Investigation of structure-function relationship of TRPM2

Ph.D. thesis

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Introduction

Transient receptor potential melastatin 2 (TRPM2), a Ca^{2+} permeable non-selective cation channel, belongs to the TRP protein family, and is abundantly expressed in brain neurons, bone marrow, phagocytes, pancreatic β-cells, and cardiomyocytes, where it opens under conditions of oxidative stress. TRPM2 function is required for pathogen-induced migration and chemokine production of phagocytic cells and normal glucose-evoked insulin secretion of pancreatic β-cells; in addition, TRPM2 has been linked to several pathological conditions that lead to apoptosis, including cerebral stroke, myocardial infarction, and neurodegenerative diseases.

TRP channels belong to the superfamily of voltage-gated cation channels, and share their overall tetrameric transmembrane (TM) architecture; the ion permeation pathway is built from TM helices 5 and 6, and the intervening pore-loop which forms the selectivity filter. The TRPM2 pore does not discriminate between Na^+ and K^+, and is highly permeable to Ca^{2+}.

The primary activators of TRPM2 are ADP-ribose (ADPR) and Ca^{2+}: simultaneous binding of both agonists is required for channel opening. ADPR binds to the NUDT9-homology (NUDT9-H) domains located at the cytosolic C termini of the subunits, named based on sequence homology to the mitochondrial enzyme NUDT9. Both NUDT9 and isolated NUDT9-H bind ADPR and convert it to

AMP and ribose-5-phosphate, but the role in TRPM2 channel gating of the very slow ADPR hydrolase activity of NUDT9-H is unknown.

Although in intact cells ADPR activates TRPM2 in the presence of either intra- or extracellular Ca^{2+}, biophysical studies in inside-out patches have shown that the activatory Ca^{2+}-binding sites are located intracellularly of the gate, such that extracellular Ca^{2+} needs to permeate through the pore before reaching its binding sites. Nevertheless, the vicinity of these sites to the pore suggests that in intact cells extracellular Ca^{2+} is the primary source of this activating ligand.

What are the signals that initiate TRPM2 channel activity in a cellular context? Early on TRPM2 currents have been linked to the redox status of the cell, and several studies have pinpointed oxidative stress, such as that inducible by H_2O_2, as a causative agent in TRPM2 activation. One early report of H_2O_2-activated whole-cell currents from a truncated TRPM2 construct no longer responsive to ADPR could not be reproduced. On the other hand, several mutations and deletions in the conserved Nudix motif (RILRQE) of the NUDT9-H domain that abrogated ADPR binding yielded channels that also failed to activate in response to H_2O_2, leading to the conclusion that H_2O_2 acts indirectly by increasing intracellular [ADPR]. Intriguingly, recent reports of synergistic effects between subthreshold concentrations of ADPR and H_2O_2, and of differential inhibitory profiles of ADPR- and H_2O_2-induced whole-cell TRPM2

currents again raised the possibility that H_2O_2 might directly affect TRPM2 activity by a mechanism independent of ADPR. Several additional compounds, mostly adenine nucleotides, have been found to modulate TRPM2 whole-cell currents in different cell types, but there is also substantial disagreement between the various reports. Cyclic ADPR (cADPR) fully stimulated TRPM2 currents in some studies, whereas it had little or no effect in others. In addition, small (subthreshold) concentrations of cADPR resulted in large, up to 100-fold, sensitization toward activation by ADPR, whereas others have found no such synergy. More recently nicotinic acid adenine dinucleotide phosphate (NAADP) was reported to fully activate endogenous TRPM2 currents in Jurkat T-lymphocytes and neutrophil granulocytes, and to potentiate ADPR-mediated channel activation, just as reported for cADPR. Low affinity activation of TRPM2 currents by NAD^+ is likely due to contamination by ADPR, and the significance of TRPM2 activation by O-acetylated ADPR remains to be elucidated. Finally, ADPR-activated whole-cell TRPM2 currents are inhibited by intracellular dialysis of AMP, raising the possibility that AMP – a degradation product of ADPR hydrolysis – might competitively inhibit ADPR induced TRPM2 current. Although the above whole cell patch-clamp studies have identified several interesting compounds as candidates that might directly affect TRPM2 channels, the conclusions of these reports are limited by the fact that in whole-cell recordings the entire cellular machinery

involved in nucleotide and Ca^{2+} homeostasis is in place. Thus, intracellular dialysis of a compound might affect TRPM2 activity either directly, by binding to the TRPM2 protein, or indirectly, by altering the local concentrations of the primary ligands ADPR and Ca^{2+}. Possible perturbations of other signaling pathways that ultimately affect TRPM2 activity also have to be considered. Regulation of cytosolic [ADPR] is the result of a complex interplay between several cytosolic, nuclear, and mitochondrial enzymes that produce and break down ADPR or are involved in its translocation between compartments. It is likely that intracellular dialysis of high concentrations of adenine nucleotides, which are substrates and/or products of several of these enzymes, will ultimately affect cytosolic [ADPR]; just as a bulk overload of intact cells with Ca^{2+} is likely to cause generation of reactive oxygen species and consequent ADPR production, as suggested by gradual activation of whole cell TRPM2 currents under such conditions. Likewise, intracellular [Ca^{2+}] is dynamically regulated by Ca^{2+} release from intracellular stores and plasma membrane Ca^{2+} influx, counteracted by transporters that extrude Ca^{2+} from the cytosol. Acting on ryanodine receptors and on two pore channels (TPC), respectively, both cADPR and NAADP are potent Ca^{2+} mobilizing agents, which likely alter both bulk intracellular [Ca^{2+}] and its microdomains when dialyzed into cells. Therefore, from whole-cell experiments a direct action of these compounds on the TRPM2 protein cannot be concluded.

Although previous studies identified ADPR and Ca^{2+} as the main activators of TRPM2, the biophysical details of TRPM2 gating are largely unknown. A major hurdle in addressing such questions has been the inability to obtain steady-state single-channel recordings, because TRPM2 currents decay rapidly in excised patches, even in the maintained presence of both activating ligands. This "rundown" has a time constant of <1 min, reflects a progressive decline in the number of active channels in the patch, and has so far defied attempts to prevent or reverse it. Beyond presenting a technical hurdle, rundown of several classes of ion channels in inside-out patches has highlighted important regulatory mechanisms, including the role of membrane phosphoinositides as key physiological regulators of inwardly rectifying potassium channels and several TRPM family members. In inside-out patches, rundown of these channels is caused by dephosphorylation of phosphatidylinositol bisphosphate (PIP_2); the presence of PIP_2 in the inner membrane leaflet is required to open the gate formed by the TM6 helix bundle-crossing. Many more mechanisms are associated with channel rundown, including dephosphorylation of the channel protein, dissociation and washout of an essential regulatory subunit, or cysteine oxidation, but for TRPM2 these were at least partly excluded by previous studies.

Objectives

- Identification of direct and indirect effectors of TRPM2 channels.
- Dissection of the mechanism of TRPM2 channel rundown, attempt to slow this process.
- Investigation of PIP_2 effect on TRPM2 channels

Methods

Molecular Biology

TRPM2 mutations were made using QuikChange (Stratagene); pGEMHE-TRPM2 and pGEMSH-TRPM8 cDNA were linearized by Nhe1 and transcribed in vitro with T7 polymerase (Ambion); cRNA was stored at −80 °C.

Isolation and Injection of *Xenopus* Oocytes

Oocytes were isolated from anaesthetized adult female *Xenopus laevis* and injected with cRNA. Single-channel and macroscopic recordings were made 2–3 days after injection of 1–10 ng of cRNA.

Excised Inside-Out Patch Recording

Patch pipettes were pulled from borosilicate glass and had resistances of 2-4 MΩ in our pipette solution. For most recordings the tip of the pipette was filled up to 1 cm height with a sodium gluconate based solution, for permeability measurements sodium was replaced by the examined cation. The back of the pipette, containing the electrode, was filled with sodium chloride based solution of

identical osmolarity. The sodium gluconate based bath solution was supplemented with various test substances; for control experiments free [Ca^{2+}] was 125 μM and [ADPR] was 32 μM. Patches were excised and transferred into a custom-made chamber, where the continuously flowing 25-°C bath solution, exchanged using computer-driven electronic valves (solution exchange time constant <100 ms), was connected to the bath electrode through a 140-mM KCl-agar bridge. Currents were digitized at 10 kHz, filtered at 2 kHz, and recorded to disk.

Enzymatic Purification of cADPR

cADPR was purified from its ADPR contamination using nucleotide pyrophosphatase type I (P7383; Sigma). The 24 kDa enzyme was removed by 2-fold filtering through 3-kDa molecular mass cut-off filter units (Z629367; Sigma).

Analysis of Nucleotide Purity by Thin Layer Chromatography (TLC)

Purity of ADPR, cADPR, NAADP and NAAD stock solutions was analysed by blotting 10–100 nmol of nucleotide onto Polygram SIL G/UV_{254} plates (Macherey-Nagel) followed by development in 70% (v/v) ethanol, 30% (v/v) H_2O, 0.2 M NH_4HCO_3. Separated nucleotides were visualized under UV illumination.

Results

1. Although whole-cell measurements had identified several compounds as potential effectors of TRPM2, this method is not suitable to discriminate between direct and indirect modulators. To differentiate between direct and indirect effects on TRPM2, we have evaluated these compounds in cell-free inside-out patches using direct ligand superfusion. Our results indicate that H_2O_2 and AMP do not directly affect TRPM2 gating. Although TRPM2 was activated by commercially available cADPR, mass spectrometric analysis and TLC revealed significant ADPR contamination of this nucleotide. Purification through enzymatic treatment which degrades contaminant ADPR, abolished the activatory effect of this product on TRPM2. Thus, we conclude that pure cADPR does not directly affect TRPM2 channel activity. NAADP and nicotinic acid adenine dinucleotide (NAAD) are partial agonists, both capable of directly activating TRPM2, but based on their low apparent affinities it seems unlikely that they should act as direct modulators of TRPM2 currents in vivo. These results single out the primary ligands ADPR and Ca^{2+} as the final common pathway into which multiple metabolic routes converge to modulate TRPM2 activity in intact cells.

2. To explore the molecular background of TRPM2 current rundown, we compared its rate under various conditions. We found that rundown is not due to membrane PIP_2 depletion, but is state

dependent, and sensitive to the species and concentration of the permeating ion. Together, these findings suggest that TRPM2 rundown might reflect an irreversible conformational change of the selectivity filter to a state resembling that of C-type inactivated voltage-gated K^+ channels. For this reason we designed several pore mutants to try to slow this process. The idea was based on previous work on the TRPM4 selectivity filter in which removal of one of two negatively charged side chains or of an uncharged residue, present in TRPM4 and TRPM5 but absent in TRPM2, induced fast irreversible rundown. We showed that a triple-mutant TRPM2 channel with a TRPM5-like pore (T5L) does not run down in inside-out patches over the time course of hours. Although the pore diameter in T5L remained unchanged (~7 Å), the unitary conductances in various $[Na^+]$, $[Ca^{2+}]$, and $[Mg^{2+}]$ revealed increased apparent affinities for permeating cations. The increase in apparent affinities of the pore mutants was much larger toward divalent cations and was not accompanied by a change in maximal conductances, consistent with increased local cation concentrations near the mouth of the pore due to an electrostatic effect. We also compared ADPR and Ca^{2+} dependent gating of the T5L mutant with that of wild type (WT) TRPM2 in macroscopic and single channel measurements. We found that the regulation of T5L gating by ADPR and intracellular Ca^{2+} remained very similar to that of WT. The T5L mutant therefore

provides an excellent background for studying the biophysics of TRPM2 gating at steady state.

3. Although TRPM2 rundown is unrelated to the depletion of membrane PIP_2, to further address whether PIP_2 plays any role in TRPM2 regulation, we tested the effect on TRPM2 currents of polylysine, a polycation that masks PIP_2 headgroups. High concentrations (15 μg/mL) of polylysine rapidly abolished WT TRPM2 currents, and this action was indeed due to depletion of free PIP_2, because application of PIP_2 following polylysine removal restored WT TRPM2 activity. Thus, complete depletion of free PIP_2 closes the TRPM2 activation gate. Of note, T5L TRPM2 channels, which do not run down, were similarly closed by polylysine and reopened by PIP_2, suggesting that the T5L pore mutation does not decouple PIP_2 effects from the gate. Inhibition by polylysine was slowly reversible. Complete depletion of free PIP_2 by a large concentration of polylysine shifted the $K_{1/2}$ for Ca^{2+} activation from ~20 μM to >1 mM, and gradual current restoration was accompanied by a restoration of Ca^{2+} sensitivity. Moreover, for both WT and T5L TRPM2, fractional activity in 4 μM cytosolic Ca^{2+} (+ 32 μM ADPR) was significantly higher in the presence of 50 μM PIP_2 compared with control conditions, consistent with a slightly higher apparent Ca^{2+} affinity in the presence of high concentrations of PIP_2.

Conclusions

- H_2O_2, AMP, and cADPR are indirect effectors of TRPM2 channels, while NAADP and NAAD are low-affinity partial agonists.
- The molecular mechanism of TRPM2 rundown is similar to that of C-type inactivation. We designed a pore mutation which mimics the filter sequence of TRPM5 (T5L), and completely eliminates rundown: T5L channels retain unabated maximal activity for over 1 hour. Although its permeation properties are altered, ADPR/Ca^{2+}-dependent gating of T5L remains intact. This non-inactivating TRPM2 variant will be an invaluable tool for gating studies.
- TRPM2 rundown is unrelated to the depletion of membrane PIP_2. Complete depletion of free PIP_2 by a large concentration of polylysine dramatically reduces, while exposure to very high levels of PIP_2 only modestly increases the apparent Ca^{2+} affinity of TRPM2. Because membrane PIP_2 concentrations must be already very low at the beginning of our experiments, we conclude that TRPM2 channels must bind PIP_2 with very high affinity

List of publications

The PhD thesis is based on the following publications

Tóth B, Csanády L. (2010) Identification of direct and indirect effectors of the transient receptor potential melastatin 2 (TRPM2) cation channel. *J Biol Chem* **285:** 30091-30102.
IF: 5.328

Tóth B, Csanády L. (2012) Pore collapse underlies irreversible inactivation of TRPM2 cation channel currents. *Proc Nat. Acad Sci U S A* **109:** 13440-13445.
IF: 9.681

Other publications

Mándi M, **Tóth B,** Timar G, Bak J. (2006) Ca^{2+} release triggered by NAADP in hepatocyte microsomes. *Biochem J* **395:** 233-238.
IF: 4.100