

channels are inhibited by cytosolic concentrations of Ca^{2+} in a CaMKII-dependent manner. Supported by NSERC & the Jeanne Mance Foundation, Hotel Dieu Hospital.

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Characterization of Transient Receptor Potential Melastatin 7 in Bone Marrow Stem Cells

Henrique Cheng.

Louisiana State University, Baton Rouge, LA, USA.

Changes in the intracellular concentrations of Ca^{2+} and Mg^{2+} play a significant role in cell growth and differentiation. Mesenchymal stem cells (MSCs) from bone marrow are a potential source for tissue repair due to their ability to differentiate into specialized cells, including bone, fat and muscle. However, the molecular signals controlling the differentiation process remains largely unknown. In this study, we examined whether MSCs express Transient Receptor Potential Melastatin 7 (TRPM7), a member of the TRP family of ion channels and a key pathway for Ca^{2+} and Mg^{2+} entry into cells. By RT-PCR, we identified TRPM7 transcripts with the expected molecular size of 198bp, but not TRPM6 (317bp), a close family member with similar function. Electrophysiological recordings revealed that depletion of intracellular Mg^{2+} or Mg^{2+} -ATP activated TRPM7, suggesting that the channel is functionally active. Furthermore, treatment of MSCs with 2-aminoethoxydiphenyl borate (2-APB 1pM-100pM), a TRPM7 blocker inhibited TRPM7 currents in a dose-dependent manner. Our findings suggest that TRPM7 may represent an important pathway for controlling stem cell growth and differentiation by regulating the amount of Ca^{2+} and Mg^{2+} entering cells.

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The Type IV Mucopolidiosis-Associated Protein TRPML1 is an Endolysosomal Iron Release Channel

Xian-Ping Dong¹, Xiping Cheng¹, Eric Mills¹, Markus Delling², Fudi Wang³, Tino Kurz⁴, Haoxing Xu¹.

¹The Department of Molecular, Cellular, and Developmental Biology, The University of Michigan, Ann Arbor, MI, USA, ²The Department of Cardiology, Children's Hospital Boston, Boston, MA, USA, ³Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Science, Shanghai, China, ⁴The Department of Pharmacology, Faculty of Health Science, University of Linköping, Linköping, Sweden. TRPML1 (mucopolin-1/MCOLN1) is predicted to be an intracellular late endosomal and lysosomal ion channel protein belonging to the mucopolin subfamily of Transient Receptor Potential (TRP) proteins. Mutations in the human *TRPML1* gene cause mucopolidiosis type IV disease (ML4). ML4 patients exhibit motor impairment, mental retardation, retinal degeneration, and iron-deficiency anemia. Since aberrant iron metabolism may cause neural and retinal degeneration, it may be a primary cause of ML4 phenotypes. In most mammalian cells, release of iron from endosomes and lysosomes following iron uptake via endocytosis of Fe^{3+} -bound transferrin receptors, or following lysosomal degradation of ferritin-Fe complexes and autophagic ingestion of iron-containing macromolecules, is the major source of cellular iron. The Divalent Metal Transporter protein (DMT1) is the only endosomal Fe^{2+} transporter currently known and is highly expressed in erythroid precursors, but genetic studies suggest the existence of a DMT1-independent endosomal/lysosomal Fe^{2+} transport protein. Here, by measuring radiolabeled iron uptake, monitoring the levels of cytosolic and intra-lysosomal iron and directly patch-clamping the late endosomal/lysosomal membrane, we show that TRPML1 functions as a Fe^{2+} permeable channel in late endosomes and lysosomes. ML4 mutations are shown to impair TRPML1's ability to permeate Fe^{2+} at varying degrees, which correlate well with the disease severity. A comparison of TRPML1^{-/-} ML4 and control skin fibroblasts showed a reduction of cytosolic Fe^{2+} levels, an increase of intralysosomal Fe^{2+} levels, and an accumulation of lipofuscin-like molecules in TRPML1^{-/-} cells. We propose that TRPML1 mediates a mechanism by which Fe^{2+} is released from late endosomes/lysosomes. Our results suggest that impaired iron transport may contribute to both hematological and degenerative symptoms of ML4 patients.

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Activation Mutations of the TRPML1 Channel Revealed by Proline Scanning Mutagenesis

Xiang Wang¹, Xianping Dong¹, Su Chen¹, Dongbiao Shen¹, Meiling Liu¹, Eric Mills¹, Xiping Cheng¹, Markus Delling², Haoxing Xu¹.

¹University of Michigan, Ann Arbor, MI, USA, ²Children's Hospital Boston, Boston, MA, USA.

The mucopolin TRP (TRPML) proteins are a family of intracellular channels primarily localized in the late endosome and lysosome. Mutations in the human *TRPML1* gene cause mucopolidiosis type IV disease, a devastating pediatric

neurodegenerative disease. In wild-type TRPML1-expressing HEK293 cells, no significant channel activity can be detected at the plasma membrane, but a proline substitution (TRPML1^{V432P}) results in a large whole-cell current that allows characterization of TRPML1 as a Ca^{2+} and $\text{Fe}^{2+}/\text{Mn}^{2+}$ dually permeable channel. As TRPML1-mediated current can be recorded in late endolysosomes using our recently developed lysosome patch-clamp technique, it remains unknown whether large TRPML1^{V432P} mediated current has resulted from increased surface expression ("trafficking" effect), increased constitutive channel activity ("gating" effect), or both. In the current study, we systematically but individually performed the proline substitutions on 20 amino acid residues around the 432 spot, a S4-S5 linker region. These proline-substitutions were studied by whole-cell and lysosome luminal-side-out recordings in TRPML1-expressing HEK293 cells. Several proline substitutions were identified to display gain-of-function (GOF) constitutive activity at both the plasma membrane and endolysosomal membranes, and their localizations were not restricted to late endosomes and lysosomes, while wild-type TRPML1 and non-GOF substitutions were localized exclusively in these compartments. All of the proline-substituted GOF TRPML1 channels displayed inwardly rectifying currents that were carried by Ca^{2+} or $\text{Fe}^{2+}/\text{Mn}^{2+}$, but not protons. As lysosomal exocytosis is known to be Ca^{2+} -dependent, constitutive Ca^{2+} permeability of proline substitutions may have resulted in stimulus-independent intralysosomal Ca^{2+} release, hence the surface expression and whole-cell current of TRPML1. We conclude that the TRPML1 channel is an inwardly rectifying proton-impermeable cation-permeable channel, which may be gated through unknown cellular mechanisms through a conformational change in the cytoplasmic face of the TM5.

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Dynamic Properties of the TRPML3 Pore and their Modification by the Varitint-Waddler Phenotype

Hyun Jin Kim¹, Insuk So², Shmuel Muallem¹.

¹University of Texas Southwestern Medical Center, Dallas, TX, USA, ²Seoul National University College of Medicine, Seoul, Republic of Korea.

TRPML3 is a Ca^{2+} channel expressed in intracellular vesicular compartments and is regulated by H^{+} that interact with the large intravesicular loop between transmembrane domains 1 and 2. The A419P mutation in TRPML3 causes the varitint-waddler phenotype as a result of gain-of-function (GOF). The mechanism by which the A419P mutation leads to GOF is unknown. Here, we show that the TRPML3 pore is dynamic and expands when conducting Ca^{2+} to change its permeability and selectivity from a strong to a weak field strength site. Pore expansion appears to be regulated by trapping Ca^{2+} , probably within the pore. Expansion of the pore can be reversed only by conducting Na^{+} through the pore. The A419P mutation, which locks the channel in an open state results in permanently expanded pore. Notably, the TRPML3(H283A) mutation that eliminates regulation of TRPML3 by H^{+} and locks the channel in an open state shows the same pore properties as wild-type TRPML3. On the other hand, the pore mutation E449A also locks the channel in an open state and permanently expanded pore. Interestingly, the TRPML3 large intravesicular loop interacts with the pore domain composed of transmembranes 5 and 6. Although this interaction is enhanced by the A419P and E449A mutations, it is not affected by the loop mutation H283A, suggesting that pore expansion together with enhanced loop-pore communication is responsible for the GOF. These findings provide a molecular mechanism for GOF by the TRPML3(A419P) mutation to account for the varitint-waddler disease phenotype.

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Regulation by Calcium of the TRP Channel Polycystin-2 (TRPP2)

María del Rocío Cantero¹, Horacio F. Cantiello^{2,1}.

¹ININCA, UBA-CONICET, Buenos Aires, Argentina, ²Massachusetts General Hospital, Charlestown, MA, USA.

● Polycystin-2 (PC2, TRPP2) is a member of the TRP (transient receptor potential) superfamily of cation channels. Like other members of this superfamily, PC2 permeates Ca^{2+} , which is involved in both signal transduction, and Ca^{2+} entry. Previously, we showed that PC2 is normally active at intracellularly high Ca^{2+} concentrations (10-15 μM). Little is known, however, about the role intracellular Ca^{2+} plays in PC2 channel function. Here, we explored the role of physiological concentrations of intracellular Ca^{2+} in PC2-mediated channel function in reconstituted apical membranes from term human syncytiotrophoblast (hST). Addition of either EGTA (1 mM) or BAPTA (2 mM) to reach low intracellular Ca^{2+} (<5 nM) at the cytoplasmic side, elicited a complete PC2 channel inhibition. A dose response elicited by addition of increasing cytoplasmic Ca^{2+} showed that Ca^{2+} activated PC2 with an apparent half activating concentration of 4.78 nM and a Hill coefficient of ~5. Conversely, extracellular Ca^{2+} concentrations, between 0.5 mM and 5 mM, had a stimulatory effect on PC2 channel activity while higher external concentrations

(10-90 mM) were inhibitory. Further, this activating mechanism was not intrinsic to the PC2 channel but instead seems to be mediated by PC2-associated proteins. Channel function of the *in vitro* translated PC2 protein with Ca^{2+} concentrations of 10-15 mM was non-responsive to lowering cytoplasmic Ca^{2+} with either EGTA or BAPTA. Our data are consistent with a regulatory role of both cytoplasmic and external Ca^{2+} in PC2 channel function, which does not involve putative Ca^{2+} -binding sites on the channel protein, but instead external sites to the channel protein.

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Molecular Basis of Calmodulin and Ca^{2+} interaction with thermoTRP channels

Vera Moiseenkova-Bell¹, Bo Chen¹, Ben Tobe¹, Irina Serysheva², Theodore G. Wensel¹.

¹Baylor College of Medicine, Houston, TX, USA, ²University of Texas-Houston Medical School, Houston, TX, USA.

The TRP (transient receptor potential) family of proteins are relatively non-selective cation channels, most of which are highly permeable to Ca^{2+} . They participate in many sensory and physiological processes, and subdivided into seven major subfamilies: TRPV, TRPA, TRPC, TRPM, TRPP, TRPML, and TRPN. They are predicted to have six transmembrane domains per subunit, intracellular N- and C-termini, and form tetrameric assemblies. Several TRP channels have been proposed to be regulated by intracellular Ca^{2+} , and/or Calmodulin (CaM). Immunoprecipitation of TRP channels expressed in cultured cells suggests that CaM forms complexes with some of them, but questions remain about whether full-length TRP channels bind CaM directly or through some intermediary protein(s), and about the affinity and kinetics of complex formation. We have used fluorescence emission anisotropy of Alexa 488-labeled CaM to measure binding of CaM and Ca^{2+} -CaM to full-length TRP channels expressed in yeast and purified to homogeneity in detergent solution. At nanomolar concentrations, CaM bound rapidly to TRPA1 and to purified ryanodine receptor used as a positive control. CaM binding was enhanced by Ca^{2+} , but not strictly dependent on it, and binding of labeled CaM was antagonized by addition of unlabeled CaM. Dissociation of the complex occurred much more slowly than association. Under conditions in which TRPA1 and ryanodine receptor bound Ca^{2+} -CaM with high affinity, no binding of Ca^{2+} -CaM was observed for TRPV1 or TRPV2.

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Rapid Temperature Jump by Low Cost Laser Diode Irradiation

Jing Yao, Beiyang Liu, Feng Qin.

SUNY Buffalo, Buffalo, NY, USA.

Thermal TRP ion channels play important functions in somatosensation and pain. Aside from activation by agonist or voltage, they are directly gated by temperature, a novel property unique to these channels. However, the underlying mechanism of the abnormally high thermal sensitivity has remained elusive. Studies on thermal activation of these channels have been challenging, partly because of the difficulty for rapid alteration of ambient temperature in live cells. Existing approaches mostly employ resistive or thermal electric heating/cooling, and have a response time far slower than channel activation. As a result, they only allow for measurement of steady-state properties of the channel. We report here an alternative approach for rapid perturbation of temperature. The approach employs an infra-red laser diode as a heat source, and by restricting laser irradiation around a single cell, it can produce constant temperature jumps over 500C in sub-milliseconds. Experiments with several heat-gated channels (TRPV1-3) demonstrate its applicability as a general tool for local temperature stimulation of single cells. Compared to other laser heating approaches such as those based on Raman-shifting of the Nd:YAG fundamentals, it is more cost-effective while providing adequate resolution for detection of ion channel kinetics.

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Heat Activation of Temperature-Gated Ion channels Studied by Fast Temperature Jumps

Jing Yao, Beiyang Liu, Feng Qin.

SUNY-Buffalo, Buffalo, NY, USA.

Temperature-gated ion channels are distinct for their extraordinarily high thermal sensitivity. Activation by temperature is made possible with large changes in enthalpy and entropy and appropriate compensation between them, yet the source of the energy and the molecular basis of temperature sensing have remained elusive. The present knowledge on the thermal gating of these channels has resulted largely from equilibrium measurements of temperature responses. Nonsteady-state properties of the gating have been lacking because of the difficulty in rapid perturbation of ambient temperature. To address these issues, we have developed a laser-based fast-heating approach and

used it to investigate the heat activation of the vanilloid receptor TRPV1. In response to a temperature step, the channel was activated with an exponential time course, and the responses were sustained at hyperpolarization but became slowly inactivated at depolarization. The rate of activation was variable depending on cell conditions, but had a time constant generally on the order of milliseconds. The activation showed saturation in both kinetics and steady-state currents at temperatures above 500C. Strong activation occurred in saturating temperatures independent of membrane potentials. Nonlinear relaxation behavior was observed especially during the deactivation process under extreme hyperpolarization. Our data indicate that, despite large energetic changes, the temperature activation can occur rapidly, and consists of multiple states involving both temperature-dependent and independent transitions. The results provide further constraint on the locality of the energetic distributions and novel insights for understanding the mechanism of temperature sensing.

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Controlling Temperature and Chemical Environment for Patch Clamp Studies of TRP Channels

Erik T. Jansson, Maria Millingen, Aldo Jesorka, Per Lincoln, Owe Orwar.

Chemical and Biological Engineering, Chalmers University of Technology, Göteborg, Sweden.

A microfluidic device fabricated in PDMS, which permits control of local temperature and rapid exchange of solution around a single cell, has recently been developed. The system allows an experimental design where a single cell can be exposed to a large set of temperatures and concentrations of chemicals in a short time.

Transient receptor potential (TRP) channels are activated by both ligands and temperature, and synergetic effects exist between temperature and concentration of ligand. Therefore, together with patch clamp recording, the microfluidic system is used for studying the response and cooperative effects of temperature and chemicals in TRP channels.

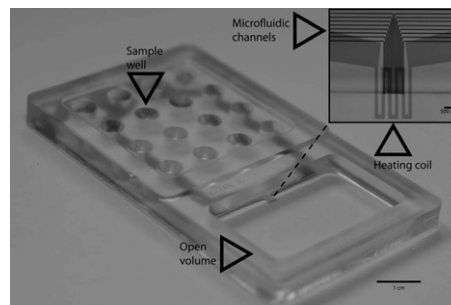


Figure 1. The microfluidic device contains 16 wells connected to separate channels, with separate outlets in the open volume. In the immediate vicinity of the outlet, there is no mixing between the flows from the different channels due to laminar flow. A thin-film Cr/Au-structure is aligned beneath the channel outlets, which yields resistive heating when a voltage is applied.

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Single-Cell RT-PCR Analysis of TRPC Channels Expressed in Rat Cholinergic, Dopaminergic, Noradrenergic, and Serotonergic Neurons in the Brain

Takeharu Kawano, Nicole M. Jones, Christina V. Floreani, Abba M. Albsoul-Younes, Pawinee Yongsatirachot, Shigehiro Nakajima, Yasuko Nakajima.

University of Illinois at Chicago, Chicago, IL, USA.

The canonical transient receptor potential (TRPC) channels comprise a family of nonselective cation channels composed of seven members (TRPC1-7). TRPC channels are widely distributed in the nervous system and contribute to neuronal excitation. In the present study, using the single-cell RT-PCR method, the distribution of TRPC channel mRNA is analyzed in cholinergic neurons in the nucleus basalis (NB), serotonergic neurons in the dorsal raphe nucleus (DRN), noradrenergic neurons in the locus coeruleus (LC), dopaminergic neurons in the substantia nigra (SN), and dopaminergic neurons in the ventral tegmental area (VTA). NB, LC, SN and VTA neurons are cultured from 3-5 day-old and DRN from 10-12 day-old Long Evans rats. Single-cell RT-PCR was performed on these cultured neurons using the previously described method (Kawano et al., 2004, Neuroscience letters, 358:63). Tyrosine hydroxylase primers were used to identify noradrenergic neurons in LC