Physiological significance of volume-regulatory transporters

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O'Neill, W. Charles. Physiological significance of volume-regulatory transporters. Am. J. Physiol. 276 (Cell Physiol. 45): C995–C1011, 1999.—Research over the past 25 years has identified specific ion transporters and channels that are activated by acute changes in cell volume and that serve to restore steady-state volume. The mechanism by which cells sense changes in cell volume and activate the appropriate transporters remains a mystery, but recent studies are providing important clues. A curious aspect of volume regulation in mammalian cells is that it is often absent or incomplete in anisosmotic media, whereas complete volume regulation is observed with isosmotic shrinkage and swelling. The basis for this may lie in an important role of intracellular Cl\(^{-}\) in controlling volume-regulatory transporters. This is physiologically relevant, since the principal threat to cell volume in vivo is not changes in extracellular osmolarity but rather changes in the cellular content of osmotically active molecules. Volume-regulatory transporters are also closely linked to cell growth and metabolism, producing requisite changes in cell volume that may also signal subsequent growth and metabolic events. Thus, despite the relatively constant osmolarity in mammals, volume-regulatory transporters have important roles in mammalian physiology.

cell volume; regulatory volume increase; regulatory volume decrease; sodium-potassium-chloride cotransporter; sodium-proton antiporter

DETERMINANTS OF CELL VOLUME

In discussing cell volume it is important to distinguish between factors that determine steady-state volume and mechanisms that correct acute perturbations in steady-state volume. This has been a source of confusion, and it is best to consider these as entirely separate processes. Transporters responsible for correcting acute changes in cell volume are usually not active at steady-state volume. Thus steady-state cell volume is not a balance between their ongoing, opposing actions. Rather, cells appear to establish a steady-state volume and then activate volume-regulatory transporters only when cell volume deviates from this "set point."
This is far more efficient than having opposing volume-regulatory transporters continuously active.

**Steady-State Cell Volume**

Mere existence in an isosmotic environment represents a continual threat to cell volume and integrity due to the large concentration of impermeant, negatively charged molecules within cells, such as proteins, nucleic acids, and other organophosphates. In addition to the osmotic force generated by the molecules themselves, there is an additional osmotic force due to an asymmetrical distribution of permeant ions created by the impermeant anions. Known as the Gibbs-Donnan equilibrium (65, 115), this is actually a steady-state disequilibrium. Unles the osmotic gradient is counter-balanced, there would be continuous influx of water and ions. In theory this counterbalancing could be accomplished by hydrostatic pressure, but it is highly unlikely that the plasma membrane can generate the tension required to balance the Donnan effect (115). In the absence of an opposing force, cells must either restrict permeability or actively extrude fluid. Because mammalian cells are generally quite permeable to water and there is no evidence for active transport of water, it must be solute that is actively transported and has restricted diffusion. This is illustrated by ionophores and other compounds that increase membrane permeability to Na\(^{+}\) and K\(^{+}\), which produce cell swelling without altering the Gibbs-Donnan "equilibrium." In addition, this confirms that membrane tension is not responsible for maintaining steady-state cell volume.

Another simple maneuver that causes cell swelling is replacement of extracellular Na\(^{+}\) with K\(^{+}\). Because neither the Gibbs-Donnan equilibrium nor other osmotic forces are altered (115), elimination of the outward K\(^{+}\) gradient is directly implicated in cell swelling. By virtue of the selective permeability of the plasma membrane to K\(^{+}\) over Na\(^{+}\), this gradient results in an outward K\(^{+}\) current and a negative membrane potential that dictates a low intracellular concentration of permeant anions, primarily Cl\(^{-}\). The source of energy is ultimately the Na\(^{+}\)-K\(^{+}\) pump, explaining why its inhibition often leads to cell swelling. This ability to extrude Cl\(^{-}\) (coupled with extrusion of Na\(^{+}\) by the Na\(^{+}\)-K\(^{+}\) pump) appears to be the principal mechanism that maintains steady-state cell volume. In cells with high anion permeability and low membrane potential, such as erythrocytes, it is the net cation extrusion by the Na\(^{+}\)-K\(^{+}\) pump coupled with low membrane permeability to both Na\(^{+}\) and K\(^{+}\) that limits Cl\(^{-}\) entry. In erythrocytes from carnivores, which lack Na\(^{+}\)-K\(^{+}\) pumps, net cation extrusion occurs through combined action of Ca\(^{2+}\) pumps (Ca\(^{2+}\)-ATPases) and Na\(^{+}\)/Ca\(^{2+}\) exchangers. The former generates a very large inward Ca\(^{2+}\) gradient that is then used to pump out Na\(^{+}\) via the latter (127). Cl\(^{-}\) accompanies Na\(^{+}\) efflux by virtue of the electronegativity of both transporters.

**Acute Changes in Steady-State Volume**

The steady-state disequilibrium that maintains cell volume can be threatened by changes in extracellular osmolarity, but a far more common threat is a change in the cellular content of osmotically active molecules, so-called isosmotic volume change. Cell volume can be viewed as a balance between the osmotic effects of high concentrations of impermeant anions and low concentrations of Cl\(^{-}\) within cells, and changes in either without offsetting changes in the other can alter cell volume. Changes in the former occur through synthesis, degradation, and fluxes, whereas changes in intracellular Cl\(^{-}\) concentration ([Cl\(^{-}\)]) arise from altered fluxes or membrane potential. Do the mechanisms described above for maintenance of steady-state cell volume protect cells from acute changes in cell volume as well? Cell shrinkage can reduce K\(^{+}\) conductance (37, 166), which should increase cell volume. However, the response would be slow and does not appear to contribute substantially to volume recovery (166). Although cell swelling has the opposite effect of increasing K\(^{+}\) conductance, this occurs through channels distinct from those responsible for basal K\(^{+}\) conductance.

Cells instead recruit additional mechanisms to correct acute deviations in their volume. These processes, termed regulatory volume increase (RVI) and regulatory volume decrease (RVD), occur through activation of specific transporters in the plasma membrane that mediate net fluxes of osmotically active molecules (and therefore water). Not surprisingly, both [Cl\(^{-}\)] and macromolecule concentration play key roles in governing these volume-regulatory responses. A further adaptation to osmotic stress is the accumulation of organic osmolytes such as sorbitol, sn-glycerol-3-phosphorylcholine, betaine, and taurine, which occurs in response to increased ionic strength rather than cell shrinkage (70, 207) and therefore is not truly a volume-regulatory mechanism. Instead, these compounds replace high intracellular salt concentrations that can perturb protein structure and impair enzyme function (18, 70), enabling cells to function in high osmolarity, such as in the renal medulla. Organic osmolytes also appear in the brain during chronic hypernatremia and comprise the so-called idiogenic osmoles (195). Organic osmolytes do have an impact on volume regulation, because they must be rapidly removed when normal osmolarity is restored in order to prevent cell swelling.

The mechanism by which cells sense changes in their volume is unknown but clearly is not through changes in osmolarity, ionic strength, or ion concentration, since isosmotic volume changes also activate RVI and RVD. Cell volume could be sensed chemically as changes in the intracellular concentration of impermeant molecules or mechanically through some form of stretch receptor. The former possibility, proposed by Minton et al. (129) and based on the concept of macromolecular crowding, is supported by direct experimental data. According to this theory, it is not cell volume per se that is regulated but rather the state of intracellular water.
In erythrocytes, volume-regulatory transporters can be activated in the absence of changes in cell volume by altering intracellular protein concentration or by adding agents that perturb protein hydration (157). These findings have been extended to nonerythroid cells, specifically renal tubular cells (83) and barnacle muscle (200). The question that remains is the nature of the sensor for macromolecular crowding. Mechanical sensing of cell volume is an attractive theory, since cell structure is directly threatened, but the evidence is indirect. Agents that perturb the actin cytoskeleton can inhibit RVD (24, 25, 42, 49, 184), and channels activated directly by membrane stretch can also be activated by cell swelling (176). Mechanical sensing of changes in cell volume remains speculative since no role for the cytoskeleton has been demonstrated in RVI, and the role of stretch-activated channels in RVD remains unproven. The issue of how cells sense their volume is far from resolved, and it is quite possible that both chemical and mechanical sensors are employed.

Consequences of Cell Shrinkage and Swelling

What exactly are the detrimental effects of changes in cell volume that volume-regulatory mechanisms are designed to prevent? Aside from extreme results such as cell lysis, this is an area that has received little attention. The most obvious effects of changing cell volume are mechanical, since the function of many cells depends on their architecture. Close examination reveals blebbing of the plasma membrane after severe hypertonic swelling (217). In addition to direct distortion, shrinkage and swelling also induce rapid changes in the actin cytoskeleton (Ref. 66 and P. B. Perry and W. C. O’Neill, unpublished observations), presumably as a protective measure to ease tension on the plasma membrane. At the tissue level, swelling of parenchymal cells can compromise regional blood flow, possibly aggravated by swelling of vascular endothelium and smooth muscle (17). Due to its rigid confines, the brain is the predominant site of morbidity from hyponatremia and hypernatremia, and, accordingly, is the tissue that exhibits the most complete volume regulation. At the cellular level, swelling and shrinkage could alter metabolic reactions through changes in the concentrations of enzymes and substrates. In this regard, cell swelling is more of a problem than cell shrinkage, which is well tolerated. For instance, in alveolar macrophages, hypertonic swelling decreases O2 consumption and increases lactate production while there is little effect of hyperosmotic shrinkage (169). In mouse L cells, halving cell volume with extracellular sorbitol does not alter growth or the oxidation or metabolism of glucose (119). In both these studies and in other studies, severe hypertonic shrinkage (osmolality >700 mosM) does impair cell growth and metabolism, but this appears to be an effect of increased ionic strength rather than cell shrinkage (18, 70).

REGULATORY VOLUME INCREASE

Volume-Regulatory Transporters

A seemingly simple way for cells to increase volume after shrinkage is by the opening of Na+ channels, with Cl− following, but this appears to be a rare event (214). Instead, volume regulation occurs primarily through electroneutral transporters, principally the Na+-K+-2Cl− cotransporter (NKCC1) and the Na+/H+ antiporter (NHE1). NKCC1 mediates coupled influx of Na+, K+, and Cl− and is found in virtually all cells. It is distinguished by its sensitivity to sulfamoylbenzoic acid derivatives, the so-called “loop” diuretics such as furosemide and bumetanide (56). NHE1, the ubiquitous NHE isoform, produces a net inward movement of ions by coupling with Cl−/HCO3− exchange (Fig. 1). The combined effect is influx of Na+ and Cl− with efflux of H+ and HCO3−. The latter two combine to form CO2, which diffuses back into the cell to regenerate H+ and HCO3−. The net result is influx of NaCl. The Na+−K+ pump is a necessary participant in RVI, exchanging Na+ for K+. The pump also provides the thermodynamic energy for RVI by maintaining a low intracellular Na+ concentration and, indirectly, a low [Cl−]. Amino acid uptake can also contribute to RVI (30), but this is probably minor at normal extracellular amino acid concentrations.

The mechanism by which cell shrinkage activates NKCC1 or NHE1 is unknown. Because activation occurs within minutes, it cannot be due to increased synthesis of transporters. Current data suggest that protein phosphorylation is involved. Activation of NKCC1 by shrinkage is associated with phosphorylation of the transporter (on serine and threonine, but not tyrosine) and can be blocked by kinase inhibitors and mimicked by inhibitors of protein phosphatases (90, 163). Although these agents have a similar effect on NHE1 activity (9), cell shrinkage does not increase phosphorylation of the transporter (53). The data imply the existence of a volume-sensitive protein kinase (or more than one) that phosphorylates NKCC1 and a
Table 1. Volume-regulatory response to hypertonic shrinkage in various mammalian tissues and cells

<table>
<thead>
<tr>
<th>Cell or Tissue</th>
<th>RVI</th>
<th>Transporter</th>
<th>Osmotic Agent</th>
<th>Notes</th>
<th>Reference</th>
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<td></td>
<td></td>
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<td>No</td>
<td>Mannitol</td>
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<td>Some RVI over many hours</td>
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<td></td>
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<td>Erythrocytes (human neonatal)</td>
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<td>NaCl</td>
<td>Some RVI over many hours</td>
<td>165</td>
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<tr>
<td>Erythrocytes (human)</td>
<td>Yes</td>
<td>NHE</td>
<td>NaCl</td>
<td>Neonatal</td>
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<tr>
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<td>Some RVI over many hours</td>
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<td></td>
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<td></td>
<td></td>
<td>67</td>
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<td>Renal epithelia</td>
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<td></td>
<td>Only with gradual shrinkage</td>
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<td></td>
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<td></td>
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<td>NKCC, NHE</td>
<td>Mannitol</td>
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<td></td>
<td>With or without ADH</td>
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<tr>
<td>CCD (rabbit)</td>
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<td>NaCl</td>
<td>Requires butyrate</td>
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<td>Raffinose</td>
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<td>?</td>
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<td>OMC (rabbit)</td>
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<td>Sucrose</td>
<td>Faster with ADH</td>
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<td>IMCD (rat)</td>
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<td>NKCC, NHE</td>
<td>NaCl</td>
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<td>Sucrose</td>
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<td>MDCK cells</td>
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<td>NaCl</td>
<td></td>
<td></td>
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<td>NaCl + osmolyte</td>
<td>NaCl</td>
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<td>MDCK</td>
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<td>Mannitol</td>
<td>Mannitol in isosmotic medium</td>
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<td>OK cells</td>
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<td>NaCl, NMDG, sucrose</td>
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<td>?</td>
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<td>220</td>
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<td>Other epithelia</td>
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<td></td>
<td></td>
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<td>Sucrose</td>
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<td>NKCC, NHE, amino acid</td>
<td>Mannitol</td>
<td>NHE in presence of CO2/HCO3</td>
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<td>Jejunal villus cells (guinea pig)</td>
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<td>NaCl</td>
<td></td>
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<td>Jejunal crypts (guinea pig)</td>
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<td>NKCC</td>
<td>NaCl</td>
<td>But not with mannitol</td>
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<td></td>
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<td>Nervous system</td>
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<td>NaCl, sucrose</td>
<td></td>
<td></td>
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<td>Whole brain (rabbit)</td>
<td>Yes</td>
<td>Glucose</td>
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<tr>
<td>Whole brain (rat)</td>
<td>Yes</td>
<td>NaCl, sucrose, mannitol</td>
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<td></td>
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<td>Neurons, ganglia (mouse)</td>
<td>Yes</td>
<td>NKCC</td>
<td>Sucrose</td>
<td>Choline in isosmotic medium</td>
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<td>Nerve terminals, brain (rat)</td>
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<td>NKCC</td>
<td>Sucrose</td>
<td>Slow, incomplete</td>
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<td>Astrocytes (rat)</td>
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<td>Mannitol</td>
<td></td>
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<td>Astrocytes (rat)</td>
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<td>NaCl</td>
<td></td>
<td>Declining baseline</td>
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<td>Sucrose</td>
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<td></td>
<td></td>
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<td>NaCl, mannitol</td>
<td></td>
<td></td>
<td>85</td>
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<td>C6 glioma cells</td>
<td>Yes</td>
<td>NKCC, NHE</td>
<td>NaCl</td>
<td>Requires additives</td>
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<td>NaCl</td>
<td></td>
<td>Additives present</td>
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<td>NaCl</td>
<td></td>
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<td>Mesenchymal</td>
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<td>Osteosarcoma</td>
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<td>Sodium gluonate</td>
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<td>Occasional RVI in situ</td>
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<td>Gingival fibroblasts</td>
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<td>Sucrose</td>
<td>Slow, amino acids required</td>
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<td>Sucrose, NaCl</td>
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<td>Skeletal muscle (rabbit)</td>
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<td>NaCl</td>
<td></td>
<td>Minimal RVI; no RVI with sucrose</td>
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<td></td>
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<td>Mannitol in isosmotic medium</td>
<td>205</td>
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<td>Cardiovascular</td>
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<td>Heart (cat)</td>
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<td>Sucrose</td>
<td>Sucrose in isosmotic medium</td>
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<td>Lung (rabbit)</td>
<td>No</td>
<td>NaCl</td>
<td></td>
<td></td>
<td>213</td>
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regulatory protein that activates NHE1. Hopefully, the recent demonstration of a volume-sensitive kinase that phosphorylates NKCC1 in vitro will lead to identification of this kinase (145). Although the identity of this kinase is unknown, other volume-sensitive kinases have been tentatively identified. One of these is myosin light chain kinase (MLCK), which is activated by shrinkage in vascular endothelial and smooth muscle cells (89), mesangial cells (203), and glial cells (188). Inhibition of MLCK blocks activation of NKCC1 in shrunken endothelial cells (89) and activation of NHE1 in shrunken astrocytes (188). However, MLCK is not the volume-sensitive kinase that phosphorylates NKCC1, and the mechanism by which myosin light chain phosphorylation influences NKCC1 and NHE1 is unknown. Hypertonicity increases phosphorylation of stress-activated protein kinases and mitogen-activated protein (MAP) kinases (45, 69). It is not known whether this is an effect of hypertonicity or cell shrinkage, and phosphorylation of MAP kinase also occurs after hypotonicity, suggesting a nonspecific stress response rather than a specific response to cell volume. However, phosphorylation and activation of the tyrosine kinases p59^gr and p56^ck occurred in neutrophils shrunken both hypertonically and isosmotically (91), directly implicating cell shrinkage.

Hypertonic vs. Isosmotic Shrinkage

It is important to distinguish between these two fundamentally different types of cell shrinkage because the response frequently differs, a fact that is often not appreciated. Hypertonic shrinkage removes only water from cells, whereas isosmotic shrinkage results from loss of osmotically active molecules, usually KCl. Examples of isosmotic shrinkage include returning cells to isotonic medium after volume regulation in hypotonic medium, substituting external Na^+ or Cl^- with impermeant molecules, and increasing intracellular Ca^{2+} concentration ([Ca^{2+}]) to open K^- and Cl^- channels. RVI almost always occurs after isosmotic shrinkage but is infrequent after hypertonic shrinkage. Although occasionally noted, absence of hypertonic RVI has largely been ignored and never systematically examined. Table 1 is an attempt at a complete listing of mammalian cells and tissues in which hypertonic RVI has been examined. For this purpose, RVI was defined as a significant volume increase occurring within 1 h. Although hypertonic RVI does correlate somewhat with tissue type or origin, being more common in glial, neural, and epithelial cells, there are clearly discrepancies between studies on the same tissue or cell type. Absence of hypertonic RVI cannot be explained by lack of volume-regulatory transporters, because most of the cells listed undergo RVI after isosmotic shrinkage. One exception is mature human erythrocytes, which do have a low complement of transporters.

Can thermodynamics explain the lack of hypertonic RVI? When hypertonicity is produced with an impermeant molecule such as sucrose or mannitol, the concentration of extracellular ions is unchanged while intracellular ions increase in concentration, thereby reducing the driving force for influx. This can actually lead to further cell shrinkage (101) and is accentuated by the occasional use of impermeant molecules in place of NaCl in the reference isotonic solution. However, hypertonic RVI is stillinfrequent when NaCl is the osmotic agent. Given reasonable estimates of intracellular ion activities, activation of NKCC1 should result in net influx of ions, primarily because of the inwardly directed Cl^- gradient. In the absence of accurate determinations of intracellular ion activities, it can always be argued that NKCC1 is at equilibrium. However, substitution of external Na^+ with K^+ to equalize their concentrations still does not result in net influx and RVI, despite the fact that the driving force for influx is increased sevenfold (143, 168). Furthermore, thermodynamics cannot explain a lack of hypertonic RVI by NHE1 and anion exchange, provided some CO2 and
HCO$_3^-$ are present. These results indicate that the lack of RVI in hypertonic medium is a regulatory event rather than a thermodynamic effect. This could be because the responses to the two types of shrinkage are inherently different (40) or could be explained by superimposed regulatory effects of ionic strength or intracellular ion concentrations. In either event it is clear that cells can sense how they are being shrunk.

Although the difference between isosmotic and hypertonic shrinkage could be sensed as a change in ionic strength or an agonist-induced signal, the most logical candidate is [Cl$^{-}$]. Cl$^{-}$ is a minority anion in most cells, yet it is the major permeant anion, with the majority of anions being impermeant proteins and organophosphates. While hypertonic shrinkage increases [Cl$^{-}$], in parallel with all other cytoplasmic constituents, [Cl$^{-}$], decreases with isosmotic shrinkage, since the fluid moving out of cells has a higher Cl$^{-}$ concentration than cytoplasm. As with hypertonic shrinkage, the concentration of all other cytoplasmic constituents increases (except for K$^+$, which should change little since it comprises the vast majority of intracellular cations). The decrease in [Cl$^{-}$] is actually greater than the decrease in cell volume, making Cl$^{-}$ concentration a more sensitive indicator of changes in cell volume than cell volume itself under isosmotic conditions. As shown in Fig. 2, if Cl$^{-}$ accounts for one third of cellular anions and a cell loses one sixth of its volume via loss of KCl, half the cell Cl$^{-}$ is lost and [Cl$^{-}$] theoretically decreases by 40% (1/2 the original Cl$^{-}$ content in 5/6 the original volume). It is not surprising then that [Cl$^{-}$] has important effects on volume-regulatory transporters.

[Cl$^{-}$] inhibits NKCC1, and this occurs through inhibition of phosphorylation and probably through a direct effect on the transporter as well. Phosphorylation of NKCC1 is stimulated by lowering [Cl$^{-}$] and inhibited by raising [Cl$^{-}$] (57, 111), probably via effects on the same kinase activated by cell shrinkage (110). However, this cannot be the principal mechanism that blocks hypertonic RVI, since there is still a substantial increase in NKCC1 phosphorylation with hypertonic shrinkage, suggesting that Cl$^{-}$ has an additional, direct effect on NKCC1. The inhibition of NKCC1 by Cl$^{-}$ in internally perfused squid axons (16) is consistent with this hypothesis. In a sense this represents classic feedback inhibition of NKCC1 and provides an ideal yet simple mechanism for regulating cell volume. The nature of this Cl$^{-}$ effect is unclear. In endothelial cells, hypertonic and isosmotic shrinkage activate unidirectional influx through NKCC1 to about the same degree, yet net influx only occurs after the latter (143). This suggests that intracellular Cl$^{-}$ blocks net influx while allowing the partial reaction [K$^+$][Cl$^{-}$][H$^+$]/[K$^+$][HCO$_3^-$] to continue. Evidence for inhibition of NKCC1 by internal Cl$^{-}$ has also been obtained in Ehrlich ascites cells (101), salivary acinar cells (168), and tracheal epithelial cells (58). In many cells NKCC1 apparently functions in a similar mode at steady-state volume as well (21, 46, 101, 116, 143, 150), essentially “running in neutral” due to inhibition by intracellular Cl$^{-}$, yet poised for rapid influx when [Cl$^{-}$] decreases. Because cells already expend energy to counteract swelling due to the Gibbs-Donnan equilibrium, it makes little sense to allow net influx via NKCC1 or NHE1 at steady-state volume.

The mechanism by which hypertonic shrinkage prevents RVI by NHE1 has not been examined. In the absence of CO$_2$/HCO$_3^-$, RVI could be limited by the alkalization that accompanies Na$^+$ influx and rapidly shuts off NHE1. For instance, if shrinkage produces a 0.2 unit alkaline shift in the pH set point for NHE1 and the internal buffering capacity is 30 mM/pH unit, NHE1 shuts off after an influx of only 6 mM Na$^+$. The fact that more substantial RVI via NHE1 can occur in the nominal absence of CO$_2$/HCO$_3^-$ (after isosmotic shrinkage) indicates that there is still enough CO$_2$/HCO$_3^-$ to permit some Cl$^{-}$/HCO$_3^-$ exchange. Because hypertonic RVI requires more Na$^+$ influx, it may be substantially slower under these conditions. However, there appears to be an additional mechanism since hypertonic shrinkage can actually suppress activation of NHE1, presumably due to an effect of internal Cl$^{-}$ (168).

Inhibition of RVI by intracellular Cl$^{-}$ may explain the variable responses to hypertonic shrinkage noted in Table 1. Epithelial cells, which frequently exhibit hypertonic RVI, may have a significantly lower [Cl$^{-}$], that is below the threshold for inhibition of RVI even after hypertonic shrinkage. Some epithelia that do not exhibit RVI under standard hypertonic conditions do so in the presence of agents that raise cAMP levels (43, 54, 202), most likely via activation of Cl$^{-}$ channels and reduction of [Cl$^{-}$]. Hypertonic RVI by C6 glioma cells appears to require additives as well, including insulin and PGE$_1$ (133). This could also be due to a decrease in [Cl$^{-}$], but the possibility that these agents might modulate the inhibitory effect of Cl$^{-}$ cannot be ruled out. An additional additive that confers hypertonic RVI on renal epithelial cells is butyrate (135, 170). The mechanism is not known, but the RVI is only partly accounted for by monovalent ions (170), suggesting that butyrate stimulates the synthesis of organic osmo-lytes. The occasional observation of hypertonic RVI in
other cells and the discrepancies between studies employing similar cells could be due to subtle differences in culture conditions or the inadvertent presence of agonists or growth factors, perhaps autocrine in nature. Hypertonic RVI may also depend on the rate of shrinkage. In renal proximal tubules, no shrinkage occurred when tonicity was increased 1.5 mosM/min, whereas the cells behaved as perfect osmometers at 3 mosM/min (104). A similar response has been described in C6 glioma cells (134), but these cells do exhibit some RVI after acute hypertonic shock (133). The basis for this so-called isovolumetric regulation is unknown but could relate to the fact that [Cl\(^{-}\)] is not markedly elevated during shrinkage. This phenomenon is probably not widespread, since most tissues do not exhibit RVI during hypertonicity in vivo, which also develops gradually.

Hypertonic Shrinkage In Vivo

The lack of RVI in most isolated or cultured cells is supported by data obtained in whole tissue and in vivo. During infusion of mannitol into dogs, the total intracellular water compartment behaves as a perfect osmometer (216), and in perfused skeletal muscle, which accounts for 75% of total intracellular water, there is no recovery of intracellular water after hypertonic shrinkage (68). A 6% increase in osmolarity in rats produced by dehydration decreased total intracellular water by 11% (137), with a decrease noted in muscle and several other tissues except brain. In dehydrated humans, loss of cellular water from skeletal muscle was precisely the other tissues except brain. In dehydrated humans, loss of cellular water from skeletal muscle was precisely the inverse of the 7% increase in osmolarity (26), with no increase in the content of K\(^{+}\) or Cl\(^{-}\). Hypertonic infusions of NaCl, sucrose, or mannitol in rabbits also produced the expected decrease in total intracellular water and muscle cell water with no increase in K\(^{+}\) content (3, 192), again indicating absence of RVI. In hypertonically perfused lung, total alveolar cell volume decreased in exact inverse proportion to osmolarity (213), indicating that the lack of RVI extends to other tissues as well. The absence of hypertonic RVI in vivo is consistent with the clinical dictum that water deficits are distributed equally across all compartments.

Based on both in vitro and in vivo data, it appears that mammalian cells are poised to undergo RVI after isosmotic shrinkage but not hypertonic shrinkage. This might seem appropriate since these cells reside in an environment of constant osmolarity. But is the osmolarity of mammalian plasma and interstitial fluid truly that constant? Despite the best efforts of the hypothalamus, kidneys, colon, and skin, significant water losses still occur during water deprivation. Thus osmolarity fluctuates with the frequency of water intake. In various rodents and ungulates, particularly those that reside in arid areas, water loss that exceeds 20% of body weight is well tolerated (112, 181). Deprivation for as short as 1 day can lead to a 6% or greater weight loss (112, 181). Some of this fluid comes from the intestinal lumen (113) and there is also excretion of electrolytes, so the corresponding rise in osmolarity is not as great. Deprivation of water increases plasma osmolarity 6–7% after 8 days in kangaroo rats (193), 4 days in dogs (224), and 3 days in Moroccan goats (76). In humans, strenuous exercise in hot conditions without water can result in water losses up to 5% of body weight (8% of total body water, 7% increase in osmolarity) without functional impairment (26, 100). Because periods of water deprivation are probably common in mammals, hypertonicity appears to be a routine occurrence that is well tolerated.

The lack of hypertonic RVI may instead be due to potential detrimental effects on the organism. Substantial water losses are often well tolerated, without circulatory collapse, because the loss is distributed across all compartments (intracellular, interstitial, and intravascular). If RVI occurred, the water loss would be concentrated in the one third of body water that is not intracellular. A 10% loss of body water would instead result in a 30% decrease in extracellular volume, including intravascular volume. Thus the absence of RVI helps maintain vascular volume during water deprivation, essentially allowing tissues to serve as a water reservoir (112). Another serious consequence of RVI would be the profound transcellular shift of K\(^{+}\). Correction of a mere 1% cell shrinkage via cellular uptake of KCl could decrease plasma K\(^{+}\) concentration as much as 3 mM.

Because of the potential detrimental effects of hypertonic RVI and the seemingly minimal detrimental effects of mild hypertonic shrinkage, RVI may be sacrificed for the benefit of the whole organism. Exceptions appear to be intestinal epithelium (138) and brain (3, 28, 29, 137, 192) and cells cultured from these sites (116, 133). Because intestinal epithelium is one of the few tissues routinely exposed to sudden changes in osmolarity, hypertonic RVI would be appropriate, particularly since cell shrinkage could disrupt the mucosal barrier. The brain is a critical organ with limited capacity to shrink without structural damage because of the inelasticity of the skull. Another exception may be chondrocytes. The extracellular fluid in cartilage is hypertonic compared with plasma, owing to the high concentration of fixed, polyanionic macromolecules, and this can change with applied load (64). Hypertonic RVI is occasionally observed in chondrocytes in situ but not in isolated chondrocytes (Ref. 63 and A. C. Hall, personal communication). The specific mechanism that allows hypertonic RVI to proceed in some tissues and cells but not in others is unknown.

Isosmotic Shrinkage In Vivo

It appears that volume-regulatory pathways in mammalian cells are designed with the dual purpose of avoiding RVI after hypertonic shrinkage yet mediating rapid volume recovery after isosmotic shrinkage. So when does the need for isosmotic RVI arise? Isosmotic shrinkage results from cellular loss of osmotically active molecules, usually K\(^{+}\) and Cl\(^{-}\), since they are the principal permeant ions. This can occur rapidly during agonist stimulation (58, 146, 168). In cultured vascular endothelial cells, cell volume decreases 16% over 10 min in response to Ca\(^{2+}\)-mobilizing agonists by virtue of
Ca\(^{2+}\)-dependent K\(^{+}\) channels (and probably Cl\(^{-}\) channels as well). Inhibition of NKCC1 doubles the shrinkage, indicating that RVI is required to stabilize cell volume during agonist stimulation. In addition to maintaining cell volume, this process also prevents large decreases in [Cl\(^{-}\)], that could impair agonist-induced Cl\(^{-}\) currents. RVI also occurs during stimulation of secretory epithelia (41, 58, 211) where large solute losses across the apical membrane would lead to rapid shrinkage if not compensated by influx across the basolateral membrane. Not surprisingly, NKCC1 and NHE1 are components of the basolateral membrane and become quite active when apical Cl\(^{-}\) channels are open (57, 58, 168). This coupling of apical efflux and basolateral influx via cell volume provides a simple mechanism for maintaining cell volume during large transepithelial fluxes, a process that has been termed homocellular volume regulation (183). Another example of isosmotic shrinkage may be the decrease in mucosal volume of the intestine during fasting (61). In rats, a 15\% volume decrease occurs during a 48-h fast and is rapidly corrected within minutes after exposure to an electrolyte solution. The restoration of cell volume after refeeding is associated with stimulation of NHE1 (61).

**REGULATORY VOLUME DECREASE**

**Volume-Regulatory Transporters**

The predominant pathway for RVD is the opening of K\(^{+}\) channels and anion channels. The K\(^{+}\) channels involved are usually large-conductance, Ca\(^{2+}\)-activated channels (so-called BK channels). The molecular basis of the anion conductance is unknown and may comprise more than one channel (196). The conductance can be quite nonselective and can include a variety of organic anions in addition to Cl\(^{-}\) (196), hence the term volume-sensitive organic anion channel (VSOAC). VSOACs are quite ubiquitous, but differences exist between cell types, particularly in the pharmacology of inhibition. Conductance of organic anions in addition to Cl\(^{-}\) is appropriate for a volume-regulatory channel because [Cl\(^{-}\)]\(_{i}\) is often low and could reach equilibrium across the cell membrane before RVD is complete. Furthermore, there is a much greater outward electrochemical gradient for organic anions than for Cl\(^{-}\}. However, organic osmolytes are very expensive metabolically, since they must be replaced by synthesis or by uptake from extracellular fluid against a steep concentration gradient. It would be best to use organic anions for RVD only when [Cl\(^{-}\)]\(_{i}\) is low and this may explain the inhibition of VSOACs by Cl\(^{-}\). When [Cl\(^{-}\)]\(_{i}\) is above a certain level, VSOACs are inhibited and RVD occurs by Cl\(^{-}\) loss through a separate pathway (34, 196). Only when [Cl\(^{-}\)]\(_{i}\) is low do cells open VSOACs and resort to organic anions for RVD. The “willingness” of cells to sacrifice these compounds attests to the importance of preventing cell swelling. Inhibition of VSOACs by Cl\(^{-}\) not only conserves Cl\(^{-}\) but also ensures that cells respond appropriately to the cause of swelling. When swelling is due to Cl\(^{-}\) uptake, RVD occurs via Cl\(^{-}\) loss. In contrast, when swelling is due to accumulation of organic compounds (such as amino acids), Cl\(^{-}\) is lost to a certain level (to accommodate the organic compounds) below which the organic osmolytes are lost.

An additional transporter that mediates RVD is the K\(^{+}\)-Cl\(^{-}\) cotransporter (KCC), which transports K\(^{+}\) and Cl\(^{-}\) stoichiometrically in either direction across the plasma membrane (98). Although structurally related to NKCC1 and inhibited by furosemide, this transporter is distinguished by its Na\(^{+}\) independence and insensitivity to bumetanide. Among physiological anions, it has a strict requirement for Cl\(^{-}\) and, not surprisingly, is the principal mechanism for RVD in cells with a high [Cl\(^{-}\)], such as erythrocytes. Studies of KCC have been hampered by the lack of a specific inhibitor, but, based on mRNA abundance, the transporter is probably more prevalent than previously thought (47, 48, 132). For instance, it appears to be responsible for about one-half the RVD in aortic endothelial cells (160).

The mechanisms by which cell swelling activates volume-sensitive channels and KCC are poorly understood. The nature of the K\(^{+}\) channels involved suggests Ca\(^{2+}\) as a signal, and evidence in support of this has been obtained primarily in epithelial cells (22, 31, 72, 121, 122, 172, 173, 189, 221). The hypothesis based on these data is that cell swelling induces Ca\(^{2+}\) influx, possibly via stretch-activated channels, leading to a rise in [Ca\(^{2+}\)]\(_{i}\) and subsequent activation of BK channels. However, in many other cells only a small rise in [Ca\(^{2+}\)]\(_{i}\) occurs and is not required for activation of K\(^{+}\) efflux (52, 74, 125, 161, 222). One possible explanation for this discrepancy is the distinction between dependence on Ca\(^{2+}\) and activation by Ca\(^{2+}\). The K\(^{+}\) channels may require Ca\(^{2+}\) for activity, but swelling could open them through a separate mechanism. Maneuvers that block increases in [Ca\(^{2+}\)], (such as removing extracellular Ca\(^{2+}\)), often lower basal [Ca\(^{2+}\)], thereby preventing channel activation by any mechanism. Further evidence against the Ca\(^{2+}\)-independence andactivation by Ca\(^{2+}\) is the theory that Ca\(^{2+}\) is not involved in the activation of anion channels or K\(^{+}\)-Cl\(^{-}\)cotransport in swollen cells. Because the evidence for Ca\(^{2+}\) signaling of RVD is quite compelling in epithelial cells, it is reasonable to hypothesize two pathways for activation of K\(^{+}\) channels by cell swelling: a mechanically sensitive Ca\(^{2+}\) influx that is the primary pathway in epithelia and a Ca\(^{2+}\)-independent pathway operational in nonepithelial cells. The nature of the Ca\(^{2+}\)-independent signal is unknown, but phospholipase A\(_2\) has been implicated in volume-sensitive K\(^{+}\) and anion channels (136, 147), whereas protein kinase A has been implicated in the activation of KCC in swollen erythrocytes (11, 82, 84). Unfortunately, it has not yet been possible to determine whether the cotransporter itself is phosphorylated.

**Hypotonic vs. Isosmotic Swelling**

Essentially all cells (with the exception of some erythrocytes) respond to hypotonic swelling with an RVD, but the response is usually incomplete and cells remain swollen. The failure to complete RVD has re-
mained a mystery, but recent studies in endothelial cells provide an explanation (Fig. 3). When these cells are swollen isosmotically by loading them with KCl, which raises [Cl\(^-\)], as opposed to the decrease in [Cl\(^-\)], after hypotonic swelling, there was a complete and far more rapid RVD. In fact there was an overshoot that was gradually corrected. Complete RVD could also be accomplished in hypotonic medium by adding bumetanide to inhibit NKCC1. These data indicate that incomplete RVD in hypotonic medium was due to a decrease in [Cl\(^-\)], that resulted in net influx via NKCC1. Hypotonic RVD in the presence of bumetanide is still slower than isosmotic RVD, which may be a manifestation of the smaller outward Cl\(^-\) gradient or stimulation of RVD pathways by Cl\(^-\). A similar phenomenon has been described in Ehrlich ascites cells, in which swelling activates Na\(^+\)/H\(^+\) exchange and amiloride enhances RVD (103). Thus, incomplete RVD in hypotonic medium does not represent inadequacy of RVD pathways or their sensitivity to cell volume but rather represents a new steady-state volume at which efflux through RVD pathways equals influx through RVI pathways that are activated by a decrease in [Cl\(^-\)]. Thus, as with RVI, RVD also depends on how volume is altered and centers on [Cl\(^-\)], and its conservation.

Hypotonic Swelling In Vivo

Despite the fact that mammalian cells regulate their volume in hypotonic medium, the need rarely arises in vivo. At the level of the whole organism, hypotonicity can only occur from excess water intake (in the absence of pathological states), which is possible since thirst is not driven exclusively by osmolarity. For instance, mild hypotonicity will develop when water is provided to rats deprived of water for 10 h (182). As a localized phenomenon, hypotonicity may be more frequent. Ingestion of water can render the intestinal lumen hypotonic, and this can even extend to the liver since portal blood can become hypotonic after water ingestion (71). The RVD exhibited by isolated cells appears to accurately reflect the occurrence of RVD in vivo. In rats made hyponatremic through administration of water and vasopressin, total intracellular water increased far less than predicted and total body K\(^+\) decreased (208, 209), consistent with RVD. This is indicative of RVD in skeletal muscle, since it contains most of the cellular water, but hypotonic RVD has also been demonstrated in specific organs including liver (59, 96), heart (151), and brain (195).

Isosmotic Swelling In Vivo

Isosmotic swelling is a much more common occurrence and results from an increase in the cellular content of osmotically active molecules. Examples include uptake of nutrients, acidosis, or depolarization of the cell membrane potential. Uptake of nutrients, particularly amino acids and sugars, can and must occur rapidly in tissues responsible for their assimilation after meals such as intestinal epithelium, liver, and skeletal muscle. In Ehrlich ascites cells, for instance, 10 mM glycine, a physiological postprandial concentration, produces a 17% increase in cell volume through Na\(^+\)-coupled uptake (77), and a similar response has been noted in intact, perfused liver (71). Nutrient-induced swelling followed by RVD is also observed in mammalian intestine (117). RVD after nutrient uptake is incomplete, probably due to dilution of intracellular Cl\(^-\).

Acidosis can also increase cell volume, a response that is distinct and opposite from the effect of pH on the Gibbs-Donnan equilibrium. By titrating negative charges on impermeant molecules, a reduction in pH decreases the tendency of cells to swell. However, this effect is more than offset by accumulation of the anions that accompany the H\(^+\) and replace impermeant anions titrated by the H\(^+\) (79). If intracellular buffering capacity is 30 mM/pH unit, lowering cell pH by 0.5 with a monovalent acid would lead to accumulation of 15 mM anion. Cell swelling occurs during all three types of acidosis: organic, inorganic, and respiratory. With organic acids such as lactic acid, the anion accumulates not only within cells in which they are synthesized but also in other cells, because the acids are weak and permeant and because the anions can be transported (105). The resulting swelling has been demonstrated in astrocytes (80, 81, 105) and erythrocytes (191). Hydrochloric acidosis, the principal inorganic acidosis in vivo, can be created in vitro by replacing HCO\(_3\)\(^-\) with Cl\(^-\). The assumption is that HCO\(_3\) leaves cells in exchange for Cl\(^-\), transmitting the acidosis to the cytoplasm. Because of intracellular buffering, the amount of HCO\(_3\) leaving the cell and replacement anions entering will be greater than the drop in HCO\(_3\) concentration, leading to an increase in osmotically active molecules. In renal proximal tubular cells, a decrease in HCO\(_3\)
In contrast, exposure of rat skeletal muscle to 30% CO2 skeletal muscle (171), implying that RVD had occurred. Such swelling occurs during vigorous exercise, problematic since it could compromise regional blood during repetitive contraction would be particularly expected swelling has in fact been demonstrated in nerve fibers (78) and in cultured myogenic cells (185). Although the Na
molecule per cycle. Thus the Na
pump is an
1
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pump can rapidly export the excess Na
, much of this is exchanged for K
, with the resulting net efflux of only one osmotically active molecule per cycle. Thus the Na
-K
 pump is an inefficient volume-regulatory transporter that cannot rapidly relieve cell swelling. Swelling of skeletal muscle during repetitive contraction would be particularly problematic since it could compromise regional blood flow. Such swelling occurs during vigorous exercise, accompanied by an increase in Cl
 content (123, 190), but the swelling cannot be ascribed solely to repetitive depolarization since acidosis could also contribute to the swelling. The increase in intracellular water is small, implying that volume regulation has occurred. Volume regulation via Ca
-activated K
 channels could serve the dual purpose of repolarizing the membrane potential (15). A dramatic example of isosmotic swelling in vivo, possibly related to depolarization and acidosis, occurs after ischemia (39, 215). Although ischemia directly swells cells in vitro (92), in vivo this may require reperfusion (215). The marked swelling of endothelium probably contributes to reduced blood flow and further ischemia, since perfusion with hypertonic solutions maintains blood vessel patency and organ perfusion (39), and prevents ischemic damage (2). The cellular events responsible for cell swelling during reperfusion and the role of volume-regulatory transporters are unknown.

### CHANGES IN THE SET POINT FOR STEADY-STATE CELL VOLUME

Steady-state volume varies over the lifespan of a cell, particularly during growth and differentiation. This obviously requires suppression of normal volume-regulatory mechanisms, but volume-regulatory transporters may also play a more direct role. Activation of NHE1 or NKCC1 in the absence of cell shrinkage could lead to cell enlargement, whereas volume-independent activation of RVD pathways could lead to cell shrinkage. Although the role of volume-regulatory transporters in adjusting steady-state cell volume has not been established, the rapid activation of NKCC1 (152, 153, 155, 156, and W. C. O’Neill, J. D. Klein, and S. T. Lamitina, unpublished observations) and NHE1 (60, 175) by growth factors lends credence to this concept. Addition of serum produces a rapid increase in cell volume (15% over 10 min) in endothelial cells, which is mediated by NKCC1 (W. C. O’Neill, J. D. Klein, and S. T. Lamitina, unpublished observations) and a similar response occurs in growth-stimulated fibroblasts (126, 152, 153). A more chronic stimulation of NKCC1 is also seen with growth factors (Ref. 19 and W. C. O’Neill, J. D. Klein, and S. T. Lamitina, unpublished observations) and contributes to the increase in cell volume during the growth cycle (19). This switch in NKCC1 from its normal mode of K
/K
 exchange at steady-state volume to a net influx mode implies that growth stimuli can modulate or overcome the putative inhibitory effect of intracellular Cl
. Activation of NHE1 by growth factors also increases cell volume (51), which, like NKCC1, requires disinhibition of the transporter at steady-state volume, in this case by shifting the pH dependence to the right (51). The mechanism by which growth factors activate NHE1 and NKCC1 is not understood. Phosphorylation of NHE1 is increased (178), but a mutated antipporter lacking phosphorylation sites is still activated by mitogens, although to a lesser degree (210). Regulation of NKCC1 by growth factors has not been examined at the molecular level and, whatever the mechanism, it must overcome the normal inhibition of net influx (presumably due to intracellular Cl
) to increase cell volume. Volume regulatory transporters may also be responsible for cell enlargement during transformation. Fibroblasts expressing the ras oncogene were found to be 32% larger than control cells, with upregulation of both NKCC1 and NHE1 (95, 126), whereas cell enlargement accompanying cytomegalovirus infection is associated with enhanced activity of NHE1 (27).

Cell shrinkage is an early and dramatic event during apoptosis (6, 7, 149). The mechanism is unknown but appears to be biphasic. In dexamethasone-treated lymphoid cells, shrinkage begins in 12 h and progresses until chromatin condensation (36 h). Although this is associated with a net loss of K
, other cytoplasmic components are lost as well, since buoyant density does not change (7). The second phase of shrinkage after...
The concept that cell volume controls cell growth is considerably strengthened by evidence that cell volume also regulates cell metabolism. Swelling (either hypotonic or isosmotic) of hepatocytes (109, 167, 194) increases protein synthesis and decreases protein degradation, whereas hypertonic shrinkage has the opposite effect (71) in liver and in other cells (162). Parallel effects are seen on glycogen synthesis and breakdown (71, 159). This process also represents a volume-regulatory mechanism, since osmotically active molecules are consumed during synthesis of protein and glycogen and released during proteolysis and glycogenolysis. In the liver, changes in cell volume serve to couple metabolism to nutrient uptake and hormone action (71). Nutrient uptake and insulin both cause cell swelling (the latter via stimulation of NKCC1 and NHE1), leading to an increase in the content of protein and glycogen. Prevention of cell swelling by inhibiting NKCC1 and NHE1 blocks this anabolic response. On the other hand, glucagon causes cell shrinkage (probably via opening of K⁺ channels), thereby inducing a catabolic response (proteolysis and glycogenolysis). There is in fact a linear relationship between hepatocyte volume and protein synthesis and proteolysis along which many anabolic and catabolic stimuli fall (Fig. 4). The anabolic and catabolic effects of cell volume may exist in skeletal muscle as well (106–108), but the role of cell volume in the action of insulin in target tissues other than liver is unclear (223). The signaling mechanisms by which cell volume controls cell metabolism and growth are unknown but could be related to known growth signaling cascades. For instance, cell swelling has been shown to activate tyrosine kinases, phosphorylate (and activate) MAP kinases (180, 204), and to increase c-jun phosphorylation (180), c-jun mRNA abundance (38), and phosphorylation of ribosomal protein S6 (109). To what extent any of these is responsible for the metabolic effects of cell volume is unknown.

SUMMARY

The purpose of this review is to dispel the notion that cell volume regulation in higher organisms is a curious but physiologically irrelevant phenomenon. Osmolarity of the milieu interieur is not as constant as is often assumed, and significant threats to cell volume still exist in an isosmotic environment under both physiological and pathological conditions. In addition to maintaining cell volume, volume-regulatory mechanisms are also employed to initiate changes in cell volume, as...
required for cell growth and differentiation. The close link between volume-regulatory transporters, cell volume, and cell growth and metabolism indicates that regulation of cell volume is a fundamental and important property of mammalian cells. The sensing and signaling mechanisms for cell volume regulation remain a mystery, and elucidation of these pathways will provide important new information on other aspects of cell physiology as well.

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