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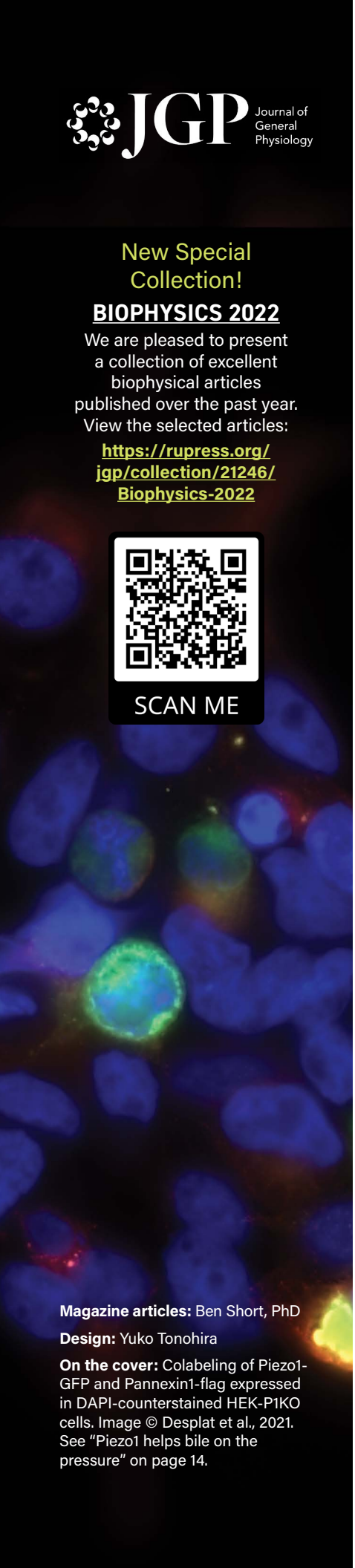

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On the cover: Colabeling of Piezo1-GFP and Pannexin1-flag expressed in DAPI-counterstained HEK-P1KO cells. Image © Desplat et al., 2021. See "Piezo1 helps bile on the pressure" on page 14.

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Single molecule imaging reveals a slice of life

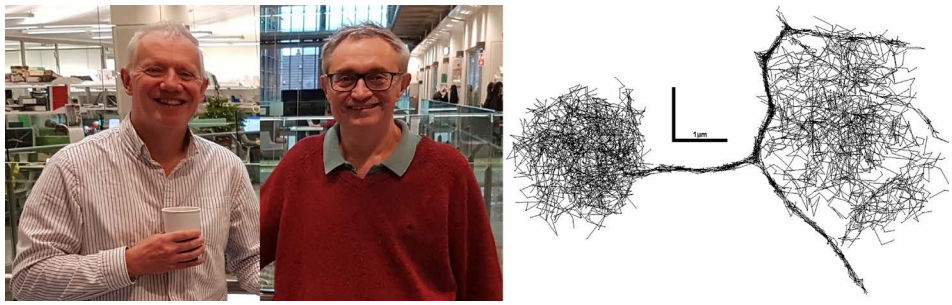
A method to trace the real-time movements of individual membrane proteins in live tissue slices

Directly observing the movements of single, fluorescently labeled molecules can provide crucial information about a molecule's interactions in living cells. Plasma membrane proteins, for example, may freely diffuse around the lipid bilayer, pausing only when they collide and interact with other proteins. These movements can be followed relatively easily in single-cell organisms or cultured mammalian cells but are much more challenging to observe in multicellular organisms, where cell–cell interactions can dramatically alter the properties of the plasma membrane. Mashanov et al. describe a new method to image and track individual plasma membrane proteins in living tissue slices (1).

Justin Molloy's group at The Francis Crick Institute in London are interested in how the M_2 muscarinic acetylcholine receptor regulates the heartbeat. This G protein–coupled receptor diffuses through the plasma membrane and, in response to acetylcholine, alters the resting potential of cardiomyocytes via a $G_{\beta\gamma}$-mediated interaction with inwardly rectifying potassium GIRK channels (2, 3).

"It's a diffusion-limited signaling cascade, so it's important to look at the movement of the molecules within the membrane," Molloy explains. "We've tracked the movements of single M_2 receptors in cultured cardiomyocytes, but we wanted to do it in tissues where the cells are in their native environment."

Molloy and colleagues, led by Gregory Mashanov, developed a technique to image single M_2 receptors in cardiac tissue slices (1). Freshly extracted mouse hearts are quickly placed in a custom-made, 3-D–printed cutting block, then sectioned by a multi-blade assembly into 1-mm-thick slices. These slices are treated with a fluorescently labeled ligand that tightly binds to M_2 receptors, before being transferred to coverslips for TIRF video microscopy.



Justin Molloy (left), Gregory Mashanov (right), and colleagues describe a method to image single plasma membrane proteins in live tissue slices. By tracking individual M_2 muscarinic acetylcholine receptors in cardiac tissue over time, the researchers can construct a super-resolution map of the tissue, encompassing both the round cardiomyocytes and the ultrathin nerve fibers that innervate them.

Mashanov immediately noticed that cardiomyocytes in living tissue are much more rounded than they are in cell culture. More remarkable still, however, were the differences Mashanov observed when he compared the movements of single M_2 receptors in cells and tissues. "The M_2 receptors move around the membrane around four times faster in tissue than they do in cultured cells," Mashanov says.

The reason for this increased mobility in tissues remains unclear, but Mashanov et al. saw a similarly rapid movement of M_2 receptors in zebrafish hearts, which the researchers were also able to dissect and prepare for TIRF microscopy with their new technique, even though these organs measure just ~0.5 mm in length.

In addition, the researchers discovered that they could use their single-molecule tracking data to create super-resolution images of the cardiac tissue slices. "When we average our tracking data over time, the paths of individual M_2 receptors combine to delineate the cellular structure of the tissue," Molloy explains.

Because neurons also express M_2 receptors, these super-resolution tissue maps include not only the cardiomyocytes but also the nerve fibers that innervate them. "These nerve fibers are only ~0.2 µM in diameter and they aren't really visible by light microscopy," Mashanov says. "But we could see hundreds of them. Every cardiomyocyte has a nerve fiber associated with it."

Mashanov et al.'s technique should be easily adapted for other tissues and membrane proteins and may even facilitate single-molecule imaging in entire model organisms like zebrafish or fruit flies. For Molloy's laboratory, though, the next step is to develop dual-color labeling of M_2 receptors and the downstream proteins in the pathway, $G_{\beta\gamma}$ and GIRK, so that the kinetics of the molecules' interactions can be studied in living tissues.

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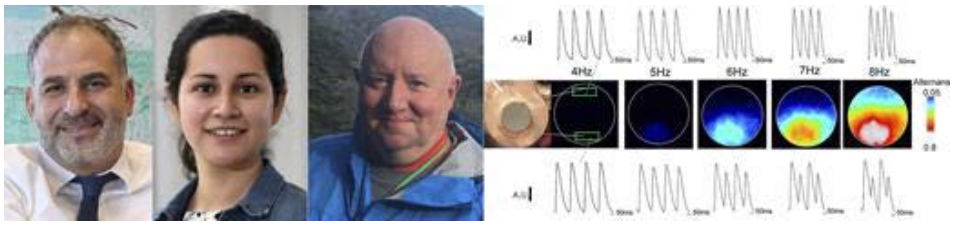
ORIGINAL PAPER

Gregory I. Mashanov, Tatiana A. Nenasheva, Tatiana Mashanova, Catherine Maclachlan, Nigel J.M. Birdsall, and Justin E. Molloy. A method for imaging single molecules at the plasma membrane of live cells within tissue slices. https://doi.org/10.1085/jgp.202012657

Understanding Ca^{2+} alternans

Insufficient reuptake of calcium into the sarcoplasmic reticulum underlies arrhythmogenic variations in cardiac calcium transients

Ca^{2+} alternans (Ca-Alts) are beat-to-beat changes in the amplitude of the Ca^{2+} transients evoked in cardiomyocytes, which can lead to arrhythmias and sudden cardiac death. Ca-Alts can be induced by an elevated heart rate (tachycardia) or metabolic impairments such as ischemia or hypothermia, but the molecular mechanisms underlying the phenomenon are unclear. Millet et al. reveal that Ca-Alts arise when SERCA pumps are unable to fully replenish Ca^{2+} levels in the SR (1).



Jose Millet, Yuriana Aguilar-Sanchez, Ariel L. Escobar (left to right), and colleagues investigate the mechanisms underlying arrhythmogenic Ca-Alts. The FLOM technique shows how these beat-to-beat changes in Ca^{2+} transients can be induced in intact hearts by increased heart rate and local reductions in temperature produced by a cold finger. The researchers find that Ca-Alts result from insufficient replenishment of SR Ca^{2+} levels by SERCA pumps.

"Ca-Alts are very arrhythmogenic," says Ariel L. Escobar, a professor at the University of California, Merced. "If you develop these alternans, you have a very high chance of suffering ventricular fibrillation."

Yet the mechanisms underlying Ca-Alts remain unclear. Though they appear to involve changes in the amount of Ca^{2+} released from the SR (2,3,4), Ca-Alts could be triggered by variations in the duration of action potentials (APD-Alts) that stimulate calcium-induced calcium release, an incomplete recovery of the ryanodine receptor that releases Ca^{2+} from the SR, or incomplete refilling of the SR by SERCA ATPases.

To investigate the phenomenon in more detail, Escobar and colleagues, including co-first authors Jose Millet and Yuriana Aguilar-Sanchez, developed a new technique called fluorescence local field optical mapping (FLOM), which uses optical conduits containing >70,000 optical fibers to map the fluorescence of calcium-sensitive or potentiometric dyes in the epicardium of intact mouse hearts. "This approach allows us to study the spatiotemporal dynamics of calcium and membrane potential changes in a functional heart," Escobar explains.

FLOM imaging confirmed that Ca-Alts can be induced by increased heart rate and/or global reductions in temperature, two conditions that also induce APD-Alts. More crucially, however, Escobar and colleagues used a small, crescent-shaped cold finger to show that local reductions in tissue temperature also induce Ca-Alts but do not cause APD-Alts, demonstrating that the two phenomena can be uncoupled and that Ca-Alts are not driven by changes in action potential duration.

Because the crescent-shaped cold finger creates a temperature gradient within the epicardium, Escobar and colleagues were able to carefully analyze the temperature dependence of Ca^{2+} dynamics. The relaxation of Ca^{2+} transients becomes gradually slower at lower temperatures, and a thermodynamic analysis of this process suggested that it involves not only active mechanisms—such as the ATPases that pump Ca^{2+} into the SR—but also passive mechanisms such as diffusion and binding to cytosolic buffers.

In contrast, the relatively steep temperature dependence of Ca-Alts indicated that they exclusively depend on an active process like SERCA-mediated Ca^{2+} reuptake into the SR. Indeed, Escobar and colleagues found that the Q_{10} temperature coefficient of Ca-Alts is remarkably similar to the Q_{10} of SERCA-mediated Ca^{2+} transport in vitro.

To confirm the importance of Ca^{2+} reuptake in Ca-Alts, Escobar and colleagues treated hearts with the SERCA inhibitor Thapsigargin. Partial blockade of SERCA-mediated reuptake enhanced the level of Ca-Alts, the researchers found, indicating that Ca-Alts are induced when SERCA pumps fail to fully replenish SR Ca^{2+} stores between heart beats. This could occur when the heart is beating particularly fast or when the metabolic activity of cardiomyocytes is impaired by, for example, low temperatures.

Escobar's team is now developing a needle-shaped optical conduit that can be used to probe any layer within the ventricular wall. "We hope to measure Ca-Alts in each layer, including the endocardium where SERCA levels are lower and Ca-Alts tend to be initiated," Escobar says.

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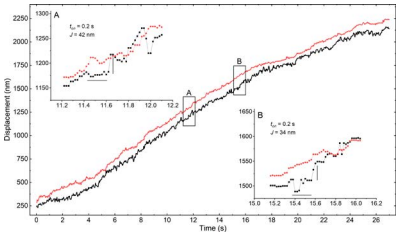
Jose Millet, Yuriana Aguilar-Sanchez, Dmytro Kornyeyev, Maedeh Bazmi, Diego Fainstein, Julio A. Copello, and Ariel L. Escobar. Thermal modulation of epicardial Ca^{2+} dynamics uncovers molecular mechanisms of Ca^{2+} alternans. https://doi.org/10.1085/jgp.202012568

S2 domain gives myosin filaments some flexibility

A microscopy study supports the idea that the region linking myosin head and tail domains can be peeled away from filament backbone to prevent actin-attached heads from impeding filament movement

Myosin II motors move along actin filaments by coupling cycles of ATP binding and hydrolysis to a repetitive process in which the myosin head domains attach to actin, undergo a conformational shift/powerstroke, and then detach. In muscle cells, myosin II molecules assemble into thick filaments containing hundreds of head domains, and any heads that remain attached to actin after completing their power stroke may impede the ability of other heads to move the filament and drive muscle contraction. Brizendine et al. provide direct evidence that this potential drag on filament movement is limited by the flexibility of myosin II's S2 subdomain (1).



(Left to right) Richard Brizendine, Christine Cremo, and Murali Anuganti provide direct evidence that the S2 domain of myosin II is a flexible structure, which would allow it to prevent actin-attached heads from impeding the movement of myosin filaments. Quantum dots labeling a head domain (black) and the filament backbone (red) mostly follow the same trajectory as a filament moves in vitro. But, in rare instances (insets), an actin-attached head briefly lags the backbone's trajectory before catching up, an event facilitated by the flexibility of the S2 region that connects the motor protein's head and tail domains.

For the past few years, Christine Cremo and colleagues at the University of Nevada, Reno, have been studying the kinetics of filament movement using fluorescently labeled myosin and actin filaments in vitro (2). Based on their data, Cremo's team, in collaboration with Josh Baker, developed a mixed kinetic model that predicted a key mechanical function for the S2 subdomain of myosin II, which links the motor protein's head domains to the C-terminal light meromyosin (LMM) domains that mediate filament assembly (3,4). According to the model, the flexibility of the S2 subdomain, and its ability to be peeled away from the filament backbone, provides some slack to actin-attached heads as the filament moves forward, giving them more time to detach before they impede the filament's progress.

"So now we wanted to see if we could directly observe this flexibility," Cremo explains. To do this, two postdocs in Cremo's laboratory, Richard Brizendine and Murali Anuganti, assembled smooth muscle myosin filaments labeled with two differently colored quantum dots, one attached to the LMM domain and the other attached to the head domain. Most of the time, these two labels should follow the same trajectory along actin filaments in vitro. If the S2 domain is flexible, however, it should be possible to occasionally observe an actin-attached head remain in place while the LMM domain continues moving forward. This brief "dwell" should then be followed by a "jump" as the head domain detaches from actin and catches up with the trajectory of the filament backbone.

"We were looking for rare events in a sea of noise," Cremo says, yet the researchers were able to identify dwells and jumps in the quantum dot trajectories consistent with the predicted flexibility of the S2 domain. The frequency and duration of these events fit the known kinetics of actomyosin motility.

Based on their data, Brizendine et al. (1) estimate that, in smooth muscle, a myosin filament can move up to ~52 nm without being impeded by an actin-attached head, a figure close to that predicted by the mixed kinetic model. To provide this flexibility, the researchers calculate that as much as 26 nm of the S2 domain can be unzipped from the filament backbone. Intriguingly, this matches the maximum length that S2 can be seen to project from thick filaments in tomograms of *Drosophila* flight muscle (5), and the forces generated by working myosin heads should be more than sufficient to achieve this unzipping.

Many cardiomyopathy-associated mutations are located in the S2 region of myosin II. However, the mixed kinetic model predicts that, compared with smooth muscle, myosin filaments in cardiac and skeletal muscle cannot move quite as far without being impeded by actin-attached heads. "What leads to these differences?" Cremo wonders. "Are there differences in the biophysical behavior of the S2 domain in different muscle types?"

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https://doi.org/10.1085/jgp.202012751

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Dissecting neurotransmission with artificial synapses

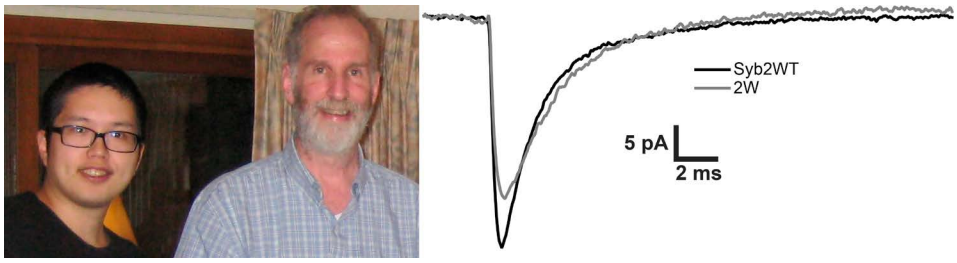
Recordings from neuron–HEK cell cocultures provide a clearer picture of the factors involved in synaptic transmission

Resolving the rapid series of steps involved in synaptic transmission and assessing the contributions of different molecules to each of them is an enormous challenge. Chiang et al. show that the process can be studied with greater resolution at the artificial synapses formed between neurons and cocultured human embryonic kidney (HEK) cells (1).

Meyer Jackson and colleagues at the University of Wisconsin School of Medicine and Public Health are particularly interested in exocytosis. Though this process can be measured directly in endocrine cells, its role in controlling the dynamics of synaptic transmission can be difficult to separate from all the downstream steps required to elicit a response in the postsynaptic neuron. "We wanted to study a surrogate synapse with a simplified response to neurotransmitter that would allow us to focus on vesicle release with greater resolution," Jackson explains.

For years, researchers have studied synaptogenesis by transfecting HEK cells with a handful of postsynaptic factors that enable them to assemble functional synapses when cocultured with neurons (2, 3). Jackson realized that these artificial synapses lack two key sources of variability that can obscure the contribution of vesicle release to synaptic transmission. First, the postsynaptic apparatus of neuron–HEK synapses is consistent and can be precisely controlled (in contrast to regular synapses, where the molecular composition may vary from synapse to synapse). Second, the compact shape of HEK cells greatly reduces the influence of dendritic filtering, the phenomenon by which synaptic inputs take varying lengths of time to reach the cell body, depending on the location of the synapse within the dendritic arbor.

Jackson and colleagues, including first author Chung-Wei Chiang, transfected



Chung-Wei Chiang (left), Meyer Jackson (right), and colleagues studied synaptic transmission between hippocampal neurons and cocultured HEK cells. Mutations in the SNARE protein synaptobrevin 2 alter the shape of mEPSCs generated in HEK cells, an effect made clearer by the absence of dendritic filtering in this artificial system."

HEK cells with four postsynaptic proteins—the AMPA receptor subunit GluA2, the adhesion molecule neuroligin 1, the scaffold protein PSD-95, and the accessory factor stargazin—and cocultured them with hippocampal neurons (1). The researchers then measured the miniature excitatory postsynaptic currents (mEPSCs) evoked in the HEK cells by the spontaneous release of single synaptic vesicles from neighboring neurons.

The mEPSCs generated at these artificial synapses were larger and faster than mEPSCs produced by regular, neuron–neuron synapses (though they involved comparable amounts of charge, suggesting that the vesicle populations are similar at both types of synapse).

Notably, the rise rate of mEPSCs in HEK cells was faster and less variable, in keeping with the absence of dendritic filtering and the consistent, shorter distance between the artificial synapses and the HEK cell body. This allowed Chiang et al. (1) to resolve the contribution of vesicle release to mEPSC dynamics using mutant versions of the SNARE protein synaptobrevin 2 that impede the flux of neurotransmitters through synaptic fusion pores (4). These mutant proteins decreased the rise rate and decay rate of mEPSCs at artificial synapses. "However, the effect was much clearer in HEK cells than we'd previously seen in neurons," Jackson says.

Chiang et al. (1) were also able to resolve the contribution of postsynaptic receptors to mEPSC shape, but Jackson is most interested in using the neuron–HEK coculture system to investigate synaptic vesicle release in more detail. "It opens up a new approach that will allow us to study synaptic exocytosis more precisely and look for much subtler effects," Jackson says. For example, Jackson hopes to explain why mutations in some exocytotic proteins have major effects on endocrine cells but little to no effect at synapses.

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ORIGINAL PAPER

Chung-Wei Chiang, Wen-Chi Shu, Jun Wan, Beth A. Weaver, and Meyer B. Jackson. Recordings from neuron–HEK cell cocultures reveal the determinants of miniature excitatory postsynaptic currents. https://doi.org/10.1085/jgp.202012849

Regional differences in arrhythmogenesis

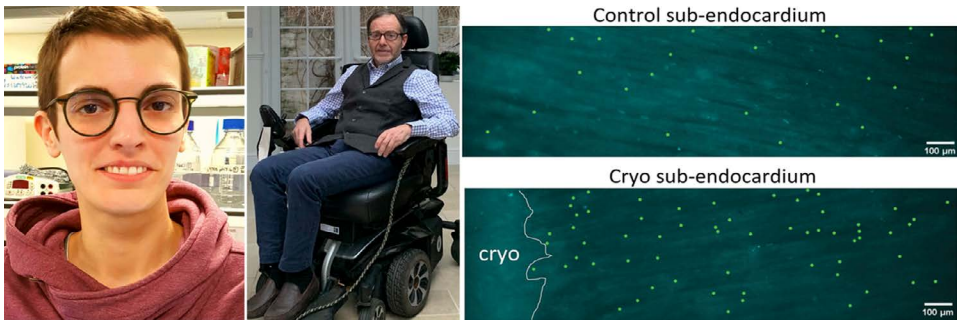
The subendocardium is more susceptible to spontaneous Ca^{2+} release events that can initiate arrhythmias, and this may be reduced by local CaMKII inhibition

Calcium release and uptake must be carefully controlled in cardiomyocytes to ensure that the heart maintains a regular beat, and spontaneous Ca^{2+} release (SCR) from the sarcoplasmic reticulum—due to leaky ryanodine receptors, for example—can trigger lethal ventricular arrhythmias. Dries et al. demonstrate that the subendocardial layer of the ventricular wall is particularly susceptible to arrhythmogenic SCR, and that this could potentially be treated by local inhibition of calcium/calmodulin-dependent kinase II (CaMKII; 1).

SCRs have been extensively studied in isolated cardiomyocytes, but arrhythmias are multicellular events (2) in which the behavior of individual cells is influenced by their interactions with neighboring cells and the extracellular matrix. "In addition, myocardial electrophysiology changes at different depths of the ventricular wall, and the vast majority of studies do not account for this transmurality," explains Cesare Terracciano, a professor at the National Heart and Lung Institute, Imperial College London.

Terracciano's group has pioneered the use of living myocardial slices prepared from different layers of the ventricular wall to study regional differences in the electrical and mechanical properties of healthy hearts (3,4). However, it is unclear how these differences are impacted by injury or disease and whether this leaves some layers of the heart wall more susceptible to SCRs and arrhythmogenesis.

Terracciano and colleagues, including first author Eef Dries, therefore prepared myocardial slices from different layers of the rat ventricular wall and subjected them to cryoinjury (1). Structural remodeling—in the form of reduced T-tubule density—was similar in both subendocardial and subepicardial slices after injury, but only subendocardial slices showed an increase in spontaneous, arrhythmic contractions.



Using living myocardial slices, Eef Dries (left), Cesare Terracciano (center), and colleagues show that, following injury, the subendocardial layer of the rat ventricular wall is more susceptible than the subepicardial layer to arrhythmogenic SCR events. High-resolution Ca^{2+} imaging of the subendocardium shows the increased number of SCRs (green dots) in the region bordering the injured tissue. The frequency of SCRs and ectopic contractions can be reduced by CaMKII inhibition.

Dries et al. used a fluorescent Ca^{2+} indicator and high-resolution imaging to examine Ca^{2+} signaling in the "border zone" surrounding the cryoinjury, as this region has been implicated in triggering arrhythmias following myocardial infarction. "Intriguingly, and only in subendocardial slices after injury, we observed a reduction in the amplitude of calcium transients that also became slower to decline, changes that are hallmarks of heart failure," Terracciano says. "SCR events were more frequent and more closely distributed when we cryoinjured the slices but, again, only in the subendocardium."

The clustering of multiple SCRs in both space and time makes them more likely to trigger an ectopic contraction. One possibility is that the open probability of ryanodine receptors is increased in subendocardial slices. This could be caused by enhanced CaMKII-mediated phosphorylation of ryanodine receptors and, indeed, Dries et al. found that, after cryoinjury, receptor phosphorylation is increased in subendocardial, but not subepicardial, slices (1).

Accordingly, Terracciano and colleagues found that the CaMKII inhibitor AIP reduced the frequency of SCRs and spontaneous contractions in cryoinjured subendocardial slices. In contrast, AIP had no effect on injured subepicardial slices or on normal, healthy cardiac tissue. CaMKII inhibitors have been proposed as potential therapies for cardiac arrhythmias, but their use has so far been limited by off-target effects. Dries et al.'s results suggest that targeting CaMKII inhibitors to specific regions of the ventricular wall (using localized gene therapy, for example) could greatly improve their efficacy.

"A picture is emerging that subendocardial slices are more susceptible to arrhythmogenic stimuli, and this can be important for understanding and treating arrhythmias," Terracciano says. He now plans to study injured myocardial slices over longer time periods and investigate the molecular changes underlying the enhanced arrhythmogenic susceptibility of the subendocardium, as well as testing localized gene therapy approaches in animal models of disease.

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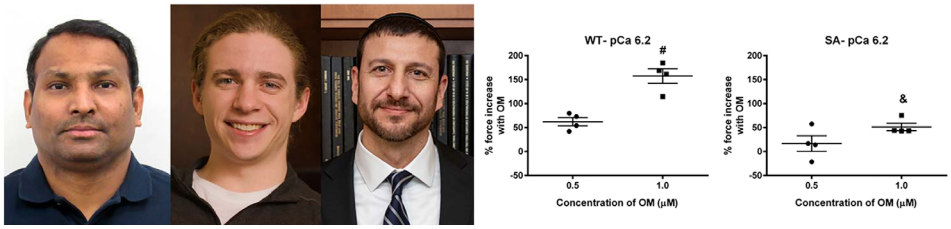
cMyBPC phosphorylation alters response to heart failure drug

The phosphorylation state of cMyBPC modulates the ability of omecamtiv mecarbil to enhance myocardial force generation

The small molecule omecamtiv mecarbil (OM) is a cardiac-specific myosin activator that is currently undergoing clinical trials for the treatment of heart failure with reduced ejection fraction. Mamidi et al. demonstrate that OM's ability to increase cardiac force production is altered by the phosphorylation state of cardiac myosin-binding protein C (cMyBPC), a target of β-adrenergic signaling that is often dysregulated in late-stage heart failure patients (1)



(Left to right) Ranganath Mamidi, Joshua Holmes, Julian Stelzer, and colleagues reveal that the effects of the heart failure drug OM are modulated by the phosphorylation state of the contractile protein cMyBPC. For example, OM's ability to increase force generation is significantly blunted in mouse myocardial preparations expressing phosphoablated (SA) rather than WT cMyBPC due to changes in myosin cross-bridge kinetics.

OM enhances myocardial force generation by increasing the number of strongly bound myosin cross-bridges (2), partly by slowing ADP release and cross-bridge detachment (3). Though the drug has progressed to phase 3 clinical trials, little is known about how its effects may be influenced by pathophysiological changes in other sarcomeric proteins, such as cMyBPC, that regulate myosin cross-bridges and force production.

During exercise or other physiological stresses, adrenaline stimulates the phosphorylation of cMyBPC by PKA, thereby accelerating cross-bridge kinetics and myocardial contractility to meet the increased demand for cardiac output (4). In late-stage heart failure patients, however, β-adrenergic signaling is dysregulated and cMyBPC phosphorylation is greatly reduced. "We wanted to test how the phosphorylation state of cMyBPC would effect OM treatment," explains Julian Stelzer, a professor at Case Western Reserve University.

Stelzer's team, including cofirst authors Ranganath Mamidi and Joshua Holmes, prepared myocardial tissue from both WT mice and mice expressing a cMyBPC mutant that lacks the three main PKA phosphorylation sites. The researchers treated the preparations with OM and found that the ablation of cMyBPC phosphorylation significantly blunted OM's ability to increase force production (1).

Dephosphorylated cMyBPC is thought to stabilize the super-relaxed state of myosin, in which the head domains are folded back toward the filament backbone and are less available to form active cross-bridges (5). Stelzer and colleagues have previously shown that ablating cMyBPC phosphorylation slows cross-bridge kinetics (6).

"This is exacerbated by the addition of OM," Stelzer says. "It creates an even slower system that limits cross-bridge recruitment, and those that are recruited can't really be detached." This may reduce the effectiveness of OM in end-stage heart failure patients with low levels of cMyBPC phosphorylation.

In contrast, phosphorylation of cMyBPC by PKA usually accelerates myosin cross-bridge kinetics. However, when Stelzer and colleagues treated their myocardial preparations with both PKA and OM, mimicking the scenario of an early-stage heart failure patient exercising or experiencing stress, the effects of the drug dominated the effects of the kinase.

"OM did not allow any acceleration and, in fact, slowed cross-bridge kinetics even further, completely negating the effect of PKA on contractility," Stelzer says. This could mean that early-stage patients on OM are unable to increase their cardiac output during exercise, elevating the risk of ischemia.

New iterations of OM are already being explored as potential next-generation treatments for heart failure. Stelzer says that it will be important to investigate how these drugs interact with cMyBPC and other components of the contractile machinery. In the meantime, Stelzer's laboratory is focused on developing novel therapeutic approaches involving the direct manipulation of cMyBPC phosphorylation.

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This work is part of a special collection on myofilament function and disease.

A TREK inhibitor takes multiple tracks

Single-channel recordings reveal that norfluoxetine inhibits the two-pore domain K^+ channel TREK-2 by a complex array of mechanisms

The TREK subfamily of two-pore domain K^+ channels are expressed throughout the central and peripheral nervous systems and are involved in a diverse range of processes such as mechanosensation, thermosensation, and nociception. Accordingly, channel gating—which is thought to involve changes in the selectivity filter of TREKs—can be regulated by a wide variety of factors, including pressure, temperature, and multiple endogenous ligands (1). Proks et al. reveal that this regulatory complexity is reflected in the fact that the TREK inhibitor norfluoxetine impairs channel activity via several different mechanisms (2).

Peter Proks (left), Stephen J. Tucker (center), and colleagues use single-channel recordings to investigate how norfluoxetine inhibits the two-pore domain K^+ channel TREK-2. Norfluoxetine binds exclusively to the "down" conformation of TREK-2 (right) and prevents the channel's transmembrane domains from transitioning to the "up" configuration. But Proks et al. find that TREK-2 can be fully active in the down conformation and that norfluoxetine works via multiple mechanisms to inhibit both the open and closed states of the channel.

Norfluoxetine is a metabolite of fluoxetine (Prozac), and both compounds are among the few known inhibitors of TREK activity (3). "TREK channels are not the principal targets of fluoxetine, which is mainly a selective serotonin reuptake inhibitor," explains Stephen J. Tucker from the University of Oxford. "But fluoxetine and norfluoxetine are useful tools to study the mechanisms of TREK channel gating."

Tucker and colleagues previously helped solve the crystal structures of TREK-2 in the presence and absence of norfluoxetine (4). The channel can adopt two distinct conformations, named "up" or "down" depending on the orientation of its transmembrane helices, and norfluoxetine was found to bind within the inner cavity of TREK-2 in a gap that is only formed when the transmembrane helices are in the down configuration. Norfluoxetine can therefore block the transition from the down to up conformation, and it was originally suggested that this might inhibit channel activity by locking the selectivity filter in its closed state. But the mechanism of filter gating appears to be more complex. Tucker's group, for example, has previously shown using macroscopic recordings that TREK-2 can adopt several open states, some of which may occur in the down conformation (5).

To learn more about the mechanisms underlying filter gating and norfluoxetine inhibition, Tucker and colleagues, including first author Peter Proks, turned to single-channel recordings of purified TREK-2 channels embedded in lipid bilayers (2). "We found that norfluoxetine affects both the open and closed states of the channel and is therefore a state-independent inhibitor of TREK-2," Tucker says. "That information is lost in macroscopic recordings."

Moreover, the fact that highly active channels are sensitive to norfluoxetine inhibition confirms that TREK channels can be fully open in the down conformation. It also indicates that, in addition to blocking changes in transmembrane conformation, norfluoxetine must inhibit TREK channels by other mechanisms as well.

"We found that there are several mechanisms involved, all of which converge on the selectivity filter gate," Tucker says. The researchers also observed a mild voltage dependence of norfluoxetine inhibition, suggesting that it can influence voltage-dependent gating as well.

"The complexity with which the drug works reflects the many different ways in which the selectivity filter can gate the channel," Tucker says. "This, in turn, reflects the polymodal regulation of TREK channels and their ability to integrate a wide variety of signals."

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JGP Journal of General Physiology

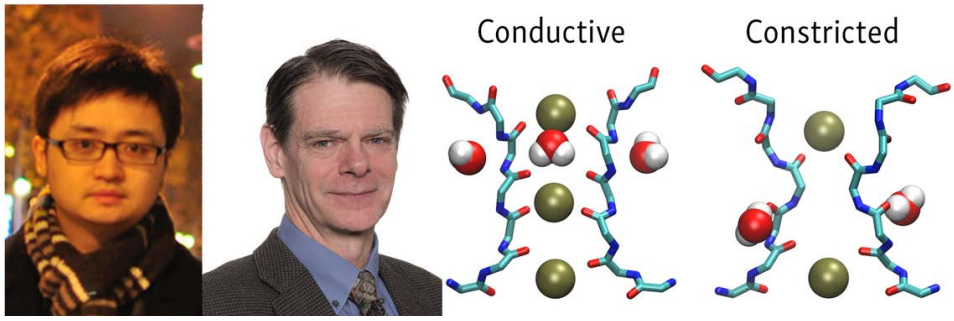
Widening the scope of constriction

Modeling study suggests that selectivity filter constriction is a plausible mechanism for C-type inactivation of the* Shaker *voltage-gated potassium channel

In response to prolonged activation, many K^+ channels spontaneously reduce the membrane conductance by undergoing C-type inactivation, a kinetic process crucial for the pacing of cardiac action potentials and the modulation of neuronal firing patterns. In the pH-activated bacterial channel KcsA, C-type inactivation appears to involve constriction of the channel's selectivity filer that prohibits ion conduction, but whether voltage-gated channels like *Drosophila Shaker* use a similar mechanism is controversial (1). A computational study by Li et al. suggests that filter constriction is indeed a plausible mechanism for the C-type inactivation of *Shaker* (2).

Various structural approaches have shown that C-type inactivation of KcsA channels is associated with the symmetrical constriction of all four channel subunits at the level of the central glycine residue in the selectivity filter. Benoît Roux and colleagues at The University of Chicago used MD simulations to show that the KcsA pore can transition from the conductive to the constricted conformation on an appropriate timescale, and that this transition is allosterically promoted by the wide opening of the pore's intracellular gate (3). Modeling by Roux and colleagues suggests that C-type inactivation of cardiac hERG channels could also involve selectivity filter constriction, though in this case it appears to be an asymmetric process in which only two of the channel's subunits move closer together (4).

"In view of the high similarity between the pore domains of *Shaker* and KcsA (almost 40% sequence identity), we wanted to examine if it's possible for the *Shaker* selectivity filter to constrict and, if so, how similar it is to KcsA," Roux explains. Led by first author Jing Li—now an assistant professor at the University of Mississippi—Roux and colleagues developed several homology models of the *Shaker* pore domain with the intracellular gate open to various degrees (2).



(Left to right) Jing Li, Benoît Roux, and colleagues use computational modeling to show that selectivity filter constriction, allosterically promoted by opening of the intracellular activation gate, is a plausible mechanism for the C-type inactivation of voltage-gated K^+ channels such as *Drosophila Shaker*. The selectivity filter is conductive (left) when the intracellular gate is partially open, but adopts a constricted conformation (right) when the gate is open wide.

MD simulations and free energy calculations revealed that the *Shaker* selectivity filter can dynamically transition from a conductive to a constricted conformation, and that this transition is allosterically coupled to the intracellular gate; the constricted conformation is stable when the gate is wide open. "Our computations strongly suggest that constriction is a plausible mechanism for the C-type inactivation of *Shaker*," Roux says. "There's no reason based on the currently available information to reject the existence of a constricted state in *Shaker* channels."

As with KcsA, *Shaker* channels appear to constrict symmetrically at the level of the selectivity filter's central glycine. But Li et al.'s simulations revealed some small variations between the two channels, including differences in the number of water molecules bound to each channel subunit and the arrangement of the hydrogen-bond network they form to stabilize the constricted state.

Li et al. also modeled the pore domain of the *Shaker* W434F mutant, which is widely assumed to be trapped in a C-type inactivated state. The simulation suggests that the mutant channel's filter adopts a stable constricted conformation even when the intracellular gate is only partially open, although the constriction is asymmetric and occurs at the level of a different filter residue (2).

Constriction may therefore be a universal mechanism of C-type inactivation, even if the exact conformation varies from channel to channel. But, says Roux, confirming this will require more experimental work using the right conditions and mutations to capture the structure of inactivated channels.

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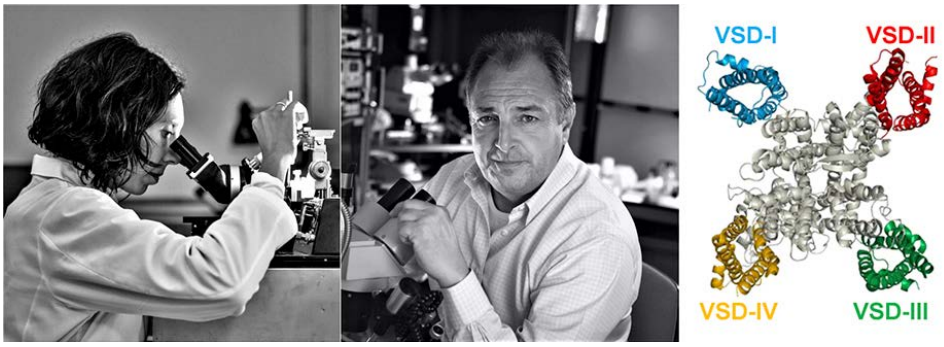
Distinct roles for $Ca_V1.1$'s voltage-sensing domains

Study reveals how a slowly activating calcium channel is able to control rapid excitation–contraction coupling in skeletal muscle

Skeletal muscle contraction is initiated by action potentials that depolarize the muscle fiber and trigger the rapid release of Ca^{2+} from the SR via RYR1 channels. This process of excitation–contraction coupling depends on voltage-gated $Ca_V1.1$ channels in the plasma membrane, or sarcolemma, of muscle fibers. But $Ca_V1.1$ channels are only slowly activated by changes in the sarcolemma membrane potential, and it is therefore unclear how they are able to trigger the much faster activation of RYR1 channels. Savalli et al. reveal that this paradox can be explained by the fact that each of $Ca_V1.1$'s four voltage-sensing domains (VSDs) have distinct biophysical properties (1).



Nicoletta Savalli (left), Riccardo Olcese (center), and colleagues reveal the distinct physical properties of the $Ca_V1.1$ channel's four voltage-sensing domains (VSD I–IV, right). VSD-I shows slow activation kinetics and is the main contributor to the opening of $Ca_V1.1$. The other VSDs activate much faster and may therefore be coupled to RYR1 to mediate the rapid release of Ca^{2+} from the SR during skeletal muscle contraction.

RYR1 channels have no voltage-sensing machinery of their own and therefore rely on a physical connection to $Ca_V1.1$ channels to release Ca^{2+} and initiate muscle contraction in response to muscle fiber depolarization. But RYR1 channels open ~25 times faster than $Ca_V1.1$ channels. "So, how can these slowly activating $Ca_V1.1$ channels trigger the rapid release of Ca^{2+} from the SR?" asks Riccardo Olcese, a professor at the David Geffen School of Medicine, UCLA.

Olcese and colleagues, including Assistant Project Scientist Nicoletta Savalli, suspected that the answer might lie in the fact that, like many other voltage-gated ion channels, $Ca_V1.1$ has four VSDs that alter their conformation in response to voltage changes. These domains are similar, but not identical, to each other, potentially enabling them to have distinct biophysical properties and perform distinct functions. Indeed, Olcese and colleagues previously demonstrated that, in the closely related channel $Ca_V1.2$, only VSDs II and III are involved in pore opening (2, 3).

Savalli et al. used voltage-clamp fluorometry to compare the properties of $Ca_V1.1$'s VSDs, expressing the channel in Xenopus oocytes and labeling each of its VSDs in turn with an environmentally sensitive fluorophore to report voltage-dependent changes in their conformation (1). "We found that the four VSDs were very heterogenous in both their kinetics and voltage dependencies," says Olcese. "VSD-I had very slow kinetics, compatible with the slow activation of the $Ca_V1.1$ pore. The other three VSDs had much faster kinetics and could, therefore, be good candidates to be the voltage sensors for RYR1 activation."

Olcese and colleagues confirmed the importance of VSD-I for $Ca_V1.1$ activation by analyzing a naturally occurring, charge-neutralizing mutation in this domain, R174W, that is linked to malignant hyperthermia (4). The team found that this mutation reduced the voltage-sensitivity of VSD-I and abolished the ability of $Ca_V1.1$ to conduct Ca^{2+} at physiological membrane potentials, but had no effect on the behavior of the other three VSDs.

Finally, Savalli et al. applied their data on both the wild-type and mutant VSDs to an allosteric model of Ca_V activation (2, 3), which predicted that VSD-I contributes most of the energy required to stabilize the open state of $Ca_V1.1$, while the other VSDs contribute little to nothing.

Thus, $Ca_V1.1$ activation is mainly driven by a single VSD—a mechanism that hasn't been seen in any other voltage-gated ion channel—leaving the other VSDs free to perform other functions, such as the rapid activation of RYR1. Olcese and colleagues now want to pinpoint exactly which VSD(s) are coupled to RYR1 and determine how they trigger rapid Ca^{2+} release from the SR.

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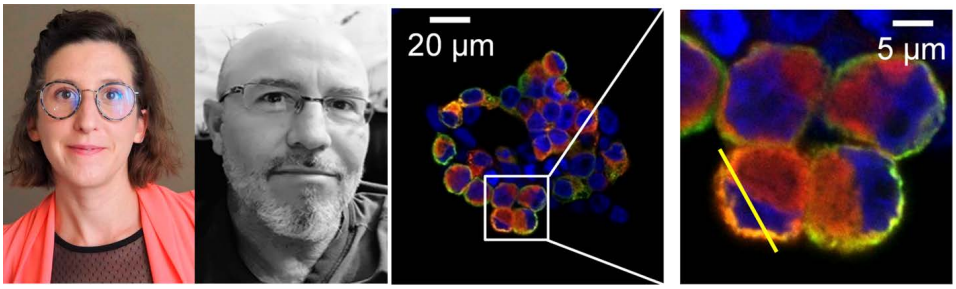
JGP Journal of General Physiology

Piezo1 helps bile on the pressure

A mechanosensitive complex containing Piezo1 and Pannexin1 couples osmotic pressure to ATP secretion in bile duct cholangiocytes.

Cholangiocytes are epithelial cells that line the bile ducts within the liver and modify the composition of hepatocyte-derived bile. Desplat et al. identify a mechanosensory complex that may help cholangiocytes respond to changes in osmotic pressure (1).



Angélique Desplat (left), Patrick Delmas (center), and colleagues identify a mechanosensitive pathway that couples hypotonic stress to calcium influx and ATP release in cholangiocytes. Cell swelling induces calcium influx through the stretch-activated ion channel Piezo, triggering ATP release by Pannexin1 channels. This leads to the activation of P2X4 receptors and further calcium influx. Piezo1 (red) and Pannexin1 (green) colocalize in cells and may interact to form a mechanosensory complex that facilitates the hypotonic stress response.

The activity of cholangiocytes can be regulated not only by chemical signals, such as hormones and bile acids, but also by mechanical cues arising from changes in bile composition and flow. "Abnormal mechanical tension is also an aggravating factor in many biliary diseases, including primary sclerosing cholangitis," explains Patrick Delmas, a Research Director at Centre National de la Recherche Scientifique/Aix-Marseille-Université. "So, identifying the molecular players in cholangiocyte force sensing could provide a step forward for better management of biliary diseases."

Current models suggest that mechanical cues trigger an influx of calcium into cholangiocytes, leading to the release of ATP, which, by stimulating purinergic receptors at the cell surface, promotes further calcium influx and induces the secretion of anions, water, and HCO_3^- to modify the tonicity and pH of hepatic bile (2, 3). To identify mechanosensitive proteins that might regulate this pathway, Delmas and colleagues, including first author Angélique Desplat, purified mouse cholangiocytes from intrahepatic bile ducts and subjected them to hypotonic stress (1). The subsequent cell swelling activates calcium influx and ATP release.

Desplat et al. found that depleting or inhibiting the stretch-activated ion channel Piezo1 significantly reduced this response to hypotonic stress. This mechanosensitive channel mediates the initial calcium influx into cholangiocytes when activated by cell swelling.

The subsequent release of ATP is mediated by a different channel, however. Desplat et al. found that cholangiocytes express high levels of the gap junction family protein Pannexin1, and that pharmacologically inhibiting Pannexin1 channels reduced the amount of ATP released in response to hypotonic stress and Piezo1 activation.

Delmas and colleagues suspect that the increase in intracellular calcium mediated by Piezo1 may activate Pannexin1 channels to release ATP, and this activation may be facilitated by a physical association between the two proteins: the researchers found that recombinant versions of the two channel proteins colocalize within the plasma membrane of cholangiocytes and can be coimmunoprecipitated.

Finally, the researchers determined that the ATP released through Pannexin1 channels amplifies the signal initiated by hypotonic stress by activating purinergic P2X4 receptors, leading to further increases in intracellular calcium levels. Transfecting Piezo1-deficient HEK293 cells, which usually don't respond to hypotonic stress, with cDNAs encoding Piezo1, Pannexin1, and P2X4R was sufficient to reconstitute the entire pathway of calcium influx and ATP release.

Cholangiocytes express other mechanosensitive channels, including TRPV4, which has previously been implicated in the cells' response to hypotonic stress (4). The functions of TRPV4 and Piezo1 may therefore be partially redundant, providing some robustness to cholangiocytes mechanical signaling pathways. However, it is also possible that, in vivo, the two channels respond to different stimuli and elicit distinct downstream effects. "Further investigation is warranted to better understand the respective roles of these two molecular players," says Delmas. "To continue our work, we would like to challenge our model in vivo by testing whether Piezo1 agonists are able to regulate bile acid secretion."

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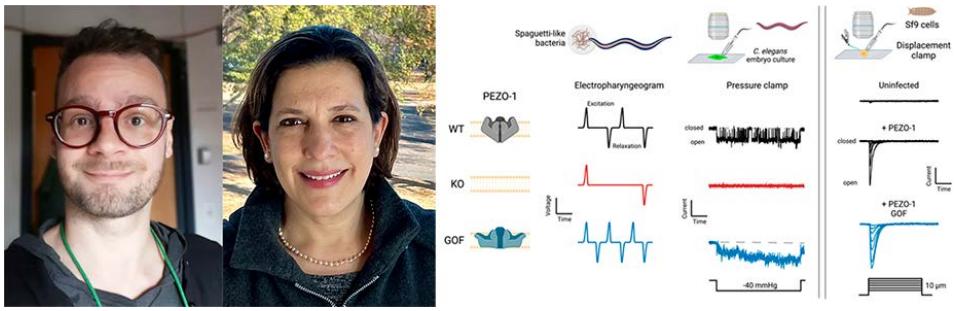
Worms find PEZO-1's function easy to swallow

The* C. elegans *orthologue of the PIEZO family is a mechanosensitive ion channel that regulates pharyngeal pumping and food sensation.

The PIEZO family of mechanosensitive cation channels has been implicated in a wide variety of physiological processes in mammals and is also associated with human disease. Mammalian genomes encode two family members, known as *Piezo1* and *Piezo2*, but invertebrates such as the nematode *Caenorhabditis elegans* only possess a single Piezo-related gene (1). The function of the *C. elegans* orthologue, known as *pezo-1*, has largely remained obscure, but Millet et al. reveal that it encodes a bona fide mechanosensitive ion channel that regulates pharyngeal activity (2).



Jonathan Millet (left), Valeria Vásquez (center), and colleagues reveal that *pezo-1*, the sole PIEZO family member in *C. elegans*, is a mechanosensitive ion channel that regulates pharyngeal pumping and food sensation, particularly when worms are fed with large and stiff bacterial filaments that are difficult to swallow (graphic created with BioRender.com).

In 2020, an elegant study demonstrated that *pezo-1* controls *C. elegans* ovulation and fertilization (3). However, explains Valeria Vásquez from the University of Tennessee Health Science Center, whether *pezo-1* encodes for a mechanosensitive ion channel was unknown. "PEZO-1 is expressed in many tissues, including the pharynx, which is the organ we decided to concentrate on in our study," Vásquez says.

Muscle cells in the *C. elegans* pharynx rhythmically contract and relax to pump food into the worm's intestine. Vásquez and colleagues, including first author Jonathan Millet, found that PEZO-1 is expressed in several different pharyngeal cell types (2), including the gland cells whose secretions lubricate the pharynx, and the proprioceptive NSM neurons that are thought to sense the presence of food within the pharynx lumen and release serotonin to increase the rate of pharyngeal pumping.

Millet et al. analyzed pharyngeal pumping in worms lacking *pezo-1*, as well as in animals expressing a *pezo-1* point mutant that, in human *Piezo1*, increases channel function by slowing channel deactivation and inactivation. Loss or gain of *pezo-1* function had surprisingly little effect on pharyngeal activity, causing only mild alterations in the duration and frequency of pumping induced by serotonin, and more obvious effects when challenged with high osmolarity solutions.

Worms cultured in the laboratory are usually fed a diet of small, easily ingested *Escherichia coli* cells and, both loss and gain of *pezo-1* function increased the pharynx's response to this type of food. In their natural habitat, however, *C. elegans* encounter bacteria of various shapes and sizes, some of which might be harder to swallow. "It occurred to me that it might make a difference if we fed the worms with bacteria that were stiffer and longer," Vásquez says.

The researchers therefore provided their *pezo-1* mutants with *E. coli* treated with cephalexin, an antibiotic that inhibits cell separation and causes the bacteria to form long, spaghetti-like filaments. Compared with wild-type worms fed with this diet, pharyngeal activity was markedly enhanced by the gain-of-function *pezo-1* mutant, but substantially reduced in the absence of *pezo-1*, almost as if the worms were "choking" on the bacterial filaments.

Crucially, by performing patch-clamp experiments on both cultured *C. elegans* cells and insect cells expressing recombinant *pezo-1*, Millet et al. confirmed that PEZO-1 is, indeed, a mechanosensitive ion channel. However, it remains to be seen exactly how PEZO-1 helps the pharynx sense the physical parameters of food and adjust its pumping activity accordingly. One possibility is that the channel acts within the proprioceptive neurons to regulate the release of serotonin.

Intriguingly, the Drosophila PIEZO orthologue controls feeding behavior in flies (4). "However, it's not known which mechanosensitive channels are important in the pharyngeal system of mammals," Vásquez says. "Our studies in *C. elegans* could therefore open an opportunity to understand food sensation in humans."

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C. elegans PEZO-1 is a mechanosensitive ion channel involved in food sensation. https://doi.org/10.1085/jgp.202112960
This work is part of a special issue on mechanotransduction by membrane proteins.

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