Dynamic regulation of TREK1 gating by Polycystin 2 via a Filamin A-mediated cytoskeletal mechanism

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Abstract

Mechanosensing is essential for several physiological functions including osmoregulation, touch and pain sensation, hearing, blood pressure regulation, salt and fluid balance, urination, tissue growth, and vestibular function. Understanding how mechanosensitive ion channels (MSCs) are gated thus brings important information regarding the aforementioned physiological processes. In this thesis, we focused specifically on the TREK1 channel and its gating by polycystin 2 (TRPP2). The TREK subfamily of two-pore domain potassium channels (K₂P) includes TREK1, TREK2, and TRAAK, all of which are mechanosensitive. When TREK1 channels are expressed in Cos7 cells, a mechanically-induced outward current is observed. In basal conditions, this current is under a partial inhibition by the F-actin cytoskeleton. Previous work has demonstrated that TRPP2 can increase the inhibitory effect of the F-actin cytoskeleton in TREK1 currents by recruiting the actin binding protein filamin A (FLNa). However, the characteristics of this inhibition are poorly understood. Our hypothesis is that this inhibition is highly dynamic and is mediated by a FLNa-dependent increase in F-actin turnover. Our results show that gradual removal of the F-actin cytoskeleton relieves TRPP2-mediated TREK1 inhibition. Moreover, the rate at which the inhibition recovers is faster in cells expressing TREK1 with TRPP2 than TREK1 alone, suggesting an increased F-actin turnover. However, through Fluorescence Recovery After Photobleaching (FRAP), we show that the F-actin dynamics are not enhanced in the presence of TRPP2. We have also determined that this process requires FLNa as TRPP2 does not alter the recovery of TREK1 inhibition in FLNa knockout (M2) cells but does so in wild type control (A7) cells. Through immunofluorescence techniques and generation of a stable S8 cell line expressing FLNa-RFP, we show that FLNa is instead recruited to the cell membrane in the presence of TRPP2. This recruitment leads to an alteration
in the subcellular reorganization of actin filaments that in turn is believed to regulate TREK1 opening.
Résumé

Le détection mécanique est essentiel pour plusieurs fonctions physiologiques incluant la régulation osmotique, la sensation de douleur et le toucher, de l'audition, la régulation de la pression artérielle, l'équilibre des fluides et le sel, la miction, la croissance des tissus, et la fonction vestibulaire. Comprendre comment les canaux ioniques et mécanosensible sont régulé apporte ainsi des informations importantes concernant les processus physiologiques susmentionnés. Dans cette thèse, nous nous sommes concentrés spécifiquement sur le canal TREK1 et son déclenchement par polycystine 2 (TRPP2). La sous-famille de TREK comprend TREK1, TREK2, et TRAAK qui sont tous des canaux de potassium contenant deux domaines de pores (K2P) et qui sont tous mécanosensible. Lorsque les canaux de TREK1 sont exprimés dans des cellules Cos7, on observe un courant induite mécaniquement vers l'extérieur. Dans des conditions basales, ce courant est sous une inhibition partielle par le cytosquelette F-actine. Des travaux antérieurs ont démontré que le TRPP2 peut augmenter l'effet inhibiteur du cytosquelette d'actine sur les courants TREK1 par le recrutement de filamine A (FLNa), une protéine qui se lie à actine. Cependant, les caractéristiques de cette inhibition ne sont pas complètement compris. Notre hypothèse est que cette inhibition est très dynamique et est médidée par une augmentation de renouvellement d’actine qui est dépendant sur le FLNa. Nos résultats montrent que l'élimination progressive du cytosquelette d'actine soulage inhibition de TREK1 par le TRPP2. De plus, la vitesse à laquelle l'inhibition récupère et plus rapides dans les cellules exprimant TREK1 avec TRPP2 que dans les cellules exprimant TREK1 seul. Ceci suggère une renouvellement de F-actine augmenté dans la présence de TRPP2. Cependant, grâce à la technique Fluorescence Recovery After Photobleaching (FRAP), nous montrons que la dynamique de F-actine n’est pas améliorée en présence de TRPP2. Nous avons également
déterminé que ce processus nécessite le FLNa car le TRPP2 ne modifie pas la reprise de l'inhibition de TREK1 dans les cellules M2 où le FLNa est génétiquement supprimé, mais le fait dans les cellules A7 de contrôle qui exprime le FLNa. Grâce à des techniques d'immunofluorescence et la génération d'une lignée de cellules S8 exprimant le FLNA-RFP, nous montrons que FLNA est plutôt recruté à la membrane cellulaire en présence de TRPP2. Ce recrutement mène à une altération de la réorganisation subcellulaire des filaments d'actine qui à son tour régulent l’ouverture de TREK1.
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| Abbreviation | Description |
|--------------|-------------|
| A7           | Human Melanoma Cell Line |
| ABD          | Actin Binding Domain |
| ADPKD        | Autosomal Dominant Polycystic Kidney Disease |
| ANP          | Atrial Natriuretic Peptide |
| ATP          | Adenoside Triphosphate |
| BKCa$^{2+}$  | Large Conductance Ca$^{2+}$-Activated K$^+$ Channel |
| Ca           | Calcium |
| cDNA         | Complementary Deoxyribonucleic Acid |
| CHO          | Chinese Hamster Ovary |
| CFTR         | Cystic Fibrosis Transmembrane Conductance Regulator |
| CNS          | Central Nervous System |
| Cos7         | African Green Monkey Kidney Cell Line |
| DMEM         | Dulbecco’s Modified Eagle’s Growth Medium |
| DRG          | Dorsal Root Ganglion |
| EMEM         | Eagle’s Minimum Essential Medium |
| ER           | Endoplasmic Reticulum |
| F-actin      | Filamentous Actin |
| FLNa         | Filamin A |
| FRAP         | Fluorescence Recovery After Photobleaching |
| G            | Gravitational Force |
| GABA         | γ-aminobutyric Acid |
| G-actin      | Globular Actin |
| GFP          | Green Fluorescent Protein |
| GPCR         | G Protein Coupled Receptor |
| GTP          | Guanosine Triphosphate |
| HEK293       | Human Embryonic Kidney Cell Line |
| HeLa         | Human Cervical Cancer Cell Line |
| HPMVEC       | Human Pulmonary Microvascular Endothelial Cell |
| Hz           | Hertz |
| IMCD         | Inner Medullar Collecting Duct Cells |
| K            | Potassium |
| K$_{2P}$     | Two-pore-domain potassium channel family |
| K562         | Human Leukemia Cell Line |
| Kv           | Voltage-Gated Outward Rectifying Potassium Channel |
| K$_{ir}$     | Two Transmembrane Domain Inward Rectifying Potassium Channel |
| M2           | Human Melanoma Cell Line |
| MDCK         | Madin-Darby Canine Kidney Cells |
| MEF          | Mechanoelectric Feedback |
| mL           | Milliliter |
| mM           | Milimolar |
| mm           | Millimeter |
| mmHg         | Millimeter of Mercury |
| MNSc         | Magnocellular Neurosecretory Cells |
| mOsm         | Miliosmolar |
ms  Millisecond  
MSC  Mechanosensitive Ion Channel  
MscL  Mechanosensitive Channel of Large Conductance  
MΩ  MegaOhm  
Na  Sodium  
P  Pressure  
PCR  Polymerase Chain Reaction  
PCT  Proximal Convoluted Tubule  
PH  Periventricular Heterotopia  
PKD  Polycystic Kidney Disease  
r  Radius of Curvature  
RFP  Red Fluorescent Protein  
RPM  Revolutions Per Minute  
s  Seconds  
SAC  Stretch-Activated Non-Selective Cationic Channel  
SAK  Stretch Activated Potassium-Selective Channel  
SIC  Stretch Inactivated Cation Channel  
SIM  Structured Illumination Microscopy  
T  Tension  
TRAAK  TWIK-Related Arachidonic Acid-Stimulated K⁺ Channel  
TREK  TWIK-Related K⁺ Channel  
TWIK  Tandem of P Domains In A Weak Inward Rectifying K⁺ Channel  
TRP  Transient Receptor Potential  
TRPA  Transient Receptor Potential, Ankyrin Subtype  
TRPC  Transient Receptor Potential, Canonical Subtype  
TRPM  Transient Receptor Potential, Melastatin Subtype  
TRPP  Transient Receptor Potential, Polycystic Subtype  
TRPP1  Polycystin 1  
TRPP2  Polycystin 2  
TRPV  Transient Receptor Potential, Vanilloid Subtype  
x  times  
α  Alpha  
β  Beta  
μg  Microgram  
μm  Micrometer  
μm²  Micrometer squared  
°C  Degrees Celsius  
%  Percentage
Chapter 1 – Introduction

1.1 Mechanotransduction

Mechanotransduction is the conversion of a mechanical stimulus into an electrical or biochemical signal that can be processed by the cell\(^1\). This process can be traced back to archaebacteria and eubacteria, where it is necessary for cell protection against hypo- or hypertonic environments through osmoregulation\(^2\). Mechanotransducers must therefore have emerged early on during evolution\(^2\). In higher organisms, mechanotransduction is the basis of several fundamental physiological functions including touch and pain sensation, hearing, blood pressure regulation, salt and fluid balance, urination, tissue growth, and vestibular function\(^3-10\).

1.1.1 Mechanosensitive Ion Channels

Mechanosensitive ion channels (MSCs) are important molecules of cellular mechanotransduction. These proteins are activated by mechanical stretch/pressure, which leads to a conformation change of the channel and a resulting change in the membrane potential following ion flux. Because MSCs respond directly to mechanical stimuli, the mechanosensory cells are capable of transducing the stimulus into electrical information within microseconds to milliseconds\(^11,12\). Two principal models of channel gating are currently proposed: the bilayer model and the tether model (Figure 1-1). The “bilayer model” reasons that the polar head groups and non-polar tails result in the anisotropic nature of the lipid bilayer that creates a force profile of the membrane and any inserted proteins. Therefore any mechanical stimulus on the cell membrane is converted into a lateral tension that is perceived by the embedded MSCs and may make it energetically more favorable for them to change their conformation\(^13,14\). The bilayer model of MSC opening is strongly supported by the fact that the bacterial mechanosensitive
channel of large conductance (MscL) found in *Escherichia coli* retains its mechanosensitivity when placed in lipid bilayers\textsuperscript{15,16}. Since no other molecules are present, the force detected by the channels when a stimulus is applied to the membrane must come directly from the lipids\textsuperscript{13}. The “dual-tether” model suggests that the MSC is tethered to intracellular cytoskeletal proteins and/or extracellular components such as the extracellular matrix. Gating of the channel would thus be controlled by the relative bending/stretching of the tethered components\textsuperscript{17-19}. The dual-tether model was first proposed to describe hair cell transduction. In this case, the MSC is tethered to the extracellular tip links that are attached to the stereocilia, as well as the actin filaments of the underlying cytoskeleton. The stereocilia bending causes the channel to be stretched between its two tether points and opens it like a “trap door”\textsuperscript{17,19}. A third model termed the “single-tether” model suggests that mechanogating involves the force profile of the membrane and a single tether. In this instance, manipulation of the tether would reposition the MSC into or out of the membrane like an “elevator” and the altered force of the membrane on the channel would allow it to alter its conductive state\textsuperscript{13,17}.

1.1.2 Types of Mechanosensitive Ion channels

All mechanosensitive ion channels can be categorized into three main classes. The stretch-activated non-selective cationic channels (SACs) are permeable to Na\textsuperscript{+}, K\textsuperscript{+}, and Ca\textsuperscript{2+} ions and lead to cellular depolarization when opened by mechanical stimuli. In contrast, stretch-activated potassium-selective channels (SAKs) are only permeable to K\textsuperscript{+} ions and are thus hyperpolarizing when activated\textsuperscript{11}. A third class of MSCs exists which are termed stretch-inactivated cation channels (SICs). These MSCs permeate Na\textsuperscript{+} and K\textsuperscript{+} ions and unlike SACs and SAKs, SICs are most active in the absence of a mechanical stimulus. Magnocellular
neurosecretory cells (MNCs) of the supraoptic nucleus normally express SICs. These channels display basal activity when the host is euhydrated. During overhydration, the extracellular fluid becomes hypo-osmotic and causes the cells to swell. A mechanical force such as membrane stretch during cell swelling inhibits the SICs, therefore removes a source of depolarization, and leads to cellular hyperpolarization. When cells shrink and mechanical force is minimal, there is a disinhibition of SIC activity, which leads to depolarization\textsuperscript{20}.

1.2 Putative Mammalian Mechanosensitive Ion Channels

While many ion channels are involved in mechanosensation, it is unknown whether the channel is intrinsically mechanosensitive or if it is indirectly regulating mechanotransduction. Certain criteria have been proposed by Nilius and Honoré to determine whether a channel can be considered a stretch-activated channel\textsuperscript{11,12}. These include the direct activation of the channel by mechanical stimuli, a rapid change in channel kinetics, a dependence of evoked current on the stimulus magnitude, and an association of mechanogating with conformational changes\textsuperscript{11,12}. Here we will review certain ion channels for which there exists evidence of mechanosensitivity.

1.2.1 Transient Receptor Potential Ion Channels

The transient receptor potential (TRP) family is a large class of ion channels where each subunit contains six transmembrane domains with a putative pore between S5 and S6, and four subunits assemble to form a channel. Most TRP channels are non-selective cation channels that are involved in several sensory pathways throughout an organism including vision, olfaction, taste, chemosensation, thermosensation, and mechnosensation\textsuperscript{21,22}. 
TRPA1 of the TRP subfamily A was initially shown to be responsive to noxious cold temperatures\textsuperscript{23}. More recent studies have suggested that TRPA1 is also mechanosensitive. When the \textit{C. elegans} ortholog TRPA1 is mutated, the worms no longer have proper escape response following tactile stimulation of the nose. Furthermore, heterogeneous expression of TRPA1 in Chinese hamster ovary (CHO) cells show that TRPA1 is responsive to mechanical stimuli\textsuperscript{24}. In one mammalian study, TRPA1\textsuperscript{−/−} mice had no altered sensitivity to mechanical stimuli whereas another study showed mice having a lowered sensitivity to such stimuli\textsuperscript{25,26}. Whole cell recordings of dorsal root ganglion (DRG) neurons also showed that \textit{trpa1} deletion significantly reduced mechanically induced currents\textsuperscript{27}. Together, these studies show that TRPA1 is involved in mechanotransduction; however further experiments are required to determine if TRPA1 is in fact a MSC as opposed to an accessory protein.

Like TRPA1, the canonical TRPC1 is also considered to have mechanosensitive properties. By reconstituting membrane proteins of CHO cells into liposomes, Maroto et al. identified TRPC1 as a candidate for the cell’s endogenous SAC. Overexpression of TRPC1 resulted in a 10-fold increase in the total current with the single cell conductance remaining the same as endogenous channels suggesting that TRPC1 is at least involved in the cell’s mechanosensation. TRPC1 knockdown using antisense RNA expectedly reduced stretch induced currents therefore supporting TRPC1’s mechanosensitive role. Moreover, because the liposomes contained only membrane proteins and still retained mechanosensitivity, TRPC1 may function as a MSC through the bilayer model\textsuperscript{28}. Overexpression of TRPC6 in CHO cells also leads to an increase in mechanosensitive currents that are blocked by the known inhibitor of mechanosensitive ion channels, GsMTx4, thus suggesting that TRPC6 is also a MSC candidate\textsuperscript{29}. More recent findings however, suggest that TRPC1 and TRPC6 are not directly
activated by membrane stretch and are instead indirectly activated during mechanotransduction\textsuperscript{30}. Moreover, another study shows that the overexpression of TRPC1 or TRPC6 does not alter the amplitude of mechanosensitive currents thus decreasing the likelihood of TRPC1 and TRPC6 being actual MSCs\textsuperscript{31}.

The transient receptor potential vanilloid type 1 channel (TRPV1) is expressed widely in primary afferent neurons, in particular, nociceptors as well as in other organs such as the kidneys\textsuperscript{32}. The channel is activated by noxious temperatures (above 43°C), low pH, and capsaicin, which lead to an inward nonselective cation current\textsuperscript{33}. In one study, afferent renal nerve activity was increased following mechanostimulation by elevated intrapelvic pressure. This effect was attenuated in the presence of the TRPV1 antagonist, capsazepine, therefore suggesting a role for TRPV1 in mechanotransduction\textsuperscript{32}. Another member of the vanilloid type channels is TRPV4 which is activated by warm temperatures and acidic pH and is involved in the transduction of osmotic and mechanical stimuli\textsuperscript{34}. Because TRPV4 knockout in mice only displays a moderate loss of mechanosensation despite a strong desensitization to noxious mechanical stimulation, it is suggested that TRPV4 is not itself a MSC but is instead involved in mechanotransduction indirectly\textsuperscript{1,35,36}. Furthermore, TRPV4 channel opening to hypotonic stress occurs through the phospholipase A\textsubscript{2}-dependent formation of arachidonic acid therefore confirming that TRPV4 activation is not directly activated by membrane stretch\textsuperscript{37}.

In the melastatin subfamily of TRP channels, both TRPM4 and TRPM7 are candidates for MSCs\textsuperscript{38,39}. In HEK cells, overexpression of TRPM4 increased the extent of channel activation following mechanical stimulation\textsuperscript{38}. In HeLa cells, knockdown of TRPM7 reduced SAC activity and the rate of cell volume recovery\textsuperscript{39}. While both phenomena propose mechanosensitive function, it was shown that both channels are inhibited when extracellular
calcium is removed. Therefore TRPM4 and TRPM7 may simply be responding to calcium instead of membrane stretch\textsuperscript{38,39}.

1.2.2 Na\textsuperscript{+} Channels

Na\textsubscript{v}1.5 is a voltage-sensitive sodium channel that is found in the human heart and the gut, which are both mechanical systems\textsuperscript{40,41}. Expression of the sodium channel in human embryonic kidney cells 293 (HEK293) reveals that there is a leftward shift in voltage dependent activation and inactivation curves. Therefore Na\textsubscript{v}1.5 has mechanosensitive properties\textsuperscript{42}. Moreover, these channels are no longer mechanosensitive when the actin cytoskeleton is abolished by cytochalasin D therefore showing that mechanosensation is dependent on an intact actin cytoskeleton\textsuperscript{43}. However, mechanical stimuli appear to simply modulate Na\textsubscript{v}1.5 channel opening instead of directly gating it. Therefore Na\textsubscript{v}1.5 is not likely to be a MSC\textsuperscript{42}.

1.2.3 Ca\textsuperscript{2+} Channels

Ca\textsubscript{v}1.2 is a voltage sensitive L-type calcium channel that may give intestinal smooth muscle cells their mechanosensitive properties. When these channels are expressed in heterologous systems, an increased voltage-dependent current is observed following mechanical shear stress\textsuperscript{44}. Similar experiments in expression system show a similar phenomenon in N-type calcium channels whereas T-type channels (Ca\textsubscript{v}3.3) appear to be insensitive to membrane stretch\textsuperscript{45}. However, others have reported that T-type calcium channels are linked to the mechanical sensitivity of the D-hair mechanoreceptors that populate dorsal root ganglia (DRG)\textsuperscript{46}. These voltage gated calcium channels are similar to voltage gated sodium channels in that they are simply modulated by mechanical stimulation as opposed to directly opened by it. Though
conversely, L-type (CaV1.2) calcium channels elicit an electrical current following mechanical stimulation therefore suggesting that this subtype of calcium channel is a MSC\textsuperscript{47}.

1.2.4 Cl\textsuperscript{−} Channels

The cystic fibrosis transmembrane conductance regulator (CFTR) is an anion selective channel that is ligand-gated by cAMP. When a mechanical stimulus is applied to cells expressing these channels, a current is evoked that is increased as the stimulus magnitude is increased. Interestingly, the mechanical force also increased single channel conductances, suggesting the channel may have subconductive states. Similar pressure dependent responses were seen in excised, inside-out patches in the absence of ATP thus showing that CFTR mechanosensitivity is not dependent on intracellular mediators\textsuperscript{48}.

1.2.5 Piezo Channels

The latest MSC candidates are the Piezo1 and Piezo2 proteins discovered by the Patapoutian lab in 2010. Both proteins contain over 30 putative transmembrane domains and therefore do not resemble any other ion channel. Regardless, overexpression of either protein in various cell types leads to stretch activated currents with nonselective cationic conductances. Moreover, Piezo2 knockdown in mouse DRG neurons reduced rapidly adapting stretch activated currents\textsuperscript{49}. In subsequent reports, Piezo1 was reconstituted into asymmetric bilayers containing no intracellular components and retained its ability to pass a current thus confirming its pore-forming properties but its mechanical sensitivity was not tested\textsuperscript{50}. Piezo1 and Piezo 2 are also inhibited by GsMTx4 and ruthenium red, which are known blockers of MSC, and thus provide other evidence in support of their candidacy as MSCs\textsuperscript{50,51}. 

7
1.2.6 K⁺ Channels

TREK1, TREK2, and TRAAK are confirmed mechanosensitive ion channels pertaining to the TREK subfamily of two-pore-domain K⁺ (K₂P) channels that have previously been well characterized. Expression of purified subunits in lipid bilayers has confirmed that TREK1 channels are in fact directly sensitive to membrane tension⁵². These channels are potassium selective and thus hyperpolarize the cell following mechanical activation⁵³,⁵⁴. The TREK channels will be the main focus throughout this thesis.

1.3 Cytoskeleton

The eukaryotic cell cytoskeleton is composed of actin microfilaments, microtubules, and intermediate filaments. Together, these filaments create an intracellular infrastructure that is crucial for maintaining cell shape and responding to mechanical forces. Here we will review the roles of the actin and microtubule cytoskeleton in modulating MSC gating⁵⁵.

1.3.1 Actin Cytoskeleton

Monomeric actin subunits (G-actin) assemble into two twisted polymers to form actin microfilaments (F-actin). These filaments are highly dynamic since subunits are constantly added and removed at both ends of the filament. Moreover, one end (barbed) of the actin filament undergoes a faster rate of polymerization compared to the other (pointed) end therefore creating polarity within the fiber⁵⁶,⁵⁷. The overall rate of actin polymerization can effectively be enhanced or inhibited using pharmacological agents to study the effects of the actin cytoskeleton on MSCs⁵⁸.
When human gingival fibroblasts are mechanically stimulated at the cell membrane, a large influx of calcium ions through MSCs is observed. The actin-barbed end capping toxin, cytochalasin D, promotes depolymerization of the F-actin and leads to a disruption of the actin cytoskeleton. When the fibroblasts are first pre-treated with cytochalasin D (1 μM), the stretch-activated calcium transients have a 3-fold higher amplitude compared to controls\textsuperscript{58}. Cytochalasin D effects can also be replicated on SACs found in Cos7 cells and myotubes while alternatively disrupting the actin cytoskeleton of Cos7 cells using the G-actin sequestering agent latrunculin A causes a similar 2-fold increase in stretch-induced currents\textsuperscript{59,60}. Conversely, stabilizing and polymerizing F-actin with jasplakinolide (3 μM) inhibits the activity of SACs\textsuperscript{59}. Together, these results insinuate that MSCs are under tonic inhibition by the underlying actin cytoskeleton.

In 2005, Staruschenko showed that the actin cytoskeleton might sometimes be capable of reinforcing MSC activity. Human leukemia k562 cells were treated with either cytochalasin D, cytochalasin B, or latrunculin B to degrade the actin cytoskeleton. Each toxin led to a significant decrease in the single channel conductance of SACs compared to control conditions. It is also worth mentioning that two populations of SACs were observed in the leukemia cells and the cytochalasins and latrunculin had similar effects on both groups\textsuperscript{61}. Another group performed calcium imaging experiments on human pulmonary microvascular endothelial cells (HPMVECs) following mechanical stretch and observed a rise in intracellular calcium levels. Pre-treatment with cytochalasin D (0.1 μM) nearly completely abolished the calcium response whereas jasplakinolide (0.5 μM) enhanced the stretch induced calcium response\textsuperscript{62}. In 2007, the Bourque lab studied the effects of the actin cytoskeleton on SICs. When recording from isolated magnocellular neurosecretory cells (MNCs) in the whole-cell configuration, the group was able to apply negative pressure through the recording pipette to lower the cell volume and activate
SICs. Pre-treatment with cytochalasin D (195-250 μM) decreased the current response to stretch removal (lowered cell volume) whereas pre-treatment with jasplakinolide (2.5 μM) led to a significantly greater response compared to control conditions. Together, these findings propose that the actin cytoskeleton instead reinforces the MSC activity in response to mechanical stimuli.

It is unclear as to whether the differing effects of the actin cytoskeleton are due to the different cell types being examined or perhaps different MSCs are regulated differently by the actin cytoskeleton. One thing that remains consistent throughout each study however is that the actin cytoskeleton plays a crucial role in the modulation of MSC mechanogating.

1.3.2 Microtubule Cytoskeleton

Microtubules are composed of α- and β-tubulins that together form heterodimers. These dimers polymerize with the α-subunit (-) end of one dimer contacting the β-subunit (+) end of another and therefore result in a protofilament with a (+) and (-) end. Thirteen protofilaments bundle together in parallel to form a microtubule with a hollow core. During polymerization, both α- and β-tubulins are both bound to GTP. However, once bound to the polymerizing protofilament, the GTP on the β-tubulin may be hydrolyzed to GDP. If the GDP-bound tubulin is in the middle of the microtubule, the structure remains stable. In contrast, if the GDP-bound tubulin is at the (+) tip of the microtubule, then rapid depolymerization and shrinking of the microtubule occurs. The importance of microtubules in MSC regulation can be linked to the findings of Howard et al. who showed a loss of electron-dense material in the microtubule cytoskeleton in mutant *Drosophila* that led to dysfunctional mechanical sensation. Like the actin cytoskeleton, the rate of microtubule growth or shrinkage can be altered using...
pharmacological techniques to then examine the microtubule cytoskeleton’s role on regulation MSCs.

In the same study performed in 2005 by the Staruschenko lab, the role of microtubules on MSC activity was also assessed pharmacologically. Both colchicine and nocodazole bind to tubulin and therefore prevent microtubule polymerization. When human leukemia k562 cells were treated with either compound to disrupt to microtubule cytoskeleton, no changes in the single channel conductance or open probability of SACs were observed\(^6\)\(^1\). A later study measured the current passing through SAC expressed in Cos7 cells. Pre-treatment with nocodazole (10 μM) for 1.5 hours or with colchicine (500μM) for 2 hours did not show any changes in the elicited current compared to control cells following a pressure stimulus\(^5\)\(^9\). Finally, a different study on the large conductance Ca\(^{2+}\)-activated K\(^+\) channel (BK\(_{\text{Ca}}^{2^+}\)) found in coronary artery smooth muscle cells did not show any changes in open probability to membrane stretch when pre-treated with taxol, a microtubule stabilizer that protects from disassembly\(^6\)\(^8\). The findings together show that microtubules may play a role in mechanotransduction as observed in \textit{Drosophila}, however there is no evidence suggesting that microtubules directly affect MSC mechanogating.

1.4 TREK Channels

While the identity of the mammalian stretch-activated non-selective cationic channel (SAC) remains elusive, the TWIK-related K\(^+\) channel 1 (TREK1) is a well-characterized stretch-activated potassium-selective channel (SAK)\(^6\)\(^9\). The discovery of TREK1 in 1996 came as a result of an exploratory investigation that took place soon after the identification of the tandem of P domains in a weak inward rectifying K\(^+\) channel 1 (TWIK1), a potassium channel whose
structure and function differed from that of previously described voltage-gated outward rectifying (K_v) and two transmembrane domain-containing inward rectifying (K_ir) potassium channels\textsuperscript{70-73}. After alignment of TWIK-1 and related orthologues from \textit{C.elegans}, conserved amino acid sequences were identified and used for degenerative PCR. The amplified fragment was then used to probe a mouse cDNA library and identify TREK1. Like TWIK1, TREK1 subunits contain four transmembrane domains and two pore domains and their discovery provided evidence for a third family of potassium channels termed the two-pore-domain K\textsuperscript{+} (K\textsubscript{2P}) channels\textsuperscript{71}. Despite its structural similarity to TWIK1, the amino acid identity between the two channels is only 26\% and TREK1 displays outward rectification as opposed to TWIK1’s inward rectification\textsuperscript{71}.

In 1998, the same group investigated the biophysical, pharmacological, and regulatory properties of TREK1 and compared it to its hypothesized ortholog, the S-type K\textsuperscript{+} channel of \textit{Aplysia californica}\textsuperscript{69}. The molluscan S channel, which is involved in the sensitization of the gill-withdrawal reflex, is notably characterized by its sensitivity to membrane stretch in addition to its other properties of regulation and gating\textsuperscript{69,74,75}. When heterologously expressed in Cos7 cells and \textit{Xenopus} oocytes, TREK1 displayed similar properties to the \textit{Aplysia} S channel including its sensitivity to membrane stretch\textsuperscript{69}. Disruption of the cytoskeletal integrity via colchicine, which disrupts microtubules, and cytochalasin D, which disrupts F-actin, did not prevent stretch activation of TREK1 whereas modification of the lipid bilayer altered stretch-induced TREK1 currents, suggesting the mechanogating of this channel functions via a bilayer model as opposed to a tethered model\textsuperscript{69}. Later experiments reconstituting TREK1 in lipid bilayers confirmed this hypothesis\textsuperscript{52}. Moreover, insertion of crenators into the external leaflet of the bilayer, which cause the membrane to bulge outwards and stretch the outer lipid layer as opposed to the inner layer,
increased the sensitivity of TREK1 to stretch while cup-forming amphipathic molecules, which insert into the inner leaflet and cause stretching of the inner lipid layer, inhibit TREK1 activity\textsuperscript{69,76}. This data suggests that the opening of the outer half of the lipid bilayer specifically controls TREK1. The higher efficiency of channel opening with negative versus positive pressure applied through cell-attached patch pipettes further favors this hypothesis\textsuperscript{69}.

1.4.1 Structure

The \textit{KCNK2} (K\textsubscript{2p}2.1) gene, located on human chromosome 1, encodes the 370 amino acid subunit that dimerizes to form a functional TREK1 (Figure 1-2)\textsuperscript{71,77,78}. Like other K\textsubscript{2p} members, TREK1 contains four transmembrane segments (M1-M4) with two pore domains (P1 and P2) located between segments M1 and M2 (P1), and segments M3 and M4 (P2). The short N-terminal and long C-terminal are both located within the cytoplasm while an extended loop between M1 and P1 is situated extracellular. This M1P1 domain appears to be necessary for the dimerization of subunits to form functional homo- or possibly heterodimers\textsuperscript{79,80}. Unlike the conventional GYG (glycine, tyrosine, glycine) selectivity motif found in the pore of conventional K\textsuperscript{+} channels, both pore regions of TREK1 contain a GFG motif instead. The substituting phenylalanine (F) in the GFG motif of P1 interacts with a conserved aspartate residue following the GFG motif of P2 to retain K\textsuperscript{+} selectivity and determine gating properties\textsuperscript{81,82}.

1.4.2 Expression Pattern

TREK1 is widely expressed in the mammalian central nervous system (CNS). In the brain, TREK1 is abundant in \textgamma-aminobutyric (GABA)-ergic projection neurons and interneurons\textsuperscript{83}. Most notably, high expression of TREK1 is observed in the putamen, amygdala,
caudate nucleus, thalamus, hypothalamus, hippocampus, frontal, occipital, and temporal cortices\textsuperscript{54,83}. High TREK1 expression is also found in the spinal cord and sensory dorsal root ganglia neurons\textsuperscript{54}. Outside the nervous system, TREK1 can be found in the gastrointestinal system, with rich expression in the stomach, and other peripheral tissues such as bone\textsuperscript{54,84}. TREK1 is also found in the heart atrial and ventricular myocytes as well as myocytes and endothelial cells of various arteries\textsuperscript{85-88}. Other members of the TREK1 family, TREK2 (KCNK10) and TRAAK (TWIK-related arachidonic acid-stimulated K\textsuperscript{+} channel, KCNK4), show similar expression patterns to TREK1. TREK2 does however show increased expression in the periphery such as the kidneys, pancreas, spleen and testis. In contrast, TRAAK expression mostly occurs in the brain and DRGs\textsuperscript{54}.

1.4.3 Mechanosensitive Functions of TREK1 in Different Physiological Systems

Nearly 60\% of pain sensing TRPV1-expressing dorsal root ganglia (DRG) neurons also express TREK1, suggesting that the latter may be involved in nociception\textsuperscript{89}. When KCNK2 is deleted in genetically knocked out mice, they become more sensitive to low threshold mechanical stimuli compared to their wild-type littermates when assessed by stimulating the hindpaw with von Frey filaments of increasing stiffness\textsuperscript{89}. The mechanical hypersensitivity observed after TREK1 knockout suggests that the channel is, at least in part, involved in tuning the mechanosensitivity of pain sensing neurons\textsuperscript{78,89}. In fact, TREK1 is likely to act as a brake to nociceptive stimuli and promote cellular hyperpolarization while other nociceptive sensing SACs are inducing membrane depolarization. Moreover, inflammation-induced mechanical hyperalgesia is greater in TREK1 knockout mice which indicates that TREK1 is partly involved in the sensitization of peripheral nociceptors to inflammation\textsuperscript{89}. 
Mouse knockout studies of TREK1 and its isoforms (TREK2 & TRAAK) revealed that proximal convoluted tubule epithelial cells (PCT) of the kidney are more susceptible to cell death in response to elevated intrarenal pressures. TREK channels therefore have a protective role against cell death during mechanical stresses.90.

TREK1 is also highly expressed in rat atrial myocytes where its outward rectifying current regulates the duration of the cardiac action potential.87 Since the main stimulus for atrial natriuretic peptide (ANP) release is stretch, TREK1 may possibly control blood pressure by regulating ANP secretion.87,91 The most supported theory is that ANP secretion is triggered by stretch activation of SACs whereas TREK1 would act as a negative feedback for secretion.87,92 Moreover, TREK1 is expressed on the plasma membrane of rat ventricular myocytes. When these cells get stretched during the cardiac cycle, TREK1 would get activated and provide a feedback signal.88,93 This mechanoelectric feedback (MEF) is the process in which mechanical forces on the myocardium alter the myocardium’s electrical properties via SACs and SAKs.94 TREK1 may thus function in detecting the mechanical forces in atrial and ventricular myocytes to maintain proper cardiac function.93.

TREK1 currents are also modulated by changes in cell volume. TREK1 current amplitudes are significantly increased by cell swelling in response to hypotonic extracellular solutions whereas cell shrinking hypertonic solution decrease TREK1 current amplitudes. This phenomenon insinuates that TREK-1 mechanogating is involved in detecting changes in cell volume.69.

TREK1 is functionally expressed in human osteoblasts suggesting that it may be involved in bone remodeling in response to mechanical loads.84,95 Pharmacologically inhibiting TREK1
via the local anesthetic, bupivacaine, reduces osteoblast proliferation and demonstrates how mechanical forces can directly alter the bone remodeling osteoblasts\textsuperscript{84,96}.

1.4.4 Activation and Modulation of TREK1

Although TREK1 is a background $K^+$ channel responsible for maintaining the resting membrane potential, the channel is remarkable for its unconventional polymodal activation\textsuperscript{71,78}. Aside from its stretch sensitivity, TREK1 is stimulated by progressive rises in temperature whereas cold temperatures effectively inhibit channel activity. Interestingly, excision of the patch during channel recordings results in a loss of thermal sensitivity, indicating that cytosolic factors are necessary for the channel’s thermal activation. In contrast, TREK1 still maintains stretch sensitivity in excised patches suggesting that mechanosensitivity is an intrinsic property of the channel\textsuperscript{78,97}. Unlike typical leak $K^+$ channels, TREK1 is voltage dependent and shows preferential opening at depolarized potentials, making it an outward rectifier\textsuperscript{71}. Deletion and mutation studies show that the carboxy (C)-terminal domain is responsible for the voltage-dependent gating\textsuperscript{98}. Removal of the C-terminal domain also renders the channel more resistant to stretch, indicating that the domain is necessary for TREK1 mechanogating\textsuperscript{53}. A lowered intracellular pH also stimulates TREK1 activation through a negatively charged glutamic acid (Glu306) residue located on the C-terminus that acts as a proton sensor\textsuperscript{78,99}. Polyunsaturated fatty acids, most notably arachidonic acid, are also capable of reversibly opening TREK1\textsuperscript{69}. Phospholipids including phosphatidylinositol, phosphatidylethanolamine, and phosphatidylserine enhance TREK1 activity by interacting with the C-terminal domain including Glu 306\textsuperscript{78,100}. Also, extracellular lysophospholipids activate TREK1 and intracellular lysophospholipids inhibit TREK1 due to their triggered curvature of the cell membrane following insertion\textsuperscript{78,101,102}. 
Downstream effects of G protein coupled receptor (GPCRs) stimulation including, phosphorylation of Serine 333 by protein kinase A, phosphorylation of Serine 300 and Serine 333 by protein kinase C, and direct binding of diacylglycerol, all inhibit TREK1 activity\(^69,78,103,104\). Finally, numerous volatile general anesthetics stimulate TREK1. These include chloroform, diethyl ether, halothane, isoflurane, nitrous oxide, xenon, cyclopropane, and chloral hydrate. The C-terminus is also essential for the modulation of TREK1 by these anesthetics\(^78,105-107\).

1.4.5 Interaction with the Cytoskeleton

When TREK1 is overexpressed in neurons, an induction of numerous filopodia is observed in dendrites and the axon. Moreover, strong colocalization between TREK1 and actin occurs in these structures, suggesting an interaction between these two molecules. When actin polymerization is disrupted using latrunculin A, TREK1 exhibits increased sensitivity to membrane stretch. Separating TREK1 from the actin cytoskeleton via patch excision from the cell-attached to the inside-out configuration also shows a similar effect and demonstrates that actin inhibits TREK1 mechanogating. Disrupting the microtubule cytoskeleton with nocodazole has no effect. The sites Glutamate 306 and Serine 333 of TREK1’s C-terminal domain are both necessary for the actin cytoskeleton to interact with TREK1 and tonically inhibit it\(^108\). The results and discussion chapters of this thesis will further explore the importance of the actin cytoskeleton in regulating TREK1 gating.
1.4.6 TREK1 Regulation via Polycystins

Recently, novel modulators of TREK channels have been identified. Polycystins 1 (TRPP1) and 2 (TRPP2) are both linked to autosomal dominant polycystic kidney disease and interact together to regulate TREK channel mechanogating\(^9\),\(^10\). The following section will highlight the physiological roles of the polycystins and their involvement in the regulation of TREK1 channel gating.

1.5 Polycystins

1.5.1 Autosomal Dominant Polycystic Kidney Disease

Autosomal dominant polycystic kidney disease (ADPKD) is among the most common hereditary diseases and is a leading cause of renal failure with patients frequently necessitating dialysis or kidney transplantation since they are the only available cures. ADPKD occurs in 1 in 800 live births and affects approximately 4 to 6 million people worldwide\(^11\). The disease is characterized by the presence of several hundreds to thousands of cysts in the kidneys in addition to the liver, pancreas and intestine. Patient symptoms often include hypertension, hematuria, polyuria, abdominal pain, urinary tract infections, and kidney stones. Patients also have a greater likelihood of developing aortic aneurysms and defects with heart valves\(^11\),\(^11\). Two types of ADPKD are currently defined. Type 1 is caused by a mutation in the \(PKD1\) gene and accounts for 85-90% of cases whereas type 2 is caused by a mutation in the \(PKD2\) gene and is responsible for 10-15% of cases\(^11\),\(^11\),\(^11\). Type 1 and type 2 are pathologically similar with the most significant difference being the later onset in type 2. Patients suffering from ADPKD type 2 thus have a longer life expectancy (69 years) compared to those with type 1 (53 years). Both patients still have a lower life expectancy compared to controls (78 years)\(^11\). Over 100 \(PKD1\) mutations
and 75 PKD2 mutations causing ADPKD have been identified and include deletions, insertions, frame shifts, splicing, nonsense, missense, and point mutations\textsuperscript{115,116}. Such mutations may lead to a decreased/absent production of polycystins or an overexpression of polycystins, but the most common consequence is the expression of polycystins with a loss of function mutation\textsuperscript{110,117,118}.

1.5.2 Polycystin 1

Polycystin 1 is a large (460 kD), 4302 amino acid-containing glycoprotein encoded by the PKD1 gene of chromosome 16. It contains 11 transmembrane domains, a short intracellular C-terminal domain and a sizable extracellular N-terminal domain. This large domain contains a multitude of binding motifs for other proteins, lipids, and carbohydrates and is thus involved in cell-cell and cell-matrix interactions and signaling pathways\textsuperscript{110,119,120}. Furthermore, TRPP1 forms multiprotein complexes within the cell for signaling purposes\textsuperscript{121}. TRRP1 is expressed on the plasma membrane of cells in the kidneys, liver, pancreas, heart, intestine, lungs, and brain\textsuperscript{122,123}.

1.5.3 Polycystin 2

Unlike Polycystin 1, Polycystin 2 is a member of the transient receptor potential (TRP) superfamily as it structurally resembles other TRP channels, including its six transmembrane domains, intracellular N- and C- termini, and a pore region\textsuperscript{124}. It is a 968 amino acid-containing protein (110kD) encoded by the PKD2 gene located on chromosome 4\textsuperscript{113}. \textit{PKD2} is expressed in most tissues including the kidneys, liver, pancreas, heart, lungs, and brain\textsuperscript{125}. A coiled-coil domain on the C-termini of both TRPP1 and TRPP2 enables the two polycystins to interact\textsuperscript{126}. Aside from TRPP1 binding, TRPP2 interacts with TRPC1 and multiple components of the actin and microtubule cytoskeleton\textsuperscript{127,128}. TRPP2’s function as a channel is controversial. Some
studies report that TRPP2 is incapable of permeating ions whereas some evidence supports TRPP2’s function as a calcium permeable, non-selective cationic channel\textsuperscript{129}. Reports have shown that TRPP2 functions as a Ca\textsuperscript{2+} dependent intracellular release channel on the endoplasmic reticulum (ER) where much of the protein is located due to an ER retention signal\textsuperscript{130,131}. Others have shown modest yet functional TRPP2 channels on the plasma membrane of inner medullar collecting duct (IMCD) cells and Madin-Darby canine kidney (MDCK) cells\textsuperscript{132}. The extent to which TRPP1 plays in TRRP2’s channel activity at the plasma membrane is currently under investigation. Co-assembly of TRPP2 with TRPP1 may be necessary to form a functional TRPP2 channel at the plasma membrane or TRPP1 may only be required to recruit TRPP2 channels to the plasma membrane\textsuperscript{132,133}. The controversies regarding TRPP2’s localization and function may be due to differences in adapter proteins and other intracellular components that exist between cell types, differentiation status, or environmental components\textsuperscript{132,134}.

1.5.4 Polycystins as Mechanotransducers

Polycystin 1 and 2 both co-localize to the primary cilium of renal epithelial cells where they have been reported to together function as a mechanical transducer\textsuperscript{135}. When kidney epithelial cells are exposed to a flow stimulus, the resulting bending of their primary cilium is converted to a calcium signal that develops in the cilium, but then triggers a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release in the cytosol\textsuperscript{136}. However, when TRPP1 activity is blocked, using function-blocking antibodies, or genetically replaced with a truncated mutant, the flow-induced Ca\textsuperscript{2+} response is no longer observed. Alternatively blocking TRPP2 with function-blocking antibodies also elicits a similar loss in the flow-induced rise in the intracellular Ca\textsuperscript{2+} concentration. Like renal epithelial
cells, vascular endothelial cells have primary cilia which contain both TRPP1 and TRPP2\textsuperscript{137-140}. Again, interfering with TRPP1 or TRPP2 prevents any observable flow-induced calcium response\textsuperscript{139,140}. Furthermore, disrupting TRPP1 and TRPP2 in endothelial cells abolishes nitric oxide production, a vasodilator and subsequently crucial regulator of the cardiac vascular tone, and may thus explain extrarenal vascular abnormalities associated with PKD\textsuperscript{139-142}. Interestingly, exposure to shear flow induces proteolytic cleavage of TRPP1 thereby rendering the cell insensitive to further stimuli. This phenomenon exposes a complex feedback mechanism that occurs during mechanotransduction of shear stresses\textsuperscript{135}. Together, the findings propose that TRPP1 functions as the mechanical sensor to shear stress via its large extracellular N-terminal domain. Detection of shear flow by TRPP1 would regulate the opening of TRPP2 to elicit a Ca\textsuperscript{2+} response. Failure of flow detection in ADPKD would effectively block any downstream calcium signaling\textsuperscript{135,139}.

1.5.5 Polycystins as Regulators of Mechanosensitive Ion Channels

Instead of acting as direct mechanotransducers, TRPP1 and TRPP2 may in fact be regulators of endogenous mechanosensitive channels. When overexpressed in Cos7 cells, TRPP2 inhibits the activity of endogenous SACs. This inhibition is remarkably reversed when TRPP1 is co-expressed with TRPP2. Conversely, knockout of PKD1 in arterial myocytes reduces SAC activity suggesting that the dosage between TRPP1 and TRPP2 is important since only TRPP2 that is unbound to TRPP1 can inhibit SACs. The PKD1 knockout cardiomyocytes display an increased threshold for intraluminal pressure to induce a myogenic contraction thus signifying that the altered SAC activity reduces the effectiveness of the myogenic tone, which is the contraction of vascular smooth muscle cells in response to mechanical stretch. Knockdown of
TRPP2 in these cells effectively recovers SAC activity and myogenic tone thereby confirming that the activity of SACs is regulated by the relative dosage and binding of TRPP1 and TRPP2. Disrupting the F-actin cytoskeleton using cytochalasin D or latrunculin A removes TRPP2’s inhibitory effects whereas microtubule-disrupting agents had no effect. In contrast, stabilization of actin filaments by jasplakinolide inhibited SAC activity similarly to TRPP2. Therefore, excess TRPP2 that is not bound to TRPP1 inhibits SAC by a mechanism involving the F-actin cytoskeleton\(^5^9\). Because TRPP2 and TRPP1 regulate SACs indirectly via the F-actin cytoskeleton, then their regulation may not be specific to one type of mechanosensitive ion channel. Indeed, TRPP2 was later shown to be capable of inhibiting endogenous SAKs of PCT cells and heterologously expressed TREK1, TREK2, and TRAAK channels in Cos7 cells through a mechanism that also requires an intact F-actin cytoskeleton\(^9^0\). Interestingly, TRPP2’s mechanism for SAC and SAK inhibition necessitates Filamin A (FLNa), an actin crosslinking protein that binds to TRPP2\(^5^9,12^9,14^3,14^4\).

1.6 Filamin A

Filamin is an actin cross-linking protein that also serves as a scaffold for over 90 binding partners including channels, receptors, intracellular signaling molecules, and transcription factors\(^1^4^5,14^6\). Three isoforms filamin are expressed in mammals. Filamin A (FLNa) is encoded on the X chromosome and is the most abundantly expressed isoform in non-muscle cells\(^1^4^7\). Filamin B is also a non-muscle filamin and encoded on chromosome 3 whereas Filamin C is on chromosome 7 and is primarily found in adult cardiac, skeletal, and smooth muscle cells\(^1^4^8,14^9\). Functional FLNa consists of two 280 kDa dimers, each containing an N-terminal spectrin family F-actin binding domain (ABD) and an elongated flexible segment composed of immunoglobulin-
like repeats that make up rod 1 (repeats 1-15), rod 2 (repeats 16-23), and a self association domain (repeat 24). The 24th repeat at the C-terminal end allows dimerization of the two subunits to form a V-shaped complex. This arrangement of FLNa subunits results in the orthogonal branching of F-actin when bound to the ABDs. A second lower affinity ABD is located on the rod 1 domain to permit high avidity binding between FLNa and F-actin. Conversely, the rod 2 domain is not involved in F-actin binding and is instead the site where the majority of partner interactions occur.

1.6.1 Cell Lines

Two important cell lines are currently used to advance the study of Filamin A. The M2 cell line is derived from malignant human melanomas with undetectable levels of FLNa when assayed via immunoprecipitation, immunoblotting, and immunostaining. The cells have minute amounts of FLNa mRNA with apparently normal FLNa DNA gene structure suggesting that the protein deficiency is due to a defect in the regulation of gene expression or a decrease in mRNA stability. The M2 cells have reversible but continuous extensive blebbing when ambient temperatures fall below 30°C or if serum is removed. An A7 cell line was subsequently developed by stably transfecting the M2 cell line with FLNa. The new cell line only displayed transient localized blebbing that was more resembling of native FLNa+/+ cell lines. Furthermore, the A7 cells regained a asymmetrical shape with the presence of focal lamellae. The discovery of the M2 cell line and the development of the A7 cell line thus introduced an invaluable tool for the study of Filamin A’s cellular functions.
1.6.2 Cellular Functions

M2 cells deficient in FLNa have reduced motility suggesting that FLNa is involved in promoting cellular locomotion\textsuperscript{153}. FLNa is thus hypothesized to localize to growing lamellipodia and recruit interacting signaling molecules for efficient signal transduction that is necessary for cellular movement\textsuperscript{146}. In contrast, FLNa plays an inhibitory role in cellular migration. By negatively regulating integrin, a necessary component for cellular migration, FLNa can effectively prevent the atypical migration of cells throughout the body\textsuperscript{146,155,156}. Consistently, decreased FLNa expression in breast cancer cells results in increased migration, invasion, and metastasis\textsuperscript{157}. Furthermore, FLNa-deficient M2 melanoma cells display membrane blebbing\textsuperscript{153}. These blebbing cells can squeeze through the extracellular matrix and can more easily migrate and metastasize\textsuperscript{156}. FLNa thus functions as a regulator of cellular migration by controlling integrin activation and maintaining proper membrane structure\textsuperscript{146}.

FLNa plays an important role in maintaining cellular integrity and shape. Cells lacking FLNa (M2 cells) are softer/less stiff than cells expressing physiological concentrations of FLNa. This occurrence is due to the inability of FLNa-lacking cells to generate large enough internal contractile stresses. Moreover, these cells have an impaired ability to tune their stiffness to match that of a changed substrate, rendering the cells unable to properly probe their local environment\textsuperscript{158}. This mechanical feedback from the extracellular matrix is a crucial step implicated in biological processes including development, differentiation, regeneration, and disease\textsuperscript{159}.
1.6.3 Filamin A Mutations and Disease

Mutations and deletions in the FLNa gene have been linked to several diseases including a multitude of congenital disorders. Periventricular heterotopia (PH) is one disorder which first becomes evident when seizures appear in teenagers. PH is a disorder in which neurons fail to migrate to the cerebral cortex in the fetal brain. Common indicators may include brain malformations, microcephaly, recurrent infections, and developmental delays or intellectual disability. In one study, 83% of patients with regular PH symptoms showed mutations in the FLNa gene thus showing a link between FLNa and PH and highlighting FLNa’s importance in brain development. A second related congenital disorder is FG syndrome which is characterized by mental development delays, congenital heart disease, anal atresia, inguinal hernia, and cryptorchidism among other defects. A previous report has linked a FLNa missense mutation as a cause for FG syndrome. Furthermore, FLNa mutations have been linked to various cancers including breast, colon, and prostate cancers. Finally missense and deletion mutations of the FLNa gene have also been found in patients with myxomatous valvular dystrophy, a disorder leading to incompetent cardiac valves.

1.6.4 Mechanoprotection and Mechanosensitive Channels

When a repeated mechanical force is applied to the cell membrane via a magnetic bead, an accumulation of F-actin is observed at the site of membrane stress. Interestingly, FLNa is the only actin-interacting protein also found at the site suggesting that FLNa recruits F-actin in response to membrane stress. Indeed, minimal force-induced actin accumulation to the membrane occurs in M2 cells whereas a strong recruitment of F-actin is observed in A7 cells. The actin accumulation at the site of stress is correlated with increased membrane rigidity and a
dampened sensitivity of SACs to membrane stretch\textsuperscript{165}. Disrupting the F-actin cytoskeleton using cytochalasin D ablated the inhibition of SAC activity thereby confirming the necessity of the actin cytoskeleton\textsuperscript{166}. Moreover, after successive mechanical stimulation, FLNa-lacking M2 cells displayed significantly less SAC inhibition when compared to FLNa-containing A7 cells thus confirming FLNa’s role in recruiting actin to block SAC activity\textsuperscript{166}.

Under the same conditions, repeated mechanical forces induces cell death as assessed by membrane leakage\textsuperscript{166}. However, the cell death induced by mechanical force was minimal in A7 cells as compared to M2 cells therefore highlighting FLNa’s mechanoprotective role\textsuperscript{166}. Repeated experiments in Rat-2 cells confirmed the importance of FLNa’s actin cross-linking function as FLNa constructs lacking the actin-binding domain did not render transfected cells less susceptible to mechanical force-induced death whereas full length FLNa did\textsuperscript{167}. The studies link mechanoprotection to the regulation of SACs since overstimulation of these channels by mechanical forces would lead to the excessive influx of calcium and subsequent cell death\textsuperscript{166,168}. It is therefore believed that under mechanical stress, FLNa recruits actin to the plasma membrane where it stiffens the membrane to inhibit SAC activity and prevents apoptosis by pathological levels of intracellular calcium\textsuperscript{166,167}.

1.6.5 Filamin A-dependent regulation of TREK1 via Polycystin 2

As previously mentioned, the Honoré lab described how SACs expressed in Cos7 cells respond with a markedly reduced current to a negative pressure pulse when co-expressed with TRPP2\textsuperscript{59}. In M2 cells lacking FLNa however, the net channel probability of opening is higher compared to FLNA\textsuperscript{+/-} A7 cells suggesting that FLNa is inhibiting channel opening. More importantly, the effects of TRPP2 are completely nonexistent in M2 cells suggesting that Filamin
A is a required downstream component of TRPP2’s mechanism of SAC inhibition\textsuperscript{59}. In a follow-up study, the group highlighted Filamin A’s role on TREK1 channels\textsuperscript{90}. TREK1 current was similarly inhibited by TRPP2 however this effect was again lacking when FLNa\textsuperscript{−/−} cells were used. Furthermore, excision of the recording patch in FLNa\textsuperscript{+/+} had a similar effect on the elicited current to using FLNa\textsuperscript{−/−} cells in that TRPP2 was no longer inhibiting the inward TREK1 current. This observation suggested that intracellular components were a necessary factor for Filamin A to have its effects. Treating the cells with the actin polymerization inhibitor, latrunculin A, had the same effect as membrane excision and therefore proposes that actin is the necessary downstream component for Filamin A’s effects\textsuperscript{90}.
Figure 1-1: Bilayer and tether models of SAC gating. (A) Channels may open in direct response to tension in the lipid bilayer. (B) Intracellular and/or extracellular components may instead transmit force to the channel via a tether to control gating. Adapted from: Nilius et al. 2012.
**Figure 1-2:** Topology of two-pore domain K\(^+\) channels. (A) Illustration of K\(_{2P}\) subunit with four transmembrane segments and two pore-forming domains (P1 & P2) arranged in tandem (top). The M1P1 domain is necessary for dimerization of subunits. (B) Pore sequence of 3 K\(_{2P}\) channels (TWIK1, TREK1, and TASK1) showing differences in the selectivity motifs compared to that of the conventional GYG motif found in voltage gated K\(^+\) channels (Kv1.1 & KcsA). A, alanine; F, phenylalanine; G, glycine; I, isoleucine; L, leucine; S, serine; T, threonine; V, valine; Y, tyrosine. Adapted from Honoré 2007.
Chapter 2 – Hypothesis

Previous studies have already begun to investigate the mechanical regulation of TREK1 mechanosensitive potassium channels. They have since proposed a model termed “The Upholstery Model” originally for SAC but then adapted for TREK1 channels. According to the model, Filamin A binds to TRPP2 and alters the underlying actin cytoskeleton. It does so by binding to two F-actin filaments and, because of its V-shaped structure, holds the filaments in an orthogonal crosslink. The altered arrangement of the actin cytoskeleton is believed to pull segments of the cell membrane closer together and create bulges of the membrane at the site of TREK1 channels. These micro domains have smaller radii of curvature compared to a flatter membrane. According to Laplace’s Law (T=Pr/2), a lower radius of curvature results in a lowered lateral tension on TREK1 channels when a given pressure stimulus is applied. A lowered perceived lateral tension on the channels results in a lower probability of opening and ultimately causes channel inhibition. When TRPP2 is no longer available at the plasma membrane possibly due to interaction with TRPP1, Filamin A is not recruited to alter the actin cytoskeleton and TREK1 thus perceives a greater lateral tension following a mechanical stimulus (Figure 2-1) \(^{59,90}\). This proposed model has been portrayed as a static one and the characteristics of this mechanism are not fully understood. Our central hypothesis is that the mechanical regulation of TREK1 is a highly dynamic one in which there is an increase in actin dynamics at the plasma membrane through TRPP2 and filamin A.
Figure 2-1: The Upholstery Model. For a given pressure stimulus (P), TREK1 channels perceive a lateral tension (T) which is dependent on the radius of curvature (I) of the cell membrane. Binding of FLNa to local TRPP2 increases actin crosslinking and creates microdomains with smaller radii of curvature. According to Laplace’s Law (T=Pr/2), a reduced tension is perceived by TREK1 channels for a given pressure which results in a diminished probability of opening. When TRPP2 of FLNa is absent, TRPP1 interacts with TRPP2, or when the actin cytoskeleton is disrupted, no microdomains are formed and the larger radius of curvature results in a stronger activation of TREK1 channels for every pressure stimulus. Adapted from: Sharif-Naeini, R. et al. 2009.
Chapter 3 – Methods

3.1 Cell Culture

The fibroblast-like Cos-7 cell line derived from African green monkey kidneys were used in addition to the human melanoma derived M2 and A7 cell lines. Cos-7 cells were maintained with Dulbeco’s Modified Eagle’s Growth Medium (DMEM; Wisent) supplemented with 10% Fetal Bovine Serum and 1% penicillin/streptomycin. M2 and A7 cells were maintained in Eagle’s Minimum Essential Medium (EMEM; Wisent) supplemented with 2% Newborn Calf Serum, 8% Fetal Bovine Serum, and 1% penicillin/streptomycin. A7 cells were additionally grown with 200 μg/mL of geneticin (Wisent) to maintain stable expression of Filamin A. One day prior to transfection, cells were plated at ~50% confluency onto 35 mm plastic dishes. All constructs were transfected using FuGene 6 (Promega) according to manufacturer’s instructions using 2 μg of DNA. Cells co-transfected with two plasmids received 1 μg of each plasmid. One day following transfection, cells were plated onto 35 mm glass bottom dishes. All recordings were performed 48 hours after transfection.

3.2 Electrophysiology

Cell-attached recordings were performed on transiently transfected Cos7, M2, and A7 cells. The extracellular recording medium contained 155 mM KCl, 5 mM, EGTA, 3 mM MgCl2, and 10 mM HEPES (pH 7.22, 310 mOsm). The pipette solution contained 150 mM NaCl, 5 mM KCl, 1 mM CaCl2, and 10 mM HEPES (pH 7.4, 310 mOsm). The pipette solution also contained 10 mM Tetraethyl-ammonium (TEA), 5 mM 4-Aminopyridine (4AP), and 10 μM glibenclamide to block contaminating potassium channels. After achieving a gigaseal in the cell-attached configuration and voltage clamping at 0 mV, membrane patches were stimulated with repeated
200 ms pressures pulses (-70 mmHg at 2Hz) through the recording electrode/pipette setup using a high-speed pressure clamp device (ALA Scientific Instruments). After the 10s stimulation, the membrane was allowed to rest for 2s, 15s, and 30s before a following pressure pulse was given. All recordings were performed with an Axon MultiClamp 700B amplifier (Molecular Devices) using non-coated fire polished glass pipettes (1.4-2.4 MΩ). Clampex 10.3 (Molecular Devices) was used for data acquisition. Data analysis and figure preparation was performed using Clampfit 10.3 (Molecular Devices), Microsoft Excel, and Prism 6 (GraphPad Software).

3.3 Fluorescence Recovery After Photobleaching (FRAP)

FRAP experiments were performed on a spinning disk confocal microscope (Quorum WaveFX, Leica) using a 63x objective. A 100 ms laser pulse was used to photobleach a 6.53 μm² area on the cell membrane. Images were captured with MetaMorph (Molecular Devices) before and for ~17s after photobleaching to acquire fluorescence recovery at the cell membrane. Data was corrected for the overall bleaching of the background signal resulting from image acquisition. Fluorescence data was fit to an exponential function to extrapolate the recovery time constant using Prism 6 (GraphPad Software).

3.4 Filamin A Subcellular Localization

The S8 cell line was generated by stably transfecting M2 cells with a red fluorescent form of Filamin A (Filamin A-RFP; Stossel Lab). Transiently transfected (TRPP2 or Mock) S8 cells were placed in a 6-well plate and centrifuged for 20 minutes at 0RPM (0G), 500RPM (63G), or 2000RPM (1010G) to apply a mechanical force. Immediately after centrifugation, cells were fixed with 4% paraformaldehyde and then treated with WGA-350 to label the plasma membrane.
Images were captured at 63x on a spinning disk confocal microscope (Quorum WaveFX, Leica). The WGA-350 signal was used as a mask to determine the Filamin A-RFP signal at the membrane, which was then normalized to the overall Filamin A-RFP cell intensity. ImageJ (National Institutes of Health) was used to perform data analysis.

3.5 Structured Illumination Microscopy (SIM)

Transiently transfected M2 and A7 cells were fixed with 4% paraformaldehyde and treated with phalloidin-488 (Cytoskeleton) to stain actin filaments. Cos7 were similarly prepared with the addition of a 20 minute mechanical stimulation via centrifugation at 2000RPM (1010G) prior to fixation and staining. Super resolution images were collected using an OMX V4 microscope (Applied Precision/GE Deltavision) using a 100x objective. OMX and softWoRx softwares (Applied Precision/GE Deltavision) were used for image acquisition and structured illumination reconstruction, respectively. Analysis of SIM data was performed using ImageJ (NIH) and the plugin FibrilTool to calculate anisotropy, the property of being directionally dependent, amongst actin filaments. The cell anisotropy values were measured by analyzing 4 μm thickness of actin staining just deep to the entire plasma membrane.

3.6 Statistical Analysis

Results are represented as mean ± SEM. Statistical significance was tested using unpaired t-tests for comparison of means. Differences were considered significant for p < 0.05 (*).
Chapter 4 – Results

4.1 Recovery of TREK1 Current Inhibition by TRPP2 in Cos7 Cells

Cos7 cells transiently expressing TREK1 with Mock or TREK1 with TRPP2 displayed an increasing peak potassium current following 10s of a 2Hz pressure pulse train, initially starting at 51.74 ± 6.80 % and 42.37 ± 4.66 % of the 20th pulse current, respectively (n= 40 for control group and n=38 for TRPP2 group, Figure 4-1A-B). After the cell membrane was permitted to rest, a recovery of the TREK1 current inhibition was observed in both conditions. By t=57s (47s after the pressure pulse train), control cells (n=28) recovered to only 92.94 ± 14.43 % of the 20th pulse current whereas cells expressing TRPP2 (n=36) recovered to 63.72 ± 5.58 % current (Figure 4-1C, p<0.05).

4.2 Recovery of TREK1 Current Inhibition by TRPP2 in FLNa+/+ and FLNa−/− Cells

To determine the importance of Filamin A in TRPP2 mediated TREK1 inhibition, we used M2 FLNa−/− cells and A7 FLNa+/+ control cells. In M2 cells transfected with TRPP2, no significant changes were observed in the current inhibition recovery curves when compared to control conditions (Figure 4-2A). At 47s after the pressure pulse train, TRPP2 transfected M2 cells (n=38) recovered to 70.70 ± 6.05 %, similar to control cells (n=51), which recovered to 68.63 ± 5.65 % (Figure 4-2C). In contrast, A7 cells expressing Filamin A displayed rescued TRPP2 mediated TREK1 current inhibition akin to Cos7 cells expressing TRPP2 (Figure 4-2B). At 47s post pulse pressure train, TRPP2 expressing A7 cells (n=17) recovered to 47.36 ± 6.00 % current while control cells (n=23) recovered to 70.16 ± 7.44 % (Figure 4-2C, p<0.05).
4.3 Actin Dynamics at the Plasma Membrane

To determine whether TRPP2 functions via a change in actin dynamics at the plasma membrane, we transfected Cos7 cells with LifeAct-RFP (to stain F-actin) and performed FRAP imaging to observe the rate of actin membrane recruitment and polymerization\textsuperscript{170}. Photobleaching the cell membrane did not demonstrate any significant differences in actin dynamics at 37°C in cells expressing TRPP2 (n=34) versus controls (n=24, Figure 4-3A). Fitting the data to an exponential function revealed time constants of 3.40 ± 0.38 seconds for TRPP2 expressing cells and 3.29 ± 0.39 seconds for control cells (Figure 4-3C). In an attempt to tease out possible minor differences in actin dynamics, we slowed kinetics and performed the experiment again at room temperature (Figure 4-3B). Time constants were indeed slower but no different among TRPP2 (n=20) and control (n=15) conditions (tau = 7.58 ± 1.46 s for TRPP2 transfected cells and tau = 6.53 ± 0.83 s for GFP transfected cells, Figure 4-3D).

FRAP experiments were then performed on cells transfected with GFP-Actin as opposed to LifeAct-RFP to more directly measure actin monomer recruitment (Figure 4-4A). Recordings at room temperature again showed no significant differences in time constants between TRPP2 and mock transfected cells (3.88 ± 0.47 s, n=21, in TRPP2 transfected cells compared to 3.11 ± 0.50 s, n=22, in mock transfected cells, Figure 4-4B). No differences in mobile fraction were observed either between conditions (0.42 ± 0.06 in TRPP2 transfected cells versus 0.41 ± 0.07 in mock transfected cells, Figure 4-4C).

4.4 Polycystin 2 Mediated Recruitment of Filamin A to the Plasma Membrane

We next suspected that Filamin A may itself be recruited to the cell membrane via TRPP2 to regulate TREK mechano-gating. In order to track the subcellular localization of FLNa,
we generated a new S8 cell line expressing the Filamin A-RFP fusion protein (Figure 4-5A). Transiently transfecting the cells with TRPP2 resulted in a significant increase in the RFP fluorescence signal at the cell membrane when compared to mock transfected cells (0.844 ± 0.047, n=10, in TRPP2 transfected S8 cells compared to 0.582 ± 0.046, n=10, in mock transfected cells) indicating that Filamin A is in fact recruited to the membrane following TRPP2 overexpression (Figure 4-5B). As previously described by the Glogauer lab, FLNa is found localized at sites of membrane stress following repeated mechanical force stimulation with magnetic beads.166 We thus suspected that mechanically stimulating the cell membrane via a centrifugal force would also lead to increased membrane RFP intensity in mock transfected cells and would potentiate the rise in intensity observed in TRPP2 transfected cells. Centrifuging the cells at 500RPM (63G) for 20 minutes did not result in an increased FLNa membrane localization (0.687 ± 0.035, n=10, in TRPP2 transfected S8 cells and 0.627 ± 0.040, n=10, in mock transfected cells) and neither did centrifugation at 2000RPM (1010G) for 20 minutes (0.812 ± 0.058, n=10, in TRPP2 transfected S8 cells compared to 0.714 ± 0.045, n=10, in mock transfected cells, Figure 4-5B). Furthermore, no significance difference was found between S8 cells transfected with TRPP2 and control cells at any of the mechanical stimulation exercises.

4.5 TRPP2-Induced Actin Reorganization at the Plasma Membrane

Since actin dynamics at the plasma membrane were not affected by TRPP2 overexpression, we hypothesized that the organization of the actin cytoskeleton may instead be altered via the recruitment of the actin crosslinking protein, Filamin A. To study the organization of the actin meshwork, we resorted to structured illumination microscopy, a form of super resolution microscopy, and measured the degree of anisotropy amongst actin filaments. Since
anisotropy is the property of being directionally dependent, a high anisotropy value with regards to actin filaments is suggestive of filaments being organized in a more parallel fashion whereas lower anisotropy values suggest an actin meshwork with more orthogonal crosslinking. When SIM was performed on A7 control cells, a $0.1905 \pm 0.0171$, $n=15$, anisotropy value was measured (Figure 4-6A). In contrast, A7 cells overexpressing TRPP2 displayed a significantly lowered $0.1397 \pm 0.0082$, $n=15$, anisotropy value which proposes a rearranged actin cytoskeleton from a more parallel to a more orthogonal state in the presence of TRPP2. No difference however was found between FLNa deficient M2 cells expressing a control vector ($0.1135 \pm 0.0082$, $n=15$) and those expressing TRPP2 ($0.1121 \pm 0.0103$, $n=13$). Therefore Filamin A appears to play an intricate role in mediating actin cytoskeleton rearrangement by Polycystin 2.

As mentioned before, the literature suggests FLNa localization to membrane patches subjected to mechanical forces\textsuperscript{166}. We therefore mechanically stimulated Cos7 via centrifugal force and observed for actin cytoskeleton rearrangement (Figure 4-6B). Unlike what was observed in A7 cells, TRPP2 transfected Cos7 cells did not display a change in anisotropy ($0.1062 \pm 0.0075$, $n=14$) compared to mock transfected cells ($0.1081 \pm 0.0065$, $n=15$). When exposed to centrifugation at 2000RPM (1010G), TRPP2 transfected Cos7 cells unexpectedly showed a raised anisotropy value ($0.1408 \pm 0.0098$, $n=15$) compared to centrifuged mock transfected cells ($0.1119 \pm 0.0084$, $n=15$) and non stimulated TRPP2 transfected cells.
Figure 4-1: TRPP2 induces an accelerated recovery of TREK1 current inhibition in Cos7 cells following sensitization via a -70 mmHg 2Hz pressure pulse train. (A) Representative traces showing elicited current during 2Hz pressure pulse train and after 2s, 15s, and 30s of rest. (B) Normalized stimulus response curves of mechanically activated TREK1 current during (0-10 s) and after (10-57 s) pressure pulse train in TRPP2 (n=38) expressing and mock (n=40) cells. (C) Recovered TREK1 current inhibition at 47s post pressure pulse train showing a significant increase in TREK1 current inhibition when TRPP2 is expressed. All data expressed as a percentage to the 20th (final) pulse at 10s. Statistical significance at *=p<0.05.
Figure 4-2: TRPP2 requires Filamin A to induce an accelerated recovery of TREK1 current inhibition following sensitization via a -70 mmHg 2Hz pressure pulse train. 
(A) Stimulus response curves of mechanically activated TREK1 currents of M2 FLNa⁻/⁻ cells expressing TRPP2 (n=38) or mock (n=51) vectors and (B) of A7 FLNa⁺/+ cells expressing TRPP2 (n=17) or mock (n=23). (C) Recovered TREK1 current inhibition at 47s post pressure pulse train showing a significant recovering of TREK current inhibition in Cos7 and A7 FLNa⁺/+ cells when expressing TRPP2 but not in M2 FLNa⁻/⁻ cells. All data expressed as a percentage to the 20th (final) pulse at 10s. Statistical significance at *=p<0.05.
Figure 4-3: Rate of actin recruitment to the cell membrane is not altered following expression of TRPP2. (A) Averaged traces showing recovery of LifeAct-RFP fluorescence signal following membrane photobleaching at t=1.5s in GFP (n=24) or TRPP2 (n=34) expressing Cos7 cells at 37°C and (B) at 22°C. (C) Tau value of fluorescence recovery trace at 37°C and (D) at 22°C showing no significant difference in recovery time constants between GFP transfected and TRPP2 transfected cells.
Figure 4-4: FRAP using GFP-Actin expressing Cos7 cells shows no change in actin dynamics following TRPP2 expression. (A) Averaged fluorescence recovery traces following membrane photobleaching of GFP-Actin in mock transfected (n=22) and TRPP2 transfected (n=21) cells at 22°C. (B) Time constant and (C) mobile fraction unaltered following TRPP2 expression.
Figure 4-5: Filamin A localizes to the plasma membrane without mechanical stimulation following TRPP2 transfection but not in the presence of mechanical stimulation. (A) S8 cells expressing Filamin A-RFP in red transfected with (i) TRPP2 or (ii) GFP control. (B) Filamin A-RFP fluorescence intensity expressed as membrane signal normalized to whole cell signal in S8 cells transfected with or with TRPP2 under resting or mechanically stimulating conditions. n=10. Statistical significance at ***=p<0.001.
Figure 4-6: SIM data suggesting actin reorganization in Filamin A expressing cells following transfection with TRPP2. (A) A7 and M2 cell anisotropy values following transfection with TRPP2 or mock plasmid (n=13-15). (B) Comparison of anisotropy values for Cos7 cells with or without mechanical stimulation following transfection with TRPP2 or mock plasmid (n=14-15). Statistical significance at *=p<0.05, **=p<0.01.
Chapter 5 – Discussion

5.1 Dynamic Inhibition of TREK1 Channels via Polycystin 2

The TREK1 two-pore-domain K⁺ channels play an intricate role in detecting mechanical stresses and to mediate the appropriate cellular responses such as volume regulation and mechanoprotection⁶⁹,⁷¹,⁹⁰. Previous studies demonstrate how Polycystin 2 inhibits TREK1 channels through a mechanism that involves an intact actin cytoskeleton and the actin binding protein Filamin A. The proposed model however is one that is static and does not investigate whether any dynamic cellular processes such as protein recruitment are involved⁵⁹,⁹⁰. In our electrophysiology experiments, we designed a protocol that is capable of exercising the plasma membrane through repeated negative pressure pulses to disrupt the underlying cytoskeleton¹⁷¹. As expected, the elicited TREK1 current increases progressively and nearly doubles as the membrane is exercised up until the 20th and final pulse. When the membrane is allowed to rest we see minimal recovery of current inhibition with the TREK1 current remaining elevated at 92.94 ± 14.43 % of the 20th pressure pulse even after 47s of membrane rest. Cos7 cells transfected with TRRP2 show the same rising induced current during membrane exercise but instead recovered to 63.72 ± 5.58 % of the 20th pulse after membrane rest. Ideally, recordings would have been continued until TREK1 currents have been fully inhibited back to baseline pre-exercise conditions however a limiting factor was a low success rate at maintaining a cell-attached configuration for longer than 1 minute. This is to be expected considering the rapid successive pulses used for membrane stimulation. Regardless, this greater degree of the recovered current inhibition observed in the same timespan suggests cells expressing TRPP2 recover to pre-membrane stimulation conditions at a faster rate than cells simply
expressing a mock vector. This novel finding is the first evidence that supports the notion that TRPP2 regulates TREK1 channels through a dynamic process.

We next investigated whether the dynamic regulation of TREK1 channels by TRPP2 required the actin binding protein Filamin A as suggested in the previously proposed static model\(^{59}\). The M2 cell line was a perfect tool for testing the involvement of Filamin A; not only because the gene encoding FLNa was completely knocked out, but because the A7 cell line is a perfect FLNa\(^{+/+}\) control since it originated from transfected M2 cells\(^{153}\). Similar to Cos7 cells, membrane exercise elicited a rising outward TREK1 current in A7 cells until a plateau was reached. Membrane rest allowed a degree of current inhibition recovery in control cells whereas TRPP2 transfected cells showed a complete recovery of inhibition by 47s post pressure pulse train. M2 cells also showed recovery post exercise but failed to display any differences between TRPP2 transfected and mock transfected conditions. In fact, both conditions recovered to the same degree as A7 cells lacking TRPP2 thus highlighting Filamin A’s key role as a player in the TRPP2 pathway. The fact that mock transfected A7 and M2 cells still recovered to about 70% of the 20\(^{th}\) pressure pulse shows that an intrinsic mechanism of actin re-polymerization at the plasma membrane not dependent on TRPP2 is involved in dynamically inhibiting the TREK1 currents whereas TRPP2 likely serves to speed up the process. The faster recovery rate observed in A7 cells expressing TRPP2 compared to that of Cos7 cells may be explained by A7 cells potentially having an intrinsically faster actin cytoskeleton recovery rate or due to an increased Filamin A expression pattern since the A7 cells are in fact an overexpression system. Regardless, TRPP2 accelerates the recovery rate of TREK1 current.
inhibition, therefore confirming that the inhibition is dynamic, and that the process is clearly dependent on Filamin A availability.

5.2 Actin Recruitment to the Cell Membrane

Seeing that TREK1 current inhibition recovers following disruption of the actin cytoskeleton and that polymerized actin has previously been reported as crucial for TRPP2’s mechanism, we suspected that actin might be actively recruited to the plasma membrane by TRPP2\textsuperscript{59,90}. Fluorescence Recovery After Photobleaching (FRAP) was the ideal technique to measure the recruitment rate of actin to the cell membrane after bleaching the fluorescence signal at a small patch of membrane. In our first set of experiments we used cells transfected with LifeAct-RFP to visualize F-actin as opposed to G-actin since we reasoned that only actin that was recruited to the cell membrane and polymerized could affect cell membrane structure and therefore regulate TREK1 channel opening\textsuperscript{170}. Measurements fit to an exponential curve to extrapolate times constants revealed no difference in LifeAct-RFP fluorescence recovery between TRPP2 and control transfected cells. We then hypothesized that TRPP2’s effect on actin recruitment may be too small to be detected under such experimental conditions. We therefore re-performed the experiments at 22°C as opposed to 37°C to slow down the kinetics of actin recruitment. Any difference in time constants would thus be more evident when comparing transfection conditions. Indeed, colder experimental conditions resulted in overall slower time constant values however no difference was evident with cells expressing TRPP2 compared to controls (Figure 4-3).
We next attempted FRAP experiments with GFP-actin to observe directly the trafficking of G-actin monomers to the plasma membrane (Figure 4-4). Again, the experiments suggested that actin was not recruited at an accelerated rate to the plasma membrane due to TRPP2. We speculated that the mobile fraction of actin could be altered in TRPP2 conditions. A lower actin mobile fraction at the cell membrane could suggest that TRPP2 holds actin in place to inhibit local TREK1 channels. Calculation of mobile fractions from FRAP curves however yielded no significant differences. Our data thus far suggest that actin dynamics are not altered in TRPP2 expressing conditions. It would be worth it however for future experiments to investigate actin dynamics directly following mechanical stimulation at the cellular membrane. Perhaps such stimulus is a necessary factor for TRPP2 to recruit actin to the membrane.

5.3 Filamin A Localization

The clear evidence of Filamin A’s necessity for the TRPP2 to regulate TREK1 channels lead to the postulation that Filamin A was preferentially localized to the membrane via TRPP2. To track the subcellular localization of Filamin A, we developed a new cell line by stably transfecting M2 cells with Filamin A-RFP. This new cell line, termed the S8 line, is essentially an A7 cell but with a fluorescently tagged Filamin A. The morphology of the S8 cell line resembled a hybrid of M2 and A7 cells which is assumed to be due to a Filamin A expression level subpar to A7 cells (data not shown). Regardless, all S8 cells displayed adequate RFP fluorescence signal to track the subcellular localization of Filamin A. In cells subsequently transfected with TRPP2, we observed a greater fluorescence signal at the cell membrane compared to control cells, therefore suggesting
that TRPP2 does indeed alter the subcellular localization of FLNa (Figure 4-5). TRPP2 likely holds FLNa at the membrane due to the direct interaction between FLNa’s C-terminal domain with the intracellular N- and C- termini of TRPP2.144

As previously mentioned, FLNa has been found to concentrate to sites of local mechanical stress.166 We aimed to apply a mechanical stress to the entire cell in an attempt to determine whether a greater degree of Filamin A is localized to the cell membrane with TRPP2. Mechanically stimulating the cells via centrifugation yielded unexpected results in that there was no longer a greater proportion of FLNa at the cell membrane in TRPP2 cells compared to mock cells. This unanticipated observation may be due to our method of mechanical stimulation which contrasts that of the Glogauer lab. The aforementioned group used magnetic beads to mechanically stimulate local regions of the cell membrane whereas our centrifugation technique stimulates the entire cell.166 This includes not only the complete cell membrane but also all the intracellular components which may lead to inaccurate results. Our results without any mechanical stimulation still stand however and show that the inhibition of TREK1 channels via TRPP2 function through a recruitment of FLNa to the plasma membrane.

5.4 Actin Reorganization

Our recent findings are not suggestive of faster actin recruitment to the cell membrane however the current literature and our electrophysiology findings validate actin’s role in TRPP2-induced TREK1 inhibition.59,90 Since we observe a subcellular localization of Filamin A to the cell membrane in cells co-expressing TRPP2 (Figure 4-5), we suspected that the localized concentration of FLNa was capable of reorganizing the
actin cytoskeleton due to FLNa’s ability to crosslink actin in an orthogonal fashion\textsuperscript{143}. Using structured illumination microscopy, we were able to acquire super resolution images of phalloidin stained actin filaments and then measure the anisotropy values at the membrane as a technique to determine how orthogonal the F-actin was arranged. As we expected, A7 cells displayed a decrease in anisotropy following transfection with TRPP2 (Figure3-7A). This measured decrease in anisotropy articulates that the microfilaments are less directionally dependent, or in other words, more orthogonal instead of parallel. This finding is reasonable since greater FLNa levels are found at the membrane with TRPP2 expression which allows for a greater degree of actin crosslinking just deep to the cell membrane. M2 cells did not exhibit any changes in anisotropy value during TRPP2 transfection which further validates the necessity of Filamin A in crosslinking actin. Further experimentation with Cos7 cells yielded results that were less expected. No change in anisotropy was found when transfection with TRPP2 was performed compared to mock control cells. We correlate these findings to the lower expression level of Filamin A in Cos7 cells compared to that of A7 cells. We suspect that the expression level of Filamin A is high enough to see the functional difference in our electrophysiology recordings but too low to see any structural changes with our imaging techniques. Furthermore, we showed that TREK1 current inhibition recovered to a lesser degree with and without TRPP2 in Cos7 cells compared to A7 cells which may potentially be explained by non visual changes in the actin reorganization due to lower Filamin A expression. Future studies will require the overexpression of Filamin A in Cos7 cells to see if electrophysiology results are enhanced and whether changes in actin reorganization become evident.
Finally, we subjected the Cos7 cells to mechanical stimulation via centrifugation in a similar manner to our S8 cells for analyzing the subcellular localization of FLNa. When SIM was performed of Cos7 cells subjected to mechanical stimulation we observed an increase in the anisotropy with cells expressing TRPP2 therefore showing a reorganization of actin to more parallel bundles instead of orthogonal structures (Figure 4-6B). Although not originally expected, this finding is consistent with the decrease in Filamin A localization found at the cell membrane following centrifugal stimulation. As we develop better techniques for mechanically stimulating the plasma membrane independently from the entire cell such as performed in the previous literature, we will then be able to stimulate just a local region of the membrane in A7, M2, and Cos7 cells and compare that region to the rest of the cell membrane\textsuperscript{166}.

5.5 Revised Upholstery Model

Our current findings remain consistent with the Upholstery Model first described by Sharif-Naeini et al. in 2009 for SACs and then applied to TREK1 by the same group in 2012\textsuperscript{59,90}. The model originally proposed that uninterrupted TRPP2 is capable of recruiting actin and crosslinking the filaments of the cytoskeleton to create microdomains of the lipid bilayer with relatively small radii of curvature. Thus according to Laplace’s Law, lower tension is perceived by TREK1 channels for a given transmembrane pressure in the presence of TRPP2 with FLNa and an intact actin cytoskeleton. Our studies confirm that Filamin A shows preferential subcellular localization to the cell membrane in the presence of TRPP2. Moreover, while the rate of actin recruitment is not altered, we show that actin is reorganized orthogonally which was originally suspected for the Upholstery Model to
create the microdomains. As an update to the model, through our electrophysiology data, we show that the process of TREK1 inhibition occurs through a dynamic process since inhibition recovers at a faster rate in TRPP2 transfected cells following disruption of the actin cytoskeleton (Figure 4-1). We show that this process occurs not because actin is recruited at a faster rate, but because of the way it is organized following recruitment (Figure 4-6). Future experiments will however be required to prove that the orthogonal reorganization of actin does indeed cause a rigidification of the membrane that can impede channel opening. This question can be solved using Atomic Force Microscopy whereby a probe is pressed against the cell membrane and a feedback force is measured for a given indentation. Ultimately, we can calculate of the membrane tension of cells under different conditions. Cells overexpressing FLNa and TRPP2 will likely display a higher effective membrane tension compared to cells with lower levels of FLNa and TRPP2.

Chapter 6 – Conclusion

Our study features the mechanical regulation of the TREK1 mechanosensitive potassium channel by Polycystin 2. We show that while co-expressing TRPP2 in heterologous systems with TREK1 greatly inhibits the elicited current following mechanical stimulation, this inhibition can be removed by disrupting the underlying actin cytoskeleton by exercising the membrane with repeated pressure pulses. Furthermore, during membrane rest, the inhibition of TREK1 occurs at a faster rate when TRPP2 is expressed. We then show that this process is not due to an increased recruitment of actin to the cell membrane, but because Filamin A is preferentially localized at the cell membrane and crosslinks actin filaments in an orthogonal fashion. Because TREK1 is widely
expressed in the human body, especially the nervous system, and has several mechanosensory functions, understanding how it is regulated may prove useful in future therapy development. The data presented in this thesis refine the roles of Polycystin 2, Filamin A, and the actin cytoskeleton in regulating TREK1 mechanosensitivity.
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