Vascular TRP Channels: Performing Under Pressure and Going with the Flow

Endothelial cells and smooth muscle cells of resistance arteries mediate opposing responses to mechanical forces acting on the vasculature, promoting dilation in response to flow and constriction in response to pressure, respectively. In this review, we explore the role of TRP channels, particularly endothelial TRPV4 and smooth muscle TRPC6 and TRPM4 channels, in vascular mechanosensing circuits, placing their putative mechanosensitivity in context with other proposed upstream and downstream signaling pathways.

Circulating blood exerts mechanical forces on the walls of blood vessels. Most notable among these are shear stress on the vascular wall associated with blood flow and intravascular pressure resulting from resistance to flow (225) (FIGURE 1). These forces are sensed through cell type-specific mechanisms and signal through different intracellular molecular pathways to elicit distinct vascular responses.

In resistance arteries (the small arteries that contribute to peripheral vascular resistance), intravascular pressure induces vasoconstriction, a process termed the myogenic response. As the name suggests, this response is intrinsic to vascular smooth muscle cells (SMCs) and serves to regulate arterial diameter, and thus blood flow, in response to changes in pressure. In myogenic arteries, pressure induces vasoconstriction by depolarizing the SMC membrane potential ($V_m$), activating voltage-dependent calcium ($Ca^{2+}$) channels (VDCCs) in the plasma membrane (92). This allows extracellular $Ca^{2+}$ to flow down its electrochemical gradient into the cell, where it acts on the $Ca^{2+}$-dependent molecular machinery that drives myosin-actin cross-bridge cycling to promote contraction of SMCs (48, 73, 110, 124, 172, 174). $Ca^{2+}$-dependent mechanisms that act to enhance the $Ca^{2+}$ sensitivity of the contractile apparatus further contribute to the myogenic response, especially at higher pressures (33, 173, 214).

The shear stress associated with blood flow has predominantly the opposite effect, acting on endothelial cells (ECs) to exert a vasodilatory influence on adjacent smooth muscle. Here too, the intracellular intermediary is elevated intracellular $Ca^{2+}$, which acts through multiple $Ca^{2+}$-dependent molecular mechanisms in ECs to elaborate factors and/or initiate cellular processes that serve to hyperpolarize SMC membrane potential, suppress VDCC activity, and induce dilation.

A growing body of evidence has implicated members of the TRP (transient receptor potential) family of “nonselective” cation channels (see Sidebar 1) in both pressure-induced constriction and flow-induced dilation. Much of the initial enthusiasm for a TRP-dependent basis for these processes stemmed from the reported mechanosensitivity of certain TRP family members. Although some members of this family are indisputably capable of responding directly to mechanical stimuli, whether they actually function under physiological conditions as the kind of one-man-band originally envisioned is not entirely clear. Recent work places TRP channels squarely in the molecular circuitry that mediates vascular responses to flow and pressure, but suggests a more nuanced picture.

Prominent Vascular TRP Channels

Most of the research on TRP channels in the vasculature has centered on three subfamilies: TRPC (canonical or classical), TRPV (vanilloid), and TRPM (melastatin). In this review, we focus primarily on individual members of these three families that have consistently been implicated in the regulation of vascular tone in response to...
The term “nonselective” is somewhat of a misnomer in describing TRP channels, since individual members of this family exhibit variable selectivity for different cations and patterns of differential ion selectivity that vary between one TRP subfamily and another. Beyond this seeming semantic distinction is a potentially more meaningful distinction: which ionic selectivities are “real” and which are products of recording conditions that bear little relationship to physiological conditions. Typically, ion permeabilities of different TRP channel family members have been estimated from the Goldman-Hodgkin-Katz equation by measuring the reversal potential (i.e., zero-current; E_{rev}) of the whole-cell TRP current and its change with substitution of external Na\(^+\) or Ca\(^{2+}\). Using this approach, the Ca\(^{2+}\)/Na\(^+\) permeability ratios for TRPV4 and TRPC6 have been estimated to be ~6 (182) and 5 (76), respectively. However, even though TRP channels have been shown (or are presumed) to have a very significant K\(^+\) permeability, cesium has typically been used as the major intracellular cation (to minimize currents through K channels). This approach has two major limitations: (1) the nature of the permeant ion (Ca\(^{2+}\) vs. K\(^+\)) could affect the selectivity of Na\(^+\) and Ca\(^{2+}\); and (2) the reversal potential of channel effects of channel activation on membrane potential will be minimal. Indeed, the combination of high Ca\(^{2+}\) and K\(^+\) permeability (e.g., TRPV4) may be well suited for delivering Ca\(^{2+}\) without causing a significant decline in the electrical driving force through membrane potential depolarization. In contrast, TRPM4 channels are Na\(^+\) and K\(^+\) permeable (Na\(^+\)-to-K\(^+\) ratio ~ 1) but largely impermeable to Ca\(^{2+}\) (148), with an E_{rev} of ~0 mV. Thus activation of TRPM4 channels would depolarize the membrane potential of vascular SMCs.

The single-channel conductance of TRPV4 and TRPC6 channels at physiological membrane potentials with Na\(^+\) and Ca\(^{2+}\) as charge carriers (with internal cesium) are ~60 and 35 pS, respectively, or ~3–6 pA at ~40 mV (76). By comparison, the L-type VDCC has a single-channel conductance of 3.5 pS (with physiological Ca\(^{2+}\) as the charge carrier), corresponding to a unitary current of 0.18 pA at ~40 mV (159). The upshot of these properties is that the Ca\(^{2+}\) influx rate through a single TRPV4 channel is substantially greater than that through a single TRPC6 channel and equivalent to that through ~10 VDCCs (129).
Although a number of studies support the involvement of TRP channels, including TRPC6, TRPM4, and TRPV4, in transducing mechanical stimuli into a change in vascular tone, it is not clear whether TRP channels act as direct mechanosensors or are engaged by some other upstream stimulus. Mechanisms of mechanical activation of TRP channels have been suggested to include the following (83): 1) direct activation by deformation of the lipid bilayer by mechanical forces, such as cell stretch, shear, and friction (57, 120, 179), which produce membrane thinning or bending that, in turn, leads to a conformational change in the embedded proteins toward an energetically stable open state; 2) tethering of mechanosensory TRP channels to specific cellular structures, such as cytoskeletal (10, 125) and/or regulatory/adaptor proteins (191), which transduce physical forces that open the channel; and 3) mechano-biochemical conversion, which involves engagement of upstream biochemical signals, such as enzymes or second messengers that, in turn, activate TRP channels (94, 114, 127). The first two mechanisms are predicated on the intrinsic mechanosensitive properties of TRP channels, whereas the third general mechanism can operate independently of them, as discussed in greater detail below. These mechanisms are not mutually exclusive; thus it is possible, perhaps even likely, that more than one mechanism operates in a given vascular bed.

Mechanosensing in Endothelial Cells: Vasodilation

Shear stress is nearly universally associated with vasodilation in the peripheral vasculature, where research has consistently demonstrated a predominant vasodilatory response to blood flow in conduit arteries, such as femoral (205) and carotid (6, 15, 16, 116, 156) arteries, as well as resistance arteries and arterioles, including mesenteric arteries (16, 23, 98, 116, 123, 188, 234), coronary arteries (22, 54, 78, 79, 90, 91, 99, 101, 105, 112, 113, 133, 134, 178, 203, 204, 236, 237), skeletal muscle feed arteries/arterioles (5–7, 55, 77, 86–89, 96, 97, 166, 171, 180, 186, 187, 189, 190, 196, 202, 208, 228–230), and adipose arterioles (153). Interestingly, shear stress is clearly capable of causing vasoconstriction in the cerebral circulation (20, 145, 201). Here, the response to flow appears to depend on pressure and flow rate, with flow inducing dilation at lower pressure (59, 197) or higher flow rates (170), and constriction at higher pressure (20, 59, 119, 145, 197) or lower flow rates (170).

As a rule, flow-induced dilation is endothelium dependent (17, 26, 37, 95, 162) and is associated with an increase in EC Ca2+ (22, 51, 94, 128). Although exceptions have been reported (9, 156), the...
preponderance of recent work supports this generalization. It is also important to note that recent studies using more sophisticated techniques have established the centrality of local EC Ca\(^{2+}\) signaling in endothelium-dependent vasodilation (4, 155, 177) rather than global elevations in intracellular Ca\(^{2+}\), the traditional metric used to assess Ca\(^{2+}\) dependence. Endothelial Ca\(^{2+}\) levels are increased through two mechanisms: extracellular Ca\(^{2+}\) influx and release of Ca\(^{2+}\) from intracellular ER stores. This latter mechanism is presumed to be central to the action of neurohumoral factors, which typically signal through the canonical Gq-protein-coupled receptor (GqPCR)/phospholipase C\(\beta\) (PLC\(\beta\)) pathway to hydrolyze phosphoinositide bisphosphate (PIP\(_2\)). This yields DAG and inositol trisphosphate (IP\(_3\)), respectively. However, this pathway does not likely contribute directly to the elevation of intracellular Ca\(^{2+}\) in response to flow that drives dilation. In fact, available evidence suggests the opposite, with reports showing that flow-induced dilation is actually increased by depletion of intracellular Ca\(^{2+}\) stores, an effect attributable to increased extracellular Ca\(^{2+}\) influx (102, 111).

**Endothelial TRPV4 Channels in the Promotion of Vascular Dilation**

How extracellular Ca\(^{2+}\) enters ECs has been a longstanding question in endothelial biology. One endothelial Ca\(^{2+}\)-influx pathway that has been clearly identified is nonselective cation channels of the TRPV family, particularly TRPV4. TRPV4 is prominently expressed in vascular ECs, where it has been detected at mRNA and protein levels (22, 128, 239). Electrophysiological approaches have further demonstrated the presence of functional TRPV4 currents (characterized by their outward rectification and voltage-dependent block by Ruthenium Red of inward, but not outward, current) in ECs from mesenteric arteries (177) and the aorta (217).

A number of lines of evidence have linked endothelial TRPV4-mediated Ca\(^{2+}\) entry with vasodilatory responses. Several laboratories have demonstrated that selective activators of TRPV4 channels induce Ca\(^{2+}\) influx-dependent vasodilation (22, 94, 114, 128). Studies using TRPV4-knockout mice, in particular, have provided compelling support for the functional importance of this Ca\(^{2+}\)-entry pathway. For example, endothelium-dependent relaxation responses to the vasodilator acetylcholine are attenuated in superior mesenteric arteries of TRPV4\(^{-/-}\) mice (239). Moreover, endothelium-dependent dilation to flow is absent in carotid arteries from TRPV4\(^{-/-}\) mice (71), providing support for a role for TRPV4 in shear stress-induced vasodilation. These observations confirm the results of previous reports from wild-type animals showing that flow-induced vasodilation in conduit (carotid) and resistance (gracilis) arteries is sensitive to inhibition by the TRPV blocker Ruthenium Red. Interestingly, endothelial TRPV4 channels have also been reported to mediate dilation in response to pressure, in particular low pressure (<50 mmHg) (4). This mechanism, which selectively engages TRPV4 channels localized to a microdomain at myoendothelial projections (MEPs) through the internal elastic lamina (IEL; see FIGURE 3, INSET), could act to oppose or reverse myogenic tone at the low end of the myogenic response range (30–50 mmHg). Moreover, in conjunction with conducted vasodilation, it could also serve to actively dilate arteries under conditions in which blood flow was dramatically reduced, for example, due to ischemia or an upstream blockage.

Recent development of potent, selective TRPV4 agonists (GSK1016790A) and antagonists (e.g., HC06704, GSK2193874) have made it possible to directly probe the functional role of TRPV4 in regulating vascular function. Using these tools, our laboratory has shown that activation of TRPV4 channels in ECs from resistance-size (~100-\(\mu\)m diameter) mesenteric arteries can be monitored optically by detecting Ca\(^{2+}\) influx through individual TRPV4 channels (177) (Sidebar 2), providing a powerful new approach for directly studying responses of these channels to mechanical stimulation. Underscoring the utility of this optical-detection approach and reaffirming results from TRPV4-KO studies, we have found that TRPV4 channels are rapidly activated by the mechanical force associated with picospritzing, primarily a local increase in flow/shear stress (FIGURE 2).

**TRPV4 in the Mechanosensing Circuity**

The proposed mechanosensitivity of TRPV4 channels has led to the suggestion that this channel directly senses changes in the plasma membrane bilayer caused by shear stress and/or indirectly responds to tethered cytoskeletal elements that act as proximate mechanosensors, and transduces this stimulus into an increase in open probability. There is strong evidence that TRPV4 is, in fact, mechanosensitive. Originally cloned independently in 2000 by several groups and variously named OTRPC4 (OSM-9-like TRP channel 4) (182), VRL-3 (vanilloid receptor-like 3) (38), VR-OAC (vanilloid receptor-related osmotically activated channel) (109), and TRP12 (227), TRPV4 has been shown to confer mechanosensitivity upon transient transfection in a number of different cell lines (HEK393, CHO-K1, RBL), manifesting as the
appearance of a cation current (109, 182) and increased intracellular Ca\(^{2+}\) (109, 182, 227) in response to hypotonicity. At one time, it was thought that the repertoire of mammalian TRPV4 might be limited to osmoregulation, in contrast to its osmo-/chemo-/mechanosensitive Caenorhabditis elegans counterpart (osm-9), with early evidence suggesting an absence of responsiveness to negative (or positive) pressure-induced membrane stretch (182). However, subsequent work identified temperature as key to the mechanical responsiveness of TRPV4 channels, showing that exposure of TRPV4-expressing HEK293 cells to increasing shear stress within the physiological range (from 0 to 10 dyne/cm\(^2\)) resulted in a substantial (approximately fourfold) increase in peak TRPV4-dependent intracellular Ca\(^{2+}\) accumulation at 37°C but evoked a negligible change at room temperature (57).

For ion channels, which activate rapidly, the kinetics of the response can provide an important indicator of whether a mechanical stimulus is sensed directly or indirectly: a delayed response would suggest the existence of a separate upstream sensing mechanism, whereas an immediate response would indicate that the channel itself likely senses the stimulus directly. Because the molecular circuitry that lies between channel activation and vasodilation introduces a variable-length delay, this kinetics concept is best applied to an analysis of changes in intracellular Ca\(^{2+}\). In an intriguing series of experiments using both parental and engineered capillary endothelial cell lines, Ingber and colleagues demonstrated “ultra-rapid” TRPV4 channel activation in response to a mechanical stimulus, in this case, a mechanical strain induced by applying force to β1 integrins in the cytoskeletal framework of focal adhesions using magnetic pulling cytometry (125). Ca\(^{2+}\) elevation, measured using Fluo-4, was detected within ~4 ms of stimulus application and was substantially reduced by treatment with Ruthenium Red and virtually eliminated in cells transfected with small interfering (siRNA) against TRPV4. These studies

**FIGURE 2.** Flow-induced TRPV4 sparklets in resistance-sized mesenteric arteries

Endothelial cells in an en face preparation of resistance-size mesenteric arteries from GCaMP mice were stimulated by a brief, parallel-oriented flow of bath solution delivered by picospritzing (pressure, 1.5 bar; tip diameter, 5–6 μm; distance from surface, ~20 μm). Ca\(^{2+}\) signals were imaged by confocal microscopy. Picospritzing-induced Ca\(^{2+}\) signals were largely abolished by the TRPV4 antagonist HC067047, establishing their identity as TRPV4-mediated events. Top: representative raw trace. Bottom: summary data (n = 3 fields from 3 arteries) (previously unpublished data).
further showed that membrane deformation alone was not sufficient to induce TRPV4-mediated Ca\(^{2+}\) elevation; integrins were strictly required. To our knowledge, a similar detailed kinetic analysis of flow-induced, TRPV4-mediated Ca\(^{2+}\) elevation in intact artery segments has not been reported.

It has also been suggested that endothelial TRPV4 channels form a complex with TRPC1 channels that respond to shear stress (118). This study by Yao and colleagues provided evidence that these channels interact with each other when co-expressed in an exogenous expression system (HEK cells) and demonstrated that flow-induced Ca\(^{2+}\) elevations in TRPV4-expressing cells are enhanced by TRPC1 coexpression. Importantly, the kinetics of the response were changed: in the absence of TRPC1, flow-induced Ca\(^{2+}\) increases occurred on a time scale of seconds, whereas, at the resolution shown, Ca\(^{2+}\) appeared to increase almost immediately upon initiation of flow in the presence of TRPC1. Coexpression of a dominant-negative TRPC1 construct or treatment with a TRPC1-blocking antibody inhibited the potentiating effect of TRPC1. Importantly, TRPC1 and TRPV4 were found to coimmunoprecipitate in native mesenteric ECs, and treatment of mesenteric artery segments with a TRPC1-blocking antibody blunted flow-induced dilation. Whether TRPV4-TRPC1 complexes function as proposed in native cells is not yet clear since most of the details of this overall model were developed using cultured ECs.

Collectively, these observations suggest that integrins and TRPV4, perhaps in complex with TRPC1, act as a functional unit to rapidly (within milliseconds) transduce a mechanical stimulus into Ca\(^{2+}\) influx in a manner that relies on the intrinsic mechanosensitivity of the TRPV4 channel. However, this view is complicated by the results of certain pharmacological interventions that suggest a mechano-biochemical conversion mechanism involving upstream signaling elements. In this context, Kohler et al. reported that TRPV4-dependent, flow-induced dilation was blocked by inhibition of phospholipase A2 (PLA\(_2\)) with arachidonyl trifluoromethyl ketone (AACOCF\(_3\)) (94). In contrast, dilation induced by direct activation of TRPV4 with 4α-PDD was unaffected by AACOCF3, suggesting that flow triggers activation of PLA\(_2\), increasing levels of an AA metabolite that mediates activation of TRPV4. Consistent with this, previous studies have reported that PLA\(_2\) activity is enhanced directly by membrane deformation (24, 25, 106).

PLA\(_2\) sits atop multiple downstream pathways that utilize AA as a substrate. AA, generated from membrane lipids by the action of PLA\(_2\), is a substrate for CYP epoxygenases, which generate EETs. In a series of studies employing cultured ECs (53, 114, 213) and intact arterial preparations (114) from humans as well as wild-type and TRPV4\(^{-/-}\) mice, Nilius and colleagues showed that shear stress induces the production of EETs, notably 5,6-EET and 8,9-EET (213), both of which directly activate TRPV4 channels (220). These findings suggest that the CYP epoxygenase responsible for the generation of EETs is an upstream element in the TRPV4-dependent pathway, leading to flow-induced dilation (114). Specifically, these authors showed that flow-induced dilation was blunted by inhibition of epoxygenase (MS-PPOH) or TRPV channels (Ruthenium Red). Moreover, when used together, these inhibitors were no more effective than either alone, suggesting a serial linkage between epoxygenase-mediated EET production and TRPV4 activation. Interestingly, shear stress was also found to induce the translocation of TRPV4 protein from a perinuclear location to the plasma membrane in cultured human ECs (114), presumably due to the direct action of EETs, as suggested previously by these authors (53).

A research thread that often, and oddly, goes unacknowledged in primary studies as well as in reviews on fluid shear stress mechanotransduction mechanisms is the role of the endothelial glycocalyx layer (EGL), a thin (<0.5 μm), gelatinous endothelial surface boundary layer composed of proteoglycans, glycosaminoglycans (GAGs), and associated plasma proteins (31, 100, 115, 195, 222, 231). Although most of this work has been conducted in microvessels (i.e., capillaries and postcapillary venules), functional studies have provided evidence for a hemodynamically relevant EGL in cremaster muscle (165) and coronary (80) arterioles as well as in mesenteric (154), coronary (206), and femoral (72, 135) arteries. One implica-
tion of this line of research is that hypotheses based on the presumption that the EC surface itself directly senses the shear stress associated with blood flow will have to be revisited. In fact, all three major models that have been proposed to describe the behavior of this boundary layer predict that the fiber matrix that makes up the EGL greatly attenuates shear stress, with the result being that the level of shear stress experienced by the EC membrane is “vanishingly small” (Ref. 222, and references therein). Instead, these models suggest that deformation of GAGs in the EGL by the hydrodynamic drag associated with blood flow is relayed to the underlying cytoskeleton at the apical surface and/or to the extracellular matrix at the basal surface via actin stress fiber-mediated linkages to integrins and/or syndecans. This suggestion is fully compatible with the requirement for B1 integrins reported for ultra-rapid TRPV4 channel activation by shear stress, noted above (125), as well as previous studies that have posited a role for integrins in flow-induced dilation (115, 121).
In a sense, the absence of detectable shear stress at the luminal EC surface implied by the presence of the EGL is conceptually liberating in that it eliminates constraints on the cellular position of the stress-responsive molecular machinery. Accordingly, targets localized to the basal surface of ECs could be as readily linked to the lever action of pressure-sensing elements embedded in the EGL as apical surface targets. Potential “beneficiaries” of this liberation are TRPV4 channels colocalized with their target IK (intermediate-conductance K⁺) channels within myoendothelial projections at the basal surface of the EC (see Figure 3, Inset). Whereas this microdomain architecture could facilitate transmission of local TRPV4 Ca²⁺ signals (sparklets) to smooth muscle in the form of IK-dependent hyperpolarization, its connection to shear stress at the apical surface would be difficult to rationalize in the context of a mechanism that assumed direct exposure to shear stress. Whether flow actually triggers activation of TRPV4 channels in this microdomain or at other locations within the cell, or both, is not currently known.

**Downstream of TRPV4**

The influx of extracellular Ca²⁺ mediated by TRPV4 channels acts directly on Ca²⁺-sensitive EC targets to release soluble vasodilatory factors or initiate processes that promote dilation through hyperpolarization of the membrane of underlying vascular SMCs. Broadly speaking, there are three general classes of endothelial-derived, Ca²⁺-dependent dilator influences: 1) nitric oxide (NO), 2) cyclooxygenase (COX)-derived products, and 3) a non-NO/non-COX influence termed endothelial-derived hyperpolarization (EDH) (Sidebar 3).

Only a handful of studies have explicitly investigated the signaling pathways downstream of TRPV4 in the context of flow-induced dilation. Zhang and colleagues (128), working with mesenteric arteries from mice, found that luminal flow induced vasodilation through both NO and EDH mechanisms, and showed that both components were substantially diminished in TRPV4⁻/⁻ mice. Loot and coworkers demonstrated a critical role for CYP450 epoxygenases and no effect of NO or COX inhibition on flow-induced TRPV4 signaling in mouse carotid arteries (114), suggesting a prominent role of EDH in the TRPV4 response to shear stress. Consistent with this, Saliez et al. (160) showed that Ca²⁺ entry via TRPV4 initiated the EDH response, which required colocalization of TRPV4 and caveolin-1 in ECs. The importance of an EDH component in flow-induced dilation has been previously demonstrated by others (77, 193). A starkly different result was obtained by Kohler et al. (94), who reported that TRPV4-dependent vasodilation induced by shear stress acts strictly through NO generation in rat gracilis muscle arteries without any apparent EDH component. Similar results were obtained in carotid arteries. Studies on the role of the glycocalyx in flow-mediated dilation have also emphasized the role of NO as a signal mediator to the virtual exclusion of all other mechanisms. In fact, in an early study on shear-stress-induced dilation in small (~200 μm) rabbit mesenteric arteries, Bassenge and colleagues (154) reported that inhibition of NO or disruption of the glycocalyx by treatment with neuraminidase abolished flow-induced dilation, suggesting that flow acts exclusively through a glycocalyx-dependent, NO-mediated pathway in these vessels. Under pathological conditions (coronary arteries from patients with coronary artery disease), TRPV4 may induce vasodilation in response to flow through a novel mechanism involving mitochondrial release of hydrogen peroxide (22).

**FIGURE 3. Possible mechanisms of flow-induced TRPV4 activation**

Blood flow through the vessel lumen exerts frictional forces on to the endothelial membrane that could activate TRPV4 directly via membrane deformation, indirectly through a biochemical conversion mechanism involving upstream signaling elements (e.g., GPCRs, PLA₂), or through a lever-like action involving cytoskeletal linkages to molecules embedded in the glycocalyx. In addition to acting on TRPV4 channels on the apical surface, this mechanism could indirectly engage TRPV4 channels within MEP microdomains (inset) on the basal surface to induce endothelial-dependent vasodilation through an SK/IK channel-dependent EDH mechanism or do so indirectly through a Ca²⁺-induced Ca²⁺-release mechanism involving an internal IP₃R-mediated signaling cascade. Alternatively, the TRPV4-mediated rise in intracellular Ca²⁺ might act via eNOS-mediated production of NO or elaboration of PGI₂ through COX action on PLA₂-derived AA. Although pictured as a single channel, TRPV4 channels most likely function in the cells as a four-channel metastructure.
NO-mediated vasodilation. In ECs, NO, originally referred to as endothelium-derived relaxing factor (EDRF), is generated as a by-product of the eNOS-mediated reaction that converts L-arginine to L-citrulline. eNOS is a complex, constitutively expressed enzyme that requires a number of cofactors but is activated by the Ca\(^{2+}\)-dependent binding of calmodulin (45). Once released from the endothelium, NO stimulates soluble guanylate cyclase in VSMCs, resulting in a rise in 3’,5’ cyclic-guanosine monophosphate (cGMP), activation of cGMP-dependent kinase (PKG) and SM relaxation through multiple mechanisms (reviewed in Ref. 52). Because it is a lipophilic gas, NO is uniquely capable of diffusing readily across multiple cellular membranes to cause vasodilation. It also has a short half-life (\(<6\) s), providing the potential for fine temporal vasodilatory control.

Cyclooxygenase-dependent vasodilation. The second classic EC-derived mediator of vasorelaxation, prostacyclin (PGI\(_2\)), is a product of the COX pathway. This pathway is initiated by activation of the Ca\(^{2+}\)-dependent enzyme phospholipase A2 (PLA\(_2\)), which hydrolyzes cellular membrane lipids to produce AA. In addition to serving as a substrate for lipoxygenases and cytochrome P450 (CYP) monooxygenases, AA is acted on by COX, which generates a number of vasoactive prostanoids. Some of these products are vasoconstrictive, but a prominent and potent vasodilatory product of COX activity is PGI\(_2\).

EDH-mediated vasodilation. A third process for endothelium-mediated control of vascular tone distinct from endothelium-derived NO or COX metabolites is EDH, which promotes vasodilation by hyperpolarizing ECs and VSMCs (50). EDH is essentially a catch-all term that refers to an EC-dependent hyperpolarizing influence whose nature is defined by virtue of what it is not, i.e., the component of EC-dependent vasorelaxation that remains after inhibiting NOS and COX (14, 15, 21, 37, 50, 111). A defining feature of the EDH mechanism is its dependence on activation of endothelial IK and SK channels (3, 62, 63). Although a number of soluble factors have been suggested to contribute to EDH activity, including K\(^+\) ions (9, 73, 88), cytochrome P450 metabolites [e.g., EETs (61, 75)], lipoxygenase products (15, 77), hydrogen peroxide (93), and C-type natriuretic peptide (18), the current view increasingly favors a predominant role for direct electrical coupling via gap junctions (13, 14, 29, 41, 161, 163, 233).

Studies investigating TRPV4-dependent pathways engaged following direct activation of TRPV4 may shed additional light on signaling pathways downstream of TRPV4. We have found that, in small resistance-sized mesenteric arteries, direct activation of endothelial TRPV4 channels promotes vasodilation strictly through an EDH mechanism. In these arteries, dilation induced by the TRPV4 agonist GSK1016790A was largely inhibited by charybdotoxin (IK inhibitor) alone and was completely eliminated by combined treatment with charybdotoxin and apamin (SK inhibitor); inhibitors of NOS (l-NNA) and COX (indomethacin) had no effect (177). Similar results, also in mesenteric arteries, have been reported for activation of TRPV4 channels with the newly isolated, plant-derived flavone apigenin, which induced a dilatory response that was only slightly diminished by inhibition of NOS and COX, but was dramatically reduced by inhibition of IK/SK channels with charybdotoxin/apamin (117). Consistent with this, systemic activation of TRPV4 with intravenously administered 4a-PDD has been shown to induce profound hypotension, an action that is markedly inhibited by combined treatment with charybdotoxin/apamin but is unaffected by inhibition of NOS and COX with l-NNA/indomethacin (56). Similarly, hypotension induced by the TRPV4 agonist GSK1016790A is unaffected by loss of eNOS (226).

Interestingly, in the studies of Kohler et al. described above (94), direct activation of TRPV4 channels with 4a-PDD caused dilation through both EDH and NO mechanisms in gracilis muscle arteries, whereas flow-induced dilation acted exclusively through NO. The implication of these results is that activation of TRPV4 by flow in these arterial beds somehow bypasses the EDH machinery in favor of the NO machinery. One possible explanation for these observations is that shear stress may only activate a subset of TRPV4 channels, for example those positioned near eNOS-containing caveolae (131). In support of selective activation of TRPV4 channels, we have recently discovered that muscarinic agonists activate TRPV4 channels only at MEPs to engage the EDH pathway, but not the eNOS pathway (176). In any case, these results serve as a caveat against generalizing about signaling pathways downstream of flow based on the results of pharmacological activation of the (presumed) molecular target of flow.

Suggested TRPV4-dependent vasodilatory pathways are summarized schematically in FIGURE 3.

Mechanosensing in Smooth Muscle Cells: Vasoconstriction

It has long been known that an elevation of intracellular pressure leads to membrane potential depolarization, activation of VDCCs, and influx of extracellular Ca\(^{2+}\), a cascade that ultimately engages the Ca\(^{2+}\)-dependent molecular machinery responsible for actin-myosin cross-bridge cycling and SMC contraction. However, pressure-induced depolarization is not affected by inhibition of L-type VDCCs (93), implying that another ion channel is responsible for initiating SMC depolarization in response to pressure. Despite the centrality of this mechanism in the regulation of vascular tone, the identity of the molecular entity that responds to pressure to produce the initiating depolarization has remained elusive.
Mechanosensitivity of TRPC6 and TRPM4 Channels

In recent years, the search for a sensor-cum-depolo-
zarizing entity has increased significantly turned to the
large and mechanistically diverse TRP family of non-
selective cation channels, in part because several
TRP family members are known to be activated by
mechanical stimuli. A number of studies have fo-
cused on TRPC channels, which are widely
expressed in SMCs, as possible mediators of the
myogenic response. Early studies by Brayden and
colleagues (223, 224) demonstrated the presence of
an osmotically sensitive cation current with bio-
physical and pharmacological properties consist-
ent with a channel of the TRPC family in isolated
SMCs from resistance (cerebellar and cerebral) ar-
teries. These authors further showed that acute
knockdown of TRPC6 using an antisense strategy
substantially reduced this current and attenuated
pressure (40–100 mmHg)-induced depolarization and
constriction in intact cannulated arteries, sug-
gest ing a role for TRPC6 in translating pressure
into a myogenic response. Similar results were ob-
tained by Park et al. (152), who demonstrated that
negative pressure (−7.5, −15, and −45 mmHg)
induced the opening of single cation channels in
cell-attached patches from freshly isolated pulmo-

nary artery SMCs. Notably, this increase was im-
mediate and reversible, kinetics that suggest that,
under these conditions, the channel acts directly as
a mechanosensor without any intervening up-
stream elements. However, this interpretation is
undercut by the authors’ demonstration that
stretch-induced activation was eliminated by acute
exposure to the phospholipase C inhibitor U73122,
an effect that argues instead for a mechano-bio-
chemical conversion mechanism in which DAG
derived from PLC-mediated hydrolysis of PIP2 is
responsible for activating the channel. This inter-
pretation is supported by the demonstration that
the current was induced by direct application of
the DAG analog DOG, and stretch-induced in-
creases in the tension of isolated basilar arteries
was significantly inhibited by pretreatment with
U73122. Collectively, these studies indicate that
native pulmonary SMCs express a nonselective cat-
ion channel with TRPC-like properties that can
activate immediately upon stimulation (notwith-
standing its suggested PLC dependence), and
siRNA-mediated knockdown of TRPC6 or inhibi-
tion of PLC in resistance arteries attenuates pres-
sure-induced constriction.

A subsequent study by Spassova et al. (179) and
very recent work by Earley and colleagues (66a)
have provided additional evidence for direct acti-
vation of TRPC6 by stretch, induced by either neg-
ative pressure or hypoosmotic challenge (240
mosM), in HEK cells exogenously expressing
TRPC6. Spassova and coworkers further showed
that negative pressure applied to isolated mem-
brane patches from CHO cells expressing TRPC6
activated a current with properties similar to those
previously reported for this channel. The pressure
threshold for this effect was 84 ± 15 mmHg, al-
though, curiously, they used a 5-s pulse of ~75
mmHg to activate the current in isolated patches.
However, in contrast to the findings of Park et al.
(152), they found that stretch-induced activation of
TRPC6 was unaffected by inhibition of PLC.

Gudermann and colleagues (127) reached an en-
tirely different conclusion, arguing that TRPC6 is
not stretch sensitive per se under physiological
conditions. Using TRPC6-expressing HEK cells,
they showed that negative pressures of 10 or 70
mmHg [i.e., below the threshold reported by
Spassova et al. (179)] did not evoke a current, nor
did hypo-osmotic challenge with 250 mosM (i.e.,
10 mosM less hypo-osmotic than conditions used
by Spassova et al.). The authors of this latter study
note that the pressure threshold determined by
Spassova et al. is comparable to that of a large
conductance channel in E. coli that acts as a “last-
ditch” safety valve and argue that “mechanosensi-
tive channels in mammalian cells should be tuned
to lower-pressure set points to be able to partici-
pate in the dynamic regulation of physiological
processes” (127). Gottlieb et al. (67) similarly con-
cluded that TRPC6 expressed in COS or CHO cells
lacked mechanosensitivity. In a similar vein, Inoue
et al. found that a mechanical stimulus alone did
not activate TRPC6; however, they reported that it
did substantially potentiate TRPC6 activation by
muscarinic receptor stimulation (81). On the basis
of their findings, they proposed the operation of
a mechanism in which mechanical stimuli syn-
ergize with GqPCR activation to activate TRPC6
channels via a pathway involving PLC, DAG, CYP
omega-hydroxylase, and 20-hydroxyeicosatetra-
enic acid (20-HETE).

Compelling evidence also links the TRPM4 chan-
pel to the myogenic response (45, 46, 66). In the
first of these studies (46), Earley et al. showed that
mRNA for TRPM4, but not TRPM5, is expressed in
rat cerebral arteries. They also identified a current
in isolated arterial myocytes that exhibited a
current-voltage relationship characteristic of a Na+
current and showed dose-dependent activation by
intracellular Ca2+. Working with intact pressurized
cerebellar and cerebral arteries, these authors
demonstrated that antisense-mediated TRPM4
knockdown substantially reduced pressure-in-
duced depolarization and myogenic tone. This
study also reproduced the intriguing finding, re-
ported previously (69), that treatment with PKC-
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Sidebar 4. GPCR conformation and pressure sensitivity

GPCRs are seven-transmembrane spanning receptors that link ligand binding to signal transduction through heterotrimeric G-proteins comprising an α-subunit and tightly associated β- and γ-subunits that effectively form a single βγ-subunit. The α-subunit is a GDP-/GTP-binding protein that possesses intrinsic GTPase activity. In its inactive GDP-bound state, the α-subunit remains tightly associated with the βγ-subunit. Upon ligand binding, the GPCR undergoes a conformational change that is translated to the associated heterotrimeric G-protein, inducing exchange of α-subunit-bound GDP for GTP, which reduces the affinity of the α-subunit for the βγ-subunit. The GTP-bound α-subunit and βγ-subunit dissociate from one another (and the GPCR), and both subunits independently transduce intracellular signals by interacting with cellular effectors (e.g., enzymes, channels).

Importantly, pharmacological studies employing a class of antagonists known as inverse agonists have demonstrated that GPCRs are typically not functionally silent in the absence of stimulation; instead, they mediate a basal level of ligand-independent signal transduction. This constitutive activity reflects the stochastic adoption of an active conformation. Inverse agonists, which eliminate constitutive GPCR activity, work by raising the energy barrier to interconversion between inactive and active conformations, effectively “locking” the receptor in its inactive form. The ligand-independent, GPCR-mediated pressure sensor mechanism postulated by Mederos y Schnitzler et al. (127) likely reflects a shift in the equilibrium between inactive and active conformations in response to pressure that favors adoption of the active form. The single-channel conductance of TRPV4 and TRPC6 channels at physiological membrane potentials with Na⁺ and Ca²⁺ as charge carriers (with internal cesium) are ~60 and 35 pS, respectively, or ~3–6 pA at −40 mV (76). By comparison, the L-type VDCC has a single-channel conductance of 3.5 pS (with physiological Ca²⁺ as the charge carrier), corresponding to a unitary current of 0.18 pA at −40 mV (159). The upshot of these properties is that the Ca²⁺ influx rate through a single TRPV4 channel is substantially greater than that through a single TRPC6 channel and equivalent to that through ~10 VDCCs (129).

Activating phorbol esters induced a substantial increase in the frequency of cells expressing TRPM4 currents (45, 58). In a subsequent study, Earley and colleagues provide a mechanistic explanation for this phenomenon, demonstrating that PKC activity dynamically regulates surface expression of the TRPM4 channel, promoting its rapid insertion into the sarcolemmal membrane and substantially increasing TRPM4-mediated depolarization and vasoconstriction. Despite one report that TRPM4 is activated by membrane stretch (139), our recent work suggests that TRPM4 does not respond directly to mechanical stimuli in exogenous expression systems (66a), suggesting TRPM4 is not mechanosensitive per se. TRPM4 activation likely is a secondary event mediated by elevations in intracellular Ca²⁺.

An important property of the TRPM4 channel that distinguishes it from most other TRP family members is its activation by intracellular Ca²⁺. Because antisense-mediated knockdown experiments indicate a contribution of both TRPC6 and TRPM4 to the myogenic response (46, 223), it had been suggested that TRPC6 and TRPM4 might act together as a functional unit in which TRPM4 amplifies the effects of TRPC6 (12, 18). According to this conceptually attractive model, activation of TRPC6 in response to mechanical stress mediates an influx of Ca²⁺ that activates nearby TRPM4 channels, resulting in Na⁺ entry, depolarization of the smooth muscle membrane, and activation of VDCCs. However, in subsequent studies, Earley and colleagues showed that TRPM4 is not activated by extracellular Ca²⁺ influx. Instead, transient inward TRPM4 currents are sensitive to inhibitors of sarcoplasmic reticulum (SR)/ER Ca²⁺-ATPase (SERCA) or IP₃R activity, implicating Ca²⁺ released from SR stores in TRPM4 activation (64, 65).

TRPC6 and TRPM4 in the Mechanosensing Circuitry of Smooth Muscle Cells

An implicit assumption motivating much of the research on the role of TRP channels in the myogenic response has been that the pressure sensor and depolarizing component are one and the same entity. Interestingly, although a number of TRP channels are mechanosensitive and thus might logically be considered candidate mediators of both pressure-sensor and depolarizing functions, evidence for the intrinsic mechanosensitivity of TRPC6 and TRPM4, the two TRP channels most frequently cited for their potential role in the myogenic response, is mixed at best.

A recent study by Mederos y Schnitzler et al. (127) offers a paradigm with the potential to resolve this seeming paradox and crystallize a number of separate, free-floating observations into a unified view that places TRPC channels, specifically TRPC6, downstream of a distinct pressure-sensor element. These authors identify GqPCRs as the vascular mechanosensor, showing that increased intravascular pressure induces an agonist-independent conformational change in Gq-coupled receptors that results in effective G-protein coupling (Sidebar 4). This, in turn, leads to stimulation of PLC, generation of IP₃ and DAG, and activation of DAG-sensitive TRPC6 channels. This work builds on a number of previous studies suggesting that GPCRs (or G-proteins) could be activated in response to membrane deformation (reviewed in Ref. 181, and formalizes concepts advanced by Inoue et al. (81). Consistent with this model, the myogenic response in cerebral arteries is inhibited by the angiotensin II receptor inverse agonist losartan, as well as by PLC inhibitors, as reported.
previously by Park et al. (152). Mechanosensitivity
is also imparted to HEK293 cells by co-expression
of TRPC6 with G_{q/11} -coupled histamine H1, m5
muscarinic, endothelin A, or V_{1a} vasopressin re-
ceptors, suggesting that this mechanosensitivity is
a general property of G_{q/11} -type GPCRs. By
posing an upstream G_{q/11} -coupled receptor as
the smooth muscle mechanosensor, this model
reconciles the results of a number of studies that
have identified PLC-, DAG-, and/or PKC-depen-
dent features of the myogenic response (81, 82,
151, 199). Recent work by Brayden and colleagues
reinforces the generalizability of this model, show-
ing that the G_{q/11} -coupled purinergic receptors
P2Y4 and P2Y6 are critical for the myogenic re-
sponse in cerebral parenchymal arterioles (19).

Importantly, this mechanism is compatible with
previous studies that have implicated both TRPC6
and TRPM4 in the myogenic response. In the sim-
plest formulation of this TRPC6/TRPM4-depen-
dent pathway, pressure signals sensed by Gq-
coupled receptors are transduced in parallel to
TRPC6 and TRPM4 channels through PLC-derived
DAG and IP_{3}, respectively. In this extension of the
Mederos y Schnitzler model, the combined effects
of TRPC6-mediated Ca^{2+} influx and TRPM4-medi-
ated Na^{+} influx are responsible for the membrane
potential depolarization that activates VDCCs and
leads to initiation of the classic myogenic response.

This otherwise appealing formulation does not
fully accommodate all published reports on the
subject. One important “outlier” observation is
that the myogenic response persists in mice lack-
ing either TRPM4 (122) or TRPC6 channels (40).
One explanation for these apparent contradictory
findings lies in the proposed parallel mechanism:
in the absence of one branch, the flux through the
other branch is sufficient to maintain a normal
myogenic response. However, in the studies of
Brayden and colleagues (18, 19), knockdown of
either TRPC6 or TRPM4 eliminated the vast major-
ity of the myogenic response, suggesting that these
channels act in series rather than in parallel. The
recent work of Gonzales et al. confirms his latter
view, defining a serial mechanism linking TRPC6
and TRPM4 activation and shedding new light on
the upstream signaling pathways initiated by pres-
sure (66a). According to this refined model,
TRPC6-mediated Ca^{2+} influx sensitizes IP_{3}R_{S} to IP_{3}
liberated from PIP_{2} by the action of PLC, with the
resulting release of Ca^{2+} by IP_{3}R_{S} activating
TRPM4 channels.

An important upstream element in this Ca^{2+} -
induced Ca^{2+} -release mechanism is the gamma
isoform of PLC, which is specifically activated by
the tyrosine kinase Src, a target of the G_{q}-coupled
angiotensin II receptor (AT_{1}R). Although this
mechanism assumes that TRPC6 is activated di-
rectly by pressure, and thus acts in parallel with
pressure-induced activation of the AT_{1}R-Src-PLCy
pathway, it is also compatible with an indirect
TRPC6 activation mechanism (FIGURE 4). Since
these latter observations tend to rule out parallel
pathways as an explanation for the persistence of
the myogenic response in TRPM4- or TRPC6-KO
mice, the answer might lie instead with the classic
problem of compensatory changes that occur dur-
ing development in germline knockouts, a problem
that is especially common where the target is a
member of an extended family with functionally
overlapping members, such as the TRP family. In-
terestingly, in the case of TRPC6-KO mice, the
myogenic response is actually augmented. Consis-
tent with a potential compensatory mechanism,
TRPC3 and TRPC7 channels (DAG-sensitive rela-
tives of TRPC6) are upregulated in TRPC6-KO mice
and may functionally replace TRPC6 in the myo-
genic response. However, this is not the end of the
story. The myogenic response (in rat cerebral ar-
teries) is reported to be unaffected by acute knock-
down of TRPC3 channels (158), indicating that
TRPC3 does not likely contribute to the myogenic
response under physiological conditions and cast-
ing at least some doubt on its ability to compensate
for the loss of TRPC6. This observation is also per-
plexing in light of the proposed role of TRPC3 in
mediating UTP-induced depolarization (158):
given its responsiveness to G_{q}-coupled receptor
agonists, TRPC3 would have been predicted to fit
comfortably within the confines of the mecha-no-
sensor model proposed by Mederos y Schnitzler et
al. (127). Clearly, additional research and more
precise tools, notably conditional knockout mice,
will be required to fully reconcile these and other
incongruities in the literature and create a refined
model of the perennially elusive myogenic re-
sponse mechanism.

**Balls in the Air**

Activation of Ca^{2+} signaling pathways in endothe-
lial and SMCs tends to have opposite effects on
vascular tone. The opposite polarity of vascular
responses to activation of endothelial TRPV4 chan-
nels and SMC TRPC6/TRPM4 channels reflects this
fundamental dynamic. In each case, endothelial
and smooth muscle TRP channels are well inte-
grated into mechanosensing circuitry, mediating
responses to flow and pressure, respectively. Iron-
ically, however, given the oft-touted mechanore-
sponsiveness of TRP family members, including
those considered here, the available evidence falls
short of unambiguously establishing these chan-
nels as bone-fide, proximate mechanosensors.
Conflicting reports in the literature relating to the
role of indirect biochemical-conversion pathways
upstream of TRP channels tend to further cloud the issue.

In the case of flow-induced dilation, a linkage between cytoskeletal elements and TRPV4 channels is almost certainly involved in mediating mechanotransduction, whereas mechanisms that rely on direct sensing of shear pressure at the surface of the EC membrane are made unlikely by the presence of the glycocalyx boundary layer. Interesting in this latter context, selective degradation of the sialic acid component of the glycocalyx with neuraminidase eliminates shear stress-induced NO production without affecting PGI2 production (72). This observation forces the important conclusion that efforts to identify the shear stress sensor may be misguided: rather than a single mechanotransduction mechanism, multiple mechanisms, and multiple shear-stress sensors, likely exist. Consistent with this idea, there is evidence for the involvement of ion channels apart from TRPV4 in mediating flow-induced dilation. In addition to channels mentioned above (Mechanosensor Candidates), ligand-gated cation channels of the P2X purinergic receptor family (232) have been implicated in this function. Provocative data also link members of the polycystin family of TRP channels, specifically TRPP1 and TRPP2, to NO-dependent, endothelial cell-mediated dilation in response to flow (1, 142). Moreover, other members of the TRP family expressed in the vasculature, including TRPV2 (140) and TRPA1 (34, 103), have been reported to possess mechanosensitive properties and thus could potentially contribute to flow-induced dilation. In addition, a role for endothelial GqPCRs as proximate sensors of shear stress, analogous to that of smooth muscle GqPCRs in the myogenic circuitry, has not been addressed.

The situation is somewhat clearer with respect to the pressure-induced myogenic response, where a strong case can be made for GqPCRs as direct mechanosensors. However, here too questions remain about downstream linkages to TRPM4 and TRPC6 and potential parallel pressure-sensitive pathways (e.g., TRPC6). TRPC6 may not be alone in this latter context. Recent studies have also suggested the involvement of TRP channels of the polycystin family (TRPP1 and TRPP2) (141, 169) and TMEM16A chloride channels (23a) in pressure sensing and myogenic tone. Notably, the Mederos y Schnitzler model implicitly assumes that GqPCRs signal via PLCB isoforms, the typical targets of Gα11 proteins, but this has not been directly tested. This question is particularly relevant given the ability of GPCRs to transactivate receptor and nonreceptor tyrosine kinases (70, 84), which couple predominantly to PLCγ isoforms and have been implicated in transducing pressure signals. In addition to stimulating the canonical PLCB-DAG/IP3 pathway and the tyrosine kinase-PLCγ pathway, activation of GqPCRs depletes membranePIP2 (130), which has been reported to modulate the activity of both TRPM4 (147, 240) and TRPC6 (2, 107) channels. A possible contribution of PIP2 depletion to the GqPCR-TRPC6/TRPM4 signaling pathway, described above, has not been studied.

Implicit in this review is the concept that local Ca2+ signaling matters. The Ca2+-permeant ion channels within the molecular circuits discussed here (TRPV4, TRPC6, IP3Rs, RyRs, VDCCs) deliver high local Ca2+ to affect nearby targets. Local Ca2+
signals can oppose global changes, as is the case with ryanodine receptor-mediated Ca\textsuperscript{2+} sparks in smooth muscle (144), or enhance transcellular communication, as we have shown for TRPV4 sparklets in the endothelium (177). Local Ca\textsuperscript{2+} signals, such as those through VDCCs, have been shown to engage Ca\textsuperscript{2+}-dependent transcription factors (146, 164). Therefore, the flexibility and versatility of mechanosensing molecular circuits can be leveraged and enhanced by proximity relationships to Ca\textsuperscript{2+}-sensitive targets, an area ripe for investigation.

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