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The Role of Swelling-Induced Anion Channels During Neuronal Volume Regulation

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Abstract

Regulation of cell volume is an essential function of most mammalian cells. In the cells of the central nervous system, maintenance of cell osmolarity and, hence, volume, is particularly crucial because of the restrictive nature of the skull. Cell volume regulation involves a variety of pathways, with considerable differences between cell types. One common pathway activated during hypo-osmotic stress involves chloride (Cl^-) channels. However, hypo-osmotically stimulated anion permeability can be regulated by a diverse array of second messengers. Although neuronal swelling can occur in a number of pathological and nonpathological conditions, our understanding of neuronal volume regulation is lir ıited. This article summarizes our current understanding of the role of anion channels during neuronal volume regulation.

Index Entries: Volume regulation; regulatory volume decrease (RVD); Cl^- channels; amino acids; calcium; arachidonic acid; stretch-sensitive channels; P-glycoprotein.

Introduction

Virtually all types of mammalian cells possess mechanisms of cell volume regulation to protect against hypertonic or hypotonic shock. Initially, there would seem to be few situations (hyponatremia, water intoxication, dehydration) in which osmotic changes can influence the brain. The brain is protected by the skull and its extracellular fluid environment is shielded by the blood–brain barrier and secretion of cerebrospinal fluid (CSF). However, common situations exist in which swelling can occur, particularly in response to blunt or ischemic injury. Trauma leading to changes in blood flow and hypoxic damage leading to acidosis will cause cell swelling. In addition, following volleys of action potentials or in biculline-induced status epilepticus, swelling of nerve cells (Iwasa et al., 1980) or dendrites (Soderfeldt et al., 1981) has been observed. Modest alterations in the brain volume can have profound effects on the signaling and transmission process as the spatial relationship of neurons, astrocytes, and the extracellular space is modified (Strange, 1992). It is therefore

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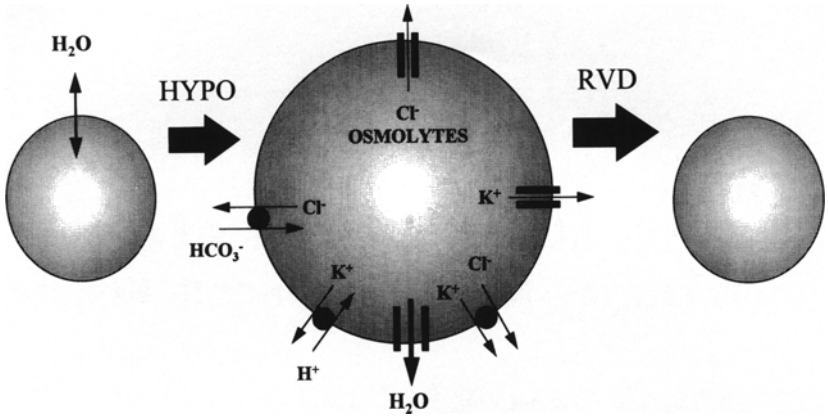

Fig. 1. Membrane transport during RVD. Exposure to hypo-osmotic stress results in rapid cell swelling to activate subsequently one of four transport pathways leading to RVD. (1) Activation of K^+ and Cl^- channels; (2) a transport pathway for organic osmolytes; (3) a K^+-Cl^- cotransporter; and (4) electroneutral exchangers, such as K^+/H^+ and Cl^-/HCO_3^-.

relevant to investigate which mechanisms exist in neuronal cells to protect against cell swelling and return cell volume toward normal via a regulatory volume decrease (RVD).

Cell volume regulation involves a variety of pathways, with considerable differences among cell types for specific mechanisms of RVD. Four principal classes of membrane transport systems may be involved (Fig. 1) (for review, *see* Hoffmann and Simonsen, 1989; Parker, 1993).

1. Activation of K^+ and Cl^- channels;
2. A transport pathway for organic osmolytes, including amino acids, and polyols, such as myoinositol and sorbitol;
3. A K^+-Cl^- cotransporter; and
4. Electroneutral exchangers, such as K^+/H^+ and Cl^-/HCO_3^-.

The activation of Cl^- channels during RVD is particularly important in neurons to prevent damage to the brain.

Chloride channels are ubiquitous in the plasma membrane of mammalian cells. In addition to their pivotal role in cell volume regulation, they participate in a variety of other cellular functions, including affecting excitability in nerve and muscle. Cl^- channels show a diversity in voltage dependence and can be activated by a variety of second messengers, including Ca^{2+}, cAMP, G-proteins, ATP, cGMP, leukotrienes, or arachidonic acid (for review, *see* Valverde et al., 1995).

The brain contains a nonhomogenous cellular population, and difficulties exist in isolating primary neurons. Thus, our understanding of volume regulatory mechanisms in the CNS has been limited. Yannet (1940) provided important early in vivo observations of the brain's ability to respond to altered osmolarity. However, the structural complexities of studying the cell in the CNS in vivo mean few data are available. Cell volume regulation in neurons has, therefore, been studied with primary cell cultures without the complex natural connections to other brain cells or with neuroblastoma cell lines. Although both K^+ and Cl^- permeability may be activated in response to hypo-osmotic stress, the present short article will focus on activation of the anion permeability pathway during neuronal RVD.

RVD

The ability of cells to regulate their volume during hypo-osmotic insult has been demonstrated in many cell types, including astrocytes (Kempski et al., 1983; Kimelberg and Frangakis, 1986; Pasantes-Morales and Schousboe, 1988), frog retinal pigment epithelial cells (Adorante,

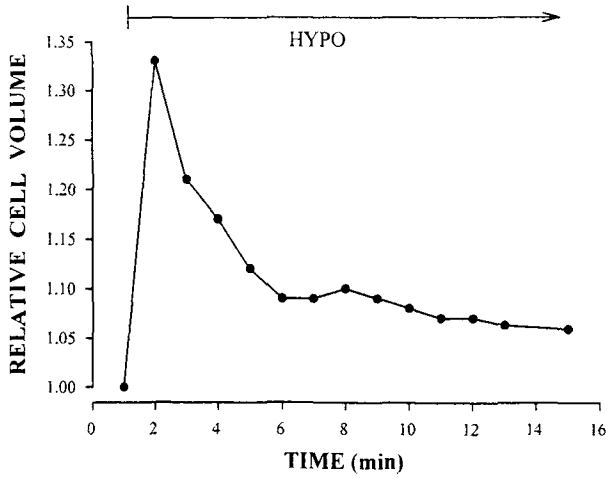

Fig. 2. Effects of hypo-osmotic stress on cell volume in CHP-100 cells. Changes in cell volume were measured after reducing the extracellular osmolarity from 290 to 190 mOsm/kg H_2O. In this representative study with the Coulter Multisizer, hypo-osmotic stress resulted in rapid cell swelling, followed by RVD. Results are expressed as change in relative cell volume compared to iso-osmotic conditions.

1995), synaptosomes (Babila et al., 1990), and most recently in neuroblastoma cells (Lippmann et al., 1995; Basavappa et al., 1996). In primary neurons, very little evidence of RVD has been reported. However, Pasantes-Morales et al. (1993) have observed RVD in rat cerebellar granule neurons. In rat synaptosomes, exposure to a hypotonic solution caused a rapid swelling and recovery to within 5% of the original cell volume within 2 min (Babila et al., 1990). In rat cerebellar granular neurons, RVD was complete within 15 min of exposure to hypo-osmotic shock (Pasantes-Morales et al., 1993). Using the Coulter Multisizer and measurements of intracellular ^3H_2O, Basavappa et al. (1996) have demonstrated in the human neuroblastoma cell line CHP-100 that decreasing the extracellular osmolarity from 290 to 190 mOsm/kg H_2O resulted in an initial rapid cell swelling followed by a biphasic RVD response that was completed within 15 min (Fig. 2). Similar changes in cellular volume were detected using the confocal microscopy technique in N1E115 neuroblastoma cells (Lippmann et al., 1995). The rapid RVD response is vital in neurons to avoid ischemic damage consequent on swelling in the confines of the cranium.

Anion Permeability During RVD

Before we can consider characterization of the anion permeability pathways during RVD, it is essential to discuss briefly the effects of anion channel blockers during RVD. Using confocal microscopic techniques to monitor changes in cell volume, Lippmann et al. (1995) in N1E115 neuroblastoma cells have demonstrated that anion channel blockers 4,4'-diisothiocyanatostilbene-2,2'disulfonic acid (DIDS) and indanyloxyacetic acid-94 (IAA-94) were effective in preventing RVD. Similar results have been reported in astrocytes and in human nonpigmented ciliary epithelial cells, where anion channel blockers were effective in reducing the RVD response (Kimelberg et al., 1989; Civan et al., 1992; Pasantes-Morales et al., 1994), whereas in rat cerebellar granule neurons RVD was abolished with intracellular depletion of Cl^- (Pasantes Morales et al., 1993). These studies are in agreement with observations in CHP-100 cells, where the classical chloride channel blocker 5-nitro-2-(3-phenylpropylamino)-benzoic acid (NPPB) significantly blocked the RVD response (Basavappa et al., unpublished results).

The above data indicate a role for anion conductance pathways during RVD. Two techniques have been utilized to identify the role of anion-selective channels in the RVD response. Electrophysiological data from patch-clamp studies (whole-cell and single-channel recordings) and radiotracer studies, principally using ^{125}I fluxes. With these two methods, characterization has been extended to substrate selectivity, pharmacological characterization, and signaling second messenger pathways. These will be considered below.

In several brain cell types, chiefly astrocytes, activation of anion permeability pathways during hypo-osmotic stress has been reported (Strange et al., 1993; Jalonen, 1993; Pasantes-Morales et al., 1994; Bakhramov et al., 1995). In

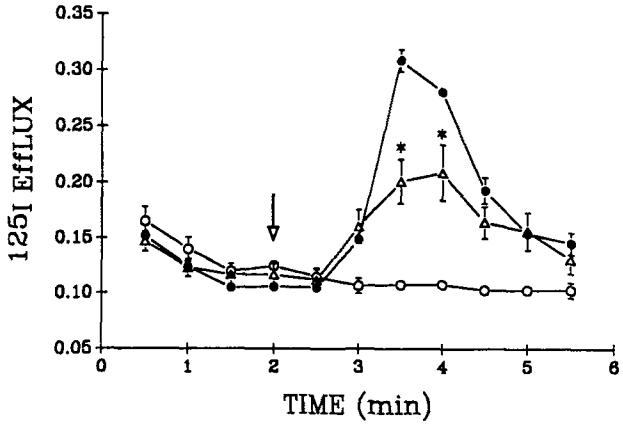

Fig. 3. Effects of hypo-osmotic stress on ^{125}I efflux. In this representative study, CHP-100 cells under iso-osmotic conditions (○) showed a gradual decline in efflux. Addition of hypo-osmotic solution (at arrow) caused an increase in ^{125}I efflux within 1 min of exposure (●). Cells pretreated with the Cl^- channel blocker NPPB prior to hypo-osmotic solution, and during hypo-osmotic stress, showed an inhibition of ^{125}I stimulated efflux (△). The bath solution contained 3 m*M* 4-AP to block K^+ permeability. $*p < 0.05$ with respect to hypo-osmotic conditions (reproduced from Basavappa et al., 1995a, with permission of the publisher.)

order to understand this process better, we have utilized the Cl^- permeability marker $^{125}I^-$ as a complementary technique to patch-clamp studies. When CHP-100 cells are exposed to hypo-osmotic stress, an increased efflux of ^{125}I was observed. This efflux was inhibited by the Cl^- channel blocker NPPB (Fig. 3). Sanchez-Olea et al. (1995a) have similarly used this tracer to monitor changes in chloride efflux during RVD in primary rat astrocytes.

Swelling-activated Cl^- currents have been described in many cell types. However, in cells of the CNS, there are few data and they are mostly confined to astrocytes. Published studies include experiments in F11 cells, a mouse neuroblastoma (NG18tG2) × rat dorsal root ganglion (DRG) hybrid cell line (Pollard, 1993), N1E115 cells (Falke and Mister, 1989), C6 glioma cells (Jackson and Strange, 1993), and astrocytes (Bakhramov et al., 1995). The hypo-osmotically induced Cl^- currents in most cell types have several common characteristics, including:

1. Rapid activation;
2. An outwardly rectifying current–voltage (I–V) relationship that tends to inactivate at high depolarizing membrane potentials;
3. Sensitivity to anion channel blockers, including DIDS, DPC, NPPB, MK196, and SITS; and
4. A similar anion selectivity ($SCN^- > I^- > Br^- > Cl^- >>$ gluconate).

For example, we have in the neuroblastoma cell line CHP-100 characterized the swelling-induced whole-cell Cl^- currents with the above characteristics (Fig. 4) (Basavappa et al., 1995, 1996). The swelling-induced Cl^- currents were sensitive to NPPB, displayed outward rectification, and exhibited a similar anion selectivity. The major difference in the swelling-activated anion conductance observed among different cell types is the voltage dependence of channel inactivation and its time-course. For instance, in the neuroblastoma × DRG cell hybrid inactivation has been reported at modest depolarizations (Pollard, 1993), whereas in C6 glioma cells (Jackson and Strange, 1995a) and the human astrocytoma cell line U373 MG (Bakhramov et al., 1995), the swelling-activated anion currents were inactivated at a relatively high membrane depolarization (above +90 and +50 mV, respectively). Jackson and Strange (1995a) have characterized further the kinetics of channel inactivation, and their model indicates that inactivation may be attributed to transitions of the channel from a single open state into the first inactivated state. The electrophysiological differences observed in the inactivation of the swelling-activated anion current may reflect the existence of more than one type of swelling-activated anion channel or may merely reflect differences that are cell-specific.

The specific Cl^- channel type participating in volume regulation varies with the cell type. In nonneuronal cells, swelling-activated Cl^- channels with conductances from 1–300 pS (Solc and Wine, 1991; Ho et al., 1994; Schwiebert et al., 1994) have been reported. In cell-attached patch recordings from Ehrlich cells, a small Cl^- channel termed the "minichloride channel," with a conductance of 7 pS is activated after

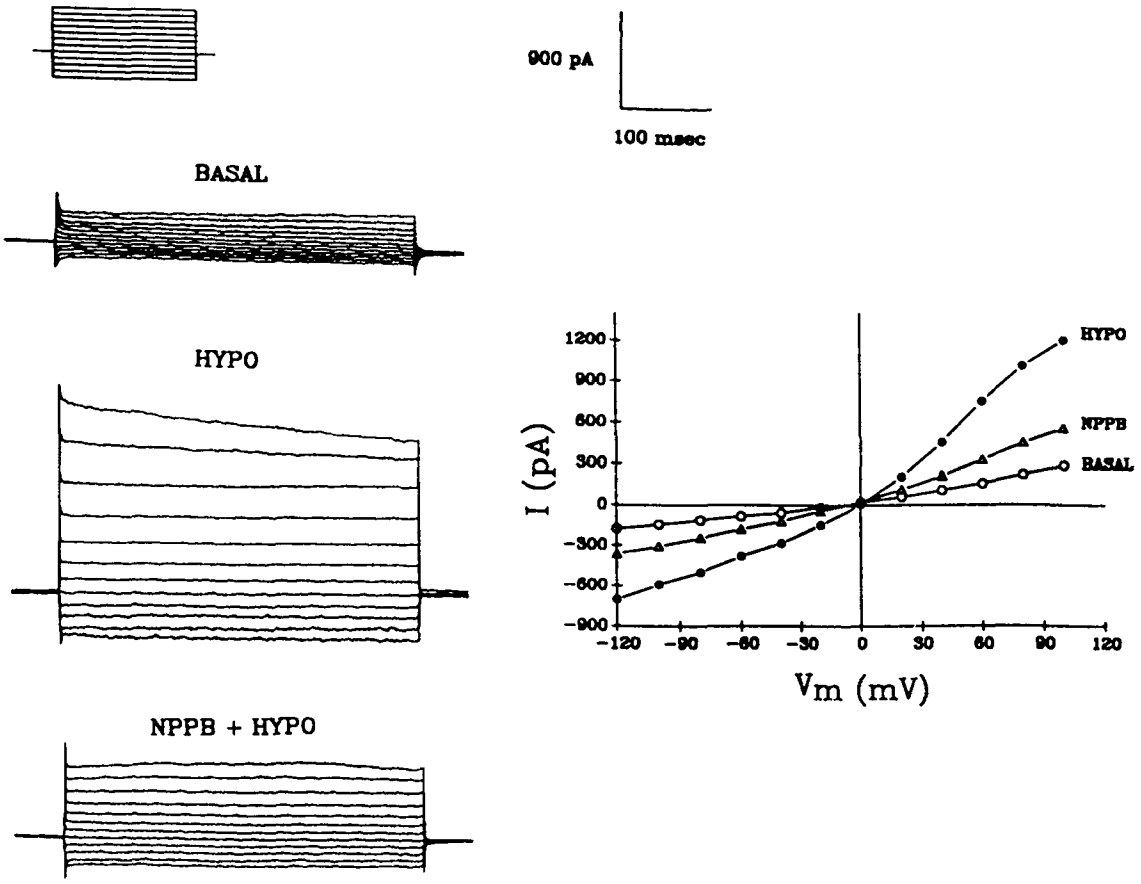

Fig. 4. Hypo-osmotic stress activates Cl^- currents. In this representative nystain perforated-patch recording, the extracellular solution was supplemented with 5 m*M* $BaCl_2$ to block K^+ currents. Membrane voltage was held at –40 mV and stepped to test potentials between –120 and 100 mV in 20-mV increments (400-ms duration) (top). Membrane currents (left) and current–voltage relationship (right) are illustrated under iso-osmotic conditions (○), after exposure to hypo-osmotic solution (●), and subsequent to NPPB (△), which inhibited the hypo-osmotically activated Cl^- currents (reproduced from Basavappa et al., 1995a, with permission of the publisher.)

hypotonic exposure (Christensen and Hoffmann, 1992). In the few reported single Cl^- conductance measurements in neuronal cell lines, Falke and Misler (1989) report an anion channel between 300 and 400 pS activated by reducing the bath osmolarity, and in CHP-100 cells, we have recently described in cell-attached patches a swelling-activated Cl^- channel with a conductance of ~50 pS (Basavappa et al., 1996). Jackson and Strange (1995b) in C6 glioma cells used stationary and nonstationary noise analysis to predict the unitary conductance of volume-activated anion channels and found conductances of ~1 and ~15 pS, respectively.

Permeability of the Volume-Activated Anion Channel

Efflux of several amino acids, including glutamate, aspartate, glycine, and taurine, as well as polyols such as myoinositol and sorbitol, has been observed during RVD in brain cells (Law, 1994; McManus et al., 1995; Gonzalez et al., 1995). The amino acids involved during RVD are all classified metabolically as nonessential and are often present in high intracellular concentrations. Furthermore, these amino

acids are known neurotransmitters, putative neurotransmitters, or neuromodulators. In particular, the sulfonic amino acid taurine is present in high concentrations (5–50 m*M*) in neuronal cells (Huxtable, 1989) where it may function as an osmoregulator. In vivo studies have demonstrated increased efflux of taurine following exposure to hypotonic conditions (Lehman, 1989). Wade et al. (1988), using intracranial microdialysis, have demonstrated in the piriform cortex of rats a loss of cellular amino acids, including taurine during hypo-osmotic stress. In vitro studies have confirmed taurine efflux from a variety of neuronal cells during RVD, including Muller cells (Faff-Michalak et al., 1994), cerebellar granular cells (Schousboe et al., 1991), and astrocytes (Pasantes-Morales and Schousboe et al., 1988; Kimelberg et al., 1990; Jackson and Strange, 1993). Taurine released from rat cerebellar Purkinje cells by hypo-osmotic shock has been observed to be taken up supporting glial cells (Nagelhus et al., 1993). However, the identity of the permeability pathway for these amino acids during hypo-osmotic stress is still controversial.

One potential candidate pathway for the amino acid efflux is via the volume-activated anion pathway. Roy and Malo (1992) were among the first to suggest that anion channels may mediate amino acid efflux from MDCK cells in response to swelling. Subsequently, Banderali and Roy (1992) reported using the patch-clamp technique inside-out configuration in the presence of a swelling-activated outwardly rectifying anion channel with a high permeability to taurine, glutamate, and aspartate in MDCK cells. Kirk et al. (1992), using tracer measurements with flounder erythrocytes, proposed that efflux of organic osmolytes may be mediated via a hypo-osmotically induced anion-selective pathway. Recent studies by Huang et al. (1996) indicate that hypo-osmotically activated taurine efflux in the human erythrolukemia cell line K562 is mediated via an anion permeability pathway. From their studies of swelling-activated taurine and inositol efflux in C6 glioma cells, Strange et al. (1993) and Strange and Jackson (1995), have termed the swelling-activated anion channel as volume-sensitive organic osmolyte/anion channel (VSOAC), which they demonstrate is permeable to other anions, amino acids, and osmolytes. VSOAC, swelling-activated myoinositol, and taurine efflux were all blocked to the same extent by classical anion channel inhibitors, such as NPPB, whereas verapamil and oleic acid, two agents that did not inhibit whole-cell Cl^- currents, also did not affect osmolyte efflux. In neuroblastoma CHP-100 cells, hypo-osmotic stress significantly increased taurine and glycine efflux (Basavappa et al., 1996). This hypo-osmotically activated taurine efflux was decreased by several anion channel blockers, including NPPB, DIDS, and niflumic acid, and by the volume-activated Cl^- channel blocker tamoxifen (Valverde et al., 1993). Furthermore, this efflux was sensitive to removal of extracellular Ca^{2+}. Anion channel blockers inhibited the swelling-induced Cl^- current in CHP-100 cells, which was also sensitive to extracellular Ca^{2+} removal. Taken together, these studies argue in favor of the participation of a single anion-permeation pathway during RVD. In contrast, studies by Lambert and Hoffmann (1994) in Ehrlich ascites tumor cells, suggest that hypo-osmotically activated efflux of taurine may be mediated via a separate "taurine channel," which has a different activation time and sensitivity to DIDS and arachidonic acid when compared to the hypo-osmotically activated "mini Cl^- channel."

If a single anion permeability pathway is activated during hypo-osmotic stress, is it physically feasible for the swelling-activated anion channel to mediate transport of a very diverse group of molecules that vary in their molecular mass as well as in shape? The pore diameter of the anion channel has been estimated to be 5.2–6.0Å in outwardly rectifying anion channels in T84 cells, and in glycine and GABA-receptor-linked Cl^- channels (Bormann et al., 1987; Halm and Frizzell, 1992). Haynes and Goldstein (1993) using erythrocytes of skate *Raja erinacea*, estimate that the volume-activated DIDS-sensitive amino acid channel to be between 5.7 and 6.3Å. The crystal diameter

Table 1
Role of Ca^{2+} During RVD in Brain Cells

Cell type	Ca^{2+} dependent RVD	Reference
Nonpigmented ciliary cell line	Yes	Civan et al. (1992) Adorante and Cala (1995)
Necturus choroid plexus epithelium	?	Christensen (1989)[a]
N1E115 neuroblastoma cells	No	Falke and Mister (1989)
Primary astrocytes	Yes	O'Connor and Kimelberg (1993)
Primary astrocytes	No	Pasantes-Morales et al. (1994)
Cortical neurons	No	Pasantes-Morales et al. (1993)[a]
UC-11MG astrocytoma cell line	No	Sanchez et al. (1993)[a]
CHP-100 neuroblastoma cells	Yes	Medrano and Gruenstein (1993)

[a]Elevation in $[Ca^{2+}]_i$ has been observed during hypoosmotic stress.
[b]Unpublished results.

of Cl^- anion is 3.6Å (Franciolini and Nonner, 1987), whereas the diameters of glycine and inositol range from 4.4–5.9Å (Haynes and Goldstein, 1993; Strange and Jackson, 1995). Thus, the above data support the concept that disparate osmolytes and ions can share a common pathway during osmotic stress.

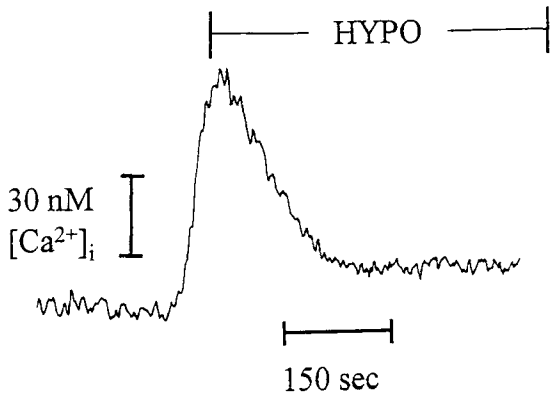

Fig. 5. Increase in cytosolic Ca^{2+} in response to hypo-osmotic stress. In CHP-100 cells loaded with the Ca^{2+} fluorescence dye indicator fura2 (AM), exposure to hypo-osmotic solution rapidly increased the cytosolic Ca^{2+} level.

Regulation of the Swelling-Activated Anion Permeability Pathway

Calcium

Intracellular messengers may be involved in the signaling pathway and in regulation of the hypo-osmotically activated anion channel. Several candidates have been proposed, including cAMP, Ca^{2+}, leukotrienes, and arachidonic acid. In several different cell types, intracellular Ca^{2+} levels ($[Ca^{2+}]i$,) are increased in response to hypo-osmotic stress (for review, *see* McCarty and O'Neil, 1992). Table 1 illustrates the different effects of Ca^{2+} during RVD in brain cells.

The role of Ca^{2+} in volume-activated Cl^- permeability is dependent on the cell type examined. Studies in MDCK cells (Rothstein and Mack, 1990), eccrine clear cells (Ohtsuyama et al., 1993), and other cell types (McCarty and O'Neil, 1992) indicate a clear role for Ca^{2+} in swelling-induced activation of Cl^- channels. In astrocytes and nonpigmented ciliary epithelial cells (NPE) of the aqueous humor (Civan et al., 1992; O'Conner and Kimelberg, 1993), influx of Ca^{2+} is required for RVD. In contrast, Pasantes-Morales et al. (1994), also working with astrocytes, reported that RVD is independent of extracellular Ca^{2+}. In CHP-100 neuroblastoma cells, exposure to hypo-osmotic stress increased $[Ca^{2+}]_i$ as measured by fura2 fluorescence (Fig. 5) (Basavappa et al., 1995a). Removal of extracellular Ca^{2+} decreased the osmotic shock-dependent increase in cytosolic Ca^{2+}, ^{125}I efflux, and whole-cell Cl^- currents. Further studies with Ca^{2+} channel antagonists indi-

cated that ω-conotoxin MVIIC, a P-type Ca^{2+} channel blocker (Hillyard et al., 1992), inhibits the hypo-osmotically activated whole-cell Cl^- currents and increases its $[Ca^{2+}]_i$ (Basavappa et al., 1995a). In contrast, the volume-activated Cl^- conductance in neuroblastoma F11 cells was not sensitive to Ca^{2+} (Pollard, 1993). In the human UIC-11MG astrocytoma cell line (Medrano and Gruenstien, 1993) and in clonal neuroblastoma N1E115 cells (Falke and Mister, 1989; Lippmann et al., 1995), swelling-activated K^+ and Cl^- pathways appear to be independent of extracellular Ca^{2+}. However, studies in nonneuronal cells, such as acinar cells (Kotera and Brown, 1993) and in pancreatic duct cells (Verdon et al., 1995), find a similar requirement for extracellular Ca^{2+} in the activation of swelling-activated Cl^- currents.

Calmodulin and Protein Kinase C (PKC)

An increase in intracellular Ca^{2+} may activate Ca^{2+}-dependent kinases, such as Ca^{2+}/calmodulin-dependent protein kinases (CaM K) and PKC. CaM K may affect a variety of cellular functions, including regulation of neurotransmitter release and phosphorylation of ion channels, which may subsequently affect cell swelling and RVD (for review, *see* Hanson and Schulman, 1992). These kinases are particularly abundant in the brain and may play a role in volume regulation. Recently in cerebral cortical slices, Law (1995) has demonstrated inhibition of hypo-osmotically mediated taurine efflux with the CaM inhibitor trifluoperazine, whereas in astrocytes (Bender et al., 1992), CaM antagonists blocked RVD. In NPE cells, Civan et al. (1992, 1994) observed that RVD was incomplete with inhibition of Ca^{2+} – CaM, whereas activity of volume-activated chloride channels was increased at high Ca^{2+} CaM K levels. Although the data in neuronal cells are limited, in nonneuronal cells, such as Ehrlich ascites tumor cells (Cornet et al., 1993), CaM antagonists inhibited RVD. In HeLa cells, Kirk and Kirk (1994) reported that CaM may participate in volume regulation, since there was good correlation between binding to CaM and inhibition of the hypo-osmotically induced taurine efflux. Similarly, blockers of CaM (at very high doses) blocked volume regulation and taurine efflux in *Noetia ponderosa* red blood cells after hypo-osmotic stress (Pierce et al., 1989). In contrast, in airway epithelial cells, swelling-activated Cl^- currents were unaffected by the inclusion of a specific inhibitor of CaM K in the pipet solution (Chan et al., 1992). In preliminary studies in the neuroblastoma CHP-100 cells, KN-62, a cell-permeable specific antagonist of Ca^{2+}-CaM KII, did not affect hypo-osmotically induced ^{125}I efflux (Basavappa et al., unpublished results). Additional studies with specific peptide inhibitors of Ca^{2+}-CaM KII may help to delineate whether this pathway is associated with regulation of the hypo-osmotically activated anion currents in neurons.

PKC also serves as a Ca^{2+}-dependent messenger and plays an important role in many physiological processes in the brain, as shown by the reduction in brain edema following inhibition of PKC activity in rats (Joo et al., 1989). However, a role for PKC during RVD in neuronal cells has not been defined specifically. In astrocytes, Bender et al. (1992) reported a potentiation in cell swelling with PKC-stimulating agents, whereas in pig jejunal enterocytes, RVD and swelling-induced Cl^- conductance were independent of PKC (MacLeod et al., 1992). In contrast, in proximal tubule cells of *Rana temporaria*, inhibition of PKC blocked the swelling-induced Cl^- conductance, whereas stimulation of PKC increased the Cl^- conductance in these cells (Robson and Hunter, 1994). In GH-4C-1 rat pituitary cells, downregulation of PKC decreases the swelling-induced rise in $[Ca^{2+}]_i$ and promotes cell swelling (Sato et al., 1992). However, in NPE cells, Civan et al. (1994) reported that inhibitors of PKC enhanced RVD, whereas PKC activators downregulated RVD. These investigators concluded that cell swelling reduces endogenous PKC activity to increase the upregulation of Cl^- channels. In C6 glioma cells, Strange et al. (1993) conclude that although PKC may modulate the activity of VSOAC, it is not essential for its activation. At present, the role of PKC in cell volume regulation is unclear and may be very cell-specific.

Other Second Messengers

In addition to Ca^{2+}, other second messengers may play an important role in the volume regulatory process. Many of the examples cited in this section pertain to nonneuronal cells since there are limited data on neuronal cells. Leukotriene synthesis increases after hypo-osmotic stress in Ehrlich ascites tumor cells have been suggested to participate in increases in Cl^- permeability and the subsequent RVD (Lambert, 1994). Hoffmann and Lambert (1993) suggest further that the 5-lipoxygenase product of arachidonic acid, leukotriene D_4 (LTD_4), may act as a second messenger for activating taurine efflux, as well as the hypo-osmotically activated Cl^- permeability. In contrast, in the same cell type, arachidonic acid inhibited swelling-induced Cl^- efflux, but stimulated taurine efflux (Lambert and Hoffmann, 1994). These investigators observed similar effects with oleic acid (an unsaturated fatty acid), and suggested that fatty acid-induced activation of "taurine channels" may be important in ischemia owing to the increased levels of fatty acids found during this condition. However, in brain cells, such as C6 glioma cells, arachidonic acid inhibited the swelling-induced inositol and taurine efflux (Strange et al., 1993). Similar effects were observed in rat cerebellar astrocytes in culture, where arachidonic acid blocked RVD, swelling-activated ^{125}I, taurine, aspartate, and inositol efflux (Sanchez-Olea et al., 1995b). Thus, the consequences of the hypo-osmotically stimulated 5-lipoxygensase pathway during RVD may be dependent on the cell type.

In some cell types, increases in cell volume are associated with elevated levels of intracellular cAMP concentration (Watson, 1991). However, increased cAMP levels following cell swelling did not participate in RVD in S49 cells (Watson et al., 1991). In contrast, cAMP levels were unaffected by cell swelling in C6 glioma cells (Strange et al., 1993). Similarly, in CHP-100 cells exposed to hypo-osmotic stress, cAMP levels did not increase (Basavappa et al., unpublished results). Heterotrimeric GTP-binding proteins (G-proteins) may also play a central role in coupling membrane receptors to effector mechanisms, such as membrane anion channels. During volume regulation, RVD was inhibited in human platelets by the nonhydrolyzable GDP analog GDPβS and by pertussis toxin (PTX), which irreversibly ADP-ribosylates $G_{i\alpha}$ and G_o (Margalit et al., 1993). Similarly, in astrocytes glutamate-induced cell swelling was reduced by PTX (Hansson et al., 1994), and in proximal tubule cells, RVD involves a PTX-sensitive Ca^{2+} influx (Suzuki et al., 1990). In an interesting study by Oz and Sorota (1995) in human cardiac myocytes, the swelling-induced Cl^- currents were enhanced by forskolin. In neurons, there are very few results regarding these second messengers and the swelling-activated anion permeability pathway.

ATP

Investigations with several nonneuronal cell types, such as T84 cells (Ho et al., 1994), pancreatic duct cells (Verdon et al., 1995), human endothelial cells (Nilius et al., 1994), and human sweat gland cells (Solc and Wine, 1991), have reported the requirement for ATP in the activation of the swelling-induced anion currents. In rat C6 glioma cells, Jackson et al. (1994) have similarly demonstrated the prerequisite for ATP in activating volume-sensitive anion channels. Zhang et al. (1993) reported in cardiac cells that using conventional whole-cell patch technique without ATP, no observable volume regulation occurred, whereas in nystatin-perforated patch-clamp studies, where the intracellular messengers are conserved (Horn and Marty, 1988), volume regulation was detected. This is further corroborated by experiments with the lung cancer cell line H69AR, for which ATP was required for activation swelling-activated anion currents, but not for volume-sensitive anion currents in outside-out patches (Jirsch et al., 1993). This role for ATP is presumably separate from functioning as a source for cAMP and may reflect a direct interaction of the ligand with the channel.

Table 2
SA Ion Channels

Cell type	Type of SA channel	Reference
Neurons of *Cepaea*	Cation	Bedard et al. (1988)
Leech central neurons	K^+ selective	Pellegrino et al. (1990)
Snail neurons	Cation	Sigurdson and Morris et al. (1989)
Human retinal muller cells	Cation	Erxleben (1989)
Outer hair cells	Cation and K^+ selective	Ding et al. (1991)
N1E115 neuroblastoma	Anion and cation	Falke and Misler (1989)
Molluscan neurons	Anion and K^+ selective	Bedard and Morris (1992)
Liver cells	Cation	Bear (1990)
Chick heart cells	Cation and K^+ selective	Ruknudin et al. (1993)
Osteoblasts	K^+ selective	Davidson et al. (1990)
Choroid plexus epithelium	Cation	Christensen (1987)
Renal proximal tuble	K^+ selective	Sackin (1987)
Escherichia coli spheroblasts	Anion	Martinac et al. (1987)
Tobacco protoplasts	Anion	Falke et al. (1988)
Cortical collecting duct	Anion	Stanton et al. (1990)
Necturus proximal tubule	Cation	Filipovic and Sackin (1991)

Stretch

The increase in cell volume brought about by a decrease in osmolarity results in an increased shear force on the plasma membrane. This increase in membrane stress can activate stretch-sensitive ion (SA) channels (Christensen, 1987). SA cation channels have been demonstrated in a variety of cells (*see* Table 2), and one of its putative roles may be associated with cell volume regulation (Sackin, 1995). A common feature of SA nonselective cation channels is their permeability to Ca^{2+} (as well as Na^+ and K^+), which may subsequently activate anion channels that participate in RVD. In astrocytes, it has been proposed that cell swelling caused by hypotonic media leads to opening of nonselective cation channels, causing membrane depolarization, which subsequently activates voltage-sensitive L-type channels. The increased $[Ca^{2+}]_i$ triggers the activation of ion channels (O'Connor and Kimelberg, 1993). A similar mechanism may be occurring in CHP-100 cells, in which activation of the hypo-osmotically induced anion current is dependent on Ca^{2+} (Basavappa et al., 1995a).

Although not as prevalent, SA anion channels also have been detected and usually have high unitary conductance (Sackin, 1995). In RCCT-28A cells, an SA anion-selective channel has been demonstrated with a conductance of 305 pS, which can be activated by hypo-osmotic stress (Stanton et al., 1990). Schwiebert et al. (1992) reported that the SA anion channel, in addition to being activated by stretch, can also be activated by an inositol lipid cascade involving diacyl-glycerol, PKC, and G-proteins, resulting in an increased Cl^- efflux during RVD. SA cation channels have been observed in crustacean stretch receptor neurons (Erxleben, 1989) and in N1E115 neuroblastoma cells (Falke and Milser, 1989). In N1E115 neuroblastoma cells, SA cation channels are activated in response to hypo-osmotic stress. The resultant cell depolarization activates an anion channel of 300–400 pS, which may facilitate RVD in these cells. However, in human retinal Muller cells, the activation of SA channels in response to cell swelling increases the activity of Ca^{2+}-activated K^+ channels, which mediate the efflux of K^+ and decrease the glial cell volume (Puro, 1991).

Structure

Although pharmacological and biophysical characterization has been performed in extensive studies, the identification of the membrane proteins involved in the regulatory volume response is still not complete. Together with P-glycoprotein, two other candidate peptides have been cloned and sequenced that have been proposed as possible molecular candidates and are considered below.

Since the initial reports by Valverde et al. (1992) that P-glycoprotein, the product of the multidrug resistance gene (MDR1), whose overexpression has been linked with an increase in Cl^- channel activity in response to hypo-osmotic stress, may either function as a volume-sensitive Cl^- channel or as an ATP-dependent pump, the field has been controversial. It belongs to a family of ABC transporters and is contained in the plasma membrane. Several studies have reported a lack of correspondence between expression of P-glycoprotein and of volume-sensitive Cl^- channels (Jirsch et al., 1993; Altenberg et al., 1994; Rasola et al., 1994; Wang et al., 1994). However, no functional studies have been reported in neuronal cells regarding the role of P-glycoprotein in volume regulatory response. Although Jackson et al. (1994) report in C6 glioma cells swelling-activated whole-cell anion currents with similar characteristics as that arising from P-glycoprotein, the pharmacological inhibitor of the P-glycoprotein was without effect. However, it is now widely believed that P-glycoprotein is a channel regulator, rather than having inherent channel activity (Higgins 1995).

The *Torpedo* electric organ was used to clone the first voltage-gated Cl^- channel (ClC-0) (Jentsch et al., 1990). Subsequently, by homology screening, the ClC-2 protein was isolated from rat brain, and was found to be sensitive to changes in cell volume (Thiemann et al., 1992). The rat ClC-2 contains 907 amino acids and is ubiquitously expressed, with the highest levels found, in addition to the brain, intestine, and kidney (Thiemann et al., 1992). It was suggested that this channel contains 12 transmembrane domains, with a 13th domain being intracellular. When expressed in *Xenopus* oocytes, ClC-2 is observed to be active following hypo-osmotic stress or strong hyperpolarization (E_m < –90 mV) (Grunder et al. 1992). This channel shows an anion selectivity of $Cl^- > Br^- > I^-$ (which differs from $I^- > Br^- > Cl^-$ swelling-induced anion permeability sequence observed in most cells), with a slight inward I–V rectification. However, no such swelling-activated Cl^- channels have been detected in native tissue, although they have been identified by Northern analysis in a variety of tissue types, including neurons (Thiemann et al., 1992).

Paulmichi et al. (1992) cloned a protein termed pI_{Cln}, which was postulated to be involved in the activation of a volume-sensitive Cl^- conductance pathway. Expression of pI_{Cln} in *Xenopus* oocytes produced a prominent Cl^- current with similar characteristics to the hypo-osmotically induced Cl^- current described earlier (*see* Anion Permeability During RVD). This pI_{Cln}-associated current was observed in the absence of hypo-osmotic stress. From these observations, Paulmichi et al. (1992) proposed that pI_{Cln} was forming a β barrel pore. A similar Cl^- current was detected in a small percentage of control oocytes, suggesting the existence of an endogenous anion channel-like protein. Further studies indicated that this Cl^- current could be induced by hypotonicity in 99% of native uninjected oocytes (Ackerman et al., 1994). However, studies by Krapivinsky et al. (1994) with monoclonal antibodies (Mab) indicated that the endogenous pI_{Cln} does not form a Cl^- channel, but is necessary for the activation of the endogenous swelling-induced Cl^- current. In addition, immunochemistry studies indicated that pI_{Cln} is predominately immunolocalized to the cytosol and not to the plasma membrane (Krapivinsky et al., 1994). Although the present concept of pI_{Cln} is that it functions as a regulator of the swelling-induced Cl^- channel, Strange and Jackson (1995) propose that VSOAC in C6 glioma cells may be coded for by the I_{Cln} gene. They argue that the formation of a β barrel structure by I_{Cln} has structural characteristics of porins, which are primitive chan-

nels permeable to organic solutes, and that VSOAC, like porin, is permeable to several osmolytes, has brief spontaneous channel closures, and has a high open probability.

Cell Swelling and Metabolism

Several authors have considered the consequences of cell swelling for the metabolic machinery of the cell. In particular, recent observations demonstrate that cell swelling stimulates protein and glycogen synthesis to convert amino acids into "less active" osmolytes during volume regulation (Haussinger et al., 1994; Hue, 1994; Lang et al., 1995). In addition, cell swelling has been observed to inhibit glycolysis and stimulate glycine oxidation, glutamine breakdown, and formation of NH_4^+ and urea from amino acids, while also decreasing the mRNA for the principal enzymes involved in gluconogenesis (Haussinger et al., 1994). Haussinger et al. (1994) also report increased expression mRNA levels for β-actin and tubulin to help stabilize the cytoskeleton, since it may have been altered by cell volume perturbations.

Conclusions

Anion permeability pathways are an important feature during RVD in neuronal cells can be modulated by a diverse array of second messengers. Importantly, the swelling-activated anion pathway is also permeable to amino acids and organic osmolytes. Since the neuron is obligated to regulate its volume rapidly, swelling activation of a single pathway permeable to many substances may offer the fastest route for a return to the iso-osmotic state.

Although our understanding of volume regulation in the brain is increasing, many questions yet remain to be addressed—in particular, the volume-sensing mechanism Several candidates have been proposed for the volume-sensing mechanism, including membrane tension, cytoskeletal changes, macromolecular crowding, and changes in ionic strength. Similarly, the second messengers involved remain complex in many areas. With the advent of new technology, it is probably relevant to return to the whole-brain in vivo situation to investigate and characterize the processes involved in neuronal volume regulation.

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