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TRPV4 Channels in Mouse Skeletal Muscle: Gene Expression, Role for Ca^{2+} Homeostasis and Muscle Force and Fatigue

Thom Lange[1], Markus Princk[1], Yaxin Zhang[1], Bernd W. Pritschow[1], Saskia Teucher[1], Wolfgang Liedtke[2], **Heinrich Brinkmeier**[1].
[1]Institute of Pathophysiology, University of Greifswald, Greifswald, Germany, [2]Duke University, Center for Translational Neuroscience, Durham, NC, USA.

The cation channel TRPV4 is one of the most abundant TRP channels in mouse skeletal muscle. Here, we investigated the contribution of TRPV4 to Ca^{2+} homeostasis, muscle force and fatigue. For this we used muscles of wildtype (WT) and TRPV4$^{-/-}$ mice. To estimate background Ca^{2+} influx we applied the Mn^{2+} quench technique. Muscle force was tested on isolated soleus muscles. Gene expression was analyzed by RT-PCR and data normalized to that of 18s rRNA. In WT fibers application of 4a-PDD, a TRPV4 activator, caused an increase in the Mn^{2+} quench rate of about 45 % (4.27 ± 0.24 %/min vs. 6.28 ± 0.39 %/min, $p<0.01$). This effect was completely absent in TRPV4$^{-/-}$ fibers. However, basic quench was not reduced in TRPV4 deficient fibers. Decay time of KCl induced Ca^{2+}-transients was increased in WT fibers after application of 4-αPDD (from 4.46 ± 0.2 s, n=67 to 5.92 ± 0.28 s, n=23, $p < 0.01$). This effect was likewise not observed in fibers of TRPV4$^{-/-}$ animals. Muscle fatigue was attenuated in the presence of 4α-PDD, an effect which was not observed in muscles of TRPV4$^{-/-}$ mice. No difference between TRPV4$^{-/-}$ and WT animals was seen in wire hang test. Gene expression analysis showed that TRPV4 and TRPV2 were the most abundant TRPV channels in muscle, their mRNA concentration being about 5-fold higher in soleus muscle that in fast twitch EDL. In soleus muscles of TRPV4$^{-/-}$ mice TRPV2 mRNA was significantly increased. We conclude that TRPV4 is functionally expressed in mouse skeletal muscle, contributes to background calcium entry and can modulate Ca^{2+} transients. TRPV4 activity can attenuate fatigue, but its lack does not affect gross motor skills. TRPV4 knockout causes a compensatory upregulation of TRPV2.

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Oncogenic Potential of TRPV6 Channel in Prostate Cancer

Vyacheslav Lehen'kyi[1], Maylis Raphael[1], Banjamin Beck[1], Matthieu Flourakis[1], Dmitro Gordienko[1,2], Roman Skryma[1], **Natalia Prevarskaya**[1].
[1]INSERM U-1003, Villeneuve d'Asq, France, [2]Laboratory of Molecular Pharmacology and Biophysics, Bogomoletz Institute of Physiology, Kiev, Ukraine.

The transient receptor potential channel, subfamily V, member 6 (TRPV6), is strongly expressed in advanced prostate cancer and significantly correlates with the Gleason >7 grading, being undetectable in healthy and benign prostate tissues. However, the role of TRPV6 as a highly Ca^{2+}-selective channel in prostate carcinogenesis remains poorly understood. Here, we report that TRPV6 is directly involved in the control of prostate cancer cells proliferation by decreasing the proliferation rate, cell accumulation in the S-phase of cell cycle and proliferating cell nuclear antigen (PCNA) expression. We demonstrate that the Ca^{2+} uptake into prostate cancer cells is mediated by TRPV6, with the subsequent downstream activation of the transcription factor nuclear factor of activated T-cell. TRPV6- mediated Ca^{2+} entry is also involved in apoptosis resistance of prostate cancer cells. One of the mechanisms by which TRPV6 regulates apoptosis is related to the translocation of channel to the plasma membrane. We conclude that the upregulation of TRPV6 channel in prostate cancer cells may represent a mechanism for maintaining a higher proliferation rate, increasing cell survival and apoptosis resistance as well.

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Molecular Mechanisms of Calmodulin Regulation of TRPV6

Chike Cao, Eleonora Zakharian, Tibor Rohacs.
UMDNJ, Newark, NJ, USA.

Transient Receptor Potential Vanilloid 6 (TRPV6) is an epithelial Ca2+ channel that is responsible for active Ca2+ absorption in the intestines. We have recently demonstrated that this channel is activated directly by phosphatidylinositol 4, 5-bisphosphate (PIP2) and that cytoplasmic ATP stimulates TRPV6 channel activity indirectly by providing substrates for lipid kinases and the generation of PIP2. TRPV6 undergoes Ca2+-induced inactivation, preventing toxic Ca2+ overload of the cell. This inactivation has been shown to be mediated both by Ca2+-calmodulin (CaM) and depletion of PIP2. In this current study, we investigated how Ca2+-CaM inhibits channel activity upon increased cytoplasmic Ca2+ concentration. First we found that in excised inside-out patch-clamp measurements, Ca2+-CaM inhibited TRPV6 activity that was stimulated by dic8-PIP2. Further, we confirmed that CaM binding site was located in the distal C-terminus of human TRPV6 using an in vitro CaM-binding assay. We found that the C-terminal region binds to CaM in a Ca2+-dependent manner. This was observed on the full-length TRPV6 protein, transiently expressed in HEK293 cells, and maltase binding protein (MBP)-fusion fragments that were expressed in E. coli cells. Deletion of the distal C-terminal region about 30 amino acid residues diminished CaM binding to TRPV6. We also found that two highly conserved amino acid residues in this region were responsible for interacting with CaM. Mutation at these sites dramatically inhibited CaM binding to the full-length human TRPV6 proteins expressed in HEK293 cells. Our findings indicate the distinct binding site for CaM-TRPV6 interaction, which may be implicated in the specific channel inactivation.

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Effects on TRPM2 Channel Gating of Mutations in the NUDT9-H Domain

Balázs Tóth, László Csanády.
Semmelweis University, Budapest, Hungary.

TRPM2 is a Ca2+ permeable cation channel involved in physiological and pathophysiological processes linked to oxidative stress. TRPM2 channels are co-activated by intracellular Ca2+ and ADP ribose (ADPR). ADPR binds to the C-terminal NUDT9-H domain, which shows high sequence similarity to the mitochondrial ADPR hydrolase NUDT9. However, the mechanism by which ADPR binding/hydrolysis regulates TRPM2 channel gating is unclear. To investigate this mechanism, we designed mutations in the NUDT9-H domain expected to alter ADPR binding affinity or ADPRase activity based on a crystal structure of NUDT9. The effects of the mutations on TRPM2 channel gating were tested in inside-out patch recordings.
To impair binding of the adenine base, mutations M1286A and N1345A remove side chains which in NUDT9 contact the adenine base. To facilitate binding, S1391M reconstructs such a side chain. By restoring a hydrogen bond to the 2'-OH group Y1349D might facilitate binding of the distal ribose, while H1488A might impair it by removing a hydrogen-bond to the 3'-OH group. Mutants Y1485F and R1433A, respectively, remove a hydrogen bond and a salt bridge to the β-phosphate, expectedly impairing ADPR binding. An opposite effect is expected from L1379R which recreates a salt bridge to the β-phosphate. To enhance catalysis, I1405E recreates the side chain which coordinates Mg2+ in NUDT9. Finally, we tested whether D1468 might act as the catalytic base − if so ADPRase activity should be abolished in D1468A mutant.
All mutants formed functional channels. To assay for possible effects on ligand binding and closing rate, we determinted apparent affinities for ADPR and current decay rates upon ADPR removal. Neither was dramatically altered by any of the mutations. We conclude that ADPR hydrolysis is either not required or not rate limiting for channel closure.

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Zinc Inactivates Melastatin Transient Receptor Potential 2 Channels via the Outer Pore

Wei Yang[1,2], Paul T. Manna[2], Jie Zou[2], Jianhong Luo[1], David J. Beech[2], Asipu Sivaprasadarao[2], Linhua Jiang[3].
[1]Institute of Neuroscience, Zhejiang University, Hangzhou, China, [2]IMSB, FBS, University of Leeds, Leeds, United Kingdom, [3]IMSB, FBS, Univerisity of Leeds, Leeds, United Kingdom.

Zinc ion (Zn2+) is an endogenous allosteric modulator that regulates the activity of a wide variety of ion channels in a reversible and concentration-dependent fashion. Here we used patch-clamp recording to study the effects of Zn2+ on the TRPM2 channel. Zn2+ inhibited the human (h) TRPM2 channel currents, and the steady-state inhibition was largely not reversed upon washout and concentration-independent in the range of 30-1000 μM, suggesting that Zn2+ induces channel inactivation. Zn2+ inactivated the channels fully when they conducted inward currents, but only by half when they passed outward currents, indicating profound influence of the permeant ion on Zn2+-inactivation. Alanine substitution scanning mutagenesis of twenty Zn2+-interacting candidate residues in the outer pore region of the hTRPM2 channel showed that mutation of Lys952 in the extracellular end of the fifth transmembrane segment and Asp1002 in the large turret strongly attenuated or abolished Zn2+-inactivation, and mutation of several other residues dramatically changed the inactivation kinetics. The mouse (m) TRPM2 channels were also inactivated by Zn2+ but the kinetics were remarkably slower. Reciprocal mutation of His995 in the hTRPM2 channel and the equivalent Gln992 in the mTRPM2 channel completely swapped the kinetics, but no such opposing effects resulted from exchanging another pair of species-specific residues Arg961/Ser958. We conclude from these results that Zn2+ inactivates the TRPM2 channels and residues in the outer pore are critical determinants of the inactivation.

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On the Potential Interaction between Non-Selective Cation Channel TRPM4 and the Sulfonylurea Receptor SUR1

Monica Sala-Rabanal, Shizhen Wang, Colin G. Nichols.
Washington University School of Medicine, Saint Louis, MO, USA.

The sulfonylurea receptor SUR1, of the ABC protein superfamily, associates with inward-rectifying K channel subunits Kir6.2 or Kir6.1 to form K_{ATP} channels, which link metabolism to electrical activity in multiple cell types. The

strong coupling of SUR1 with Kir6 appears exclusive, but recent studies argue that SUR1 also associates with and regulates TRPM4, of the transient receptor potential (TRP) channel family of non-selective cation (NSC) channels. It has been reported that, following stroke, brain or spinal cord trauma, SUR1 is expressed *de novo* in neurovascular cells at the injury site. In these cells, there appears to be dramatic upregulation of a NSC conductance with TRPM4-like properties, which seems to be reduced by sulfonylureas and enhanced in presence of the K_{ATP} channel opener diazoxide, leading to the postulation that post-traumatic NSC currents are determined by SUR1-modulated TRPM4 channels (Simard et al., *J Neurosurg* 113: 622-9; 2010). However, direct evidence for functional association of TRPM4 and SUR1 is lacking. To address this, we performed inside-out membrane patch-clamp of COSm6 cells expressing TRPM4 channels with or without SUR1. TRPM4-mediated, non-selective currents were Ca^{2+}-activated, voltage-dependent, underwent desensitization, and were inhibited by ATP, but were insensitive to diazoxide, glibenclamide and tolbutamide. These properties were not affected by co-transfection with SUR1 albeit functional, sulfonylurea-sensitive K_{ATP} channels were formed when the same SUR1 was co-transfected with Kir6.2. In cells co-transfected with Kir6.2, SUR1 and TRPM4, we measured Ca^{2+}-independent, K_{ATP}-mediated K^+ currents and Ca^{2+}-activated, sulfonylurea-insensitive Na^+ currents in the same patch, further showing that SUR1 subunits control K_{ATP} channel activity, without affecting TRPM4 channel properties. Co-transfection of TRPM4 and SUR1 does not elicit sulfonylurea-sensitive NSC currents as reported in stroke and brain injury models, suggesting that functional association of TRPM4 and SUR1 is unlikely.

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Cardiac-Targeted TRPM7 Deletion Induces Heart Block and Cardiomyopathy via Disrupted Embryonic Ventricular Development

Rajan Sah[1,2], Christopher Bates-Withers[2], Xenos Mason[2], Marjolein Van den Boogert[2], William Gibson[3], Jie Jin[2], William Pu[2], David E. Clapham[2].
[1]Brigham and Women's Hospital, Boston, MA, USA, [2]Children's Hospital/Harvard Medical School, Boston, MA, USA, [3]Harvard Medical School, Boston, MA, USA.

Transient Receptor Potential Melastatin-7 (Trpm7) is a divalent permeant channel-kinase of unknown function that is ubiquitously expressed, concentrated in myocardium during embryonic development, and forms a functional current in adult murine cardiac myocytes. Using multiple cardiac-targeted knock-out lines we show that the timing of Trpm7 deletion during embryonic development variably disrupts adult atrio-ventricular conduction, ventricular morphology and function without altering total myocardial magnesium, calcium or zinc content. Late embryonic cardiac Trpm7 deletion in αMHC-Cre-TRPM7fl/fl mice resulted in normal ventricular size and function, while slightly earlier deletion in αMHC-Cre-TRPM7fl/- mice yielded a bimodal phenotype in which ~50% develop a dilated, fibrotic cardiomyopathy with impaired function and high grade atrioventricular block. Microarray analysis performed on αMHC-Cre-TRPM7fl/- ventricular tissue revealed 184 differentially expressed genes including significant elevations in Nppa (33-fold), Nppb (15-fold), TIMP1 (13-fold), Itgb1b3 (13-fold), Postn (12-fold) and reductions in Lgi-1 (20-fold), Pfkb1 (7-fold), Kcnv2 (Kv8.2) (5-fold), Mme (5-fold), Kcnj3 (GIRK1) (4-fold) and Kcnd2 (Kv4.2) (4-fold). To probe the importance of Trpm7 in early embryonic cardiac development, we crossed Trpm7flfl mice with Isl1-Cre and TnT-Cre lines. These early cardiac knockouts developed congestive heart failure and died by E11 due to impaired formation of the compact myocardium secondary to arrested myocardial proliferation. Taken together, these results define an essential role for Trpm7 in normal embryonic myocardial development and suggest that adult cardiac phenotypes associated with disruptions in Trpm7 arise from defects in myocardial differentiation.

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Complex Regulation of the TRPM8 Cold Receptor Channel: Role of Arachidonic Acid Release following M3 Muscarinic Receptor Stimulation

Alexis Bavencoffe[1], Artem Kondratskyi[2,3], Dimitra Gkika[2,3], Brigitte Mauroy[2,4], Yaroslav Shuba[5], Natalia Prevarskaya[2,3], Roman Skryma[2,3].
[1]University of Texas Health Science Center at Houston, Houston, TX, USA, [2]Inserm U1003, Villeneuve d'Ascq, France, [3]Université des Sciences et Technologies de Lille (USTL), Villeneuve d'Ascq, France, [4]Université Catholique de Lille, Lille, France, [5]Bogomoletz Institute of Physiology and International Center of Molecular Physiology, Kyiv, Ukraine.

TRPM8 (transient receptor potential melastatin 8) channels are well established as the main sensor of cool temperatures in sensory neurons. Recent studies indicate that its regulation is a complex integration of multiple influences such as pH, calcium, phosphorylation (PKA, PKC), lipids (PIP2, lysophospholipids). Among lipid modulators of TRPM8, the physiological pathway underlying the inhibitory effect of arachidonic acid (AA) remains unknown. Here, we examined how stimulation of M3 muscarinic acetylcholine receptors by a non-selective agonist, oxotremorine methiodide (Oxo-M), leads to inhibition of TRPM8 activity. Using HEK-293 cells heterologously co-expressing M3 receptors and TRPM8, we demonstrated that activation of M3 receptors triggers a cascade of events including stimulation of cytosolic calcium dependant phospholipase A2 (cPLA2), degradation of plasma membrane phospholipids, production of AA, and decrease of TRPM8 channels activity. The inhibition of plasma membrane TRPM8-mediated current was mimicked by a direct exposure to AA, a blockade of cPLA2 activity by pharmacological agent AACOCF3 or an siRNA-mediated cPLA2 silencing strategy. Our work elucidates the intracellular functional link between M3 receptor and TRPM8 channel via cPLA2/AA and suggests a novel physiological mechanism of arachidonate-mediated regulation of TRPM8 channel activity through Gq/11 protein coupled receptors.

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Polyhydroxybutyrate (PHB) is an Essential Structural Compound of TRPM8 Channel Required for its Function

Chike Cao[1], Yevgen Yudin[1], Wei Chen[2], Hong Li[2], Evgeny Pavlov[3], Tibor Rohacs[1], **Eleonora Zakharian**[1].
[1]New Jersey Medical School - UMDNJ, Newark, NJ, USA, [2]New Jersey Medical School Cancer Center - UMDNJ, Newark, NJ, USA, [3]Dalhousie University, Halifax, NS, Canada.

The transient receptor potential channel of the melastatin subfamily TRPM8 is the cold and menthol receptor. Our recent findings indicate that the functional TRPM8 channels exist in a form of supramolecular complex of protein and two ubiquitous homopolymers - inorganic polyphosphate (polyP) and polyhydroxybutyrate (PHB) (Zakharian et al., 2009). In the current study we are investigating the role of PHB in TRPM8 channel function. Our studies have shown that PHB associates with the TRPM8 protein covalently and does not dissociate from TRPM8 during SDS-PAGE, as has been shown in Western blot analysis with PHB-antibodies. Further, to identify the PHB binding sites on the protein, the purified TRPM8 protein was analyzed in matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) experiments (UMDNJ Proteomics Facility). The results were analyzed with Swiss Proteomics and indicated putative binding sites for PHB on TRPM8. We have identified a number of peptides, which are covalently bound to PHB. Also, we performed Ca2+-imaging experiments on dorsal root ganglion (DRG) neurons, expressing TRPM8 protein alone or with PHB-depolymerase, the enzyme that cleaves the short-chain PHB. Reduction of PHB in DRG neurons was evidenced by confocal microscopy, using the fluorescent dye BODIPY 493/503 as an indicator of the levels of PHB and by immunohistochemical analysis using PHB-IgG antibodies. We found that co-expression of the PHB depolymerase in DRG neurons expressing TRPM8 channels significantly inhibited intracellular Ca2+ signals induced by cold, menthol, and icilin. Our findings suggest that PHB is a structural compound of the TRPM8 channel which is covalently attached to its protein part and its presence is essential for normal channel function.

Zakharian E, Thyagarajan B, French RJ, Pavlov E, Rohacs T (2009) Inorganic polyphosphate modulates TRPM8 channels. PLoS ONE 4:e5404.

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EC50 and IC50 Measurements of TRPM8 in Lipid Bilayers

Carl Salazar, Ahmad M. El-Arabi, Jacob J. Schmidt.
UCLA, Los Angeles, CA, USA.

Ensemble measurements of ion channel currents, made in the presence of pharmaceutical compounds, enable determination of compound activity and potency in the form of IC50 and EC50 values, and are traditionally performed with Patch Clamp. An alternative platform for ion channel study involves the formation of artificial bilayers from the contact of lipid monolayers at aqueous/oil interfaces. This technique has the potential for automation and parallel measurements. Previous work with this platform has demonstrated measurements of physiologically relevant ion channels at the single molecule level. Here we present measurements of the cold and menthol receptor TRPM8 at the single channel and ensemble level. We studied the effect of menthol and temperature on the open probability of TRPM8 and found a single-channel conductance of 64 ± 6 pS, comparable to previous work. In addition, we measured ensemble activity of TRPM8 as a function of temperature and varying PI(4,5)P2 concentration. Our apparatus enables introduction of pharmaceutical compounds to the solution