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Review Article

TRPM2, calcium and neurodegenerative diseases

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Abstract: NMDA receptor overactivation triggers intracellular Ca^{2+} dysregulation, which has long been thought to be critical for initiating excitotoxic cell death cascades associated with stroke and neurodegenerative disease. The inability of NMDA receptor antagonists to afford neuroprotection in clinical stroke trials has led to a re-evaluation of excitotoxic models of cell death and has focused research efforts towards identifying additional Ca^{2+} influx pathways. Recent studies indicate that TRPM2, a member of the TRPM subfamily of Ca^{2+}-permeant, non-selective cation channel, plays an important role in mediating cellular responses to a wide range of stimuli that, under certain situations, can induce cell death. These include reactive oxygen and nitrogen species, tumour necrosis factor as well as soluble oligomers of amyloid beta. However, the molecular basis of TRPM2 channel involvement in these processes is not fully understood. In this review, we summarize recent studies about the regulation of TRPM2, its interaction with calcium and the possible implications for neurodegenerative diseases.

Keywords: TRPM2, oxidative stress, neurodegeneration, amyloid beta, stroke, NMDA receptor

Introduction

Traditional models of neuronal excitotoxicity have focused on the glutamate-mediated overactivation of receptors such as the ionotropic N-methyl-D-aspartate (NMDA) glutamate receptor subtype (NMDAR). However, glutamate receptor blockade, antioxidant or anti-inflammatory therapy has failed to provide neuroprotection in clinical stroke trials. Accordingly, the focus of recent research has shifted towards identifying neurotoxic signalling molecules positioned further downstream of NMDAR stimulation. These efforts have yielded exciting implications as to specific strategies for treating excitotoxic disorders. Specifically, several previously overlooked Ca^{2+} influx pathways have recently been identified. These include acid sensing and pannexin channels, as well as a member of the melastatin subfamily of transient receptor potential (TRP) channels [1-3]. Increasing evidence has shown that TRP channels represent an exciting new family of cation channels, several members of which are highly expressed in the brain [4-7]. The mammalian TRP family consists of six main subfamilies termed the TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin), and TRPA (ankyrin) subfamilies. Collectively, the broad and varied physiological roles of TRP channels are being increasingly appreciated as is their contribution to a number of diseases [5;6;8;9]. TRPM2, one member of the TRPM subfamily, is a Ca^{2+}-permeable, non-selective cation channel, that plays a critical role in mediating cellular responses to a wide range of physiological stimuli that can also induce cell death in response to oxidative stress [9-11].

TRPM2 channel structure

TRPM2 consists of six putative transmembrane domains, a re-entrant pore forming loop and intracellular N- and C- termini. Channels, thought to form as homotetramers, have high monovalent cation permeability with variable permeability for Ca^{2+}, Mg^{2+} and other divalent cations [12-15] (**Figure 1**). Intriguingly, the divalent cation permeability appears to vary substantially with heat [16]. The transmembrane

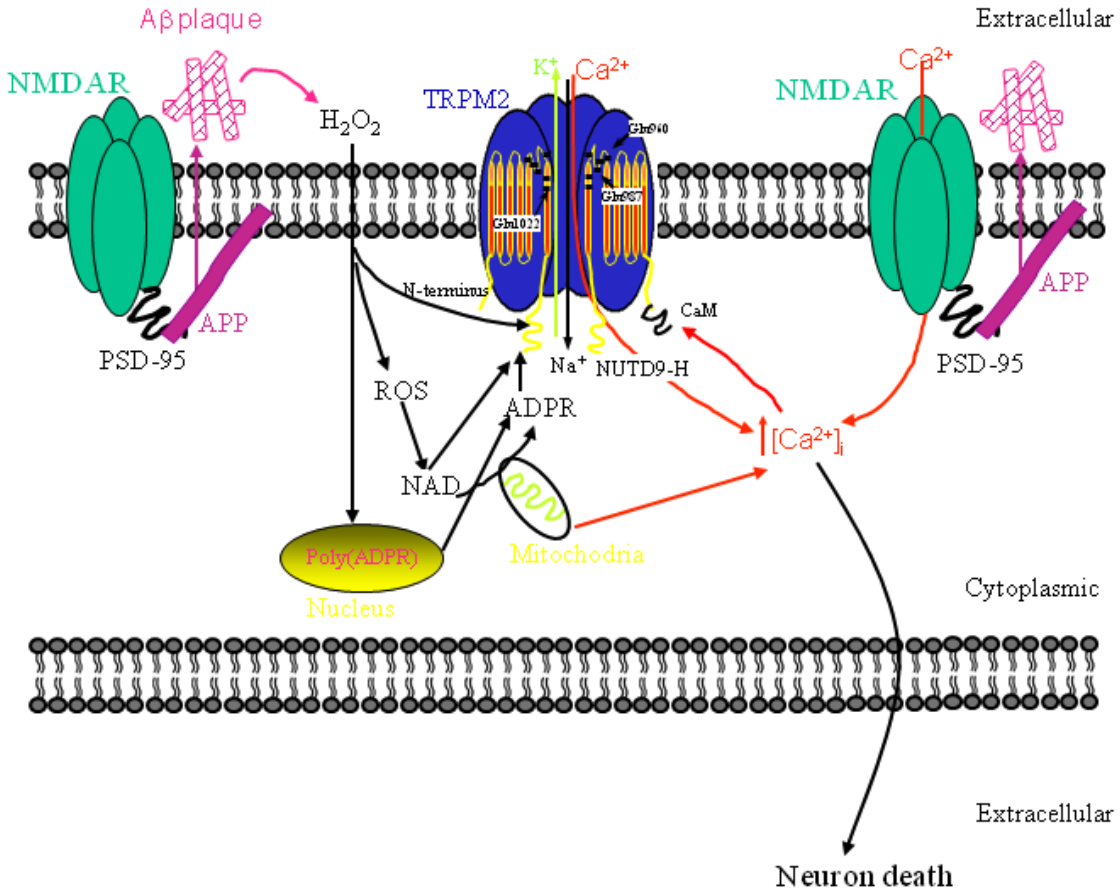

Figure 1. Schematic diagraph showing the signal pathway of TRPM2 and NMDAR. Abbreviations see text.

segments TM5 and TM6 are highly conserved with its related family member TRPM8, and the distal part of S6 is crucial for charge discrimination [17]. Furthermore, the Glu-960, Gln-981, Asp-987, and Glu-1022 residues of TRPM2 channel are found to be engaged in determining divalent cation permeation properties [13] while two conserved cysteine residues (at positions 996 and 1008), located within the putative pore region of the human TRPM2, are obligatory for TRPM2 channel function [18] (**Figure 1**). The C-terminus of TRPM2 can be further subdivided into an approximately 100 amino acid region of high coiled coil character, a short perhaps 30 amino acid linker region, and the approximately 270 amino acid NUDT9-homology domain (NUDT9-H) [11]. Within NUDT9-H lies the so-called Nudix box, a region of 22 amino acids that is conserved among a family of enzymes that catalyze the hydrolysis of nucleoside diphosphates. The recognition of this motif led Scharenberg's group [19] to identify a unique mode of channel activation: TRPM2 is gated by cytosolic ADPR. Accordingly, TRPM2 has been dubbed a "chanzyme" (channel-enzyme) possessing both ion conducting and enzyme domains. However, it should be noted that the NUDT9-H enzyme activity of TRPM2 is severely restricted relative to that of NUDT9 due to specific amino acid substitutions (EF → IL) within the Nudix box [20].

TRPM2 distribution

TRPM2 is widely distributed in the CNS including the hippocampus, cerebral cortex, thalamus, and midbrain [4, 12, 21-24]. Although initial reports suggested that the expression of TRPM2

within the CNS was restricted to non-neuronal cells, subsequent more exhaustive examination leaves little doubt that TRPM2 is also expressed within neurons [24-26]. However, that is not to say that neuronal expression is ubiquitous throughout all CNS regions and within all neuronal subtypes. Indeed, transcripts for TRPM2 could not be detected within cultured granule cells of the cerebellum [12] nor could we find functional evidence of TRPM2 expression in stratum radiatum interneurons of the hippocampus [24]. Thus, though broadly distributed, TRPM2 expression within specific cell types (e.g. neurons, astrocytes and microglia) may vary regionally which, correspondingly, likely influences the functional consequences of TRPM2 activation in each region.

At the subcellular level, limited information is available to date. In cultured cerebral cortical neurons from fetal rat, TRPM2 could be detected within cell bodies and neurites. Interestingly, molecular cloning of TRPM2 from rat brain cDNA revealed several differences in amino acid sequence within the Nudix box region as compared with those of human and mouse TRPM2 [27]. As a result, the sensitivity of rat TRPM2 to ADPR appears somewhat greater with all other properties being similar to human and mouse TRPM2. Within cultured hippocampal neurons we reported a similar expression profile within soma and neurites [24]. In addition, we found that the expression pattern appeared diffuse and did not specifically co-localize with PSD-95, a marker of excitatory synapses. Although more detailed co-localization is needed, the evidence thus far suggests that TRPM2 may preferentially be expressed within extrasynaptic regions of hippocampal pyramidal neurons.

In addition to full length transcripts, four splice variants of TRPM2 have been identified: TRPM2 -S, TRPM2-ΔN, TRPM2-ΔC and SSF-TRPM2. TRPM2-S (short) has a deletion of the entire C-terminus, including four of six C-terminal transmembrane domains and the putative channel pore [28]. TRPM2-ΔN has a deletion of amino acids 538–557 in the N-terminus. TRPM2-ΔC has a deletion of amino acids 1292–1325 in the C-terminal CAP domain of NUDT9-H that decreases the affinity for ADPR [29]. Finally, a short form lacking the first 214 amino acids of the N-terminal was identified as being expressed uniquely within the striatum and has thus been termed the striatum short form protein (SSF-TRPM2)[30]. The functional significance of these naturally occurring variants is not presently fully appreciated nor have the relative expression levels by region been determined. It is known however, that the expression of TRPM2-S can suppress the channel activity of the full-length variant [22, 28]. Thus, the relative expression level of each variant could have important consequences for TRPM2 function.

TRPM2 gating mechanisms

In addition to ADPR, evidence has accumulated suggesting that TRPM2 may be gated by several means in a coordinated manner. Several adenine intracellular second messengers, metabolically related to ADPR, have been proposed to gate TRPM2 channels. These include nicotinamide adenine dinucleotide (NAD^+), which serves as a precursor for the formation of ADPR, as well as cyclic ADPR (cADPR), a well recognized regulator of intracellular Ca^{2+} homeostasis. The ability of each of these adenine metabolites to gate the channels directly has been controversial [19, 31, 32]. Indeed, it is now generally agreed that activation by NAD^+ at room temperature may be due to the presence of contaminating ADPR. Similarly, while two groups have reported that cADPR is incapable of gating TRPM2 directly [19, 31], another has reported that relatively high concentrations (e.g. > 100 μM) may do so [32]. Perhaps more interestingly, in the latter study the authors reported that modest concentrations of cADPR (10 μM) may facilitate TRPM2 function such that nM concentrations of ADPR may gate the channel. Previously considered to regulate Ca^{2+}-induced Ca^{2+} release via ryanodine receptors, the findings suggest an expanded role for cADPR in regulating intracellular Ca^{2+} concentration. More recently, TRPM2 gating has been shown to be heavily influenced by temperature and is in fact directly gated by heat, the threshold for activation being 35°C [16]. Moreover, heat was shown to greatly facilitate gating by ADPR and, interestingly, by NAD^+ as well as by cADPR, suggesting that TRPM2 channel activity may not be solely regulated by the intracellular levels of ADPR. A further layer of complexity is added by the finding that ADPR's breakdown product, adenosine monophosphate (AMP) specifically inhibits ADPR, but not cADPR-mediated gating of TRPM2, whereas the cADPR antagonist 8-Br-cADPR exhibits the reverse block specificity [32]. Collectively, these studies suggest that

the regulation of TRPM2 channel activity is tightly regulated by the relative levels of these intracellular messengers.

TRPM2 has also been reported to be gated by oxidative and nitrosative stress (ROS/RNS). Gating by ROS/RNS requires an intact ADPR binding cleft in the C-terminal domain, suggesting that gating occurs through the intracellular production of ADPR. More definitive evidence comes from recent studies showing that ROS/RNS induces mitochondria to produce free ADPR and release it to the cytosol where subsequent accumulation of ADPR induces TRPM2 gating [33,34] (**Figure 1**). Oxidative stress, may also induce the formation of ADPR in the nucleus following activation of DNA repair enzymes [34, 35] (as shown in **Figure 1**) poly(ADP-ribose) polymerases (PARPs) and glycohydrolases (PARG). These enzymes first poly-ADP-ribosylates nuclear proteins and then liberate monomers of ADPR, respectively. In addition to being sensitive to ROS/RNS, more recently TRPM2 was shown to be responsive to cell stress induced by puromycin through the recruitment of Sir2 family of NAD^+-dependent protein deacetylases and the production of the metabolite 2'-*O*-acetyl-ADP-ribose (OAADPR) [36].

Irrespective of the activating mechanism, TRPM2 currents possess a linear current–voltage relationship with a reversal potential of about 0 mV. Single channel recordings reveal a slope conductance of 60-80 pS and distinctly long open times (in the range of several hundred milliseconds to seconds). TRPM2 channels are permeable to monovalent cations as well as Ca^{2+} and Mg^{2+} and have no apparent voltage dependence. However, at high intracellular Na^+ concentrations TRPM2 channels undergo a hyperpolarization-dependent inactivation [19].

In addition to ADPR, cADPR, H_2O_2, intracellular ions typically used in patch-clamp experiments such as Sr^{2+}, Ca^{2+}, Cs^+ or Na^+ can alter ADPR sensitivity and voltage dependence of TRPM2 channels, complicating the evaluation of the roles of the various modulators in a physiological context. Lastly, TRPM2 currents are not suppressed by La^{3+}, a broad spectrum blocker of non-selective cation channels, but can be inhibited by several blockers including flufenamic acid, clotrimazole, econazole and ACA in the micromolar range [7, 34].

TRPM2 and calcium

TRPM2 gating by ADPR is influenced by the intracellular concentration of Ca^{2+}. This was initially reported by Scharenberg's group when they showed that raising intracellular Ca^{2+} lowered the EC_{50} for channel activation by ADPR [19]. The importance of Ca^{2+} in regulating TRPM2 channel opening is highlighted by recordings in leucocytes demonstrating that the endogenous concentration of ADPR is insufficient to activate TRPM2 when $[Ca^{2+}]_i$ is at resting levels. However, when $[Ca^{2+}]_i$ is elevated, these same levels of intracellular ADPR are now sufficient to permit gating [7]. Importantly, subsequent ion substitution experiments allowed the identification of one of the most distinctive features of TRPM2, namely that extracellular Ca^{2+} acts as a positive regulator of TRPM2 [31]. Indeed, omission of Ca^{2+} from external solutions prevents or rapidly abrogates TRPM2 activation. To the best of our knowledge, no other channel displays this unique property. Thus, in the absence of specific channel blockers for TRPM2, lowering of extracellular Ca^{2+} provides a means of distinguishing TRPM2 generated currents from other potential contributing conductances. This can be useful when studying the function of endogenously expressed TRPM2 channels; especially in neurons where numerous voltage- as well as ligand-gated conductances can contribute to cationic membrane flux [24].

Therefore, TRPM2 supports Na^+ and Ca^{2+} influx, thereby modulating membrane potential as well as intracellular Ca^{2+} levels. On the other hand, the sensitivity of TRPM2 to ADPR is also facilitated by intracellular Ca^{2+} [37-39] via positive feedback. Rather than acting at an extracellular site, Ca^{2+} entering via TRPM2 acts upon an intracellular calcium sensor closely associated with the channel [31, 38]. Once opened, Ca^{2+} entering through the channel pore may slow channel closure by keeping a Ca^{2+} activating site saturated. This activating site is proposed to lie intracellularly from the gate, but in a shielded crevice near the pore entrance [40]. The activating site in question appears to correspond to a Ca^{2+}-CAM binding site located within the N-terminus [39;41], see **Figure 1**. Importantly, Ca^{2+}-CAM may do more that simply modulate the sensitivity of TRPM2 for ADPR. Indeed, a recent study, investigating the gating mechanisms in TRPM2 splice variants and mutants in which ADPR-binding has been dis-

rupted, revealed that elevations of intracellular Ca^{2+} may be sufficient, in and of themselves, to gate TRPM2 channels [39, 41].

TRPM2 and Diseases

In the brain, Ca^{2+} is fundamental in the control of synaptic activity and memory formation, a process that leads to the activation of specific Ca^{2+} -dependent signal transduction pathways. The identification of several modulators of Ca^{2+} homeostasis as potential factors involved in the pathogenesis of Alzheimer's (AD), Parkinson's (PD), and Huntington's (HD) diseases, provides strong support for a role of Ca^{2+} dysregulation in neurodegeneration [42, 43]. The influx of Na^{+} and Ca^{2+} via TRPM2 channels, which promotes membrane depolarization and increases in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), and the wide expression profile of TRPM2 renders it a potentially significant therapeutic target in a variety of pathological settings including cardiovascular and neurodegenerative diseases [9-11, 44-48].

TRPM2 and neuronal injury and death

Activation of TRPM2 by a variety of means, including TNFα, ROS/RNS and ADPR, has been linked to cell death, suggesting that TRPM2 is likely to be a key player downstream of several signalling pathways mediating cell death in response to cerebral ischemia and reperfusion injury [49]. In the rat transient middle cerebral artery occlusion (tMCAO) stroke model, TRPM2 mRNA expression increased at 1 and 4 weeks following ischemic injury, partially as a consequence of glial activation since the profile is temporally consistent with the pattern of microglial activation seen in this model. This suggests that TRPM2, expressed in glia, may play a role in the pathophysiology produced following a transient period of ischemia [23]. However, another study indicated that oxidative stress, induced by exogenous application of H_2O_2, caused preferential damage of pyramidal neurons in hippocampus [25]. Consistent with this, treatment of cultured cerebral cortex neurons with small interfering RNA against rat TRPM2 efficiently suppressed TRPM2 immunoreactivity, the H_2O_2-induced Ca^{2+} influx and neuronal death, suggesting that TRPM2 plays a pivotal role in H_2O_2-induced neuronal death [27]. During a period of acute ischemia *in vivo* or oxygen-glucose deprivation (OGD) *in vitro*, a TRP-like channel is activated by cellular stress and contributes to ischemia-induced membrane depolarization, intracellular Ca^{2+} accumulation and cell swelling in CA1 neurons [4]. Moreover, clotrimazole, a TRPM2 channel blocker [7, 10], was found to block TRPM2 currents in hippocampal pyramidal neurons [24], be neuroprotective in spinal cord clip compression injury [50] and reduce the death in cultured rat cerebellar granule and hippocampal neurons induced by excitotoxic insults [51]. While cognizant of the fact that clotrimazole is far from being selective for TRPM2 and that other targets may have been involved in the effects described above, a collective body of work is nevertheless supportive of a role for TRPM2 as a mediator of neuronal cell death.

TRPM2 and related neurodegenerative diseases

Cognitive impairment and emotional disturbances in AD result from the degeneration of synapses and neuronal death in the limbic system and associated regions of the cerebral cortex. The accumulation of beta amyloid protein (Ab) in the brain is considered to be a key factor that causes AD. Aβ can render neurons vulnerable to excitotoxicity and apoptosis by disruption of cellular Ca^{2+} homeostasis and membrane-associated oxidative stress [46, 52]. Recent findings, suggest that, in addition to its role in H_2O_2 induced-neuronal death of striatal neurons, TRPM2 may also be implicated in Aβ-induced death of cultured striatal neurons [22]. Transfection with a splice variant (TRPM2-S) that acts as a dominant negative inhibitor of TRPM2 function was shown to inhibit both H_2O_2- and Aβ-induced increases in intracellular-free Ca^{2+} and cell death. Furthermore, small interfering RNA targeting TRPM2 reduced TRPM2 mRNA levels and the toxicity induced by H_2O_2 and Aβ [22]. More recently, Aβ has been suggested to directly incorporate into neuronal membranes where it may form Ca^{2+} -permeable ion channels (amyloid channels) that cause abnormal elevations of intracellular Ca^{2+} [53]. This supplemental Ca^{2+} entry would be expected to facilitate further TRPM2 channel activation and consequent rise in intracellular Ca^{2+}. More broadly, the specific coupling of TRPM2 activation to pathways leading to increased oxidative stress might implicate TRPM2 in other neurodegenerative diseases. Specific examples have recently been revealed by studies suggesting that TRPM2 plays important role in motor neu-

ron death associated with Guamanian Amyotrophic Lateral Sclerosis (ALS-G) and parkinsonism dementia (PD-G) [44, 45].

In addition, the finding that reduced mRNA levels of TRPM2 channel in B lymphoblast cell lines (BLCL) from bipolar I disorder (BD-I) patients showing elevated basal $[Ca^{2+}]_i$, an index of altered intracellular Ca^{2+} homeostasis, implicates the involvement of this gene in the Ca^{2+} abnormalities and the pathophysiology of bipolar disorder [54].

Potential link between Amyloid β protein, NMDA receptor and TRPM2

Acute brain injuries have been identified as a risk factor for developing AD and glutamate plays a pivotal role in these pathologies. In primary cultures of cortical neurons, sublethal NMDAR activation was found to alter the processing of the amyloid precursor protein (APP). Indeed, Ca^{2+} entry through NMDARs increased the expression of neuronal Kunitz protease inhibitory domain (KPI) containing APP (KPI-APP). Expression of KPI-APPs favoured β-secretase processing, instead of the normally dominant α-secretase processing, leading to increased production and secretion of Aβ [55]. Interestingly, the salient features of these NMDAR-mediated changes in APP processing could be replicated in a mouse model of traumatic brain injury. A further link between NMDAR function and the production Aβ comes from studies examining the localization of APP and Aβ in relation to markers of excitatory synapses. For example, a pool of APP is found in the postsynaptic compartment in cortical neurons where it partially co-localizes with both NR1 and PSD-95 [56, 57], as illustrated schematically in **Figure 1**. In addition, immunolocalization of soluble oligomers of Aβ, bound to cultured hippocampal neurons, reveals a pattern of staining suggesting that Aβ associates with excitatory synapses [58, 59]. Intriguingly, the association of Aβ with excitatory synapses could be prevented by pre-treatment with an antibody recognizing the extracellular N-terminus of the NMDAR subunit, NR1. Moreover, the rapid rise in intracellular Ca^{2+} and ROS formation, observed during acute applications of soluble oligomers of Aβ to cultured neurons, could be prevented by NMDAR inhibition [60]. We have shown that hippocampal pyramidal neurons possess functional TRPM2 channels whose activation by ADPR is functionally coupled to voltage-dependent Ca^{2+} channels (VDCCs) and NMDARs through a rise in $[Ca^{2+}]_i$ [24]. In light of the findings discussed above, we suggests that TRPM2, acting in concert with NMDARs, may provide the basis for a positive feedback loop in which Ca2+ influx is facilitated through a pathway involving aberrant NMDAR activation, the production of Aβ and the formation of ROS all of which leads to the activation of TRPM2. A schematic figure summarizing this proposed model is shown in **Figure 1**.

Prospective

Neuroprotection via glutamate receptor blockade, antioxidant or anti-inflammatory therapy has not proven effective in the clinical treatment of brain damage due to narrow therapeutic windows, poor pharmacokinetics and blockade of the signalling essential for normal excitatory neurotransmission and neuronal survival. In light of the wide-spread expression and growing work indicating that TRPM2 may play important role in neuronal death that is activated by oxidative stress and downstream from excitotoxic signal pathways, useful therapeutic applications are expected for future drugs that block TRPM2 channels or inhibit their activation. This will provide an exciting new avenue for research into the pathophysiology and treatment of neuronal death and related CNS diseases.

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