Irena Levitan, PhD
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Vascular Ion Channels in Physiology and Disease

Foreword by
Dr. Mark T. Nelson

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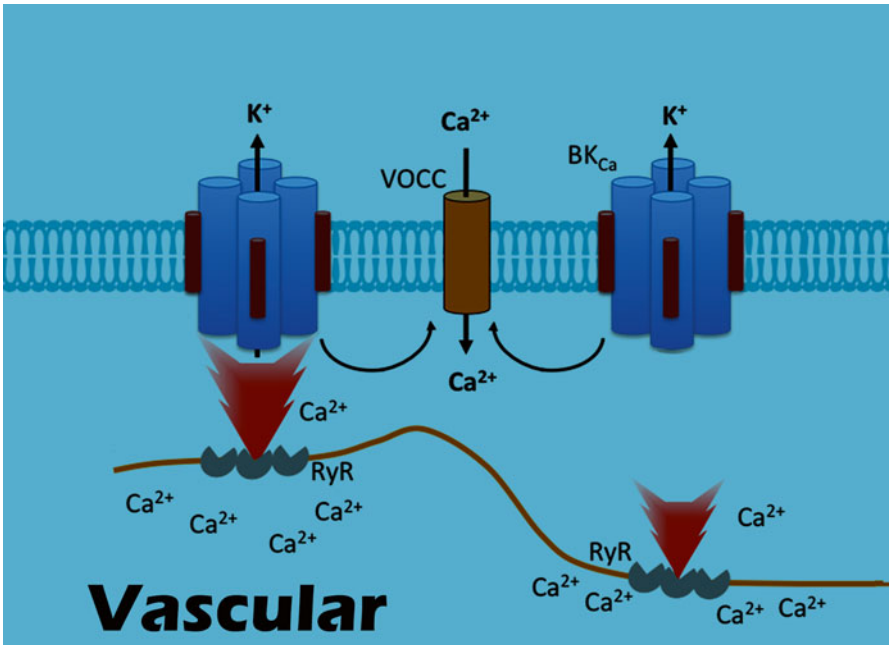

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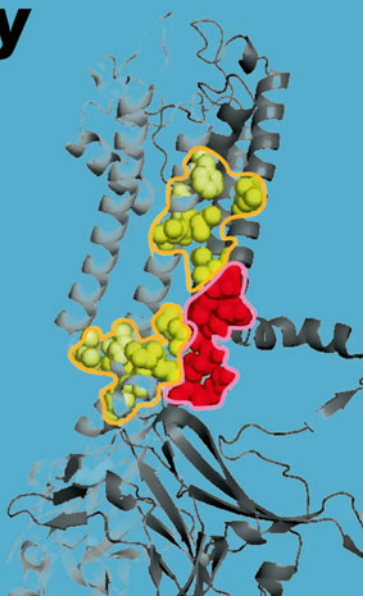

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Foreword

The central role of the vasculature in the function of the human body has been appreciated, at least in certain circles, since the time of ancient Greece, when the first known anatomical treatise that was not based on mysticism was produced. Early anatomists got much wrong, of course, crediting blood vessels with carrying tears and urine (but not sweat) in addition to blood, and suggesting the more fantastical role of conveying "breaths" of life and death (curiously these were said to be directed to right and left ears, respectively). Aristotle and his fourth century BC colleagues helped bring more empiricism to the subject, but their strictly anatomical approach based on animal dissections left much of vascular function to the imagination. Detailed studies of human cadavers, notably those performed by Leonardo da Vinci, the first to describe atherosclerosis, and later by Vesalius, considered by many to be the founder of modern human anatomy, dramatically advanced our understanding of the structure and basic responsibilities of the vascular system. However, medieval tools would prove to be no match for the twentieth century question of how various pieces and parts of the vascular system, though exquisitely detailed in exacting drawings, actually functioned.

If there is a consensus start to the modern era of functional vascular biology research, it is the publication in 1902 of a paper by William Bayliss, titled "On the local reactions of the arterial wall to changes of internal pressure," which reported the counterintuitive finding that intravascular pressure causes healthy arteries to constrict. More than 100 years later, this process, termed the myogenic response, remains an area of active investigation—a testament to the complexity of the underlying mechanism. The concept that ion channels are fundamentally involved in this and other vascular processes—the topic of this book—would be developed much later, after the seminal work of Hodgkin and Huxley on action potentials in the 1950s, made possible by the experimentally accessible neurons of their giant squid axon preparation, and after the confirmation of the existence of ion channels as specific molecular entities by Katz and Miledi in the 1970s. In fact, it wasn't until the development of the "patch-clamp" technique by Neher and Sakmann in the late 1970s and early 1980s that the study of ion channels in vascular cells would begin in earnest.

Since that time, the body of literature on the subject has grown at a rate that threatens our ability to keep pace. No single volume can possibly convey any more than a small subset of the available information, and this book is no exception. However, our hope is that those with different backgrounds will find something to like here, whether it be big-picture topics of potential interest to general readers, such as ion channel repertoires in endothelial cells (Chap. 1), regulation and function of calcium channels in smooth muscle cells (Chap. 2), regional variation in arterial myogenic responsiveness (Chap. 6), ion channel trafficking in the control of contractility (Chap. 7), and calcium mobilization from intracellular stores (Chap. 11), or more specific offerings, such cholesterol-potassium channel interactions (Chap. 15). As reflected in the title of this book, a number of chapters address the role of ion channels in the etiology of vascular diseases, including hypertension (Chaps. 8 and 14), cancer (Chap. 12), and metabolic diseases (Chaps. 17 and 18), or their potential as therapeutic targets in the treatment of disease (Chaps. 5 and 9). The book is organized into three main parts. Part I, "Ion channel regulation of vascular tone and blood flow," addresses aspects of endothelial cells (Part I) and smooth muscle cells (Part II) that regulate moment-to-moment changes in contractile state (tone) of arteries, and hence blood flow, as well as alterations associated with hypertension. Part III, "Ion channels in the regulation of cell proliferation, remodeling, hypertrophy and angiogenesis," focuses on longer-term dynamics, particularly vascular remodeling, under pathological (tumor angiogenesis, pulmonary hypertension) and physiological conditions. The final part (Part IV) presents selected topics in "Ion channel regulation by lipids and channel modifications in metabolic disease."

Each chapter is the product of scientists who are actively engaged in the cutting-edge work described and is a reflection of their individual research interests. The resulting book is thus an eclectic offering that allows each contributor to speak with his or her own voice, a feature that we like to think is part of its charm.

Burlington, VT, USA

Mark T. Nelson
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Preface

Ion channels are the major class of membrane proteins responsible for rapid and regulated transport of ions across biological membranes and for the generation and propagation of electrical signals in excitable tissues. Ion channels are also known to play critical roles in the regulation of cell proliferation, insulin secretion, and intracellular signaling in a variety of cell types. During the last decade, since the first atomic structure of a potassium-selective channel has been solved in 2000 by the group of Roderick MacKinnon, a discovery that led to a Nobel Prize in Chemistry shortly after, there has been an explosion of studies on the structure-function relationship of ion channels leading to an unprecedented level of mechanistic understanding of channel function. This new era of ion channel research provided the basis for detailed analysis of the roles of ion channels in cellular and tissue physiology. This book focuses on the roles of ion channels in vascular tissues under normal and pathological conditions.

Vascular abnormalities are known to underlie a plethora of severe pathological conditions, such as atherosclerosis, systemic and pulmonary hypertension, coronary or cerebral vasospasm, and diabetes. In addition, dysregulation of angiogenesis is one of the major contributors to tumor development. Therefore, it is clearly imperative to obtain a better understanding of the molecular mechanisms that contribute to vascular disorders. This book is the first comprehensive assembly of assays to present the studies that have been conducted during the last decade to elucidate the roles of ion channels in vascular physiology and in different vascular diseases.

Among the major concepts discussed in this volume are: the diversity of ion channels expressed in endothelial and vascular smooth muscle cells, and their roles in regulating arterial contractility and vascular tone in systemic, cerebral, and pulmonary circulation, as well as in the regulation of cell proliferation, barrier function, and vascular remodeling. It is also highlighted that both plasma membrane and intracellular ion channels, particularly in mitochondria, contribute significantly to the regulation of vascular functions. Emerging topics include the roles of channel trafficking in regulation of vascular tone, local control and co-ordination of ion channel function

in different domains, and description of novel positive and negative gating modulators of the channels as innovative therapeutic tools to treat cardiac disorders and as anti-vasospastic agents. The last part of the volume describes the latest insights into the mechanisms underlying lipid regulation of ion channels and the implications of these mechanisms for cardiovascular dysfunction in metabolic disease.

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Part I
Ion Channel Regulation of Vascular Tone and Blood Flow. Changes with Hypertension: Endothelial Cells

Chapter 1
Endothelial Cell Ion Channel Expression and Function in Arterioles and Resistance Arteries

William F. Jackson

Abstract Ion channels importantly contribute to the function of endothelial cells. They serve as the major source of intracellular Ca^{2+}, which, in turn, controls the production of endothelium-derived vasodilators, the permeability of the endothelium, gene expression, and other properties of endothelial cells. In addition, the activity of ion channels determines the membrane potential of endothelial cells that serves as an important signal for cell-cell communication between endothelial cells and between endothelial cells and overlying smooth muscle cells, and may feedback to regulate the activity of the ion channels themselves. This review provides an overview of the expression and function of endothelial ion channels that contribute to Ca^{2+} and membrane potential signaling that is involved in the regulation and modulation of vasomotor tone of resistance arteries and arterioles. Channels discussed include inositol 1,4,5 trisphosphate receptors that mediate agonist-induced Ca^{2+} release from endoplasmic reticulum stores; members of the transient receptor potential family and other channels that mediate agonist-induced Ca^{2+} influx through the plasma membrane; Ca^{2+}-activated K^+ channels that mediate agonist-induced membrane hyperpolarization; and inward rectifier K^+ channels that serve as sensors for changes in extracellular K^+ and amplifiers of hyperpolarization induced by the activity of other ion channels. It is emphasized that all of these channels exist as members of macromolecular signaling complexes providing a rich environment for regulation of their activity and the function of endothelial cells in resistance arteries and arterioles.

Keywords Vascular endothelium • Ion channels • Endothelium-dependent vasodilatation • Conducted dilatation • Potassium channels • Transient receptor potential channels • Calcium ions • Inositol 1,4,5 trisphosphate receptors • Calcium waves • Calcium sparklets

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Introduction

Endothelial cells express a diverse array of ion channels in their plasma membranes and in the membranes of intracellular organelles that contribute to the function of these cells. These channels provide the major source of intracellular Ca^{2+} that serves as an important second messenger controlling the activity of Ca^{2+}-dependent ion channels and cell membrane potential [40], endothelial cell production of NO, prostaglandins and epoxides of arachidonic acid (EETs) [40] and regulating barrier function of the endothelium [52, 90]. Intracellular Ca^{2+} is also an important signal controlling gene expression in [117, 130] and proliferation of [120, 123] endothelial cells. Ion channels also participate in cell volume regulation [70]. In addition, plasmalemmal ion channel activity importantly contributes to the membrane potential of endothelial cells that serves as a major signal for cell-cell communication between adjacent endothelial cells and as well as overlying smooth muscle cells due to the expression of homocellular and heterocellular gap junctions in the vascular wall [29]. Membrane potential may also feedback to affect Ca^{2+} influx through plasmalemmal Ca^{2+} permeable ion channels by influencing the electrochemical gradient for Ca^{2+} influx [65, 66, 69, 123], although this topic remains controversial [23, 34, 108, 113, 158]. Thus, ion channels importantly contribute to the function of endothelial cells in health and disease. This review will focus on the expression and function of endothelial ion channels involved in the regulation of vasomotor tone in resistance arteries and arterioles. Because there are considerable changes in ion channel expression and function during proliferation of cells in culture [11, 12, 20, 123, 135], emphasis will be placed on evidence from intact blood vessels and from freshly isolated endothelial cells from the peripheral circulation. The reader is also directed to a number of outstanding earlier reviews of ion channels in endothelial cells for access to earlier literature on this topic [123–125].

Setting the Stage

Most vasodilators that produce endothelium-dependent vasodilatation (see [79]) act on $G\alpha_q$-protein-coupled receptors which are linked to phospholipase C (PLC)-β producing inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) from membrane phospholipids [13] (Fig. 1.1). The released IP_3 activates IP_3R in the membranes of the smooth endoplasmic reticulum, releasing stored Ca^{2+} and increasing cytosolic Ca^{2+} [13]. The activation of IP_3R, loss of Ca^{2+} from intracellular Ca^{2+} stores and the DAG produced by PLC-β, activate plasma membrane Ca^{2+}-permeable ion channels [51], allowing Ca^{2+} to diffuse down its electrochemical gradient into the cells, producing a steady-state increase in intracellular Ca^{2+} (Figs. 1.1 and 1.2). The sum of these two major events (the release of ER-stored Ca^{2+} via IP_3R and the influx of Ca^{2+} via plasmalemmal ion channels) produces the well-described, agonist-induced cytosolic Ca^{2+} transient in endothelial cells (see Fig. 1.2). The increase in

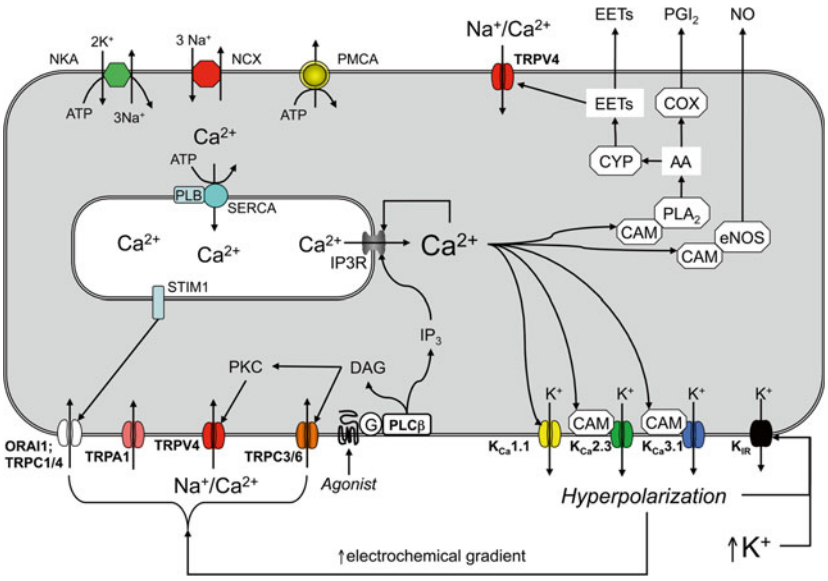

Fig. 1.1 Endothelial cell ion channel and Ca^{2+} signaling overview. Shown is a schematic representation of an endothelial cell and the ion channels and transporters relevant to agonist-induced Ca^{2+} signaling. Agonists of $G\alpha_q$-coupled receptors activate PLC-β producing $IP_3$and DAG. IP_3 activates IP_3R in the membrane of the endoplasmic reticulum (ER), releasing stored Ca^{2+} and raising cytosolic Ca^{2+} as shown. The released Ca^{2+} and Ca^{2+} entry through overlying plasma membrane Ca^{2+} permeable channels further stimulate Ca^{2+} release via Ca^{2+}-induced-Ca^{2+}-release. The elevated cytosolic Ca^{2+} will then activate plasma membrane K_{Ca} channels to produce membrane hyperpolarization, an important signal for cell-cell communication in resistance arteries and arterioles. This hyperpolarization also has the potential to increase the electrochemical gradient for diffusion of Ca^{2+} (and other cations) into the endothelial cell counter-acting the depolarizing effect of this cation influx. The DAG produced by the action of PLCs can activate TRPC3 and/or TRPC6 channels in the membrane, contributing to steady-state, agonist-induced Ca^{2+} influx into the cells. The DAG can also activate PKC, which phosphorylates TRPV4 channels increasing their activity, also contributing to Ca^{2+} influx. Loss of Ca^{2+} from the ER is sensed by STIM1, which clusters, interacts with and activates membrane ORAI1, TRPC1 and/or TRPC4 channels. The resultant Ca^{2+} influx contributes to steady-state, agonist-induced Ca^{2+} influx. The elevated cytosolic Ca^{2+} from these processes also activates nitric oxide synthase (eNOS) to stimulate NO production, and phospholipase A_2 (PLA_2) to produce arachidonic acid (AA) from membrane phospholipids. Arachidonic acid is then converted into vasodilator prostanoids, such as prostacyclin (PGI_2), by cyclooxygenase (COX), and epoxides (EETs) by cytochromes P450 (CYP). EETs may contribute to activation of TRPV4. Upon removal of agonist, Ca^{2+} is pumped back into the ER by the smooth endoplasmic reticulum Ca^{2+} ATPase (SERCA) and extruded from the cell by the Na^+/Ca^{2+} exchanger (NCX) and the plasma membrane Ca^{2+} ATPase (PMCA). Sodium that enters the cells via TRP channels is extruded by the Na^+/K^+ ATPase (NKA) and NCX. *CAM* calmodulin. *PLB* phospholamban

intracellular Ca^{2+} then activates plasmalemmal Ca^{2+}-activated K^+ channels to produce hyperpolarization of the endothelial cell membrane as shown in Fig. 1.2c, as well as activation of other Ca^{2+}-dependent processes such as NO production and release of arachidonic acid from membrane phospholipids resulting in increased production of prostacyclin and epoxides of arachidonic acid [40] (Fig. 1.1).

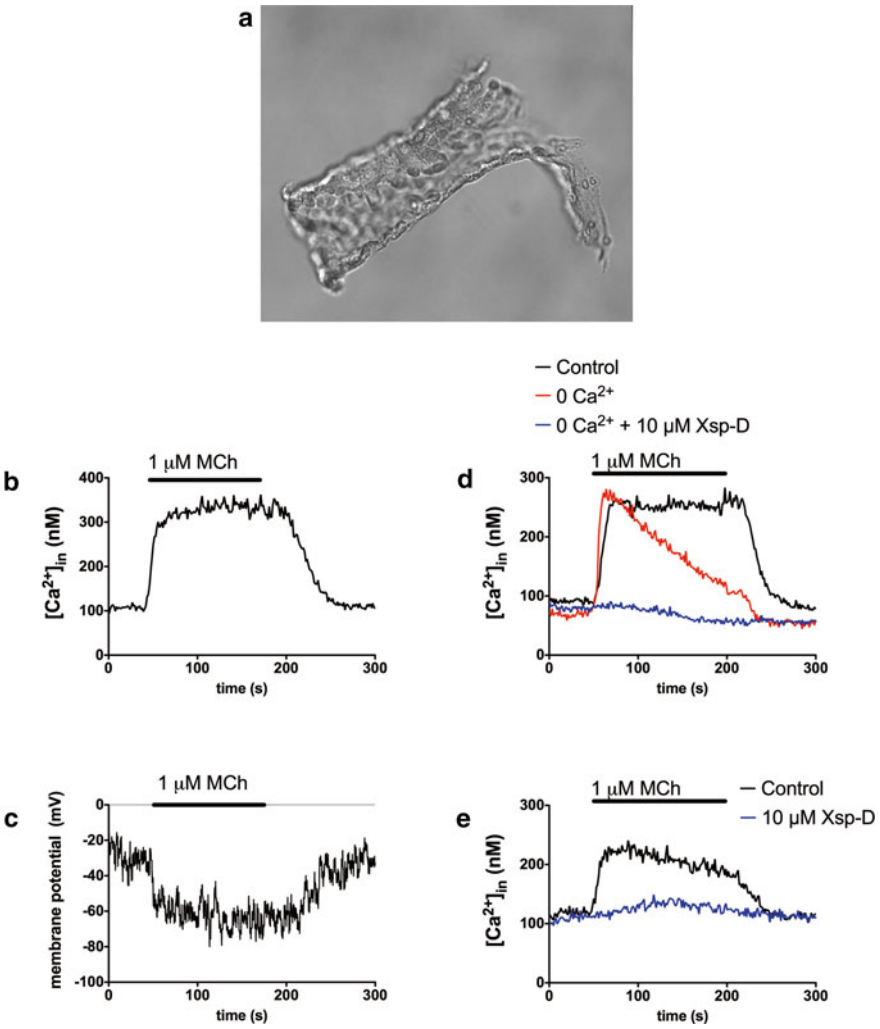

Fig. 1.2 Methacholine-induced global Ca^{2+} transients in arteriolar endothelial cells. Panel **a** shows an image of an endothelial cell tube enzymatically isolated from a second order hamster cremaster arteriole as described [23]. Panel **b** shows a representative Ca^{2+} transient elicited by the muscarinic receptor agonist, methacholine (MCh) from an endothelial cell tube loaded with the ratiometric calcium indicator, Fura-2AM. Panel **c** shows the MCh-induced membrane hyperpolarization of an endothelial cell tube loaded with the potentiometric indicator, di-8-ANEPPs as described [23]. Panel **d** shows MCh-induced Ca^{2+} transients, as in **a**, under Control conditions, after brief exposure to solutions containing 0 mM Ca^{2+} demonstrating the loss of the plateau phase of the Ca^{2+} transient. After return to Ca^{2+}-replete conditions (to obviate depletion of intracellular Ca^{2+}), subsequent exposure to 0 mM Ca^{2+} and the IP_3R-antagonist, xestospongin-D (Xsp-D; 10 μM) abolished the effects of MCh. Panel **e** shows inhibition of a MCh-induced Ca^{2+} transient by Xsp-D in the presence of extracellular Ca^{2+} (2 mM). Data shown in **a**–**e** are modified from [23]

The remainder of this review will focus on the expression and function of ion channels responsible for agonist-induced Ca^{2+} signals and membrane hyperpolarization in endothelial cells as shown in Fig. 1.2, as well as other endothelial cell ion channels that appear important to the regulation of myogenic tone in resistance arteries and arterioles.

What Endoplasmic Reticulum Ion Channels Mediate Agonist-Induced Ca^{2+} Signals?

Inositol-1,4,5-trisphosphate Receptors

Early studies demonstrated that agonists of $G\alpha_q$-coupled receptors increased intracellular Ca^{2+} in endothelial cells that was due to an initial release of Ca^{2+} from internal stores followed by Ca^{2+} influx [18, 25, 59, 139, 142, 143] (Figs. 1.1 and 1.2). Pharmacological studies subsequently identified IP_3Rs as the primary Ca^{2+} release channel responsible (see [23, 143] and Fig. 1.2 for examples). Inositol-1,4,5-trisphosphate receptors are large (350 kDa) tetrameric Ca^{2+} release channels found in the endoplasmic reticulum of all mammalian cells [47, 104]. Each monomer contains an IP_3-binding domain that is located in cytoplasmic N-terminus of the proteins [47, 104]. Calcium appears to be the trigger for gating IP_3 channels to open (see Table 1.1 for EC_{50} values for activation of the channels) [47, 104]. However, the response to increases in Ca^{2+} is biphasic, with higher concentrations of Ca^{2+} becoming inhibitory (Table 1.1) [47, 104]; Ca^{2+} activates the channels at low concentrations, but inhibits Ca^{2+} release at high concentrations (see Fig. 7 in [47] for examples). It has been proposed that the concentration of IP_3 determines the affinity of IP_3Rs for the inhibitory effects of elevated Ca^{2+}; as the concentration of IP_3 increases, higher levels of Ca^{2+} are required to inhibit the channels (Table 1.1) [47, 104]. Thus, the concentration of IP_3 effectively determines the range of cytosolic Ca^{2+} concentration over which the IP_3Rs will be active [47]. However, this has not been observed in all systems [171]. Nonetheless, in the presence of physiologically relevant concentrations of IP_3, IP_3R can undergo Ca^{2+}-induced-Ca^{2+} release (CICR) providing a positive feedback mechanism for release of Ca^{2+} from adjacent IP_3R (as in the case of Ca^{2+}waves), as well as amplification, with a limit, of Ca^{2+} signals originating from Ca^{2+} influx through overlying plasma membrane ion channels.

There are three isoforms of IP_3R, IP_3R1, 2 and 3 originating from three genes with modestly different characteristics (see Table 1.1); IP_3R2 and 3 appear to have the highest sensitivity for Ca^{2+}-induced activation with IP_3R3 having the lowest sensitivity for IP_3 [47, 104, 171]. In addition to IP_3 and Ca^{2+}, the activity of IP_3Rs is also sensitive to cytoplasmic ATP concentrations [47, 104, 171]. In IP_3R1 and 3, ATP produces a leftward shift in the Ca^{2+}-activity relationship increasing the affinity of the channels for Ca^{2+}, with little effect on the maximal open-state probability [47, 171]. In distinct contrast, ATP has no effect on Ca^{2+}-sensitivity of IP_3R2 receptors,

Table 1.1 Microvascular endothelial ion channels and their pharmacology

Channel	Gene	Alternative names	Accessory subunits	Antagonists (IC_{50})	Agonists (EC_{50})
CaV1.2	CACANA1C	L-type	β, $\alpha_2\delta$	Nifedipine (10–100 nM) [97] Nimodipine (139 nM) [183] Diltiazem (500 nM) [72] Verapamil (60 nM) [72] Mibefradil (1.4–13 μM) [67, 110] Cd^{2+} (7 μM) [121] Ni^{2+} (280 μM) [121]	BayK 8644 (6 nM) [188] FPL64176 (211 nM) [188]
CaV3.1	CACNA1G	T-type		Mibefradil (0.4–1.2 μM) [67] Cd^{2+} (160 μM) [94] Ni^{2+} (167–250 μM) [94]	
CaV3.2	CACNA1H	T-type		Mibefradil (1.1–1.2 μM) [67] Cd^{2+} (160 μM) [94] Ni^{2+} (5.7–12 μM) [94]	
$K_{Ca}1.1$	KCNMA1	BK_{Ca}, Slo1	β1-4 (KCNMB1-4)	Iberiotoxin (1.7 nM) [176] Charybdotoxin (2.9 nM) [176] Paxilline (1.9 nM) [176] TEA (0.14 mM) [176]	NS1619 BMS204352 DHS-1 Estradiol
$K_{Ca}2.3$	KCNN3	$SK_{Ca}3$, SK3	Calmodulin	Apamin (10 nM) [176] UCL1684 (9.5 nM) [176] TRAM-34 (20 μM) [181]	EBIO (87–600 μM) [181] NS309 (120–900 nM) [181] SKA-31 (3 μM) [181]
$K_{Ca}3.1$	KCNN4	$IK_{Ca}1$, IK1	Calmodulin	Charybdotoxin (5 nM) [181] Clotrimazole (70 nM) [176] TRAM-34 (10–25 nM) [181] NS6180 (11 nM) [181]	EBIO (24–80 μM) [181] NS309 (10–27 nM) [181] SKA-31 (260 nM) [181]
KIR2.1	KCNJ2			Ba^{2+} (2 μM at −100 mV; 19–30 μM at −40 mV) [6, 99] Intracellular Mg^{2+} and polyamines [68] ML133 (1.9 μM) [172]	Extracellular K^+ (3–20 mM) [101]

KIR2.2	KCNJ12			Ba^{2+} (0.5 μM at −100 mV; 9 μM at -40 mV) [99] ML133 (2.9 μM) [172] Intracellular Mg^{2+} and polyamines [68]	Extracellular K^+ (3–20 mM) [101]
KIR2.3	KCNJ4			Ba^{2+} (10.3 μM at −100 mV; 70 μM at −40 mV) [99] ML133 (4 μM) [172] Intracellular Mg^{2+} and polyamines [68]	Extracellular K^+ (3–20 mM) [101]
KIR6.1	KCNJ8		SUR2b	Glibenclamide (20–100 nM) [122, 129] Tolbutamide (350 μM) [129]	Diazoxide (32 μM) [106] Pinacidil (0.6 μM) [106] Levcromakalim (79 nM) [106]
KIR6.2	KCNJ11		SUR2b	Glibenclamide (20–100 nM) [122, 129] Tolbutamide (350 μM) [129] ML133 (7.7 μM) [172]	Diazoxide (32 μM) [106] Pinacidil (0.6 μM) [106] Levcromakalim (79 nM) [106]
IP_3R1	ITPR1		See [47] for list of interacting proteins	Ca^{2+} (1.3–52 μM) [47] Heparin (4.1 μg/ml) [133] Xestospongin C/D (358–844 nM) [49] 2-Aminoethoxydiphenyl borate (2-APB) (42 μM) [111]	Ca^{2+} (57–348 nM) [47, 171] IP_3 (34 nM) [133] Adenophostin A (4.5 nM) [133]
IP_3R2	ITPR2		See [47] for list of interacting proteins	Ca^{2+} (1.3–52 μM)[a] [47] Heparin (22 μg/ml) [133] 2-Aminoethoxydiphenyl borate (2-APB) (~100 μM) [133]	Ca^{2+} (58 nM) [171] IP_3 (151 nM) [133]
IP_3R3	ITPR3		See [47] for list of interacting proteins	Ca^{2+} (0.3–39 μM) [47] Heparin (2.8 μg/ml) [133] 2-Aminoethoxydiphenyl borate (2-APB) (≫100 μM) [133]	Ca^{2+} (77 nM) [47] IP_3 (219 nM) [133] Adenophostin A (19.5 nM) [133]

(continued)

Table 1.1 (continued)

Channel	Gene	Alternative names	Accessory subunits	Antagonists (IC_{50})	Agonists (EC_{50})
RyR1	RYR1		See [46, 102] for list of interacting proteins	Ryanodine (100 nM to 1 μM)[b] [192] Tetracaine (100 μM) [192]	Ryanodine (>10 μM)[b] [192] Caffeine (0.2–0.5 mM) [192]
RyR2	RYR2		See above	See above	See above
RyR3	RYR3		See above	See above	See above
TRPA1	TRPA1	NAKTM1, TRPN1		Allyl isothiocyanate (AITC) (4.4–16.2 μM) [154]	HC-030031 (0.7–6.3 μM) [112]
TRPC1	TRPC1	TRP1	TRPC3, TRPC4 IP_3R1 STIM1, ORAI1	Gd^{3+} (1–10 μM) [10] La^{3+} (1–10 μM) [10] 2-Aminoethoxydiphenyl borate (2-APB) (80 μM) [10]	
TRPC3	TRPC3	TRP3	TRPC1 IP_3R1	Pyr-3 (0.7 μM) [84] Gd^{3+} (0.1 μM) [58] La^{3+} (4 μM) [58] SKF96365 (8 μM) [58]	OAG (100 μM) [71]
TRPC4	TRPC4	CCE1, TRP4	TRPC1, STIM1	ML-204 (0.96 μM) [116]	La^{3+} (100–300 μM peak effect) [138]
TRPC6	TRPC6	TRP6		La^{3+} (13–50 μM) [14, 42] SKF96365 (5–25 μM) [14, 42] ML-9 (36 μM) [64]	Hyperforin (1.5 μM) [96] OAG (117 μM) [71]
TRPV3	TRPV3		Calmodulin	Ruthenium red (10 μM) [38]	Carvacrol (4–34 μM) [38] 6-*Tert*-butyl-**m**-cresol (370 μM) [168]
TRPV4	TRPV4	TRP12, VRL-2	Calmodulin	HC-067047 (17–133 nM) [43]	GSK1016790A (18 nM) [161]

[a]The inhibitory effect of Ca^{2+} on Ca^{2+} release through IP_3R depends on the concentration of IP_3 to which the channel is exposed. The values shown are for $[IP_3] = 10–100$ nM for IP_3R1 [105] and 20 nM to 10 μM for IP_3R3 [103]

[b]Concentrations required to block the channel

but has large effects on the maximum open-state probability of the channels; low ATP severely reduces the maximal activity of IP_3R2 [104, 171]. Given that IP_3Rs exist in signaling microdomains adjacent to ATPases in the ER and in the plasma membrane, local ATP concentrations could have a profound effect on IP_3R function in an isoform-specific fashion. Finally, IP_3R interact with a large number of proteins in the cytosol and in the lumen of the ER that can modulate the activity of these channels by protein-protein interactions, and phosphorylation/dephosphorylation in the case of protein kinases and phosphatases [47, 159].

Freshly isolated rat aortic endothelial cells express transcripts for all three isoforms of IP_3R with IP_3R1 being most highly expressed [118, 119]. Endothelial cells from mouse mesenteric arteries express transcripts for all three isoforms with IP_3R2 being most highly expressed [93]. Mouse cremaster arteriolar endothelial cells also express transcripts for all three isoforms, but IP_3R3 appears to be most highly expressed (Fig. 1.4a), and protein expression for all three isoforms has been reported [77]. All three IP_3R isoforms were detected in the endothelium of Wistar rat basilar and mesenteric arteries by immunocytochemistry [56]. Thus, it appears that there may be regional or species-dependent differences in the expression of IP_3R isoforms, and little is known about the localization or function of the individual IP_3R isoforms in native endothelial cells.

In co-cultures of smooth muscle and endothelial cells from mouse cremaster arterioles, and in intact vessels, it has been reported that IP_3R1 localizes at sites of myoendothelial gap junctions (MEJs) [77]. Similarly, in intact mouse mesenteric resistance vessels, there are clusters of IP_3Rs near holes in the internal elastic lamina [93], sites that have been correlated with projections of endothelial cells (myoendothelial projections, MEPs, Fig. 1.3) towards overlying smooth muscle cells and the localization of MEJs [136]. However, the IP_3R isoform expressed in these clusters was not identified. Importantly, these sites were shown to generate localized endothelial cell Ca^{2+} events that have been termed Ca^{2+} pulsars [93]. These events are too small and rapid to be detected by global Ca^{2+} measurements made with Fura 2 (Fig. 1.2, for example), but can be detected using Fluo-4 or genetic Ca^{2+}sensors such as GCaMP2 and high-speed confocal imaging [83, 93]. Previous studies have shown that $K_{Ca}3.1$ channels also are clustered in the same microdomain [137] providing a means for Ca^{2+} pulsars to be translated into, for example, changes in membrane potential (see below for more on this topic). In addition, a growing list of proteins congregate in the vicinity of MEJs including TRPA1 channels [37], TRPV4 channels [149, 150], anchoring proteins (e.g., AKAP150 [150]), protein kinases (e.g., PKC [150]), nitric oxide synthase [151], Na^+/K^+ ATPase [33] and other proteins [152].

Calcium pulsars [83, 93] and Ca^{2+}waves [36, 83] are present under resting conditions in endothelial cells of pressurized vessels [83, 93] and likely contribute to the resting activity of $K_{Ca}3.1$ channels (in the case of pulsars and waves) and $K_{Ca}2.3$ channels (Ca^{2+} waves) and endothelial cell membrane potential. Endothelium-dependent vasodilators, such as acetylcholine [93, 147] or adenosine [36], increase the number and frequency of Ca^{2+} pulsars [93], and also recruit IP_3R located throughout endothelial cells to produce asynchronous [93, 147] or synchronous [36,

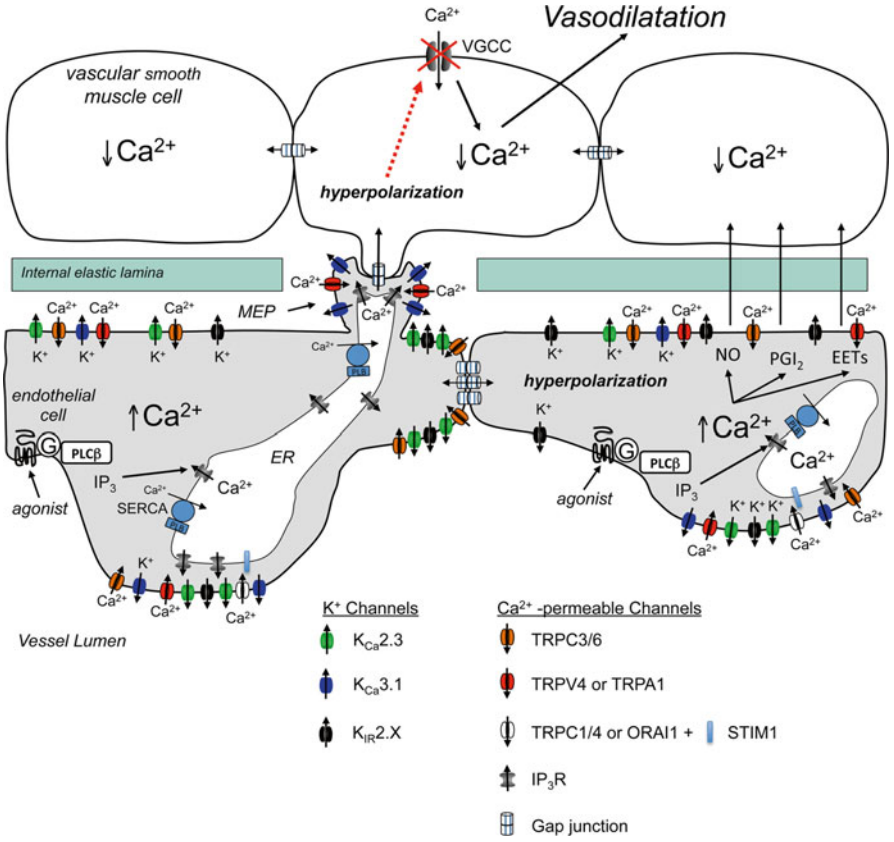

Fig. 1.3 Endothelial cell Ion channels and cell-cell communication in the vessel wall. Shown is a schematic representation of a longitudinal cross section through two endothelial cells and their relationship to overlying smooth muscle cells. Endothelial cells communicate with overlying smooth muscle cells via myoendothelial projections (MEPs), that pass through the internal elastic lamina to make contact with overlying smooth muscle cells, as shown. Gap junctions may form at MEPs to yield myoendothelial junctions (MEJs) allowing endothelial cell hyperpolarization to be conducted to the smooth muscle cells, closing smooth muscle voltage-gated Ca^{2+} channels (VGCCs) and leading to vasodilatation. Ion channels such as TRPV4, $K_{Ca}3.1$ and IP_3R (as shown) may cluster in MEPs to form signaling complexes to direct the endothelial cell responses to vasodilator agonists. Other ion channels such as TRPC3 and $K_{Ca}2.3$ may cluster elsewhere to form other signaling complexes. Abbreviations are as in Fig. 1.1

147] Ca^{2+}waves and increases in global Ca^{2+} (Fig. 1.2). Thus, the global Ca^{2+} signals that have been reported in native microvascular endothelial cells [23, 32, 107, 148] represent a complex mixture of local Ca^{2+} pulsars and Ca^{2+} waves in addition to more homogeneous increases in cytosolic Ca^{2+}. Additional studies are needed to define the precise localization of IP_3R isoforms and their function related to endothelium-dependent vasomotor activity.

Ryanodine Receptors

Ryanodine receptors (RyR) are composed of very large protein subunits (~500 kDa) that form Ca^{2+}-sensitive-Ca^{2+}-release channels in the endoplasmic reticulum [61]. Similar to IP_3R, they are tetramers with three isoforms from three distinct genes: RyR1, RyR2 and RyR3 [61, 62, 185]. Skeletal muscle expresses predominantly RyR1, the heart expresses predominantly RyR2 and RyR3 is expressed in the brain and other tissues [61, 62, 185]. Vascular smooth muscle cells may express all three isoforms, with RyR2 being predominant in resistance artery [166] and arteriolar smooth muscle cells [178]. Immunofluorescence demonstrated RyR expression in guinea pig endocardium and aortic endothelial cells [95], and ryanodine binding sites have been reported in porcine coronary artery endothelial cells [54]. Studies of cultured endothelial cells suggest expression of functional ryanodine receptors [191], and freshly isolated endothelial cells from rabbit aorta display caffeine-induced Ca^{2+} transients implying the presence of RyR in these cells [132]. In porcine coronary artery endothelial cells, caffeine elicits a Ca^{2+} transient in only 37 % cells studied suggesting heterogeneity of the distribution and function of endothelial RyR [55]. Transcripts for RyR3, but not RyR1 or RyR2 have been reported in endothelial cells freshly isolated from human mesenteric arteries [89]. Thus, RyR appear to be expressed and functional in macrovascular endothelial cells. However, there is little evidence for expression of ryanodine receptors in microvascular endothelial cells. In mouse mesenteric resistance arteries, where expression of IP3Rs are readily detected, no message for the three RyR isoforms were found, and ryanodine had no effect on basal or acetylcholine-stimulated Ca^{2+} events [93]. We have also found lack of expression of the three RyR isoforms in endothelial cells from mouse cremaster arterioles (Fig. 1.4b), and caffeine does not elicit a Ca^{2+} transient in freshly isolated hamster cremaster arteriolar endothelial cells [23] (Fig. 1.5a), although lack of effect of caffeine on global Ca^{2+} levels does not completely exclude a role for

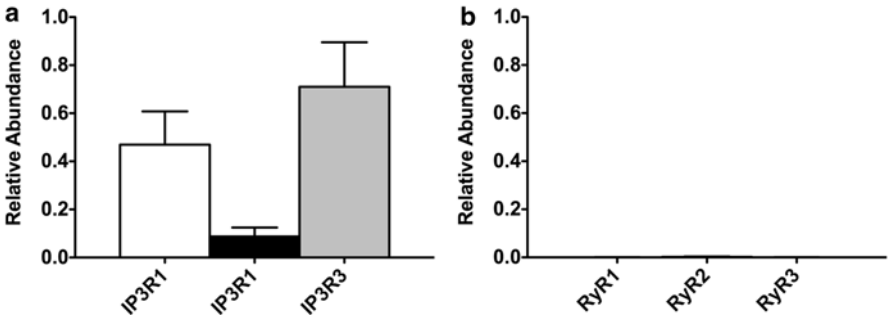

Fig. 1.4 Expression of transcripts for IP_3R and RyR in freshly isolated mouse cremaster arteriolar endothelial cells. Shown are means ± SE (n = 5 cell isolates for IP_3R and n = 7 for RyR) abundance of transcripts for IP_3R (panel **a**) and RyR (panel **b**) isoforms relative to eNOS in endothelial cell tubes isolated from second-order mouse cremaster arterioles. See [23, 178] for methodological details

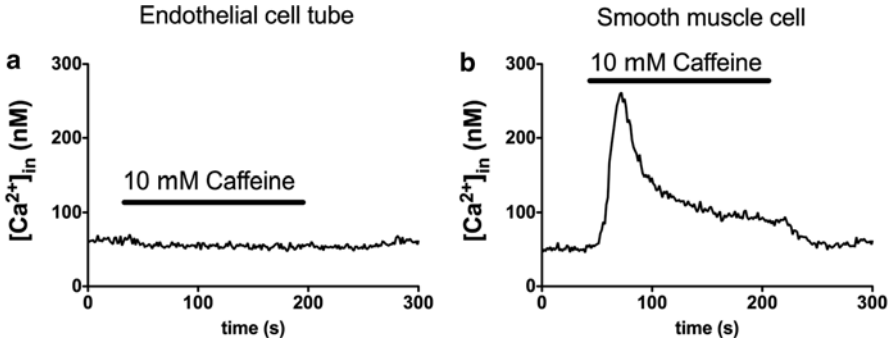

Fig. 1.5 Failure of caffeine to elicit Ca^{2+} transients in arteriolar endothelial cells but not smooth muscle cells. Shown are representative responses of an endothelial cell tube (panel **a**) and a smooth muscle cell (Panel **b**) isolated by the same method from hamster cremaster arterioles [23], to the RyR agonist, caffeine (10 mM). Consistent with the lack of expression of RyR in arteriolar endothelial cells (see Fig. 1.4b), caffeine failed to elicit a Ca^{2+} transient above baseline in endothelial cells in endothelial cells (n = 5 isolates from five arterioles), but produced the expected response from smooth muscle cells isolated from the same vessels

RyR [126]. A lack of effect of the RyR antagonist, ryanodine, on Ca^{2+} signals in endothelial cells in rat mesenteric arteries also has been observed [83]. Although species and regional heterogeneity in the expression of RyR cannot be excluded, these data suggest that RyR do not play a major role in Ca^{2+} signaling in endothelial cells of resistance arteries and arterioles.

What Ion Channels Mediate Agonist-Induced Ca^{2+} Influx?

TRP Channels

Endothelium-dependent vasodilators not only increase the activity of IP_3R, by stimulating the production of IP_3, they also result in the activation of ion channels in the plasmalemma of endothelial cells that conduct Ca^{2+} and are responsible for steady-state increases in intracellular Ca^{2+} (i.e., the plateau phase of the Ca^{2+}transient shown in Fig. 1.2). Early studies in cultured endothelial cells provided evidence that agonist-induced Ca^{2+} entry was electrophysiologically and pharmacologically similar to the Ca^{2+} entry induced by depletion of intracellular Ca^{2+} stores [139, 142, 143, 164, 165]. In primary cultures of porcine coronary artery endothelial cells, substance P activates a non-selective, inward whole-cell cation current that can be completely inhibited by blocking IP_3-dependent activation of IP_3R with heparin [143]. Similarly, block of IP_3R with xestospongin-D abolishes methacholine-induced global Ca^{2+} transients in arteriolar endothelial cells [23] (Fig. 1.2e). These data suggest that, at least under the conditions of these experiments, agonist-induced activation of IP_3R, and likely release of Ca^{2+} from internal stores is required to activate the

Ca^{2+} influx pathway that is responsible for the plateau phase of the agonist-induced global Ca^{2+} transients, and hence the steady-state phase of agonist-induced endothelial cell hyperpolarization. This does not exclude the activation of receptor, or second-messenger activated Ca^{2+} influx as the currents activated may be too small to detect by conventional whole-cell methods, or may produce only local changes in Ca^{2+} that do not influence global Ca^{2+}, particularly as detected by Fura-2.

The ion channels that are responsible for agonist-induced Ca^{2+} influx in native endothelial cells remain in question. Several members of the transient receptor potential (TRP) family of ion channels, in particular TRPC1, TRPC3, TRPC4, TRPC6 and TRPV4, along with members of the stromal interaction molecule (STIM) and ORAI families appear to be likely candidates, and it is also likely that multiple channels are activated and contribute to the Ca^{2+} influx activated by endothelium-dependent vasodilators (Fig. 1.1).

The TRP channel family form, in general, cation channels that are weakly Ca^{2+} selective (permeability for Ca^{2+}/permeability for Na^{2+} <10) [179]. The channel monomers are assumed to have six membrane spanning domains with the pore between segments 5 and 6, and both the C- and N-termini of the channels located intracellularly, with four monomers forming a functional channel [179].

Endothelial cells express TRPC1, which may serve as store-operated Ca^{2+} channels in endothelial cells [124]. However, their function in agonist-induced endothelial hyperpolarization and vasodilatation remains unclear. This may partly be due to the observation that TRPC1 heteromultimerize with other members of the TRPC family (especially TRPC4) as well as STIM/ORAI containing channels [30]. Studies of cultured human pulmonary artery endothelial cells revealed expression of TRPC1, and that antisense oligonucleotide knockdown reduced Ca^{2+} influx induced by Ca^{2+} store depletion by about 50 % suggesting that TRPC1 contributes to the Ca^{2+} influx pathway in these cells [17]. A similar conclusion was drawn in cultured bovine aortic endothelial cells using a TRPC1 antibody to inhibit basic fibroblast growth factor-induced Ca^{2+} entry [7]. Studies of human and mouse pulmonary microvascular endothelial cells also support a role for TRPC1 in agonist- and Ca^{2+} store depletion-induced Ca^{2+} entry and microvascular permeability [5, 92, 114, 155, 156]. However, the roles played by TRPC1 in agonist-induced endothelial cell hyperpolarization and vasodilatation is not as clear. Study of carotid artery endothelial cells from TRPC1 knock-out mice reveal enhanced acetylcholine-induced hyperpolarization, rather than decreased responses predicted based on studies of cultured cells and lung models [140]. Although compensatory upregulation of expression of other channels might explain these results, prior experiments failed to detect upregulation of other TRPC channels [31]. At the least, these results indicate that TRPC1 is not essential for agonist-induce endothelial cell hyperpolarization and that additional channels contribute to the Ca^{2+} influx induced by agonists in native endothelial cells, although regional heterogeneity in expression and function cannot be excluded. In contrast, studies of native aortic endothelial cells from TRPC1$^{-/-}$ mice demonstrated a small reduction of endothelial cell Ca^{2+} transients induced by acetylcholine [87], suggesting that TRPC1 channels do participate, to a small extent, in agonist-induced Ca^{2+} signals. A small reduction in thrombin-induced

Ca^{2+} transients was also observed after siRNA knock down of TRPC1 in cultured pulmonary microvascular endothelial cells [155]. Taken together, these data do not support a major role for TRPC1 in agonist-induced Ca^{2+} entry into endothelial cells relevant to endothelial cell hyperpolarization and vasodilatation.

TRPC4 is another channel that has been implicated in agonist and store-depletion-induced Ca^{2+} entry into endothelial cells [48, 155, 162]. In cultured aortic endothelial cells isolated from TRPC4$^{-/-}$ mice, the plateau phase of agonist-induced Ca^{2+} transients and related endothelial cell hyperpolarization was substantially depressed (but not eliminated) [48], suggesting a major role for TRPC4 in agonist-induced Ca^{2+} transients in macrovascular endothelial cells. Similarly, use of cultured, pulmonary microvascular endothelial cells isolated from TRPC4$^{-/-}$ mice, as well as siRNA knock down of endogenous TRPC4 from cells isolated from wild-type mice demonstrated a major role for TRPC4 in thrombin- and Ca^{2+}-store-depletion-induced Ca^{2+} signals [155]. The authors also demonstrated that expression of STIM1 was necessary for normal store-operated Ca^{2+} entry and showed that STIM1 and TRPC4 interacted and that expression of both proteins was required for normal Ca^{2+} signaling [155]. Endothelium-dependent vasodilatation is also substantially reduced in vessels from TRPC4$^{-/-}$ mice [48]. These data support a role for TRPC4 as a Ca^{2+} influx pathway during agonist-induced Ca^{2+} signaling, with activity triggered by loss of Ca^{2+} from internal stores as sensed by STIM1 (Fig. 1.1).

In human umbilical vein endothelial cells, the Ca^{2+} influx induced by agonists or depletion of intracellular stores depends on expression of STIM1 and ORAI1 with STIM1 serving as the sensor of ER Ca^{2+} and ORAI1 forming the pore of the store-operated channels [1, 155, 190]. Furthermore, in contrast to the studies outlined above, it was shown that effective knock down of TRPC1 or TRPC4 had no effect on Ca^{2+} store depletion-induced Ca^{2+} signals in this model [1]. However, studies in murine pulmonary microvascular endothelial cells indicate that expression of ORAI1 is not required for normal endothelial cell Ca^{2+} signaling [155]. These data suggest that there may be regional or species-dependent differences in the ion channels responsible for agonist-induced Ca^{2+} signaling in endothelial cells. The role played by STIM1 and ORAI1 in agonist-induced endothelial cell hyperpolarization of endothelial cells in resistance arteries and arterioles has not been reported.

TRPV4 channels also participate in agonist- and shear-stress-induced Ca^{2+} signals in endothelial cells, and endothelium-dependent vasodilatation in intact vessels [8, 35, 39, 109, 149, 150, 187]. These channels are temperature sensitive [175], stretch-sensitive [170] and can be activated by EETs (Fig. 1.1) and phorbol ester derivatives [170]. Calmodulin interacts with the C-terminal domain and mediates Ca^{2+}-dependent activation of these channels [153]. Activation of TRPV4 channels in endothelial cells increases intracellular Ca^{2+} [109, 149, 150, 174, 187] and produces vasodilatation [39, 134, 149, 150, 187]. Importantly, the plateau-phase of vasodilator agonist-induced endothelial cell Ca^{2+} transients are reduced in the endothelium of vessels from TRPV4$^{-/-}$ mice [149, 150, 187]. In mouse mesenteric arteries, TRPV4 channels cluster in the same microdomain as IP_3R at MEPs (Fig. 1.3), and endothelium-dependent agonists activate these channels to produce TRPV4-Ca^{2+}sparklets at the sites of MEPs [149, 150]. Activation of Ca^{2+} influx through only

a few TRPV4 channels per endothelial cell activates endothelial $K_{Ca}2.3$ and $K_{Ca}3.1$ channels and can produce maximal vasodilatation, with activation of $K_{Ca}3.1$ channels, which also cluster at MEJ's, occurring preferentially at low levels of TRPV4 activation [149]. Block of TRPV4 channels with HC-067047 in mouse mesenteric arteries substantially inhibits the component of acetylcholine-induced vasodilatation that is mediated by activation of endothelial $K_{Ca}2.3$ and $K_{Ca}3.1$ channels, suggesting that TRPV4 channels play a major role in agonist-induced Ca^{2+} influx that contributes to endothelial cell hyperpolarization and subsequent vasodilatation [149]. In mouse mesenteric arteries, muscarinic receptor agonists activate TRPV4 channels through a signaling pathway involving PLC-β and activation of PKC that is targeted to the channel by the scaffolding protein, AKAP150 [150] (Fig. 1.1). Thus, TRPV4 appears to play a major role in agonist-induced endothelial cell Ca^{2+} signaling that is related to hyperpolarization and vasodilatation.

Evidence also has been presented suggesting that TRPC3 is involved in agonist-induced Ca^{2+} signaling in endothelial cells [75, 86, 87, 98, 141, 184]. These TRP channels can be activated by DAG formed through the action of PLCs on membrane phospholipids [71] (Fig. 1.1). In cerebral vascular smooth muscle, activation of IP_3R1, independent from release of Ca^{2+}, results in activation of TRPC3 [4]. It is not known if a similar interaction occurs in endothelial cells. TRPC3 is involved in flow- and bradykinin-induced vasodilatation in rat small mesenteric arteries, but not dilation induced by histamine, ATP or cyclopiazonic acid [98]. As with TRPV4, TRPC3 appears to cluster near MEPs in rat mesenteric resistance artery endothelial cells [141]. In rat mesenteric arteries, the TRPC3 blocker, Pyr3, inhibited endothelial cell hyperpolarization and the portion of acetylcholine-induced endothelium-dependent vasodilatation mediated by activation of $K_{Ca}2.3$ and $K_{Ca}3.1$ channels and the consequent endothelial cell hyperpolarization [141]. These data suggest a close physical and functional relationship between TRPC3 and the K_{Ca} channels that mediate agonist-induced endothelial cell hyperpolarization. In contrast, in porcine coronary arteries, the TRPC3 antagonist Pyr3 inhibited the portion of bradykinin-induced vasodilatation that is mediated by NO, NO production in isolated endothelial cells and bradykinin-induced endothelial cell Ca^{2+} transients suggesting that TRPC3 channels contributed to Ca^{2+} signaling directed at NO production in this system [75]. In murine cerebral arteries, ATP-induced endothelial cell Ca^{2+} transients are reduced by Pyr3 or in cells isolated from $TRPC3^{-/-}$ mice, and Ca^{2+} entry through TRPC3 appears to selectively activate $K_{Ca}2.3$ channels during the plateau-phase of agonist-induced endothelial cell hyperpolarization [86]. Thus, there appear to be regional and likely species-dependent differences in the roles played by TRPC3 in endothelial cell Ca^{2+} signaling and hyperpolarization. This likely represents differences in the localization of the channels and the signaling microdomains in which they are expressed.

Endothelial cells in cerebral arteries also express TRPA1 channels that, when activated, produce endothelial cell hyperpolarization and endothelium-dependent vasodilatation [37, 154]; the endothelium of mouse, rat or human coronary, renal or mesenteric arteries do not express transcripts or protein for TRPA1 [154]. The TRPA1 subunits have 14–18 ankyrin repeats at their amino-terminal that give the

channels their name, are activated by a diverse array of chemicals including pungent substances found in mustards and garlic and are heavily expressed in sensory nerves [9]. In endothelial cells of cerebral arteries, these channels cluster at MEPs and co-localize with $K_{Ca}3.1$ channels [37, 38, 154] (Fig. 1.3). Activation of TRPA1 leads to TRPA1-Ca^{2+}sparklets [154]. Vasodilatation induced by activation of TRPA1 channels with allyl isothiocyanate (AITC) in substantially inhibited by the $K_{Ca}3.1$ blocker, TRAM34 and abolished by the combination of TRAM34 and the $K_{Ca}2.3$ blocker, apamin, but is unaffected by blockade of nitric oxide synthase or cyclooxygenase [37, 154]. These data indicate that Ca^{2+} influx through TRPA1 primarily activates endothelial cell K_{Ca} channels to produce vasodilatation. Furthermore, as with TRPV4 channels [149], only a small number of active TRPA1 channel clusters per endothelial cell are required for maximal vasodilatation [154]. It was also shown that vasodilatation induced by AITC could be inhibited by Ba^{2+} suggesting that inward rectifier K^+ channels either amplify the hyperpolarization induced by activation of endothelial cell K_{Ca} channels, or transduce the endothelial cell K_{Ca} channel activation by detecting the K^+ released through the K_{Ca} channels [37] (Fig. 1.1). In the endothelium of cerebral arteries, TRPA1 co-localizes with NADPH oxidase (NOX) isoform 2, and lipid peroxides produced by NOX2 activate these channels to produce vasodilatation [154].

Expression and function of TRPV3 channels in rat cerebral artery endothelial cells also has been reported [38]. These channels appear to be more uniformly expressed than TRPV4 or TRPA1 [38]. Activation of TRPV3 with agents such as the oregano monoterpenoid phenol, carvacrol, increases endothelial cell Ca^{2+} and activates endothelial cell K_{Ca} channels to produce endothelial cell hyperpolarization and vasodilatation [38].

Finally, endothelial cells also express TRPC6 that appears to contribute to Ca^{2+} signaling [21, 60, 100, 127, 145]. As for TRPC3, TRPC6 is activated by DAG produced simultaneously with the formation of IP_3 by PLCs [71] (Fig. 1.1 and Table 1.1). Human pulmonary artery endothelial cells express TRPC6, and siRNA knockdown of this channel impairs Ca^{2+} signaling and increases monolayer permeability induced by thrombin [145]. Human dermal microvascular endothelial cells in culture express TRPC6, but not TRPC3, and the plateau phase of the histamine-induced increase in Ca^{2+} is reduced by SKF96365 in these cells. Furthermore, histamine-induced increases in microvascular permeability are abolished in $TRPC6^{-/-}$ mice supporting a role for this channel in regulation of microvascular Ca^{2+} signaling and permeability [21]. Increased microvascular permeability induced by vascular endothelial growth factor (VEGF) appears to be mediated by TRPC6 [127], and VEGF-induced Ca^{2+} transients are reduced in cultured human microvascular endothelial cells expressing a dominant-negative form of TRPC6 [60]. Mouse aortic endothelial cells express TRPC6 and TRPC3, but carbachol-induced Ca^{2+} transients in isolated endothelial cells as well as carbachol-induced endothelium-dependent relaxation of aortas are reduced only in vessels isolated from $TRPC6^{-/-}$ mice [100]. The role of TRPC6 in agonist-induced Ca^{2+} signaling related to vasodilatation of arterioles and resistance arteries remains to be established.