1521-0081/66/2/513–569$25.00
PHARMACOLOGICAL REVIEWS

http://dx.doi.org/10.1124/pr.112.007351
Pharmacol Rev 66:513–569, April 2014

ASSOCIATE EDITOR: CHRISTOPHER J. GARLAND

Regulation of Cellular Communication by Signaling Microdomains in the Blood Vessel Wall

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This work was supported by National Institutes of Health National Heart, Lung, and Blood Institute [Grants HL088554 (to B.E.I.) and HL107963 (to B.E.I.)]; and an American Heart Association predoctoral fellowship (to A.W.L.).

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dx.doi.org/10.1124/pr.112.007351.

ABBREVIATIONS: 4αPDD, 4-α-phorbol-12,13-didecanoate; ADAMTS13, A Disintegrin And Metalloproteinase with Thrombospondin type1 Motifs, member 13; Ang II, angiotensin II; α-SNAP, α-soluble NSF attachment protein; bEnd, immortalized mouse brain endothelial cell line; BH_4, tetrahydrobiopterin; BK_{Ca}, calcium-dependent large conductance potassium channels; Ca^{2+}, calcium; $[Ca^{2+}]_I$, intracellular calcium concentration; $[Ca^{2+}]_{ER}$, calcium concentration in the endoplasmic reticulum; $[Ca^{2+}]_{mi}$, mitochondrial calcium concentration; CaM, calmodulin; CaR, calcium-sensitive receptor; CAT1, cationic amino acid transporter 1; Cav1, caveolin-1; CICR, calcium-induced calcium release; CIRB, calmodulin and inositol 1,4,5-phosphate receptor binding domain; Cl^-, chloride ion; CO, carbon monoxide; Cx, connexin; CytB5R3, cytochrome B_5 reductase 3; DHHC, aspartate-histidine-histidine-cystine motif; EC, endothelial cells; EDH, endothelium-dependent hyperpolarization; EDHF, endothelium-derived hyperpolarizing factor; EDRF, endothelium-derived relaxant factor; eNOS, endothelial nitric-oxide synthase; ER, endoplasmic reticulum; ERα, estrogen receptor α; GPCR, G protein-coupled receptor; GSNOR, *S*-nitrosoglutathione reductase; H_2O_2, hydrogen peroxide; H_2S, hydrogen sulfide; Hbα, hemoglobin α; HDL, high-density lipoprotein; HUVEC, human umbilical vein endothelial cells; ID4, intracellular domain 4; IK_{Ca}, calcium-dependent intermediate conductance potassium channels; IP_3, inositol 1,4,5-phosphate; IP_3R, IP_3 receptors; K^+, potassium; KO, knockout; L-NAME, N^{ω}-nitro-L-arginine methyl ester; MAPK, mitogen-activated protein kinase; MβCD, methyl β-cyclodextrin; MCU, mitochondrial calcium uniporter; MEJ, myoendothelial junction; MyRIP, myosin VIIA and Rab-interacting protein; NCX, sodium/calcium exchanger; nNOS, neuronal nitric-oxide synthase; NO, nitric oxide; Nox, NADPH oxidase; NSF, *N*-ethylmaleimide sensitive factor; $O_2^{\bullet-}$, superoxide anion; ONOO, peroxinitrite; P450, cytochrome P450; PA, phosphatidic acid; Panx, pannexin; PASMC, pulmonary arterial smooth muscle cells; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PLD1, phospholipase D_1; PM, plasma membrane; PMCA, plasma membrane calcium ATPase; Rab, Ras-related protein; RalGDS, Ral guanine-nucleotide dissociation stimulator; RyR, ryanodine receptor; S1P, sphingosine-1-phosphate; SERCA, sarco/endoplasmic reticulum calcium ATPase; shRNA, short hairpin RNA; siRNA, small interfering RNA; SK_{Ca}, calcium-dependent small conductance potassium channels; Slp4-a, synaptotagmin-like protein 4; SMC, smooth muscle cell; SNARE, soluble NSF attachment protein receptor; SOD, superoxide dismutase; STIM, stromal interaction molecule; SR, sarcoplasmic reticulum; SR-B1, scavenger receptor type B1; TGN, *trans*-Golgi network; TRAM 34, 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole; TRAM 39, 2-(2-chlorophenyl)-2,2-diphenylacetonitrile; TRP, transient receptor potential channels; U-46619, (5*Z*)-7-[(1*R*,4*S*,5*S*,6*R*)-6-[(1*E*,3*S*)-3-hydroxy-1-octenyl]-2-oxabicyclo[2.2.1]hept-5-yl]-5-heptenoic acid; VGCC, voltage-gated calcium channels; VSMC, vascular smooth muscle cells; vWF, von Willebrand factor; WPB, Weibel-Palade bodies; ZO-1, zonula occludens-1.

***Abstract*——It has become increasingly clear that the accumulation of proteins in specific regions of the plasma membrane can facilitate cellular communication. These regions, termed signaling microdomains, are found throughout the blood vessel wall where cellular communication, both within and between cell types, must be tightly regulated to maintain proper vascular function. We will define a cellular signaling microdomain and apply this definition to the plethora of means by which cellular communication has been hypothesized to occur in the blood vessel wall. To that end, we make a case for three broad areas of cellular communication where signaling microdomains could play an important role: 1) paracrine release of free radicals and gaseous molecules such as nitric oxide and reactive oxygen species; 2) role of ion channels including gap junctions and potassium channels, especially those associated with the endothelium-derived hyperpolarization mediated signaling, and lastly, 3) mechanism of exocytosis that has considerable oversight by signaling microdomains, especially those associated with the release of von Willebrand factor. When summed, we believe that it is clear that the organization and regulation of signaling microdomains is an essential component to vessel wall function.**

I. Introduction

It has become clear that proteins do not randomly accumulate at cellular foci but are instead organized at particular regions of the cell to exert their function in a more efficient manner. The vast majority of proteins does not act alone but are highly coordinated by a network of associated molecules that can modify, activate, or inhibit the protein's function. Concordantly, the numerous signaling molecules involved in intracellular signaling pathways often have a short half-life; thus their target must frequently be spatially localized to their site of production. For example, the half-life of inositol 1,4,5-trisphosphate (IP_3) produced by phospholipase C (PLC) is of the order of 30 ms with a diffusion coefficient of approximately 300 $\mu m^2/s$ because of its rapid degradation by localized 5-phosphatases (Wang et al., 1995). Therefore, having IP_3 receptors (IP_3R) in close proximity to the region where the IP_3 is produced maximizes the effect of the messenger (Berridge, 2006). This has been shown to be the case with PLC, because the enzyme has been found to reside in close proximity to IP_3R on the endoplasmic reticulum (ER) (e.g., Nomura et al., 2007; Weerth et al., 2007). Overall, there are few proteins that can diffuse to notable distances within the cell without being modified, activated, or inhibited in some way. Thus, it is important to have associated proteins within close proximity to efficiently maintain their function.

However, the question arises as to how the proteins associated with a particular function congregate to a precise location within the cell. It is now recognized that this can be accomplished by multiple factors, including but not exclusive to 1) differing lipid composition of the membrane, 2) unique addressing sequences within proteins directing them to the apical, basal, or lateral regions of the cells, 3) sequestration of proteins transcribed in local regions of the cell, and/or 4) associated protein-protein interaction into macromolecular structures (Lippincott-Schwartz et al., 2000). This last example forms the basis of signaling microdomains, where a group of proteins form a macromolecular complex that in turn can regulate cell-to-cell (paracrine) or cell-to-self (autocrine) signaling processes. There is currently no specific definition for a signaling microdomain, and so we have put forth a set of guidelines to define these nexuses (Table 1).

A. Definition of a Signaling Microdomain

The first characteristic of a signaling microdomain is that proteins are concentrated to a specific region within the cell (Table 1). As mentioned above, it would be difficult for proteins at opposite ends of a cell to have rapid, nonrandom associations, because they are not located in the same cellular location. The closer the protein association is, the more the effect could be deemed nonrandom and deliberate. This is especially true in specialized cellular structures such as the myoendothelial junction where hemoglobin α (Hbα) has been shown to accumulate and regulate nitric oxide diffusion to surrounding smooth muscle cells (Straub et al., 2012).

The next characteristic of a signaling microdomain is that the proteins are within specific regions of the plasma membrane (Table 1). The plasma membrane is composed of a variety of lipids, and it is now well understood that specialized lipid regions, especially those enriched with cholesterol, can harbor proteins together to create a signaling platform at the plasma membrane. Perhaps the most well-known of these specialized plasma membrane regions are lipid rafts and caveolin 1 (Cav1)-enriched caveolae, which are known to concentrate membrane receptors, transporters, and other signaling proteins (for review, see Popescu et al., 2006). The

TABLE 1
Rubrics defining a signaling microdomain

In this review, at least two rules listed below are required to consider a group of proteins as part of a *signaling microdomain* that can regulate cellular communication. Examples of signaling microdomain applicable to each guideline are indicated in the right column.

Guideline	Example
1. Proteins are concentrated to a region of the cell (i.e., apical membrane, myoendothelial junction) and altogether participate in a specific cellular function.	Exocytosis at the apical membrane (section IV) Endothelium-dependent hyperpolarization-mediated response (section III.C)
2. There is an accumulation of two or more proteins contained within a membranous phospholipid region (i.e., lipid raft, caveolae), and the loss of this structure alters cell-cell communication.	eNOS localized in caveolae (section II.A)
3. There is a direct protein-protein interaction, and disruption of this interaction alters cell-cell communication.	eNOS and caveolin 1 (section II.A.1) eNOS and Hbα (section II.A.4) Cx43 and ZO-1 (section III.A.4)
4. There is evidence for close localization of proteins, with a loss of one of the proteins (function or expression) altering the way in which cell-cell communication occurs.	Endothelium-dependent hyperpolarization-mediated response (section III.A)

association of endothelial nitric-oxide synthase (eNOS) with Cav1 can be spatially enriched in the vicinity of a number of cofactors and substrates (Mineo and Shaul, 2012). The membrane compartmentalization then can serve an important role in keeping proteins organized that together can regulate a particular function.

The third rule is that two or more proteins directly interact with each other (Table 1). There are numerous instances where two proteins actually bind together, and their tertiary states can affect one another's activation or inhibition (e.g., Jones, 2012). This would indicate that the proteins require direct association. An example of this can be found with connexin 43 (Cx43) and zonula occludens-1 (ZO-1) (see section III.A). There is a unique binding sequence for ZO-1 on Cx43 via a PDZ domain, and this interaction dictates both Cx43 trafficking to the plasma membrane and actual function of the fully formed gap junction (see section III.A). Thus the direct interaction of these two proteins serves as a model of how the discreet direct interaction of two proteins plays an important part in cellular communication.

Lastly, proteins may regionally associate without direct protein-protein interaction (Table 1). In this instance, the proteins may not be directly associated, but may be part of a larger macromolecular complex or spatially localized to a similar region of the cell where they work in concert. In this case, the functional association of several proteins is usually revealed using pharmacological tools and often involves an intracellular messenger with a short half-life (e.g., nitric oxide, superoxide anion, IP_3, calcium ion).

With a definition in hand, the focus of this review is centered on the organization of signaling microdomains and how they are functionally used by cells of the blood vessel wall, specifically the endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), for cellular communication (Fig. 1). The areas of cellular communication this review will focus on include 1) paracrine release of molecules such as nitric oxide or superoxide anion, 2) channel communication via gap junctions and other ion channels, and 3) exocytosis. There are other examples of released molecules, channels, transporter, or other proteins that play a role in cellular communication, but because they have yet to be fully recognized as being part of a signaling microdomain we have not discussed them in detail because they do not fit into the focus of this review (e.g., pannexin channels). There are other aspects of cellular communication including the role of integrins and chemotaxis that are not discussed here but can be found in multiple reviews (Herbert and Stainier, 2011; Hoffman et al., 2011).

B. On the Importance of Calcium Compartmentalization

Calcium is a ubiquitous second messenger that controls numerous cellular functions. How calcium is compartmentalized in cells and the consequences on vascular function

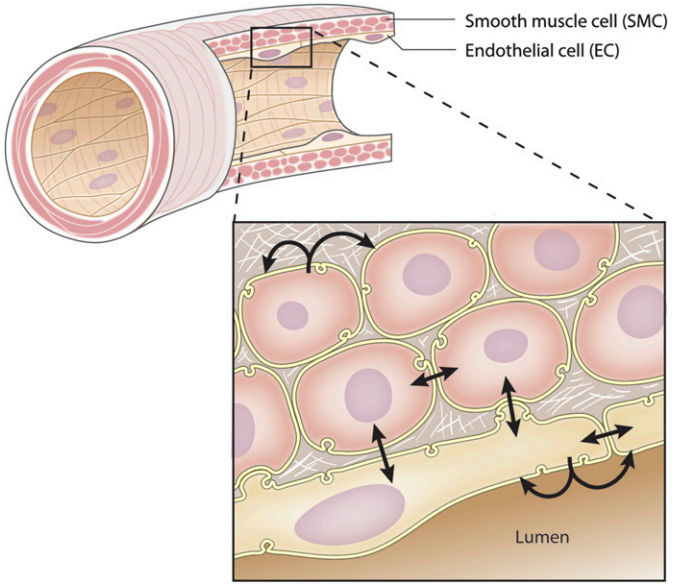

Fig. 1. Schematic representation of intercellular communication in the arterial wall. The SMCs and ECs composing the vascular wall can communicate with each other either by releasing molecules to neighboring cells (paracrine communication) or directly via gap junction channels that link the cytoplasm of two adjacent cells. The different types of intercellular communications are represented by the arrows.

must be discussed because such compartmentalization directly relates to both the coordination of intercellular communication between cells and their corresponding signaling microdomains. In addition, the calcium signaling microdomains at the origin of such compartmentalization are an excellent example of how cells accumulate proteins together to efficiently regulate cellular functions. The concept of calcium compartmentalization described in this section is by no means exhaustive and will not cover in detail the dynamics of calcium homeostasis; we invite the reader to refer to excellent reviews on this topic (Berridge, 2006; Bolton, 2006; Parekh, 2008; Putney and Bird, 2008; Hill-Eubanks et al., 2011; van Breemen et al., 2013).

Cells need to maintain a low intracellular calcium concentration ($[Ca^{2+}]_i$) to avoid inappropriate and random activation of signaling pathways. The cytosolic calcium concentration is maintained at a low level ($[Ca^{2+}]_i$ ~100 nM) through a multitude of pumps and calcium transporters at the plasma membrane and at the ER membrane, respectively, extruding calcium outside of the cell or storing calcium in the ER [or in the sarcoplasmic reticulum (SR), a specialized ER in muscle cells] (ZhuGe et al., 1999). Because the equivalent of the total free cytosolic calcium enters the cells every half second, these pumps and transporters have to be constitutively active to maintain a low $[Ca^{2+}]_i$ (Lee et al., 2002). The extracellular calcium concentration and the calcium concentration within the ER ($[Ca^{2+}]_{ER}$) are higher (approximately 2 mM and 200 μM, respectively). Additionally, it is now accepted that the mitochondria constitutes another buffer organelle to regulate cytoplasmic calcium concentration (for review, see McCarron et al., 2012).

Because calcium is a central molecule for cellular functions, the dynamics of calcium are complex to temporally and spatially control specific signaling pathways. Thus, it has become evident that cells do not regulate their $[Ca^{2+}]_i$ as a whole but more in discrete regions to activate specific, localized signaling pathways (i.e., the calcium signaling is compartmentalized). The activity level of the multiple calcium pumps, channels, and transporters are thus responsible for high calcium concentrations that can develop close to the plasma membrane as well as close to the ER membrane (Cheng et al., 1993; Nelson et al., 1995; Perez et al., 2001; Berridge et al., 2003; Navedo et al., 2005; Berridge, 2006; Niggli and Shirokova, 2007; Feletou, 2011b). To help with this, the different intracellular calcium compartments are close to one another and/or to the plasma membrane to form a restricted space, where the calcium can be confined. The best known examples of such intracellular membrane junctions are found in cardiac myocytes where the T-tubules of the sarcolemma and the terminal cisternae of the SR come into contact, a contact that is central in the excitation-contraction coupling of the cardiac muscle (Fabiato and Fabiato, 1972; McNutt, 1975).

Some of the best examples of compartmentalized calcium are in SMCs because they are the best described to date. Compartmentalization of calcium allows for the activation of specific cellular pathways, mainly through the activation of calcium-dependent enzymes located close to the sources of calcium, near the ER or the plasma membrane (Berridge, 2006). In contrast, other calcium-dependent enzymes located further from the sources of calcium (i.e., further from the ER or the plasma membrane) are not activated because of the rate of calcium diffusion (Berridge, 2006). A striking example of calcium compartmentalization is the observation that, although increases in whole $[Ca^{2+}]_i$ cause contraction of SMCs, a local subplasmalemmal increase in calcium facilitates relaxation (Nelson et al., 1995); this example will be discussed in detail in this section (see section I.A.1.a). Calcium homeostasis has particularly been investigated in vascular SMCs (VSMCs) because of the central role of calcium in the contractile process (Nelson et al., 1990; Fleischmann et al., 1994). Heterogeneous and high local calcium concentrations have been observed in VSMCs in multiple reports (e.g., Deth and van Breemen, 1977; Van Breemen, 1977; van Breemen et al., 1986; Laskey et al., 1992; Kargacin, 1994; Nelson et al., 1995; Rembold et al., 1995), and computer modeling of calcium signaling within the VSMCs showed that high calcium concentrations could occur in restricted spaces and persist for 100–200 ms (Kargacin, 1994, 2003; Naraghi and Neher, 1997). Based in part on these reports, calcium compartmentalization was conceptualized where localized $[Ca^{2+}]_i$ could activate the contractile apparatus without altering other calcium-dependent pathways (Karaki, 1989).

1. Spatial Organization of Intracellular Organelles Is Crucial For Efficient Calcium Compartmentalization. There is evidence that spatial localization of organelles can contribute to calcium compartmentalization, including (but not limited to) proximity of the SR and plasma membrane and proximity of the SR and mitochondria (Fig. 2A). Thus, the location of calcium entry from the outside of the cell or the location of the release of calcium from the SR into the cytoplasm is not only important in regard to the signaling proteins that are surrounding the calcium channel but also in regard to the intracellular organization of organelles (Poburko et al., 2004). Indeed, if calcium influxes occur at a location where intracellular organelles come into close contact with the plasma membrane, the latter will prevent free diffusion of calcium, making localized calcium concentration persist for longer periods of time. Conversely, if calcium influxes occur in a region of the plasma membrane where there are no intracellular organelles, the calcium will diffuse freely and dilute in the cytoplasm, and its effect on the surrounding signaling proteins will be lower (Kargacin, 1994). One example of importance in cell-cell communication is the localization of ER at the myoendothelial junctions (MEJ), a cellular structure linking ECs and SMCs that is embedded in extracellular matrix (for review, see Heberlein et al., 2009). The local release of calcium in this compartment, presumably from ER, has

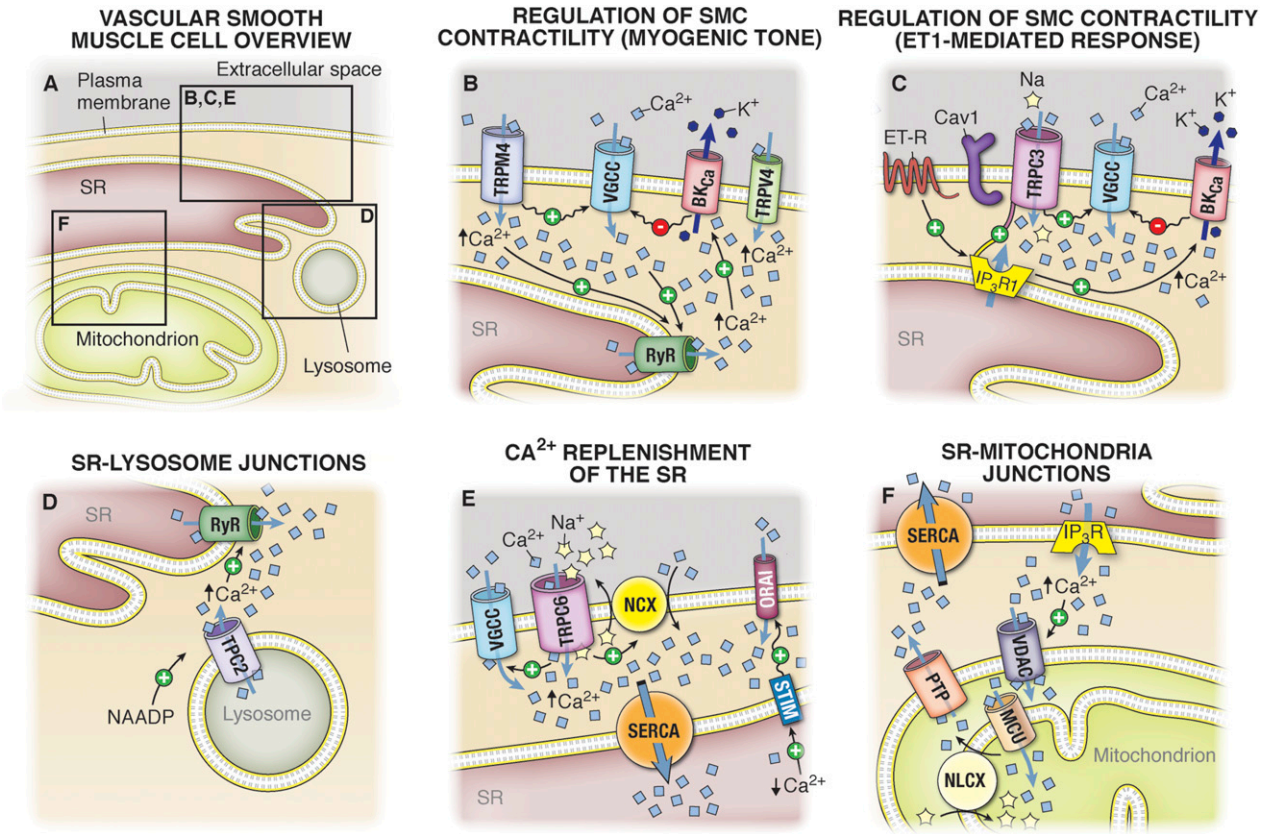

Fig. 2. Schematic representation of calcium compartmentalization. (A) In VSMCs, calcium (Ca^{2+}) can be stored in both the SR and in the mitochondria. Calcium release from both organelles is tightly coordinated to calcium influx at the PM, and this coordination is facilitated by the close proximity between the organelles and the PM. (B) In cerebral VSMCs, after increased intravascular pressure, there is a coordinated action of TRPV4, TRPM4, VGCC, and large conductance potassium channels (BK_{Ca}) at the plasma membrane and ryanodine receptors RyR at the SR membrane. In this configuration, increased pressure activates calcium influx in VSMCs via TRPV4, which stimulates calcium release from the SR through RyR, whereas opening of TRPM4 results in calcium influx through VGCC also activating calcium release from the SR through RyR. Calcium release from RyR (also termed calcium "sparks") further activates potassium (K^+) efflux via BK_{Ca} channels. The hyperpolarization resulting from potassium efflux reduces the activity of VGCC, making BK_{Ca} key in the autoregulation of calcium homeostasis in VSMCs. (C)Upon cerebral VSMC stimulation with ET-1, activation of IP_3-R1 at the SR membrane activates calcium influx through TRPC3 independently of calcium release via IP_3R but via a direct protein interaction between IP_3-R and TRPC3. This IP_3R/TRPC3 interaction is facilitated by the presence of Cav1. Calcium release via IP_3R upon ET-1 stimulation further activates BK_{Ca} channels at the plasma membrane in a similar manner as RyR activates BK_{Ca} channels in (B). Activation of BK_{Ca} induces hyperpolarization of the plasma membrane, thus attenuating the activation of VGCC by cation influx through TRPC3. (D) After stimulation with ET-1, nicotinic acid adenine dinucleotide phosphate (NAADP) activates the release of calcium from intracellular lysosomes via the two pore calcium channel (TCP2). The calcium released from the lysosome further activates calcium release from the SR via RyRs. (E) The compartmentalization of VGCC, TRP channels, the NCX, and the SERCA are part of a signaling microdomain controlling calcium replenishment of the PM-SR junction. In this configuration, calcium and Na^+ influxes via TRPC6 activate the adjacent VGCC and the NCX in reverse mode. Calcium influx via the VGCC and the NCX provide sources of calcium for ER/SR replenishment via the SERCA pump. Additionally, the STIM present at the SR membrane is capable of sensing decreased levels of calcium in the SR and activates calcium influx via Orai at the plasma membrane, again providing calcium for SR replenishment via the SERCA pumps. (F) Mitochondria also play a major role as a buffer and as a source of calcium for the SR. After stimulation of VSMCs, mitochondria take up the calcium released from IP_3R via the VDAC on the outer mitochondrial membrane and the mitochondrial calcium uniporter (MCU) on the inner mitochondrial membrane. The buffering role of mitochondria is essential to prevent the formation of high local calcium concentrations surrounding the IP_3-R, which would inhibit the IP_3-R activity. The release of calcium from the mitochondria via the mitochondrial sodium/calcium exchanger (NLCX) present on the inner mitochondrial membrane and the permeability transition pore (PTP) on the outer mitochondrial membrane provides a source of calcium for SR replenishment by the SERCA pumps. Straight arrows with positive and negative signs indicate activation and inhibition by Ca^{2+}, respectively. Wavy arrows with a positive or negative sign indicate activation by depolarization or an inhibition by hyperpolarization respectively.

been observed in numerous instances (Ledoux et al., 2008; Bagher et al., 2012), which could act to regulate eNOS or other localized channels such as IK_{Ca} channels (see sections II.A and III.B).

Observations of plasma membrane-SR junctions (PM-SR junctions) were reported nearly 50 years ago (Fawcett, 1961; Rosenbluth, 1962; Franzini-Armstrong, 1964; McNutt, 1975). In these articles, the authors observed the close proximity of two biological membranes separated by a cytoplasmic space of 10–30 nm wide over a few hundred nanometers in both skeletal and cardiac muscle cells. These junctions were functionally associated with the calcium-induced calcium release (CICR) present in cardiac and skeletal muscles (Fawcett, 1961; Rosenbluth, 1962; Franzini-Armstrong, 1964; McNutt, 1975). In the VSMCs, the close apposition of the SR to the plasma membrane has been suggested by several investigators (Somlyo et al., 1971, 1979; Somlyo, 1985; Benham and Bolton, 1986; Hermsmeyer and Sturek, 1986), and the presence of a CICR and

very high local calcium concentrations in the cytoplasmic space between the two membranes were both evidenced in this cell type (Van Breemen, 1977; van Breemen et al., 1986). Since the presence of PM-SR junctions were identified, van Breemen et al. (2013) proposed that the SR in VSMCs is capable of forming at least eleven types of junctions with other intracellular organelles and with itself. However, the junctions formed by apposition of the plasma membrane and the SR membrane are the most abundant SR junctions in the VSMCs (van Breemen et al., 2013).

Structurally, various reports have identified a series of proteins that play an important role in the architectural organization of PM-SR junctions. Junctophilins (Takeshima et al., 2000; Komazaki et al., 2002) and junctate (Treves et al., 2010; Srikanth et al., 2012) are two transmembrane proteins expressed at the SR membrane that constitute protein bridges that keep the SR and plasma membranes close and may prevent their fusion (Carrasco and Meyer, 2011). For example, PM-SR junctions are absent in cardiac myocytes from mice deficient in the cardiac isoform of junctophilins gene (Takeshima et al., 2000). Further studies on these proteins could help reveal another key component to localization of calcium-related organelles.

2. Examples of Calcium Compartmentalization Involved in Vascular Smooth Muscle Cells Contractile State. When VSMCs are stimulated, the coordination between all of the molecular players responsible for calcium entry into the cell and calcium release from the intracellular organelles is crucial for a homogeneous and regulated contraction. In this section, we describe three examples where compartmentalization of calcium signaling plays an important role in the regulation of VSMC contraction.

a. Regulation of myogenic tone in cerebral arteries. Calcium release from the SR and coordination with channels and other transporters at the plasma membrane of cerebral VSMCs was first described in 1995 by Nelson et al. (1995). In the seminal article, the group defined calcium sparks as a temporal and spatial release of intracellular calcium from the SR via the ryanodine receptors (RyR), which further activate calcium-dependent large conductance potassium channels (BK_{Ca}) at the plasma membrane (Nelson et al., 1995) (Fig. 2B). Activation of BK_{Ca} channels induced hyperpolarization of the plasma membrane, making the coordination between RyR and BK_{Ca} central to the regulation of voltage gated calcium channel (VGCC) expressed at the plasma membrane (Fig. 2B) (Nelson et al., 1995; Jaggar et al., 1998b). Conversely, calcium influx from channels at the plasma membrane can also activate RyR at the SR membrane. Calcium influx through channels from the transient receptor potential (TRP) family, namely TRPV4 channels, is expressed at the plasma membrane of cerebral VSMCs and activates calcium release from the SR via RyR channels, making TRPV4 and RyR part of a CICR mechanism (Earley et al., 2005) (Fig. 2B). Of note, high calcium concentrations between RyR and BK_{Ca} were demonstrated in stomach SMCs where the calcium concentration between both channels could reach 10 μM in an area of 1 μm^2 during a calcium spark (ZhuGe et al., 1999; Zhuge et al., 2002). At the arterial level, the coordination between RyR, BK_{Ca}, and TRPV4 is crucial in the regulation of smooth muscle contraction as shown specifically in pressure-induced constriction, where the hyperpolarization induced by BK_{Ca} channels negatively feeds back on the depolarization occurring during increases in intravascular pressure (Jaggar et al., 1998a,b; Knot and Nelson, 1998; Knot et al., 1998; Jaggar, 2001; Wellman et al., 2002; Ledoux et al., 2006). Accordingly, cerebral arteries isolated from mice deficient in the β subunit of BK_{Ca} channels are significantly more constricted at a given intraluminal pressure compared with control mice (Brenner et al., 2000). This negative feedback is key in the autoregulation of cerebral blood flow, a process that is impaired during subarachnoid hemorrhage, thus resulting in a decreased activation of the BK_{Ca} and a higher constriction of cerebral arteries (Koide et al., 2011).

During pressure-induced contraction of cerebral arteries, other calcium channels expressed at the plasma membrane of cerebral VSMCs are activated by calcium release from the SR, specifically TRPM4 channels (Earley et al., 2005). However, as opposed to BK_{Ca} channels that are activated by calcium sparks released via RyR channels, TRPM4 channels are activated by calcium release via IP_3R present at the SR membrane (Fig. 2B) (Gonzales et al., 2010a). In cerebral VSMCs, TRPM4 channels at the plasma membrane are less than 50 nm from the SR membrane but are not physically coupled to the IP_3R, as shown by immunofluorescence overlap and immuno-fluorescence resonance energy transfer (Zhao et al., 2010; Gonzales and Earley, 2012). It is noteworthy that translocation of the TRPM4 channels at the plasma membrane via a PKC-dependent pathway is key for the channel activation by calcium release through IP_3R (Crnich et al., 2010; Garcia et al., 2011). Because activation of TRPM4 by a PKC-dependent pathway is involved in the myogenic response to increased intravascular pressure (Earley et al., 2004, 2007; Gonzales et al., 2010b), it has been hypothesized that the functional complex formed by IP_3R, TRPM4, and PKC could play a role in the depolarization of VSMCs observed upon increase in intravascular pressure (Earley, 2013). However, neither the origin of IP_3R activation by increased levels of IP_3 (Narayanan et al., 1994) nor the origin of PKC activation upon increase intravascular pressure has been elucidated (Earley, 2013). Mechanical activation of G_q receptors by increased intravascular pressure has been suggested (Mederos y Schnitzler et al., 2008; Brayden et al., 2013) and could reconcile the ideas that

both PKC and IP_3R are activated during increased intravascular pressure, which would, respectively, result in relocation of the TRPM4 at the plasma membrane and in its activation. Further investigation is needed, because activation of G_q receptors upon increased intravascular pressure is controversial (Anfinogenova et al., 2011; Earley, 2013).

b. Agonist-induced constriction in cerebral arteries. As opposed to the functional but indirect interactions between channels at the plasma membrane and at the SR membranes described above, TRPC3 channels expressed at the plasma membrane of VSMCs have been shown to be physically coupled to IP_3R1 on the SR. This direct interaction was demonstrated using coimmunoprecipitation in intact rat cerebral arteries and by immuno-fluorescence resonance energy transfer (Adebiyi et al., 2011). Functionally, TRPC3:IP_3R1 coupling is important in the endothelin-1 (ET-1)-mediated response where activation of IP_3R1 directly activates a cation influx via TRPC3 channels at the plasma membrane, producing a sustained constriction (Xi et al., 2008; Adebiyi et al., 2010, 2011). It is noteworthy that activation of TRPC3 by IP_3R1 occurs independently of calcium release from the SR via the IP_3R1, because exogenous IP_3 or ET-1 applied to isolated cerebral myocytes induces a cation influx via TRPC3, even when the SR was depleted of calcium (Xi et al., 2008). The TRPC3:IP_3R1 interaction occurs via a calmodulin and IP_3R binding domain (CIRB) that is present on the TRPC3 channels and can be disrupted using a peptide corresponding to the N-terminal sequence of the IP_3R1 known to interact with CIRB domain (Adebiyi et al., 2010). Conversely, the functional effect of TRPC3:IP_3R1 interaction can be mimicked by a peptide corresponding to the CIRB domain of TRPC3, which simulates IP_3R1 interaction to TRPC3 and results in the activation of TRPC3 at the plasma membrane (Adebiyi et al., 2010).

The presence of Cav1 is key in the assembling of IP_3R and TRPC3 complex as shown by the decrease in IP_3-induced cation influx via TRPC3 when VSMCs were treated with methyl β cyclodextrin (MβCD) or with shRNA targeting Cav1 (Adebiyi et al., 2011). The same group also demonstrated that IP_3-induced cation influx via TRPC3 was inhibited by a peptide that competes with endogenous Cav1 for interaction with protein partners (Adebiyi et al., 2011). Concordantly, MβCD, shRNA targeting Cav1, and the competing peptide all abolished IP_3-induced constriction of cerebral arteries (Adebiyi et al., 2011). In parallel, it was also shown that local calcium release via IP_3R1 could activate BK_{Ca} channels, similarly to BK_{Ca} activation by calcium sparks (see above) (Zhao et al., 2010).

These observations clearly demonstrate the impact of compartmentalized calcium signaling, especially as it relates to VSMCs. These areas of compartmentalized calcium signaling have also been demonstrated in the systemic circulation, where IP_3R1, TRPC3, and Cav1 also interact together (Adebiyi et al., 2012). Additionally, another TRP channel can activate BK_{Ca} at the plasma membrane of systemic VSMCs, namely TRPC1, which coimmunoprecipitate and colocalize with BK_{Ca} channels in freshly isolated aortic SMCs (Kwan et al., 2009). Functionally, the authors demonstrated that TRPC1 channels are involved in the responses to several contractile agonists including ET-1, but also phenylephrine and U-46619. Agonist-induced activation of TRPC1 further activates a potassium efflux via BK_{Ca} at the plasma membrane, thus controlling the contractile state of VSMCs (Kwan et al., 2009). It is noteworthy that the IP_3R:TRPC3 coupling is increased in spontaneously hypertensive rats, along with an increase in TRPC3 expression and ET-1-induced vasoconstriction (Adebiyi et al., 2012).

c. Lysosome-sarcoplasmic reticulum junctions. The membrane appositions between the lysosomes and the SR have important implications in processes such as autophagy and cholesterol metabolism (van Breemen et al., 2013). Recent studies reported a role of these junctions in the regulation of CICR from the SR induced by the second messenger nicotinic acid adenine dinucleotide phosphate. This second messenger, which can be produced in response to agonists such as ET-1, stimulates the release of calcium from the lysosomes via the two pore segment channel subtype 2 (TPC2; Fig. 2D) (Calcraft et al., 2009). The released calcium further activates release of calcium from the SR via RyR3 found at SR-lysosomes nanojunctions in a CICR manner (Kinnear et al., 2004, 2008). After activation of RyR3, the RyR2 isoform is activated in a CICR manner, and the calcium released from the SR is propagated as a wave in the cytoplasm to activate contraction of SMCs (Kinnear et al., 2004, 2008; Clark et al., 2010). By use of a lysosome marker and labeled ryanodine, Kinnear et al. (2008) demonstrated a close proximity between the lysosomes and ryanodine receptors. These studies strongly point to a calcium compartmentalization between the SR and the lysosome that may play important roles in the regulation of calcium homeostasis.

3. Calcium Signaling Microdomains Involved in the Regulation of Calcium Concentration in the Sarcoplasmic Reticulum. The SR is able to autoregulate its own calcium content and maintain a constant calcium concentration. The capacity of SR to store calcium is attributed to the presence of high-capacity, low-affinity calcium-binding proteins in its lumen such as calsequestrin and calreticulin (Michalak et al., 1992; Milner et al., 1992; Raeymaekers et al., 1993). After stimulation of a cell, the SR is able to replenish its content by pumping calcium via the sarco/endoplasmic reticulum calcium ATPase (SERCA) pumps localized strategically in close apposition to the plasma membrane and the mitochondria (Fig. 2, E and F) (Putney, 1986; Floyd

and Wray, 2007; Satoh et al., 2011). Thus, the SR is able to refill its calcium stock from both the extracellular space and the mitochondria. To do this, the cells have established different mechanisms to transfer calcium from the extracellular space or the mitochondrial matrix to the SR in a highly coordinated manner to avoid diffusion into the cytosol and/or unwanted activation of calcium-dependent signaling pathways.

a. Signaling microdomains at the plasma membrane-sarcoplasmic reticulum junctions. Upon VSMC stimulation, the opening of TRPC6 channels at the plasma membrane allows for the entry of sodium along with calcium, which reverses the sodium and calcium exchanger (NCX) by increasing subplasmalemmal concentration of sodium and activating calcium entry via VGCC, respectively (Lee et al., 2001; Lemos et al., 2007; Poburko et al., 2007; Fameli et al., 2009) (Fig. 2E). Activation of VGCCs can be induced solely by cation influx from TRPC6 and independently of the NCX; however, reversal of the NCX offers an additional source of calcium specifically for calcium refilling (Poburko et al., 2008). Disruption of the PM-SR junctions using the cytoskeleton-disrupting agent calyculin A prevents calcium refilling of the SR, inhibits calcium influx, but increases sodium entry presumably due to a disruption of the refilling mechanism involving NCX, TRPC6, and SERCA presented in Fig. 2E (Dai et al., 2005a; Lemos et al., 2007). At the mitochondrial level, a similar mechanism involving the mitochondrial NCX has been demonstrated (see section I.B.3.b).

In parallel, the SR can also replenish its calcium content by stromal interaction molecule (STIM) and Orai expressed, respectively, at the SR and plasma membrane (Williams et al., 2001; Liou et al., 2005; Roos et al., 2005; Zhang et al., 2005; Feske et al., 2006; Prakriya et al., 2006). In this system, STIM serves as a $[Ca^{2+}]_{SR}$ sensor due to an EF-hand located in the SR lumen (Zhang et al., 2005) and relocate to SR regions that are close to the plasma membrane (approximately 10–25 nm) when $[Ca^{2+}]_{SR}$ decreases (Luik et al., 2006; Wu et al., 2006; Calloway et al., 2009). After STIM relocation at the PM-SR junctions, STIM interact physically with the Orai channels at the plasma membrane and activate Ca^{2+} entry (Fig. 2E) (Liou et al., 2005; Zhang et al., 2005; Prakriya et al., 2006; Ong et al., 2007; Muik et al., 2008; Navarro-Borelly et al., 2008; Park et al., 2009a; Zheng et al., 2013a). Recently, the store operated channel entry associated regulatory factor was found to associate with STIM at the plasma membrane so as to regulate calcium influx via Orai to avoid excessive refill of the SR (Palty et al., 2012).

b. Calcium signaling microdomains at the sarcoplasmic reticulum-mitochondria junctions. Fifty years ago, mitochondria were shown to accumulate calcium (Deluca and Engstrom, 1961; Vasington and Murphy, 1962; Lehninger et al., 1963), but the physiologic relevance of the process was initially dismissed because of the discordance between the mitochondria's low affinity for the ion (in the millimolar range) and the measured physiologic cytosolic values of calcium ($<1\ \mu M$) (Patron et al., 2013). The role of mitochondria in calcium homeostasis re-emerged in the early 1990s with the development of calcium probes targeted to the mitochondria (Rizzuto et al., 1992). Since then, the discrepancy between the low affinity of the mitochondria for calcium and the low cytosolic calcium concentration has been explained by the close proximity between the organelle and channels that release calcium both at the SR and at the plasma membrane (Mannella et al., 1998; Rizzuto et al., 1998; Csordas et al., 1999).

In VSMCs, the functional role of mitochondria in calcium homeostasis was demonstrated using mitochondria protonophores, which cause the mitochondrial membrane potential to collapse or pharmacologically block the mitochondrial calcium uniporter (MCU) known to drive calcium influx into the mitochondria (Drummond and Fay, 1996; McCarron and Muir, 1999). Because the mitochondrial calcium uptake relies on the large proton electrochemical driving force, these mitochondrial inhibitors were shown to increase cytosolic calcium concentration upon depolarizing stimulation (Drummond and Fay, 1996; McCarron and Muir, 1999; Kamishima and Quayle, 2002; Cheranov and Jaggar, 2004). Blockers of the mitochondrial ATP synthase, however, did not affect mitochondrial calcium uptake, suggesting that the role of mitochondria in calcium homeostasis was not due to a depletion of cellular ATP and subsequent inactivation of the calcium pumps present at the SR (e.g., SERCA) or at the plasma membrane (e.g., PMCA) (Drummond and Fay, 1996; McCarron and Muir, 1999). It recently became more clear that the mitochondria acts as a buffer of the calcium released from the SR, because an increase in mitochondrial calcium concentration ($[Ca^{2+}]_{mit}$) occurs after release of the ion from the SR (Drummond and Fay, 1996). Indeed, application of caffeine or a G_qPCR agonist, which stimulate the RyRs and the IP_3R, respectively, or flash photolysis of caged IP_3 induced increases in $[Ca^{2+}]_{mit}$ (Drummond and Fay, 1996; Drummond and Tuft, 1999; McCarron and Muir, 1999; Gurney et al., 2000; Kamishima and Quayle, 2002; Chalmers and McCarron, 2008). This increase in $[Ca^{2+}]_{mit}$ was sensitive to mitochondrial protonophores (Drummond and Tuft, 1999). Given the buffering role of mitochondria, their contribution to the return of $[Ca^{2+}]_i$ to baseline levels after stimulation has also been demonstrated (Drummond and Tuft, 1999; McCarron and Muir, 1999; Kamishima and Quayle, 2002; Chalmers and McCarron, 2008). For example, mitochondrial protonophores increased the time of recovery of $[Ca^{2+}]_i$ after caffeine application in rat PASMCs (Drummond and Tuft, 1999).

The temporal buffering of calcium by the mitochondria was also demonstrated using ATP in rat PASMCs, where application of the purine induced oscillations in $[Ca^{2+}]_i$ synchronized with oscillations of $[Ca^{2+}]_{mit}$ (Drummond and Tuft, 1999). More direct evidence has been observed in HeLa cells where $[Ca^{2+}]_{mit}$, $[Ca^{2+}]_{SR}$, and $[Ca^{2+}]_i$ have been measured simultaneously (Arnaudeau et al., 2001). These studies indicated a larger ER depletion when calcium uptake by the mitochondria was blocked by inhibitors of the mitochondrial respiratory chain (Arnaudeau et al., 2001). The same study also demonstrated a larger ER depletion when calcium efflux from the mitochondria by the mitochondrial Na^+-Ca^{2+} exchanger was pharmacologically blocked, indicating that mitochondria calcium stores aid in the refilling of the ER by locally extruding calcium proximal to the SERCA pump on the ER (Arnaudeau et al., 2001). Interestingly, ER regions that are close to mitochondria refilled and released more calcium than ER regions that are more distant from mitochondria (Arnaudeau et al., 2001).

Because calcium has been shown to accumulate in the mitochondrial matrix, the ion has to traverse both the external mitochondrial membrane and the inner mitochondrial membrane. It is presumed that calcium ions, driven by the negative charge of the mitochondrial membrane potential established by the respiratory chain, cross the external mitochondrial membrane via the voltage-gated anion channel. Calcium residing in the intermembrane space is then imported into the mitochondrial matrix via the MCU (Madesh and Hajnoczky, 2001; Rapizzi et al., 2002; Kirichok et al., 2004). Calcium is slowly released from the mitochondria via the Na^+-Ca^{2+} exchanger and the mitochondrial permeability transition pore and is used to refill the SR via the SERCA pump (Landolfi et al., 1998; Szado et al., 2003; Ishii et al., 2006; Chalmers and McCarron, 2009; Poburko et al., 2009; Giacomello et al., 2010) (Fig. 2F). Several reports demonstrated that calcium buffering by the mitochondria is especially important in the spatial area surrounding the IP_3R to prevent its inhibition by cytosolic calcium accumulation (Hajnoczky et al., 1999; Olson et al., 2010).

4. Calcium Signaling Microdomains Involved in Protein Expression and Cell Proliferation. During cellular proliferation, the transcription factor nuclear factor of activated T-cells can be activated by calcium-bound calcineurin, which induces the translocation to the nucleus (Hogan et al., 2003; Aubart et al., 2009). Several SR and plasma membrane calcium channels have been involved in the calcium-induced translocation of nuclear factor of activated T-cells in the nuclei, and it appears that the STIM/Orai complex described above is central in this process (Aubart et al., 2009; Baryshnikov et al., 2009; Zhang et al., 2011). Interestingly, in contractile quiescent VSMCs, STIM and Orai are expressed at very low levels; however, when VSMCs dedifferentiate and transition to a proliferative phenotype, the expression of these two proteins is significantly increased (Aubart et al., 2009; Potier et al., 2009; Bisaillon et al., 2010). For example, several reports have demonstrated an increased expression of the STIM1 and Orai1 isoforms in VSMCs after carotid balloon injury, where VSMCs adopt a highly proliferative phenotype (Aubart et al., 2009; Guo et al., 2009; Bisaillon et al., 2010; Zhang et al., 2011). In these studies, the genetic knock down of STIM1 and Orai1 in vivo resulted in significant inhibition of neointimal growth (Aubart et al., 2009; Guo et al., 2009; Bisaillon et al., 2010; Zhang et al., 2011).

Calcium influx secondary to calcium release from the SR (i.e., calcium capacitive influx) plays a key role in the increased PASMC proliferation observed during pulmonary hypertension (Sylvester et al., 2012; Firth et al., 2013). Both TRPC1 and the STIM/Orai molecular complex are key in this capacitive calcium influx (Ng et al., 2009, 2010a,b) and have been shown to play a critical role in the calcium response of PASMC under hypoxic conditions (Lu et al., 2008, 2009; Ng et al., 2012). Although multiple lines of in vitro data demonstrate the importance of the STIM/Orai/TRPC1 complex in PASMCs, the physiologic relevance of the complex in vivo remains unclear. However, because it is well accepted that the capacitive calcium entry in PASMCs in vivo is involved in the development of pulmonary arterial hypertension, the STIM/Orai/TRPC1 complex could be an important molecular component in this pathology. Indeed, a recent investigation reported that platelet-derived growth factor, a growth factor known to be elevated in patients with pulmonary arterial hypertension, enhances the expression of STIM and Orai in human PASMCs, along with an increase in capacitive calcium entry and proliferation of the cells (Ogawa et al., 2012).

The main conclusion that can be drawn from the work described above is that calcium is highly regulated and compartmentalized by the cell. To do so, cells harbor a complex organization of intracellular organelles but also assemble calcium pumps, calcium channels, and calcium-binding proteins in calcium signaling microdomains. The result of such highly organized microdomains is a very efficient regulation of calcium homeostasis. Therefore, calcium signaling microdomains provide a valuable example to both understand and provide a basis for signaling microdomains that regulate intercellular communication work.

II. Gaseous Molecule Cellular Communication by Signaling Microdomains

The biologic prevalence of physiologic and pathologic signaling cascades utilizing diffusible gaseous molecules in the blood vessel wall has been extensively documented. Of particular note, a number of reactive

nitrogen and oxygen species have been implicated in a vast array of cellular signaling pathways in the vascular wall. Among these gaseous molecules, nitric oxide (NO) and its oxidized derivatives, nitrate, nitrite, and peroxinitrite, have been studied the most extensively, with a number of other free radical species [notably superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2)] having come into focus over the last decade with conclusive work demonstrating an equally important role in cellular communication (reviewed by Ronson et al., 1999; Wolin, 2000; Ignarro, 2002; Ardanaz and Pagano, 2006; Bian et al., 2008; Touyz et al., 2011; Sparacino-Watkins et al., 2012; Bueno et al., 2013). How these molecules are regulated by signaling microdomains is discussed below, as well as potential contributions from carbon monoxide (CO) and hydrogen sulfide (H_2S).

A. A Case for Endothelial Nitric-Oxide Synthase and Nitric Oxide

The initial discovery by Murad in 1977 that exogenous NO can act as a bioactive messenger to activate soluble guanylate cyclase (sGC) in SMCs (Katsuki et al., 1977), along with the work by Furchgott and Zawadzki (1980) identifying the presence of an endothelium derived relaxing factor that has since been identified as NO (Ignarro et al., 1987; Palmer et al., 1987), fueled the concept that NO is an essential player in regulating blood vessel physiology. Since then, a number of intra- and intercellular targets for bioactive NO have been identified with the biological effects ranging from enzyme activation or inhibition, posttranslational modifications altering protein function, including S-nitrosylation and tyrosine nitrosation, and the generation of complex reactive nitrogen and oxygen species through the rapid spontaneous reaction of NO with other gaseous molecules in the cell (Ignarro, 1991; Davidge et al., 1995; Xu et al., 1998; Handy and Loscalzo, 2006; Yoshida et al., 2006; Kang-Decker et al., 2007; Selemidis et al., 2007; Zuckerbraun et al., 2007; Illi et al., 2008; Briones et al., 2009; Fernhoff et al., 2009; Lima et al., 2010; Thibeault et al., 2010; Bess et al., 2011; Choi et al., 2011; Straub et al., 2011; Marin et al., 2012; Haldar and Stamler, 2013; Korkmaz et al., 2013).

It has now become evident that the spatial and temporal regulation of reactive nitrogen and oxygen species generation can dictate the functional impact of these signaling molecules on the homeostatic maintenance of vascular function, where dysregulation can lead to complications including, but not limited to, endothelial dysfunction, inflammation, and atherosclerosis (reviewed by Giles, 2006; Pacher et al., 2007; Muller and Morawietz, 2009). The biologic half-life of NO is extremely short (<5 seconds), because of the rapid diffusion to surrounding cells, chemical reactions with other cellular oxidants, and scavenging by heme-containing proteins, most notably hemoglobin (Nathan, 1992; Archer, 1993; Hakim et al., 1996). These observations suggest that the generation of NO may be spatially confined to microdomains within the cell where induction of NO-dependent signaling cascades can be poised in close proximity to downstream targets.

Although the potent effects of NO as a vasodilator and anti-inflammatory mediator were first recognized nearly 30 years ago, the enzymes responsible for its synthesis were not identified until the early 1990s. Three nitric-oxide synthase (NOS) enzymes have since been cloned and characterized with differential tissue distributions and regulatory elements. These NOS isoforms were subsequently termed nNOS (neuronal NOS; NOS1) (Bredt et al., 1990; Bredt and Snyder, 1990), inducible NOS (NOS2) (Charles et al., 1993; Sherman et al., 1993; Maier et al., 1994), and eNOS (endothelial NOS; NOS3) (Busse and Mulsch, 1990; Lamas et al., 1992; Marsden et al., 1992). With respect to the cells comprising the blood vessel wall, eNOS is the most abundant isoform with robust expression in the ECs lining the vascular intima under physiologic conditions. The other two NOS isoforms have also been identified in the vessel wall, with nNOS expression being detectable at low levels in the VSMCs in certain vascular beds (Boulanger et al., 1998; Brophy et al., 2000) and inducible NOS expression increasing in both ECs and VSMCs in response to vascular damage or cellular activation by proinflammatory cytokines (Hansson et al., 1994; Kanno et al., 1994; Ikeda et al., 1997; Hecker et al., 1999). In addition to the vascular cells comprising the blood vessel wall, sympathetic perivascular nerves innervate the resistance arteries express nNOS, providing another source of NO for the regulation of vascular function (Sosunov et al., 1995; Faraci, 2002). Based on the current myriad of literature implicating eNOS in vascular NO generation and the complex regulatory networks dictating compartmentalized eNOS signaling, this section of the review will focus on the signaling microdomains important for control of eNOS activity and signal transduction cascades in the vascular endothelium and how these domains impart discrete control over NO production in this tissue.

1. Structural Organization and Functional Regulation of Endothelial Nitric-Oxide Synthase. Endothelial NOS is a highly regulated enzyme in the vasculature, with a multifaceted control of its enzymatic activity conferred by numerous factors including local substrate and cofactor availability, regulatory protein binding partners, and dynamic posttranslational modifications, predominantly by phosphorylation of specific serine, tyrosine, and threonine residues. The eNOS enzyme has a constitutive low level of activity for NO generation that is tightly modulated by each of the aforementioned factors (Palmer et al., 1988; Bredt and Snyder, 1990; Busse and Mulsch, 1990; Lamas et al., 1992; Garcia-Cardena

et al., 1996; Michel et al., 1997; Presta et al., 1997). The enzyme contains an N-terminal oxygenase domain harboring a heme prosthetic group and a C-terminal reductase domain with the latter containing binding sites for flavin mononucleotide, flavin adenine dinucleotide, and NADPH (Sessa et al., 1992; Fleming and Busse, 1999) (Fig. 3A). The oxygenase and reductase domains are separated by a linker region containing a calmodulin binding domain (Sessa et al., 1992; Fleming and Busse, 1999) (Fig. 3A). Translated eNOS forms a homodimer where the N-terminal oxygenase domain from one monomer participates in oxidation-reduction reactions with the C-terminal reductase domain of the second monomer (Fig. 3B). As a dimer, eNOS uses NADPH, L-arginine, and O_2 to synthesize NO and the byproduct L-citrulline through a reaction driven by electron transport from the enzyme's reductase domain to the heme moiety located in the oxygenase domain of the second monomer (Fig. 3B). This reaction by the heme iron promotes binding and subsequent reduction of O_2 and incorporation into L-arginine, ultimately terminating with the production of bioactive NO (Palmer et al., 1988; Presta et al., 1997; Fleming and Busse, 1999). This process is dependent on a number of cofactors including 1) tetrahydrobiopterin (BH_4), which binds to the oxygenase domain and is required for eNOS dimerization; 2) CaM, which binds to the linker region of dimeric eNOS conferring an activating conformational change; and 3) heat shock protein 90. Transient increases in intracellular Ca^{2+} recruit CaM to its regulatory binding site in the eNOS linker, promoting NADPH-dependent electron flux between the reductase domain of one eNOS monomer to the oxygenase domain of the second monomer (Abu-Soud and Stuehr, 1993; Chen et al., 1997; List et al., 1997; Presta et al., 1997). In addition, BH_4 is required for coordinating the electron transport from the reductase to oxygenase domains and a decrease in the bioavailability of this essential cofactor leads to eNOS uncoupling and the production of superoxide anion ($O_2^{\bullet -}$) instead of NO, imparting oxidative stress on the cell (Wever et al., 1997; Stuehr et al., 2001) (Fig. 3B).

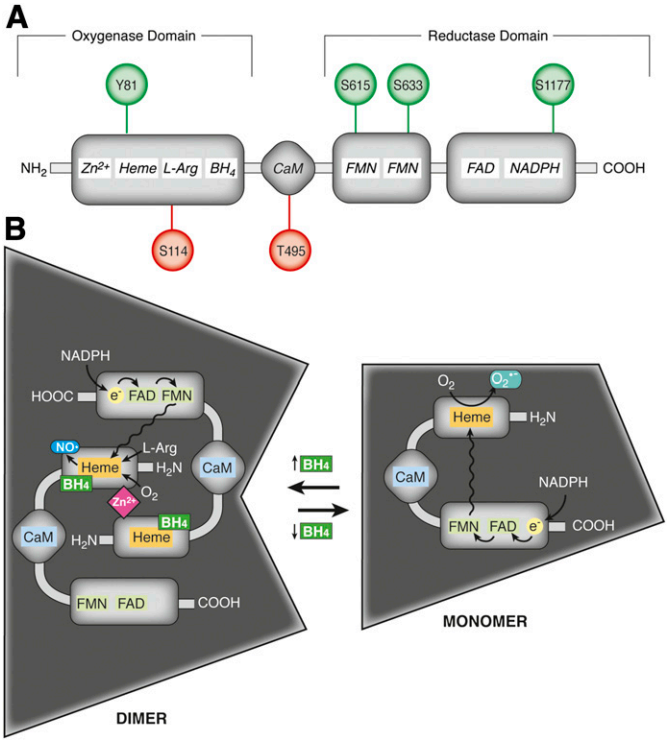

Fig. 3. eNOS: protein domains, phosphorylation sites, and higher order organization. (A) eNOS is composed of an N-terminal oxygenase domain containing binding sites for tetrahydrobiopterin (BH_4), Zn^{2+}, heme, and L-arginine and a C-terminal reductase domain containing NADPH, flavin adenine dinucleotide, and flavin mononucleotide binding sites. The oxygenase and reductase domains are separated by a linker region that harbors a regulatory CaM binding domain. Binding domains are indicated in italics. eNOS also harbors several serine, threonine, and tyrosine residues that are targeted for phosphorylation. The most extensively characterized phosphorylated residues are depicted with those that promote enzyme activation in green (Tyr81, Ser615, Ser633, and Ser1177; human sequence) and sites imparting inhibition in red (Ser114 and Thr495). (B) eNOS forms a homodimer coordinated by BH_4 and Zn^{2+} binding in the N-terminal oxygenase domains of each monomer. Dimeric eNOS synthesizes NO from L-arginine and O_2 through NADPH-dependent electron flux from the C-terminal reductase domain of one monomer to the heme moiety located on the oxygenase domain. Depletion of BH_4 promotes eNOS uncoupling, leaving the enzyme in a monomeric form, resulting in the production of superoxide rather than NO.

In addition to the array of regulatory cofactors that control eNOS activity, the enzyme is dynamically modulated by phosphorylation, with modification of multiple serine, threonine, and tyrosine residues influencing NO synthesis (Fig. 3A). Although there are several potential phosphorylation sites on eNOS, Tyr81, Ser615, Ser633, and Ser1177 have been identified as target residues for enzyme activation by phosphorylation, and Ser114 and Thr495 for enzyme inhibition (Dimmeler et al., 1999; Fleming et al., 2001; Scotland et al., 2002; Chen et al., 2003; Fulton et al., 2005; Li et al., 2007; Fisslthaler et al., 2008; Loot et al., 2009; Watts and Motley, 2009). Phosphorylation of Ser1177 in the C-terminal reductase domain has been the most extensively characterized, and this activating modification increases electron flux and NO synthesis by eNOS (Dimmeler et al., 1999; Scotland et al., 2002). Ser1177 is modified by a number of kinases in a context-dependent manner. For instance, shear-stress induces the activation of the kinases Akt and protein kinase A (PKA), which phosphorylate eNOS at Ser1177 and promote NO-dependent arterial relaxation (Dimmeler et al., 1999; Gallis et al., 1999; Boo et al., 2002b). Akt-mediated phosphorylation of eNOS has also been observed in response to VEGF and estrogen stimulation, whereas the vasodilation observed in response to bradykinin is controlled by phosphorylation of eNOS Ser1177 by CaM kinase II (Papapetropoulos et al., 1997; Bernier et al., 2000; Yang et al., 2000; Fleming et al., 2001; Chambliss and Shaul, 2002; Chen et al., 2006; Gentile et al., 2013). Site-directed mutagenesis studies have found that

mutating this residue to an alanine prevents Akt-dependent NO synthesis, whereas a phosphomimicking mutation of Ser1177 to aspartate renders eNOS constitutively active (Dimmeler et al., 1999; Fulton et al., 1999). In addition to Ser1177, Ser633 can be phosphorylated in response to shear stress and adiponectin stimulation in a PKA and 5′-AMP-activated protein kinase-dependent manner, respectively, leading to eNOS activation and NO synthesis in arterial endothelial cells (Boo et al., 2002a; Chen et al., 2003; Osuka et al., 2012). More recently, phosphorylation of Tyr81 in the N-terminal oxygenase domain has been reported to increase eNOS activity in a c-Src-dependent manner (Fulton et al., 2005). Phosphorylation of Ser615 has also been observed in response to bradykinin stimulation and has been associated with increased eNOS activity (Michell et al., 2002). In contrast to these activating modifications, targeted phosphorylation of Ser114 or Thr495 exerts inhibitory effects on eNOS. Modification of Ser114 renders eNOS less active in response to VEGF stimulation, and mutagenesis studies have concurrently revealed that phosphorylation of this residue promotes eNOS interaction with its scaffolding regulatory protein Cav1 (Li et al., 2007). Of the most studied inhibitory residues in eNOS, Thr495 located in the CaM binding site plays a dynamic role in regulating eNOS activity. In resting ECs, Thr495 is constitutively phosphorylated by 5′-AMP-activated protein kinase and PKC, antagonizing CaM binding in response to Ca^{2+}-mobilizing agonists and functionally inhibiting NO synthesis by the enzyme (Fleming et al., 2001; Watts and Motley, 2009). The myriad of evidence for eNOS regulation by phosphorylation has made it clear that the dynamic balance between phosphorylation of activating and inhibiting residues of eNOS imparts strict control over the enzyme's ability to produce NO and propagate NO-dependent signaling cascades. Based on the growing literature for phosphorylation in regulating interactions of eNOS with its cofactors, these posttranslational modifications may prove to play an important role in the regulation of eNOS in distinct signaling microdomains by controlling the localization and binding interactions between other known signaling partners that are discussed in the following sections. Characterization of the catalytic activity of eNOS and the requirement of indispensable cofactors and substrates have prompted numerous investigations into the key factors conferring specificity to localization of the enzyme to specific regions in the cell where these substrates are concentrated and interactions between eNOS and other protein binding partners that can regulate its activity. At the axis of eNOS regulation in the blood vessel wall, evidence has emerged suggesting distinct signaling microdomains containing the enzyme at the level of the Golgi, plasma membrane caveolae, and the MEJ.

2. Compartmentalized Endothelial Nitric-Oxide Synthase Regulation in the Endothelial Cells Golgi Apparatus. eNOS resides in several distinct locations within ECs, notably the Golgi apparatus, cholesterol-enriched microdomains at the plasma membrane (including lipid rafts and caveolae), and the MEJ where heterocellular communication can occur through direct cell-to-cell coupling. Proper trafficking of eNOS to these domains requires the coordinated cotranslational modification of eNOS by N-myristoylation at amino acid residue Gly2 and posttranslational modification by S-palmitoylation at residues Cys15 and Cys26 (Sessa et al., 1993; Liu and Sessa, 1994; Liu et al., 1995; Robinson and Michel, 1995; Shaul et al., 1996; Fernandez-Hernando et al., 2006). N-Myristoylation is an absolute requirement for eNOS trafficking through the Golgi because a deficiency in the covalent attachment of a myristoyl group to Gly2 results in cytoplasmic sequestration of the enzyme and decreased catalytic activity for NO generation. Retention of eNOS in the cytoplasmic compartment of the cell probably results in decreased NO generation because of suboptimal exposure of the enzyme to its required cofactors and substrates for NO synthesis. Following N-myristoylation, eNOS is targeted to the *trans*-Golgi, where a class of acetyltransferases of the Asp-His-His-Cys motif (DHHC) palmitoyltransferase family palmitoylate eNOS at Cys15 and Cys26 (Fernandez-Hernando et al., 2006).

The eNOS enzyme has been shown to polarize to the Golgi along with five members of the DHHC family of palmitoyl transferases including DHHC 2, 3, 7, 8, and 21. In particular, regional colocalization of eNOS and the DHHC 21 isoform has been observed by coimmunoprecipitation and immunofluorescence overlap studies, suggesting that these two enzymes may form a functional complex required for eNOS palmitoylation and activity (Fernandez-Hernando et al., 2006). Modification of eNOS by S-palmitoylation confers proper trafficking and subcellular localization of the enzyme to cellular membranes where eNOS activity has been shown to be maximal, notably at plasma membrane caveolae and lipid rafts (Michel, 1999; Sessa, 2004). Dysregulated acylation of eNOS by DHHC 21 results in diminished NO production both basally and in response to vasodilatory agonists such as ATP, which may ultimately perturb communication between the endothelium and smooth muscle in the blood vessel wall (Fernandez-Hernando et al., 2006). This is particularly evident because eNOS constructs with mutations at the myristoylation and/or palmitoylation sites are less active than wild-type eNOS, and genetic knockdown of DDHC 21 in ECs impairs trafficking of eNOS to plasma membrane compartments and reduces NO generation in response to the calcium ionophore ionomycin and adenosine triphosphate (ATP) (Fernandez-Hernando et al., 2006). Taken together, these studies suggest that a signaling microdomain poised in the EC Golgi is

important for eNOS processing and subsequent trafficking to peripheral membranes in the cell where its biologic activity is optimal. Improper lipid modification of eNOS can therefore have detrimental effects on signaling events in the vasculature, which can lead to pathologies including hypertension, impairments in angiogenesis and atherosclerosis.

3. Signaling Microdomains Involving Endothelial Nitric-Oxide Synthase Regulation at the Plasma Membrane.

a. Endothelial nitric-oxide synthase and caveolin 1 interactions in the endothelium. The major fraction of eNOS in ECs is localized to specialized regions of the plasma membrane, including cholesterol-rich lipid rafts and caveolae where its activity has been shown to be optimal (Zhang et al., 2006b). In ECs, eNOS colocalizes with the caveolin 1 (Cav1) isoform in caveolae, anchoring the enzyme at the cytoplasmic face of the invagination. A direct protein-protein binding interaction between eNOS and Cav1 has been observed both in vitro and in vivo, and characterization of this interaction identified the ability of Cav1 to impart an inhibitory clamp on the catalytic activity of eNOS, acting as an allosteric inhibitor controlling NO production in the endothelium (Garcia-Cardena et al., 1997; Ju et al., 1997; Bucci et al., 2000). Furthermore, studies aimed at mapping the interacting domains of eNOS and Cav1 identified this protein-protein interaction to occur between the N-terminal oxygenase domain of eNOS and the intracellular N-terminal scaffolding domain of Cav1 (Ju et al., 1997; Bucci et al., 2000; Bernatchez et al., 2005). In addition, a synthetic peptide corresponding to residues 82–101 of Cav1 called cavtratin was capable of binding to and inhibiting eNOS activity (Ju et al., 1997; Bucci et al., 2000).

Functionally, Cav1 binding to eNOS antagonizes the interaction of the enzyme with its activating cofactor calmodulin under resting conditions. Further investigation of the Cav1/eNOS interaction has identified three residues (Thr90, Thr91, and Phe92) in the Cav1 scaffolding domain that impart the negative regulation of Cav1 on eNOS activity (Bernatchez et al., 2005). The inhibitory clamp can be relieved by stimulating endothelial cells with agonists that mobilize intracellular Ca^{2+}, including fluid shear stress on the endothelium and a number of vasodilator agonists. This process leads to dissociation of eNOS from Cav1, binding of CaM to its allosteric site on eNOS, and activation of the enzyme to facilitate NO production (Fig. 4A). Disruption of the eNOS/Cav1 interaction by genetic deletion of Cav1 from ECs increases NO production by eNOS and facilitates SMC relaxation. To this end, $Cav1^{-/-}$ mice develop systemic hypotension due to increased activity of eNOS (Murata et al., 2007). On the basis of these observations, it has been proposed that Cav1 binding to eNOS in caveolae serves to impart allosteric inhibition of the enzyme under basal conditions to prevent excessive NO production and nitrosative stress. In addition to the systemic vascular effects of Cav1 depletion, mice lacking Cav1 acquire pulmonary hypertension attributed to dysregulated eNOS activity (Zhao et al., 2009). The mechanism responsible for this pathology is proposed to be mediated through increased protein kinase G tyrosine-nitration due to nitrosative stress, which inhibits protein kinase G, thus preventing smooth muscle

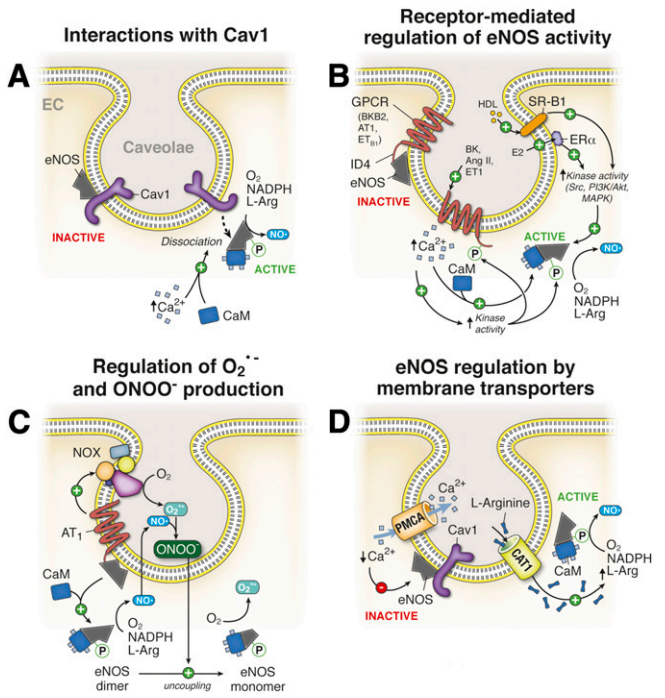

Fig. 4. Nitric oxide regulation at plasma membrane caveolae. (A) eNOS localizes to plasma membrane caveolae, where it directly binds to Cav1. This interaction inhibits basal eNOS activity and NO synthesis. Increases in Ca^{2+} facilitate activation of CaM, which is recruited to eNOS and promotes dissociation of the enzyme from Cav1. Binding of CaM to free eNOS increases its enzymatic activity, resulting in NO production from the substrates L-arginine, NADPH, and O_2. (B) eNOS colocalizes with a number of membrane receptors in endothelial cell caveolae, including the angiotensin II type 1 receptor (AT_1), the bradykinin B_2 receptor (BKB2), the endothelin-1 type B receptor, the estrogen receptor (ERα), and the scavenger receptor (SR-B1). The GPCRs bind eNOS directly through an interaction with their fourth intracellular domain (ID4) and inhibit basal eNOS activity. Binding of GPCR ligands to their complement receptors promotes eNOS dissociation from these receptors, relieving the inhibitory clamp that is mediated through increases in intracellular Ca^{2+} and phosphorylation of eNOS and the GPCR, leading to NO production by eNOS. Activation of SR-B1 by high-density lipoprotein (HDL) or ERα by estradiol promotes activation of protein kinases, including Src, PI3K/Akt, and MAPKs, which promote eNOS phosphorylation and enzyme activation. (C) Activation of the AT1 receptor in caveolae signals eNOS activation as described in (B) as well as the recruitment and activation of NOX to caveolae. NOX activation produces superoxide anion ($O_2^{\bullet-}$), which uncouples dimeric eNOS to its monomeric form, resulting in $O_2^{\bullet-}$ production. NO and $O_2^{\bullet-}$ generated at EC caveolae rapidly react to form the free radical peroxinitrite ($ONOO^-$). See Fig. 6 for NOX regulation. (D) eNOS enriched at EC caveolae colocalizes with and is regulated by the activity of the cationic amino acid transporter 1 (CAT1) and the plasma membrane Ca^{2+} ATPase (PMCA). In caveolae, PMCA functions to extrude Ca^{2+} from the local cytosolic compartment and the subsequent reduction in free intracellular Ca^{2+} prevents CaM recruitment and Cav1 dissociation from eNOS, inhibiting NO production. Conversely, CAT1 facilitates the cellular uptake of the eNOS substrate L-arginine in spatial proximity to the synthase, providing local enrichment in the precursor for NO synthesis.

relaxation and increasing vascular resistance in the pulmonary vasculature. This pulmonary hypertension has been shown to be ablated in mice lacking both Cav1 and eNOS or by eNOS inhibition in $Cav1^{-/-}$ mice with L-NAME treatment (Zhao et al., 2009). In addition to the effects of Cav1 deficiency on the vascular tone, ECs isolated from $Cav1^{-/-}$ mice have an impaired barrier function due in part to increased nitrosative stress and nitration of regulatory proteins involved in adherens junction assembly and maintenance (Siddiqui et al., 2011).

After the characterization of this signaling complex in caveolae, the Sessa laboratory developed a non-inhibitory analog of cavtratin that contains alanine substitutions at Thr90, Thr91, and Phe92 called cavnoxin (Bernatchez et al., 2011). Cavnoxin competes with Cav1 for binding the oxygenase domain of eNOS but, unlike cavtratin, does not inhibit eNOS activity. Instead, cavnoxin increases basal NO production in eNOS-expressing cells, decreases vascular resistance in isolated arterioles, and reduces blood pressure in mice (Bernatchez et al., 2011).

b. Endothelial nitric-oxide synthase interactions with membrane receptors localized to caveolae in endothelial cells. Multiple plasma membrane receptors have been implicated in signal transduction pathways that regulate the activity of eNOS in caveolae including the bradykinin receptor B2, the angiotensin receptor, the endothelin 1 receptor, estrogen receptor α (ERα), and the scavenger receptor class B type 1 (SR-B1) (Fig. 4B) (Ju et al., 1998; Chen et al., 1999; Bernier et al., 2000; Chambliss et al., 2000; Golser et al., 2000; Haynes et al., 2000; Waid et al., 2000; Hisamoto et al., 2001; Yuhanna et al., 2001; Suzuki et al., 2006). The G-protein coupled receptors (GPCRs) have been shown to bind to eNOS in caveolae through a C-terminal intracellular domain (ID4) and inhibit the enzyme under basal conditions (Ju et al., 1998). Activation of these GPCRs by their complement ligands relieves the inhibitory clamp on eNOS by inducing an increase in intracellular Ca^{2+} (see above) and recruitment of CaM to eNOS or it can stimulate phosphorylation cascades, ultimately targeting activating residues in eNOS or the receptor itself causing dissociation of the receptor from eNOS (McDuffie et al., 1999; Bernier et al., 2000; Golser et al., 2000; Waid et al., 2000; Suzuki et al., 2006). Both mechanisms result in eNOS activation and NO production in ECs. Incubation of purified ID4 domains with purified eNOS decreases the enzyme's catalytic activity and NO bioavailability, providing direct evidence for a GPCR:eNOS signaling complex (Ju et al., 1998; Golser et al., 2000).

Of particular note, eNOS regulation at caveolae in ECs by the AT_1 receptor provides complex control over the production of NO and its oxidized derivative peroxinitrite ($ONOO^-$) (Fig. 4C). Activation of the caveolae angiotensin receptor by angiotensin II (Ang II) promotes eNOS activation through the mechanisms described above but may also promote recruitment and activation of NADPH oxidase (NOX) to this microdomain (Pueyo et al., 1998; Lobysheva et al., 2011). This process results in the generation of $O_2^{\bullet -}$, which can readily react with eNOS-derived NO to form $ONOO^-$. Upon $ONOO^-$ accumulation, eNOS dimers uncouple to their monomeric form in the caveolae resulting in the synthesis of $O_2^{\bullet -}$ rather than NO. In addition, $ONOO^-$ may diffuse within the cell or to surrounding cells in the vascular wall, imparting oxidative stress (Huie and Padmaja, 1993; Hogg et al., 1994).

Additional evidence has supported a role for high-density lipoprotein (HDL) mediated eNOS activation by binding to and activating SR-B1, conferring atheroprotective NO production (Yuhanna et al., 2001). Binding of HDL to SR-B1 localized to EC caveolae leads to activation of several protein kinases including Src, MAPK, and PI3K/Akt, which function to phosphorylate and activate eNOS. Likewise, the estrogen receptor ERα colocalizes with eNOS in plasma membrane caveolae, and binding of estradiol to this receptor activates eNOS and promotes NO generation in ECs through the coordinated action of protein kinase-mediated eNOS phosphorylation (Chambliss et al., 2000; Haynes et al., 2000; Hisamoto et al., 2001). This signaling mechanism has been suggested to be an important determinant for atheroprotection in females that can be mimicked in the male population by estrogen supplementation.

c. Regulation of endothelial nitric-oxide synthase by membrane transporters and channels in caveolae. The sequestration of eNOS to caveolae in ECs poises the enzyme in a spatial region of the cell where specific membrane channels and transporters have been shown to colocalize and regulate eNOS activity by tightly controlling the abundance of essential cofactors and substrates. Notably, the cationic amino acid transporter 1 (CAT1) colocalizes with eNOS and Cav1 in endothelial cell caveolae, where its function has been proposed to regulate the cellular import of L-arginine, the prerequisite substrate for NO synthesis by eNOS, in close proximity to the enzyme (Fig. 4D). Studies have shown that the CAT1 coimmunoprecipitates with eNOS and Cav1 from isolated endothelial cell membranes, suggesting a regionalized interaction between these proteins in caveolae (McDonald et al., 1997). Therefore, the regulated activity of CAT1 in vascular endothelial cell caveolae may impart functional effects on NO synthesis through localized modulation of substrate bioavailability.

In addition to CAT1, the plasma membrane calcium ATPase (PMCA) has also been found to localize to plasma membrane caveolae. Notably, PMCA is concentrated 18- to 25-fold higher in caveolae compared with noncaveolae plasma membrane fractions (Fujimoto, 1993; Schnitzer et al., 1995). PMCA is a P-type ATPase

that plays a crucial role in the regulation of cell calcium homeostasis (Di Leva et al., 2008). As its name indicates, it utilizes energy from ATP hydrolysis to extrude calcium from the cytosol to the extracellular space (Di Leva et al., 2008). In human umbilical vein endothelial cells (HUVECs), treatment with methyl β cyclodextrin (MβCD), which disrupts caveolae by sequestering cholesterol from the compartment, significantly reduces Ca^{2+} efflux mediated by PMCA (Zhang et al., 2009). Conversely, replenishment of cholesterol to MβCD-treated cells restored PMCA-mediated calcium efflux from ECs (Zhang et al., 2009). Because eNOS activity is highly dependent on calcium, PMCA was shown to negatively regulate eNOS activity by extruding Ca^{2+} from the cytosol, decreasing the local concentration of Ca^{2+} in spatial proximity to eNOS in caveolae (Holton et al., 2010). This active shuttling of Ca^{2+} to the extracellular compartment decreases CaM recruitment to Cav1 bound eNOS, preventing dissociation of the two proteins and ultimately preventing NO synthesis (Fig. 4A). In addition to the control of local Ca^{2+} availability at the caveolae membrane, recent work has identified a novel binding interaction between endothelial cell PMCA and eNOS that may directly antagonize eNOS activity and NO production partly through effects on eNOS phosphorylation status (Holton et al., 2010). Taken together, these observations provide evidence for a regulated signaling microdomain between CAT1, PMCA, and eNOS isoforms in EC caveolae.

4. Compartmentalized Nitric Oxide Signaling at the Myoendothelial Junction. The MEJ is a distinct anatomic structure in the blood vessel wall where (predominantly) ECs send cellular projections through small holes in the internal elastic lamina separating the intima and media that allow for direct cell-to-cell contact with the overlying smooth muscle cell layer (Moore and Ruska, 1957; for review, see Heberlein et al., 2009). In small resistance arteries, MEJs are numerous compared with larger conduit arteries and coordinate signal transduction between the smooth muscle and endothelium by facilitating intercellular transport of small molecules and ions as well as harboring polarized proteins involved in vascular cell crosstalk (Sandow and Hill, 2000; Dora et al., 2003a; Isakson and Duling, 2005; Isakson et al., 2007; Isakson, 2008; Straub et al., 2011).

a. Nitric oxide regulation of gap junction permeability at the myoendothelial junction. The MEJ has emerged as an important signaling microdomain in the vasculature with a number of signaling proteins localized to the junction that influence vascular homeostasis, most notably gap junctions comprised of connexins.

The presence of gap junctions at the MEJ influences smooth muscle-endothelial cell coupling, and the permeability of connexin 43 (Cx43)-based gap junctions is tightly regulated by nitric oxide (Straub et al., 2011). Use of a vascular cell coculture model has revealed an enrichment of Cx43 and eNOS localized to the MEJ in vitro, and characterization of this enrichment in vivo by immunolabeling coupled to transmission electron microscopy has confirmed this observation in the intact arterial wall (Straub et al., 2011). Concurrent with a localized enrichment of these two proteins at the MEJ, a novel signaling microdomain involving eNOS and Cx43 has been identified in which the post-translational modification of Cx43 at the MEJ by S-nitrosylation of Cys271 regulates the permeability of these intercellular channels. S-Nitrosylation of Cx43 promotes an open-channel conformation allowing exchange of cytosolic constituents between smooth muscle and endothelium. Cx43 is constitutively S-nitrosylated at the MEJ due to colocalization of eNOS, which harbors a low level of basal activity (Straub et al., 2011). In small resistance arteries, stimulation of smooth muscle α_1-adrenergic receptors promotes induction of G_q-dependent signaling cascades, leading to the activation of phospholipase C that cleaves the phospholipid phosphatidylinositol 4,5-bisphosphate to IP_3 and diacylglycerol, increasing the cytosolic inositol triphosphate (IP_3) concentration. The generated IP_3 induces Ca^{2+} release from the smooth muscle cell SR (see above) but can also directly traverse gap junctions at the MEJ to activate the IP_3 receptor type 1 (IP_3R1) in ECs (Isakson et al., 2007; Isakson, 2008). This has been proven in vivo in mesenteric and cremasteric arteries, where IP_3 diffusion from SMCs can elicit a local increase in $[Ca^{2+}]_i$ in ECs specifically at the MEJ where a subset of the IP_3R1 isoform is poised at extensions of the ER within the MEJ (Isakson, 2008; Ledoux et al., 2008). The initial rise in endothelial calcium activates another enzyme polarized and enriched at the MEJ, the *S*-nitrosoglutatione reductase (GSNOR), whose activation facilitates denitrosylation of Cx43 and channel closure (Straub et al., 2011) (Fig. 5, top). After activation of GSNOR and denitrosylation of Cx43, the rise in endothelial cell $[Ca^{2+}]_i$ promotes eNOS activation and subsequent generation of NO at the MEJ, which diffuses to and relaxes the SMCs and ultimately renitrosylates Cx43 at the MEJ, presumably providing rapid spatial and temporal control of heterocellular communication between the vascular cells (Straub et al., 2011). In addition to the role of smooth muscle derived IP_3 in regulating endothelial cell $[Ca^{2+}]_i$ signaling at the MEJ, smooth muscle Ca^{2+} released from the SR downstream of IP_3 mobilization may also influence heterocellular communication by traversing the gap junctions at the MEJ to control eNOS and GSNOR activity (Isakson et al., 2007). Regardless of the second messenger to elicit the increase in $[Ca^{2+}]_i$, the NO-related dynamics on the EC side of the MEJ likely remain the same. These studies have shown a novel interaction between Cx43, IP_3R1, eNOS, and GSNOR localized to the MEJ, providing evidence for a signaling microdomain that can regulate

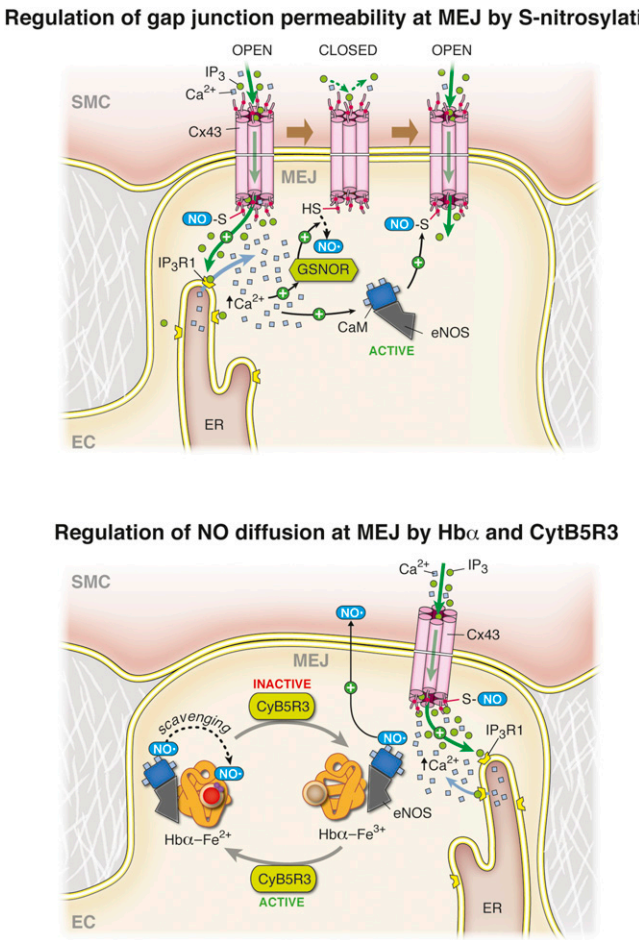

Fig. 5. Nitric oxide regulation at the MEJ. Top, Nitric oxide posttranslationally modifies the gap junction protein connexin 43 (Cx43) at the MEJ through S-nitrosylation. Under basal conditions, Cx43 is constitutively S-nitrosylated, which renders the channel in an open, permeable state. IP_3 and Ca^{2+} from the smooth muscle cell layer diffuse through these open gap junctions and bind to IP_3 receptor type 1 (IP_3R1) on the EC endoplasmic reticulum that is poised at the MEJ, resulting in Ca^{2+} release from the internal store. This local increase in Ca^{2+} promotes the activation of the denitrosylase enzyme *S*-nitrosoglutathione reductase (GSNOR) whose activity leads to denitrosylation of Cx43. Reduction of the *S*-nitrosothiols on Cx43 closes the channel, preventing additional ion and metabolite diffusion into the ECs. After this event, the local rise in Ca^{2+} activates the eNOS localized at the MEJ, resulting in increased NO production and renitrosylation of Cx43 gap junctions, restoring gap junctional communication between ECs and SMCs in the arterial wall. The black dashed arrow indicates reduction of the nitrosothiol, and the red dots on Cx43 correspond to the cysteine residue on the carboxyl tail of Cx43 that is S-nitrosylated. Bottom, Hemoglobin α (Hbα) is synthesized by vascular ECs and is enriched at the MEJ, where it forms a complex with eNOS and the reductase CytB5R3. NO generated by eNOS at the MEJ is able to diffuse to the overlying smooth muscle cell layer when Hbα resides in the Fe^{3+} state (methemoglobin, maroon). Reduction of Hbα to the Fe^{2+} state (oxyhemoglobin, red) by the activity of CytB5R3 promotes NO scavenging by Hbα and prevents NO diffusion.

cellular communication between the EC and SMC in the blood vessel wall.

b. Nitric oxide signaling regulation at the myoendothelial junction by hemoglobin α and cytochrome B_5 reductase 3. Polarization and enrichment of eNOS at the EC side of the MEJ poises the enzyme in close proximity to the overlying SMC layer, allowing for rapid NO diffusion upon eNOS activation. Recent evidence has shown that the alpha chain of hemoglobin (Hbα) is actively synthesized by arterial ECs and polarizes to the MEJ in small resistance arteries (Straub et al., 2012). In addition, the Hbα subunit has been shown to be expressed in small mesenteric arteries (Burgoyne et al., 2012). In red blood cells, hemoglobin is known to scavenge NO through reactive chemistry involving its heme center (Cassoly and Gibson, 1975). Recent work has also found a similar function of Hbα at the MEJ, where monomeric Hbα acts to regulate NO diffusion to SMCs and functionally impacts arterial reactivity (Straub et al., 2012).

NO scavenging by hemoglobin in the red blood cell can be regulated by the reducing activity of the enzyme cytochrome B_5 reductase 3 (CytB5R3), where the enzyme acts to reduce the heme iron in Hb from the Fe^{3+} state to the Fe^{2+} state (Hultquist and Passon, 1971). Importantly, the reaction kinetics of NO with heme iron in the Fe^{2+} state is extremely rapid ($2.4 \times 10^7\ M^{-1}{\cdot}s^{-1}$) (Cassoly and Gibson, 1975), whereas the control of NO diffusion by heme iron in the Fe^{3+} state occurs more slowly ($3.3{\times}10^3\ M^{-1}{\cdot}1{\cdot}s^{-1}$) (Sharma et al., 1987). As a result, Hbα residing at the MEJ in the Fe^{2+} state may scavenge NO rapidly, preventing diffusion to SMCs and maintaining a more constricted state. Conversely, Hbα in the Fe^{3+} state may allow for greater NO diffusion to SMCs, enhancing vasorelaxation. Concurrently, NO scavenging by heme iron in the Fe^{2+} state can limit the amount of NO diffusing to the overlying SMC layer, whereas heme iron in the Fe^{3+} binds NO at a slower rate. It is noteworthy that CytB5R3 was also present at the MEJ and that coimmunoprecipitation studies using in vitro MEJ fractions, intact arteries, and purified proteins have indicated that eNOS, Hbα, and CytB5R3 form a protein complex enriched at this signaling nexus (Straub et al., 2012).

In isolated resistance arteries, siRNA knockdown of Hbα in EC potently increased NO diffusion through the arterial wall, indicating the functional importance of Hbα in scavenging endothelium-derived NO (Straub et al., 2012). Furthermore, pressure myography studies on these arteries have provided functional evidence for NO scavenging by EC Hbα in response to phenylephrine and acetylcholine stimulation, two agonists that induce NO synthesis by the endothelium (Straub et al., 2012). Further analysis revealed that CytB5R3 actively reduces Hbα to the Fe^{2+} state at the MEJ, imparting strict control over the ability of Hbα to scavenge diffusible NO at the interface of endothelium and smooth muscle (Straub et al., 2012). These observations have identified a novel signaling microdomain at the MEJ in small resistance arteries where Hbα, eNOS, and CytB5R3 work in concert to regulate arterial tone (Fig. 5, bottom).

The identification of multiprotein signaling microdomains at the MEJ has shed light on the regulation of

heterocellular communication in the resistance vasculature and provides insight into the complex control of arterial tone. At one axis, it is now clear that gap junctions at the MEJ can be direct targets of bioactive NO that is synthesized in close proximity to the junction by eNOS. Furthermore, the eNOS:Hbα:CytB5R3 ternary complex that resides at the MEJ plays a role in regulating the diffusion of NO to the junction and surrounding SMCs to influence arterial tone. These new insights into signal propagation between ECs and SMCs may prove to be intimately associated where Hbα may not only function to control the amount of NO diffusing to the SMC to activate cGMP-dependent relaxation, but also regulate the extent of Cx43 S-nitrosylation and ultimately gap-junction-mediated heterocellular communication. In this respect, decreased NO scavenging by Hbα at the MEJ may favor S-nitrosylation of Cx43 poising the junctions in an open conformation and allow diffusion of IP_3 and calcium from the SMC to EC, promoting vasodilation (Fig. 5).

In conclusion, eNOS activation and NO bioavailability results from a complex regulating process involving direct protein interaction with multiple players (Cav1, GPCR, Hbα, CytB5R3, PMCA, etc.) that accumulate within a specialized phospholipid region, caveolae, and, in some cases, are polarized to a specific region of the cells, the MEJ. With regard to our definition in Table 1 and the work described above, eNOS and its protein partners are an important signaling microdomain for intercellular communication in the blood vessel wall.

B. Reactive Oxygen Species Are Signaling Molecules Involved in Intercellular Communication

Oxygen is a necessary molecule for cellular function by fueling the respiratory chain in the mitochondria. Paradoxically, oxygen is also the main source of reactive oxygen species (ROS) that cause a multitude of cellular damages within the cell. The superoxide anion ($O_2^{\bullet -}$) is at the origin of the formation of other ROS such as hydrogen peroxide (H_2O_2) or the hydroxyl radical (·OH). The superoxide anion can also react with NO and form peroxynitrite (ONOO·), which can further generate reactive nitrogen species that also have deleterious cellular effects (for review, see Martinez and Andriantsitohaina, 2009). When ROS are produced in large amount, due to upregulation of the proteins orchestrating their synthesis and/or decreased ROS degradation or scavenging, it results in cellular oxidative stress. Oxidative stress occurs in multiple pathologic conditions, including hypertension, atherosclerosis, and myocardial infarction where ROS are involved in VSMC proliferation and migration, monocyte infiltration, endothelial dysfunction, and remodeling of the extracellular matrix (for review, see Touyz et al., 2011; Montezano and Touyz, 2012; Sedeek et al., 2012). The plasma level of the ROS H_2O_2 is increased in patients with hypertension compared with healthy patients, suggesting its importance as a paracrine molecule mediating oxidative stress (Varma and Devamanoharan, 1991; Lacy et al., 1998; Halliwell et al., 2000).

The short half-life of ROS and their high reactivity has led to the hypothesis that these oxidant molecules may be produced and exert their effects in a restricted space (Davidson and Duchen, 2006). Although the concept of a "ROS microdomain" has never been clearly established, $O_2^{\bullet -}$ production was observed in discrete regions at the plasma membrane and in the area surrounding the mitochondria (Zorov et al., 2000; Aon et al., 2003; Brady et al., 2004; Davidson and Duchen, 2006; Yi et al., 2006; Garcia-Perez et al., 2012; Siddall et al., 2013). Superoxide anion can be produced via several pathways, both intracellularly and extracellularly. The main intracellular source of $O_2^{\bullet -}$ is the mitochondria, which "leaks" electrons from the respiratory chain that react with molecular oxygen. At the plasma membrane, NADPH oxidase is the principal source of $O_2^{\bullet -}$ and produces $O_2^{\bullet -}$ extracellularly. Given the extremely short half-life of the superoxide anion and the presence of an extracellular form of superoxide dismutase (EC-SOD), it has been hypothesized that $O_2^{\bullet -}$ is rapidly dismutated to H_2O_2 in the extracellular space. Because H_2O_2 is a lipophilic molecule, it can readily diffuse through the plasma membrane and exert its oxidative effects intracellularly. Conversely, due to the lipophilic nature and relative stability of H_2O_2 compared with $O_2^{\bullet -}$, it has been suggested that H_2O_2 is the central molecule imparting oxidative stress. However, it is becoming increasingly clear that $O_2^{\bullet -}$ produced extracellularly by Nox has an effect intracellularly, which has subsequently led several groups to investigate candidate proteins for the transport of the $O_2^{\bullet -}$ across the plasma membrane.

1. NADPH Oxidase as a Signaling Microdomain. The NADPH oxidase (Nox) was first characterized in phagocytic cells where it plays an important role in the antimicrobial defense via the production of superoxide anion (Parkos et al., 1987; Rotrosen et al., 1992). Nox is a multimeric protein complex that could be considered in itself a signaling microdomain because it is composed of two distinct enzymatic subunits that interact with multiple small regulating proteins. The intrinsic transmembrane component of the Nox is formed by a heterodimeric flavocytochrome, which comprises two subunits, gp91phox and p22phox (Fig. 6) (Parkos et al., 1987; Rotrosen et al., 1992). This heterodimeric flavocytochrome is also called cytochrome b_{558}, based on its spectroscopic properties. These subunits were named after their molecular mass on gel electrophoresis whereas the letters indicate a protein (p) or glycoprotein (gp) of the phagocyte oxidase (phox) (Dinauer et al., 1987; Parkos et al., 1987, 1988; Rotrosen et al., 1992). Several homologs of gp91phox have been identified and are termed Nox1, Nox3, Nox4, Nox5, Duox1, and Duox2,

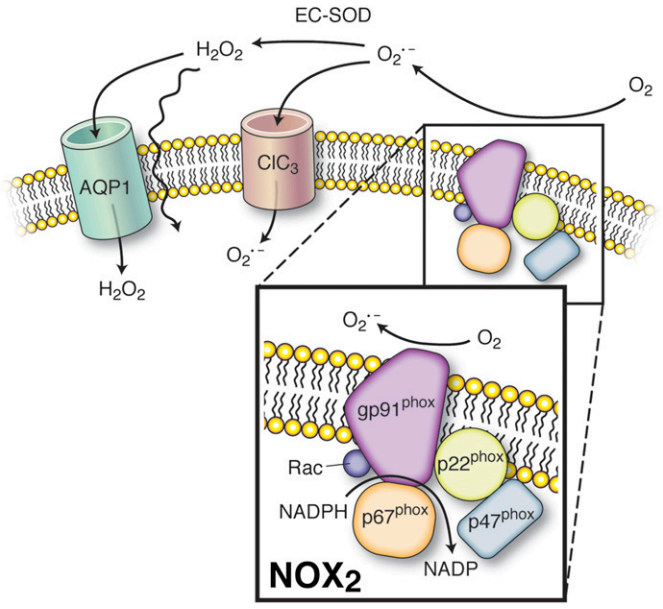

Fig. 6. ROS microdomain at the plasma membrane. NADPH oxidase is the main source of $O_2^{\bullet-}$ at the plasma membrane where it produces $O_2^{\bullet-}$ in the extracellular space. The NADPH oxidase protein complex represented here corresponds to the Nox2 isoform (previously termed gp91phox). Upon stimulation, the regulators of gp91phox, including gp22phox, gp67phox, gp47phox, and Rac assemble with the gp91phox. The $O_2^{\bullet-}$ produced extracellularly can either be dismuted by the extracellular superoxide dismutase (EC-SOD) or traverse the chloride channel ClC3 at the plasma membrane. In the extracellular space, the dismutation of $O_2^{\bullet-}$ results in the production of H_2O_2, which can either cross the plasma membrane directly because of its lipophilic property or via the aquaporin channels (AQP1).

with the isoform gp91phox subsequently renamed Nox2 (for review, see Bedard and Krause, 2007; Montezano et al., 2011). These isoforms have different modes of regulation, expression, and activity but all harness the capacity to produce $O_2^{\bullet-}$, with the exception of Nox4, which has been shown to mainly produce H_2O_2 (Wu et al., 2010). The Nox1, Nox2, Nox4, and Nox5 isoforms have all been identified in the vasculature, with differential expression in both ECs and VSMCs (Touyz et al., 2011). Although each Nox isoform (with the exception of Nox5) requires the presence of the enzymatic subunit p22phox, their activity is differentially regulated: Nox1 is regulated by NOX1A, NOX1B, and Rac1, whereas Nox2 is regulated by p47phox, p67phox, and the small GTPase (Rac) (Nunoi et al., 1988; Volpp et al., 1988; Abo et al., 1991; Bedard and Krause, 2007). Although the regulators of Nox1 and 2 have been well characterized, the mechanisms controlling Nox4 and Nox5 activity are not as well understood; however, it appears that Nox4 is constitutively active and Nox5 does not require the subunit p22phox (Montezano et al., 2011).

In resting conditions, the different elements composing the Nox enzymatic complex are colocalized at the plasma membrane, but they only interact with each other upon stimulation, which brings each subunit and regulating protein in close apposition (Sumimoto et al., 1996; Han et al., 1998; Groemping et al., 2003). Once activated, two heme groups present in the transmembrane portion of the gp91phox subunit allow for the transfer of electrons from a cytosolic NADPH to a molecular oxygen on the extracellular side of the plasma membrane, thus producing $O_2^{\bullet-}$ in the extracellular milieu (Bedard and Krause, 2007). In the vasculature, production of $O_2^{\bullet-}$ can be activated by a variety of stimuli such as Ang II, inflammatory mediators, and shear stress (Fisher, 2009). The activation of Nox, specifically Nox2, by Ang II has been at the center of a multitude of investigations, and it is now well described that Ang II induces the phosphorylation of the regulatory subunits p47phox and p67phox, causing them to relocate to the plasma membrane with the heterodimeric flavocytochrome b_{558} (Griendling et al., 1994). In these conditions, p38 MAPK is activated by the Nox2-derived ROS and results in VSMC hypertrophy (Ushio-Fukai et al., 1998).

The superoxide anion produced extracellularly by the Nox can exert its effect via several pathways. The most well described pathway involves dismutation of $O_2^{\bullet-}$ to H_2O_2 in the extracellular space by the EC-SOD. Thus, H_2O_2 has been considered a second messenger that is involved in intercellular communication as well as in intracellular signalization (Bedard and Krause, 2007). The role of H_2O_2 as a paracrine molecule has been demonstrated in the vascular wall, where H_2O_2 is key in the communication between the vascular adventitia and VSMCs and is involved in VSMC hypertrophy (Liu et al., 2004). In parallel, studies indicate that H_2O_2 derived from the adventitia plays a critical role in the relaxation of rat carotid artery in response to acetylcholine by activating p38 MAPK and inhibiting the protein tyrosine phosphatase SHP-2 in VSMCs (Cascino et al., 2011). In addition to the damaging effect of $O_2^{\cdot-}$-dependent H_2O_2 production, it has become evident that despite its short half-life, $O_2^{\bullet-}$ may itself play an active role in oxidative cellular damage. Studies incorporating exogenous SOD to deplete $O_2^{\bullet-}$ have aided in the investigation of a primary role for the free radical during oxidative stress. For example, mice deficient in EC-SOD exhibit higher systemic blood pressure upon Ang II infusion compared with control mice (Welch et al., 2006). Likewise, a recombinant heparin-binding form of SOD acutely decreased blood pressure in spontaneously hypertensive rats (Chu et al., 2003). These observations have raised questions regarding the exact mechanism responsible for intracellular oxidative damages caused by the extracellular production of $O_2^{\bullet-}$, and several investigators suggested different mechanisms that may facilitate the transport of $O_2^{\bullet-}$ across the plasma membrane, including the voltage-gated chloride channel ClC-3 and aquaporin (Fig. 6).

2. Candidates for Transport of Reactive Oxygen Species across the Plasma Membrane.

a. ClC-3 channels. ClC-3 is a member of the family of voltage-gated chloride channels that is abundantly

expressed in the VSMCs and ECs where it can be activated by increases in cell volume or cellular stretch (Duan, 2011). Additionally, ClC-3 channels have been shown to open in conditions where the cells are stimulated by Ang II, ET-1, CaM kinase II, and ROS (for review, see Duan, 2011). Evidence for a flux of $O_2^{\bullet -}$ across the plasma membrane has been demonstrated in pulmonary microvascular ECs using the superoxide-sensitive dye hydroethidine (Hawkins et al., 2007). When $O_2^{\bullet -}$ was added to the extracellular milieu, the fluorescence of EC loaded with hydroethidine increased significantly, and this was abolished when cells were pretreated with the anion channel blocker (4,4′-diisothiocyanato-2,2′-stilbenedisulfonic acid disodium salt) or with siRNA targeting ClC-3 (Hawkins et al., 2007). The authors observed an increase in $[Ca^{2+}]_i$ when cells were treated with exogenous superoxide anion (Hawkins et al., 2007). The calcium appeared to be originating from the ER, and in turn, calcium altered the mitochondrial membrane potential, inducing more superoxide anion production from the mitochondria (Madesh et al., 2005; Hawkins et al., 2007).

In pulmonary hypertension, increased ROS production has been correlated with increased Nox expression and/or increased activity of Nox (for review, see Freund-Michel et al., 2013). Additionally, the increased ROS production has been shown to be involved in VSMC proliferation and endothelial dysfunction (Freund-Michel et al., 2013). Interestingly, in a model of monocrotaline-induced pulmonary hypertension, the expression of the gene coding the ClC-3 channels was upregulated in rat pulmonary arteries and cultured canine PASMCs (Dai et al., 2005b). Although no direct link has been established between the overexpression of Nox4 and ClC-3, the in vitro data reported by Hawkins et al. (2007) could lead to the hypothesis that the channel overexpression contributes to the influx of extracellular $O_2^{\bullet -}$ produced by the enzyme.

b. Aquaporin. As discussed above, H_2O_2 has been assumed to be the best candidate for intracellular damage given its longer half-life and lipophilic properties. Because of these characteristics, it was assumed that H_2O_2 exerts its intracellular effects by diffusing across the plasma membrane. This notion was recently challenged by Miller et al. (2010), who reported that aquaporin can mediate H_2O_2 uptake in mammalian cells. In this study, human embryonic kidney-293 and HeLa cells overexpressing different isoforms of aquaporin showed increased uptake of the chemoselective H_2O_2 indicator Py1-ME (Miller et al., 2010).

In the vasculature, Pagano's laboratory was the first to describe the transport of H_2O_2 through aquaporin-1 channels at the plasma membrane of VSMCs (Al Ghouleh et al., 2013). In their model, H_2O_2 applied exogenously on rat aortic SMCs penetrated into the cells via aquaporin-1 and activated superoxide anion production by Nox1 (Al Ghouleh et al., 2013). This signaling cascade leads to the phosphorylation of the protein Ask1, which results in hypertrophy of SMCs.

The presence of multiple regulators directly interacting with the different Nox isoforms makes the NADPH oxidase complex a signaling microdomain by itself. Additionally, because the structural characteristic of the enzyme results in extracellular production of $O_2^{\bullet -}$ and the presence of 1) channels that allow the transfer of the ROS into the cell and 2) EC-SOD that degrades the $O_2^{\bullet -}$ into H_2O_2, it could be argued that a ROS signaling microdomain is an important component of vascular cell communication.

C. Signaling Microdomains in the Release of Hydrogen Sulfide and Carbon Monoxide?

In addition to the well-defined nitrogen-related gaseous species described above, carbon monoxide (CO) and hydrogen sulfide (H_2S) have also been shown to have potent effects in the blood vessel wall as specific cellular signaling molecules that can alter local vasoconstriction/dilation and blood pressure [e.g., CO (Suematsu et al., 1994; Johnson et al., 1997; Caudill et al., 1998; Leffler et al., 1999); H_2S (Zhao et al., 2001; Yang et al., 2008; Leffler et al., 2011a; Liang et al., 2011)]. Both gases are produced by ubiquitously expressed enzymes. Although their production and activation have yet to be identified as being regulated by signaling microdomains, emerging evidence may indicate a possible role in CO production.

Heme oxygenase [HO; found in two isoforms, HO-1 (inducible) and HO-2 (constitutive)] catalyzes the O_2-dependent degradation of heme into free iron, biliverdin IXa, and CO. NADPH-cytochrome P450 reductase provides the electrons for this catalysis (for review, see Kim et al., 2006; Leffler et al., 2011b). Although both HO-1 and HO-2 are primarily found in the endoplasmic reticulum (e.g., Tenhunen et al., 1968), there is now sufficient evidence that both enzymes can associate with Cav1 at the plasma membrane of mesengial (Jung et al., 2003) and endothelial cells (Kim et al., 2004). Further reports indicated that Cav1 is a competitive inhibitor for HO-1, identifying a minimum sequence required for binding (Taira et al., 2011) and possibly regulating activation of BKCa channels in endothelium (Riddle and Walker, 2012) [although this Cav1 interaction has yet to be detected in smooth muscle cells where the link between BK and CO was originally identified and well-described (Wang et al., 1997; Jaggar et al., 2002, 2005; Xi et al., 2004)]. One report indicated that cytochrome P450 reductase is also associated with Cav1 (Jung et al., 2003), potentially forming a compartmentalized signaling microdomain for CO production. Further work on this concept is required to elucidate the role for sequestration of these proteins into microdomains and the importance in cellular signaling. In addition, because CO is potently

scavenged by hemoglobin, it will be interesting to determine how the Hbα expressed in the endothelium of small arteries (Burgoyne et al., 2012; Straub et al., 2012) may interact and modulate CO function.

The production of H_2S is by a series of steps starting with serine and homocysteine that proceeds through cystathionine beta synthase (with H_2S as a substrate) producing cystathionine, which feeds into cystathionine γ-lyase (with H_2S as a substrate) producing cysteine; this is the trans-sulfuration pathway (Hildebrandt and Grieshaber, 2008). The cysteine produced from this pathway or other endogenous sources can then feed into the cysteine catabolism pathway, which converts cysteine into mercaptopyruvate via cysteine aminotransferase (Hildebrandt and Grieshaber, 2008). Lastly, mercaptopyruvate sulfurtransferase converts the meraptopyruvate into pyruvate and H_2S (although the exact acceptors remain unidentified) (Hildebrandt and Grieshaber, 2008; Kabil and Banerjee, 2010). There remains much to understand in regard to H_2S, including the following. 1) How does H_2S penetrate the plasma membrane? Under physiologic pH, H_2S does not readily move through plasma membranes and there are currently no known transports for this molecule (Mathai et al., 2009), 2) How can H_2S specifically be inhibited? The current pharmacological inhibitors have been called into question (Kabil and Banerjee, 2010). 3) What is the physiologic concentration of H_2S? The amount of H_2S needed to produce biologic effects is an order of magnitude above physiologically relevant levels (Furne et al., 2008; Whitfield et al., 2008; Olson, 2009). The answers to these questions may lead to a novel understanding of how such a fundamental gas could function in the vasculature. Currently there is no known localization of the H_2S enzymes together in a particular region of the cell that may constitute a signaling microdomain.

III. Channel-Based Cellular Communication by Signaling Microdomains

In section II, we described intercellular communication via free diffusion across the plasma membranes or extracellular production of signaling molecules by cells within the vascular wall. In this section, we will first discuss intercellular communication through the direct cytoplasmic transfer of molecules from one cell to another via gap junctions. We will further describe the multiple ion channels structured in a signaling microdomain, especially those involved in the endothelium-derived hyperpolarization (EDH) mediated response.

A. Gap Junction Channels

One of the first indications of a physiologic role for gap junctions in the vasculature came from studies by Segal and Duling (1986) that identified a "conducted vasodilation" in arterioles upstream of a vasodilator stimulus, suggesting that some form of direct communication was involved. Gap junction channels coordinate responses through the passage of small molecules between cells and are important in normal vascular physiology but are also highly implicated in vascular pathophysiology such as hypertension and arteriogenesis. The gap junctions as membrane proteins contain highly flexible intracellular regions that make them ideal for forming protein-protein interactions to regulate their function, and decades of studies have established their roles in signaling microdomains.

The gap junctions as a cellular structure were described nearly 50 years ago (Dewey and Barr, 1964). These membrane channels permit direct cell-to-cell (intercellular) transfer of ions, metabolites, and small molecules between two different cell cytoplasms and are essential for the maintenance of normal vascular functions. The small molecules include (but are not limited to) ATP, IP_3, nicotinamide adenine dinucleotide, and other metabolites up to 1 kDa in size (see Table 2). Gap junctions are ubiquitously expressed in almost all cells of the body, existing transiently at the cell surface and at points of cell-to-cell contact (Laird, 2006; Johnstone et al., 2009a). The protein subunit comprising gap junctions is the connexin (Cx), which assembles in hexameric channels at the plasma membrane. In this state, the hexameric channel is referred to as a connexin hemichannel or connexon, which is transported to the plasma membrane. Two connexin hemichannels residing on apposed cells further dock to each other, forming a gap junction channels. In total, 21 human connexin isoforms have been identified with a primary role of direct intercellular communication. The classification of connexin isoforms is based on their molecular weights, with further classification based on isoform interaction (Eastman et al., 2006). Within the cells of the blood vessel wall, four connexins have been well characterized: Cx37, Cx40, Cx43, and Cx45. In addition, it was recently shown that Cx32 is expressed within vascular cells and may contribute to direct cell-to-cell communication through gap junctions (Okamoto et al., 2009, 2011; Fowler et al., 2013). Typically, ECs predominantly express Cx37, Cx40, and Cx43, whereas VSMCs have been shown to express Cx37, Cx43, and Cx45 (Johnstone et al., 2009a,b; Okamoto et al., 2009). Additionally, within the resistance vasculature at sites of endothelial to smooth muscle contact (i.e., MEJ), Cx37, Cx40, and Cx43 have all been shown to be expressed (Haddock et al., 2006; Sandow et al., 2006; Isakson et al., 2008; Straub et al., 2010, 2011). However, connexin expression across the vascular wall varies widely depending on the animal species and the vascular bed examined. A number of factors may influence this, including sheer stress or changes in vessel wall structure (as can be found in vascular disease states such as atherosclerosis and hypertension) (Kwak et al., 2002; Johnson and Nerem, 2007; Vorderwulbecke et al., 2012). A more

TABLE 2
Substrates and ions permeable to gap junctions

Table summarizes molecules reported to traverse gap junction channels. Key publication(s) showing evidence for transfer of a molecule via gap junctions is indicated in the right column

	References
Substrate	
Glucose	Lavado et al., 1997; Goldberg et al., 1999, 2002; Qu and Dahl, 2004; Ma and Dahl, 2006
10 mer or < siRNA, shRNA, micro RNA	Neijssen et al., 2005; Valiunas et al., 2005; Wolvetang et al., 2007; Kizana et al., 2009; Brink et al., 2010; Katakowski et al., 2010; Lim et al., 2011
cAMP	Lawrence et al., 1978; Murray and Fletcher, 1984; Spray and Burt, 1990; Stagg and Fletcher, 1990; Locke et al., 2004; Bedner et al., 2006; Kanaporis et al., 2008
1-IP and 1,4-IP_2,1,4,5-IP_3, 1,4,6-IP_3, 1,3,4-IP_3, 1,3,4,5-P_4	Saez et al., 1989; Hansen et al., 1993; Kam et al., 1998; Niessen et al., 2000; Locke et al., 2004; Ayad et al., 2006; Straub et al., 2010; Decrock et al., 2012
cGMP	Bevans et al., 1998; Taimor et al., 2000; Locke et al., 2004
Ions/Small Molecules	
K^+	Veenstra et al., 1994a,b; Beblo and Veenstra, 1997
Ca^{2+}	Saez et al., 1989; Christ et al., 1992; Tour et al., 2007
Na^+	Veenstra et al., 1994a,b; Beblo and Veenstra, 1997; Behringer et al., 2012
Cl^-	Beblo and Veenstra, 1997
Glutamate	Beblo and Veenstra, 1997
$Nitrate^-$	Garant, 1972; Beblo and Veenstra, 1997
Lithium	Beblo and Veenstra, 1997

detailed examination of vascular specific differences in connexin expression profiles in EC and VSMC in vitro and in vivo can be found in Table 3.

1. Intrinsic Characteristics of Connexins Allowing for Protein Interactions. Connexin proteins possess an intracellular N terminus, four transmembrane-spanning domains, two extracellular loops, a single intracellular loop, and a highly flexible intracellular C-terminal region containing multiple sites for potential protein interactions and posttranslational modifications (Evans and Martin, 2002; Kovacs et al., 2007). Within the connexin isoforms expressed in the vasculature, C-terminal regions are essentially unstructured, allowing for rapid changes as a result of protein modifications such as phosphorylation and nitrosylation events (Sorgen et al., 2004a; Bouvier et al., 2009; Straub et al., 2012). These random coil or disordered domains are the main sites of connexin protein-protein interactions, and a diverse array of proteins have now been demonstrated to regulate connexin protein assembly, trafficking, and gating, ultimately affecting cellular communication involved in cell proliferation and migration (Wei et al., 2004; Johnstone et al., 2012b). Eliminating the C-terminal domain of Cx43, the most ubiquitously expressed isoform, significantly reduces the formation of large gap junction plaques but does not alter the proper trafficking and insertion into the plasma membrane, suggesting that interactions between the C-terminal domain and other proteins are required (but not essential) for efficient gap junction plaque formation (Duffy et al., 2004; Simek et al., 2009).

A further unique feature of connexin-protein interactions that can be crucial for incorporation into signaling microdomains is the apparent requirement for dimerization of two connexin proteins to allow for interactions such as interactions between the C terminus and the intracellular loop within the same Cx protein (Ponsaerts et al., 2010), interactions between Cx and the cell cycle regulating protein cyclin E (Johnstone et al., 2012b), and interactions with other connexins (Sorgen et al., 2004b; Zhou et al., 2007b). An alteration in the C-terminal structural conformation resulting from phosphorylation may play a central role in connexin C-terminal dimerization and interactions with other proteins, although the exact nature of this dimerization has not been determined (Kopanic and Sorgen, 2013; Grosely et al., 2013).

Oligomerization of the connexins primarily occurs within the ER for Cx37, Cx40, and Cx45 or later in the *trans*-Golgi network (TGN) for Cx32 and Cx43 (Das Sarma et al., 2001; Vanslyke et al., 2009; Smith et al., 2012). Composition of a connexin hemichannel can either be formed of six of the same connexin isoform (homomeric) or a mixture of multiple isoforms (heteromeric). Within the connexin isoforms expressed in the vasculature, heteromeric channels have been identified for Cx37/Cx40 (Ayad et al., 2006; Laird, 2006; Smith et al., 2012), Cx37/Cx43 (Brink et al., 1997; Larson et al., 2000; Wang et al., 2005; Beyer et al., 2013), Cx40/43 (He et al., 1999; Stergiopoulos et al., 1999; Bouvier et al., 2009), Cx40/Cx45 (Martinez et al., 2002); Cx43/Cx45 (Moreno et al., 1991; Koval et al., 1995), and Cx43:Cx32 (Lagree et al., 2003; Vanslyke et al., 2009). Typically, compared with homomeric hemichannels, these heteromeric associations produce channels with altered gating sensitivities to voltage, pH, or Ca^{2+}; altered channel opening; different ion selectivity; and

TABLE 3
Connexin expression in vascular cells

Key references indicating the expression of Cx32, 37, 40, 43, and 45 reported in ECs, SMCs, and at the MEJ in cell culture and in vivo. [Apologies to authors of any relevant studies missing from this table.]

System	Cell Type/ Location	Cx32	Cx37	Cx40	Cx43	Cx45
Cell culture (static/ with flow)	EC	HUVEC [Im, mRNA, WB (Okamoto et al., 2009, 2011)]	HUVEC [Im (Kameritsch et al., 2005)]	Mouse cremaster EC [Im, WB (Isakson and Duling, 2005)]	Mouse EC [Im, WB (Isakson and Duling, 2005; Straub et al., 2010)]	ND (Isakson and Duling, 2005)[e]
		HAEC, HMVEC, PAEC [mRNA (Okamoto et al., 2009)]	bEnd [Im (Pfenniger et al., 2012)]		HUVEC [Im, mRNA, WB (Okamoto et al., 2009, 2011; Straub et al., 2011)]	Bovine aortic EC [mRNA (Hirschi et al., 2003)]
			Mouse EC [Im (Isakson and Duling, 2005)]		HAEC, HMVEC, PAEC [mRNA (Okamoto et al., 2009)]	
			bEnd, increased with flow [Im, mRNA, WB (Pfenniger et al., 2012)]		Decreased expression in sheer stress models [WB (Feaver et al., 2008)]	
					Sheer stress and stretch increases Cx43 in bEnd cells (Kwak et al., 2005)	
	MEJ[a]	ND	Mouse cremaster VCCC, EC side only [Im (Isakson and Duling, 2005)]	Mouse cremaster VCCC, EC side only [Im (Isakson and Duling, 2005; Isakson, 2008)]	EC and VSMC [Im, WB (Isakson and Duling, 2005; Straub et al., 2010)]	**ND**
					HUVEC/HUVSMC VCCC [WB (Straub et al., 2011)]	
	SMC	ND	Mouse aortic [Im, WB (Isakson and Duling, 2005; Johnstone et al., 2012b)]	**NP** mouse cremaster VSMC [Im (Isakson and Duling, 2005; Isakson, 2008)]	HUVEC [WB (Straub et al., 2011)]	Mouse aortic (Johnstone et al., 2012b)
					Mouse and rat aortic [Im, WB (Johnstone et al., 2009b, 2012b; Straub et al., 2010)]	
Resistance/ muscular arteries[b]	EC	ND	Mouse/rat cremaster [Im (Sandow et al., 2006; Isakson et al., 2008)]	Mouse/ Rat cremaster [Im (Mather et al., 2005; Sandow et al., 2006; Isakson et al., 2008)]	Mouse cremaster [Im (Isakson et al., 2008; Straub et al., 2010)]	Mouse cremaster [Im (Isakson et al., 2008)]
			Mouse renal [Im (Braunstein et al., 2009)]	Mouse renal [Im (Braunstein et al., 2009)]	Mouse renal [Im (Braunstein et al., 2009)]	NP in transgenic mouse GFP reporter (Schmidt et al., 2012)
			Rat basilar artery [Im (Haddock et al., 2006)]	Mouse afferent arteriole [Im (Zhang et al., 2006a; Sorensen et al., 2012)]	Rat basilar artery [Im (Haddock et al., 2006)]	Mouse lung [Im (Wang et al., 2012)]
			Mouse afferent arteriole [Im (Zhang et al., 2006a; Sorensen et al., 2012)]	Rat Basilar artery [Im (Haddock et al., 2006)]	Mouse efferent arteriole ECs, lost in transgenic eNOS-null diabetic mice [Im (Zhang et al., 2006a)]	
	MEJ[f]	ND	Mouse/rat cremaster [Im (Sandow et al., 2006; Isakson et al., 2008)]	Mouse/rat cremaster [Im (Mather et al., 2005; Sandow et al., 2006; Isakson et al., 2008)]	Mouse/rat cremaster, also present as Cx43-pS368 [Im (Isakson et al., 2008; Straub et al., 2010)]	NP, Mouse/ rat cremaster [Im (Isakson et al., 2008)]
			Rat Basilar artery [Im (Haddock et al., 2006)]	Rat basilar artery [Im (Haddock et al., 2006)]	NP Rat basilar artery [Im (Haddock et al., 2006)]	
	SMC	ND	Mouse cremaster [Im (Isakson et al., 2008)].	**NP**-mouse cremaster [Im (Isakson et al., 2008)]	Mouse cremaster [Im (Isakson et al., 2008; Straub et al., 2010)]	Mouse cremaster [Im (Isakson et al., 2008), present in transgenic mouse GFP reporter (Schmidt et al., 2012)]
			Rat basilar artery [Im (Haddock et al., 2006)]	**NP**-rat basilar artery [Im (Haddock et al., 2006)]	Rat basilar artery [Im (Haddock et al., 2006)]	
			Mouse afferent arteriole [Im (Zhang et al., 2006a)]	Mouse afferent arteriole [Im (Zhang et al., 2006a)]		

(*continued*)

TABLE 3—*Continued*

System	Cell Type/ Location	Cx32	Cx37	Cx40	Cx43	Cx45
Conduit *arteries*[c]	EC	NP-mouse aorta [Im (Lopez et al., 2009)]	Mouse carotid, decreased in mice at regions of turbulent flow [Im (Pfenniger et al., 2012)] Human femoral [Im (Bol et al., 2013)] Decreased in ApoE$^{-/-}$ mice [Im (Johnstone et al., 2009b)].	Mouse aorta and Carotid [Im (Kwak et al., 2002; Johnstone et al., 2009b; Koval et al., 2011)] Human femoral [Im (Bol et al., 2013)]	Mouse carotid [Im (Johnstone et al., 2009b)] Cx43 is increased in aorta of hypertensive rat carotid [Im (Haefliger et al., 2004)] Human femoral artery [Im (Bol et al., 2013)]	Mouse aorta and carotid [Im (Johnstone et al., 2009b; Koval et al., 2011)]
	SMC	NP, mouse aorta [Im (Lopez et al., 2009)]	**NP**, mouse carotid [Im (Kwak et al., 2002; Johnstone et al., 2009b)] Low levels in human femoral artery [Im (Bol et al., 2013)] Increased in ApoE$^{-/-}$ mouse carotid and aorta [Im (Johnstone et al., 2009b)]	NP, mouse carotid [Im (Johnstone et al., 2009b)] Human femoral artery [Im (Bol et al., 2013)] Increased. Expression in ApoE$^{-/-}$ mouse aorta/carotid [Im (Johnstone et al., 2009b, 2012b)]	Mouse carotid [Im (Johnstone et al., 2009b)] Human femoral artery [Im (Bol et al., 2013)]	Mouse carotid [Im (Johnstone et al., 2009b)]
Conduit *veins*	EC	Mouse inferior vena cava [Im (Okamoto et al., 2009)]	Rat cranial/ caudal vena cava [Im (Inai and Shibata, 2009)] Human saphenous vein [Im (Bol et al., 2013)]	Rat cranial/caudal vena cava [Im (Inai and Shibata, 2009)] Human saphenous vein [Im (Bol et al., 2013)] Expression lost in 14 week ApoE$^{-/-}$ mice aorta/ carotid [Im (Kwak et al., 2002)]	Rat cranial/caudal vena cava [Im (Inai and Shibata, 2009)] Low levels in human saphenous vein [Im, WB (Deglise et al., 2005; Bol et al., 2013)]	Rat cranial/caudal vena cava [Im (Inai and Shibata, 2009)] human saphenous vein [Im, WB (Deglise et al., 2005)]. Expression lost in 14 week ApoE$^{-/-}$ mice aorta/ carotid [Im (Kwak et al., 2002)]
	SMC	Mouse inferior vena cava [Im (Okamoto et al., 2009)]	Low levels in human saphenous vein [Im, mRNA (Deglise et al., 2005; Bol et al., 2013)]	Human saphenous vein [Im, mRNA, WB (Deglise et al., 2005; Bol et al., 2013)]	Rat cranial/caudal vena cava [Im (Inai and Shibata, 2009)]. Significant increase in human saphenous vein [Im, mRNA, WB (Deglise et al., 2005; Inai and Shibata, 2009; Bol et al., 2013)]	Low levels in human saphenous vein]mRNA (Deglise et al., 2005)]

Im, immunodetection, e.g., immunofluorescence, immunohistochemistry, immunolabeling coupled to transmission-electron microscopy, ND, not determined; NP, not present; VCCC, vascular cell coculture (in vitro); WB, Western blot.

[a] Refers to the in vitro MEJ as described (Heberlein et al., 2010).

[b] Resistance/muscular arteries refers to vessels where EC and VSMC make contacts, i.e., MEJ. These include cremasteric, mesenteric, pulmonary and thoracodorsal.

[c] Conduit vessels, e.g., aorta, carotid, coronary artery

[d] Typically only found in sheer stress regions of the vascular.

[e] Several reports suggest poor antigenicity and specificity of Cx45 antibody (Theis et al., 2004; Isakson et al., 2006b).

[f] For table of MEJ identified in vivo, see review (Sandow et al., 2012).

permeability properties, but their membrane targeting is not altered (Beyer et al., 2000, 2001; Cottrell and Burt, 2001; Wei et al., 2004). The specific characterization of the functional properties of these heteromeric channels has been extensively reviewed by others (e.g., Moreno, 2004).

2. Microdomains Regulating the Intracellular Trafficking of Connexins. Once formed, vascular connexin hemichannels are actively transported to the plasma membrane through direct binding of their C-terminal and intracellular loop regions with proteins such as tubulin (α and β) (Giepmans et al., 2001b; Thomas et al., 2005; Kang et al., 2009; Saidi Brikci-Nigassa et al., 2012). Associations between Cx43 and tubulins (α and β) have been shown through coimmunoprecipitation, immunofluorescence overlap, analytical size exclusion chromatography, nuclear magnetic resonance, pull-down assays, and site-directed mutagenesis in a variety of studies (e.g., Giepmans et al., 2001b; Saidi Brikci-Nigassa et al., 2012). The interaction between Cx43 and tubulins occurs in a 26 amino acid region (in the region of aa 228–262) in the Cx43 C terminus, which results in the formation of helical regions at the point of interaction between the two proteins. This process was recently demonstrated to be regulated through v-src phosphorylation at the Tyr247 of the Cx43 C terminus (Giepmans et al., 2001b; Saidi Brikci-Nigassa et al., 2012). The interaction between connexins and tubulins regulates membrane targeting, localization within the plasma membrane, gap junction plaque formation, and TGF-β signaling (Shaw et al., 2007; Saidi Brikci-Nigassa et al., 2012). Despite the presence of this binding site, microtubule blockers do not block trafficking or gap junctions assembly, which can be explained by redundancies that allow for alternative trafficking pathways to the cell membrane (Jordan et al., 1999; Majoul et al., 2009; del Castillo et al., 2010). Additionally, removal of the microtubule-binding domain or complete removal of the Cx43 C terminus does not stop plasma membrane trafficking or gap junction assembly (although gap junction plaques are smaller) (Jordan et al., 1999; Omori and Yamasaki, 1999; Rhee et al., 2009). However, under these conditions, there is a reduction in available hemichannels at the plasma membrane and subsequent transition to gap junctions (Johnson et al., 2002; Maass et al., 2007). Although the connexins can bypass the tubulin pathway to target the membrane, it may be a critical domain for normal development. In transgenic mice, expression of a mutated form of Cx43 where there is a truncated Cx43 C terminus (δ258, within the tubulin binding domain) produces a lethal phenotype in neonatal mice (Maass et al., 2004, 2007). Many deletion and mutagenesis studies have shown that the C terminus is at least in part dispensable for membrane trafficking of Cx43. However, a recent study demonstrated that deletion mutations within the tubulin binding domain i.e., aa 235–242 (unlike previous mutations that retain this domain) inhibit gap junctions and gap junction intercellular communication (Wayakanon et al., 2012). These studies clearly define that interactions between connexins and the microtubule network result in efficient signaling microdomain formation but demonstrate that the C-terminal binding site is not essential.

The trafficking of Cx43 in monomer form to the TGN is facilitated through interactions with the GTPase protein rab20 (Das Sarma et al., 2008). By use of GST pull-down or yeast-2-hybrid assays, connexins have also been found to specifically interact with consortin within the TGN. Consortin further interacts with clathrin adapter proteins to regulate vesicular trafficking of proteins (del Castillo et al., 2010). Reduced interaction with consortin alters the membrane transport of Cx43, Cx45, and Cx32 (del Castillo et al., 2010). The ability of connexin hemichannels to reach the membrane significantly dictates their ability to form functional gap junction channels with the ultimate outcome being reduced gap junctional activity through reduced membrane targeting. Altogether, consortin and tubulins are key protein partners to connexin proteins and ensure the trafficking of connexin hemichannels to the plasma membrane.

3. The "Functional" Connexin Hemichannel Versus Pannexin Channel. Connexin hemichannels are inserted into the membrane in an arbitrary fashion in that that they are not directed to specific cellular regions prior to membrane insertion (Lauf et al., 2002; Simek et al., 2009). Once at the membrane, connexin hemichannels are then lateralized to areas of cell-to-cell contact where they aggregate in clusters based on plasma membrane lipid composition, thus forming direct interactions with opposing connexin hemichannels to form gap junctions (Wang et al., 2013). Before their insertion into a gap junction cluster, connexin hemichannels are in a closed conformation under physiologic conditions.

Connexin hemichannels at the cell surface have also been extensively studied for their singular functional role (besides that of a gap junction). Many studies have suggested that connexin hemichannels can act by releasing a number of small ionic molecules such as ATP, IP_3, or NAD^+ and by uptake of a number of fluorescent molecules. However, the ability for connexin hemichannels to function in a physiologic context remains contentious (Spray et al., 2006; Bosco et al., 2011; Fasciani et al., 2013). The primary concerns center around whether these gap junction intermediaries are really signaling hemichannels, with only a few studies directly indicating that certain connexin hemichannels open under physiologic conditions (Bukauskas et al., 2002, 2006; Contreras et al., 2003; Bukauskas and Verselis, 2004), with calls to clearly demonstrate the signaling potentials of these channels (Spray et al., 2006; Sosinsky et al., 2011). Every experiment involving

connexin hemichannels require that no extracellular calcium be present for the connexin hemichannels to be opened (Spray et al., 2006). In addition, alterations in intercellular ionic concentrations of K^+ and variation in pH have been showed to regulate the opening and closing of connexin hemichannels (Retamal et al., 2007). Specific pathways for the regulation of channel opening include intracellular loop and C-terminal regions interactions (Ponsaerts et al., 2010) and the specific post-translational modifications of connexin C-terminal amino acids (Bao et al., 2004a,b; Retamal et al., 2006; De Vuyst et al., 2007) as recently well reviewed (Saez et al., 2010; Ek-Vitorin and Burt, 2013; Fasciani et al., 2013; Herve and Derangeon, 2013; Verselis and Srinivas, 2013; Wang et al., 2013).

The role of connexin hemichannels in a physiologic setting, particularly within the vasculature, has been further polarized by the fact that connexin-mimetic peptides as well as a large number of chemical agents that have been used to demonstrate connexin hemichannels specific activity also block pannexin channel activity (Wang et al., 2013) (see Table 4). The pannexin class of proteins was only recently discovered in the early 2000s (Panchin et al., 2000). The pannexins have similar membrane topology to connexins, with four membrane-spanning domains and intracellular N terminus, loop, and C terminus (MacVicar and Thompson, 2010). Similar to connexin hemichannels, six pannexin proteins assemble to form hexameric membrane channels that have been shown to be expressed in virtually all cell types, including vascular cells (Billaud et al., 2011; Sosinsky et al., 2011; Lohman et al., 2012b). Significantly, pannexin channels are not currently known to form intercellular channels (as opposed to connexin hemichannels that form gap junctions) but act as a paracrine channel for the generation of directional signaling across the surface of the cell (Dahl and Locovei, 2006; Sosinsky et al., 2011; Lohman et al., 2012a). To date, their role as a purine-releasing channel as well as nonspecific anion channel has been clearly demonstrated (Chekeni et al., 2010; Lohman et al., 2012c; Wang et al., 2013). In the vascular wall, the three pannexin isoforms are expressed with variation depending on the type of circulation (systemic, pulmonary, coronary, hepatic) (Lohman et al., 2012b). To date, only a few studies have investigated the role of pannexin channels in vascular functions, and it is now clear that they participate in the adrenergic signaling pathway in SMCs as well as in the thrombin signaling pathway in ECs (Billaud et al., 2011; Godecke et al., 2012). Lastly, although pannexins have not been identified in any clear microdomains and are not localized within caveoli, as demonstrated in a rat mammary tumor cell line (Gehi et al., 2011), they have been associated with a number of receptors that can initiate their opening, including the α1D-adrenergic receptor, the NMDA receptor, and PAR-1 (Billaud et al., 2011; Godecke et al., 2012; Weilinger et al., 2012). Ongoing and future studies of these channels could provide interesting clues into Panx1 integration in signaling microdomains in the blood vessel wall.

4. Gap Junctional Plaque as a Signaling Microdomain. Gap junctions form through accretion and docking of opposing connexin hemichannels between adjacent cells and have been identified between EC, VSMC, and at the MEJ in a large number of vascular beds (Brisset et al., 2009). Although gap junctional communication has been clearly identified between ECs in numerous vascular beds, the presence of gap junction between VSMCs and at the MEJ seems to differ according to the vascular beds and the species (Johnstone et al., 2009a; Billaud et al., 2011). Within the blood vessel wall, gap junctions play an integral role in key physiologic responses, including vascular resistance, vascular growth, and cell differentiation, and are highly adapted during diseases, implicating them as key members in modulating different aspects of disease responses (Kwak et al., 2002; Liao et al., 2007; Chadjichristos et al., 2008) (see Table 5). The formation of gap junctions and gap junction channel properties are highly regulated through intracellular environment but also through direct protein interactions in signaling microdomains.

a. Caveolin. At the plasma membrane, connexin hemichannels are held within discrete cholesterol- and sphingolipid-rich membrane regions known as lipid rafts (Schubert et al., 2002; Locke et al., 2005; Locke and Harris, 2009; Defamie and Mesnil, 2012). Connexins are differentially expressed in a number of different lipid rafts of variable composition characterized by their sensitivity to detergents such as Triton X-100, Nonidet P-40, or Brijj and by the presence of Cav1 (Locke et al., 2005). Multiple interaction studies (e.g., immunofluorescence overlap, coimmunoprecipitation, Far Western) have now identified that vascular connexins, including Cx32, Cx37, Cx43, and Cx40, can interact with both Cav1 and Cav2 (Schubert et al., 2002; Locke et al., 2005; Langlois et al., 2008; Saliez et al., 2008). Protein truncation studies have revealed that Cav1 and Cx43 interact at residues 244–256 on Cx43 C terminus (Langlois et al., 2008). Although caveolin interactions are primarily considered at the plasma membrane in caveolae, newly synthesized Cx43 can also interact with Cav1 and Cav2 in the Golgi, indicating that both isoforms of caveolin may also be involved in the transport of connexins to the plasma membrane (Langlois et al., 2008). Connexins within caveolae at the plasma membrane are presumably connexin hemichannels not gap junctions but are an integral step in gap junction channel assembly at the plasma membrane (Locke et al., 2005; Langlois et al., 2008). In experimental models, the expression levels of Cx37, Cx40, and Cx43 and gap junction formation are significantly reduced in $Cav1^{-/-}$ mouse arteries (Saliez et al., 2008). Additionally, interactions

TABLE 4
Pharmacology of connexins and pannexins

Table summarizing the pharmacological inhibitors of connexin and pannexin channels. For each drug, the range of concentration tested as well as their potency on pannexin channels, and connexin hemichannels and gap junctions is indicated.

Drug	Characteristics	Concentration	Magnitude of inhibition		References
			Panx	Cx	
Mefloquine	Prevention and treatment of malaria (many side effects reported). Interacts with a number of ion channels and proteins with nonspecific effects and targets, e.g., neuronal calcium homeostasis, the endoplasmic reticulum, calcium pump, acetylcholinesterase, P-glycoproteins in the blood-brain barrier, connexins pannexins, A2A receptors, potassium, and anion channels.	1–10 μM	++(S)	+(R)	Dow et al., 2005; Iglesias et al., 2008, 2009a; Li et al., 2010; Nevin, 2011
Probenecid (Pro)	Used in the treatment of gout and hyperuricemia, works by interfering with the kidneys organ anion transporter (OAT). Blocks channel based release of cAMP, cGMP, and ATP from various cell types. Reportedly specific to Panx1 by Silverman et al. (2008).	150 μM–1 mM	++	−/+ (Cx46/Cx50)	Hsyu et al., 1988; Paul et al., 1991; Beahm and Hall, 2002; Silverman et al., 2008, 2009; Ma et al., 2009; Li et al., 2010
^{10}Panx1	Used experimentally, acts to specifically inhibit Panx1 channels, targeted to the extracellular loop region of Panx1. Some effects demonstrated for Cx46 channel inhibition.	100 μM	++	−/+ (Cx46)	Pelegrin and Surprenant, 2006; Wang et al., 2007; Bargiotas et al., 2011; Billaud et al., 2011
18-α/β-Glycyrrhetinic acid	Derived from licorice herb. Inhibits several channels, e.g., IK_{Ca}/$SK_{Ca,}$ voltage-dependent channels, pannexin, and connexin channels.	25 μM	+	++/+++	Bruzzone et al., 2005; Ozkan and Uma, 2009; Johnstone et al., 2010; Boedtkjer et al., 2013
Carbenoxolone	Glycyrrhetinic acid derivative. Used in treatment of oral ulcerations and lesions, nonspecific ion channel inhibitor, suppresses hyperpolarization.	50–100 μM	+++	++	Sagar and Larson, 2006; Thompson et al., 2006; Iglesias et al., 2008, 2009a; Chekeni et al., 2010; Behringer et al., 2012; Gairhe et al., 2012; Boedtkjer et al., 2013; Zhang et al., 2013
^{40}Gap27, ^{37,43}Gap27	Used experimentally to inhibit connexin (Cx37, Cx43) and has been showed to affect pannexin channels. Inhibition of Ca^{2+} oscillations, depolarization, and contraction of smooth muscle cells	300 μM	+	+	Dora et al., 1999; Haddock et al., 2006; Pelegrin and Surprenant, 2006; Wang et al., 2007; Islam et al., 2012
Flufenamic acid	Fenamate, nonsteroidal anti-inflammatory drugs. Nonspecific ion channel inhibitor, including: nonselective cation channels, voltage-gated NA^+/K^+/Ca^{2+} channels, connexins (e.g., Cx46), pannexins (Panx1), and calcium activated channels.	100–300 μM	−/+	++ (reversible)	Harks et al., 2001; Bruzzone et al., 2005; Iglesias et al., 2008
Arachidonic acid and eicosatetraynoic acid (ETYA)	Nonspecific effects on voltage-gated potassium channels, blocker of connexins (e.g., Cx43) and pannexin channels. Arachidonic acid promotes endothelium-induced vasorelaxation, ETYA inhibits endothelium-dependent vasodilation.	100 μM	+++	+++	De Mey et al., 1982; Martinez and Saez, 1999; Kehl, 2001; Samuels et al., 2013
Ouabain	Cardiotonic steroid, identified in human blood. Widely used experimentally as a Na^+/K^+-ATPase inhibitor and inhibitor of connexins (not known for pannexins).	100 μM–1 mM	n/a	−/++	Weingart, 1977; Martin et al., 2004; Turner et al., 2004; Aperia, 2007; Boedtkjer et al., 2013
Heptanol	Aliphatic alcohol, gap junction uncoupler/inhibitor	200 μM–3 mM	−/+	++	Takens-Kwak et al., 1992; Garcia-Dorado et al., 1997; Pelegrin and Surprenant, 2006; Nishida et al., 2008; Li et al., 2010; Li et al., 2012[b]

+, Partial inhibition; −/+, conflicting reports; ++, greater than 50% inhibition; −, no effect; +++, complete inhibition; n/a, no information available

[a] R and S represent the two stereoisomers of mefloquine (Iglesias et al., 2009b)

[b] Evidence that heptanol effect is not specific to gap junctions in smooth muscle cells (Chaytor et al., 1997).

TABLE 5
Examples of physiologic and pathophysiological role of gap junction in the vasculature

There is a plethora of physiologic functions for gap junctions in the vasculature having been described, with a sample of the more recent referenced below.

Physiologic Function	References
Endothelium-derived hyperpolarizing factor	Edwards et al., 1998; Hutcheson et al., 1999; Yamamoto et al., 1999; Beny and Schaad, 2000; Kansui et al., 2004; Mather et al., 2005; Rath et al., 2012; Howitt et al., 2013
Vascular conducted responses	Kruger et al., 2002; Dora et al., 2003b; Simon and McWhorter, 2003; Wolfle et al., 2007; Figueroa and Duling, 2008
Feedback on vasoconstriction	Dora et al., 1997; Straub et al., 2011
Smooth muscle cell proliferation	Chadjichristos et al., 2006; Liao et al., 2007; Johnstone et al., 2010; Zhong et al., 2012[a]
Smooth muscle cell differentiation	Kwak et al., 2003; Chadjichristos et al., 2008; Shen et al., 2010; Gairhe et al., 2011, 2012[b]
Endothelial cell proliferation	Larson et al., 1997; Kwak et al., 2001; Yeh et al., 2006; Nakano et al., 2008; Wang et al., 2008[c]
Endothelial cell dysfunction	Xie and Hu, 1994; Makino et al., 2008; Chadjichristos et al., 2010; Wang et al., 2012; Ebong and Depaola, 2013
Endothelial cell migration	Pepper and Meda, 1992; Pepper et al., 1989; Kwak et al., 2001

[a] Evidence that gap junction independent pathways are involved in this regulation (Johnstone et al., 2012b).
[b] Evidence that although connexin expression and/or gap junction communication levels correlated to disease they do not appear to be directly linked to phenotypic modulation (Matsushita et al., 2007; Behringer et al., 2012).
[c] Evidence that gap junctions are not involved in EC wound repair migration or proliferation (Bearden et al., 2010).

between Cx43 and Cav1 and Cav2 are regulated via PKCγ in a model, suggesting that phosphorylation of Cx43 by PKCγ may lead to a redistribution of Cx43 hemichannels in the lipid rafts at the cell membrane, reducing their accumulation into gap junctional plaques (Lin et al., 2003). Therefore, it is likely that Cav1 and Cav2 interact with connexins in a manner that promote their incorporation into a signaling microdomain to regulate their function.

b. Zonula occludens-1. At the point of insertion to the plasma membrane, connexin hemichannels become directly associated with the tight junction proteins ZO-1/2/3 (Toyofuku et al., 1998, 2001; Singh et al., 2005), as recently reviewed in Palatinus et al. (2012). Direct interactions between ZO-1 and Cx43 and Cx45 have been demonstrated at the plasma membrane (Kausalya et al., 2001; Laing et al., 2001, 2005a,b). These interactions have been extensively studied and shown by numerous biochemical techniques such as coimmunoprecipitation, immunofluorescence, proximity ligation assay, nuclear magnetic resonance, peptide competition, and pull-down assays (Toyofuku et al., 1998, 2001; Kausalya et al., 2001; Sorgen et al., 2004a; Laing et al., 2005a,b; Bouvier et al., 2008; Chen et al., 2008; O'Quinn et al., 2011; Tence et al., 2012). The interaction between Cx43 and ZO-1 is not required for proper gap junction channel functionality per se but instead appears to negatively regulate accumulation of gap junction channels at the plasma membrane, limiting plaque size and stability (Giepmans, 2004, 2006; Hunter et al., 2005; Rhett et al., 2011; Arora et al., 2012). Plaque size is regulated by this interaction in two main ways: 1) by limiting incorporation of connexins into gap junction plaques and 2) by promoting endocytosis of connexins from gap junction plaques (Palatinus et al., 2012). Accordingly, it has been proposed that ZO-1 acts as a "connexon switch," regulating the transition from nonjunctional to junctional form of Cx43 hemichannels (Rhett et al., 2011). Newly formed connexons aggregate at the cell surface in noncoupled plaques called "perinexus" (Rhett and Gourdie, 2012). Interaction of Cx43 with ZO-1 facilitates movement between the perinexus and gap junction plaque in this way, regulating the size of the plaque. Indeed, when Cx43/ZO-1 interactions are inhibited (e.g., through siRNA approaches, inclusion of a Cx43-CT-GFP, or site directed mutation of the PDZ domain of ZO-1 or of the C-terminal region of Cx43), the ability of these two proteins to interact is abolished, resulting in decreased size of gap junction plaques (Hunter et al., 2003, 2005; Palatinus et al., 2011a, 2012).

The second function for Cx43/ZO-1 interactions is to increase gap junction disassembly and endocytosis. Phosphorylation of Cx43 at the Ser368 residue is known to decrease gap junction communication and is associated to internalization of the protein; in other words, it is associated with reduced plaque stability. This phosphorylation is reportedly dependent on Cx43 interaction with ZO-1, because in the absence of ZO-1, Cx43 can interact with but cannot be phosphorylated by PKCε and efficiency of disassembly of gap junctions is reduced (Akoyev and Takemoto, 2007). These results suggest that ZO-1 interactions cause structural changes in the Cx43-CT that allow for PKC phosphorylation (Akoyev and Takemoto, 2007; O'Quinn et al., 2011). Thus, although ZO-1 interaction with Cx43 may not be critical in the formation of gap junction channels, ZO-1 may act to regulate their function through membrane targeting and molecular inhibition by limiting gap junction formation and promoting endocytosis from the gap junction plaque.

As mentioned above, ZO-1 plays a role in maintaining gap junction stability not only by binding to Cx43 directly but also through binding to actin (Giepmans

et al., 2001c; Kostin, 2007). In addition, drebrin, another actin-binding protein originally identified as neuronal cell-specific, has since been detected in ECs and VSMCs where it is involved in gap junction function (Peitsch et al., 1999, 2005; Yamada et al., 2005). Recent studies using coimmunoprecipitation, immunofluorescence, and immunolabeling coupled to transmission electron microscopy have identified that Cx43 and drebrin directly interact, and drebrin acts to stabilize actin cytoskeleton association that is required for the maintenance of stable and functional gap junctions (Butkevich et al., 2004; Majoul et al., 2007; Park et al., 2009b). Taken together, studies of connexins reveal an intricate signaling microdomain involving tubulins, ZO proteins, and binding partners, including drebrin and actin, that maintain the ability of connexins to traffic to the plasma membrane and form functional gap junctions.

c. Calmodulin. It has been well documented that connexin hemichannels and gap junctions are sensitive to calcium environments, although the exact mechanisms of the interaction are not fully defined (Harris, 2001; Lurtz and Louis, 2007; Zhou et al., 2007b; Herve et al., 2012; Herve and Derangeon, 2013). The first observation made in the early 1980s using freeze fracture studies indicated that connexins twist the gap junction channel shut in response to supraphysiological levels of Ca^{2+} (Peracchia, 1978; Unwin and Ennis, 1983; Bruzzone et al., 1996). Several studies now suggest that this may be regulated through binding of the calcium sensing subunit calmodulin. Calmodulin interacts with both Cx32 and Cx43 to maintain a closed gap junction channel, potentially by altering channel conformation or through physical interactions with calmodulin or calcium itself (Van Eldik et al., 1985; Torok et al., 1997; Sotkis et al., 2001; De Vuyst et al., 2006; Stauch et al., 2012; Xu et al., 2012). This interaction is not homologous between connexin isoforms: gap junction channels formed of Cx40 isoforms are insensitive to high levels of calcium, potentially because of a lack of the putative binding sites for calmodulin (Lurtz and Louis, 2007; Xu et al., 2012).

d. Posttranslational modifications. It has become increasingly evident that the C-terminal region of Cx43 acts as a binding domain for many molecules that are involved in its trafficking, membrane stability, and gap junctional communications (Niger et al., 2010) (see section III.A.1). The C terminus is an unstructured, highly dynamic region that contains multiple sites for posttranslational modifications, including phosphorylation, nitrosylation, palmitoylation, sumoylation, and ubiquitination (Palatinus et al., 2011b; Straub et al., 2011; Johnstone et al., 2012a; Chen et al., 2013). As described in section III.A.1, the connexin C terminus is primarily unfolded and rich in serine, threonine, and tyrosine residues (Ser/Thr/Tyr) that are targeted for posttranslational modifications (Solan et al., 2003, 2009; Chen et al., 2013). Additionally, through a number of techniques including coimmunoprecipitation, immunofluorescence overlap, and pull-down assays, direct interactions have been identified within the Cx43 C terminus, with the suggestion that this can reduce the signaling properties of the gap junctional pore (Niger et al., 2010). Kinases and sites within Cx43 have been well characterized, including phosphorylation by kinases from the src family of Tyr265, and phosphorylated by MAPK of Ser255/(Ser262)/Ser279/Ser282 residues (Solan and Lampe, 2009; Johnstone et al., 2012a; Chen et al., 2013). Presumptively, these posttranslational modifications induce structural changes within the connexin C-terminal region that allow for further protein interactions with this region (Saidi Brikci-Nigassa et al., 2012). However, given the lack of data on the effect of posttranslational modifications on the structure of connexin C terminus, the exact consequences of these modifications remain unclear.

Further studies have shown that connexins form aggregate called "formation plaques" prior to accretion as gap junction plaques in a process that appears highly regulated by PKC phosphorylation of the C-terminal domain of Cx43 (Johnson et al., 2012). As with previously mentioned studies, removal of the C-terminal region after the tubulin-binding domain demonstrates that these sites are not essential but promote efficient gap junction assembly (see section III.A.1). In the heart, phosphorylation of the Ser368 residue (the main PKC-associated site in Cx43 C terminus) allows for the interaction with the ZO-1 proteins, which facilitates Cx43 aggregation in gap junctions but may also be a key factor in Cx43 cellular distribution. For example, phosphorylation of Cx43 Ser368 in cardiac myocytes induces lateralization of Cx43 hemichannels by removal from the intercalated disc where Cx43 is known to interact with ZO-1, desmin, and interleukins (Giepmans, 2004; Severs, 2007). These proteins form a stable signaling microdomain that facilitate Cx43 cellular localization and gap junctional signaling in the myocardium.

The src kinase family has been demonstrated to interact with and modulate Cx43 gap junctions through tyrosine phosphorylation of its C-terminal region (Warn-Cramer and Lau, 2004). The Cx43 C terminus contains two binding sites for the SH2 and SH3 domains of v-src and c-src (Kanemitsu et al., 1997; Loo et al., 1999; Giepmans et al., 2001a; Lin et al., 2006). Interaction with these kinases and subsequent phosphorylation of Cx43 decreases gap junctional communication (Duffy et al., 2004; Duffy, 2012; Geletu et al., 2012). One potential mechanism for this is through disruption of the interaction between ZO-1 and Cx43 (Toyofuku et al., 2001). Although Cx40 does not contain the consensus sequence for binding to src kinases, it has been proposed that src interacts with proline-rich

domains that are found in Cx40 (Bouvier et al., 2008). As with previous reports on the C terminus, interactions of Cx43 with diverse kinases seem to be involved in the overall regulation of gap junction plaque formation and function but do not present as an absolute requirement given that gap junction plaque can form in the absence of phosphorylation.

Taken together, it is clear that vascular gap junction proteins are regulated through specific protein interactions in specialized signaling microdomains (Fig. 7). The studies mentioned in this section report that connexins 1) can interact with numerous protein partners, 2) are polarized to specific region of the cells (e.g., at the MEJ), and 3) can accumulate in specialized areas of the plasma membrane as they have been detected in caveoli. However, although the "gap junction microdomain" can be defined in terms of the protein partners, the consequences of the protein-protein interactions still need to be clarified. This is hindered by the lack of specificity of pharmacological agents currently available to inhibit gap junctions (see Table 4). Indeed, a number of inhibitors of gap junctions are widely used but are generally considered to be nonspecific, and their mechanism of inhibition have not been clearly defined (Evans and Boitano, 2001; Evans et al., 2012). For example, studies of the functional role of connexins in arterioles have extensively used inhibitors such as glycyrrhetinic acid and carbenoxolone, but recent studies have identified nonspecific effects of these blockers, including inhibition of calcium-activated potassium channels (Behringer et al., 2012). The development of transgenic animals was first thought to be a solution to cope with the lack of specific pharmacological inhibitors, but it is now clear that a number of compensations and/or redistribution of other connexin isoforms occur in Cx KO animals, which could further hinder interpretation of connexins on physiologic function (e.g., Kruger et al., 2002; Simon and McWhorter, 2003; Isakson et al., 2006a). It appears more specific molecular (e.g., small molecule inhibitors) or genetic (inducible cell line-specific knockouts) studies are required to more clearly delineate connexin function in the vasculature.

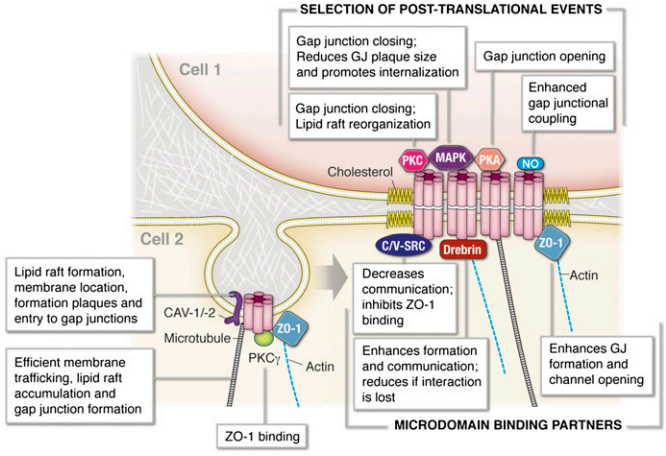

Fig. 7. Connexins and gap junction signaling microdomains. The formation of gap junctional structures is regulated through organization of connexin hemichannels in caveolae that further assemble to a gap junction plaque through association with multiple protein partners. The C-terminal domain of connexins interacts with microtubule, PKC, and ZO-1, and these interactions promote integration of connexin hemichannels to the plasma membrane at lipid-enriched caveolae. Assembling of hemichannels in the lipid raft structures surrounding a gap junction plaque is enhanced through interactions with ZO-1/2 and drebrin. The different steps leading to the formation of gap junction assembly as well as gap junctional permeability are modulated through a dynamic process of posttranslational modifications at the C-terminal regions of connexins including phosphorylations by PKC and MAPK, which both reduce gap junction communication, and by PKA, which increases gap junctional signaling.

B. Potassium and Calcium Channels: A Case for the Endothelium-Derived Hyperpolarization-Mediated Response

In the late 1970s, the Kuriyama group reported that acetylcholine was capable of inducing a hyperpolarization of the VSMC plasma membrane in guinea pig coronary and mesenteric artery as well as in rabbit mesenteric artery (Kuriyama and Suzuki, 1978; Karashima and Kuriyama, 1981; Takata and Kuriyama, 1980). In their preparation, VSMC hyperpolarization was paradoxically simultaneous to arterial constriction, which was the mechanical response commonly observed in in vitro arterial preparations at the time (Kitamura and Kuriyama, 1979; Karashima and Kuriyama, 1981). Over the same period of time, the identification of an endothelium-derived relaxant factor (EDRF, see section II.A) led scientists to realize that the contractile effect of acetylcholine on in vitro preparation was caused by damage to the endothelial layer during arterial isolation (Furchgott and Zawadzki, 1980; Furchgott, 1999). After this groundbreaking work, care was taken to work on in vitro preparation with intact endothelium, and in 1984, Bolton et al. (1984) demonstrated that the VSMC hyperpolarization induced by acetylcholine also depended on the integrity of the endothelium. In the late 1980s, a clear distinction between EDRF [then identified as NO (Ignarro et al., 1987; Khan and Furchgott, 1987; Palmer et al., 1987)] and the endothelium-dependent hyperpolarization (EDH) of VSMCs was drawn. In their seminal article, Chen et al. (1988) reported that hemoglobin and methylene blue (which, respectively, scavenges NO and blocks the guanylate cyclase) reduced acetylcholine-induced relaxation and abolished cGMP production but had no effect on the VSMC hyperpolarization or on the ^{86}Rb efflux (a marker for potassium) in rat aorta and pulmonary artery (Chen et al., 1988). With the discovery of L-arginine analogs as specific inhibitors of NO production (Hibbs et al., 1987a,b; Marletta et al., 1988; Knowles et al., 1989; Stuehr et al., 1989), it became clear that the EDH was resistant to prostacyclin blockers and NO-synthase blockers, alone or combined (Ishii et al., 1990; Nagao and Vanhoutte, 1992; Rand and

Garland, 1992; Cowan et al., 1993; Waldron and Garland, 1994a). These observations pointed to the fact that NO and prostacyclin derived from ECs could not be responsible for the endothelium-dependent hyperpolarization of VSMC observed in acetylcholine dilation, which resulted in a surge of interest in this NO- and prostacyclin-independent vasodilation.

The concept of an endothelium-derived hyperpolarizing factor (EDHF) emerged in the 1980s (Taylor and Weston, 1988). Since then, numerous laboratories have tried to identify the "hyperpolarizing factor" itself and have worked on characterizing the hyperpolarization response induced by acetylcholine and other endothelial agonists. It was generally accepted that the endothelium-dependent hyperpolarization involved potassium ions (K^+), because 1) the amplitude of hyperpolarization was inversely correlated to the extracellular concentration of K^+ (Chen et al., 1989; Chen and Suzuki, 1989; Nagao and Vanhoutte, 1992), 2) K^+ efflux was observed in cells preloaded with a radioactive form of K^+ (^{42}K) or a marker for K^+ ions (^{86}Rb) upon stimulation with acetylcholine (Chen et al., 1988; Taylor et al., 1988), and 3) the EDH was abolished by potassium channels blockers (Chen et al., 1991; Nagao and Vanhoutte, 1992; Van de Voorde et al., 1992). The role of extracellular calcium as well as calcium from the intracellular stores in EDH-mediated relaxation was also demonstrated by a number of laboratories (e.g., Chen and Suzuki, 1990). The role of extracellular calcium was also confirmed by application of the calcium ionophore A23187, which also resulted in an EDH that was insensitive to eNOS blockers and to inhibitors of the prostacyclin pathway (Chen and Suzuki, 1990; Parsons et al., 1994; Plane et al., 1995; Zygmunt and Hogestatt, 1996).

1. Role of Potassium Channels. The role of K^+ in acetylcholine response was first proven by the Kuriyama group's work on guinea pig coronary arteries that showed that the amplitude of acetylcholine-induced hyperpolarization was reduced in low K^+ solutions, whereas removal of other ions (namely Na^+ and Cl^-) did not affect the hyperpolarization (Kitamura and Kuriyama, 1979). When the concept of EDHF emerged 25 years later, the identification that K^+ efflux is a fundamental step in the EDH-mediated response became a major milestone in our understanding of the pathway (Chen et al., 1988; Taylor and Weston, 1988). Later, treatment with potassium channel blockers such as glibenclamide, an inhibitor of ATP-sensitive potassium channel (Chen et al., 1991; Garland and McPherson, 1992; Van de Voorde et al., 1992; Plane and Garland, 1993, 1994; Plane et al., 1995; Corriu et al., 1996), or 4-aminopyridine, an inhibitor of voltage-dependent potassium channels (Zygmunt and Hogestatt, 1996; Hashitani and Suzuki, 1997), failed to consistently inhibit EDH in several vascular beds. Conversely, nonselective K_{Ca} blockers such as tetraethylammonium or tetrabutylammonium were shown to inhibit EDH of VSMCs (Chen et al., 1991; Van de Voorde et al., 1992; Cowan et al., 1993; Zygmunt and Hogestatt, 1996). Several laboratories further observed that the EDH was attenuated by drugs such as apamin, a blocker of small conductance K_{Ca} channels (SK_{Ca},), and charybdotoxin, a nonspecific blocker of large conductance K_{Ca} (BK_{Ca}), and intermediate conductance K_{Ca} (IK_{Ca}) (Adeagbo and Triggle, 1993; Cowan et al., 1993; Holzmann et al., 1994). However, in 1994, the EDH-mediated response for the first time was abolished using a combination of apamin and charybdotoxin (Waldron and Garland, 1994b). This observation was followed by the demonstration that iberiotoxin, a blocker of BK_{Ca}, had no effect on the EDH-mediated response (Zygmunt and Hogestatt, 1996). Because specific blockers of IK_{Ca} were not available at the time, it was then concluded that both IK_{Ca} and SK_{Ca} were involved in the EDH-mediated response. This was proven to be true with the further development of the IK_{Ca} inhibitors TRAM39 and TRAM34 (Wulff et al., 2000, 2001), which clarified a role of these channels in the EDH-mediated relaxation (Crane et al., 2003; Hinton and Langton, 2003). In parallel, IK_{Ca} channel openers such as 1-ethyl-2-benzimidazolinone (1-EBIO) or SK_{Ca} openers (riluzole) were able to reproduce the EDH induced by acetylcholine (Edwards et al., 1999a,b; Walker et al., 2001; Crane and Garland, 2004). These pharmacological studies led to the conclusion that the hyperpolarization observed in the EDH pathway reflects the activation of two potassium channels, IK_{Ca} and SK_{Ca}, which was a key step in our understanding of the EDH-mediated response.

In 1998, the exact role, location, and temporal activation of these channels became more clear as a result of an investigation published in *Nature* by Edwards et al. (1998). In this study, the authors measured the membrane potential of ECs and demonstrated that the K^+ efflux observed in EDH-mediated response was due to the activation of SK_{Ca} and IK_{Ca} channels at the plasma membrane of ECs and not of SMCs as it was assumed at this time (Garland et al., 2011). Edwards et al. (1998) further showed that this K^+ efflux creates an accumulation of K^+ in the extracellular space between ECs and VSMCs, which in turn activates the Na/K/ATPase as well as the K_{IR} channels at the plasma membrane of the VSMC, thus hyperpolarizing and relaxing the VSMCs (Edwards et al., 1998). This K^+ accumulation was demonstrated using potassium-selective electrodes, allowing for the measurement of local K^+ concentration in the blood vessel wall measuring a concentration of approximately 10 mM in the space between ECs and VSMCs in intact rat hepatic arteries stimulated with acetylcholine (Edwards et al., 1998). In parallel, another study confirmed the role of IK_{Ca} and SK_{Ca} located at the plasma membrane of EC in rat mesenteric arteries (Doughty et al., 1999).

It became clear that although both IK_{Ca} and SK_{Ca} were involved in the EDH-mediated response, their

location, mode of activation, and their role were different. Several studies from the early 2000s showed that the role of K_{Ca} channels appears to differ depending on the contractile state of the smooth muscle: when no depolarizing preconstrictor is present (i.e., when the contractile state of the smooth muscle is due to basal tone), acetylcholine produces a "true" hyperpolarization, which is due to activation of SK_{Ca}, whereas in the case of agonist-induced SMC depolarization (in presence of phenylephrine, for example), EDH-mediated relaxation in response to acetylcholine can be separated in two components reflecting SK_{Ca} and IK_{Ca} activities (Dora et al., 2000; Dora and Garland, 2001; Crane et al., 2003; Takano et al., 2004).

Because both IK_{Ca} and SK_{Ca} are activated by the calcium-calmodulin sensor (Xia et al., 1998) with similar sensitivity to calcium (Carignani et al., 2002), their functional difference has been suggested to be the result of a differential intracellular location in the ECs. The development of isoform-specific antibodies as well as knockout mouse models helped to increase our knowledge regarding the contribution of each isoform of K_{Ca} channels. Several reports observed a spatial separation of IK_{Ca} and SK_{Ca} in ECs with SK_{Ca} predominantly expressed at EC-EC junctions, whereas IK_{Ca} channels are mainly present at the points of contacts between ECs and VSMCs and in close proximity to the ER (Sandow et al., 2006; Dora et al., 2008; Ledoux et al., 2008). It is not clear how this channel polarization occurs, but it is possible that it may be due to local plasma membrane composition because IK_{Ca} is not located in caveolae, whereas SK_{Ca} is located in caveolae in ECs as demonstrated by coimmunoprecipitation and experiments using sucrose gradient (Absi et al., 2007). The SK_{Ca} portion of the EDH-mediated relaxation can be inhibited in presence of the caveolae disrupting agent MβCD, an inhibition that was reversed by addition of cholesterol (Graziani et al., 2004; Absi et al., 2007).

With the development of transgenic mouse models, the importance of the EDH-mediated response at the whole animal level has become more clear. Carotid and resistance arteries isolated from the global $K_{Ca}3.1$ (= IK_{Ca}) knockout (KO) mice exert decreased hyperpolarization of ECs and VSMCs in response to acetylcholine, as well as decreased associated vasodilation (Si et al., 2006; Wolfle et al., 2009; Milkau et al., 2010). Interestingly, $K_{Ca}3.1$ KO mice are hypertensive [approximately 10 to 15 mm Hg higher compared with wild-type mice), highlighting a crucial role for this potassium channel in the control of vascular tone and blood pressure (Si et al., 2006)]. The double KO of both $K_{Ca}2.3$ (= SK_{Ca}) and $K_{Ca}3.1$ exhibited an impaired acetylcholine-induced EDH and dilation in conduit and resistance arteries measured in vivo (Brahler et al., 2009). In a mouse model in which the expression level of SK_{Ca} can be manipulated with dietary doxycycline, the amount of SK_{Ca} expression in the EC was inversely correlated with the blood pressure (Taylor et al., 2003). It is noteworthy that several studies in small and large animals demonstrated that activation of IK_{Ca} and SK_{Ca} channels using the drug SKA-31 could decrease blood pressure, making these channels a potential therapeutic target for treatment of hypertension (Sankaranarayanan et al., 2009; Hasenau et al., 2011; Damkjaer et al., 2012).

2. Importance of Local Calcium Release: Role of Calcium Channels. In addition to being an important second messenger in the regulation of the contractile state of the VSMCs during the EDH-mediated response, calcium has been identified as a key element in the induction of the endothelial hyperpolarization itself in the late 1990s. Suzuki's group used submucosal arterioles loaded with Fura-2 for 1 hour or for 3 hours to investigate the calcium response from ECs and VSMCs, respectively, and demonstrated for the first time that acetylcholine elevates fluorescence in ECs, whereas it has the ability to reduce Ba^{2+}-induced increase in $[Ca^{2+}]_i$ in VSMCs (Fukuta et al., 1999). More importantly, their experiments showed that the decrease in $[Ca^{2+}]_i$ in VSMCs was not blocked by a combination of inhibitors of the EDRF/NO and the prostaglandin pathways (Fukuta et al., 1999). Lastly, although charybdotoxin had no effect on the acetylcholine-induced increase in $[Ca^{2+}]_i$ in ECs, it reduced the acetylcholine-induced decrease in $[Ca^{2+}]_i$ in VSMCs, suggesting that acetylcholine may modulate $[Ca^{2+}]_i$ in VSMCs indirectly via activation of potassium channels in the ECs (Fukuta et al., 1999).

Once the K_{Ca} channels involved in the EDH-mediated response were identified in the ECs (see above), the source of calcium activating the endothelial K_{Ca} channels has been at the center of numerous investigations. In mouse mesenteric arteries, IK_{Ca} are directly activated by calcium released from IP_3R upon stimulation with acetylcholine (Ledoux et al., 2008). The role of extracellular calcium has also been suggested by several investigations, initially showing that a capacitive entry of calcium is involved in the EDH-mediated relaxation (e.g., in Taylor et al., 2001). These initial observations have since been confirmed by other groups, identifying channels from the TRP family as the molecular protein responsible for extracellular calcium influx. Namely, the isoforms TRPV1, TRPV3, TRPV4, TRPC3, and TRPC1 have all been reported in the EDH-mediated response (Kohler and Hoyer, 2007; Loot et al., 2008; Earley et al., 2009; Schmidt et al., 2010; Senadheera et al., 2012; Ma et al., 2013).

Among the vanilloid family of TRP channels, TRPV1, TRPV3, and TRPV4 all appear in some way to be involved in the EDH-mediated response, with TRPV4 being the most studied. The isoform TRPV4 is of particular interest because 1) TRPV4 KO mice exhibit

an impaired EDH-mediated relaxation and 2) the TRPV4 specific opener 4αPDD induced an endothelium-dependent hyperpolarization (Kohler and Hoyer, 2007). A recent study in rats showed that TRPV4 and SK_{Ca} coimmunoprecipitate and colocalize in isolated ECs, suggesting a functional interaction between the two channels (Ma et al., 2013). Interestingly, Fleming's group established a correlation between the EDH-mediated relaxation and the translocation of TRPV4 channel from a perinuclear localization to the cell membrane (Loot et al., 2008). At the whole animal level, activation of TRPV4 channels using 4αPDD resulted in an increase in local blood flow in the mesenteric vascular bed as well as in a decreased blood pressure (Ma et al., 2013). Lastly, when TRPV4 KO mice were treated with an inhibitor of eNOS, the increased in blood pressure was greater compared with control mice, showing a prominent role of these channels in the regulation of blood pressure (Earley et al., 2009). Regarding other TRPV channels, stimulation of TRPV3 appears to induce an endothelium-dependent hyperpolarization of adjacent VSMCs as well as a decreased $[Ca_{2+}]_i$ (Earley et al., 2010). The EDH-mediated response observed upon stimulation of TRPV3 was further reduced by blockers of SK_{Ca} and IK_{Ca} (Earley et al., 2010). Although a lot of important information has been gained in regard to TRPV channels with EDH, the more recent experiments have relied on knockout mice. A future critical point in these experiments would be to demonstrate the lack of other TRPV channels when one is deleted, because developmental compensation has been clearly demonstrated in a very large variety of germline knockout animals.

In addition to channels from the TRPV family, TRP channels from the canonical family such as TRPC1 and TRPC3 have been studied in the EDH-mediated response. The role of TRPC3 was recently demonstrated using Pyr3, which inhibited hyperpolarization generation (Senadheera et al., 2012). In this study, the authors concluded that TRPC3 activity is involved in the triggering of SK_{Ca} and IK_{Ca} at the EC plasma membrane, making TRPC3 a possibly important component to the EDH-mediated response. Conversely, TRPC1 channels appeared to have an inhibitory effect on the EDH-mediated response, because the TRPC1 KO mice exhibited increased EDH-mediated relaxation and a greater hyperpolarization of ECs (Schmidt et al., 2010). In this study, the authors also showed that TRPC1 KO mice exhibited a reduced arteriolar tone as well as reduced blood pressure.

In addition to being an activator of the hyperpolarization on the EC side, calcium is also central in the relaxation process in the EDH-mediated response. As mentioned above, investigations of calcium dynamics simultaneously in the ECs and in the SMCs showed that the EDH-mediated component of acetylcholine response is characterized by an increase in calcium in the ECs, whereas the calcium in VSMCs decreases (Bolz et al., 1999; Fukuta et al., 1999). The K_{Ca} blockers tetrabutylammonium and charybdotoxin abolished calcium decrease and hyperpolarization of VSMCs but did not affect calcium increase and hyperpolarization in ECs (Bolz et al., 1999; Fukuta et al., 1999). These observations suggest that the decrease of calcium in VSMCs is due to the activity of K_{Ca} channels in the ECs, which presumably close voltage-gated calcium channels in the VSMCs by reducing the membrane potential of the VSMCs (Nelson et al., 1990; Bolz et al., 1999).

Another potentially important component in the EDH-mediated response is the calcium-sensitive receptor (CaR or CaSR). CaR is a G protein-coupled receptor activated by millimolar concentrations of calcium, which results in the release of calcium from intracellular stores via IP_3R (for review, see Ward et al., 2012). The role of CaR in the EDH-mediated response was demonstrated in rat mesenteric arteries where activation of CaR in ECs induced a hyperpolarization of the VSMCs, which was abolished by the IK_{Ca} inhibitor TRAM34 or by denudation of the endothelium (Weston et al., 2005). The same group also demonstrated that CaR and IK_{Ca} are both located in noncaveolae fractions. Conversely, CaR does not colocalize with SK_{Ca} channels (Weston et al., 2005). The colocalization of CaR and IK_{Ca} might be involved in the differential activation of IK_{Ca} and SK_{Ca} in the EDH-mediated response (Dora et al., 2008).

3. Gap Junction Channels. The central role of gap junction channels in the EDH-mediated response has been observed for several decades with the use of nonspecific blockers such as carbenoxolone, 18α-glycyrrhetinic acid, octanol, D-mannitol, sucrose, or heptanol, which reduced the NO-independent relaxation in response to vasodilators in numerous vascular beds (Kuhberger et al., 1994; Yamamoto et al., 1998, 1999; Brandes et al., 2000; Sandow and Hill, 2000; Dora et al., 2003a). With the development of gap junction mimetic peptides such as $^{37\text{-}43}$Gap 27, ^{40}Gap27, $^{37\text{-}40}$Gap26, and ^{43}Gap26, the role of specific connexin isoforms was initially thought to be further clarified. Several reports demonstrated an inhibitory effect of these connexin-mimetic peptides on indomethacin- and L-NAME-resistant relaxation to acetylcholine (Chaytor et al., 1998, 2001, 2003, 2005; Dora et al., 1999; Sandow et al., 2002; Ellis et al., 2009). It is noteworthy that the $^{37\text{-}43}$Gap 27, ^{40}Gap27, and $^{37\text{-}40}$Gap26 were shown to inhibit VSMCs hyperpolarization upon acetylcholine stimulation, whereas they had no effect on the hyperpolarization of ECs (Sandow et al., 2002; Chaytor et al., 2003, 2005; Ellis et al., 2009). In contrast, the use of ^{43}Gap 26, ^{40}Gap 27, and ^{37,43}Gap 27 in a different study failed to inhibit the EDH-mediated response, which is in line with other studies that have now called into

question the specificity of the connexin mimetics [e.g., Table 4 (Mather et al., 2005; Dahl, 2007; Wang et al., 2007)]. However, loading ECs of intact pressurized mesenteric arteries with an antibody blocking Cx40 resulted in an impaired EDH-mediated dilation, whereas loading of EC with antibodies against Cx37 or Cx43 had no effect on EDH-mediated response (Mather et al., 2005). Lastly, the use of Cx40 KO mice also provided possible evidence that in particular vascular beds, the Cx40 isoform may be important (Figueroa et al., 2003; Milkau et al., 2010).

Structurally, the presence of gap junction communication in the vessel wall has been reported by numerous investigators (see above). Transmission electron microscopy as well as immunolabeling for different connexin isoforms (mostly Cx37, Cx40, and Cx43) considerably enhanced our knowledge on the location and the nature of gap junctions within the vascular wall of arteries presenting EDH-mediated responses. By use of transmission electron microscopy, the presence of the typical gap junction pentalaminar structure has been observed between endothelial and smooth muscle layers as well as between ECs in several vascular beds (Sandow and Hill, 2000; Sandow et al., 2002, 2003b; Straub et al., 2011; Billaud et al., 2012). Although the presence of functional gap junctions between VSMCs has been observed consistently in large vessels, their occurrence in smaller arteries presenting EDH-mediated responses is more debated (Little et al., 1995; Welsh and Segal, 1998; Yamamoto et al., 2001; Sandow et al., 2002; Looft-Wilson et al., 2004a,b; Fanchaouy et al., 2005; Hakim et al., 2008). There are reports of Cx37 and Cx43 immunostaining in VSMCs, with variable expression of both isoforms depending on the vascular bed studied (Yamamoto et al., 2001; Chaytor et al., 2003, 2005; Sandow et al., 2003b). Consequently, the role of gap junctional communication between VSMCs in the transfer of hyperpolarization in the EDH-mediated response is unclear. In contrast, the presence and the role of gap junction communication between ECs in the EDH-mediated response is well accepted, with Cx40 being key in the conduction of the hyperpolarization along the endothelial layer (Welsh and Segal, 1998; Emerson and Segal, 2000; Yamamoto et al., 2001; Chaytor et al., 2003, 2005). Indeed, several reports using Cx40 KO mice observed a decreased EDH-mediated response as well as a decreased conduction of the hyperpolarization and relaxation along the endothelial layer (de Wit et al., 2000; Figueroa and Duling, 2008; Milkau et al., 2010). In addition, the presence and the contribution of Cx37 and Cx43 at the EC junctions in the EDH-mediated response have been reported, but their exact role is not clear (Haddock et al., 2006; Sandow et al., 2006).

The presence of gap junctional communication at the junctions between ECs and SMCs was also demonstrated by measuring membrane potentials of both cells types in vascular beds with and without EDH-mediated responses (Yamamoto et al., 1998, 1999; Emerson and Segal, 2000; Sandow et al., 2002; Haddock et al., 2006). In these studies, there was a strong EDH-mediated response upon acetylcholine stimulation, and the resting membrane potentials of ECs and SMCs were similar. In contrast, the rat femoral artery does not present EDH-mediated response when stimulated with acetylcholine, and the resting membrane potentials of ECs and VSMCs are significantly different, strongly suggesting an absence of gap junctional coupling between both cell types (Sandow et al., 2002). At the electron microscopy level, most of the gap junctions between endothelial and smooth muscle layers are organized in one single plaque that is smaller in size compared with the plaques observed between ECs (Little et al., 1995; Sandow and Hill, 2000; Dora et al., 2003a; Sandow et al., 2003b).

With regard to all of these observations, the role of gap junctions in the EDH-mediated response is clear. It is noteworthy that a recent study highlighted the influence of the methodology in the investigation of gap junctional coupling in the EDH-mediated response by comparing the EDH-mediated response in arteries in vivo and in arteries mounted in a wire myograph or in a pressure myograph using global Cx40 KO mice as well as mice deficient in Cx40 specifically in ECs (Boettcher and de Wit, 2011). The EDH-mediated response was completely dependent on Cx40 in arteries mounted in a wire myograph (isometric conditions), whereas the EDH-mediated responses studied in a vessel mounted in a pressure myograph (isobaric conditions) or in vivo were not affected by the deletion of Cx40 (Boettcher and de Wit, 2011). Although the role of Cx40 is clear in the EDH-mediated response (Mather et al., 2005; Boettcher and de Wit, 2011), it appears that the EDH-mediated response is a highly sensitive mechanism [e.g., changes in post-translational modification of connexins after artery manipulation (Straub et al., 2010)] that requires careful interpretation according to the methodology used.

4. Other Important Players in the Endothelium-Dependent Hyperpolarization-Mediated Response. Since the first demonstration of an EDRF/NO^- and prostaglandin-independent component of endothelial relaxation in the late 1980s, it was assumed that this component was due to the release of a hyperpolarizing factor from ECs (Taylor and Weston, 1988). Since then, it is debated whether the endothelium-induced hyperpolarization of VSMCs is induced by a diffusible factor released by ECs and targeting VSMCs and/or is attributable to direct transfer of hyperpolarization from the endothelium via myoendothelial gap junctions. However, several molecules such as arachidonic acid metabolites and hydrogen peroxide (H_2O_2) have also been suggested to constitute the endothelium-derived hyperpolarizing factor, EDHF.

a. Metabolites of arachidonic acids. Metabolites of arachidonic acid such as epoxyeicosatrienoic acids have

been shown to contribute to EDH-mediated response in the late 1990s (Campbell and Harder, 1999; Fisslthaler et al., 1999). Several authors were able to reproduce an endothelium-dependent hyperpolarization of the VSMC and/or arterial vasodilation by applying exogenous arachidonic acid itself or its metabolites (Pinto et al., 1987; Rosolowsky and Campbell, 1993; Oltman et al., 1998; Campbell and Harder, 1999; Fisslthaler et al., 1999). In parallel, inhibitors of the enzymes involved in the arachidonic acid metabolism [phospholipase A_2, cytochrome P450 (P450)] are able to significantly decrease EDH-mediated relaxation in several vascular beds (Pinto et al., 1987; Rubanyi and Vanhoutte, 1987; Rosolowsky and Campbell, 1993). Given that the role of TRPV4 channels in the EDH-mediated response had been discovered earlier (see section III.B.2) but their gating was still unknown, the discovery that arachidonic acid activates TRPV4 channels in ECs later helped understanding the role of these lipid compounds in the EDH-mediated response (Watanabe et al., 2003). Later work found that arachidonic acid metabolites such as epoxyeicosatrienoic acids produced via cytochrome P450 could be an endogenous activator of TRPV4, and thus an important player in the EDH-mediated response in many vascular beds (e.g., Earley et al., 2005). It is now accepted that arachidonic acid metabolites activate TRPV4 and calcium influx in ECs, leading to the activation of K_{Ca} channels and thus to hyperpolarization (Earley et al., 2005; Vriens et al., 2005; Marrelli et al., 2007). However, the exact isoform of K_{Ca} channels activated in this case remains under debate and is likely vascular bed-dependent (Campbell and Fleming, 2010; Dora, 2010; Feletou, 2011a). Activation of arachidonic acid metabolism is thought to happen after activation of the phospholipase A_2 by diacylglycerol produced by phospholipase C when GPCR (such as muscarinic receptors) are stimulated (Saliez et al., 2008; Ella et al., 2010). More recently, a study demonstrated that TRPV4 is phosphorylated by PKA in EC, a phosphorylation that seems to be required for arachidonic activation of the channel in human coronary arteries (Zheng et al., 2013b).

b. Hydrogen peroxide. Although it is not clear whether H_2O_2 is a "true" EDHF, this reactive oxygen species has also been suggested to be produced and may be further released by the endothelium to contribute to the EDH-mediated response. Supporting this hypothesis, the addition of exogenous H_2O_2 or the combination of xanthine and xanthine oxidase induces a typical EDH-mediated dilation that is dependent on IK_{Ca} and/or SK_{Ca} channels and that is characterized by VSMCs hyperpolarization (Sobey et al., 1997; Pomposiello et al., 1999; Matoba et al., 2000, 2002, 2003; Chaytor et al., 2003; Matoba and Shimokawa, 2003; Miura et al., 2003; Rabelo et al., 2003). In parallel, numerous studies using H_2O_2-sensitive probes have demonstrated that H_2O_2 is produced in arteries stimulated with agonists such as acetylcholine, the calcium ionophore A23187, or bradykinin (Matoba et al., 2000, 2003; Chaytor et al., 2003; Miura et al., 2003). Functional experiments using catalase that degrades H_2O_2 also resulted in a decreased EDH-mediated dilation (Sobey et al., 1997; Pomposiello et al., 1999; Matoba et al., 2000, 2002; Edwards et al., 2008). In contrast, the evidence against H_2O_2 as an EDHF is that, although exogenous addition of H_2O_2 is capable of hyperpolarizing VSMCs (Beny and von der Weid, 1991; Chaytor et al., 2003), catalase often failed to reduce the smooth muscle hyperpolarization (Beny and von der Weid, 1991; Matoba et al., 2002; Chaytor et al., 2003; Gluais et al., 2005). However, research on this work is ongoing.

The exact contribution of H_2O_2 in the EDH-mediated response is still unclear and appears variable depending on the vascular bed investigated. Recently, experiments in culture ECs supported that H_2O_2 potentiates calcium release from EC stores, probably via redox modification of the IP_3R (Edwards et al., 2008). The calcium release is suggested to further activate K_{Ca} channels and induce the EDH-mediated response (Sobey et al., 1997). The source of H_2O_2 production is also variable according to the literature, because NADPH oxidase, uncoupled eNOS, and P450 have all been involved in H_2O_2 production in the EDH-mediated response (Shimokawa, 2010). It has been suggested that NADPH oxidase, uncoupled eNOS, and P450 generate H_2O_2 indirectly by first producing superoxide anion, which is further transformed in H_2O_2 via superoxide anion dismutase, mostly the soluble isoform Cu,Zn SOD (Morikawa et al., 2003).

The identification of a single solitary factor mediating the EDH response has been at the center of multiple investigations in the past decades, and its identity has been debated because it seems to be highly dependent on the vascular bed and the species in question, the preconstrictor used, or the methodology. In addition, the necessity of the term "factor" is highly debated "because it masks the real identity of the signal(s) involved" (Feletou and Vanhoutte, 2013).

5. Altogether at the Myoendothelial Junction. The importance of MEJ in the EDH-mediated response has been reported by a number of investigators in the past 20 years, and it is now well accepted that the EDH-mediated signaling pathway is likely located at the MEJ (Mulvany and Aalkjaer, 1990; Hwa et al., 1994; Shimokawa et al., 1996; Sandow and Hill, 2000; Berman et al., 2002). Whether the EDH-mediated response requires an EDH factor or simple transfer of hyperpolarization from the ECs to the VSMCs, the presence of these close contacts between ECs and VSMCs seems to be essential in the EDH-mediated response since a lot of the molecular players described above are present at the MEJ.

The potassium channel IK_{Ca} and to a lesser extent the SK_{Ca}; the plasma membrane calcium channels TRPV4

and TRPC3; the $\alpha 2/\alpha 3$ subunits of Na/K/ATPase; and the gap junction proteins Cx37 and Cx40 have all been detected at the MEJ in arteries (Isakson et al., 2006a; Sandow et al., 2006, 2012; Dora et al., 2008; Ledoux et al., 2008; Chadha et al., 2011; Bagher et al., 2012; Senadheera et al., 2012; Sonkusare et al., 2012; Kirby et al., 2013). Functionally, the presence of gap junctions at the MEJ is thought to be key in the transfer of the hyperpolarization from ECs to VSMCs (Edwards et al., 2010). In parallel, the close apposition of the two cell types at the MEJ is believed to be essential for the accumulation of potassium released from EC via the SK_{Ca} and the IK_{Ca} channels and the further activation of K_{IR} and Na/K/ATPase in the VSMC, inducing their hyperpolarization (Edwards et al., 2010). With regard to calcium, its dynamics at the MEJ are crucial in the EDH-mediated response, because calcium concentration is specifically increased at the MEJ (Dora et al., 2008; Ledoux et al., 2008; Bagher et al., 2012; Sonkusare et al., 2012). The presence of a source of calcium (ER) at the MEJ, as well as the close localization of the calcium release channels IP_3R and the calcium-dependent IK_{Ca} channels at the MEJ is also of importance in the EDH-mediated response. Indeed, this aggregation at the MEJ is crucial for the activation of IK_{Ca} by calcium release from the ER via IP_3R in presence of acetylcholine (Isakson, 2008; Ellis et al., 2009; Sonkusare et al., 2012; Kirby et al., 2013). Lastly, given the functional role of TRP channels in the EDH-mediated response, it has been suggested that TRPC3 and TRPV4 channels present at the MEJ could either participate in the ER replenishment or in the activation of the K_{Ca} channels (Bagher et al., 2012; Senadheera et al., 2012; Sonkusare et al., 2012)

Although the MEJ is a key region for EDH-mediated response, the points of contact between ECs are equally important in this response. Namely, the spread of the hyperpolarization throughout the endothelium is central to ensure a coordinated response along the vascular bed (de Wit, 2010; Bagher and Segal, 2011). Accordingly, SK_{Ca}, Cx37, Cx40, and Cx43 have been evidenced at the points of contact between ECs (Emerson and Segal, 2000; Isakson et al., 2006a; Sandow et al., 2006, 2012; Dora et al., 2008; de Wit, 2010).

Structurally, it appears that caveolae play an important role in maintaining a functional EDH signaling microdomain at both EC contacts and the MEJ, because the EDH-mediated response is virtually abolished in mesenteric arteries from Cav1 KO mice (Saliez et al., 2008). In this study, the authors also observed an impaired calcium homeostasis in ECs, possibly resulting from a decreased activity of TRPV4, because the function of TRPV4 channels in HUVECs was impaired when cells were treated with Cav1 siRNA (Saliez et al., 2008). In parallel, Cav1 KO mice also exhibit decreased Cx37, 40, and 43 expressions at the MEJ (Saliez et al., 2008). Lastly, TRPV4, Cx37, Cx40, Cx43, and SK_{Ca} are localized in caveolae and also all colocalize and/or coimmunoprecipitate with Cav1 (Graziani et al., 2004; Absi et al., 2007; Saliez et al., 2008).

The importance of the MEJ in the EDH-mediated response has also been highlighted in pathologic conditions where their number increases along with the degree of EDH-mediated relaxation (Sandow et al., 2003a; Chadha et al., 2011). Additionally, IK_{Ca} channels and connexin expression at the MEJ is upregulated in arteries of obese rats (Sandow et al., 2003a; Haddock et al., 2011). In these studies, endothelium-dependent relaxation tends to shift from a NO component to an EDH component (Sandow et al., 2003a; Haddock et al., 2011). Thus, it is tempting to speculate that the increased number of MEJ in disease states compensate for other dysfunction in the arterial wall (Sandow et al., 2003a; Heberlein et al., 2009).

Several reports observed that the calcium released by the ER in EC and the calcium entering via TRP channels seem to be selectively involved in the hyperpolarization of ECs and appear not to induce NO generation from eNOS (e.g., Sonkusare et al., 2012). This fits well with the demonstration that eNOS and Hbα are closely localized at specific regions in the EC [i.e., the MEJ (see above)] where they regulate the amount of NO generated and released (Straub et al., 2012). With Hbα being expressed only in MEJs from resistance arteries and not conduit arteries, it is tempting to suggest that the Hbα "allows" for EDH to occur by binding any NO produced by eNOS activation after localized calcium release. Further studies will be required to tease this potentially important mechanism apart.

The studies described above involve a number of ion channels that are located in close proximity at the MEJ working in tandem to communicate a specific response in a spatially and temporally restricted manner (Fig. 8). Because the EDH-mediated response involves proteins that are 1) polarized to the MEJ, 2) accumulate in caveolae, and 3) are closely located to each other as demonstrated by coimmunoprecipitation or double immunolabeling, the EDH-mediated response is likely mediated by a signaling microdomain so as to regulate cellular communication in the blood vessel wall.

IV. Vesicular Communication: The Exocytosis Microdomain

Thus far, we have discussed communication involving diffusion of signals across the membrane or through plasma membrane channels. We now turn to the process of exocytosis, which allows for the storage and movement of signaling molecules in specialized vesicles. In the vascular wall, exocytosis provides ECs with a mechanism of rapid response to changes in the microenvironment to potentiate thrombosis, hemostasis,

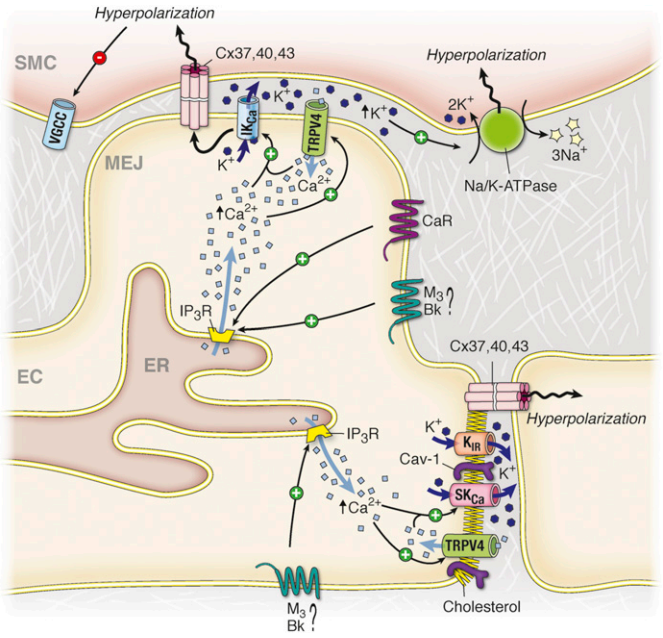

Fig. 8. The endothelium-dependent hyperpolarization microdomain. The EDH response starts with the activation of IP_3R at the ER in EC by a G_qPCR [for example, muscarinic (M3), BK, or the calcium sensing receptor (CaR)]. The calcium released from the ER activates the calcium-sensitive potassium channels SK_{Ca} at the EC-EC junctions and IK_{Ca} at the MEJ, which induces hyperpolarization of the EC. In parallel, the calcium release from the ER activates the capacitive entry of calcium at both the EC-EC junctions and the MEJ via TRPV4 channels, which sustains the opening of the IK_{Ca} and SK_{Ca} channels. The activation of SK_{Ca} and IK_{Ca} at the plasma membrane of ECs activates the efflux of potassium and its accumulation in the extracellular space between the VSMCs and the ECs. This potassium accumulation further activates the sodium/potassium ATPase (Na/K/ATPase) at the plasma membrane of VSMC, producing hyperpolarization and relaxation of the VSMC by closing the voltage-gated calcium channels (VGCC). The hyperpolarization of EC can also be transferred to the adjacent ECs and VSMCs via gap junctions channels located at the MEJ and at the EC-EC junctions. Wavy arrows indicate hyperpolarization.

or an inflammatory response. The secretory vesicles of the endothelium, known as Weibel-Palade bodies (WPB), were originally discovered as the storage depot of von Willebrand factor (vWF) (Weibel and Palade, 1964). Since their initial discovery via electron microscopy, WPBs have also been found to store and release P-selectin (Bonfanti et al., 1989; McEver et al., 1989), interleukin-8 (Utgaard et al., 1998; Wolff et al., 1998), endothelin-1 (Ozaka et al., 1997; Russell et al., 1998), angiopoietin-2 (Fiedler et al., 2004), tissue-type plasminogen activator (Huber et al., 2002), and other proteins that play important roles in the signaling pathways of inflammation, hemostasis, and tissue repair (for review, see Rondaij et al., 2006b; Valentijn et al., 2011).

A. Components of Exocytosis Signaling Microdomains

Exocytosis of the WPBs takes place in a series of discrete steps. The process starts with the synthesis of the WPBs, which is mostly driven by the presence of vWF. Once the vesicle has been formed, interactions with the cytoskeleton either keep the WPBs in the perinuclear space or move them to a more peripheral position. To have its contents released into the extracellular space, the vesicle is primed and then fused to the plasma membrane. Upon secretion of its contents, the WPB dissociates from the membrane (Fig. 9). These steps require the coordinated movements of several key proteins organized into signaling microdomains.

1. von Willebrand Factor. von Willebrand factor is a 2050-amino acid-long protein synthesized as a prepro–vWF precursor protein in the ER and transported to the Golgi, where pro-vWF assembles in ultra-large multimers (Sadler, 1998). The vWF multimers are stored in WPBs, which act to transport and release vWF into the lumen of blood vessels. Once released, vWF multimers anchor themselves to the ECs where they are proteolyzed by ADAMTS13 (Dong et al., 2002). The vWF allows platelets to adhere to ECs and can travel via blood to sites of blood vessel injury and attach to exposed collagen fibers. These functions ascribe vWF an important role in the responses of ECs, which cause coagulation, form thrombi, and repair damaged tissue (for a detailed review on vWF, see Lenting et al., 2012; De Ceunynck et al., 2013).

2. Synthesis of Weibel-Palade Bodies. The presence of vWF drives the synthesis of WPBs, as shown by the reduced number of WPBs in animal models lacking vWF and in humans with von Willebrand disease, a condition where the protein is mutated or not produced (Denis et al., 2001; Haberichter et al., 2005). In parallel, a study in dogs with von Willebrand disease showed that formation of WPBs was restored upon administration of vWF (Haberichter et al., 2005). Although the WPB is an EC-specific structure in vivo, the presence of vWF is enough to induce the formation of cigar-shaped organelles similar to WPBs in other cell types in vitro, such as monkey kidney CV-1 cells (Wagner et al., 1991; Voorberg et al., 1993).

The WPBs originate from clathrin and clathrin adaptor-protein 1-coated vesicles, filled with vWF multimers, that bud from the *trans*-Golgi network [TGN (Lui-Roberts et al., 2005; Zenner et al., 2007)]. The WPBs lose these clathrin/AP-1 protein coats as they mature (Lui-Roberts et al., 2005; Zenner et al., 2007), and the vWF contained within them becomes highly multimeric so it is able to efficiently anchor platelets once secreted into the lumen (Wagner and Marder, 1984).

The physiologic importance of proper WPB synthesis can be observed in the various disease states associated with release of immature, altered, or higher levels of vWF. In humans, several genetic mutations have been shown to affect the biosynthesis of vWF by altering its multimerization in the Golgi and its tubular packing in the WPB. In addition, the recruitment of other proteins stored within the vesicles is altered in these genetic disorders (for review, see Valentijn et al., 2011).

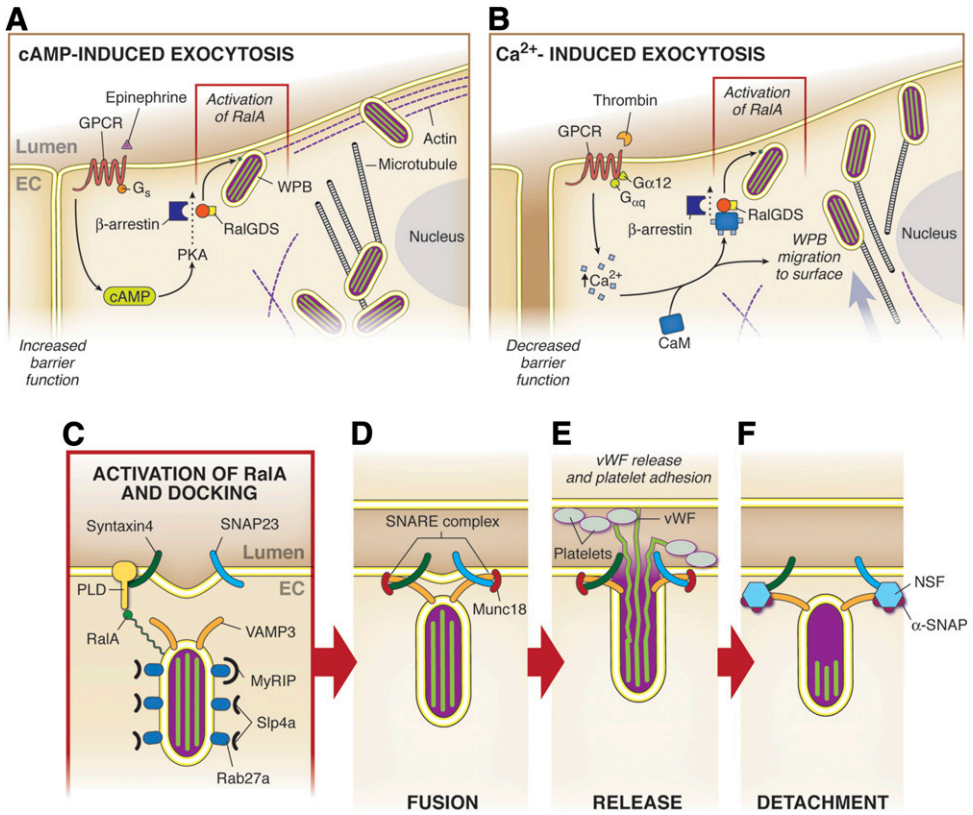

Fig. 9. The exocytosis microdomain. (A) Epinephrine induces increased cAMP via G_s protein-coupled receptors, resulting in the activation of RalA by RalGDS. The exocytosis of the Weibel-Palade bodies present at the PM is activated, whereas the progression of the WPBs located in the perinuclear region is inhibited by the presence of a more prominent peripheral actin rim. This process is accompanied by an increase in barrier function. (B) Upon activation of G_qPCR in ECs by agonists such as histamine or thrombin, there is an increase in $[Ca^{2+}]_i$, which associates with CaM. The Ca^{2+}-CaM further binds to the amino terminus of Ral GDS, which leads to the dissociation from the inhibitory beta-arrestin and to activation of RalA. This whole process allows WPB migration to the surface and concurrently decreases the strength of the barrier function between ECs. (C) RalA promotes fusion of the membrane by increasing PLD activity. Rab27a helps to determine when exocytosis will occur via its ratio of fractional occupancy by MyRIP and Slp4a. (D) The V-SNARE VAMP3 and the t-SNAREs syntaxin4 and SNAP23 interact to pull the two membranes in close proximity for fusion to occur. Munc18 acts to inhibit the SNAREs from binding prematurely. (E) vWF is released into the lumen, where it can bind to and attract platelets in addition to exerting effects on neighboring ECs. (F) NSF/α-SNAP bind to SNAREs to facilitate their disassembly.

3. Direct Protein-Protein Interactions of the Weibel-Palade Bodies. After the WPB leaves the TGN, it moves to the outer regions of the cell. As the WPBs mature, they recruit proteins important in the exocytosis process to the membranes. This includes small monomeric G proteins of the Rab family (members of the Ras superfamily of proteins) and vesicle soluble *N*-ethylmaleimide sensitive factor (NSF) protein receptor proteins (v-SNAREs). These components in conjunction with additional proteins help the vesicles attach to the membrane.

The Rabs are a family of over 60 members of small GTPases that help to control membrane identity and actions of intracellular vesicles; each secretory vesicle of the cell hosts a unique set of Rab family members (for review, see Stenmark, 2009). Rab proteins found in association with the WPBs are Rab3b (Bierings et al., 2012) Rab3d (Knop et al., 2004), Rab27a (Hannah et al., 2003), Rab3a, Rab15, Rab33a, and Rab37 (Zografou et al., 2012). Rab27a can either inhibit the secretion of WPBs by associating with its effector MyRIP (myosin VIIA and Rab interacting protein), thus anchoring WPBs to actin filaments and keeping them from attaching to the plasma membrane (Nightingale et al., 2009) or activate the secretion of WPBs by associating with an alternate effector, Slp4-a (synaptotagmin-like protein 4), which links the secretory granules to the plasma membrane (Gomi et al., 2005). Rab27a is central to the process of vesicle secretion, because the ratio of fractional occupancy of Rab27a by Slp4-a and MyRIP plays a role in determining the balance for or against exocytosis (Bierings et al., 2012). Furthermore, Rab27a may work in conjunction with Rab15 to help control exocytosis; Zografou et al. (2012) recently showed that simultaneous knockdown of the two Rabs using siRNA in HUVECs leads to a greater reduction in vWF secretion compared with knockdown of either Rab individually. Their experiments further showed that Munc13-4, an already known effector of Rab27a, colocalized with Rab15 at WPBs. Taken together, this information suggests that the three proteins Rab27a, Rab15, and Munc13-4 likely work in tandem to help regulate exocytosis of vWF (Zografou et al., 2012).

RalA, a small GTP-binding protein, is another central actor in the process of exocytosis that has been shown to cosediment with WPBs in density gradients (de Leeuw et al., 1999). RalA helps to promote exocytosis in two ways: 1) it promotes fusion of the membranes by increasing phospholipase D_1 activity (Vitale et al., 2005) and 2) it interacts with components of the exocyst (Moskalenko et al., 2002). The exocyst is an octameric protein complex consisting of Sec and Exo protein subunits (Hsu et al., 1996; TerBush et al., 1996) identified as having a role in vesicle tethering and fusion in nonendothelial cells (Grindstaff et al., 1998; Bao et al., 2008; Grote et al., 2000). However, to our knowledge, this complex has not been well characterized in ECs. In neutrophil, RalA is activated by its exchange factor, Ral guanine-nucleotide dissociation stimulator (RalGDS), which acts to promote the loss of the bound GDP and the uptake of GTP. This small protein is kept inactive by its attachment to β-arrestin, which uncouples from RalGDS upon stimulation of a GPCR, leaving the exchange factor in an active state (Bhattacharya et al., 2002) (Fig. 9). Downregulation of RalGDS expression using siRNA in HUVECs leads to a significant increase in the number of WPBs remaining in the cell after thrombin and noradrenaline stimulation, showing the importance of the exchange factor in the exocytosis process (Rondaij et al., 2008). Therefore, the RalGDS/β-arrestin complex is an important component of the signaling microdomain because of its role in linking GPCR activation with WPB exocytosis.

SNAREs are a family of transmembrane proteins that fuse to both vesicle (v-SNARE) and target (t-SNARE) membranes; they are defined by an extended coiled-coil stretch known as the "SNARE motif" (Jahn and Fasshauer, 2012). The three SNAREs that are known to target WPBs to the endothelial cell membrane are syntaxin 4 (t-SNARE) (Matsushita et al., 2003; Fu et al., 2005), SNAP-23 (t-SNARE), and VAMP3 (v-SNARE) (Pulido et al., 2011). When the vesicle is shuttled to the target membrane with the help of myosin and actin, it is aligned in such a way that the SNAREs from both membranes assemble in a α-helix to the forming bundle, and these helices "zipper" pull the membranes together (Fig. 9) (Sutton et al., 1998; Stein et al., 2009). This zippering model has been more fully characterized at neuronal synapses, but the players are analogous to those in the endothelium. Other proteins within the signaling microdomain potentially help to hold the SNAREs in a stabilized state before the zippering interaction, which facilitates exocytosis at a rate much faster than kinetics of the zippering would allow from a nonprimed state (Pobbati et al., 2006). Although this stabilized complex has not been clearly shown in ECs, complexes of SNAP23, syntaxin4, and VAMP3 have been coimmunoprecipitated, showing that at least these SNAREs interact to promote exocytosis (Pulido et al., 2011).

Munc18 is one such factor thought to interact with SNARE proteins to render the zippering model more efficient and decrease the time required for fusion. An inhibitory role has been ascribed to Munc18, because its binding to syntaxin isoforms keeps the SNARE in a closed conformation (Dulubova et al., 1999; Misura et al., 2000), but its function remains somewhat elusive. It has been suggested that a modification of Munc18, such as phosphorylation by PKC, is necessary before WBP exocytosis can occur (Fu et al., 2005). This suggests that Munc18 may play a role beyond pure inhibition and instead contribute to the stability of the SNARE complex before exocytosis.

Disassembly of the SNARE complex is required before the vesicle can be released from its membrane attachment. *N*-Ethylmaleimide sensitive factor (NSF) is an ATPase that binds to SNARE complexes to facilitate the disassembly of the "zippered" bundles (Zhao et al., 2012). Because NSF lacks a direct binding domain for members of the SNARE family, it connects to the complex via an effector, the α-soluble NSF attachment protein (α-SNAP) (Clary et al., 1990). Six NSF assemble together at the plasma membrane, and each NSF hexamer requires three α-SNAPs to mediate binding to the SNAREs (Wimmer et al., 2001). Once the NSF/αSNARE complex is formed, the NSF hydrolyzes ATP, providing the energy necessary for the disassembly of the SNARE complex (Whiteheart et al., 1994). Interestingly, α-SNAP may do more than just mediate the disassembly of SNAREs, because G_α proteins were recently shown to work in conjunction with α-SNAP to aid in the exocytosis process. Indeed, Rusu et al. (2013) provided strong evidence through the use of HUVECs and knockout animal models that $G_{\alpha 12}$ subunits interact with α-SNAP to promote the docking and fusion of WPBs. $G_{\alpha 12}$ binding sites for α-SNAP were mutated and evaluated for promotion of exocytosis by testing for a direct interaction between the two (Rusu et al., 2013). They further showed that $G_{\alpha 12}$ and $G_{\alpha q}$ subunits may regulate actin polymerization via activation of RhoA (a Ras-related GTPase), which leads to WPB docking, providing a direct link between GPCR activation and SNARE complex formation (Rusu et al., 2013).

4. Importance of Cellular Structure. Cytoskeletal elements are important to the exocytotic process because they facilitate the transport of WPBs from the TGN to more peripheral locations and eventually to the plasma membrane (Manneville et al., 2003). The cytoskeleton is also integral to the idea of the exocytotic machinery being organized into a signaling microdomain, because it serves as the scaffolding, which keeps the exocytosis complex confined to a specific area of the cell. Microtubules are polarized within the ECs, with the "minus" ends oriented perinuclearly and the "plus" ends extending toward the cell periphery; these microtubules help to move the WPBs over long distances (Nightingale et al., 2009). Motor proteins of the kinesin family help

facilitate movement in the plus-end direction, and dynein motors facilitate movement in the minus-end direction (Rondaij et al., 2006a). Actin combines with cross-linking proteins and with myosin II motors to form stress fibers (Burridge, 1981). Stress fiber components rearrange to either cause cell retraction or a strengthening of the cortical actin rim in response to different mediating pathways of exocytosis, providing structural changes that affect WPB movement and interaction with the membrane (Vischer et al., 2000).

The role of actin in exocytosis of WPBs has been given much attention. Many studies concluded that the actin filaments function as a physical barrier to prevent secretion (Doreian et al., 2008; Berberian et al., 2009; Bittins et al., 2009; Deng et al., 2009), whereas others proposed a different, although not mutually exclusive, idea that the stress fibers interact directly with the WPBs to anchor them in the cell until their complete maturity is reached (Desnos et al., 2003; Waselle et al., 2003; Hume et al., 2007; Nightingale et al., 2009). Recently, Nightingale et al. (2011) proposed a new function for actin in WPB exocytosis: after fusion of the WPB with the membrane, actin filaments and myosin II are recruited to form a ring around the base of the WPB. They believe that this ring acts to exert force at the base of the open granule, pushing vWF out into the lumen (Nightingale et al., 2011). The same group further demonstrated that myosin Va (MyoVa), an actin dependent motor, is capable of binding MyRIP, which, as described in section IV.A.3, works in unison with Rab27a to anchor WPBs in the periphery until they are fully matured (Fukuda and Kuroda, 2002; Desnos et al., 2003; Nightingale et al., 2009). In their study, Rojo Pulido et al. (2011) showed that knockdown of MyoVa in HUVECs using siRNA leads to a significant increase in the amount of immature vWF released after histamine stimulation (Rojo Pulido et al., 2011). They further demonstrated that Rab27a, MyRIP, and MyoVa are colocalized and coprecipitate in HUVECs (Rojo Pulido et al., 2011). This indicates that MyoVa is responsible for holding WPBs at the membrane until they are fully matured and ready for exocytosis (Rojo Pulido et al., 2011), at which point the Rab27a switches from MyRIP to the exocytosis activator Slp4-a, as detailed in section IV.A.3.

The lipid composition of the plasma membrane appears to be crucial in the exocytotic process, as illustrated by the specific changes in the phospholipid composition of the plasma membrane that occur before exocytosis takes place in ECs. For example, there is an increase in phosphatidic acid (PA) in HUVECs upon histamine-induced exocytosis of WPBs (Disse et al., 2009). The increased in PA observed was caused by recruitment to the membrane and activation of phospholipase D_1 (PLD1), an enzyme that hydrolyzes phosphatidylcholine to produce PA (Disse et al., 2009). The central role of this enzyme in WPB exocytosis was further evidence using shRNA to reduce the expression of PLD1, which resulted in a reduced secretion of vWF upon histamine stimulation (Disse et al., 2009). PLD1 is commonly thought as a general promoter of membrane fusion because of its role in producing fusogenic cone-shaped lipids such as PA (Roth, 2008). Depending on cell type and activation pathway, PLD1 requires activation by one or more factors, including small GTPases such as those of the ADP-ribosylation factor, Rho families as well as RalA, RalGDS, or protein kinase C (for review, see Jenkins and Frohman, 2005). Thus, RalA and RalGDS not only play a role in the exocytosis process itself, as discussed in section IV.A.4, but are additionally associated with WPBs and help to increase PLD1 activity (Rondaij et al., 2008). Taken together, this information suggests that RalA could serve as an upstream activator of PLD1, causing its movement to the membrane and the subsequent generation of PA-enriched domains, which could aid in the membrane fusion process.

In addition to PA, annexin-A2 has been found to promote the formation of microdomains with increased cholesterol content, which correlates with sites of exocytosis in chromaffin cells (Chasserot-Golaz et al., 2005). Likewise, expression of annexin-A2 has been shown via immunofluorescence in the cytoplasm and at the plasma membrane in HUVECs (Knop et al., 2004). Functionally, when HUVECs were depleted of the annexin-A2 along with its main protein partner S100A10, secretion of vWF was decreased (Knop et al., 2004). This suggests that annexin-A2 is necessary for EC exocytosis, possibly because of its role in formation of cholesterol-rich microdomains as demonstrated in other cell types.

B. Activation Pathways for Exocytosis

Exocytosis of vWF from ECs can be triggered by many events such as hypoxia, ischemia, or acute injury. Exocytosis in response to a biologic stimulus is often induced via activation of GPCR through the binding of ligands such as histamine (Hamilton and Sims, 1987), thrombin (Levine et al., 1982), serotonin (Schluter and Bohnensack, 1999), epinephrine (Vischer and Wollheim, 1997), and vasopressin (Kaufmann et al., 2000). The components of the GPCRs are of particular importance, because they bind to downstream signaling effectors such as heterotrimeric G protein complexes, kinases, and arrestins (Rasmussen et al., 2011; Katritch et al., 2012). Depending on the nature of the G_α protein coupled to a GPCR, exocytosis of WPBs can be activated via a calcium-dependent pathway (if the stimulated GPCR is coupled to a G_q protein) or via a cAMP-dependent pathway (if the stimulated GPCR is coupled to a G_s protein).

1. Calcium-Dependent Activation of Exocytosis. Calcium's ability to stimulate exocytosis has been demonstrated experimentally using Ca^{2+} ionophores, which were sufficient to promote the release of vWF

(Loesberg et al., 1983; van den Eijnden-Schrauwen et al., 1997). In parallel, experiments in HUVECs using the caged Ca^{2+} chelator DM-nitrophen showed that the cell capacitance increases upon elevation of intracellular calcium via photolysis, which is indicative of exocytosis (Zupancic et al., 2002). Calmodulin inhibitors and intracellular calcium chelators are also able to decrease thrombin-induced exocytosis of vWF in HUVECs (Birch et al., 1992; van den Eijnden-Schrauwen et al., 1997). The blocking effect of calmodulin inhibitors was explained recently using a peptide that mimics the calmodulin-binding domain present in the N terminus of RalGDS, showing that RalGDS association to calmodulin is crucial for RalA activation (Rondaij et al., 2008). When HUVECs are treated with the mimetic peptide, RalA activation is inhibited along with WPB exocytosis initiated by thrombin (Rondaij et al., 2008). Thus, it appears that calcium indirectly activates RalGDS by binding to calmodulin, further activating RalA and WPB exocytosis.

Thrombin and histamine stimulation, angiotensin II (Ge et al., 2007), sphingosine-1-phosphate (S1P) (Matsushita et al., 2004), and ATP/ADP (Vischer and Wollheim, 1998) all cause increases in $[Ca^{2+}]_i$ via GPCR activation, which is a prime potentiator of exocytosis (Tse et al., 1997), but there is also evidence for extracellular calcium involvement. For example, influx of extracellular calcium via the T-type calcium channels Cav3.1 occurs upon stimulation of pulmonary microvascular ECs with thrombin, resulting in a procoagulant endothelial phenotype (Wu et al., 2003). In a more recent work, the same group linked exocytosis to external calcium influx via Cav3.1 using the Cav3.1 channels blocker mifbefradil and by direct targeting with shRNA, which both inhibit thrombin-induced vWF secretion in pulmonary microvascular ECs (Zhou et al., 2007a). In this study, the authors also showed that there is differential regulation of exocytosis between microvessels and conduit arteries in the pulmonary circulation, with exocytosis being entirely calcium dependent in smaller vessels, whereas exocytosis is regulated by two distinct pathways involving calcium or cAMP in larger vessels (Zhou et al., 2007a). This flexibility is thought to be a result of different populations of WPBs and might allow for a faster or slower response to inflammation (Zhou et al., 2007a). Further evidence for the relevance of T-type calcium channels in mediating exocytosis is that the mRNA of Cav3.1 in EC is increased upon Ang II stimulation (Wang et al., 2006).

The sphingolipid S1P is similar to thrombin and histamine in that it can potentiate inflammation in the vasculature via exocytosis of vWF and angiopoietin 2 from WPBs (Matsushita et al., 2004; Jang et al., 2009). Matsushita et al. (2004) showed that S1P-mediated exocytosis of WPBs occurs in part via PLCγ but also involves extracellular calcium, because both PLC inhibitors and calcium-free media blocked S1P-induced secretion of vWF from human aortic ECs. Given that the receptor was pertussis toxin sensitive, the authors concluded that the S1P must act via a G_i-linked receptor (Matsushita et al., 2004).

2. Cyclic AMP Activation of Exocytosis. The regulation of vWF secretion by cAMP was first evidenced in HUVECs in the late 1990s by two different groups (Vischer and Wollheim, 1997; Hegeman et al., 1998). In both studies, the authors were able to measure an increase in cAMP concomitant with vWF secretion after stimulation with epinephrine and the adenylate cyclase activator forskolin (Vischer and Wollheim, 1997; Hegeman et al., 1998). In parallel, pharmacological blockers of the cAMP pathway completely abolished vWF secretion (Vischer and Wollheim, 1997; Hegeman et al., 1998). Other agonists are now known to activate WPBs exocytosis in a cAMP-dependent manner, including serotonin (Schluter and Bohnensack, 1999) and vasopressin (Kaufmann et al., 2000), all of which induce vWF secretion independently of a rise in $[Ca^{2+}]_i$. The exocytosis of WPBs caused by an increase in cAMP is partly due to a PKA-dependent signaling pathway, which leads to the activation of RalA (de Leeuw et al., 1999; Rondaij et al., 2004, 2008). Exocytosis can also be activated independently of PKA activity through a cAMP-induced activation of Rap1 (Ras-related protein 1) mediated by the guanine nucleotide exchange factor Epac (van Hooren et al., 2012). It is noteworthy that activation of both Rap1 and RalA pathways may be coordinated, allowing both pathways to contribute to the exocytotic process in a synchronous fashion (van Hooren et al., 2012).

3. Differential Activation of Weibel-Palade Bodies Exocytosis by Calcium and cAMP. Although both calcium- and cAMP-mediated pathways lead to release of vWF from WPBs in ECs, there are subtle differences that may have important consequences. The two pathways lead to different rearrangements of cytoskeletal elements as demonstrated by Vischer et al. (2000) in HUVECs, where stress fibers are rearranged differently upon activation of cAMP or calcium pathways. Namely, histamine and thrombin, which both activate calcium-mediated exocytosis, induced the formation of more prominent stress fibers that aligned in a parallel, longitudinal fashion, whereas the myosin II was redistributed to underlying stress fibers (Vischer et al., 2000). However, when cAMP was increased via forskolin or IBMX, there was a rapid disappearance of stress fibers, and the peripheral actin rim became more prominent at the basis of WPBs (Vischer et al., 2000). The secretion of vWF in response to Ca^{2+}-elevating agonists was inhibited in HUVECs treated with the microtubule disrupters colchicine or nocodazole but was potentiated when treated with the actin disrupter cytochalasin E (Vischer et al., 2000). However, the secretion of vWF in response to cAMP-elevating

agonists is not affected by either treatment (Vischer et al., 2000; Manneville et al., 2003; Nightingale et al., 2009;).

Interestingly, calcium- and cAMP-dependent pathways lead to differential WPB exocytosis. Specifically, vWF depletion of almost all WPBs is observed when an increase in Ca^{2+} occurs, whereas an increase in cAMP leads to a clustering of WPB in the perinuclear space with only the most peripherally located vesicles releasing their contents (Cleator et al., 2006). This perinuclear clustering of WPBs seen with increased cAMP is due to movements facilitated by the dynein-dynactin complex (Rondaij et al., 2006a).

These different responses also lead to opposite consequences on the barrier function of endothelial cells: increased Ca^{2+} leads to the disassembly of tight and adherens junctions via RhoA activation (van Nieuw Amerongen et al., 2000; Wojciak-Stothard et al., 2001), whereas activation of Rap1 through a cAMP-dependent pathway leads to improved barrier function due to promotion of cell-cell contacts mediated by VE-cadherin (Cullere et al., 2005; Fukuhara et al., 2005; Kooistra et al., 2005). The differing responses in cytoskeletal rearrangement and barrier function could play a role in determining the effects of the exocytotic process in response to different stimuli. Although the machinery involved in getting the content of WPBs to the lumen is similar, cAMP-mediated WPB release is slower (>10 minutes), whereas calcium-dependent WPB release is rapid (<5 minutes) (Vischer et al., 2000; Rondaij et al., 2004). Taken together, these differences provide mechanisms by which ECs can use the same process of exocytosis to bring about proper physiologic change in response to varying triggers.

Although there are many molecular players and different modes of activation in this complex choreography, and although the details of the entire pathway are still elusive, it is known that the components work closely together to form a macromolecular complex to ensure proper coordination of the exocytosis pathway. From the activation of a GPCR to the fusing of the vesicle at the membrane, this process is tightly regulated by a dependence on several protein-protein interactions and polarization of the individual components to a specific cellular location, making the multiple proteins involved in the exocytotic process part of an important signaling microdomain involved with cellular communication.

V. Conclusion

In the vascular wall, cells have to act in a highly coordinated manner to ensure proper function. To do this, cellular communication must be present and highly regulated. Behind this tight regulation lies a highly coordinated intracellular machinery organized as signaling microdomains where the second messenger calcium is of utter importance. In this review, we described and defined examples of intracellular proteins that act in an orchestrated manner to ensure proper intercellular communication.

The signaling microdomains described in this review are the results of considerable work using pharmacological and molecular tools coupled, in a majority of cases, to high-resolution microscopy. However, in some instances, it was difficult for us to evaluate whether two or more proteins are part of the same signaling microdomain. The main reason for this was the lack of correlation between observed functional data coupled to detection of the proximity of the different proteins involved. Recently, several technical advances have been made in the field, allowing scientists to observe the function of a single protein. For example, the scanning ion conductance microscopy (Lab et al., 2013), measurements of elementary calcium events (Sonkusare et al., 2012), or evaluation of the proximity of proteins directly in tissues (Gullberg et al., 2003) considerably enhanced our understanding of signaling microdomains. The expansion of these, as well as several other unique molecular techniques, will certainly open even more possibilities in the future to understand how cellular interactions regulate their ability to efficiently communicate.

Acknowledgments

The authors sincerely apologize to those who were not cited in this review. Please be assured that this was not attributed to any other reason than the need to condense the vastness of the field or the adherence to the rubrics laid out in Table 1.

Authorship Contributions

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