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The Role of TRP Channels in Disease

**Non-selective cation channels, transient receptor potential channels and
ischemic stroke**

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Abstract

Several pathways to neural cell death are involved in ischemic stroke, and all require monovalent or divalent cation influx, implicating non-selective cation (NC) channels. NC channels are also likely to be involved in the dysfunction of vascular endothelial cells that leads to formation of edema following cerebral ischemia. Two newly described NC channels have emerged as potential participants in ischemic stroke, the acid sensing ion channel (ASIC), and the sulfonylurea receptor-1 (SUR1)-regulated $\text{NC}_{\text{Ca-ATP}}$ channel. Non-specific blockers of NC channels, including pinokalant (LOE 908 MS) and rimonabant (SR141716A), have beneficial effects in rodent models of ischemic stroke. Evidence is accumulating that NC channels formed by members of the transient receptor potential (TRP) family are also up-regulated in ischemic stroke and may play a direct role in calcium-mediated neuronal death. The nascent field of NC channels, including TRP channels, in ischemic stroke is poised to provide novel mechanistic insights and therapeutic strategies for this often devastating human condition.

1. Introduction

A number of different mechanisms have been implicated in cell death in CNS ischemia and stroke, including excitotoxicity, oxidative stress, apoptosis, and oncotic (necrotic) cell death. Each of these mechanisms is thought to propagate through largely distinct, mutually exclusive signal transduction pathways [1]. However, in some measure, each of these mechanisms requires cation influx into neural cells. Unchecked influx of Na^+ gives rise to oncotic cell swelling (cytotoxic edema) which predisposes to oncotic cell death (Fig. 1). Unchecked influx of Ca^{2+} can trigger apoptotic as well as necrotic death. Because cation channels are responsible for most cation influx, it is evident that cation channels are key to life-death processes in neural cells during ischemic stroke.

A variety of cation channels have been implicated in neural cell death induced by ischemia/hypoxia. Among them are channels that are highly selective for permeant cations, such as voltage-dependent Na^+ and Ca^{2+} channels, as well as channels that are not selective for any given cation – non-selective cation (NC) channels. In ischemic stroke, much attention has been directed to dihydropyridine-sensitive L-type voltage-dependent Ca^{2+} channels ($\text{Ca}_v1.2$), but block of this channel in patients with acute ischemic stroke has shown little benefit [2]. Arguably, the best studied channels in ischemic stroke belong to the group of receptor operated cation channels opened by glutamate, including N-methyl-D-aspartate (NMDA) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptor channels, which are involved in excitotoxic

cell death [3,4]. Again, however, clinical trials with agents targeting these mechanisms have been disappointing [5-7]. Unfavorable experiences with mechanisms involving L-type Ca^{2+} channels and NMDA receptor channels have resulted in redirection of attention to other cation channels, namely, glutamate receptor-independent, NC channels.

Apart from neural cell death, other critically important pathophysiological processes that contribute to adverse outcome in ischemic stroke include formation of ionic edema, vasogenic edema and hemorrhagic conversion – all processes involving capillary endothelial cells [8] (Fig. 1). Molecular mechanisms involved in these processes are only beginning to be elucidated. In the case of ionic edema formation, transcapillary flux of Na^+ constitutes the seminal process that drives inflow of H_2O into brain parenchyma, resulting in edema and swelling. It is likely that NC channels play a crucial role in this process. Thus, NC channels may be implicated not only in primary neural cell death but in secondary neural cell death caused by endothelial dysfunction.

In recent years, study of ischemia/hypoxia-induced cell death has been dominated by discussion of apoptosis, a form of “delayed” programmed cell death that involves transcriptional up-regulation of death-related gene products such as caspases. However, in stroke, only a fraction of cells undergo apoptotic death, with the majority of cells dying by oncotic/necrotic death [9]. The lesson from studies on apoptosis is that death, like so many other cellular events, is driven by gene expression and synthesis of new gene products, a concept that has not been fully embraced in studies on oncotic/necrotic death.

Comprehensive understanding of the pathophysiology of ischemic stroke requires a focus not only on constitutively expressed NC channels in neurons, astrocytes and endothelial cells, but perhaps more importantly, on newly expressed NC channels whose transcription is driven by mechanisms involved in ischemic stroke, namely, hypoxia and oxidative stress.

The purpose of this review is to examine evidence that NC channels, including TRP channels, are involved in CNS ischemia and ischemic stroke. In reviewing this literature, it becomes apparent that for stroke, the potential contribution of NC channels including TRP is far less well understood than for many other normal and pathological conditions. The relative dearth of hard data forces consideration of somewhat weaker evidence, including indirect or suggestive data. First, we review potential involvement of two important, recently identified NC channels that are up-regulated in ischemic stroke, ASIC and the SUR1-regulated NC_{Ca-ATP} channel. Next, we consider studies using non-specific NC channel blockers, specifically pinokalant (LOE 908 MS), the fenamates, SKF 96365 and rimonabant (SR141716A). Finally, we consider evidence for involvement of NC channels of the TRP family, reviewing not only data documenting their role in neuronal death and their up-regulation in cerebral ischemia, but also data that would predict potential involvement based on the transcriptional drivers, hypoxia and oxidative stress.

2. NC channels in ischemic stroke

2.1. Acid sensing ion channel (ASIC)

Acid sensing ion channels (ASIC) are not members of the TRP family, but are included here because they are NC channels relevant to ischemic stroke. ASIC are members of the epithelial Na⁺ channel/degenerin family of ion channels, and they are expressed throughout the mammalian nervous system [10,11]. Six different ASIC subunits have been cloned to date, which are encoded by four genes (ASIC1-ASIC4) [12]. Members of this supergene family are permeable to Na⁺ (P_{Na}/P_K : 8–40) and, to a lesser degree, to Ca²⁺, and they are blocked by amiloride (IC₅₀, 0.2–10 μM).

ASIC were first described a quarter of a century ago in sensory neurons by Krishtal and coworkers [13-15]. Recently, ASIC have enjoyed renewed interest in the context of neural cell death and ischemic stroke. It is well known that ischemia results in a marked reduction in tissue pH, and that acidosis is an important determinant of neurological injury [16,17]. Oxygen depletion necessitates a switch from aerobic metabolism to anaerobic glycolysis, leading to generation of lactic acid as well as protons, resulting in acidification of tissues.

ASIC are ligand-gated cation channels that respond to acidic stimuli. In contrast to most Ca²⁺-permeable neurotransmitter-receptor channels such as nicotinic acetylcholine receptors and glutamate receptors, which are inhibited as pH falls, ASIC are activated as pH falls. They are generally inactivated at physiological pH (7.4). The probability of opening of ASIC1a increases as the pH falls below 7.0, and activation is half maximal at a pH of 6.2 – a pH range that can occur within the penumbra and core of an infarct, especially in the context of hyperglycemia [17]. Activation of ASIC is promoted by stretching of the

membrane, release of arachidonic acid, production of lactate [18,19] or a drop in extracellular Ca^{2+} concentration [20] – conditions that occur within an infarct as cells swell, Ca^{2+} -dependent phospholipases are activated, and Ca^{2+} influx occurs [21].

ASIC1a, the major ASIC subunit with Ca^{2+} permeability, is highly expressed in neurons. Recent evidence suggests that activation of Ca^{2+} -permeable amiloride-sensitive ASIC1a may be responsible for acidosis-mediated, glutamate receptor-independent, neuronal injury [22,23]. *In vitro*, activation of ASIC results in an increase in intracellular Ca^{2+} and induces time-dependent neuronal injury that occurs in the presence of the blockers of voltage-gated Ca^{2+} channels and glutamate receptors.

ASIC1a may also have a role in cerebral ischemia *in vivo* [22]. In rodent models of ischemic stroke, intracerebroventricular administration of the ASIC1a blockers, amiloride and tarantula toxin psalmotoxin 1 prior to onset of ischemia, as well as knockout of the ASIC1a gene, reportedly protects from ischemic injury [22]. Also, transient global ischemia induces expression of ASIC2a in rat brain, including in neurons of the hippocampus and cortex [24].

2.2. SUR1-regulated $\text{NC}_{\text{Ca-ATP}}$ channel

The SUR1-regulated $\text{NC}_{\text{Ca-ATP}}$ channel is a 35 pS cation channel that conducts all inorganic monovalent cations (Na^+ , K^+ , Cs^+ , Li^+ , Rb^+), but is impermeable to Ca^{2+} and Mg^{2+} [25]. The fact that it conducts Cs^+ makes it easy to distinguish from K_{ATP} channels with which it shares several properties (see

below). Channel opening requires nanomolar concentrations of Ca^{2+} on the cytoplasmic side. Channel opening is blocked by ATP (EC_{50} , 0.79 μM), but is unaffected by ADP or AMP. Studies using a variety of organic monovalent cations indicate that the channel has an equivalent pore radius of 0.41 nm (Table 1).

The SUR1-regulated $\text{NC}_{\text{Ca-ATP}}$ channel is believed to be composed of pore-forming and regulatory subunits. The regulatory subunit is sulfonylurea receptor 1 (SUR1), the same as that for K_{ATP} channels in pancreatic β cells [26], and so the two channels have pharmacological profiles that resemble each other closely. Opening of SUR1-regulated $\text{NC}_{\text{Ca-ATP}}$ channels is blocked by tolbutamide (EC_{50} , 16.1 μM at pH 7.4) and glibenclamide (EC_{50} , 48 nM at pH 7.4). Block by sulfonylurea is due to prolongation of and an increase in the probability of long closed states, with no effect on open channel dwell times or channel conductance. The potency of block by glibenclamide is increased ~8-fold at pH 6.8 (EC_{50} , 6 nM), consistent with the weak acid needing to enter the lipid phase of the membrane to cause block [27]. In the presence of ATP, channel opening is increased by diazoxide, but not pinacidil or cromakalin, as expected for SUR1 but not SUR2. The inhibitory effect of glibenclamide on opening of the SUR1-regulated $\text{NC}_{\text{Ca-ATP}}$ channel is prevented by antibody directed against one of the cytoplasmic loops of SUR1. Knockdown of SUR1 using antisense-oligodeoxynucleotide reduces SUR1 expression [27] and prevents expression of functional SUR1-regulated $\text{NC}_{\text{Ca-ATP}}$ channels (unpublished, Simard and Chen).

The SUR1-regulated NC_{Ca-ATP} channel is not constitutively expressed, but is expressed in the CNS under conditions of hypoxia or injury. The channel was first discovered in freshly isolated reactive astrocytes obtained from the hypoxic inner zone of the gliotic capsule [25,26]. Since then, it has also been identified in neurons from the core of an ischemic stroke [27]. In rodent models of ischemic stroke, the SUR1 regulatory subunit is transcriptionally up-regulated in neurons, astrocytes and capillary endothelial cells.

The consequence of channel opening has been studied in isolated cells that express the channel, by depleting ATP using Na azide or Na cyanide plus 2-deoxyglucose, or by using diazoxide. These treatments induce a strong inward current that depolarizes the cell completely to 0 mV. Morphological studies demonstrate that cells subsequently undergo changes consistent with cytotoxic edema (oncotic cell swelling), with formation of membrane blebs. Bleb formation is reproduced without ATP depletion by diazoxide [25]. Cells later die predominantly by non-apoptotic, propidium iodide-positive necrotic death [27].

The effect of channel block by glibenclamide has been studied *in vitro* in reactive astrocytes that express the channel [26,27]. In cells exposed to Na azide to deplete ATP, glibenclamide blocks membrane depolarization, significantly reduces blebbing associated with cytotoxic edema, and significantly reduces necrotic cell death.

The effect of channel block by glibenclamide has also been studied in 2 rodent models of ischemic stroke [27]. Specificity of the drug for the target was based on administering a low dose by constant infusion (75-200 ng/h), which was

predicted to yield serum concentrations of ~1–3 ng/ml (2–6 nM), coupled with the low pH of the ischemic tissues, to take advantage of the fact that glibenclamide is a weak acid that would preferentially target acidic tissues. In a rodent model of massive ischemic stroke with malignant cerebral edema associated with high mortality (68%), glibenclamide reduced mortality and cerebral edema (excess water) by half. In a rodent model of stroke induced by thromboemboli with delayed spontaneous reperfusion, glibenclamide reduced lesion volume by half, and its use was associated with cortical sparing attributed to improved leptomeningeal collateral blood flow due to reduced mass effect from edema.

Recently, the outcome from stroke in humans was retrospectively evaluated in patients with diabetes mellitus (DM) who were taking a sulfonylurea such as glibenclamide and who continued on it during their hospitalization for stroke [28]. The primary outcome was a decrease in National Institutes of Health Stroke Scale (NIHSS) of 4 points or more from admission to discharge or a discharge NIHSS score = 0. When compared to controls (DM patients not on sulfonylurea), patients in the sulfonylurea group fared significantly better, with the primary outcome being reached by 36.4% of patients in the treatment group versus 7.1% in the control group ($p=0.007$).

In summary, the salient features of the SUR1-regulated NC_{Ca-ATP} channel are that: (i) it is not constitutively expressed, but is transcriptionally up-regulated in association with an hypoxic insult; (ii) when expressed, it is not active but becomes activated when intracellular ATP is depleted, leading to cell depolarization, cytotoxic edema and necrotic cell death; (iii) block of the channel

in vitro results in block of depolarization, cytotoxic edema and necrotic cell death induced by ATP depletion; (iv) block of the channel *in vivo* results in significant improvement in rodent models as well as in humans with ischemic stroke.

3. NC channel blockers in ischemic stroke

A number of studies have shown that pharmacological inhibition of NC channels reduces focal ischemic injury in rodent models of ischemic stroke. Although none of these pharmacological agents is uniquely specific for any single molecular entity, some have been shown to block TRP channels. Because these agents are not specific, it is not known whether the NC channel that is targeted is constitutively expressed, is newly expressed by ischemia/hypoxia or oxidative stress, or whether the drug is acting on a TRP channel or some other NC channel. Nevertheless, such studies provide important insights into the potential role of NC channels in ischemic stroke, and point to the importance of continued studies in these areas.

3.1. Pinokalant

The isoquinoline derivative pinokalant (LOE 908 MS, (R,S)-(3,4-dihydro-6,7-dimethoxy-isoquinoline-1-yl)-2-phenyl-N,N-di[2-(2,3,4-trimethoxyphenyl)ethyl]-acetamide) blocks a variety of NC channels, including both receptor- and store-operated NC channels that mediate Ca^{2+} -entry, including:

(i) norepinephrine-activated Ca^{2+} -entry channels in adrenergic receptor-expressing Chinese hamster ovary cells [29];

(ii) endothelin-1 (ET-1)-activated Ca^{2+} -entry channels in rat aorta myocytes [30], A7r5 cells [31,32], rabbit internal carotid artery myocytes [33], in C6 glioma cells [34], in ET-1-expressing CHO cells [35,36] and in bovine adrenal chromaffin cells [37];

(iii) ATP- and N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-stimulated cation currents in HL-60 cells [38];

(iv) vasopressin-induced cation current in A7r5 cells [39];

(v) store-operated NC channels in human endothelial cells [40]; (however, in some cells, store-operated NC channels are resistant to pinokalant, reflecting a significant diversity of molecular constituents of these channels [41,42]).

The primary candidate subunits of mammalian receptor- and store-operated NC channels are TRP proteins. Some of the above receptor- and store-operated NC channels that are blocked by pinokalant have been shown to be mediated by members of the TRP family, suggesting that pinokalant may, at least in part, be targeting some TRP channels. Thus, TRPC6 is an essential component of the norepinephrine-activated channel in rabbit portal vein, and it is believed that TRP6 plays an important role in mediating Ca^{2+} influx in vascular smooth muscle [43-45]. TRPC1 has been implicated in ET-1-evoked arterial contraction [46]. TRPC are thought to function as Ca^{2+} entry channels operated by store-depletion as well as receptor-activated channels in a variety of cell types, including endothelial cells [47]. In the cockroach, *Periplaneta Americana*,

the TRP γ (pTRP γ) channel is blocked by pinokalant [48]. However, block by pinokalant cannot be taken as evidence in and of itself that a TRP channel is involved in any given cationic current. Voltage-activated delayed rectifier K⁺ channels in PC12 cells and cortical neurons [49] and in HL-60 cells [38] are also blocked by pinokalant .

Given its pharmacological profile as an inhibitor of NC channels, pinokalant has been evaluated as a potential neuroprotectant in rodent models of stroke [50-54].

Magnetic resonance imaging (MRI) was used to study the effect of pinokalant in a permanent (suture occlusion) middle cerebral artery occlusion (MCAO) model [51]. In untreated animals, the ischemic lesion volume [defined as the region in which the apparent diffusion coefficient (ADC) of water decreased to below 80% of control] steadily increased by approximately 50% during the initial 6 h of vascular occlusion. In treated animals, the ADC lesion volume decreased by approximately 20% during the same interval. After 6 h of vascular occlusion, blood flow was significantly higher in treated animals, and the volume of ATP-depleted and morphologically injured tissue representing the infarct core was 60-70% smaller. The volume of severely acidic tissue did not differ, indicating that pinokalant does not reduce the size of ischemic penumbra. These findings were interpreted as demonstrating that post-occlusion treatment delays the expansion of the infarct core into the penumbra for a duration of at least 6 h.

MRI was also used to study the effect of pinokalant in a temporary (90-min suture occlusion) MCAO model [52-54]. Before treatment, the DWI-derived

infarct volume did not differ between the groups, whereas at 4 h after MCAO, it was significantly smaller in the treated group. A significant difference in ischemic lesion size was detected beginning 1.5 h after treatment. The size of the ischemic core was significantly smaller in the treatment group, while the size of the ischemic penumbra was similar in the two groups at 85 min after arterial occlusion. Postmortem, 2,3,5-triphenyltetrazolium chloride (TTC)-derived infarct volume was significantly attenuated in the pinokalant group and the neurological scores at 24 h were significantly better among the treated rats.

3.2. Fenamates

The fenamates, flufenamic acid, mefenamic acid and niflumic acid, block Ca^{2+} -activated non-selective cation channels in a variety of cells [55-57]. Flufenamic acid inhibits several TRP members, including TRPC3 [58], TRPC5 [59,60], TRPM2 [61-63], TRPM4 [64,65], TRPM5 [65], whereas it increases activity of TRPC6 [66-68]. However, channels other than TRP are also blocked by these compounds [63].

Three fenamates (flufenamic acid, meclofenamic acid and mefenamic acid) were examined for their protective effect on neurons under ischemic (glucose/oxygen deprivation) or excitotoxic conditions, using the isolated retina of chick embryo as a model [69]. The fenamates protected the retina against the ischemic or excitotoxic insult, with only part of the neuroprotection attributed to inhibition of NMDA receptor-mediated currents, implicating non-NMDA NC channels in the response.

The effect of pre-treatment or post-treatment with mefenamate was evaluated in a rodent model of transient focal ischemia [70]. Neither pre- nor post-ischemic administration of a dose previously shown effective in preventing epileptic neuronal necrosis was found to reduce necrosis in cortex, nor in any subcortical structures, which forced the authors to conclude that NC channel blockade with mefenamate affords no neuroprotection in this model.

3.3. SKF 96365

SKF 96365 (SK&F 96365) (1-(beta-[3-(4-methoxy-phenyl)propoxy]-4-methoxyphenethyl)-1H-imidazole hydrochloride) is structurally distinct from the known Ca^{2+} antagonists and shows selectivity in blocking receptor-mediated Ca^{2+} entry, compared with receptor-mediated internal Ca^{2+} release [71]. However, SKF 96365 is not as potent ($\text{IC}_{50} \sim 10 \mu\text{M}$) or selective (also inhibits voltage-gated Ca^{2+} entry) as would be desirable, so caution has been advised when using this compound [71].

Measurements of intracellular Ca^{2+} in human embryonic kidney (HEK)293 cells that stably expressed human TRPC3 were used to show that SKF 96365 blocks TRP channels [72]. Expression of TRPC3 in these cells forms a non-selective cation channel that opens after the activation of phospholipase C, but not after store depletion. Increased Ca^{2+} entry in TRPC3-expressing cells is blocked by high concentrations of SKF 96365 [72].

The blood-brain barrier (BBB) serves as a critical organ in the maintenance of CNS homeostasis and is disrupted in a number of neurological

disorders, including ischemic stroke. SKF 96365 was used to determine if Ca^{2+} flux was important in mediating hypoxic/aglycemic effects on endothelial cells of the BBB [73-75], which do not express voltage-dependent Ca^{2+} channels. Expression of the tight junction protein occludin increased after hypoxic/aglycemic stress when cells were exposed to SKF 96365, which correlated with inhibition of the hypoxia-induced increase in permeability. Treatment with SKF 96365 increased intracellular Ca^{2+} under normoglycemic conditions, and was protective against hypoxia-induced BBB disruption under normoglycemia.

3.4. Other blockers – rimonabant and capsaicin

Rimonabant (SR141716A) is a compound that interacts with the G-protein coupled cannabinoid 1 (CB1) receptor [76]. Rimonabant has also been suggested to block TRP channel vanilloid subfamily member 1 (TRPV1) [77]. The link between CB1 and TRPV1 is reinforced by evidence that anandamide, an endogenous CB1 ligand, also activates TRPV1 [78]. Capsaicin as well as H^+ (pH ≤ 5.9) are agonists known to activate TRPV1 [79,80].

In a rat model of ischemic stroke, rimonabant, given 30 min after initiation of permanent MCAO, reduced infarct volume by ~40% [81].

The effects of rimonabant and capsaicin were investigated, with the aim of assessing the potential role of TRPV1 in a model of global cerebral ischemia in gerbils [77,82]. Both compounds were found to antagonize the electroencephalographic changes, hyperlocomotion and memory impairment

induced by global ischemia, and both were associated with a progressive survival of pyramidal cells in the CA1 subfield of the hippocampus. Notably, capsazepine, a selective TRPV1 antagonist, reversed both rimonabant-induced and capsaicin-induced neuroprotective effects. The authors interpreted their findings as suggesting that neuroprotection associated with capsaicin might be attributable, at least in part, to TRPV1 desensitization.

4. TRP channels in ischemic stroke

There is a dearth of studies addressing the potential role of TRP channels in ischemic stroke *in vivo*, which is attributable, in part, to a paucity of appropriate tools. Although *in vitro* studies (reviewed below) suggest a possible role, demonstrating a role *in vivo* is considerably more difficult, as it requires demonstrating the presence of one or more of the following: (i) salutary effect of pharmacological block; (ii) salutary effect of gene silencing; (iii) transcriptional up-regulation under conditions of hypoxia/oxidative stress *in vitro*; (iv) transcriptional up-regulation under conditions of ischemic stroke *in vivo*.

At present, available pharmacological tools lack the required specificity [83], and the result of silencing a single gene at a time can be difficult to interpret, since TRP proteins tend to form heteromultimers and can exhibit interdependent expression [84,85]. The physical associations between TRP and other proteins are far more complex than previously realized, and the classical TRP proteins appear to be organized into macromolecular assemblies, the functions of which are only beginning to be understood [86]. Moreover, as pointed out by Flockerzi

and colleagues [87], many of the results relating to TRP channel activity have been obtained from experiments using heterologously expressed TRP proteins and, in most cases, cells and expression vectors optimized for over-expression of the protein in question. Because they are purposely over-expressed, TRPs might act in places other than the plasma membrane and they might be forced into forming multimeric protein complexes that do not exist in their native environment. Thus, caution is required when extrapolating findings from *in vitro* experiments to *in vivo* pathological conditions.

4.1. *In vitro* studies – TRPM2/TRPM7

Restoring extracellular Ca^{2+} after a period of low Ca^{2+} concentrations has long been known to cause a paradoxical increase in intracellular Ca^{2+} levels that can lead to cell death (' Ca^{2+} paradox'). Until recently, entry of Ca^{2+} through NMDA receptor channels was considered to be the major pathway leading to the excitotoxic, delayed cell death associated with ischemia. There is now evidence, however, that TRP channels, specifically TRPM2 and TRPM7, may be important contributors to both the Ca^{2+} paradox and the delayed death of neurons following ischemia [88-92]. Aarts and coworkers studied the mechanism of death in cultures of mixed cortical neurons subjected to 1.5 h of oxygen/glucose deprivation, followed by return to normoxic conditions and treatment with an anti-excitotoxic cocktail (MK-801, CNQX, and nimodipine) [85]. Treatment unmasked a Ca^{2+} -mediated death mechanism associated with a Ca^{2+} -permeable NC conductance that was shown to be carried by TRPM7, based on siRNA-inhibition

of TRPM7 expression. Suppressing TRPM7 expression blocked TRPM7 currents, anoxic $^{45}\text{Ca}^{2+}$ uptake, production of reactive oxygen species (ROS), and anoxic death. In addition, TRPM7 suppression eliminated the need for the anti-excitotoxic cocktail to rescue anoxic neurons and permitted the survival of neurons previously destined to die from prolonged anoxia. Notably, TRPM7 current is potentiated by acidosis [93], a condition that is present with cerebral ischemia and that makes this mechanism all the more relevant in ischemic stroke.

4.2. *In vivo studies – TRPC4*

Involvement of TRPC4 was studied in a rodent model of MCAO [94]. The authors used a commercial antibody directed against TRPC4 (from Alamone Labs) for Western immunoblots and immunohistochemistry. Some have cautioned that unequivocal detection of endogenous TRPC4 proteins may be difficult [87]. In any case, TRPC4 protein was found to be significantly elevated in striatum and hippocampus 12 h – 3 d after MCAO, with TRPC4 immunoreactivity being present in neuronal membranes.

4.3. *TRPM4 – an $\text{NC}_{\text{Ca-ATP}}$ channel*

Unlike every other channel discussed so far, TRPM4 has not been directly linked to ischemic stroke. However, it is included here because, as first pointed out by Csanady and Adam-Vizi [95], many of its biophysical properties are similar to those of the SUR1-regulated $\text{NC}_{\text{Ca-ATP}}$ channel (see Table 1), which has been

implicated in ischemic stroke (see Section 2.2). TRPM4, together with TRPM5, are the only molecular candidates presently known for the class of non-selective, Ca^{2+} -impermeable cation channels that are activated by intracellular Ca^{2+} and blocked by intracellular ATP, i.e., $\text{NC}_{\text{Ca-ATP}}$ channels. Given that the pore-forming subunit of the SUR1-regulated $\text{NC}_{\text{Ca-ATP}}$ channel has not yet been identified, it is not surprising that speculation would arise regarding the possibility that TRPM4 (or TRPM5) might actually constitute the pore-forming subunit of the SUR1-regulated $\text{NC}_{\text{Ca-ATP}}$ channel.

Both the SUR1-regulated $\text{NC}_{\text{Ca-ATP}}$ channel and TRPM4 are highly selective for monovalent cations, have no significant permeation of Ca^{2+} , are activated by internal Ca^{2+} and blocked by internal ATP. The two channels have several features in common (Table 1), and some of the apparent differences may be explained by straightforward arguments. First, differences are to be expected based simply on the fact that overexpression of TRPM4 in an artificial system may not mimic native expression in reactive astrocytes. For example, in native mast cells, TRPM4 activation is independent of voltage [96] whereas expression in HEK 293 cells yields a voltage-dependent channel whose voltage dependence is removed by augmenting PIP_2 [97]. Second, the lipid environment, especially membrane levels of PIP_2 , is known to affect key properties of TRPM4, not only voltage dependence but also channel kinetics and Ca^{2+} -dependence [97]. Finally, the high sensitivity to glibenclamide exhibited by the SUR1-regulated $\text{NC}_{\text{Ca-ATP}}$ channel requires co-expression with SUR1. If SUR1 can bind to TRPM4 to increase its sensitivity to sulfonylurea, it is not unreasonable to expect that

heteromers of SUR1 and TRPM4 might exhibit somewhat different properties than homomeric TRPM4.

The postulated heteromeric assembly of SUR1 and TRPM4 is further made plausible by the fact that SUR1 is known to be a promiscuous regulatory subunit. SUR1 is best known for its role in forming K_{ATP} channels by assembling with Kir6.1 or Kir6.2 pore forming subunits. However, heterologous constructs of SUR1 with another inwardly rectifying K^+ channel, Kir1.1a, have also been described [98]. Speculation aside, however, it is not yet known whether the pore forming subunit of the SUR1-regulated NC_{Ca-ATP} channel is encoded by a gene from any member of the TRP family.

4.4. Drivers of TRP expression – hypoxia and oxidative stress

Hypoxia

Stroke is a pathological condition marked by ischemia-induced hypoxia. Thus, we consider TRP channel expression that is induced by hypoxia.

In pulmonary arterial smooth muscle cells, hypoxia induces a 2–3-fold increase in TRPC1 and TRPC6 mRNA and protein levels, but has no effect on TRPC3/TRPC4 expression [99,100]. Notably, hypoxia-induced up-regulation of TRPC1 and TRPC6 is correlated with and is dependent on the transcription factor, hypoxia inducible factor 1 (HIF -1) [99].

Redox state

Apart from ischemia/hypoxia, stroke is also a condition characterized by reperfusion, which is associated with oxidant stress. Overproduction of ROS is one of the major causes of cell death in ischemic-reperfusion injury. ROS as well as reactive nitrogen species (RNS) play a pivotal role in CNS pathophysiology, especially in the context of ischemia/reperfusion.

TRPM2, which is abundantly expressed in the brain, is a Ca^{2+} -permeant, non-selective cation channel that senses and responds to oxidative stress levels in the cell. Comprehensive reviews of the involvement of TRPM2 (a.k.a. TRPC7 or LTRPC2) in oxidative stress-induced cell death have been published [101-104]. TRPM2 functions as a cell death-mediating Ca^{2+} -permeable cation channel that possesses both ion channel and ADP-ribose hydrolase functions. Oxidative and nitrosative stress lead to the accumulation of cytosolic ADP-ribose released from mitochondria, and accumulation of ADP-ribose is required for oxidative- and nitrosative-stress-induced gating of TRPM2 cation channels [105]. Inhibition of TRPM2 function by poly(ADP-ribose)polymerase-1 (PARP-1) inhibitors protects cells from oxidative stress-induced death [102]. In heterologous cells, TRPM2 expression enhances and TRPM2 suppression reduces vulnerability to H_2O_2 toxicity [106]. Although specific involvement of TRPM2 in ischemic reperfusion injury in CNS has not been shown, its interdependent expression with TRPM7, coupled with the demonstrated role of TRPM7 in neuronal death [85], make it likely that TRPM2 indeed plays a role.

TRPC channels are likely to serve as redox sensors in vascular endothelium, where it is believed that they form the molecular basis of

endothelial oxidant-activated cation channels [107]. TRPC are increasingly recognized for their role in regulating endothelial barrier function [47], which in the brain, is crucial for formation of edema in ischemic stroke [8]. In mammalian endothelial cells, numerous TRP proteins are expressed [108,109], including TRPC3 and TRPC4, which associate to form heteromeric redox-sensitive cation channels in endothelial cells [84,110]. At present, the functional role of many endothelial TRP channels is poorly understood. Additional work will be required to advance the hypothesis that TRPC3 and TRPC4 participate in formation of cerebral edema.

Analysis of promoter regions

As reviewed above, direct evidence linking TRP proteins to ischemic stroke is limited. However, we considered that additional insights might be gained by examining transcriptional mechanisms shown to be active in ischemic stroke, and attempting to link these mechanisms with TRP protein expression. Cerebral ischemia is associated with hypoxia and oxidant stress, which activate a transcriptional program in brain that may include the transcription factors, AP-1 [111-114], Sp-1 [27], HIF-1 [115,116], NF- κ B [117,118], PPAR α & γ [119-121], egr-1 [122,123], c-Myc [124,125].

We analyzed the promoter regions of members of the TRPC and TRPM subfamilies, searching for consensus binding site sequences for the transcription factors listed above. Surprisingly, the promoter regions of all TRP proteins examined, TRPC1-7 and TRPM1-8, were found to possess multiple consensus

sites for one or more of the transcription factors linked to ischemic stroke (Table 2). Although this analysis cannot be taken as evidence for involvement of any of the factors in transcriptional regulation of the TRP proteins, it indicates that none can be excluded, suggesting that further work on the role of TRP proteins in ischemic stroke is warranted.

5. Conclusion and Perspective

Several lines of evidence converge to support the hypothesis that NC channels, including TRP, are involved in ischemic stroke. A number of non-specific NC channel blockers have been found to have salutary effects in cerebral ischemia or ischemic stroke, consistent with possible involvement of their targets, including NC and TRP channels, in pathophysiological mechanisms of stroke. At present, however, this line of evidence is only suggestive, and the specific targets of these drugs remain uncertain. The likelihood that NC channels comprised of ASIC are involved is strong, given the prominence of acidosis in ischemic stroke, coupled with our understanding of the activation mechanisms for this group of channels. Similarly, the likelihood that the SUR1-regulated NC_{Ca-ATP} channel is involved is strong, given its profile of expression and its activation by ATP depletion. However, further work will be required to validate this assertion and to obtain molecular characterization of the pore-forming component of this channel. Finally, evidence is emerging that TRP proteins may play a direct role in ischemic stroke, especially in Ca²⁺-mediated neuronal death. A growing body of evidence suggests that the burgeoning field of NC channels, including TRP

channels, is poised to provide novel mechanistic insights and therapeutic strategies for ischemic stroke.

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Table 1. Properties of the SUR1-regulated NC_{Ca-ATP} channel and of the TRPM4 channel.

	SUR1-regulated NC _{Ca-ATP}	TRPM4
channel conductance	35 pS	25 pS
divalent cation conductivity	no	no
pore radius	0.41 nm	
Ca ²⁺ activation (EC ₅₀)	0.12–1.5 μM	1.3 μM
ATP block (EC ₅₀)	0.8 μM	0.13–1.7 μM
ADP, AMP block	no	yes
voltage dependent	no	yes
PIP ₂ activation	yes*	yes
PKC activation	no*	yes
glibenclamide block (EC ₅₀)	48 nM	10–100 μM

Data for SUR1-regulated NC_{Ca-ATP} channel are from published [25,26] and unpublished observations (*Chen and Simard, unpublished). Data for TRPM4 channel [64,65,97,126-132] were selected to most closely approximate those of the SUR1-regulated NC_{Ca-ATP} channel.

Table 2. Number of consensus binding sites on promoter regions of TRPC1-7 and TRPM1-8 for transcription factors active in ischemic stroke.

	♣			♣	♣	♣	♣	♣						♣	
	C1	C2	C3	C4	C5	C6	C7	M1	M2	M3	M4	M5	M6	M7	M8
AP-1	3	0	2	3	1	3	4	2	2	5	2	2	4	5	3
c-Myc	3	2	0	0	1	2	0	1	3	3	0	0	5	3	0
egr-1	3	2	1	4	3	4	1	2	3	1	7	3	1	6	2
HIF-1	1	1	1	0	4	2	1	3	1	3	0	1	0	6	0
NF-κB	2	3	5	4	4	4	7	6	6	2	6	5	1	1	5
PPAR a&g	3	6	2	3	5	5	4	7	7	4	5	6	5	2	2
Sp-1	4	2	1	3	7	1	0	1	4	1	8	1	1	5	2

♣ – genes with multiple transcription start sequences

The promoter regions of 15 genes for the indicated TRP proteins were analyzed for potential consensus sequence binding sites for the transcription factors, AP-1, c-Myc, egr-1, HIF-1, NF-κB, PPAR a&g, and Sp-1 using Gene2Promoter and MatInspector applets (www.Genomatrix.de). The promoter region analyzed was assumed to be 2500 bp upstream and 500 bp downstream of the transcription start sequence.

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Figure 1. Schematic diagram illustrating various types of edema progressing to hemorrhagic conversion. **Normally**, Na^+ concentrations in serum and in extracellular space are the same, and much higher than inside the neuron. **Cytotoxic edema** of neurons is due to entry of Na^+ into ischemic neurons via pathways such as $\text{NC}_{\text{Ca-ATP}}$ channels, depleting extracellular Na^+ and thereby setting up a concentration gradient between intravascular and extracellular compartments. **Ionic edema** results from cytotoxic edema of endothelial cells, due to expression of cation channels on both the luminal and abluminal side, allowing Na^+ from the intravascular compartment to traverse the capillary wall and replenish Na^+ in the extracellular space. **Vasogenic edema** results from degradation of tight junctions between endothelial cells, transforming capillaries into “fenestrated” capillaries that allow extravasation (outward filtration) of proteinaceous fluid. **Oncotic death of neuron** is the ultimate consequence of cytotoxic edema. **Oncotic death of endothelial cells** results in complete loss of capillary integrity and in extravasation of blood. i.e., **hemorrhagic conversion**. (from Simard et al. [8], with permission).

