A New Trick for an Old Dogma: ENaC Proteins as Mechanotransducers in Vascular Smooth Muscle

Myogenic constriction is a vasoconstriction of blood vessels to increases in perfusion pressure. In renal preglomerular vasculature, it is an established mechanism of renal blood flow autoregulation. Recently, myogenic constriction has been identified as an important protective mechanism, preventing the transmission of systemic pressure to the fragile glomerular vasculature. Although the signal transduction pathways mediating vasoconstriction are well known, the identity of the sensor detecting VSMC stretch is unknown. Previous studies have considered the role of extracellular matrix-integrin interactions, ion conduction units (channels and/or transporters), and the cytoskeleton as pressure detectors. Whether, and how, these structures fit together in VSMCs is poorly understood. However, a model of mechanotransduction in the nematode Caenorhabditis elegans (C. elegans) has been established that ties together extracellular matrix, ion channels, and cytoskeletal proteins into a large mechanosensing complex. In the C. elegans mechanotransducer model, a family of evolutionarily conserved proteins, referred to as the DEG/ENaC/ASIC family, form the ion-conducting pore of the mechanotransducer. Members of this protein family are expressed in VSMC where they may participate in pressure detection. This review will address how the C. elegans mechanotransducer model can be used to model pressure detection in mammalian VSMCs and provide a new perspective to pressure detection in VSMCs.
features of the mechanosensory model include a membrane ion channel pore that is anchored intracellularity and extracellularly by linker proteins to the cytoskeleton and extracellular matrix. In the nematode, the ion channel pore is formed by members of a conserved family of proteins (Degenerins) that are also expressed in mammals. Extracellular force or tension is transduced through the linker proteins to modulate channel activity, which leads to activation of other signaling events. Previous studies on mechanisms of myogenic constriction have demonstrated the importance of these components (matrix proteins, ion channels, and cytoskeleton); however, this general mechanosensory model has not been applied to mechanotransduction in VSMCs. In the current review, we will briefly discuss 1) previously identified mechanisms of mechanosensing in pressure-induced constrictor responses, 2) evidence of mammalian degenerin protein involvement, and 3) how these structures may interact to form a mechanotransducer in VSMCs.

**Cellular Structures Required in Mechanosensing Pressure-Induced Stretch in Vessels**

Numerous systems and structural elements have been proposed to participate in the transduction of VSMC stretch into a cellular signaling event. These include 1) extracellular matrix-integrin interactions, 2) cytoskeletal proteins, and 3) membrane-bound enzyme and second messenger systems, ion transporters and exchangers, and direct activation of mechanosensitive ion channels on the smooth muscle cell membrane (20, 21, 47).

**Extracellular matrix/integrins**

Integrins have long been associated with mechanotransduction and thus have been identified as potential transducers. Integrins are transmembrane proteins, formed as \( \alpha \beta_1 \) and \( \alpha \beta_2 \) heterodimers. At least 11 integrin dimers are expressed in vascular tissue, and their roles in migration and growth have been well studied. Our understanding of the role of integrins in myogenic constriction is limited; however, it does suggest a critical role. The importance of \( \alpha \beta_1 \) and \( \alpha \beta_3 \) integrin dimers in pressure-induced constriction has been addressed in a recent investigation where integrin antibodies and integrin recognition peptides (RGD peptides) were shown to block pressure-induced tone in skeletal muscle arterioles (22, 67, 68). Because integrin antibodies and binding peptides also disrupt integrin binding with extracellular matrix proteins, these findings also support a role for the extracellular matrix. Additionally, anecdotal evidence that excessive removal of the adventitia and surrounding tissue from isolated vessels attenuates myogenic constrictor response is consistent with a role for the extracellular matrix in gating the response. However, there is no direct evidence demonstrating the importance of a specific extracellular matrix protein in pressure-induced constrictor responses.

**Cytoskeleton**

The cytoskeleton is another important structure in force transduction in vascular tissues. Disruption of the actin cytoskeleton inhibits pressure-induced constriction in cerebral arteries and tail arterioles (17, 32, 40). However, it does not block pressure-induced depolarization, suggesting the cytoskeleton may not be involved in the initial stretch response but is required for downstream signaling (40). The involvement of intermediate filaments and microtubules is not clear, since vimentin knockout mice do not have altered myogenic constriction in mesenteric vessels, and microtubule depolymerization does not inhibit myogenic constriction in cremaster arterioles (46, 61, 84). Because integrins and their associated extracellular matrix and cytoskeletal proteins are required for other adhesion-mediated mechanosensitive processes, they remain potential transducers of pressure-induced vessel stretch (30, 36, 51).

**Mechanosensitive enzyme systems, transporters, and channels**

Activation of a variety of ion channels, transporters, and membrane-bound enzyme systems have been
proposed to account for VSMC depolarization. Inhibition of K⁺ channels, or activation of Cl⁻ channels and voltage gated Ca²⁺ channels have been considered as mechanisms involved in the initial depolarization response. Modulation of transport pumps such as Ca²⁺ ATPase, Na⁺/K⁺-ATPase, and the Na⁺/Ca²⁺ exchanger have been considered because they can regulate membrane depolarization or ion gradients. A detailed review of the potential role of these channels, transporters, and enzyme systems can be found elsewhere (21). Many investigators favor the involvement of mechanosensitive cation channels; however, identification of molecular components of mechanosensitive ion channels in vascular smooth muscle has been difficult since there have been few potential candidates for the mechanosensor.

In recent years, at least two families of evolutionarily conserved, putative mechanosensitive proteins have been identified in mammals. These include members of the transient receptor potential (TRP) and eegenerin/epithelial sodium channel/acid-sensing ion channel (DEG/ENaC/ASIC) proteins. Members of both families are widely distributed (expressed in neuronal, muscle, and epithelial tissue) and participate in diverse functions (18, 56). The DEG/ENaC/ASIC family has strong evolutionary ties to mechanotransduction in neuronal and muscle tissues (8, 66, 90). In the nematode, members of this family form the ion-conducting pore of a mechanosensor, which is thought to be a large multi-protein complex that includes extracellular proteins, ion channels, and cytoskeletal proteins. Previous investigations into the mechanisms of myogenic constriction have considered each of these structures in isolation, and little consideration has been given to the concept that the extracellular matrix, ion channels, and cytoskeleton interact to form a large hetero-multimeric mechanosensory complex. This review will address emerging evidence demonstrating an important role for ENaC/ASIC proteins as components of mechanotransducers in VSMCs in vessels.

DEG/ENaC/ASIC Proteins: A Diverse Protein Family with Links to Mechanosensation

DEG/ENaC/ASIC proteins are a large family of proteins expressed in a diverse range of species, including the nematode, Caenorhabditis elegans (C. elegans), Drosophila, and mammals, and cell types including epithelia, neurons, and muscle cells. Members of this family are involved in diverse functions including Na⁺ transport, proprioception, acid sensation, learning, memory, and mechanosensation. Members of the family share a common protein structure: intracellular NH₂ and COOH termini and a large extracellular domain separated by two membrane-spanning domains. Many of the DEG/ENaC/ASIC proteins form homo- and heteromultimeric, non-voltage-gated, Na⁺/cation channels (8, 56, 66, 90).

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C. elegans mechanotransducer model

A large body of evidence links C. elegans degenerins to mechanotransduction. An excellent comprehensive review of this topic can be found elsewhere (90). Numerous genetic studies have led to the development of an “all-purpose” model of mechanotransducers in C. elegans neuronal and muscle tissue. The model consists of five primary components: 1) extracellular matrix proteins, 2) extracellular linking proteins, 3) pore-forming channels, 4) intracellular linking proteins, and 5) cytoskeletal proteins. In this model, nematode members of the DEG/ENaC/ASIC protein family form the ion channel pore. The application of a mechanical force is transduced through the extracellular matrix to gate the channel. Thus the interaction between the pore-forming degenerin proteins and the extracellular matrix is considered critical to channel gating. The cytoskeleton may also participate in transduction of the applied force and, along with other extracellular proteins, may also stabilize the pore-forming proteins at the cell surface. Also included in the C. elegans model are enzymes thought to stabilize the channel or regulate activity. The reader probably recognizes the similarity to certain structural components required for pressure-induced constrictor responses in blood vessels, namely the cytoskeleton, extracellular matrix, and channel pore. We suspect it is unlikely that this is a coincidence. Rather, we suspect that the mechanotransducer in VSMCs is organizationally similar to the C. elegans mechanosensor, where mammalian degenerin proteins form the ion-conducting pore.

Mammalian degenerins

In mammals, two subfamilies of DEG/ENaC/ASIC proteins have been identified: the ENaC and ASIC proteins. The most well known are the ENaC proteins for their role in Na⁺ and water transport in the kidney, lung, and colon. In these tissues, α, β, and γENaC proteins form a non-voltage-gated, Na⁺-selective ion channel critical in Na⁺ and water transport. This channel will be referred to as the “classical” ENaC channel and is inhibited by low doses (submicromolar to low micromolar range) of the diuretic amiloride and its analog benzamil. ASIC proteins are predominantly expressed in neuronal tissue where they may form homo- and hetero-multimeric cation channels (56). A
feature of ASIC channels is their sensitivity to protons and thus may play a role in pH sensing (56). Although they are also amiloride blockable, they tend to require higher concentrations than ENaC. Certain ENaC and ASIC proteins have been found at several important sites of mechanotransduction, including light touch receptors in hairy skin (whisker and hair follicles), hairless skin (Pacinian Corpuscles, Meissner Corpuscles, Merkel Cells, free nerve endings), arterial baroreceptor neurons, osteoclasts, keratinocytes, and VSMCs (24, 26, 33, 54, 59, 71, 85, 86, 89). Recently, several groups have provided genetic evidence that certain ASIC proteins are required for normal mechanosensory responses in peripheral sensory neurons (23, 73, 85, 86). Because of their close evolutionary relationship to the C. elegans degenerins, localization in mechanosensitive tissues, ability to form channels, and requirement for normal mechanosensory responses, ENaC and ASIC proteins have been considered as components of mechanosensitive ion channel complexes in vertebrate tissue.

**Are ENaC Channels Mechanically Gated?**

Early investigations into the direct stretch sensitivity of ENaC in heterologous expression systems were equivocal (4, 5, 52, 55, 59). However, subsequent studies using endogenously expressing tissue are supportive of ENaC’s mechanosensitivity. Increasing membrane tension, by the application of negative pressure, to isolated channels in cortical collecting duct cells can gate native ENaC channels (64, 82). Recent studies suggest shear stress gates ENaC channels (1, 14, 15, 75, 88). In isolated rabbit cortical collecting ducts, Na⁺ reabsorption is directly dependent on tubular flow rate/shear stress. Furthermore, shear stress can activate αβγENaC expressed in oocytes, a finding that provides direct evidence of ENaC mechanosensitivity (1, 14). Taken together, the evidence suggests ENaC channels can be mechanically activated.

**ENaC Proteins and Pressure-Induced Constriction**

To consider ENaC proteins as mechanosensors mediating stretch-induced constriction in blood vessels, two fundamental criteria need to be met. First, ENaC proteins must be expressed in VSMCs and located at the site of mechanotransduction near the cell surface. Second, the response to pressure-induced activation, i.e., ion entry and constriction, should be inhibited by ENaC blockade or disruption. Several studies show certain ENaC proteins are expressed in VSMCs isolated from myogenically active vascular beds (FIGURE 2) (25, 53, 54). VSMCs enzymatically dissociated from cerebral and renal arterial segments express β and γENaC, but not α, at or near the cell surface.
The localization pattern is significant because the cell surface is the site where the mechanosensor might be predicted to be located. The lack of αENaC in VSMCs is an important finding. The lack of αENaC and loss of high constitutive activity that this subunit confers would be consistent with a mechanosensitive channel, which tends to be quiescent or active at very low levels in the absence of mechanical activation (56, 76). Although αENaC is required for conductance properties of the “classical” ENaC channel found in epithelial tissue, β and γ can form an amiloride-sensitive, Na+-conducting channel in the absence of αENaC (13). The possibility of another subunit, such as an unidentified ENaC or an ASIC protein, interacting with β and γENaC to form a channel has not been ruled out. The latter possibility remains a very attractive hypothesis since ASIC and ENaC proteins have been identified in similar cell types (such as neurons, glia, and smooth muscle) and are capable of interacting to form functional channels (9, 42, 43, 56, 72).

The second essential criterion to consider ENaC proteins as components of a mechanosensor in VSMCs is that physiological responses to mechanical activation can be inhibited following ENaC blockade. Indeed, pressure-gated Na+ and Ca2+ fluxes and vasoconstriction in isolated mouse renal interlobar arteries are inhibited following ENaC inhibition with amiloride (5 μM) and benzamil (1 μM) (54). Recent studies by Guan et al. suggest myogenic constriction in rat afferent arterioles is also sensitive to ENaC inhibition (44). Thus the importance of ENaC in the myogenic constrictor response in renal vessels may not be limited to the renal interlobar artery.

An important factor in the interpretation of these experiments is the selectivity of the ENaC inhibitors. At submicromolar and low micromolar doses, amiloride and benzamil are very selective inhibitors of ENaC. Although amiloride can also inhibit other exchangers and nonspecifically block vasoconstriction, such as the Na+/H+ exchanger, Na+/Ca2+ exchanger, voltage-gated Na+ and Ca2+, TRPC6 (IC50 = 130 μM), and TRPA1 (IC50 = 500 μM), this occurs at concentrations 100- to 1,000-fold greater than used by Jernigan et al. and Drummond et al. (25, 54, 60). Thus it is likely that amiloride inhibition of myogenic constriction is due to ENaC inhibition rather than a nonspecific inhibition of other transporters or channels. Furthermore, following ENaC inhibition, vessels are still able to constrict to phenylephrine, an alpha-adrenergic agonist, suggesting ENaC inhibition specifically blocks pressure-induced constriction and not just the ability of the vessel to constrict (54).

Pharmacological inhibition is a great tool for screening for DEG/ENaC/ASIC involvement; however, specific subunit involvement cannot be determined. To do this, specific gene silencing approaches are required. A recent study used siRNA and dominant-negative approaches to silence β or γENaC expression in isolated mouse renal interlobar artery segments (53). With this approach, renal interlobar segments were isolated, transiently transfected overnight with siRNA and dominant-negative cDNAs, then assayed for myogenic and agonist-induced vascular reactivity. Both approaches led to a specific reduction in ENaC expression. The reduction in protein expression caused loss of pressure-induced constrictor responses without altering the ability of the vessel to constrict to phenylephrine, suggesting the loss of vasoconstriction specifically to pressure rather than a generalized loss in the ability of the vessel to constrict.

What about other DEG/ENaC family members?

ENaC proteins are not the only mammalian members of the degenerin family. ASIC proteins have also been identified as potential mechanosensors in neuronal tissue. Recently, they have been identified in VSMCs (42). ASIC1, 2, and 3 transcripts and proteins are expressed in cultured VSMCs where they participate in migration in response to wounding and chemo-attractants (42). Although these findings do not confirm a mechanosensory role for ASIC proteins in VSMCs, they do demonstrate that ASIC molecules are also expressed in vascular smooth muscle. A role for ASIC proteins in myogenic constriction is currently under investigation in our laboratory. At least one ASIC protein (ASIC2) appears to play a significant role; evaluation of myogenic constrictor responses in the ASIC2 knockout mouse model reveals an important role for ASIC2 protein in pressure-induced constriction (35).

Electrophysiology: the missing link?

Although expression and functional data indicate an important role for ENaC proteins in myogenic constriction, electrophysiological evidence of ENaC channels in VSMCs is lacking. Mechanosensitive ion channels (Na+, Ca2+, nonselective cation) have been identified in VSMCs from various tissues (21). To date, only one report of an ENaC-like current in a cultured VSMC line has been found in the literature (91). Interestingly, this channel was not blocked by amiloride but was sensitive to an amiloride analog phenamil, suggesting that the ENaC-like channel is not the “classical” epithelial ENaC channel. An obvious question is if ENaC proteins are expressed in VSMCs, why isn’t there electrophysiological evidence? There are at least two possibilities. First, it is likely that channels formed by ENaC proteins are electrically silent until gated by pressure-induced VSMC stretch. The presence of β and γ, without αENaC, suggests an isolated channel would likely be silent. Although electrically silent αβγENaC channels in tubules (1, 14, 15, 75, 88) can be gated by shear stress, it is unknown whether βγENaC channels can be gated by shear stress.
The second possibility is that the mechanosensory complex cannot be properly gated under conditions required for electrophysiological analysis. Electrophysiological analysis of VSMCs requires the cells to be free of the adventitial extracellular matrix. This process requires enzymatic digestion and dissociation of the extracellular matrix. If the extracellular matrix proteins are involved in force transduction and gating as the C. elegans mechanosensor model predicts, then the processes of preparing the cells for electrophysiological analysis may disable the gating mechanism. We speculate that both factors contribute to the lack of electrophysiological evidence of ENaC channels in VSMCs.

Are ENaC and ASIC Proteins Part of a Large Mechanosensory Complex in VSMCs?

We propose that the VSMC mechanotransducer resembles the C. elegans mechanotransducer model and that β and γ ENaC, and perhaps ASIC2, proteins form the pore of the mechanosensor (FIGURE 3). These pore-forming proteins are linked, or tethered, to the extracellular matrix and cytoskeleton, either directly or indirectly, via associated proteins. When mechanical forces are applied to the extracellular matrix, the force is transmitted to the pore, which modifies channel activity. Although the identity of the other proteins interacting with ENaC to form the mechanosensory complex in VSMCs has not been established, we suspect that many of these proteins are well known. Is it a coincidence that the C. elegans model incorporates many of the features and structures thought to be involved in vascular detection of intraluminal pressure, such as extracellular matrix, cytoskeleton, and ion channels? Or does it suggest that these structures interact with ENaC ASIC channels to form the large mechanosensory complex? We speculate VSMC ENaC proteins are part of a large, multicomponent protein complex similar to the established model of the mechanosensor in C. elegans (FIGURE 3).

Integrins have long been considered as mechanotransducers and regulate vascular tone and pressure-induced constriction. Although integrins are not part of the C. elegans model, they are expressed in touch neurons and muscle (37, 48). Along with other focal adhesion proteins, integrins have been postulated to participate in tethering mechanosensor complexes (29, 36). A direct role for integrins in mechanotransduction in C. elegans has been difficult to address because integrin mutations typically cause paralysis and/or lethality. But these findings provide a tenuous link between two mechanisms of mechanosensing, integrins and ENaC ASICs, and raise the possibility that integrins may be a component of the mechanosensory complex.

Trp’ed up?

As mentioned previously, degenerins are not the only protein family proposed to act as mechanosensors. Certain members of the transient receptor potential family (TRP) contribute to mechanosensory processes. TRP channels are comprised of six transmembrane-containing domains, intracellular NH2 and COOH termini, and very small extracellular domains. There are four subfamilies: TRPC, TRPV, TRPM, and the newly identified TRPA (18, 74). Certain members of the TRPV and TRPA subfamilies are thought to be involved in mechanosensing in C. elegans and mammalian neurons (19, 58, 77).

Two members of the TRP family, TRPC6 and TRPM4, have been considered mediators of pressure-induced depolarization in cerebral vessels. Suppression of TRP6 or TRPM4 expression in isolated middle cerebral arteries, using antisense oligonucleotides, abolishes pressure-induced depolarization and constriction (28, 93). Although evidence of their role in myogenic constriction is compelling, the manner in which they participate is not clear. The small extracellular domain of the TRP channels renders the possibility that forces can be transduced through the extracellular matrix less likely, since the small extracellular domains may leave little room for tethering of extracellular proteins directly gating the channel. It is interesting that separate investigations using similar preparations find inhibition of ENaC function or TRP channel function produces a near total loss of myogenic responsiveness. If ENaC and TRP channels function independently, why doesn’t the other channel compensate? One possibility is that TRP and ENaC channels are linked, directly or indirectly.
Clinical significance: is there a link between hypertension and vascular ENaC?

Although recent studies suggest 1) VSMC ENaC proteins mediate pressure-induced constriction and 2) pressure-induced constriction protects the delicate renal microvasculature from hypertension-related injury, the hypothesis that ENaC-mediated constriction protects the kidney has not been tested. Furthermore, there are no studies linking VSMC ENaC proteins and hypertension. It is unknown whether VSMC ENaC expression (protein expression, localization, or activity) is altered in genetic models of hypertension (spontaneously hypertensive, sabra, or Dahl salt-sensitive rat) or following the induction of chronic hypertension (angiotensin II, DOCA salt, Goldblatt). However, it is very likely that VSMC ENaC expression and/or function may be altered in hypertension for two reasons. First, many of the “usual suspects” implicated in hypertension (i.e., endothelin, aldosterone, angiotensin II, inflammatory cytokines, reactive oxygen species, and nitric oxide, dietary salt) regulate tubular ENaC and neuronal ASIC channel expression (6, 10, 16, 27, 34, 39, 49, 56, 65, 69, 70, 83, 87). Second, certain genetic models of hypertension have altered tubular ENaC expression (2, 3, 31, 57, 80). How these vasoactive factors or genetic differences regulate VSMC ENaC expression has yet to be addressed. Future studies are needed to determine the importance of VSMC ENaC in hypertension.

Summary

Recent evidence suggests pressure-induced constriction may protect the kidney from injury by preventing transmission of systemic pressure to the glomerular capillaries, a critical determinant in the progression of glomerulosclerosis in hypertension, diabetes, and end-stage renal disease (11, 12, 45, 62, 63, 81, 92). Thus understanding the mechanism(s) underlying the initiation of pressure-induced constriction is a critical step toward prevention of renal injury associated with cardiovascular disease. We propose a large mechanosensory complex in VSMCs, which includes extracellular matrix, cytoskeleton, ion channel proteins, and possibly integrins, senses pressure-induced vessel stretch. Members of the ENaC/ASIC protein family may form the ion channel pore of this mechanosensor. This large heteromeric protein complex transduces a pressure stimulus into a cellular event (i.e., depolarization). Future studies will need to identify 1) critical extracellular matrix, cytoskeletal, and linker proteins, and how they gate the channel, 2) regulation of mechanosensory complex components by hormonal, paracrine, and autocrine factors in cardiovascular disease, and 3) how dysfunction of mechanosensory complexes contributes to cardiovascular disease.

References

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