

## Mechanical transduction by ion channels: A cautionary tale

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### Abstract

Mechanical transduction by ion channels occurs in all cells. The physiological functions of these channels have just begun to be elaborated, but if we focus on the upper animal kingdom, these channels serve the common sensory services such as hearing and touch,

provide the central nervous system with information on the force and position of muscles and joints, and they provide the autonomic system with information about the filling of hollow organs such as blood vessels. However, all cells of the body have mechanosensitive channels (MSCs), including red cells. Most of these channels are cation selective and are activated by bilayer tension. There are also K<sup>+</sup> selective MSCs found commonly in neurons where they may be responsible for both general anesthesia and knockout punches in the boxing ring by hyperpolarizing neurons to reduce excitability. The cationic MSCs are typically inactive under normal mechanical stress, but open under pathologic stress. The channels are normally inactive because they are shielded from stress by the cytoskeleton. The cationic MSCs are specifically blocked by the externally applied peptide GsMtx4 (aka, AT-300). This is the first drug of its class and provides a new approach to many pathologies since it is nontoxic, non-immunogenic, stable in a biological environment and has a long pharmacokinetic lifetime. Pathologies involving excessive stress are common. They produce cardiac arrhythmias, contraction in stretched dystrophic muscle, xerocytotic and sickled red cells, *etc.* The channels seem to function primarily as "fire alarms", providing feedback to the cytoskeleton that a region of the bilayer is under excessive tension and needs reinforcing. The eukaryotic forms of MSCs have only been cloned in recent years and few people have experience working with them. "Newbies" need to become aware of the technology, potential artifacts, and the fundamentals of mechanics. The most difficult problem in studying MSCs is that the actual stimulus, the force applied to the channel, is not known. We don't have direct access to the channels themselves but only to larger regions of the membrane as seen in patches. Cortical forces are shared by the bilayer, the cytoskeleton and the extracellular matrix. How much of an applied stimulus reaches the channel is unknown. Furthermore, many of these channels exist in spatial domains where the forces within a domain are different from forces outside the domain, although we often hope they are proportional. This review is intended to be a guide for new investigators who want

to study mechanosensitive ion channels.

**Key words:** Channel; Mechanical; Patch; Force; Tension; Bilayer; Domain; Osmotic; Transduction; Biomechanics

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**Core tip:** Mechanosensitive ion channels are found in all cells and their physiological function in most cells has yet to be defined inviting new researchers to the field. This review provides some guidelines to help newcomers understand key issues and potential artifacts.

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## A WIMPY BACKGROUND

We are all familiar with many forms of mechanical transduction<sup>[1]</sup> including hearing<sup>[2]</sup>, touch<sup>[3]</sup> and mechanical pain<sup>[4-7]</sup> that feed the central nervous system. In addition, there are the unconscious motor pathways bearing information about muscle stress and joint position<sup>[8,9]</sup>. Afferents in the autonomic nervous system service blood pressure regulation<sup>[8,10-12]</sup> and the distension of hollow organs<sup>[13,14]</sup>. While these reminders may be familiar to the readers of this journal, what may be less familiar is that all cells in our body, including red cells<sup>[15,16]</sup>, are mechanically sensitive<sup>[17]</sup>. This cell sensitivity probably reflects our evolutionary origins<sup>[18]</sup>. Single cell prokaryotes like *Paramecia* have differentiated touch senses; if they bump into walls they back up, and if you bite their tails they run away<sup>[19-24]</sup>. We have the same heritage, but what are these sensors?

Mechanical sensitivity requires that forces do work on the sensor. This means that the sensors must be deformable, but that only narrows the field to all molecules since they can bend and stretch; it's a quantitative issue. Computational biology of molecular dynamics shows molecules moving under thermal and externally applied forces. How does one design a mechanical sensor? It's a matter of numbers. Sensors have a big output energy compared to the input energy. I will define mechanical sensors as those molecules or organelles in which the output energy, whatever that may be, is significantly larger than the input energy. Ion channels can do this because they are enzymes that dissipate lots of stored energy by catalyzing ion transport and their output energy is a function of the turnover number which can be  $10^7$ .

All sensors have a background noise due to the random shaking of molecules (thermal fluctuations). In this review I will focus on ion channels as sensors (since that is my background), but all structural molecules

can be viewed as mechanical sensors since they are compliant to applied force. The channels serve to couple mechanical stress to electrophysiology and biochemistry, typically by changes in calcium levels. This coupling of biochemistry to mechanics is familiar in muscle contraction, mechanically induced changes in gene expression, stem cell differentiation and changes in cell shape. We conjecture that all pathologies involve mechanics since they all involve a change in cell shape and that requires changes in force.

I will define mechanosensitive channels (MSCs), mechanically sensitive ion channels, as those channels whose dynamic range is fully accessible with physiologically relevant forces<sup>[25-37]</sup>. Many other ion channels, such as the voltage gated ion channels<sup>[38,39]</sup>, are modulated by mechanical stress, but cannot span their dynamic range with mechanics alone. Many enzymes are also mechanically sensitive<sup>[40]</sup>. There are two basic types of MSCs: those gated by stress in attached structural proteins, and those gated by tension in the bilayer. MSCs in the differentiated sensory organs seem to be gated by forces in structural proteins<sup>[30,41-45]</sup> where the channel is in series with the extracellular matrix and the cytoskeleton (Figure 1). The other class of MSCs is gated by tension in the lipid bilayer (Figure 2)<sup>[24,42,46-50]</sup>.

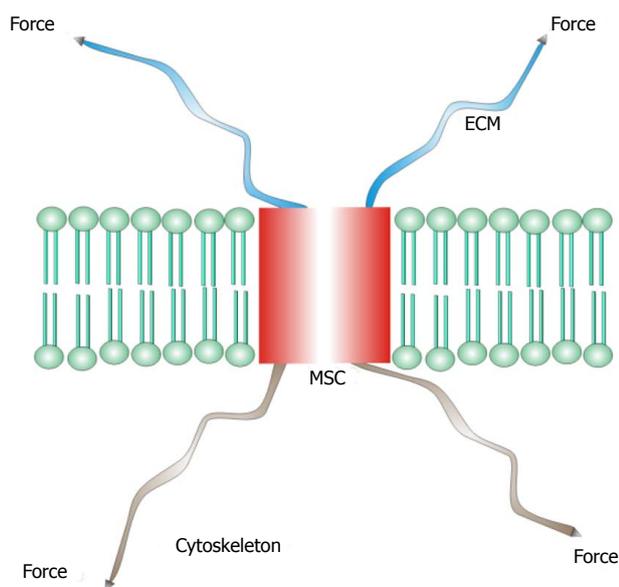
There is currently one drug known specific for MSCs of any kind, a peptide called GsMtx4 that is active on cation selective, bilayer-activated, MSCs<sup>[15,25,51-58]</sup>. Recently there has been a report of an organic molecule that tends to activate some of these channels<sup>[16]</sup>.

This review is not intended to serve as a guide to the literature of MSCs, but addresses core issues that are required to understand how they work. For access to the general literature, there may be more review articles than research papers<sup>[18,24,42,46,47,59-63]</sup>! My primary goal is to familiarize newcomers to the field about what we have learned and to warn readers about some misleading dogmas to sensitize readers to critical interpretations of research papers. Many of the references in this paper come from the work in my lab, but that is not to say that they are the most important available, merely that I remember them better.

## DEFINITION OF THE STIMULUS IN SIMPLE SYSTEMS

### *Lipid patch*

One of the critical limitations of work on MSCs is that the stimulus tends to be imprecise. We can pull, indent, swell or shrink a cell, but what does the channel feel? We don't know, although but we can guess from the channel behavior. Even in plain lipid bilayer in a patch<sup>[46,47,61,64-66]</sup>, we don't know the stresses with precision. Let's look at what might be considered the simplest of experimental systems: a patch of a liposome (Figure 3). Patches stick to the glass pipette. This adhesion produces a tension in the membrane that is a significant fraction of the lytic tension. This means that no one has ever recorded



**Figure 1** A mechanosensitive channel from a differentiated sensory organ where stress is passed through the channel from the extracellular matrix to the cytoskeleton. MSC: Mechanosensitive channel; ECM: Extracellular matrix.

patch currents of any kind except under extreme, nonphysiological, tension (there is one exception to this statement; we were able to measure MSC kinetics under low stress for short periods of time<sup>[67]</sup>). Patch tension is far in excess of anything seen in resting cells<sup>[31,33,68-70]</sup>. We know that tension can modulate many ion channels<sup>[38,39,67,71]</sup>, probably transporters<sup>[72]</sup> and other membrane bound enzymes. When you read a paper about patch clamp recording, remember that all data was obtained under extreme membrane tension<sup>[59]</sup> and the patch data may not apply to the same channels *in situ*.

Returning to our ideal lipid patch, without pressure applied to the pipette the patch is pulled flat by adhesion to the glass. When we apply pressure, the patch bends, and the average tension in the membrane can be calculated from Laplace's law that states that the tension  $T=Pr/2$  where  $P$  is the pressure across the membrane and  $r$  is the radius of curvature of the patch (not the radius of the pipette)<sup>[50,73-77]</sup>. From an image of a patch we should be able to calculate the membrane tension, and thus the stimulus that drives an embedded channel. Not so fast... notice that only the outer monolayer of the bilayer touches the glass. The inner monolayer is floating on the outer one<sup>[78-81]</sup>, and as a fluid it cannot have any tension gradients at equilibrium<sup>[82,83]</sup>.

Thus, even our simplest system has a gradient of tension normal to the membrane. You will notice that the papers on molecular dynamics simulations of MSCs apply a uniform tension across the bilayer in incorrectly compare those results to patch clamp data. While the mean tension in a membrane is related to the pressure across it by Laplace's law, models of membrane patches ignore the high membrane curvature where the patch

contacts the glass (Figure 3) at the upper end of a seal<sup>[44,68,84,85]</sup>.

### Planar bilayer

We might imagine that an even simpler experiment that gets rid of the glass, a planar bilayer where the membrane is floating in space<sup>[66,86-90]</sup>. However, these bilayer lipids are attached to a support structure such as a hole in a Teflon partition. Lipids wet the support, and this adherence creates significant tension (approximately 5 mN/m) in the bilayer (the bulk lipid lining the hole in the support is known as the Plateau-Gibbs border<sup>[91,92]</sup>, Figure 4). No one has made a measurement of channel activity in an unstressed planar bilayer<sup>[66,93]</sup>, and for MSCs, this is a serious bias. The bilayer experiments of Coste *et al.*<sup>[66]</sup> on the PIEZO channels suffer from this bias. If one is working with channels that inactivate, the resting tension in membranes, either in planar bilayers or patches, can put channels in an inactivated state making them invisible to a patch recording, and making a reconstitution experiment appear to have failed<sup>[18,43,48,94,95]</sup>.

### New method

There is one method, not currently in use, that might solve some of these problems. If the channels are reconstituted into large lipid vesicles (approximately 10  $\mu\text{m}$  in diameter), and the vesicles are patched in "whole-cell-mode" then most of the membrane is not in contact with glass. By controlling the pressure in the vesicle which is large enough to measure accurately the radius of curvature, Laplace's law will provide reliable estimates of the mean tension and there won't be significant tension gradients.

## STIMULATING CELLS

While lipid membranes are simple model systems, they are not cells. Cells are not homogenous solutions of macro molecules, but heterogeneous, anisotropic, viscoelastic/plastic structures made of multiple proteins and lipids with an extracellular matrix<sup>[43]</sup> that can carry stress between the exterior and the interior of the cells. But let's ignore these details for a moment and think of how we to experiment on cells.

### Direct mechanical stimulation

The simplest stimulus is to poke a cell, usually with a fire polished pipette<sup>[93]</sup>. What does that do to MSCs? Imagine pressing a chopstick against a clump of Jello representing the cell interior. There is an indentation (more formally called a displacement) at the site of stimulation, but the amount of displacement decreases with distance from the site of stimulation. The stimulation is not uniform even at this macro level. This variation of stress/strain with distance occurs whether one stresses the cell with a pipette, a magnetic bead<sup>[96]</sup>, a bead in a laser trap, an atomic force cantilever<sup>[97,98]</sup> or local perfusion<sup>[99]</sup>. It's

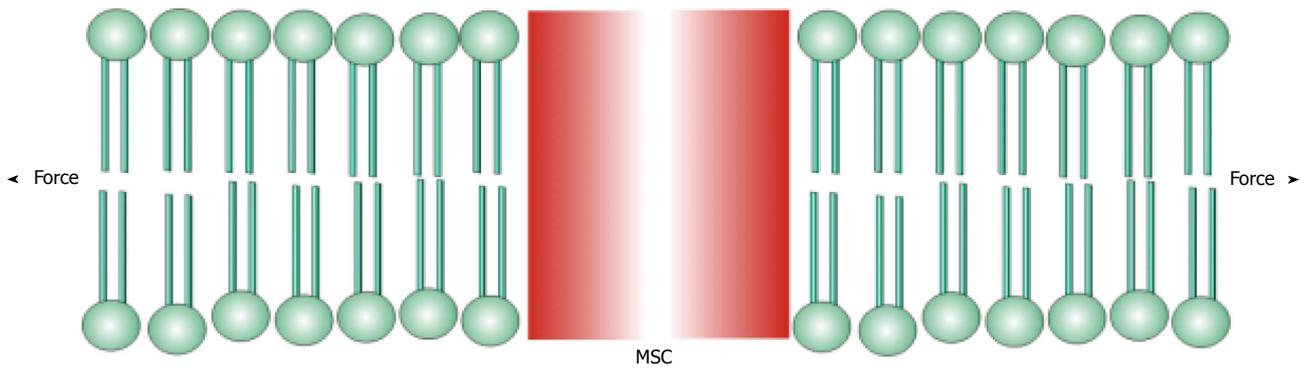


Figure 2 A mechanosensitive channel activated by tension (force) in the bilayer. MSC: Mechanosensitive channel.

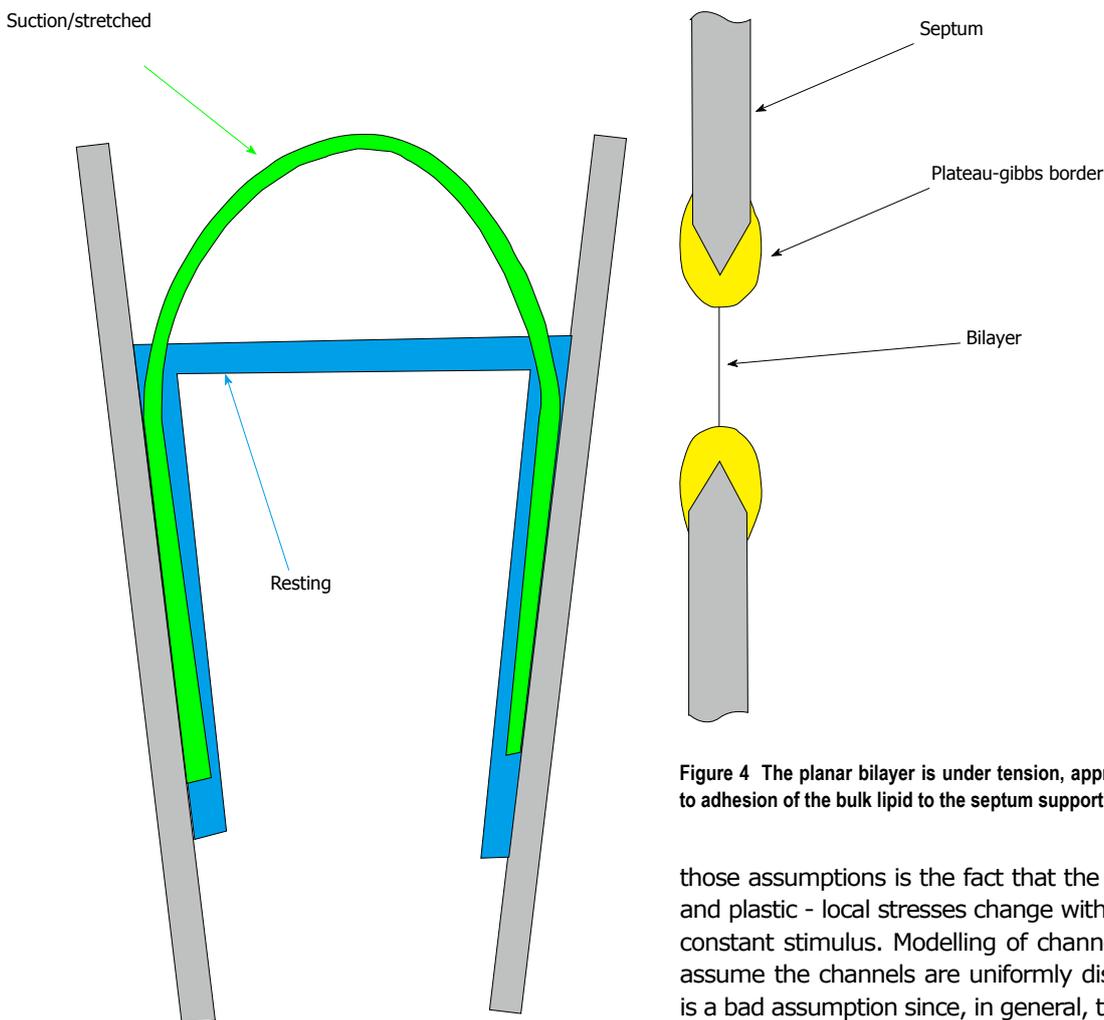


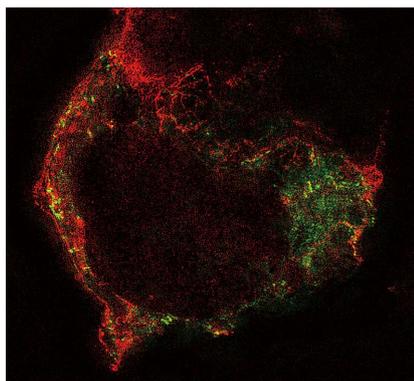
Figure 3 Cartoon of a patch pipette holding a lipid membrane at rest (pulled tight by adhesion to the glass) and under suction. Notice that suction peels the membrane off the glass a bit and functional channels.

Figure 4 The planar bilayer is under tension, approximately 6 mN/m due to adhesion of the bulk lipid to the septum support.

just a property of a deformable material<sup>[100]</sup>. When you record mechanically-induced currents from a cell, the response is represents a mean value from a distributed stress, and it decreases nonlinearly with distance but covers larger and larger areas<sup>[100]</sup>. The best you can hope for is that the response is proportional to the stimulus, and hopefully monotonic. Further complicating

those assumptions is the fact that the cell is viscoelastic and plastic - local stresses change with time even with a constant stimulus. Modelling of channels *in situ* usually assume the channels are uniformly distributed, but this is a bad assumption since, in general, they aren't (Figure 5). Remember to remain humble when interpreting your data since you really don't know the details of the stimulus.

In case I haven't yet scared you away from the field, let's look more closely at real cells. We will stick with whole-cell recordings where the local effect of the pipette glass is not significant. The key problem is determining what the channel feels. The ability to reconstitute bacterial MSCs in lipids shows the channels respond to tension in the bilayer and don't interact with a cytoskeleton, and that seems to apply to PIEZO MSCs as well<sup>[43]</sup>. What is the actual tension in the bilayer when



**Figure 5** Structured illumination image of an human embryonic kidney cell cotransfected with human PIEZO1 channels labelled green and TREK-1 channels labelled red using green fluorescent protein mutants. Notice that the channels are in different structural domains and thus feel different forces. Notice also that TREK-1 tends to follow underlying cytoskeletal fibers. (Courtesy Gottlieb P).

MSCs are activated in cells? No one has measured it. The bilayer is supported by the cytoskeleton that shields the bilayer from excess stress (known as “mechanoprotection”<sup>[36,101-103]</sup>). One experiment dealt with the distribution of stresses between the cytoskeleton and the bilayer in patches of an human embryonic kidney (HEK) cell, it there it was about 50:50. The cytoskeleton can thus alter the stress in the bilayer. Defects in the cytoskeleton can lead to diseases like muscular dystrophy<sup>[104-112]</sup>.

Laser trap measurements of bilayer tension in resting cells suggests that it is negligible<sup>[69,70,113-115]</sup>. That fits our common observation that MSCs are not active in resting cells (Figure 6)<sup>[53]</sup>. Why do cells make MSCs if they can’t be activated at normal stresses? Cortical stress is shared between the cytoskeleton and the bilayer, so bilayer stress reacts to cytoskeletal stress and *vice versa*, and these stresses are time dependent. The effective viscosity that makes responses time dependent arises from viscosity of the lipids and the dynamics of bonds in the cytoplasm<sup>[76,94,111,114,116-119]</sup>. The existence of connections between the bilayer and the cytoskeleton mean that any drugs that affect the cytoskeleton are likely to affect MSC activity, although drugs rarely are tested for these effects.

Adding to the complexity of defining the stimulus, lipids will flow under stress, and membrane lipids are not even homogeneous<sup>[46,76,116,120]</sup>. Spatial domains do exist<sup>[120-122]</sup> and physics tells us that the stress outside a domain is different from the stress inside a domain<sup>[73-75,123]</sup>. The energy gradient of stress at the edge of a domain is known as line tension. That affects the force inside the domain relative to that outside<sup>[75]</sup>. While we don’t know in detail the stimulus at an MSC, we are safe in assuming the stress is greater than zero and less than the lytic limit of the bilayer.

If the transmembrane domains of a channel are thicker or thinner than the surrounding bilayer, the bilayer will bend at the boundary and those stresses are likely to modify MSC activity<sup>[124]</sup>. This is termed a hydrophobic

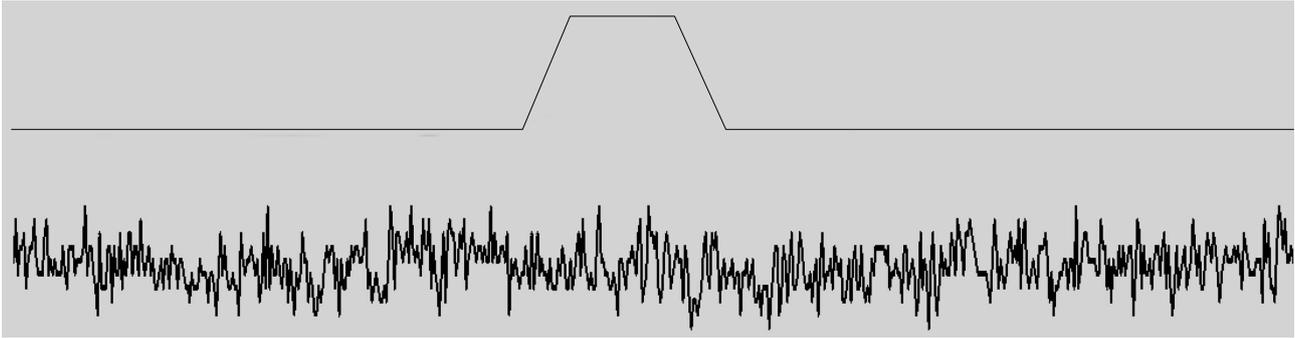
mismatch, but the local curvature does not extend more than a few lipids from the channel<sup>[90,125-127]</sup>. However, amphipaths can dissolve in the membrane<sup>[128-131]</sup> and interact locally with the channel modifying the local stress and affecting channel gating. For example, the general anesthetics at clinically relevant concentrations cause opening of two-pore domain K<sup>+</sup> selective TREK-1 channels<sup>[132]</sup>. Opening these channels hyperpolarizes neurons possibly accounting for general anesthesia. The presence of these channels may explain why people can be knocked out by a blow to the head.

### Osmotic stimulation

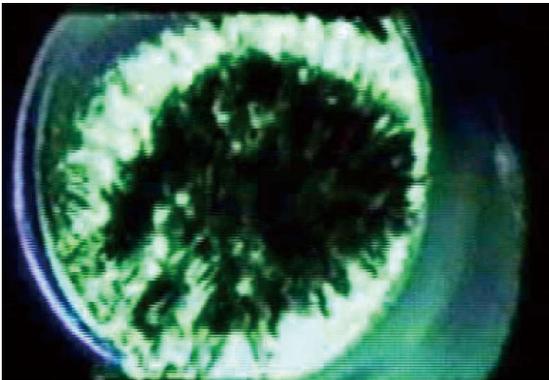
Suppose instead of these local mechanical stimuli we try for a more uniform stimulus like hypotonic stress? Cells swell with hypotonicity and we have been taught that swelling will stretch the membrane. If cells were spherical objects with a fluid cortex like red cells, that could work, but nucleated cells are filled with cross linked gels and the gels are what store most of the osmotic stress<sup>[133]</sup>. Consider the basic mechanics. Cells are not spherical so there are forces normal to the membrane. Secondly, with a given pressure across the membrane, the tension will depend upon the local radius of curvature (according to Laplace’s law), and cells do not have uniform curvature. But a more serious problem is that nucleated cells have a cytoskeleton that acts like a sponge, a three dimensional object that fills the cell volume. The mechanics of three dimensional objects are different<sup>[134]</sup> from those of two dimensional objects like membranes<sup>[98]</sup>.

We found that osmotic swelling doesn’t make the membrane tense unless the cytoskeleton is disrupted<sup>[98]</sup>, contrary to my intuition and years of textbook dogma. In fact, swelling tends to make cells softer<sup>[98]</sup>! How can that be? It turns out that everyone has done the experiment. When we pick up a dry kitchen sponge it is stiff. If we put it in the sink, it swells and soaks up water and it becomes softer. What is magic about a sponge? Nothing. It is just a set of cross-linked wettable polymers just like the cytoskeleton<sup>[26,135,136]</sup>, and cells presumably can move water the same way without the need to move solutes. The cell membrane still remains the rate limiting step for water movement, but most of the energy from an osmotic gradient is in the cytoplasm and not in the membrane<sup>[133]</sup>.

We visualized the distribution of osmotic stress in the cytoplasm using genetically coded optical stress sensors placed in structural proteins<sup>[26,27,29,30,119,137-139]</sup>. This three dimensional cross linked structure allows cells to withstand huge osmotic pressures<sup>[69]</sup>. (Ask yourself why sponges don’t lyse.) Many cells, like bovine endothelial cells (BAECs), can withstand distilled water for hours and remain viable. The predicted osmotic (hydrostatic) pressure due to exposure of cells to distilled water is about 7 Atm, twice the pressure in a car tire. The cell’s stability under this huge gradient arises because the cell interior is glued together like a sponge. In the case of BAECs, this bonding allows the cells to face severe



**Figure 6** A whole-cell current recording (lower trace) from a human embryonic kidney cell in response to indentation of a few  $\mu\text{m}$  with a fire-polished micropipette in a piezoelectric manipulator (upper trace shows the cell indentation). There is no current associated with the indentation even though human PIEZO1 was cloned from the same cells! RNA expression does not mean the presence of functional channels, and even cells with no obvious endogenous currents may have channels, but they can't be activated because of mechanoprotection. They may become visible with large and/or repeated stimuli or treatment with agents like cytochalasin. (The trace is 1 s long and the RMS current noise is about 1 pA). RMS: Root-mean-square.



**Figure 7** A frame from tomographic reconstruction of a patch of a *Xenopus* oocyte using high voltage electron microscope tomography<sup>[1]</sup>. The image shows cytoskeleton spanning the pipette and the bilayer is attached to the upper side but is not visible in this reconstruction due to its low density.

viscous drag in arteries where blood flow tries to rip the apical cortex from the basal cortex<sup>[27,140,141]</sup>. Osmotic stress does not stress the cell membrane very much, and despite many citations in the literature, osmotic stress should not be considered a "mechanical stimulus" of the cell membrane. Do not accept the results of papers that claim it is. Instead, treat those papers literally as dealing with the effects of osmotic stress.

There are a vast number of papers on cell volume regulation<sup>[55,98,142-147]</sup> invoking various ion channels such as the BK channels<sup>[148]</sup> and other  $\text{K}^+$  channels<sup>[149]</sup>, chloride channels<sup>[147,150-152]</sup> as well as neutral transporters<sup>[143,153,154]</sup> and water transporters<sup>[155-157]</sup> as well as the cytoskeleton<sup>[26-29,98,139,158]</sup> and host of calcium and other intracellular messengers<sup>[15,159-161]</sup>. Given the vast scale of modulators and potential effectors, it is unwise to think of cell volume as a specific stimulus.

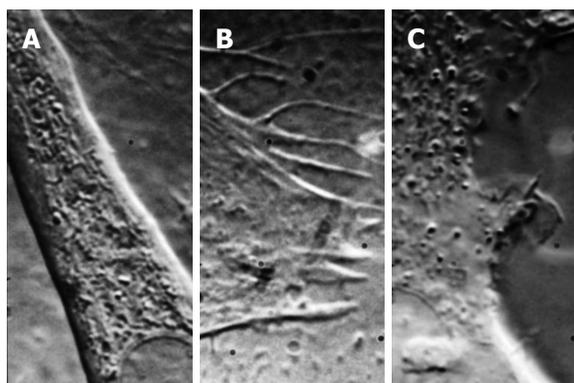
### Patch clamp stimulation

We all know about patch clamp recording and the revolution it created in our understanding of ion channels<sup>[162,163]</sup>. But what is a patch? The dogma says it is a bilayer containing channels<sup>[163]</sup> that spans the pipette.

However, unless you are working with lipid vesicles, that is incorrect; patches are pieces of the cell cortex. Microscopy of patches (light microscopy<sup>[44,164]</sup> and electron microscopy<sup>[1,165-168]</sup>) show that patches are samples of the cell cortex, including the cytoskeleton (Figure 7)<sup>[1,165]</sup>.

Whenever you make a patch, cell-attached or excised, the bilayer that contains your channels shares its stress with the cytoskeleton. How much does the bilayer feel in this composite structure? In the only published paper on the matter<sup>[169]</sup>, we compared the amount of mechanical stress required to break a patch (pipette suction) with the voltage required to break the patch (typical of patch clamp "zap" voltages). The mechanical stress measured the lytic stress of the entire cortex. Voltage measured only the stress of the bilayer since that is where the voltage drop occurs. We measured the voltage required to lyse a patch as a function of the mechanical stress; the more mechanical stress, the less voltage. Since voltage only exerts force on the bilayer, we could separate the bilayer stress from the mean stress. It turns out that the bilayer lyses with a constant energy density, whether it comes from mechanics or voltage. For our particular cells, HEK-293, about half of the applied stress was in the bilayer and the rest in the cytoskeleton, but that result is from patches and we do not know how that applies to resting cells.

Regardless of the degree of stress sharing, no one has ever measured channel currents in a patch that emulates the tension characteristic of a resting cell<sup>[50,76]</sup>. The magnitude of the resting stress in a patch was emphasized to us when we tried to use Triton-X100 to lyse patches. It doesn't work. The patches are stable. The reason is that detergents work by forming micelles. If you want to form a micelle in a patch under tension, you need to increase the membrane area since a plane plus a sphere has more area than the plane. The energy required to change the area of a membrane under tension  $T$  is  $\Delta G = T \Delta A$  where  $\Delta A$  is the change in area (Hooke's law in two dimensions). The energy available to the detergent is insufficient to form a micelle, but the



**Figure 8** Differential interference contrast images of three different cell types (mouse myotube, left; rat astrocyte, center; human embryonic kidney cell, right) showing the variability of membrane structure and how patch clamp recordings are expected to be variable. The structures above change rapidly over time (this is a frame from a movie, Courtesy Suchyna T).

same detergent works well in the resting cell the patch because its membrane is not under tension.

Is the cytoskeleton of a patch the same as that in a cell? We don't know, but we do know that the chemical composition of a patch is different from that of the cell it was taken from<sup>[44]</sup>. We labelled different components of cell membranes and then patched them. We found that some elements made it to the pipette-spanning dome, and some didn't, notoriously the extracellular matrix<sup>[44]</sup>. That never even made it into the seal region. Some ion channels made it, and some didn't. You need to think of the pipette as a silica column. Biochemists know proteins stick to silica, so after dragging a membrane up a pipette, some things will stick to the glass and get filtered out, and some will make it to the dome.

The heterogeneity of the patch emulates the heterogeneity of the cell membrane and we know that the cell membrane is not homogenous (Figure 8). Even pure bilayers may not be homogeneous. Like ice and water, there are phase separations<sup>[170]</sup>. The amount of each phase (the fraction of total membrane area) is modulated by internal and external conditions. Cell membranes are much more complicated. If you look at a time lapse movie of cells, you will be impressed by the motion of the cell surface. Imagine your patch pipette coming down on one of these cells and then try to figure out which piece of membrane you patched. The answer, of course, is that you have no idea. Furthermore, given the data showing changes in patch composition with patch formation<sup>[44]</sup>, and visible domains in a patch, you are in fact recording from a new mixture of cellular components. We suspect that patches might contain membrane from the endoplasmic reticulum and other organelles as well as the plasmalemma. Someone needs to check on that. We think that patch clamp recordings are as reproducible as they are because the formation of the patch helps to homogenize the components. In any case, be cautious about assuming that the properties of currents you get from a patch are the same as you would observe *in situ*.

## WHAT CHANNEL ARE YOU RECORDING FROM?

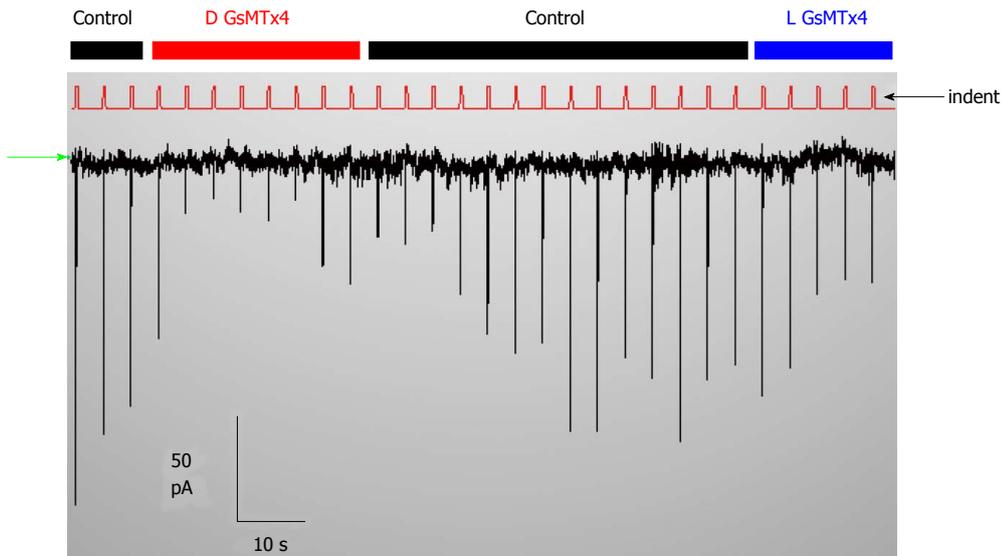
We know that most if not all types of cells have endogenous cationic MSCs<sup>[15,17,51,55,128,171-174]</sup>. You may not see them frequently as they are normally closed because bilayer stress is shielded by the cytoskeleton ("mechanoprotection")<sup>[71,93]</sup>. You must know your background channel activity if you want to examine cells containing transfected channels. Treating the cells with cytochalasin or latrunculin to break up the cytoskeleton will reduce mechanoprotection and make background channels more visible<sup>[171]</sup>. Cell lines vary from lab to lab. According to the literature Coste *et al*<sup>[93]</sup> used N2A cells to clone PIEZO1, but Lee *et al*<sup>[71]</sup> used the same cell line and found no background PIEZO1 and 2 activity. Why? I expect that the cytoskeleton changes with passage number and with different batches of serum.

Because of the nearly universal presence of background channels, seeing a cationic MSC current after transfection does not mean you are seeing the channel coded by the DNA you used to transfect the cells. Furthermore, the expression of an MSC (or probably many other proteins) can cause massive structural changes in the cytoskeleton, even if the channel is non-conducting<sup>[175]</sup>. Thus, the process of transfection alone (not the effect of the transfection reagents, *per se*) can modify the forces that reach the channels.

We can now study cytoskeletal protein stress using genetically coded stress sensors<sup>[3,25,26,28-30,119,137-139,176]</sup>. The same issues apply to siRNA since suppression of one protein can affect others. For example, we showed that cytochalasin or colchicine affects the stress in actinin, spectrin and filamin and likely other structural proteins that are not judged to be the drug targets. When we modify any protein in a cell, we modify the stresses in the elements that are coupled to that protein.

Transfection can be a dangerous game. You can easily show modified RNA expression, we know that RNA expression is not cleanly related to the presence of functional channels. We cloned the human form of PIEZO1 and 2 from HEK cells<sup>[53]</sup>, a human cell line of neural origin that usually exhibits little background MSC activity (Figure 6). The N2A cells that Coste *et al*<sup>[66]</sup> initially used to isolate PIEZO1, had no background MSC activity in other samples of the same cell line<sup>[71]</sup>. So how do we know what channels produced the currents we are looking at?

The best test would be to create a mutant channel with similar gating functions but with visibly different ionic selectivity than the endogenous channels. You cannot depend upon channel conductance alone<sup>[57]</sup> as a sound marker of expressed channels since it is easy to find situations in which the environment: cell-attached patch, inside-out or outside-out patch, or whole-cell, or planar bilayer have different conductances<sup>[66,177]</sup>. You would want a channel with big differences in selectivity, ideally a change from cation to anion selectivity!



**Figure 9** There are no PIEZO1 currents in this resting whole-cell recording unless the cell are indented (the red trace labelled indent shows the stimuli). The inhibitor GsMtx4 is effective in suppressing the stimulus-induced current but causes no change in the holding current. The stimuli are activated by computer control ([www.qub.buffalo.edu](http://www.qub.buffalo.edu)). The baseline current (green arrow at left) is not affected by adding the D or the L enantiomers of GsMtx4, but the currents that are evoked by indentation are inhibited. Thus, tension in the resting cell is insufficient to activate PIEZO1.

You also cannot trust the channel kinetics as a marker since the kinetics of the channels depend upon their environment<sup>[177,178]</sup> and you have little control of that. Furthermore, you do not know if the channel you are trying to express might have a subunit that associates with an endogenous channel subunit or accessory protein, or induces the expression of previously unexpressed endogenous protein. It is well known that mechanical stress alters gene expression<sup>[179-182]</sup>. We know that expression of two different MSCs can create currents that do not belong to expression of either one alone<sup>[71]</sup>. There are no clean solutions in cells. We arbitrarily tend to look more closely at transfections that produce currents much larger than the background channels. We also don't know that we are looking at homomers of the transfected proteins, but we often make the simplifying assumption they are (this also aids in grant funding). Expression of green fluorescent protein labelled channels on the cell surface<sup>[43,48]</sup> or in patch membranes<sup>[44]</sup> does not mean that the fluorescent object is a functional channel, simply that the protein is present. We have tested the mechanical sensitivity of labelled transient receptor potential-canonical channels in patches and found they are present but are not mechanically sensitive<sup>[183]</sup>.

## WHY BOTHER WITH MSCS?

Cationic MSCs are normally protected from cell stress, so what do they do for a living? They do not seem to participate in behavior of normal hearts<sup>[184]</sup>, but they do in stretched hearts where they seem to play a role in generating arrhythmias like atrial fibrillation<sup>[184]</sup>. They also play a role in muscular dystrophy where the channels produce a  $\text{Ca}^{2+}$  leak when the muscle is stretched<sup>[110,185]</sup>. We have come to believe that the typical cationic MSCs, like PIEZO1, serve primarily as sensors for potential

bilayer failure. They would inform the cytoskeleton that the local bilayer is under excessive stress and likely to break, and the ion fluxes through the channel are signaling for mechanical reinforcement. The channels are functioning a bit like fire alarms whose function you unaware of until disaster looms. If the channels are closed in the resting cell you will not see the effect of inhibitory drugs like GsMtx4 on the currents (Figure 9). But if the same drug is active on open MSCs, you will see an effect, but to open the channels may require pathologic stress. Since channels like PIEZO inactivate, the information about the excess stress is transitory.

PIEZO1 mutations can cause anemias<sup>[43]</sup>, and we have wondered why are these channels that inactivate quickly ( $< 30$  ms) are present in red cells<sup>[186]</sup>. When does a red cell need such a short lived channel? We guess that the only time red cell stress becomes "pathological" is upon entry and exit from a capillary or perhaps a bifurcation. It might modify ion and water concentrations to reduce stress on the membrane as it is highly deformed upon entering the capillary. The same channels may be involved in sickle cell anemia where hemoglobin crystals push out on the membrane activating PIEZO1<sup>[15]</sup>.

The universal presence of MSCs fits the common demand of all cells to avoid lysis and that occurs in disease. GsMtx4<sup>[187]</sup> and other agents that might act specifically on MSCs promise to be a new class of therapeutic agent with ideal selectivity they would only affect sick cells. We have found that GsMtx4 can be administered to mice daily for a month with no effect on behavior, and it can be injected into the CNS with no effect on behavior, but it does work to inhibit volume stimulated arrhythmias<sup>[188]</sup> and the phenotype of muscular dystrophy<sup>[109]</sup>.

There is evidence that PIEZO2 channels may serve

a sensory role in nociception<sup>[4]</sup>. Since PIEZO was only cloned a few years ago, we have a lot more work to do. A nagging problem is why is PIEZO so big<sup>[189]</sup> - it is the largest transmembrane protein (approximately 2500 amino acids) and even tends to form tetramers with a MW of about 10<sup>6</sup> with the N and C termini about 20-30 nm apart<sup>[190]</sup> making us suspicious that PIEZOs have other functions; a large size is not necessary for MSC function<sup>[43,67]</sup>. There are many kinds of MSCs<sup>[191-194]</sup>, nearly a dozen in bacteria alone, so we have lots of interesting problems to keep us busy.

## CONCLUSION

This review has two goals, nominally for investigators new to the field of mechanotransduction: (1) Be humble about your data because you generally don't know your stimulus, and be explicit about your assumptions so people can read your paper properly. Quantitative models of the data have the intrinsic appeal of making the assumptions explicit; and (2) Create new preparations that can answer some of the pressing host of new questions.

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