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COMMENTARY

Under pressure: Ano1 mediates pressure sensing in the lymphatic system

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Although identified only 11 years ago, the Ca^{2+}-activated Cl^- channel (CaCC), Ano1, is firmly established as an essential physiological protein. This channel is known by many names—TMEM16a, DOG1, ORAOV2, TAOS2—and mediates many more physiological processes. Several of these Ano1-regulated processes are seemingly disparate, ranging from mucosal secretion (Huang et al., 2012) to fertilization (Wozniak et al., 2018). However, the precise role of the channel is not necessarily as varied. Indeed, a recurring role for Ano1 in signaling the contraction of various types of smooth muscle is emerging. For example, Ano1 mediates parasympathetic-induced bronchi contraction in airway smooth muscle (Huang et al., 2012). In uterine smooth muscle, Ano1 promotes spontaneous and oxytocin-induced contractions (Bernstein et al., 2014). In some vascular smooth muscle, such as that surrounding the cerebral artery, Ano1 mediates stretch-activated constriction (Bulley et al., 2012). In this issue of the *Journal of General Physiology*, Zawieja et al. describe a new role for Ano1 as a mediator of pressure-sensitive contraction in lymphatic collecting vessels.

In the lymphatic system, contraction of the smooth muscle surrounding lymphatic vessels enables the return of fluid and macromolecules from the interstitial space to the blood (Jones and Min, 2011). These lymphatic vessels exhibit spontaneous contractions with a pressure-dependent frequency such that fluid build-up results in vessel contractions in healthy tissues. Dysfunction of these vessels causes lymphedemas, defined as the abnormal buildup of fluid in extremities (Jones and Min, 2011). Because there are no existing cures for lymphedemas, afflicted patients have to bear with this disorder for the rest of their lives. A better understanding of the molecular and ionic mechanisms underlying contraction of these lymphatic collecting vessels may pave the way for the development of pharmaceutical therapies to treat and reverse lymphatic disorders.

To explore whether Ano1 contributes to the regulation of lymphatic vessel contraction, Zawieja et al. (2019) first confirmed that this channel is expressed in inguinal-axillary lymphatic collecting vessels (IALVs) using RT-PCR, Western blots, and immunohistochemistry. They also confirmed that these cells lacked another candidate CaCC, TMEM16b (Ano2). CaCC currents were then recorded in these IALV smooth muscle cells using whole cell voltage clamp. Increased intracellular Ca^{2+} indeed increased the currents recorded in these cells, and anion replacement with either I^- or glutamate demonstrated that these currents were conducted by Cl^- channels. Moreover, replacing Cl^- with glutamate or I^- altered the reversal potential of this Ca^{2+} activated current, as predicted.

Unexpectedly, the Ano1 currents reported in this manuscript exhibited modest inward rectification. At subsaturating concentrations of intracellular Ca^{2+}, Ano1 currents are typically outwardly rectifying (Xiao et al., 2011). The Ca^{2+}-binding site for Ano1 is located within a membrane-embedded domain (Dang et al., 2017; Paulino et al., 2017) and thus within the membrane voltage field. Consequently, the apparent binding affinity of the channel for Ca^{2+} increases at depolarizing voltages, and this voltage-dependent change in Ca^{2+}-Ano1 binding has been hypothesized to mediate outward rectification (Peters et al., 2018). The inward rectification reported here could be the result of a distinct molecular mechanism that may reflect the recording conditions used for these experiments, or specific modifications or interacting proteins that are unique to IALV Ano1.

Following the demonstration that Ano1 channels are present and functional in IALV smooth muscle, Zawieja et al. (2019) investigated a possible role for Ano1-conducted currents in regulating the pace-making of these vessels, using diverse biophysical techniques including pressure myography, whole-cell recordings, and Ca^{2+} imaging. Using pharmacological and genetic depletion of Ano1 currents, the authors explored whether pressure increased IALV contraction frequency in the absence of Ano1 currents. Because Ano1 knockout mice are perinatal lethal (Rock et al., 2008), inducible or constitutive tissue-specific knockout approaches were required. Zawieja et al. (2019)

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impressively employed multiple tissue-specific Ano1 knockout mice to tackle these experiments. The CaCCs in lymphatic muscle cells were nearly abolished in one Cre-induced smooth muscle knockout. Moreover, pressure-induced changes in contraction frequency of IALVs were nearly abolished in Ano1-deficient vessels, regardless of the knockout strategy. No other differences were noted between vessels with or without Ano1, such as contraction amplitude or tone. Together, these data suggest that the inability of pressure to increase contraction frequency in IALVs in the absence of Ano1 currents was not due to a deficit in the molecular machinery of these smooth muscle cells. Rather, the effect was likely due to a difference in the ability of these cells to transform signals in tissue pressure to changes in contraction frequency.

In addition to transgenic approaches to deplete Ano1 currents, pharmacological inhibition was also employed. IALV contractions were measured under different pressures in the presence and absence of the high-affinity and reversible Ano1 inhibitor, benzbromarone. Whereas a modest change in pressure evoked a fourfold increase in the contraction frequency of untreated vessels, similar pressure changes evoked only modest changes in the contraction frequency of vessels treated with benzbromarone. Although benzbromarone has been proposed to act as a pore blocker of Ano1 (Huang et al., 2012), and consequently may not uniquely target the channel, it evoked similar changes to those seen in the transgenic animals. Therefore, this drug was likely reducing pressure-induced changes in IALV contraction by blocking Ano1 currents.

Whole-cell recordings made on dispersed IALV smooth muscle cells further substantiated a role for Ano1 in regulating contraction frequency. Between action potentials, the membrane potential of IALV smooth muscle cells is normally unstable, exhibiting a linear depolarization that steadily increases to the threshold voltage at which an action potential is initiated. Interfering with Ano1 currents by either genetic knockout of channel expression or acute benzbromarone application rendered smooth muscle cells that depolarized much more slowly between action potentials. This directly resulted in less frequent depolarizations, suggesting that activation of Ano1 shortens the time between action potentials and thereby increases contraction frequency. Additionally, action potentials recorded from cells lacking Ano1 currents had a markedly different shape; the membrane depolarized to a more positive potential and repolarized much more quickly in the Ano1 knockout cells. Presumably, these Ano1-free recordings reveal the contribution of L-type Ca^{2+} channels to spontaneous action potentials. From these data, we can predict that Ano1 currents not only contribute to the slow depolarizations between action potentials, but also to the slowly repolarizing plateau immediately following the action potential peak. Sharp electrode recordings made in the presence of benzbromarone were intermediate to those made from control and Ano1 knockout cells. These experiments used 5 µM benzbromarone, a concentration that had an intermediate effect on pressure-evoked changes in IALV contraction. Thus, the intermediate phenotype of these whole-cell recordings is likely because Ano1 currents had not been completely abolished in these experimental conditions.

In a final series of experiments, intracellular Ca^{2+} was imaged in control and Ano1 knockout smooth muscle cells using transgenic expression of the GCaMP6f reporter, a fluorescent Ca^{2+} indicator. Regular elevations of intracellular Ca^{2+} were documented in both wild-type and Ano1-deficient cells. These Ca^{2+} events were relative at frequencies similar to those of action potentials recorded by electrophysiology: more frequent for wild-type and less frequent for Ano1 knockout cells. Intriguingly, when plotting the change in GCaMP6f fluorescence versus time, the area under the curve was higher for Ano1-deficient cells relative to controls. These data reveal that the Ano1-deficient cells either have enhanced Ca^{2+} signaling or more Ca^{2+} ions are available to bind the GCaMP6f reporter.

Zawieja et al. (2019) convincingly document a critical role for Ano1-conducted current in the regulation of IALV pacemaking. This new understanding that Ano1 plays a critical role in the normal clearing of lymphatic vessels could pave the way for novel treatments for lymphedemas, which are currently incurable. Extensive structural and functional studies of Ano1 have revealed a wealth of information about how they are regulated. These multimodal channels are gated by changes in temperature (Cho et al., 2012), by direct interactions with the endoplasmic reticulum–located IP_3 receptor (Jin et al., 2013), as well as by changes in intracellular Ca^{2+}. Finally, a suite of Ano1-specific drugs are currently available that either potentiate or inhibit channel activity (Namkung et al., 2011). Novel lymphedema treatments could target Ano1 or pathways upstream of its activation.

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