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Piezo1

Properties of a cation selective mechanical channel

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Piezo ion channels have been found to be essential for mechanical responses in cells. These channels were first shown to exist in Neuro2A cells, and the gene was identified by siRNAs that diminished the mechanical response. Piezo channels are approximately 2500 amino acids long, have between 24–32 transmembrane regions, and appear to assemble into tetramers and require no other proteins for activity. They have a reversal potential around 0 mV and show voltage dependent inactivation. The channel is constitutively active in liposomes, indicating that no cytoskeletal elements are required. Heterologous expression of the Piezo protein can create mechanical sensitivity in otherwise insensitive cells.

Piezo1 currents in outside-out patches were blocked by the extracellular MSC inhibitor peptide GsMTx4. Both enantiomeric forms of GsMTx4 inhibited channel activity in a manner similar to endogenous mechanical channels. Piezo1 can adopt a tonic (non-inactivating) form with repeated stimulation. The transition to the non-inactivating form generally occurs in large groups of channels, indicating that the channels exist in domains, and once the domain is compromised, the members simultaneously adopt new properties.

Piezo proteins are associated with physiological responses in cells, such as the reaction to noxious stimulus of Drosophila larvae. Recent work measuring cell crowding, shows that Piezo1 is essential for the removal of extra cells without apoptosis. Piezo1 mutations have also been linked to the pathological response of red blood cells in a genetic disease called Xerocytosis. These finding suggest that Piezo1 is a key player in cells' responses to mechanical stimuli.

Introduction

How are mechanical forces sensed by cells? All organisms and tissues have protein mechanosensitive ion channels (MSCs) that report on local stress in their environment. Since ion channels have the highest turnover rates of all enzymes, they are efficient sensors that allow for a rapid cellular response. The best studied MSCs are those in bacteria[1] where they protect the cells against hyposmotic shock. Their physiology has been well studied[2,3] as well as the molecular structure[4,5] and three-dimensional structure.[6-9] These channels have served as models of mechanosensitive channels in general; however, there is no sequence or structural homology even within the bacterial channels, let alone the eukaryotic channels, so generalizations are limited. There is no particular domain structure associated with mechanical sensitivity, unlike the S4 domain for voltage dependent channels.[10,11] The first MSCs ever observed were in chick skeletal muscle when a membrane patch was stretched with hydrostatic pressure.[12] The protein(s) responsible for these responses have yet to be cloned but they are likely to be members of the newly discovered Piezo family. Piezo channels were identified using a combination of patch clamp, whole cell stimulation/recording and molecular biology.[13]

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The Piezo Ion Channel Family

Piezo proteins were first identified as potential channels in the cell line Neuro2A (a glial tumor line) by the Patapoutian group.[13] In whole cell mode, Coste et al. mechanically stimulated many cell types looking for a cell line with a strong and reproducible mechanical response and they found it in Neuro2A. The currents evoked by pressing on the cells with a glass probe increased with the depth of penetration, the reversal potential was near zero, and the currents rapidly inactivated.

To identify the gene(s) responsible for the current, they analyzed cellular transcripts of membrane proteins with unknown function using silencing RNA to knock out individual transcripts.[13] The screen of nearly 75 genes identified one called *Fam38A* as essential. The gene had been previously associated with senile plaque-associated astrocytes[14] and found to be associated with integrins and cell adhesion in endothelial cells.[15] The protein is approximately 2500 amino acids long and shows no homology to other channels and was named Piezo1. An analysis of hydrophobicity regions suggested that there are between 24 and 36 trans-membrane domains.

Coste et al. also cloned a homologous gene (called Piezo2) from dorsal root ganglion (DRG) cells and searches of the databases showed that there were homologous genes in many animals and plants.[13] Piezo2 currents were similar to Piezo1 but had quantitatively different kinetics and conductance; they inactivated more rapidly andhad a lower unitary conductance and lower expression level. Using quantitative PCR, the authors found that RNA coding for Piezos varied in concentration with lung, bladder and skin

having exceptionally high concentrations of Piezo1. Piezo2 was highly expressed in DRG neurons. It is important to emphasize that for MSCs, channel density is probably not the dominant means of controlling responsiveness.[16] More likely it is the regulation of cytoskeletal mechanics that changes bilayer stress.[17-20]

To begin characterizing the biophysical properties of Piezo channels, Coste et al. expressed Piezo1 and 2 in HEK293 cells and patched them.[13] The mechanical responses were similar to the whole cell currents observed in Neuro2A (Piezo1) and DRGs (Piezo2), with currents showing voltage-dependent inactivation that slowed with depolarization, and the currents reversed near zero mV. The presence of Piezo1 in the plasma membrane was confirmed using antibodies. These results provided convincing evidence that Piezo 1 and 2 were genetic products associated with the mechanical response.

The Pore-Forming Unit of Piezo

The question then arose, was the protein a channel or simply a cofactor? To answer this, Coste et al. used two complementary approaches to look for auxiliary proteins.[14] In one approach, they chemically cross-linked all closely bound proteins in the cells. On a denaturing gel, Piezo proteins appeared with discrete molecular weights representing an integral number of monomers. The maximum size was a daunting ~1.2 million Daltons suggesting that it was a tetramer. Mass spec analysis of the cross-linked proteins showed no other bound proteins. Addressing the issue of whether it was a tetramer, the authors added a GFP to Piezo1 and using single molecule imaging techniques bleached the bound GFP. They found that it bleached in four discrete steps as expected from a homotetramer. This experiment did not show that the pore-forming unit was a tetramer, only that in the membrane the protein existed primarily as a tetramer. The predictions for the behavior of a tetrameric channel, for example, are that if the channel was a tetramer of independent monomers with each monomer containing a gated channel, we would expect to see currents in a binomially distribution[21,22] arising from groups of four, but that has not been observed. If the tetramer formed the active channel cooperatively, it would appear as a single channel as observed. It remains unclear why nature might have resorted to such a gigantic structure to simply serve as a mechanical transducer, so the size suggests other functions. For example, expression of 2P mechanosensitive channels, whether conducting or not, massively alters the cytoskeletal structure.[23]

To test whether cytoskeletal proteins might be involved in gating Piezo1, Coste et al. labeled them with GST and then isolated and reconstituted them into liposomes and planar bilayers. They observed single channel activity in both preparations.[14] To make sure all the responding channels were oriented in the same way in the membrane, and to further correlate the data with cell based recordings, they inhibited channels facing one side of the membrane using ruthenium red that is known to inhibit Piezo currents in cells.[13] The reconstituted channels were constitutively active as expected since both planer bilayers and liposome patches are under high resting tension.[17] However, the channel's kinetics did not display inactivation behavior such as bursts of channel openings with burst durations on the order of 30–50 ms, the characteristic inactivation time. There was also no data to show that that channel activity was sensitive to applied membrane tension. However, these results do suggest the channel can be reconstituted and do not require cytoskeletal elements for activation and, like bacterial MSCs, is probably activated by bilayer tension.

Inhibition of Piezo1 Channels

A traditional method for studying ion channels is through the use of inhibitors. Coste et al.[13] showed that Piezo channels are inhibited by Ruthenium Red, a nonspecific inhibitor of many cation channels, but its lack of specificity limits its usefulness. GsMTx4 is a much more specific inhibitor of endogenous cationic MSCs. It is a peptide isolated from a blind search of arachnid venoms,[24,25] is about half the size of insulin and is a member of the ICK family of peptides.[26,27] The mechanism of action on mechanical channels is unique since the D and L enantiomers have equal efficacy so the key interactions are long range.[28] GsMTx4 acts as a gating modifier by inserting at the channel-lipid interface and prestressing the channel toward the closed conformation. Since GsMTx4 worked on endogenous MSCs, we tested whether GsMTx4 could inhibit Piezo1.[29]

Using outside-out patches from cells transfected with Piezo1 (clone courtesy of A. Patapoutian), we found mechanically stimulated channel activity with voltage-dependent inactivation, a reversal potential near 0 mV and a pressure dependency similar to the cell attached recordings.[29] This activity was suppressed by GsMTx4 with a K_d near 200 nM. The kinetics of association and dissociation were similar to those observed for endogenous MSCs.[25] The properties of inhibition were nearly identical to those seen for endogenous cation MSCs including weak voltage dependency, gating modifier behavior, and equal sensitivity to both enantiomers. The gating modifier appeared as a 28 mmHg left shift in the activation curve. GsMTx4 also inhibited whole cell Piezo1 currents. The dissociation kinetics in whole cell mode is similar to that measured for outside-outs, but the association rate was approximately 10-fold slower. This may reflect differences in the background tension of patches and resting cells.

Biophysical Properties of Piezo1

Patapoutian's group showed inactivation rates of ~15 ms at -80 mV for whole cell currents of Piezo1 in HEK293 cells.[13] The half-maximal pressure for channel activation (P_{50}) in cell-attached mode was approximately -30 mmHg. We repeated these measurements in outside-out patches and found inactivation relaxation times of about 40 ms at -50 mV slowing with depolarization and P_{50} was around -40 mmHg.[30] Single channel recordings showed voltage-dependent inactivation as well.

We observed a striking irreversible transition from inactivating to non-inactivating currents[30] and the transition was potentiated by repeated stimulation. The latency to make the transition cannot be precisely controlled, but the transition itself probably represents a domain failure in the cytoskeleton

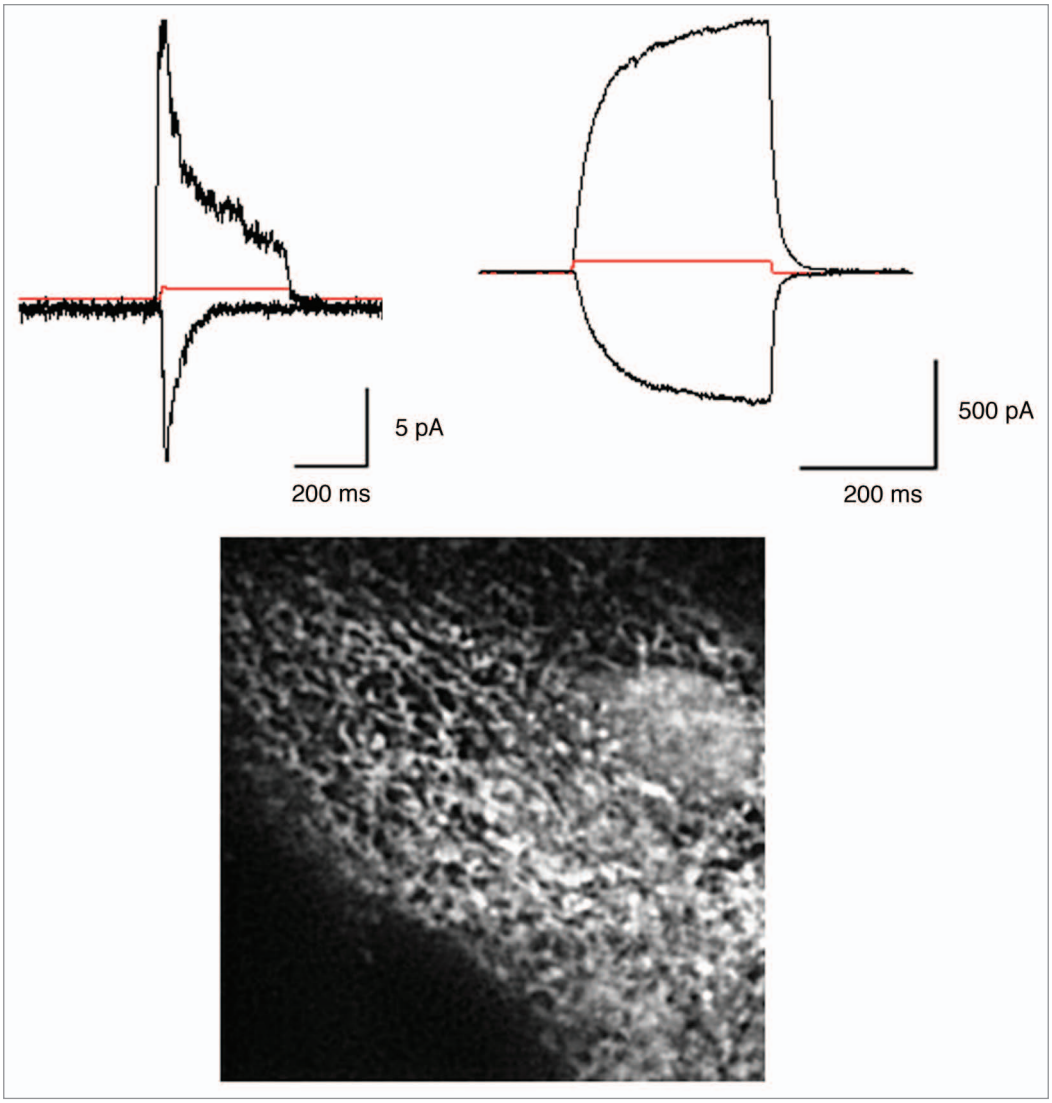

Figure 1. Top right panel: Typical response of Piezo currents in an outside-out patch exhibiting slow inactivation with depolarization and fast inactivation during hyperpolarization (pipette solution KCl saline with 3 mM Mg^{+2}, 60 mmHg at -40 mV and +40 mV). Notice the extremely fast rise time of the current. Top left panel: Replacing the Mg^{+2} with Zn^{+2} increases the peak current ~100-fold and it is non-inactivating. Bottom panel: The distribution of Piezo1 in PtK2 cells. Cell were imaged using TIRF after transfection (100 ng DNA) with a Piezo1 construct that links gfp to the N-terminal of Piezo1 (the image is 16 μm on a side). The image shows channels distributed all over the cell bottom surface but in a striking latticework distibution that may represent the mechanical domains of the channel (image courtesy of Heng Huang and Arnd Pralle, Department of Physics, SUNY at Buffalo).

that alters the stress applied to the channels. This domain property has also been observed with whole cell currents in heart cells.[31] From a functional perspective, inactivation guarantees that if the channel should open spontaneously, the response will only last about 30 ms and thus the cell is well protected against nonspecific responses. If inactivation is a functional aspect of Piezo physiology, it is unclear how such phasic responses are useful outside of the differentiated sensory organs. However, mechanical signals generated in the cell can be transmitted across the cell with sub-millisecond latency[32] suggesting that rapid inactivation may make Piezo specialized for intracellular signaling.

The irreversible loss of inactivation represents a mode of activity that can make the channel into a tonic receptor, and we do not yet know whether cells have taken advantage of this form of modulation for physiological purposes. Inactivation can be modulated by factors other than voltage and exercise. We observed that 3 mM Zn^{2+} on the intracellular face of the membrane removes inactivation and increases currents by 100-fold compared with internal Mg^{2+} ions (see **Fig. 1**). This shows that there are many more channels in the patch than are seen during a typical experiment and since the cell has about one thousand times the area of a patch there are a huge number of channels available but only a small fraction can be stimulated. Using a GFP linked human Piezo1 gene we have imaged the distribution using TIRF microscopy and remarkably the channel is distributed everywhere even on the bottom of the cell (**Fig. 1**) confirming the presence of a significant number of

channels. The images show that Piezo seems to be distributed in a latticework that may define a relevant mechanical domain.

Stress-dependent activation of Piezo is extremely fast and resolution is limited to the rise time of the pressure clamp, which is a few milliseconds.[33] This speed is very surprising given that the mechanical relaxation time of a patch requires hundreds of milliseconds.[17,34] The rapid rise time may reflect pressure driven fluid flow of bilayer lipids in the patch dome and seal[35] before the viscosity of the cytoplasm relaxes.

A remarkable property of the loss of inactivation with exercise is that it occurs simultaneously across many channels.[30] If inactivation were a property of individual channels, we would expect to see a randomized transfer from the inactivating to a non-inactivating pool that would show mean currents with a transient component and a plateau in smoothly varying proportions. The simultaneous transition of a group of channels implies that they exist in a common domain that can undergo a rapid physical change. This collective loss of inactivation or sensitization has long been observed with endogenous MSCs.[31,36-38] The domains might be rafts, corrals or caveolae that can be broken by repeated stimulation. Upon breakage, the stress in the domain will relax to more closely reflect the mean stress in the cortex. The ability of a cell to modulate the domain size, domain compliance and channel density provide a wide range of modulation potential. The domain properties are likely to be affected by cytoskeletal components in series and parallel so that local bilayer tension depends upon the status of the cytoskeleton.[39]

CytochalasinD (cytoD) disruption of f-actin surprisingly resulted in a loss of whole cell currents in HEK293 cells, but in patches of all conformations, Piezo activity was unaffected.[30] This again warns us to be cautious about predicting in situ behavior from in vitro behavior. We could pre-stress the cytoskeleton with hypotonic solutions before whole cell indentation, significantly increasing evoked currents. In resting cells most MSCs are shielded from external stress, a term called "mechanoprotection."[40,41] We know that the channels in the resting cell are not active because the inhibitor GsMTx4 has no effect on the holding current[30] or Ca^{2+} influx.[18] Mechanoprotection by parallel elastic elements of the cytoskeleton is likely to be a major form of sensitivity modulation of all MSCs.

Other Eukaryotic Cationic MSCs

Piezo channels are not the only family of eukaryotic cation MSCs. Much of what we know other cationic mechanosensors has been derived from genetic and biochemical information and includes ENaC/Deg family channels[42,43] and TRP channels[44] as well as GPCRs,[45] although direct involvement of TRPC mechanosensitivity has not been demonstrated.[46] The potassium selective P2 mechanical channels such as TREK1 have been studied for many years[47-49] and an X-ray structure has been recently published.[50] The X-ray structure does not reveal specific structures responsible for mechanical sensitivity. We would like to point out that mechanical stress is a universal modulator of membrane proteins in the same way as voltage. For example, every voltage-sensitive channel that has been so studied is also modulated by membrane tension.[40,51,52]

Physiological Roles of Piezo

The first demonstration of a physiological role for Piezo was a knockout of the nociceptive response in *Drosophila melanogaster* larvae.[53] The Drosophila channels have similar properties to the mouse Piezo with slightly different kinetics of inactivation and conductance. With the knockout, larvae lost their rollover behavior in response to poking but gentle touch sensitivity was unaffected. Genetic reintroduction of the channel restored nociceptive sensitivity. The data also showed that there was a second parallel mechanosensory pathway that utilized DEG/ENAC channels *(ppk)*. Removal of both channel types made the larvae completely unresponsive to noxious mechanical stimuli.

Piezo Channels in Homeostatic Control of Cell Number

How do cells know when to stop dividing? This is not only relevant to cancer but to the normal growth of the epithelia and other cells. Why don't cell layers keep on reproducing and buckle? This control of growth is most likely is a mechanical sensing modality. Extrusion of excess cells from fully formed epithelia involves two unrelated processes: one that prevents live cell crowding and a second that removes damaged cells. The ultimate fate of cell death from cell-crowding is sensed by Piezo1, and damaged cells are removed by apoptosis.[54]

In the above study, MDCK epithelial cells were grown on a stretched elastic sheet that was then relaxed causing cell crowding. The cells were extruded from the overcrowded core were not susceptible to apoptosis when BCL2 was overexpressed. A direct link to mechanosensitivity was that Gd^{+3}, a common inhibitor of MSCs,[55] inhibited the overcrowding response. Piezo1 involvement in the process came from zebra fish where introducing morpholino-silencing RNA to knock out Piezo1 resulted in a significantly diminished response to extrusion.[56] These experiments underscore the close relationship between cytoskeleton remodeling, cell physiology and cortical stress.

Piezo1 Mutants in Pathology

Recent work showed that hereditary xerocytosis (HX) is caused by specific mutations in Piezo1.[57] HX is an autosomal dominant hemolytic anemia involving erythrocyte dehydration. This results in an increase in concentration of corpuscular hemoglobin and an increase in osmotic fragility. This is the first work to identify a disease that may results from mutations to Piezo1. However, it has also been shown that volume regulation of sickled red cells is sensitive to GsMTx4 again suggesting Piezo involvement.[58] Based upon GsMTx4 sensitivity MSCs have been implicated in the volume regulation of some cell types and excluded from others.[59] It is not yet known how Piezo1 mutants cause xerocytosis but the authors proposed that repeated cycles of oxygenation/deoxygenation may result in their eventual dehydration. How activation of a cation channel causes dehydration needs to be examined but it likely involves Ca^{2+} permeation. The repeated changes in erythrocyte shape with changes

in oxygenation, are reminiscent of the loss of inactivation of Piezo1 with repeated stimulation. An obvious challenge is to study the biophysics of these mutants.

Summary

The newly cloned Piezo family of MSCs has opened the door to mutational analysis of eukaryotic MSC function. Key areas that require study include pharmacology, domain structures, correlation of inactivation to domain structure and the exploration of tonic and phasic sensing modalities. There remain the basic science questions of the topology of transmembrane domains and linkers, locating the channel pore, testing whether the channel functions as a monomer or tetramer with independent or dependent subunits, why the channel must be so large to do what bacterial channels can do in a much smaller volume, measuring the X-ray structure, etc. We are due for a festive time!

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