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Comparison of Computationally and Experimentally Determined Single I_{Kr} Channel Activity During Pacemaking in Sinoatrial Node Cells

Marieke W. Veldkamp, Ronald Wilders, Lennart N. Bouman, Tobias Opthof, Antoni C.G. van Ginneken.
Academic Medical Center, Amsterdam, Netherlands.

Background: The contribution of the rapidly activating delayed rectifier current (I_{Kr}) to sinoatrial (SA) node pacemaking, is mainly derived from computational models. The mathematical representation of I_{Kr} therein, is based on voltage-clamp data in SA-node cells, but to what extent computational I_{Kr} activity accurately describes true dynamic behavior of I_{Kr} during the SA-node action potential (AP), remains to be established.
Methods: With the dual electrode patch clamp technique, we simultaneously recorded spontaneous APs (whole-cell) and single I_{Kr} channel activity (cell-attached-patch) from isolated rabbit SA-node cells. To allow comparison between measured I_{Kr} channel activity and computational I_{Kr} activity, a model rabbit SA-node cell (Zhang et al. Am J Physiol Heart Circ Physiol. 2000; 279: H397-H421) was action potential clamped by our experimentally recorded APs.
Results: In experiments, I_{Kr} channel openings were detected during AP repolarization and diastolic depolarization. The open probability (P_o) was very low (<0.15) early during repolarization, but rapidly increased towards final repolarization, reaching a maximum of 0.27 ± 0.03 (mean $\pm$ SEM, $n = 4$) shortly before the maximum diastolic potential (MDP). During the subsequent diastolic depolarization, P_o gradually declined to a value of 0.08 ± 0.02 (mean $\pm$ SEM, $n = 4$) just before take-off of the next AP. P_o obtained from the computational model followed a qualitatively similar course during SA-node automaticity, but values were doubled. That is: P_o rapidly increased from zero to a maximal value of 0.63 shortly before MDP, and then declined to 0.18 towards the end of diastolic depolarization.
Conclusions: The computational model well-described the time- and voltage dependent changes in P_o of I_{Kr} channels during SA-nodal pacemaking, however, P_o was over-estimated by a factor of 2 in all phases of the pacemaker cycle.

TRP Channels

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Modelling the Membrane Potential Dependence on Non-Specific Cation Channels in Canine Articular Chondrocytes

Rebecca Lewis, Gregor Purves, Julia Crossley, Richard Barrett-Jolley.
University of Liverpool, Liverpool, United Kingdom.

In a previous report, we showed that the predominant ion channel in potassium-free solutions was a gadolinium III (Gd^{3+}) sensitive non-specific cation channel, with functional characteristics similar to that expected for transient receptor potential (TRP) type channels.
In this study reverse transcription-PCR (RT-PCR) was used to investigate the expression of TRP channels in canine articular chondrocytes. Both a mathematical model based on the Goldman-Hodgkin-Katz voltage equation and current clamp whole-cell electrophysiology were then used to investigate the effect of these channels on membrane potential (*Vm*).
Chondrocytes isolated from canine articular cartilage were cultured for 5 days in Dulbeccos Modified Eagles Medium with 10% Foetal Calf Serum. For RT-PCR analysis, total RNA was extracted from first passage cells. Electrophysiological recording was carried out on first to third passage cells.
RT-PCR analysis of chondrocyte mRNA, and subsequent sequencing of products, showed a member of the TRP vanilloid group of channels (TRPV4) to be present; sequence homology to the human TRPV4 was 94%. We have so far failed to find mRNA for the functionally similar TRPC3 and TRPC6 channels.
Using whole-cell and single-channel data from our own experiments and the literature, our model predicts the membrane to be heavily dependent on the activity of TRPV4. Simulated block of all non-specific cation channels in the chondrocyte membrane leads to a predicted −27mV change in *Vm*. This prediction closely matches our current clamp experiments with 100μM Gd^{3+} inducing a -32 ± 1mV ($n = 6$) change of *Vm*.
Previously, large-conductance chloride and voltage-sensitive potassium channels have been reported to be important in the maintenance of chondrocyte *Vm.* The data presented here showsthat the TRPV4 channel also has a significant contribution to maintenance of the chondrocyte *Vm*.

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Regulation by Calcium of Polycystin-2 (TRPP2) and Hetero-Complexes with TRPC1

María del Rocío Cantero[1], Horacio F. Cantiello[2,3].
[1]Instituto de Investigaciones Cardiológicas, Capital Federal, Argentina, [2]Massachusetts General Hospital, Charlestown, MA, USA, [3]Instituto de Investigaciones Cardiológicas, Buenos Aires, Argentina.

Polycystin-2 (PC2, TRPP2) and the canonical TRPC1 are TRP superfamily members that act as non-selective cation channels, with multiple subconductance states and permeability to calcium. Recent studies from our laboratory (Zhang et al., *Hum Mol Genet*, 2009) indicated that both channels, form homo and hetero-tetramers with distinct functional properties. Both TRP channels are implicated in calcium transport and signaling events, therefore, their regulation by calcium is relevant to their role in cell function. Here we studied the effect of cytoplasmic and external calcium on the regulation of PC2, TRPC1 and PC2/TRPC1, channel function. Lowering cytoplasmic calcium (0.3 nM) with either BAPTA or EGTA inhibited PC2 channel function. This inhibition was extrinsic to the channel complex, and was dependent of external calcium concentrations. Titration of cytoplasmic calcium recovered PC2 channel activity, with a Hill coefficient of 5 and an apparent affinity constant of 1-5 nM. The addition of either BAPTA (2 mM) or EGTA (1 mM) to TRPC1 to lower cytoplasmic calcium concentrations did not inhibit the current (single channel conductance) mediated by the TRPC1 homo-tetrameric channel. In contrast, low calcium decreased TRPC1's mean open probability by 50%. In addition, low cytoplasmic calcium did not inhibit PC2 channel function when hetero-complexed with TRPC1. These data suggest that the formation of structural hetero-complexes between PC2 and TRPC1 confers important regulatory changes to either channel's function, such as the sensory response to cytoplasmic calcium concentrations, thus providing functional diversity to their channel properties in endogenous environments such as the primary cilium or the plasma membrane, where they locate.

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Evaluation of the QPatch HT and HTX Systems As Methods for Ion Channel Screening

Mark McPate[1], Sarah Lilley[1], Martin Gosling[1], Søren Friis[2], Rasmus B. Jacobsen[2], Pamela Tranter[1].
[1]Novartis Institutes for Biomedical Research, Horsham, United Kingdom, [2]Sophion Biosciences, Ballerup, Denmark.

The introduction of high throughput planar automated electrophysiology systems has brought the potential of ion channels as targets for pharmacological modulation to the forefront. Sophion Biosciences QPatch systems are fully automated electrophysiology platforms generating high resistance seals with resultant ion channel biophysical and pharmacological properties similar to conventional patch clamp recordings. In this study, using a CHO cell line stably expressing the TRPM2 channel, the performance of the QPatch HTX, which records from a population of 10 cells per recording site, was evaluated against the QPatch HT which records from a single cell per recording site.
TRPM2 can be challenging to measure, particularly when determining inhibitor potencies, due to poor recording stability characterised by current rundown. A compound set was screened against TRPM2 on the QPatch HT and HTX to assess their relative performances. There was a good correlation between the IC_{50} values generated on the two systems ($r^2 = 0.91$). The current rundown on the QPatch HTX was considerably reduced compared to the QPatch HT (19.5 $\pm$ 1.5% and 37.5 $\pm$ 3.0% respectively, mean $\pm$ SEM, $n = 4$ experiments, 767 cells profiled). This resulted in an increase in the number of successful recordings obtained from 51.6 $\pm$ 7.1% for HT to 83.1 $\pm$ 4.5% for HTX (Mean $\pm$ SEM, $n = 4$ experiments, 912 cells profiled) for a 5 point IC_{50} assay and from 67.2 $\pm$ 8.9% for HT to 98.6 $\pm$ 1.4% for HTX (Mean $\pm$ SEM, $n = 3$-4 experiments, 480 cells profiled) for a single concentration assay.
The QPatch HT has made a significant impact on the volume of high quality electrophysiology data that can be generated. This preliminary study suggests that the QPatch HTX system can further enhance throughput, particularly with channels where current stability is problematic.

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Activating Mutations Reveal a Role of TRPML1 in Lysosomal Exocytosis

Xiang Wang, Xian-ping Dong, Dongbiao Shen, Haoxing Xu.
University of Michigan, Ann Arbor, MI, USA.

The contents of lysosomes undergo exocytosis (lysosomal exocytosis) in response to an increase of intracellular Ca^{2+}. Emerging evidence suggests that lysosomal exocytosis plays important roles in a variety of cell biological functions including neurotransmitter release, neurite outgrowth, and plasma membrane repair. The putative Ca^{2+} channel in the lysosome that mediates intralysosomal Ca^{2+} release, however, has not been identified. The mucolipin TRP (TRPML) proteins are a family of endolysosomal cation channels with genetically established importance in man and rodent. Mutations of human *TRPML1* cause type IV mucolipidosis, a devastating pediatric neurodegenerative disease. In this study, we found that although TRPML1-mediated currents can only be recorded in late endosome and lysosome (LEL) using the lysosome patch clamp technique, several proline substitutions in TRPML1 (such as $TRPML1^{V432P}$) display gain-of-function (GOF) constitutive activities at both

the plasma membrane (PM) and endolysosomal membranes. Although wild-type TRPML1 localized exclusively in LEL and were barely detectable in the PM, the GOF mutants were not restricted to LEL compartments, and most significantly, exhibited significant surface expression. As a Ca^{2+}-permeable channel, the constitutive Ca^{2+} permeability due to Pro substitutions may allow TRPML1 proteins traffic to the PM via Ca^{2+}-dependent lysosomal exocytosis, resulting in the surface expression and whole cell currents of TRPML1. Consistent with the hypothesis, surface staining of lysosome-associated membrane protein-1 (Lamp-1) was dramatically increased in cells expressing GOF TRPML1 channels. Interestingly, the extent of the lysosomal exocytosis appeared to be correlated with the degree of channel gain-of-function of TRPML1 mutants. Our results suggest that upon unidentified cellular stimulations, TRPML1 mediates intralysosomal Ca^{2+} release to trigger lysosomal exocytosis. Currently we are investigating whether inhibiting exocytosis could reduce surface expression of GOF TRPML1 mutants, and whether stimulating exocytosis could enhance surface expression of TRPML1.

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TRPM8 Near-Membrane Dynamics and Channel Stabilization after Stimulation

Luis Veliz, Carlos Toro, Luis Arias, Jenifer Villegas, **Sebastian E. Brauchi**.
Universidad Austral de Chile, Valdivia, Chile.

TRPM8 is a non-selective cation channel expressed on a subset of peripheral neurones, and is the molecular machine that allow us to detect cold signals from our surroundings. Some members of the TRP channel family changes their cellular distribution in response to agonist stimulation. Here, we will describe membrane/near-membrane dynamics of TRPM8-GFP containing particles in both, HEK-293T and F-11 transfected cells. 2D and 3D trajectories together with the velocity of individual protein containing vesicles were obtained by Total Internal Reflexion of Fluorescence Microscopy (TIRFM) and single particle tracking (SPT), and analyzed by plotting of the mean-square displacement against time. Four characteristic types of motion were observed: (a) stationary; (b) simple Brownian diffusion; (c) directed diffusion; and (d) confined diffusion, in which particles undergoing Brownian diffusion are confined within a limited area. Our data suggests that TRPM8, when inserted into the plasma membrane, is confined into small domains of about 3 um in diameter, in which receptor molecules resides in the time scale of 2-8 s. In the absence of stimuli TRPM8 vesicles constantly move along a network that cover the plasma membrane, periodically stopping, most often just briefly. Stimulation halted this hop-diffusion probably by stabilizing TRPM8 channels, as a result, release from plasma membrane became significantly slower. This slow release of TRPM8 determined the overall increase of available receptors.

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Ligand Stoichiometry of TRPM8

Thomas Voets, Annelies Janssens.
KU Leuven, Leuven, Belgium.

Temperature-sensitive TRP channels (thermoTRPs) play a key role in somatosensory thermosensation. Several thermoTRPs, including the heat-activated TRPV1 and the cold-activated TRPM8, are not only sensitive to changes in temperature but also to ligands that evoke a thermal sensation, such as the 'hot pepper' compound capsaicin (TRPV1) and the cooling agent menthol (TRPM8). Recent data indicate that the binding site of these lipophilic compounds is located in the so-called 'sensor domain' made up of transmembrane domains S1-S4. For example, mutating S4 arginine at position 842 in TRPM8 to histidine (mutant R842H) produces a channel with unaltered cold and voltage sensitivity, but with a ~100-fold reduction in menthol affinity. Given that TRP channels are tetramers and that the sensor domains are spatially apart, a functional TRPM8 channel can potentially bind four menthol molecules. However, the contribution of each individual menthol binding event to channel gating is unknown. To address this question, we made vectors encoding tandem tetramers with all possible combinations of WT and R842H subunits. Expression of these vectors in HEK293 cells exclusively produced protein of the expected tetrameric molecular weight, and whole-cell recordings further confirmed that the tandem linkage of subunits did not significantly affect channel gating. This approach will further be used to determine the exact stoichiometry of menthol-induced TRPM8 gating.

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Exploring Structural Relationships between TRP and Kv Channels

Jeet Kalia, Kenton J. Swartz.
NINDS, NIH, Bethesda, MD, USA.

Transient Receptor Potential (TRP) ion channels are homotetrameric, non-selective cation channels that are expressed in diverse cell types in eukaryotes, ranging from yeast to humans. TRP channels have been implicated in many physiological roles such as thermosensation, mechanosensation, chemesthesis, hearing, and sensing pain. Indeed, TRP channels are gated by a plethora of stimuli. For example, TRPV1 is activated by heat, voltage, and small molecules such as capsaicin, and TRPM8 is activated by cold, voltage and cooling agents such as menthol. The molecular mechanisms underlying this polymodal gating of TRP channels are poorly understood. In contrast, the mechanism of voltage-activation of the voltage-gated potassium (Kv) channels is well understood. The architectural similarity of TRP channels and Kv channels, in addition to several mutagenesis-based reports published on TRP channels, raise the possibility that the mechanisms of voltage gating of these two families of ion channels may have common features. To address this hypothesis, we have created a series of chimeras between the Kv channel, Kv2.1, and two TRP channels, TRPV1 and TRPM8, and studied them using electrophysiological techniques. Replacing the critical S3b-S4 paddle motif of Kv2.1 with analogous regions of TRPV1 or TRPM8, results in channels that activate in response to membrane depolarization. In both instances, the slopes of voltage-activation relations were decreased in the chimeras as compared to wild-type Kv2.1.These results are consistent with the hypothesis that TRP channels contain a structural motif related to the paddle motifs in Kv channels, and that this motif can sense voltage in the context of a Kv channel. We are currently testing whether this motif can sense voltage in TRP channels and exploring structural relationships between these two families of channels.

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TRPM3 Expression and Function in Vascular Smooth Muscle

Jacqueline Naylor, Jing Li, Fanning Zeng, Piruthivi Sukumar,
Yasser Majeed, Carol J. Milligan, Bhaskar Kumar, Karen E. Porter,
David J. Beech.
University of Leeds, Leeds, United Kingdom.

The Transient Receptor Potential (TRP) channels form a diverse superfamily of cation channels. Currently there are few well characterised, selective, physiological regulators of TRP channels, which can impede the discovery of native channel functions. TRPM3, a member of the melastatin subfamily of TRP channels, is reported to form constitutively active Ca^{2+}-permeable cation channels activated by sphingolipids, hypotonic shock, and the neurosteroid pregnenolone sulphate. Expression is predominantly in brain and kidney, although expression has recently been reported in pancreatic β-cells. Here we report TRPM3 expression and function in vascular smooth muscle cells. Anti-TRPM3 blocking antibody and short-interfering RNA targeted to TRPM3 were used to confirm the involvement of TRPM3 in the pregnenolone sulphate elicited Ca^{2+} influx in cultured human vascular smooth muscle cells. Although pregnenolone sulphate is a useful pharmacological and potentially therapeutic agent, the physiological significance in the vasculature is unknown. We therefore screened for novel modulators, and found that cholesterol inhibited both pregnenolone sulphate-induced and constitutive TRPM3 activity. The data suggest TRPM3 is a novel calcium-entry channel of vascular smooth muscle cells.

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1772-Pos

Clotrimazole Potentiates TRPM3 Responses to Pregnenolone Sulfate

Joris Vriens, Bernd Nilius, Thomas Voets.
K.U.Leuven, Leuven, Belgium.

Clotrimazole (CLT) is an antifungal compound commonly used in over-the-counter medications for the topical treatment of fungal infections of the skin, vagina, and mouth. CLT exerts its antifungal actions by inhibiting P450-dependent enzymes. TRPM3a2 (1), a splice variant of TRPM3, is rapidly and reversibly activated by pregnenolone sulfate (PS) and nifedipine (1). Here, we demonstrate that CLT strongly potentiates the response of TRPM3a2 to pregnenolone sulfate (PS) stimulation. Direct application of CLT to TRPM3a2 has no effect, however preapplication of CLT followed by PS stimulation strongly potentiates the TRPM3a2 response. The potentiation by CLT is reversible, repetitive and independent of external calcium. At CLT concentrations above 1 mM, the intensity of potentiation does not dependent CLT dose but rather on PS concentration. The response to PS after priming with CLT was relatively slow, suggesting a modulation in the signaling cascade rather than a direct effect. We conclude that CLT potentiates the TRPM3a2 response in an indirect manner, possibly by preventing further metabolization of pregnenolone sulfate by inhibiting P450-dependent enzymes.

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