatin 
subfamily are selective for monovalent cations (Na\textsuperscript{+}, K\textsuperscript{+}, Li\textsuperscript{+}, etc.), and are essentially 
impermeable to divalent cations (Ca\textsuperscript{2+} and Mg\textsuperscript{2+})\textsuperscript{92, 121, 122}. By contrast, TRPV5 and 
TRPV6 channels are highly selective for Ca\textsuperscript{2+}, with a relative Ca\textsuperscript{2+} to Na\textsuperscript{+} permeability 
ratio of ~100:1\textsuperscript{123, 124}. These are rather extreme examples of cation selectivities. Other 
TRP channels, though permeable to both types of cations, display varying degrees of 
ionic preference. Some of them are essentially nonselective, like TRPC1\textsuperscript{125}, whereas 
some are more permeant to Ca\textsuperscript{2+}, like TRPV3\textsuperscript{126}, which has a Ca\textsuperscript{2+} to Na\textsuperscript{+} permeability 
ratio of ~12:1. TRPM6 and TRPM7 are preferentially selective for Mg\textsuperscript{2+}\textsuperscript{127}.

1.4.1 TRP channels in vascular smooth muscle

A number of TRP channels have been found in vascular smooth muscle cells, 
contributing to various aspects of smooth muscle function, including modulation of 
vascular contractility and smooth muscle proliferation. Specifically, TRPM4\textsuperscript{19, 34}, 
TRPC6\textsuperscript{98} and TRPP2\textsuperscript{106, 107} are involved in pressure-induced vasoconstriction through 
direct or indirect regulation of smooth muscle membrane potential and intracellular 
[Ca\textsuperscript{2+}]. Moreover, TRPC3, TRPC6 and TRPM4 channels are implicated in G protein-
coupled receptor (GPCR)-initiated contractile responses in cerebral arteries\textsuperscript{128}, carotid arteries\textsuperscript{129}, mesenteric arteries\textsuperscript{130}. For instance, in brain pial arteries, TRPC3 antisense oligodeoxynucleotides significantly reduced inward currents, smooth muscle depolarization and vasoconstriction by purinergic receptor agonist UTP\textsuperscript{128}. This finding is supported by another study demonstrating that ET-1-induced Ca\textsuperscript{2+} entry and cerebral arterial constriction was largely attenuated following suppression of TRPC3 channel expression\textsuperscript{131}. TRPC6 channels are coupled to Ang II receptors\textsuperscript{18}, whereas TRPM4 channels are associated with P2Y receptor activity\textsuperscript{34}. Both mechanisms are centrally involved in mediating myogenic response\textsuperscript{34, 102}. Furthermore, TRPC1, TRPC4 and TRPC5 channels are proposed to participate in store-operated Ca\textsuperscript{2+} entry (SOCE), which is defined as Ca\textsuperscript{2+} influx occurs in response to depletion of intracellular Ca\textsuperscript{2+} stores. Via modulating intracellular Ca\textsuperscript{2+} levels, SOCE is a significant physiological process for excitation-contraction as well as cellular differentiation and proliferation\textsuperscript{47}. Contribution of TRPC1, TRPC4 and TRPC5 to SOCE activity has been investigated both in the expression system\textsuperscript{132} and in native myocytes\textsuperscript{133-135}. TRPC1 channels as an example, they were reported to mediate SOCE through interaction with STIM1 and Orai1 in mouse pulmonary artery smooth muscle cells\textsuperscript{134, 135}. However, conflicting evidence exists as smooth muscle cells of TRPC1 knockout mice isolated from thoracic aortas and cerebral arteries showed no significant change in SOCE activity compared to wildtype, suggesting TRPC1 is not an obligatory component in the SOCE mechanism\textsuperscript{136}. Several TRP channels, TRPV4 and TRPA1 channels in particular, are responsible for mediating smooth muscle-dependent vasodilation. It was illustrated by Earley et al. that Ca\textsuperscript{2+}-influx through TRPV4 stimulated directly by 11, 12-EET or through Ang II-initiated PLC-PKC
pathway leads to potentiation of Ryanodine receptor-mediated Ca\(^{2+}\) sparks and subsequent activation of large conductance Ca\(^{2+}\)-activated K\(^{+}\) channels, resulting in K\(^{+}\) efflux, hyperpolarization and vasodilation\(^{137}\).

In addition to the excitation-contraction response, alteration of cytosolic Ca\(^{2+}\) level mediated by TRP channels also stimulates other cellular processes in smooth muscle. For instance, elevated Ca\(^{2+}\) stimulates a number of proteins that are associated with cell cycle regulation and transcription factors, resulting in smooth muscle proliferation. In proliferating human pulmonary artery smooth muscle cells, TRPC1 and TRPC6 channel expression is upregulated\(^{138}\), and inhibition of either gene expression with antisense technique attenuates proliferation\(^{139, 140}\), suggesting the involvement of TRPC channels in pulmonary artery hypertrophy. Recent evidence reveals a novel role of TRPM7 in mediating aortic smooth muscle proliferation\(^{141}\).

1.4.2 TRP channels in endothelium

Endothelium-associated vascular relaxation relies on the release of nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factors (EDHF), all of which are dependent on the level of endothelial Ca\(^{2+}\). As the majority of TRP channels play a pivotal role in conducting Ca\(^{2+}\) influx, their contributions to endothelial activation have been intensely studied. To date, TRPV1, TRPV3, TRPV4, TRPA1, TRPC3 and TRPC4 channels have been reported to be involved in endothelium-derived vasodilation\(^{142-147}\). As a prime example, endothelial TRPV4 channels have been found to facilitate muscarinic\(^{146, 147}\) and purinergic\(^{148}\) agonist-induced as well as flow\(^{149-151}\)-evoked dilation. A study from Sonkusare et al. further revealed the cooperative gating of the channel to conduct Ca\(^{2+}\)
influx (Ca$^{2+}$ sparklets), which stimulates small and intermediate conductance Ca$^{2+}$-activated K$^+$ (SK$_{Ca}$ and IK$_{Ca}$) channels$^{147}$. K$^+$ efflux from endothelial cells causes hyperpolarization in smooth muscle by activating K$_{ir}$ channels, with concomitant vasodilation. Other endothelial TRP channel-associated dilatory mechanisms include TRPV1-mediated eNOS activation and elevated NO production observed in mesenteric arteries$^{152}$, renal arteries$^{153,154}$ and coronary arteries$^{155}$, muscarinic GPCR agonist-induced and TRPC3-mediated IK$_{Ca}$, SK$_{Ca}$ activation in mesenteric arteries$^{144,156}$, and reactive oxygen species-triggered TRPA1-mediated IK$_{Ca}$ channel opening in cerebral arterial endothelial cells$^{157}$.

Apart from regulation of vascular tone, the endothelium also serves as barrier that controls the passage of ions, water and macromolecules between blood and the interstitium$^{158}$. The degree of vascular permeability varies in response to various stimuli under physiological and pathological conditions. Several TRP channels have been shown to dynamically influence endothelial permeability, including TRPC1, TRPC4, TRPC5 and TRPV4 channels. Among these proteins, convincing evidence has been provided by several studies that TRPV4 channel is critically involved in regulation of pulmonary vascular permeability based on the observations that microvascular permeability can be markedly increased by TRPV4 agonists and attenuated by TRPV4 antagonists$^{159}$.

Angiogenesis is a process of new blood vessel growth under physiological circumstances of tissue damage and embryonic development, as well as pathological conditions like diabetes and cancer$^{160}$. Angiogenesis is then followed by endothelial cell proliferation and migration to generate new capillary tubes. Several studies have reported the participation of TRP channels in angiogenic mechanisms, consisting TRPC1, TRPC3,
TRPC4, TRPC5, TRPC6 and TRPV4 channels\textsuperscript{47}. Each of these TRP channels has been shown to mediate growth factor-evoked endothelial Ca\textsuperscript{2+} modulation and subsequent signaling mechanisms. Zadeh and coworkers reported that expressing a dominant-negative construct of TRPC6 substantially inhibited vascular endothelial growth factor (VEGF)-induced cytosolic Ca\textsuperscript{2+} increase, proliferation, spouting and migration\textsuperscript{161}. Moreover, activation of VEGF receptors has been shown increase endothelial TRPC6 channel currents in expression systems\textsuperscript{162}. Unfortunately, these observations are not supported by genetic techniques, as neither TRPC6 gain-of-function in humans\textsuperscript{163} nor gene deletion in mice\textsuperscript{104} showed abnormal blood vessel structure or capillary growth.

**Part II: Myogenic Tone Development**

**1.5 TRPM4 Channels in Myogenic Response**

The melastatin (M) transient receptor potential (TRP) channel TRPM4 is a non-selective cation channel that is activated by intracellular Ca\textsuperscript{2+}. It was first described in cultured cardiac myocytes, and was later discovered in multiple tissue types, including cardiac muscle, neurons and vascular smooth muscle. It regulates a variety of physiological and pathological processes that involve TRPM4-induced sodium entry, membrane depolarization and modulation of intracellular Ca\textsuperscript{2+}. In this section, the molecular and functional characterization of TRPM4 channels will be summarized with a particular focus on their roles in cerebral arterial smooth muscle.
1.5.1 TRPM4 structure

The TRPM4 gene is located on human chromosome 19, and in mouse on chromosome 7. It comprises 25 exons, spanning 54 kb in the human genome and 31 kb in the mouse genome\textsuperscript{164}. All TRP channels are expressed as six-transmembrane domain (S1-S6) subunits with a pore-forming region between the S5 and S6 domains. Four of these subunits assemble to form a functional ion channel. Heteromultimeric channels composed of two or three different subunits can form and these channels have properties quite different from homomeric channels. Formation of heteromultimeric channels has been characterized for TRPC channels, TRPM6/TRPM7, and TRPV5/V6 channels\textsuperscript{164}. However, to date, heteromultimers of TRPM4 and other TRP membrane have not been reported. Within the TRPM subfamily, TRPM4 is very closely related to TRPM5 in terms of structure since they share approximately 50\% homology\textsuperscript{165}. Several important protein domains have been identified in the TRPM4\textsubscript{b} protein sequence. The cytoplasmic N- and C- termini contain a number of potential protein-protein binding sites: The C-terminus contains coiled-coil domains, and both N- and C-termini contain calmodulin-binding sites\textsuperscript{164}. Other potentially significant sites were also reported in the TRPM4 sequence, including phosphorylation sites for protein kinases (PKA and PKC)\textsuperscript{164}, four Walker B motifs\textsuperscript{164}, a phosphatidylinositol bisphosphate (PIP\textsubscript{2}) binding site\textsuperscript{166}, and at least two functionally different divalent cation-binding sites that determines the Ca\textsuperscript{2+} sensitivity of the channel\textsuperscript{167}. 
1.5.2 TRPM4 biophysical properties and regulation

A short form splice variant of TRPM4 (TRP-MLSN-4 or TRPM4a) was first cloned and characterized by Xu et al.\textsuperscript{168}. With techniques including fura-2 Ca\textsuperscript{2+} imaging and immunofluorescence, it was claimed that expressed TRPM4a in HEK293 cells was localized very close to the plasma membrane and function as a divalent cation channel with a specificity for ions of Ca\textsuperscript{2+} \geq Ba\textsuperscript{2+} \geq Sr\textsuperscript{2+}\textsuperscript{168}. This finding was, however, not followed by any electrophysiological characterization, and was not able to be reproduced by other groups. Later, a second, longer (containing additional 174 amino acids N-terminal) splice variant was reported by Launay et al., and was then designated as TRPM4b\textsuperscript{169}. It was then suggested by Launay and colleagues that TRPM4a has a very similar length to a truncated form of TRPM4b (ΔN-TRPM4) with the first 177 amino acids in the N-terminus deleted, and could therefore act as a native dominant negative subunit\textsuperscript{170}. Hence, TRPM4b is now generally accepted to be the commonly expressed and functional isoform of the channel, and will be referred to in the future context simply as TRPM4.

To study the single-channel properties of TRPM4, several laboratories utilized inside-out configuration of patch clamp technique on HEK293 cells overexpressing TRPM4 or on native smooth muscle cells\textsuperscript{19, 121, 169}. The unitary conductance of TRPM4 is \textasciitilde\textasciitilde25\textmu S. TRPM4 and closely related channel TRPM5 display two defining biophysical properties: Ca\textsuperscript{2+}-dependent activation and specific selectivity for monovalent cations\textsuperscript{169}. The cation selectivity of TRPM4 was assessed with equimolar substitution of intracellular Na\textsuperscript{+} with other permeant cations. The relative permeability to sequence was found to be Na\textsuperscript{+} \approx K\textsuperscript{+} > Cs\textsuperscript{+} > Li\textsuperscript{+}. The study also confirmed that TRPM4 is virtually
impermeable to Ca\(^{2+}\) (P\(_{\text{Ca}}/P_{\text{Na}} < 0.001\)). The putative selectivity filter is formed by negatively charged amino acids between Glu981 and Ala986 of the human TRPM4 subunit.\(^{171}\)

**Ca\(^{2+}\)-dependence.** TRPM4 is a Ca\(^{2+}\)-activated cation channel and its activity is highly dependent on concentration of intracellular Ca\(^{2+}\). No TRPM4 activity was observed when intracellular Ca\(^{2+}\) level was buffered at or below 100 nmol/L. Human TRPM4 channels expressed in HEK cells have an EC\(_{50}\) for Ca\(^{2+}\)-dependent activation of 300 ~ 500 nmol/L in whole cell recordings, as reported by Launay et al., and much higher [Ca\(^{2+}\)] (in µmol/L range) is required as reported by Nilius et al.\(^{121}\). Though there is no explanation for this discrepancy in HEK cells constructs, µmol/L levels of Ca\(^{2+}\) are a prerequisite for TRPM4 opening in native vascular smooth muscle cells. Earley et al. reported that TRPM4 in arterial myocytes has an EC\(_{50}\) of 10 µmol/L under whole cell patching condition\(^{20}\) and approximately 200 µmol/L with inside-out configuration\(^{19}\). This sizable difference between the two patch configurations may result from loss of cytosolic factors that sustain TRPM4 channel activity, such as calmodulin, PIP\(_2\) and PKC, when plasma membrane is disrupted upon excision. In support of the observation in native tissue, TRPM4 channels in HEK cells also showed an EC\(_{50}\) of 370 µmol/L [Ca\(^{2+}\)] in excised inside-out recordings\(^{172}\). Furthermore, the Ca\(^{2+}\) sensitivity of TRPM4 channels is regulated by several cellular factors. ATP is significant for restoring the Ca\(^{2+}\) sensitivity of TRPM4 channels after rapid desensitization. With any patch configuration, TRPM4 channels exhibit a time-dependent deactivation, with currents completely inactivated in 120 s in HEK\(^{121}\) and A7r5 cell expression systems\(^{173}\). Elevation of intracellular Ca\(^{2+}\) accelerates the decay as HEK cells with 10 µmol/L [Ca\(^{2+}\)] showed a relatively slow
inactivation (120 s) compared to the cells in 100 µmol/L [Ca\(^{2+}\)]\(_i\), which completely inactivated within 90 s\(^!\). In cerebral artery smooth muscle cells, TRPM4 currents reached maximum activation approximately 4 s after whole cell conditions were established, which is followed by a rapid decay within 20 s\(^!\). However, this decay could be almost completely restored by Mg-ATP for a short period. Interestingly, ATP\(^{4+}\) was previously shown to be an effective blocker of TRPM4 channel, indicating ATP plays a dual role with regard to regulating the TRPM4 activity\(^!\).

Nilius et al. found that expressing a dominant negative calmodulin (CaM) drastically decreased TRPM4 current amplitude. Further, overexpression of functional CaM reduced TRPM4 current decay, indicating that CaM is essential for conferring Ca\(^{2+}\) sensitivity\(^!\). PKC-dependent phosphorylation also plays a key role in activating TRPM4 channels expressed in HEK cells. This is demonstrated by the evidence that the PKC activator phorbol ester PMA considerably decreased the EC\(_{50}\) of [Ca\(^{2+}\)]-dependent TRPM4 activation. Moreover, mutating the putative PKC phosphorylation sites (Serine residues: S1145 and S1152) on TRPM4 channels essentially abolished TRPM4 activation\(^!\). The key role of PKC to TRPM4 opening was also observed in native vascular smooth muscle cells, in which PMA (1 µmol/L) decreased the EC\(_{50}\) of Ca\(^{2+}\) from 10 µmol/L to 5 µmol/L in whole cell patch configuration, and brought the threshold of [Ca\(^{2+}\)]\(_i\) for TRPM4 activation to a more physiological level\(^!\). Finally, TRPM4 channel activity is regulated by the intracellular PIP\(_2\) as shown in a later study from Nilius and colleagues. They illustrated that TRPM4 channel deactivation (loss of Ca\(^{2+}\) sensitivity) is fully recovered by application of PIP\(_2\) in inside-out and whole-cell patch configurations.

On the other hand, modulation of the cellular PIP\(_2\) levels, including PLC-coupled M\(_1\)
receptor activation, pharmacological inhibition of PLC, modulation of PIP2 metabolism and scavenging of PIP2, all significantly altered TRPM4 channel activity. This is indicative of a crucial role of PIP2 in regulating TRPM4 channels. PIP2 failed to initiate channel opening under low [Ca2+]i conditions, suggesting PIP2 is a strong positive modulator rather than a direct activator of TRPM4 channels. Furthermore, putative PIP2 binding sites were discovered by neutralizing the basic residues in a C-terminal Pleckstrin homology (PH) domains, which failed to recover any current decay. Though regulation of TRPM4 Ca2+ sensitivity has been intensely investigated, the binding sites for Ca2+ remained unknown until a recent study pointed out certain negatively charged amino acids are determinant for Ca2+ sensitivity of TRPM4 channels. The C-terminal tail of TRPM4, particularly the TRP domain and TRP box located in the C-terminal region, has been demonstrated essential for the regulation of its activity. The TRP domain is a homologous block of roughly 25 residues immediately C-terminal to S6 that is loosely conserved in almost all TRP mammalian subfamilies. Moreover, the TRP domain contains a highly conserved 6-amino acid TRP box. It has been proposed that the TRP domain of TRPM5, TRPM8 and TRPV5 serves as a PIP2-interacting region, whereas in TRPM4, the TRP box does not appear to be associated with PIP2 interactions. Further investigation into the TRP domain on TRPM4 protein illustrated at least two functionally different divalent cation-binding sites. One has a comparatively higher selectivity for Ca2+, while the other is the binding site for other divalent cations (Co2+, Mn2+, Ni2+). Ca2+-binding to the former site is prerequisite for TRPM4 channel opening. A binding of a divalent cation to the latter site increases the Ca2+ sensitivity of the first binding site and makes the channel less voltage-dependent similar to the effects of PIP2.
In addition, this study also found that two negatively charged amino acids (Asp-1049, Glu-1062) near and in the TRP domain are critical regulators of Ca$^{2+}$ binding affinity for the first site, as mutation of either amino acid substantially decreased the Ca$^{2+}$ sensitivity of the first site without much effect on the affinities of the second binding site or the PIP$_2$-binding site$^{167}$. However, additional questions concerning the binding mechanisms remain to be elucidated, especially whether the TRP domain is a direct binding site or an allosteric region for Ca$^{2+}$ binding, which will require resolution of the crystal structure of the TRPM4 channel.

**Voltage dependence.** Previous reports from HEK cells and native smooth muscle showed some discrepancy in terms of the voltage dependence of TRPM4 channels. Launay et al. reported that TRPM4 channel activity exhibited both an increase in open probability as well as an increase in open times at positive potentials with single channel recording from excised membrane patches. This may in part account for the slight outward rectification at positive potentials shown from the whole-cell recordings. Furthermore, Launay and colleagues also observed that single-channel amplitudes exhibited slight rectification at both positive and negative membrane voltages, which may provide some explanation for the S-shaped appearance of both single-channel and whole-cell current-voltage relationships$^{169}$. Later, more biophysical studies of TRPM4 channel in HEK cells were carried out by Nilius et al.$^{121}$. These investigators showed with inside-out patch configuration that voltage steps applied from 0 mV to negative potentials induced small, rapidly deactivating inward currents, whereas much larger outward currents were activated during steps to positive potentials, indicating that gating of TRPM4 is a voltage-dependent process$^{121}$. However, in contrast to the previous study by
Launay et al.\textsuperscript{169}, Nilius et al. pointed out that the current-voltage relationship is linear\textsuperscript{121} rather than sigmoidal. This was further supported by the biophysical studies of TRPM4 in native smooth muscle cells. With single-channel recordings on cerebral arterial smooth muscle cells, Earley et al. also observed a linear current-voltage relationship with a calculated single channel conductance of $\approx 24$ pS\textsuperscript{19}. Nevertheless, unlike cloned TRPM4, these channels in arterial myocytes do not exhibit voltage dependence since the open probabilities were not significantly different for cells held at -80 mV and +80 mV\textsuperscript{19}. It is not clear why there is difference in voltage sensitivity between expressed and native channels. Investigation of TRPM4 channels in other tissue types suggests that TRPM4 channels exhibit tissue- and species- specific properties and regulation. For example, TRPM4 in rat ventricular cardiomyocytes\textsuperscript{175} and mouse sino-atrial node cells\textsuperscript{69} exhibits both voltage dependence and linear current-voltage relationship. This difference in voltage sensitivity may result from different splicing, specific multimerization, or distinct intracellular modulating factors.

1.5.3 Exogenous TRPM4 regulation

\textit{Molecular regulation.} Prior to the discovery of more selective TRPM4 inhibitors and activators, investigation of TRPM4 channels, particularly the functional importance of these channels in various tissue, was limited to using molecular approaches to downregulate channel expression. Launay et al. were the first to generate and utilize a dominant native mutant of TRPM4 channels by deleting the first 177 amino acids in the N terminus ($\Delta N$-TRPM4). This mutant was initially tested in HEK 293 cells, where reduced Na$^+$ entry was observed compared to the wt-TRPM4 transfected HEK cells,
indicating a dominant negative effect of ΔN-TRPM4 on endogenous TRPM4. Further, expression of ΔN-TRPM4 did not affect the homomultimerization with wt-TRPM4, suggesting that deleted N-terminus region is not required for multimerization\textsuperscript{170}. Functional roles of TRPM4 channels in immune cells (Jurkat T cells) were later investigated in this paper using the dominant negative mutant of TRPM4. ΔN-TRPM4 showed a profound influence on receptor-mediated Ca\textsuperscript{2+} mobilization, illustrating that TRPM4-mediated T cell membrane depolarization modulates Ca\textsuperscript{2+} oscillations, with downstream effects on cytokine production\textsuperscript{170}.

Genetic modulation of TRPM4 channels was adopted by Earley et al. to study the physiological implication of TRPM4 in vascular smooth muscle cells\textsuperscript{19}. To suppress the expression level of the channel, antisense oligodeoxynucleotides (ODNs) selectively targeted to TRPM4 were used. Antisense ODNs are short strands of complementary DNA that bind to the messenger RNA (mRNA) produced by the target gene and prevent transcription process by putatively recruiting ribonuclease H (RNase H) to accelerate the degradation of target mRNA, hence reducing the expression of target protein\textsuperscript{176}. The effects and specificity of TRPM4 antisense ODNs were evaluated by Earley et al.. Semiquantitative RT-PCR results showed that the antisense significantly reduced TRPM4 mRNA level without any influence on TRPC6 channels which were previously reported to regulate myogenic tone of cerebral arteries. Effects of antisense ODNs on channel activity were then confirmed with two patch clamp configurations. Firstly, inside-out membrane patch recordings on isolated arterial myocytes that were pre-exposed to the ODNs showed TRPM4 channels (~24pS) were observed more frequently in the sense- (47%) treated than the antisense- (10%) treated cells\textsuperscript{19}. Secondly, with conventional
whole-cell patch configuration, the magnitude of the peak outward current in myocytes from vessels treated with TRPM4 antisense was significantly less than that of cells from sense-treated arteries\textsuperscript{20}, both of which indicate TRPM4 antisense is a useful tool to study the functional important of TRPM4 in vascular smooth muscle. Later, Gonzales et al. also reported that commercially available TRPM4 siRNA effectively and selectively reduced TRPM4 protein expression in cerebral arteries and TRPM4 currents in siRNA-treated myocytes. However, in these studies, the approach to deliver the antisense or sense ODNs to cerebral arterial smooth muscle cells was reversible permeabilization (RP), which requires isolation of arteries, RP procedures for at least 4 hours and tissue culture for 72 hours. Though it was not studied in any detail, these laborious processes could potentially lead to shifts in the functional contribution of various cellular players, including different TRP channels.

To illustrate the significance of vascular smooth muscle TRPM4 channels in a more physiological context, Reading et al. applied an approach for antisense delivery \textit{in vivo} whereby ODNs were infused into the cerebral spinal fluid (CSF) using an osmotic pump that was surgically implanted in a subcutaneous scapular pocket\textsuperscript{177}. Enough time (7-day treatment) was allowed for distribution of ODNs throughout the CSF and uptake of ODNs by cerebral arterial smooth muscle cells before rats were euthanized and arterial segments were isolated for myograph studies. The effectiveness of this method was confirmed with semiquantitative PCR. Functionally, \textit{in vivo} suppression of TRPM4 decreased myogenic constriction of cerebral arteries and impaired autoregulation. This is consistent with prior studies where \textit{in vitro} TRPM4 gene knockdown also substantially attenuated myogenic depolarization and tone of cerebral arteries\textsuperscript{19}. 40
A recent study from our laboratory further developed the ODN delivery method into a more targeted and efficient process; TRPM4 antisense or sense ODNs were directly injected into the rat cisterna magna for two consecutive days. Rats were euthanized on the third day, and tissue was isolated for myograph experiments. RT-PCR results indicated that exposure of parenchymal arterioles (PAs) to TRPM4 antisense in vivo effectively reduced the expression level of TRPM4 channels. Control experiments on cerebral pial arteries also showed a significant reduction on TRPM4 mRNA level in the antisense group. Further specificity test showed no influence of TRPM4 antisense on TRPC6 message levels, demonstrating that the designed antisense is a useful tool for functional studies. Indeed, in vivo TRPM4 suppression using the above approach considerably diminished myogenic tone developed by PAs at different intraluminal pressures. The in vivo administration of TRPM4 ODNs was also used by other groups to study the functional roles of this channel in other systems. For example, to downregulate TRPM4 expression after spinal cord injury, Gerzanich et al. gave rats a loading dose of ODNs intravenously and implanted mini-osmotic pumps with jugular vein catheters to continue infusing ODNs for the next 24 hours. Channel suppression was also demonstrated to be very effective. Most recently, TRPM4 knockout mice have opened the door to studies of the physiological roles of TRPM4 in many systems, including the cardiac myocytes, the nervous system, the immune system, and so forth.

**Pharmacological regulation.** Before the discovery of more selective TRPM4 channel modulators, studies of this channel were restricted to using generic activators and inhibitors. Decavanadate was found to potentiate Ca$^{2+}$-dependent TRPM4 activity. Nilius

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et al. reported that application of devacanadate strongly affected the voltage-dependent gating of the channel, which sustained currents over the voltage range between -180 mV and +140 mV\textsuperscript{172}. However, lack of specificity significantly limits the use of this compound. Decavanadate was found to be a reversible and competitive antagonist of the P2X7 receptors with slight inhibitory influence on P2X2 and P2Y4 receptors as well\textsuperscript{182}. Moreover, it also binds to the IP\textsubscript{3} receptors and reduces Ca\textsuperscript{2+} release from the endoplasmic reticulum in rat pancreatic acinar cells\textsuperscript{183}. Some earlier research on TRPM4 channels also used nonselective cation channel inhibitors like flufenamic acid and clotrimazole as gating inhibitors, and Gd\textsuperscript{3+} and spermine as pore blockers\textsuperscript{69, 184, 185}. Further, high concentrations of glibenclamide, an ATP-sensitive potassium (K\textsubscript{ATP}) channel blocker, were also shown to inhibit TRPM4 channel activity by several groups. It was first reported by Demion et al. that 100 µmol/L glibenclamide significantly decreased TRPM4 activity by ~80% in HEK 293 cells\textsuperscript{69}. Glibenclamide interacts with ATP-binding cassette proteins (ABC proteins)\textsuperscript{165}. Interestingly, the TRPM4 protein contains two ABC transporter signature-like motifs, which may explain the inhibitory effects of glibenclamide\textsuperscript{165}. Later, a hydroxytricyclic compound 9-phenanthrol was found to be a much more selective modulator of TRPM4 channel activity. Inhibition of TRPM4 by 9-phenanthrol was observed initially in HEK cells with both inside-out and whole-cell configurations, with an IC\textsubscript{50} of 20 µmol/L\textsuperscript{165}. This group also reported that 9-phenanthrol (100 µmol/L) had no inhibitory effects on TRPM5, which shares 50% structural similarity and similar electrophysiological properties with TRPM4 channels\textsuperscript{165}. More detailed specificity tests were conducted by Gonzales et al. who found that 30 µmol/L 9-phenanthrol, which almost completely blocks TRPM4 currents, did not alter
the activity of ion channels in smooth muscle cells that are potentially involved in modulation of vascular membrane potential and tone (voltage dependent Ca\textsuperscript{2+} channels (VDCCs), big conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK\textsubscript{Ca}) channels, voltage-gated K\textsuperscript{+} (K\textsubscript{v}) channels, inward rectifying K\textsuperscript{+} (K\textsubscript{ir}) channels)\textsuperscript{93}; 9-phentanthrol (30 µmol/L) also has no effect on other TRP channels (TRPC3, TRPC6 and TRPM7)\textsuperscript{93, 186}. Based on the above results, 9-phentanthrol appears to be a specific and effective tool in studies of the functional roles of TRPM4 channels. However, Gonzales et al. only focused on the ion channels present in the vascular smooth muscle cells. It was recently reported by Garland et al. that 9-phentanthrol activates the intermediate conductance K\textsuperscript{+} (IK\textsubscript{Ca}) channels in mesenteric arterial endothelial cells, hyperpolarizes smooth muscle and induces vasodilation at a relatively low concentration (3 µmol/L)\textsuperscript{94}. Similar results were also obtained in cerebral parenchymal arterioles by our laboratory (unpublished data). The nonselective inhibitory effects of 9-phentanthrol were also reported on cerebral arterial myocytes TMEM16A channels\textsuperscript{187}, which were proposed to be a newly discovered mediator of myogenic membrane depolarization and constriction, a role identical to that of TRPM4 channels. The IC\textsubscript{50} of 9-phentanthrol on TMEM16A channels is 12 µmol/L, which is very close to the IC\textsubscript{50} for TRPM4. All these off-target effects of 9-phentanthrol indicate that the use of 9-phentanthrol should be handled with more caution for further TRPM4 studies. Proper control experiments should be conducted to eliminate the possibility that the observed results might actually be attributed to the off-target effects.
1.5.4 TRPM4 distribution and function

Using various molecular approaches, TRPM4 channel protein has been detected in a large number of tissues. In human, high levels of TRPM4 signal were detected in the heart, pancreas, placenta, and prostate, and lower levels of expression in the kidney, skeletal muscle, liver, intestines, thymus, and spleen. It has also been found in several hematopoietic cell lines, including T and B lymphocyte cell lines and monocyte cell lines. In the central nervous system, expression and functional roles of TRPM4 were identified in the hippocampal neurons, substantial nigra pars compacta, cerebral cortex, spinal cord motor neurons, and the pre-Bötzheimer complex in the brainstem. This channel is present in smooth muscle cells found in rat cerebral arteries and arterioles, rat and guinea pig urinary bladder and detrusor, rat aorta, rat pulmonary arteries, and monkey and human colon. TRPM4 channels are implicated in numerous physiological processes in various systems, all of which include TRPM4-mediated Na+ entry and membrane depolarization, followed by modulation of intracellular ions, mostly Ca2+. Previous studies have shown that TRPM4 channels are involved in insulin secretion from pancreatic β cells, immune response in T cells, dendritic cells and mast cells, respiratory rhythm genesis in the pre-Bötzheimer complex in the brain stem, cardiac conduction and hypertrophy in various types of cardiac myocytes, and detrusor smooth muscle contraction in the urinary bladder.
1.5.5 TRPM4 function and regulation in vascular smooth muscle

It has been well established that membrane potential is the major determinant of smooth muscle cell contractility. It is attributed to that L-type voltage-dependent calcium channel (VDCC) activity is highly dependent on membrane potential\textsuperscript{15}. VDCCs are sharply activated at membrane potential between -50 mV to -30 mV\textsuperscript{15}. Opening of VDCCs allows for Ca\textsuperscript{2+} rushing into the cells down its concentration gradient (extracellular: \textasciitilde 2 mmol/L Ca\textsuperscript{2+}, intracellular: \textasciitilde 100 nmol/L Ca\textsuperscript{2+}). Ca\textsuperscript{2+} then binds to calmodulin and activates myosin light chain kinase (MLCK), which further phosphorylates and activates myosin light chain, promotes myosin/actin interaction and smooth muscle contraction. It was reported by Knot at el. that along with a membrane potential changing from -58 mV to -23 mV, intracellular [Ca\textsuperscript{2+}] increased from 126 ± 7 nmol/L to 349 ± 12 nmol/L, and that cerebral arteries went from full dilation to maximum constriction\textsuperscript{15}. It was also well accepted that vascular smooth muscle membrane potential is regulated by intraluminal pressure. Specifically, elevation of intravascular pressure causes a graded membrane depolarization of smooth muscle cells in resistant arteries, and results in graded constriction, i.e. myogenic tone\textsuperscript{14, 15}. As a prime example, in cerebral arteries, increase in vascular pressure from 10 to 100 mmHg leads to smooth muscle depolarization from -63 to -36 mV, [Ca\textsuperscript{2+}]i elevation from 119 nmol/L to 245 nmol/L, and subsequent arterial constriction from 208 µm to 116 µm\textsuperscript{15}. This is again indicative of the central role of membrane potential regulation in the mechanism of pressure-induced vasoconstriction, blood flow and cerebral autoregulation. Nevertheless, how the mechanical stimulus (membrane stretch) is translated into a biological response (membrane depolarization) is not fully understood at the time.
As the key role of TRPM4 in regulating membrane potential has been defined in HEK cells\textsuperscript{121, 169} and in other systems\textsuperscript{170}, it became the prime suspect to mediate myogenic depolarization in cerebral circulation. As first reported by Earley et al. that TRPM4 transcript and currents are present in cerebral pial arterial smooth muscle cells\textsuperscript{19}. Those currents exhibited the hallmarks of TRPM4 channels, including Ca\textsuperscript{2+}-dependence, unitary conductance, and ion permeability. Recently, TRPM4 transcript signals were also detected in the cerebral microcirculation\textsuperscript{34}, particularly parenchymal arteriole smooth muscle. The functional significance of TRPM4 was assessed with both molecular and pharmacological approaches. Firstly, downregulation of the channel \textit{in vitro} with ODNs effectively and selectively reduced TRPM4 mRNA levels and current occurrence, and inhibited pressure-induced membrane depolarization and vasoconstriction\textsuperscript{19}. Next, administration of a commercially available TRPM4 small interfering RNA (siRNA) generated similar results; myogenic response is attenuated by TRPM4 siRNA compared to control\textsuperscript{203}. Thirdly, a selective pharmacological blocker 9-phenanthrol elicited inhibitory effects not only on whole-cell TRPM4 currents, but myogenic depolarization and constriction\textsuperscript{93}. All of the above studies provide compelling evidence that pressure-induced membrane depolarization is primarily mediated by opening of TRPM4 channels.

In order to evaluate the involvement of TRPM4 in a more physiological setting, Reading et al. adopted an \textit{in vivo} suppression technique; ODNs were administered to the cerebral spinal fluid of the third cerebral ventricle of rats using osmotic pumps\textsuperscript{177}. With RT-PCR, the expression level of TRPM4 was reduced by 7-day treatment with antisense ODNs. Functionally, myogenic constriction was decreased by 70% to 85% in cerebral arteries from antisense-treated compared to sense-treated rats. Moreover, cerebral blood
flow was also substantially greater in the TRPM4 antisense group than in the sense group\textsuperscript{177}. Cerebral blood flow was measured over a range of mean arterial pressures\textsuperscript{177}. These observations suggest that arteries lacking TRPM4 channel fail to respond properly to elevated arterial pressure, and therefore the arteries are more dilated and blood flow is greater. This also points to the physiological significance of TRPM4 as it contributes \textit{in vivo} to myogenic response and cerebral blood flow regulation.

Supporting evidence that TRPM4 is mechano-sensitive was first provided by Morita et al.. TRPM4-like currents were observed in isolated cerebral arterial myocytes using the on-cell patch configuration\textsuperscript{184}. These channels exhibited unitary conductance of approximately 22 pS, and were permeable to Na\textsuperscript{+} and Cs\textsuperscript{+} and blocked by Gd\textsuperscript{3+}. Their activity was highly dependent on [Ca\textsuperscript{2+}]\textsubscript{i}. These are all very important fingerprints of TRPM4 channels. Interestingly, the authors demonstrated that these channels could be activated by applying negative pressures to the membrane, which mimicked the membrane stretch during increase in intraluminal pressure\textsuperscript{184}. The open probability of these channels increased with increased negative pipette pressure, which is consistent with pressure-dependent depolarization and constriction observed in intact arteries\textsuperscript{184}. However, whether TRPM4 channels are directly activated by membrane stretch, or stimulated by a stretch-sensitive upstream signaling needs further investigation. A recent work from Gonzales et al. provided some insights into the mechano-sensitivity and activation mechanisms of TRPM4\textsuperscript{102}. First, using perforated whole-cell patch configuration, they observed that inward TRPM4 currents were augmented by membrane stretch generated by switching to a hypotonic bath solution, indicating that TRPM4 can be activated by mechano-stimulation\textsuperscript{102}. However, what is unexpected in the research is
that the single TRPM4 currents in HEK cells overexpressing the channel protein failed to be activated by negative pressures\textsuperscript{102}. As a comparison, TRPC6 channels transfected in HEK cells exhibited apparent stretch-activation\textsuperscript{102}. These results indicate that TRPM4 channels are not inherently mechano-sensitive, rather, they are activated by upstream signaling pathways that potentially involve TRPC6 channels.

\textit{IP\textsubscript{3} Ca\textsuperscript{2+} release.} As described in previous text that TRPM4 is a Ca\textsuperscript{2+}-activated ion channel. Its activity is highly dependent on intracellular [Ca\textsuperscript{2+}]. In experimental settings, previous studies have shown that this channel requires a Ca\textsuperscript{2+} concentration of 10-100 µmol/L to activate as indicated by both whole-cell and inside-out patch recordings. This level of Ca\textsuperscript{2+} is much higher than global Ca\textsuperscript{2+} levels in vascular smooth muscle cells under normal conditions (~ 100 nmol/L). Global Ca\textsuperscript{2+} increase through Ca\textsuperscript{2+} influx from VDCCs is unlikely to reach the µmol/L range, whereas local Ca\textsuperscript{2+} elevation resulting from Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) could be substantial enough to activate TRPM4 in proximity, a proposed mechanism similar to the well-characterized BK\textsubscript{Ca} channel activation by SR Ca\textsuperscript{2+} release from ryanodine receptors. Ca\textsuperscript{2+} sparks can reach the levels of 1- to 100- µmol/L\textsuperscript{204}. Using the perforated patch voltage-clamp technique, Gonzales et al. reported that inward TRPM4 currents were not acutely affected by removal of extracellular Ca\textsuperscript{2+}, suggesting the channel is not dependent on Ca\textsuperscript{2+} influx through VDCCs\textsuperscript{21}. However, prolonged Ca\textsuperscript{2+}-free condition eventually reduced TRPM4 activity (after a few minutes), suggesting extracellular Ca\textsuperscript{2+} is important for sustaining TRPM4 activation presumably through maintaining the SR Ca\textsuperscript{2+} stores. This hypothesis was supported by further observation that disrupting Ca\textsuperscript{2+} stores with cyclopiazonic acid (CPA) largely suppressed TRPM4 currents\textsuperscript{21}. Moreover, the authors
showed that TRPM4 activity was not affected by blocking RyR function, rather, it was significantly inhibited by an IP₃ blocker xestospongin C²¹. This is the first demonstration that TRPM4 channels interact with IP₃ receptors on the SR to mediate myogenic depolarization. This mechanism may be present not only in the vascular smooth muscle cells, but also in other systems. In fact, the coupling between TRPM4 and IP₃ receptor has also been documented in the mouse preBötzinger complex, regulating respiratory rhythms²⁰⁰.

**PKC regulation.** In expression systems, TRPM4 channels are under the regulation of PKC, which phosphorylates the channel and decreases the EC₅₀ of [Ca²⁺]-dependent channel activation¹⁷⁴. Such a regulatory mechanism was also investigated in the native smooth muscle. Earley et al. reported that, in isolated cerebral arterial myocytes, the selective PKC activator phorbol 12-myristate 13-acetate (PMA) shifted the EC₅₀ of [Ca²⁺] for TRPM4 activation from 10 μmol/L to 5 μmol/L, which, to some extent, brought the required [Ca²⁺] to a more physiological level. Consistent with this observation, PMA also enhanced myogenic tone of isolated pial arteries. To demonstrate that this effect is mediated by TRPM4, TRPM4 antisense ODNs were utilized by the authors. It was shown that PMA depolarized smooth muscle cells from sense-treated arteries, but did not significantly influence the membrane potential of antisense-treated smooth muscle. Further, TRPM4 sense-treated arteries exhibited concentration-dependent constriction to PMA. Such effects were not observed in arteries exposed to antisense, which caused oscillatory diameter changes, and significantly attenuated contractile response to PMA. These results indicate that PKC-mediated smooth muscle depolarization and constriction
involve activation of TRPM4 channels. More recently, a novel mechanism regarding PKC and TRPM4 was investigated by Crnich et al.\textsuperscript{205}. In cultured aortic smooth muscle cells (A7r5 cells), the surface expression of TRPM4 protein increased by approximately 3 fold when cells were exposed to PMA\textsuperscript{205}. This also applies to native vascular smooth muscle as PMA increased TRPM4 expression in intact cerebral arteries\textsuperscript{205}. PMA-induced translocation of TRPM4 could be inhibited by a PKCδ blocker, but it is insensitive to PKCα and PKCβ modulators\textsuperscript{205}. Additionally, molecular and pharmacological inhibition of PKCδ dramatically reduced TRPM4 expression on the plasma membrane, indicating PKCδ is required to sustain the expression and activity of TRPM4 channels. In agreement with this observation, siRNA targeted to PKCδ significantly attenuated pressure-induced depolarization and vasoconstriction of pial arteries\textsuperscript{205}, demonstrating that PKC-induced contractile response is partially attributed to increased surface expression of TRPM4.

\textit{TRPC6 coupling.} As briefly mentioned previously in this chapter, TRPC6 rather than TRPM4 appears to be directly mechano-sensitive\textsuperscript{102}. Since TRPM4 is critically involved in stretch-induced responses, this observation is very intriguing, as TRPC6 may potentially serve as an upstream signaling partner in order to activate TRPM4 channels. It has been proposed previously that TRPM4 and TRPC6 might be electrically coupled to facilitate myogenic constriction\textsuperscript{19}. The rationale is that both channels have been shown to participate in myogenic tone development. In addition, TRPC6 is a Ca\textsuperscript{2+}-permeable, non-selective cation channel\textsuperscript{206}. It was hypothesized that if TRPC6 and TRPM4 channels are in proximity, Ca\textsuperscript{2+} entry through TRPC6 could lead to Ca\textsuperscript{2+}-dependent activation of TRPM4 channels, and subsequent excitation-contraction signaling. These channels could also form heteromultimers with novel biophysical characteristics. However, though
heteromultimerization between within the TRPC family has been reported\textsuperscript{125}, such constructs have not been found between different TRP families. The interactions between TRPC6 and TRPM4 were investigated in much greater detail by Gonzales et al.\textsuperscript{102}. The authors used \textit{in situ} proximity ligation assay to show that these two channels are located within 40 nm of each other, supporting the proposal that TRPC6 is positioned to provide a Ca\textsuperscript{2+} source for TRPM4 activation\textsuperscript{102}. Moreover, stretch-induced TRPM4 activation was inhibited by a nonselective TRPC6 blocker and TRPC6 inhibitory antibody, providing supporting evidence that TRPC6 and TRPM4 are functionally connected. Further, hypotonic solution-stimulated TRPM4 currents were also attenuated when cells were pre-incubated in TRPC6 inhibitory antibody\textsuperscript{102}. These results all point to the possibility that Ca\textsuperscript{2+} influx through TRPC6 is necessary for stretch-induced, IP\textsubscript{3} receptor-involved, and Ca\textsuperscript{2+}-mediated activation of TRPM4 in the cerebral arterial smooth muscle cells. On the other hand, Earley et al. have also provided compelling evidence that PLC plays an essential role in regulating TRPM4 activity. First of all, PLC\textgamma 1, among other isoforms, was found to co-localize with TRPM4\textsuperscript{102}. Suppressing the expression PLC\textgamma 1 with siRNA reduced TRPM4 activity, which could be partially rescued by administration of Bt-IP\textsubscript{3}\textsuperscript{102}. This finding demonstrated that PLC-induced TRPM4 activation occurs through modulation of IP\textsubscript{3} levels. Consistent with this finding, PLC\textgamma 1 siRNA also significantly inhibited pressure-induced membrane depolarization and myogenic constriction\textsuperscript{102}. Taken together, Gonzales et al. have revealed a complex signaling network mediating pressure-induced depolarization. By incorporating the major players, including PLC, IP\textsubscript{3} receptor, TRPM4 and TRPC6, the stretch-induced responses appear to be a dual effect: pressure-induced PLC\textgamma 1 activation stimulates IP\textsubscript{3} release, and stretch-
activated TRPC6 mediates Ca\(^{2+}\) influx. As IP\(_3\) increases the sensitivity of IP\(_3\) receptors to cytosolic Ca\(^{2+}\), IP\(_3\) receptor work as central hub that receives excitatory signals from the two pathways and mediate Ca\(^{2+}\) release from the SR. Local Ca\(^{2+}\) elevation further activates TRPM4 channels, and leads to subsequent depolarization and myogenic response\(^{102}\).

### 1.6 Purinergic Receptors in Vascular Function

Purinergic receptors are a family of plasma membrane proteins that have been identified in almost all mammalian tissues\(^{207}\). They are involved in a large variety of cellular functions, including cytokine secretion\(^{208}\), cell proliferation\(^{208}\), vascular reactivity and remodeling\(^{209}\), apoptosis, neuromodulation\(^{41}\), and synaptic transmission and plasticity\(^{41}\), etc. Based on the molecular structure and signal transduction mechanisms, purinergic receptors are grouped into three major classes\(^{210}\): P\(_1\) (adenosine) receptors, G protein- (primarily G\(_s\) and G\(_i/o\)) coupled receptors that are activated by adenosine; P\(_{2X}\) receptors, ligand-gated ion channel receptors that are primarily stimulated by ATP; P\(_{2Y}\) receptors, G protein- (primarily G\(_q\) and G\(_{12/13}\)) coupled receptors that show different sensitivities to various nucleotides, such as ATP, ADP, UTP and UDP. Purinergic receptors are widely expressed in almost all tissue of all mammalian species. Among the purinergic receptors, P\(_1\) receptors, including A\(_1\), A\(_{2A}\), A\(_{2B}\) and A\(_3\) receptors, are present in brain, heart, liver, intestine and lungs, and involved in modulation of the cardiovascular, immune and central nervous system activity\(^{211}\). P\(_{2X}\) receptors, including P\(_{2X1}\) to P\(_{2X7}\) receptors, are mainly found in vascular smooth muscle, platelets, central nervous system and spinal cord\(^{211}\), and contribute to regulation of vascular reactivity and synaptic
transmission\textsuperscript{41}. P2Y receptors, comprising P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, P2Y14 receptors, are abundantly expressed in vascular endothelial cells and smooth muscle cells to regulate vasodilation and constriction\textsuperscript{209}; they also participate in platelet activation and aggregation, inflammation, and neuroimmune functions\textsuperscript{211}.

1.6.1 P2Y receptors in vascular function and mechanisms

P2Y receptors have been widely detected in the endothelium and smooth muscle of numerous vascular beds, contributing to regulation of vascular tone, mediation of smooth muscle proliferation, and promotion of platelet aggregation\textsuperscript{209}. P2Y receptors have different selectivities for endogenous agonists, which may be critical for fine-tuning of appropriate and accurate cellular responses. P2Y1 receptors are selectively activated by ADP and ATP; P2Y2 receptors are stimulated equipotently by UTP and ATP; P2Y4 receptors have higher selectivity for UTP, whereas P2Y6 receptors are preferentially stimulated by UDP\textsuperscript{212}. Upon physiological and pathological stimulation, these purines and pyrimidines are locally released from surrounding sympathetic nerves, myocytes, endothelial cells, erythrocytes, platelets and white blood cells, and act synergically on receptors in endothelial cells and smooth muscle cells to eventually regulate vessel contractile status\textsuperscript{212}.

\textit{Vascular smooth muscle.} The major P2Y receptors detected in arterial myocytes are P2Y2, P2Y4 and P2Y6 receptors. They play a pivotal role in mediating agonist-dependent or independent vasoconstriction in mesenteric arteries, cerebral arteries, pulmonary arteries, and renal arteries from different species\textsuperscript{212}. P2Y receptor-mediated cellular signaling transduction mechanisms also vary significantly. For example, in
mesenteric arteries, P2Y2 and P2Y6 receptors are found in arterial smooth muscle. Juul et al. reported that UTP induced sustained depolarization, increased $[\text{Ca}^{2+}]_i$ and constriction$^{213}$. In comparison, ATP-induced depolarization and constriction were transient. Further, nucleotide-elicited $[\text{Ca}^{2+}]_i$ elevation appeared to arise from both $\text{Ca}^{2+}$ release from the SR and $\text{Ca}^{2+}$ entry through VDCCs, since transient $[\text{Ca}^{2+}]_i$ increase was still detected in $\text{Ca}^{2+}$-free medium$^{213}$. In contrast to the well-established signaling mechanisms of P2Y receptors through $G_q$ protein- and PLC-dependent $\text{Ca}^{2+}$ modulation, Sauzeau et al. reported that P2Y1, P2Y2, P2Y4 and P2Y6 receptors are also coupled to RhoA-Rho kinase-dependent signaling pathways, which result in cytoskeleton reorganization and $\text{Ca}^{2+}$ sensitization$^{214}$. In agreement of this finding, Luykenaar and colleagues found that inhibiting Rho kinase significantly attenuated UTP-induced responses$^{29}$. However, rather than initiating a $\text{Ca}^{2+}$-sensitization mechanism, UTP evoked Rho kinase-mediated inhibition of $K^+$ channels, and subsequent depolarization and vasoconstriction$^{29}$. P2Y receptors have also been shown to mediate pressure-induced vasoconstriction$^4$. Brayden et al. demonstrated that P2Y2, P2Y4 and P2Y6 receptors are present in the intraparenchymal arterioles within the brain. Surprisingly, P2Y2 receptor activation has no effect on arteriolar diameter. By contrast, P2Y4 and P2Y6 receptors play a previously undiscovered role as mechanosensors and mediators of myogenic regulation in these vessels$^4$. Possible contributions of P2Y6 receptors in myogenic control were reported previously in mouse mesenteric arteries$^{16}$. Nevertheless, it was proposed and later demonstrated by another group that P2Y6 receptors were activated by pressure-dependent local release of nucleotides via an autocrine/paracrine mechanism$^{17}$. Comparatively, the observation that modulation of endogenous nucleotide activity had no
influence on myogenic responses but effectively altered exogenous nucleotide-induced vasoconstriction points to a convincing stretch-activation mechanism initiated by the P2Y receptors\textsuperscript{4}. A follow-up mechanistic investigation by Li. et al. and thoroughly presented in Chapter 2 of this dissertation, revealed that P2Y receptors mediate mechano-activation of TRPM4 channels, and concomitant smooth muscle depolarization and contraction\textsuperscript{34}. As P2Y receptors are coupled to the Rho signaling, we further investigated the role of this cellular mechanism in mediating pressure-induced and P2Y receptor-mediated activation of TRPM4 channels in parenchymal arteriolar myocytes. Chapter 3 focuses on testing this hypothesis.

\textit{Vascular endothelium.} P2 receptors are in fact more abundantly expressed in vascular endothelial cells than in smooth muscle cells. P2Y1 and P2Y2 receptors are involved in endothelium-dependent vasodilatory effects in various vascular beds\textsuperscript{209}, which counterbalance the vasocontractile effects of purines and pyrimidines directly on smooth muscle, resulting in more complex control of vascular tone. Under disease conditions when endothelium is damaged, locally secreted nucleotides may act directly on smooth muscle purinergic receptors and lead to pathological vasospasm\textsuperscript{209}. Upon stimulation by purines, endothelial cell activation leads to increase in endothelial [Ca\textsuperscript{2+}]\textsubscript{i} and triggers several pathways, including NO\textsuperscript{215}, prostacyclin\textsuperscript{216} and endothelial-derived hyperpolarizing factors (EDHF)\textsuperscript{217}, that function in concert to eventually induce smooth muscle relaxation\textsuperscript{209}. Recent evidence from Crecelius et al. showed that the vasodilator responses to ATP were significantly attenuated by BaCl\textsubscript{2}, indicating that inward rectifying potassium (K\textsubscript{ir}) channels are also involved in P2Y receptor-mediated vascular hyperpolarization\textsuperscript{218}. Interestingly, ATP release from endothelial cells is also regulated by
shear stress and hypotonicity\textsuperscript{219}. Elevation in shear stress leads to increased local concentration of ATP, which triggers endothelial P2Y receptor activation and vasodilation.

1.6.2 P2X receptors in vascular function and mechanisms

P2X receptors are ionotropic receptors that are gated by ATP. They are non-selective cation channels responsible for mediating various cellular processes, like smooth muscle contraction, platelet aggregation, macrophage activation, and numerous neuronal functions\textsuperscript{220}.

\textit{Vascular smooth muscle.} P2X1 receptors are the most abundant P2X receptors in vascular smooth muscle\textsuperscript{209}. In accord with the expression profile, the dominance of P2X1 receptor in mediating currents in smooth muscle cells of rat mesenteric arteries was also confirmed\textsuperscript{221}. Functionally, the use of P2X1-deficient mice further revealed that P2X1 is critically involved in sympathetic neurogenic vasoconstriction, which is mediated by neuronal release of ATP upon stimulation\textsuperscript{222}. ATP-evoked activation of P2X1 receptors allows for calcium (and other anion) influx, leading to smooth muscle depolarization, $\text{Ca}^{2+}$ entry through VDCCs and vasoconstriction\textsuperscript{209}. In rat cerebral parenchymal arterioles \textit{in vivo}, low concentrations of ATP produce a biphasic response including a transient contraction via P2X1 receptor activation in arterial smooth muscle followed by vasodilation due to endothelial P2Y receptor activation\textsuperscript{223}.

\textit{Vascular endothelium.} Though P2X1, P2X2 and P2X4 subtypes are identified by immunostaining in the endothelium of rat mesenteric, middle cerebral and coronary arteries\textsuperscript{209}, the functional significance of P2X receptors in vascular endothelial cells is
largely unknown. The most thoroughly characterized receptor is the P2X4 receptor, which was identified as crucial transducers in response to ATP released from shear stress-activated endothelial cells. Yamamoto et al. observed that stepwise increases in shear stress elicited a corresponding stepwise increase in [Ca\(^{2+}\)]\(_i\) in human umbilical vein endothelial cells. However, downregulation of P2X4 purinoceptors with selective antisense ODNs markedly diminished shear stress-induced Ca\(^{2+}\) influx into endothelial cells, indicating that this subtype of P2X receptor has a “shear-transducer” property through which the mechanical signal is perceived and potentially transformed into vasodilation in a physiological system\(^{224}\). Later, this laboratory continued the investigation into the physiological role of P2X4 receptors using P2X4 knockout mice. They found that P2X4-deficient mice failed to respond to changes in blood flow with endothelial cell [Ca\(^{2+}\)] increase and NO release normally observed in wild type mice\(^{225}\). Further, P2X\(^{-/-}\) mice exhibited higher blood pressures and excreted smaller amounts of NO products in their urine. These findings imply that impaired, P2X-mediated and flow-induced endothelial cell activation leads to significantly reduced NO release from endothelium and substantially blunted vasodilatory effects in smooth muscle\(^{225}\). This is suggestive that P2X4 receptors play a fundamental role in flow-induced mechanotransduction and regulation of vascular tone and blood pressure.

1.6.3 P1 receptors in vascular function and mechanisms

P1 receptors are G protein-coupled receptors that are activated by adenosine, hence the name adenosine receptors. All four types of P1 receptors (A\(_1\), A\(_{2A}\), A\(_{2B}\) and A\(_3\)) have been detected in the vasculature with a higher expression level of A\(_{2A}\) and A\(_{2B}\)
receptors\textsuperscript{209}. A number of studies have shown that P1 receptors contribute to endothelium-mediated or endothelium-independent vasorelaxation, which plays a crucial role in decreasing tissue damage under pathological conditions, including hypoxia and vasospasm after subarachnoid hemorrhage\textsuperscript{209}. It has been well-characterized that adenosine-induced vasodilation participates fundamentally in protective arterial dilations following hypoxia and ischemia\textsuperscript{209, 226}.

*Vascular smooth muscle.* Though the major role of adenosine receptors lies in endothelium-dependent mechanisms, they are also found in the vascular smooth muscle from rat and guinea pig aorta, rat and rabbit mesenteric arteries, porcine coronary arteries, rat cerebral arteries and guinea pig and rabbit pulmonary arteries\textsuperscript{209}. In mesenteric arteries, earlier studies from Prentice et al. suggested that adenosine and other adenosine analogues evoked vasodilation through A\textsubscript{2B} receptors in arteries stripped to endothelium\textsuperscript{227}. In contrast to this study, a more recent publication from Wang and colleagues proposed an interesting mechanism in small mesenteric resistance arteries, which comprises the involvement of A\textsubscript{2A} receptors in adenosine-induced inhibition of store-operated Ca\textsuperscript{2+} entry, and a subsequent decrease in cytosolic Ca\textsuperscript{2+} levels and vasorelaxation\textsuperscript{228}. It was also shown in several early studies that adenosine-triggered dilatory responses are partially mediated by ATP-sensitive K\textsuperscript{+} (K\textsubscript{ATP}) channels in intact coronary arteries\textsuperscript{229-231}. However, whether or not this is important in vascular smooth muscle is unclear. Hein et al. further illustrated using denuded coronary arterioles that glibencalmide (K\textsubscript{ATP} channel blocker) abolished adenosine- as well as CGS21680 (A\textsubscript{2A} receptor agonist)-induced smooth muscle relaxation, indicating that smooth muscle K\textsubscript{ATP} channels are coupled to A\textsubscript{2A} receptors to facilitate adenosine-stimulated dilation\textsuperscript{232}.  

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Vascular endothelium. All of the P1 receptors have been found in the endothelium from numerous vascular beds. However, $A_{2A}$ and $A_{2B}$ receptors appear to play a predominant role in endothelium-dependent dilator effects, which is mediated by endothelial release of NO\textsuperscript{209}. Interestingly, it seems that upon adenosine activation, different P1 receptors trigger distinct signaling pathways, which, however, eventually lead to the same destination - NO release and vasodilation. Specifically, Ray and colleagues found that $A_1$ receptor-initiated NO release requires extracellular Ca\textsuperscript{2+}, phospholipase $A_2$ (PLA\textsubscript{2}), and endothelial K\textsubscript{ATP} channel activation, whereas $A_{2A}$-facilitated NO release involves extracellular Ca\textsuperscript{2+} as well as small and intermediate conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (SK\textsubscript{Ca} and IK\textsubscript{Ca}) channels\textsuperscript{233}. The authors hypothesized two distinct mechanisms. First, they suggested that $A_1$ receptor-mediated K\textsubscript{ATP} activation results in K\textsuperscript{+} efflux, endothelium hyperpolarization, and increased driving force for Ca\textsuperscript{2+} influx. Increases in $[\text{Ca}^{2+}]_i$ stimulate PLA\textsubscript{2} to liberate arachidonic acid and stimulate cyclooxygenase to generate prostacyclin (PGI\textsubscript{2}), which acts on its endothelial receptors to evoke cAMP-PKA pathway that subsequently activates eNOS and produce NO\textsuperscript{233}. The second mechanism starts with activation of $A_{2A}$-coupled K\textsubscript{Ca} channels, which also facilitates Ca\textsuperscript{2+} entry into endothelial cells, thereby eventually activating eNOS and NO release\textsuperscript{233}. Though the mechanisms are still elusive, it is rather clear that $[\text{Ca}^{2+}]$ regulation in endothelial cells is crucial to P1 receptor-mediated NO release. In cerebral arteries, $A_{2A}$ receptors were found to mediate both hypoxia- and glutamate-induced vasodilation in the cerebral cortex\textsuperscript{234, 235}, thus contributing essentially to regulation of cerebral blood flow. Additionally, $A_1$ and $A_3$ receptor transcript messages have also been detected on endothelial cells in rat pial and intraparenchymal arterioles\textsuperscript{236}. It was reported by Lin et al.
that an A₁ receptor agonist effectively attenuated subarachnoid hemorrhage-induced vasospasm\textsuperscript{236}, pointing to A₁ receptors as potential therapeutic targets for hemorrhagic stroke and even other cerebral pathologies. In addition to vasodilation, it was also shown that A₃ receptors are involved in endothelium-dependent contraction of aorta\textsuperscript{237} based on the observation that A₃ receptor agonist failed to contract aorta from A₃ knockout mice, and that removing the endothelium abolished the contractile response\textsuperscript{237}. Since different COX-1 inhibitors showed common attenuating effects on A₃ receptor-mediate constriction, Ansari et al. revealed a novel mechanism that endothelial A₃ receptors induce aorta constriction through a COX-1 signaling pathway\textsuperscript{237}.

1.7 G protein-Coupled Receptors as Mechanosensors

The involvement of G protein-coupled receptors (GPCRs) in regulation of vascular tone has long been established. GPCRs comprise seven-transmembrane domains, and transduce the extracellular stimuli into cellular responses via intracellular signaling pathways. Typically, these mechanisms on a cellular level involve ligand binding to the GPCR, GPCR conformational change, activation of G protein (GDP-bound to GTP-bound) and Gα binding/activating downstream effectors. The intracellular signaling pathways are, to a large extent, dictated by the Gα subunit type, including Gαs, Gαq/11, Gα12/13, etc. In blood vessels, vascular tone is modulated by a variety of GPCRs that are present in smooth muscles and endothelial cells, and also in other cell types in the vicinity, such as neurons and astrocytes. It is widely accepted that GPCRs in vascular smooth muscle respond to neuronal and hormonal stimuli with contraction and relaxation of smooth muscle cells via the Gq protein (and G₁₂/₁₃ protein) signaling cascades or the Gs
pathway, respectively. For instance, the circulating hormone angiotensin II (Ang II) binds to Ang II (AT1) receptors and activates the G_4 protein-associated signaling pathways that involve activation of phospholipase C (PLC), diacylglycerol (DAG), protein kinase C (PKC) and inositol trisphosphate (IP_3), promotion of Ca^{2+} release from the sarcoplasmic reticulum (SR) and Ca^{2+} influx through voltage-dependent calcium channels (VDCCs), and ultimately vasoconstriction. On the other hand, the neurotransmitter epinephrine stimulates the G_s protein-coupled β2 receptors in arterial smooth muscle, which subsequently causes activation of adenylyl cyclase, cAMP and PKA, concomitant reduction of cytosolic Ca^{2+} level and smooth muscle relaxation.

It has already been suggested in this chapter that GPCRs are implicated in myogenic tone development; several downstream signaling effectors of GPCRs have been shown to participate in myogenic regulation. For example, phospholipase C (PLC) plays a key role in the genesis of myogenic responses via membrane depolarization and increased calcium influx^{238}. A contribution of DAG in pressure-evoked vasoconstriction has also reported^{239}. Additionally, PKC elicits substantial influence on various ion channels^{239}, like TRPM4^{20} and VDCCs^{240}, that are clearly involved in myogenic control. These observations all point to an important contribution of GPCRs to the modulation of myogenic contractility. Since GPCRs are ligand-stimulated, it was postulated that the mechanical stretch imposed on the vascular wall resulting from an increase in intraluminal pressure might evoke local release of vasoactive substance, including peptides, amines, lipids and nucleotides, which in turn activates corresponding receptors in smooth muscle in an autocrine or paracrine manner, and causes vasoconstriction^{241}. In accord with this hypothesis, Harder et al. reported that in cerebral arteries, the formation
of an arachidonic acid metabolite 20-hydroxycosatetraenoic acid (20-HETE) is positively associated with elevation of intravascular pressure\textsuperscript{242}. 20-HETE is a potent vasoconstrictor, which has been shown to activate both PKC-dependent pathways\textsuperscript{242} and Rho signaling cascades\textsuperscript{243}. Therefore, pressure-induced formation of 20-HETE from phospholipids significantly potentiates myogenic response and regulates cerebral blood flow\textsuperscript{242}. Other reports observe that myogenic tone of mouse mesenteric arteries is attenuated by a P2Y6 receptor inhibitor\textsuperscript{16}. The mechanism proposed by these authors is that P2Y6 receptors are activated by local UTP release from mesenteric arteries in response to pressure. This theory was corroborated by another laboratory. Kauffenstein et al. took advantage of the ectonucleotidase NTPDase1, a membrane-bound enzyme found abundantly expressed in vascular smooth muscle which hydrolyzes and inactivates extracellular nucleotides like UTP and UDP\textsuperscript{17}. They found that the mesenteric arteries from NTPDase knockout mice exhibited enhanced pressure-evoked constriction, consistent with greater local concentrations of endogenously released nucleotides\textsuperscript{17}. This effect was inhibited by a P2Y receptor antagonist, indicating that local release of nucleotides is essential for myogenic regulation via activating P2Y receptors in mesenteric arteries\textsuperscript{17}.

Recently, the classic perspective that GPCRs need to be stimulated by extracellular ligands has been challenged by evidence pointing to GPCRs as mechanosensors, which mediate myogenic response in an agonist-independent manner. It was first reported by Mederos Schnitzler et al. that angiotensin II AT1 receptors, along with several other \(G_q/11\)-coupled receptors, are mechanosensitive\textsuperscript{18}. In that study, they first demonstrated that TRPC6 channels are not directly activated by mechanical force.
based on the observation that neither hypotonicity nor negative pressures on the cell membrane activated TRPPC6 currents in TRPC6-transfected HEK cells. However, when co-expressing TRPC6 with AT1 receptors, membrane stretch could markedly activate TRPC6 currents. Interestingly, these currents could be prevented by pretreating the cells with inhibitors of AT1 receptor (losartan and candesartan), suggesting it is AT1 receptors rather than TRPC6 channels that are directly associated with pressure sensing. Similar results were obtained with several other Gq-coupled receptors, including H1 histamine and muscarinic receptors, but not with Gs-coupled receptors, such as β2 receptors. These experiments were conducted in an expression system, where theoretically endogenous agonists could not be synthetized or released. In other words, these Gq-coupled receptors were stimulated independently of their endogenous ligands. In A7r5 cells derived from a vascular source (embryonic rat thoracic aorta), mechano-activity of TRPC6 channels was similarly tightly coupled to AT1 receptor activity. To test the possibility that Ang II is stored in these cells and evoked the AT1 receptors in an autocrine fashion, the authors inactivated Ang II with a neutralizing antibody and found it had no significant influence on hypotonicity-induced TRPC6 activation, strongly indicating that local ligands are not involved in the mechano-stimulation mechanism. To further confirm the physiological relevance of their mechanosensitivity, the authors then switched to studying myogenic responses in isolated rat cerebral arteries. The AT1 receptor inhibitor losartan clearly inhibited cerebral artery myogenic vasoconstriction, suggesting that AT1 receptors are also important in sensing native smooth muscle membrane stretch. Moreover, the observation that the angiotensin-converting enzyme (ACE) inhibitor failed to affect the
losartan-sensitive myogenic activity provided definitive evidence that locally formed Ang II does not contribute to developing or maintaining myogenic tone\textsuperscript{18}.

Consistent with this study, recent work from Brayden et al. showed that purinergic receptors, particularly P2Y4 and P2Y6 receptors, contribute essentially to myogenic regulation in the cerebral microcirculation, which appears to be unrelated to local levels of nucleotides\textsuperscript{4}. The major evidence was that myogenic tone of intraparenchymal arterioles was reduced by selective molecular suppression on either receptor. In addition, P2Y receptor inhibitors also attenuated pressure-induced constrictions of these vessels. Interestingly, administration of an exogenous ectonucleotidase, which effectively abrogated UTP-induced constriction, failed to modulate myogenic response. Similarly, application of an endogenous ectonucleotidase inhibitor, which potentiated UTP-induced contraction, also elicited no influence on pressure-induced tone, strongly suggesting that P2Y receptors in the penetrating arterioles are activated by mechanical stimulation and not by autocrine/paracrine mechanisms\textsuperscript{4}. Since P2Y receptors are all G\textsubscript{q}-coupled receptors, this finding is in agreement with the previous discovery by Mederos Schnitzler et al.. It is rather surprising to see that neither Ang II nor AT1 receptor antagonist modulated PA contractility. The explanation for this apparent shift of functional mechanosensors from brain surface arteries to penetrating arterioles is unknown, but it may in part be the unique environment where parenchymal arterioles are encased by neurons and astrocytes, and are intrinsically innervated\textsuperscript{2}. It may also be attributed to different receptor profiles\textsuperscript{5}, distinct Ca\textsuperscript{2+} signaling\textsuperscript{6}, and diverse intracellular mechanisms in regulation of vascular tone. Chapter 3
of the dissertation will concentrate on an intracellular signaling mechanism that appears rather unique to the microvasculature.

Extensive structural analysis on different GPCRs has established that ligand binding to GPCRs shifts the conformation of the receptors to an active state, which subsequently activates G protein signaling. Yet how GPCRs are activated mechanically in the absence of agonists is essentially unknown. In order for a membrane protein to be recognized as a mechanosensor, a few criteria should be strictly followed\textsuperscript{243}. Firstly, the kinetics between the mechanical stimulation and a cellular response should be fast enough and comparable to already established ligand activation. Secondly, the extent of response should be closely related to the amplitude of mechanical stimulation. In short, bigger mechanical force should be able to evoke greater biological response in the same amount of time. Third, a conformational change of the protein should be detected upon machano-activation. Fourth, mechano-stimulation of the protein should be able to be attenuated by protein inhibitors or inverse agonists. Finally, since mechanosensitivity is an intrinsic property of the protein, then purified protein inserted into an expression system should still be activated by stretch\textsuperscript{244}. Judging by these criteria, G\textsubscript{q}-coupled receptors, as defined by Mederos y Schnitzler et al., are mechanosensitive. This group went one step further in terms of understanding the mechanism of mechano-activation and demonstrated that membrane stretch allows the receptor to shift to an active conformation, which effectively recruit β-arrestin-2 with very similar intensity as compared to agonist-induced conformational change and β-arrestin-2 binding. This finding suggests stretch does result in an activated conformation, which is sensitive to receptor antagonists\textsuperscript{18}. However, whether or not ligand-activated and stretch-initiated
conformational changes are the same has to be resolved by analysis of crystal structures. Currently, there are two separate hypotheses regarding how GPCRs perceive membrane stretch\textsuperscript{244}. The first one is a “tethered” theory that postulates a molecular spring anchoring to GPCR. Membrane stretch would dislocate the spring and force a conformational modification of the GPCR. The second one focuses on the altered lateral pressure profile, which translates membrane stretch into conformation changes of GPCRs. Unfortunately, the precise mechanistic model is still elusive. Clearly, a combination of molecular study, structural analysis and biophysical investigation will be required to clarify this novel mechanism.

1.8 RhoA/ROCK signaling

Recently, along with emerging evidence that inhibition of ROCK effectively attenuates agonist- and pressure-induced vasoconstriction, the physiological and pathological significance of smooth muscle ROCK and Ca\textsuperscript{2+}-sensitization mechanisms has begun to receive more attention.

1.8.1 RhoA/ROCK signaling in health and disease

\textit{Physiological roles of RhoA/ROCK signaling pathway.} With a cranial window approach to monitor vascular diameter of anesthetized rats, two ROCK inhibitors (Y-27632 and HA1077) caused marked concentration-dependent dilations of basilar arteries\textsuperscript{22}, pointing to a key role of ROCK to cerebral artery tone in vivo. Later, several studies showed Rho mediates different vasoactive agent-initiated contractile responses. For instance, vasoconstriction induced by endothelin-1 (ET-1) has been found to involve
Rho signaling in various beds. In rabbit basilar arteries, ET-1 enhanced RhoA activity and stimulated Y-27632-sensitive contraction. Rat internal pudendal and clitoral arteries are both sensitive to ET-1, which also signals through ROCK. In porcine retinal arterioles, the ROCK inhibitor H-1152 effectively abolished ET-1-induced vasoconstriction. Another potent ROCK inhibitor SAR407899 reduces constriction of rat renal arteries to ET-1. Other Rho-mediated vasoconstrictor responses have also been investigated. Briefly, serotonin triggers constriction in bovine middle cerebral arteries and cerebral arteries, both of which are sensitive to Rho inhibition. ROCK signaling also plays an important role in eliciting UTP-induced constriction in cerebral arteries. In addition to these receptor ligands, vascular tone is also greatly influenced by changes in intraluminal pressure, and ROCK is tonically active to regulate this influence in several vascular beds. In cerebral arteries, Y-27632 and HA 1077 were reported to suppress pressure-induced tone in a Ca\(^{2+}\)-independent fashion, supporting a significant role for RhoA/ROCK pathway in cerebral artery stretch-activated constriction mechanisms that augment smooth muscle calcium sensitivity. ROCK-regulated myogenic activity was also observed in rat tail small arteries and rabbit facial veins, both of which dilated when exposed to Y-27632.

**Pathological roles of RhoA/ROCK signaling pathway.** Along with increased focus on the physiological importance in vascular smooth muscle, ROCK is also receiving progressively more recognition in various cardiovascular diseases and as a therapeutic target. It is not surprising that this signaling pathway and subsequent hyper-vasoconstriction has been rigorously studied in human and animal models of hypertension, cerebral and coronary vasospasm, and subarachnoid hemorrhage. In three
different models of hypertension (spontaneous hypertensive rats, renal hypertensive rats and deoxycorticosterone acetate (DOCA)-salt hypertensive rats), intravenous administration of Y-27632 effectively lowered blood pressure\textsuperscript{255}, suggesting Rho/ROCK signaling contributes to blood pressure regulation \textit{in vivo} and that it is augmented in hypertension. Other evidence include that vascular ROCK mRNA levels are elevated in the spontaneously hypertensive rat model\textsuperscript{256}, and levels and activity of GTP-RhoA are increased in aortic smooth muscle from angiotensin II-induced hypertensive rats, spontaneously hypertensive rats, and L-NAME-treated rats\textsuperscript{257, 258}. Vasodilator responses of basilar arteries to Y-27632 were observed in vivo in spontaneous hypertensive rats, and significantly augmented in normotensive rats that were pre-exposed to L-NAME\textsuperscript{22}, indicating that ROCK activity is abnormally elevated in hypertension and cerebral circulation may be largely affected by this pathological alteration. In agreement with this proposal, isolated cerebral arteries from hypertensive rats were significantly more sensitive to the dilating effects of Y-27632 and HA-1077 than those from normotensive rats\textsuperscript{252}, which, again, emphasizes on the elevated contribution of ROCK to the cerebral myogenic reactivity in hypertension.

In the context of Rho in the cerebral circulation, several studies have reported its involvement in cerebral vasospasm after subarachnoid hemorrhage (SAH). Increased RhoA and ROCK mRNA levels were observed in rat basilar arteries of a rat double cisternal blood-injection SAH model\textsuperscript{259}. Elevated ROCK and myosin light chain phosphorylation were also found in canine basilar arteries following SAH\textsuperscript{260}. Further, enhanced ROCK activity was found to account for augmented contractile responses of cerebral arteries to serotonin after experimental SAH\textsuperscript{261}. In SAH patients, a potent ROCK
inhibitor fasudil hydrochloride exhibited promising clinical results with alleviated cerebral vasospasm\textsuperscript{262}. Relaxation of cerebral vasospasm by fasudil is accompanied by a decrease in myosin light chain phosphorylation, consistent with ROCK inhibition\textsuperscript{263}, and it is now clinically used in Japan and China for treating this symptom\textsuperscript{264}.

\textbf{1.8.2 RhoA/ROCK signaling mechanisms}

\textit{Ca\textsuperscript{2+} sensitization.} Rho-mediated smooth muscle contraction happens primarily through increasing the calcium sensitivity of the contractile apparatus, whereby myosin light chain (MLC) can be highly activated even with much lower levels of intracellular [Ca\textsuperscript{2+}]. It was initially accepted that, similar to striated muscle contraction, the force of smooth muscle contraction is exclusively dictated by the levels of cytosolic Ca\textsuperscript{2+}. Elevation of [Ca\textsuperscript{2+}]\textsubscript{i} enhances the binding of Ca\textsuperscript{2+} to calmodulin (CaM), which subsequently stimulates myosin light chain kinase (MLCK) to phosphorylate MLC\textsuperscript{265}. It was therefore widely accepted that the degree of MLC phosphorylation, tightly regulated by cytosolic Ca\textsuperscript{2+}, is the essential factor that determines the extent to which smooth muscle contracts. However, several earlier studies with Ca\textsuperscript{2+} indicators observed that the [Ca\textsuperscript{2+}]\textsubscript{i} does not always parallel to the degree of MLC phosphorylation and constriction. Constrictions induced by a variety of agonists are higher than depolarization (high K\textsuperscript{+})-induced contraction, even with lower [Ca\textsuperscript{2+}], a process called Ca\textsuperscript{2+} sensitization\textsuperscript{266, 267}. It was later revealed that the calcium sensitivity of the contractile apparatus of smooth muscle, i.e. the levels of MLC phosphorylation, is regulated not only by MLCK but also by myosin light chain phosphatase (MLCP), which dephosphorylates and inactivates MLC independent of [Ca\textsuperscript{2+}]\textsubscript{i}. Subsequent studies revealed that MLCP is physiologically
inhibited by the intracellular Rho signaling, which promotes contraction that is uncoupled to the changes in cytosolic Ca^{2+} levels^{268}.

Stimulation of the small GTPase Rho following activation of certain heterotrimeric G protein coupled receptors (GPCRs) or receptor tyrosine kinases, triggers the Ca^{2+} sensitization mechanism. Similar to other GTP binding proteins, the small GTPase Rho shows binding affinity for both GDP and GTP, and exhibits intrinsic GTPase activity. Rho continuously goes through the cycle of inactivated state (GDP-Rho) and active state (GTP-Rho), which is tightly regulated by various proteins. In myocytes under resting conditions, the inactive Rho GDP dissociation inhibitor (Rho GDI) binds to inactive GDP-Rho and stabilizes GDP-Rho in the cytosol. Activation of receptors leads, through activity of guanine nucleotide exchange factor (GEFs), to the GTP-GDP exchange reaction, whereby inactive GDP bound Rho is converted to active GTP-Rho. Rho GDI dissociates and RhoA-GTP is then targeted to the cell membrane through its C-terminal geranyl-geranylated tail and interacts its downstream effectors, one of which is ROCK. ROCK activation, in turn, phosphorylates the myosin phosphatase target subunit (MYPT-1), the regulatory subunit of MLCP, and inhibits its activity. Suppressed MLCP activity leads to reduced dephosphorylation of MLC, resulting in increased MLC activity and promoting smooth muscle contraction^{255, 268}. Active Rho (GTP-Rho) is terminated by intrinsic and GTPase-activating proteins (GAPs)-induced GTPase activity of Rho and converted to inactive GDP-Rho.

In addition to inhibiting MLCP, ROCK also directly phosphorylates MLC and activates myosin ATPase. Amano et al. were the first to report that isolated MLC is phosphorylated by ROCK in a cell-free system. In the same study, phosphorylation sites
on MLC by ROCK were investigated. It was found that the MLC kinase preferentially phosphorylates MLC at Ser-19, and surprisingly, ROCK phosphorylates MLC mainly at the exact same site and activates its MgATPase activity of myosin in a GTP•Rho-dependent manner as an added mechanism to facilitate Ca\(^{2+}\)-sensitization and smooth muscle contraction\(^{269}\).

*Ion channel regulation.* Though Ca\(^{2+}\) sensitization accounts primarily for Rho/ROCK-mediated regulation of vasomotor activity, emerging evidence suggests that RhoA and ROCK can regulate smooth muscle contraction by controlling the activity of ion channels, mostly potassium channels.

Several groups have reported that RhoA/ROCK-signaling regulates the activity and expression of voltage-dependent delayed rectifier potassium (K\(_v\)) channels in different systems. With co-immunoprecipitation, Cachero et al. were the first to show close association between RhoA and K\(_v\)1.2 in a native mammalian system. Overexpression of RhoA markedly reduced K\(_v\)1.2 basal currents in expression systems. Additionally, in HEK cells, M1 muscarinic receptor-initiated suppression of K\(_v\)1.2 channel activity was prevented when RhoA was inhibited by the C3 exoenzyme, strongly suggesting that RhoA links the signal transduction from GPCRs to ion channels\(^{270}\). Later, the mechanisms through which RhoA suppresses K\(_v\)1.2 channels were investigated by Stirling et al.\(^{271}\). They found that treating HEK-K cells with increasing concentrations of C3 exoenzyme (RhoA inhibitor) produced a concentration-dependent increase in steady-state K\(_v\)1.2 levels at the cell surface. In addition, inhibition of RhoA showed a decrease in steady-state channel endocytosis. Also, activation of RhoA by stimulation of endogenous LPA receptors triggered K\(_v\)1.2 channel endocytosis, which was reversed by ROCK
inhibitor Y27632, strongly indicating that the level of Kv1.2 expression is modulated by RhoA/ROCK-dependent steady-state channel endocytosis. Further exploration of this mechanism revealed that LPA receptor activation elicits clathrin-dependent Kv1.2 endocytosis and consequent attenuation of Kv1.2 currents. Furthermore, a dual role of ROCK was discovered as it inhibits the recycling of endocytosed channel back to the plasma membrane, which further ensures the reduction of Kv1.2 channel expression on the cell surface271. Studies of ROCK and delayed rectifier potassium channels were not limited to expression systems. Luykenaar et al. reported that, in rat cerebral arterial smooth muscle, two ROCK inhibitors (Y27632 and H1152) not only abolished the attenuating effects of uridine triphosphate (UTP) on Kv channel activity, but also suppressed UTP-induced vasoconstriction28. This group further deciphered the mechanism by which Rho/ROCK inhibits Kv channels, and found that ROCK inhibition prevents UTP-induced actin polymerization, and that disruption of actin cytoskeleton rescues UTP-induced suppression of Kv channel activity. In addition, both ROCK inhibition and cytoskeleton disruption suppress UTP-induced depolarization and constriction of cerebral arteries. Together, these observations indicate that UTP initiates ROCK-mediated remodeling of the actin cytoskeleton and consequently suppresses the Kv currents, thereby facilitating depolarization and constriction of cerebral arteries29. This important finding provides physiological and mechanistic evidence that Rho-associated constriction happens partially through regulation of membrane potential and presumably intracellular [Ca2+].

Inward rectifying potassium channels (Kir2.1-2.3), which are also important regulators of membrane potential and cell excitability, are also negatively regulated by
RhoA. In HEK cells, cotransfection with the activated mutant of the Rho reduced Kir current density, while C3 exoenzyme (RhoA inhibitor) attenuated M1 muscarinic receptor-induced reduction of Kir2.1-Kir2.3 activity\textsuperscript{26, 27}. Interestingly, this effect does not appear to be mediated by ROCK since Y27632 did not block the inhibitory effects of carbachol on Kir2.1\textsuperscript{30}. Another ROCK-regulated potassium channel, the TASK1 (K\textsubscript{2P}3.1) two-pore-domain K\textsuperscript{+} channel, contributes to resting membrane potential in human pulmonary artery smooth muscle cells. Endothelin-1 inhibited TASK1-mediated I\textsubscript{KN} currents in human pulmonary artery myocytes, which was reversed by Y27632. Mutation of either one of the putative ROCK phosphorylation sites (Ser\textsuperscript{336} and Ser\textsuperscript{393}) on TASK1 essentially reversed the inhibition of endothelin-1 on the channel, revealing a mechanism whereby regulation of vascular TASK1 currents by endothelin-1 is mediated by activation of ROCK and direct channel phosphorylation\textsuperscript{272}.

Rho signaling has also been shown to regulate other ion channel activities. Staruschenko et al. reported that co-expression of constitutively active RhoA and epithelial Na\textsuperscript{+} channels (ENaC) markedly increased ENaC activity. Pretreating the cells overexpressing ENaC and RhoA with Y27632 significantly decreased RhoA-dependent ENaC activity. Further investigation into this mechanism revealed that phosphatidylinositol 4,5-bisphosphate (PIP2) is necessary for RhoA/ROCK-dependent ENaC activation\textsuperscript{25}. Furthermore, it was reported that T-type calcium channels are regulated by Rho signaling as well. Lysophosphatidic acid (LPA) significantly inhibits the peak current amplitudes of expressed rat Ca\textsubscript{v}3.1 and Ca\textsubscript{v}3.3 channels without affecting the voltage dependence of activation or inactivation. This inhibitory effect was sensitive to ROCK inhibitors (fasudil and Y27362) as well as RhoA inhibition (C3 exoenzyme),
indicating LPA-mediated activation of Rho leads to ROCK-mediated suppression of Ca\textsubscript{v}3.1 channels\textsuperscript{273}.

Together these studies indicate that in addition to Ca\textsuperscript{2+} sensitization, the RhoA/ROCK signaling pathway also regulates ion channel activity and can control smooth muscle contractility. The influence of this signaling on TRPM4 channels and its participation in arteriolar myogenic depolarization and constriction were the topic of studies described in chapter three of this dissertation.

**Stress fiber formation.** Additional mechanisms by which Rho regulates smooth muscle contractility have been revealed. There is substantial evidence to support the notion that smooth muscle contraction and relaxation involve actin polymerization and depolymerization, respectively. For example, decreased G-actin content, or a rise in F- to G- actin ratio, consistent with the utilization of G-actin and increased polymerization, were observed in blood vessels exposed to vasoconstrictors, osmotic challenge, or increased intravascular pressure. It was also shown that disruption of actin filament pharmacologically would significantly affect force generation\textsuperscript{274}. Several studies have shown that ROCK regulates actin dynamic, and thus modulates actin-myosin interactions. For instance, LIM kinase 1 and 2 are phosphorylated by ROCK at Thr508 and Thr505, respectively, resulting in increased cofilin phosphorylation at Ser3. Cofilin is an actin-depolymerizing factor and regulates actin dynamics, and its activity is attenuated by phosphorylation\textsuperscript{275}. Therefore, through inhibition of cofilin and stabilization of actin filaments, ROCK further contributes to regulation of smooth muscle contractility. Moreno-Dominguez et al. have assessed the contribution of dynamic reorganization of the actin cytoskeleton and thin filament regulation to the myogenic response and
serotonin-induced constriction of pressurized rat middle cerebral arteries. They reported that the ROCK inhibitor (H1152) significantly increased the level of G-actin and caused a loss of myogenic tone. Additionally, the involvement of cofilin, which was previously identified as a mediator of ROCK-dependent control of actin dynamics, in myogenic responses has been studied. The level of cofilin-S3 phosphorylation was substantially elevated by an increase in pressure, which was effectively inhibited by H1152, indicating that dynamic reorganization of the cytoskeleton involving increased actin polymerization in response to ROCK/cofilin contributes significantly to force generation in myogenic constriction of cerebral resistance arteries\textsuperscript{274}.

**1.8.3 RhoA structure, regulation and substrates**

At least ten members of the Rho family of GTPases are present in the mammals: Rho (isoforms A-E, and G), Rac (isoforms 1 and 2), Cdc42 and TC10, where Rho, Rac1 and Cdc42 have been most intensively characterized. Among them, RhoA, RhoB and RhoC share the same amino acid sequence and very similar cellular functions. Therefore, most of the studies on Rho were based on the investigation of the function and regulation of RhoA\textsuperscript{268}.

*RhoA structure.* As summarized by Huret and colleagues, Rho-GTP-binding proteins are comprised of an effector domain, four separate guanosine phosphate binding regions that span the length of the core structure, a hypervariable region and a CAAX box motif (C: cysteine, A: aliphatic residue; X: any amino acid). The effector domain (residues 26-45) changes conformation between the GTP bound and GDP bound states. All Rho proteins possess conserved residues at Gly14, Thr19, Phe30 and Gln93, which
participate in binding, stabilization or regulation of GTP hydrolysis. The N-terminus region also contains switch 1 (residues 27-40) and switch 2 (residues 59-78) regions that change conformation between GTP-bound and GDP-bound states, and may mediate changes in effector region necessary for binding to downstream targets. The hypervariable region of Rho is made up of residues 173-189, and is the region with most diversity between individual Rho family members. The C-terminus of RhoA is essential for correct localization of the protein. It is post-translationally modified by prenylation of a conserved C-terminal cysteine and followed by methylation and proteolytic removal of the last three amino acids. These are essential processes as the prenyl group anchors RhoA into membrane and facilitates its stability, cell growth, transformation and cytoskeletal organization.

**RhoA regulation.** Rho GTPases can be regulated intrinsically and extrinsically. They behave as molecular switches that shuffle between active (GTP-bound) state and inactive (GDP-bound) state. This cycle is under the control of two regulatory proteins: guanine nucleotide-exchange factors (GEFs), which catalyze the exchange of GDP for GTP to activate Rho GTPases, and GTPase-activating proteins (GAPs), which potentiate the intrinsic GTPase activity and accelerate the inactivation of Rho by hydrolyzing GTP to GDP. Moreover, in resting cells, RhoA can be sequestered in the cytoplasm by guanine nucleotide-dissociation inhibitors (GDIs), which interact with prenylated GDP-bound Rho, allowing for translocation of Rho GTPases between membrane and cytosol.

It is well characterized that RhoA is stimulated by various agonists through coupling to heterotrimeric G proteins. Specifically, \(G_{\alpha12}\) and \(G_{\alpha13}\) are involved in numerous Rho-associated responses, such as stress fiber formation and cytoskeletal
alterations. Agonists initially stimulate G-protein coupled receptor (GPCRs) on the plasma membrane, which in turn activate \( G_{\alpha12/13} \) proteins and subsequent Rho signaling. The GPCRs that have been shown to initiate Rho signaling include \( \alpha \)-adrenergic, muscarinic, prostanoid, purinergic, endothelin, thrombin, vasopressin, oxytoncin, epidermal growth factor and angiotensin receptors. Most of these receptors are also coupled to the \( G_q \) protein and activate phospholipase C (PLC)-modulated responses.

RhoA GTPases are not only regulated by endogenous stimulation, but also affected by bacterial toxins and exoenzymes that have been proved valuable for experimental investigations. The C3-exoenzyme and the *Escherichia coli* protein ENIN are the most widely used RhoA inhibitors. They are ADP-ribose transferases that ADP-ribosylate the Asn-41 residue of Rho, thus inactivating RhoA and downstream signaling. The C3-exoenzyme is highly selective for RhoA without any effect on Rac or Cdc42, leading to its extensive experimental application. A less specific inhibitor, *Clostridium difficile* toxin B, is also used in several studies. Its limited selectivity lies in that it glycosylates not only Thr-37 of RhoA, but also the counterparts (Thr-35) of Rac and Cdc42. Toxin B inhibits GTPases by glycosylating and blocking the residues that contribute to \( Mg^{2+} \) coordination, and thereby preventing the GTP/GDP exchange necessary for activation. Interestingly, a RhoA activator CN03 was recently developed in order for better understandings of the Rho pathways. It was developed based on the cytotoxic necrotizing factor 1 (CNF-1), which has been shown to activate Rho, Rac, and Cdc42 by deamidating Gln-63 to Glu-63, which significantly inhibits the intrinsic and GAP-activated GTPase activity of Rho, leaving these proteins constitutively active.
Based on CNF-1, CN03 contains a covalently attached cell penetrating moiety, allowing for cell penetration within 2-4 hours in the culture media. The targeted action on RhoA with CN03 makes it a more attractive tool for the study of Rho GTPase signaling than the classic indirect activators, like LPA.

1.8.4 ROCK structure, expression, regulation and substrates

**ROCK structure.** ROCK is a serine/threonine kinase with a molecular mass of about 160 kDa, and it is a major downstream effector of the small GTPase RhoA. It is expressed in invertebrates (C elegans, Drosophila and mosquito) and in vertebrates (zebrafish, Xenopus, chicken, mouse, rat and human). ROCK consists of an amino-terminal kinase domain that is followed by a potential coiled-coil forming region and other functional motifs at the carboxyl terminus. These motifs include a Rho-binding domain, and Pleckstrin homology (PH) domain, which has an internal cysteine-rich domain. These carboxy-terminal domains constitute an autoinhibitory region that reduces the kinase activity of ROCKs. The Rho-binding domain (RBD) is located in the predicted coiled-coil region of ROCK, and it exhibits sequence homology to the Rho-interaction domain of kinectin, which is a protein that regulates microtubule-based organelle motility. The coiled-coil region is thought to interact with other α-helical proteins, whereas the PH domain in the carboxyl terminus might participate in protein localization.

**ROCK expression.** There are two isoforms of ROCK encoded by two different genes have been identified: ROCK-1, also known as ROCK I, P160-ROCK, or ROKβ) and ROCK-2 (ROCK II or ROKα). Human ROCK-1 and ROCK-2 genes locate on
chromosome 18 (18q11.1) and chromosome 2 (2p24), respectively\textsuperscript{294}. They are highly homologous, with an overall amino acid sequence identity of 65\%. Identity in the RBD region is 58\% and approaches 92\% in the kinase domain\textsuperscript{295}. ROCK-1 and ROCK-2 are ubiquitously expressed in mouse and rat tissues, with a preferential expression of ROCK-2 in the brain and skeletal muscle, suggesting the protein might have a specialized role in these cell types. In terms of the cardiovascular system, both of the kinases are found in the heart and vascular smooth muscle. Additional studies on the localization of ROCK-2 using immunofluorescence techniques, show that ROCK-2 is primarily localized to the cytoplasm\textsuperscript{296}. Another study supported this finding with cell-fractionation studies, which indicated that the main proportion of ROCK-2 is located in cytosol, whereas only a small proportion of ROCK-2 is found in the membrane fraction\textsuperscript{297}. When active RhoA is overexpressed, ROCK-2 translocates from the cell cytoplasm to plasma membrane\textsuperscript{297}. Moreover, some immunostaining of ROCK-2 is detected at the cell periphery and membrane of growing cells\textsuperscript{298}.

**ROCK regulation.** ROCK is endogenously activated/regulated by RhoA in an activated and GTP-bound form, thereby ROCK kinase activity is enhanced. Additionally, it was demonstrated by Feng et al. that ROCK can be markedly stimulated by lipids, including arachidonic acid and some acidic phospholipids\textsuperscript{299}. Protein oligomerization might also regulate ROCK activity, presumably through amino-terminal transphosphorylation\textsuperscript{300}. A recent study pointed out that ROCK is negatively regulated by other small GTP-binding proteins, including Gem and Rad\textsuperscript{301}. Recently, another small GTPase, RhoE, was identified as an inhibitor of ROCK-1. It binds to the amino-terminal section of ROCK-1 and presumably physically interfere with the kinase activity\textsuperscript{302}.
Interestingly, Gem and Rad associate with the cytoskeleton and RhoE relates with the Golgi, the proteins could therefore function at specific intracellular subdomains to modulate ROCK-mediated processes\(^{302}\).

In order to manipulate ROCK activity for experimental purposes, several ROCK inhibitors were developed that show a relatively high degree of specificity over the past few years. The most widely used is Y-27632, which is very useful in assessing the cellular and functional roles of ROCK. Y-27632 has a very high selectivity for ROCK over other protein kinases, with an inhibitory constant (\(K_i\)) at 0.14 µmol/L and 26 µmol/L for PKC. An important feature of Y-27632 is that, though it competes with ATP for binding, it is highly effective in cells even with high ATP levels\(^{303}\). Other ROCK inhibitors include HA-1077 and another pyridine derivative, Wf-536. These compounds are non-isoform-selective ROCK inhibitors that target the ATP-dependent kinase domains and are therefore equipotent in terms of suppressing both ROCK-1 and ROCK-2\(^ {304}\). And because of the essential contributions of ROCK to various cardiovascular pathologies and inflammatory conditions, these compounds have gained favorable interest from the pharmaceutical industry for novel drug development. And excitingly, the active metabolite of HA-1077, hydroxyfasudil, has been approved in Japan and China for treating cerebral vasospasm after SAH. Animal studies of numerous cardiovascular disease models have shown that ROCK inhibitors are beneficial for diseases including atherosclerosis and arterial remodeling following vascular injury\(^{305}\), angiogenesis\(^{306}\), cerebral ischemia\(^{307}\), erectile dysfunction\(^{308}\), myocardial ischemia-reperfusion injury\(^{309}\), etc. A most recent inhibitor is H-1152P, which has a \(K_i\) of 0.012 µmol/L for ROCK-2, 3.03 µmol/L for PKA, and 5.68 µmol/L for PKC. It is a more potent inhibitor than either
Y-27632 or HA-1077. Its effectiveness has been rigorously tested by large numbers of studies examining the roles of ROCK in vascular reactivity, stress fiber assembly and neurite extension.

**ROCK substrates.** ROCK mediates a broad range of cellular responses. For instance, it regulates cell contractility and actin cytoskeleton by phosphorylating a variety of proteins, such as MLCP, LIM kinases, adducin, and ezrin-radixin-moesin (ERM) proteins. These proteins are also phosphorylated by other serine/threonine kinases including PKA, PKC and PKG. The consensus amino acid sequences for phosphorylation are R/KXS/T or R/KXXS/T (R: arginine, K: lysine, X: any amino acid, S: serine, T: threonine). ROCK-1 and ROCK-2 share the majority of the amino acid sequence in the kinase domain. Though substrate studies were conducted focusing on individual ROCK isoforms, for example, ROCK-2 on MLCP and ROCK-1 on LIMK, thus far there is no direct evidence that they phosphorylate different substrates. ROCK directly phosphorylates MLC at Ser-19, the same phosphorylation site for MLCK, and activates MLC. The direct excitatory effects on MLC may be physiologically significant as it largely enhances smooth muscle contractility independent of changes in intracellular [Ca\(^{2+}\)]. Additionally, the phosphorylation and inhibition of MLCP by ROCK are very well established. The MLCP consists of three subunits: a catalytic subunit, a myosin phosphatase target subunit (MYPT), and a small non-catalytic subunit of unknown functions. Phosphorylation of residues in the carboxyl-terminal of MYPT results in inhibition of MLCP activity and a concomitant increase in phosphorylation of MLC and cell contraction. ROCK-2 phosphorylates MYPT at Thr697, Ser854 and Thr855. Furthermore, another major type of substrates for ROCK is LIM kinases. LIM kinases 1
and 2 are serine/threonine kinases that are involved in the regulation of actin-filament dynamics. ROCK I phosphorylates LIMK1 at Thr508 and LIMK2 at Thr505, and potentiates the ability of LIMKs to phosphorylate cofilin, which in turn promotes actin assembly and polymerization.

1.9 Protein Kinase C and Myogenic Tone

Protein kinase C, also known as PKC, is a family of protein kinase enzymes that are involved in regulating the function of other proteins through phosphorylation. PKC plays important roles in \( G_q \) protein-initiated signal transduction. PKC has been found in numerous cell types and mediates cell-type specific effects. Expression of PKC in smooth muscle cells from arteries, bronchi, urinary bladder, uterus and the gastrointestinal tract is uniformly associated with smooth muscle contraction, indicating that PKC positively regulates the excitation-contraction mechanism. This section briefly summarizes the functional involvement and signaling mechanisms of PKC in myogenic vasoconstriction. The crucial roles of PKC in myogenic tone development have been established by earlier studies based on the evidence that pharmacological inhibition of PKC significantly reduces myogenic response, and that PKC activators potentiate pressure-induced contraction. These observations were made in different types of vessels from different species, including rat cerebral arteries, rat cremaster arteries, rat skeletal muscle arterioles, rat coronary arteries, human coronary arterioles and human subcutaneous resistance arteries. Following from these reports, mechanisms of PKC-mediated mechanoactivation have been heavily investigated. Primarily, PKC-modulated proteins in vascular smooth muscle can be primary divided into two groups, including ion...
channels that are essential for regulation of membrane potential and intracellular $[\text{Ca}^{2+}]$, and the contractile apparatus that is not dependent on increased cytosolic $\text{Ca}^{2+}$ levels.

1.9.1 PKC regulates membrane potential

Several studies have reported that modulation of PKC activity is associated with changes in smooth muscle membrane potential and cytosolic $[\text{Ca}^{2+}]$. In cerebral arteries, the PKC activator PMA depolarized smooth muscle cells by $\sim 15$ mV and concomitantly caused robust vasoconstriction$^{20}$. In support of this finding, the PKC inhibitor chelerythrine reduced myocyte $[\text{Ca}^{2+}]_i$ in pressurized porcine coronary arteries, which was, not surprisingly, coupled to attenuated vasoconstriction$^{320}$. Increased cellular $\text{Ca}^{2+}$ leads to activation of calmodulin and subsequent myosin light chain kinase activity, which gives rise to greater myosin-actin interaction and smooth muscle contraction. Therefore, regulation of membrane potential and $[\text{Ca}^{2+}]_i$ is a fundamental process underlying PKC-initiated responses. Several ion channels that are centrally implicated in modulation of smooth muscle membrane potential are shown to be regulated by PKC.

*TRPM4 channels.* Regulation of TRPM4 by PKC was first reported by Nilius et al.. They found that phosphorylation of TRPM4 by PKC not only markedly enhanced the activation of TRPM4 currents by $\text{Ca}^{2+}$ but also elevated the whole-cell current density recorded in HEK cells expressing TRPM4 channels$^{174}$. Similarly, using native cerebral arterial myocytes, Earley et al. showed that TRPM4 currents were substantially elevated by the PKC activator PMA, confirming an excitatory influence of PKC on TRPM4 activity$^{20}$. Moreover, application of PMA increased TRPM4 $\text{Ca}^{2+}$ sensitivity by way of decreasing the EC$_{50}$ of $[\text{Ca}^{2+}]_i$ for TRPM4 activation$^{20}$. Further studies from this group
demonstrated that a particular isoform of PKC (PKCδ) is involved in maintaining localization of TRPM4 protein at the plasma membrane\textsuperscript{321}. It was concluded from these observations that either inhibiting PKCδ pharmacologically or molecularly largely attenuated TRPM4 distribution on the membrane as well as whole-cell TRPM4 currents\textsuperscript{321}. As TRPM4 is an important depolarizing contributor in cerebral arterial\textsuperscript{19} and arteriolar\textsuperscript{34} smooth muscle, PKC, by activating TRPM4 channels, effectively augments myogenic Ca\textsuperscript{2+} entry through VDCCs and subsequent pressure-induced vasoconstriction.

**BK\textsubscript{Ca} channels.** BK\textsubscript{Ca} channels are important regulators of membrane potential. During myogenic tone development, depolarization-mediated BK\textsubscript{Ca} channel opening and K\textsuperscript{+} efflux function as negative feedback mechanisms to reduce the degree of excitation by pressure\textsuperscript{50}. As PKC is tightly coupled to vasoconstriction, it is therefore not surprising to find that PKC phosphorylation inhibits BK\textsubscript{Ca} channels in vascular smooth muscle\textsuperscript{322-324}. Subsequent molecular studies focusing on the phosphorylation sites on BK\textsubscript{Ca} channels revealed a dynamic regulation of the channel by PKC activity. In this study, phosphorylation of Ser695 inhibited both basal BK\textsubscript{Ca} channel open-state probability and stimulatory effects of PKG and PKA on BK\textsubscript{Ca} channels\textsuperscript{325}. BK\textsubscript{Ca} channels contribute not only to regulation of myogenic tone\textsuperscript{50} but also to modulating vasoactive substance-induced contraction\textsuperscript{64,65}, and it is not surprising that modulation of BK\textsubscript{Ca} activity by PKC during agonist-evoked vasoconstriction has been documented. As one example, Kunduri and coworkers reported that activation of adenosine (A\textsubscript{1}) receptor inhibits BK\textsubscript{Ca} activity in a PKC\textsubscript{α}-dependent manner in aortic smooth muscle\textsuperscript{326}. Thus, by reducing BK\textsubscript{Ca} channel open probability, PKC potentiates vascular smooth muscle depolarization and vasoconstriction induced by various excitatory stimuli.
**K<sub>ir</sub> channels.** Smooth muscle K<sub>ir</sub> channels are critically involved in K<sup>+</sup>-induced membrane hyperpolarization and vasodilation. This is a pivotal regulatory mechanism for vascular reactivity mediated by endothelium-derived hyperpolarizing factor (EDHF) and neuronal activation. Inhibition of K<sub>ir</sub> channels reduces K<sub>ir</sub>-mediated K<sup>+</sup> efflux and smooth muscle hyperpolarization. It has been shown by several studies that PKC can evoke a vasoconstrictor response by inhibiting K<sub>ir</sub> channels. This was first observed by Chrisoobolis et al. that K<sup>+</sup> (5 to 15 mmol/L)-elicited vasodilation of rat basilar arteries was inhibited by PKC activation and augmented by PKC inhibition, indicating a suppressive influence of PKC on K<sup>+</sup>-induced responses.<sup>327</sup> Further, Park and colleagues published two consecutive studies on rabbit coronary arteries to elucidate the contribution of PKC in vasoconstrictor-triggered inhibition of K<sub>ir</sub> channels.<sup>328</sup>,<sup>329</sup> They reported that both endothelin-1 (ET-1) and Ang II markedly inhibited K<sub>ir</sub> currents, and that pretreating cells with PKC inhibitors prevented this response. PKC stimulation reduced K<sub>ir</sub> activity in this study, further confirming the inhibitory effects PKC on K<sub>ir</sub> channels.<sup>328</sup>,<sup>329</sup> Interestingly, PKC-regulated K<sub>ir</sub> channels appear to be involved in a unique cellular function in cerebral arterial smooth muscle. Studies on the mechano-regulation of K<sub>ir</sub> channels by Wu et al. showed that Ba<sup>2+</sup>-sensitive K<sub>ir</sub> channels are unresponsive to various vasoconstrictors that are known to constrict and depolarize cerebral arteries. However, K<sub>ir</sub> currents were strongly attenuated by hyposmotic challenges, suggesting that K<sub>ir</sub> might be sensitive to mechanical stimuli.<sup>330</sup> In agreement of these electrophysiological results, the authors also observed substantial inhibition on membrane depolarization and tone by Ba<sup>2+</sup> at a low intraluminal pressure, but much less effects were observed from Ba<sup>2+</sup> at a high pressure, suggesting that K<sub>ir</sub> channel activity is sensitive to membrane stretch.
Moreover, PKC inhibition abolished hyposmotic challenge-induced suppression of $K_{ir}$ currents, indicating PKC plays an important role in mechano-regulated $K_{ir}$ activity in the cerebral circulation$^{330}$.

**VDCCs.** A common denominator of vascular tone regulation is intracellular $Ca^{2+}$ alterations, and a fundamental ion channel involved in regulation of cytosolic $[Ca^{2+}]$ is the L-type voltage-dependent $Ca^{2+}$ channel (VDCC). VDCCs are activated by membrane depolarization, and they facilitate $Ca^{2+}$ influx, leading in turn to vasoconstriction. In many smooth muscle preparations, PKC has been shown to increase VDCC activity, thus posing an excitatory influence on smooth muscle reactivity. Chik et al. reported that pituitary adenylate cyclase activating protein (PACAP) causes an increase in VDCC activity in rat tail arterial smooth muscle cells, which was reduced by PKC inhibition and mimicked by PMA. Moreover, Cobine et al. showed that the PKC activator PDBu also enhanced L-type VDCC currents, which was abolished by different PKC inhibitors$^{331}$. Using more advanced $Ca^{2+}$-fluorescent imaging techniques, Navedo and coworkers illustrated that PKCα is required for basal persistent L-type VDCC activity in arterial myocytes$^{332}$. Biochemical investigation into the activating mechanism of PKC on VDCC suggests that phosphorylation of residues on the NH$_2$-terminus of the α-subunit relieves a tonic inhibitory control on channel activation$^{333}$.

In summary, the activating effects of PKC on smooth muscle electrical excitation are mediated by an array of pathways; PKC-stimulated activation of TRPM4 and inhibition of $K^+$ channels cause membrane depolarization, which activates L-type VDCCs that are also potentiated by PKC, leading to enhanced $Ca^{2+}$ influx and myogenic vasoconstriction.
1.9.2 PKC and Ca\textsuperscript{2+}-sensitization

With the development and utilization of Ca\textsuperscript{2+}-sensitive fluorescent dyes, e.g., Fura-2, uncoupling between [Ca\textsuperscript{2+}]\textsubscript{i} and the magnitude of myogenic contraction has been reported. This was first observed in hamster cheek pouch arterioles, where augmented myogenic response was tightly related to the magnitude of step increases in pressure by comparing 40-60 cm and 40-100 cm H\textsubscript{2}O step increases, yet cytosolic [Ca\textsuperscript{2+}] was not significantly different between the two step increases, clearly displaying an uncoupling between intracellular [Ca\textsuperscript{2+}] and myogenic response. Subsequently, more evidence was obtained by different groups showing that stretch-evoked contraction is greater than that stimulated by high K\textsuperscript{+} at a given [Ca\textsuperscript{2+}], indicating the existence of other signaling pathways to sensitize the contractile apparatus to Ca\textsuperscript{2+} \textsuperscript{334, 335}. It is widely accepted that the [Ca\textsuperscript{2+}]-tone relationship gives direct assessment of the Ca\textsuperscript{2+} sensitivity. According to the work of Schubert et al. using rat tail arteries, the relationship between tone and [Ca\textsuperscript{2+}] is much larger at 80 mmHg than at 10 mmHg, further confirming that stretch increases the sensitivity of the myofilament to Ca\textsuperscript{2+} in vascular myocytes\textsuperscript{253}. Involvement of PKC in the Ca\textsuperscript{2+} sensitization mechanisms has been reported\textsuperscript{336-338}. Karibe et al. found that pressure-dependent [Ca\textsuperscript{2+}]\textsubscript{i} increase and vasoconstriction were not abolished by a VDCC blocker (nimodipine). However, application of a PKC inhibitor abolished myogenic tone without affecting cytosolic [Ca\textsuperscript{2+}] increases in rat skeletal muscle arterial myocytes\textsuperscript{336}. In agreement with this finding, Lagaud and coworkers found that, after using 60 mmol/L K\textsuperscript{+} bath solution to clamp membrane potential and [Ca\textsuperscript{2+}]\textsubscript{i}, stepwise pressure increases evoked myogenic constriction without significant changes in [Ca\textsuperscript{2+}]\textsubscript{i}\textsuperscript{337}. This appears to be an effective means to separate the Ca\textsuperscript{2+}-dependent signaling from the Ca\textsuperscript{2+}-
sensitization mechanisms. Not surprisingly, these authors found that pressure-induced vasoconstriction on top of 60 mmol/L K+ was largely attenuated by the PKC inhibitor, which suggests that PKC is implicated in regulating Ca\(^{2+}\) sensitivity of the myofilament\(^{337}\). This mechanism is corroborated in several other studies which point out that PKC mediates smooth muscle Ca\(^{2+}\) sensitization by activating CPI-17, a 17-kDa polypeptide that specifically and potently diminishes myosin light chain phosphatase (MLCP) activity\(^{339-341}\), leading to smooth muscle contraction. PKC phosphorylates CPI-17 at Thr-38 to enhance its potency for inhibition of MLCP\(^{340, 341}\).

Taken together, PKC elicits an excitatory influence on myogenic behavior through synergistically activating two independent yet tightly associated pathways. On one hand, PKC potentiates membrane depolarization and Ca\(^{2+}\) entry through VDCCs to elevate cytosolic [Ca\(^{2+}\)]; On the other hand, PKC modulates smooth muscle Ca\(^{2+}\)-sensitivity by indirectly inhibiting myosin light phosphatase and can enhance contraction in the absence of changes in [Ca\(^{2+}\)]. Both mechanisms ultimately lead to augmented responses to increased intravascular pressure.
Part III: Hypotheses

1. **P2Y4 and P2Y6 purinergic receptors activate TRPM4 channels to mediate pressure-induced depolarization and myogenic vasoconstriction in cerebral parenchymal arterioles.**

The overarching objective of this research project described in Chapter two was to determine the expression and functional roles of TRPM4 channels in myogenic regulation of cerebral PAs. Previously, our laboratory made an important discovery that P2Y4 and P2Y6 purinergic receptors mediate PA myogenic constriction⁴, presumably through regulation of smooth muscle membrane potential and intracellular [Ca²⁺]. Therefore, a central goal of this project was to explore the interactions of P2Y4/P2Y6 receptors with TRPM4 channels in facilitating pressure-initiated depolarization and constriction of PAs. An array of techniques allowed for evaluation of TRPM4 channel expression in PAs, vascular reactivity, smooth muscle membrane potential, and TRPM4 channel activity in PA myocytes. Predictions were that TRPM4 channels are expressed in PAs and that inhibiting this channel with either molecular or pharmacological tools would reduce myogenic responsiveness. Examinations of arteriolar tone and membrane potential of endothelium-denuded PAs indicated TRPM4 channels mediate P2Y4 and P2Y6 receptor ligand-stimulated depolarization and contraction. Direct measurements of channel activity provided definitive evidence that TRPM4 channels are regulated by P2Y receptor activity, further supporting the hypothesis that TRPM4 channels couple mechano-activation of P2Y receptors and myogenic vasoconstriction in cerebral PAs. Dysfunction of the cerebral microcirculation has been implicated in numerous cardiovascular and neurological pathologies, including subarachnoid hemorrhage
(SAH)\textsuperscript{10}, hypertension\textsuperscript{13}, vascular dementia\textsuperscript{12} and Alzheimer’s disease\textsuperscript{342}. Thus, a more thorough understanding of the vasomotor control mechanisms in PAs, as revealed in the present work, may lead to development of novel therapeutic strategies in treating the microvascular diseases.

2. **RhoA/ROCK signaling contributes to pressure-induced, P2Y receptor-mediated TRPM4 channel activation, myogenic depolarization and vasoconstriction in PA myocytes.**

Following from the interesting findings described in Chapter two, cellular mechanisms, specifically the signaling pathways that couple mechanical stretch to myogenic activation of TRPM4 channels, were explored (Chapter three). The initial focus was on the PLC-DAG-PKC pathway since all three signaling partners have been shown to participate in myogenic regulation of vascular tone in various vascular beds, particularly in cerebral arteries\textsuperscript{238, 239}. Additionally, P2Y receptors have been reported to couple to the G\textsubscript{q} protein, whose association with the PLC-PKC pathway has been well documented. More importantly, TRPM4 channels are regulated by PKC\textsuperscript{20}, as demonstrated by previous research from our laboratory, which further increased the plausibility that this signaling cascade may play a key role in PA smooth muscle. Interestingly, diameter measurements showed that myogenic constriction was barely affected by PLC or PKC inhibition (see Chapter four), which is consistent with a recent study concerning the contractile mechanisms of pial and parenchymal arteries\textsuperscript{343}. This is suggestive of a fundamental shift of intracellular signaling mechanisms in the microcirculation. The present study demonstrated that ROCK plays a role in myogenic mechanisms in PAs\textsuperscript{343}. Although the mechanism of Rho-mediated constriction is
generally accepted as Ca$^{2+}$ sensitization$^{303}$, ROCK also contributes to electrical/mechanical coupling by regulation of ion channel activities and modulation of smooth muscle membrane potential$^{25,28}$. Therefore, the overall goal of these studies was to investigate the functional significance of Rho signaling in pressure-induced and P2Y receptor-mediated activation of TRPM4 channels in PA smooth muscle cells. A combination of techniques was employed, including measurements of arteriolar diameter, intracellular [Ca$^{2+}$], membrane potential and TRPM4 channel activity. Consistent with the hypothesis, our data demonstrated that inhibiting ROCK reduced both pressure-induced and receptor ligand-initiated vascular and cellular responses, whereas activating RhoA exerted the opposite effects on TRPM4 activity, membrane potential and tone. This finding brings new appreciation of the RhoA/ROCK signaling in regulation of smooth muscle contraction, which may contribute to identification of new therapeutic targets for preventing and treating cerebral microvascular diseases.
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CHAPTER 2: JOURNAL ARTICLE

TRPM4 channels couple purinergic receptor activation and myogenic tone development in cerebral parenchymal arterioles

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Running headline: TRPM4 couples P2Y receptors and myogenic tone
Abstract

Cerebral parenchymal arterioles (PAs) play a critical role in assuring appropriate blood flow and perfusion pressure within the brain. PAs are unique in contrast to upstream pial arteries, as defined by their critical roles in neurovascular coupling, distinct sensitivities to chemical stimulants, and enhanced myogenic tone development. The objective of the present study was to reveal some of the unique mechanisms of myogenic tone regulation in the cerebral microcirculation. Here we report that in vivo suppression of TRPM4 channel expression, or inhibition of TRPM4 channels with 9-phenanthrol substantially reduced myogenic tone of isolated PAs, supporting a key role of TRPM4 channels in PA myogenic tone development. Further, downregulation of TRPM4 channels inhibited vasoconstriction induced by the specific P2Y4 and P2Y6 receptor ligands (UTPγS and UDP) by 37% and 42%, respectively. In addition, 9-phenanthrol substantially attenuated purinergic ligand-induced membrane depolarization and constriction of PAs, and inhibited ligand-evoked TRPM4 channel activation in isolated PA myocytes. In concert with our previous work showing the essential contributions of P2Y4 and P2Y6 receptors to myogenic regulation of PAs, the current results point to TRPM4 channels as an important link between mechanosensitive P2Y receptor activation and myogenic constriction of cerebral parenchymal arterioles.

Keywords

Cerebral arteriole, myogenic tone, P2Y-receptor, TRPM4, vasoconstriction
Introduction

While the regulation of vascular tone in cerebral pial arteries has been intensely investigated, mechanistic studies of the intracerebral vessels branching from the surface arteries, i.e. the cerebral parenchymal arterioles (PAs), are uncommon, despite the fact that the PAs have unique functional roles and properties. PAs contribute to as much as 40% of cerebrovascular resistance and play a key role in regulating local blood flow within the brain\(^1\). By maintaining appropriate local perfusion pressure, PAs serve to protect downstream capillaries from the potential damaging effects of high intravascular pressure\(^1\). When these small arterioles branch from pial arteries to perfuse the cerebral cortex, they enter a unique environment where surrounding astrocytes and neurons modulate vascular diameter and blood flow through a process called neurovascular coupling\(^2, 3\). In addition, compared to pial arteries, the distinct features of PAs involve different responses to vasoconstrictors\(^4, 5\), altered ion channel function\(^6\), and enhanced myogenic responsiveness\(^2\). It is therefore likely that some of the fundamental contractile mechanisms in PA smooth muscle are unique as well. Since PAs are major sites of cerebral small vessel diseases (SVD)\(^7\), a better understanding of vascular tone regulation in PAs may reveal potential therapeutic targets for treatment of cerebral microvascular diseases.

As one of the essential processes that modulate vascular tone, myogenic reactivity represents the ability of small arteries and arterioles to constrict and reduce their diameter in response to increased intravascular pressure. Myogenic regulation ensures that blood flow remains relatively constant despite moment-to-moment fluctuations in arterial pressure. In the brain, myogenic tone is an essential contributor to cerebral
autoregulation. Mechanisms of myogenic tone development have been extensively studied and it is well established that vascular smooth muscle cells respond to increased intraluminal pressure via membrane depolarization⁸, calcium entry through voltage-dependent calcium channels and subsequent vasoconstriction⁹. However, the signaling pathways responsible for pressure-induced membrane depolarization have not been fully elucidated. Prior research in our laboratory demonstrated a critical role of transient receptor potential (TRP) channels, specifically TRPM4, in this mechanism involving monovalent cation influx (mainly Na⁺) and membrane depolarization in the smooth muscle cells of cerebral pial arteries¹⁰. Further studies of this channel revealed that it is regulated by protein kinase C (PKC) and Ca²⁺ release from the sarcoplasmic reticulum¹¹,¹². However, neither the significance nor the regulation of TRPM4 channels has been investigated in the PAs.

Another recent finding from our laboratory indicates the essential contribution of G protein-coupled receptors (GPCRs), specifically P2Y4 and P2Y6 purinergic receptors, to myogenic responsiveness of PAs via mechanosensitive receptor activation, which triggers vascular smooth muscle contraction⁵, similar to a proposed role for angiotensin II receptors in pial arteries¹³. Following from this novel finding, a major goal of the current study was to explore the possible interactions between TRPM4 channels and P2Y receptors. We hypothesized that P2Y4 and P2Y6 receptors regulate TRPM4 channel activity to mediate pressure-induced depolarization and myogenic vasoconstriction in cerebral PAs. The results of the present study support this hypothesis by demonstrating that downregulation of TRPM4 channel expression or pharmacologically blocking TRPM4 channels substantially attenuates pressure- and P2Y receptor ligand-induced
vascular and cellular responses, indicating a key role of TRPM4 channels in the P2Y receptor-mediated myogenic regulation of cerebral PAs.
Materials and Methods

Animals

All protocols and experimental procedures used in this research project were approved by the University of Vermont Institutional Animal Care and Use Committee. Male, Sprague-Dawley rats (15 to 20 weeks old; Charles River Laboratories, Saint Constant, QC, Canada) were used for all experiments.

Suppression of TRPM4 Channels in vivo

Antisense and sense oligodeoxynucleotides (ODNs) were designed based on the published rat TRPM4 sequence (RGD: 620244). Antisense ODN sequences were: TRPM4 AS-1, 5’-GTGTGCACCGCGGTACCCCG-3’; TRPM4 AS-2, 5’-GCCCTCATGAATCCCAGTAA-3’. Control animals were treated with sense ODNs: TRPM4 S-1, 5’-CGGGGTACCGCGGTGCACAC-3’; TRPM4 S-2, 5’-TTACTGGGATTCATGAGGGC-3’. The last four bases on the 5’ and 3’ ends were phosphorothioated to limit ODN degradation. All ODNs were synthesized by Integrated DNA Technologies Inc. (San Diego, CA, USA). ODNs were dissolved in bicarbonate-free artificial cerebral spinal fluid solution (in mmol/L): 130 NaCl, 3 KCl, 2 CaCl₂·2H₂O, 1 MgCl₂, 3 Na₂HPO₄·12H₂O, 0.45 NaH₂PO₄·H₂O. Rats were initially anesthetized by isoflurane (5%) in an induction chamber and then maintained on isoflurane (2-3%) inhaled via a nose cone. A small (0.5-1.5 cm) longitudinal, midline incision was centered over the foramen magnum, and the neck muscle was dissected until the dura was visualized. ODNs (100 µL, 0.8 mmol/L) were injected through the dura into the cisterna magna (day 0) using an insulin syringe with a 31-gauge needle. Muscle and
skin were then sutured and the rat was placed on a heating pad until recovery from anesthesia. Twenty-four hours later (day 1), a second injection of ODNs solution (100 µL, 0.8 mmol/L) was delivered by repeating the above procedure. Buprenorphine (0.01 mg/kg) was injected every 12 h (for 36 h) as an analgesic. Rats were euthanized on day 2 for further experimental procedures.

Tissue Preparation
Rats were euthanized by an overdose of sodium pentobarbital (120 mg/kg, intraperitoneal injection) followed by exsanguination. The brain was rapidly removed and placed in cold (4 °C) MOPS buffered physiological salt solution (PSS) (in mmol/L): 3 MOPS, 145 NaCl, 5 KCl, 1 MgSO₄, 2.5 CaCl₂, 1 KH₂PO₄, 2 sodium pyruvate, 5 glucose, 1% albumin from bovine serum, pH 7.4. Middle cerebral arteries (MCAs) and attached parenchymal arterioles (PAs) were isolated from the brain as previously described and transferred to a small volume of MOPS buffered PSS for further experimental use.

RT-PCR for TRPM4 Channel Message
Total RNA was prepared from isolated cerebral pial and parenchymal arteries using RNA STAT-60 total RNA isolation reagent (Tel-test inc., Friendswood, TX, USA). First-strand cDNA for pial and parenchymal arteries was prepared from 1320 ng total RNA, and 620 ng total RNA, respectively, using the Qiagen (Valencia, CA, USA) Sensiscript Reverse Transcriptase kit. Semi-quantitative PCR was performed using HotStarTaq DNA Polymerase (Qiagen). Amplification of the same templates for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as internal control, and TRPC6 channel
transcript expression served to indicate the specificity of TRPM4 antisense ODNs. PCR reactions were hot started at 94°C for 15 minutes and exposed to 38 cycles of 94°C for 60 s, 60°C for 60 s, and 72°C for 60 s, followed by final extension at 72°C for 10 minutes. All reaction products were resolved on 1.8% agarose gels. Primer sequences are listed in Table 1.

Pressure Myography

Parenchymal arteriolar segments were cannulated on glass pipettes in an arteriograph chamber (Living Systems, Burlington, VT, USA) containing a bicarbonate-buffered artificial cerebral spinal fluid (aCSF) of the following composition (in mmol/L): 136 NaCl, 3 KCl, 15 NaHCO₃, 1.25 NaH₂PO₄, 1 MgCl₂, 4 glucose, 2 CaCl₂ (temperature 37 °C and pH 7.3). For most experiments, in order to eliminate endothelial responses to P2Y receptor agonists, arterioles were stripped of endothelium by passing air bubbles through the arteriolar lumen. Arterioles were pressurized at 5 mmHg (with no flow), and superfused with warmed (37 °C), gassed (20% O₂/5% CO₂/balance N₂) aCSF. As an initial check of tissue viability, arterioles were reversibly constricted with the thromboxane receptor activator U46619 (100 nmol/L) (Enzo Life Sciences Inc., Farmingdale, NY, USA). Vessels were rejected for study if constrictions to U46619 were less than a 60% decrease in diameter. In experiments where myogenic constriction was not desired, e.g. agonist-induced vasoconstriction, intraluminal pressure was controlled at 3-5 mmHg. In experiments where steady myogenic tone was desired, PAs were pressurized to 40 mmHg. After steady myogenic tone developed, PAs were exposed to
IK$_{Ca}$ and SK$_{Ca}$ channel activator NS 309 (1 µmol/L). Absence of a dilator response to NS 309 indicated successful endothelial cell removal.

Membrane Potential
Arterioles were cannulated as described above and pressurized to 5 mmHg. The endothelium was removed from all vessels to eliminate any contribution of endothelial components to membrane potential. Nimodipine (300 nmol/L) (Calbiochem, San Diego, CA, USA) was included in aCSF prior to membrane potential measurements to prevent excessive vessel movement. Smooth muscle membrane potential was measured by insertion of a sharp glass microelectrode (~200 MΩ resistance when filled with 0.5 mol/L KCl) into the vessel wall. The criteria for successful impalement were 1) an abrupt negative potential deflection upon entry, 2) a stable membrane potential for at least 30s, and 3) an abrupt positive potential deflection to 0 mV upon electrode withdrawal. Measurements were made with an electrometer (World Precision Instruments) and recorded via computer with AxoScope and Dataq software.

Single Cell Isolation
Arterioles were placed in a cell isolation solution containing (in mmol/L): 55 NaCl, 80 Na Glutamate, 5.6 KCl, 2 MgCl2, 10 HEPES, 10 Glucose, (pH 7.3). Arteriolar segments were initially incubated in 0.5 mg/ml papain (Worthington Biochemical Corp. Lakewood, NJ, USA) and 1.0 mg/ml dithioerythritol for 12 min at 37 °C and then in 1.0 mg/ml collagenase type F for 10 mins at 37 °C. The digested segments were washed three times in ice-cold cell isolation solution and incubated on ice for 30 min. Digested arterioles
were triturated to liberate smooth muscle cells and stored in ice-cold cell isolation solution for use. Smooth muscle cells were studied within 5 h following isolation.

Patch Clamp Recordings

Isolated smooth muscle cells were placed into a recording chamber and allowed to adhere to glass coverslips for 20 minutes at room temperature. Whole cell currents were recorded with an AxoPatch 200B amplifier (Axon instruments) equipped with an Axon CV 2032wBU headstage (Molecular Devices). Recording electrodes (3-5 MΩ) were pulled from borosilicate glass (Sutter Instrument, Navato, CA, USA). Gigaohm seals were obtained in bath solution of the following composition (in mmol/L): 134 NaCl, 6 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES and 10 glucose, pH 7.4. The pipette solution contained (in mmol/L): 85 K-aspartate, 1 MgCl₂, 30 KCl, 10 NaCl, 10 HEPES, 5 µmol/L EGTA with pH adjusted to 7.2 with KOH. Nystatin (200 µg/ml) was included in the pipette solution to perforate the cell membrane. Perforation was deemed acceptable when series resistance was less than 50 MΩ. Currents were filtered at 1 kHz, sampled at 2 kHz, and stored for subsequent analysis. Clampex and Clampfit version 9.2 (Molecular Devices) were utilized for data acquisition and analysis, respectively. For all experiments, membrane potential was held at -70 mV, and all recordings were performed at room temperature (22 °C). TRPM4 channel activity was calculated as the sum of the open probability (NPo) of multiple open states of 1.75 pA. This value was based on the reported unitary conductance of TRPM4 (25 pS). NPo was calculated using the following equation:

\[ NPo = \sum_{j=1}^{N} \frac{(t_j \cdot j)}{T} \]
where \( P_0 \) is the open state probability, \( t_j \) is time spent (in seconds) with \( j = 1,2,\ldots,N \) channels open. \( N \) is maximum number of channels observed, and \( T \) is duration of measurement.

**Chemical and Reagents**

Buffer reagents, dissociation enzymes, nystatin, UDP and 9-phenanthrol were purchased from Sigma-Aldrich (St Louis, MO, USA). UTP\( \gamma S \) was purchased from Tocris (Minneapolis, MN, USA). Nimodipine was dissolved in DMSO to a final solvent concentration of 0.1%. All other compounds were dissolved in the appropriate salt solution.

**Statistical Analysis**

Values are expressed as Mean ± SEM, and \( n \) indicates the number of animals. Myogenic tone under different conditions was typically normalized as percentage tone, and calculated as follows:

\[
\% \text{ Tone} = \left( \frac{\text{Passive diameter} - \text{Constricted diameter}}{\text{Passive diameter}} \right) \times 100,
\]

where “Passive diameter” is the diameter in \( \text{Ca}^{2+} \)-free aCSF.

For P2Y receptor agonist responses, constrictions were expressed as percent of the maximum contraction induced by the thromboxane receptor activator U46619 (100 nmo/L). Dilation to NS309 (1 \( \mu \)mol/L) was calculated as percent of maximum dilation as follows:
Dilation (% maximum) = \[\text{Change in diameter}/(\text{Passive diameter}-\text{Initial diameter})\] \times 100.

Student’s t-test was used to compare two experimental groups. One way ANOVA analysis of variance followed by Tukey multiple comparison test was used in the comparison of multiple groups. Mean values were considered significantly different at \(p \leq 0.05\).
Results

Inhibition of TRPM4 channels reduces pressure-induced vasoconstriction of parenchymal arterioles.

Previous studies have shown that TRPM4 channels are involved in pressure-induced (myogenic) membrane depolarization and constriction of pial arteries. However, the roles of these channels in PAs have not been investigated. Therefore, we first determined the presence of TRPM4 channel message in parenchymal arteriolar extracts. In agreement with previous studies examining the localization of TRPM4 channels in cerebral pial arteries, TRPM4 message was detected in PAs (Figure 1A). Next, to explore the functional significance of TRPM4 channels, we tested the effects of suppressing TRPM4 expression on myogenic responses of PAs. Exposure of PAs to TRPM4 antisense oligodeoxynucleotides (ODNs) in vivo effectively reduced the expression level of TRPM4 channels as indicated by semi-quantitative PCR (Figure 1B). Control experiments on cerebral pial arteries also showed a significant reduction on TRPM4 mRNA level in the TRPM4 antisense group (Supplementary Figure 1). As an additional control, we also assessed the effects of TRPM4 antisense on TRPC6 expression level, which was previously shown to contribute to myogenic tone development in cerebral pial arteries. TRPC6 message level was not affected by TRPM4 antisense (Supplementary Figure 1).

Functional assessment of contractility revealed that PAs exposed in vivo to TRPM4 antisense ODNs developed 59%, 55% and 39% less myogenic tone at 20, 40 and 60 mmHg, respectively, compared with PAs in the sense group (Figure 1C, 1D, 1E). In control tests, we found no significant difference in endothelial cell K\(^+\) channel function.
between TRPM4 sense and antisense-treated PAs, which was indicated by the similar dilator responses to NS 309 (1 µmol/L), an activator of small and intermediate conductance Ca$^{2+}$-activated K$^+$ (SK$_{Ca}$ and IK$_{Ca}$) channels (Figure 1F). In parallel pharmacological studies, we found that 9-phenanthrol, which specifically inhibits TRPM4 channels, substantially attenuated pressure-induced tone of endothelium-denuded PAs, with an IC$_{50}$ of 27.9 µmol/L (Figure 2A, 2B). Thus, the results of both a genetic and a pharmacological approach indicated that TRPM4 channels are involved in the development of myogenic tone in cerebral PAs.

Inhibition of TRPM4 channels suppresses P2Y purinergic receptor ligand-evoked vasoconstriction and membrane depolarization of parenchymal arterioles.

Interestingly, previous studies from our laboratory have demonstrated the significant contributions of P2Y4 and P2Y6 purinergic receptors to myogenic responses of PAs through coupling of membrane stretch to vasoconstriction, which is primarily mediated by calcium entry through voltage-gated calcium (Ca$\text{v}$) channels(5). Since TRPM4 channels are also implicated in the myogenic constriction of PAs, presumably through depolarization and activation of Ca$\text{v}$ channels, it is logical to hypothesize that TRPM4 channels are involved in the coupling between activation of mechano-sensitive P2Y receptors and pressure-evoked vasoconstriction of PAs.

To establish a possible link between P2Y receptors and TRPM4 channels, we first examined the effects of suppressing TRPM4 expression on the contractile responses of endothelium-denuded PAs to P2Y4 and P2Y6 receptor ligands. We found that the selective P2Y4 receptor agonist UTP$\gamma$S (3 µmol/L) triggered 37% less constriction in the
TRPM4 antisense-treated PAs than sense-treated PAs. Similarly, constrictions induced by the P2Y6 receptor ligand UDP (3 μmol/L) were 42% lower in PAs from the antisense group (Figure 3A). Control tests revealed no significant difference between sense and antisense groups in the constrictions evoked by the thromboxane receptor ligand U46619 (100 nmol/L) (Figure 3B). In parallel, we explored the effects of pharmacologically blocking TRPM4 channels on the constrictor responses. We found that the TRPM4 channel antagonist 9-phenanthrol (10 μmol/L) inhibited UTPγS (0.5 μmol/L) and UDP (0.5 μmol/L)-induced constriction of denuded PAs by 44% and 57%, respectively (Figure 4A, 4B). Since there was no inhibitory effect of 9-phenanthrol (10 µmol/L) on vasoconstriction induced by elevation of bath K⁺ (30 mmol/L K⁺-aCSF) (Supplementary Figure 2), these results suggest a key role of TRPM4 channels in P2Y receptor-mediated contractile responses of PAs.

The proposed link between P2Y receptor activation, TRPM4 channels, and constriction of PAs implies that activation of P2Y receptors should induce a depolarization of PA myocytes that is inhibited by TRPM4 channel block. Thus, in the next series of experiments, we measured smooth muscle membrane potential using intracellular microelectrodes before and after the administration of P2Y receptor ligands, in the absence and presence of 9-phenanthrol. At 5 mmHg, PA smooth muscle cells depolarized from -43 ± 1 mV to -25 ± 1 mV in response to UTPγS (0.5 μmol/L). Subsequent administration of 9-phenanthrol (10 μmol/L) reversed the depolarization to -40 ± 2 mV. Similarly, UDP (0.5 μmol/L) depolarized PA smooth muscle from a resting potential of -42 ± 1 mV to -33 ± 1 mV, which did not occur in the presence of 10 μmol/L 9-phenanthrol (-47 ± 2 mV) (Figure 5A, 5B). These findings clearly demonstrate that
TRPM4 channels participate in P2Y4 and P2Y6 receptor-mediated membrane depolarization and vasoconstriction of PAs. The critical importance of P2Y receptor-induced membrane depolarization in the vasomotor response of PAs is strongly reinforced by the observation that inhibition of voltage-dependent calcium (Ca\textsubscript{v}) channels in these arterioles abolished P2Y receptor-mediated vasoconstrictor responses (Supplementary Figure 3).

**Activation of P2Y receptors potentiates TRPM4 channel activity in parenchymal arteriolar smooth muscle cells.**

In a final series of experiments, direct coupling between P2Y receptors and TRPM4 channels was assessed using the nystatin-perforated whole cell patch clamp technique. The P2Y4 receptor specific ligand UTP\textsubscript{γ}S (0.5 µmol/L) enhanced TRPM4 channel open probability by about 3 fold, which was reversed by blocking TRPM4 channels with 9-phenanthrol (30 µmol/L) (Figure 6A, 6B). Due to the somewhat transient nature of UDP current activation, the sequence of administering UDP and 9-phenanthrol was reversed in order to determine the effects of TRPM4 channel block. In the absence of 9-phenanthrol, the P2Y6 receptor agonist UDP (0.5 µmol/L) elicited a nearly 5-fold increase in TRPM4 channel activity, which was absent in cells pre-treated with 9-phenanthrol (Figure 6C, 6D, 6E). These results indicate that TRPM4 channels are regulated by P2Y4 and P2Y6 receptor activity, further supporting the hypothesis that coupling between P2Y receptors and TRPM4 channels is critical in the development of myogenic tone in PAs.
**Discussion**

Here, we investigated the expression and functional roles of smooth muscle TRPM4 channels in cerebral intraparenchymal arterioles. The major findings of the present study are 1) TRPM4 channels are expressed in rat parenchymal arterioles; 2) administration of TRPM4 antisense oligodeoxynucleotides (ODNs) *in vivo* effectively reduced TRPM4 expression in both pial and parenchymal arteries, and inhibited myogenic tone and P2Y purinergic receptor agonist-evoked vasoconstriction of PAs; 3) the specific TRPM4 channel blocker 9-phenanthrol reduced myogenic responses, and purinergic ligand-stimulated depolarization and vasoconstriction; 4) purinergic ligands strongly activated 9-phenanthrol-sensitive TRPM4 currents in isolated PA smooth muscle cells. Given that P2Y4 and P2Y6 receptors have been demonstrated to be important contributors of PA myogenic vasoconstriction(5), collectively these results indicate that TRPM4 channels are involved in P2Y purinergic receptor-mediated myogenic responses of cerebral PAs.

*TRPM4 Channel Presence and Function in Parenchymal Arteriole*

Myogenic tone development is a process by which arterial pressure regulates vascular diameter in order to maintain constant blood flow. The mechanisms underlying the myogenic response are not entirely clear. However, the primary roles of membrane potential and voltage-dependent Ca\(^{2+}\) (Ca\(_v\)) channels have been established. It is clear that increased intraluminal pressure is sensed by vascular smooth muscle cells, which subsequently depolarizes the cell membrane potential and increases the open probability of Ca\(_v\) channels, resulting in Ca\(^{2+}\) influx into smooth muscle cells and vasoconstriction\(^9\). Nevertheless, how a mechanical signal (i.e. membrane stretch) is converted to a cellular
response (i.e. membrane depolarization) remains obscure. Previous studies from our laboratory focused on the roles of transient receptor potential channels and demonstrated that both TRPM4 and TRPC6 channels are involved in myogenic tone development of cerebral pial arteries. This work concluded that smooth muscle TRPM4 and TRPC6 channels are activated by increased intravascular pressure and mediate cation influx, which leads to membrane depolarization and vasoconstriction\textsuperscript{10, 15}. Subsequently, other depolarizing contributors implicated in this mechanism have been reported in cerebral pial arteries, including TRPP2 channels\textsuperscript{16}, epithelial Na\textsuperscript{+} channels (ENaC)\textsuperscript{17} and TMEM-16A chloride channels\textsuperscript{18}, which further emphasize the significance of membrane potential regulation in myogenic tone development in cerebral arteries.

Interestingly, compared to pial arteries, downstream parenchymal arterioles (PAs) are considerably more sensitive to mechanical forces. Specifically, we found that PA smooth muscle is more depolarized and more constricted compared to pial arteries over the same range of intraluminal pressures\textsuperscript{2, 14}, suggesting reduced contribution of hyperpolarizing influence (primarily K\textsuperscript{+} channels) and/or enhanced activity of depolarizing factors on the smooth muscle. For instance, it has been shown that at normal pH (7.4), PAs display little activity of large-conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK\textsubscript{Ca}) channels\textsuperscript{6}. On the other hand, a more depolarized membrane potential may be partially attributed to enhanced depolarizing influences. The TRPM4 channel is a very promising candidate for this role. However, neither the significance nor the regulation of TRPM4 channels in PAs has been studied in any detail. The present study provides the first evidence that TRPM4 channels are centrally involved in myogenic tone development of PAs. In agreement with previous studies testing the presence of TRPM4 in cerebral pial
arteries, transcripts of this channel were also detected in PAs, suggesting a similar functional role in the myogenic response. Consistent with our initial hypothesis, we found that knocking down TRPM4 channels significantly inhibited the myogenic responsiveness of PAs. The present study validated the efficacy of a new technique that introduces antisense ODNs in vivo targeted against the TRPM4 gene. Given that subarachnoid cerebral spinal fluid (CSF) rapidly flows into the brain parenchyma along the outside of cortical surface arteries and penetrating arterioles, intracisternally injected antisense ODNs had physical access to parenchymal smooth muscle cells and effectively reduced TRPM4 channel message in the present study. One important advantage of this approach is that it allows for acute (48-hour) message knockdown, which largely limits the possibility of compensatory pathways being upregulated. In the present study, this approach reduced PA myogenic vasoconstriction by 40 to 60%. However, one of our previous studies showed 70 to 85% reduction in pial artery myogenic tone following TRPM4 knockdown using another in vivo approach (osmotic mini pumps). One possible explanation for this apparent smaller effect on PAs is that the overall amount of antisense ODNs introduced into rat brain was less in the present study (~160 nmoles) compared to the previous publication (~ 350 nmoles), which might result in reduced channel suppression. Another possibility is that TRPM4 channels play a somewhat less prominent role in the cerebral parenchymal arterioles than in the surface arteries. It is likely that other depolarizing pathways, perhaps including other TRP channels (TPRC3, TRPC6 and/or TRPP2), exist and function in parallel with TRPM4 to facilitate membrane potential depolarization. Although the involvement of other TRP channels in this response has not yet been resolved for PAs, results of the present study
will provide a better understanding of the diversity and functional significance of various TRP channels in vascular smooth muscle.

9-Phenanthrol was used as an alternative tool to confirm the involvement of TRPM4 in myogenic regulation. This selective TRPM4 channel blocker\textsuperscript{21} elicited concentration-dependent inhibition of pressure-induced tone in endothelium-denuded arterioles, supporting an essential role of smooth muscle TRPM4 channels in the coupling of mechanical stimulation and vasoconstriction in PAs. The specificity of 9-phenanthrol has been tested by various groups. 9-phenanthrol does not inhibit TRPM5 channels\textsuperscript{22}. At a concentration of 30 µmol/L, which almost completely blocks TRPM4 activity, 9-phenanthrol does not alter the activity of ion channels in smooth muscle cells that are involved in modulation of vascular tone (Ca\textsubscript{v} channels, BK\textsubscript{Ca} channels, voltage-gated K\textsuperscript{+} channels, inward rectifying K\textsuperscript{+} channels\textsuperscript{21}; it has no effect on other TRP channels (TRPC3, TRPC6 and TRPM7)\textsuperscript{21, 23}. In the present study, 10 µmol/L 9-phenanthrol was used on isolated arterioles. Control experiments showed no inhibition of 9-phenanthrol at this concentration on membrane depolarization (30 mmol/L K\textsuperscript{+}-aCSF)-induced vasoconstriction (Supplementary Figure 2). Thus, 9-phenanthrol appears to be a specific and very useful tool in studies of the functional roles of TRPM4 ion channels.

\textit{P2Y4 and P2Y6 Receptors Are Coupled to TRPM4 Channels to Mediate Myogenic Constriction in Parenchymal Arterioles.}

Purinergic signaling plays an important role in control of vascular contractility in a variety of blood vessels\textsuperscript{24}. Although different vessels in various species express distinct purinergic regulatory mechanisms, it is rather clear that vascular tone is under the control
of both the dilator effects of endothelial purinergic receptors and contractile responses of smooth muscle-associated purinergic signaling. In rat cerebral arteries, P2Y1 and P2Y2 receptors are located on endothelial cells and mediate endothelium-dependent vasodilation 25, whereas P2Y2, P2Y4 and P2Y6 receptors are abundantly expressed in smooth muscle and mediate vasoconstriction in response to purine and pyrimidine stimulation 5, 26.

Several earlier studies have explored the possible contributions of pyrimidine receptors in myogenic regulation. For instance, P2Y6 receptors likely play an important role in mouse mesenteric arteries 27, as the P2Y6 antagonist (MRS2567) inhibits myogenic vasoconstriction of these blood vessels 28. Moreover, recent findings from our laboratory revealed the central involvement of P2Y4 and P2Y6 receptors in myogenic tone development of cerebral PAs 5. Interestingly, rather than endogenous release of nucleotides from local sources as was reported for systemic arteries 27, pressure-induced activation of these receptors in the PAs appeared to be caused by direct mechanoactivation, since neither potentiation nor suppression of ectonucleotidase activity has any effect on the myogenic behavior of PAs 5. This observation is in fact consistent with a previous study on cerebral pial arteries that myogenic tone is mediated by mechanosensitive G protein-coupled receptors (GPCRs). In that study, an inverse agonist of angiotensin II (Ang II) receptor attenuated myogenic responses by 50%. Further, because pretreatment of isolated pial arteries with an angiotensin I converting enzyme (ACE) inhibitor had no effect on myogenic tone development, it was proposed that myogenic vasoconstriction is facilitated by direct mechanical activation of Ang II receptors 13.
The mechanisms of direct mechanoactivation of GPCRs (Ang II receptors, P2Y receptors, etc.) are not clear. Presently, a few possibilities have been proposed as to how GPCRs perceive mechanical force and initiate a conformational alteration to an active state. First, a “tethered” model has been proposed suggesting that intra- and/or extracellular anchorage to other proteins acts as a molecular spring to activate GPCRs upon membrane stretch. Second, a so-called “membrane” model proposes that the altered lateral pressure profile at the protein/phospholipid bilayer boundary can directly force a conformational change of the GPCRs. Additional mechanisms including the interactions between receptors and cytoskeletal elements to facilitate stimulation of GPCRs have also been presented. Clearly, identification of the specific mechanisms involved in GPCR regulation of myogenic tone will require deeper and more detailed biochemical and biophysical investigations.

A major finding of the present study is that TRPM4 channels are regulated by P2Y4 and P2Y6 receptors, providing the first direct evidence that TRPM4 channels are associated with mechano-sensitive GPCRs, and this coupling is essential for pressure-evoked responses of the cerebral PAs. Knocking down TRPM4 channels or blocking them with 9-phenanthrol significantly inhibited the constrictor responses to both UTPγS and UDP (specific P2Y4 and P2Y6 receptor ligands). Consistent with the functional studies, 9-phenanthrol also attenuated ligand-induced membrane potential depolarization. Furthermore, activation of these receptors largely enhanced TRPM4-like spontaneous inward currents in isolated smooth muscle cells. The properties of these currents were consistent with previously reported transient inward cation currents (TICCs) in cerebral artery myocytes. Additionally, the currents are sensitive to 9-phenanthrol, indicating
these are TRPM4 currents. Though regulation of this channel by protein kinase C (PKC) and Ca$^{2+}$ release from the endoplasmic reticulum has been previously reported$^{11,12}$, the present study provides the first evidence that this channel is a downstream effector of G$_{q/11}$-coupled receptor signaling, and that this mechanism plays an crucial role in myogenic regulation of vascular contractility. Involvement of other TRP channels in GPCR signaling has been previously proposed. For example, TRPC3 was demonstrated to mediate pyrimidine receptor-induced depolarization of rat pial arteries$^{31}$ and TRPC6 was proposed to participate in angiotensin II receptor-mediated vasoconstriction in response to mechanical stimuli$^{13}$. Together with the present findings, it is clear that the vasomotor mechanisms in resistance arteries involve multiple players, including different GPCRs, various TRP channels and possibly distinct signaling pathways. Though the mechanisms are not fully elucidated, the present study provides important insights regarding contractile signaling in the smooth muscle of the cerebral penetrating arterioles.

Regulation of TRPM4 Channels upon Myogenic Stimulation

Previous studies in cerebral arteries have demonstrated that TRPM4 channel activity is regulated by PKC: activation of PKC increases the sensitivity of TRPM4 to intracellular Ca$^{2+}$$^{11}$. Moreover, Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) through inositol trisphosphate (IP$_3$) receptors is required for sustained TRPM4 activity$^{12}$. Both findings suggest that TRPM4 channels may be physically associated with GPCR signaling partners to mediate myogenic regulation in smooth muscle cells. Furthermore, there is abundant evidence in support of a role of GPCR downstream signaling mechanisms in myogenic tone development. For instance, phospholipase C (PLC) plays a key role in the
genesis of myogenic responses via membrane depolarization and increased calcium influx\textsuperscript{32}. Contribution of diacylglycerol (DAG) in pressure-evoked vasoconstriction has also been reported in cerebral arteries\textsuperscript{33}. Additionally, PKC exerts substantial influence on various ion channels in smooth muscle of different vascular beds, including Ca\textsubscript{v}\textsuperscript{34}, K\textsubscript{v}\textsuperscript{35} and TRPM4\textsuperscript{11} channels, which are clearly involved in myogenic regulation. Moreover, IP\textsubscript{3} receptors and intracellular levels of IP\textsubscript{3} have been demonstrated to correlate with mechanical stimulation of arteries or arterioles\textsuperscript{36}. Since P2Y4 and P2Y6 are associated with G\textsubscript{q/11} protein signaling, PLC-DAG-PKC and IP\textsubscript{3} are logical candidate pathways that couple mechano-stimulation of purinergic receptors to activation of TRPM4 channels in PA smooth muscle.

In addition to G\textsubscript{q/11} protein signaling, P2Y receptors are also coupled to the Rho-associated protein kinase (ROCK) pathway\textsuperscript{37}. Interestingly, correlation between ROCK signaling and myogenic responses has been investigated in various vascular beds, including rat cerebral arteries\textsuperscript{38}. Although Ca\textsuperscript{2+} sensitization through inhibiting myosin light chain phosphatase is generally accepted as a major mechanism of ROCK-associated myogenic vasoconstriction\textsuperscript{38}, it has also been shown that ROCK regulates the activity of various ion channels, including Ca\textsubscript{v} and K\textsubscript{v} channels\textsuperscript{39, 40}. Additionally, ROCK-mediated modulation of membrane potential\textsuperscript{40} serves as further evidence that ROCK signaling contributes to the depolarizing mechanisms within smooth muscle, suggesting a possible role of ROCK in myogenic activation of TRPM4 channels. Understanding the relative contributions of these pathways may reveal therapeutic targets that can be manipulated in clinical settings.
Conclusion

The current results demonstrate that TRPM4 channels contribute substantially to P2Y4 and P2Y6 receptor-mediated myogenic tone development of cerebral intraparenchymal arterioles. A more thorough understanding of the vasomotor mechanisms within the brain may contribute to the development of novel medical approaches and revealing new therapeutic targets that could help patients with cerebral microvascular diseases.
Acknowledgements

The authors thank Dr. Fabrice Dabertrand and Sheila Russell for their advice and assistance with the study.

Disclosure/Conflict of Interest

The authors declare no conflict of interest.

Supplementary information is available at the Journal of Cerebral Blood Flow & Metabolism website – www.nature.com/jcbfm
References


**Figures**

**Figure 1.** Effects of *in vivo* suppression of TRPM4 channels on myogenic tone of parenchymal arterioles. (A) RT-PCR indicates the presence of TRPM4 channel message in cerebral pial arteries and parenchymal arterioles. (product size: 148 bp) (B) Semi-quantitative PCR reveals a substantial reduction of TRPM4 expression level in cerebral parenchymal arterioles from rats exposed to TRPM4 antisense versus sense oligodeoxynucleotides (ODNs). Sense or antisense ODN-treated PAs from three animals were pooled to assess relative message abundance. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to demonstrate uniformity of reaction conditions.
between the two groups. NT denotes reactions performed without DNA template. (C-E) Myogenic constriction in response to step-increased intraluminal pressure was significantly reduced in arterioles exposed to TRPM4 antisense ODNs (D) than sense ODNs (C). n=3 for both groups. * p < 0.05, **** p < 0.0001 versus sense group. (F) NS 309-mediated, endothelium-dependent vasodilations of TRPM4-sense and TRPM4-antisense treated arteries. n=3 for both groups.
Figure 2. Effects of 9-phenanthrol on myogenic tone of endothelium-denuded parenchymal arterioles. (A) 9-phenanthrol substantially reduces myogenic constriction of PAs held at 40 mmHg. Lack of dilatory effects of NS309 (1 µmol/L) indicates successful endothelium removal. (B) Summary data of 9-phenanthrol concentration/response relationship in PAs held at 40 mmHg. IC\textsubscript{50}=27.9 µmol/L. n=6 at each concentration.
**Figure 3.** Effects of *in vivo* TRPM4 channel suppression on P2Y4- and P2Y6- specific ligand-induced vasoconstriction in endothelium-denuded parenchymal arterioles. (A) TRPM4 antisense ODNs significantly suppress vasoconstriction induced by specific P2Y4 receptor ligand (UTPγS: 3 µmol/L), and specific P2Y6 receptor ligand (UDP: 3 µmol/L). n=3 for both groups. ** p < 0.01 versus sense group. (B) There is no significant difference in U46619 (100 nmol/L)-triggered vasoconstriction between PAs exposed to TRPM4 sense and antisense ODNs. n=3 for both groups.
Figure 4. 9-Phenanthrol (10 µmol/L) significantly reduces (A) P2Y4 receptor agonist (UTPγS: 0.5 µmol/L, n=6) and (B) P2Y6 receptor agonist (UDP: 0.5 µmol/L, n=6)-induced vasoconstriction in endothelium-denuded parenchymal arterioles. ** p < 0.01 versus response without 9-phenanthrol.
Figure 5. Effects of P2Y receptor ligands and 9-phenanthrol on parenchymal arteriolar smooth muscle membrane potential (Em). (A) 9-Phenanthrol inhibits P2Y4 receptor agonist (UTPγS: 0.5 µM)-induced depolarization in endothelium-denuded parenchymal arterioles. n=6, **** p < 0.0001 versus control and UTPγS. (B) The presence of 9-Phenanthrol attenuates the depolarization triggered by P2Y6 receptor ligand (UDP: 0.5 µM) in endothelium-denuded parenchymal arterioles. n=7, **** p < 0.0001 versus control and UDP.
Figure 6. Effects of P2Y receptor ligands and 9-phenanthrol on TRPM4 channel activity in isolated parenchymal arteriolar smooth muscle cells. (A, B) UTPγS (0.5 µmol/L) significantly increases TRPM4 channel open probability and 9-phenanthrol (30 µmol/L) inhibits UTPγS-activated TRPM4 currents. n=11 for control, n=7 for UTPγS, and n=4 for UTPγS + 9-phenanthrol. ** p < 0.01 versus control and response without 9-phenanthrol. (C-E) UDP (0.5 µmol/L) triggers substantial increase in TRPM4 channel activity in the absence of 9-phenanthrol (C), but significantly less activation in the presence of 9-
phenanthrol (30 µmol/L) (D). n=10 for control group, n=5 for UDP and UDP + 9-phenanthrol groups. *** p < 0.001 versus control, ** p < 0.01 versus response without 9-phenanthrol.
Table 1. Primer information for PCR studies examining TRPM4 channel transcript expression

<table>
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<tr>
<th>Target</th>
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<td>TRPM4F</td>
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<tr>
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<td>5'-AATCCAGTCCCTCCCAC-3’</td>
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<td>5'-GTGCCAAGTCCCAAGTCCCTGC-3’</td>
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<tr>
<td>TRPC6R</td>
<td>5'-CTGGGCGCTGCAGTACGTATC-3’</td>
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Supplementary Figure 1. Semi-quantitative PCR indicates a substantial reduction of TRPM4 expression level in cerebral pial arteries from rats exposed to TRPM4 antisense versus sense oligodeoxynucleotides (ODNs). Lack of effects on TRPC6 expression level indicates specificity of TRPM4 antisense ODNs. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to demonstrate uniformity of reaction conditions between the two groups. NT denotes reactions performed without DNA template.
**Supplementary Figure 2.** 9-phenanthrol (10 µmol/L) has no significant effect on membrane depolarization (30 mM K⁺-aCSF)-induced vasoconstriction of endothelium-denuded parenchymal arterioles. n = 3.
Supplementary figure 3. Effects of the voltage-dependent calcium (Ca_v) channel inhibitor nimodipine on P2Y4 and P2Y6 receptor-mediated constriction of endothelium-denuded parenchymal arterioles. (A), Representative recording showing block of UTPγS (0.5 µM)-induced vasoconstriction by nimodipine (300 nM). (B), Summary data showing nimodipine (300 nM) inhibition of P2Y4 receptor agonist (UTPγS: 0.5 µM, n=4) and P2Y6 receptor agonist (MRS2693: 1 µM, n=4)-induced vasoconstriction of PAs. ** p < 0.01 versus response without nimodipine, *** p < 0.001 versus response without nimodipine.
CHAPTER 3: JOURNAL ARTICLE

Rho Kinase Activity Governs Arteriolar Myogenic Depolarization

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This study was supported by NIH grant P01 HL095488, the Totman Trust for Medical Research and an American Heart Association predoctoral fellowship (Yao Li)

Running headline: Rho kinase and myogenic depolarization
**Abstract**

Cerebral arterioles contribute critically to regulation of local and global blood flow within the brain. Dysfunction of these blood vessels is implicated in numerous cardiovascular diseases. However, treatments are limited due to incomplete understanding of fundamental control mechanisms at this level of circulation. Emerging evidence points to a key role of Rho-associated protein kinase (ROCK) in regulation of microvascular contractility. This study sought to decipher the mechanisms of ROCK-mediated myogenic vasoconstriction in cerebral parenchymal arterioles (PAs). Here we report that the ROCK inhibitor H1152 strongly attenuated pressure-induced constriction, cytosolic $[\text{Ca}^{2+}]$ increases, and depolarization of isolated PAs. Further, the RhoA activator CN03 potentiated PA myogenic constriction and depolarization, indicating important involvement of RhoA/ROCK signaling in myogenic excitation-contraction mechanisms. Because of the well-established role of TRPM4 in pressure-induced depolarization, possible modulatory effects of ROCK on TRPM4 currents were explored using patch clamp electrophysiology. TRPM4 currents were suppressed by H1152 and enhanced by CN03. Finally, H1152 elevated the apparent $[\text{Ca}^{2+}]$-threshold for TRPM4 activation, suggesting that ROCK activates TRPM4 by increasing its $\text{Ca}^{2+}$-sensitivity. Our results support a novel mechanism whereby ROCK-mediated myogenic vasoconstriction occurs primarily through activation of TRPM4 channels, smooth muscle depolarization and cytosolic $[\text{Ca}^{2+}]$ increases in cerebral arterioles.

**Keywords**

Cerebral arteriole, depolarization, myogenic, Rho kinase, TRPM4
Introduction

Cerebral blood flow is precisely regulated through the vasomotor activity of surface pial arteries and penetrating arterioles. As a prime example, arterioles within the brain parenchyma contribute to as much as 40% of cerebrovascular resistance, playing a crucial role in global cerebral autoregulation. Furthermore, these parenchymal arterioles (PAs) modulate local blood flow via dilation in response to increased neuronal activity, in the process known as neurovascular coupling. Compared to upstream pial arteries, PAs exhibit unique functional properties and anatomical structure. Notably, distinct vasoconstrictor responses, altered ion channel functions and diverse Ca$^{2+}$ signaling events in PA smooth muscle all strongly suggest that mechanisms of vascular tone regulation might also be quite different from the signaling pathways in the pial vessels.

Structurally, in contrast to the ramifying and anastomotic networks formed by surface arteries and capillaries, the topology of the PA network is more one-dimensional. This creates a “bottleneck” effect in the cerebral microcirculation, which renders PAs and surrounding brain tissue especially vulnerable to changes in local blood flow. Accordingly, arteriolar dysfunction is implicated in numerous cerebral vascular pathologies, including cerebral and coronary small vessel diseases, ischemic and hemorrhagic stroke, hypertension, and vascular dementia. However, effective measures for disease prevention and treatment are limited due to incomplete understanding of the factors and mechanisms that regulate these blood vessels.

Myogenic tone represents one of most fundamental functions of resistance arteries in maintaining a relatively constant blood flow despite moment-to-moment fluctuations of arterial pressures. In the brain, myogenic tone sets the arteries and arterioles at a
partially constricted background so that blood flow can be tightly regulated by extracellular stimuli, particularly vasodilatory signals from neurovascular coupling and endothelial cell activation. Myogenic tone also contributes to controlling appropriate perfusion pressure and protecting downstream capillaries. The primary mechanism underlying pressure-induced vasoconstriction involves smooth muscle membrane depolarization and subsequent Ca\(^{2+}\) entry through voltage-dependent calcium channels (VDCCs)\(^{12}\). Yet how an extracellular mechanical stimulus is converted into intracellular membrane depolarization is not fully understood. Interestingly, compared to pial arteries, myogenic response plays a more prominent role in regulating PA reactivity, since these blood vessels exhibit significantly enhanced pressure-evoked depolarization\(^{8}\), intracellular [Ca\(^{2+}\)] increase\(^{13}\), and contraction\(^{13}\) versus pial arteries over the same range of vascular pressures. Hence, elucidating the mechano-stimulated cellular pathways is particularly important for the cerebral microcirculation. Previous studies from our laboratory have provided important inroads in this area. P2Y4 and P2Y6 receptors appear to be mechanosensors in PAs and mediate myogenic responses in a ligand-independent manner\(^{4}\). TRPM4 channels couple the mechanoactivation of P2Y4 and P2Y6 receptors to membrane depolarization and vasoconstriction in PAs\(^{14}\). However, the intracellular signaling cascades that mediate the stretch-induced, P2Y receptor-mediated activation of TRPM4 remain unknown.

Emerging evidence points to the dynamic role of the RhoA/Rho associated protein kinase (ROCK) pathway in regulating cerebrovascular function, including myogenic control\(^ {9,15,16}\). Though it is generally accepted that ROCK promotes contraction by inhibiting myosin light chain phosphatase, i.e. via “Ca\(^{2+}\) sensitization” mechanisms\(^{17}\),
recent findings have revealed that ROCK also regulates ion channel activity, including activation of epithelial Na\(^+\) channels (ENaC)\(^{18}\) and inhibition of voltage-dependent K\(^+\) (K\(_v\)) channels which modulates smooth muscle membrane potential\(^{19}\), suggesting ROCK may be involved in electromechanical coupling pathways. Following from these interesting findings, a major goal of the current study was to explore the roles of RhoA/ROCK signaling in myogenic reactivity of PAs. We hypothesized that RhoA/ROCK signaling mediates stretch activation of TRPM4 channels in the brain microcirculation. The results from this work support this hypothesis by demonstrating that pressure-induced and P2Y receptor agonist-triggered effects on TRPM4 activity, membrane potential, cytosolic [Ca\(^{2+}\)] and vascular tone are all attenuated by ROCK inhibition and potentiated by RhoA activation, revealing a previously underappreciated contribution of ROCK signaling to myogenic regulation through depolarization and Ca\(^{2+}\)-dependent pathways.
Materials and Methods

Animals

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont and performed in accordance with the National Institutes of Health Policy on the care and use of laboratory animals. Male, Sprague-Dawley rats (15 to 20 weeks old; Charles River Laboratories, Saint Constant, QC, Canada) were used for all experiments.

Tissue Preparation

Rats were euthanized by an overdose of sodium pentobarbital (120 mg/kg, intraperitoneal injection) followed by exsanguination. The brain was rapidly removed and placed in cold (4 °C) MOPS buffered physiological salt solution (PSS) (in mmol/L): 3 MOPS, 145 NaCl, 5 KCl, 1 MgSO₄, 2.5 CaCl₂, 1 KH₂PO₄, 2 sodium pyruvate, 5 glucose, 1% albumin from bovine serum, pH 7.4. Middle cerebral arteries (MCAs) and attached parenchymal arterioles (PAs) were isolated from the brain as previously described and transferred to a small volume of MOPS buffered PSS for further experimental use.

Pressure Myography

Parenchymal arteriolar segments were cannulated on glass pipettes in an arteriograph chamber (Living Systems, Burlington, VT, USA) containing a bicarbonate-buffered artificial cerebral spinal fluid (aCSF) of the following composition (in mmol/L): 136 NaCl, 3 KCl, 15 NaHCO₃, 1.25 NaH₂PO₄, 1 MgCl₂, 4 glucose, 2 CaCl₂ (temperature 37 °C and pH 7.3). In order to eliminate endothelial responses to P2Y receptor agonists,
arterioles were stripped of endothelium by passing air bubbles through the arteriolar lumen. PAs were initially pressurized at 3 mmHg (with no flow), and superfused with warmed (37 °C), gassed (20% O\textsubscript{2}/5% CO\textsubscript{2}/balance N\textsubscript{2}) aCSF. As an initial check of tissue viability, arterioles were reversibly constricted with the thromboxane receptor activator U46619 (100 nmol/L) (Enzo Life Sciences Inc., Farmingdale, NY, USA). Vessels were rejected for study if constrictions to U46619 were less than a 60% decrease in diameter. In experiments where myogenic constriction was not desired, e.g. agonist-induced vasoconstriction, intraluminal pressure was controlled at 3 mmHg. In experiments where steady myogenic tone was desired, PAs were pressurized to 40 mmHg. After myogenic tone developed, PAs were exposed to the IK\textsubscript{Ca} and SK\textsubscript{Ca} channel activator NS309 (1 \textmu mol/L), which mediates endothelium-dependent vasodilator responses in these arterioles\textsuperscript{21}. Absence of dilation by NS309 indicated successful endothelial cell removal.

Membrane Potential Measurements

The endothelium was removed from all vessels to eliminate any contribution of endothelial components to membrane potential. Arterioles were cannulated as described above and pressurized to 3 mmHg. In these experiments, the myosin light chain kinase inhibitor wortmannin (800 nmol/L) was included in aCSF prior to membrane potential measurements to prevent excessive vessel movement. Smooth muscle membrane potential was measured by repeated insertion of a sharp glass microelectrode (~200 M\textOmega resistance when filled with 0.5 mol/L KCl) into the vessel wall. The criteria for successful impalement were 1) an abrupt negative potential deflection upon entry, 2) a stable membrane potential for at least 30s, and 3) an abrupt positive potential deflection.
to 0 mV upon electrode withdrawal. Measurements were made with an electrometer (World Precision Instruments, Sarasota, FL, USA) and recorded via computer with AxoScope and Dataq software. At least three repeated impalements were made and the average membrane potential was calculated under each pharmacological treatment for every PA.

RhoA Activation of Isolated PAs

MCAs with PAs attached were isolated as previously described and incubated with the selective RhoA activator CN03 (1 µg/mL, equivalent to 8.5 nmol/L) (Cytoskeleton, Inc., Denver, CO, USA) for 3 hours in 2 ml DMEM/F-12 (serum-free) culture medium (37 °C). Control arterioles were incubated for 3 hours in DMEM/F-12 culture medium in the absence of CN03. Myogenic tone and membrane potential were then assessed as described above.

Intracellular Ca\(^{2+}\) Concentration Measurements

Freshly isolated PAs were cannulated on glass micropipettes and the endothelium was removed as described above. Cannulated arterioles were initially pressurized to 5 mmHg and equilibrated in aCSF for 1h, and then loaded with the ratiometric Ca\(^{2+}\)-sensitive dye fura-2 [acetoxyethyl ester (AM) membrane-permeant form] by incubating in aCSF containing fura-2 AM (5 µmol/L) (Invitrogen, Carlsbad, CA, USA) with pluronic acid (0.1%) (Invitrogen) for 1 hour at room temperature (~22 °C). The myograph chamber was mounted on a Nikon TE2000-S inverted fluorescence microscope. Arterioles were washed with aCSF 3 times and de-esterification of fura-2 AM occurred by superfusing
with warmed (37 °C), gassed (20% O₂/5% CO₂/balance N₂) aCSF for 20 mins before recording. Bath pH was closely monitored and maintained at 7.30-7.35.

For pressure-response tests, studies were performed in which intravascular pressure was increased to 10, 20, 30 and 40 mmHg. Fluorescence ratio was obtained from the background-corrected ratio of the 510 nm emission from arterioles alternately excited at 340 and 380 nm with hardware and software developed by IonOptix (Milton, MA, USA). [Ca²⁺]ᵢ was estimated using the following equation: 

\[ [\text{Ca}^{2+}]_i = K_d \times \beta \times \frac{(R - R_{\text{min}})}{(R_{\text{max}} - R)} \]

where \( R_{\text{min}} \) and \( R_{\text{max}} \) are the ratios of emission signals under Ca²⁺-free and Ca²⁺-saturated conditions, respectively, were measured from a separate set of ionomycin-treated arterioles, and \( \beta \) was determined as the ratio of \( F_{380} \) intensities at \( R_{\text{min}} \) and \( R_{\text{max}} \). Calibration values were pooled for calculation of intracellular \([\text{Ca}^{2+}]_i\) using an apparent dissociation constant \((K_d)\) of 282 nmol/L of fura-2 for Ca²⁺.

Single Cell Isolation

Arterioles were placed in a cell isolation solution containing (in mmol/L): 55 NaCl, 80 Na Glutamate, 5.6 KCl, 2 MgCl₂, 10 HEPES, 10 Glucose, (pH 7.3). Arteriolar segments were initially incubated in 0.5 mg/ml papain (Worthington Biochemical Corp. Lakewood, NJ, USA) and 1.0 mg/ml dithioerythritol for 11 min at 37 °C and then in 1.0 mg/ml collagenase type F for 13 mins at 37 °C. The digested segments were washed three times in ice-cold cell isolation solution and incubated on ice for 30 mins. Digested arterioles were triturated to liberate smooth muscle cells and stored in ice-cold cell isolation solution prior to use. Smooth muscle cells were studied within 5 h following isolation.
Patch Clamp Recordings

General Methods. Isolated smooth muscle cells were placed into a recording chamber and allowed to adhere to glass coverslips for 20 minutes at room temperature. Currents were recorded with an AxoPatch 200B amplifier (Axon instruments) equipped with an Axon CV 2032wBU headstage (Molecular Devices). Recording electrodes (3-5 MΩ for perforated patch recordings, 5-8 MΩ for conventional whole-cell and inside-out patch recordings) were pulled from borosilicate glass (Sutter Instrument, Novato, CA, USA). All patch clamp experiments were performed at room temperature (22 °C).

Perforated whole-cell patch recording. Gigaohm seals were obtained in bath solution of the following composition (in mmol/L): 134 NaCl, 6 KCl, 1 MgCl₂, 2 CaCl₂, 10 HEPES and 10 glucose (pH 7.4 adjusted with NaOH). The pipette solution contained (in mmol/L): 85 K-aspartate, 1 MgCl₂, 30 KCl, 10 NaCl, 10 HEPES, 5 μmol/L EGTA (pH 7.2 adjusted with KOH). Nystatin (200 μg/ml) was included in the pipette solution to perforate the cell membrane. Perforation was deemed acceptable when series resistance was less than 50 MΩ. Currents were filtered at 1 kHz, sampled at 2 kHz, and stored for subsequent analysis. Clampex and Clampfit version 9.2 (Molecular Devices) were utilized for data acquisition and analysis, respectively. For all experiments, membrane potential was held at -70 mV, and all recordings were performed at room temperature (22 °C). TRPM4 channel activity was calculated as the sum of the open probability (NPo) of multiple open states of 1.75 pA. This value was based on the reported unitary conductance of TRPM4 (25 pS). NPo was calculated using the following equation:

$$NPo = \sum_{j=1}^{N} [(t_j - j)/T]$$
where Po is the open state probability, \( t_j \) is time spent (in seconds) with \( j = 1,2,\ldots,N \) channels open. \( N \) is maximum number of channels observed, and \( T \) is duration of measurement.

**Conventional whole-cell patch recording for TRPM4 currents.** Cells were initially held at a membrane potential \( (V_m) \) of 0 mV, and currents were recorded during voltage ramps between -120 and +80 mV. Voltage ramps were initiated as soon as whole cell conditions were established and were repeated every 2 s during the experiment. The bath solution contained (in mmol/L) 156 NaCl, 5 CaCl\(_2\), 10 HEPES, and 10 glucose (pH 7.4 adjusted with NaOH). The bath solution also contained a selective voltage-dependent Ca\(^{2+}\) channel (VDCC) blocker nimodipine (200 nmol/L) (Calbiochem, San Diego, CA, USA), and a selective large-conductance Ca\(^{2+}\)-activated K\(^+\) (BK\(_{Ca}\)) channel blocker iberiotoxin (300 nmol/L). To prevent smooth muscle contraction under the condition of high intracellular [Ca\(^{2+}\)], arteriolar myocytes were pretreated with the myosin light chain kinase inhibitor wortmannin (10 \( \mu \)mol/L) 5 mins prior to patch clamp experiments. The pipette solution contained (in mmol/L) 156 CsCl, 1 MgCl\(_2\), 10 HEPES (pH 7.2 adjusted with CsOH). The Ca\(^{2+}\) concentration was adjusted between 1 \( \mu \)M and 100 \( \mu \)M by adding appropriate amounts of CaCl\(_2\) to 5 mmol/L EGTA calculated using the program Maxchelator (http://maxchelator.stanford.edu/). In some experiments, cells were treated with the ROCK inhibitor H1152 (1 \( \mu \)mol/L) or the TRPM4 channel blocker 9-phenanthrol (30 \( \mu \)mol/L) prior to patch recordings. For experiments with RhoA activation, the RhoA activator CN03 (0.1 ng/mL, equivalent to 0.85 pmol/L) was included in the pipette solution to allow for direct access to the inside of the smooth
muscle cells and rapid activation of RhoA. Currents were filtered at 1 kHz, sampled at 5 kHz, and stored for subsequent analysis.

**Conventional whole-cell patch recording for VDCC currents.** The bath solution contained (in mmol/L): 125 NaCl, 10 BaCl₂, 5 KCl, 1 MgCl₂, 0.1 CaCl₂, 10 HEPES, 10 glucose (pH 7.4 adjusted with NaOH). The pipette solution contained (in mmol/L) 130 CsCl, 1 MgCl₂, 10 HEPES, 10 EGTA, 10 glucose, 2 ATP, 0.5 GTP, 5 phosphocreatine (pH 7.2 adjusted with NaOH). Cells were initially voltage-clamped at -60 mV, and Caᵥ channel currents were recorded by stepping the membrane potential to +10 mV. Ca²⁺ currents were compared in the same cell before and after application of the Rho kinase inhibitor H1152 (1 µmol/L). Currents were filtered at 0.5 kHz, sampled at 2 kHz, and stored for subsequent analysis.

**Excised inside-out patch recording for TRPM4 channel currents in HEK cells.** The bath solution contained (in mmol/L): 156 CsCl, 10 HEPES, 1 MgCl₂, 0.1 CaCl₂ (pH 7.2 adjusted with CsOH). The pipette solution contained (in mmol/L): 156 NaCl, 10 HEPES, 1 MgCl₂, 5 CaCl₂, 10 Glucose (pH 7.4 adjusted with NaOH). Patches were initially held at a holding potential of 0 mV during patching and excision. Single channel currents were recorded at a membrane potential of -60 mV. TRPM4 channel activity was calculated as the sum of the open probability (NPo) of multiple open states of 1.50 pA based on the reported unitary conductance of TRPM4 (25 pS). NPo was obtained using the method described above for perforated-patch recordings. Single TRPM4 currents were compared in the presence and absence of H1152 (1 µmol/L) and CN03 (0.1 ng/mL, equivalent to 0.85 pmol/L), respectively. Currents were sampled at 10 KHz, filtered at 0.5 KHz and stored for subsequent analysis.
HEK 293T Cell Culture and Transient DNA Transfection

Human embryonic kidney (HEK) 293T cells were cultured in Dulbecco’s high glucose Modified Eagle Medium (Gibco, Grand Island, NY, USA) supplemented with 0.5% penicillin-streptomycin. Cells were maintained at 37 °C with 5% CO₂, and sub-cultured when confluent using 0.25% trypsin. HEK cells were transiently transfected with a plasmid encoding a mouse TRPM4 protein (TransOMIC technologies Inc., Huntsville, AL, USA). Cells were cultured for 1-2 days prior to electrophysiology experiments.

Chemical and Reagents

Buffer reagents, dissociation enzymes, NS309, nystatin, UDP, 9-phenanthrol, wortmannin, ionomycin and iberiotoxin were purchased from Sigma-Aldrich (St Louis, MO, USA). UTPγS and H1152 were purchased from Tocris (Minneapolis, MN, USA). Nimodipine was dissolved in DMSO to a final solvent concentration of 0.1%. All other compounds were dissolved in the appropriate salt solution.

Statistical Analysis

Values are expressed as Mean ± SEM, and n indicates the number of animals. Myogenic tone under different conditions was normalized as percentage tone, and calculated as follows:

\[
\% \text{ Tone} = \left( \frac{\text{Passive diameter} - \text{Constricted diameter}}{\text{Passive diameter}} \right) \times 100,
\]

where “Passive diameter” is the diameter in Ca²⁺-free aCSF at the corresponding pressure level.
For P2Y receptor agonist responses, constrictions were expressed as percent of the maximum contraction induced by the thromboxane receptor activator U46619 (100 nmo/L).

Student’s t-test was used to compare two experimental groups. One way ANOVA followed by the Tukey multiple comparison test was used in the comparison of multiple groups. Mean values were considered significantly different at $p \leq 0.05$. 
Results

ROCK is involved in pressure-induced and P2Y receptor-mediated vasoconstriction and membrane depolarization

We first examined the functional roles of ROCK in cerebral PAs. We found that a selective ROCK inhibitor (H1152) greatly attenuated pressure-induced tone with a half inhibitory concentration (IC\textsubscript{50}) of 0.24 µmol/L (Figure 1A, 1B), indicating a key role of ROCK in mediating myogenic response in the brain microcirculation, consistent with its contribution in pial arteries\textsuperscript{16, 22}. We have previously established the essential involvement of P2Y4 and P2Y6 receptors in cerebral arteriolar myogenic regulation\textsuperscript{4}. Therefore, the effects of ROCK inhibition on P2Y receptor-mediated responses were also explored. We found that H1152 (1 µmol/L) inhibited UTP\textsubscript{γ}S- (P2Y4 receptor agonist) (0.5 µmol/L) and UDP- (P2Y6 receptor ligand) (0.5 µmol/L) induced vasoconstriction by 85% and 87%, respectively (Figure 1C, 1D).

To determine whether ROCK-mediated myogenic constriction of PAs depends on cytosolic Ca\textsuperscript{2+}, changes in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) following stepwise increases in intraluminal pressure were measured. We found that H1152 (1 µmol/L) significantly suppressed [Ca\textsuperscript{2+}]\textsubscript{i} elevations by 30%-45% over a range of pressures from 5-30 mmHg (Figure 2A, 2B). [Ca\textsuperscript{2+}]\textsubscript{i} oscillations observed at different pressures in control PAs were attenuated by H1152 (Figure 2A). To eliminate the possibility that these effects result from direct inhibition of H1152 on L-type VDCCs, whole-cell VDCC activity before and after applying H1152 was compared and no significant difference was found (Supplementary Figure 1). Further, the observation that myogenic [Ca\textsuperscript{2+}]\textsubscript{i} elevation could be abolished by blocking VDCCs with nimodipine (100 nmol/L) (data not shown).
suggests that this response is mediated by Ca\(^{2+}\) entry through VDCCs presumably due to membrane depolarization of PA smooth muscle.

Therefore, we next examined the influence of ROCK inhibition on membrane potential. Consistent with our hypothesis, H1152 (1 µmol/L) hyperpolarized PAs held at 20 mmHg from -35 ± 1 mV to -43 ± 1 mV (Figure 2C, 2D), indicating that ROCK-mediated myogenic activation occurs in part through membrane depolarization and Ca\(^{2+}\) influx in PAs. To confirm this novel ROCK-related mechanism, P2Y receptor agonist-evoked changes in [Ca\(^{2+}\)]\(_i\) and membrane potential were studied. We found that H1152 (1 µmol/L) markedly attenuated UTP\(_{\gamma}\)S- (0.5 µmol/L) and UDP- (0.5 µmol/L) triggered [Ca\(^{2+}\)]\(_i\) increases by 61% and 50%, respectively (Figure 3A, 3B). H1152 also reversed agonist-induced smooth muscle depolarization in endothelium-denuded arterioles (Figure 3C, 3D). Thus, in contrast to the generally accepted view that ROCK influences smooth muscle contractility exclusively through Ca\(^{2+}\)-sensitization\(^{17}\), these findings demonstrate that membrane depolarization and Ca\(^{2+}\) entry are also a crucial part of ROCK-associated modulation of vascular tone in the cerebral microcirculation.

**RhoA activation enhances myogenic tone and depolarization**

To further elucidate the mechanisms of Rho signaling in PAs, we next investigated the effects of RhoA activation on tone and membrane potential. Arterioles pretreated with the RhoA activator CN03\(^{16,23}\) (1 µg/mL) for 3 hours developed very high levels of myogenic tone (Figure 4A, 4B), which were diminished by H1152 (1 µmol/L) (data not shown). Control PAs (cultured for 3 hours in the absence of CN03) also developed substantial myogenic tone, but significantly less than RhoA-activated arterioles. In addition, CN03-
treated vessels exhibited a more depolarized membrane potential than control (-31 ± 1 mV versus -39 ± 0.5 mV), which was reversed by 1 µmol/L H1152 (-44 ± 1 mV) (Figure 4C, 4D), indicative of an important role of RhoA/ROCK signaling in modulating membrane potential. In light of the well-established contribution of TRPM4 channels to myogenic depolarization, we hypothesized that RhoA-stimulated depolarization is mediated by TRPM4. In accord with our prediction, the selective TRPM4 blocker 9-phenanthrol (30 µmol/L) hyperpolarized CN03-treated arterioles to -44 ± 3 mV (Figure 4C, 4D), strongly supporting the involvement of TRPM4 in Rho-mediated depolarizing mechanisms in PA myocytes.

**RhoA/ROCK-signaling activates TRPM4 channels**

In this series of experiments, we studied the direct coupling between RhoA/ROCK signaling and TRPM4 channels using the perforated patch voltage-clamp technique. UTPγS (0.5 µmol/L) elicited a 3-fold increase in TRPM4 channel activity, which was diminished by H1152 (1 µmol/L) (Figure 5A, 5B). Similar inhibition was also observed for UDP-activated (0.5 µmol/L) TRPM4 currents (Supplementary Figure 2), pointing to an excitatory role of ROCK on TRPM4. To ensure that these results are not due to nonspecific effects of H1152 on the TRPM4 channel itself, we utilized the excised inside-out patch clamp configuration to study single TRPM4 channels expressed in human embryonic kidney (HEK) 293 cells. H1152 (1 µmol/L) had no effect on single TRPM4 currents in these membrane patches (Supplementary Figure 3), indicating no direct channel inhibitory action of H1152.
Next, effects of RhoA on TRPM4 activity were examined using the conventional whole-cell patch configuration, whereby RhoA activation was achieved by directly including CN03 (0.1 ng/mL) in the pipette solution. Whole-cell TRPM4 currents were potentiated by 4 fold in the presence of CN03 (Figure 5C, 5D). As CN03 had no direct influence on TRPM4 channel per se (Supplementary Figure 3), the RhoA activator-stimulated channel activity is potentially mediated by ROCK activation. Indeed, it was partially (68%) inhibited by H1152 (1 µmol/L) and nearly abolished (95%) by blocking TRPM4 channels with 9-phenanthrol (30 µmol/L) (Figure 5C, 5D). These data strongly support the proposal that RhoA/ROCK signaling couples the mechano-stimulation of P2Y receptors to TRPM4 channel opening, which leads to myogenic vasoconstriction in cerebral PAs.

**ROCK increases the Ca$^{2+}$-sensitivity of TRPM4 channels**

Our laboratory previously established that protein kinase C (PKC) activates TRPM4 by increasing channel sensitivity to intracellular Ca$^{2+}$ in pial arterial myocytes$^{24}$. In the present study, we hypothesized that ROCK plays a similar role in the PAs, which appear to be highly dependent on RhoA/ROCK signaling for myogenic regulation$^{13}$. In accord with past studies$^{24, 25}$, TRPM4 activity was influenced by [Ca$^{2+}$]$_{i}$ (Figure 6). We found that in 1 µmol/L [Ca$^{2+}$]$_{i}$, TRPM4 current activity levels were very low in both control (similar to our previous report$^{24}$) and in H1152- (1 µmol/L) treated myocytes with peak current density at 2.1 ± 0.3 pA/pF and 1.8 ± 0.3 pA/pF, respectively (Figure 6A, 6B). In 10 µmol/L [Ca$^{2+}$]$_{i}$, there was no apparent increase in TRPM4 activity in the presence of H1152 (2.1 ± 0.3 pA/pF), whereas there was near maximal current activation in the
absence of H1152 (4.3 ± 0.9 pA/pF). In 100 µmol/L [Ca\textsuperscript{2+}]_i, currents were maximally activated in the presence and absence of H1152 with peak amplitude at 4.6 ± 1.3 pA/pF and 4.9 ± 0.9 pA/pF, respectively (Figure 6B). These findings suggest that ROCK inhibition increased the apparent threshold of [Ca\textsuperscript{2+}]_i for TRPM4 activation, supporting the hypothesis that ROCK enhances the Ca\textsuperscript{2+} sensitivity of TRPM4 channels in PA smooth muscle cells.
**Discussion**

Here, we investigated the contributions of the RhoA/ROCK signaling pathway to myogenic tone development in cerebral intraparenchymal arterioles. The major findings of the present study are 1) the RhoA/ROCK signaling is involved in pressure-induced and P2Y receptor-mediated smooth muscle depolarization, cytosolic $[\text{Ca}^{2+}]$ increase and vasoconstriction of cerebral PAs; 2) RhoA/ROCK signaling couples P2Y receptor activation to TRPM4 channel opening in PA myocytes; 3) ROCK enhances the apparent $\text{Ca}^{2+}$-sensitivity of TRPM4 channels in PA smooth muscle cells. Collectively, these results demonstrate that in addition to the traditional $\text{Ca}^{2+}$-sensitization pathway, the Rho signaling also facilitates mechano-activation of TRPM4 channels, leading to $\text{Ca}^{2+}$-entry through VDCCs and associated myogenic responses in PAs (Figure 7).

*Mechanisms of RhoA/ROCK signaling in myogenic vasoconstriction*

A correlation between ROCK function and myogenic reactivity has been previously reported in the cerebral circulation$^{9, 22}$, with a generally accepted mechanism being a ROCK-mediated inhibition of myosin light chain phosphatase. This results in greater myosin light chain phosphorylation and activation, and enhanced smooth muscle contractility, which is not dependent on increased $\text{Ca}^{2+}$ delivery to the contractile apparatus$^{17}$. However, several recent studies point to a regulatory role of Rho signaling on the activity of ion channels, including $\text{ENaC}^{18}$, $\text{K}_{v}^{19}$ and inward rectifying $\text{K}^{+}$ ($\text{K}_{\text{ir}}$) channels$^{26}$. ROCK has also been reported to mediate UTP-induced smooth muscle depolarization by inhibiting $\text{K}_{v}$ channels$^{19}$, further supporting an important yet underappreciated role of ROCK in regulating the excitation-contraction coupling in...
vascular smooth muscle. In the present study, we observed that the selective ROCK inhibitor H1152 significantly suppressed pressure-induced responses in membrane potential, cytosolic [Ca\(^{2+}\)] and tone. Furthermore, the RhoA activator CN03 potentiated myogenic tone and membrane depolarization, both of which were sensitive to ROCK inhibition, providing the first evidence that the RhoA/ROCK signaling pathway participates critically in pressure-induced depolarizing mechanisms in cerebral PAs.

Our laboratory has recently established the coupling between mechano-sensitive P2Y4/P2Y6 receptors and TRPM4 channels, and its contribution in mediating myogenic depolarization and vasoconstriction in PAs\(^4\), \(^14\). Consistent with ROCK-associated mechanisms in myogenic control, here we also observed that P2Y4 and P2Y6 receptor ligand-evoked constrictions, [Ca\(^{2+}\)], elevations and depolarization were attenuated by H1152. Furthermore, our prior study demonstrated that TRPM4 channels are stimulated by P2Y receptor agonists\(^14\). In the current work, UTP\(_\gamma\)S and UDP-activated TRPM4 currents were reduced by H1152. We also found that the RhoA activator CN03 elicited a substantial increase in TRPM4 activity in PA myocytes in a ROCK-dependent manner. These findings demonstrate that RhoA/ROCK signaling facilitates pyrimidine receptor-mediated activation of TRPM4 channels in PA smooth muscle.

Several mechano-sensitive G\(_{q/11}\) protein-coupled receptors have been reported by us and others\(^4\), \(^27\). P2Y4 and P2Y6 receptors appear to mediate stretch-evoked vasoconstriction in a ligand-independent manner, because myogenic behavior of PAs is not affected by altering ectonucleotidase activity, which regulates the local levels of purines and pyrimidines\(^4\). This is similar to the involvement of Angiotensin II (Ang II) receptors in cerebral pial arterial myogenic tone, which is sensitive to the Ang II receptor
inhibitor losartan but not influenced by Ang II converting enzyme (ACE) inhibition\textsuperscript{27}. Ang II receptor-mediated myogenic responses rely on \(G_{q/11}\) protein-coupled, phospholipase C (PLC)-dependent signaling pathways\textsuperscript{27, 28}. This is supported by the observations that the PLC inhibitor U73122 diminishes membrane stretch-induced responses in an Ang-II receptor/ion channel expression system\textsuperscript{27}, and relaxes pressurized rat pial arteries\textsuperscript{29}. In addition, molecular suppression of the \(\gamma_1\) isoform of PLC significantly attenuates myogenic depolarization and vasoconstriction, and also inhibits hypotonicity-stimulated TRPM4 currents. Interestingly, in our preliminary experiments, PLC did not appear to participate in myogenic regulation of PAs since U73122 had little effect on arteriolar diameter at a concentration that causes near maximal dilations in pial vessels (data not shown). Instead, current data illustrate the novel involvement of the Rho-associated mechanisms in P2Y receptor-mediated mechanical responses. Interestingly, in addition to \(G_{q/11}\)-protein, P2Y receptors are also coupled to \(G_{12/13}\) and initiate Rho signaling cascades in vascular myocytes\textsuperscript{30}. Moreover, several apparent \(G_{q/11}\) protein-coupled mechanosensors identified in expression systems\textsuperscript{27}, such as ET\textsubscript{A} endothelin and V\textsubscript{1A} vasopressin receptors, also couple to \(G_{12/13}\) to stimulate RhoA/ROCK-dependent smooth muscle contraction\textsuperscript{31}. Together with the key observations in the present study, these data strongly suggest that stretch-induced contraction of vascular myocytes is dynamically regulated not only by \(G_{q/11}\)-related signaling pathways but also by \(G_{12/13}\)-mediated and Rho-dependent cellular mechanisms.

\textit{Contribution of RhoA to myogenic constriction of cerebral PAs}
To further investigate the mechanistic involvement of RhoA in myogenic regulation, we utilized the commercially available RhoA activator CN03. This cell permeable recombinant protein functions by converting glutamine-63 to glutamate, thus disrupting both intrinsic- and GAP-stimulated GTPase activity, leaving RhoA constitutively active. The effectiveness of CN03 has been confirmed in several studies, where RhoA activity in native smooth muscle cells is significantly increased following a 2 to 4 hour-incubation with CN03 at concentrations ranging from 1 to 5 µg/mL. Functionally, we detected a significant augmentation of pressure-induced tone in arterioles cultured with 1 µg/mL (or 8.5 nmol/L) CN03 for 3 hours, which was 25% to 30% higher than control, indicating the magnitude of responsiveness to pressure is directly associated with RhoA activity levels. This is consistent with a previous study on mouse cerebral arteries where CN03 (5 µg/mL, 4 hours) induced a substantial elevation of myogenic tone. In the present study, control PAs (cultured for 3 hours in the absence of CN03) also developed significant amount of tone. This may be due to upregulation of unknown cellular factors during the culture process that potentiate myogenic reactivity of PAs. In addition to contractility, activation of RhoA also depolarized the smooth muscle membrane through an H1152-sensitive pathway, further supporting the notion that myogenic depolarization and vasoconstriction in PAs are regulated by upstream RhoA/ROCK signaling cascade.

A major finding of the present study is that Rho activates TRPM4 channels as illustrated with conventional whole-cell patch recordings. Channel activity detected with this configuration has been well characterized. These currents exhibit the hallmarks of TRPM4 channel properties: [Ca^{2+}]_{i} dependence, outward rectification and time-sensitive decay. Additionally, these currents are diminished by suppressing TRPM4 expression
with antisense\textsuperscript{24}. We also found that the selective TRPM4 blocker 9-phenanthrol nearly abolished the channel activity, indicating they are TRPM4 currents. In the present series of experiments, CN03 (0.1 ng/mL or 0.85 pmol/L) was included in the pipette to obtain direct access to the inside of the cells and induce acute RhoA activation upon membrane rupture. TRPM4 currents were markedly activated by CN03. Because CN03 has no direct effects on TRPM4 itself (Supplementary Figure 3), this is indicative of an excitatory influence of Rho on the depolarizing mechanisms, leading to myogenic activation of PA myocytes.

\textit{Regulation of TRPM4 channels by ROCK}

In the present study, ROCK regulated the responses of TRPM4 channels to Ca\textsuperscript{2+}. Under control conditions, the EC\textsubscript{50} of [Ca\textsuperscript{2+}]\textsubscript{i} for TRPM4 activation appeared to lie between 1 to 10 \(\mu\)mol/L. Nevertheless, when myocytes were treated with H1152, the threshold for TRPM4 stimulation was elevated beyond 10 \(\mu\)mol/L [Ca\textsuperscript{2+}]\textsubscript{i}, suggesting that ROCK regulates the Ca\textsuperscript{2+} sensitivity of the channel itself. The exact mechanisms underlying ROCK-associated TRPM4 activation are still unknown. However, several early studies showed that ROCK regulates K\textsubscript{v}\textsuperscript{3.5} and ENaC\textsuperscript{18} channel trafficking and expression on the plasma membrane to modulate membrane excitability. Although it is not clear whether TRPM4 is subjected to this mechanism, the observations that TRPM4 currents can be acutely potentiated and attenuated by CN03 and H1152, respectively, point to immediate modulatory effects from ROCK, presumably through direct phosphorylation of the Ca\textsuperscript{2+}-sensing sites on TRPM4. Importantly, PKC plays a similar role in pial artery myocytes, where the PKC activator PMA increases Ca\textsuperscript{2+} sensitivity of TRPM4 channels\textsuperscript{24}. However,
our preliminary experiments showed that PKC inhibition had little effect on PA vascular tone (data not shown), which is compatible with minimal contribution of PLC to myogenic regulation in cerebral PAs. Several prior studies concerning the roles of PKC and ROCK in the cerebral circulation reported similar observations that myogenic control in cerebral pial arteries is mediated by PKC and ROCK together\textsuperscript{24, 36, 37}, while only ROCK participates in pressure-evoked response in the penetrating arterioles\textsuperscript{9}. Interestingly, both PKC and ROCK are serine/threonine kinases with similar consensus substrate phosphorylation sites\textsuperscript{38}, further supporting the results that TRPM4 channels can be regulated by both enzymes. Although explanations for the apparent shift of functional kinases from large to small cerebral arteries requires further investigation, the unique contributions of ROCK in the cerebral microcirculation may provide important insights in preventing and treating microvascular diseases.

\textit{Conclusion}

Collectively, our data reveal a novel role of RhoA/ROCK-signaling in cerebral vasoconstriction, which is determined primarily via activation of TRPM4 channels, smooth muscle depolarization and Ca\textsuperscript{2+} influx through VDCCs. As emerging evidence points towards important physiological and pathological contributions of ROCK in the cerebral circulation\textsuperscript{9, 16}, a more complete understanding of the cellular mechanisms whereby RhoA/ROCK signaling is controlled may lead to development of innovative therapeutic strategies for microvascular pathologies in the brain.
Acknowledgement

We thank Drs. George Wellman and Anthony Morielli for valuable advice, and Arsalan Syed and Sharath Madasu for helpful technical assistance.

Disclosure/Conflict of Interest

The authors declare no conflict of interest.

Supplementary information is available at the journal of Cerebral Blood Flow & Metabolism website – www.nature.com/jcbfm
References


Figures

Figure 1. ROCK inhibition suppresses pressure-induced and P2Y receptor-mediated vasoconstriction in endothelium-denuded PAs. (A-B), A representative diameter recording and summary data showing a selective ROCK inhibitor H1152 reduces myogenic constriction of PAs held at 40 mmHg (IC\textsubscript{50}=0.24 µmol/L). Lack of dilation by NS309 (1 µmol/L) indicates successful endothelium removal. (n=6). (C), A representative diameter recording showing H1152 (1 µmol/L) reduces P2Y4 receptor ligand (UTP\textsubscript{γS}: 0.5 µmol/L)-induced vasoconstriction in denuded PAs. (D), Summary data showing H1152 reduces P2Y4 (UTP\textsubscript{γS}: 0.5 mol/L) and P2Y6 (UDP: 0.5 mol/L) receptor ligand-initiated constriction of PAs. (n=6). **P<0.01, ***P<0.001 versus responses without H1152.
Figure 2. ROCK inhibition attenuates myogenic [Ca^{2+}]_i elevation and depolarization in endothelium-denuded PAs. (A-B), representative diameter recordings and summary data showing H1152 (1 µmol/L) inhibits [Ca^{2+}]_i elevation and oscillations induced by step-wise pressure increase. (n=5 for control, n=4 for H1152) (C-D), H1152 (1 µmol/L) attenuates pressure-induced depolarization in arterioles held at 20 mmHg. This pressure is selected based on the greatest difference in [Ca^{2+}]_i observed in fura-2 Ca^{2+} measurement tests. (n=5) *P<0.05, **P<0.01 versus control.
Figure 3. ROCK inhibition suppresses P2Y4 and P2Y6 receptor-mediated [Ca^{2+}]_{i} increase and depolarization in endothelium-denuded PAs. (A), A representative recording of [Ca^{2+}]_{i} measurement showing H1152 (1 μmol/L) inhibits UTPγS-(0.5 μmol/L) induced [Ca^{2+}]_{i} increase in denuded PAs. (B), Summary data showing H1152 inhibits UTPγS (0.5 μmol/L) and UDP (0.5 μmol/L)-initiated [Ca^{2+}]_{i} elevation in PAs. (n=5). *P<0.05 versus responses without H1152. (C), Representative membrane potential recordings showing H1152 (1 μmol/L) attenuates UTPγS-(0.5 μmol/L) induced membrane depolarization in denuded PAs held at 3 mmHg. (D), Summary data showing H1152 attenuates PA smooth muscle depolarization initiated by UTPγS (n=5) and UDP (0.5 μmol/L) (n=4). **P<0.01 versus Control and Agonist.
Figure 4. RhoA activation enhances myogenic constriction and depolarization in endothelium-denuded PAs. (A-B), Representative recordings and summary data showing PAs pretreated with the RhoA activator CN03 (1 µg/mL) develop enhanced myogenic tone compared to vehicle-treated arterioles. (n=4) *P<0.05 versus control. (C-D), CN03-(1 µg/mL) treated arterioles exhibit depolarized membrane potential (n=5) compared to control (n=5), which is attenuated by H1152 (1 µmol/L) (n=4) and the TRPM4 channel blocker 9-phenanthrol (30 µmol/L) (n=4). Intraluminal pressure=3 mmHg., **P<0.01 versus CN03, ***P<0.001 versus control and CN03.
Figure 5. TRPM4 channel activity is reduced by ROCK inhibition and enhanced by RhoA activation in PA myocytes. (A-B), A representative nyastatin-perforated patch recording and summary data showing H1152 (1 µmol/L) inhibits UTPγS-activated (0.5 µmol/L) TRPM4 currents (n=7). ***P<0.001 versus control and UTPγS. (C-D), Representative conventional whole-cell recordings and summary data showing CN03 (0.1 ng/mL) potentiates TRPM4 currents (n=7) versus control (n=8), which is reversed by H1152 (1 µmol/L) (n=8) and 9-phenanthrol (30 µmol/L) (n=7). The representative currents are selected from cells with membrane conductance of 7-8 pF. [Ca^{2+}]i = 1 µmol/L. ****P<0.0001 versus control and CN03.
Figure 6. ROCK inhibition decreases the Ca\(^{2+}\)-sensitivity of TRPM4 channels in PA myocytes. (A), Representative current-voltage relationships from individual cells showing TRPM4 channel activity is Ca\(^{2+}\)-activated under control conditions (left) and that H1152 (1 µmol/L) inhibits TRPM4 activation by Ca\(^{2+}\) (right). The representative currents are selected from cells with membrane conductance of 7-8 pF. (B), Summary data of peak outward currents showing H1152 increases the [Ca\(^{2+}\)]\(_i\)-threshold for TRPM4 activation. H1152 significantly inhibits TRPM4 currents at 10 µmol/L [Ca\(^{2+}\)]\(_i\). (n=5-9 cells from 2-3 rats per group). *P<0.05 versus control.
**Figure 7.** Proposed mechanism of pressure-induced, P2Y-dependent activation of TRPM4 through the Rho signaling pathway in cerebral arterioles. Mechano-sensitive P2Y receptors are stimulated by increase in intravascular pressure, which initiates the activation of RhoA and ROCK. ROCK subsequently activates TRPM4 channels, and causes membrane depolarization, Ca²⁺ entry through VDCCs, and activation of MLCK, which phosphorylates MLC and results in smooth muscle contraction. Additionally, ROCK increases the Ca²⁺-sensitivity of the contractile machinery via inhibiting MLCP.

ROCK, Rho-dependent protein kinase; VDCC, voltage-dependent calcium channels; CaM, calmodulin; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; Vm, membrane potential.
Supplemental Figure 1. ROCK inhibition has no effect on VDCC currents in PA myocytes. (A), representative VDCC currents at +10 mV recorded from PA smooth muscle cells before (left) and after (right) application of H1152 (1 µmol/L). (B), Summary of VDCC current density before and after application of H1152. n = 4.
Supplemental Figure 2. ROCK inhibition reduces P2Y6 receptor-mediated TRPM4 channel activation. (A-C), Representative perforated whole-cell patch clamp recordings and summary data showing UDP (0.5 µmol/L) elevates TRPM4 currents (A), and H1152 (1 µmol/L) suppresses UDP (0.5 µmol/L)-activated TRPM4 currents (B). (n=7) Membrane potential is held at -70 mV. *P<0.05 versus UDP, and **P<0.01 versus Control.
Supplemental Figure 3. TRPM4 channel activity is not directly affected by the ROCK inhibitor H1152 or the RhoA activator CN03. (A), Representative single channel recordings from inside-out patches showing TRPM4 activity in human embryonic kidney (HEK) cells overexpressing TRPM4 channels. Membrane potential is held at -60 mV. The amplitude of the single channel currents is -1.5 pA. (B), Summary data of TRPM4 single channel recordings showing that neither H1152 (1 µmol/L) (n=10) nor CN03 (0.1 ng/mL) (n=11) affected TRPM4 activity compared to control (n=20). The selective TRPM4 blocker 9-phenanthrol (30 µmol/L) significantly inhibited H1152- (n=10) and CN03- (n=9) insensitive currents. *P<0.05 versus H1152 and CN03.
CHAPTER 4: UNPUBLISHED DATA

4.1 Involvement of PLC and PKC in Myogenic Constriction of Cerebral PAs

Rationale

Results presented in Chapter two have illustrated that the coupling between mechano-sensitive P2Y4/P2Y6 receptors and TRPM4 channels contribute critically to pressure-evoked vasoconstriction of PAs. Since P2Y4 and P2Y6 receptors are Gq-coupled, the initial hypothesis was that TRPM4 activation by P2Y receptors depends on the PLC-DAG-PKC signaling pathway, which is further supported by a previous study showing that TRPM4 activity is regulated by PKC1. It is likely that pressure-induced membrane depolarization and myogenic tone in cerebral PAs follow the similar PLC-DAG-PKC-TRPM4 signaling pattern since PKC has been shown to exert substantial influence on myogenic responses through sensitizing Ca2+ and inhibiting K1, in other vascular beds. In addition, PLC participates in myogenic tone development in rat cerebral arteries4. Together, these results suggest the potential significance of the PLC-DAG-PKC signaling cascade in pressure-induced and P2Y receptor-mediated activation of TRPM4 in PA myocytes.

Results

We unexpectedly observed that the PLC inhibitor U73122 showed minimal inhibitory influence on myogenic reactivity of PAs at a concentration of 3 µmol/L.
Figure 1. Comparatively, 3 µmol/L U73122 substantially reduced pressure-evoked tone to about 10% of its original level in cerebral pial arteries\(^4\), suggesting that PLC activity can be nearly abolished by the inhibitor at this concentration. Although PA myogenic tone was completely inhibited by a higher concentration (10 µmol/L), it has been demonstrated that U73122 at this level elicits significant blocking effects on VDCCs, resulting in smooth muscle relaxation\(^5\). We also verified this effect on intact vessels by demonstrating that 60 mmol/L K\(^+\)-aCSF solution failed to constrict PAs in the presence of 10 µmol/L U73122 (data not shown). These results argue against the initial hypothesis that PLC plays an important role in mediating mechano-activated responses in the cerebral microvasculature.

Next, involvement of PKC in PA myogenic regulation was tested with the specific PKC inhibitor chelerythrine. Similar to PLC inhibition, we found that chelerythrine (3 µmol/L) only attenuated myogenic tone of PAs by approximately 25% (Figure 2A, 2B), whereas the same level of PKC inhibition achieved almost 90% suppression on myogenic response in upstream pial vessels\(^1\). A previous study from our laboratory has also shown that 3 µmol/L chelerythrine completely blocked DAG analogue (DOG)-activated as well as cell swelling-stimulated cation currents in pial arterial myocytes, indicating that 3 µmol/L is adequate to effectively inhibit PKC activity in vascular smooth muscle\(^6\). In support of this notion, we also observed that P2Y4 and P2Y6 receptor agonist-induced constrictions of denuded PAs could be markedly attenuated by chelerythrine (3 µmol/L) (Figure 2C, 2D). It is not clear why PKC inhibition elicits a much smaller attenuating effect on pressure-induced constrictions compared to agonist-initiated responses, especially since we have established that
P2Y4/P2Y6 receptors are critical mechanosensors in PA myocytes. It is likely that stimuli-triggered contractile responses are differentially regulated by diverse cellular signaling mechanisms. In other words, activation of PKC can be substantially evoked by P2Y receptor ligands, but not so much by wall tension. This may result from different conformational changes on the G protein-coupled receptors (P2Y receptors in this case) stimulated by different stimuli, leading to activation of distinct G protein-associated signaling pathways. However, resolving this issue requires extensive biochemical studies on the analysis of crystal structures of GPCRs, which is beyond the scope of this dissertation.

Figure 1. Effects of the PLC inhibition on pressure-induced vasoconstriction in endothelium-denuded PAs. (A-B), representative diameter recordings showing the PLC
inhibitor U73122 has little effect on myogenic vasoconstriction at lower concentrations (≤ 3 µmol/L). Pressure-induced tone is substantially inhibited by U73122 at 10 µmol/L or higher through non-specific inhibitory effects on VDCCs.

Figure 2. PKC inhibition suppresses P2Y receptor agonist-induced vasoconstriction, but has little effect on myogenic tone in endothelium-denuded PAs. (A-B), A representative diameter recording and summary data showing a PKC inhibitor chelerythrine only partially reduces myogenic constriction of PAs at lower concentrations (≤ 3 µmol/L). A higher concentration (10 µmol/L) of Chelerythrine has non-specific blocking effects on VDCCs and nearly abolishes myogenic tone. (n=3) (C), A representative diameter recording showing Chelerythrine (3 µmol/L) reduces P2Y4 receptor ligand (UTPγS: 0.5 µmol/L)-induced constriction in denuded PAs. (D), Summary data showing chelerythrine (3 µmol/L) reduces P2Y4 (UTPγS: 0.5 µmol/L)
and P2Y6 (UDP: 1 µmol/L) receptor ligand-initiated constriction of denuded PAs. (n=4).

\*P<0.05, \*\*P<0.01 versus responses without chelerythrine.

4.2 The Constitutive Activity of ROCK in PA Smooth Muscle Cells

Rationale

Basal activity of TRPM4 channels in PA myocytes were previously reported by us and others using perforated whole-cell patch technique\(^8,9\). Interestingly, constitutive activity of TRPM4 channels appear to be more prominent in the myocytes isolated from PAs than those from pial arteries. As results from Chapter three of this dissertation have demonstrated that ROCK plays a key role in regulating TRPM4 in PAs, we hypothesize that ROCK is basally active to sustain TRPM4 activity, leading to depolarization and constriction in the absence of mechanical stimuli.

Results

In agreement with our prediction, the selective ROCK inhibitor significantly reduced constitutive TRPM4 currents in PA myocytes (Figure 3), indicating that basal ROCK activity contributes critically to sustaining channel activity in unstimulated smooth muscle cells. Because H1152 has no direct blocking effect on TRPM4 channels per se (see Supplementary Figure 3 in Chapter three), the reduced whole-cell currents should result from inhibited ROCK activity. These results are in concert with other observations by us that denuded PAs are quite depolarized and constricted at very low intraluminal pressures (3~5 mmHg) as illustrated in Chapters two and three. Additionally, H1152 significantly reduced intracellular [Ca\(^{2+}\)] in PAs held at 5 mmHg.
These observations point to ROCK being constitutively active in PA smooth muscle. This could be an essential vascular control element \textit{in vivo} for tight regulation of local blood flow. In that scenario, constitutive ROCK induces arteriolar constriction, even at low intravascular pressures, and positions the arterioles to be highly responsive to dilator or to additional constrictor stimuli. In accord with this proposal, several studies have shown that cerebral arterioles exhibit enhanced myogenic depolarization and tone compared to the larger diameter pial arteries\textsuperscript{10,11}, and that ROCK plays a critical part in regulating cerebral vascular tone \textit{in vivo}\textsuperscript{12}.

\textbf{Figure 3.} ROCK inhibition reduces basal TRPM4 channel activity in PA smooth muscle cells. (A-B), A representative recording and summary data showing H1152 (1 \textmu mol/L) reduces basal TRPM4 channel activity. (n=5) *P<0.05 versus Control.
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CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

Novelty and Significance

Results presented in Chapter two and Chapter three illustrate several novel aspects of myogenic control mechanisms in the cerebral microcirculation. These observations provide the first direct evidence that TRPM4 channels are coupled to mechano-sensitive G protein coupled receptors (GPCRs) to mediate pressure-evoked responses. This is demonstrated by the findings that P2Y receptor-mediated depolarization and constriction are highly dependent on the levels of TRPM4 channel expression and activity, and that P2Y receptor ligands markedly activate TRPM4 currents in PA smooth muscle cells. The important link between TRPM4 and GPCRs is corroborated by Gonzales et al., who recently reported that in cerebral pial arteries, TRPM4 channels are associated with upstream Ang II receptor activity, since the Ang II receptor antagonist losartan attenuates TRPM4 currents in pial arterial myocytes. To our surprise, the Ang II receptor does not seem to participate in tone regulation in the microvasculature since neither Ang II nor Ang II receptor inhibitor modulates arteriolar diameter. Several possibilities could account for the apparent lack of involvement of Ang II receptors. This may be attributed to reduced or absent receptor expression. It could also result from the effects of dilatory mechanisms (e.g. TRPV4) stimulated by Ang II receptor activation, which counterbalance the contractile influence of Ang II on the PAs. Either way, the functional mechanosensors seem to have shifted from Ang II receptor to P2Y receptors in the cerebral microcirculation, supporting the notion that PAs are
uniquely regulated by cellular signaling mechanisms that are distinct from other vascular beds. Notably, although different mechanosensors are present, both Ang II and P2Y receptor-initiated responses converge on activation of TRPM4 channels, indicating that TRPM4-mediated depolarization represents the basis for myogenic excitation-contraction coupling mechanisms. Presently, this mechanism has only been reported in the cerebral circulation. However, activation of depolarizing TRPM4 channels may be a common feature of most vascular beds as this channel has been detected in pulmonary, aortic and renal arteries.

The most recent work in this thesis project, described in Chapter three, reveals a novel contribution of ROCK to pressure-induced vasoconstriction. ROCK is widely accepted as a mediator of “Ca\(^{2+}\)-sensitization” via inhibiting myosin light chain phosphatase, thus promoting myosin light chain activation and smooth muscle contraction that is not dependent on increased Ca\(^{2+}\) delivery to the contractile machinery. Interestingly, contrasting results from Chapter three of this dissertation provide the first evidence that RhoA/ROCK signaling also regulates cytosolic [Ca\(^{2+}\)] through TRPM4-dependent smooth muscle depolarization. Therefore, at least in cerebral PAs, by both elevating average [Ca\(^{2+}\)] and increasing Ca\(^{2+}\)-sensitivity of the contractile apparatus, ROCK stimulates dual effects to modulate myosin-actin interaction and smooth muscle contractility. This may partially account for the significantly enhanced myogenic depolarization, [Ca\(^{2+}\)]\(_{i}\) elevations, and constrictions observed in PAs compared to pial vessels over the same range of intravascular pressures. Though regulation of ion channel activity and membrane potential by ROCK has been previously reported, Chapter three provides the first definitive evidence that in cerebral PAs, RhoA/ROCK
activity plays an important yet underappreciated role in the Ca\textsuperscript{2+}-dependent electromechanical coupling mechanisms.

Several G\textsubscript{q/11} protein-coupled receptors have been recently identified as mechanosensors in expression systems. One in particular, the Ang II receptor was found to mediate stretch-induced responses through phospholipase C (PLC)-associated pathways in both HEK cells and pial arterial smooth muscle cells\textsuperscript{1,11}. However, these signaling pathways do not appear to regulate myogenic response in PAs based on the evidence that neither PLC nor PKC has any sizable effect on PA myogenic tone at concentrations that cause near maximal relaxation in the upstream pial vessels. Instead, Chapter three of this dissertation unveils the key involvement of G\textsubscript{12/13} protein and downstream RhoA/ROCK signaling in the myogenic control mechanisms. This thesis work has advanced the concept that mechano-sensitivity is an important feature of G\textsubscript{q/11}-coupled receptors. Specifically, several stretch-sensitive G\textsubscript{q/11}-coupled receptors, such as P2Y purinergic and endothelin-1 receptors, can also stimulate G\textsubscript{12/13} protein and elicit Rho-associated signaling cascades\textsuperscript{12,13}. As myogenic tone represents one of the most fundamental vascular functions, it is therefore not surprising to find that its transduction mechanisms are dynamically regulated by both G\textsubscript{q/11} protein-associated pathways and G\textsubscript{12/13} protein-mediated, Rho-facilitated signaling. Notably, that PA myogenic response is largely dependent on Rho reactivity further emphasizes that PAs rely on unique signaling pathways to facilitate pressure-induced responses.

As numerous cardiovascular diseases influence the reactivity of cerebral PAs, which in turn lead to further deterioration of brain function\textsuperscript{14-18}, characterization of distinct cellular signaling factors and pathways in the cerebral microcirculation may
provide useful therapeutic targets specific for treating microvascular diseases in the brain without triggering side effects on the surface brain arteries or peripheral circulation.

**Future Directions**

Following from these interesting findings, one major aim of future work will be to determine the underlying molecular mechanisms responsible for Rho-elicited activation of TRPM4 channels. There are several possibilities. First of all, the observations that TRPM4 currents can be acutely attenuated and potentiated by ROCK inhibition and RhoA activation, respectively, strongly point to direct modulatory effects of RhoA/ROCK on TRPM4 channels. A previous study from Nilius and coworkers has shown that PKC enhances the Ca\(^{2+}\)-sensitivity of TRPM4 channels by phosphorylating two serine residues in the C-terminus\(^{19}\). Since both PKC and ROCK are serine/threonine kinases with similar consensus substrate phosphorylation sites\(^{20}\), it is therefore postulated that ROCK also directly phosphorylates TRPM4 to decrease the threshold of \([\text{Ca}^{2+}]_i\) for channel activation.

Moreover, potentiation of TRPM4 activity by PKC also involves increased channel translocation to the plasma membrane\(^{21}\), indicating that PKC triggers two major effects on the channel protein; not only does it acutely augment channel activity by modulating its sensitivity to Ca\(^{2+}\), but it also elevates channel density as a mechanism to increase membrane excitability. As PAs are highly dependent on ROCK to govern TRPM4-mediated myogenic responses, it is plausible that ROCK also contributes to sustaining a high level of TRPM4 expression in arteriolar smooth muscle, which may account for the physiologically enhanced myogenic control in the microcirculation. In
agreement with this possibility, RhoA/ROCK signaling has been reported to regulate ion channel expression in vascular smooth muscle. For example, RhoA significantly increases the percentage of ENaC in the plasma membrane versus the cytosol in expression systems\textsuperscript{8}. In addition, RhoA/ROCK was found to activate K\textsubscript{v}1.2 channel endocytosis pathways and block recycling of endocytosed channel back to the plasma membrane, leading to a significantly diminished level of functional channels\textsuperscript{10}.

Thirdly, participation of actin-regulatory systems in vascular smooth muscle contraction has long been established\textsuperscript{22-26}. Recent evidence suggests that actin dynamics are implicated in RhoA/ROCK-associated responses in vascular smooth muscle. In expression systems, RhoA/ROCK was shown to affect K\textsubscript{v}1.2 channel trafficking through an actin-dependent mechanism that encompasses the LIM-kinase/cofilin pathway\textsuperscript{10}. This concept was further verified in rat cerebral pial arterial myocytes, where ROCK facilitates UTP-induced actin polymerization\textsuperscript{27}. Pharmacological disruption of actin cytoskeleton substantially prevents UTP-initiated, ROCK-dependent K\textsubscript{v} current inhibition\textsuperscript{27}. Moreover, this study provides important evidence that the force-generating actin dynamics also contribute to membrane excitability regulation, since disrupting actin structure significantly inhibits UTP-evoked smooth muscle depolarization in cerebral arteries\textsuperscript{27}. Intriguingly, early studies have demonstrated that actin polymerization is stimulated by increased intravascular pressure, and subsequently brings about smooth muscle contraction\textsuperscript{25}. Based on these converging evidence, it is possible that, in addition to direct force generation, ROCK-mediated actin remodeling may also determine TRPM4 channel activity, hence mediating pressure-induced responses in cerebral PAs. Although the mechanisms underlying TRPM4 stimulation in the vasculature have been the subject
of substantial research effort, it is clear that additional, extensive and detailed molecular, biochemical and biophysical assessments will be required to advance this field.

As previously mentioned, identifying the unique features of physiological control mechanisms in the cerebral PAs is indispensable for developing novel medical strategies regarding prevention and treatment of microvascular diseases in the brain. Even though the pathological implications of P2Y receptors and TRPM4 have not been reported in cerebral microcirculation, impaired PA myogenic regulation observed in several stroke-related diseases implies that these essential signaling factors may be pathologically altered. Therefore, another major aim of future research will be to investigate these signaling pathways in vascular disease models, and to discover possible new targets that may allow for “bench to bedside” transition, which eventually leads to new drugs and practical treatment options.

One possible intervention is restoring myogenic tone of PAs after subarachnoid hemorrhage. Subarachnoid hemorrhage (SAH), commonly resulting from aneurysm rupture, is defined as the extravasation of blood into the subarachnoid space. Rapid discharge of blood immediately after aneurysm rupture may result in an acute elevation of intracranial pressure, leading to cessation of blood flow, global cerebral ischemia and death. Further, a significant portion of patients who survive the initial hemorrhage are still severely impacted by delayed and sustained constriction of cerebral arteries and penetrating arterioles (cerebral vasospasm), which is the major contributor to death and disability following SAH. An experimental rat SAH model is widely used in research of this type. Following repeated injections of tail blood into the cisterna magna on two consecutive days, rats exhibit many of the features of SAH found in humans, including
vasospasm, behavioral deficits, and impaired neurovascular coupling\textsuperscript{6, 28, 29}. Using the above SAH model, Nystoriak et al. observed that isolated PAs from SAH rats exhibited markedly enhanced pressure-induced vasoconstriction compared to control rats\textsuperscript{6}. Interestingly, this increase in myogenic responsiveness is tightly correlated with augmented smooth muscle depolarization and arteriolar wall Ca\textsuperscript{2+}. Tone/[Ca\textsuperscript{2+}] relationships are very much similar between PAs from control and SAH animals, indicating that the Ca\textsuperscript{2+} sensitivity of the contractile apparatus is unaffected by SAH\textsuperscript{6}. Rather, increased myogenic reactivity can be attributed to SAH-induced membrane depolarization, which may result from activation of depolarizing ion channels (TRPM4, TRPC6) or suppression of hyperpolarizing K\textsuperscript{+} conductance.

Wellman and colleagues have demonstrated that inhibition of K\textsubscript{v} currents contributes to SAH-triggered membrane depolarization and constriction of cerebral pial arteries through a mechanism involving oxyhemoglobin-evoked activation of tyrosine kinase EGF receptor and K\textsubscript{v} channel internalization\textsuperscript{30-32}. However, effects of SAH on cerebral PAs may also depend on RhoA/ROCK-TRPM4-mediated depolarizing mechanisms. In support of this hypothesis, mRNA levels of RhoA and ROCK are elevated in basilar arteries that exhibit vasospasm after SAH\textsuperscript{33}. As the Ca\textsuperscript{2+}-sensitization mechanisms appears to be unaffected by SAH in PA myocytes\textsuperscript{6}, the enhanced RhoA/ROCK expression may logically elicit two parallel effects: activating TRPM4 channels and inhibiting K\textsubscript{v} channels, both of which lead to potentiated myogenic depolarization and vasoconstriction of cerebral PAs. Furthermore, results from clinical and experimental investigations show that endothelin-1, a potent vasoconstrictor, is a major cause of cerebral vasospasm after SAH\textsuperscript{34}. Elevated levels of ET-1 have been
verified in the cerebrospinal fluid and plasma of patients after SAH and cerebral infarction\textsuperscript{34, 35}. In addition, antagonizing ET\textsubscript{A} receptors can reverse ET-1-elicted vasoconstriction\textsuperscript{34}, suggesting that activation of smooth muscle ET\textsubscript{A} receptors is indispensable for sustained vasospasm.

Among the ET-1-triggered cellular mechanisms involving Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent contractions, RhoA/ROCK pathway is stimulated by ET-1 as observed in rabbit basilar arteries\textsuperscript{36}. It is thus plausible that ET-1-triggered ROCK signaling subsequently activates TRPM4 channels. More supporting evidence comes from the observation that ET\textsubscript{A} receptor expression level is remarkably increased following a canine two-hemorrhage SAH model\textsuperscript{37}. Since the ET\textsubscript{A} receptor has been identified as a mechanosensor in expression systems, what might be happening in the cerebral microcirculation is that, following SAH, expression of ET\textsubscript{A} receptor is elevated in the PA myocyte plasma membrane, which confers increased pressure-induced responses, as well as dramatically enhanced ET-1-evoked vasoconstriction due to increased circulating levels of ET-1. Both mechanisms converge on activation of RhoA/ROCK signaling, which could lead to TRPM4-mediated depolarization and pronounced vasoconstriction. Further, prolonged stimulation of ET\textsubscript{A} receptors further increases ROCK expression, leading to sustained vasospasm.

As illustrated above, RhoA/ROCK signaling, TRPM4 channels and VDCCs appear to be the common denominators in the proposed mechanisms. Thus, inhibiting this signaling pathway may be very effective in treating or preventing cerebral vasospasm. The specific VDCC blocker nimodipine is clinically approved for this pathology. In clinical trials, nimodipine markedly reduced the incidence of delayed neurological
deficits and cerebral infarcts\textsuperscript{38}. However, it did not significantly influence the incidence of moderate to severe angiographic vasospasm\textsuperscript{39}. Also, prophylactic use of nimodipine revealed minimal improvements on neurological outcome\textsuperscript{38}. Further, use of fasudil after SAH, a selective ROCK inhibitor, is also a matter of controversy. It was reported in one randomized clinical trial that this drug exhibited significant reductions in angiographic vasospasm, symptomatic vasospasm and radiographic infarcts with improved clinical outcome\textsuperscript{40}. Nevertheless, another clinical trial showed that fasudil failed to improve the incidence of symptomatic vasospasm and radiographic infarcts\textsuperscript{41}, indicating that clinical application of fasudil requires further validation. Judging by the current situation, more effective measures are needed.

The side effects of oral administration of nimodipine range from dizziness, lightheadedness, flushing, swelling ankles/feet to fainting and arrhythmia, most of which result from unwanted systemic effects of nimodipine on peripheral vessels and the heart. The ROCK inhibitor fasudil also relaxes vascular beds in addition to the ones undergoing vasospasm. One way to resolve the issues mentioned above would be to limit the affected region to the brain by directly administrating drugs to the cerebral spinal fluid (CSF) via the cisterna magna. Since CSF enters the parenchyma along paravascular spaces that surround penetrating arterioles\textsuperscript{42}, drugs applied this way gain direct access to the cerebral PA smooth muscle cells. This method was initially used to create an SAH model by injecting blood into CSF. Our laboratory has advanced the use of this method of drug delivery and demonstrated that in vivo injection of TRPM4 antisense oligodeoxynucleotides effectively reduced TRPM4 channel expression in both pial and parenchymal arteries, and functionally suppressed PA myogenic tone\textsuperscript{43}. One advantage of
this method is that the medicine is locally delivered to the brain, thus concentrations can be adjusted to a much lower level, which further reduces possible side effects. Because the administered drugs are in CSF, they can now elicit effects without passing through blood-brain barrier, making drug development less complicated. Further, in addition to using pharmacological compounds, such as VDCC blockers and ROCK inhibitors, this method also opens the door to using molecular constructs, such as antisense or siRNA, which could effectively decrease the expression levels of RhoA, ROCK, TRPM4 or VDCC in the cerebral vasculature and prevent vasospasm.

However, this method is not without disadvantages. The biggest problem of this approach is that it is extremely invasive. Currently, it may only be applied to lab animals to determine the effects of different compounds on sustained vasospasm. Furthermore, as CSF cycles through the ventricles in the brain and spinal cord, drugs in the CSF may also affect the central nervous system and lead to severe adverse effects. Nevertheless, it is possible that with careful and extensive characterization and validation, this method will eventually become a useful therapeutic strategy for treating cerebral vascular and microvascular pathologies.

**Conclusions**

In this dissertation, data are presented in support of the central role of TRPM4 channels in facilitating mechano-sensitive P2Y receptor-initiated, RhoA/ROCK signaling-dependent myogenic depolarization, \([\text{Ca}^{2+}]\), elevation and vasoconstriction in cerebral PAs. The unique involvement of ROCK in the \(\text{Ca}^{2+}\)-dependent
mechanochemical coupling mechanisms in PA myocytes may represent a novel target for prevention and treatment of cerebral microvascular diseases.

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APPENDIX: OTHER PUBLISHED WORK


Introduction

Myogenic tone is a fundamental aspect of vascular behavior in resistance arteries. Despite its importance, little is known about the mechanisms involved in control of myogenic regulation in the cerebral microcirculation. A recent study comprising heterologous expression of G protein coupled receptors and TRPC6 channels as well as examination of stretch-induced mechanisms in brain pial and systemic arteries, lead to the important proposal that Angiotensin II receptors function as mechanosensors to mediate myogenic reactivity in vascular smooth muscle cells. Interestingly, although parenchymal arterioles display substantial pressure-induced tone, the Ang II receptor inhibitor candesartan showed no effect on vascular diameter of the PAs. This was in agreement with an absence of vasoconstrictor responses to Ang II itself in these vessels. In light of the lack of contribution of Ang II receptors in vascular reactivity in PAs, other GPCRs in myogenic tone regulation were investigated. The primary focus was on P2Y receptors, as previous evidence in mesenteric resistance arteries indicated that pyrimidine receptors may contribute to pressure-evoked responses of systemic arteries. Therefore, in the present study, expression and functional involvement of P2Y receptors in PA myogenic behavior were explored. My contribution to this journal article involved RT-
PCR experiments for P2Y receptor message. Methods and results of this component in the study are as follows.

**Methods**

RNA was prepared from isolated arterioles with endothelium present. First-strand cDNA was prepared from 720 ng total RNA using the Sensiscript Reverse Transcriptase kit (Qiagen). PCR reactions were hot started at 94 °C for 10 minutes and exposed to 38 cycles of 94 °C for 60 seconds, 60 °C for 90 seconds, and 72 °C for 60 seconds, and followed by 72 °C final extension for 10 minutes. All reaction products were resolved on 1.8% agarose gels. GAPDH transcript was used as a positive control. Primer sequences are shown in Table 1.

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>P2Y2</td>
<td>5'-TTCCACGTCAACCCGACACCCTTTATTACT-3'</td>
<td>5'-CGATCCCCAAGCCACACATACAAATGTTG-3'</td>
</tr>
<tr>
<td>P2Y4</td>
<td>5'-GTAACTGCCACCTCCTACTA-3'</td>
<td>5'-GTCCGCCACCTGCTGAT-3'</td>
</tr>
<tr>
<td>P2Y6</td>
<td>5'-CGCAACCGCACTGTCTG-3'</td>
<td>5'-TCTCTGCCCTGCACTTG-3'</td>
</tr>
</tbody>
</table>

**Results**

I first determined that message for predominant vascular P2Y receptors (P2Y2, P2Y4 and P2Y6) is present in parenchymal arteriolar extract. In agreement with previous studies examining localization of P2Y receptors in coronary and cerebral pial arteries, message for each of these receptors is present in PAs (Figure 1). P2Y2 and P2Y6 appeared to be more abundantly expressed than P2Y4 receptors in parenchymal arterioles. However, because RT-PCR tests were performed on intact arterioles, whether
the mRNA message came from smooth muscle cells or endothelium remains an open question. Further myography experiments from this study illustrated that P2Y2 receptors are not involved in regulation of vascular tone, suggesting either P2Y2 receptors are not present in PA smooth muscle, or they are not coupled to contractile cellular signaling pathways. By contrast, both P2Y4 and P2Y6 receptor agonists elicited concentration-dependent vasoconstriction in endothelium-denuded PAs, indicating that these two receptors are present and functionally active in PA smooth muscle cells (data not shown).

![Image of RT-PCR gel]

**Figure 1.** Message for P2Y2, P2Y4, and P2Y6 receptors is present in PA homogenates as measured using RT-PCR. NT = No Template control.

To test the possible link between P2Y receptor activity and myogenic tone in PAs, we next investigated the effects of P2Y4 and P2Y6 antisense ODNs on pressure-induced vasoconstriction in PAs. I found that expression of P2Y4 and P2Y6 receptors was substantially reduced by corresponding antisense ODNs, as indicated by semi-quantitative PCR (Figure 2). These ODNs were quite selective for their targets as antisense designed against P2Y4 receptors had no effect on P2Y6 receptor expression (Figure 2A) and similarly, P2Y6 antisense significantly reduced P2Y6 receptor
expression without affecting P2Y4 message levels (Figure 2B). Further functional studies illustrated that myogenic tone in PAs treated with antisense ODNs targeting the two receptors was reduced by ~50%, respectively, compared with control (sense-treated) arterioles (data not shown), indicating that P2Y4 and P2Y6 receptors play critical roles in mediating myogenic responses in cerebral PAs.

**Figure 2. Effects of antisense oligodeoxynucleotides on P2Y receptor message levels.** (A-B), Semi-quantitative PCR indicates a specific and substantial decrease in P2Y4- or P2Y6- message levels in arteries exposed to antisense versus sense ODNs. Sense or antisense ODN-treated PAs from three animals were pooled in each case to assess relative message abundance.

**Significance**

The RT-PCR results in Figure 1 indicated the existence of the vasoactive P2Y receptors in cerebral PAs. It set the ground for further investigation of their functional significance in pressure-induced responsiveness. Semi-quantitative PCR data in Figure 2 supported this study by validating the effectiveness and specificity of designed antisense
ODNs targeted to the corresponding receptors. As the subsequent functional studies showed significant reduction of myogenic constriction by either P2Y4 or P2Y6 receptor antisense, these RT-PCR tests serve as proper control experiments to preclude the possibility of cross-suppressing effects of ODNs on these two proteins, and provide more confidence in drawing the conclusion that both P2Y4 and P2Y6 receptors are essential contributors to myogenic tone development in cerebral PAs.

References

