Theoretical Investigation of Intra- and Inter-cellular Spatiotemporal Calcium Patterns in Microcirculation

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DOI: 10.25148/etd.FI15032102
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THEORETICAL INVESTIGATION OF INTRA- AND INTER-CELLULAR SPATIOTEMPORAL CALCIUM PATTERNS IN MICROCIRCULATION

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

BIOMEDICAL ENGINEERING

by

Jaimit Parikh

2015
To:  Dean Amir Mirmiran  
     College of Engineering and Computing  

This dissertation, written by Jaimit Parikh, and entitled Theoretical Investigation of Intra- and Inter-cellular Spatiotemporal Calcium Patterns in Microcirculation, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Florida International University, 2015
DEDICATION

Dedicated to my parents Bharat and Minakshi Parikh
ACKNOWLEDGMENTS

I would like to express my sincere thanks to my advisor, Dr. Nikolaos Tsoukias for being such a great mentor; he has been a huge part in the accomplishment of this dissertation. I also want to thank Dr. Adam Kapela for his continued guidance throughout my career at Florida International University, and my lab members, Sridevi Nagaraja, Shabnam Namin, Daniel Rodriguez, Jiali Lei, Florencia Goluboff, Tushar Gadkari, and Kumpal Madrasi for making my time fly.

I extend my appreciation to the Biomedical Engineering Department and the department chair, Dr. Ranu Jung, as well as my committee members, Dr. Anthony McGoron, Dr. Jessica Ramella-Roman, Dr. Malek Adjouadi and Dr. Sharan Ramaswamy for the continued support.

I want to acknowledge the University Graduate School at Florida International University for awarding me the Dissertation Year Fellowship (DYF) and Doctoral Evidence Acquisition Fellowship (DEA). It allowed me to completely focus on completing my dissertation and finishing in a timely manner.

I wish to thank my parents, Bharat and Minakshi, and my sisters, Rachana and Kruti Parikh, for their unconditional love and support. I would also like to thank Samantha Dages for being a fantastic friend and for all the wonderful memories, as well as Sasmita Rath, Ashesh Shah, Chintan Kinariwala, Jacky Gangwani, Jigar Seth, Nikita Shah, Rutvi Trivedi, and all others who made my time more enjoyable. Finally, I would like to thank my cousins, Chirayu and Himanshu Shah, for all their support during my initial years in the United States.
ABSTRACT OF THE DISSERTATION

THEORETICAL INVESTIGATION OF INTRA- AND INTER-CELLULAR SPATIOTEMPORAL CALCIUM PATTERNS IN MICROCIRCULATION

by

Jaimit Parikh

Florida International University 2015

Miami, Florida

Professor Nikolaos Tsoukias, Major Professor

Microcirculatory vessels are lined by endothelial cells (ECs) which are surrounded by a single or multiple layer of smooth muscle cells (SMCs). Spontaneous and agonist induced spatiotemporal calcium (Ca\(^{2+}\)) events are generated in ECs and SMCs, and regulated by complex bi-directional signaling between the two layers which ultimately determines the vessel tone. The contractile state of microcirculatory vessels is an important factor in the determination of vascular resistance, blood flow and blood pressure. This dissertation presents theoretical insights into some of the important and currently unresolved phenomena in microvascular tone regulation. Compartmental and continuum models of isolated EC and SMC, coupled EC-SMC and a multi-cellular vessel segment with deterministic and stochastic descriptions of the cellular components were developed, and the intra- and inter-cellular spatiotemporal Ca\(^{2+}\) mobilization was examined.

Coupled EC-SMC model simulations captured the experimentally observed localized subcellular EC Ca\(^{2+}\) events arising from the opening of EC transient receptor vanilloid 4 (TRPV4) channels and inositol triphosphate receptors (IP\(_3\)Rs). These localized EC Ca\(^{2+}\)
events result in endothelium-derived hyperpolarization (EDH) and Nitric Oxide (NO) production which transmit to the adjacent SMCs to ultimately result in vasodilation. The model examined the effect of heterogeneous distribution of cellular components and channel gating kinetics in determination of the amplitude and spread of the Ca\(^{2+}\) events. The simulations suggested the necessity of co-localization of certain cellular components for modulation of EDH and NO responses. Isolated EC and SMC models captured intracellular Ca\(^{2+}\) wave like activity and predicted the necessity of non-uniform distribution of cellular components for the generation of Ca\(^{2+}\) waves. The simulations also suggested the role of membrane potential dynamics in regulating Ca\(^{2+}\) wave velocity. The multi-cellular vessel segment model examined the underlying mechanisms for the intercellular synchronization of spontaneous oscillatory Ca\(^{2+}\) waves in individual SMC.

From local subcellular events to integrated macro-scale behavior at the vessel level, the developed multi-scale models captured basic features of vascular Ca\(^{2+}\) signaling and provide insights for their physiological relevance. The models provide a theoretical framework for assisting investigations on the regulation of vascular tone in health and disease.
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activate directly ClCa and BKCa channels, and indirectly NSC channels through Ca^{2+}-
sensitive PLC. Variations in channels’ activity generate Vm oscillations, which
spread through gap junctions to neighboring SMCs (right) and modulate VOCC
channels and RyRs. Oscillatory IP3, generated by Ca^{2+}-dependent PLC, can diffuse
to other cells, and may also have a synchronizing effect by acting on IP3 receptors.
Ca^{2+} elevation in one cell may increase Ca^{2+} in neighboring cells by direct diffusion
of Ca^{2+} through the gap junctions if SMC-to-SMC gap junctions have sufficient
permeability. .................................................................................................................. 175

Figure 5.5 (A) Bifurcation diagrams of Ca^{2+} versus NE for single SMC model
without EC (solid line) and with EC stimulated by Ach (dashed-dotted line). (B)
Representative distribution of oscillation periods in a model with 10 oscillatory
SMCs before coupling (o), after coupling without synchronization (×) and with
synchronization (*). (C) Forced Ca^{2+} oscillations in non-oscillatory SMC induced by
IP3 and electrical coupling with an oscillatory SMC. (D) Unsynchronized Ca^{2+}
oscillations induced by 0.3 μM NE were synchronized transiently by a short 10 ms
pulse of high extracellular K+ (35 mM) resetting simultaneously all SMC oscillators. 186

Figure 5.6 A population of five SMCs and five ECs is stimulated by NE (0.8 μM)
and Ach (1 a.u.). Ca^{2+} oscillations in the SMCs are shown. Clamping ClCa channels

xviii
did not elicit desynchronization (A), but total block of $\text{Cl}_{\text{Ca}}$ current can lead to loss of synchronization (B).

Figure 5.7 Simulations in a population of 80 SMCs and 80 underlying ECs arranged into a cylinder. In the presence of 0.8 NE and 1 a.u. Ach, inhibition of intercellular IP$_3$ diffusion desynchronized the oscillations, similarly to results with five SMCs from Figure 5.2-A.

Figure 5.8 Cluster formation in a population of 80 identical SMCs arranged into a cylinder. When stimulated by 0.4 µM NE from the same initial conditions, the cells produce in-phase oscillations (left). A short local perturbation of the system (10% NE increase for 1 minute at cell #1) destabilizes the oscillations and initiates formation of clusters (right). Cells with the same color are in-phase. The curve indicates circular boundary conditions.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
</tr>
<tr>
<td>MEGJ</td>
<td>Myoendothelial gap junction</td>
</tr>
<tr>
<td>MP</td>
<td>Myoendothelial projections</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>$V_m$</td>
<td>membrane potential</td>
</tr>
<tr>
<td>$Ca^{2+}$</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>$K^+$</td>
<td>Potassium</td>
</tr>
<tr>
<td>$Na^+$</td>
<td>Sodium</td>
</tr>
<tr>
<td>$Cl^-$</td>
<td>Chloride</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>Inositol (1,4,5)-trisphosphate</td>
</tr>
<tr>
<td>CM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CQSN</td>
<td>Calsequestrin</td>
</tr>
<tr>
<td>MLCP</td>
<td>Myosin light-chain phosphatase</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>Phosphatidylinositol-4-5-biphosphate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>Hb</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------</td>
</tr>
<tr>
<td>Hbα</td>
<td>Hemoglobin α</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylate cyclase</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>PGI₂</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>EET</td>
<td>Epoxyeicosatrienoic acids</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptors</td>
</tr>
<tr>
<td>IP₃R</td>
<td>IP₃ receptor</td>
</tr>
<tr>
<td>P2XR</td>
<td>Purinergic P2X receptor</td>
</tr>
<tr>
<td>VOCC</td>
<td>Voltage gated calcium channels</td>
</tr>
<tr>
<td>SK&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>Small conductance calcium activated potassium channel</td>
</tr>
<tr>
<td>IK&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>Intermediate conductance calcium activated potassium channel</td>
</tr>
<tr>
<td>BK&lt;sub&gt;Ca&lt;/sub&gt;</td>
<td>Large conductance calcium activated potassium channel</td>
</tr>
<tr>
<td>NaK</td>
<td>Sodium-potassium ATPase pump</td>
</tr>
<tr>
<td>NCX</td>
<td>Sodium-calcium exchanger</td>
</tr>
<tr>
<td>PMCa</td>
<td>Plasma membrane Ca&lt;sup&gt;2+&lt;/sup&gt; ATPase</td>
</tr>
<tr>
<td>SOC</td>
<td>Store operated Ca&lt;sup&gt;2+&lt;/sup&gt; channels</td>
</tr>
<tr>
<td>SERCA</td>
<td>Sarcoplasmic reticulum Ca&lt;sup&gt;2+&lt;/sup&gt; ATPase</td>
</tr>
<tr>
<td>NSC</td>
<td>Non-selective cation channels</td>
</tr>
<tr>
<td>Kir</td>
<td>Inward rectifying potassium channels</td>
</tr>
<tr>
<td>Kᵥ</td>
<td>Voltage gated potassium channels</td>
</tr>
<tr>
<td>VRAC</td>
<td>Volume regulated anion channels</td>
</tr>
<tr>
<td>CaCC</td>
<td>Calcium-activated Cl⁻ channels</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>ClCa</td>
<td>Calcium activated Cl⁻ channels</td>
</tr>
<tr>
<td>TRPV4</td>
<td>Transient receptor potential vanilloid channel 4</td>
</tr>
<tr>
<td>TRPM4</td>
<td>Transient receptor potential melastatin 4</td>
</tr>
<tr>
<td>R⁣gi</td>
<td>Gap junction resistance</td>
</tr>
<tr>
<td>CCE</td>
<td>Capacitive calcium entry</td>
</tr>
<tr>
<td>EDH</td>
<td>Endothelium derived hyperpolarization</td>
</tr>
<tr>
<td>EDHF</td>
<td>Endothelium derived hyperpolarizing factor</td>
</tr>
<tr>
<td>EDRF</td>
<td>Endothelium derived relaxing factor</td>
</tr>
<tr>
<td>CICR</td>
<td>Calcium induced calcium release</td>
</tr>
<tr>
<td>GHK</td>
<td>Goldman-Hodgkin-Katz</td>
</tr>
<tr>
<td>FEM</td>
<td>Finite element method</td>
</tr>
<tr>
<td>RMA</td>
<td>Rat mesenteric arteries</td>
</tr>
<tr>
<td>CPA</td>
<td>Cyclopiazonic acid</td>
</tr>
<tr>
<td>P₀</td>
<td>Open probability</td>
</tr>
<tr>
<td>NP₀</td>
<td>Average number of open channels</td>
</tr>
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</table>
CHAPTER 1-INTRODUCTION
1.1 Motivation

High blood pressure or hypertension affects 33.5% of US adults (equivalent to around 76.4 million people) and poses a serious health concern. Estimations predict an additional 27 million people (10% increase) to suffer from hypertension by 2030 and projects a 100% increase in the medical cost (to reach $240 billion) for hypertension (Roger et al. 2012). High blood pressure can lead to increased risk of added cardiovascular diseases including stroke, heart attack and others. Peripheral vascular resistance is increased in virtually all models of hypertension. The total peripheral resistance in turn depends on the arteriolar diameter. Calcium (Ca\(^{2+}\)) mobilization in microvascular cells plays a central role in the regulation of microcirculatory diameter. Complex mechanisms at the molecular and cellular levels participate in the regulation of vascular diameter. Elucidating the spatiotemporal Ca\(^{2+}\) signaling pathways in the vasculature will provide better understanding of the mechanisms that regulate vascular resistance, blood flow and pressure in health and disease and in future promote better treatments for hypertension.

1.2 Background

The increase in intracellular Ca\(^{2+}\) in the smooth muscle cell (SMC) induces contraction of these cells through modulation of MLCK (myosin light-chain kinase) and MLCP (myosin light-chain phosphatase) activities, while in the endothelial cell (EC), Ca\(^{2+}\) increases regulate the release of vasoactive substances. Complex bi-directional myoendothelial interactions via paracrine/gap junction signaling (Yashiro and Duling 2000; Dora et al. 2000; Dora, Doyle, and Duling 1997) modulate the Ca\(^{2+}\) and membrane potential (\(V_m\)) dynamics in both the cells. The myoendothelial interactions often include diffusion of endothelium derived relaxing factors such as nitric oxide (NO) (Palmer,
prostacyclin (PGI$_2$) (Flower 2006; Vane 1964) and endothelium derived hyperpolarization (EDH) (Chen, Suzuki, and Weston 1988; Garland, Hiley, and Dora 2011) which transmit to adjacent SMCs and result in vessel relaxation (Figure 1.1).

**Figure 1.1** Illustration of major endothelial mediated vasodilatory signaling pathways

Since the Ca$^{2+}$ levels in the vascular cells are governed through complex cellular processes it is expected that these changes are highly organized and occur at various spatial (global or local) and temporal levels (sustained or transient) (Sanders 2001; Berridge 1997). Ca$^{2+}$ signals range from subcellular events confined to a small region (locally confined events arising from stochastic opening of Ca$^{2+}$-permeable channels) to whole cell Ca$^{2+}$ events. Moreover the Ca$^{2+}$ signals can spread in between the cells to result in inter-cellular Ca$^{2+}$ mobilization. Figure 1.2 highlights different subcellular localized Ca$^{2+}$ events observed in the vascular cells mediated through Ca$^{2+}$-permeable channels (such as voltage gated Ca$^{2+}$ channels (VOCC), store operated channels (SOC),
Ryanodine receptor (RyR), inositol 1,4,5-triphosphate receptors (IP₃R), Transient receptor potential channel (TRPV4), and Purinergic P2X receptor (P2XR) channels. Earlier reviews on Ca²⁺ dynamics in a wide variety of cell types can be found in the literature and provide an extensive of this field (Berridge 1997; Berridge, Bootman, and Roderick 2003; Berridge, Lipp, and Bootman 2000; Combettes, Dupont, and Parys 2004; Dupont, Combettes, and Leybaert 2007; Dupont et al. 2000; Falcke 2004; Schuster, Marhl, and Hofer 2002) including some specifically for vascular cells (Amberg and Navedo 2013; Hill-Eubanks et al. 2011; Sanders 2001; Tsoukias 2011; Moccia, Berra-Romani, and Tanzi 2012).

![Diagram](image)

**Figure 1.2** Schematic of localized Ca²⁺ events reported in the ECs and SMCs

Subcellular to whole cell Ca²⁺ events (i.e. puffs, sparks, sparklets, waves and whole cell events) arising in the microcirculatory vascular ECs and SMCs, and their regulation of the vascular tone are reviewed in this chapter. The characteristics of various Ca²⁺ events are described in Table 1.1.
Table 1.1 Characteristics of subcellular to whole cell Ca^{2+} events

<table>
<thead>
<tr>
<th>Ca^{2+} Event Type</th>
<th>Freq (Hz)</th>
<th>Spread (µm²)</th>
<th>Rise time (ms)</th>
<th>Decay time (t_{1/2}) (ms)</th>
<th>Velocities</th>
<th>Cell Type</th>
<th>REF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca^{2+} sparks (RyRs)</td>
<td>0.2-1.3</td>
<td>14</td>
<td>20</td>
<td>50-60</td>
<td>NA</td>
<td>SMCs</td>
<td>(Jaggar et al. 2000)</td>
</tr>
<tr>
<td>Ca^{2+} puffs (IP_{3}Rs)</td>
<td>0.02-0.2</td>
<td>2-4</td>
<td>57-160</td>
<td>107-375</td>
<td>NA</td>
<td>ECs and SMCs</td>
<td>(Ledoux et al. 2008; Narayanan, Adebiyi, and Jaggar 2012; Tovey et al. 2001)</td>
</tr>
<tr>
<td>Ca^{2+} pulsars (IP_{3}Rs)</td>
<td>0.008-0.1</td>
<td>14-16</td>
<td>163-212</td>
<td>146-168</td>
<td>NA</td>
<td>ECs</td>
<td>(Ledoux et al. 2008)</td>
</tr>
<tr>
<td>Ca^{2+} wavelets (IP_{3}Rs)</td>
<td>0.2-0.23</td>
<td>40-51</td>
<td>Longer duration than pulsars</td>
<td>40</td>
<td>ECs</td>
<td>(Tran et al. 2012)</td>
<td></td>
</tr>
<tr>
<td>Ca^{2+} sparklet (VOCC)</td>
<td>NA</td>
<td>0.8</td>
<td>( \tau_{fast}=23 ) ( \tau_{slow}=200 )</td>
<td>NA</td>
<td>SMCs</td>
<td>(Santana et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>Ca^{2+} Sparklet (TRPV4)</td>
<td>0.02-0.2</td>
<td>11</td>
<td>( \tau=37 ) Duration few seconds</td>
<td>NA</td>
<td>ECs</td>
<td>(Sonkusare et al. 2012)</td>
<td></td>
</tr>
<tr>
<td>Junctional Ca^{2+} Transient (P2XR)</td>
<td>-</td>
<td>25</td>
<td>Duration: ( t_{1/2} =150 )</td>
<td>NA</td>
<td>SMCs</td>
<td>(Lamont and Wier 2002)</td>
<td></td>
</tr>
<tr>
<td>Ca^{2+} waves</td>
<td>0.06-0.16 (EC) 0.04-1.41 (SMC)</td>
<td>8.8-50</td>
<td>500-2000</td>
<td>2000-10000</td>
<td>1-126</td>
<td>ECs and SMCs</td>
<td>(Beliveau, Lapointe, and Guillemette 2011; Jaffe 2010; Narayanan, Adebiyi, and Jaggar 2012; Socha et al. 2012)</td>
</tr>
</tbody>
</table>

NA denotes not applicable

The modeling approaches in synergy with experimental approaches undertaken for individual Ca^{2+} event types are also presented. Mathematical modeling offers a systematic approach for the analysis of varied Ca^{2+} signaling mechanisms, and ranges from minimal models with phenomenological descriptions to models with detailed kinetic descriptions of transmembrane channel currents with deterministic or stochastic implementation. The models also differ in the amount of spatial heterogeneity
incorporated, with compartmental models incorporating single cytosolic compartment with single or multiple internal stores to continuous models with cytosolic sub-compartments, micro-domains and spatial gradients of cellular components. Lastly, a generalized modeling approach to account for cellular heterogeneities and membrane potential dynamics that could reproduce varied multi-cellular Ca\(^{2+}\) events is presented.

1.2.1 Global Ca\(^{2+}\) events – Ca\(^{2+}\) waves and whole cell oscillations

Vascular ECs and SMCs exhibit oscillatory Ca\(^{2+}\) behavior arising spontaneously or after agonist stimulation. The underlying mechanisms and cellular components involved in the generation and modulation of the Ca\(^{2+}\) waves and oscillations might be related (Keener and Sneyd 1998), i.e. in both cases the Ca\(^{2+}\) dynamics is mainly associated with Ca\(^{2+}\) release through the store receptors, Ca\(^{2+}\) influx from the membrane channels, and Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR). Ca\(^{2+}\) released from a cluster of store receptors (RyR, IP\(_3\)R) can act on itself and diffuse to subsequent adjacent clusters of store receptors to induce regenerative CICR which appears as a propagating Ca\(^{2+}\) wave that traverses across the cell (Figure 1.3).

Cell to cell communication through homo-cellular gap junctions may synchronize oscillatory Ca\(^{2+}\) waves across adjacent vascular cells (Peng et al. 2001; Neylon and Irvine 1990) and induce nearly simultaneous intracellular transient Ca\(^{2+}\) elevation in individual vascular cells referred to as whole cell calcium oscillations. In the SMC, the transition of agonist induced intracellular Ca\(^{2+}\) waves (0.05 Hz to 0.1 Hz) to high-frequency (0.17 to 0.33 Hz) whole cell oscillations has been reported (Peng et al. 2001) and is suggested to initiate the phenomenon of vasomotion.
Figure 1.3 Schematic of regenerative calcium release and propagating waves

Under certain stimulatory conditions activation of ionic channels (such as activation of Ca$^{2+}$-activated cGMP-dependent chloride channels (Cl$_{Ca}$) (Matchkov, Aalkjaer, and Nilsson 2004)) can induce depolarizing currents which spreads rapidly across the cell and can result in simultaneous opening of the voltage regulated Ca$^{2+}$ channels to result in rapid Ca$^{2+}$ increases throughout the cell and synchronization of localized oscillators to result in whole cell oscillations (Figure 1.4).
1.2.1.1 Physiological role of calcium waves and whole cell oscillations

$Ca^{2+}$ signals can regulate different intracellular responses through varying the frequency of $Ca^{2+}$ oscillations. For example, the CamKII activity increases exponentially with increase in the frequency of $Ca^{2+}$ spikes (De Koninck and Schulman 1998). An oscillatory $Ca^{2+}$ signal than constant steady $Ca^{2+}$ levels induces higher gene expression in B-lymphocytes (Dupont, Combettes, and Leybaert 2007; Dolmetsch, Xu, and Lewis 1998). In vascular cells $Ca^{2+}$ waves and oscillations may play a role in the regulation of vessel tone directly through modulation of the contractile apparatus or through regulation of $Ca^{2+}$ dependent channels like $Ca^{2+}$ activated chloride channels (Stewart et al. 2012). $Ca^{2+}$ wave blockade has been shown to result in dilation in cerebral artery SMC (Mufti et al. 2010). Oscillatory $Ca^{2+}$ waves are suggested to produce significantly less contraction than whole-cell $Ca^{2+}$ oscillations in airway SMC (Wang et al. 2010). Transient $Ca^{2+}$ dependent NO production in the EC (Buerk and Riva 1998; Kanai et al. 1995) can be a consequence of $Ca^{2+}$ waves and oscillations and may improve the efficacy of NO in
maintaining vascular tone (Tsoukias, Kavdia, and Popel 2004). Moreover, SMC Ca\(^{2+}\) waves may synchronize in neighboring cells to initiate the phenomenon of vasomotion (Peng et al. 2001). On the contrary it has also been suggested that Ca\(^{2+}\) waves have no significant impact on global cytosolic Ca\(^{2+}\) levels and are insignificant to force generation in the myogenic response (Jaggar 2001). The necessity for these two different types of oscillatory Ca\(^{2+}\) pattern (wave-like vs. global transients), their underlying cellular mechanisms and physiological roles need to be better understood.

### 1.2.1.2 Ca\(^{2+}\) oscillations

#### 1.2.1.2.1 Experiments

Temporal changes in cytosolic Ca\(^{2+}\) levels under constant agonist stimulations have been observed in the form of repetitive spikes in vascular ECs (Jacob et al. 1988; Socha et al. 2012; Moccia et al. 2003) and SMCs (Jaggar 2001; Lee et al. 2002; Peng et al. 2001; Mauban et al. 2001; Iino, Kasai, and Yamazawa 1994). A regenerative discharge of stored Ca\(^{2+}\) may be responsible for these Ca\(^{2+}\) oscillations (Woods, Cuthbertson, and Cobbold 1986). Although the mechanisms for Ca\(^{2+}\) oscillations in variety of cells have been studied for nearly few decades, ambiguity and controversy still exist on their exact cellular mechanisms. A number of reviews have discussed these issues in detail (Berridge and Galione 1988; Dupont et al. 2011; Dupont, Combettes, and Leybaert 2007; Falcke 2004; Schuster, Marhl, and Hofer 2002). Sequential Ca\(^{2+}\) release from sarcoplasmic/endoplasmic reticulum (SR/ER) via IP\(_3\)Rs (Berridge and Irvine 1989) have been the most studied mechanism for generation of Ca\(^{2+}\) oscillations. IP\(_3\)Rs can generate sustained calcium oscillations thanks to their bell-shaped Ca\(^{2+}\) dependence (Bezprozvanny 1994; Bezprozvanny, Watras, and Ehrlich 1991) i.e., at low Ca\(^{2+}\) levels
they are activated rapidly and result in \( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \) release (CICR) providing the rising phase of the spike. At higher \( \text{Ca}^{2+} \) levels, their slow inhibition results in falling phase of the spike. Constant vs. oscillatory IP\(_3\) levels may accompany \( \text{Ca}^{2+} \) oscillations, and different isoforms of IP\(_3\)R receptors may play a role in modifying the frequency and sustenance of these oscillations. Both experimental techniques and theoretical models describing the role of IP\(_3\)R in \( \text{Ca}^{2+} \) oscillations have been discussed in detail in (Dupont et al. 2011). In addition to IP\(_3\)R, RyR alone (Heppner et al. 2002) or the interplay between IP\(_3\)Rs and RyR (Iino, Kasai, and Yamazawa 1994; Boittin et al. 1999; Jaggar and Nelson 2000; Lee et al. 2002) can provide regenerative \( \text{Ca}^{2+} \) release. RyR are most extensively characterized in the SMCs, but few studies have suggested their influence on \( \text{Ca}^{2+} \) oscillations in some EC types (Jacob et al. 1988; Neylon and Irvine 1990). Mitochondria can also play a role in maintenance of EC \( \text{Ca}^{2+} \) oscillations by modulating cytosolic \( \text{Ca}^{2+} \) levels (Ishii, Hirose, and Iino 2006). In addition to oscillations through the store release, \( \text{Ca}^{2+} \) oscillations may arise from the \( \text{Ca}^{2+} \) influx through the transmembrane channels. In isolated rabbit arteries \( \text{Ca}^{2+} \) oscillations through interaction between the large-conductance \( \text{Ca}^{2+} \)-activated potassium channels (BK\(_{\text{Ca}}\)) and the VOCC channels have been reported (Omote, Kajimoto, and Mizusawa 1992).

1.2.1.2.2 Models

Experimental techniques performed to assess all elements of the regulatory mechanism of \( \text{Ca}^{2+} \) oscillations suggest involvement of different channels, pathways and feedback processes that cannot be fully approached by qualitative reasoning. Mathematical models in addition to experimental techniques provide a better tool for understanding the overall \( \text{Ca}^{2+} \) dynamics. Many computational models have been
developed to investigate or simulate oscillations in different cell types, including the EC and SMC. The models differ in the underlying assumption of the type of receptors and channels involved and the oscillatory mechanism, i.e. oscillations through store IP_{3}Rs and RyR (cytosolic oscillators) (Koenigsberger et al. 2004; De Young and Keizer 1992; Keizer and Levine 1996), membrane oscillators i.e. oscillations induced through membrane channels (for example voltage gated channels Ca^{2+} or K^{+} channels (Fioretti, Franciolini, and Catacuzzeno 2005; Zeng, Li, and Chen 2009)) or oscillations arising from periodic variations in metabolic IP_{3} concentrations (Meyer and Stryer 1988). Moreover they differ in the detail of description ranging from microscopic (stochastic modeling) to macroscopic descriptions (deterministic modeling). The different models have been nicely tabulated in (Dupont et al. 2011). The increase in intra-cellular Ca^{2+} via store channels in case of cytosolic oscillators or through the membrane channels (membrane oscillators) in the presence of positive feedback (CICR) results in the rising phase of the oscillations in the models. The decline phase of oscillation in these models can be a result of inactivation of IP_{3}Rs by high [Ca^{2+}] (Bezprozvanny 1994), membrane potential repolarization from slow rundown of IK_{Ca} channels (Fioretti, Franciolini, and Catacuzzeno 2005) or termination of RyR-mediated Ca^{2+} release by store depletion (Kapela, Bezerianos, and Tsoukias 2008). Moreover, theoretical investigation has been carried out to examine the effect of IP_{3}R isoforms on cytosolic Ca^{2+} oscillations through the development of phenomenological models (Dupont and Combettes 2006).

The existing literature for Ca^{2+} dynamics models in vascular cells (EC and SMC) is reviewed in (Tsoukias 2011). SMCs differ from the electrically non-excitable ECs in their channel and receptor compositions. Although L-type Ca^{2+} channels and CICR from
RyR and/or IP$_3$-sensitive stores play a predominant role in SMC, the phosphatidylinositol pathway and capacitive calcium entry (CCE) have a significant role in ECs. Furthermore, the type and density of membrane ion channels in the cells exhibit significant differences among different species and tissues and probably change with the size of the vessel even within the same vascular bed. This heterogeneity suggests a need for cell- and vessel-specific detailed models of Ca$^{2+}$ signaling and dynamics in the vasculature.

A detailed model of Ca$^{2+}$ dynamics and oscillations in the SMC have been presented in (Kapela, Bezerianos, and Tsoukias 2008). The model predicts spontaneous Ca$^{2+}$ oscillations generated by repetitive CICR through RyRs and emptying of a small release compartment as release termination mechanism (Kapela, Bezerianos, and Tsoukias 2008). A detailed EC model that integrates both EC Ca$^{2+}$ dynamics and plasmalemma electrical activity to investigate EC responses to various stimulatory conditions and the relationship between Ca$^{2+}$ and membrane potential ($V_m$) have been presented in (Silva, Kapela, and Tsoukias 2007). However, the model did not predict Ca$^{2+}$ oscillations.

Majority of the models for intracellular Ca$^{2+}$ oscillations are deterministic in nature. Recently, Falcke et al. (Falcke 2003) developed stochastic models for Ca$^{2+}$ oscillations, where oscillations are generated by local stochastic events arising from opening of an IP$_3$ receptor channel. Moreover, Falcke and coworkers have shown that Ca$^{2+}$ oscillations in some cell types do not obey deterministic dynamics and rather are a sequence of random spikes (Falcke 2003; Skupin et al. 2008).
1.2.1.3 \textit{Ca}^{2+} \textit{waves}

1.2.1.3.1 Experiments

Spatially organized Ca$^{2+}$ waves with diverse propagation velocities have been observed in a variety of cell types (Berridge 1993; Rottingen and Iversen 2000; Berridge and Dupont 1994; Jaffe 2010; Narayanan, Adebiyi, and Jaggar 2012; Huser and Blatter 1997). The observed sarcoplasmic (SR) and endoplasmic reticulum (ER) released Ca$^{2+}$ waves in vascular cells can be characterized broadly into two distinct types based on the experimental protocol carried out to generate them. In the first type, Ca$^{2+}$ waves appear from a localized Ca$^{2+}$ increase via store receptors (IP$_3$Rs / RyRs) (Halidi et al. 2011; Marchant, Callamaras, and Parker 1999; McCarron et al. 2010) acquired experimentally through photolysis of caged IP$_3$ in a small region of the cell (Wang and Augustine 1995). If the localized Ca$^{2+}$ release is sufficiently strong, it can diffuse across the cell and have amplified response via CICR to result in a propagating Ca$^{2+}$ wave that traverses the cell. Sensitization of store receptors with presence of basal levels of Ca$^{2+}$ and/or IP$_3$ might be essential for the propagation via CICR (McCarron et al. 2010). The initiation of such waves usually occurs from the site of the local IP$_3$ release. The second type is characterized as, repetitive or oscillatory Ca$^{2+}$ waves in the EC and SMC obtained experimentally through uniform agonist stimulus applied throughout the cell. Such waves appear to originate from a fixed site, and might be a result of higher density of membrane or store receptors in that region or microdomain (Olson et al. 2012). Endothelial Ca$^{2+}$ waves are reported to preferentially originate at specific loci in caveolin-rich cell edges upon uniform stimulation with ATP, bradykinin and thrombin (Isshiki et al. 1998).
Agonist-induced oscillatory Ca\(^{2+}\) waves have been observed in both the SMCs (Peng et al. 2001; Iino, Kasai, and Yamazawa 1994; Boittin et al. 1999; Jaggar and Nelson 2000; Dai et al. 2007; Dai et al. 2006; Lee et al. 2001; Ruehlmann et al. 2000; Syyong et al. 2009) and ECs (Beliveau, Lapointe, and Guillemette 2011; Huser and Blatter 1997; Isshiki et al. 1998; Socha et al. 2012) of different species. Ca\(^{2+}\) waves can also be classified based on their proposed underlying mechanism. In one scenario, a localized Ca\(^{2+}\) oscillator provides necessary Ca\(^{2+}\) which through diffusion and CICR results in a propagating wave (Keener and Sneyd 1998). In another scenario, the whole cell is oscillatory, and spatial cellular heterogeneity can cause the wave like pattern appearance due to phase differences in oscillatory activity in different region of the cells (Jafri and Keizer 1994). A combination of the two scenarios is also possible. In the later mechanism, the diffusion of Ca\(^{2+}\) or IP\(_3\) might not be essential for generation of Ca\(^{2+}\) transients but may provide their phase lock.

Stimulus strength, Na\(^+-\)Ca\(^{2+}\) exchanger (NCX), non-selective cation channels (NSCs), VOCC channels, and store receptor channels are suggested to modulate both the frequency and speed of Ca\(^{2+}\) waves and reviewed in (Narayanan, Adebiyi, and Jaggar 2012) The modulation of Ca\(^{2+}\) waves through the plasmalemmal channels and pumps can be a result of altering membrane potential dynamics electrochemical gradient for Ca\(^{2+}\) influx.

1.2.1.3.2 Models

Modeling Ca\(^{2+}\) waves requires examination of both spatial and temporal scales and necessitates to account for the description of cytosolic Ca\(^{2+}\) diffusion. Several minimal deterministic reaction-diffusion models of Ca\(^{2+}\) waves, describing Ca\(^{2+}\) and IP\(_3\) dynamics
have been undertaken. Similar to the models of Ca\textsuperscript{2+} oscillations, the simulations of Ca\textsuperscript{2+} waves differ in the underlying assumption of the oscillatory mechanism. (For example Ca\textsuperscript{2+} waves arising from IP\textsubscript{3}R are consider in (Atri et al. 1993; Dupont and Goldbeter 1994; Sneyd et al. 1995), oscillation through combination of IP\textsubscript{3}R and RyR in (Wang et al. 2010)). Moreover, the models differ in type of stimulus i.e. Ca\textsuperscript{2+} waves generated from localized Ca\textsuperscript{2+} increase (Atri et al. 1993; Dupont and Goldbeter 1994) which propagates across the cell or under uniform stimulus throughout the cell (Wang et al. 2010) (e.x. through rise of IP\textsubscript{3} levels throughout the cell). Non-uniform cellular component distribution (e.x. store receptors) or introduction of noise in the model was essential for spatial heterogeneity for Ca\textsuperscript{2+} wave generation under uniform stimulus, in the models (Wang et al. 2010; Jacobsen et al. 2007). The initial models were deterministic in nature and provide a good understanding of generation and propagation of these waves. Falcke et al., based on the experimental observations in Xenopus oocytes (Sun et al. 1998) developed stochastic models of Ca\textsuperscript{2+} waves (Falcke, Tsimring, and Levine 2000; Falcke 2003; Thul and Falcke 2004). They simulated the transition from puffs to waves to fast oscillations with increasing stimulus strength. The waves were a result of nucleation, i.e. opening of at least few clusters of IP\textsubscript{3}Rs resulting in localized Ca\textsuperscript{2+} increase high enough to activate the adjacent clusters inside the cells. Detailed three-dimensional model of ventricular myocytes comprising of anisotropic distribution of RyR clusters within a few sarcomeres reconstructed directly from confocal imaging measurements, and simple stochastic CICR release dynamics have shown to produce global Ca\textsuperscript{2+} wave propagations (Li et al. 2010)
Limited models have examined the role of membrane potential dynamics in regulation of the Ca\(^{2+}\) waves. Jacobsen et al. developed a multi-compartmental vascular SMC model describing both \(V_m\) and Ca\(^{2+}\) dynamics (Jacobsen et al. 2007). Modulation in frequency of Ca\(^{2+}\) wave with varying mean \(V_m\) was observed in the model. We have recently developed multi-scale model of integrated Ca\(^{2+}\)-\(V_m\) dynamics for modeling regulation of vascular tone (Kapela and Tsoukias 2011), appropriate for investigating the role of both cytosolic and membrane components in intracellular Ca\(^{2+}\) waves. The model incorporates diffusion of Ca\(^{2+}\), Na\(^+\), K\(^+\), Cl\(^-\) and IP\(_3\) and all membrane and store components from our original lumped models of EC and SMC (Kapela, Bezerianos, and Tsoukias 2008; Silva, Kapela, and Tsoukias 2007).

Examination of experimentally observed (Peng et al. 2001) transition of intra-cellular Ca\(^{2+}\) waves to whole-cell oscillations in the SMC, associated with the phenomenon of vasomotion have been analyzed using computational model in (Jacobsen et al. 2007). Based on their observations, they supported the role of cGMP-dependent Ca\(^{2+}\)-activated chloride channels (Cl\(_{Ca}\)) (Matchkov, Aalkjaer, and Nilsson 2004) in synchronizing local Ca\(^{2+}\) oscillators into whole-cell oscillations. According to the model, activation of chloride channels by Ca\(^{2+}\) transient’s results in whole-cell membrane potential depolarization and Ca\(^{2+}\) influx via L-type voltage regulated Ca\(^{2+}\) channels resulting in the coordination of Ca\(^{2+}\) changes. Other study (Postnov et al. 2011) used a reductionist approach to reveal the dynamical features and non-linear mechanisms involved in this transition from Ca\(^{2+}\) waves to oscillations. Synchronization of SMCs has been investigated in a number of computational studies (Jacobsen et al. 2007; Kapela, Parikh, and Tsoukias 2012; Postnov et al. 2011; Koenigsberger et al. 2008; Jacobsen et al. 2007;
Koenigsberger et al. 2006). The role of NSC channels in addition to the Cl⁻ channels, and weak intercellular IP₃ fluxes to promote SMCs synchronization is suggested in (Kapela, Parikh, and Tsoukias 2012). Similar to synchronization between cells, these channels may also provide pathways for transition from intracellular waves to whole-cell oscillations.

1.2.2 Localized Ca²⁺ events

Localized Ca²⁺ events are observed in a wide variety of cell types at rest or low agonist stimulation levels. These events are highly restricted both in space and time and originate from random opening of few SR/ER or plasmalemmal Ca²⁺ channels. Advancement in Ca²⁺ imaging techniques with the development of sensitive cameras and better dyes allow optical imaging systems to routinely achieve detailed spatial and temporal resolution, significant for characterization of local Ca²⁺ events. Moreover, selective loading of the ECs without interference from surrounding SMCs (Kansui, Garland, and Dora 2008), development of transgenic mouse expressing the genetically encoded Ca²⁺ indicator GCaMP2 (Nakai, Ohkura, and Imoto 2001; Ji et al. 2004) provide better visualization and understanding of localized Ca²⁺ events in the vascular ECs and SMCs. The local Ca²⁺ events can be classified based on whether the release is from a single channel or a cluster of channels, the type of channels or receptors involved such as RyR, IP₃R or membrane channels, and the underlying characteristics of the events. In particular, Ca²⁺ sparks denote localized Ca²⁺ increase via RyR, Ca²⁺ puffs are localized Ca²⁺ events via IP₃R, Ca²⁺ pulsar is a localized Ca²⁺ increase via IP₃R located in vicinity of endothelial microprojections, and Ca²⁺ events mediated by plasmalemmal channels are termed as Ca²⁺ sparklets. In vascular cells the localized events have been
shown to modulate the vessel tone. Moreover these random local events at microscopic levels can transform into coordinated global Ca$^{2+}$ responses like waves and oscillations. Examining the properties of these events, their mechanisms and transition to global Ca$^{2+}$ responses will provide a more comprehensive understanding of Ca$^{2+}$ dynamics.

### 1.2.2.1 Ca$^{2+}$ sparks (Localized Ca$^{2+}$ event via RyRs)

RyRs are expressed at varying levels in different smooth muscle cells tissues. Dissimilar expression and/or function of three RyR subtypes is suggested to result in the diverse spatiotemporal characteristics of the Ca$^{2+}$ sparks in different vascular SMCs (Zheng et al. 2008). Localized Ca$^{2+}$ events via RyR termed Ca$^{2+}$ sparks were first observed in cardiac cells (Cheng, Lederer, and Cannell 1993) and suggested to influence excitation-contraction coupling. These events have been reported in variety of vascular SMCs (Jaggar et al. 2000; Nelson et al. 1995; Sieck, Kannan, and Prakash 1997). Majority of the vascular ECs are found to be devoid of RyRs (Ledoux et al. 2008) except few studies which have reported their presence in the ECs and, hence may lack Ca$^{2+}$ sparks. A single Ca$^{2+}$ spark results in increase of local Ca$^{2+}$ level to around 10-100 µM (Jaggar et al. 2000) near the site of release and spread to around 14 µm$^2$. Other properties of Ca$^{2+}$ sparks are listed in Table 1.1.

Due to their limited spread, Ca$^{2+}$ sparks have been suggested to play a minimal influence on the global Ca$^{2+}$ levels (Nelson et al. 1995). Ca$^{2+}$ sparks occurring in regions of the cell with SR in close proximity to the plasma membrane can result in indirect regulation of contraction and relaxation of the SMC through activation of co-localized Ca$^{2+}$-dependent membrane channels. Ca$^{2+}$ spark can result in relaxation of rat cerebral artery SMCs (Nelson et al. 1995) through co-localization of RyR with BK$_{Ca}$. Localized
Ca\(^{2+}\) increase in the microdomain can activate several BK\(_{Ca}\) channels, resulting in SMC hyperpolarization, global Ca\(^{2+}\) decrease, and in turn SMC relaxation. On contrary, in SMCs from rabbit portal vein tissues RyRs are juxta-posed with the Cl\(_{Ca}\) and localized Ca\(^{2+}\) sparks in these microdomains cause SMC depolarization (Wang, Hogg, and Large 1992), global Ca\(^{2+}\) increase via opening of L-type voltage gated Ca\(^{2+}\) channels, and SMC contraction. Reduction in amplitude and frequency of Ca\(^{2+}\) sparks in SMCs from cerebral artery of type-2 diabetes model have been reported and suggested to be involved in cerebral artery dysfunction in diabetic patients (Rueda et al. 2013).

1.2.2.2 \(Ca^{2+}\) puffs (localized Ca\(^{2+}\) events via IP\(_3\)Rs)

Three IP\(_3\)R isoforms (IP\(_3\)R1, IP\(_3\)R2 and IP\(_3\)R3) are expressed at varying levels in both ECs and SMCs (Ledoux et al. 2008; Tovey et al. 2001; Tasker, Michelangeli, and Nixon 1999). Spontaneous Ca\(^{2+}\) release via single IP\(_3\)R is referred as Ca\(^{2+}\) blip. Ca\(^{2+}\) blips in the EC have shown to last less than 100 ms with an average amplitude of 23 nmol/L corresponding to 0.3 pA influx and a spread of 1- to 3-\(\mu\)m width (Huser and Blatter 1997). Sufficiently high Ca\(^{2+}\) release from a single channel can activate other IP\(_3\)Rs in a cluster through passive diffusion, and produce a slightly larger but still random Ca\(^{2+}\) event termed as Ca\(^{2+}\) puff. Ca\(^{2+}\) puffs have been observed in ECs (Kansui, Garland, and Dora 2008; Doria and Sarelius 2004, 2004). They are observed in variety of cell types with different IP\(_3\)R1-3 levels with similar amplitude, spatial spread, and kinetics, and hence suggested to be generic elementary Ca\(^{2+}\) signals with minimal functional variation of the three IP\(_3\)R isoforms at the level of subcellular Ca\(^{2+}\) signaling (Tovey et al. 2001). Ca\(^{2+}\) puffs have not been examined as widely as Ca\(^{2+}\) sparks in vascular SMCs, with exception of few studies on colonic and cerebral artery SMCs (Bayguinov et al. 2000;
Ca\textsuperscript{2+} dynamics in the SMC at the macroscopic level has been suggested to be significantly modulated by the expression levels of various subtypes of IP\textsubscript{3}Rs (see Ca\textsuperscript{2+} waves section). The effect of different IP\textsubscript{3}R receptors have been reviewed in Dupont et al (Dupont, Combettes, and Leybaert 2007).

A single Ca\textsuperscript{2+} puff results in increase of local Ca\textsuperscript{2+} level to around 50-500 nM (Ledoux et al. 2008; Tovey et al. 2001) near the site of release. Other properties of Ca\textsuperscript{2+} puffs are listed in Table 1.1. Localized Ca\textsuperscript{2+} events via IP\textsubscript{3}Rs in vascular cells have been shown to modulate the vessel tone. The presence of microdomains where IP\textsubscript{3}Rs are co-localized with specific membrane channels TRPM4 (transient receptor potential melastatin 4), IK\textsubscript{Ca} (intermediate conductance Ca\textsuperscript{2+} activated potassium channels)) permits spatially limited Ca\textsuperscript{2+} release to be sufficient to activate these channels and modulate vessel tone. In rat cerebral SMCs Ca\textsuperscript{2+} puffs have been shown to promote opening of TRPM4 channels, resulting in SMC membrane depolarization, opening of VOCC channels and ultimately contraction. Role of Ca\textsuperscript{2+} puffs in regulation of vascular tone have been reviewed in (Amberg and Navedo 2013).

1.2.2.3 Ca\textsuperscript{2+} pulsars and wavelets

Spontaneous localized Ca\textsuperscript{2+} increases through IP\textsubscript{3}Rs in the EC near myoendothelial projections (MPs) are reported in unstimulated rat mesenteric arteries and referred as Ca\textsuperscript{2+} pulsars (Ledoux et al. 2008). Several studies since then have reported existence of Ca\textsuperscript{2+} pulsars at the sites of MPs (Sonkusare et al. 2012; Nausch et al. 2012; Bagher et al. 2012). MPs are cellular extensions from ECs and/or SMC that extend over the internal elastic lamina and come in close contact with the other cell type (Heberlein, Straub, and Isakson 2009; Sandow et al. 2009). Although these localized Ca\textsuperscript{2+} events are associated
with IP₃Rs, their spatial spread and kinetic properties are significantly different from Ca²⁺ puffs and more similar to Ca²⁺ sparks (Table 1.1). Increased frequency of Ca²⁺ pulsars under acetylcholine stimulation and activation of intermediate conductance potassium channels (IKₐ) co-localized around myoendothelial projections can significantly hyperpolarize the EC (Ledoux et al. 2008) which may spread to adjacent SMCs to ultimately induce vessel relaxation.

Localized Ca²⁺ increase via a discrete pool of IP₃Rs localized in the endothelial projections have been reported in muscle feed arteries under stimulation of the SMC with norepinephrine, and are referred as Ca²⁺ wavelets (Tran et al. 2012). The duration and spread of Ca²⁺ wavelets are similar to those of Ca²⁺ pulsars and significantly higher than of Ca²⁺ puffs (Table 1.1). The Ca²⁺ wavelet are suggested to activate IKₐ channels within or near endothelial projections, resulting in hyperpolarization which feeds back on the SMC to moderate agonist-induced depolarization and constriction (Kerr et al. 2012; Tran et al. 2012). Endothelial mediated feedback was initially suggested in (Dora, Doyle, and Duling 1997).

1.2.2.4 **Ca²⁺ sparklets and junctional Ca²⁺ transients**

Localized Ca²⁺ events via Ca²⁺-permeable membrane channels are usually referred as Ca²⁺ sparklets. Ca²⁺ sparklets are found to occur in persistent, specific region of the plasmalemma, unlike localized Ca²⁺ events from store channels, which usually occur randomly and uniformly throughout the cell. Initially Ca²⁺ sparklets were observed in cardiac muscle cells, where local Ca²⁺ increase results from opening of a single or a cluster of L-type VOCC (Wang et al. 2001). VOCC-induced Ca²⁺ sparklets are observed in vascular SMCs, using total internal reflection (TIRF) microscopy, and reported to
modulate global Ca\textsuperscript{2+} levels indirectly through activation of RyRs (Navedo et al. 2005). The average area of VOCC-induced Ca\textsuperscript{2+} sparklets is small and approximately 0.8 µm\textsuperscript{2} (Santana et al. 2008). Santana et al. described low and high activity ("persistent") Ca\textsuperscript{2+} sparklets and necessity of protein kinase C (PKC) activation for increased frequency of persistent sparklets. Events via VOCC channels and their role in modulation of vascular tone have been summarized in (Hill-Eubanks et al. 2011).

Localized Ca\textsuperscript{2+} events have also been observed from Ca\textsuperscript{2+}-permeable non-selective cation channels (NSC) such as TPRV4 and ATP-gated P2X receptors, which allow influx of Ca\textsuperscript{2+} and other extracellular cations like Na\textsuperscript{+}. Stimulation of P2X receptors by ATP revealed localized Ca\textsuperscript{2+} influx events referred as junctional Ca\textsuperscript{2+} transients have been observed in pressurized rat mesenteric small arteries (Lamont and Wier 2002). The properties of these events are described in Table 1.1. In addition to the localized Ca\textsuperscript{2+} events, activation of P2X receptors may induce depolarizing excitatory junctional potential due to Na\textsuperscript{+} influx, and global Ca\textsuperscript{2+} increase via activation of VOCC (Zang et al. 2006). Localized quantal Ca\textsuperscript{2+} increases through TRPV4 channels, referred as TRPV4 sparklets, have been identified recently in mice mesenteric arteries ECs, using genetically encoded Ca\textsuperscript{2+} biosensor (GCaMP2) for Ca\textsuperscript{2+} imaging (Sonkusare et al. 2012). The TRPV4 sparklets have a spatial spread of 11 µm\textsuperscript{2} and reported to occur at the same sites. Activation of few (~3) TRPV4 channels per cell is suggested to induce maximal vessel dilation through activation of co-localized EC IK\textsubscript{Ca} and small-conductance Ca\textsuperscript{2+}-activated potassium (SK\textsubscript{Ca}) channels. Low intraluminal pressure may also stimulate TRPV4-associated EC Ca\textsuperscript{2+} sparklet events (Bagher et al. 2012). AKAP150, a protein kinase C (PKC) anchoring protein mediated cooperative opening of a 4-channel TRPV4 cluster.
fashioned Ca$^{2+}$ sparklet near the EC MPs site have been suggested to be central to endothelium dependent vasodilation during muscarinic-receptor stimulation (Sonkusare et al. 2014).

1.2.2.5 Mathematical modeling

Computational studies aim at quantification and better understanding of the characteristics of these localized Ca$^{2+}$ events and their vasoregulatory mechanisms. Although the majority of models developed are based on data from nonvascular cells, their results and methodology can be also utilized to investigate the localized Ca$^{2+}$ events reported in vascular ECs and SMCs. The initial models of Ca$^{2+}$ sparks, puffs were simple reaction-diffusion models involving Ca$^{2+}$ release from the stores (SR/ER), cytosolic Ca$^{2+}$ diffusion, reaction with cytosolic Ca$^{2+}$ buffers and re-sequestration of Ca$^{2+}$ into the store via pumps. Point source release of Ca$^{2+}$ calcium was achieved in the models using a time-dependent point source $\sigma(\mu\text{mole/s})$ times a Dirac-delta function ($\delta(r)$) (Smith et al. 1998; Smith 1996). Fast buffering approximations similar to in (Wagner and Keizer 1994) were considered. Another approach have also been carried out to model localized Ca$^{2+}$ puffs and sparks events using stochastic descriptions of single or cluster IP$_3$R and RyR (implemented using Markov chain modeling (Colquhoun and Hawkes 1981)) coupled with deterministic cytosolic Ca$^{2+}$ diffusion, Ca$^{2+}$ buffering, store uptake, and membrane Ca$^{2+}$ fluxes (Swillens et al. 1998; Falcke, Tsimring, and Levine 2000; Keizer et al. 1998). Stochastic transitions among the different channel states were characterized by the number of IP$_3$ and Ca$^{2+}$ molecules bound to their respective binding sites (Swillens et al. 1998). The models are able to predict the amount of localized increase from opening of a single or cluster of channels, number of channels in a cluster, and separation distances.
between clusters. Several reviews have described in details stochastic modeling of the localized events (Dupont et al. 2000; Dupont, Combettes, and Leybaert 2007; Jafri 2012). To improve computational efficiency, some models of $\text{Ca}^{2+}$ puffs implemented hybrid Gillespie method for $\text{Ca}^{2+}$- or IP$_3$-dependent transitions, and neglected $\text{Ca}^{2+}$ diffusion and spatial distributions (Keener and Sneyd 1998; Cao et al. 2013). On the other hand, computationally expensive detailed models have been formulated. For example, model of $\text{Ca}^{2+}$ sparks in cardiac cells have been implemented using 3D finite elements method (FEM) with geometry imported from electron tomographic images of $\text{Ca}^{2+}$ release units (CRU) (Hake et al. 2012).

Deterministic continuum models of coupled vascular EC-SMC have been developed and arising local and global $\text{Ca}^{2+}$ transients examined in (Nagaraja et al. 2013; Brasen, Jacobsen, and Holstein-Rathlou 2012). Brasen et al. (Brasen, Jacobsen, and Holstein-Rathlou 2012) used a 2-D axisymmetric model and predicts localized spatiotemporal $\text{Ca}^{2+}$ responses in EC MP during SMC stimulation, and suggest the rectification of $\text{Ca}^{2+}$ signals by microprojections geometry. A detailed 2D continuum model using finite element techniques to investigate integrated $\text{Ca}^{2+}$ and $V_m$ responses is presented in (Nagaraja et al. 2013). The model incorporates exact geometries of myoendothelial projections (MP) from experiments and localized pool of IP$_3$Rs and IK$_{\text{Ca}}$. A cartoon illustration of the cellular components implemented in the model is shown in Figure 1.5. Kir, inward rectifier $\text{K}^+$ channel; VRAC, volume-regulated anion channel; SK$_{\text{Ca}}$, IK$_{\text{Ca}}$, and BK$_{\text{Ca}}$, small-, intermediate-, and large-conductance $\text{Ca}^{2+}$-activated $\text{K}^+$ channels; SOC, store-operated channel; NSC, nonselective cation channel, CaCC and ClCa, $\text{Ca}^{2+}$-activated chloride channel; NaK, Na$^+\text{-K}^+$-ATPase; PMCA, plasma-membrane $\text{Ca}^{2+}$-
ATPase; NCX, Na\(^+\)/Ca\(^{2+}\) exchanger; NaKCl, Na\(^+\)-K\(^+\)-Cl\(^-\) cotransport; Kv, voltage-dependent K\(^+\) channel; \(K_{\text{leak}}\), unspecified K\(^+\) leak current; VOCC, voltage-operated Ca\(^{2+}\) channels; SR/ER, sarco/endoplasmic reticulum; IP\(_3\)R, IP\(_3\) receptor; RyR, ryanodine receptor; SERCA, SR/ER Ca\(^{2+}\)-ATPase; CSQN, calsequestrin; CM, calmodulin; R, receptor; G, G protein; DAG, diacylglycerol; PLC, phospholipase C; sGC, soluble guanylate cyclase; cGMP, cyclic guanosine monophosphate were considered in the model.

**Figure 1.5** Cartoon illustration of coupled EC-SMC model with MP

Enriched distribution of IK\(_{\text{Ca}}\) and IP\(_3\)Rs around the MP was implemented in the model and the cells were coupled by myoendothelial gap junctions (MEGJ) permeable to Ca\(^{2+}\), Na\(^+\), K\(^+\), and Cl\(^-\) ions and IP\(_3\). The model simulations predicted local Ca\(^{2+}\) release in and around EC MP and activation of spatially localized IK\(_{\text{Ca}}\) channels to produce hyperpolarizing currents which can moderate smooth muscle constriction. Few mV of feedback was observed in the model during SMC stimulation. Global EC Ca\(^{2+}\) response was not observed during SMC stimulation.
1.2.3 Integrated cytosolic and membrane potential modeling

The above review of the variety of spatiotemporal Ca\textsuperscript{2+} patterns demonstrates that many Ca\textsuperscript{2+} events are tightly coupled with changes in membrane potential. For example, $V_m$ depolarization may increase the activity of Ca\textsuperscript{2+} sparklets through VOCC and activate CICR through RyRs, which in turn can feedback on $V_m$ through Ca\textsuperscript{2+}-activated membrane channels.

A general computational framework for modeling spatiotemporal Ca\textsuperscript{2+} events integrated with plasma membrane electrophysiology in single or coupled vascular cells has been proposed in (Kapela and Tsoukias 2011). It is based on the FEM approach, and allows implementing continuous models (as opposed to discrete compartmental models) in full three dimensions, two dimensions with axial symmetry, two dimensions with lumped third dimension, or only one dimension. Transport of ionic species $S = Ca^{2+}, Na^+, K^+, Cl^-$ across the cell cytosol is given by the electrodiffusion equation,

$$\frac{\partial [S]}{\partial t} = \nabla \left( D_s \nabla [S] + Z_s u_{ms} [S] \nabla V + I_{rel,s} \right)$$

Equation 1.1

where $D_s$ is the diffusion coefficient of ionic species, $u_{ms}$ is the ionic mobility, $Z_s$ is the charge number of ionic species $S$, $I_{rel,s}$ is the net release and uptake flux of ion $S$ from the internal stores ($I_{rel,s} = 0$ for $S = Na^+, K^+$, and $Cl^-$). The ionic currents through membrane channels, pumps, and exchangers can be implemented as boundary fluxes. The net boundary flux ($N_s$) (Equation 1.2) for each ionic species $S$ is calculated from the sum of $K$ component currents ($I_{s,k}$), corresponding to all channel types permeable to $S$. The FEM approach allows introduction of spatial heterogeneity of cellular components by defining
the maximum conductance of release and membrane fluxes as functions of spatial coordinates. Moreover, 2D or 3D FEM models allow incorporation of microdomain structures with geometries imported from experimental images, e.x. electron or confocal microscopy.

\[- n . N_s = \frac{1}{Z_s F} \sum_k I_{s,k} \]

Equation 1.2

Electrodiffusion equation (Equation 1.1) takes into account the effect of both concentration and electrical potential gradients across the cell cytosol on the transport of all the ionic species. Although the cytosol is largely isopotential, the electrodiffusion equation can capture possible heterogeneities in electrical potential between cytosol and some microdomains generated during current conduction and limited bulk-microdomain diffusivity, e.x. in endothelial microprojection during EDHF response. The electrodiffusion model in the cytosol also allows simple implementation of voltage and concentration dependent membrane currents as boundary fluxes. Furthermore, the use of electrodiffusion equations, rather than diffusion equations, permits implementation of intercellular coupling through gap junctions as ionic currents proportional to electrochemical gradients. The implementation of intercellular coupling and the role of intercellular fluxes have been discussed in detail in Nagaraja et al. (Nagaraja, Kapela, and Tsoukias 2012). The release and membrane currents in Equations 1.1 and 1.2 can be implemented as stochastic processes, rather than with deterministic equations, to study localized events such as Ca^{2+} puffs, sparks and sparklets.
1.3 Outline of the dissertation

- **Chapter 2** presents the formulation of a coupled stochastic-continuum model of integrated calcium (Ca^{2+}) and membrane potential ($V_m$) dynamics in combined endothelial (EC) and smooth muscle (SMC) cell domains with microprojections (MP). The model describes the localized Ca^{2+} events arising from stochastic opening of a cluster of TRPV4 channels in the EC and its contribution to the regulation of vessel tone.

- **Chapter 3** describes the development of a detailed continuum model of coupled EC-SMC with MP, incorporated with the NO-cGMP pathway. We examine the NO mediated vasodilatory feedback response. The model predicts local NO production under the preferential distribution of endothelial nitric oxide synthase (eNOS) around the MP domain. Moreover, the model examines the modulation of SMC NO bioavailability in the presence of NO scavengers such as endothelial Hemoglobin α (Hbα) and erythrocytes.

- **Chapter 4** presents the development of individual spatiotemporal models of isolated SMC and the EC to examine the repetitive intracellular Ca^{2+} wave activity frequently observed in both the cell types. It also discusses the role of heterogeneity of cellular components, diffusion of Ca^{2+} and inositol triphosphate (IP$_3$), and electrical signaling ($V_m$ modulation) in the generation of oscillatory Ca^{2+} waves. Moreover it examines the effects of membrane potential dynamics on the modulation of intracellular Ca^{2+} wave frequency and velocity.
Chapter 5 provides a multi-cellular temporal model of a vessel segment (consisting of a layer of ECs with multiple layer of SMCs) and describes the role of Ca\(^{2+}\) and second messenger (IP\(_3\)) gap junction fluxes and electrical coupling in synchronizing SMC Ca\(^{2+}\) oscillations and producing oscillations in vessel diameter.

Chapter 6 provides a summary of the dissertation and,

Chapter 7 lists the equations of the models.

1.4 References


CHAPTER 2- LOCALIZED CALCIUM EVENTS ARISING FROM OPENING OF CLUSTER OF TRPV4 CHANNELS

This chapter is accepted in Biophysical Journal (in press) as Parikh, J., A. Kapela and N. M. Tsoukias. “Stochastic model of a TRPV4 Calcium sparklet: Effect of bursting and cooperativity on endothelial-derived hyperpolarization”
2.1 Abstract

We examined the endothelial transient receptor vanilloid 4 (TRPV4) channel vasodilatory signaling in the regulation of vessel tone using mathematical modeling. The model analyzes experimental data by Sonkusare et al. (Science 336: 597-601, 2012) on TRPV4-induced endothelial Ca\(^{2+}\) events (sparklets). A previously developed continuum model of an endothelial and a smooth muscle cell coupled through microprojections (MPs), was extended to account for the activity of a TRPV4 channel cluster. Different stochastic descriptions for the TRPV4 channel flux were examined using finite state Markov chains. The model also took into consideration recent evidence for the co-localization of intermediate-conductance calcium-activated potassium channels (IK\(_{\text{Ca}}\)) and TRPV4 channels near the MPs. A single TRPV4 channel opening resulted in a stochastic localized Ca\(^{2+}\) increase in a small region (i.e. few \(\mu\text{m}^2\) area) close to the channel. We predict micromolar Ca\(^{2+}\) increases lasting for the open duration of the channel, sufficient for the activation of low-affinity endothelial K\(_{\text{Ca}}\) channels. Simulations of a cluster of four TRPV4 channels incorporating burst and cooperative gating kinetics, provided quantal Ca\(^{2+}\) increases (i.e. steps of fixed amplitude), similar to the experimentally observed Ca\(^{2+}\) sparklets. These localized Ca\(^{2+}\) events result in endothelium-derived hyperpolarization (EDH) (and SMC relaxation) with magnitude that depends on event frequency. The gating characteristics (bursting, cooperativity) of the TRPV4 cluster enhance Ca\(^{2+}\) spread and the distance of K\(_{\text{Ca}}\) channel activation. This may amplify the EDH response by the additional recruitment of distant K\(_{\text{Ca}}\) channels.
2.2 Introduction

A complex bidirectional communication between endothelial (EC) and smooth muscle (SMC) cells regulates SMC constriction and vessel tone. Cytosolic calcium (Ca^{2+}) regulates the ability of ECs to induce the release of vasoactive signals including the discharge of hyperpolarizing current to the SMC. In small resistance vessels, this NO-independent endothelium-derived hyperpolarizing (EDH) signaling is typically mediated by the activation of intermediate- (IK_{Ca}) and small- (SK_{Ca}) conductance Ca^{2+}-activated potassium channels in response to an increase in Ca^{2+} concentration (Edwards, Feletou, and Weston 2010; Garland, Hiley, and Dora 2011). Global EC Ca^{2+} mobilization results from transmembrane Ca^{2+} influx and/or Ca^{2+} release from the intracellular stores. Recent evidence suggest that localized Ca^{2+} influx from spontaneous or agonist-induced opening of the transient receptor vanilloid 4 (TRPV4) channel can also induce SMC hyperpolarization and vessel dilation through the EDH mechanism in resistance vessels (Bagher et al. 2012; Sonkusare et al. 2012; Sonkusare et al. 2014).

TRPV4 channels are sensitive to a wide array of stimuli including, Epoxyeicosatrienoic acids (EETs), Diacylglycerol and phorbol esters via PKC dependent and independent pathways, osmotic changes, mechanical stimuli, Ca^{2+} levels and temperature (Heller and O'Neil 2007; Strotmann, Schultz, and Plant 2003; Nilius et al. 2004). In the vasculature, EC agonists like acetylcholine (Sonkusare et al. 2012; Sonkusare et al. 2014; Earley et al. 2009), shear stress (Mendoza et al. 2010) and sustained low pressures (Bagher et al. 2012), in addition to exogenous agonist like GSK1016790A (GSK), 4-α phorbol 12,13-didecanoate (4αPDD) or 11-12 EET, may activate TRPV4 channels to produce a transient stationary Ca^{2+} burst (sparklet) within the
EC that may ultimately result in vasodilation. Single channel patch clamp data reveals stochastic TRPV4 opening with pA current amplitude at resting membrane potential (-50 mv) (Watanabe et al. 2002). Activation of as few as three channels per EC may be sufficient to induce maximal vessel dilation (Sonkusare et al. 2012). The role of individual activators and pathways resulting in TRPV4 channel openings is currently under investigation. Recent evidence suggests bursting activity and cooperative opening of TRPV4 channels in a cluster (Sonkusare et al. 2012). The physiological relevance of such gating mechanisms has not been elucidated.

EC extensions over the internal elastic lamina towards SMCs, termed myoendothelial projections (MPs), have been observed in small diameter vessels (Sandow et al. 2009; Heberlein, Straub, and Isakson 2009) and can play a role in the modulation of vascular tone (Kerr et al. 2012). Recent studies provide evidence for the localization of TRPV4 channels with IKCa, inositol 1,4,5-triphosphate receptors (IP3Rs), and connexins in/near MPs (Bagher et al. 2012; Sandow et al. 2006; Ledoux et al. 2008; Isakson 2008; Dora et al. 2008). Myoendothelial gap junctions (MEGJs) are often found at the tip of the MP, and allow for electrochemical communication between ECs and SMCs (Heberlein, Straub, and Isakson 2009; Sandow et al. 2009). Spontaneous and agonist-induced localized Ca2+ events mediated by transmembrane Ca2+ influx (TRPV4 sparklets (Bagher et al. 2012; Sonkusare et al. 2012; Sonkusare et al. 2014)) or intracellular store Ca2+ release (‘pulsars’(Ledoux et al. 2008) and ‘wavelets’(Tran et al. 2012)) have been observed in ECs near the MP sites.
Mathematical modeling offers a systematic approach for the analysis of complex signaling mechanisms, and it can serve as a tool for data interpretation and for guiding new experimental studies. Few theoretical studies have been carried out to investigate the role of ECs and MPs in the modulation of SMC Ca\(^{2+}\) and membrane potential (\(V_m\)) dynamics. We have previously examined SMC Ca\(^{2+}\) and \(V_m\) changes during EC stimulation through the development of an EC-SMC compartmental model (Kapela, Bezerianos, and Tsoukias 2009). This model integrates detailed single cell EC (Silva, Kapela, and Tsoukias 2007) and SMC (Kapela, Bezerianos, and Tsoukias 2008) models with electrical, chemical and NO coupling pathways. Acetylcholine (Ach) stimulation of ECs in the model increased global EC Ca\(^{2+}\) levels, activated EDH and NO pathways to hyperpolarize the SMC which ultimately reduced global Ca\(^{2+}\) concentration in the SMC. We have also extended the compartmental model into a 2D continuum model which incorporates accurate MP geometry from electron microscopy images and spatial localization of IK\(_{Ca}\) and IP\(_3\)Rs in the MP. This new formulation was utilized to investigate the role of feedback in EC-SMC communication (Nagaraja et al. 2013). Similarly, Brasen et al. have developed a 2D-axisymmetric model incorporating the anatomical structure of MPs into a two cell system (Brasen, Jacobsen, and Holstein-Rathlou 2012). Their results show that MPs may rectify the signal between the EC and SMC. Previous models did not examine the role of TRPV4 channels. Moreover, they considered deterministic whole cell current descriptions for membrane channels and pumps, and did not account for localized and stochastic channel openings.
In this study, we present the development of a computational model to examine the localized Ca$^{2+}$ mobilization, in the vicinity of the MP, arising from a single or a cluster of TRPV4 channels. The TRPV4s were incorporated in a previously developed continuum EC-SMC model with MPs. The model accounts for preferential presence of the TRPV4s near the MPs as suggested in experimental studies. Stochastic opening of a TRPV4 channel was captured using a finite state Markov chain. We utilize this model to examine the contribution of these channels to the regulation of vessel tone.

2.3 Methods

2.3.1 Continuum model

We have presented a general computational framework for modeling spatiotemporal Ca$^{2+}$ events integrated with plasma membrane electrophysiology in single or coupled vascular cells in (Nagaraja et al. 2013; Kapela and Tsoukias 2011). The model assumes EC and SMC as simplified rectangular domains with dimensions as shown in Figure 2.1-A and implements only half of the EC and SMC by assuming symmetry for the other half. Moreover, the model incorporates an accurate MP geometry from experimental images and assumed high density of IK$_{Ca}$ (25 % of total, under control conditions) and IP$_3$Rs (10% of total) within the MP. The continuum model takes into account concentration gradients of Ca$^{2+}$ and other ions within the EC and MP. The transport for individual ionic species is influenced by both electrical and concentration gradients, and was described using the Nernst-Planck electrodiffusion equation (Equation. 2.1)

$$\delta_{\text{eff}} \frac{\partial [S]}{\partial t} = \nabla \cdot (D_s \nabla [S] + z_FS [S] \nabla V) - R_s$$

Equation. 2.1
where $S = Na^+, K^+, Cl^-, Ca^{2+}$, $D_s$ is the diffusion coefficient of ionic species $S$, $z_s$ is the valence of ionic species $S$, $\nabla V$ is the electrical gradient, $F$ is the Faraday constant and $u_{ms}$ is the ionic mobility given by $\frac{D_s}{RT}$ ($R$ is the ideal gas constant (8341 mJ·mol$^{-1}$·K$^{-1}$) and $T$ is the absolute temperature). $R_s$ is the source/sink term which includes the expressions for cytosolic $Ca^{2+}$ exchange with the ER/SR, and $Ca^{2+}$ buffering in the EC and MP. $\delta_{buff}$ accounts for the $Ca^{2+}$ buffering in the SMC using a fast buffering approximation. Transport of cytosolic IP$_3$ and $Ca^{2+}$ within a uniformly distributed ER/SR store was described using Equation. 2.2.

$$\delta_{buff} \frac{\partial [S]}{\partial t} = \nabla . (D_s \nabla [S]) - R_s$$

Equation. 2.2

where $[S]$ is the concentration of either species (IP$_3$, $Ca^{2+}$ ER and SR) in the store. $R_s$ is the source/sink term and includes $Ca^{2+}$ exchange between stores and the cellular domains, and IP$_3$ production and degradation. A uniform distribution of transmembrane channels and pumps was considered along the boundary of the cellular domains. The membrane currents were defined as boundary fluxes (Equation. 2.3) across the top and bottom boundaries of EC, SMC and the MP boundaries.

$$-nN_S = \frac{1}{z_s F} \sum_K I_{S,K}$$

Equation. 2.3

where $n$ is the normal to the surface and $N_S$ is the membrane flux given by summation of all the transmembrane currents species for species $S$ ($I_{S,K}$). The membrane currents were distributed between the MP and bulk cell according to their respective volumes. The
membrane current definitions and parameters are identical to the original models (Kapela, Bezerianos, and Tsoukias 2008, 2009; Kapela and Tsoukias 2011; Silva, Kapela, and Tsoukias 2007).

To estimate the characteristics of the TRPV4 mediated Ca\(^{2+}\) event in the EC and resulting vessel tone modulation, the model in Nagarja et al. (Nagaraja et al. 2013) was modified through the introduction of the single or a cluster of four TRPV4 channels in a localized region on the EC boundary 0.5 µm away from the MP (Figure. 2.1-B). The current carried by each cation S (\(I_{\text{TRPV4,S}}\)) through a single TRPV4 channel is described using Equation. 2.4.

\[
I_{\text{TRPV4,S}}(t, V_m, S_i) = P_{\text{TRPV4,S}} \frac{z_i^2 F^2}{RT} V_m \frac{S_i - S_0 e^{-\frac{-z_i V_m F}{RT}}}{1 - e^{-\frac{-z_i V_m F}{RT}}}
\]

Equation. 2.4

where \(S_i\) is the concentration of Ca\(^{2+}\), K\(^{+}\), Na\(^{+}\) inside the EC, \(S_o\) is the extracellular concentration, \(F\) is the Faradays constant, \(P_{\text{TRPV4,S}}\) is the ionic permeability and \(V_m\) is the membrane potential.

The net current (\(I_{\text{TRPV4,total}}\)) through the channel (Equation. 2.5) modulates the membrane potential (\(V_m\)) described using standard Hodgkin Huxley formalism (Silva, Kapela, and Tsoukias 2007).

\[
I_{\text{TRPV4,total}} = \sum I_{\text{TRPV4,S}}
\]

Equation. 2.5
A single TRPV4 channel-conductance of 45 pS for inward currents at -100 to 0 mV was reported using inside-out patch clamp under symmetric intra- and extra-cellular ionic concentrations (Loukin et al. 2010), and a single channel-conductance of 56 to 66 pS for inward currents at -100 to 0 mV were observed under near physiological ionic concentrations using cell-attached patch clamp technique (Watanabe et al. 2002). We estimated the permeability for each ion \( P_{TRPV4,i} \) to match these conductance’s, using the \( I_{TRPV4,\text{total}} \) description (Eqs. 4&5) and reported permeability ratios \( P_{TRPV4,Ca} : P_{TRPV4,K} : P_{TRPV4,Na} = 7.1:1.42:1 \) (Ma et al. 2011)). The calculated \( \text{Ca}^{2+} \) permeability for a single
TRPV4 channel $P_{TRPV4,Ca}$ was $6.8 \times 10^{-13} – 8.5 \times 10^{-13}$ cm$^3$/s (which corresponds $4 \times 10^{-8} – 5 \times 10^{-8}$ cm/s assuming $1 \mu$F/cm$^2$ standard membrane capacitance and total EC membrane capacitance of 17 pF). In the model we use $P_{TRPV4,Ca}$ control value of $4.5 \times 10^{-8}$ cm/s. This corresponds to control values for $P_{TRPV,Na}$ and $P_{TRV4,K}$ of $6.5 \times 10^{-9}$ cm/s and $8.77 \times 10^{-9}$ cm/s respectively.

2.3.2 Stochastic opening of TRPV4 channel

2.3.2.1 Two-state model

Individual channels are not constantly open and the ionic currents fluctuate stochastically. A single channel can be represented using a continuous-time homogenous finite state Markov chain, which describes reversible transitions between a finite number of distinct states in which the ion channel can reside (Colquhoun and Hawkes 1982; Ball and Rice 1992). The probability of transitions from one state to another is assumed to be independent of the current state. As a simplest case, we considered the TRPV4 channel to be either in conducting (open) state or non-conducting (close) state (Mechanism. 1)

\[
\begin{array}{ccc}
\alpha & \rightarrow & \beta \\
O & \leftarrow & C
\end{array}
\]

Mechanism 1

where O is the open state, C the close state and $\alpha$, $\beta$ define the rate of transition from one state to another. The probability density function (pdf) for the closed-channel lifetime ($f_c$) and the open-channel lifetime ($f_o$) are distributed exponentially (Equation. 2.6).

\[
f_c = \beta e^{-\beta t} \\
f_o = \alpha e^{-\alpha t}
\]

Equation. 2.6
The mean lifetime in any state is given by the reciprocal of sum of the transition rates that lead away from the state (Colquhoun and Hawkes 1981). This provides a mean open lifetime and mean closed lifetime of $1/\alpha$ and $1/\beta$ respectively for the two-state model in Mechanism 1. The transition rates $\alpha$, $\beta$ were calculated based on the reported mean open time (37ms; $1/\alpha$) in Sonkusare et al. (Sonkusare et al. 2012) and an assumed mean closed time of 780 ms ($1/\beta$). This estimate for the mean closed time provides an open probability $(P_o; \beta/(\alpha+\beta))$ for an individual two-state TRPV4 channel of 0.045 as observed in (Sonkusare et al. 2012).

### 2.3.2.2 Three-state model

$\text{Ca}^{2+}$ data obtained from a cluster of TRPV4 channels (Bagher et al. 2012; Sonkusare et al. 2012) reveals a $\text{Ca}^{2+}$ burst of few seconds followed by longer inactivity periods, suggesting burst kinetics (i.e. openings are separated by short closed periods, before a long closed period). The two-state model cannot capture the experimentally observed $\text{Ca}^{2+}$ burst. We simulated stochastic opening of a single TRPV4 channel using a simple three-state model (Colquhoun and Hawkes 1982) as given in Mechanism 2.

![Mechanism 2 Diagram](B) where B is the block state (intraburst short closed state), O is the open state and S is the shut state (interburst long closed state). $k_1$, $k_2$, $k_3$ and $k_4$ define the rates of transition from one state to another. The TRPV4 channel can reside in either of these three states. The model in Mechanism 2 results in mean open time, mean blocked time, mean shut time and burst length given by $1/(k_3+k_1)$, $1/k_2$, $1/k_4$ and $(1+k_1/k_2)/k_3$ respectively. The
transition rates $k_1$ to $k_4$ were calculated based on the reported mean open time (37ms) of
the TRPV4 channel, and observed long inactivity (shut time) of about 1 min and burst
duration of around 5 sec from the Ca$^{2+}$ data in Sonkusare et al. (Sonkusare et al. 2012).
We assumed a mean channel blocked time (~33ms) to obtain an overall channel $P_o$ of
0.045 as in the two-state model. The overall probability $P_0$ was calculated as described in
(Colquhoun and Hawkes 1982). The three-state model provides a significantly higher
open probability during the burst ($P_o^{\text{burst}}$) of about 0.5.

2.3.2.3 Cluster of independent or cooperative TRPV4 channels

Stochastic opening of a cluster of four independent TRPV4 channels, with each
individual channel represented by the Markov two-state and three-state chain described in
Mechanism 1 and Mechanism 2 respectively, were simulated using a reduced transition
rate matrix (see supplement section 2.8). Given a group of $N$ independent and identical
TRPV4 channels, the probability that $k$ of these channels are open, $p(k)$, is described by
the binomial distribution (Equation 2.7).

$$p(k) = \binom{N}{k} P_o^k (1 - P_o)^{N-k}$$

Equation 2.7

A two minute long simulation of a cluster of four independent channels followed the
binomial distribution (see results). The $P_o$ of each individual channel in a cluster of four
independent channels was observed to be 0.045 as before, using both the two- and three-
state models. The average number of open (active) TRPV4 channels ($NP_o$) in this
simulation was 0.17 (close to the theoretical value of 4*P_o of 0.18).
Data in (Sonkusare et al. 2012) suggest that opening of two, three or four TRPV4 channels are significantly more frequent than expected based on the binomial distribution, indicating an interaction between the channels within a cluster. The cooperative two- and three-state models account for these channel interactions in a cluster through modulation of the transition rate from closed to open state (β), and shut state to open state (k₄) respectively, when at least one other channel in the cluster is in the open state (see supplement). For example, as shown in Figure 2.2 for a cluster of four channels described using the three-state model, if the channel 3 is in open state, the transition rate k₄ for the channel 1, 2 and 4 was increased to k₄' to allow for cooperativity between the channels. Cooperativity increased average number of open (active) TRPV4 channels (NPₒ) to 0.27 in both the two- and three-state models.

**Figure 2.2** Cooperativity implementation of four TRPV4 channels, (A) Four TRPV4 channel in a cluster described using a simple two-state (open and close) Markov chain model. The rate parameter β, for transition of a channel from the closed state to the open state, was increased in the presence of at least one other channel in the open state. (B) TRPV4 channels implemented using a three-state (shut, block and open) Markov chain to capture burst opening of the channel. The rate parameter k₄ describing the transition of a channel from the shut state to the open state was increased in the presence of at least one other channel in the open state.
2.4 Results

2.4.1 Continuous TRPV4 opening

A single TRPV4 channel opening was simulated through the introduction of TRPV4 current (Equation. 2.5). Ca\(^{2+}\) levels arising from continuous opening of the TRPV4 channel for 10, 100, 200, and 4000 ms are depicted color-coded in Figure. 2.3-A. A micromolar increase in Ca\(^{2+}\) levels was observed within few milliseconds in a small area (0.25 µm\(^2\)) around the TRPV4 channel location. Channel opening for longer durations, increases the spread area of the Ca\(^{2+}\) event. The resulting Ca\(^{2+}\) spread can activate Ca\(^{2+}\)-activated potassium channels (K\(_{Ca}\)) in the vicinity of the TRPV4 location. The contour lines in Figure. 2.3-B (black, yellow, red and brown) represent the region of Ca\(^{2+}\) spread for 10, 100, 200 and 4000 ms TRPV4 open duration respectively. The region was defined by Ca\(^{2+}\) concentrations higher than the half activation of the IK\(_{Ca}\) channel [(EC\(_{50}\) 740 nM (Ahn et al. 2004)]. Simulations predict Ca\(^{2+}\) activity spreading up to 0.3-6 µm radial distances for 10-4000 ms open time durations. The increase in the Ca\(^{2+}\) spread area did not increase linearly with channel open time.

2.4.2 Stochastic TRPV4 opening

Stochastic opening of a TRPV4 channel represented by a simple two-state Markov model (Mechanism 1) was simulated using a transition rate matrix (Equation. 2.11 in section 2.8) with \(\alpha=2.7\times10^{-2}\) ms\(^{-1}\) and \(\beta=1.3\times10^{-3}\) ms\(^{-1}\). A representative single channel record of 120 second duration is shown in supplement (Figure. 2.9-A), displaying random opening and closing of the TRPV4 channel with an overall P\(_o\) of 0.045. Simulations with two-state model could not capture the long quiescent period of TRPV4 channel inactivity observed in the experiment (Sonkusare et al. 2012).
Figure 2.3 Spatial Ca\textsuperscript{2+} profiles resulting from a single TRPV4 channel opening. (A) Continuous opening of the TRPV4 channel for open times of 10, 100, 200 and 4000 ms resulted in \( \mu \text{M} \) Ca\textsuperscript{2+} concentrations progressively spreading over a larger area. (B) Contour lines (Ca\textsuperscript{2+} concentration equal to EC\textsubscript{50} of IK\textsubscript{Ca}) indicate increasing Ca\textsuperscript{2+} spatial spread for 10,100,200 and 4000 ms of continuous opening of a TRPV4 channel and highlight the time evolution of the cell regions with at least 50\% IK\textsubscript{Ca} activity.

2.4.2.1 Three-state model

Three-state Markov chain model (Mechanism 2) was used to describe a single TRPV4 channel exhibiting bursting activity. Simulations were performed using a transition rate matrix obtained from the three-state model with \( k_1 = 2.35 \times 10^{-2} \text{ ms}^{-1} \), \( k_2 = 4.35 \times 10^{-2} \text{ ms}^{-1} \), \( k_3 = 3.23 \times 10^{-4} \text{ ms}^{-1} \) and \( k_4 = 1.69 \times 10^{-5} \text{ ms}^{-1} \) (Equation. 2.12 in section 2.8). These values were derived from the observed open time, open probability, interburst interval and burst duration in Sonkusare et al (Sonkusare et al. 2012).as described in the methods section. A
representative single channel record with burst openings is shown in Figure 2.4-A.

TRPV4 superposition records for a cluster of four independent channels were simulated (data not shown) using the reduced transition rate matrix of the three-state model (Equation 2.15 in section 2.8). Comparison between a single and a cluster of TRPV4 channels enable us to examine the physiological relevance of channel clustering in mediating Ca^{2+} signaling and EDH.

![Figure 2.4](image)

**Figure 2.4** Stochastic opening of a cluster of four TRPV4 channels implemented using a three-state Markov chain to simulate burst opening of the channel, (A) Illustrative example of a temporal profile of a single TRPV4 channel transition between the conducting (open) and non-conducting states (shut, block). (B) Example of a superposition temporal profile of the TRPV4 cluster, with the level number describing the number of open channels at a given time. Insert shows zoomed in view of a segment of the total simulation time for better visualization. (C) Experiment (Sonkusare et al. 2012) (black solid bar) and cooperative channel gating in the Markov model (black checkered) demonstrated increased open probabilities of second, third, and fourth channel openings respectively relative to the binomial distribution (grey solid) and a Markov model (grey checkered) considering independent channels.

Cooperativity was implemented by increasing the transition rate $k_4$ to $k_4'$ when at least one other channel is in the open state. An 8-fold increase in $k_4'$ was considered to match the experimentally observed NP_0 value (Figure. 2.8, in section 2.8). An illustrative
superposition record of a cooperative TRPV4 cluster is displayed in Figure.2.4-B. A significant number of 2\textsuperscript{nd}, 3\textsuperscript{rd} and 4\textsuperscript{th} level opening is observed during the burst period (compare with Figure. 2.9-B) followed by a long period of channel idleness. Similar to the two-state model, a cluster of independent channels resulted in an open probability distribution (Figure. 2.4-C, grey checkered bar) which follows a binomial distribution (Figure. 2.4-C, grey solid bar). An increase in the probabilities for second, third and fourth level openings was observed for simulations with cooperativity (Figure. 2.4-C, black checkered bar), in agreement with the experimental observation (Figure. 2.4-C, black solid bar). Statistical chi-squared test described in (Draber, Schultze, and Hansen 1993) was carried out to confirm for cooperativity in the simulations. The three-state model with cooperativity is able to closely capture the salient features of the TRPV4 cluster from the experiments. The different model implementations for the TRPV4 cluster allow us to examine the role of bursting and cooperativity in the modulation of vessel tone.

2.4.3 \( \text{Ca}^{2+} \) and \( V_m \) dynamics: stochastic continuum model

In the absence of agonist stimulation (i.e. no \( \text{Ca}^{2+} \) release from the stores), we examined the \( \text{Ca}^{2+} \) and \( V_m \) changes induced from opening of a cluster of EC TRPV4s in the continuum model. Stochastic current \( (I_{\text{stochastic,TRPV4},S}) \) from the TRPV4 channel cluster is provided by Equation. 2.8.

\[
I_{\text{stochastic,TRPV4},S}(t) = I_{TRPV4,S}(t) * N(t)
\]

Equation. 2.8
where $I_{TRPV4,i}(t)$ is the TRPV4 flux as described by the GHK equation (Equation. 2.5) and $N(t)$ is the number of open TRPV4 channels at time $t$ given by superposition records such as the records in Figure. 2.4-B and 2.5-B.

A two minute long representative simulation using the three-state cooperative model is displayed in Figure. 2.5. The stochastic TRPV4 openings (Figure. 2.5-A) and the average Ca$^{2+}$ concentration in a small area (0.25 µm$^2$) around the cluster’s location (Figure. 2.5-B) are depicted. The TRPV4 current resulted in quantal Ca$^{2+}$ increases with fixed amplitude of approximately 4 µM. [Note: Average Ca$^{2+}$ levels depend on the sampled region around the cluster and for an area of 2 µm$^2$ the amplitude was 1.8 µM]. Long inactivity periods follow these stochastic Ca$^{2+}$ transient bursts. Ca$^{2+}$ events in different simulations are shown in Figure. 2.5-D. The Ca$^{2+}$ transients closely resemble in duration and spread area the fluorescence recording of Ca$^{2+}$ sparklets in the experiments (Sonkusare et al. 2012; Sonkusare et al. 2014).

**Figure 2.5** Representative example of observed temporal EC Ca$^{2+}$ and EC $V_m$ profiles from the stochastic opening of the TRPV4 cluster in the continuum model, (A) Superposition temporal profile of TRPV4 cluster with cooperative gating kinetics implemented using the three-state model. Insert on right shows zoomed in view for better visualization, (B) EC Ca$^{2+}$ concentration around the TRPV4 cluster (0.25 µm$^2$ area) arising from the TRPV4 openings in (A), (C) EC $V_m$ transients follow the EC Ca$^{2+}$ events, (D) Local EC Ca$^{2+}$ concentration around the TRPV4 cluster (0.25 µm$^2$ area) observed in different stochastic simulations.
The simulated Ca\textsuperscript{2+} sparklets activated the localized IK\textsubscript{Ca} channels (25\% of total in the vicinity of the TRPV4s) to produce EC (and SMC) hyperpolarization. EC $V_m$ profile shows hyperpolarization’s corresponding to each Ca\textsuperscript{2+} sparklet (Figure. 2.5-C). An average EC hyperpolarization of around $\sim$2.3 mV was estimated in the 2 minute long simulation.

Simulations were carried out using the alternative model implementations for the TRPV4 cluster openings. Spatial Ca\textsuperscript{2+} profiles obtained, at the time of maximum Ca\textsuperscript{2+} spread, using: a) the two-state model with independent channels (Top: No bursting activity), b) the three-state model with independent channels (Middle: bursting activity) and c) the three-state model with interacting channels (Bottom: bursting activity with cooperativity) are depicted color-coded in Figure. 2.6-A. The contour lines represent the region of the EC where the Ca\textsuperscript{2+} concentration is above the IK\textsubscript{Ca} EC\textsubscript{50}. Model simulations with no bursting activity or cooperativity (two-state independent channel model) resulted in approximately 2 $\mu$m maximum radial distance for half activation of the IK\textsubscript{Ca} channels (Figure. 2.6-B). Bursting activity (three-state independent channel model) and bursting activity with cooperativity (three-state cooperative model) increased this radial distance by about 3.2 and 4.5 fold respectively (Figure.2.6-B).

In the model we simulate a single EC coupled to a single SMC. We also assumed that under control conditions, 25 \% of the total IK\textsubscript{Ca} channels are localized in close proximity to the TRPV4 cluster (i.e. within 1$\mu$m). This corresponds to 36 IK\textsubscript{Ca} channels, based on whole-cell IK\textsubscript{Ca} conductance ($G_{IK_{Ca}}$) of 1.7 nS, single IK\textsubscript{Ca} channel conductance of 17pS, and open probability of 0.7 (Bychkov et al. 2002; Silva, Kapela, and Tsoukias 2007).
Figure 2.6 Increase in distance of IK_{Ca} channel activation arising from burst and cooperative gating kinetics in the TRPV4 cluster. (A) Ca^{2+} concentration profile in the EC and the SMC at the time of maximum Ca^{2+} spread. TRPV4 channel cluster implemented with a two-state model (top: no bursting or cooperative gating kinetics), three-state model (middle: bursting activity) and three-state model with channel interactions (bottom: bursting and cooperativity). Contour lines indicate the Ca^{2+} concentration equivalent to EC_{50} of IK_{Ca} channels. (B) Radial distance for half-maximum K_{Ca} channel activation in the simulations in (A).

Figure. 2.7-A shows temporal changes in the EC V_{m} induced by the TRPV4 Ca^{2+} sparklet in a representative simulation. [Note: Similar levels of SMC hyperpolarization were obtained for a MEGJ resistance of 0.9 GΩ (Yamamoto et al. 2001; Kapela, Bezerianos, and Tsoukias 2009)]. Mean EC V_{m} hyperpolarization of around 6 mV is predicted during sparklet activity. Under the assumed arrangement of the TRPV4 and IK_{Ca} channels in the model, second, third and fourth level TRPV4 opening resulted in ~1.5-4.5 mV depolarization, attenuating the Ca^{2+} induced hyperpolarization.

In vivo, the hyperpolarization induced by TRPV4 sparklet in individual ECs will spread through homocellular gap junctions to neighboring ECs and through MEGJs to SMCs, causing vessel hyperpolarization and dilation. The bursting response in an individual cell from the IK_{Ca} channel activation seen in the model (Figure. 2.5-C, 2.7-A) will be smoothed giving rise to a steady change in vessel’s membrane potential. The mean hyperpolarization of the endothelium will be determined by the frequency and
duration of the sparklet events. Assuming TRPV4 sparklets with a mean duration, $\tau_{burst}$, an average frequency of occurrence per EC site, $f_{burst}$, and an average number of active sites per EC, $n_{sites}$, yields an average number of ECs, $N=1/(\tau_{burst} f_{burst} n_{sites})$, having an active event and open $I_{KCa}$ channels at any given time.

![A) EC-SMC mode](image1)

![B) Multi-cellular vessel model](image2)

**Figure 2.7** Predicted $V_m$ hyperpolarization induced by a localized Ca$^{2+}$ increase through TRPV4 channels (Ca$^{2+}$ sparklet). (A) Temporal EC $V_m$ profile (bottom) indicates an average EC hyperpolarization of $-6$ mV during the bursting activity of the TRPV4 cluster (top) in the single EC- single SMC model. (B) Hyperpolarization of the endothelium as a function of sparklet frequency and $I_{KCa}$ localization, predicted for an intact vessel with EC and SMC layers coupled by MEGJ.

Thus, hyperpolarizing current from each sparklet will spread, on average, in $N$ cells. The resulting hyperpolarization of the endothelium can be approximated from a simplified electrical equivalent of an intact vessel segment (Figure. 2.10) (Equation. 2.17 in the section 2.8). For a representative scenario of $\tau_{burst} = 5$ sec, $f_{burst} = 2$ bursts per min per active site and one active site per cell $n_{sites} = 1$ (i.e. $N=6$), $I_{KCa}$ conductance $g_{I_{KCa}} = \gamma_{I_{KCa}}$.

$G_{I_{KCa}} = 0.25 \times 1.72$ nS (representing 25% of $I_{KCa}$ channels or 36 channels activated by a single TRPV4 sparklet), and net EC-SMC membrane resistance $R_{m} = \cdots$
of \( \frac{1}{R_m^{EC}} + \frac{1}{R_m^{SMC} + R_m^{ME}} \) of \( \approx 1.2-2.2 \) GΩ (for \( R_m^{EC} = 2 - 10 \Omega \), \( R_m^{SMC} = 2 \Omega \) and

\( R_m^{ME} = 0.9 \Omega \); (Nilius and Droogmans 2001; Yamamoto et al. 2001; Nagaraja, Kapela, and Tsoukias 2012; Kapela et al. 2011; Yuan et al. 1993)), the estimated vessel hyperpolarization is \( \Delta V_m \approx 2-3.4 \) mV (Equation. 2.17). Figure 2.8-B shows how this hyperpolarization can increase as a function of the sparklet frequency per EC (\( f_{burst} \cdot n_{sites} \)) and the fraction of \( \text{IK}_{Ca} (\gamma_{IK_{Ca}}) \) localized around the TRPV4 cluster. In the presence of 36 (25% of total), 72 (50%) and 144 (100%) localized \( \text{IK}_{Ca} \) channels around the TRPV4 cluster, the TRPV4 mediated \( Ca^{2+} \) sparklet (with assumed mean duration of 5 sec) results in a vessel hyperpolarization of 0.3-15 mV for sparklet frequency between 0.1-12 bursts/min per EC (Figure. 2.7-B). Similar results were obtained with a multicellular computational model of an intact vessel segment (Kapela, Nagaraja, and Tsoukias 2010). The vessel model predicted a similar \( \Delta V_m \) value of 2.3 mV in the endothelium [simulating opening of 25% of \( \text{IK}_{Ca} \) channels in one EC out of 6 ECs (i.e. N=6)].

According to Equation. 2.17, hyperpolarization also depends on the membrane resistance, and in microvessels with higher input resistance, achieved levels of hyperpolarization may be greater relative to larger vessels.

2.5 Discussion

The primary aim of the study was to examine the regulatory mechanism of EC TRPV4 channels in inducing vessel relaxation. Using the 2D EC-SMC continuum model coupled with MP, we examined the localized \( Ca^{2+} \) events arising from activation of a single and a cluster of TRPV4 channels in the EC and the resulting vasodilatory response.
Simulations showed the effect of TRPV4 and IK_{Ca} channel distribution, and burst and cooperative gating kinetics of TRPV4 channels in defining the localized Ca^{2+} event (sparklet), and the resulting SMC hyperpolarization.

Endothelial control of vascular tone is attributed to EC Ca^{2+} mobilization that may manifest as events with different spatiotemporal characteristics. Agonist or mechanical stimulation, for example, may increase global Ca^{2+} levels, the frequency of Ca^{2+} waves/oscillations, the presence and frequency of Ca^{2+} pulsars (localized mediated by IP_{3}Rs in the vicinity of MP (Ledoux et al. 2008; Nilius and Droogmans 2001)), or of Ca^{2+} sparklets (events mediated by EC TRPV4 channels (Bagher et al. 2012; Sonkusare et al. 2012; Sonkusare et al. 2014)). Recent evidence suggests a central role of TRPV4 channels in agonist and mechanical induced vasoactive signaling in the microcirculation (Bagher et al. 2012; Sonkusare et al. 2012; Sonkusare et al. 2014).

2.5.1 **TRPV4 Ca^{2+} sparklet**

The model predicts local micromolar Ca^{2+} increases from a single TRPV4 channel opening for the duration of the channel’s open time (Figure. 2.3). The obtained Ca^{2+} levels in a small region around the TRPV4 channel are significantly higher than the nanomolar global Ca^{2+} increases usually observed experimentally (Ledoux et al. 2008; Nilius and Droogmans 2001) or predicted theoretically (Silva, Kapela, and Tsoukias 2007) in response to agonist or mechanical stimuli. These high local Ca^{2+} concentrations may be required to fully activate cellular components with low Ca^{2+} affinity including IK_{Ca} channels whose affinity may be in the high nanomolar range (reported EC_{50} values as high as 300-740 nM (Ahn et al. 2004; Ishii et al. 1997)).
Multiple TRPV4 channel opening in a cluster resulted in localized quantal Ca$^{2+}$ increases (Figure. 2.5-B & 2.5-D). Second, third and fourth level openings in the TRPV4 cluster lead to essentially constant amplitude increases in Ca$^{2+}$ concentrations that return fast to the previous baseline following channel closure, consistent with the experimental data (Bagher et al. 2012; Sonkusare et al. 2012; Sonkusare et al. 2014). This allows examining channel gating characteristics based on the changes in Ca$^{2+}$ levels as was done in the experimental studies (Sonkusare et al. 2012; Sonkusare et al. 2014). The model corroborates the methodology utilized in these earlier analyses and shows that Ca$^{2+}$ diffusion, extrusion and buffering have minimal effect on the interpretation of the data.

In the experiments, TRPV4 channels with an average open time of milliseconds (Sonkusare et al. 2012), generate a Ca$^{2+}$ burst event (i.e. sparklet) of 1-10 second duration, followed by a long inactivity period before the occurrence of the next event. The significant increase in duration of the Ca$^{2+}$ event, compared to channel open times, can be a result of bursting activity of the TRPV4 channel. Bursting kinetics of TRPV4s have been observed in single channel patch clamp data (Loukin et al. 2010; Watanabe et al. 2002). Model implementation of bursting in a cluster of four TRPV4 channels (Figure. 2.4 & 2.5) resulted in Ca$^{2+}$ events lasting for seconds followed by longer inactivity periods (Figure. 2.5-B & 2.5-D) as observed in the experiments. Burst activity in a cluster of independent TRPV4 channels enhanced the Ca$^{2+}$ spread and activated IK$_{Ca}$ channels at longer distances (Ca$^{2+}$ concentrations >50% activation within 5µm) (Figure. 2.6-A and 2.6-B).
Cooperative activation of TRPV4 channels in a cluster has been suggested in experiments as multilevel openings appear much more frequently than suggested by the binomial distribution for random events (Sonkusare et al. 2012). We have accounted for cooperativity in channel kinetics, capturing the increased probability of multi-level openings seen in the experiments. Cooperativity further enhances the Ca$^{2+}$ spread. The combined effect of bursting and cooperativity is a Ca$^{2+}$ spread of approximately 7µm (defined as the radial distance with Ca$^{2+}$ levels greater than IK$_{Ca}$ EC$_{50}$). Thus simulations suggest that the gating characteristics of TRPV4 cluster increase the sparklet’s area and presumably the resulting hyperpolarization by the recruitment of additional IK$_{Ca}$ channels. It will be interesting to examine the effect of clustering of other TRP channel such as of TRPA1- and TRPV3 which have been shown to facilitate endothelium-dependent dilation in cerebral arteries (Earley, Gonzales, and Crnich 2009; Earley, Gonzales, and Garcia 2010). The coupled stochastic-continuum model presented here could be extended towards the analysis of experimental data from stimulation of such channels.

2.5.2 TRPV4 mediated hyperpolarization

EC hyperpolarization through the activation of IK$_{Ca}$ and SK$_{Ca}$ channels is a major contributor in NO-independent endothelium-derived vasodilation in microvessels (Garland, Hiley, and Dora 2011). TRPV4 Ca$^{2+}$ sparklets may activate nearby IK$_{Ca}$ channels (Sonkusare et al. 2012; Bagher et al. 2012) to induce hyperpolarization and dilation. We tested the potential of TRPV4 sparklets to mediate EDH responses. The presence of high density cellular components including the K$_{Ca}$ channels near microdomain structures like MPs have been reported in small resistance vessels
Micromolar localized Ca$^{2+}$ increases around the MPs can potentially fully activate the nearby $K_{Ca}$ channels (assuming an $EC_{50}$ as high as 740 nM). In the model of a single EC coupled to a single SMC, a TRPV4 sparklet activated co-localized $IK_{Ca}$ channels in the vicinity of the cluster to generate about 6 mV of hyperpolarization in the EC (Figure. 2.7-A).

Although cooperative gating in the cluster, increased the Ca$^{2+}$ spread (Figure. 2.6-A), it showed no significant amplification of hyperpolarization (data not shown). This was attributed to the positioning of a high density of $IK_{Ca}$ channels within a very close proximity to the cluster (<1 µm). Within this area even a single TRPV4 channel produces saturating Ca$^{2+}$ concentrations so multi-level opening in a cluster does not offer any additional advantage. On the contrary, at physiological ionic concentrations and around resting $V_m$, significant sodium influx currents (pA) are predicted from each TRPV4 opening. This can result in few mV depolarization which in the absence of additional $K_{Ca}$ channel recruitment will have an attenuating effect on EDH signaling. Thus, the high Na$^+$ influx and the micromolar Ca$^{2+}$ levels in the vicinity of the TRPV4 cluster arising from a single channel opening suggest that bursting and cooperatively may benefit EDH signaling only if $K_{Ca}$ channels are positioned at intermediate distances (3-10 µm) away from the cluster. Under the assumed arrangement of TRPV4 and $IK_{Ca}$ channels in the model, we observed that second, third and fourth TRPV4 channel opening from cooperative gating kinetics, result in a ~1.5-4.5 mV depolarization (Figure. 2.7-A).
Sustained vessel hyperpolarization results from the spread of transient hyperpolarization generated in a single EC cell to its neighbors. The achieved vessel hyperpolarization will depend on many parameters including membrane resistances of the EC and SMC layers, the MEGJ resistance, the transient hyperpolarizing current, as well as the frequency and duration of the events. Simulations suggest a significant EC hyperpolarization through activation of TRPV4 channels, which increases with increasing the frequency and duration of the event and the amount of the $K_{Ca}$ channels in proximity to the TRPV4 cluster (Figure. 2.7-A). Our calculations suggest that experimentally observed TRPV4 Ca$^{2+}$ sparklets that typically have an average duration of about 5 sec and frequency of occurrence of 2 bursts/min per EC should induce about 2.0 mV hyperpolarization; assuming that 36 channels (25% of total) are located near the cluster (Figure. 2.7-B). This is lower than what has been observed in experiments (~10 mV) (Sonkusare et al. 2012). This discrepancy may be attributed to a higher number of sparklet events/sites than observed experimentally, a very high number of $K_{Ca}$ activated by each sparklet (i.e. the majority of $I_{K}\text{Ca}$) or to mechanisms amplifying TRPV4/$I_{K}\text{Ca}$ mediated hyperpolarization. Moreover, the predicted level of hyperpolarization depends on parameters, such membrane resistances (Equation. 2.17), that have not been accurately characterized, that may change with stimulation or varied between vessels. Particularly as the size of vessel decreases input resistance typically increases and this may result in significantly higher hyperpolarization for a given transmembrane current.
2.5.3 Local versus global Ca\(^{2+}\) activity

It is well established that a variety of stimuli can elicit global Ca\(^{2+}\) increases in ECs. A typical Ca\(^{2+}\) response has an initial transient that is mediated by store Ca\(^{2+}\) release (with peak concentrations in the order of 0.4-1.5 \(\mu\)M (Nilius and Droogmans 2001; Buchan and Martin 1991)) followed by a lower plateau phase that is sustained by Ca\(^{2+}\) influx through Ca\(^{2+}\) permeable channels in the plasma membrane. This global Ca\(^{2+}\) mobilization is thought to mediate vasoactive signaling, including EDH responses. Recent studies, however, showed activation of IK\(_{Ca}\) channels by localized Ca\(^{2+}\) events such as pulsars and sparklets (Sonkusare et al. 2014; Sonkusare et al. 2012; Ledoux et al. 2008; Bagher et al. 2012) and a moderate global Ca\(^{2+}\) increase (\(~30\%\) above resting) by cyclopiazonic acid (CPA) did not lead to a significant activation of K\(_{Ca}\) channels in (Ledoux et al. 2008). Thus, recent evidence points towards a preferential activation of IK\(_{Ca}\) channels and EDH response to local rather than global Ca\(^{2+}\) increases.

Is there a mechanism that would allow for a portion of K\(_{Ca}\) channels to be relatively immune to global Ca\(^{2+}\) mobilization so that their activity to be regulated preferentially/exclusively by localized Ca\(^{2+}\) signaling? One possibility is that compartmentalization of TRPV4 and K\(_{Ca}\) channels may provide a locally confined, regulatory unit relatively inaccessible to global Ca\(^{2+}\) changes. Restriction of Ca\(^{2+}\) signals to subplasmalemma regions has been previously reported suggesting the presence of cytosolic compartments (Saliez et al. 2008; Nilius and Droogmans 2001; Ledoux et al. 2008; Rizzuto and Pozzan 2006; Brasen, Jacobsen, and Holstein-Rathlou 2012).
Simulation results provide an alternative possibility. The preferential activation of the KCa channels to local rather than global Ca\textsuperscript{2+} increases may be a result of significantly higher Ca\textsuperscript{2+} concentrations in localized events (micromolar levels are predicted in the model (Figure. 2.6-B and 2.6-D)) relative to nanomolar global Ca\textsuperscript{2+} increases during EC stimulation (Silva, Kapela, and Tsoukias 2007; Nilius and Droogmans 2001). Provided that the KCa EC\textsubscript{50} is in the high nanomolar range, global Ca\textsuperscript{2+} levels may not be sufficient to fully activate KCa and thus a significant portion of the channels is activated only by the high concentration local Ca\textsuperscript{2+} event. However, reported values for IKCa affinity for Ca\textsuperscript{2+} range from approximately 100 nM to 740 nM (Nilius and Droogmans 2001; Ahn et al. 2004; Crane et al. 2003; Ishii et al. 1997). The predicted high local Ca\textsuperscript{2+} concentrations will be beneficial only if the EC\textsubscript{50} is in the high end of this range. [A high EC\textsubscript{50} (740 nM) (i.e. low affinity) for IKCa channels was assumed in the simulations].

Interestingly, a high Ca\textsuperscript{2+} affinity for IKCa channels can make these channels significantly (if not fully) activated by global Ca\textsuperscript{2+} events. In this case, subsequent TRPV4 activation may result in EC depolarization that will attenuate rather than promote EDH. [Recruitment of saturated KCa channels will not compensate for the depolarizing TRPV4 current]. An intermediate scenario where an IKCa channel is activated by both local events and global transients might also be possible. Further experimentation is required to determine the relative contribution of global and local Ca\textsuperscript{2+} events on IKCa channel activation.
2.6 Limitations

A number of parameter values utilized have not been previously quantified or may vary between different vessels. In this study, we try to remain consistent with our previous EC–SMC models with respect to parameter values and whole cell currents and examine responses over a range of values for parameters with significant uncertainty. The model is limited by the absence of quantitative data for the spatial distribution of important cellular components. Such data may significantly improve the predictive ability of the proposed models. The TRPV4 channel current was modeled using a GHK equation and ignores the effects of temperature, shear and modulatory pathways on the channel. Moreover simple Markov chain models were considered for simplicity and can capture the observed channel behavior, however, the actual gating mechanism could be more complex.

2.7 Conclusions

The developed model describes TRPV4 mediated signaling for the regulation of vessel dilation. Model simulations are in good agreement with experiments in isolated vessels, corroborating data for the role of TRPV4 signaling in vascular control. The model predicts a micromolar localized Ca$^{2+}$ increase through activation of cluster of four TRPV4 channels. This is significantly higher than the nanomolar global Ca$^{2+}$ levels typically observed during EC stimulation. The model shows an enhanced Ca$^{2+}$ spread as a result of burst opening and cooperative channel gating in the TRPV4 cluster. Ca$^{2+}$ mobilization over several micron radial distances can recruit $K_Ca$ channels and produce millivolts of EDH. Model predicts that amplification of EDH signaling by cooperativity in the TRPV4 cluster is more likely if $K_Ca$ channels are not immediately adjacent to the
TRPV4 channels, but at distances that can reach 10 microns away. The magnitude of the resulting hyperpolarization, dependents on a variety of parameters including the sparklet frequency and the effective membrane resistance of the vessel wall. Nevertheless, for a wide range of parameter values predicted TRPV4-induced hyperpolarization’s underestimate what has been recorded in some experiments. This may suggest additional mechanisms contributing to the observed EDH response.

2.8 Supplement

2.8.1 Transition rate matrix

Simulating single-channel behavior can be achieved through matrix formulations which describes the rates of transition in between plausible kinetic states that a channel could reside, as illustrated in (Colquhoun and Hawkes 1982). Consider a continuous time Markov chain with state space (1, 2, ..., r), the r x r transition rate matrix (Q) is defined as Q = [q_{ij}], where, q_{ij} is the transition rate from state i to state j and the diagonal elements q_{ii} satisfy,

\[ q_{ii} = - \sum_{i \neq j, j=1}^{r} q_{ij} \]

Equation 2.9

The probability of transition from state i to state j (\( \pi_{ij} \)) is given by,

\[ \pi_{ij} = - \frac{q_{ij}}{q_{ii}} \]

Equation 2.10
For the simplest two state model with one open and one closed state as described in Mechanism 1 the transition rate matrix is defined as,

\[
Q = \begin{bmatrix}
1:O & 2:C \\
1:O & -\alpha & \alpha \\
2:C & \beta & -\beta
\end{bmatrix}
\]

Equation 2.11

Similarly for the three state model (Equation. 2.6), the transition rate matrix is given by,

\[
Q = \begin{bmatrix}
1:O & 2:B & 3:S \\
1:O & -(k_3 + k_1) & k_1 & k_3 \\
2:B & k_2 & -k_2 & 0 \\
3:S & k_4 & 0 & -k_4
\end{bmatrix}
\]

Equation 2.12

### 2.8.2 Reduced transition rate matrix

A system of two channels implemented with two state model will result in a 4 x 4 transition matrix. However a system of four channels implemented with three state model will result in 81 x 81 matrix and necessities the formation of reduced transition matrix to implement the behavior of a group of channels. A approach similar to described in Ball et al. (Ball and Rice 1992) was undertaken to obtain a reduced state matrix for a system of four TRPV4 channel described by the three state model. As an example let’s consider a system of two channel described using the two state kinetic model of Mechanism 1. The number of possible states are \(2^2\) resulting in \(2^2 \times 2^2\) transition matrix (Equation. 2.13)
Equation 2.13

Of the four possible states i.e. both channels open (O₁O₂), one channel open and other closed ((O₂C₁),(C₂O₁)) and both channels closed (C₁C₂), the state (O₂C₁) and (C₂O₁) are indistinguishable as it is difficult to determine which of the two channels is in the open state. Utilizing these symmetries and considering only the number of channels in each state it is possible to reduce the size of the state space to 3 states (Equation 2.14) or in general for a N channel system described using r state model reduces to \( \binom{N + r - 1}{N} \) (Ball and Rice 1992).

Equation 2.14

We utilized similar approach for a system of four channels described by the three state model to reduce it to a 15 x 15 matrix given by Equation 2.15, where the number is the brackets in the first row represent the number of symmetrical states to the corresponding
state provided in the second row. For example state 7 in second row (OBBB) has four other symmetrical states depending on which of channel one to four is in the open state. The diagonal elements \(d_i\) are equal to negative of sum of elements in row \(i\), where \(i=1,2,3\ldots15\).

\[
\begin{align*}
\begin{array}{ccccccccccccccccc}
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 & 11 & 12 & 13 & 14 & 15 \\
\text{OOOO:1} & d_1 & 4k_1 & 4k_3 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\text{OOOB:2} & k_2 & d_2 & 0 & 6k_1 & 12k_3 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\text{OOOS:3} & k_4 & 0 & d_3 & 0 & 12k_1 & 6k_3 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\text{OOBB:4} & 0 & 4k_2 & 0 & d_4 & 0 & 0 & 4k_1 & 12k_3 & 0 & 0 & 0 & 0 & 0 & 0 \\
\text{OOBS:5} & 0 & 4k_4 & 0 & d_5 & 0 & 0 & 0 & 0 & 12k_1 & 12k_3 & 0 & 0 & 0 & 0 \\
\text{OSSS:6} & 0 & 0 & 4k_4 & 0 & 0 & d_6 & 0 & 0 & 12k_1 & 4k_3 & 0 & 0 & 0 & 0 \\
\end{array}
\end{align*}
\]

\[
Q_{\text{channels}} = \begin{array}{ccccccccccccccccc}
\text{OOBB:7} & 0 & 0 & 0 & 6k_2 & 0 & 0 & d_7 & 0 & 0 & 0 & k_1 & 4k_3 & 0 & 0 & 0 \\
\text{OBBS:8} & 0 & 0 & 0 & 6k_4 & 12k_2 & 0 & 0 & d_8 & 0 & 0 & 0 & 4k_1 & 6k_3 & 0 & 0 \\
\text{OBSS:9} & 0 & 0 & 0 & 0 & 12k_4 & 6k_2 & 0 & 0 & d_9 & 0 & 0 & 0 & 6k_1 & 4k_3 & 0 \\
\text{OSSS:10} & 0 & 0 & 0 & 0 & 0 & 6k_4 & 0 & 0 & 0 & d_{10} & 0 & 0 & 0 & 4k_1 & k_3 \\
\text{BBBB:11} & 0 & 0 & 0 & 0 & 0 & 0 & 4k_2 & 0 & 0 & 0 & d_{11} & 0 & 0 & 0 & 0 \\
\text{BBBS:12} & 0 & 0 & 0 & 0 & 0 & 0 & 4k_4 & 12k_2 & 0 & 0 & 0 & d_{12} & 0 & 0 & 0 \\
\text{BBSS:13} & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 12k_4 & 12k_2 & 0 & 0 & 0 & d_{13} & 0 & 0 \\
\text{BSSS:14} & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 12k_4 & 4k_2 & 0 & 0 & 0 & d_{14} & 0 \\
\text{SSSS:15} & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 4k_4 & 0 & 0 & 0 & 0 & d_{15}
\end{array}
\]

Equation 2.15

2.8.3 Cooperativity implementation

Consider system of two interacting channels as illustrated in (Keleshian et al. 1994) each described by a simple two state model (Mechanism 1) with transition rates \(\alpha\) and \(\beta\). The channels have an opening rate \(\beta'\) during a period in which the other channel is open, and \(\beta\) otherwise. The conditional densities can be written as in Equation. 2.16, where, for example, \(f_{2\rightarrow1\leftarrow}\) is the density of closed sojourn time of second channel given that the first channel is in closed state.
\[ f_{2|1|c} = f_{1|2|c} = \beta e^{-\beta k} \]
\[ f_{2|0|1} = f_{2|0|2} = f_{1|1|2} = \alpha e^{-\alpha} \]
\[ f_{2|0|0} = f_{1|1|0} = \beta' e^{-\beta'} (\beta' > \beta) \]

Equation 2.16

To implement cooperativity we modified the reduced transition matrix defined in Equation 2.14 and Equation 2.15 through increasing the parameter value of \( \beta \) (to \( \beta' \)) and \( k_4 \) (to \( k_4' \)) respectively during presence of at least one channel in open state, i.e. we increase the probability of opening of a channel in a shut or a blocked state only in the presence of one or more open channels in a cluster. For example, in Equation 2.15 consider the transition from state OOOS to OOOO. We increase the parameter \( k_4 \) as transition of the only shut channel occurs to open state in the presence of three open channels, however for the transition from SSSS to OSSS state the parameter \( k_4 \) in the transition matrix would not be modified as one of the shut channel transits to open state in the presence of three other channels which are all in shut state. The values of \( \beta' \) and \( k_4' \) were chosen to obtain an \( NP_o \) observed in the experiments (Sonkusare et al. 2012) as shown in Figure 2.8.

![Figure 2.8 Plot of NP_o obtained with 1-20 fold increase in beta (yellow line) and k_4 (red dotted line)](image)
2.8.4 Stochastic TRPV4 opening (Two-state model)

Stochastic opening of a TRPV4 channel was simulated using a transition rate matrix (Equation 2.11) with $\alpha = 2.7 \times 10^{-2}$ ms$^{-1}$ and $\beta = 1.3 \times 10^{-3}$ ms$^{-1}$. A representative single channel record of 120 second duration is shown in Figure 2.9-A, displaying random opening and closing of the TRPV4 channel with an overall $P_o$ of 0.045. The histogram of the dwell time in the open state (O) was constructed (data not shown) and matched the experimentally observed channel open time distribution in (Sonkusare et al. 2012).

Superposition records of a cluster of four TRPV4 channels, with each individual channel represented by the two-state model (Mechanism 1), were simulated using the reduced two-state four-channel transition rate matrix (Equation 2.14) with $\alpha$ ($2.7 \times 10^{-2}$ ms$^{-1}$) and $\beta$ ($1.3 \times 10^{-3}$ ms$^{-1}$) as before. A two min long representative simulation of a cluster of four independent TRPV4 channels is shown in Figure 2.9-B. A few second and third level opening are observed with overall $NP_o$ of 0.17. Cooperative opening in a cluster of four TRPV4 channels was simulated through increasing the transition rate $\beta$ to $\beta'$ in the presence of at least one open channel in the cluster (data not shown). A range of $\beta'$ was examined (Figure 2.8) and $\beta'$ equal to $6\beta$ was used in the model to match the experimentally observed $NP_o$ value (~0.27).

In a cluster of four independent TRPV4 channel, the probabilities for one, two, three or four channels being open at a time (Figure 2.9-C, Grey checkered bars) followed closely the expected binomial distribution with a single channel $P_o$ of 0.045 (Figure 2.9-C, Grey solid bars).
Simulations with a cluster of TRPV4s with cooperative gating revealed significantly higher probabilities for second, third and fourth level opening (Figure. 2.9-C, Black checkered bars) that closely follow those observed in experiments (Figure. 2.9-C, Black solid bars).

**Figure 2.9** Stochastic opening of TRPV4 channels described by a two-state Markov chain. (A) Illustrative example of a temporal profile of a single TRPV4 channel transitioning between the close and the open states. (B) A superposition temporal profile of a cluster of four independent TRPV4 channels. The level number describes the number of open channels at a given time point. (C) Open probabilities of first, second, third and fourth level channel openings in a TRPV4 cluster of four channels. The two-state Markov model considering independent channels (grey checkered bars) matches the binomial distribution of independent events (grey solid bar; Equation. 2.7, \( P_{o}=0.045 \)). Experimental data from (Sonkusare et al. 2012) (black solid bar) show increased probability of second, third, and fourth channel openings relative to the binomial distribution. The two-state model with cooperative channel gating (black checkered bar; \( \beta' = 6\beta \)) matches the experimentally observed open probabilities.

### 2.8.5 Electrical equivalent of multi-cellular vessel segment

Figure 2.10 shows a simplified electrical equivalent circuit used to estimate the hyperpolarization of the endothelium in intact vessels induced by the activity of TRPV4.

From Figure. 2.10

\[
\Delta V_m = \frac{E_K - V_{rest}}{R_m} + \frac{\gamma_{IK_{tra}}}{R_{IK_{tra}}} \quad R_m = \frac{E_K - V_{rest}}{N \cdot R_{IK_{tra}}} + \frac{\gamma_{IK_{tra}}}{N \cdot R_{IK_{tra}}} \cdot R_m
\]
where $\Delta V_m$ is the change in endothelial $V_m$, $E_K = -79$ mV is the Nernst potential for $K^+$, $V_{\text{rest}} = -54$ mV is the resting EC $V_m$ coupled to a SMC, $R_m \approx 1.2 \text{ G} \Omega$ is the net membrane resistance of EC connected through myoendothelial gap junction to SMC, $R_{IK_{Ca}}$ is the total resistance of IK$_{Ca}$ channels, $\gamma_{IK_{Ca}}$ (0-1) is the fraction of IK$_{Ca}$ activated by single TRPV4 sparklet, $N = \frac{1}{\tau_{\text{burst}} \cdot f_{\text{burst}} \cdot n_{\text{sites}}}$, is the number of ECs having an active event and open IK$_{Ca}$ channels at any given time point., $\tau_{\text{burst}}$ (assumed mean of 5 sec) is the average duration of TRPV4 sparklet, $f_{\text{burst}}$ is the frequency of TRPV4 Ca$^{2+}$ burst per site and $n_{\text{sites}}$ is the number of active sites per EC.

*Figure 2.10* Electrical equivalent of a vessel segment with activated IK$_{Ca}$. $r_m$ is the net membrane resistance of ECs coupled to SMCs and $R_m$ is the effective resistance of a EC coupled to a SMC, $R_{IK_{Ca}}$ is the total resistance of IK$_{Ca}$ channels in a single EC and $r_{IK_{Ca}}$ is the net resistance of IK$_{Ca}$ channels in the vessel activated by TRPV4 sparklets.
2.9 References


compartmentation in endothelium-derived hyperpolarizing factor-mediated relaxation: Ca2+ signals and gap junction function are regulated by caveolin in endothelial cells. *Circulation* 117 (8):1065-74.


CHAPTER 3-NITRIC OXIDE (NO) MEDIATED VASODILATORY FEEDBACK RESPONSE AND ITS MODULATION BY ENDOTHELIAL HEMOGLOBIN α AND ERYTHROCYTES

This chapter is to be submitted to *Journal of Physiology* (with modifications) as Parikh, J., A. Kapela and N. M.Tsoukias. “Localized endothelial nitric oxide-mediated smooth muscle cell hyperpolarization; regulation by endothelial Hemoglobin α and erythrocyte’s”
3.1 Abstract

Nitric oxide (NO) derived from endothelial nitric oxide synthase (eNOS) is known to modulate the vessel tone in response to both vasoconstrictors and vasodilators. We utilize mathematical modeling to investigate NO mediated vasodilatory feedback response and address experimental limitations in quantifying vascular NO concentration. We analyze recently reported hypothesis governing NO signaling through consumption by hemoglobin α (Hbα) colocalized with eNOS in the endothelial microprojections (MP) by Straub et al. (Nature 491: 473-477, 2012). We developed detailed continuum cellular models of Ca\(^{2+}\) dynamics, membrane electrophysiology and NO signaling for coupled smooth muscle (SMC) and endothelial cell (EC). The model takes into consideration the recently reported enriched expression of the IP\(_3\) receptors, eNOS, Ca\(^{2+}\) activated potassium (K\(_{Ca}\)) channels and Hbα in the MP. Localized EC Ca\(^{2+}\) mobilization is induced during SMC stimulation in the MP. NO mediated feedback response through this localized Ca\(^{2+}\) signaling is observed in the model in the presence of localized eNOS in the vicinity of the MP. EC Hbα reduced the feedback through the consumption of NO as observed in the experiments. Modulation of NO bioavailability by Hbα is enhanced by their colocalization in the MP. Moreover model simulations suggest that RBC perfusion will decrease the ability of Hbα to modulate NO levels, and µM levels of EC Hbα are necessary for a significant modulation of SMC NO availability. The model explores the NO signaling mechanism in response to the local Ca\(^{2+}\) mobilization and also aids in quantification of Hbα concentrations essential for modulating NO mediated feedback response.
3.2 Introduction

In microvasculature, complex bidirectional communication between the endothelial (EC) and smooth muscle cell (SMC) regulates SMC constriction and vascular tone. EC extensions over the internal elastic lamina towards SMC, termed myoendothelial projections (MPs), have been observed in resistance vessels (Heberlein, Straub, and Isakson 2009; Sandow et al. 2009) and can play a role in the modulation of vessel tone (Kerr et al. 2012). Recent studies provide evidence for enriched expression of inositol 1,4,5-triphosphate receptors (IP3Rs), connexins, caveolae, eNOS and Hbα in/near MPs (Straub et al. 2011; Straub et al. 2012; Isakson 2008; Ledoux et al. 2008; Heberlein, Straub, and Isakson 2009) to form a powerful signaling unit. Myoendothelial gap junctions (MEGJs) are often found at the tip of the MP, and allow for electrochemical communication between ECs and SMCs (Heberlein, Straub, and Isakson 2009; Sandow et al. 2009). Agonist stimulation of the SMC induces inositol triphosphate (IP3) production and Ca²⁺ increase to result in vasoconstriction. Ca²⁺ and or second messenger IP3 diffusion to the EC through myoendothelial gap junctions (MEGJs) may lead to Ca²⁺ response in the EC to result in Ca²⁺ dependent vasodilatory feedback responses, including NO signaling to modulate the vessel tone (Dora, Doyle, and Duling 1997; Isakson, Ramos, and Duling 2007; Yashiro and Duling 2000; Kerr et al. 2012; Tran et al. 2012; Sandow et al. 2009). Different aspects of the NO mediated feedback response remains unclear and elucidation of the mechanisms regulating this process could provide novel insight into microvascular reactivity.
Nitric oxide (NO) is produced in the endothelial cells (ECs), which line blood vessels, through the degradation of L-arginine by endothelial nitric oxide synthase (eNOS) enzyme. The activity of eNOS depends on the cytosolic EC calcium (Ca\(^{2+}\)) concentration (Busse and Mulsch 1990). The NO produced in the EC diffuses to the adjacent smooth muscle cell (SMC) to activate a cascade of signaling mechanisms including activation of soluble guanylate cyclase (sGC) to result in SMC relaxation. In the vasculature, NO have been identified as an endothelium derived relaxing factor (EDRF) and a key regulator of vascular tone and blood flow (Furchgott and Zawadzki 1980; Ignarro et al. 1987; Palmer, Ferrige, and Moncada 1987).

EC are in close proximity with the RBCs in the blood which are abundant in Hemoglobin (Hb) concentrations. NO is scavenged rapidly through fast reactions with both oxy- and deoxy-Hb (Eich et al. 1996; Herold, Exner, and Nauser 2001) forming met-Hb (Hb-Fe\(^{3+}\)) and nitrate (NO\(_3^-\)), which may severely limit NO diffusion and bioavailability of NO in the SMC. Several experimental studies and mathematical models have been carried out to examine how NO escapes scavenging through the complex interaction of NO with substances, such as heme-containing proteins and reactive oxygen species and are reviewed in (Tsoukias 2008; Buerk, Barbee, and Jaron 2011). Recent experimental studies on isolated vessels and vascular cell co-culture models in the absence of the blood revealed localized NO production through spatial localization of eNOS in the MP (Straub et al. 2012; Straub et al. 2011) as one of the safeguard for significant scavenging in the RBCs (Rahaman and Straub 2013; Gladwin and Kim-Shapiro 2012) during the feedback. Straub et al. recently identified enriched expression of Hb\(_\alpha\) at the MP (Straub et al. 2012) and revealed its role as a NO signaling switch.
(through scavenging of NO depending on its heme iron oxidation state) to control NO bioavailability and vascular tone during agonist induced stimulation (Straub et al. 2012; Rahaman and Straub 2013).

Mathematical modeling can assist in examination of NO pathway physiology and quantify vascular NO concentrations. Mass transport models for NO transport have been developed to examine its fate in the vasculature. We recently reported a comprehensive review of different mathematical models undertaken to describe NO transport in vasculature (Tsoukias 2008). We have previously developed coupled EC-SMC compartmental model integrated with NO signaling pathway to investigate the effect of myoendothelial communication on modulation of SMC Ca\(^{2+}\) and membrane potential \((V_m)\) dynamics. Very few theoretical models have examined the role of MP with spatially localized channels, proteins and molecules in the regulation of the vessel tone. We extended the coupled EC-SMC model compartmental model into a 2-D continuum model which incorporates accurate MP geometry from electron microscopy images and spatial localization of IK\(_{Ca}\) and IP\(_3\)Rs in the MP. This new formulation was utilized to investigate the role of localized signals in EC-SMC communication in absence of NO signaling (Nagaraja et al. 2013). Similarly, Brasen et al. have developed a 2-D axisymmetric model incorporating the anatomical structure of MPs into a two cell system (Brasen, Jacobsen, and Holstein-Rathlou 2012). Their results show that MPs may rectify the signal between the EC and SMC. Previous models did not examine the NO mediated feedback signaling during the stimulation of the SMC and its modulation by EC Hba.
In this chapter, we present a computational model to examine the EC mediated NO signaling. Moreover we analyzed the relative contribution of enriched EC Hbα in the MP and the RBCs in the modulation of NO feedback response. The NO signaling pathways was incorporated in the previously developed continuum EC-SMC model with MPs (Figure 3.1-A). The model accounts for preferential presence of the Hbα, eNOS and IP₃Rs in the MPs as suggested in experimental studies. We utilize this model to examine the contribution of NO feedback signaling in the regulation of vessel tone.

**Figure 3.1** Schematic of the EC-SMC model, (A) Cartoon illustration describing all the channels, pumps and pathways considered in the model. (B) 2D axisymmetric model geometry with SMC, EC and RBC layer as rectangular domains. SMC coupled with EC with MP whose shape is imported from electron microscopy image by (Sandow et al. 2009) and MEGJs. Ion channel currents are uniformly distributed along the top and bottom boundaries of each cell. Inset contains zoomed view of the MP with mesh. eNOS, IP₃Rs, Kₐ₃ and Hbα channels are localized in the MP. All other channels and pumps are also present in MP membrane, but proportionally to MP volume. Nitric oxide (NO) is produced in the EC and can react with Hbα in the EC, diffuse into the lumen, where it reacts with hemoglobin (Hb) of red blood cells (RBCs). It can also diffuse abluminally and react with oxygen or substrates such as soluble guanylate cyclase (sGC) in smooth muscle.
3.3 Methods

3.3.1 Continuum model

We have previously developed detailed compartmental models of integrated Ca\textsuperscript{2+} and $V_m$ dynamics in an isolated EC and SMC (Kapela, Bezerianos, and Tsoukias 2008; Silva, Kapela, and Tsoukias 2007), based primarily on data from RMA. The isolated models were combined into a two-cell (EC-SMC) and multicellular compartmental models to investigate the myoendothelial communication and conducted vasoreactivity respectively (Kapela, Bezerianos, and Tsoukias 2009; Kapela, Nagaraja, and Tsoukias 2010). We updated the compartmental models to capture the spatiotemporal nature of Ca\textsuperscript{2+} mobilization through the development of a continuum EC–SMC model, incorporated with the accurate MP geometry from experimental studies, and examined the role of MP in endothelial derived hyperpolarizing feedback response for the regulation of the vascular tone (Nagaraja et al. 2013), in the absence of nitric oxide (NO) signaling.

To examine the NO mediated myoendothelial feedback response for the regulation of vascular tone, and modulation of NO signaling by Hemoglobin α (Hbα) (which are recently reported to be expressed abundantly in MP (Straub et al. 2012)), we updated the model in (Nagaraja et al. 2013) to include NO signaling pathway. A layer representing the lumen of artery, which consist of RBCs that scavenge NO, and RBC free layer of 6 µm (Figure 3.1-A) between the EC and RBC layer were considered as in (Tsoukias, Kavdia, and Popel 2004). NO signaling pathway was implemented through introduction of Ca\textsuperscript{2+} dependent NO generation (Kapela, Bezerianos, and Tsoukias 2009) in the EC and MP, NO diffusion across SMC, EC, cell free layer (cfl) and RBC layer (rbl), and NO scavenging. The EC, SMC, cfl and rbl were modeled as simplified rectangular domains.
with dimensions as shown in Figure 3.1-B. The model implemented only half of the EC and SMC by assuming symmetry for the other half. Majority of the IKCa channels and around 10% IP3Rs were localized in the MP as in (Nagaraja et al. 2013). Abundant expression of Hba in the MP and its co-localization with eNOS, reported in the recent experimental data (Straub et al. 2012), was tested with localization of range of Hba (0-1 µM (The Hba concentrations are reported based on the EC volume of 400 µm³ throughout the manuscript)) and eNOS (0-100% of the total eNOS) in MP in the model. All the other EC and SMC channels and pumps of the were uniformly distributed as in our previous model (Nagaraja et al. 2013).

### 3.3.2 Ionic and IP3 transport

The transport for individual ionic species is influenced by both electrical and concentration gradients, and was described using the Nernst-Planck electrodiffusion equation (Equation 3.1)

\[
\delta_{\text{buff}} \frac{\partial [S]}{\partial t} = \nabla \left( D_S \nabla [S] + z_S F u_{ms} [S] \nabla V \right) - R_s
\]

Equation 3.1

where \( S = Na^+, K^+, Cl^-, Ca^{2+} \), \( D_S \) is the diffusion coefficient of ionic species \( S \), \( z_S \) is the valence of ionic species \( S \), \( \nabla V \) is the electrical gradient, \( F \) is the Faraday constant and \( u_{ms} \) is the ionic mobility given by \( \frac{D_S}{RT} \) (R is the ideal gas constant (8341 mJ·mol⁻¹·K⁻¹) and T is the absolute temperature). \( R_s \) is the source/sink term which includes the expressions for cytosolic \( Ca^{2+} \) exchange with the ER/SR, and \( Ca^{2+} \) buffering in the EC and MP. \( \delta_{\text{buff}} \) accounts for the \( Ca^{2+} \) buffering in the SMC using a fast buffering approximation. Transport of IP3 and \( Ca^{2+} \) in ER/SR was described using Equation 3.2.
where \([S]\) is the concentration of either species (IP$_3$, Ca$^{2+}$ ER and SR). \(R_s\) is the source/sink term and includes Ca$^{2+}$ exchange between stores and the cellular domains, and IP$_3$ production and degradation. A uniform distribution of transmembrane channels and pumps was considered along the boundary of the cellular domains. The membrane currents were defined as boundary fluxes (Equation 3.3) across the top and bottom boundaries of EC, SMC and the MP boundaries.

\[
-n.N_s = \frac{1}{z_s F} \sum_k I_{S,K}
\]

Equation 3.3

where \(n\) is the normal to the surface and \(N_s\) is the membrane flux given by summation of all the transmembrane currents species for species \(S\) (\(I_{S,K}\)). The membrane currents were distributed between the MP and bulk cell according to their respective volumes. The membrane current definitions and parameters are identical to the original models (Kapela, Bezerianos, and Tsoukias 2008, 2009; Kapela and Tsoukias 2011; Silva, Kapela, and Tsoukias 2007).

### 3.3.3 NO transport

NO transport across the EC, SMC, cfl and rbl domains was implemented using unsteady reaction diffusion equation (Equation 3.4)

\[
\frac{\partial[NO]}{\partial t} = \nabla \cdot (D_{NO} \nabla [NO]) - R_{NO,j}
\]

Equation 3.4
where \([\text{NO}]\) is the concentration of NO, \(D_{\text{NO}}\) is the diffusion coefficient of NO, \(R_{\text{NO},i}\) is the consumption rate of NO in each region and \(i = \text{EC, SMC, cfl and rbl}\). Continuity of concentration at the interface between adjacent layers was assumed. A zero concentration gradient boundary condition was imposed at the center of the arteriole considering axis of symmetry, and far from the SMC layer (50 \(\mu\text{m}\) away)

\[
D_{\text{NO}} \frac{\partial [\text{NO}]}{\partial x} \bigg|_{x=0} = D_{\text{NO}} \frac{\partial [\text{NO}]}{\partial x} \bigg|_{x=\infty} = 0
\]

Equation 3.5

An effective first-order reaction rate mechanism (Equation 3.6) based on the previous NO transport model (Tsoukias, Kavdia, and Popel 2004) was used to describe the consumption rate of NO in RBC layer.

\[
R_{\text{NO,rb}} = k_{\text{blood}} [\text{NO}]
\]

Equation 3.6

Consumption rate of NO in the EC and MP by Hb\(\alpha\) was implemented as a first order reaction rate directly proportional to concentration of Hb\(\alpha\) ([Hb\(\alpha\)]) and \([\text{NO}]\) in EC (Equation 3.7). In the control model all the Hb\(\alpha\) was localized in the projection.

\[
R_{\text{NO,ECandMP}} = k_{\text{Hb\(\alpha\)}} [Hb\alpha][\text{NO}]
\]

Equation 3.7

The consumption through reaction with oxygen was consider in SMC and cfl, and implemented through a second order reaction rate proportional to concentration of oxygen ([O\(_2\)]) and square of \([\text{NO}]\) (Equation 3.8) as described in (Lewis and Deen 1994).

\[
R_{\text{NO,SMC & cfl}} = 4k_1 [O_2][\text{NO}]^2
\]

Equation 3.8
EC Ca\(^{2+}\) dependent NO production by endothelial nitric oxide synthases (eNOS) enzyme was implemented using (Equation 3.9) as in our previous model (Kapela, Bezerianos, and Tsoukias 2009), and incorporated in the EC and MP domains.

\[
Q_{NO} = \frac{Q_{NO,\text{max}}}{[Ca^{2+}]^{4.2} + k_{m,\text{Ca}}^{NO}}
\]

Equation 3.9

The flux was distributed between the MP and bulk cell according to their respective volumes. A significant percentage (30 % under control conditions) of the total eNOS was localized in the MP and remaining distributed in the bulk EC. The distribution of the eNOS was controlled by the maximum NO flux parameter \((Q_{NO,\text{max}})\) (Table 3.1).

**Table 3.1 Parameter values**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
<th>Description</th>
<th>REF</th>
</tr>
</thead>
<tbody>
<tr>
<td>(D_{NO}(\text{cm}^2/\text{s}))</td>
<td>3.3x10(^{-5})</td>
<td>Diffusion coefficient of NO</td>
<td>(Tsoukias, Kavdia, and Popel 2004)</td>
</tr>
<tr>
<td>(k_{blood}(\text{s}^{-1}))</td>
<td>6400</td>
<td>Blood reaction constant</td>
<td>(Tsoukias and Popel 2002)</td>
</tr>
<tr>
<td>(k_{Hb}(\text{µM}^{-1}\text{s}^{-1}))</td>
<td>100</td>
<td>NO-oxyHb reaction constant</td>
<td>(Tsoukias, Kavdia, and Popel 2004)</td>
</tr>
<tr>
<td>(k_{f}(\text{M}^2\text{s}^{-1}))</td>
<td>2.1x10(^6)</td>
<td>NO-O(_2) reaction constant</td>
<td>(Lewis and Deen 1994)</td>
</tr>
<tr>
<td>(<a href="%5Ctext%7B%C2%B5M%7D">\text{Hb}\alpha</a>)</td>
<td>0.5 (0-1)</td>
<td>Hb(\alpha) concentration in EC</td>
<td>Assumed</td>
</tr>
<tr>
<td>(<a href="%5Ctext%7B%C2%B5M%7D">\text{O}_2</a>)</td>
<td>277</td>
<td>(\text{O}_2) concentration in SMC and cfl</td>
<td></td>
</tr>
<tr>
<td>(Q_{NO,\text{max}}(\text{nmolcm}^{-2}\text{s}^{-1}))</td>
<td>2.65x10(^{-3})</td>
<td>Maximum NO production rate (equivalent to 53 (\mu\text{Ms}^{-1})(assuming EC thickness of 0.5 (\mu\text{m}))</td>
<td>(Tsoukias, Kavdia, and Popel 2004; Chen, Pittman, and Popel 2008; Tsoukias 2008)</td>
</tr>
<tr>
<td>(K_{m,\text{Ca}}^{NO}(\text{nM}))</td>
<td>300</td>
<td>Ca(^{2+}) concentration for half-maximum NO release rate.</td>
<td>(Kapela, Bezerianos, and Tsoukias 2009)</td>
</tr>
</tbody>
</table>
NO-cGMP pathway (Figure 3.1-B) and its modulation of large-conductance \( \text{Ca}^{2+} \)-activated potassium channels in the SMC was implemented as in our previous model (Kapela, Bezerianos, and Tsoukias 2009). The parameter values used in the model are provided in Table 3.1.

3.4 Results

3.4.1 No profiles arising from agonist stimulation of the EC and SMC

Agonist stimulation of the EC with Ach (1 a.u.- IP\(_3\) production rate: \(5.5 \times 10^{-8}\) M/s) increased IP\(_3\) concentrations in the EC bulk and MP (Figure 3.2-A, red and green dotted lines), causing store \( \text{Ca}^{2+} \) release to elevate global and local EC \( \text{Ca}^{2+} \) concentrations (Figure 3.2-A, red and green solid lines), similar to our previous model (Nagaraja et al. 2013).

![Figure 3.2](image)

**Figure 3.2** \( \text{Ca}^{2+} \) (continuous lines) and IP\(_3\) (dashed line) concentrations in the EC MP (red lines) and EC bulk (green lines) during (A) NE stimulation of SMC and (B) ACh stimulation of EC.
This resulted in the production of NO through Ca$^{2+}$ dependent activation of eNOS. The resulting near steady state spatial NO profiles in the EC and SMC with uniform distribution of eNOS, in the presence and absence of uniformly distributed 0.5 μM Hbα in the EC, are depicted color-coded and shown in Figure 3.3-A and B respectively. Illustrative spatial profiles near steady state with 100 % localized eNOS in the presence and absence of Hbα are shown in Figure 3.3-C and D.

**Figure 3.3** Illustrative spatial NO profiles during EC stimulation. (A) Uniform eNOS, Hbα (-), (B) Uniform eNOS, Uniform Hbα (0.5 μM), (C) Localized eNOS, Hbα (-), and (D) Localized eNOS, Localized Hbα (0.5 μM).

Significant amount of NO produced in the EC was scavenged in the RBC layer. The rest of NO diffuses towards across the SMC to result in a steady state average NO concentration of 92-100 nM (Figure 3.4) in the SMC with uniformly distributed eNOS,
similar to those observed in the previous 1-D model of NO transport in arterioles (Tsoukias, Kavdia, and Popel 2004). Localization of 30 and 100% eNOS in the MP in the absence of Hbα resulted in increased steady state NO levels experienced by the SMC to around 124 and 154 nM respectively (Figure 3.4). Localized Hbα (0.5 μM (concentration is per EC volume)) reduced the SMC NO concentrations to around 59 and 89 nM respectively (Figure 3.4) in the presence of localized Hbα (0.5 μM (concentration is per EC volume)) in the MP.

![Figure 3.4](image)

**Figure 3.4** Average SMC NO concentration during EC stimulation in presence of uniform eNOS, and localized eNOS (30% and 100% of total eNOS) in the MP. Blue bar represent concentration in the absence of Hbα and red bars are in the presence of Hbα (0.5 μM).

Norepinephrine (NE) (1 μM) stimulation of SMC in the model resulted in large amplitude (μM) Ca^{2+} (Figure 3.2-B, red solid line) and IP_{3} transient (Figure 3.2-B, red dotted line) in EC MP arising mediated by intercellular IP_{3} diffusion from the SMC to the EC, similar to our previous model (Nagaraja et al. 2013). The EC bulk IP_{3} and Ca^{2+} concentration did not increase during NE stimulation (Figure 3.2-B, green solid and dotted lines). Illustrative steady spatial profiles of NO concentration obtained during the
stimulation of SMC are depicted color-coded and shown in Figure 3.5. Uniformly
distributed eNOS in the EC yielded insignificant NO levels in the EC (Figure 3.5-A) due
to lack of increase in global EC Ca^{2+} concentrations. Localization of eNOS (100 %)
(Figure 3.5-B) in the MP produced sustained NO production in the MP which spreads
across the SMC on abluminal side and scavenged in the RBC layer on the luminal side.
Presence of uniformly distributed Hbα (0.5 μM) in the EC and the MP attenuated slightly
the amplitude of NO levels produced the in MP through consumption by Hbα as shown in
Figure 3.5-C. Localization of 100 % Hbα (0.5μM) in the MP further reduced the NO
levels through higher consumptions by Hbα (Figure 3.5-D) due to its close proximity
with eNOS in the MP.

Figure 3.5 Illustrative spatial NO profiles during SMC stimulation. (A) Uniform eNOS, Hbα (-), (B)
Localized eNOS, Hbα (-), (C) Localized eNOS, Uniform Hbα (0.5 μM), and (D) Localized eNOS,
Localized Hbα (0.5 μM)
3.4.2 eNOS localization during SMC stimulation

eNOS have been shown to enriched and active in the MPs (Straub et al. 2011), but their density and activation characteristics remain unclear and it is likely that they vary across different vascular beds. Average SMC NO concentrations arising from NE stimulation of the SMC for 200 sec (near steady state) were observed in the model. Lack of increase in the global Ca$^{2+}$ levels during NE stimulation of SMC resulted in insignificant steady state average NO levels of around 5 nM in the SMC (Figure 3.6-A, red bar), in the presence of uniformly distributed eNOS in the EC. Hence, eNOS localization in the MP may be necessary for generation of any significant NO mediated myoendothelial feedback. Localized Ca$^{2+}$ mobilization in the MP arising from NE stimulation of SMC in the model, yielded significantly higher SMC NO concentrations in range of 50-150 nM (Figure 3.6-A) in presence of 30-100% eNOS localized in the MP, in the absence of Hba. A control value of 30% eNOS distribution in the MP was considered in the model.

![Figure 3.6](image-url) Average SMC NO concentration observed during SMC stimulation, in the presence of (A) uniform (U) distributed eNOS in the EC or localized (L) eNOS distribution in the MP(30% blue bar), 50% (orange bar) and 100% (black bar) of total eNOS) and in the absence (-) of Hba or presence of 0.5 μM Hba distributed uniformly (U) or localized (L). in the MP (B) 30% eNOS localized in the MP and range of Hba (0-1 μM) localized in the MP.
3.4.3 Hbα localization during SMC stimulation

Enriched Hbα expression in the MP have been reported to regulate of eNOS mediated control of vascular reactivity through NO consumption (Straub et al. 2012). We analyzed a range of Hbα concentration (0-1 µM (concentrations per EC volume of 400 µm³)) through their localization in the MP, to observe its effect on the available NO concentrations in the SMC. The initial range of Hbα concentration was chosen on the basis of reported 0.01 to 0.04 µM eNOS concentration (Figure 3.6-B, purple bar) in the EC but had no significant effect on the NO levels in the SMC during NE stimulation (Figure 3.5-B). Increased concentrations of Hbα between 0.05 µM -1 µM localized in the MP resulted in exponential decrease in the mean NO concentrations generated in the SMC. 0.5 µM Hbα resulted in reduction of mean NO levels in the SMC during NE stimulation by more than 60 % (Figure 3.6-A and B), for varied eNOS localization (30-100 % of total eNOS), and was considered as the control value in the model. Uniformly distributed 0.5 µM Hbα in the EC had no significant modulation on the SMC NO concentrations (less than 20 % change (Figure 3.6-A)).

3.4.4 SMC V_m feedback

Under the control conditions (localized IP₃Rs, IK_Ca eNOS and Hbα in the MP), NE stimulation of the SMC for 200 s, resulted in the SMC depolarization to about -48 mV (Figure 3.7-A, green solid line). Block of the NO signaling pathway in the control model reduced the myoendothelial feedback and enhanced SMC depolarization to about -46mV (Figure 3.7-A, purple dashed-dotted line). The absence of Hbα (zero concentration in the model) enhanced NO mediated myoendothelial feedback to result in reduction of SMC depolarization to around -50 mV (Figure 3.7-A, red dotted line), bringing it close to the
resting SMC $V_m$ levels. Lack EC mediated NO signaling and hyperpolarization (EDH), resulted in enhanced SMC depolarization to around -43.5 mV (Figure 3.7-A, orange dashed line). This was implemented through the block of IP$_3$ diffusion from the SMC to the EC which is the major signaling molecule generating significant Ca$^{2+}$ transient in the MP during SMC stimulation in the model as shown in our previous study (Nagaraja et al. 2013). Lack of the MP Ca$^{2+}$ transient resulted in insignificant NO production and inactivity of the EC IK$_{Ca}$ and SK$_{Ca}$ channels (data not shown).

![Figure 3.7 SMC hyperpolarization during SMC stimulation in presence of spatially enriched eNOS in the MP (30% of total eNOS), (A) under control conditions (green continuous line), block of NO signaling (purple dashed dotted line), absence of Hb$\alpha$ (red dotted line) and block of K$_{Ca}$ channels and NO signaling (orange dashed line). (B) Amount of feedback from NO mediated signaling (purple bar) during K$_{Ca}$ block, activation of K$_{Ca}$ channels during NO signaling block (green bar) and presence of both NO and K$_{Ca}$ mediated hyperpolarization.](image)

We summarized the NO and EDH mediated feedback observed in the model in the presence or absence of localized Hb$\alpha$ in the MP (Figure 3.7-B). We observed around 3 mV feedback through activation of the IK$_{Ca}$ channels, in the absence of NO signaling, similar to our previous model (Nagaraja et al. 2013) in both the presence and absence of localized Hb$\alpha$ (Figure 3.7-B, Green bars). NO signaling pathway, in the absence of IK$_{Ca}$
mediated hyperpolarization and localized Hbα, resulted in a feedback of around 6.5 mV
which was significantly diminished (to around ~ 2mV) in the presence of localized Hbα
in the MP (Figure 3.7-B, purple bars). Localized Hbα reduced the feedback (of both IKCa
mediated hyperpolarization and NO signaling) from about 8 mV to 5 mV in the model
(Figure 3.7-B, yellow bars).

3.4.5 Erythrocyte versus EC Hbα NO consumption

Hbα modulation of NO bioavailability have been recently reported in isolated vessels
in the absence of NO consumption by erythrocytes (Straub et al. 2012). We examined the
relative contribution of erythrocyte in the lumen which are distant (about 6 µm) from the
site of NO production, and Hbα colocalized with eNOS in the consumption of the NO
(Figure 3.8). The absence of both erythrocyte (kblood=0) and EC Hbα ([Hbα]=0) resulted
in mean NO concentrations in the SMC around 8 and 11 µM during SMC and EC
stimulation respectively suggesting a slightly diminished NO production during SMC
stimulation, occurring in the model from the lack of EC Ca2+ bulk increase. The NO
concentrations took a long time (~60 sec) to approach steady state in the presence of only
NO consumption being reaction with oxygen in the model (Figure 3.8-A and B, red
dotted line). In the absence of erythrocytes consumption, localized Hbα (0.5 µM)
provided a significant reduction in the steady state mean NO concentration in the SMC to
around 650 nM (~6 % of 11µM) and 120 nM (~1.5% of 8 µM ) (Figure 3.8-A and B
,yellow dashed line) for the agonist stimulation EC and SMC respectively suggesting
enhanced Hbα consumption during feedback. Although not intuitive at the first,
consumption through erythrocytes which are located away from NO production site, in
the absence of localized Hbα, resulted in 2-fold (to around 50 nM) and 5-fold higher (to
around 120 nM) reduction in mean SMC NO concentrations than observed through consumption from only localized Hbα in the absence of erythrocytes (Figure 3.8-A and B, orange dashed-dotted line) during SMC and EC stimulation respectively. Analytical calculations using mass balance equation also revealed 5 fold higher NO consumption from the erythrocyte layer than from the EC Hbα similar to that observed in the model (see supplement). In the control model (30 % eNOS localized in the MP) in the presence of both erythrocyte and localized Hbα (0.5 µM in the MP), agonist stimulation of the EC and SMC resulted in the mean NO concentrations in the SMC of 81nM and 17 nM respectively (Figure 3.8-A and B, green solid line). Reduced mean SMC NO concentration during the SMC stimulation comparted to EC stimulation was from the lack of NO production in the bulk EC in the absence of EC bulk Ca^{2+} increase and enhanced Hbα scavenging of the locally produced NO during NE stimulation.

To further analyze the effect of Hbα scavenging, we reported the effect of NO consumption from a rage of Hbα concentration localized in the MP both in the presence and absence of NO consumption in the RBC layer. In the absence of erythrocyte consumption, Hbα concentration comparable to observed eNOS concentration (Figure 3.8-C, purple bar) resulted in significant drop in the mean NO concentration in the SMC to 16% and 30% of the NO levels in the absence of Hbα during the stimulation of SMC and EC cell respectively (Figure 3.8-C, black and blue dotted lines). Higher Hbα concentration reduced further the NO levels. However, in the presence of erythrocyte consumption Hbα concentration in the range (0.01 to 0.05 µM) had no significant modulation of SMC NO levels for both the stimulation of EC and SMC cell. 50 to 100 fold higher concentrations than experimentally observed eNOS concentrations were
essential to reduce the SMC NO by more than 50% from its levels observed in the absence of Hbα (Figure 3.8-C, black and blue solid lines). Moreover we observed enhanced scavenging of locally produced NO by Hbα during feedback.

![Graph](image)

**Figure 3.8** Erythrocyte versus EC Hbα regulation of mean SMC NO levels, (A) after 200 s stimulation of the EC, (B) after 200 s stimulation of the SMC. (C) Percentage change in mean NO levels in the SMC with increase in localized Hbα concentration in the MP during the presence of NO scavenging in the RBC layer (blue lines) or absence of NO scavenging in the RBC layer (black lines).

### 3.5 Discussion

The primary aim of the study was to examine the EC cell generated NO signaling and its modulation during agonist stimulation of vascular cells. Using the 2-D continuum model with enriched localization of eNOS and Hbα in the MP, we examined EC Ca^{2+} mediated NO signaling and its regulation of SMC Ca^{2+} and $V_m$ alterations during contractile stimuli. Simulations showed significant feedback (few mV) arising from NO signaling and its modulation through localized Hbα and the RBCs.
NO mediated regulation of vascular tone is attributed to an increase in EC Ca\(^{2+}\) concentrations. We observed IP\(_3\) and Ca\(^{2+}\) increase in the EC (Figure 3.2-B) confined to the MPs as in our previous model (Nagaraja et al. 2013) during SMC stimulation, consistent with recent experimental evidence of local EC Ca\(^{2+}\) events in and around EC MPs following SMC stimulation (Tran et al. 2012; Kansui, Garland, and Dora 2008). Our previous modeling efforts and theoretical estimations predict suggest that passive diffusion of Ca\(^{2+}\) and/or IP\(_3\) into EC is insufficient for a global Ca\(^{2+}\) mobilization (Nagaraja et al. 2013; Nagaraja, Kapela, and Tsoukias 2012). Significantly smaller surface area and volume of the MP with respect to the bulk of EC, restricted diffusivity and spatial polarization of store receptors (RyRs and/or IP\(_3\)Rs) around the MP can amplify the weak fluxes of Ca\(^{2+}\) and IP\(_3\) to induce local Ca\(^{2+}\) transients (Ledoux et al. 2008; Nagaraja et al. 2013; Isakson 2008).

In the model the absence of significant EC bulk Ca\(^{2+}\) increase during SMC stimulation necessitates spatial localization of eNOS around the MP for NO production through Ca\(^{2+}\) dependent eNOS activation. Uniformly distributed eNOS in the EC resulted in insignificant NO production. Increased localized NO production with rise in the amount of eNOS localized in the MP was observed in the model (Figure 3.5 and Figure 3.6-A). The locally produced NO diffused to the adjacent SMC in the model to provide few mV feedback through the NO-cGMP pathway (Figure 3.7-A and B). Spatially enriched eNOS in specific cellular regions resulting in local NO production have been observed experimentally (Qian et al. 2010; Iwakiri et al. 2006). Recent experimental studies revealed abundant expression of eNOS in the MP and suggest paracrine release of localized NO in providing feedback SMC constriction (Straub et al. 2011).
Several mechanisms have been proposed to account for a more efficient delivery of endothelium-derived NO to the SMC and for NO preservation and reduced scavenging by the hemoglobin in the blood (Buerk, Barbee, and Jaron 2011; Tsoukias 2008). Localized NO production at the EC-SMC junction (i.e. near the MP) has been suggested as one of the possible mechanism for reduced NO scavenging in the blood (Gladwin and Kim-Shapiro 2012; Rahaman and Straub 2013; Straub et al. 2012). In the model local NO production during EC stimulation from spatially enriched eNOS around the MP increased mean SMC NO concentration by 25-50 nM (from uniform NO production) in the presence of Hb consumption in the RBC layer (Figure 3.4) suggesting reduced scavenging of the locally produced NO. Moreover, local NO production during SMC stimulation increased mean SMC concentration by 50-140 nM (Figure 3.5) which can be ascribed to diminish NO scavenging in the blood through localized production and insignificant EC Ca$^{2+}$ increase in the presence of uniform eNOS distribution.

The fast reaction of NO with Hb and the resulting short half-life of NO make experimental measurements in presence of blood perfusion difficult. Mathematical modeling can help address the limitation and predict the NO bioavailability in the presence of erythrocyte consumption. Overproduction of NO in the absence of Hb scavengers (knockout of EC Hb$\alpha$ and absence of erythrocyte consumption) have been observed in isolated arteries and co-culture models (Straub et al. 2011; Straub et al. 2012) during contractile stimuli. In the model lack of consumption in the RBC layer and zero Hb$\alpha$ concentration resulted in µM NO concentration in the SMC (Figure 3.8). Regulation of NO bioavailability by EC Hb$\alpha$, which are preferentially located around the EC MP, through fast reactions with NO depending on Hb$\alpha$ heme iron oxidation state have been
suggested in the absence of NO consumption in RBC (Straub et al. 2012). The model explored this hypothesis and significant NO scavenging by Hbα concentrations (0.01 to 0.05 μM) were observed in the absence of NO consumption in RBC layer (Figure 3.8-C). However in the presence of consumption in the RBC layer, Hbα modulation of NO signaling in the model required significantly higher μM concentrations spatially localized around the MP (Figure 3.8-C). Unlike EC stimulation which results in both localized and bulk NO production, SMC stimulation results in locally produced NO from the lack of EC bulk Ca\(^{2+}\) increase in the model. Enhanced role of Hbα regulation was observed during SMC stimulation compared to EC stimulation through scavenging of local NO production in the absence of bulk NO production. However this predictions needs to be tested experimentally. Inhibition of contractile response after transfection of ECs with Hbα siRNA in isolated vessels have been documented (Straub et al. 2012). In the model NO scavenging by Hbα significant reduced the NO mediated SMC hyperpolarization feedback in the model.

### 3.6 Limitation

A number of parameter values have not been accurately quantified. In this study, we try to remain consistent with our previous 2D continuum EC–SMC model with respect to parameter values and whole cell currents. The 2D model is limited by the absence of quantification for the spatial distribution of important cellular components (eNOS, Hbα, IP\(_3\)Rs and K\(_{Ca}\)). Ca\(^{2+}\) independent NO production was not considered.
3.7 Conclusion

In conclusion, the developed model describes Ca$^{2+}$ mediated NO signaling for regulation of vessel dilation. Model simulations were in good agreement with experiments in isolated vessels, verifying the hypotheses for the role of localized NO signaling in vascular control. The model quantifies the NO bioavailability in the SMC during agonist stimulation of vascular cells. Model predicts localized Ca$^{2+}$ signaling during the SMC stimulation around the MP and resulting NO production, and feedback response in the presence of enriched distribution of eNOS around the MP. Diminished NO feedback in the presence of EC Hb$\alpha$ was observed in the model and was amplified from its spatial localization around the MP. Moreover, the model analyzes the relative contribution of EC Hb$\alpha$ and erythrocyte scavenging in the regulation of the SMC NO bioavailability and suggest that RBC perfusion will decrease the ability of Hb$\alpha$ to modulate NO levels, and µM levels of EC Hb$\alpha$ are necessary for a significant modulation of SMC NO availability.

3.8 Supplement

We performed analytical analysis using mass balance (Equation 3.9) for the NO consumption by Hb$\alpha$ in EC and its consumption by the RBCs (Figure 3.9) to validate the profiles observed in the simulations.

\[ Q_{NO} V - K_{Hb}[Hb\alpha][NO] V - \frac{D_{NO}[NO]}{L} S_{area} = 0 \]

Equation 3.10
where V is the volume of the EC, \( S_{area} \) is the surface area of the EC, L is the distance between EC and start of RBC layer. Rearranging Equation 3.10 provides NO concentration obtained in the EC (Equation 3.11).

\[
[NO] = \frac{Q_{NO}}{K_{Hb}[Hb\alpha] + \frac{D_{NO}}{Lt_{EC}}} 
\]

Equation 3.11

where \( t_{EC} \) is the length of the EC.

\( K_{Hb}[Hb\alpha] \) represents the rate of NO consumption through scavenging by EC Hb\( \alpha \) and

\( \frac{D_{NO}}{Lt_{EC}} \) represents the rate of consumption through erythrocyte layer. Using the dimension of EC considered in the model (\( t_{EC} = 2 \mu m \)), distance between the RBC layer and EC (6
µm), NO-Hbα reaction rate $k_{Hb}$ of 100 (µM$^{-1}$s$^{-1}$) and Hbα concentration of 0.5 µM and using Equation 3.11 we obtain the NO consumption rate ($K_{Hb}[Hb]$) of 50 s$^{-1}$ by EC Hbα and consumption rate in the erythrocyte layer ($\frac{D_{NO}}{L_{EC}}$) of 275 s$^{-1}$. Hence a 5.5 fold higher consumption can be expected in the erythrocyte layer for the above parameter values.

3.9 References


CHAPTER 4-INTRACELLULAR CALCIUM WAVES IN VASCULAR CELLS

This chapter is to be submitted to Biophysical Journal (with modifications) as Parikh, J., A. Kapela and N. M.Tsoukias. “Oscillatory calcium waves in vascular cells: wave velocity dependence on membrane electrophysiology”
4.1 Abstract

Agonist stimulation of both smooth muscle (SMC) and endothelial (EC) cells results in calcium (Ca$^{2+}$) mobilization that is associated with diverse cellular functions. Spatiotemporal Ca$^{2+}$ events in the form of intracellular Ca$^{2+}$ waves have been reported in SMCs and ECs of different vascular beds. The underlying mechanisms and physiological roles are under investigation. Here we use detailed mathematical models of Ca$^{2+}$ dynamics and electrophysiology to investigate the mechanisms that initiate and modulate Ca$^{2+}$ waves in these two cell types. The models reproduce wave like activity when a gradient in store (i.e. inositol triphosphate, ryanodine) or membrane (i.e. adrenergic, muscarinic) receptor density is assumed. The resulting intracellular waves depend on the intrinsic ability of the cells for store-dependent oscillations. These repetitive/oscillatory waves require a weak propagating signal to synchronize neighboring domains, balancing uneven receptor distribution and/or store dynamics. Small Ca$^{2+}$ fluxes in the axial direction are adequate to maintain wave activity. Model predicts that the membrane potential ($V_m$) can affect wave velocity through modulation of transmembrane Ca$^{2+}$ influx (i.e. via voltage sensitive Ca$^{2+}$ channels in SMCs or by altering the electrochemical gradient for Ca$^{2+}$ influx in ECs). Depolarizing currents in SMCs (i.e. via non-selective cation channels and Ca$^{2+}$ activated chloride channels) and hyperpolarizing currents in ECs (i.e. small and intermediate conductance Ca$^{2+}$ activated K$^+$ channels) promote an increase in wave velocity and transition to whole-cell oscillations. The model predicts that the frequency and velocity of these oscillatory Ca$^{2+}$ waves may depend on agonist stimulus strength and thus waves may encode information of physiological importance.
4.2 Introduction

Diverse cellular events like contraction, relaxation, gene expression, cell death and proliferation are associated with cytoplasmic calcium (Ca\textsuperscript{2+}) mobilization (Foskett et al. 2007). Transmembrane Ca\textsuperscript{2+} fluxes, store uptake and release generate local or global Ca\textsuperscript{2+} events. In the vasculature, spatio-temporal Ca\textsuperscript{2+} events ranging from localized Ca\textsuperscript{2+} activity like sparks, puffs, sparklets or pulsars to well organized signaling events like waves or whole cell oscillations have been reported (Amberg and Navedo 2013). Moreover Ca\textsuperscript{2+} events may also propagate to adjacent cells forming intercellular Ca\textsuperscript{2+} waves (Halidi et al. 2011; Seppey et al. 2010; Socha et al. 2012). Intracellular Ca\textsuperscript{2+} waves have been observed in vascular cells with different propagation velocities (Jaffe 2010; Narayanan, Adebiyi, and Jaggar 2012). The physiological relevance of Ca\textsuperscript{2+} wave activity is still unclear, although, a role in cell proliferation and gene expression (Narayanan, Adebiyi, and Jaggar 2012) or in the regulation of vascular tone(Wang et al. 2010; Amberg and Navedo 2013) has been suggested.

The reported intracellular Ca\textsuperscript{2+} waves be attributed two different mechanisms based on the experimental technique undertaken to generate them; Stochastic Ca\textsuperscript{2+} waves and repetitive/oscillatory Ca\textsuperscript{2+} waves. The first type is often a result of localized Ca\textsuperscript{2+} release due the stochastic/random opening of store receptors (McCarron et al. 2010; Marchant, Callamaras, and Parker 1999; Halidi et al. 2011). If the localized release is sufficiently high, Ca\textsuperscript{2+} diffusion away from this point may cause calcium induced calcium release (CICR) resulting in single or frequently appearing Ca\textsuperscript{2+} wave traversing across the cell. The point of origin of these waves can be random depending on the site of localized increase of Ca\textsuperscript{2+}. In experiments localized increase in Ca\textsuperscript{2+} through photolysis of caged
IP₃ has often been utilized to examine the underlying mechanism of Ca²⁺ wave propagation (McCarron et al. 2010) and several mathematical models examining the generation of Ca²⁺ puffs and their conversion to waves have been presented (Falcke 2003; Falcke, Or-Guil, and Bar 2000; Falcke, Tsimring, and Levine 2000). On the contrary the second type of Ca²⁺ wave i.e. repetitive/oscillatory Ca²⁺ waves often appear as a result of constant agonist stimulation of cultured vascular cells/intact vessel to produce a global Ca²⁺/IP₃ response. Such Ca²⁺ waves usually have a fixed initiation site and frequency of appearance.

Agonist induced oscillatory Ca²⁺ waves have been observed in SMCs (Dai et al. 2007; Dai et al. 2006; Lee et al. 2001; McCarron et al. 2010; Peng et al. 2001; Ruehlmann et al. 2000; Syyong et al. 2009) and ECs (Huser and Blatter 1997; Isshiki et al. 1998; Socha et al. 2012; Beliveau, Lapointe, and Guillemette 2011) of different species. Agonist stimulus results in elevated Ca²⁺ and IP₃ levels and may induce stable localized or whole cell Ca²⁺ oscillations in the cells by bringing the Ca²⁺ levels within the oscillatory domain (Kapela, Bezerianos, and Tsoukias 2008). Ca²⁺ oscillations depend on calcium induced calcium release (CICR) through either of IP₃ receptor (IP₃R) channel (Bai, Edelmann, and Sanderson 2009; Lamont and Wier 2004; McCarron et al. 2010) and/or Ryanodine receptor (RyR) channels (Lagaud et al. 1999; Dai et al. 2006; Balemba et al. 2006). The spatial heterogeneity essential for generation of Ca²⁺ waves may result for non-uniform distribution of the cellular components. Gradient distribution of both IP₃ and RyRs i.e. higher density of these receptors on one side of cell compared to other was shown to be essential for generation and propagation of Ca²⁺ waves in airways SMC model (Wang et al. 2010). Both Ca²⁺ and IP₃ mobilization was required for Ca²⁺ wave generation and
propagation in (McCarron et al. 2010). $V_m$ can modulate Ca$^{2+}$ levels, directly through activation/inactivation of voltage dependent Ca$^{2+}$ channels or indirectly by affecting the electrochemical gradient for Ca$^{2+}$ entry via non-selective cation channels (NSC).

Wide variation in Ca$^{2+}$ wave velocities have been reported (Jaffe 2010; Narayanan, Adebiyi, and Jaggar 2012). While several experiments and models have been undertaken to understand the origin and mechanism of oscillatory Ca$^{2+}$ waves the mechanism(s) that modulates wave velocities has not been elucidated. Different cellular components have been suggested to modulate this oscillatory Ca$^{2+}$ waves and wide variation in velocity (1-126 µm/sec) and propagation distances (8.8-50 µm) have also been reported (Jaffe 2010; Narayanan, Adebiyi, and Jaggar 2012). The physiological significance of accelerating/decelerating wave velocity is not yet understood but theoretical consideration suggest that high velocities and transition to whole cell oscillations may increase contractility of vascular SMCs (Wang et al. 2010). Transition from oscillatory calcium waves to whole cell oscillations have also been observed experimentally (Peng et al. 2001) and mathematical models explaining these transitions have also been reported (Jacobsen et al. 2007; Peng et al. 2001; Postnov et al. 2011). Calcium activated chloride channels (Cl$_{Ca}$) have been shown to be the main determinant for this conversion to whole cells waves.

Several minimal deterministic reaction-diffusion models of Ca$^{2+}$ waves, describing Ca$^{2+}$ and IP$_3$ dynamics have been undertaken. They differ in the underlying assumption of the oscillatory mechanism. (For example Ca$^{2+}$ waves arising from IP$_3$R are consider in (Atri et al. 1993; Dupont and Goldbeter 1994; Sneyd et al. 1995), oscillation through combination of IP$_3$R and RyR in (Wang et al. 2010). Moreover, the models differ in type
of stimulus i.e. Ca^{2+} waves generated from localized Ca^{2+} increase (Atri et al. 1993; Dupont and Goldbeter 1994) which propagates across the cell or under uniform stimulus throughout the cell (Wang et al. 2010) (e.x. through rise of IP_{3} levels throughout the cell). Very few models have examined the effect of V_{m} on generation and modulation of the oscillatory Ca^{2+} waves. Jacobsen et al. (Jacobsen et al. 2007) incorporates V_{m} dynamics and transmembrane currents in their cytosolic oscillator model to examine the effect of Ca^{2+} activated chloride channels (Cl_{Ca}) in transition from waves to whole cell oscillations, however they lack the presence of NSC channels, RyRs, Ca^{2+}-dependent PLC activity, intracellular IP_{3} diffusion, or activation of BK_{Ca} channels by cGMP.

Postnov et al. (Postnov et al. 2011) went with a minimal model to understand the transition to whole cell oscillations. The proposed mathematical models (Jacobsen et al. 2007; Postnov et al. 2011; Wang et al. 2010) provides valuable insights into generation and propagation of the intracellular Ca^{2+} waves and also transition to whole cell oscillations. These earlier models can be further advanced by incorporation of potentially important components and pathways and improve quantitative descriptions.

In this theoretical study, we develop detailed mathematical models of integrated Ca^{2+} and V_{m} dynamics to try and understand the mechanism generating and mediating these oscillatory Ca^{2+} waves and attempt to unravel the underlying pathways modulating the velocities and frequency of the agonist induced oscillatory intracellular Ca^{2+} wave in SMCs and ECs. Simulations suggest that complex pathways exist in the generation and propagation of oscillatory Ca^{2+} wave and different cellular components may play important roles in the two cell types. Moreover the model reveals that several pathways may simultaneously regulate the velocity and frequency of the oscillatory Ca^{2+} waves and
the relative importance of the pathways depend on the simulation type and intensity. The model also assists in understanding the effect of non-homogenous spatial distribution of cellular components in both the cell types.

4.3 Methods

We have previously developed detailed models of integrated Ca$^{2+}$ and $V_m$ dynamics in isolated EC and SMC (Kapela, Bezerianos, and Tsoukias 2008; Silva, Kapela, and Tsoukias 2007), based primarily on data from rat mesenteric arteries (RMA). The single cells models were extended to incorporate spatial heterogeneity of cellular components and intracellular gradients for Ca$^{2+}$, IP$_3$ and other ions (Cl$^-$, Na$^+$ and K$^+$). We developed a FEM model to account for the spatial gradients of cellular components inside the EC and SMC essential for Ca$^{2+}$ wave generation. While a 3D cylindrical axisymmetric model of EC and SMC was implemented in (Kapela and Tsoukias 2011), here we assumed a simplified geometry with EC and SMC being represented as rectangular structures 100 μm long and 6 μm wide. The model implements only half of the EC and SMC. The results in the remaining half are assumed to be symmetrical. Electro-diffusion for the ionic species was taken into account. This allows implementation of voltage and concentration dependent membrane currents as boundary equations. The resulting differential equations describing the intracellular gradients for each ionic species A (Ca$^{2+}$, Cl$^-$, Na$^+$ and K$^+$) and second messenger IP$_3$ (Kapela, Bezerianos, and Tsoukias 2008) in the two cells SMC/EC (Equation 4.1- 4.4): The transport of Ca$^{2+}$ across the cell is given by Equation 4.1
\[ \delta_{\text{buff,Ca}} \frac{\partial [Ca]}{\partial t} = \nabla \left( D_{Ca} \nabla [Ca] \right) + z_{Ca} F \frac{D_{Ca}}{RT} [Ca] \nabla V_i \big) + R_{\text{rel}} - R_{\text{uptake}} \]

Equation 4.1

where \( \delta_{\text{buff,Ca}} \) accounts for rapid buffering in cytosol and store, \( R_{\text{rel}} \) defines the release of \( \text{Ca}^{2+} \) from store via IP\(_3\)R and RyR receptors and a leak current through the stores, \( R_{\text{uptake}} \) defines the re-sequestration of \( \text{Ca}^{2+} \) by the store through uptake by SERCA. The expressions for buffering, release and uptake are same as in our previous models (Kapela, Bezerianos, and Tsoukias 2008; Silva, Kapela, and Tsoukias 2007). \( D_{Ca} \) is the diffusion coefficient of \( \text{Ca}^{2+} \) (TABLE 1). \( z_{Ca} \) is the valence of \( \text{Ca}^{2+} \) ion. \( F \) is the Faradays constant. \( V_i \) denotes intracellular electrical potential. The molar fluxes on the membrane for \( \text{Ca}^{2+} \) are calculated using the sum of all \( \text{Ca}^{2+} \) membrane currents (\( \sum K \text{Ca}_K \)) and implemented as a boundary condition (Equation 4.2)

\[ -n N_{Ca} = \frac{1}{Z_{Ca} F} \sum K \text{Ca}_K \]

Equation 4.2

where \( n \) is the normal to the surface, \( N_{Ca} \) is the flux of \( \text{Ca}^{2+} \) across the membrane, \( K \) refers to all membrane currents for each ionic species.

The transport of IP\(_3\) is assumed to be independent of the membrane potential dynamics and implemented using simple diffusion equation (Equation 4.3)
\[
\frac{\partial [IP_3]}{\partial t} = \nabla \left( D_{IP_3} \nabla [IP_3] \right) - R_{\text{degradation}}
\]

Equation 4.3

where \( D_{IP_3} \) is the diffusion coefficient of \( \text{Ca}^{2+} \) (TABLE 1). \( R_{\text{degradation}} \) is sink term which includes expression for cytosolic degradation of IP_3 (Kapela, Bezerianos, and Tsoukias 2008; Silva, Kapela, and Tsoukias 2007).

The molar flux for IP_3 production (\( J_{\text{PLC,IP}_3} \)) is fulfilled as a boundary condition (Equation 4.4).

\[
-n \cdot n_{IP_3} = J_{\text{PLC,IP}_3}
\]

Equation 4.4

Transport of rest of the ionic species \( S \) where \( S = Na^+, K^+, Cl^- \) is described by Equation 4.5.

\[
\frac{\partial [S]}{\partial t} = \nabla \left( D_S \nabla [S] + z_S F \frac{D_S}{RT} [S] \nabla V_i \right)
\]

Equation 4.5

\( D_S \) is the diffusion coefficient of ionic species \( S \), \( z_S \) is the charge number of ionic species \( S \).

The molar fluxes on the membrane for ionic species \( S \) are calculated using the sum of all membrane currents (\( \sum I_{S,K} \)) for each ionic species \( S \) and implemented as a boundary condition (Equation 6).
\[-n.N_S = \frac{1}{Z_S F} \sum_k I_{S,K}\]

Equation 4.6

The change in membrane potential across the length of cell is given by (Equation 4.7) and is dependent on sum of all membrane currents (K) for each ionic species A (\(\sum_k I_{A,K}\))

\[-n.F \sum A \left( D_A \nabla [A]_i + Z_A F u \frac{D_A}{RT} [A]_i \nabla V_i \right) = \sum_k I_{A,K}\]

Equation 4.7

where A= Ca\(^{2+}\), Na\(^+\), K\(^+\) and Cl\(^-\), D\(_A\) is the diffusivity of the individual ions (Table 4.1)

FEM models for both the cells were developed using the chemical engineering module of the COMSOL multiphysics software. The details of the FEM models are given in supplement.

In order to reduce the computational overheads of a 2-D FEM model and to better understand/estimate the role diffusional fluxes of ions and second messenger, we reduced the 2-D model by neglecting the concentration gradients across the width of the cell and assuming only the axial fluxes, to result in 1-D axial geometry for examining oscillatory Ca\(^{2+}\) waves. Reducing to 1-D resulted in the boundary conditions (membrane fluxes) of the 2-D model to be incorporated into the equation of transport of each species itself. The resulting equations are described in Equation 4.8-4.11 (supplement (section 4.9)).

Intracellular transport for Ca\(^{2+}\) in 1-D is described using Equation 4.8

\[
\delta_{\text{buff,Ca}} \frac{\partial [Ca]}{\partial t} = D_{\text{Ca}} \frac{\partial^2 [Ca]}{\partial x^2} + \frac{\partial}{\partial x} \left( D_{\text{Ca}} \frac{Z_{\text{Ca}} F [Ca]}{RT} \frac{\partial [V]}{\partial x} \right) + \frac{I_{\text{Ca,ref}}(x) - I_{\text{Ca,uptake}}(x)}{Z_{\text{Ca}} F} + \frac{\sum_k I_{\text{Ca,K}}(x)}{Z_{\text{Ca}} F}
\]

Equation 4.8
where $I_{Ca.rel}(x), I_{Ca.uptake}(x)$ are the current densities $\left( \frac{\text{pA}}{\mu\text{m}^2} \right)$ for $\text{Ca}^{2+}$ release and uptake from the store respectively at any axial point $x$ across the length of the cell. $\sum_k I_{Ca,k}(x)$ is the summation of membrane current density $\left( \frac{\text{pA}}{\mu\text{m}^2} \right)$ of all the membrane $\text{Ca}^{2+}$ channel currents at any point $x$ across the length of the cell.

Intracellular transport for IP$_3$ in 1-D is given by Equation 4.9

$$\frac{\partial [\text{IP}_3]}{\partial t} = D_{\text{IP}_3} \frac{\partial^2 [\text{IP}_3]}{\partial x^2} - R_{\text{degradation}}(x) - R_{\text{PLC,IP}_3}(x)$$

Equation 4.9

where $R_{\text{PLC,IP}_3} = \frac{R_{h.G}[\text{PIP}_2]}{\gamma_G}, R_{\text{degradation}} = K_{\text{deg,G}}[\text{IP}_3]$ (Kapela, Bezerianos, and Tsoukias 2008)

The transport in 1-D for rest of the ionic species $S$ is reduced to Equation 4.10

$$\frac{\partial [S]}{\partial t} = D_S \frac{\partial^2 [S]}{\partial x^2} + \frac{\partial}{\partial x} \left( D_S Z_S F[S] \frac{\partial [V]}{\partial x} + \sum_k I_{S,k}(x) \right) / Z_S F$$

Equation 4.10

where $\sum_k I_{S,k}(x)$ is the summation of membrane current densities for each ionic species $S \left( \frac{\text{pA}}{\mu\text{m}^3} \right)$.

Finally the change in membrane potential with respect to time across the length of cell in 1-D is given by Equation 4.11
\[ C_m \frac{\partial V}{\partial t} = \frac{\partial}{\partial x} \left( F \sum Z_A \left( -D_A \frac{\partial [A]}{\partial x} - \frac{Z_d F u_m [A]}{RT} \frac{\partial V}{\partial x} \right) \right) - \sum \frac{1}{k} \cdot I_{A,k}(x) \]

**Equation 4.11**

Here, \( \sum_{k} I_{A,k}(x) \) is the summation of total membrane current density for all ions including second messenger, A \( \left( \frac{pA}{\mu m^3} \right) \). Discretization of the single cell models of EC and SMC (Figure 4.1-A) to form a multi-compartmental model as discussed in supplement (section 4.9) was performed to solve for oscillatory Ca\(^{2+}\) wave model. The cellular components and pathways examined for the individual compartments in SMC and EC has been shown in Figure 4.1-B and C.

A two pool model have been suggested for generating histamine induced Ca\(^{2+}\) oscillations in human endothelial cells (Paltaufl-Doburzynska et al. 2000). In their study RyR are shown to cause the calcium induced calcium release following opening or IP\(_3\)Rs. However recent studies suggest the absence of RyRs in particular EC types but have shown the presence of more than one of subtypes of IP\(_3\)Rs (Ledoux et al. 2008) (IP\(_3\)R-1, IP\(_3\)R-2, and IP\(_3\)R-3). Varying proportions of these subtypes depending on cell type and species are observed. To the best of our knowledge no detailed models for Ca\(^{2+}\) oscillation in vascular EC has yet been reported. We formulated a two pool model(Paltaufl-Doburzynska et al. 2000; Parthimos, Edwards, and Griffith 1999) to account for more than one these subtypes of IP\(_3\) receptor in EC as shown in Figure 4.1-C and resulted in generate the Ca\(^{2+}\) oscillation which were absent in the original single cell model (Silva, Kapela, and Tsoukias 2007). The first pool maintains the steady state Ca\(^{2+}\) levels with the second pool inducing the oscillations on the top of this steady state level.
The current formulations (Equation 4.12–4.14) and parameter values describing the second pool in the model (Table 4.1) were chosen from Parthimos et al. (Parthimos, Edwards, and Griffith 1999).

**Figure 4.1** (A) Schematic diagram of single cell EC and SMC model divided into compartments (B) Zoomed in view of a single SMC compartment showing all the incorporated components. BKCa – large-conductance Ca$^{2+}$-activated K$^+$ channels; SOC – store-operated channel; NSC – nonselective cation channel, CaCC and Cl$_{Ca}$ – Ca$^{2+}$-activated chloride channel; NaK – Na$^+$-K$^+$-ATPase; PMCA – plasma membrane Ca$^{2+}$-ATPase; NCX – Na$^+/Ca^{2+}$ exchanger; NaKCl – Na$^+$-K$^+$-Cl$^-$ cotransport; Kc – voltage-dependent K$^+$ channel; K$^{leak}$ – unspecified K$^+$ leak current; VOCC – voltage-operated Ca$^{2+}$ channels; SR – sarcoplasmic reticulum; IP$_3$R – IP$_3$ receptor; RyR – ryanodine receptor; SERCA – SR Ca$^{2+}$-ATPase; CSQN – calsequestrin; CM – calmodulin; R – receptor; G – G-protein; DAG – diacylglycerol; PLC – phospholipase C; sGC – soluble guanylate cyclase; cGMP – cyclic guanosine monophosphate. (C) Zoomed in view of a single EC compartment revealing all the incorporated components. K$_{ir}$ – inward rectifier K$^+$ channel; VRAC – volume-regulated anion channel; SKCa, IKCa and BKCa – small-, intermediate-, and large-conductance Ca$^{2+}$-activated K$^+$ channels; SOC – store-operated channel; NSC – nonselective cation channel, CaCC and Cl$_{Ca}$ – Ca$^{2+}$-activated chloride channel; NaK – Na$^+$-K$^+$-ATPase; PMCA – plasma membrane Ca$^{2+}$-ATPase; NCX – Na$^+/Ca^{2+}$ exchanger; NaKCl – Na$^+$-K$^+$-Cl$^-$ cotransport; Kc – voltage-dependent K$^+$ channel; ; $K^{leak}$ – unspecified K$^+$ leak current; ER – endoplasmic reticulum; IP$_3$R1 and IP$_3$R2 – IP$_3$ receptor isoforms.
The parameter values were scaled (to convert from fluxes (μM/s) to currents (pA)) and modified to generate Ca\(^{2+}\) oscillations in the original EC model. IP\(_3\) activation was also introduced in the CICR current (Equation 4.14). Moreover, the maximum current via IP\(_3\)R in the first pool was set to its initial estimate i.e. \(I_{IP3R} = 1170\ pA/mM\) (Silva, Kapela, and Tsoukias 2007).

\[I_{SERCA2} = I_{SERCAmax} \left( \frac{[Ca]_i^2}{[Ca]_i^2 + K_{SERCA2}^2} \right)\]

Equation 4.12

\[I_{leak2} = K_{leak2} ([Ca]_{IS2} - [Ca]_i)\]

Equation 4.13

\[I_{CICR2} = I_{CICRmax} \left( \frac{[IP_3]^{3.8}}{[IP_3]^{3.8} + K_{CICR2}^{3.8}} \right) \left( \frac{[Ca]_i^4}{[Ca]_i^4 + K_{CICR}^{4}} \right) \left( \frac{[Ca]_{IS2}^2}{[Ca]_{IS2}^2 + K_{CICR2}^2} \right)\]

Equation 4.14

The Ca\(^{2+}\) balance in the 2\(^{nd}\) pool is described by Equation 4.15.

\[\frac{d[Ca]_{IS2}}{dt} = \frac{I_{SERCA2} - I_{leak2} - I_{CICR2}}{Z_{Ca} Fvol_{IS2}}\]

Equation 4.15

The modified equation for cytosolic calcium change due to incorporation of second pool is given in Equation 4.16.

\[\frac{d[Ca]_i}{dt} = \frac{I_{Caom} + I_{SERCA,IS} - I_{leak,IS} - I_{IP3,IS} + I_{SERCA2} - I_{leak2} - I_{CICR2}}{Z_{Ca} Fvol_{Ca}} - \frac{d[Ca]_b}{dt}\]

Equation 4.16

All other model components and parameters are the same as in the previous studies, except 1) Random variations simulating biological noise were implemented; 2)
Introducing non-uniformity in the store and membrane receptor distribution. Randomized variation with an average gradient in the store and membrane receptor distribution was introduced i.e. on an average the receptors were higher on one side of the cell compared to other as shown in the Figure 4.2-A,4.3-A,4.3-B, 4 4-A,4.7-A and 4.7-B. Gear’s backward differential formula method for stiff systems in FORTRAN 90 with maximum time step of 4ms was used to solve the system of ordinary differential equations describing the model.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Model Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{Ca}$</td>
<td>Diffusion coefficient for Ca$^{2+}$ (Allbritton, Meyer, and Stryer 1992)</td>
<td>300 $\mu$m$^2$/s</td>
</tr>
<tr>
<td>$D_{Na}$</td>
<td>Diffusion coefficient for Na$^+$ (Keener and Sneyd 1998)</td>
<td>505 $\mu$m$^2$/s</td>
</tr>
<tr>
<td>$D_{K}$</td>
<td>Diffusion coefficient for K$^+$ (Keener and Sneyd 1998)</td>
<td>744 $\mu$m$^2$/s</td>
</tr>
<tr>
<td>$D_{Cl}$</td>
<td>Diffusion coefficient for Cl$^-$ (Keener and Sneyd 1998)</td>
<td>900 $\mu$m$^2$/s</td>
</tr>
<tr>
<td>$D_{IP_{3}}$</td>
<td>Diffusion coefficient for second messenger IP$_3$ (Keener and Sneyd 1998)</td>
<td>283 $\mu$m$^2$/s</td>
</tr>
</tbody>
</table>

EC Store: 2$^{nd}$ pool parameters (Parthimos, Edwards, and Griffith 1999)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Model Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[Ca]_{IS2}$</td>
<td>Initial Ca$^{2+}$ concentration in the second pool of IP$_3$ sensitive store.</td>
<td>0.08 mM</td>
</tr>
<tr>
<td>$I_{SERCA_{max}}$</td>
<td>Maximum current via SERCA in second pool of IP$_3$ sensitive store.</td>
<td>54.7 pA</td>
</tr>
<tr>
<td>$K_{SERCA_2}$</td>
<td>$[Ca^{2+}]$, for half activation of SERCA in second pool of IP3-sensitive store.</td>
<td>0.4 $\mu$M</td>
</tr>
<tr>
<td>$K_{leak_{2}}$</td>
<td>ER leak constant for second pool</td>
<td>6.7 pA/mM</td>
</tr>
<tr>
<td>$I_{CICR_{max}}$</td>
<td>Maximum current via CICR channel in second pool of IP$_3$ sensitive store.</td>
<td>14850 pA</td>
</tr>
<tr>
<td>$K1_{CICR_2}$</td>
<td>$[Ca^{2+}]$, for half activation of CICR in second pool of IP3-sensitive store</td>
<td>0.54 $\mu$M</td>
</tr>
<tr>
<td>$K2_{CICR_2}$</td>
<td>$[Ca^{2+}]_{IS2}$ for half activation of CICR Ca$^{2+}$ efflux in second pool of IP3-sensitive store</td>
<td>120 $\mu$M</td>
</tr>
<tr>
<td>$K3_{CICR_2}$</td>
<td>IP$_3$ concentration for half-maximal CICR activation</td>
<td>0.5 $\mu$M</td>
</tr>
<tr>
<td>$vol_{IS2}$</td>
<td>Store volume for the second pool.</td>
<td>0.28 pL</td>
</tr>
</tbody>
</table>

| Table 4.1 Parameter values considered in the model |
Long simulated times were used (up to 1000 s) to eliminate transient effects. Ca$^{2+}$ events observed over space and time were plotted as a kymograph i.e. line scans where spatial Ca$^{2+}$ concentrations are depicted color coded at individual times and are stacked on top of each other to form a 2-D image with x-axis representing cell length and the y-axis representing time and color map showing the Ca$^{2+}$ concentration at any point on the image. The slopes from the kymograph were used to quantify the Ca$^{2+}$ wave velocities. Spatial heterogeneity of receptors was also introduced in the 2-D FEM models resulting in oscillatory Ca$^{2+}$ waves (result shown in supplement (section 4.9)) as in the compartmental model; however all of the results here are from the compartmental model.

4.4 Results -SMC

4.4.1 Oscillatory Ca$^{2+}$ wave generation

4.4.1.1 Store/Membrane receptor distribution

Step distribution of adrenergic receptors, through increase in density of receptors in small region (Figure 4.2-A yellow section) and reduced density of receptors in rest of the cell (Figure 4.2-A purple section) to maintain the overall receptor density as in the original model without introduction of the heterogeneity, was introduced in the model. Agonist (280-450 nM) induced Ca$^{2+}$ elevation within the concentration window for Ca$^{2+}$ oscillations (Kapela, Bezerianos, and Tsoukias 2008) in the presence of the step distribution of adrenergic receptors (Figure 4.2-A green line) resulted in oscillatory Ca$^{2+}$ waves. An illustration example of the obtained oscillatory waves at 300 nM agonist concentration is plotted as a kymograph in Figure 4.2-B. To evaluate the role of diffusion of Ca$^{2+}$ and IP$_3$, and electrical coupling in shaping the oscillatory Ca$^{2+}$ wave, we blocked
the inter-compartmental currents for all the species individually and under different combinations. Agonist stimulation of the SMC under the same conditions in Figure 4.2-B, with block of inter-compartmental Ca\(^{2+}\) currents (\(I_{ic,Ca} = 0\)) resulted in loss of oscillatory Ca\(^{2+}\) waves with either individual compartments oscillating out of phase and at different intrinsic frequencies or complete deficiency of oscillations in few or all the compartments (Figure 4.2-C). Mean steady state levels of Ca\(^{2+}\), IP\(_3\) and V\(_m\) potential arising across the cell under different scenarios were recorded and the peak to peak amplitude of their oscillation measured and represented by error bars on the top of the steady state value to visualize the change in levels under oscillations from the mean value.

**Figure 4.2** (A) Step variation in adrenergic receptors resulting in generation of oscillatory Ca\(^{2+}\) wave in the SMC. (B) Ca\(^{2+}\) concentration amplitude depicted color coded and plotted with time vertically and distance from left to right (Kymograph) arising from step variation of adrenergic receptor distribution, shown in (A). (C) Kymograph of Ca\(^{2+}\) concentration after Ca\(^{2+}\) diffusion block showing the importance of intracellular Ca\(^{2+}\) signaling for maintenance of oscillatory Ca\(^{2+}\) wave (B). Mean Ca\(^{2+}\) concentrations (D), mean IP\(_3\) concentrations (E) and mean V\(_m\) changes (F) across the cell length arising from adrenergic receptor distribution under control conditions (green line) and block of inter-compartmental currents (red line). The bars represent the amplitude of oscillations in the individual compartments across the cell.
In the absence of inter-compartmental flux for all species, Ca$^{2+}$ and IP$_3$ concentrations in the compartments with low density of membrane receptors (Figure 4.2-D and E yellow section of the cell) did not attain sufficient levels (Figure 4.2-D and E red line) to reach the oscillatory window. On the other hand compartments with high density of membrane receptors (Figure 4.2-D and E purple section of the cell) produced Ca$^{2+}$ and IP$_3$ levels sufficient (Figure 4.2-D and E red line) for Ca$^{2+}$ oscillations in those compartments (Figure 4.2-D and E red error bars). Moreover a step change in membrane potential was also observed across the cell (Figure 4.2-F red line) from the block of all inter-compartmental fluxes. Such an intervention (uncoupling sections of a cell) is difficult to achieve experimentally but can be easily carried out in the model and will aid us in better understanding the role of these individual species.

Electrical coupling alone (i.e. having the inter-compartmental currents for Na$^+$, K$^+$ and Cl$^-$ present) in the absence of inter-compartmental Ca$^{2+}$ currents resulted in cell becoming iso-potential (uniform mean $V_m$ profile) (Figure 4.2-F green solid line). Oscillatory Ca$^{2+}$ and IP$_3$ activity was retained in all the individual compartments (by bringing the Ca$^{2+}$ and IP$_3$ levels in the low receptors density compartments also in the oscillatory window) but no whole cell oscillatory Ca$^{2+}$ wave was observed (data not shown). Allowing $I_{ic,Ca}$ current in addition to electrical coupling synchronized individual compartments to result in formation of whole cell oscillatory waves (Figure 4.2-B). However, it was the oscillatory component of the diffusion Ca$^{2+}$ current that resulted in synchronization of the individual compartments. The presence/absence of inter-compartmental IP$_3$ currents ( $I_{ic,IP_3}$ ) had no significant effect on the simulations suggesting negligible role for IP$_3$
diffusion. The heterogeneity for the wave generation was provided by the IP₃ gradient resulting from non-uniform membrane receptor distribution. Figure 4.2-E (green line) shows the gradient in the mean/steady state IP₃ concentrations across the cell under control simulation conditions. The steady state Ca²⁺ levels under control simulation were nearly uniform (Figure 4.2-D green line).

Similar to adrenergic receptors, non-uniform spatial distribution of store (RyR) receptors (Figure 4.3-A and 4.3-B) resulted in oscillatory Ca²⁺ waves as shown in Figure 4.3-C and 4.3-D. Completely randomized distribution of both membrane and store receptors in the absence of average gradient did not result in generation of an oscillatory Ca²⁺ wave (result not shown) suggesting the presence of non-uniform spatial distribution of store/membrane receptor with a gradient in receptor density distribution.

**Figure 4.3** Linearized variation in RyR density in (A) and normalized variation in (B) resulted in SMC oscillatory Ca²⁺ wave’s depicted color coded as a kymograph in (C) and (D) respectively.
However experimental test to validate the presence of such distributions are necessary. The site of initiation of the wave was always the one with the highest density of these receptors. The average slope of distribution of the store and membrane receptors modulated the velocity component of the Ca\(^{2+}\) waves with zero slope (uniform distribution) corresponding to infinite velocities or whole cell oscillations. Increasing the slopes lowered the Ca\(^{2+}\) wave velocity to certain point before which the waves were lost (results not shown).

4.4.2 Effect of \(V_m\) dynamics on wave velocity

Modulation in \(V_m\) coupled to Ca\(^{2+}\) oscillations can feedback on the Ca\(^{2+}\) oscillators in individual compartments to modulate Ca\(^{2+}\) wave velocity through \(V_m\)-dependent Ca\(^{2+}\) channels and alteration of electrochemical gradient for calcium influx. To visualize the influence of \(V_m\) dynamics on oscillatory Ca\(^{2+}\) waves we introduced a stimulus current (\(I_{stim}\)) in the model with RyR heterogeneity. Stimulus current forced \(V_m\) to follow the average of calcium levels in all compartments (\(Ca_{osc}(t)\)) with time. The stimulus current forces \(V_m\) oscillations (\(V_m(t)\)) to be either in phase or anti-phase of \(Ca_{osc}(t)\) described by Equation 4.17.

\[
I_{stim} = K \left( \pm \frac{V_{max}Ca_{osc}(t)}{lnM} \right) \left( V_m(t) - \bar{V}_m \right)
\]

Equation 4.17

where

\[
Ca_{osc}(t) = \left( \frac{\sum_i^N Ca_i(t)}{N} \right) - \bar{Ca}_i
\]

Equation 4.18
is the AC component of average calcium oscillation in the cell. $V_m(t) - \overline{V}_m$ is the AC component of mean $V_m$ oscillation in the cell. $K$ and $V_{max}$ are the scaling factors that regulate the strength of stimulus and amplitude of $V_m$ oscillation respectively, $N$ is the number of compartments. Both the amplitude of $V_m$ oscillations and its phase with the average Ca$^{2+}$ concentration ($Ca_{osc}(t)$) determined the oscillatory Ca$^{2+}$ wave velocity as shown in Figure 4.4. The wave velocities increased exponentially with the increase in the amplitude of $V_m$ oscillations when $V_m(t)$ was made to follow $Ca_{osc}(t)$ in phase ($V_m$-Ca$^{2+}$ in phase) whereas reduced velocities were observed with increase in amplitude of $V_m$ oscillation when $V_m$-Ca$^{2+}$ were anti-phase i.e. increasing the amplitude of $V_m$ oscillations resulted in exponential decrease of wave velocity under this condition.

![Figure 4.4](image)

**Figure 4.4** Increase in oscillatory Ca$^{2+}$ wave velocity was observed with increased amplitude of forced $V_m$ oscillations in phase with the average Ca$^{2+}$ oscillations while decrease in wave velocities with increased amplitude of forced $V_m$ oscillations anti-phase to the average Ca$^{2+}$ oscillations (Negative $V_m$ indicates anti-phase with Ca$^{2+}$).

$V_m$ dynamics is modulated by the balance of current through transmembrane channels and pumps. All the model components that influence $V_m$ dynamics were tested to evaluate their role in wave velocity modulation. NE concentration of 280-450 nM under
step membrane receptor distribution resulted in oscillatory Ca\(^{2+}\) waves with varying velocities. The velocities were calculated from the slope of kymograph and are shown in Figure 4.5-A. Exponentially decreasing velocities were obtained with increase in mean agonist concentration. Clamping of \(V_m\) resulted in significant decrease in the wave velocities at low agonist concentration and a relatively smaller change at high agonist levels (Figure 4.5-B). The amplitude of \(V_m\) oscillations and its phase with overall intracellular Ca\(^{2+}\) oscillation (the mean of all compartmental Ca\(^{2+}\) concentration at individual time points) in the cell (\(V_m\)-Ca\(^{2+}\) phase) were recorded and quantified using correlation coefficient.

![Figure 4.5 (A)](image)

**Figure 4.5 (A)** Exponential decrease in oscillatory Ca\(^{2+}\) wave velocity with increased agonist (NE=0.29-0.4 \(\mu\)M) stimulus in SMC model with NE step variation. (B) Plot of correlation of average \(V_m\) and Ca\(^{2+}\) oscillations against increased NE concentration. Increasing agonist (NE) stimulus resulted in more out of phase (\(V_m\)-Ca\(^{2+}\) oscillations). The bars indicate the range of correlation coefficients observed in the individual compartments of the cell and green diamond indicates the mean value of correlation at particular agonist concentration.

The amplitude of \(V_m\) oscillations were nearly the same around 2mV for the range of NE concentrations. \(V_m\) and Ca\(^{2+}\) oscillations were in phase (high correlation coefficient) at lower agonist levels. The phase difference started growing with increasing agonist concentrations (reducing correlation coefficient) as shown in Figure 4.5-B. The in-phase
\( V_m \)–Ca\(^{2+}\) at lower agonist concentrations resulted in higher wave velocities compared to out of phase or anti-phase \( V_m \)–Ca\(^{2+}\) oscillations at higher agonist concentrations which agrees to prediction by injected stimulus current.

Simulations were performed clamping the individual channels and pumps to their mean steady state currents to analyze the relative importance of each of them in regulation of wave velocities. Clamping NSC channels reduced significantly the amplitude of \( V_m \) oscillation from the control (Figure 4.6-A). Simultaneously it resulted in out of phase \( V_m \)–Ca\(^{2+}\) oscillations (Figure 4.5-B error bar lower values) resulting in significantly lower wave velocities compared to control at low agonist levels (Figure 4.6-B, green triangle). Due to the Ca\(^{2+}\) dependency of the PLC pathway, Ca\(^{2+}\) oscillations generate concomitant oscillations in DAG concentration. Oscillatory DAG in turn modulates NSC channel activation, depolarizes \( V_m \), and activates, through electrical coupling, VOCC channels throughout the cell bringing the average Ca\(^{2+}\) oscillations in cell in phase with \( V_m \) resulting in increased wave velocities. On the other hand, clamping BK\(_{Ca}\) channels resulted in significant increase in the amplitude of \( V_m \) oscillations (Figure 4.6-A) with higher \( V_m \)–Ca\(^{2+}\) correlation (Figure 4.5-B error bar upper values) i.e. in phase \( V_m \)–Ca\(^{2+}\) oscillations and consequently significantly higher wave velocities (Figure 4.6-B, purple squares) compared to control. BK\(_{Ca}\) channels generate hyperpolarizing current in response to Ca\(^{2+}\) elevation. At higher agonist concentrations the BK\(_{Ca}\) channels are more sensitive to Ca\(^{2+}\) changes compared to NSC in the model and induce \( V_m \) hyperpolarization which drive Ca\(^{2+}\) oscillations out of phase with \( V_m \).
The changes in $V_m$ dynamics directly modulate the wave velocities through activation / inactivation of VOCC channels and indirectly by affecting the electrochemical gradient for calcium entry in the cell. Clamping of VOCC channels results in significant reduction in wave velocity at lower range of agonist concentrations as shown in (Figure 4.6 -C).

**Figure 4.6** In the SMC model with adrenergic receptor step variation (A) clamp of BK$_{Ca}$ and NSC channels at different agonist stimulus concentrations (NE=0.29 – 0.4 µM) resulted in significant increase and decrease respectively in oscillatory $V_m$ amplitude from the control. Error bars indicate variation in $V_m$ (P-P) for varied NE concentrations (NE= 0.29 -0.4 µM) (B) The variation in oscillatory $V_m$ amplitude due to NSC clamp were translated to significant increase in wave velocity due to BK$_{Ca}$ clamp and significant decrease in wave velocities under NSC clamp. (C) Clamping VOCC channels resulted in significant decrease in wave velocities at lower range of agonist concentrations but no significant effect at higher agonist concentrations whereas NCX (Na$^+$--Ca$^{2+}$ exchanger) resulted in increase in oscillatory wave velocities at lower concentration and a very slight decrease at higher agonist concentrations.

However, at higher concentration of agonist stimulations (NE – 0.33 -0.39 µm) BK$_{Ca}$ channels were more dominant resulting in $V_m$ hyperpolarization and no significant effect of VOCC clamp was observed as a result. NCX clamp simulations (Figure 4.6-C) on the
other hand reveal that at low agonist concentrations it reduces the Ca\textsuperscript{2+} wave velocities in $V_m$ independent fashion. The flux through NCX depends on sodium concentrations and hence their levels in the cytosol were recorded. In the control simulations 0.5 mM ([$Na^+]_i = 14.70 \text{mM}$) amplitude oscillations in $[Na^+]_i$ were observed. Clamping sodium concentrations in the NCX flux equation to its average value resulted in identical response as NCX current clamp (data not shown) indicating the role of sodium oscillations in generation of the desynchronizing signal through NCX modulation.

4.5 Results-EC

4.5.1 Oscillatory Ca\textsuperscript{2+} wave generation

In non-excitable cells (ECs); generation of Ca\textsuperscript{2+} waves required spatial heterogeneity of membrane / agonist receptors with an overall average gradient, similar to Ca\textsuperscript{2+} wave production in the SMC model. Agonist stimulation of the EC with muscarinic Ach receptor (Ach\_R) step distribution (Figure 4.7-A), through increase in density of receptors in small region (Figure 4.7-A yellow section) and reduced density of receptors in rest of the cell (Figure 4.7-A purple section) to maintain the overall receptor density as in the original model without introduction of the heterogeneity, resulted in generation of oscillatory Ca\textsuperscript{2+} wave as shown in Figure 4.7-B. Block of inter-compartmental Ca\textsuperscript{2+} current ($I_{\text{ic,Ca}}$) resulted in loss of oscillatory. Ca\textsuperscript{2+} wave (Figure 4.7-C) with individual compartments oscillating at random frequencies or complete lack in oscillations. Similar to the SMC model, it was the oscillatory component of $I_{\text{ic,Ca}}$ current which resulted in synchronization of individual compartments to form the whole cell agonist induced oscillatory waves. Electrical coupling (in the absence of Ca\textsuperscript{2+} and IP\textsubscript{3} inter-
compartmental currents) resulted in cell becoming iso-potential (Figure 4.7-F green line) with intrinsic Ca\(^{2+}\) oscillations observed in individual compartments but no whole cell wave (data not shown).

**Figure 4.7** (A) Step variation in muscarinic receptors resulting in generation of oscillatory Ca\(^{2+}\) wave in the EC. (B) Ca\(^{2+}\) concentration amplitude depicted color coded and plotted with time vertically and distance from left to right (Kymograph) arising from step variation of muscarinic receptor distribution, shown in (A). (C) Kymograph of Ca\(^{2+}\) concentration after Ca\(^{2+}\) diffusion block showing the importance of intracellular Ca\(^{2+}\) signaling for maintenance of oscillatory Ca\(^{2+}\) wave (B). Mean Ca\(^{2+}\) concentrations (D), mean IP\(_3\) concentrations (E) and mean \(V_m\) levels (F) across the cell length arising from muscarinic receptor distribution under control conditions (green line) and block of inter-compartmental currents (red line). The bars represent the amplitude of oscillations in the individual compartments across the cell.

In the EC, non-uniform muscarinic receptor density produced both steady state IP\(_3\) and Ca\(^{2+}\) concentration gradients (Figure 4.7-D and E) are observed, and provide the necessary spatial heterogeneity for oscillatory Ca\(^{2+}\) wave generation in contrast too only IP\(_3\) gradient observed in the SMC model simulations with non-uniform adrenergic receptors.
Uniform Ach in the presence of gradient in density of IP$_3$Rs (Figure 4.8-A) resulted in oscillatory Ca$^{2+}$ waves similar to waves obtained under RyR gradients in the SMC. Oscillatory Ca$^{2+}$ waves obtained for agonist concentrations of 0.9 and 0.65 (a.u.) are shown in Figure 4.8-B and D respectively. Increase in the frequency of the Ca$^{2+}$ waves (Figure 4.9-B) was observed at higher agonist concentrations with no significant variation in velocity. $V_m$ clamp resulted in reduced wave velocity through modulation of electrochemical gradient for Ca$^{2+}$ influx.

**Figure 4.8** (A) Linear variation in IP$_3$R density introduced in the model produced oscillatory Ca$^{2+}$ waves. (B) Kymograph of Ca$^{2+}$ concentration from stimulus of EC (0.9 a.u. Ach) with linear distribution of IP$_3$ receptors as shown in (A). (C) Kymographs of Ca$^{2+}$ concentration after oscillatory $V_m$ clamp with same stimulatory conditions as in (B) reduced the Ca$^{2+}$ wave velocity due to change in electrochemical gradient from the $V_m$ clamp. (D) Kymograph of Ca$^{2+}$ concentration showing the reduction in frequency of oscillatory Ca$^{2+}$ waves in the EC with reduction in strength of agonist stimulus (0.65 a.u. Ach).
4.5.2 Effect of $V_m$ dynamics on wave velocity

Although not intuitive at first place due to the absence of voltage regulated Ca$^{2+}$ channels in the EC, our simulations suggests a significant role of $V_m$ dynamics in the regulation of velocity of EC Ca$^{2+}$ wave. Simulations with clamped $V_m$ in the IP$_3$R gradient model resulted in significant lower wave velocities (Figure 4.8-B, Figure 4.9-A). $V_m$ dynamics in EC are also affected by the relative contribution of currents through individual transmembrane channels and pumps similar to SMC. Agonist (Ach) induced increase in Ca$^{2+}$ levels in EC results in activation of IK$_{Ca}$ and SK$_{Ca}$ channels and hence $V_m$ hyperpolarization. The oscillatory component of $V_m$ dynamics will modulate the electrochemical gradient for Ca$^{2+}$ influx/efflux via the NSC channels and further the regulation of oscillatory Ca$^{2+}$ wave velocities.

![Figure 4.9](image)

**Figure 4.9** stimulation of EC cell with IP$_3$R gradient resulted in, (A) Significant reduction in wave velocity (red bar) under $V_m$ clamp condition from control (yellow bar) during. (B) Increase in wave frequency with increase agonist concentration

4.6 Discussion

The physiological significance of Ca$^{2+}$ waves is still unclear however it has been suggested that oscillatory Ca$^{2+}$ waves cause less contraction than whole-cell Ca$^{2+}$ oscillations induced by the same agonist concentrations in airway SMC (Wang et al.
Ca^{2+} wave blockade in cerebral artery SMC has been shown to reduce the level of myosin regulatory light chain (LC20) phosphorylation resulting in dilation (Mufti et al. 2010) however on the contrary it has also been suggested that Ca^{2+} waves do not contribute significantly to global [Ca^{2+}]_i and are therefore not important to force generation in the myogenic response (Jaggar 2001). A possible explanation for these differences is suggested in Cole et al. (Cole and Welsh 2011) which depends on the rate of LC20 dephosphorylation and inter-wave interval. Spatio-temporal Ca^{2+} wave signaling in the EC can regulate vasomotor tone (Socha et al. 2012) however the exact physiological roles of individual signaling mechanisms still need to be discerned.

4.6.1 Oscillatory Ca^{2+} wave generation

We formulated detailed models for both the EC and SMC to understand the underlying regulatory mechanism for oscillatory Ca^{2+} wave generation and its modulations. The models predicted the necessity of non-homogeneity of the store and/or membrane receptor distributions for the generation of the oscillatory calcium waves. Specific distributions for these receptors have not yet been reported in literature but their requirement has been suggested in airway smooth muscle cell model (Wang et al. 2010). Higher density of store receptors have been reported near the nucleus in certain cell type’s (Vermassen et al. 2003). In the EC layer preparations presence of purinergic/membrane receptors in only 30 % cells have been reported resulting in Ca^{2+} increase in only one third of the total cells in the absence of gap junctions which increased to 95 % cells in the presence of gap junctions (Kameritsch et al. 2012). The sites with co-localization of Ach receptors and IP3R have been suggested to be the site of initiation for the Ca^{2+} wave propagation in SMC (Olson et al. 2012). Such intra and inter
cellular variations and presence of microdomains can result in localized regions of cells oscillating at varying frequencies and wave generation through synchronization of these individual oscillators. It can also determine the position of origin of the waves and the length of propagation. In the model the waves always appeared to originate from the site with highest density of the store/membrane receptors (Figure 4.2-A, 4.3-A and B, 4.7-A and 4.8-A). Overall gradient of receptors was necessary to generate oscillatory Ca^{2+} waves in our model. Completely randomized variation in receptor density resulted in no wave generation. However further validation for the presence of such specific distributions through experimentation is required.

Both IP_{3} and Ca^{2+} are shown to play an important role in Ca^{2+} wave generation and propagation (McCarron et al. 2010). In McCarron et al. elevated IP_{3} levels have been suggested to sensitize the IP_{3} receptors and determines the distance of Ca^{2+} wave progression. Ca^{2+} diffusion may induce the propagation through directly acting on store receptors or indirectly through increase in local IP_{3} levels. Our model suggest that under certain stimulatory conditions electrical coupling in addition to Ca^{2+} and IP_{3} might be essential for wave generation (Figure 4.10-A and B). Electrical coupling can drive localized regions of the cell into firing mode by bringing the Ca^{2+} levels close to oscillatory window. Inter-compartmental oscillatory Ca^{2+} currents provide synchronizing signals to generate whole cell Ca^{2+} waves. In addition to sensitization of receptors steady state Ca^{2+}/IP_{3} gradient in the model provided the spatial heterogeneity for the oscillatory Ca^{2+} wave generation.
4.6.2 Wave velocities modulation

A wide range of $\text{Ca}^{2+}$ wave velocities have been reported both in SMC and EC in different experimental condition and cell types (Beliveau, Lapointe, and Guillemette 2011; Huser and Blatter 1997; Jaffe 2010; Narayanan, Adebiiyi, and Jaggar 2012). Modulation of wave velocity and frequency with the change in the strength of the agonist stimulus have been shown (Ruehlmann et al. 2000; Socha et al. 2012; Syyong et al. 2009). The underlying mechanisms regulating these changes are unclear yet. In the present study we show that the modulation of the wave velocities may occur through membrane potential ($V_m$) dependent pathways. Membrane potential fluctuations regulate
calcium dynamics directly through voltage regulated L-type calcium channels (VOCC) and indirectly through affecting the electrochemical gradient for calcium influx/efflux in the cell. In endothelial cells no voltage regulated Ca\textsuperscript{2+} channels has been reported hence direct modulation of Ca\textsuperscript{2+} in ECs via $V_m$ is not feasible however indirect modulation through changing electrochemical gradient for Ca\textsuperscript{2+} entry and exit from the cell is possible. Although membrane potential dynamics has been shown to be unimportant for the generation of the Ca\textsuperscript{2+} wave (Peng et al. 2001) in particular SMCs, it might significantly modulate the already generated waves. In the present model both the amplitude and phase of the membrane potential oscillations with Ca\textsuperscript{2+} oscillations determines the wave velocity in SMC. Higher amplitude $V_m$ and in-phase $V_m$-Ca\textsuperscript{2+} results in whole cells oscillations or higher wave velocities. However out of phase or anti-phase $V_m$-Ca\textsuperscript{2+} will result in lower wave velocities. The stimulus strength and relative contributions of depolarizing/hyperpolarizing currents through transmembrane channels and pumps will determine these phase and amplitude of $V_m$ oscillations. In the SMC, $V_m$ oscillations will cause opening/closing of the L-type voltage channels which can speed up or slow down the waves through modulating the Ca\textsuperscript{2+} entry in the cytosol. VOCC channels blockers have shown to significantly reduce the frequency of Ca\textsuperscript{2+} waves (Balemba et al. 2006; Dai et al. 2007; Lee et al. 2001) but it does not mean that these channels were directly involved in the frequency modulation as channel block perturbs directly the ionic balance of the cell and the frequency modulation can be indirect consequence of it. Clamping the channel to its average current value gives a better idea of its direct involvement but such experimental intervention is not easy to implement. In the model VOCC clamp resulted in significant decrease in wave velocity at low agonist
concentrations however it had no impact the frequency of waves. In endothelial cells the voltage regulated Ca\(^{2+}\) channels are absent but \(V_m\) oscillations modulates the electrochemical gradient for Ca\(^{2+}\) entry in the cell via NSC channel.

Cl\(_{Ca}\) channels through depolarization and activation of VOCC have been proposed to bring Ca\(^{2+}\) in phase with \(V_m\) oscillations and conversion from asynchronous waves to whole cell oscillations (Jacobsen et al. 2007). Moreover NSC and BK\(_{Ca}\) channels have been suggested as important candidates for vasomotion (Kapela, Parikh, and Tsoukias 2012). DAG-activated TRPC-like nonselective cation channels are expressed in RMA SMCs and mediate depolarizing Na\(^{+}\) influx during \(\alpha_1\)-adrenergic stimulation (Hill et al. 2006). Block of NSC channels using a non-specific blocker resulted in loss of repetitive wave like Ca\(^{2+}\) oscillations in intact rabbit inferior vena cava (Dai et al. 2007) suggesting its role in maintaining oscillations. Our model predicts NSC channels to be good candidate for modulating wave velocities assuming the DAG oscillations. In the model oscillatory DAG was the result of PLC Ca\(^{2+}\) dependency. Concomitant oscillations in DAG, IP\(_3\) and Ca\(^{2+}\) induced by NE were reported in Chinese hamster ovary cell culture (Bartlett et al. 2005). Recently a robust sensor which can detect DAG and Ca\(^{2+}\) oscillations simultaneously have also been reported (Tewson et al. 2012). Agonist stimulation in the model results in activation of NSC channels resulting in highly correlated average \(V_m\)–Ca\(^{2+}\) oscillations and hence higher wave velocities. However, the BK\(_{Ca}\) channels counteract the NSC channels and depending on the agonist concentration and dominance of these channels the amplitude and phase of \(V_m\) oscillations with respect to Ca\(^{2+}\) oscillations is maintained and significant variation in wave velocities could be observed. A summary figure highlighting the chief components
involved in generation and modulation of oscillatory Ca\textsuperscript{2+} waves in both EC and SMC are shown in Figure 4.10-A and B. Figure 4.10-C illustrates the role of $V_m$ dynamics in both excitable and non-excitable cells in modulation of oscillatory Ca\textsuperscript{2+} velocities and exhibits the main difference between the two cell types for cytosolic Ca\textsuperscript{2+} level modulation. In excitable cells depolarization of $V_m$ (SMC) will result in increase in Ca\textsuperscript{2+} levels via entry through voltage regulated Ca\textsuperscript{2+} channels whereas in non-excitable cells it is the hyperpolarization of $V_m$ that will result in increase in Ca\textsuperscript{2+} levels through modulation of electrochemical gradient for more Ca\textsuperscript{2+} influx.

NCX blockers have been reported to abolish repetitive calcium waves and significantly reduce tonic contraction through Ca\textsuperscript{2+} entry block in SMC (Lee et al. 2001). Block of any channel can significantly modulate the system over all and the resultant change cannot be attributed to that particular channel. In the SMC model NCX clamp produced a desynchronizing signal due to $\left[\text{Na}^{+}\right]_i$ oscillations resulting in reduction of wave velocities at lower agonist concentrations through a $V_m$ independent pathway. At higher agonist concentrations BK\textsubscript{Ca} being more dominant results in no significant effect of NCX.

4.7 Model limitations

Due to lack of relevant experimental data and computational limitation several necessary simplifying assumptions were carried out in the model. The underlying mechanism for Ca\textsuperscript{2+} oscillation in EC and SMC have not been resolved definitively with lack of any models for Ca\textsuperscript{2+} oscillations in EC. Both IP\textsubscript{3} and RyR individually and together have been suggested to be the main components for Ca\textsuperscript{2+} oscillations. In this study regular Ca\textsuperscript{2+} oscillations are generated by RyRs and slow refilling in SR for SMC
as in (Koenigsberger et al. 2004) and with a help of a novel two pool model with oscillations generated by IP$_3$Rs and slow refilling in SR for EC as in (Paltauf-Doburzynska et al. 2000). We account for non-homogenous distributions of RyRs and IP$_3$Rs and membrane receptors however their specific distributions have not been reported experimentally hence the choice of these distributions were take on arbitrarily. Compartmentalization of certain channels for generation of Ca$^{2+}$ sparks, puffs, sparklets have been reported in the literature but not incorporated here which may have a significant effect on model predictions. Moreover the current model is deterministic however the stochastic opening of individual channels have been reported and suggested to play an important role however the current model does not account for it.

4.8 Conclusion

The model predicts the presence of non -homogenous distribution of store/membrane receptors for the generation of oscillatory Ca$^{2+}$ waves in both EC and SMC. It suggests the requirement of steady state IP$_3$/Ca$^{2+}$ gradient and oscillatory Ca$^{2+}$ diffusion for origin and propagation of oscillatory Ca$^{2+}$ waves. Moreover it explains the role of electrical coupling/$V_m$ dynamics in the generation and modulation of Ca$^{2+}$ waves. Stimulatory conditions and relative channel contributions will determine the speed and frequency of the oscillatory Ca$^{2+}$ waves. The model also reveals the presence of several physiologically feasible pathways for modulation of wave velocities. This may explain inconsistent experimental results for generation of oscillatory Ca$^{2+}$ wave and its regulation.
4.9 Supplement

4.9.1 Compartmental model for oscillatory Ca\(^{2+}\) wave

The single cell models of EC and SMC were discretized into \( N = 200 \) compartments with each compartment incorporating the cellular components as in the original models. All the fluxes in the individual compartments were scaled by \( N \) (total number of compartments) and the compartments were coupled by the electro-diffusion equations (Nagaraja, Kapela, and Tsoukias 2012) as described in Equation 4.19

\[
I_{ic,S} = P_{ic,S} z_S F \left( \Delta[S]_{ic} + \frac{z_S F}{RT} [\bar{S}]_{ic} \Delta V_{ic} \right)
\]

Equation 4.19

where \( z_S, F, R \) and \( T \) are the valence of ion \( S \), Faraday’s constant, gas constant and temperature, respectively, \( I_{ic,S} \) is the inter-compartmental flux of the species \( S \), \( \Delta[S]_{ic} = [S]^n - [S]^m \), and \( [\bar{S}]_{ic} = ([S]^n - [S]^m)/2 \) is the average concentration across the compartments \( m \) and \( n \). \( \Delta[V]_{ic} = [V]^n - [V]^m \) is the change in membrane potential across the compartments \( m \) and \( n \), \( P_{ic,S} \) is the inter-compartmental permeability to \( S \). The permeability’s for each of the ions (Ca\(^{2+}\), Cl\(^-\), Na\(^+\) and K\(^+\)) and second messenger (IP\(_3\)) were calculated by:

\[
P_{ic,S} = \frac{D_S A}{L/N}
\]

Equation 4.20

where \( A, L, N \) are cross sectional area of cell, length of cell and number of compartments in the cell respectively. \( D_S \) is the intra-cellular diffusion coefficient’s for species \( S \) where \( S \) is Ca\(^{2+}\), K\(^+\), Na\(^+\), Cl\(^-\) and IP\(_3\) and their respective values are
Oscillatory Ca\(^{2+}\) wave obtained under control conditions in SMC with N=200 is shown in Figure 4.11-A. However N=25 resulted in similar results to discretization into 200 compartments and hence all the further simulations were carried using N=25 to reduce computational overheads.

### 4.9.2 Finite element model (FEM) for oscillatory Ca\(^{2+}\) wave

FEM models provide Continuous 2-D spatial resolution within cytosol and microdomains. They will enable us to study localized events such as Ca\(^{2+}\) sparks and puffs or intra- and inter-cellular Ca\(^{2+}\) waves, which are now recognized as important aspects of Ca\(^{2+}\) signaling. We have implemented EC and SMC models on 2-D rectangular domains and cylindrical domain considering axial symmetry using COMSOL Multiphysics software (Kapela and Tsoukias 2011).

**Figure 4.11** (A) Agonist (300 nM) induced oscillatory Ca\(^{2+}\) wave in the SMC under step distribution of adrenergic receptors, obtained using discretized compartmental model (N=200 compartments) Mean steady state Ca\(^{2+}\) profiles (B) and IP\(_3\) profile in (C) across the cell in 2-D FEM SMC model under agonist (300 nM). Bars indicated the amplitude of oscillations
Electrodiffusion equations with the electroneutrality condition were used to calculate intracellular and extracellular concentrations of ionic species $S = \text{Ca}^{2+}, \text{Na}^+, \text{K}^+, \text{Cl}^-$. The voltage and concentration dependent membrane channel currents were implemented as boundary conditions. The details of the model is described in Kapela et al. 2011 (Kapela and Tsoukias 2011). The models were modified to introduce spatial distribution of membrane/store receptors similar to the compartmental model. The oscillatory Ca$^{2+}$ wave obtained from the FEM model were similar to compartmental model. The mean profiles of Ca$^{2+}$ and IP$_3$ obtained from the FEM model are as shown in Figure 4.11-B and C. They are similar to the mean profiles obtained from the compartmental model (Figure 4.2).

4.10 References


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CHAPTER 5-SYNCHRONIZATION OF CALCIUM OSCILLATIONS IN VASCULAR SMOOTH MUSCLE CELLS

This chapter is published in Biophysical Journal (with slight modifications) as A. Kapela, Parikh, J, and N. M. Tsoukas. “Multiple Factors Influence Calcium Synchronization in Arterial Vasomotion”
5.1 Abstract

The intercellular synchronization of spontaneous calcium (Ca\textsuperscript{2+}) oscillations in individual smooth muscle cells (SMC) is a prerequisite for vasomotion. A detailed mathematical model of Ca\textsuperscript{2+} dynamics in rat mesenteric arteries shows that a number of synchronizing and desynchronizing pathways may be involved. In particular, Ca\textsuperscript{2+}-dependent phospholipase C, the intercellular diffusion of inositol trisphosphate (IP\textsubscript{3}) (and to a lesser extend of Ca\textsuperscript{2+}), IP\textsubscript{3} receptors, DAG-activated nonselective cation channels, and Ca\textsuperscript{2+}-activated chloride channels can contribute to synchronization, while large-conductance Ca\textsuperscript{2+}-activated potassium channels have a desynchronizing effect. Depending on the contractile state and agonist concentrations, different pathways become predominant, and can be revealed by carefully inhibiting the oscillatory component of their total activity. The phase shift between the Ca\textsuperscript{2+} and membrane potential oscillations can change, thus electrical coupling through gap junctions can mediate either synchronization or desynchronization. The effect of the endothelium is highly variable because it can simultaneously enhance the intercellular coupling and affect multiple SMC components. The model outlines a system of increased complexity and proposes potential synchronization mechanisms that need to be experimentally tested.

5.2 Introduction

Vessels from many vascular beds and species exhibit rhythmic oscillations in vessel diameter called vasomotion. These oscillations occur both in vitro and in vivo, independently from the heartbeat, respiration and sympathetic stimulation (Duling et al. 1981; Nilsson and Aalkjaer 2003). The ubiquity of vasomotion suggests an important role in the microcirculation, but further investigation is required to clarify its physiological
relevance. Vasomotion results from coordinated contractile activity of many smooth muscle cells (SMCs), but the cellular mechanisms leading to the underlying SMC calcium (Ca^{2+}) oscillations and to synchronization are not fully understood. Intercellular coordination is of importance in other physiological systems, as well. Insights acquired from the investigation of vascular smooth muscle may reveal underlying mechanisms in other systems and particularly in tissues that share common characteristics with vascular SM (i.e. airway, gastrointestinal or lymphatic SM) (Berridge 2008; Imtiaz, von der Weid, and van Helden 2009).

In this study, we focus on vasomotion in rat small mesenteric arteries (RMA), which has been studied extensively at both experimental and theoretical levels. Differences in the underlying mechanisms may exist between vascular beds and even within a bed with vessels of different size. In RMA vasomotion, Ca^{2+} oscillates uniformly within individual SMCs, rather than in the form of intracellular waves (Peng et al. 2001; Mauban and Wier 2004). The regular whole-cell Ca^{2+} oscillations are generated spontaneously by the majority of SMCs, and only a small remaining fraction of SMCs exhibits Ca^{2+} oscillations as a result of the coupling with other cells. Under control conditions, the self-sustained regular Ca^{2+} oscillations originate from the internal stores with ryanodine (RyRs) or inositol trisphosphate (IP_{3}Rs) receptors (Aalkjaer and Nilsson 2005; Haddock and Hill 2005).

The natural frequencies of Ca^{2+} oscillators (frequencies of isolated SMCs) are relatively steady under a given set of conditions but they differ between SMCs even within the same vascular segment due to the biological variability. The biological noise adds to the phase fluctuations between SMCs, and some cellular mechanisms may
actively promote desynchronization. Therefore, sustained vasomotion requires synchronizing mechanisms that will override these desynchronizing effects and phase lock Ca\(^{2+}\) oscillations in a population of SMCs. Experimental studies have demonstrated that gap junction un-couplers abolish vasomotion (Matchkov et al. 2004; Aalkjaer and Nilsson 2005). Since gap junctions are nonselective channels permeable to ions and small molecules, they can mediate ionic coupling and transfer of signaling molecules. In general, a synchronizing signal can be very weak and produce no significant forced oscillations. It may only affect the phase rather than the amplitude of oscillations (weakly-coupled oscillators) (Pikovsky, Rosenblum, and Kurths 2001). Because a synchronizing signal can be superimposed on a larger sustained signal, its experimental inhibition is often difficult without affecting the whole system. For example, Ca\(^{2+}\)-activated chloride channels (Cl\(_{Ca}\)) activity may be responsible for synchronization (Peng et al. 2001). However, it is the pulsatile increases in channel activity that cause synchronization and not the sustained current through this channel. A Cl\(_{Ca}\) channel blocker can inhibit the current and thus block the synchronizing signal but it may also affect membrane potential (\(V_m\)) levels and the intrinsic SMC oscillations. Thus, a more appropriate test will be to clamp the current to its average value, but such experimental intervention is not easy to implement. Furthermore, it is apparent that there can be several synchronizing mechanisms interacting in complex ways (Aalkjaer and Nilsson 2005). These and other factors make difficult to elucidate and control vasomotion experimentally. In this respect, computational models can provide a significant assistance to experimental studies.
Based on a series of mathematical models of RMA vasomotion, Koenigsberger and coworkers concluded that synchronization is mediated by direct Ca\textsuperscript{2+} coupling between SMCs (Koenigsberger et al. 2004; Koenigsberger et al. 2005, 2006; Koenigsberger, Sauser, and Meister 2005). In the absence of Ca\textsuperscript{2+} coupling, identical SMCs were desynchronized by Ca\textsuperscript{2+}-activated large conductance K\textsuperscript{+} channels (BK\textsubscript{Ca}) and Gaussian noise in membrane conductances. Possible synchronizing effects of oscillatory IP\textsubscript{3}, generated by Ca\textsuperscript{2+}-dependent phospholipase C (PLC)-\(\delta\), and of stress-activated channels were suggested but predicted to be negligible. The controversial role of endothelial cells (ECs) and wall stress in vasomotion was ascribed mostly to their modulatory effect on the average SMC Ca\textsuperscript{2+} levels, leading to transitions into or out of the Ca\textsuperscript{2+} window for self-sustained SMC Ca\textsuperscript{2+} oscillations. In this scenario, the endothelium and wall stress do not affect the synchronization mechanism, and loss of vasomotion is caused by transition of SMCs from regular Ca\textsuperscript{2+} oscillations to irregular noise-induced Ca\textsuperscript{2+} flashes. On the other hand, Jacobsen et al. used theoretical modeling and experimental data to show that cGMP induces vasomotion in RMA through activation of cGMP-dependent Cl\textsubscript{Ca} channels (Jacobsen et al. 2007, 2007; Jacobsen et al. 2008). In a model with a heterogeneous population of SMCs, Cl\textsubscript{Ca} channels, activated by cGMP, depolarize \(V_m\) in phase with Ca\textsuperscript{2+} transients, and gap junctions transmit the depolarization to neighboring cells where voltage-operated Ca\textsuperscript{2+} channels (VOCC) coordinate Ca\textsuperscript{2+} oscillations. Increasing electrical coupling between SMCs enhanced synchronization, whereas intercellular Ca\textsuperscript{2+} diffusion was not important in this study. In this scheme, endothelium can promote synchronization through the nitric oxide (NO)-stimulated cGMP production and by reducing effective electrical resistance between SMCs. Other theoretical studies have

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focused on vasomotion in vessels other than RMA (Gonzalez-Fernandez and Ermentrout 1994; Parthimos, Edwards, and Griffith 1999, 2003; Parthimos et al. 2007). These studies are based on single cell models and examine the mechanism and modulation of oscillations but not their synchronization.

The theoretical studies by Koenigsberger et al. and Jacobsen et al. proposed novel descriptions of population dynamics in the vascular wall and provided valuable insights into possible synchronization mechanisms. These earlier studies can be further advanced by integrating potentially important components and pathways and improve quantitative descriptions. In (Koenigsberger et al. 2004; Koenigsberger et al. 2005, 2006; Koenigsberger, Sauser, and Meister 2005), for example, the models do not account for nonselective cation (NSC) and Cl\textsubscript{Ca} channels activity or cGMP effects, and significant intercellular permeabilities for Ca\textsuperscript{2+} are assumed. In (Jacobsen et al. 2007, 2007; Jacobsen et al. 2008), the models do not incorporate NSC channels, RyRs, Ca\textsuperscript{2+}-dependent PLC activity, intercellular IP\textsubscript{3} diffusion, or activation of BK\textsubscript{Ca} channels by cGMP. The effect of endothelium-derived factors or vasomotion in the absence of cGMP is not examined. Interestingly, Ca\textsuperscript{2+} transients are associated with \(V_m\) hyperpolarizations in the model of one group and with depolarizations in the model of the other group, and intercellular Ca\textsuperscript{2+} fluxes are significant in one study but not in the other. Thus, two fundamentally different models of synchronization in the same vascular bed have been proposed.

In this chapter, the previous hypotheses about the synchronization of self-sustained Ca\textsuperscript{2+} oscillators in RMA is tried to be reconciled and attempt is made to identify new candidate pathways for synchronization. Simulations suggest a complex and variable
process that depends not only on vessel type but also on experimental conditions and stimuli. Several synchronizing/desynchronizing pathways appear to be involved simultaneously, and their relative magnitudes determine the initiation/termination of vasomotion.

5.3 Methods

We have previously developed detailed models of integrated $\text{Ca}^{2+}$ and $V_m$ dynamics in isolated EC and SMC, based primarily on data from RMA (Silva, Kapela, and Tsoukias 2007; Kapela, Bezerianos, and Tsoukias 2008). The isolated models were also combined into a two-cell (EC-SMC) and multicellular models to investigate myoendothelial communication and conducted vasoreactivity (Kapela, Bezerianos, and Tsoukias 2009; Kapela, Nagaraja, and Tsoukias 2010). To study the phenomenon of vasomotion, a multicellular model was implemented, similar to (Kapela, Nagaraja, and Tsoukias 2010). The individual SMCs and ECs are modeled as single compartments (lumped models) and SMCs can generate spontaneously self-sustained $\text{Ca}^{2+}$ oscillations as a result of the nonlinear dynamics of the intracellular stores (Haddock and Hill 2005). Figure 5.1 shows a schematic diagram of the model. The reader is referred to our earlier publications for a more detailed description of the cellular models and cell coupling mechanisms (Silva, Kapela, and Tsoukias 2007; Kapela, Bezerianos, and Tsoukias 2008, 2009; Kapela, Nagaraja, and Tsoukias 2010). The model equations are summarized in the Appendix (Chapter 7) (Pg-190-205).

All cellular components and parameter values (including homocellular and heterocellular couplings) are the same as in our previous studies. The maximum SERCA rate, $I_{\text{SERCA}} = 20 \text{ pA}$, and the sarcoplasmic reticulum (SR) leak parameter, $R_{\text{leak}} = 5.3 \times 10^{-6}$.
allowed for oscillations generated at intermediate agonist stimulations by Ca\(^{2+}\)-induced Ca\(^{2+}\) release via RyRs and slow refilling of SR (section 7.2.2 and Figure 5.5-A) (Kapela, Bezerianos, and Tsoukias 2008).

**Figure 5.1** Schematic diagram of the ECs and SMCs coupled by nonselective homocellular and heterocellular gap junctions permeable to Ca\(^{2+}\), Na\(^{+}\), K\(^{+}\), and Cl\(^{-}\) ions, and IP\(_{3}\). K\(_{\text{leak}}\) – inward rectifier K\(^{+}\) channel; VRAC – volume-regulated anion channel; SK\(_{\text{Ca}}\), IK\(_{\text{Ca}}\) and BK\(_{\text{Ca}}\) – small-, intermediate-, and large-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels; SOC – store-operated channel; NSC – nonselective cation channel, CaCC and Cl\(_{\text{Ca}}\) – Ca\(^{2+}\)-activated chloride channel; NaK – Na\(^{+}\)-K\(^{-}\)-ATPase; PMCA – plasma membrane Ca\(^{2+}\)-ATPase; NCX – Na\(^{+}\)/Ca\(^{2+}\) exchanger; NaKCl – Na\(^{+}\)-K\(^{-}\)-Cl\(^{-}\) cotransport; K\(_{\text{V}}\) – voltage-dependent K\(^{+}\) channel; K\(_{\text{leak}}\) – unspecified K\(^{+}\) leak current; VOCC – voltage-operated Ca\(^{2+}\) channels; SR/ER – sarco/endoplasmic reticulum; IP\(_{3}\)R – IP\(_{3}\) receptor; RyR –ryanodine receptor; SERCA – SR/ER Ca\(^{2+}\)-ATPase; CSQN – calsequestrin; CM – calmodulin; R – receptor; G – G-protein; DAG – diacylglycerol; PLC – phospholipase C; sGC – soluble guanylate cyclase; cGMP – cyclic guanosine monophosphate.

IP\(_{3}\)Rs are neither necessary nor sufficient for self-sustained oscillations in our model.

To account for the natural variability between SMCs, selected cell parameters (e.g., \(R_{\text{leak}}\) or \(I_{\text{SERCA}}\)) were multiplied by the perturbation coefficient \(\delta\):
\[ \delta(1: N) = \delta_{\text{max}} \frac{\text{rand}(1 : N) - \min(\text{rand}(1 : N))}{\max(\text{rand}(1 : N)) - \min(\text{rand}(1 : N))} \]

Equation 5.1

where \( N \) is the total number of SMCs, \( \delta_{\text{max}} \) – maximum perturbation (control \( \delta_{\text{max}} = 1.2 \)), \( \text{rand}(1:N) \) – vector of pseudo-random values drawn from the unit interval. The resulting oscillations in individual SMCs have natural frequencies within a predetermined physiological range (Figure 5.5-B) (Jacobsen et al. 2007; Jacobsen et al. 2008).

Simulations were repeated with different sets of perturbations and the average behavior of the model was considered. Random variations simulating time-dependent biological noise were also implemented similar to (Koenigsberger et al. 2004). Simulations with either intercellular variability or time-dependent noise show similar desynchronizing effects. Thus, only simulations with intercellular variations are presented. Models with different number of cells (2-100 SMCs and ECs), SMC layers (1-5), and arrangement (serial or cylindrical) were implemented and displayed similar synchronization properties. To reduce computational time, the control model is composed of five SMCs connected in series and overlapping five identical ECs arranged perpendicular to the SMCs, so that each SMC connects to five ECs.

Endothelial gap junctions are more prevalent than smooth muscle and myoendothelial gap junctions. This translates into a low EC-EC resistance \( R_{ij}^{\text{EC-EC}} = 3.3 \, \text{M} \Omega \), an intermediate SMC-SMC resistance \( R_{ij}^{\text{SMC-SMC}} = 87.4 \, \text{M} \Omega \), and a high EC-SMC resistance per SMC \( R_{ij}^{\text{EC-SMC}} = 900 \, \text{M} \Omega \) (Kapela, Nagaraja, and Tsoukias 2010). The permeability of the gap junctions to \( K^+ \), \( Cl^- \), \( Na^+ \) and \( Ca^{2+} \) ions was calculated according to section 7.1.1 from the corresponding intercellular resistance, as described previously.
(Kapela, Bezerianos, and Tsoukias 2009). This yields $P_{S}^{EC-EC} = 0.384 \text{ pL/s}$, $P_{S}^{SMC-SMC} = 0.0145 \text{ pL/s}$, and $P_{S}^{EC-SMC} = 0.0014 \text{ pL/s}$, where $S = K^+, Cl^-, Na^+$, and $Ca^{2+}$. A myoendothelial IP$_3$ permeability of 0.05 s$^{-1}$ (i.e., $P_{IP3}^{EC-SMC} = 0.05 \text{ pL/s}$) per cell was assumed in agreement with (Koenigsberger et al. 2005), and the corresponding SMC-to-SMC IP$_3$ permeability was adjusted based on the gap junction resistance ratio to 0.53 pL/s. This value has not been experimentally derived and represents a rough approximation. Furthermore IP$_3$ flux is assumed proportional to the chemical and not the electrochemical gradient between two cells (section 7.1.2). The uncertainty in IP$_3$ permeability, particularly, can have a significant impact on model predictions and thus a wide range of values was examined.

To better monitor transitions in and out of synchronization, a mean correlation coefficient of $Ca^{2+}$ oscillations was calculated from all SMC-SMC combinations at each time step within 300 s time window:

$$
\overline{corr}(t) = \frac{2}{N(N-1)} \sum_{n \neq m} corr(Ca^{n}(t : t+w), Ca^{m}(t : t+w)) \quad n,m = 1...N
$$

Equation 5.2

where $t$ - simulation time, $w = 100$ s – time window, $Ca^{n}(t:t+w)$ – $Ca^{2+}$ signal from time $t$ to $t+w$ in $n$-th SMC, $corr$ – the correlation coefficient. For a single SMC-SMC pair, the correlation coefficient ranges from -1 to 1, where -1, 0, and 1 correspond to complete anti-phase, uncorrelated, and in-phase oscillations, respectively. For a large population, the mean correlation varies from 0 to 1. The cells are considered synchronized when the mean correlation is close to 1 and remains stable over a significant number of oscillation periods.
The system of ordinary differential equations describing the model (11 ODEs per EC and 26 ODEs per SMC) was solved numerically in FORTRAN 90 using the Gear’s backward differential formula method for stiff systems with maximum time step of 4 ms. Long simulated times were used (up to 80 min) to eliminate transient and resetting effects (Figure 5.5-D), and thus, considerable computation times were required for models with many cells. The computations were significantly slower in the presence of time dependent noise.

5.4 Results

5.4.1 IP₃ and Ca²⁺ Coupling

In the presence of the endothelial layer, synchronized and unsynchronized oscillations in the SMCs were observed, depending on the level of smooth muscle and endothelial stimulation. For example, 0.8 µM norepinephrine (NE) and 1 a.u. acetylcholine (Ach) gave rise to synchronized Ca²⁺ oscillations (Figure 5.2-A and Figure 5.5-A). Under the specific conditions in Figure 5.2-A, synchronization was lost by blocking IP₃ diffusion between SMCs ( \( P_{\text{IP}_3}^{\text{SMC-SMC}} = 0 \) in section 7.1.2). Similar behavior was observed in a larger ensemble of SMCs and ECs (Figure 5.7). In the presence of IP₃ diffusion removal of electrical coupling ( \( P_S^{\text{SMC-SMC}} = 0; \ S = \text{Ca}^{2+}, \text{Na}^+, \text{K}^+, \text{Cl}^- \) in section 7.1.1) had no effect (data not shown). However, when both the ionic and IP₃ couplings are present, clamping NSC channels to the mean value of their DAG-induced fluctuating activity (section 7.2.1.5) causes loss in synchronization (DAG clamp; Figure 5.2-B) (see also NSC channel section below).
Figure 5.2 (A) A population of five SMCs and five ECs is stimulated by NE (0.8 μM) and Ach (1 a.u.). Ca\(^{2+}\) oscillations in the SMCs are shown. Inhibition of intercellular IP\(_3\) diffusion desynchronized SMC Ca\(^{2+}\) oscillations. A correlation coefficient (solid line) quantifies level of synchronization with total synchronization having a value of 1. (B) Same conditions as in (A). \(V_m\) (Top) and Ca\(^{2+}\) oscillations in SMCs (Bottom) are shown. Clamping DAG concentration to its mean value leads to desynchronization and demonstrates the importance of NSC channels. The synchronization was restored by clamping BK\(_{Ca}\) activation with mean cytosolic Ca\(^{2+}\). (C) In a population of five SMCs (ECs absent), NE (0.3 μM) induced synchronized \(V_m\) (Top) and Ca\(^{2+}\) oscillations (Bottom). Increasing NE concentration (0.4 μM) desynchronized SMCs, while exogeneous cGMP hyperpolarizes and resynchronized Ca\(^{2+}\) oscillations through its action on Cl\(_{Ca}\) channels. (D) Same conditions as in (C). Cl\(_{Ca}\) channels are clamped with mean cytosolic Ca\(^{2+}\). Removal of clamping restores synchronization which is lost after blocking intercellular IP\(_3\) diffusion between SMCs.

Hence, the IP\(_3\) coupling was a necessary but not the only factor contributing to synchronization under these conditions. The IP\(_3\)-mediated synchronization occurs through the following series of events: Ca\(^{2+}\) oscillations in one cell generate IP\(_3\) oscillations in the cell -as a result of the Ca\(^{2+}\) dependence of the PLC activity (\(r_{h,G}\) in section 7.2.3) - and by diffusion through gap junctions in neighboring cells. This modulates the IP\(_3\)Rs (section 7.2.2), cytosolic Ca\(^{2+}\) levels and accelerates or delays CICR through RyR in the neighboring cell (i.e., Ca\(^{2+}\) \(\rightarrow\) PLC \(\rightarrow\) IP\(_3\) \(\rightarrow\) GJ \(\rightarrow\) IP\(_3\)R \(\rightarrow\) Ca\(^{2+}\)).

Through a parametric analysis we determined the minimum value of IP\(_3\) permeability (
$P_{IP3}^{SMC-SMC} = 0.058 \text{ pL/s}$) for intercellular IP$_3$ diffusion to be able to synchronize the cells under these conditions. In the absence of ECs, strong IP$_3$-mediated synchronization was possible if the permeability of gap junctions to IP$_3$ was increased three fold from its control value (i.e., $P_{IP3}^{SMC-SMC} = 1.59 \text{ pL/s}$) (Results not shown). Direct Ca$^{2+}$ diffusion between SMCs did not play a significant role. However, Ca$^{2+}$ coupling alone could provide good synchronization if Ca$^{2+}$ permeability was increased a hundred-fold from its control value ($P_{Ca}^{SMC-SMC} = 1.45 \text{ pL/s}$). A change in the gap junctional resistance from $84 \text{ M}\Omega$ to less than $1 \text{ M}\Omega$ between SMCs is needed to justify such value.

5.4.2 **Electrical Coupling**

Electrical coupling can have a synchronizing or desynchronizing effect. The net effect of intercellular current flux on synchronization depends on a number of parameters such as the level of agonist stimulation and can be altered by channel inhibition/activation. This model behavior is attributed to the Ca$^{2+}$ dependency of three channels, the Cl$_{Ca}$, the NSC, and the BK$_{Ca}$. Current spread through gap junctions will synchronize/desynchronize depending on the balance between the synchronizing potential of these three currents. [Note: not simply the balance of magnitude of these currents].

5.4.2.1 **NSC Channels**

Due to the Ca$^{2+}$ dependency of the PLC pathway (section 7.2.3), Ca$^{2+}$ oscillations generate concomitant oscillations in DAG concentration. In simulations with or without ECs, elimination of DAG oscillations by clamping DAG to its average concentration can yield loss of synchronization. For example, clamping DAG terminated synchronization in
a population of SMCs and ECs stimulated by 0.8 µM NE and 1 a.u. Ach (Figure 5.2-B). The termination was consistent in simulations (n = 10) with sets of SMCs of different frequency distributions. The NSC clamping also desynchronized SMCs under the conditions of Figure 5.2-C and 5.3-A, but not of Figure 5.3-C. The coordination is restored by concurrent clamping of BKCa channels to their activity at mean Ca2+ levels. The results suggest that NSC channels contribute to synchronization by counteracting BKCa channels. The NSC-mediated synchronization involves oscillatory DAG, generated by the Ca2+ dependency of PLC, which in turn modulates NSC channel activation. Every time that Ca2+ increases in one cell, NSC activates, $V_m$ depolarizes, and this activates, through electrical coupling, VOCC in neighboring cells to increase their cytosolic Ca2+ (i.e., $Ca^{2+} \rightarrow PLC \rightarrow DAG \rightarrow NSC \rightarrow GJ \rightarrow VOCC \rightarrow Ca^{2+}$).

5.4.2.2 ClCa Channels

Figure 5.2-C depicts $Ca^{2+}$ oscillations in a population of SMCs, in the absence of ECs, at two different NE concentrations and after addition of cGMP. Synchronized oscillations appear at 0.3 µM NE and unsynchronized at 0.4 µM NE. The higher NE produces stronger depolarization of SMCs mainly through the NSC channel but causes desynchronization through the activity of BKCa channels (see BKCa section below). Addition of cGMP in the absence of ECs activates depolarizing ClCa channels but also hyperpolarizing BKCa. In simulations presented in Figure 5.2-C, 1 µM cGMP repolarized SMCs by increasing BKCa conductance more than ClCa conductance. This is consistent with the relaxing effect of cGMP and experimental data showing a two-fold increase in whole cell conductance after cGMP administration to isolated SMC (probably due to activation of K+ channels) (Rahman et al. 2005). cGMP, however, synchronized $Ca^{2+}$

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oscillations through the activity of Cl\textsubscript{Ca} channels (Figure 5.2-C). Thus, the channel responsible for transition in/out of synchronization is not necessarily the same as the channel mostly affecting mean membrane potential.

The importance of Cl\textsubscript{Ca} channels was confirmed by loss of coordination after clamping Cl\textsubscript{Ca} activity to its value at mean cytosolic Ca\textsuperscript{2+} levels during the oscillations (Figure 5.2-D). cGMP-induced synchronization was lost after blocking the SMC-SMC IP\textsubscript{3} coupling under the conditions of Figure 5.2-D. If the cGMP dependence of BK\textsubscript{Ca} was removed, 1 \textmu M cGMP depolarized (rather than hyperpolarized) \( V_m \) and did not synchronize the cells (data not shown). However, the synchronization was restored if cGMP was accompanied with repolarization of SMCs by 1.3-fold increase of the maximum NaK pump rate (section 7.2.1.10), similarly to (Jacobsen et al. 2007) (data not shown). In this case, electrical coupling alone was sufficient for synchronization, indicating that the depolarizing Cl\textsubscript{Ca} and NSC channels were dominant over BK\textsubscript{Ca} channels. Thus, Cl\textsubscript{Ca} channels were necessary but not always sufficient for cGMP-dependent synchronization and the ability of cGMP to synchronize a population of SMCs may depend on \( V_m \).

The ability of exogenous cGMP to synchronize EC denuded vessels may not be always indicative for the role of Cl\textsubscript{Ca} in intact vessel synchronization. In the Supplement (section 5.7), we show representative simulations after Cl\textsubscript{Ca} clamping or blocking, when the EC layer is present. For moderate levels of Ach and NE stimulation (i.e. Ach=1 au; NE=0.8uM), clamping Cl\textsubscript{Ca} did not elicit desynchronization (Figure 5.6-A). Blocking Cl\textsubscript{Ca} under the same conditions was effective in terminating synchronization in many simulations with different sets of SMCs (Figure 5.6-B). Terminations of synchronization
with Cl\textsubscript{Ca} block when Cl\textsubscript{Ca} clamp has no effect, is attributed to changes in \(V_m\) and suggests that synchronization was not due to Cl\textsubscript{Ca} current under this particular conditions.

### 5.4.2.3 BK\textsubscript{Ca} channels

BK\textsubscript{Ca} channels generate hyperpolarizing current in response to Ca\textsuperscript{2+} elevation, and thus have a desynchronizing effect. The importance of this effect depends on model configuration and experimental conditions. In Figure 5.2-C (absence of ECs) higher concentration of NE produces stronger depolarization of SMCs by further activation of NSC channels, but also sensitizes \(V_m\)-dependent BK\textsubscript{Ca} channels to Ca\textsuperscript{2+}. The sensitivity of BK\textsubscript{Ca} conductance to Ca\textsuperscript{2+} becomes more significant than the sensitivity of NSC and Cl\textsubscript{Ca} to Ca\textsuperscript{2+}. As a result \(V_m\) repolarizes (instead of depolarizing) with Ca\textsuperscript{2+} transients and synchronization is lost (Figure 5.2-C). Application of cGMP counteracts BK\textsubscript{Ca} activity by increasing the synchronizing effect of Cl\textsubscript{Ca} (Figure 5.2-C). The significant desynchronizing role of the BK\textsubscript{Ca} predicted by the model depends on the assumed Ca\textsuperscript{2+} sensitivity of the BK\textsubscript{Ca} current (Benham et al. 1986), and in some tissues BK\textsubscript{Ca} activity may be less sensitive to Ca\textsuperscript{2+} than assumed in this study.

### 5.4.3 Role of EDHF

Figure 5.3-A investigates the effect of endothelium-derived hyperpolarizing factor (EDHF) on synchronization. The SM layer was prestimulated with NE and the ECs were stimulated with Ach at appropriate levels to induce synchronized oscillations. Blockade of the SK\textsubscript{Ca} and IK\textsubscript{Ca} channels in the ECs (section 7.3.1.2) (i.e., blockade of EDHF response) has a desynchronizing effect under these conditions. This is attributed to the resulting depolarization and an increased role of BK\textsubscript{Ca} channels as described above. Thus, simulations show that EDHF can affect cell synchronization although the effect is
indirect, through the modulation of \( V_m \) and the alteration of \( \text{BK}_{\text{Ca}} \) channel contribution. Experimental studies in RMA have demonstrated that the inhibition of \( \text{SK}_{\text{Ca}} \) and \( \text{IK}_{\text{Ca}} \) channels terminated vasomotion by desynchronizing \( \text{Ca}^{2+} \) oscillations (Mauban and Wier 2004). However, Seppey et al. suggested that the EDHF effect is only to induce transition from more contracted state to less contracted state, and depending on NE concentration this may induce or stop vasomotion (Seppey et al. 2008).

**Figure 5.3** (A) Model simulations in five SMCs and five ECs stimulated with NE (1 \( \mu \)M) and Ach (0.7 a.u.). Inhibition of endothelial \( \text{SK}_{\text{Ca}} \) and \( \text{IK}_{\text{Ca}} \) channels depolarizes \( V_m \) (Top) and desynchronizes \( \text{Ca}^{2+} \) oscillations (Bottom). (B) In a population of five SMCs prestimulated with NE (1 \( \mu \)M) (ECs absent), exogenous NO (150 nM) reduces mean SM \( \text{Ca}^{2+} \) from non-oscillatory to oscillatory domain and this yields synchronized oscillations. (C) Top: Five ECs and five SMCs are stimulated with Ach (0.6 a.u.) and NE (1.8 \( \mu \)M). \( \text{Ca}^{2+} \) oscillations in SMCs are shown. Clamping the endothelium-derived NO to its average concentration reduced \( \text{Ca}^{2+} \) oscillations and abolished synchronization. Bottom: Oscillatory components of EC \( \text{Ca}^{2+} \) (solid line) and endothelium-derived NO (dashed line) show phase shift. (D) In the presence of Ach (1 a.u.) and endothelial NO release, inhibition of cGMP production by block of SM sGC reduced amplitude and synchronization of \( \text{Ca}^{2+} \) oscillations through depolarization and \( \text{BK}_{\text{Ca}} \) channels. The effect of sGC block could be compensated by NE adjustment from 0.8 \( \mu \)M to 0.4 \( \mu \)M.

### 5.4.4 Role of NO

The net result of NO on synchronization is difficult to predict due to multiple targets of NO/cGMP pathway and their differential effects on \( \text{Ca}^{2+} \) dynamics. In simulations, addition of exogenous NO (0.15-0.4 \( \mu \)M) in the presence of NE (0.8-1.2 \( \mu \)M) repolarized
SMCs to their oscillatory domain and gave rise to synchronized oscillations (Figure 5.3-B and Figure 5.5-A). This is in agreement with experimental data showing vasomotion induced by NO in endothelium denuded RMA (Seppey et al. 2008). Some combinations of NO and NE induced uncorrelated oscillations, predicting a concentration-dependent NO effect.

Endogenous, endothelium-derived NO (section 7.1.3) (i.e., endothelium-derived relaxing factor (EDRF)) may have different effect on vasomotion than similar levels of exogenous NO. EDRF can oscillate with SM Ca$^{2+}$ as a result of myoendothelial communication, and can affect synchronization dynamics in parallel to EDHF. Indeed, at certain combinations of NE and Ach (for example 1.8 µM NE and 0.6 a.u. Ach), clamping NO to its time-averaged concentration terminated synchronization (Figure 5.3-C). Diffusion of IP$_3$ from SMCs to ECs through myoendothelial gap junctions generated EC Ca$^{2+}$ and NO oscillations (Figure 5.3-C). Corresponding Ca$^{2+}$ and NO oscillations in SMC may have a significant phase-shift due to delays in IP$_3$ diffusion, NO production, and NO diffusion. The out-of-phase NO acting on BK$_{Ca}$ can then inhibit the desynchronizing effect of BK$_{Ca}$.

At some concentrations of NE and Ach, synchronized Ca$^{2+}$ oscillations were possible with complete EDRF inhibition (data not shown). Thus, the model predicts possibility of vasomotion with EDRF present or blocked. Similarly in RMA, inhibition of NO by L-NAME did not prevent Ca$^{2+}$ synchronization (Sell, Boldt, and Markwardt 2002), although it reduced significantly the amplitude of vasomotion (Mauban and Wier 2004; Rahman et al. 2005). Total inhibition of EDRF (i.e., inhibition of NO production) is different than blockade of sGC/cGMP pathway since NO can also activate BK$_{Ca}$ directly.
Inhibition of sGC/cGMP pathway in the model \( (V_{cGMP,\max} = 0 \) in section 7.2.4) reduced the amplitude and synchronization of \( \text{Ca}^{2+} \) oscillations, although adjustment of NE concentration could compensate for the changes and restored the coordination (Figure 5.3-D). In RMA, ODQ (sGC inhibitor) did not eliminate vasomotion but had modulatory role (Mauban and Wier 2004).

### 5.5 Discussion

The study shows how synchronization in a population of SMCs can be achieved/lost as a result of the competition between synchronizing and desynchronizing factors. Previously recognized pathways are evaluated in a more complex cellular model system and under different stimulatory conditions. The study also suggests two alternative pathways for synchronization. Intercellular IP\(_3\) diffusion and current flux generated through NSC channels have the potential to affect synchronization. Thus, there can be several synchronizing mechanisms that work individually, in synergy or redundancy, depending on stimulatory conditions. The model simulations depict a system of increased complexity, where the relative contribution of different pathways is very sensitive to experimental conditions.

The synchronizing pathways share certain common features and can be grouped into diffusion-mediated and electrically-mediated (Figure 5.4). Synchronization mediated by small species requires: a) \( \text{Ca}^{2+}\)-induced oscillations in the concentration of the mediator, if the mediator is not \( \text{Ca}^{2+} \), b) sufficient intercellular flux and c) modulation by the mediator of a component participating in the oscillatory cycle in the neighboring cell. Diffusion of the mediator over long distances or the ability to generate propagating waves is not required.
Figure 5.4 Schematic diagram of signaling pathways that may be involved in Ca\(^{2+}\) synchronization, as predicted by the model. Ca\(^{2+}\) increase in one SMC (left) can activate directly Cl\(_{Ca}\) and BK\(_{Ca}\) channels, and indirectly NSC channels through Ca\(^{2+}\)-sensitive PLC. Variations in channels’ activity generate \(V_m\) oscillations, which spread through gap junctions to neighboring SMCs (right) and modulate VOCC channels and RyRs. Oscillatory IP\(_3\), generated by Ca\(^{2+}\)-dependent PLC, can diffuse to other cells, and may also have a synchronizing effect by acting on IP\(_3\) receptors. Ca\(^{2+}\) elevation in one cell may increase Ca\(^{2+}\) in neighboring cells by direct diffusion of Ca\(^{2+}\) through the gap junctions if SMC-to-SMC gap junctions have sufficient permeability.

Through synchronization of immediate neighbors, a large population can be synchronized. For example, oscillatory IP\(_3\) can be induced by various isoforms of Ca\(^{2+}\)-dependent PLC, or even by \(V_m\) modulation of IP\(_3\) release (Imtiaz, von der Weid, and van Helden 2009). IP\(_3\) can diffuse through gap junctions and modulate RyR or IP\(_3\)Rs. In current-mediated synchronization, oscillatory \(V_m\) needs to be generated through membrane channels that depend directly or indirectly on Ca\(^{2+}\). Current can spread
relatively fast and over long distances and can modulate a population of cells through its action on VOCCs. A prerequisite for achieving synchronization is that the net effect of depolarizing and hyperpolarizing currents yields $V_m$ oscillations in phase with the $\text{Ca}^{2+}$ oscillations. Oscillatory $V_m$ can be generated by $\text{Ca}^{2+}$ dependent channels such as the NSC, the $\text{BK}_{\text{Ca}}$ and the $\text{Cl}_{\text{Ca}}$.

The model suggests that coordinating signals are likely to be weak and superimposed on larger constant components. Synchronization is determined not by the relative magnitude of competing pathways but by their sensitivity to $\text{Ca}^{2+}$ fluctuations. Thus, the component (i.e., an ion channel) that is affected the most by a system perturbation (i.e., agonist stimulation or channel block) may not necessarily be the component that allows synchronization. For example, VOCC block can prevent vasomotion by changing ionic balances and the ability of a cell for self-sustained oscillations and not necessarily by a direct effect on the synchronizing pathway. PLC inhibition is similarly inadequate to experimentally probe the role of IP$_3$ in synchronization, because it prevents NE-induced signaling (Lamboley et al. 2005). A molecular IP$_3$ buffer that does not change the steady state component of free IP$_3$ would be more appropriate (Politi et al. 2006). [IP$_3$ buffering that attenuates IP$_3$ oscillations can approximate selective block of intercellular IP$_3$ diffusion]. An appropriate experimental intervention to probe “current-mediated synchronization” would be to clamp $V_m$ to mean oscillatory value along the entire vessel segment. Clamping of $V_m$ would effectively block electrical coupling. However, such interventions are difficult to perform experimentally. For these reasons experimental determination of the pathways contributing to synchronization can be very challenging.

An ideal intervention should target a specific component or species and prevent its
oscillation without modifying mean activity and self-sustained oscillations. Moreover, combinations of different targets should be used to elucidate potential synergies or redundancies under various stimuli. Such scenarios can be easily examined in silico. Through our simulations we evaluated the synchronizing potential of three major mechanisms.

5.5.1 Diffusion mediated synchronization

5.5.1.1 Synchronization mediated by Ca\(^{2+}\) diffusion

Ca\(^{2+}\) diffusion through nonselective SMC-SMC gap junctions is the most basic and direct pathway to provide coordination of the intracellular oscillators in neighboring cells. In a series of models for vasomotion in RMA, intercellular Ca\(^{2+}\) flux was the only significant mechanism for cell synchronization (Koenigsberger et al. 2004; Koenigsberger et al. 2005, 2006; Koenigsberger, Sauser, and Meister 2005). Other studies, however, have argued against Ca\(^{2+}\) diffusion as a synchronizing signal in RMA. Sell et al. estimated the Ca\(^{2+}\) propagation velocity in single SMC to be of 25 µm/s and concluded that it is too slow for intercellular Ca\(^{2+}\) synchronization (Sell, Boldt, and Markwardt 2002). Jacobsen and coworkers have also argued that the diffusion of Ca\(^{2+}\) (as well as of other second messengers) is too slow and that the intercellular Ca\(^{2+}\) flux is too small (Jacobsen et al. 2007). In their model, Ca\(^{2+}\) fluxes had no effect on wave pattern at the control gap junction permeability \(P = 5 \times 10^{-8}\) m/s as well as for values up to 500\(\times 10^{-8}\) m/s, beyond which cells behaved effectively like single cell.

Unfortunately, the intercellular permeability for Ca\(^{2+}\) has not been experimentally derived and previous theoretical studies have used values that differ by two to three orders of magnitude. In our models, we described Ca\(^{2+}\) fluxes based on electrochemical
gradient and Ca\(^{2+}\) permeability is estimated from the intercellular gap junctional resistance \(R_{gj}\). \(R_{gj}\) is a parameter that has been previously estimated from electrical and observational data. Ca\(^{2+}\) permeabilities values utilized in this study are in agreement with the permeabilities used by Jacobsen and coworkers (Jacobsen et al. 2007). Thus, it is not surprising that a similar conclusion was reached regarding the role of Ca\(^{2+}\) diffusion in cell synchronization. On the other hand, \(P_{Ca}\) value is uncharacteristically high in the studies that suggest an important role for Ca\(^{2+}\) diffusion (Koenigsberger et al. 2004). It can be argued that in lumped cell models, lacking spatial resolution intracellularly, an “effective” Ca\(^{2+}\) permeability larger than actual gap junction permeability may account for regenerative mechanisms in Ca\(^{2+}\) spread. However, the SMC model of Jacobsen et al. accounts for intracellular spatial resolution and CICR waves, and still intercellular Ca\(^{2+}\) diffusion was not important unless nonphysiological coupling was assumed (i.e., \(R_{gj}\) below 1 M\(\Omega\)). Better experimental data is required for a more conclusive determination.

5.5.1.2 Synchronization mediated by IP\(_3\) diffusion

Model simulations show that intercellular IP\(_3\) diffusion can contribute to intercellular synchronization. If the IP\(_3\) coupling is sufficient to coordinate immediate neighbors, it can also synchronize a larger population (Figure 5.7). In the model, a contributing role for IP\(_3\) could be seen for \(P_{IP3}^{SMC\rightarrow SMC}\) values as low as 0.058 pL/s. This value is below our control permeability value (0.53 pL/s). For \(R_{gj}^{SMC\rightarrow SMC} = 87.4\, M\Omega\), we estimate an ionic permeability of \(P_{S}^{SMC\rightarrow SMC} = 0.015\, pL/s\) (section 7.1.1) and comparable permeabilities for ions and IP\(_3\) are possible. Thus, \(P_{IP3}^{SMC\rightarrow SMC}\) in the range of 0.01-0.5 pL/s is expected. We conclude that the contribution of IP\(_3\) in cell synchronization is conditional to a sufficient
IP₃ permeability and adequate cell coupling in the SMC layer. IP₃ oscillations associated with Ca²⁺ oscillations is a necessary condition for IP₃-mediated synchronization. Ca²⁺ oscillations may be accompanied by IP₃ oscillations in various cell types, but further evidence is required to confirm such dependence in the vasculature (Politi et al. 2006; Bartlett et al. 2005). In RMA, IP₃ oscillations can be generated by α₁-adrenoreceptors and Ca²⁺-dependent PLC activity (Clarke et al. 2008). [Agonist-independent isoforms of PLC that was not explicitly described in the model can also contribute to IP₃ oscillations in RMA SMCs (Lamboley et al. 2005; LaBelle and Polyak 1996)]. In the model of Koenigsberger et al. (Koenigsberger et al. 2004), the amplitude of IP₃ oscillations was predicted to be too small to coordinate Ca²⁺ oscillations. Although there are no direct measurements, indirect experimental evidence suggests that IP₃ may oscillate significantly in RMA SMCs during vasomotion. EC Ca²⁺ has been observed to oscillate in phase with SM Ca²⁺, and the more likely explanation for this behavior is the diffusion of oscillatory IP₃ from SM to the endothelium (Schuster et al. 2001; Lamboley et al. 2005). [Movement of IP₃, rather than Ca²⁺ between cells seems to play a more significant role in Ca²⁺ wave propagation and IP₃ diffuses faster than Ca²⁺]. Consistently with these data, our model predicts that IP₃ has the potential to play an important role in synchronization. In some SMCs, IP₃ synthesis may be Vₘ-dependent (Itoh et al. 1992; Imtiaz, Smith, and van Helden 2002). This provides an alternative mechanism for IP₃ oscillations and thus, could allow for a contribution of IP₃ in synchronization independently of the Ca²⁺-dependent PLC pathway kinetics.
5.5.2 Electrically mediated synchronization

5.5.2.1 Synchronization mediated by electrical coupling

Ionic coupling through gap junctions transmits $V_m$ changes from one cell to another. This, however, can have a synchronizing effect only if $Ca^{2+}$ elevation generates adequate and in-phase $V_m$ depolarization. The transmitted depolarization will then activate VOCCs and increase $Ca^{2+}$ in neighboring cells. $Cl_{Ca}$ channels have been proposed to mediate the necessary $Ca^{2+}$ and $V_m$ coupling in vasomotion and electric current was suggested as the only signal fast enough (i.e., > 1 cm/s) to synchronize several millimeters long vessels (Nilsson and Aalkjaer 2003; Matchkov, Aalkjaer, and Nilsson 2004). Nonetheless, analysis of experimental data and model results indicate that additional mechanisms may be contributing. In vasomotion, SMC $V_m$ oscillations are typically in phase with $Ca^{2+}$ and proceed oscillations in SMC tension (Aalkjaer and Nilsson 2005). However, $V_m$ fluctuations are not always synchronous to diameter changes (Schuster et al. 2004), and a theoretical analysis even predicts $V_m$ oscillations in antiphase with $Ca^{2+}$ oscillations (Koenigsberger et al. 2004). Furthermore, in RMA $Ca^{2+}$ oscillations are normally generated by the SR and $V_m$ oscillations are secondary to the $Ca^{2+}$ oscillations. Thus, $V_m$ may have an arbitrary phase shift relative to $Ca^{2+}$, depending on the type and number of $Ca^{2+}$ modulated channels. This suggests that electrical coupling may not always have a synchronizing effect.

DAG-activated TRPC-like nonselective cation channels are expressed in RMA SMCs and mediate depolarizing $Na^+$ influx during $a_1$-adrenergic stimulation (Hill et al. 2006). Our model predicts that NSC channels are good candidates for generating synchronizing $V_m$ changes, assuming that the $Ca^{2+}$ dependence of PLC leads to oscillatory DAG. Indeed,
synchronous oscillations of DAG, IP$_3$ and Ca$^{2+}$ induced by NE were measured in Chinese hamster ovary cell culture (Bartlett et al. 2005). Unlike some vessels with spontaneous vasomotion, RMAs require depolarization that opens VOCCs and maintains mean Ca$^{2+}$ levels within the oscillatory window (Schuster et al. 2004). In the model, NE opens NSC channels, reduces significantly membrane resistance and depolarizes SMCs in agreement with experiments (Sell, Boldt, and Markwardt 2002). Thus, the agonist-activated NSC channels have a major effect on the electrical responses that has not been examined in previous models (Koenigsberger et al. 2004; Jacobsen et al. 2007). Mechanosensitive NSC channels modulated by wall stress were predicted to have only minor synchronizing effect (Koenigsberger et al. 2006). The effectiveness of NSC and Cl$_{Ca}$ channels to synchronize depends on the amplitude of induced $V_m$ oscillations, (i.e., the Ca$^{2+}$-dependent variation of conductance has to be significant compared to total membrane resistance). Under the conditions of Figure 5.2-B, where EC and SMC layers are moderately stimulated by Ach and NE, the fluctuations in NSC and Cl$^-$ conductances are similar, but the predicted oscillatory NSC and Cl$^-$ currents differ significantly (i.e. 15 pA and 2.5 pA, respectively). This is attributed to a larger electrochemical gradient for Na$^+$ ions. Consequently, clamping NSC channels resulted in large amplitude antiphase $V_m$ oscillations and desynchronization (Figure 5.2-B), while clamping Cl$^-$ currents had no significant effect under these conditions.

Experimental evidence supports a role for Cl$^-$ current in vasomotion. Exogenous cGMP administration in endothelium-denuded vessels restored vasomotion, suggesting a role for cGMP dependent Cl$^-$ channels (Peng et al. 2001). In intact vessels, Cl$_{Ca}$ blockade has not consistently abolished vasomotion (i.e. in intact small RMA, vasomotion was
inhibited by Cl$^-$ substitution, but not by DIDS and Zn$^{2+}$ (blockers of cGMP-dependent Cl$_{Ca}$ channels) (Boedtkjer et al. 2008)). In a recent study however, transfecting rat mesenteric small arteries *in vivo* with siRNA specifically targeting bestrophin-3, inhibits cGMP dependent Cl$_{Ca}$ current and abolishes vasomotion in isolated arteries (Broegger et al. 2011). Thus, although Cl$^-$ current is the only synchronizing mechanism that has been experimentally tested, the conditions under which Cl$^-$ channels induce vasomotion, particularly in the presence of endothelium, is a topic of continuing investigation. Our simulations suggest that exogenous cGMP administration can enhance the potential for synchronization by activation of Cl$_{Ca}$ and this might be the predominant synchronizing mechanism under some conditions (Figures 5.2-C and D). When endothelium derived factors, however, lead to endogenous cGMP production, the cGMP increase is not necessarily as effective as in EC denuded vessels (Figure 5.6-A). According to the model, this is attributed to other EC-induced pathways that interfere with Cl$_{Ca}$ mediated synchronization (i.e. the parallel activation of BK$_{Ca}$ by NO) and to a different SM $V_m$ that alters sensitivity of currents to Ca$^{2+}$ changes. Simulations also show that Cl$_{Ca}$ blockade can inhibit synchronization (Figure 5.6-B) when Cl$_{Ca}$ clamping has no effect (Figure 5.6-A). Thus, under some conditions, blocking Cl$_{Ca}$ may be able to inhibit vasomotion even when Cl$^-$ current is not the synchronizing signal. Model simulations do not prove a limited role for Cl$_{Ca}$ in synchronization in intact vessels. They show however, that under some conditions Cl$_{Ca}$ may not be the predominant synchronizing pathway even when exogenous cGMP administration or Cl$_{Ca}$ blockade can affect vasomotion.
In the model, a synchronizing mechanism has to overcome cellular heterogeneity and the effect of $\text{BK}_{\text{Ca}}$ current which tends to counteract NSC and $\text{Cl}_{\text{Ca}}$ channels. This prediction is consistent with the findings that blockade of $\text{BK}_{\text{Ca}}$ with IbTx or $\text{Kv}$ with 4-AP did not eliminate vasomotion, and blockade of $\text{K}^+$ channels with TEA in some vessel types promotes vasomotion (Mauban and Wier 2004; Aalkjaer and Nilsson 2005). TEA was suggested to enhance vasomotion by increasing membrane resistance and/or gap junction formation. In a model with identical self-oscillatory SMCs in which $\text{BK}_{\text{Ca}}$ currents are dominant, electrical coupling tends to produce clusters of synchronous oscillations. The clusters may consist of adjacent as well as distant cells, and there can be many configurations depending on initial perturbation of the system. Figure 5.8 shows cluster formation in a population of eighty SMCs arranged into a cylinder. Such clustering can arise whenever $\text{Ca}^{2+}$ increase in one cell tends to reduce $\text{Ca}^{2+}$ in other cells. In this particular case, $\text{Ca}^{2+}$ elevation activates $\text{BK}_{\text{Ca}}$ channels and repolarizes $V_m$, which in turn closes VOCC in neighboring cells due to electrical coupling. For non-identical cells or in the presence of noise, the formation of clusters decreases as the heterogeneity increases, until no patterns are formed (Koenigsberger, Sauser, and Meister 2005). The desynchronizing effect of $\text{BK}_{\text{Ca}}$ in the model is significant and $\text{BK}_{\text{Ca}}$ clamp increased synchronization under most stimulatory conditions examined.

5.5.3 Role of endothelium

The effect of endothelium on vasomotion remains controversial and both synchronizing and desynchronizing effects of endothelium have been reported even in the same vessel types, including in RMA (Mauban and Wier 2004; Peng et al. 2001; Rahman et al. 2005; Sell, Boldt, and Markwardt 2002; Lamboley et al. 2003; Aalkjaer and Nilsson
Some of these discrepancies can be explained by the modulatory role of ECs on mean SM Ca\textsuperscript{2+} and transitions between oscillatory and nonoscillatory domains, similar to the effect of pressure (Mauban and Wier 2004; Koenigsberger et al. 2005, 2006). A synchronizing effect of the endothelium through some unidentified mechanism was also suggested by Rahman et al. (Rahman et al. 2005) based on the finding that cGMP produced only partial synchronization in endothelium denuded RMA, while intact endothelium allowed full synchronization even with L-NAME and ODQ.

According to our model, the inconsistent role of endothelial layer may also result from multiple effects of endothelium-derived factors on the synchronization mechanisms and their sensitivity to experimental conditions. Endothelium has relatively low electrical resistance and thus can provide global coupling (mean field) for SMCs through myoendothelial gap junctions. However, this can enhance or attenuate synchronization depending on the phase between SMC $V_m$ and Ca\textsuperscript{2+} as shown and discussed above. Inhibition of EDHF (IK\textsubscript{Ca} and SK\textsubscript{Ca} channels) can desynchronize Ca\textsuperscript{2+} oscillations by depolarizing SM $V_m$ and activating BK\textsubscript{Ca} channels (Figure 5.3-A). NO can potentially induce vasomotion by bringing Ca\textsuperscript{2+} levels into the oscillatory window (Figure 5.3-B). However, the synchronizing effect of NO is complex and difficult to predict. NO can activate directly or through sGC/cGMP depolarizing and hyperpolarizing currents that can affect synchronization (Figure 5.3-D). NO can potentially have steady-state and oscillatory components. The model predicts that an oscillatory component of NO can influence synchronization by its effect on BK\textsubscript{Ca} (Figure 5.3-C). The effect depends on the phase shift between SM Ca\textsuperscript{2+} and NO.
5.6 Model limitations

The model contains a number of simplifying assumptions and estimates for several parameter values which limit model’s predictive ability. Some of these assumptions can significantly affect model predictions. The primary purpose of the presented simulations is to demonstrate the feasibility of a synchronizing pathway and less emphasis should be placed on the parameter values and conditions where vasomotion occurs (i.e. agonist concentrations etc). Specific limitations of the model are discussed in the Supplement section (Section 5.8).

5.7 Conclusions

The model predicts several physiologically feasible pathways for synchronization of SMCs in vasomotion. These include current generated through $Cl_{Ca}$ and NSC channels, or pathways that involve the intercellular diffusion of IP$_3$ and perhaps of Ca$^{2+}$ (Figure 5.4). Their relative importance is sensitive to many parameters and stimulatory conditions. Competing pathways exist and synchronization is determined by the relative strength of each pathway (i.e., the pathway’s sensitivity to Ca$^{2+}$ fluctuations). This may explain controversies in experimental and theoretical results about vasomotion. Despite the increased complexity of the model and the utilization of a large amount of electrophysiological data, many aspects of Ca$^{2+}$ signaling remain unclear and have not been incorporated. The further development of models and experiments, in parallel, will enable us to elucidate the mechanisms underlying the phenomenon of vasomotion.
5.8 Supplement

5.8.1 Oscillations in SMCs

Figure 5.5-A shows the oscillatory domain for an isolated SMC (solid line). The model generates self-sustained Ca\textsuperscript{2+} oscillations whenever the cytosolic SMC Ca\textsuperscript{2+} level is in the range of 160-290 nM. The corresponding norepinephrine (NE) concentrations to achieve such levels depend on the presence and stimulation state of the EC as the endothelium modulates SMC Ca\textsuperscript{2+} levels. Thus, a stimulated endothelium yields a significantly larger NE concentration window for oscillations (dotted line). In a multicellular vessel, the synchronization domain will be a subset of the oscillatory domain because unsynchronized oscillations may occur.

Figure 5.5 (A) Bifurcation diagrams of Ca\textsuperscript{2+} versus NE for single SMC model without EC (solid line) and with EC stimulated by Ach (dashed-dotted line). (B) Representative distribution of oscillation periods in a model with 10 oscillatory SMCs before coupling (o), after coupling without synchronization (×) and with synchronization (*). (C) Forced Ca\textsuperscript{2+} oscillations in non-oscillatory SMC induced by IP\textsubscript{3} and electrical coupling with an oscillatory SMC. (D) Unsynchronized Ca\textsuperscript{2+} oscillations induced by 0.3 μM NE were synchronized transiently by a short 10 ms pulse of high extracellular K\textsuperscript{+} (35 mM) resetting simultaneously all SMC oscillators.
Figure 5.5-B shows representative distribution of oscillation periods in a multicellular model comprised of 10 oscillatory SMCs before and after intercellular coupling. A distribution in the natural frequencies of Ca\(^{2+}\) oscillations accounts for biological variability and was achieved as described in the methods section. The ability of cells to synchronize decreased with increasing dispersion in their natural frequencies. In our simulations, the ratio of minimum to maximum period was maintained below 0.9 in all simulations. Frequency differences of this magnitude have been observed experimentally in two oscillatory regions during beating vasomotion (26). When cells are coupled, oscillatory periods are modified \((\text{blue line})\) and under some conditions synchronization may occur and cells assume a common, often reduced, period of Ca\(^{2+}\) oscillations \((\text{red line})\).

Figure 5.5-C examines the strength of coupling \((\text{i.e., the amplitude of Ca}^{2+}\text{ oscillations in one cell due to the Ca}^{2+}\text{ oscillations in another cell})\). A non-oscillatory SMC was created \((\text{i.e. by appropriate selection of store parameters})\) and was coupled to an oscillatory SMC. The non-oscillatory SMC exhibited only small forced Ca\(^{2+}\) oscillations \((\text{dotted line})\), consistently with the observation that in the artery wall Ca\(^{2+}\) transients in one SMC do not typically produce Ca\(^{2+}\) transients in a neighboring cell (26). In the oscillatory cell, Ca\(^{2+}\) generates IP\(_3\) and DAG oscillations through Ca\(^{2+}\) feedback on the PLC, and \(V_m\) oscillations through DAG-dependent NSC channels and Ca\(^{2+}\)-dependent \(\text{BK}_{\text{Ca}}\) and \(\text{Cl}_{\text{Ca}}\) channels. The intercellular IP\(_3\) coupling gives rise to small IP\(_3\) oscillations in the non-oscillatory cell, opening of IP\(_3\)Rs and periodic Ca\(^{2+}\) release. Electrical coupling imposes \(V_m\) oscillations in the secondary cell and modules activity of VOCC. The net
effect of these mechanisms is not significant enough to induce forced oscillations in the second cell.

A rapid addition of an agonist, like NE, K\(^+\) or NO, can cause simultaneous depolarization/repolarization of the whole population of SMCs, and thus may initiate a transient Ca\(^{2+}\) synchronization. The effect of a short pulse of extracellular K\(^+\) (35 mM KCl) applied uniformly to several SMCs is shown in Figure 5.5-D. Unsynchronized oscillations were transiently synchronized by the short pulse, consistently with experiments (5). The synchronization was caused by phase resetting of the Ca\(^{2+}\) oscillators. To ensure that Ca\(^{2+}\) coordination is mediated by intercellular communication (and is not merely a result of resetting), sufficiently long times must be considered in simulations and experiments.

5.8.2 Role of ClCa when ECs are present

In the presence of endothelium and moderate levels of Ach and NE stimulations (i.e., Ach = 1 a.u.; NE = 0.8 \(\mu\)M), clamping Cl\(_{Ca}\) channels did not elicit desynchronization (Figure 5.6-A). To better compare model predictions with experimental data, we also performed simulations with total block of Cl\(_{Ca}\) currents. Under the same stimulatory conditions, total inhibition of Cl\(_{Ca}\) channels desynchronized a population of SMCs (Figure 5.6-B). The result was not consistent in all of the examined SMC sets with random oscillatory frequencies.
A population of five SMCs and five ECs is stimulated by NE (0.8 μM) and Ach (1 a.u.). Ca$^{2+}$ oscillations in the SMCs are shown. Clamping Cl$_{Ca}$ channels did not elicit desynchronization (A), but total block of Cl$_{Ca}$ current can lead to loss of synchronization (B).

5.8.3 Simulations with large ensembles

Representative simulations with large number of cells arranged into a cylinder (vessel wall) are shown in Figures 5.7 and 5.8. Each SMC within the cylinder is coupled to its four neighbors through $R_{gl} = 87.4$ MΩ. In Figure 5.7, SMCs are coupled to ECs arranged perpendicularly to SMCs through the total myoendothelial $R_{gl} = 900$ MΩ per SMC.

5.8.4 Model Limitations

In this study regular Ca$^{2+}$ oscillations are generated by RyRs and slow refilling of SR as in (Koenigsberger et al. 2004). However, the exact mechanism and even the deterministic nature of the oscillations has not been definitively resolved, and oscillators based on IP$_3$Rs have also been used in other models (Jacobsen et al. 2007).
Some studies suggested that the natural frequencies of SMCs within a vascular segment are very similar and only noise contributes significant variability in the system, whereas others reported that the frequencies of individual SMCs may differ significantly (Koenigsberger et al. 2004; Jacobsen et al. 2007; Jacobsen et al. 2008). Furthermore, the period of vasomotion in RMA varies from few seconds (Peng et al. 2001; Matchkov et al. 2004; Boedtkjer et al. 2008) to almost half a minute (Mauban and Wier 2004; Sell, Boldt, and Markwardt 2002; Seppey et al. 2008, 2010). In this study, oscillatory frequency was predicted based on previously determined kinetic constants and parameter values, and it falls within the range of previously reported values. Significant differences in frequency can potentially affect the synchronization properties of the system, thus, future studies need to examine the effect of oscillation frequency on synchronization.

Our lumped model does not account for heterogeneous intracellular distribution of RyRs and IP$_3$Rs and their compartmentalization with membrane channels. All cellular components experience the same cytosolic Ca$^{2+}$ concentration, disregarding possible differential activation of PLC, Cl$_{Ca}$ and BK$_{Ca}$. Although vasomotion is usually associated with global (whole cell) Ca$^{2+}$ oscillations, intracellular Ca$^{2+}$ waves may play a role in
initiation and maintenance of vasomotion (Jacobsen et al. 2007). A limitation of lumped models is that they do not account for intracellular diffusion of species (Ca$_{2+}$ and IP$_3$) and may not accurately reproduce inter-cellular wave (Seppey et al. 2010). Continuum, spatially-distributed cellular models are needed to capture some of these phenomena.

Mechanical feedback on SM and endothelium may also play a role, but predictions require integration of this model with biomechanical models. NE can initiate vasomotion in both endothelium-denuded and intact RMA. In the model synchronized oscillations in the presence of EC required background Ach stimulation. The prestimulation with Ach compensated for changes in resting $V_m$ caused by EC coupling, and may account for background IP$_3$ levels and resting EC activity under experimental conditions.

5.9 References


CHAPTER 6-SUMMARY OF THE DISSERTATION
Ca\textsuperscript{2+} mobilization in vascular cells (SMCs and ECs) range from locally confined subcellular events to global whole-cell Ca\textsuperscript{2+} increases. Moreover, Ca\textsuperscript{2+} increases in individual cells may spread across the adjacent vascular cells and are regulated by a network of intracellular signaling pathways and complex inter-cellular interactions. Intracellular Ca\textsuperscript{2+} concentrations in the SMCs determine the vessel tone through modulation of the contractile apparatus (global Ca\textsuperscript{2+} rise in the SMCs produces vessel constriction). Under certain conditions, transient oscillatory Ca\textsuperscript{2+} changes in the SMCs can synchronize along the vessel segment to result in spontaneous oscillations in vessel diameter (vasomotion). On the other hand, ECs influence vessel tone regulation through production of Ca\textsuperscript{2+} dependent vasoactive substances such as NO and endothelial derived hyperpolarizing factors (EDHF). Myoendothelial projections (i.e. extension of EC which reach towards the SMC side and allow communication between the EC and SMC) are reported to retain enriched expression of a variety of channels, proteins, receptors and molecules. In the presence of the enriched expressions of cellular components, localized sub-cellular Ca\textsuperscript{2+} signaling in these spatially restricted sites is suggested to be critical in the regulation of vessel tone.

6.1 **Theoretical Models**

In an attempt to unravel, quantify and reconcile certain aspects of spatiotemporal Ca\textsuperscript{2+} signaling and its regulation of vessel tone, we developed detailed mathematical models of individual cells (EC and SMC), coupled cells (EC-SMC) and a multi-cellular vessel segment (layer of ECs coupled to one or more layer of SMCs). Lumped/compartmental models (i.e. models which assume uniform and well mixed distribution of cellular components in individual cells), which lack spatial resolution, were utilized for the
examination of synchronization of global whole cell Ca\(^{2+}\) and \(V_m\) oscillations. Continuum models using finite element techniques (which are computationally expensive) were developed to incorporate spatial heterogeneity of cellular components and examine localized sub-cellular Ca\(^{2+}\) events. The total whole cell currents through individual channels and receptors were modeled using deterministic descriptions, whereas opening of a single/cluster of these channels was simulated using stochastic MARKOV chains. Theoretical investigations using the developed multi-scale models can assist in the experimental analysis, and provide quantification and elucidation of the complex regulatory mechanism underlying the spatiotemporal Ca\(^{2+}\) patterns observed in the vascular cells. This will lead to a better understanding of the mechanisms that regulate vascular resistance, blood flow and pressure in health and hypertension. Identifying critical signaling pathways that regulate vessel tone will provide targeted therapeutic strategies for diseases such as hypertension.

6.2 Outcomes

6.2.1 EC TRPV4 mediated subcellular Ca\(^{2+}\) increase

Endothelium derived hyperpolarization (EDH) is a major mechanism for the regulation of vascular diameter in small-resistance vessels. Localized Ca\(^{2+}\) increase via activation of as few as three TRPV4 channels per EC have been suggested to induce maximal vessel dilations (through the generation of EDH). Random/stochastic openings of a TRPV4 channel cluster, implemented using stochastic Markov chains, resulted in a localized quantal Ca\(^{2+}\) increase (in a region 7-46 µm\(^2\) in the vicinity of the cluster location site), consistent with experimental data. The model quantifies a 4-16 µM localized Ca\(^{2+}\) increase at the site of the channel cluster that spreads 2-7 µm (radial
distances) along the EC. The model predicts that a localized Ca$^{2+}$ increase via a cluster of TRPV4 channels can result in 4-7 mV EC hyperpolarization in the model when 25-30 IK$_{Ca}$ channels are placed in close proximity of the TRPV4 channel cluster. The endothelial hyperpolarization can spread to adjacent SMCs to result in vessel dilation. Moreover, the model predicted a 2-4 fold increase in the Ca$^{2+}$ spread arising from bursting activity and cooperative gating kinetics of TRPV4s. However, the model suggests that the amplified Ca$^{2+}$ response from the burst and cooperative gating kinetics might be counterproductive and can lead to diminished hyperpolarization (if most of the KCa channels are present adjacent to the TRPV4 cluster). The model predicts that amplification of EDH signaling by cooperativity in the TRPV4 cluster is more likely to occur if the KCa channels are not located immediately adjacent to the TRPV4 channels, but at distances that can reach 10 microns away. Further experimentation needs to be carried out to examine the exact distribution of these channels in the vascular cells.

6.2.2 NO feedback response

NO is a potent vasodilator. Cytosolic Ca$^{2+}$ levels in ECs regulate NO production. NO is significantly scavenged by Hemoglobin (Hb) in the red blood cells (RBCs). SMC stimulation has been suggested to cause an IP$_3$-mediated Ca$^{2+}$ transient in the MPs with limited global spread in the bulk EC. Continuum model of coupled EC and SMC (EC-MP-SMC), incorporated with NO-cGMP pathway, suggested the necessity of spatially localized eNOS in the MP for significant NO production during SMC stimulation. This was a consequence of limited bulk EC Ca$^{2+}$ increases during SMC stimulations. In vitro experimental studies (in the absence of blood) have shown presence of Hbo (a scavenger of NO) in the EC and suggested its role as switch regulating NO signaling. In the absence
of RBCs, 0.05-0.1 µM Hbα localized in the MP resulted in diminished NO feedback response, consistent with the experimental reports. However, the simulations suggested that RBC perfusion will decrease the ability of EC Hbα to modulate NO levels, and high µM (around 0.5-1 µM) levels of EC Hbα (10-100 fold higher than observed eNOS concentration) were necessary for a significant modulation of SMC NO availability. Further experimentation quantifying the EC Hbα concentration will aid in better understanding the role of spatially localized EC Hbα in regulation of the NO mediated vasodilatory feedback response.

6.2.3 Oscillatory intracellular Ca\textsuperscript{2+} waves in EC and SMC

In addition to the subcellular localized Ca\textsuperscript{2+} events, agonist stimulated repetitive intracellular Ca\textsuperscript{2+} wave like activity in the EC and SMC is consistently observed in the experiments and are suggested to play a role in regulation of vascular tone. Moreover, a wide range in velocities (1-100 µm/s) for the intracellular Ca\textsuperscript{2+} waves have been reported. Continuum 1-D and 2-D models of the EC and SMC were developed and oscillatory wave like activity was reproduced, if a gradient in store (i.e. inositol triphosphate, ryanodine) or membrane (i.e. adrenergic, muscarinic) receptor density was assumed. Diffusion of Ca\textsuperscript{2+} and/or IP\textsubscript{3} provided a weak propagating signal to synchronize neighboring domains, to result in the appearance of the propagating Ca\textsuperscript{2+} waves. Model predicts that \( V_m \) can affect wave velocity through modulation of transmembrane calcium influx and by altering the electrochemical gradient for calcium influx.

6.2.4 Ca\textsuperscript{2+} synchronization in arterial vasomotion

Rhythmic oscillations in vessel diameter (vasomotion) is observed in variety of vascular beds and suggested to arise from intercellular Ca\textsuperscript{2+} synchronization in individual
SMCs, and can be influenced by the endothelium. The phenomenon of vasomotion is thought to improve tissue oxygenation and enhance blood flow. A multi-cellular model of coupled SMCs with ECs to form a vessel segment with individual SMCs oscillating at varied intrinsic frequencies was developed and the synchronization mechanism were examined. Model simulations predicts several physiologically feasible pathways for synchronization of \(\text{Ca}^{2+}\) oscillations. These include depolarizing current generated through \(\text{Cl}_{\text{Ca}}\) and NSC channels, or pathways that involve the intercellular diffusion of \(\text{IP}_3\) and perhaps of \(\text{Ca}^{2+}\).

\(\text{Ca}^{2+}\) events in the vascular cells occur at varied spatial and temporal scales. Isolated or repetitive \(\text{Ca}^{2+}\) transients lasting for few milliseconds to \(\text{Ca}^{2+}\) events with half-life in the order of seconds are observed occurring randomly or in persistent regions of the vascular cells. The \(\text{Ca}^{2+}\) events are either confined to small regions or appear to propagate across or in between cells. The developed models capture such events and provide insights for their physiological relevance. Further research is required to elucidate and better comprehend the complex spatio-temporal nature of \(\text{Ca}^{2+}\) signaling. This will allow us gaining a deeper understanding of vascular tone regulation in health and disease.
CHAPTER 7-APPENDIX
7.1 Model Equations

7.1.1 Intercellular coupling

7.1.1.1 Ionic coupling

\[ I_{ji} = \sum S I_{gj,S} \]

\[ I_{gj,S} = P_{S} z_{S}^{2} \frac{F^{2}}{RT} \frac{[S]^{n} - [S]^{m}}{1 - \exp \left( - z S V_{ji} F / RT \right)} \]

\[ P_{S} = \frac{RT}{F^{2} R_{gj} \sum S \left( z_{S}^{2} [S] \right)} \]

\[ S = \text{Ca}^{2+}, \ K^{+}, \ \text{Na}^{+}, \ \text{Cl}^{-}; \ V_{ji} = V_{n}^{m} - V_{m}^{n}, \ n, m - \text{cell index}; \]

\[ R_{gj} - \text{gap junction resistance.} \]

\[ z_{K} = z_{\text{Na}} = 1; \ z_{\text{Ca}} = 2; \ z_{\text{Cl}} = -1; \ N_{A} = 6.022 \times 10^{23}; F = 96485 \text{ C/mol}; \]

\[ R = 8314 \text{ mJ/mol} \cdot \text{K}; \ T = 293.0 \text{ K}; \ [\text{Ca}]_{o} = 2.0 \text{ mM}; \ [\text{Na}]_{o} = 140.0 \text{ mM}; \ [\text{Cl}]_{o} = 129.0 \text{ mM}; \ [\text{K}]_{o} = 5.0 \text{ mM}. \]

7.1.1.2 IP3 coupling

\[ J_{\text{IP3}} = P_{\text{IP3}} ([\text{IP3}]^{n} - [\text{IP3}]^{m}) \]

7.1.1.3 NO coupling

\[ \bar{Q}_{NO,ss}^{EC} = \frac{Q_{NO,ss}^{EC}}{Q_{NO,max}^{EC}} = \frac{\left( [\text{Ca}^{2+}]^{i} \right)^{n}}{\left( [\text{Ca}^{2+}]^{i} \right)^{n} + \left( k_{m,Ca}^{NO} \right)^{n}} \]

\[ \frac{d\bar{Q}_{NO}^{EC}}{dt} = \frac{\bar{Q}_{NO,ss}^{EC} - \bar{Q}_{NO}^{EC}}{\tau_{eNOS}} \]

\[ [\text{NO}]^{SM} = [\text{NO}]_{\text{max}}^{EC} \bar{Q}_{NO}^{EC} \]

\[ \tau_{eNOS} = 2 \text{ s}; \ k_{m,Ca}^{NO} = 300 \text{ nM}; \ n = 4.2; \ [\text{NO}]_{\text{max}}^{EC} = 380 \text{ nM}. \]
7.1.2 Smooth muscle cells

7.1.2.1 Membrane electrophysiology

\[
\frac{dV_m}{dt} = \frac{-(I_{VOCO} + I_{Kv} + I_{BKCa} + I_{Kleak} + I_{NSC} + I_{SOC} + I_{CICa} + I_{PMCA} + I_{NaK} + I_{NCX} + \sum I_{gi}) + I_{stim}}{C_m}
\]

\[C_m = 25 \text{ pF}.
\]

7.1.2.1.1 L-type voltage-operated Ca\textsuperscript{2+} channels

\[
I_{VOCO} = A_m P_{VOCO} d_L f_L V_m \frac{(z_{Ca} F)^2}{RT} \frac{[Ca]_o - [Ca]}{1 - \exp \left( - \exp \left( \frac{V_m}{RT} \right) \right)}
\]

\[
\frac{dL}{dt} = \frac{dL - L}{\tau_{Ca}}; L = \left( 1 + \exp \left( - \frac{V_m}{8.3 \text{ mV}} \right) \right)^{-1}; \tau_{Ca} = 2.5 \exp \left( - \exp \left( \frac{V_m + 40 \text{ mV}}{30 \text{ mV}} \right) \right) + 1.15 \text{ [ms]}
\]

\[
\frac{dI_L}{dt} = \frac{I_L - f_L - f_L}{\tau_{Ca}}; I_L = \left( 1 + \exp \left( \frac{V_m + 42.0 \text{ mV}}{9.1 \text{ mV}} \right) \right)^{-1}; \tau_{Ca} = 65 \exp \left( - \exp \left( \frac{V_m + 35 \text{ mV}}{25 \text{ mV}} \right) \right) + 45 \text{ [ms]}
\]

\[P_{VOCO} = 1.88 \times 10^{-5} \text{ cm/s}; A_m = C_m(10^{-6} \text{ cm}^2/\text{pF}).
\]

7.1.2.1.2 Large conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} channels

\[I_{BKCa} = A_m N_{BKCa} P_{KCa} i_{KCa}
\]

\[P_{KCa} = 0.17 p_L + 0.83 p_S; \frac{dp_L}{dt} = \frac{p_L - p_L}{\tau_{pL}}; \frac{dp_S}{dt} = \frac{p_S - p_S}{\tau_{pS}}\]

\[\bar{p}_o = \left( 1 + \exp \left( - \frac{V_m - V_{1/2KCa}}{18.25 \text{ mV}} \right) \right)^{-1}
\]

\[V_{1/2KCa} = (-41.7 \text{ mV}) \log_{10} ([Ca]_o / (1 \text{ mM})) - 128.2 \text{ mV} - dV_{1/2KCa} \text{ NO} - dV_{1/2KCaGMP} \text{ NO}
\]

\[R_{NO} = \frac{[\text{NO}]}{[\text{NO} + 200 \text{nM}}; R_{cGMP} = \frac{[cGMP]^2}{[cGMP]^2 + (1.5 \text{ mM})^2}
\]
\[ i_{KCa} = P_{BKCa} V^2 \frac{F [K]_o - [K], \exp\left(\frac{V_m F}{RT}\right)}{1 - \exp\left(\frac{V_m F}{RT}\right)} \]

\[ P_{BKCa} = 3.9 \times 10^{-13} \text{ cm}^3/\text{s}; \ dV_{1/2KCaNO} = 46.3 \text{ mV}; \ N_{BKCa} = 6.6 \times 10^6 \text{ cm}^{-2}; \ \tau_{p1} = 0.84 \text{ ms}; \ \tau_{p2} = 35.9 \text{ ms}; \]

\[ dV_{1/2KCaGMP} = 76 \text{ mV}. \]

7.1.2.1.3 Voltage-dependent K\(^+\) channels

\[ I_{Kv} = g_{Kv} p_K (0.45q_1 + 0.55q_2)(V_m - E_K) \]

\[ \bar{p}_K = \frac{1}{1 + \exp\left(-\frac{V_m + 11.0 \text{ mV}}{15.0 \text{ mV}}\right)}; \ \tau_{pK} = 61.5 \exp(-0.027V_m) [\text{ms}]; \ \frac{dp_K}{dt} = \frac{\bar{p}_K - p_K}{\tau_{pK}} \]

\[ \bar{q} = \frac{1}{1 + \exp\left(-\frac{V_m + 40.0 \text{ mV}}{14.0 \text{ mV}}\right)}; \ \frac{dq_1}{dt} = \frac{\bar{q} - q_1}{\tau_{q1}}; \ \frac{dq_2}{dt} = \frac{\bar{q} - q_2}{\tau_{q2}} \]

\[ g_{Kv} = 1.35 \text{ nS}; \ \tau_{q1} = 371 \text{ ms}; \ \tau_{q2} = 2884 \text{ ms}. \]

7.1.2.1.4 Unspecified K\(^+\) leak channels

\[ I_{Kleak} = g_{Kleak} (V_m - E_K) \]

\[ g_{Kleak} = 0.067 \text{ nS}. \]

7.1.2.1.5 Nonselective cation channels

\[ I_{NSC} = I_{NaNSC} + I_{KNSC} + I_{CaNSC} \]
\[ I_{\text{NaNSC}} = A_m \left( \frac{[\text{DAG}]}{[\text{DAG}]+K_{\text{NSC}}} + d_{\text{NSCmin}} \right) P_{\text{ONSC}} P_{\text{NaNSC}} V_m \frac{F^2 [\text{Na}]_o - [\text{Na}]}{RT} \frac{\exp \left( \frac{V_m}{RT} \right)}{1 - \exp \left( \frac{V_m}{RT} \right)} \]

\[ I_{\text{KNSC}} = A_m \left( \frac{[\text{DAG}]}{[\text{DAG}]+K_{\text{NSC}}} + d_{\text{NSCmin}} \right) P_{\text{ONSC}} P_{\text{KNSC}} V_m \frac{F^2 [\text{K}]_o - [\text{K}]}{RT} \frac{\exp \left( \frac{V_m}{RT} \right)}{1 - \exp \left( \frac{V_m}{RT} \right)} \]

\[ I_{\text{CaNSC}} = A_m d_{\text{NSCmin}} P_{\text{ONSC}} P_{\text{CaNSC}} V_m \left( \frac{[\text{Ca}]}{RT} \right) \frac{z_{\text{Ca}} V_m F^2}{1 - \exp \left( \frac{z_{\text{Ca}} V_m F}{RT} \right)} \]

\[ P_{\text{ONSC}} = 0.43 + \frac{0.57}{1 + \exp \left( - \frac{V_m - 47.12 \text{mV}}{24.24 \text{mV}} \right)} \]

\[ K_{\text{NSC}} = 3 \text{ } \mu\text{M}; \quad P_{\text{NaNSC}} = 5.11 \times 10^{-7} \text{ cm/s}; \quad P_{\text{KNSC}} = 1.06 \times P_{\text{NaNSC}}; \quad P_{\text{CaNSC}} = 4.54 \times P_{\text{NaNSC}}; \]

\[ d_{\text{NSCmin}} = 0.0244. \]

7.1.2.1.6 Store-operated nonselective cation channels

\[ I_{\text{SOC}} = I_{\text{SOCCa}} + I_{\text{SOCNa}} \]

\[ I_{\text{SOCCa}} = g_{\text{SOCCa}} P_{\text{SOC}} (V_m - E_{\text{Ca}}) ; \quad I_{\text{SOCNa}} = g_{\text{SOCNa}} P_{\text{SOC}} (V_m - E_{\text{Na}}) \]

\[ P_{\text{SOC}} = \left( 1 + \frac{[\text{Ca}]}{100 \text{nM}} \right)^{-1} \]

\[ g_{\text{SOCCa}} = 0.0083 \text{ nM}; \quad g_{\text{SOCNa}} = 0.0575 \text{ nM}. \]

7.1.2.1.7 Calcium-activated chloride channels

\[ I_{\text{ClCa}} = C_m g_{\text{ClCa}} P_{\text{Cl}} (V_m - E_{\text{Cl}}) \]
\[ P_{\text{Cl}} = 0.0132 \frac{([\text{Ca}])^{n_{\text{ClCa}}} + (K_{\text{ClCa}})^{n_{\text{ClCa}}}}{([\text{Ca}])^{n_{\text{ClCa}}} + (K_{\text{ClCa}})^{n_{\text{ClCa}}}} + \alpha_{\text{Cl}} \frac{([\text{Ca}])^{n_{\text{ClCa}}} + (K_{\text{ClCa}})^{n_{\text{ClCa}}}}{([\text{Ca}])^{n_{\text{ClCa}}} + (K_{\text{ClCaGMP}})^{n_{\text{ClCa}}}} \]

\[ \alpha_{\text{Cl}} = \frac{([\text{cGMP}])^{n_{\text{ClGMP}}} + (K_{\text{ClGMP}})^{n_{\text{ClGMP}}}}{([\text{cGMP}])^{n_{\text{ClGMP}}} + (K_{\text{ClCaGMP}})^{n_{\text{ClGMP}}}} K_{\text{ClCaGMP}} = (1 - 0.9\alpha_{\text{Cl}}) \cdot 400\text{nM} \]

\[ g_{\text{ClCa}} = 0.23 \text{nS/pF}; n_{\text{ClCa}} = 2; K_{\text{ClCa}} = 365 \text{nM}; n_{\text{ClGMP}} = 3.3; K_{\text{ClGMP}} = 6.4 \text{ \mu M}. \]

7.1.2.1.8 Plasma membrane Ca\(^{2+}\) pump

\[ I_{\text{PMCA}} = \tilde{I}_{\text{PMCA}} \frac{[\text{Ca}]}{[\text{Ca}] + K_{m,\text{PMCA}}} \]

\[ \tilde{I}_{\text{PMCA}} = 5.37 \text{ pA}; K_{m,\text{PMCA}} = 170 \text{ nM}. \]

7.1.2.1.9 Plasma membrane Na\(^{+}\)-Ca\(^{2+}\) exchange

\[ I_{\text{NCX}} = g_{\text{NCX}} R_{\text{NCX,GMP}} \frac{[\text{Na}]^3[\text{Ca}]_o \Phi_F - [\text{Na}]^3[\text{Ca}]_i \Phi_R}{(1\text{mM})^4 + d_{\text{NCX}} ([\text{Na}]^3[\text{Ca}]_i + [\text{Na}]^3[\text{Ca}]_o)} \]

\[ R_{\text{NCX,GMP}} = 1 + 0.55 \frac{[\text{cGMP}]}{[\text{cGMP}] + 45\mu M} \]

\[ \Phi_F = \exp \left( \frac{\gamma V_m F}{RT} \right); \Phi_R = \exp \left( \frac{(\gamma - 1) V_m F}{RT} \right); \gamma = 0.45; d_{\text{NCX}} = 0.0003; g_{\text{NCX}} = 4.87 \times 10^{-9} \text{ pA} \]

7.1.2.1.10 Sodium-potassium pump

\[ I_{\text{NaK}} = C_{m} \tilde{I}_{\text{NaK}} Q \frac{([K]_o)^{n_{\text{HKo}}} + (K_{d\text{Ko}})^{n_{\text{HKo}}} [\text{Na}]_o^{n_{\text{HNai}}} + (K_{d\text{Nai}})^{n_{\text{HNai}}} [\text{Na}]_i^{n_{\text{HNai}}}}{V_m + 150\text{mV}} \]

\[ Q = Q_{10} \frac{T-309.15K}{10}; Q_{10} = 1.87 \]

\[ \tilde{I}_{\text{NaK}} = 2.31 \text{ pA/pF}; K_{d\text{Ko}} = 1.6 \text{ mM}; K_{d\text{Nai}} = 22 \text{ mM}; n_{\text{HKo}} = 1.1; n_{\text{HNai}} = 1.7. \]

7.1.2.1.11 Sodium-potassium-chloride cotransport

\[ I_{\text{NaKCl}}^{\text{CI}} = -R_{\text{NaKCl,GMP}} z_\text{Cl} A_m L_{\text{NaKCl}} \frac{\text{RT} \log_e \left( \frac{[\text{Na}]_o [K]_o [\text{Cl}]_o^2}{[\text{Na}]_i [K]_i (\text{[Cl]})} \right)}{R_{\text{NaKCl,GMP}} z_\text{Cl} A_m L_{\text{NaKCl}}} \]
\[ R_{NaKCl,cGMP} = 1 + 3.5 \frac{[cGMP]}{[cGMP] + 6.4\mu M} \]

\[ I_{Na}^{NaKCl} = I_{K}^{K} \quad I_{NaKCl}^{Cl} = -0.5I_{NaKCl}^{Cl} \]

\[ L_{NaKCl} = 1.79 \times 10^{-11} \text{ (mmol)}^2/(\text{J} \cdot \text{s} \cdot \text{cm}^2). \]

Reversal potentials

\[ E_A = \frac{RT}{z_A F} \ln \left( \frac{[A]_o}{[A]_i} \right), \text{ where } A \text{ denotes K, Na, Ca or Cl.} \]

7.1.2.2 Sarcoplasmic reticulum

\[ I_{SERCA} = I_{SERCA} \frac{[Ca]_i}{[Ca]_i + K_{m,up}} \]

\[ I_{tr} = ([Ca]_u - [Ca]_t) z_{Ca} F v_{volu} / \tau_{tr} \]

\[ I_{rel} = (R_{i0} + R_{\text{leak}}) ([Ca]_u - [Ca]_i) z_{Ca} F v_{volr} / \tau_{rel} \]

\[ \frac{d[Ca]_u}{dt} = \frac{I_{SERCA} - I_{tr} - I_{IP3}}{z_{Ca} F v_{volu}} \]

\[ \frac{d[Ca]_i}{dt} = \frac{I_{tr} - I_{rel}}{z_{Ca} F v_{volr}} \left( 1 + \frac{[CSQN]K_{CSQN}}{\left( K_{CSQN} + [Ca]_i \right)^2} \right)^{-1} \]

\[ \tau_{tr} = 1000 \text{ ms}; \quad \tau_{rel} = 0.033 \text{ ms} \]

\[ K_{m,up} = 1 \mu M; \quad I_{SERCA} = 20.04 \text{ pA}; \quad R_{\text{leak}} = 5.35 \times 10^{-5}; \quad K_{CSQN} = 0.8 \text{ mM}; \quad [CSQN] = 15 \]

\[ \text{mM}; \quad v_{volu} = 0.07 \text{ pL}; \]

\[ v_{volr} = 0.007 \text{ pL}. \]
7.1.2.2.1 Ryanodine receptor

\[ R_{00} = 1 - R_{01} - R_{10} - R_{11} \]

\[ \frac{dR_{10}}{dt} = K_{r1}[Ca]^2 R_{00} - (K_{r1} + K_{r2}[Ca]) R_{10} + K_{r2} R_{11} \]

\[ \frac{dR_{11}}{dt} = K_{r2}[Ca] R_{10} - (K_{r1} + K_{r2}) R_{11} + K_{r1}[Ca]^2 R_{01} \]

\[ \frac{dR_{01}}{dt} = K_{r2}[Ca] R_{00} + K_{r1} R_{11} - (K_{r2} + K_{r1}[Ca]^2) R_{01} \]

\[ K_{r1} = 2500 \text{ mM}^{-2}\text{ms}^{-1}; K_{r2} = 1.05 \text{ mM}^{-1}\text{ms}^{-1}; K_{r1} = 0.0076 \text{ ms}^{-1}; K_{r2} = 0.084 \text{ ms}^{-1} \]

7.1.2.2 IP3 receptor

\[ I_{IP3} = \bar{I}_{IP3} z_{Ca} \text{vol}_{Ca} C_{\alpha} \left( \frac{[IP3]}{[IP3] + K_{IP3}[Ca]} + K_{act,IP3} h_{IP3} \right)^{3} ([Ca]_{i} - [Ca]_{t}) \]

\[ \frac{dh_{IP3}}{dt} = k_{on,IP3}(K_{inh,IP3} - ([Ca]_{i} + K_{inh,IP3}) h_{IP3}) \]

\[ \bar{I}_{IP3} = 2880 \times 10^{-6} \text{ ms}^{-1}; K_{act,IP3} = 170 \text{ nM}; K_{inh,IP3} = 100 \text{ nM}; K_{IP3} = 120 \text{ nM}; k_{on,IP3} = 1.4 \text{ mM}^{-1}\text{ms}^{-1} . \]

7.1.2.3 \( \alpha_{1} \)-adrenoceptor activation and IP3 and DAG formation

\[ \frac{d[R_{G}^{S}]}{dt} = k_{r,G} \xi_{G} [R_{T,G}] - \left( k_{t,G} + \frac{k_{p,G}[NE]}{K_{1,G} + [NE]} \right) [R_{G}^{S}] - k_{r,G}[R_{P,G}^{S}] \]

\[ \frac{d[R_{P,G}^{S}]}{dt} = [NE] \left( \frac{k_{p,G}[R_{G}^{S}]}{K_{1,G} + [NE]} - \frac{k_{e,G}[R_{P,G}^{S}]}{K_{2,G} + [NE]} \right) \]

\[ \rho_{r,G} = \frac{[NE][R_{G}^{S}]}{\xi_{G} [R_{T,G}][K_{1,G} + [NE]]} \]
\[ \frac{d[G]}{dt} = k_{a,G} \left( \delta_{G} + \rho_{r,G} \right) [G_{T,G}] - [G] - k_{d,G} [G] \]

\[ r_{h,G} = \alpha_{G} \frac{[Ca]_{i}}{[Ca]_{i} + K_{c,G}} \]

\[ \frac{d[IP_{3}]}{dt} = \frac{r_{h,G} [PIP_{2}]}{\gamma_{G}} - k_{deg,IP_{3}} [IP_{3}] + \sum J_{IP_{3}} \]

\[ \frac{d[DAG]}{dt} = \frac{r_{h,G} [PIP_{2}]}{\gamma_{G}} - k_{deg,DAG} [DAG] \]

\[ \frac{d[PIP_{2}]}{dt} = -\left( r_{h,G} + r_{r,G} \right) [PIP_{2}] - r_{r,G} \gamma_{G} [IP_{3}] + r_{r,G} [PIP_{2,T}] \]

\[ [R_{T,G}] = 2 \times 10^{4}; \quad K_{1,G} = k_{1}^{-} / k_{1}^{+} = 0.01 \text{mM}; \quad K_{2,G} = k_{2}^{-} / k_{2}^{+} = 0.2 \text{mM}; \quad k_{r,G} = 1.75 \times 10^{-7} \text{ms}^{-1} \]

\[ k_{c,G} = 6 \times 10^{-6} \text{ms}^{-1}; \quad \xi_{G} = 0.85; \quad [G_{T,G}] = 10^{5}; \quad k_{deg,G} = 1.25 \times 10^{-3} \text{ms}^{-1}; \quad k_{a,G} = 0.17 \times 10^{-3} \text{ms}^{-1} \]

\[ k_{d,G} = 1.5 \times 10^{-3} \text{ms}^{-1}; \quad [PIP_{2,T}] = 5 \times 10^{7}; \quad r_{r,G} = 0.015 \times 10^{-3} \text{ms}^{-1}; \quad K_{c,G} = 0.4 \times 10^{-3} \text{mM} \]

\[ \alpha_{G} = 2.781 \times 10^{-8} \text{ms}^{-1}; \quad \gamma_{G} = 10^{-15} N_{AV} \text{vol}_{i}; \quad k_{p,G} = 0. \]

7.1.2.4 sGC activation and cGMP formation

\[ \bar{V}_{cGMP} = V_{cGMP,max} \frac{B_{5,sGC} [NO] + [NO]^{2}}{A_{0,sGC} + A_{1,sGC} [NO] + [NO]^{2}} \]

\[ \tau_{sGC} = \begin{cases} \tau_{sGC}^{a} & \text{if } (\bar{V}_{cGMP} - V_{cGMP}) \geq 0 \\ \tau_{sGC}^{d} & \text{otherwise} \end{cases} \]

\[ \frac{dV_{cGMP}}{dt} = \frac{\bar{V}_{cGMP} - V_{cGMP}}{\tau_{sGC}} \]

\[ \frac{d[cGMP]}{dt} = V_{cGMP} - k_{pde,cGMP} \frac{[cGMP]^{2}}{[cGMP] + K_{m,pde}} \]

\[ k_{1,sGC} = 2 \times 10^{3} \text{mM}^{-1} \text{ms}^{-1}; \quad k_{1,sGC} = 15 \times 10^{-3} \text{ms}^{-1}; \quad k_{2,sGC} = 0.64 \times 10^{-5} \text{ms}^{-1} \]

\[ k_{2,sGC} = 0.1 \times 10^{-6} \text{ms}^{-1}; \quad k_{3,sGC} = 4.2 \text{mM}^{-1} \text{ms}^{-1}; \quad k_{D_{p},sGC} = 0.4 \times 10^{-3} \text{ms}^{-1}; \quad k_{D_{p},sGC} = 0.1 \times 10^{-3} \text{ms}^{-1} \]

\[ B_{5,sGC} = \frac{k_{2,sGC}}{k_{3,sGC}} \]

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\[ \begin{align*}
A0_{sGC} &= (k_{-1,sGC} + k_{2,sGC}) k_{D,sGC} + k_{-1,sGC} k_{-2,sGC}) / (k_{1,sGC} k_{3,sGC}) \\
A1_{sGC} &= (k_{1,sGC} + k_{3,sGC}) k_{D,sGC} + (k_{2,sGC} + k_{-2,sGC}) k_{1,sGC}) / (k_{1,sGC} k_{3,sGC}) \end{align*} \]

\[ k_{pde,GMP} = 0.0695 \times 10^{-3} \text{ ms}^{-1}; \tau_{sGC}^a = 0.23 \text{ s}; \tau_{sGC}^d = 10 \text{ s}; V_{cGMP,\text{max}} = 1.26 \times 10^{-7} \text{ mM/ms}; \]

\[ K_{m,pde} = 10^{-3} \text{ mM} \]

### 7.1.2.5 Ionic balances

\[ I_{\text{Catotm}} = I_{\text{SOCCa}} + I_{\text{VOCC}} - 2I_{\text{NCX}} + I_{\text{PMCA}} + I_{\text{CaNSC}} \]

\[ \frac{d[Ca]}{dt} = -I_{\text{Catotm}} + I_{\text{SERCA}} - I_{el} - I_{IP3} + \sum I_\text{gi,Na} \left( 1 + \frac{[\Sigma_{CM}]}{([K]_d + [Ca])^2} + \frac{[\Sigma_F]}{([K]_d + [Ca])^2} \right) \]

\[ I_{\text{Natotm}} = I_{\text{NaKCl}} + I_{\text{SOClNa}} + 3I_{\text{NaK}} + 3I_{\text{NCX}} + I_{\text{NaNSC}} \]

\[ \frac{d[Na]}{dt} = -I_{\text{Natotm}} + \sum I_\text{gi,Na} \]

\[ I_{\text{Ktotm}} = I_{\text{NaKCl}} + I_{\text{Kv}} + I_{\text{BKCa}} + I_{\text{KNSC}} + I_{\text{Kleak}} - 2I_{\text{NaK}} \]

\[ \frac{d[K]}{dt} = -I_{\text{Ktotm}} + \sum I_\text{gi,K} \]

\[ I_{\text{Cltotm}} = I_{\text{NaKCl}} + I_{\text{ClCa}} \]

\[ \frac{d[Cl]}{dt} = -I_{\text{Cltotm}} + \sum I_\text{gi,Cl} \]

\[ [\Sigma_{CM}] = 0.1 \text{ mM}; [\Sigma_F] = 0.1 \text{ mM}; [K]_d = 260 \text{ nM}; [K]_dB = 530 \text{ nM}; \text{vol}_i = 1.0 \text{ pL}; \text{vol}_{Ca} = 0.7 \text{ pL}. \]

### 7.1.2.6 Initial conditions

\[ V_m = -52.7 \text{ mV}; [Ca]_i = 96 \text{ nM}; [Ca]_u = 0.77 \text{ mM}; [Ca]_r = 0.73 \text{ mM}; [Na]_i = 9.4 \text{ mM}; [K]_i = 121 \text{ mM}; [Cl]_i = 42.0 \text{ mM}; [IP_3] = [DAG] = [cGMP] = 0; V_{cGMP} = 0 \]

\[ I_{\text{VOCC}}: d_L = 1/(1+exp(-V_m/8.3\text{mV})); f_L = 1/(1+exp((V_m+42.0\text{mV})/9.1\text{mV})) \]

\[ I_{\text{BKCa}}: p_t = p_s = 1/(1+exp(-(V_m-V_{1/2}\text{KCa})/18.25\text{mV})) \]

\[ I_{\text{Kv}}: p_K = 1/(1+exp(-(V_m+11)/15)) \]

\[ q_1 = q_2 = 1.0/(1+exp((V_m+40)/14)) \]

\[ \text{RyR}: R_{01} = 0.0012; R_{10} = 0.003; R_{11} = 3.62 \times 10^{-6} \]

\[ IP_3: h_{IP_3} = K_{inh,IP_3} / ([Ca]_i + K_{inh,IP_3}) \]
\[ \alpha1\text{-adrenoceptor: } [R^S_G] = [R^T,G] \xi G; [R^S_{P,G}] = 0; [PIP_2] = [PIP_{2,T}] - (1 + k_{deg,G} / r_{G}) \gamma_G [IP_3]; \]

\[ r_{h,G} = k_{deg,G} \gamma_G [IP_3] / [PIP_2]; G = r_{h,G} (K_{c,G} + [Ca]) / (\alpha_G [Ca]); \delta_G = k_{d,G} \gamma_G / (k_{a,G} ([G_{T,G}] - G)) \]

### 7.1.3 Endothelial cells

#### 7.1.3.1 Membrane electrophysiology

\[
\frac{dV_m}{dt} = -\frac{1}{C_m} \left( I_{\text{SOC}} + I_{\text{NSC}} + I_{VRAC} + I_{CaCC} + I_{\text{Ks}} + I_{\text{Kc}} + I_{\text{NaK}} + I_{NCX} + I_{\text{PMCA}} + \sum I_{ij} - I_{\text{stim}} \right)
\]

\[ C_m = 14 \text{ pF}. \]

##### 7.1.3.1.1 Inward rectifier potassium channel

\[
I_{\text{Kir}} = \frac{G_{\text{Kir},\text{max}} (V_m - E_K)}{1 + e^{\frac{\Delta V - \Delta V_{\text{Kir},h}}{v_{\text{Kir}}}}}
\]

\[ G_{\text{Kir},\text{max}} = G_{\text{Kir}} (\text{[K}^+])^{m_{\text{Kir}}}, \quad \Delta V_{\text{Kir},h} = 39.42 \text{ mV}; \quad v_{\text{Kir}} = 7.084 \text{ mV}; \quad G_{\text{Kir}} = 0.1423 \text{ nS/mM}^{0.5}; \quad m_{\text{Kir}} = 0.5. \]

##### 7.1.3.1.2 Calcium-activated potassium channels

\[
I_X = G_X P_{O,X} (V_m - E_K)
\]

\[
P_{O,X} = \frac{[Ca^{2+}]_i^{n_X}}{[Ca^{2+}]_i^{n_X} + K_{X,Cai}^{n_X}}
\]

where X denotes SK\text{Ca} or IK\text{Ca}.

\[ G_{\text{SKCa}} = 0.62 \text{ nS}; \quad G_{\text{IKCa}} = 1.72 \text{ nS}; \quad n_{\text{SKCa}} = 5; \quad n_{\text{IKCa}} = 4; \quad K_{\text{SKCa,Cai}} = 237 \text{ nM}; \quad K_{\text{IKCa,Cai}} = 740 \text{ nM}. \]
7.1.3.1.3 Calcium-activated chloride channel

\[ I_{\text{CaCC}} = G_{\text{CaCC}} P_{O,\text{CaCC}} \frac{1}{1 + \left( \frac{K_{\text{CaCC,ai}}}{[Ca^{2+}]} \right)^{n_{\text{CaCC}}}} (V_m - E_{Cl}) \]

\[ \frac{dP_{O,\text{CaCC}}}{dt} = \frac{P_{O,\text{CaCC,SS}} - P_{O,\text{CaCC}}}{\tau_{\text{CaCC}}} \]

\[ P_{O,\text{CaCC,SS}} = \frac{1}{1 + e^{-\left( \frac{V_m - 9.19 \text{ mV}}{88.9 \text{ mV}} \right)^2}} \]

\[ \tau_{\text{CaCC}} = 386.2e^{-\left( \frac{V_m - 19.9 \text{ mV}}{88.9 \text{ mV}} \right)^2} \text{ [ms]} \]

\[ V_{\text{CaCC,h}} = 662 \text{ mV}; \nu_{\text{CaCC}} = 132 \text{ mV}; G_{\text{CaCC}} = 37.38 \text{ nS}; n_{\text{CaCC}} = 1.89; K_{\text{CaCC,ai}} = 287 \text{ nM}. \]

7.1.3.1.4 Volume-regulated anion channel

\[ I_{\text{VRAC}} = G_{\text{VRAC}} (V_m - E_{Cl}) \]

\[ G_{\text{VRAC}} = 0.381 \text{ nS} \]

7.1.3.1.5 Store-operated cation channel

\[ I_{\text{SOC,Na}} = P_{\text{SOC,Na}} A_m \frac{F^2}{RT} V_m \frac{[Na^+]_i - [Na^+]_o e^{-\frac{z_{Na}F}{RT}}}{1 - e^{-\frac{z_{Na}F}{RT}}} \]

\[ I_{\text{SOC,Ca}} = P_{\text{SOC,Ca}} A_m \frac{z_{Ca}^2 F^2}{RT} V_m \frac{[Ca^{2+}]_i - [Ca^{2+}]_o e^{-\frac{z_{Ca}F}{RT}}}{1 - e^{-\frac{z_{Ca}F}{RT}}} \]

\[ I_{\text{SOC}} = P_{\text{OSOC}} (I_{\text{SOC,Na}} + I_{\text{SOC,Ca}}) \]

\[ P_{\text{SOC,Na}} = \frac{P_{\text{SOC,Na,max}}}{1 + \left( \frac{[Ca^{2+}]_o}{K_{\text{SOC,Ca}}} \right)^{n_{\text{SOC,Na}}}} \]
\[ P_{O,SOC} = \frac{0.25}{1 + \left( \frac{[Ca^{2+}]_{IS}^{n_{SOC}}}{K_{SOC,CaIS}} \right)^{n_{SOC}}} + 0.083 \]

\[ P_{SOC,Na,max} = 3.95 \times 10^{-7} \text{ cm/s}; \quad P_{SOC,Ca} = 1.15 \times 10^{-7} \text{ cm/s}; \quad n_{SOC,Na} = 0.622; \quad n_{SOC} = 3.2; \]

\[ K_{SOC,CaIS} = 0.47 \text{ mM}; \quad K_{SOC,CaO} = 2.0 \times 10^{-4} \text{ mM}; \quad A_m = 14 \times 10^{-6} \text{ cm}^2. \]

7.1.3.1.6 Nonselective cation channel

\[ I_{NSC,Na} = P_{NSC,Na} A_m \frac{F^2}{RT} V_m \frac{[Na^+]_{i} - [Na^+]_{o} e^{-\frac{-F \phi}{RT}}}{1 - e^{-\frac{-F \phi}{RT}}} \]

\[ I_{NSC,K} = P_{NSC,K} A_m \frac{F^2}{RT} V_m \frac{[K^+]_{i} - [K^+]_{o} e^{-\frac{-F \phi}{RT}}}{1 - e^{-\frac{-F \phi}{RT}}} \]

\[ I_{NSC,Ca} = P_{NSC,Ca} A_m \frac{Z_{Ca}^2 F^2}{RT} V_m \frac{[Ca^{2+}]_{i} - [Ca^{2+}]_{o} e^{-\frac{-F \phi}{RT}}}{1 - e^{-\frac{-F \phi}{RT}}} \]

\[ I_{NSC} = I_{NSC,Na} + I_{NSC,K} + I_{NSC,Ca} \]

\[ P_{NSC,Na} = \frac{P_{NSC,Na,max}^{n_{NSC}}}{1 + \left( \frac{[Ca^{2+}]_{o}}{K_{NSC,CaO}} \right)^{n_{NSC}}} \]

\[ P_{NSC,Na,max} = 5.34 \times 10^{-8} \text{ cm/s}; \quad P_{NSC,K} = 0.49 \times 10^{-7} \text{ cm/s}; \quad P_{NSC,Ca} = 2.4 \times 10^{-8} \text{ cm/s} \]

7.1.3.1.7 Sodium-calcium exchanger

\[ I_{NCX} = \frac{1}{1 + \left( \frac{K_{NCX,CaI}}{[Ca^{2+}]_{i}} \right)^{n_{NCX}}} \times g_{NCX} \left( \frac{[Na^+]_{i}^{n_{NCX}} [Ca^{2+}]_{o} \phi_F - [Na^+]_{o}^{n_{NCX}} [Ca^{2+}]_{i} \phi_R}{(1 \text{mM})^4 + d_{NCX} [Na^+]_{o}^{n_{NCX}} [Ca^{2+}]_{i} + [Na^+]_{i}^{n_{NCX}} [Ca^{2+}]_{o}} \right) \]

\[ \phi_F = e^{\gamma_{NCX}(\alpha_{NCX} - 2) \mu_{NCX,F}/RT} \]

\[ \phi_R = e^{(\gamma - 1) \gamma_{NCX}(\alpha_{NCX} - 2) \mu_{NCX,F}/RT} \]
\[ g_{\text{NCX}} = 1.99 \text{ pA}; n_{\text{NCX,h}} = 1.50; n_{\text{NCX}} = 3; \gamma = 0.4834; d_{\text{NCX}} = 3 \times 10^{-4}; z_{\text{NCX}} = 1; \]

\[ K_{\text{NCX,Cai}} = 0.502 \text{ mM}. \]

### 7.1.3.1.8 Sodium-potassium (Na⁺/K⁺) ATPase

\[
I_{\text{NaK}} = I_{\text{NaK}} \frac{[K^+]_o + K_{\text{NaK,Ko}}}{[K^+]_o} \times \frac{[\text{Na}^+]_{1.5}^5}{[\text{Na}^+]_{1.5} + K_{\text{NaK,Na}}^{1.5}} \times \frac{V_m + 135 \text{ mV}}{V_m + 300 \text{ mV}}
\]

\[ \bar{I}_{\text{NaK}} = 20.18 \text{ pA}; K_{\text{NaK,Ko}} = 1.32 \text{ mM}; K_{\text{NaK,Na}} = 14.52 \text{ mM}. \]

### 7.1.3.1.9 Sodium-potassium-chloride (Na⁺/K⁺/2Cl⁻) cotransport

\[
I_{\text{NaKCl}_C} = -L_{\text{NaKCl}_C} \frac{RTF_{\text{Cl}}}{A_m} \ln \frac{[\text{Na}^+]_{1.5}^5}{[\text{Na}^+]_{1.5}} \frac{[\text{K}^+]_o}{[\text{K}^+]_o} \left( \frac{[\text{Cl}^-]_o}{[\text{Cl}^-]_o} \right)^2
\]

\[ I_{\text{NaKCl}_K} = I_{\text{NaKCl}_Na} = -0.5I_{\text{NaKCl}_C}
\]

\[ L_{\text{NaKCl}} = 3.2 \times 10^{-9} \text{(mmol)}^2 \text{J}^{-1} \text{s}^{-1} \text{cm}^{-2}. \]

### 7.1.3.1.10 Plasma membrane calcium ATPase

\[
I_{\text{PMCA}} = I_{\text{PMCA}} \frac{[\text{Ca}^{2+}]_{\text{PMCA}}^{n_{\text{PMCA}}}}{[\text{Ca}^{2+}]_{1.5}^{n_{\text{PMCA}}}} + K_{\text{PMCA,Cai}}^{n_{\text{PMCA}}}
\]

\[ \bar{I}_{\text{PMCA}} = 2.67 \text{ pA}; K_{\text{PMCA,Cai}} = 0.260 \times 10^{-3} \text{ mM}; n_{\text{PMCA}} = 1.4. \]

Nernst potential

\[ E_A = \frac{RT}{z_A F} \ln \frac{[A]_o}{[A]_i} \]  

where A denotes K⁺, Na⁺, Ca²⁺ or Cl⁻.

### 7.1.3.2 Fluid compartment model

### 7.1.3.2.1 IP₃ receptor

\[
I_{\text{IP₃R}} = I_{\text{IP₃R}} \frac{[\text{IP₃}]^{3.8}}{[\text{IP₃}]^{3.8} + K_{m,\text{IP₃}}^{3.8}} P_{\text{IP₃R}} ([\text{Ca}^{2+}]_{1.5} - [\text{Ca}^{2+}]_i)
\]

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\[ P_{i,IP3R} = \frac{K_{i,Cai}^{3.8}}{[\text{Ca}^{2+}]} + K_{i,Cai}^{3.8} \]

\[ I_{IP3R} = 4.67 \times 10^6 \text{ pA/mM}; \; K_m,IP3 = 1.6 \times 10^{-3} \text{ mM}; \; K_{i,Cai} = 10^{-3} \text{ mM}. \]

7.1.3.2.2 Endoplasmic reticulum calcium ATPase and ER leak

\[ I_{\text{SERCA,IS}} = I_{\text{SERCA,IS}} \left( \frac{[\text{Ca}^{2+}]}{[\text{Ca}^{2+}] + K_{\text{SERCA,IS}}} \right)^2 \]

\[ I_{\text{leak,IS}} = k_{\text{leak,IS}} \left( [\text{Ca}^{2+}]_{\text{IS}} - [\text{Ca}^{2+}] \right)^2 \]

\[ I_{\text{SERCA,IS}} = 0.88 \text{ pA}; \; K_{\text{SERCA,IS}} = 0.15 \times 10^{-3} \text{ mM}; \; k_{\text{leak,IS}} = 0.0176 \text{ pA/(mM)}^2. \]

7.1.3.3 Intracellular ionic and material balances

\[ I_{\text{Catom}} = I_{\text{SOC,Na}} - 2I_{\text{NCX}} + I_{\text{PMCA}} + I_{\text{NSCCa}} \]

\[ \frac{d[\text{Ca}^{2+}]}{dt} = -\frac{I_{\text{Catom}} + I_{\text{SERCA,IS}} - I_{\text{leak,IS}} - I_{\text{IP3R}} + \sum I_{b,j} \text{Ca}}{z_{\text{Ca}F\text{vol}_{\text{Ca}}} \frac{d[\text{Ca}^{2+}]}{dt}} \]

\[ \frac{d[\text{Ca}^{2+}]}{dt} = k_{\text{Ca}} [\text{Ca}^{2+}] \left( B_T - [\text{Ca}^{2+}] \right) - k_{\text{off}} [\text{Ca}^{2+}] \]

\[ \frac{d[\text{Na}^+]}{dt} = -\frac{I_{\text{SOC,Na}} + 3I_{\text{NCX}} + I_{\text{NSCCa}} + 3I_{\text{NaK}} + I_{\text{NaCl,Na}} + \sum I_{b,j} \text{Na}}{z_{\text{Na}F\text{vol}_{\text{Na}}} \frac{d[\text{Na}^+]}{dt}} \]

\[ \frac{d[K^+]}{dt} = -\frac{I_{\text{SK,Na}} + I_{\text{KNa}} + I_{\text{NSCCa}} - 2I_{\text{NaK}} + I_{\text{NaCl,K}} + \sum I_{b,j} K}{z_{\text{K}F\text{vol}_{\text{K}}} \frac{d[K^+]}{dt}} \]

\[ \frac{d[\text{Cl}^-]}{dt} = -\frac{I_{\text{NaCl,Cl}} + I_{\text{VRAC}} + I_{\text{CaCl}} + \sum I_{b,j} \text{Cl}}{z_{\text{Cl}F\text{vol}_{\text{Cl}}} \frac{d[\text{Cl}^-]}{dt}} \]

\[ \frac{d[\text{IP}_3]}{dt} = Q_{\text{IP3}} - k_{\text{IP3}}[\text{IP}_3] + \sum I_{b,j} \text{IP}_3 \]

\[ \frac{dQ_{\text{IP3}}}{dt} = \frac{Q_{\text{GIP3SS}} - Q_{\text{GIP3}}}{\tau_{\text{IP3}}} \]
kB_{on} = 100 \text{ (mM)}^{-1} \text{ (ms)}^{-1}; \quad kB_{off} = 0.300 \text{ (ms)}^{-1}; \quad B_T = 0.120 \text{ mM}; \quad K_{CSQN} = 15 \text{ mM}; \quad K_{CSQN} = 0.8 \text{ mM}; \quad Q_{GIP3,SS} = 5.5 \times 10^{-8} \text{ mM/ms}; \quad k_{DIP3} = 2.0 \times 10^{-3} \text{ (ms)}^{-1}; \quad \tau_{I_{IP}} = 5 \text{ s}; \quad \text{vol}_i = 1.173 \text{ pL}; \quad \text{vol}_{CA} = 0.912 \text{ pL}; \quad \text{vol}_{IS} = 0.335 \text{ pL}.

7.1.3.4 Initial conditions

\[ V_m = -49.8 \text{ mV}; \quad [\text{Ca}^{2+}]_i = 131 \text{ nM}; \quad [\text{Ca}^{2+}]_{IS} = 3.3 \text{ mM}; \quad [\text{Na}^+]_i = 18.7 \text{ mM}; \quad [\text{K}^+]_i = 116 \text{ mM}; \quad [\text{Cl}^-]_i = 46.3 \text{ mM}; \]

\[ P_{O,CaCC} = \frac{1}{1 + e^{(V_m - V_{CaCC})/V_{CaCC}}} \]

\[ [\text{Ca}^{2+}]_b = \frac{kB_{on}[\text{Ca}^{2+}]_i}{(kB_{on}[\text{Ca}^{2+}]_i + kB_{off})B_T}. \]

7.2 Model Innovations

We developed detailed mathematical models describing Ca^{2+} and V_m dynamics in vascular SMCs and ECs based on data mostly from rat mesenteric arterioles (RMAs). The models incorporated the most significant membrane currents identified in the particular cell types. Moreover, the models took into account effect of microdomains structures (such as microprojections), the uneven distribution of membrane and store components, preferential localization of cellular structures in the microdomains (such as high density distribution of IK_{Ca}, TRPV4s, I_{P3}Rs in the miroprojections) and the resulting spatial concentration gradients for ionic species and second messengers. An integrative model approach undertaken here accounts for the complex interactions at subcellular levels to reproduce macroscale responses. Similarly to other modeling approaches, our methodology uses typical Hodgkin-Huxley formalism at the base of the cellular models. The methodology presented here has several novel features including: a) Models that can examine of Ca^{2+} signaling spanning from subcellular to inter-cellular Ca^{2+} events; b) cell-
coupling formulations to monitor ionic and second messengers exchange and the weak intracellular currents; c) integration of signaling pathways (such as NO signaling from EC to SMC); d) Models incorporating accurate microdomain geometries from electron microscopy images.

### 7.3 Model Validation and Sensitivity Analysis

The models integrate membrane electrophysiology with dynamics for the major ionic species ($K^+$, $Na^+$ $Cl^-$) and second messengers ($Ca^{2+}$, IP$_3$, NO). We incorporated major components of membrane electrophysiology in typical resistance arterioles. Models include kinetic descriptions for channels, pumps, membrane and store receptors such as non-selective cation (NSC), transient receptor vanilloid 4 (TRPV4), store-operated cation (SOC), small (SK$_{Ca}$), intermediate (IK$_{Ca}$) and large (BK$_{Ca}$) conductance calcium-activated $K^+$, inward-rectifier $K^+$ (KIR), volume-regulated anion (VRAC) and calcium-activated $Cl^-$ (Cl$_{Ca}$) channels, $Na^+-Ca^{2+}$ exchanger (NCX) and $Na^+/K^+$-ATPase pump (NaK), IP$_3$ (IP$_3$R) and ryanodine (RyR) receptors, SERCA and PMCA pumps. The EC model incorporates a $Ca^{2+}$-dependent NO release in response to agonist (i.e. Acetylcholine; ACh) stimulation. Compartmental EC model contains 12 Ordinary Differential Equations (ODEs) and approximately 60 parameters. In the SMC model, a description for agonist activation of $\alpha_1$-adrenoceptor leading to IP$_3$ and DAG formation is incorporated to simulate the effect of (Norepinephrine; NE). The vasodilatory action of NO is simulated through an effect on four main targets (i.e. BK$_{Ca}$, NCX, Cl$_{Ca}$, NaKCl cotransporter). The model contains 24 ODEs and approximately 100 parameters, the majority of which are estimated from independent experimental studies. Uncertainty in parameter values exists and this poses a significant limitation for the model. Sensitivity
analysis using the method of Latin Hypercube Sampling is utilized to address this limitation. Most importantly, only a relatively small number of unknown parameters is optimized to fit a variety of integrated responses. Each cellular model is then compared with documented features of cell behavior for validation (i.e. resting concentrations, resting $V_m$, $Ca^{2+}$ and $V_m$ transients in response to agonist stimulation etc).
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