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BAKALÁŘSKÁ PRÁCE

**Properties and regulation of volume-sensitive anion
channels in astrocytes**



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Abstract

Animal cells need to preserve constant volume in the face of osmolarity perturbations to function properly. To regain their original volume after hyposmotically induced swelling, most cell types extrude intracellular electrolytes and organic osmolytes accompanied by osmotically driven water. This process is termed regulatory volume decrease and is ensured by various ion channels and transporters. Recently, much attention has been focused on the ubiquitous volume-regulated anion channels activated by cell swelling. VRACs are moderately outwardly rectifying with intermediary conductance, permeable to inorganic anions and organic osmolytes and sensitive to broad-spectrum anion channels blockers. Functional properties of VRACs in astrocytes are particularly interesting, because many brain pathologies, such as ischemia, traumatic brain injury or hyponatremia, are associated with marked astrocytic swelling and VRACs could thus constitute a possible target for therapy of cerebral edema. Furthermore, VRACs are thought to play a role in cell cycle progression, cell migration, apoptosis and intercellular communication. Despite intensive research, VRACs molecular identity and mechanism of their activation and regulation are still unclear. This work summarizes known facts about VRACs, accentuating their possible functions in astrocytes.

Key words: cell volume regulation, volume-regulated anion channels, astrocytes, brain edema

Abstrakt

Živočišné buňky musí být schopné udržet si stálý objem navzdory měnící se osmolaritě vnějšího či vnitřního prostředí, aby mohly správně fungovat. Buňky v hypotonickém prostředí nasávají vodu a zvětšují svůj objem. Ke snížení objemu na původní hodnotu pak u většiny buněčných typů slouží vypouštění intracelulárních anorganických a organických osmoticky aktivních částic, což vede k výtoku vody z buňky. Tento proces nazývaný regulační snižování objemu je zajišťován různými iontovými kanály a přenašeči. Relativně nedávno se do centra pozornosti dostaly objemově regulované aniontové kanály (VRAC). VRAC se vyznačují mírnou vnější usměrností, střední vodivostí, propustností pro anorganické anionty a organické osmolyty a citlivostí k širokospektrým blokátorům aniontových kanálů. Funkce a vlastnosti VRAC v astrocytech jsou obzvláště zajímavé, protože mnoho patologií, jako například ischemii, traumatické poranění mozku či hyponatremii, doprovází výrazné zvětšování objemu astrocytů. VRAC jsou proto hypotetickým cílem léčby edému mozku. Kromě toho se VRAC účastní průběhu buněčného cyklu, buněčné migrace, apoptózy a mezibuněčné komunikace. Molekulární identita a mechanismus aktivace VRAC jsou navzdory intenzivnímu výzkumu stále nejasné. Tato práce shrnuje dosud objevené poznatky o VRAC, s důrazem na jejich možné funkce v astrocytech.

Klíčová slova: regulace buněčného objemu, objemově regulované aniontové kanály, astrocyty, edém mozku

List of abbreviations

AQP4	aquaporin 4
ATP	adenosine 5'-triphosphate
AVD	apoptotic volume decrease
CaM	calmodulin
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
ClC-3	voltage-gated Cl ⁻ channel
CNS	central nervous system
CSWS	cerebral salt-wasting syndrome
DCPIB	4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)oxybutyric acid
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid
DNA	deoxyribonucleic acid
EAA	excitatory amino acid
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
FAK	focal adhesion kinase
GluR	glutamate receptor
GODE	gradual osmolarity decrease
GPCR	G-protein coupled receptor
GTPγS	guanosine 5'-[γ-thio]triphosphate
IP3	inositol 1,4,5-triphosphate
IVR	isovolumetric volume regulation
JNK	c-Jun N-terminal kinase
Kir	inwardly rectifying K ⁺ channel
MAPK	mitogen activated protein kinase
MDR1	multidrug resistance-1 gene
MEK	MAP kinase kinase
MLCK	myosin-light-chain kinase
MRP	multidrug resistance protein
NPPB	5-nitro-1-(3-phenylpropylamino) benzoic acid
PAR	proteinase activated receptor
P-gp	P-glycoprotein
pl _{Cl⁻}	putative Cl ⁻ channel
PKC	protein kinase C
PLC	phospholipase C
RhoK	Rho-kinase
rMCAO	rat middle cerebral artery occlusion
ROS	reactive oxygen species
RTK	receptor tyrosine kinase
RVD	regulatory volume decrease
RVI	regulatory volume increase
SODE	sudden osmolarity decrease
SON	supraoptic nucleus
Tau	taurine
TK	tyrosine kinase
TRPV4	transient receptor potential vanilloid channel 4
VRAC	volume-regulated anion channel
VSOAC	volume-sensitive organic osmolyte/anion channel
VSOR	volume-sensitive outwardly rectifying Cl ⁻ channel

Table of contents

1. Introduction	7
2. Volume-regulated anion channels	7
2.1. Basic properties	7
2.2. Activation	8
2.3. Molecular candidates	10
2.3.1. P-glycoprotein	10
2.3.2. Putative Cl ⁻ channel	10
2.3.3. ClC-3	11
2.4. Modulation	11
2.4.1. Extracellular ATP	11
2.4.2. Calcium	11
2.4.3. Protein kinases	12
2.5. Interactions with other channels	15
2.5.1. Aquaporin-4	16
2.5.2. Transient receptor potential vanilloid channel-4	16
2.5.3. Potassium channels	17
3. Roles for volume-regulated anion channels	17
3.1. Regulatory volume decrease	17
3.2. Apoptosis	19
3.3. Cell proliferation and migration	20
3.4. Astrocyte-neuron signaling	21
3.5. Brain swelling	22
4. Conclusions	24
5. References	25

1. Introduction

Most cell types are provided with mechanisms allowing them to control and adjust their volume. This ability is underlined by expression of specific ion channels which allow extrusion of intracellular osmolytes. Probably the best described ion channels involved in cell volume regulation are volume-regulated anion channels (VRACs). VRACs, activated by cell swelling, were described in most animal cell types, including astrocytes. In CNS, the pathological stages, such as ischemia or traumatic injury, are accompanied by marked cell swelling, especially of astrocytes.

Astrocytes are the most abundant cell type in central nervous system (CNS) of phylogenetically higher species, such as primates, and substantially outnumber neurons. They are interconnected by their processes via gap junctions into functional syncytia forming separate anatomical domains. Astrocytic processes envelop synapses and contact blood vessels. Astroglia had been long regarded as mere structural element of the brain tissue; however, it becomes evident that they play an important role in CNS signal transduction and modulation. Their main responsibility is the maintenance of the homeostasis of the extracellular space to ensure proper neuronal transmission and excitability. This involves clearance of excessive extracellular K^+ , uptake of neurotransmitters from synaptic space and pH buffering. Moreover, astrocytes support neurons metabolically, assist neuronal migration and synaptogenesis, regulate blood flow in brain, participate in the blood-brain barrier formation and maintenance and they might modulate neurotransmission. Given the various functions astrocytes fulfill, it is not surprising that they play an important part in most neurological diseases (reviewed in Benarroch 2005; Kimelberg 2005).

The aim of this work is to describe the properties and regulation of VRACs with emphasis on their manifestation in astroglia and their possible functions under physiological and pathological conditions.

2. Volume-regulated anion channels

2.1. Basic properties

The channel is known under several different names, e.g. volume regulated anion channel (VRAC) (Kimelberg 2004), volume-sensitive organic osmolyte/anion channel (VSOAC) (Jackson and Strange 1995) and volume-sensitive outwardly rectifying Cl^-

channel (VSOR) (Okada et al. 1998) and the current through putative VRACs is sometimes called $I_{Cl,swell}$ or $I_{Cl,vol}$ for swelling/volume-activated chloride current (Belsey et al. 2007).

VRACs are found in most animal cells and their properties may somewhat vary in different cell types; however, some features are characteristic for them: they are moderately outwardly rectifying, the single-channel conductance is 50-90 pS at depolarized voltages and ~ 10 pS at hyperpolarized voltages, they exhibit Eisenman type 1 permeability sequence ($NO_3^- > I^- > Br^- > Cl^- > F^- > gluconate$) and open-channel block by extracellular adenosine 5'-triphosphate (ATP). The activation is voltage-independent, and the current shows time-dependent inactivation at positive potentials. VRACs are permeable to various monovalent inorganic anions (mainly Cl^-) and organic osmolytes, such as amino acids (e.g. glutamate, aspartate, taurine) and polyols (myoinositol, sorbitol) (as reviewed by Nilius and Droogmans 2003).

The pore diameter was estimated to be approximately 0,6 – 0,7 nm (Ternovsky et al. 2004). VRACs are sensitive to broad spectrum Cl^- channels inhibitors, such as DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid) and NPPB (5-nitro-1-(3-phenylpropylamino) benzoic acid) (Abdullaev et al. 2006), anti-oestrogens, such as tamoxifen and nafoxidine (reviewed by d'Anglemont de Tassigny et al. 2003), and selective serotonin reuptake inhibitors, such as fluoxetine, sertraline and paroxetine (Maertens et al. 2002). The most selective VRAC blocker so far reported is a derivative of ethacrynic acid DCPIB (4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)oxybutyric acid) (Decher et al. 2001).

2.2. Activation

VRACs have been mainly studied in cultures, e.g. in endothelial cells (Nilius et al. 1998), HeLa cells (Shimizu et al. 2004), C6 glioma cells (Belsey et al. 2007), neuroblastoma N1E115 cells (Bond et al. 1999) and primary astrocyte cultures (Kimmelberg et al. 1990). Under experimental conditions, VRACs are activated by cell swelling induced by exposing the cells to extracellular hyposmotic solution, dialyzing the cells with intracellular solution of increased osmolarity or increased extracellular concentration of potassium. VRAC activation requires intracellular ATP (Rutledge et al. 1999) and might involve phosphorylation of the channel or accessory proteins (Crepel et al. 1998) or non-hydrolyzable ATP binding (reviewed by Okada 1997).

It is not clear how cells sense alterations in volume and transduce them into VRAC opening. The decrease in the intracellular ionic strength was suggested as the primary trigger for VRAC opening (Voets et al. 1999). Influx of water during cell swelling causes dilution of intracellular constituents and thus reduces intracellular ionic strength. However, other authors proposed that intracellular ionic strength has rather modulatory effect on yet unidentified volume sensor, i.e. it shifts the volume threshold of the channel (Cannon et al. 1998). Reduced intracellular ionic strength could affect the activity of signaling protein molecules by changing their surface potential (Okada et al. 2009).

VRACs are activated only above a threshold level of cell swelling, at a relative cell volume approximately 1.4, (Okada 1997); however, some authors reported activation of VRACs in the absence of cell swelling by reducing the intracellular ionic strength (Nilius et al. 1998) or intracellular application of GTP γ S (Voets et al. 1998). This could be due to the fact, that even under presumably isotonic conditions the osmolarity of extracellular and intracellular solution can slightly differ and this can cause imperceptible swelling. Reduced intracellular ionic strength and GTP γ S can shift the volume set point of VRACs and mediate its activation under seemingly isovolumetric conditions (Okada et al. 2009).

In addition, alteration in cytoskeleton organization induced by cell swelling has been suggested as a possible activator of VRACs. Actin cytoskeleton is a dynamic structure essential for cell motility and shape changes and it affects several membrane transporters. Cultured astrocytes with normal flat polygonal shape do not express Cl⁻ conductance under isosmotic condition, but Cl⁻ currents can be elicited by their swelling or changes in their morphology (Lascola and Kraig 1996; Parkerson and Sontheimer 2004). Cell swelling alters the structure of F-actin (stress fibres) and this could therefore serve as a trigger for VRAC activation, interacting with the channel directly, i.e. the channel protein would be connected to the actin cytoskeleton, or indirectly via signaling molecules associated with actin. However, disruption of F-actin with cytochalasin B under isosmotic conditions did not lead to activation of VRACs in B lymphocytes (reviewed by Eggermont et al. 2001). In vascular endothelial cells, full VRAC activation requires Rho/Rho kinase/myosin light-chain phosphorylation pathway, which regulates the formation of F-actin stress fibers (Nilius et al. 1999; Nilius et al. 2000); however, constitutively active Rho pathway did not lead to spontaneous VRAC activation under isotonic conditions and had no effect

on the activation properties during cell swelling (reviewed by Eggermont et al. 2001). Overall, it seems that F-actin rearrangement is not crucial for VRAC activation but may have a modulatory effect on the volume sensitivity of the channel.

2.3. Molecular candidates

Despite an intensive research, the molecular counterpart of VRAC remains unknown. Lack of highly specific channel ligand precludes purification of the channel protein, and endogenous expression and house-keeping activity of the channel in virtually all cell types hinders the expression cloning of VRAC. Several proteins were proposed as molecular candidates for VRAC, namely P-glycoprotein (Valverde et al. 1992), putative Cl⁻ channel (pI_{Cl⁻}) (Paulmichl et al. 1992) and voltage-gated Cl⁻ channel (ClC-3) (Duan et al. 1997). The molecular candidate for VRAC should meet following criteria: transfection with the gene for candidate protein should induce anionic current identical with VRAC current, candidate mRNA and protein should be present in cells functionally expressing VRAC current, abrogation of expression of the candidate protein should abolish VRAC current and the mutation of candidate gene should change significantly the properties of the channel (Okada et al. 1998).

2.3.1. P-glycoprotein

P-glycoprotein (P-gp), a product of multi drug resistance-1 (MDR1) gene, is an ATP-dependent transporter responsible for cell resistance to antineoplastic drugs. Valverde (1992) suggested that P-gp can function both as a transporter and as a swelling-activated Cl⁻ channel. This hypothesis was discarded because the abolition of P-gp expression by antisense oligonucleotides, antibodies against P-gp or verapamil, P-gp inhibitor, had no effect on VRAC currents (Tominaga et al. 1995).

2.3.2. Putative Cl⁻ channel

The putative chloride channel (pI_{Cl⁻}) and VRAC currents share some characteristics, such as outward rectification, slow inactivation at positive potentials, and block by extracellular nucleotides. However, pI_{Cl⁻} is localized primarily in cytoplasm (Emma et al. 1998) and biophysical properties of the currents through pI_{Cl⁻} differ from VRAC currents in rectification pattern, anion-selectivity, pharmacology and kinetics (Voets et al. 1996).

2.3.3. CIC-3

CIC-3, a protein belonging to the CIC family of voltage-gated chloride channels, has properties similar to VRACs. It is activated by cell swelling, sensitive to VRAC blockers tamoxifen and DIDS and has a permeability sequence $P_{I^-} > P_{Cl^-}$, which is untypical for CIC-family channel. However, it is localized mainly in intracellular organelles and disruption of CIC-3 gene has no effect on swelling-activated VRAC currents (Stobrawa et al. 2001). On the other hand, CIC-3 has been reported to be a component of VRAC by other groups (Hermoso et al. 2002) and its role remains to be clarified.

2.4. Modulation

2.4.1. Extracellular ATP

ATP is released from astrocytes during swelling induced by exposure to hyposmotic media, possibly through the multidrug resistance protein (MRP) (Darby et al. 2003) or maxi-anion channels (Liu et al. 2008). In hepatoma cells, ATP released during cell swelling was reported to be necessary for VRAC activation, stimulating purinergic receptors in autocrine manner and thus coupling increases in cell volume to the VRAC opening (Wang et al. 1996). However, in astrocytes, it has been shown that ATP release is not necessary or sufficient to activate VRACs. Instead, it positively modulates already active channels via a Ca^{2+} -dependent mechanism, which is discussed later (Mongin and Kimelberg 2002; Mongin and Kimelberg 2003; Kimelberg 2004). At high (10 mM) concentration, extracellular ATP blocks VRAC by interacting directly with the channel pore (Jackson and Strange 1995).

2.4.2. Calcium

In the majority of studied cell types, hyposmotic swelling causes immediate changes in the concentration of intracellular calcium ($[Ca^{2+}]_i$). In astrocytes, exposure to hyposmotic medium leads to a substantial $[Ca^{2+}]_i$ increase followed by a decrease to a sustained plateau phase, which is still above the basal level. Elevated Ca^{2+} originates from both extracellular space and intracellular stores (Schliess et al. 1996). This common cell response to swelling makes calcium a likely candidate for a signaling element transducing changes in cell volume into the activation of volume regulatory mechanisms (Morales-Mulia et al. 1998). The calcium dependency of

volume regulation varies with cell types – in general, it seems that in epithelial cells the mechanisms leading to cell volume recovery are Ca^{2+} -dependent whereas in non-epithelial cells they are Ca^{2+} -independent (reviewed by Pasantes-Morales and Morales Mulia 2000). In astrocytes, VRACs mediating the efflux of chloride ions and organic osmolytes require permissive submicromolar (40 – 50 nM) $[\text{Ca}^{2+}]_i$ for normal function (Mongin et al. 1999).

Calcium and calmodulin further participate in modulation of VRAC activity by protein kinases, as discussed later.

2.4.3. Protein kinases

VRAC activation depends upon intracellular ATP, but no phosphorylation reaction seems to be required, as the channel can be activated in the presence of non-hydrolyzable ATP analogues (reviewed by Okada 1997). However, various protein kinases exert modulatory effect on VRAC activity.

Tyrosine kinases

The protein tyrosine kinases (TKs) are members of numerous signaling pathways regulating various cellular functions, such as motility, proliferation, differentiation and survival. They can be divided into receptor protein tyrosine kinases (e.g. growth factor receptors) and non-receptor protein tyrosine kinases. The role of TKs in cell volume regulation is supported by multiple studies. In many cell types, hyposmolarity induces activation of TKs, such as focal adhesion kinase (FAK), kinases of the Src family and the growth factor receptors (summarized in Pasantes-Morales et al. 2006). TK blockers, such as genistein or herbimycin A, largely reduce currents through VRAC in human Intestine 407 cell line, whereas increased tyrosine phosphorylation provoked by inhibiting protein tyrosine phosphatase potentiates them (Tilly et al. 1993). Similar effect of TK blockers on VRACs activity was observed in many other cell types including cultured astrocytes (Crepel et al. 1998) and also *in vivo* in ischemic/reperfused rat brain, where genistein substantially suppressed efflux of aspartate and glutamate mediated by VRACs (Phillis et al. 1996).

Specific TKs and signaling cascades regulating VRACs remain unidentified; however, kinases of the Src family are probably involved. *Src*-like tyrosine kinase p56^{lck} mediates hyposmotically induced VRAC activation in lymphocytes (Lepple-Wienhues et al. 1998). Moreover, in cultured astrocytes, peroxynitrite (ONOO^-)

potentiates VRAC activity via a Src-kinase pathway (Haskew et al. 2002; Kimelberg 2004).

As for receptor tyrosin kinases (RTKs), stimulation of epidermal growth factor (EGF) receptor, which belongs to RTKs, upregulates VRAC activity in murine mammary cells (Abdullaev et al. 2003), and in human Intestine 407 cell line (Tilly et al. 1993). EGF is a member of the signaling pathway leading to the activation of mitogen activated protein kinases (MAPK), which belong to the serine/threonine kinase family (Pelech and Sanghera 1992).

Serine/threonine protein kinases

Several serine/threonine protein kinases seem to participate in modulation of VRAC activity, namely extracellular signal-regulated kinases (ERK-1 and ERK-2), c-Jun N-terminal kinase (JNK), protein kinase C (PKC) and Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII).

In cultured astrocytes, the MAP kinase kinase (MEK) inhibition, which blocks activation of ERK-1 and ERK-2, reversibly inhibits hyposmotically activated VRAC current (Crepel et al. 1998). In C6 glioma cells, which have many characteristics of astrocytes from primary cultures, inhibition of both ERK and JNK decreases cell swelling-induced taurine release (Belsey et al. 2007). The exact signaling pathway leading to hyposmotically induced MAP kinase activation remains unclear; in astrocytes, hyposmolarity-induced activation of ERK-1/2 is Ca^{2+} -dependent and possibly involves Ras/Raf pathway (Schliess et al. 1996) and TKs (Crepel et al. 1998), and in human Intestine 407 cell line hyposmolarity-induced activation of ERK-1/2 is mediated by ATP, released during osmotically induced cell swelling, and acting through purinergic P2Y receptors (Van der Wijk et al. 1999).

As mentioned above, micromolar extracellular ATP greatly enhances VRAC activity in Ca^{2+} -dependent manner. The suggested mechanism is that activation of purinergic P2Y receptors, belonging to the family of G-protein-coupled receptors (GPCR), coupled to phospholipase C (PLC) leads to mobilization of intracellular Ca^{2+} from inositol 1,4,5-triphosphate (IP3)-sensitive stores. Increased $[Ca^{2+}]_i$ then activates PKC, which is crucial for ATP potentiation of VRAC activity (Mongin and Kimelberg 2005). Two isoforms of PKC are involved – PKC α and PKC β 1 (Rudkouskaya et al. 2008). ATP also acts via additional Ca^{2+} -dependent pathway

involving CaMKII and inhibition of both PKC and CaMKII completely abolishes ATP effect (Rudkouskaya et al. 2008; Mongin and Kimelberg 2005).

PKC is also involved in upregulation of VRAC activity by thrombin, which acts through proteinase activated receptors (PAR), a subfamily of GPCRs (Cheema et al. 2005). Similar effect of GPCR agonists on VRAC activity was described in various neural and non-neural cells (reviewed by Fisher et al. 2008). Overall, there is evidence that activation of GPCR, especially from the Gq family, promotes swelling-induced efflux of osmolytes through VRACs. Proteins from the Gq family mediate PLC activation, mobilization of Ca^{2+} from intracellular stores and activation of PKC. GPCRs activation and subsequent signaling pathways could lead to phosphorylation of auxiliary protein(s) or hypothetic volume sensor, facilitating VRAC opening or decreasing the volume set point of the channel. Although GPCRs effects on VRACs are all more or less Ca^{2+} -dependent, the extent of dependency may vary depending on the cell type, agonist used, osmolyte traced or the degree of osmolarity reduction (reviewed in Fisher et al. 2008; Vazquez-Juarez et al. 2008).

Other compounds modulating VRACs in Ca^{2+} -dependent manner are ionomycin, a Ca^{2+} mobilizing agent, and hydrogen peroxide (H_2O_2). Both strongly upregulate VRAC activity via CaMKII-dependent mechanism in hyposmotically swollen astrocytes. In the case of ionomycine, phosphatidylinositol-3-kinase (PI3K) is also involved (Cardin et al. 2003; Haskew-Layton et al. 2005).

Taken together, various protein kinases positively modulate VRACs through diverse signaling pathways, which may or may not intersect. Figure 1 represents a hypothetic scheme of signaling pathways involved in VRAC modulation.

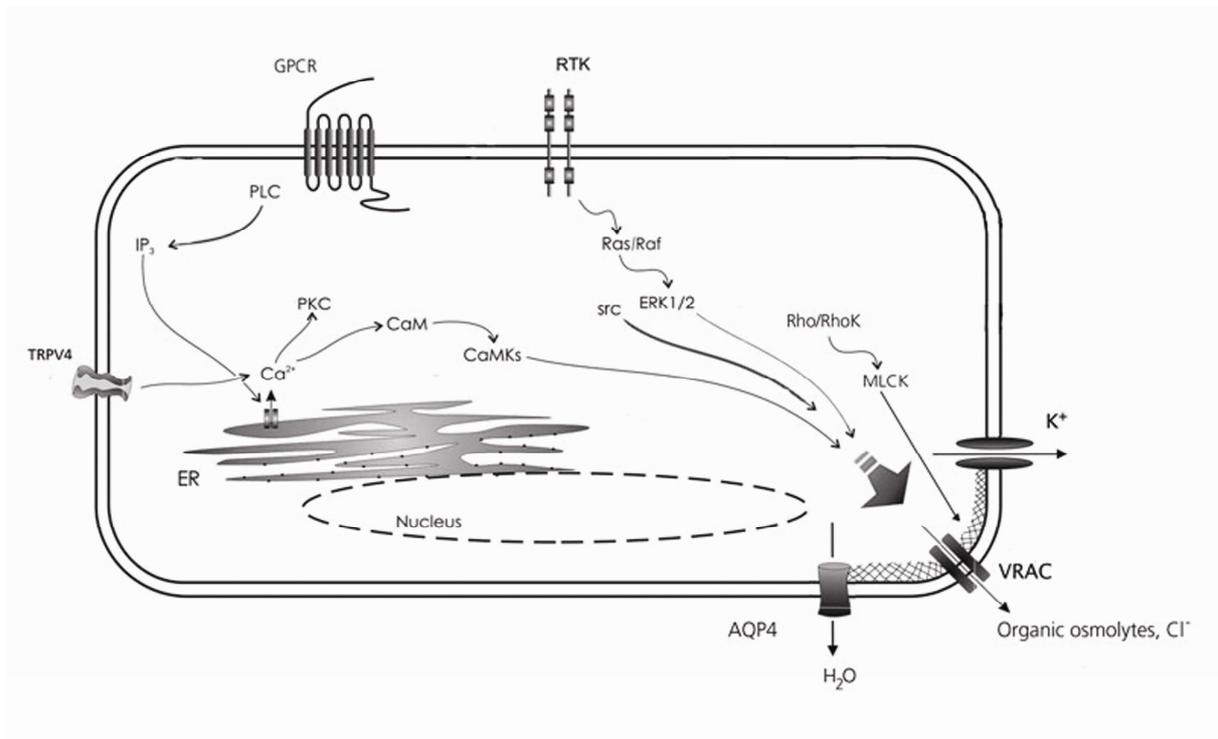


Figure 1 Molecular mechanisms activated by hypotonicity and possible interplay of signaling elements leading to cell volume adjustment and adaptive response to hyposmotic stress (adapted from Vazquez-Juarez et al. 2008). See text for details.

ER – endoplasmic reticulum, TRPV4 – transient receptor potential vanilloid channel 4, GPCR – G-protein coupled receptor, RTK – receptor tyrosine kinase, PLC – phospholipase C, IP₃ – inositol 1, 4, 5-triphosphate, PKC – protein kinase C, CaM – calmodulin, CaMK – Ca²⁺/calmodulin-dependent protein kinase, ERK – extracellular signal-regulated kinase, src – tyrosine kinase of the Src family, Rho – Rho GTPase, RhoK – Rho-kinase, MLCK – myosin-light-chain kinase, AQP4 – aquaporin 4, VRAC – volume-regulated anion channel, criss-crossed – actin

2.5. Interactions with other channels

Most cells are able to counterbalance changes in their volume caused by disturbances in the osmolarity of their surroundings. Cell shrinkage or swelling are counteracted by complex mechanisms termed regulatory volume increase (RVI) and regulatory volume decrease (RVD), respectively. Recovery of cell volume after swelling, discussed in detail in the chapter Regulatory volume decrease, involves efflux of ions, mainly K⁺ and Cl⁻ and organic osmolytes, such as amino acids and polyols, followed by osmotically driven water. This implies functional interactions of involved channels.

2.5.1. Aquaporin-4

Aquaporins (AQP) are a superfamily of related transmembrane proteins widely expressed in animal and plant cells. They mediate bidirectional water flux and some subtypes are also permeable to glycerol and possibly other small molecules (reviewed by Verkman 2005). There are at least 13 members of AQP family identified in mammals, the most common in brain being AQP4. In astrocytes, this channel is localized predominantly in end-feet adjoining blood vessels, and it is the main channel responsible for water movement in astroglia. AQP4 was suggested to functionally support astrocytic inwardly rectifying K⁺ channels (Kir) in the process of maintaining extracellular K⁺ homeostasis by facilitating water flux through the plasma membrane (reviewed by Nagelhus et al. 2004).

Knockdown of aquaporin-4 with small interfering RNA (siRNA) results in substantial down-regulation of VRAC activity in cultured astrocytes but this effect is abolished when intracellular ATP is augmented. Therefore it seems that some mechanisms necessary for VRAC functioning dependent on intracellular ATP are altered by AQP4 knockdown (Benfenati et al. 2007).

2.5.2. Transient receptor potential vanilloid channel-4

The transient receptor potential (TRP) ion channels participate in signal transduction in response to mechanical, thermal, chemical and osmotic stimuli. A subfamily of TRP, transient receptor potential vanilloid channels (TRPV) are involved in osmotransduction (reviewed by Liedtke and Kim 2005). TRPV4 is a weakly Ca²⁺-selective cation channel, which has been shown to be expressed in astrocytes *in vitro* and *in situ*. It is mainly localized in astrocytic end-feet contacting pial surface and enwrapping blood vessels. TRPV4 is activated by hyposmolarity (Benfenati et al. 2007). Mice lacking TRPV4 show abnormal osmotic sensing and regulation (Mizuno et al. 2003).

TRPV4 mediates Ca²⁺ influx upon hyposmotic stimulation, and is a plausible candidate for the channel(s) responsible for [Ca²⁺]_i rise seen in most cells challenged with hyposmolarity. Under hyposmotic conditions, TRPV4 is regulated by src-kinases and phosphorylation of the channel is enhanced by H₂O₂ (Wegierski et al. 2009). Src-kinases and H₂O₂ are also involved in VRAC modulation as discussed above. TRPV4 may thus act as an osmosensor in astrocytes, working in concert with channels involved in volume regulation, i.e. aquaporins, VRACs and potassium channels.

2.5.3. Potassium channels

Channels mediating K^+ efflux from astrocytes during RVD remain unidentified and no direct or indirect interaction between them and VRACs has been reported so far. However, RVD is mediated by simultaneous efflux of Cl^- and K^+ , which opens the possibility that VRACs and K^+ channels functionally interact.

Potassium efflux from cultured astrocytes in response to cell swelling has a Ca^{2+} -dependent component activated by small osmolarity decrease and Ca^{2+} -independent component, activated by larger osmotic gradients. The Ca^{2+} -independent pathway is responsible for 55% – 70% of swelling activated K^+ fluxes. The Ca^{2+} -dependent pathway mediates remaining 30 – 45% and its activation involves calcium/calmodulin-dependent step (Quesada et al. 1999). In C6 glioma cells, osmosensitive Ca^{2+} -dependent K^+ outflow is activated early after cell swelling and has lower volume set point, whereas activation of Ca^{2+} -independent K^+ efflux is delayed and volume threshold for activation is higher (Ordaz et al. 2004). In hippocampal astrocytes *in situ*, Kir 4.1 is involved in normosmotic volume regulation of astrocytic somata and mediate regulatory volume decrease in astrocytic processes (Hirrlinger et al. 2008).

3. Roles for volume-regulated anion channels

3.1. Regulatory volume decrease

Plasma membrane of most animal cells is highly permeable to water and any changes in intracellular or extracellular osmolarity are accompanied by water movement in order to restore the osmotic equilibrium. Water efflux or influx induce corresponding changes in cell volume, i.e. cell shrinkage or swelling, respectively. The extracellular environment of most cells is more or less constant; however, cell volume homeostasis can be compromised by ordinary cellular activities such as uptake of nutrients, transport across membrane or metabolic processes (Okada et al. 2001). Changes in cell volume can affect signaling networks crucial for cell functioning and communication, and maintenance of constant volume is therefore imperative for most cells (reviewed by Pasantes-Morales et al. 2002).

Mechanisms of regulatory volume decrease have been studied mainly in cell cultures exposed to sudden and substantial decrease in extracellular osmolarity. To recover

their original volume, cells extrude solutes, mainly K^+ and Cl^- and organic osmolytes, such as amino acids, polyols and other compounds, e.g. creatine. Efflux of solutes is accompanied by osmotically driven water. RVD in cultured astrocytes is rapid and cells recover 70% – 80% of their volume within few minutes after the initiation of swelling (reviewed by Pasantes-Morales et al. 2002).

Efflux of inorganic ions provides approximately 60% – 70% of RVD and organic osmolytes contribute to the remaining 30% – 40% (reviewed by Massieu et al. 2004). VRACs have been shown to mediate fluxes of both Cl^- and organic osmolytes, mainly excitatory amino acids glutamate and aspartate and sulfonic amino acid taurine, and they play crucial role in RVD (reviewed by Pasantes-Morales et al. 2000). Mechanism of regulatory volume decrease is schematically depicted in Figure 2.

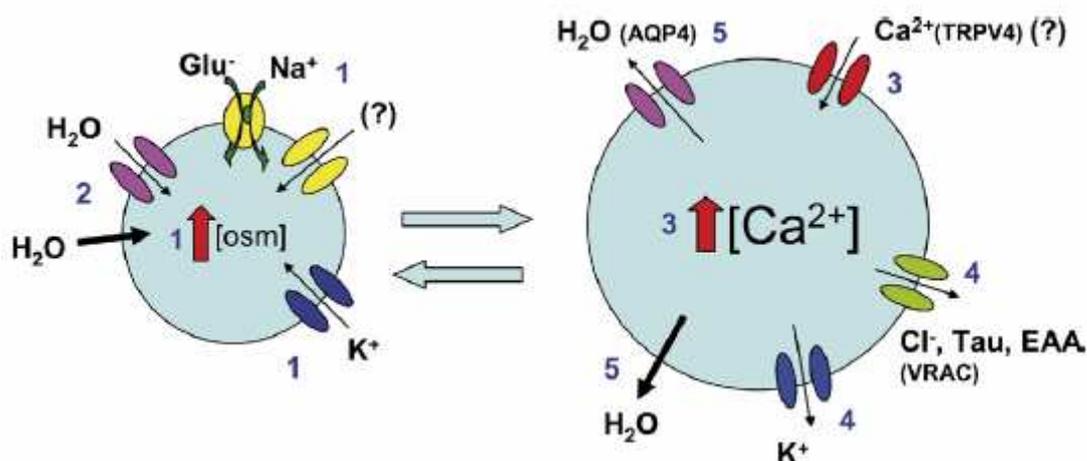


Figure 2 Schematic illustration of regulatory volume decrease (Benfenati and Ferroni 2009).

1) Rise in intracellular osmolyte concentration via specific ion channels and transporters, 2) Water influx through AQP4 and osmotic swelling, 3) Volume sensing and osmotransduction, possibly mediated by rise in $[Ca^{2+}]_i$, 4) Inorganic and organic osmolyte efflux through appropriate channels, 5) Osmotic water efflux and volume recovery

AQP4 – aquaporin 4, TRPV4 – transient receptor potential vanilloid channel 4, VRAC – volume-regulated anion channel, Tau – taurine, EAA – excitatory amino acids

Large and sudden osmolarity decrease (SODE) employed under experimental conditions probably never occur in brain *in vivo*. Instead, even in pathological states, extracellular osmolarity is likely to decrease gradually. In proximal renal tubules, cells exposed to small and gradual osmolarity decrease (GODE) were able to maintain

their original volume even when osmolarity was decreased by 50%, probably by adjusting their volume constantly. This response was termed isovolumetric volume regulation (IVR) (Lohr and Grantham 1986). Cultured astrocytes and C6 glioma cells exposed to gradual reduction in external osmolarity (-18 mOsm/min) showed constant increase in their volume, but they swelled substantially less than cells exposed to SODE of the same magnitude which suggests that they actively counterbalanced hyposmolarity induced swelling (Ordaz et al. 2004; Ordaz et al. 2004). However, the mechanism of IVR is not well understood and possible VRACs involvement was not explored.

3.2. Apoptosis

Apoptosis, a type of programmed cell death, is essential for embryogenesis, immune system maturation, organ development and somatic cell turnover and plays a role in various pathological processes. It is characterized by biochemical and morphological changes including cell shrinkage, cytochrome c release, caspase activation, nuclear condensation, DNA laddering and cell fragmentation into apoptotic bodies. In contrast to necrosis, cell content is not released during apoptosis.

Normotonic whole-cell volume reduction, a characteristic feature of the early phase of apoptosis, is termed apoptotic volume decrease (AVD). It is achieved by release of osmolytes, mainly K^+ and Cl^- ions via separated ion channels, followed by water efflux. AVD is necessary for the progress of apoptosis. Cell shrinking is normally counter-balanced by RVI mediated by uptake of solutes (mainly Na^+ and Cl^-). This process is impaired in apoptotic cells, due at least in part to dysfunction of Na^+/H^+ -cotransporter (Maeno et al. 2006).

The role of VRACs in AVD is supported by the fact, that cells undergoing AVD show facilitated RVD, and that AVD can be abolished by VRAC inhibition by phloretin, DIDS and NPPB. Furthermore, these compounds also inhibit caspase activation, DNA laddering, and prevent apoptotic cell death. It was found, that AVD actually precedes these apoptotic events. It is noteworthy, that blocking K^+ efflux has the same effect as blocking anion efflux through VRACs, which suggests, that AVD is mediated by K^+ channels and VRACs working in concert (Maeno et al. 2000).

As mentioned before, VRAC activation is usually elicited by hyposmotic shock and it occurs when cell volume exceeds a certain threshold value. However, during

apoptosis VRACs are activated in non-swollen or even shrunken cells. The channel volume set point must therefore be shifted to a lower level. In HeLa cells, reactive oxygen species (ROS) are generated upon stimulation with staurosporine, mitochondrion-mediated apoptosis inducer, and H₂O₂ activates VRAC-like currents in non-swollen cells (Shimizu et al. 2004), thus ROS could be responsible for lowering VRAC volume set point. In brain, ROS are copiously generated during ischemia and reperfusion, contributing to apoptotic neuronal death (Mongin 2007).

Intracellular ATP concentration is significantly increased in apoptotic cells (Zamaraeva et al. 2005). Given that VRACs are dependent on cytosolic ATP, its role in modulating the channel volume set point during apoptosis is plausible (Okada et al. 2006).

3.3. Cell proliferation and migration

Cell cycle is highly regulated chain of macromolecular events leading to cell division. Proliferating cells normally have higher rates of metabolism, increased uptake of metabolic substrates and undergo morphological changes. All these processes are accompanied by volume alterations, which open a possibility of VRAC involvement.

Indeed, VRAC activity is cell cycle dependent and correlates with cell proliferation. In C6 glioma cells, VRAC blockers induce cell cycle arrest in G₀/G₁ transition phase (Belsey et al. 2007). In human cervical cancer cells (SiHa cells) VRAC activity is significantly downregulated in G₀/G₁ phase and vice versa, VRAC blockers induce cell cycle arrest in this phase. When cells re-enter the cell cycle and continue to S phase, VRAC activity is enhanced. Taken together, it is plausible, that VRACs play a role in G₁/S checkpoint progression (Shen et al. 2000).

Migrating cells must be able to change their shape and volume to fit in narrow extracellular spaces. Highly invasive glioma cells actively accumulate chloride ions and their extrusion allows cells to rapidly decrease their volume (Habela et al. 2009). Interestingly, glioma and neuroblastoma cells were reported to display constitutively active VRAC currents under isotonic conditions, which opens a possibility that increased basal Cl⁻ conductance is an adaptive mechanism of malignant cells contributing to their uncontrolled proliferation and invasive migration. Evidence supporting VRAC involvement in cell migration is that VRAC inhibitors, such as

tamoxifen and NPPB, inhibit both cell migration and hyposmotically activated VRAC current with similar dose dependencies (Ransom et al. 2001).

3.4. Astrocyte-neuron signaling

Astrocytes are able to communicate with each other as well as with neurons. The membranes of perisynaptic astrocytes are equipped with ion channels, neurotransmitter receptors and various transporters. When stimulated with neurotransmitters, astrocytes generate transient increases in their intracellular Ca^{2+} levels. Those Ca^{2+} waves can propagate through astrocytic syncytium via gap junctions and trigger release of neuroactive compounds, such as ATP or glutamate, and thus modulate synaptic activity. This process has been called gliotransmission (reviewed by Belanger and Magistretti 2009).

VRACs were proposed to work in parallel with this Ca^{2+} -dependent mechanism in mediating neuron-glia signaling. Uptake of ions and neurotransmitters is accompanied by water, therefore causing slight astrocytic swelling, and astrocytes were also shown to swell in response to stimulation of astrocytic metabotropic glutamate receptors (Hansson 1994). In addition, ATP, which is often released from neurons as a co-transmitter, greatly potentiates glutamate release through VRACs from moderately swollen astrocytes (Mongin and Kimelberg 2005). Taken together, VRACs could constitute an important pathway for glutamate-mediated astrocyte-neuron communication. The possible mechanism is illustrated in figure 3.

VRACs have also been suggested to play a role in the whole-body osmoregulation. Decreased extracellular osmolarity triggers release of taurine from astrocytes in isolated supraoptic nucleus (SON) of the hypothalamus. This taurine release is inhibited by VRAC blockers. Since neurons in SON are responsible for vasopressin secretion, taurine released from astrocytes could inhibit their activity via glycine receptors, and thus participate in maintenance of the whole-body fluid balance (Deleuze et al. 1998).

However, the question if astrocytes modulate synaptic activity *in vivo* remains to be answered.

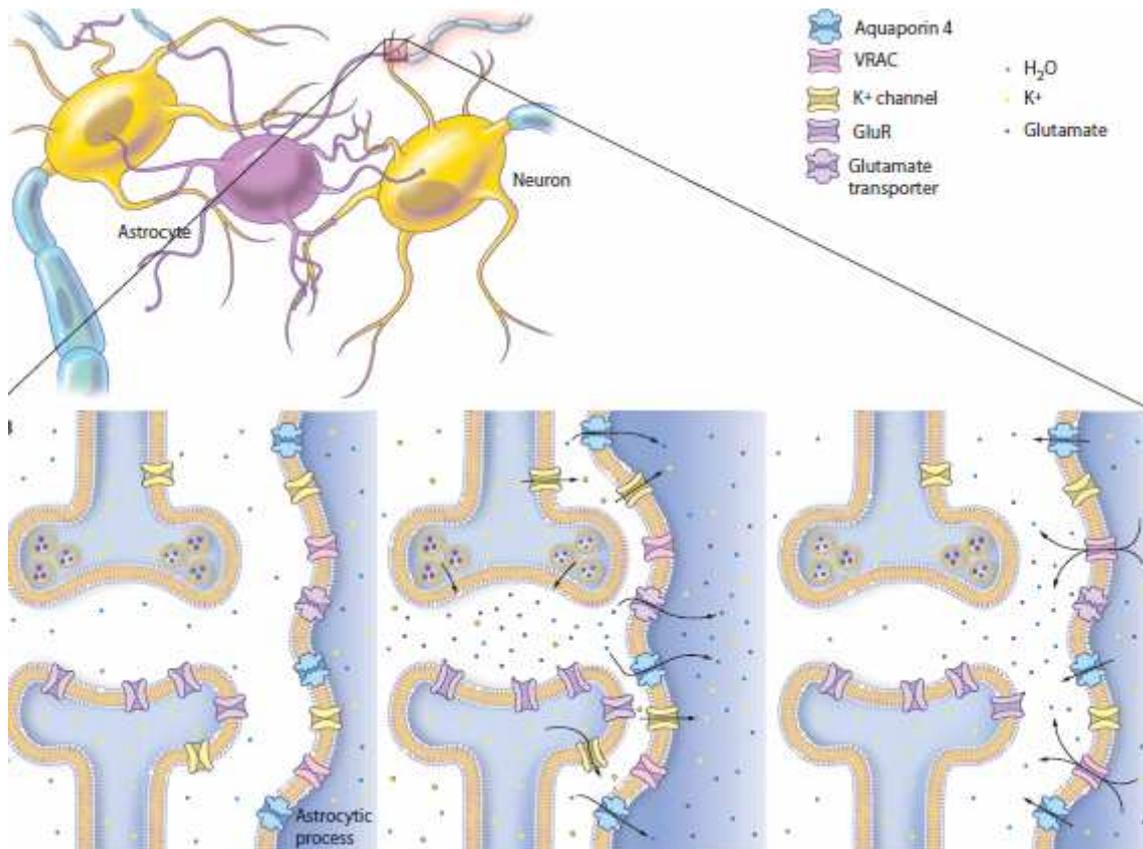


Figure 3 Astrocytic swelling in response to synaptic activity. (Left) Inactive glutamatergic synapse with relatively distant astrocytic process. Postsynaptic glutamate receptors and astrocytic VRACs are inactive. (Middle) During synaptic activity, K⁺ is released from neurons and taken up by the astrocyte. Water flows through the aquaporin channels into the astrocyte to balance the osmotic pressure due to the increase in astrocytic K⁺, leading to swelling of the astrocyte and a decrease in the extrasynaptic space. Astrocytes also take up glutamate through glutamate transporters. (Right) Osmotic swelling triggers opening of VRACs, which allow amino acids, such as glutamate, and Cl⁻ (not shown), to be released from the astrocyte. Water follows, thereby reducing astrocyte swelling. The released glutamate may influence synaptic activity. (Mulligan and MacVicar 2006)

3.5. Brain swelling

Cell swelling is particularly dangerous in brain. The rigid skull limits expansion of the tissue which can lead to blood vessels constriction, caudal displacement of brain parenchyma and compression of autonomic centers in brain stem, resulting in respiratory and cardiac arrest. Moreover, cell swelling decreases extracellular space volume, causing neuronal hyperexcitability, due to reduced diffusion of neurotransmitters and increased extracellular K⁺ concentration (reviewed by Pasantes-Morales et al. 2002). Cell swelling accompanies several pathologies, such as ischemia, traumatic brain injury, acute liver failure or hyponatremia (reviewed by Kimelberg 2005; Massieu et al. 2004).

In brain slices and *in vivo*, neurons do not swell in response to acute hyposmolarity, probably because they lack functional aquaporins (Andrew et al. 2007; Risher et al. 2009). In contrast, astrocytes swell readily. They possess high levels of AQP4, selectively localized at astrocytic end-feet contacting blood vessels and therefore constitute the main route for water entry from blood to brain cells (Nase et al. 2008). The main cause of hyposmotic brain swelling *in vivo* is hyponatremia, which can be caused by inappropriate secretion of antidiuretic hormone, glucocorticoid or mineralocorticoid deficiency, hypothyroidism, use of some diuretics, renal or hepatic failure or cerebral salt-wasting syndrome (CSWS). Acute and severe hyponatremia results in brain edema and increased intracranial pressure (reviewed by Pasantés-Morales et al. 2002). In animal models of chronic hyponatremia, brain cells were shown to actively decrease their volume over a period of several days by RVD mechanism (extrusion of K^+ , Cl^- and organic osmolytes, mainly taurine, glutamate and aspartate), which suggests VRAC involvement (reviewed by Massieu et al. 2004). Interestingly, in contrast with astrocytes from primary cultures, astrocytes *in situ* and *in vivo* do not show any immediate (i.e. within the first hour) RVD during acute hyposmotic stress (Risher et al. 2009; Hirrlinger et al. 2008).

Astrocytic VRACs were also suggested to play a significant role in neuronal damage during ischemia. Cerebral ischemia is a transient or permanent reduction of blood flow in the CNS tissue causing decreased oxygen and glucose supply to neurons. In focal ischemia (stroke), affected tissue can be divided into the infarct core, characterized by no or minimal blood flow, and the ischemic penumbra, where blood flow is preserved to some extent. The extent of brain tissue damage depends on severity and duration of stroke. In the ischemic core, energy depletion leads to rapid disruption of transmembrane ionic gradients, elevation of intracellular Ca^{2+} , activation of proteolytic cascades and subsequent cell death. In the penumbra, cells are more or less viable, although neuronal functions are also impaired. The goal of ischemia treatment is to prevent neuronal death in this region of partial ischemia (or prevent the expansion of the ischemic core into the penumbra). Importantly, extracellular K^+ and neurotransmitters concentrations are significantly increased in ischemia (reviewed by Mongin 2007; Phillis and O'Regan 2003). Astrocytes swell during stroke, mainly because of elevated extracellular K^+ and accumulation of Na^+ and Cl^- due to dysfunction of Na/K-ATPase. As discussed above, swelling activates VRACs, which are permeable to amino acids glutamate and aspartate. Glutamate,

the main excitatory neurotransmitter in the CNS is thus released from astrocytes and can activate neuronal glutamate receptors, thus allowing influx of Ca^{2+} and other cations and further exacerbating neuronal damage – a phenomenon called excitotoxicity. However, VRAC activity in the infarct core is improbable, because they require intracellular ATP. Glutamate release observed in the ischemic core is rather mediated by reversal of astrocytic glutamate transporters caused by ionic gradient disruption. In the penumbra, on the other hand, ATP levels and ionic gradients are less affected and VRACs contribute significantly to the excitatory amino acids release as shown in animal model of reversible middle cerebral artery occlusion (rMCAO) (Feustel et al. 2004). VRACs involvement further supports the finding, that DCPIB, a selective VRAC blocker, reduces infarct size and glutamate release in rMCAO (Zhang et al. 2008).

4. Conclusions

The facts summarized in this work suggest, that astrocytic VRACs actively participate in the maintenance of cell volume homeostasis, modulation of synaptic activity, cell cycle progression and apoptosis, and that they are involved in pathologies connected with brain swelling.

However, VRACs have been mainly studied in cultured cells, whose characteristics can differ from those of cells in the intact tissue. In culture, VRAC activity is usually measured by patch-clamp technique as a whole-cell anion conductance (mainly carried by Cl^-) (Parkerson and Sontheimer 2004; Crepel et al. 1998) or as an efflux of preloaded radiolabeled amino acids, D- $[\text{}^3\text{H}]$ -aspartate, L- $[\text{}^3\text{H}]$ -glutamate and $[\text{}^3\text{H}]$ -taurine. (Kimelberg et al. 1990; Abdullaev et al. 2006). The assumption, that amino acids permeate through VRACs is mainly based on similar biophysical and pharmacological profiles of VRAC currents and amino acids efflux (Abdullaev et al. 2006). *In vivo*, VRAC activity has been indirectly assessed from the level of amino acids released into dialysate (Feustel et al. 2004; Estevez et al. 1999; Zhang et al. 2008). However, amino acids can be released from astrocytes by other mechanisms, such as Ca^{2+} -dependent exocytosis, reversal of amino acids transporters (Feustel et al. 2004), connexin hemmichannels (Ye et al. 2009) or maxi-anion channels (Liu et al. 2006), and there are doubts about whether amino acids

permeate VRACs at all (Shennan 2008). The issue cannot be settled until putative VRACs are identified at the molecular level.

Molecular identification of the channel(s) responsible for VRAC currents and comprehensive *in vivo* studies are needed to fully understand VRACs contribution to cellular functioning under both physiological and pathological conditions, and subsequently, evaluate their relevance as a target for therapy in pathologies accompanied by cerebral edema.

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