

American Heart Association Research Proposal, Example 1

Specific Aims

The major goal of my research is to understand the molecular mechanisms that regulate thermal sensitivity in mammalian somatosensory neurons. TRPV1, a member of the transient receptor potential (TRP) family of ion channels, plays a critical role in mammalian thermosensation as a molecular sensor of noxious heat and pain-producing chemicals. TRPV1 activity can be altered under pro-inflammatory conditions, but the cellular mechanism of TRPV1 modulation is not entirely clear. This proposal seeks to understand how TRPV1 is activated by noxious stimuli, and how its activation can be modulated by different physiological inputs. My specific aims are:

Aim 1: ***To Identify Modulators of TRPV1 Using a Novel Genetic Screen***

Aim 2: ***To Study TRPV1 Gating Using High-throughput Random Mutagenesis***

Background and Significance

An organism's ability to sense temperature, and distinguish noxious from innocuous stimuli, is crucial to its survival under complex and constantly changing environmental conditions. In mammals, distinct somatosensory neurons in the trigeminal and dorsal root ganglia, known as nociceptors, respond directly to noxious hot and cold stimuli. Members of the TRP superfamily of cation channels expressed in these ganglia play major excitatory roles in thermosensation (1, 2). TRPV1, found in a subset of heat-sensitive nociceptors, is directly activated by capsaicin (the pungent ingredient in chili peppers), protons (pH < 6.0), and noxious heat (temperature > 43° C). Heterologous expression of TRPV1 in *Xenopus* oocytes or human embryonic kidney (HEK) cells confers responsiveness to all of these stimuli, even in excised membrane patches (3, 4). Furthermore, the temperature-response profile for TRPV1 in heterologous systems is remarkably similar to that of isolated sensory neurons (5). TRPV1-deficient mice show complete insensitivity to capsaicin and marked alterations in pH and temperature sensation, particularly in the setting of tissue injury and inflammation (6, 7). Taken together, these studies indicate that TRPV1 plays a crucial role in mammalian thermosensation as a polymodal detector of noxious stimuli.

Although TRPV1 clearly plays an important role in mammalian nociception, the molecular mechanism by which TRPV1 responds to noxious thermal and chemical stimulation remains poorly understood. TRP channels possess six predicted transmembrane domains, sharing some architectural similarity with the well-studied voltage-gated K⁺ channels (8). However, the sequence homology between these two families is poor and their functional characteristics are highly divergent. Despite advances in the structural biology of voltage-gated K⁺ channels (9, 10), crystallographic data on TRPV1 is not yet available. This has made it difficult to apply structural and mechanistic models of K⁺ channels to TRPV1. Structure-function studies have identified separate regions of TRPV1 required for binding of and activation by vanilloid ligands (11), protons (12), and regulatory phospholipids (13). However, little is known molecularly about how binding to these regions induces conformational changes that open the TRPV1 pore to allow ion flux, a fundamental process known as gating.

Since TRPV1 plays such a critical role in thermosensation and inflammatory hyperalgesia, it is likely regulated *in vivo* by multiple mechanisms. Most six transmembrane domain ion channels, including Na⁺, K⁺, Ca²⁺, and cyclic nucleotide gated (CNG) channels, interact with accessory subunits, signaling enzymes, and other modulatory factors that regulate their behavior (14, 15, 16, 17). In addition, TRP channels reside in elaborate signaling complexes in other organisms. This has been best characterized in the *Drosophila* eye, where the canonical TRP channel associates with multiple signaling molecules to mediate

phototransduction. Disruption of this TRP-containing signaling complex results in severe impairment of responses to photons (18, 19). It remains to be seen whether a similar situation exists for TRPV1 in mammalian nociceptors, or for any mammalian TRP channel.

Previously, I proposed to identify factors involved TRPV1 modulation using a forward genetic approach. Through a comprehensive functional screen for channel modulators in yeast, I have identified twelve candidate genes that are required for TRPV1 activation. I will confirm and characterize the mechanisms by which these genes modulate TRPV1 activation in mammalian cells. Additionally, I have adapted my screening methodology to allow unbiased systematic identification of sites on TRPV1 that are critical for gating.

Aim 1: To Identify Modulators of TRPV1 Using a Novel Genetic Screen

Background and Significance:

TRPV1 is modulated under different physiological conditions, but the underlying regulatory mechanisms are only beginning to be revealed. To search for proteins involved in TRPV1 regulation, I developed a functional assay to measure activation of TRPV1 heterologously expressed in the budding yeast *Saccharomyces cerevisiae*. Because many yeast genes have homologs in higher organisms, a comprehensive functional screen for TRPV1 modulators in yeast might allow me to identify ubiquitously expressed factors that are crucial for channel activation in mammalian cells. The tools available for rapid genomic manipulation make yeast superior to any mammalian system for comprehensive genetic screens. A set of five thousand mutant strains, each harboring a precise deletion of a single yeast open reading frame, has been constructed (20, 21). This knockout collection represents nearly all nonessential genes and allows for efficient genome-wide loss-of-function analysis.

Although yeast cells do not have an endogenous TRPV1 gene, I have found that they nevertheless support functional expression of mammalian TRPV1. As in mammalian cells, yeast expressing TRPV1 die upon prolonged stimulation with capsaicin, and yeast expressing a constitutively active TRPV1 mutant (TRPV1 Δ PIP₂) die even in the absence of ligand (fig. 1 of progress report). The TRPV1 pore blocker ruthenium red fully reverses toxicity, indicating that toxicity is dependent on channel activity and not simply an overexpression artifact. I used this assay to screen the yeast knockout collection described above for genes that suppress TRPV1-mediated toxicity in yeast and therefore might be required for efficient channel activation. The TRPV1 Δ PIP₂ gene was transformed into all five thousand knockout strains and plated on medium without ruthenium red, a condition which normally causes death. Surviving strains were identified, and the TRPV1 Δ PIP₂ plasmid was subsequently retransformed into a fresh culture of each putative suppressor strain to confirm the observed phenotype.

Twelve genes were identified whose deletion severely impairs TRPV1-mediated death (fig. 2 of progress report). The majority of these genes have homologs in higher organisms including mammals. One particularly intriguing candidate is a gene encoding a membrane protein with unknown function. Humans contain a homolog of this candidate expressed in a diverse range of tissue types, but its physiological role has not been reported. The gene might be required for the stability or functionality of TRPV1 at the cell surface, similar to the role played by auxiliary subunits for other mammalian ion channels. Here I describe experiments to identify the role that candidate genes play in modulating TRPV1.

Research Design and Methods:

The majority of genes identified in my suppressor screen possess one or more mammalian homologs. Those homologs expressed in the native environment of TRPV1 are the most physiologically relevant to pain sensation, and therefore the most worthwhile to study further. I will use RT-PCR to determine which homologs are expressed in sensory neurons.

Deletion of a yeast open reading frame could suppress TRPV1-mediated toxicity by a number of mechanisms, including loss of TRPV1 protein production. We are not interested in studying transcriptional or translational effects on TRPV1 in yeast, since they are unlikely to be relevant to channel regulation in a system where the channel is expressed on a plasmid under the control of a yeast promoter. To determine whether TRPV1 protein has been lost in any of the suppressor strains, I will prepare lysates from liquid cultures and assess protein expression via standard immunoblotting techniques. Immunoblot signals will be compared to those obtained from a wild-type strain transformed with TRPV1, and any suppressor strains showing significant reduction in TRPV1 protein levels will be excluded from further analysis.

Next, I will use RNA-interference technology to assess the effects on TRPV1 functionality of deleting suppressor homologs in mammalian cells. Modulation of channel activity will be measured by introducing siRNAs against the suppressor into a TRPV1-expressing stable HEK293 cell line and subjecting the cells to electrophysiological and calcium imaging analysis. Changes in the concentration-response curves for various ligands as well as the temperature-response curve, relative to cells transfected with a scrambled siRNA, will be noted. Simultaneously, the effectiveness and specificity of knockdown will be assessed by RT-PCR. In parallel, I will analyze each set of cells for suppression of capsaicin-evoked toxicity. Capsaicin will be applied to the cells for several hours, viability will be scored by trypan blue exclusion, and any changes relative to cells transfected with a scrambled siRNA will be recorded. I will conduct similar siRNA analysis on dissociated sensory neurons. Although sensory neurons are difficult to transfect, recent studies have reported successful transfection using viruses or the Amaxa system (22, 23). As a control to determine the receptor specificity of any observed regulatory effects, I will conduct analogous RNAi experiments in cell lines stably expressing other sensory neuronal ion channels, such as the cold/menthol receptor TRPM8, the serotonin receptor 5HT₃, and the ATP receptor P2X₂.

To complement the loss-of-function experiments, I will determine the effect of overexpression of each suppressor gene on TRPV1-mediated responses in heterologous expression systems. The suppressor will be coexpressed with TRPV1 in HEK293 cells and *Xenopus* oocytes, and channel activity in the presence or absence of the suppressor will be assessed electrophysiologically. Any changes in chemically- or thermally-evoked responses will be noted. It will also be interesting to examine whether the overall number of channels at the membrane is increased when the suppressor gene is overexpressed. Such an effect on expression level is often observed when the pore forming subunits of ion channel complexes are coexpressed with key accessory factors, such as ionotropic glutamate receptors with their accessory subunit stargazin (24). A similar finding could provide evidence that the suppressor exerts a profound regulatory effect on TRPV1 functionality, possibly as an accessory subunit.

The RNAi and overexpression studies will help to establish a clear link between levels of the modulator gene and alterations in TRPV1 functionality. Future biochemical and cell biological experiments will address whether the modulators reside in a complex with TRPV1, and what role such a complex might play in channel trafficking and modulation.

Expected Results and Caveats:

The experiments outlined above assume that a single mammalian homolog exists for each yeast gene identified in my screen. Some single yeast genes may have evolved into families of mammalian genes. Thus, RNAi experiments targeting a single gene in mammalian cells might not produce robust phenotypes due to genetic redundancy. In cases where all of the family members are known in mammalian cells, it might be possible to target combinations of genes with RNAi to circumvent the redundancy issue. RT-PCR analysis, as outlined above, may be useful in determining which combinations of genes within a single family are

physiologically relevant to TRPV1 regulation. In cases where RNAi experiments are inconclusive, either due to unmanageable redundancy or failure to achieve reliable knock-down, experiments involving expression of a dominant-negative version of the candidate gene may provide a viable alternative strategy.

The mammalian cell siRNA experiments will help delineate the mechanism by which a deletion of a given gene alters TRPV1-mediated cell death. The deletion may affect channel functionality, such that the response of TRPV1 to agonist stimulation is drastically altered. Alternatively, a deletion could affect the ability of cells to tolerate persistent TRPV1-mediated cation influx. Parallel analysis using assays that measure acute TRPV1 activation versus long-term cell death will help distinguish between these possibilities. I would expect a gene that modifies channel activation to display altered characteristics in electrophysiological and cell death assays compared to control cells. On the other hand, genes affecting long-term cation tolerance would show altered characteristics in the cell death assay but leave the electrophysiological properties of TRPV1 unchanged.

It is possible that some modulatory factors will alter both the ligand- and temperature-response profiles of TRPV1 when knocked down. Proteins involved in channel trafficking and assembly are likely to display this behavior. Some genes may be selectively involved in thermal but not chemical responsiveness. Such modality-specific suppression has never been demonstrated for TRPV1 and could be extremely informative for elucidating mechanisms of channel regulation. Preliminary analysis shows that several of the suppressor strains retain capsaicin- but not thermal-sensitivity when transformed with the constitutively active TRPV1 mutant. This indicates that some suppressors may indeed be modality-specific, and would represent a particularly intriguing topic for further investigation.

Aim 2: To Study TRP Channel Gating Using High-throughput Random Mutagenesis

Background and Significance:

All ion channels must efficiently couple stimulatory events, such as the interaction of a chemical agonist with its binding site, to opening of the pore. This process is known as gating. The gating mechanisms of some ion channel families, most notably voltage-dependent K^+ channels, have been probed in great detail by electrophysiological, biochemical, and structural methods. As a result, much is understood about how the movements of critical gating domains link voltage-dependent stimulation with pore opening. In contrast, the process by which TRP channels are gated by chemical or thermal stimuli remains poorly understood. Although TRP channels are believed to possess the same transmembrane architecture as K^+ channels (8), the two families have poor sequence homology and divergent functional characteristics. Therefore, it is unclear if previously established gating models may be applied to TRPV1.

A first step in molecular analysis of TRP channel gating is to identify residues that are involved in the gating process. Site-directed mutagenesis of candidate residues, followed by electrophysiological analysis of resulting mutants, has been the method of choice to approach this problem. Unfortunately, conventional ion channel mutagenesis is slow, labor intensive, and usually limited by practical considerations to only a handful of mutations at a time. It would be more efficient to develop an unbiased high-throughput screen that allowed thousands of channel mutants to be assayed simultaneously for gating effects. This approach has been used successfully to analyze structure-function relationships in inward rectifier K^+ channels (25, 26, 27). However, such a strategy has not yet been employed to study TRPV1.

I developed a strategy to efficiently identify TRPV1 gating mutants via a genetic screen in yeast. In this screen, certain types of mutations within critical residues will cause inappropriate gating and constitutive channel activation, leading to cell death *in vivo*. To pinpoint these residues, I constructed a library of $\sim 2 \times 10^5$ randomly mutagenized TRPV1

genes using error-prone PCR, in which each copy of TRPV1 possesses on average one or two amino acid changes scattered randomly throughout the gene. I transformed the library into yeast and grew the transformants on medium containing the TRPV1 pore blocker ruthenium red. I selected constitutively active mutants by replica plating the transformants to medium lacking ruthenium red, comparing the replicated plates, and picking any colonies showing decreased survival in the absence of channel blocker. From 30,000 independent clones, I identified 25 mutants that reproducibly display ligand-independent toxicity (fig. 3 of progress report). Sequence information gleaned from the yeast screen will pinpoint residues and domains within TRPV1 that are critical for gating by chemical and thermal stimuli.

Research Design and Methods:

To confirm the gain-of-function phenotype of the 25 clones identified in my screen, I will rescue the TRPV1 plasmid DNA from the positive clones, retransform each into freshly grown yeast cells, and retest the transformants for constitutive activation. This ensures that the observed toxicity is due to the TRPV1 plasmid and not to an unlinked genomic mutation. Plasmid DNA from all gain-of-function clones will be sequenced to determine the positions of mutations causing constitutive activation. Clustering within a spatial region or favoring of a specific class of amino acid substitution will be noted.

I will verify the altered gating properties of the TRPV1 mutants by functional analysis in mammalian cells. Mutant TRPV1 genes from the screen will be expressed in HEK293T cells and analyzed by electrophysiology and calcium imaging. I will look for alterations in the ligand and temperature responsiveness of the mutant channels in comparison to wild-type channels. Single-channel recording will be used to measure biophysical parameters associated with channel gating such as unitary conductance and kinetic properties. I will assess expression of wild-type and mutant channels by surface biotinylation to ensure that the gain-of-function phenotype is not simply due to increased levels of channel protein at the plasma membrane.

Libraries generated by error-prone PCR are often sufficiently diverse that every residue in the encoded protein is mutated many times (28). However, it is unlikely that all twenty amino acids will be represented for a given position because some mutations require simultaneous alteration of multiple nucleotides within a codon. I will mutate each gating position identified in the screen to all nineteen others so that its structural environment can be fully probed. This is easily accomplished by screening a small "library" of TRPV1 mutants containing a randomized codon (introduced into the gene via synthetic oligonucleotides) corresponding to the amino acid in question. I will use my yeast assay to efficiently determine whether the resulting channels are nonfunctional, wild-type, or constitutively active in nature. The pattern of tolerated substitutions at a given residue can show whether that residue faces the pore, the protein interior, or the lipid bilayer (25, 26). These experiments provide valuable structural information in the absence of crystallographic data.

Finally, I will use classical second-site suppressor analysis to address the spatial relationship between critical gating residues and other channel domains. If mutation of a gating residue locks TRPV1 into a permanently open state by perturbing a critical electrostatic interaction with a "partner residue" elsewhere on the protein, a compensatory mutation within the partner residue should restore normal channel activity. This type of analysis has been exploited for K⁺ channels to map interactions required for packing of transmembrane helices (25, 29, 30). I will obtain second-site suppressors by introducing a constitutive gating mutation into my random mutant library and selecting transformants on medium lacking ruthenium red. Surviving colonies will be tested for ligand-dependent activation by replica plating to capsaicin-containing medium, in order to eliminate any mutations that suppress toxicity by simply inducing gross defects in channel functionality such as misfolding. Remaining mutants will be

tested for suppression of constitutive gating using functional assays in mammalian cells. In the future, direct evidence for spatial proximity between a gating residue and its second site suppressor can be obtained from biochemical crosslinking experiments. These experiments provide information about which channel regions move close together during gating, and therefore begin to reveal the dynamics of the gating process.

Expected Results and Caveats:

I would expect that a gating mutant recovered in yeast should display similar properties in mammalian cells, since TRPV1-mediated toxicity in yeast appears to be directly related to channel activation (fig. 1 of progress report). Despite this conservation of channel functionality between expression systems, some mutations in the channel might affect association with a regulatory protein or lipid element found only in yeast; these mutants may not show a robust phenotype in mammalian cells. I will focus on mutations that produce functional changes in both sets of expression systems, since these are likely to affect fundamental processes such as gating. Channel mutations producing toxicity in yeast might be similarly lethal in mammalian cells, which could preclude further functional analysis. The XXX lab has successfully circumvented this problem by using the *Xenopus* oocyte expression system to analyze toxic TRPV1 mutants. Toxicity in oocytes is less pronounced than in mammalian cells, presumably due to their lower incubation temperature (16°C vs. 37°C) (12).

TRPV1 is modulated by chemical agonists, temperature, protons, regulatory lipids, and phosphorylation (2). Mutations in the channel could affect activation by any of these processes independent of gating. How are such effects distinguished from those involving gating? Previous studies have defined channel regions required for some of these processes, which will provide guideposts for my analysis. It may also be informative to examine whether a given TRPV1 mutant can be further activated by the modalities listed above. Assuming that the structural elements regulating pore opening are common to all modalities, a true gating mutant may not be further activated by another stimulus because gating is downstream of all conformational changes arising from stimulus detection. In contrast, a constitutively active capsaicin binding site mutant may exhibit increased basal activity but is susceptible to further activation by protons or heat. Of course, other types of mutations besides those purely involved in gating could still be extremely interesting. For example, it is unclear which domains within TRPV1 sense changes in ambient temperature to allow heat activation. A “temperature binding site” mutation, which can be further opened by capsaicin but not by heating, would be a valuable tool to investigate the enigmatic process of TRP channel temperature activation.

Ethical Aspects of Proposed Research

One objective of this research is to understand how TRPV1 is regulated in its neuronal context. Some experiments can be carried out in previously established cell lines, but others (preparation of oocytes and isolation of sensory neurons) require laboratory frogs and rodents. All animals and biohazards will be used according to strict institutional guidelines.

Conclusion

The experiments outlined in this proposal will allow me to identify and characterize the molecular determinants of TRPV1 activation and modulation. These studies will provide novel insights into TRPV1 regulation *in vivo*, a topic highly relevant to mammalian nociception and thermosensation in both normal and inflammatory states. Moreover, these studies will shed light on general aspects of TRP channel structure and function. Because TRP channel family members are widely expressed, these studies will be of further direct relevance to cardiovascular function and the regulation of muscle, nerve, and vascular cell excitability.

REFERENCES

1. Julius, D. and A.I. Basbaum, *Molecular mechanisms of nociception*. Nature, 2001. **413**(6852): p. 203-10.
2. Jordt, S.E., D.D. McKemy, and D. Julius, *Lessons from peppers and peppermint: the molecular logic of thermosensation*. Curr Opin Neurobiol, 2003. **13**(4): p. 487-92.
3. Caterina, M.J., et al., *The capsaicin receptor: a heat-activated ion channel in the pain pathway*. Nature, 1997. **389**(6653): p. 816-24.
4. Tominaga, M., et al., *The cloned capsaicin receptor integrates multiple pain-producing stimuli*. Neuron, 1998. **21**(3): p. 531-43.
5. Cesare, P. and P. McNaughton, *A novel heat-activated current in nociceptive neurons and its sensitization by bradykinin*. Proc Natl Acad Sci U S A, 1996. **93**(26): p. 15435-9.
6. Caterina, M.J., et al., *Impaired nociception and pain sensation in mice lacking the capsaicin receptor*. Science, 2000. **288**(5464): p. 306-13.
7. Davis, J.B., et al., *Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia*. Nature, 2000. **405**(6783): p. 183-7.
8. Clapham, D.E., *TRP channels as cellular sensors*. Nature, 2003. **426**(6966): p. 517-24.
9. Long, S.B., E.B. Campbell, and R. Mackinnon, *Crystal structure of a mammalian voltage-dependent Shaker family K⁺ channel*. Science, 2005. **309**(5736): p. 897-903.
10. Long, S.B., E.B. Campbell, and R. Mackinnon, *Voltage sensor of Kv1.2: structural basis of electromechanical coupling*. Science, 2005. **309**(5736): p. 903-8.
11. Jordt, S.E. and D. Julius, *Molecular basis for species-specific sensitivity to "hot" chili peppers*. Cell, 2002. **108**(3): p. 421-30.
12. Jordt, S.E., M. Tominaga, and D. Julius, *Acid potentiation of the capsaicin receptor determined by a key extracellular site*. Proc Natl Acad Sci U S A, 2000. **97**(14): p. 8134-9.
13. Prescott, E.D. and D. Julius, *A modular PIP2 binding site as a determinant of capsaicin receptor sensitivity*. Science, 2003. **300**(5623): p. 1284-8.
14. Pongs, O., et al., *Functional and molecular aspects of voltage-gated K⁺ channel beta subunits*. Ann N Y Acad Sci, 1999. **868**: p. 344-55.
15. Catterall, W.A., et al., *Regulation of sodium and calcium channels by signaling complexes*. J Recept Signal Transduct Res, 2006. **26**(5-6): p. 577-98.
16. Cantrell, A.R. and W.A. Catterall, *Neuromodulation of Na⁺ channels: an unexpected form of cellular plasticity*. Nat Rev Neurosci, 2001. **2**(6): p. 397-407.
17. Craven, K.B. and W.N. Zagotta, *CNG and HCN channels: two peas, one pod*. Annu Rev Physiol, 2006. **68**: p. 375-401.
18. Tsunoda, S., et al., *A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade*. Nature, 1997. **388**(6639): p. 243-9.
19. Chevesich, J., A.J. Kreuz, and C. Montell, *Requirement for the PDZ domain protein, INAD, for localization of the TRP store-operated channel to a signaling complex*. Neuron, 1997. **18**(1): p. 95-105.
20. Winzeler, E.A., et al., *Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis*. Science, 1999. **285**(5429): p. 901-6.
21. Giaever, G., et al., *Functional profiling of the Saccharomyces cerevisiae genome*. Nature, 2002. **418**(6896): p. 387-91.
22. Durham, P.L. and A.F. Russo, *Stimulation of the calcitonin gene-related peptide enhancer by mitogen-activated protein kinases and repression by an antimigraine drug in trigeminal ganglia neurons*. J Neurosci, 2003. **23**(3): p. 807-15.
23. Leclere, P.G., et al., *Effective gene delivery to adult neurons by a modified form of electroporation*. J Neurosci Methods, 2005. **142**(1): p. 137-43.

24. Tomita, S., et al., *Stargazin modulates AMPA receptor gating and trafficking by distinct domains*. Nature, 2005. **435**(7045): p. 1052-8.
25. Minor, D.L., Jr., et al., *Transmembrane structure of an inwardly rectifying potassium channel*. Cell, 1999. **96**(6): p. 879-91.
26. Yi, B.A., et al., *Yeast screen for constitutively active mutant G protein-activated potassium channels*. Neuron, 2001. **29**(3): p. 657-67.
27. Sadjia, R., et al., *Coupling Gbetagamma-dependent activation to channel opening via pore elements in inwardly rectifying potassium channels*. Neuron, 2001. **29**(3): p. 669-80.
28. Cadwell, R.C. and G.F. Joyce, *Mutagenic PCR*. PCR Methods Appl, 1994. **3**(6): p. S136-40.
29. Papazian, D.M., et al., *Electrostatic interactions of S4 voltage sensor in Shaker K⁺ channel*. Neuron, 1995. **14**(6): p. 1293-301.
30. Lai, H.C., et al., *The S4 voltage sensor packs against the pore domain in the KAT1 voltage-gated potassium channel*. Neuron, 2005. **47**(3): p. 395-406.1.