Transient Receptor Potential Channels of the Melastatin Family and Ischemic Responses of Central Neurons

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Abstract—The excitotoxic theory of stroke, which implicated N-methyl-D-aspartate (NMDA) receptors as mediators of excessive Ca\(^{2+}\) entry and neuronal death, generated a great deal of enthusiasm for the prospect of using NMDA receptor antagonists to prevent the associated brain injury. Unfortunately, these receptor antagonists failed to provide effective treatments for human stroke. In part, the failure is likely a consequence of having to administer these drugs within a very short therapeutic window after stroke and to the intolerable psychomimetic side effects associated with their use. However, new possibilities for therapeutic intervention are revealing themselves as our understanding of excitotoxicity evolves. We now recognize that ischemia and Ca\(^{2+}\) toxicity in central neurons can be attributed to a variety of mechanisms recruited downstream of NMDA receptor activation. These include the activation of Ca\(^{2+}\)-permeable transient receptor potential channels of the melastatin family. The more-delayed activation of these channels offers the tantalizing possibility that drugs targeting selected members of this family may possess a wider therapeutic window for preventing the debilitating consequences after stroke onset. (Stroke. 2007;38[part 2]:665-669.)

Key Word: brain ischemia

Cerebral stroke is one of the leading causes of morbidity and mortality, and our aging population is most at risk of this disease. It is therefore of great importance to identify therapeutic means to protect the brain from the damage attributable to stroke. After the loss of oxygen and nutrients in the region of stroke, a massive and inappropriate release of the excitatory transmitter glutamate is known to occur. The resulting pathological activation of postsynaptic glutamate receptors is one of the early triggers that ultimately leads to the subsequent intracellular Ca\(^{2+}\) overload and cell death. Paradoxically, the physiological release of glutamate mediates most excitatory synaptic transmission in the central nervous system, and therein lay the challenge for early investigators looking for means of preventing the brain injury resulting from stroke; ie, how to strictly prevent the pathological and not the physiological activation of these receptors. A potential resolution to this conundrum was immediately appreciated when the N-methyl-D-aspartate (NMDA) receptor was identified as a glutamate receptor subtype having high permeability to Ca\(^{2+}\). In addition, these receptor channels had the distinction of being constitutively blocked in a voltage-dependent manner by physiological concentrations of the extracellular divalent cation Mg\(^{2+}\). Importantly, the block by Mg\(^{2+}\) can be relieved through membrane depolarization. This feature was essential in distinguishing between the physiological and pathophysiological activation of glutamate receptors. Indeed, excitatory synaptic transmission is predominantly mediated through the \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) subtype of glutamate receptors. These inotropic receptors form nonselective cation channels that are most typically permeable only to the monovalent cations Na\(^+\) and K\(^+\). After the physiological release of glutamate from presynaptic vesicles, postsynaptic AMPARs are gated open, resulting in an influx of Na\(^+\) that generates an inward current, the excitatory postsynaptic current, which depolarizes and excites the postsynaptic cell. Importantly, the resulting depolarization is normally insufficient to completely relieve the voltage-dependent block by Mg\(^{2+}\). As a result, only modest activation of NMDARs can occur. In contrast, the pathological release of glutamate induced by ischemia stimulates both synaptic and extrasynaptic AMPARs, resulting in a strong and prolonged depolarization of the neurons. This depolarization provides the trigger to activate voltage-dependent Ca\(^{2+}\) channels and also serves to relieve the constitutive voltage-dependent blockade of NMDARs by extracellular Mg\(^{2+}\). The resulting pathological activation of NMDARs and voltage-dependent Ca\(^{2+}\) channels leads to a large decrease in the extracellular concentration of Ca\(^{2+}\) (and Mg\(^{2+}\)) and a prolonged and inappropriate rise in intracellular Ca\(^{2+}\). The entry of Ca\(^{2+}\) through NMDARs has been shown to be particularly neurotoxic, resulting in the activation of intracellular signal pathways implicated in delayed neuronal death. Although NMDARs play a central role in the excitotoxicity theory of neuron death, additional mediators have been identified that contribute to the neurotoxic cascade. For example, acidification of the extracellular compartment also occurs, leading to stimulation of proton-gated channels, which further contribute to the Ca\(^{2+}\) influx. In addition,
reversal of the $\mathrm{Na}^+$/Ca$^{2+}$ exchanger can contribute to intracellular Ca$^{2+}$ overload.\footnote{6}

During the last 2 decades, the initial excitement\footnote{6} over the possible use of glutamate receptor antagonists to prevent cell death after stroke has been replaced by strong pessimism as clinical trials of antiepileptic drugs have failed to benefit patients.\footnote{10} The exception may be the drug memantine, which is currently used to treat Alzheimer disease. However, the negative side effects of these antagonists have continued to mount, and even memantine may not be an exception to this undesirable aspect of NMDAR antagonists.\footnote{17} As a result of the failure of NMDAR antagonists, new strategies for preventing excitotoxicity at some point downstream of the receptors have been sought (eg, retain NMDAR synaptic function but block an intracellular toxic signal). For example, oxygen-glucose deprivation (OGD)–induced cell death can be reduced by disrupting the coupling of synaptic NMDARs to the cytoskeleton and a nitric oxide synthase (NOS)–dependent signal pathway.\footnote{18} This coupling occurs through the intermediary PSD-95, a postsynaptic density protein that binds both NMDARs and NOS, and small peptides that interfere with this association can “rescue” neurons in vitro and in vivo.\footnote{19} (NMDARs induce the generation by NOS of peroxynitrite, or ONOO$^-$.) These results imply that NMDARs can act as the trigger for, but may not be the only causative factor underlying, excitotoxicity. Indeed, NMDAR antagonists can protect neurons from a 1-hour period of OGD but fail to offer protection after longer exposure periods.\footnote{20} Similarly, although the antagonists prevent the Ca$^{2+}$ overload during the first hour, their ability to block this overload fails with longer periods of OGD. Note that this failure occurs, even though NMDARs, AMPARs, voltage-dependent Ca$^{2+}$ channels, and Na$^+$/Ca$^{2+}$ exchange are all suppressed. These results led us to hypothesize that there is an alternative and more sustained mechanism of Ca$^{2+}$ overload, potentially involving an unidentified Ca$^{2+}$-permeable cation channel. If this is the case, then several predictions could be made about the properties of this channel. Clearly, it should be expressed in neurons, and it should be subject to prolonged activation during ischemia or anoxia. Channel activity might also be enhanced by NMDAR-induced increases in intracellular Ca$^{2+}$ and/or by the generation of ONOO$^-$. Perhaps a more novel possibility is that channel activity is linked in some way to the fall in the extracellular concentrations of Ca$^{2+}$ and Mg$^{2+}$ (ie, relief of divalent cation block).

There is no shortage of potential, nonselective cation channel candidates, but only a relatively small number of these are known to be activated by oxidative stress and lead to cell death. One possibility arises from observations that in Drosophila photoreceptors, anoxia leads to cell degeneration via stimulation of a transient receptor potential-like channel (TRPL).\footnote{22} This channel and the related transient receptor potential channel (TRP) set the stage for the cloning of a wide variety of mammalian TRP channels. These channels constitute members of >3 major families; however, 2 members of the “melastatin” family (TRPM2 and TRPM7) are associated with oxidative stress and/or cell death.\footnote{23} TRPM7 and TRPM2 channels are ubiquitously expressed in many cell types, but for the most part, their physiological functions in the nervous system are poorly understood. Interestingly, both proteins share with their related family member TRPM6 the unique ability to function as both an ion channel and enzyme. The TRPM7 catalytic domain is homologous to that of $\alpha$-kinase family members (Figure 1A), known to phosphorylate their substrates within $\alpha$-helixes.\footnote{24} Although the function of this

![Figure 1. A. Predicted transmembrane topology of TRPM7. The protein is characterized by long cytoplasmic N- and C-terminal tails and 6 membrane-spanning domains, as well as a presumed pore-forming reentrant loop located between transmembrane domains 5 and 6. The presence of the $\alpha$-kinase domain is indicated within the C terminus. The pore domains from at least 4 subunits likely assemble to form a nonselective cation channel, which, as indicated, is permeable to the divalent cations Mg$^{2+}$ and Ca$^{2+}$ as well as the monovalent cations Na$^+$ and K$^+$. Paradoxically, the presence of divalent cations (divalent cation-containing [DVC] solutions) reduces the permeability of the channel to monovalent cations at negative membrane potentials. As a result, the current-voltage (I–V) relation for TRPM7 (cartoon) displays prominent outward rectification (B). In contrast, when the concentration of divalent cations is reduced or omitted altogether (divalent-free [DVF] solutions), the outward and especially the inward flow of current carried by monovalent cations is greatly increased.](image-url)
activity remains unknown, one substrate, annexin 1, has been identified to date. In contrast, the catalytic domain of TRPM2, homologous to that of the NUDT9 ADP-ribose (ADPR) pyrophosphatase and termed NUDT9-H (Figure 2A), is intimately associated with its ability to function as a channel. Indeed, this region of the channel serves as a ligand-binding domain, which allows channel gating by intracellularly generated ADPR. Although both TRPM2 and TRPM7 are expressed in the mammalian brain, little is known about their function in central neurons.

We used a well-characterized in vitro model of anoxia/aglycemia that uses a period of OGD to mimic the conditions of stroke. Patch-clamp recordings were done in parallel with applications of NaCN (chemical anoxia) to induce similar periods of anoxia but yielding experimental conditions amenable to electrophysiological analysis. This approach allowed us to assess the role of TRP-like channels in the induction of excitotoxicity. OGD causes a delayed death of cultured neurons (tested 24 hours after exposure). This toxicity and the associated Ca\(^{2+}\) influx were strongly inhibited by NMDAR antagonists (together with AMPARs and voltage-dependent Ca\(^{2+}\) channels), provided that the period of OGD was restricted to \(\approx 1\) hour. However, these antagonists were virtually ineffective in preventing either the Ca\(^{2+}\) loading or the toxicity as the period of OGD was increased to 2 hours and beyond, suggesting that another source of Ca\(^{2+}\) entry was involved.

We also assessed the responses of individual cultured neurons during the periods of anoxia and glucose deprivation. Prolonged chemical anoxia (NaCN, 2 to 3 hours) caused an initial and abrupt depolarization of individual neurons together with an increase in excitability (firing of action potentials) associated with a substantial increase in excitatory synaptic activity. In other recordings, action potentials and evoked synaptic activity were blocked by including tetrodotoxin in the dish, and we were able to monitor the activity of miniature excitatory postsynaptic currents (glutamatergic) as a measure of the spontaneous release of glutamate. The increase in excitability was maintained for variable periods of time, but by 2 hours, action potentials were absent and synaptic activity was lost. Despite having exhausted glutamate release, all neurons remained nevertheless depolarized as a consequence of the activation of a sustained inward current. This inward current was not mediated by glutamate receptors (eg, NMDARs), voltage-dependent calcium channels, Na\(^+/\)Ca\(^{2+}\) exchange, etc.

We demonstrated that the sustained inward current induced by chemical anoxia was mediated by a previously unidentified, nonselective cation current. This current is attenuated by free-radical scavengers (as is the associated delayed cell death) and activated by the production of NOS-dependent free radicals, or reactive oxygen/nitrogen species (ROS, RNS), and it characteristically shows outward rectification in its current-voltage relation. This led us to hypothesize that the sustained inward current could be mediated, at least in part, by a member of the melastatin family of the transient receptor potential channels (ie, TRPMs). Two members of this family are prime candidates. For example, TRPM2 channels can be activated by conditions that produce ROS, leading to cell death in an expressed cell line, and TRPM7 channels are enhanced by the production of ROS. TRPM2 and TRPM7 channels are both permeable to Ca\(^{2+}\), but TRPM7-mediated currents demonstrate a marked outward rectification attributable to the assumed voltage-dependent block on inward monovalent cations by extracellular divalent cations (Figure 1B), whereas TRPM2 channels are insensitive to such a block and therefore, lack rectification in their current-voltage relation (Figure 2B). Our initial experiments

**Figure 2.** A, Predicted transmembrane topology of TRPM2. As with TRPM7, the protein is characterized by long cytoplasmic N- and C-terminal tails and 6 membrane-spanning domains, as well as a presumed pore-forming reentrant loop located between transmembrane domains 5 and 6. The NUDT9-H domain located within the C terminus allows gating of the channel by intracellular ADPR. Subunits for TRPM2 assemble to form a nonselective cation channel, which is permeable to the divalent cation Ca\(^{2+}\) as well as the monovalent cations Na\(^+\) and K\(^+\). Unlike TRPM7, however, ion conduction through TRPM2 channels is not affected by the presence of divalent cations. Consequently, the channels exhibit ohmic current-voltage (I-V) (cartoon) behavior (B) over a broad range of applied voltages (±100 mV).
were targeted at the possibility of TRPM7 involvement in the anoxic response because of the similarity in rectification properties between these currents and the sustained current and because decreases in extracellular divalent cations dramatically reduced rectification of the inward sustained current. Given the lack of very selective pharmacological blockers of TRPM channels, we developed a series of “gene silencing” RNAs, which were transfected into cultured cortical neurons. Using conventional polymerase chain reaction measurements, we were able to demonstrate a strong suppression of TRPM7 message in these cultures, concurrent with a reduction in the sustained inward currents. These results were strengthened by using single-cell polymerase chain reaction techniques to show the loss of the outwardly rectifying sustained current in TRPM7-silenced neurons. Indeed, “gene silencing” of TRPM7 dramatically extended the survival period of neurons exposed to OGD well beyond that of NMDAR antagonists. Our experiments on stroke have so far focused on the use of an in vitro preparation, but we are currently using viral vectors to deliver siRNA to neurons in vivo.

TRPM7 and TRPM2 channels are therefore potential therapeutic targets for reducing the ischemic cell damage and excitotoxicity that follow stroke. The release of glutamate during stroke is likely limited by the eventual depletion of glutamate from cellular stores. In contrast to this relatively transient activation of NMDARs, the activation of both TRPM7 and TRPM2 is tied to factors generated by continuing oxidative stress and cell damage. These include stimulation by RNS, intracellular Ca2+ overload, and, in the case of TRPM2 channels, the production of ADPR, which gates these channels. ADPR is likely derived from mitochondria subjected to oxidative stress and potentially as a by-product of attempts by the cells to repair DNA damage through stimulation of poly(ADPR) polymerase I and poly(ADPR) glycohydrolase. In either case, the synthesis of ADPR occurs at the expense of α-nicotinamide adenine dinucleotide and contributes to the metabolic failure of the cell. It is tempting to then suggest that a TRPM-selective channel blocker ought to be developed as a potential therapeutic agent. This may prove to be a particularly difficult selectivity to achieve, given that existing nonselective cation channel blockers are already notoriously nonspecific. Of course, blocking these channels may itself prove a problem. For example, the knock-out TRPM7 channels result in cell death, likely because this channel is required for Mg2+ homeostasis in cells. The teleological argument that these channels are present to perform yet-to-be-delineated physiological roles seems compelling. For example, TRPM7 channels are known to be recruited to the plasma membrane of vascular cells in response to fluid flow, suggesting that these channels might also play a role in the vascular side of stroke. Given the failure of NMDA channel blockers in therapeutic trials, an alternative approach may be to target the intracellular sites of gating of TRPM channels by ROS/RNS and/or ADPR.

Disclosures
None.

References
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