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Physiology of Cell Volume Regulation in Vertebrates

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Hoffmann EK, Lambert IH, Pedersen SF. Physiology of Cell Volume Regulation in Vertebrates. *Physiol Rev* 89: 193–277, 2009; doi:10.1152/physrev.00037.2007.—The ability to control cell volume is pivotal for cell function. Cell volume perturbation elicits a wide array of signaling events, leading to protective (e.g., cytoskeletal rearrangement) and adaptive (e.g., altered expression of osmolyte transporters and heat shock proteins) measures and, in most cases, activation of volume regulatory osmolyte transport. After acute swelling, cell volume is regulated by the process of regulatory volume decrease (RVD), which involves the activation of KCl cotransport and of channels mediating K^+, Cl^-, and taurine efflux. Conversely, after acute shrinkage, cell volume is regulated by the process of regulatory volume increase (RVI), which is mediated primarily by Na^+/H^+ exchange, Na^+-K^+-$2Cl^-$ cotransport, and

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Na^+ channels. Here, we review in detail the current knowledge regarding the molecular identity of these transport pathways and their regulation by, e.g., membrane deformation, ionic strength, Ca^{2+}, protein kinases and phosphatases, cytoskeletal elements, GTP binding proteins, lipid mediators, and reactive oxygen species, upon changes in cell volume. We also discuss the nature of the upstream elements in volume sensing in vertebrate organisms. Importantly, cell volume impacts on a wide array of physiological processes, including transepithelial transport; cell migration, proliferation, and death; and changes in cell volume function as specific signals regulating these processes. A discussion of this issue concludes the review.

I. INTRODUCTION

The animal cell membrane is, with a few exceptions, highly permeable to water. Cell water content and cell volume are thus determined by the cellular content of osmotic active compounds and by the extracellular tonicity. As detailed below, the osmotic water permeability of animal cells is several orders of magnitude greater than the permeability towards Na^+, K^+, and Cl^-. In many cell types, a high water permeability reflects the presence of aquaporins (AQPs), and it has been shown that the presence of AQP2 (cortical collecting duct cells) or AQP5 (salivary gland cells) dramatically increases the rate of initial cell swelling following hypotonic exposure (264, 579). For a detailed description of the physiology of AQPs, see Reference 448. Cells contain impermeable, polyvalent anionic macromolecules and are, even under isotonic steady-state conditions, constantly threatened by colloid osmotic cell swelling due to entrance of diffusible ions and water. According to the "pump and leak" concept, cell swelling and lysis are avoided because a combination of a low Na^+ permeability and active Na^+ extrusion via the Na^+-K^+-ATPase renders the plasma membrane effectively impermeable to Na^+ (see Refs. 530, 1000, 1018). Most cell types are able to counteract volume perturbations following a shift in extra- or intracellular osmolarity. Osmotically swollen cells release KCl, nonessential organic osmolytes, and cell water, thereby reducing the cell volume towards the original value, the process of regulatory volume decrease (RVD). Osmotically shrunken cells generally initiate a net gain of KCl and cell water, thereby increasing cell volume towards the original value, the process of regulatory volume increase (RVI). Most cells have low leak permeabilities for Cl^- and organic osmolytes. RVD is dependent on increases in the net efflux of Cl^-, K^+, and organic osmolytes, whereas RVI involves the activation of Na^+-K^+-$2Cl^-$ cotransport, Na^+/H^+ exchange, and nonselective cation channels.

Under normal physiological conditions, the osmolarity of the extracellular fluid is kept constant by body fluid homeostasis (~285 mosmol/kgH_2O), and cell volume is most commonly perturbed by changes in intracellular, rather than extracellular, osmolarity. This may occur during, e.g., *1*) transepithelial transport, *2*) accumulation of nutrients and metabolic waste products, and *3*) neuronal, hormonal, and autocrine activation of the volume-sensitive transporters and channels for ions or organic osmolytes, or a shift in volume set point for their activation (see Ref. 510). Changes in extracellular osmolarity do, however, occur under physiological conditions. For instance, intestinal epithelial cells and blood in intestinal capillaries are exposed to low extracellular osmolarity after water or hypotonic food intake, and kidney medullar cells and blood cells in kidney medulla capillaries are exposed to very high extracellular osmolarity during antidiuresis. Cell volume is also challenged under a variety of pathophysiological conditions. For instance, cell swelling occurs during *1*) hypoxia/ischemia; *2*) hyponatremia, which occurs in situations where hormonal and renal function is impaired; *3*) hypothermia, which inhibits the Na^+-K^+-ATPase; *4*) increases in the extracellular K^+ concentration ($[K^+]_o$); and *5*) intracellular acidosis/diabetic ketoacidosis. Conversely, cell shrinkage occurs during, e.g., *1*) hypernatremia, following excessive Na^+ intake or water loss; *2*) reduced $[K^+]_o$; and *3*) hyperglycemia and alkalosis (see Ref. 510). Notably, an anisotonic state under pathophysiological conditions usually develops slowly and gradually with time, which gives the volume-restoring mechanisms a chance to preserve cell volume continuously. Therefore, the resulting shift in cell volume is in some cases negligible, a phenomenon designated isovolumetric volume regulation (583). Long-term exposure to anisosmotic conditions elicits not only changes in the volume set point but also in expression levels of volume-sensitive transporter proteins and enzymes involved in the synthesis/degradation of organic osmolytes. Such conditions also impact on signaling events that control cell growth, cell proliferation, and cell death. Similarly, dysfunction of volume-sensitive membrane transport proteins is associated with pathophysiological conditions related to control of cell proliferation, migration, invasion, and cell death.

The purpose of this review is to provide the reader with an overview of the fundamental properties of cell volume homeostasis, including the possible volume sensors, the ensuing signal transduction events, the osmolyte transporters and channels involved in RVI and RVD, the mechanism of cellular adaptation to long-term volume perturbation, and finally, how change in cell volume function as signals in a variety of physiological processes.

II. FUNDAMENTALS: THE DONNAN EQUILIBRIUM, CELL VOLUME MAINTENANCE, OSMOTIC VOLUME PERTURBATIONS, AND CELL VOLUME REGULATION

A fundamental property of cells is that they contain a significant amount of large-molecular-weight anionic colloids (mostly proteins and organic phosphates) to which the plasma membrane is impermeable. About 70 and 38% of all proteins in living systems have isoelectric points (pI) below pH 7 and pH 6, respectively, i.e., have a negative net charge at physiological pH (277). In contrast, the extracellular fluid has a low concentration of nondiffusible anion(s). These conditions, if nothing else interferes, will lead to a Gibbs-Donnan equilibrium across the cell membrane where at equilibrium, electroneutrality will be attained on both sides of the membrane and osmolarity will be higher intracellularly than extracellularly. Thus an osmotic pressure gradient will develop as described in the following.

Donnan equilibrium [the product of diffusible ion(s) is the same on both side of the membrane]

$$(C^i_{Na}+C^i_K)\cdot C^i_{Cl} = (C^o_{Na} + C^o_K)\cdot C^o_{Cl} \tag{1}$$

Electroneutrality

$$C^o_K + C^o_{Na} = C^o_{Cl} \tag{2}$$

$$C^i_K + C^i_{Na} = C^i_{Cl} + |z|C^i_A \tag{3}$$

where A is the polyvalent, impermeable macromolecule; C is the concentration; $|z|$ is the numerical value of the mean valence (a negative number) of the intracellular, impermeable charged macromolecules; and the intracellular and extracellular compartments are indicated by i and o, respectively.

From *Equations 1* and *2* is obtained

$$(C^i_{Na} + C^i_K)\cdot C^i_{Cl} = C^o_{Cl}\cdot C^o_{Cl} \tag{4}$$

The minimum for a given sum, $x + y$, is obtained for $x = y = \sqrt{k}$ when the product $x \times y = k$ is kept constant. Thus

$$C^i_{Na} + C^i_K + C^i_{Cl} > 2C^o_{Cl} \tag{5a}$$

$$C^i_{Na} + C^i_K + C^i_{Cl} + C^i_A > 2C^o_{Cl} + C^i_A \tag{5b}$$

$$C^i_{Na} + C^i_K + C^i_{Cl} + C^i_A - 2C^o_{Cl} > C^i_A \tag{5c}$$

The osmotic pressure across the membrane (Π) is defined according to the classical Boyle-van't Hoff equation as

$$\Pi = RT\cdot\Sigma C_j \tag{6}$$

where R and T are the gas constant and the temperature, respectively.

From *Equations 5c* and *6* it follows that

$$\Pi = RT\cdot(C^i_{Na} + C^i_K + C^i_{Cl} + C^i_A - 2C^o_{Cl}) > RT\cdot C^i_A \tag{7}$$

In other words, an osmotic pressure will unavoidably develop across the cell membrane. Under these conditions, water will enter the cell down its concentration gradient according to the osmotic pressure gradient described in *Equation 7*, and the cell will swell unless a strong hydrostatic pressure can develop. The mammalian plasma membrane cannot withstand hydrostatic pressure, i.e., the cell will swell and thus upset the Gibbs-Donnan equilibrium, whereafter more ions enter the cell according to *Equation 1*. This will continue until the cell bursts by colloid osmotic lysis. Nonetheless, cells do not burst. What is the mechanism preventing that? The answer is the Na^+-K^+-ATPase in the plasma membrane which, together with the low plasma membrane Na^+ permeability, makes the cell effectively impermeable to Na^+. This sets up a "double Donnan equilibrium" with Na^+ as the extracellular "functionally impermeable" charged species. This balanced passive and active movement of ions across the plasma membrane, by which the Na^+-K^+-ATPase offsets the colloid pressure of the impermeable, negatively charged macromolecules, was denoted the "pump and leak" concept (see Refs. 530, 1000, 1018). In some cell types, the plasma membrane Ca^{2+}-ATPase (PMCA) and the Na^+/Ca^{2+} exchanger (NCX) participate in (976) or, in the case of dog red blood cells (RBCs), are entirely responsible for (768), steady-state maintenance of cell volume.

A. Volume Maintenance: Steady State

The "pump and leak" concept implies that the osmotic pressure across the plasma membrane is nil. Taking intracellular ions, charged macromolecules, and uncharged solutes (X, organic osmolytes) into consideration, one obtains

$$C^i_{Na} + C^i_K + C^i_{Cl} + C^i_X + C^i_A = C^o_t \tag{8}$$

where t is the total osmolyte content in the extracellular compartment.

Multiplying the osmolyte concentrations with the cellular water content (V), we get the cellular content of osmolytes (M), and from *Equations 3* and *8* we get

$$V = [2M^{i}_{Cl} + M^{i}_{X} + (1 + |z|) \cdot M^{i}_{A}]/C^{o}_{t} \qquad (9)$$

From *Equation 9* it is seen that the intracellular content of Cl^- and organic osmolytes and the net charge of intracellular impermeable macromolecules determine the cell volume. Thus normally *1*) a decrease in the extracellular osmolarity, *2*) a net uptake of osmolytes (amino acids, Cl^-), or *3*) an increase in $|z|$ will lead to cell swelling. A decrease in intracellular pH, which leads to a decrease in the negative charge of macromolecules (a decrease in $|z|$) will, however, lead to cell swelling, due to a redistribution of Cl^- via the anion exchanger (for a discussion, see Ref. 374). The transporters and channels responsible for the net movement of ions and organic osmolytes across the plasma membrane during volume regulation are described in detail in subsequent sections of this review.

B. Osmotic Behavior Following Osmotic Perturbation and Subsequent Volume Regulation

As described above, most mammalian cells have a very high permeability for water compared with their permeabilities to Na^+, K^+, and Cl^-. For instance, the water permeability in Ehrlich ascites tumor (EAT) cells is 10^5 and 10^6 times higher than the permeability to sodium/potassium and chloride, respectively (354, 501). If cells are transferred from a standard solution with osmotic pressure π_o to a solution with a different osmotic pressure, π, and the molal osmotic coefficient for each solute is unchanged, the Boyle-van't Hoff equation (*Eq. 6*) can be modified to give the cell volume (V) at the new osmotic pressure relative to the original cell volume (V_o):

$$V = (\pi_o/\pi) \cdot (V_o - b) + b$$

where b is the nonsolvent or osmotically inactive volume of the cell. It has been estimated that the ratio between the osmotically active volume and the total cell volume is in the range of 0.7–0.9 (see Ref. 353).

1. *Cell volume regulation*

As briefly introduced in section I, most cell types have the capacity for counteracting volume perturbations, in the volume recovery processes known as RVD and RVI (for an in-depth description of these processes, see Refs. 318, 370).

In some cells, an RVI response is only observed following pretreatment of cells in hypotonic medium, the so-called RVI-after-RVD protocol (see, e.g., Ref. 360). Figure 1 shows a typical RVI-after-RVD in EAT cells after a twofold reduction in the extracellular osmolarity and an ensuing period of volume regulation, followed by reestablishment of isotonicity, which now leads to osmotic cell shrinkage followed by an RVI response. Whereas the initial phases of osmotic water influx/efflux are rapid in most cells due to the high water permeability, the duration of the subsequent recovery phases varies considerably within different cell types (see Ref. 353). While the difference is less dramatic, the rate of initial

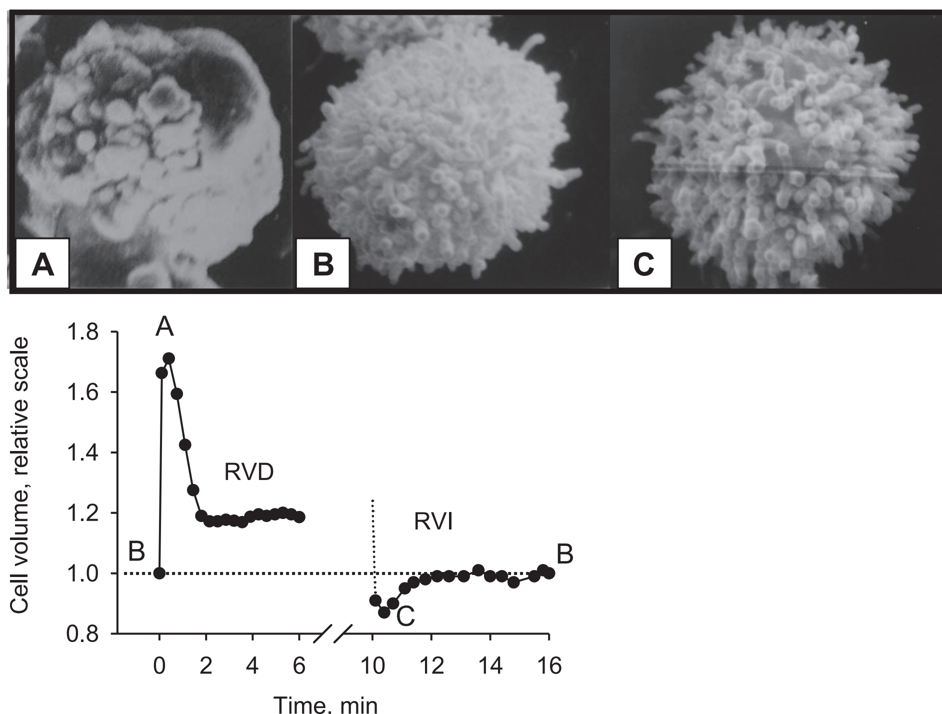

FIG. 1. Regulatory volume decrease (RVD) and regulatory volume increase (RVI) in EAT cells. RVD: EAT cells, preincubated in isotonic (300 mosM) medium for 40 min, were at zero time transferred to hypotonic medium (150 mosM) and the cell volume followed with time in a Coulter counter. Cell volume is given relative to the initial isotonic volume. [Data from Hoffmann (358).] RVI: cells preincubated in hypotonic (225 mosM) medium for 10 min were returned to isotonic medium and cell volume followed with time (Hoffmann, unpublished). Images were taken by scanning electron microscopy at time points indicated under the RVD/RVI time trace (*A*, *B*, and *C*). [Images from Hoffmann (354).]

osmotic shrinkage/swelling also varies between cell types, being very high in EAT cells (see Fig. 1) and low in, e.g., rat cortical collecting duct cells (264). From the scanning electron micrographs in Figure 1, it is seen that the initial increase/decrease in cell volume involves unfolding/folding of the plasma membrane. Notably, the steady-state volume obtained after RVD is higher than the initial cell volume, reflecting a change in volume set point (see next section) after major cell volume changes (shown for EAT cells in Figs. 1 and 2). Grygorczyk and co-workers (301) have shown that during a twofold reduction in extracellular tonicity, all increase in surface area could essentially be attributed to unfolding of the plasma membrane, whereas more dramatic cell swelling was associated with endomembrane insertion (301).

2. *Volume set point*

The cell volume "set point" for activation of a volume regulatory transport system is, by definition, the cell volume above/below which the transport system is activated. In other words, the swelling- and shrinkage-activated transport systems are inactive or marginally active when the cell volume is below and above, respectively, the set point for their activation. The magnitude of the volume changes necessary to activate the various volume-sensitive transporters and channels have not been studied in detail. For human intermediate conductance K^+ (hIK) channels expressed in *Xenopus* oocytes, it was found that $<$3.5% swelling was enough to activate the channel (305). In EAT cells, the steady-state volume obtained after RVD is greater than the initial cell volume (Figs. 1 and 2), which could reflect that the volume set point is elevated in cells which have undergone an RVD or RVI response (358, 706). As seen in Figure 2*B*, the recovery after swelling in 225 mosM medium is almost complete, whereas the "missing" recovery is $>$20% after swelling in 150 mosM medium (358, 706). One possible interpretation is that the volume set point increases with decreasing intracellular ionic strength in these cells, although obviously many other conditions in the swollen cells could affect the open

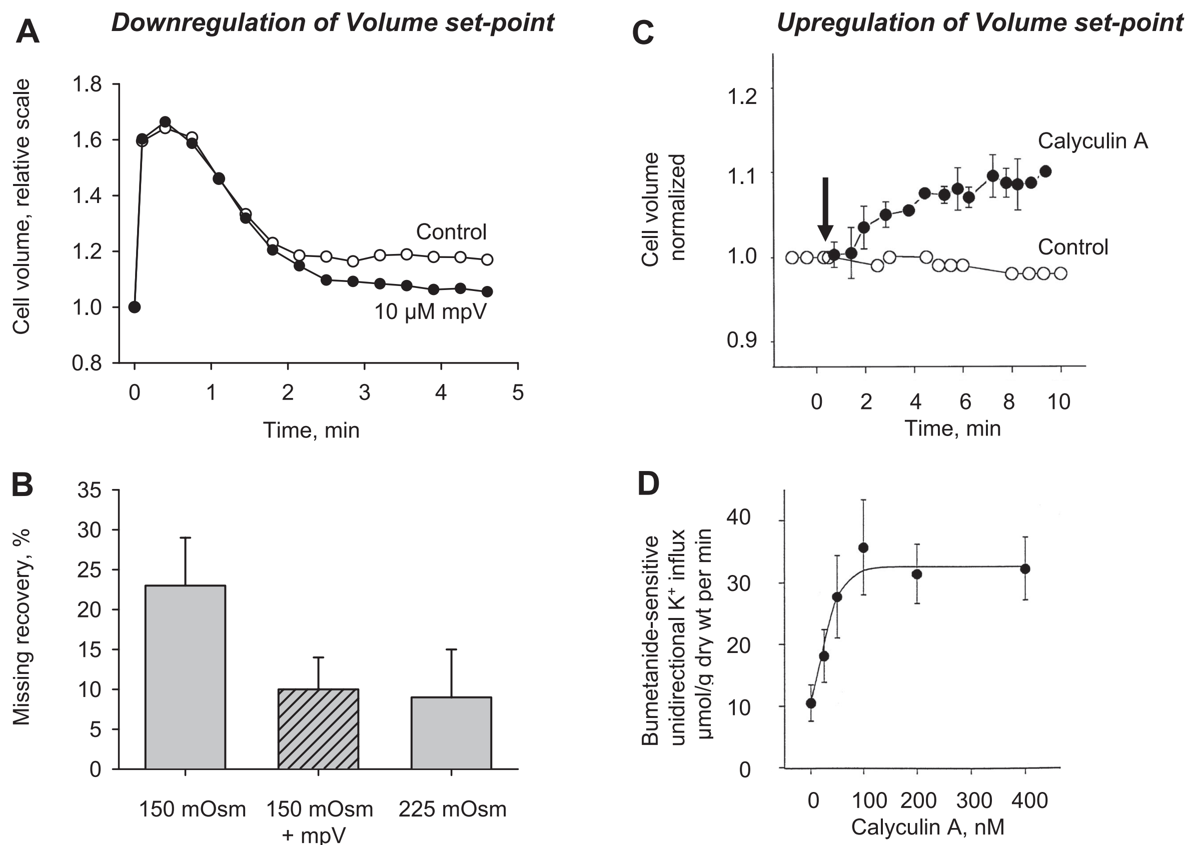

FIG. 2. Downregulation and upregulation of the volume set point in EAT cells. The experimental protocol for the experiments in *A* and *B* is identical to the RVD protocol in Fig. 1. *A* and *B*: RVD was performed at two osmolarities (150 and 225 mosM) in the presence and absence of the tyrosine phosphatase inhibitor mpV (10 μM). *B*: the missing recovery is estimated 5 min after hypotonic exposure and given as mean values ± SE. [*A* and *B* redrawn from Hoffmann (358).] The experiments in *C* and *D* are performed under isotonic conditions with or without addition of calyculin A (100 nM). *C*: cell volume was followed with time in control cells and cells treated with calyculin A (indicated by an arrow). *D*: the bumetanide-sensitive K^+ influx was estimated by tracer technique and represents the difference in K^+ influx in the absence and presence of the NKCC blocker bumetanide. [*C* and *D* from Krarup et al. (472).]

probability of the volume-sensitive channels. In apparent contrast, in Chinese hamster ovary cells, the set point for the volume-regulated anion channel (VRAC) decreased with decreasing ionic strength (110). Similarly, in endothelial cells, VRAC was activated by a decrease in ionic strength, which was interpreted as the activation of single-channel currents through VRAC rather than as a change in the set point of a volume sensor (873).

As discussed elsewhere (374), cells do not have one preferred volume. Rather, the volume set point may depend on the functional state of the cell, examples being the role of altered cell volume in control of, e.g., cell proliferation or programmed cell death (PCD) (see sect. IX). Several signaling molecules have been shown to affect the volume set point. For instance, in EAT cells, the volume set point is increased by inhibition of Ser/Thr protein phosphatases (472; Fig. 2, *C* and *D*), and decreased by the tyrosine phosphatase inhibitor mpV (Fig. 2, *A* and *B*). Phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], the concentration of which is decreased after cell swelling and increased after shrinkage, respectively (see sect. IV*C*), was also recently suggested to orchestrate the volume set point in EAT cells (706).

III. NATURE OF THE VOLUME STIMULUS

A. Sensing of Volume Changes: Principles and Hypotheses

Broadly speaking, one can separate the events elicited by cell volume changes into three major categories, namely, those initiated in response to changes in *1*) macromolecular crowding, *2*) cellular ionic strength or concentrations of specific ions, or *3*) mechanical/chemical changes in the lipid bilayer. As will be discussed below, roles for all three categories in the response to volume perturbations in vertebrates have been clearly demonstrated. There is evidence to suggest that a given response is often fine-tuned to the particular physiological/pathophysiological condition through integrated regulation via all three categories of signals. One example is the activation of Rho and Rac by cell shrinkage, which can be initiated independently by either an increase in ionic strength or a decrease in cell volume (138, 546; see sect. IV*D2*). Another is the activation of KCl cotransport in RBCs by urea-induced cell swelling, which appears to involve both a mechanical and a macromolecular crowding signal (210).

Macromolecular crowding refers to the phenomenon that the concentrations of proteins, nucleic acids, and/or polysaccharides in intracellular compartments are so high that steric exclusion can significantly affect the chemical reactivity of individual macromolecules as well as of cell water (648, 1104). Consequently, e.g., enzyme function can be modified through swelling- or shrinkage-induced changes in macromolecular crowding. Especially studies in RBCs have suggested that such changes may play important roles in the modulation of some cellular processes by volume perturbations (210, 766). The role of macromolecular crowding as a signal of volume perturbation has been discussed excellently elsewhere (94, 649, 1104), and since evidence from other cell types than RBCs is still scarce, the phenomenon will not be further discussed here.

There is also substantial evidence that the change in intracellular ionic strength associated with volume perturbation is the signal initiating the activation of many signaling cascades [e.g., modulation of PtdIns(4,5)P_2 levels; see sect. IV*C2* and Ref. 706], and the activation of some (e.g., VRAC; see sect. VII*A* and Ref. 720) albeit not all (e.g., NHE1; see sect. VI*A2* and Ref. 478) volume-activated transport proteins. The intracellular concentration of specific ions may also be a major determinant of the activity of the volume-sensitive transporter, as exemplified by the regulation of the Na^+-K^+-$2Cl^-$ cotransporter NKCC1 by intracellular Cl^- (see sect. VI*B2*). Although we restrict our discussion in this review to vertebrate organisms, it should also be noted that both ionic strength and concentrations of specific ions are important mechanisms of osmosensing for bacterial transporters and channels. This is of general importance because, whereas in eukaryotes the molecular mechanisms are often poorly understood, specific amino acid motifs responsible for activation of osmosensitive bacterial transporters and channels by, e.g., changes in ionic strength or $[K^+]_i$ have been identified (1104, 1105).

In recent years, studies of cell volume sensing have focused on the roles of mechanical/chemical changes in the lipid bilayer occurring as a result of osmotic perturbations. Such changes can regulate membrane transport proteins, receptors, and enzymes by a number of mechanisms that are still incompletely understood (for recent reviews on general aspects of mechano- and/or osmosensitivity, see Refs. 322, 482, 796, 808). As shown in Figure 3, such mechanisms include *1*) a change of forces acting within the lipid membrane (Fig. 3*A*), exemplified by the direct activation of an ion channel by membrane stretch or physical bending, leading to changes in membrane tension, thickness, and curvature. Such mechanisms have been shown to play important roles in the activation of stretch-activated (SA) channels in bacteria (322, 482, 808). In vertebrates, however, proteins residing in the plasma membrane may experience little or no changes in tension during cell swelling due to the cortical cytoskeleton and the substantial membrane reservoir in the form of, e.g., invaginations and microvilli (see Ref. 322). Nonetheless, channels regulated by membrane stretch exist in the eukaryotic plasma membrane and may be of importance after sudden, large changes in cell volume, or under con-

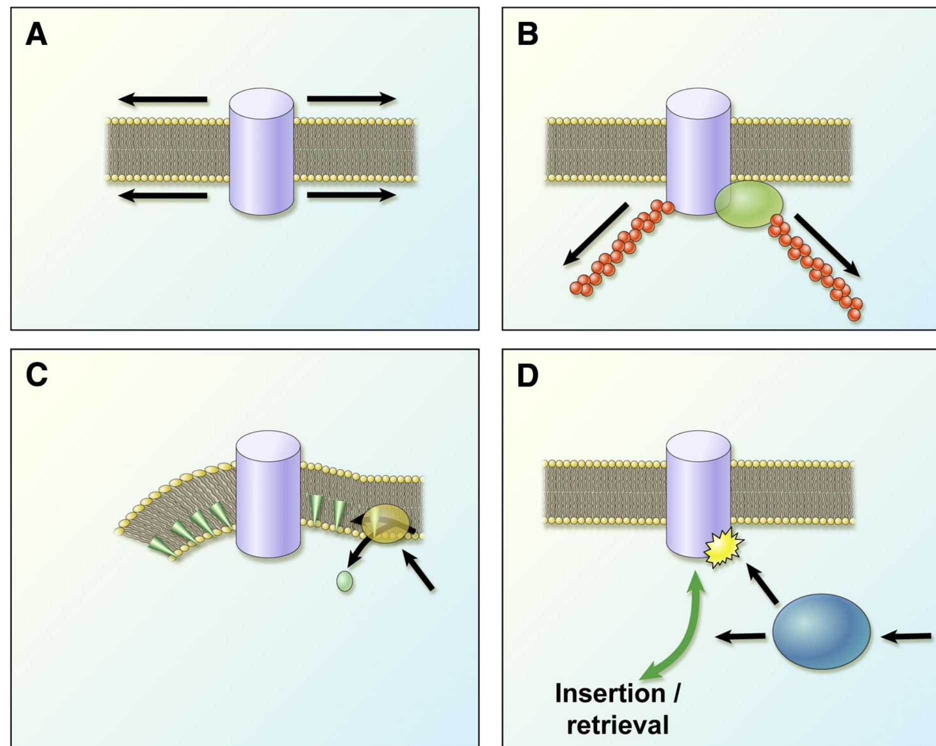

FIG. 3. Possible mechanisms of, direct and indirect, mechanosensing by membrane proteins after osmotic cell volume perturbations. In all panels, the blue cylinder illustrates a volume-sensitive protein, which could be a membrane transport protein, a receptor, or an enzyme, and hence could be either an effector involved in ion transport, as a volume sensor, or part of a signaling pathway as relevant for the protein in question. The figure illustrates general possible mechanisms by which this protein may be activated by mechanical/chemical changes occurring as a consequence of a change in cell volume. *A*: a change of forces acting within the lipid membrane can regulate the protein directly via changes in membrane tension, thickness, and curvature, i.e., in this case, the protein itself serves as the volume sensor. *B*: a protein interacting with cytoskeletal components (red) that are reorganized by the cell volume perturbation may be regulated through tether forces, i.e., in this case the sensing mechanism is the cytoskeleton or upstream of it. *C*: changes in membrane curvature leading to regulation of the protein can also result from the (direct or indirect) osmosensitivity of phospholipases and phospholipid kinases, i.e., in this case the sensor mechanism is at or upstream of the level of these enzymes. *D*: the protein may also be activated via volume-dependent posttranslational modifications such as phosphorylation/dephosphorylation events, or by vesicular insertion or removal of the channel to/from the plasma membrane (blue), in which case the sensor mechanism is at or upstream of the level of the relevant protein kinases/phosphatases and/or exocytotic machinery. See text for details.

ditions when cells have exhausted their membrane reservoirs, although this is a controversial issue (see below). The dramatic changes in endo- and exocytosis rates observed in osmotically perturbed cells (747, 852, 1024) could also be initiated at least in part via mechanical/chemical changes in the lipid bilayer (see Ref. 322). *2*) Membrane proteins may be regulated through tether forces, due to their, direct or indirect, attachment to cytoskeletal components that are reorganized by cell volume perturbations (Fig. 3*B*; sect. IV*D1*). *3*) Changes in membrane lipid composition resulting from the, direct or indirect, osmosensitivity of specific phospholipases and phospholipid kinases can alter membrane curvature, which then in turn can alter the function of transporters and channels (Fig. 3*C*; sect. IV*C*). It is interesting to note that in this manner, a physical (stretch or bending) and a chemical (e.g., activation of a lipase) signal can exert the same final effect on a given channel transporter, as recently suggested for the cation channel TRPC6 (943; sect. IV*B*). Finally, other volume-sensitive signaling events, in principle activated by either of the above-mentioned mechanisms, can mediate posttranslational modifications such as phosphorylation/dephosphorylation events, which in turn activate transporters/channels or other volume-sensitive proteins, or initiate the vesicular insertion or removal of these proteins to/from the plasma membrane (Fig. 3*D*). An important caveat is, however, that it is difficult, and sometimes impossible, to distinguish between mechanical versus other osmotic stimuli in activation of the processes outlined above, both for technical reasons and because some degree of mechanical stimulation is inherently associated with osmotic perturbation (for a discussion, see Refs. 563, 796).

B. Role of the Lipid Bilayer: Biophysical Properties of the Plasma Membrane

The distribution and functions of the proteins embedded in the phospholipid bilayer are influenced by the lipid

composition, i.e., the particular hydrocarbon chains and polar head groups of the phospholipids, and the amount of cholesterol, in the plasma membrane. Several features of the membrane lipids are important in this regard. First, the lipids are arranged with a transverse asymmetry (310). Second, the movement of some lipids is laterally restricted, and as a result, the lipid bilayer is not homogeneous. In the last decade, there has been a surge of interest in caveolae and lipid rafts, specific membrane microdomains enriched in particular lipids and proteins. These membrane microdomains are essential for efficient signal transduction, and many membrane receptors are enriched and clustered in such domains. In this manner, lipid rafts and caveolae play important roles in many cellular processes, including the control of signaling events, membrane ion transport, and membrane trafficking (935). Specifically, caveolae and/or lipid rafts have been reported to be affected by cell volume perturbations (430, 1047) and other mechanical stresses (765) and have been implicated in the regulation of several volume-sensitive transporters and channels (40, 456, 544, 1004–1006, 1015). Sequestering of acidic lipids (phosphatidylserine, phosphatidylinositols) or cholesterol into caveolae and rafts has turned out to have an impact on protein binding to the membrane bilayer and thus on signal transduction (935), cytoskeletal organization (115, 528), and membrane fusion (518). In agreement with this, changes in membrane cholesterol content modulate the function of plasma membrane receptors (98), and of several transporters and channels (256, 314, 594, 857). This has been especially well studied for VRAC (101, 456, 544, 568, 857), which has been shown to be potentiated by cholesterol depletion in EAT cells (Fig. 4*A*, Ref. 457), and BAE cells (544). Cholesterol depletion disrupts the structure of both rafts and caveolae (88, 936, 1092), but it is important to note that the effects of changes in membrane cholesterol content are not limited to effects on rafts and caveolae. For instance, an increase in cholesterol content is generally expected to decrease the fluidity of the membrane and increase bilayer thickness (125) and stiffness (700). As a result, membrane deformation energy is modulated (388, 704), and this again is important for the energy cost of channel opening/closing, as shown for voltage-dependent Na^+ channels and gramicidin channels (593, 594). Importantly, however, cholesterol depletion appears to exert at least some of its effects through alterations in the organization of the actin cytoskeleton, leading to an increased cell stiffness in cholesterol-depleted cells, rather than the reduced stiffness expected based on effects on the lipid bilayer alone (for a discussion, see Refs. 100, 456). In any case, it should be stressed that to implicate caveolae in a specific process, results from cholesterol depletion experiments must be substantiated by, e.g., knockdown or knockout of caveolins.

The shape of the bilayer can be changed by addition of amphiphatic molecules that can act either as crenators or cup formers, depending on their shape and whether they localize to the external or internal leaflet (916). Such changes can have a dramatic effect on at least some mechanosensitive channels, e.g., in *Escherichia coli* (626) as well as in mammalian two pore, mechano-gated K^+ channels (TREK-1) in mammalian cells, the opening of which seems to be dependent on expansion of the outer leaflet of the bilayer (774). Activation of phospholipase A_2 generates lysophospholipids (sect. IV*C1*), and early work indicated that lysophospholipids lower the electrical resistance and increases the permeability of membranes (535). Lysophosphatidylethanolamine (LPE) and lysophosphatidylcholine (LPC) tend to stress lipid packing and introduce curvature tension and thus change the membrane deformation energy (253, 592). This may in turn impact both enzyme and transporter activity; for

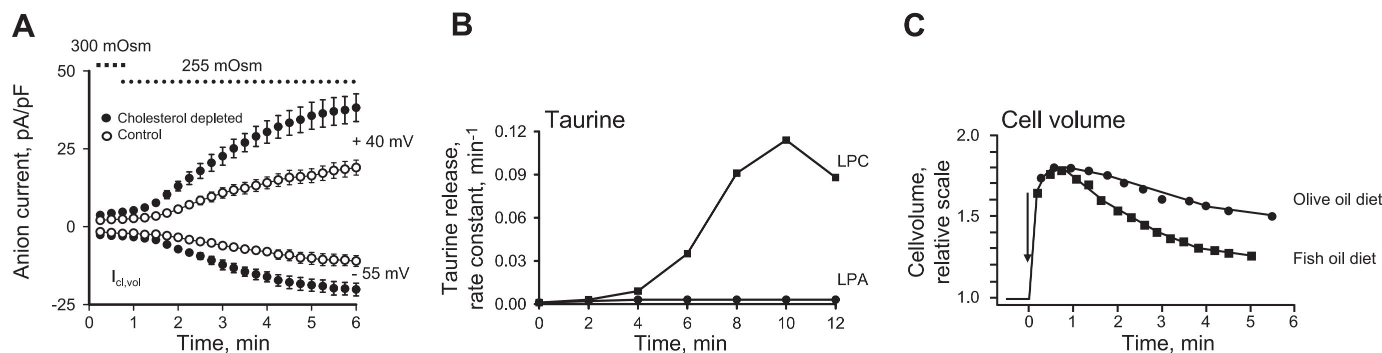

FIG. 4. Effect of lipid modification on swelling-activated anion current ($I_{Cl,vol}$), taurine efflux, and RVD. *A*: effect of cholesterol depletion on $I_{Cl,vol}$ in ELA cells under hypotonic conditions (225 mosM) was estimated by whole cell patch-clamp technique at −55 mV and +40 mV using a fast ramp protocol. Cells were cholesterol depleted by preexposure to empty MβCD. [Data from Klausen et al. (456).] *B*: taurine efflux was estimated in HeLa cells under isotonic conditions using tracer technique, and the efflux is shown as the fractional rate constant at given time points following addition of 25 μM LPC or LPA. [Data from Lambert and Falktoft (495).] *C*: effect of dietary fish oil/olive oil on RVD in EAT cells was estimated on cells isolated from mice that had been fed 2 wk on a diet supplemented with *n*-3 fish oil (MaxEPA) or virgin olive oil. The cell volume was followed with time under hypotonic conditions (150 mosM) using the Coulter counter technique. [Data from Lauritzen et al. (527).]

instance, it has been demonstrated that the ability of LPC and LPE to modulate local curvature in a membrane correlates with their ability to potentiate PKC activity (24) and to activate K^+ channels (TREK-1, TRAAK; Ref. 612) and taurine leak pathways (Fig. 4*B*).

The fluidity of the membrane is determined not only by its cholesterol content but also by the saturation of the fatty acids. Increasing the number of double bonds increases the number of kinks in the structure, causing the fatty acids to fit less closely together and the membrane to become more fluid. In EAT cells, an increase in the membrane content of the polyunsaturated fatty acid eicosapentaenoic acid increases the swelling-activated Cl^- and K^+ permeabilities (527) and accelerated RVD (Fig. 4*C*). In addition, the polyunsaturated docosahexaenoic acid increases gramicidin channel current and lifetime in artificial bilayers and decreases the free energy of channel formation (90). However, because a fraction of the fatty acids incorporated in the membrane lipids serves as precursors for the synthesis of lipid-derived second messengers, a shift from ω-6 fatty acids (linoleic acid, γ-linolenic acid, arachidonic acid) to ω-3 fatty acids (α-linolenic acid, eicosapentaenoic acid) might not only affect the physical properties of the membrane but also the substrate availability for downstream signaling (sect. V).

IV. UPSTREAM SENSORS AND TRANSDUCERS OF CELL VOLUME CHANGES

The mechanism(s) of osmosensing in mammalian cells are still incompletely understood. Two-component systems, consisting of proteins with histidine kinase and/or response regulator domains, are well described as osmosensors in bacteria, Archaea, fungi, slime molds, and plants (see, e.g., Refs. 29, 554). As the name indicates, two components mediate this response: a sensor/kinase, consisting of an extracellular domain and a histidine kinase activity which functions as a transmitter, and a response regulator with a receiver domain. This system regulates the HOG1 osmotic response MAPK pathway (see also sect. V*C1*). Although MAPK pathways also play major roles in osmosensory signaling in vertebrates (sect. V*C1*), they lack the two-component histidine kinase system, and thus must depend on other mechanisms of osmosensing.

A. Receptors and Cell Adhesion Proteins

A number of integral membrane proteins, including integrins, growth factor receptors (GFRs), cytokine receptors, and calcium-sensing receptors (CaRs), have been assigned roles as upstream sensors of cell volume perturbations. Direct evidence for roles in osmo-/volume sensing is so far limited with respect to cytokine receptors (859) and CaRs (698, 744; see Ref. 236); hence, only the possible roles of integrins and growth factor receptors will be discussed here.

1. *Integrins*

Integrins are a highly conserved family of heterodimeric adhesion molecules that connect the extracellular matrix to intracellular signaling proteins and the cytoskeleton. Integrin receptors consist of an α- and a β-subunit, each containing one transmembrane domain (419). There are currently 18 α- and 8 β-subunits identified in vertebrates, forming at least 24 different α,β-receptor combinations. Integrins are not signaling proteins per se; rather, they are the structural foundation of complexes involving at least two dozen cytoplasmic proteins. One example is the focal adhesion (FA) complex, which links the cytoskeleton and a plethora of signaling molecules including the FA kinase (FAK, see sect. V*F*) (108). In addition to their important roles in mechanosensory transduction and growth factor signaling (18, 396, 651), there is evidence implicating integrins as cell volume sensors in mammalian cells (334, 427, 587, 895, 1050), after both cell swelling (85–87, 334, 1050, 336) and shrinkage (427, 658, 918, 919; see Refs. 336, 791, 174). It should, however, be noted that in spite of considerable evidence pointing to their involvement in the events activated by cell volume perturbations, unequivocal evidence as to a role for integrins as primary sensors of cell volume change is still lacking; for instance, inside-out signaling to integrins could well be secondary to other volume-dependent events, including even the volume-dependent ion fluxes, such as an increase in $[Ca^{2+}]_i$.

Browe and Baumgarten (85–87) investigated for the role of integrins in the swelling-induced activation of VRAC. Integrin stretch was found to activate FAK, Src, and other signaling pathways that transactivate the epidermal growth factor (EGF) receptor, resulting in activation of phosphatidylinositol 3-kinase (PI3K) and Rac. Next, Rac was proposed to translocate to the membrane and be involved in forming the active NADPH oxidase complex (see also sect. V*G*), resulting in production of $O_2^{\bullet -}$, and thus H_2O_2, finally leading to activation of a current similar to VRAC (85–87). However, to our knowledge, it remains to be shown whether osmotic cell swelling induces integrin stretch, leading to activation of VRAC. Moreover, β1-integrins have been implicated in the regulation of a variety of K^+ channels relevant to cell volume regulation, including Kv1.3 (22, 545), Kv4.2, and Kv1.4 channels (1032) and Ca^{2+}-activated K^+ channels (436). In rat liver, both hypotonic and insulin-induced cell swelling increased the plasma membrane level of activated β1-integrin, and inhibition of insulin-induced swelling by furosemide abolished activation of β1-integrin, Src, and p38 MAPK (895). Conversely, the shrinkage activated Na^+/H^+ exchanger, NHE1, is also activated by integrin

activation (911), in a manner involving the Rho-Rho-kinase pathway (998, 999). This pathway is, however, unlikely to account for shrinkage-induced NHE1 activation, which is unaffected by inhibition of Rho kinase (790, 837). Finally, the hypertonicity-induced increase in tonicity-responsive enhancer-binding protein (TonEBP) expression was strongly impaired in the kidney medulla of α_1 integrin-null mice, leading to defective osmolyte accumulation (658).

2. Growth factor receptors

Several reports have pointed to a role for receptor tyrosine kinases in osmosensing, although, similar to integrins, these receptors have been implicated in both the swelling- and shrinkage-activated responses. The EGF rececptor in Swiss 3T3 fibroblasts (247) and keratocytes (449) and other cell types (771), and the ErbB4 EGF receptor in cerebellar granular neurons (548), are activated by hypotonic cell swelling, resulting in activation of the PI3K-PKB and MEK1/2-ERK1/2 pathways and volume regulatory taurine efflux (247).

Hypertonic cell shrinkage has also been found to phosphorylate the EGF receptor, EphA2, and the insulin receptor in some cell types (128, 838, 840, 859). The mechanism of growth factor receptor activation by shrinkage is not clear, but a mechanism involving shrinkage-induced, ligand-independent receptor clustering has been proposed (859). On the other hand, in hyperosmotically stressed rat hepatocytes, EGF receptor activation occurred but was clearly not the volume-sensing mechanism per se, as it was downstream of ROS-dependent activation of the Src kinase Yes (838). Hypertonic cell shrinkage has, however, also been shown to inhibit growth factor receptor signaling in several cell types. In kidney cells, shrinkage inhibited EGF receptor-mediated signaling, ostensibly downstream of Ras rather than at the receptor level (153). In NIH3T3 cells, hyperosmolarity strongly inhibited ligand-dependent activation of platelet-derived growth factor (PDGF) receptor β, as well as the downstream PI3K-PKB and MEK1/2-ERK1/2 pathways (708).

It seems likely that GFRs and integrins cooperate in cell volume signaling, both potentially functioning as a part of a volume sensory unit. Many of the pathways activated downstream from GFR activation are also activated by integrins, and it has been suggested that signaling events downstream from integrins and GFRs converge in a manner involving cytoskeleton-dependent scaffolding within the FA complex (818). In addition, integrin activation is known to transactivate GFRs (460, 678). A specific example discussed above is the coupling between integrin stretch and EGF receptor activation in the activation of VRAC (86).

B. Stretch-Activated Channels and Transient Receptor Potential Channels

In bacteria, the stretch-activated nonselective channels MscL and MscS were first identified at the electrophysiological level by Martinac and co-workers (627). These channels have since been cloned and crystallized, and their mechanisms of mechanosensing analyzed in detail, although still not fully elucidated (see Refs. 322, 482). While the presence of mechanosensory channels in vertebrate cells has been widely demonstrated in excised patches, the extent to which these channels are relevant in the intact cell is a contentious issue. This is due both to the "excess membrane area" in most animal cells, which makes it unlikely that the lipid bilayer is stretched upon osmotic swelling (sect. III*A*; see also Ref. 322), and to the fact that more recent findings have challenged the previously proposed mechanosensitivity of at least some of these channels (194, 286). Nonetheless, it has been proposed that although the excess membrane area likely buffers tension development, local areas of increased tension may arise in osmotically swollen cells, as may changes in cytoskeletal tethering of membrane proteins, given the extensive cytoskeletal reorganization under these conditions (322). Rapid activation of nonselective cation currents by swelling-induced stretch has also been demonstrated in patch-clamp studies of a wide range of cell types from vertebrate organisms (e.g., Refs. 49, 134, 144, 820, 1129; see also Ref. 626). For the most part, the molecular nature of these currents remains to be identified in vertebrates. However, similar to what was directly demonstrated in yeast cells (1147), some members of the transient receptor potential (TRP) channel family have been proposed as candidates in vertebrates, as will be discussed in more detail below.

TRP channels are polymodal sensors of a wide variety of chemical and physical stimuli (see Refs. 718, 798, 833, 1045). Seven vertebrate TRP-subfamilies have been defined, based on sequence homology: the canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), mucolipin (TRPML), ankyrin (TRPA), polycystin (TRPP), and NOMPC (TRPN) families (see Refs. 718, 798, 833, 1045). The TRPs are generally nonselective cation channels with permeability ratios P_{Ca}/P_{Na} of 0.3–10. Exceptions to this rule are TRPV5 and TRPV6, which are highly Ca^{2+} selective, and TRPM4 and TRPM5, which are only permeable for monovalent cations (see Refs. 718, 798). The channels are homo- or heterotetramers, each with six predicted transmembrane (TM) domains, cytosolic NH_2 and COOH termini, and a pore-forming region between TM5 and TM6 (see Refs. 755, 756, 798, 833). Many TRPs exhibit specific interactions with multiple binding partners including several of potential relevance to osmosensing, e.g., PLCγ-1 (1025), PtdIns(4,5)P_2, calmodulin, caveolin-1, aquaporin 5 (AQP5), and a number of scaffolding proteins, Ser/Thr-

and tyrosine protein kinases, and cytoskeletal proteins (579, 1025; see Refs. 718, 798). In addition, some TRP subfamilies have an NH_2-terminal ankyrin domain (ARD) of variable length, which has been assigned roles in mechanosensing, although later work has cast doubts on this hypothesis (see Refs. 133, 154). The tissue distribution of the TRPs varies widely, with some members (e.g., TRPC1, TRPV4, TRPM7) being essentially ubiquitous and some exhibiting much more restricted expression patterns (see Refs. 718, 798, 833).

1. *Osmosensitivity of TRPs*

Members of several TRP subfamilies have been shown to be activated by osmotic stimuli and at least in some cases contribute to the ensuing volume regulatory response. Thus, as will be described below, at least TRPV2, TRPV4, TRPM3, TRPM7, TRPC6, and TRPP2 appear to exhibit sensitivity to cell volume and/or to physical parameters coupled to membrane expansion, and there is also evidence implicating TRPV1 in osmosensory signaling (123, 292, 663, 683, 730, 943; see Refs. 286, 561). In addition, many other TRPs have been shown to be activated by other forms of mechanical stress (468, 736, 798, 1045; see Ref. 798). Here, we will focus on studies pointing explicitly to osmosensitivity. However, as noted in section III*A*, mechanical and osmotic stimuli are often inherently linked in a number of ways, making experimental distinction of such effects challenging, and for the TRPs, this has been a particularly contentious issue (for further discussion, see Refs. 133, 482, 563, 796). As will be discussed below, there is evidence to suggest that regulation of TRPs by osmotic volume perturbations involves all of the four types of mechanisms defined above (sect. III*A*, Fig. 3).

Studies of trpv1$^{-/-}$ mice strongly indicate that *TRPV1* is involved in responses to osmotic stimuli, although no evidence is available at the heterologous expression level (60, 913; see Ref. 561). Notably, the splice variant TRPV1b appears to be shrinkage-activated and mediates responses to hypertonicity in osmosensitive neurons (141, 913). PtdIns(4,5)P_2, the levels of which are rapidly altered in osmotically perturbed cells (sect. IV*C2*), appears to regulate several TRPs, including TRPV1. TRPV1 moreover interacts directly with PI3K, the activity of which has also been reported to be osmosensitive (948; sect. IV*C2*). *TRPV2* was shown to be involved in the swelling-activated nonselective cation current and consequent increase in $[Ca^{2+}]_i$ in murine aortic myocytes, and is also activated by mechanical stretch (683). The mechanism by which TRPV2 is activated by cell swelling has not been identified; however, it is interesting to note that activation of PI3K stimulates TRPV2 (807) and that these channels are also regulated by vesicular insertion in the plasma membrane (see Ref. 798). *TRPV4* is the mammalian homolog of the osmo-sensitive *Caenorhabditis elegans* channel (OSM-9), and was the first TRP to be identified as a swelling-activated channel (181, 562, 958, 1098). Further substantiating the role of TRPV4 in osmosensory signaling are the following*:* *1*) the RVD response is impaired in trpv4$^{-/-}$ mice (see Refs. 563, 736, 561); *2*) TRPV4 rescues *C. elegans* OSM-9 mutants from deficits in osmo- and mechanosensing (564); and *3*) TRPV4 mediates swelling-activated Ca^{2+} influx (see Refs. 147, 736). Interestingly, it was recently demonstrated that trpv4$^{-/-}$ mice are incontinent, due to a major role for TRPV4 in control of bladder function (275).

The swelling activation of TRPV4 is independent of ionic strength (722) and of direct membrane stretch (562, 958), and is not mediated by the NH_2-terminal ankyrin repeats (562). This does not, however, preclude a role for the actin cytoskeleton. In fact, the recently demonstrated close interaction between the actin cytoskeleton and TRPV4 (832), and the dependence of the swelling-induced increase in $[Ca^{2+}]_i$ on an intact actin cytoskeleton (sect. IV*D*) are in accordance with the view that the actin cytoskeleton may play a role in volume sensing by at least some TRPs.

Importantly, it has been clearly shown that swelling activation of TRPV4 is dependent on the arachidonic acid metabolite 5′,6′-epoxyeicosatrienoic acid (EET) (725, 1076), and hence, ostensibly on swelling activation of a phospholipase (PL) A_2 (sect. IV*C1*). Similarly, shear stress activates TRPV4 in a manner blocked by inhibition of PLA_2 (329, 465). TRPV4 phosphorylation by Src family kinases was proposed to play a role in its volume sensitivity (see Ref. 147), a notion which was, however, disputed by later studies (1054). Another kinase which might potentially link TRPV4 to cell volume is WNK4 (sect. V*D*), the activity of which decreased the surface level of TRPV4 in HEK-293 cells (251). A number of protein-protein interactions may be important in TRPV4 osmosensitivity. Thus direct interaction of TRPV4 with AQP5 may be required for its swelling-induced activation in salivary gland epithelial cells (579). Moreover, the interaction of TRPV4 with pacsin 3, a protein involved in regulation of endocytosis, may negatively regulate TRPV4 activation by hypotonicity (162; see Ref. 796). Finally, the existence of a mechanosensory complex of TRPV4 and TRPP2 in the primary cilium has been suggested (276).

TRPM3 has a daunting number of splice variants, some of which have been shown to exhibit substantial functional differences. After heterologous expression of a splice variant from human kidney in HEK293 cells, the channel was constitutively active, yet further stimulated hypotonicity (292; see Ref. 739). TRPM7 was recently proposed to be activated both by stretch and by osmotic swelling, and TRPM7 knockdown attenuated RVD in HeLa cells (730). The mechanism of osmosensitive activation of TRPM7 is not known, yet it is interesting in this regard

that shear stress increased TRPM7 activity in a manner associated with increased trafficking of the channel to the plasma membrane (738). This mechanism has, however, been questioned by Okada and collaborators (731) who proposed that the TRPM7 channel, heterologously expressed in HEK-293T cells can be directly activated by mechanical stress in a manner independent of exocytosis-mediated incorporation of the channel into the membrane. TRPC6 heterologously expressed in HEK293 cells was recently reported to be activated by both stretch and hypotonic cell swelling, by a mechanism involving PtdIns(4,5)P_2 hydrolysis in the vicinity of the channel and a concomitant increase in local membrane curvature (943). Similarly, TRPC1 was proposed to be involved in the SA cation current activated by swelling in *Xenopus* oocytes (623). It should, however, be noted that especially heterologous expression of TRPs is subject to substantial misinterpretations due to the confounding effects of endogenous currents, and that subsequent studies have questioned whether TRPC6, and also TRPC1, really mediated mechanosensitive currents (194, 286). Finally, TRPP2 was assigned a role in osmosensing after hypotonic swelling of human syncytiotrophoblast, in a manner involving the actin cytoskeleton (663).

2. Role of TRPs in cell volume regulation

The generally presumed role of TRPs in cell volume regulation is to mediate an increase in $[Ca^{2+}]_i$ which subsequently stimulates RVD by activating efflux of K^+ and Cl^- via Ca^{2+} activated K^+ and Cl^- channels (sect. VII, *A* and *B*). Accordingly, the swelling-induced increase in $[Ca^{2+}]_i$ in a variety of cell types appears to be TRP mediated (19, 730, 958). Moreover, as noted above, the absence or knockdown of TRPV4 (see Refs. 563, 736) or TRPM7 (730) was shown to attenuate RVD. Since many cells neither exhibit a detectable swelling-induced increase in $[Ca^{2+}]_i$ nor a dependence of RVD on Ca^{2+} (sect. V*B*), TRPs are unlikely to be required for RVD in all cell types. It has, to our knowledge, not been considered whether TRP-mediated fluxes of other cations than $[Ca^{2+}]_i$ may play a role in modulation of RVD, and this would be an interesting question for further investigation. Finally, it may be noted that a TRP-related channel was tentatively proposed to be involved in the shrinkage-activated cation current (HICC; see also sect. VI*D*) in hepatocytes (555).

C. Phospholipases and Lipid Kinases

1. PLA_2

Glycerophospholipids constitute a significant fraction of biological membranes. Their *sn*-2 ester bonds are hydrolyzed by members of the PLA_2 family, providing membrane-bound lyso-phospholipids and free fatty acids for downstream signaling. Released arachidonic acid is in itself a bioactive lipid, which can directly activate transport pathways for, e.g., K^+ (450, 749) and organic osmolytes (491, 868). In contrast, at higher concentrations, arachidonic acid directly inhibits swelling-activated K^+, Cl^-, and taurine efflux (316, 489, 672, 881). Arachidonic acid is also an important regulator of cellular signaling processes. For instance, arachidonic acid has been demonstrated to activate the mitogen-activated protein kinases (MAPKs) ERK1/2, JNK, and p38 MAPK (9), and to trigger ROS production in phagocytes (169, 588). Arachidonic acid can be oxidized via the cytochrome *P*-450, the cyclooxygenases (COX1/COX2), or the lipoxygenases (5-LO, 12-LO, 15-LO) into highly potent lipid-derived second messengers, the roles of which in volume regulation are described in section V*A* (see Fig. 8).

A) THE PLA_2 FAMILY. The PLA_2 family is divided into subgroups according to pharmacological profile, substrate specificity, sequence, molecular weight, Ca^{2+} sensitivity, and presence of a histidine or a serine in the catalytic site (see Refs. 480, 682, 892, 1097). The secreted PLA_2 members ($sPLA_2$) are low-molecular-weight proteins (14–19 kDa), which require Ca^{2+} in the millimolar range for catalytic activity and exhibit no acyl chain specificity. Some $sPLA_2$ isoforms are cationic and bind to anionic heparanoids, e.g., heparan sulfate proteoglycan (HSPG), which are anchored to glycosyl phosphatidylinositol (GPI) at the outer leaflet of the plasma membrane (682, 1097). As GPI-anchored proteins are concentrated in caveolae and rafts (262), it is possible that $sPLA_2$ could be activated by changes in caveolae organization during swelling-induced reorganization of the cytoskeleton or unfolding of the membrane (see Ref. 480).

The cytosolic PLA_2 members, the 85- to 110-kDa Ca^{2+}-dependent PLA_2 ($cPLA_2$), the 85- to 90-kDa Ca^{2+}-independent PLA_2 ($iPLA_2$), and the platelet-activating factor-acylhydrolases (PAF-AH) (892), contain a serine in their catalytic site. Cloning of $cPLA_2$ has revealed the presence of three isoforms ($cPLA_2$-IVA, $cPLA_2$-IVB, $cPLA_2$-IVC) plus a number of isoforms that form gene clusters with $cPLA_2$-IV (143, 469). The $cPLA_2$-IVA has a high affinity towards phospholipids with arachidonic acid in the *sn*-2 position (327). The $cPLA_2$-IVA and $cPLA_2$-IVB contain a Ca^{2+}-dependent lipid binding domain (CalB) responsible for translocation of the enzyme from the cytosol to, e.g., the endoplasmatic reticulum, the Golgi complex, and to the perinuclear membrane with subsequent docking at the head domain of vimentin (33, 682, 693, 813, 893, 1016). The differential membrane targeting of $cPLA_2$ appears to be controlled by the absolute amplitude and duration of the $[Ca^{2+}]_i$ elevation (221). The $cPLA_2$-IVA contains several consensus sites for phosphorylation, and the activity of several MAPKs (ERK1/2, p38 MAPK) and of MAPK-activated kinases (MAPKAP) is required to gain full catalytic activity (see Refs. 480, 1097). Two mamma-

lian isoforms of $iPLA_2$ ($iPLA_2$-VIA, $iPLA_2$-VIB) have been cloned. $iPLA_2$ has no apparent demands for a specific head group or fatty acid in the *sn*-2 position, and likely functions in membrane remodeling, protection against oxidative stress/damage, phospholipid reorganization during PCD (480, 1097), and, as described below, in cell volume regulation. Each $iPLA_2$ monomer contains a conserved lipase motif and several ankyrin repeats involved in their oligomerization and activation (1097). $iPLA_2$-VIA contains consensus sites for phosphorylation by casein kinase (CK) 1 and 2, PKA, PKC, and MAPKs (644, 946, 982), a nuclear localization signal (NLS) that targets the enzyme to the nucleus following insulin stimulation (601) and during cell shrinkage (929), as well as a glycine-rich, nucleotide binding motif important for ATP-mediated protection of catalytic activity (1097). $iPLA_2$ activity is moreover stimulated by ROS (628) and by caspase-mediated $iPLA_2$ cleavage (480, 628).

B) PLA_2 IN CELL VOLUME REGULATION. PLA_2-mediated hydrolysis of phospholipids to free fatty acids and lysophospholipids has been demonstrated to be an essential event in the swelling-induced signaling cascade that leads to osmolyte release in a variety of cell types, including EAT cells (989), human platelets (620), mudpuppy RBCs (566), CHP-100 neuroblastoma cells (47), HeLa cells (504), and NIH3T3 cells (492, 503, 800). Addition of the bee venom melittin, which increases the substrate availability for the PLA_2 (104), stimulates arachidonic acid mobilization (compare Fig. 5, *G* and *H*) and elicits taurine loss under isotonic conditions (492) via a signaling cascade/pathway

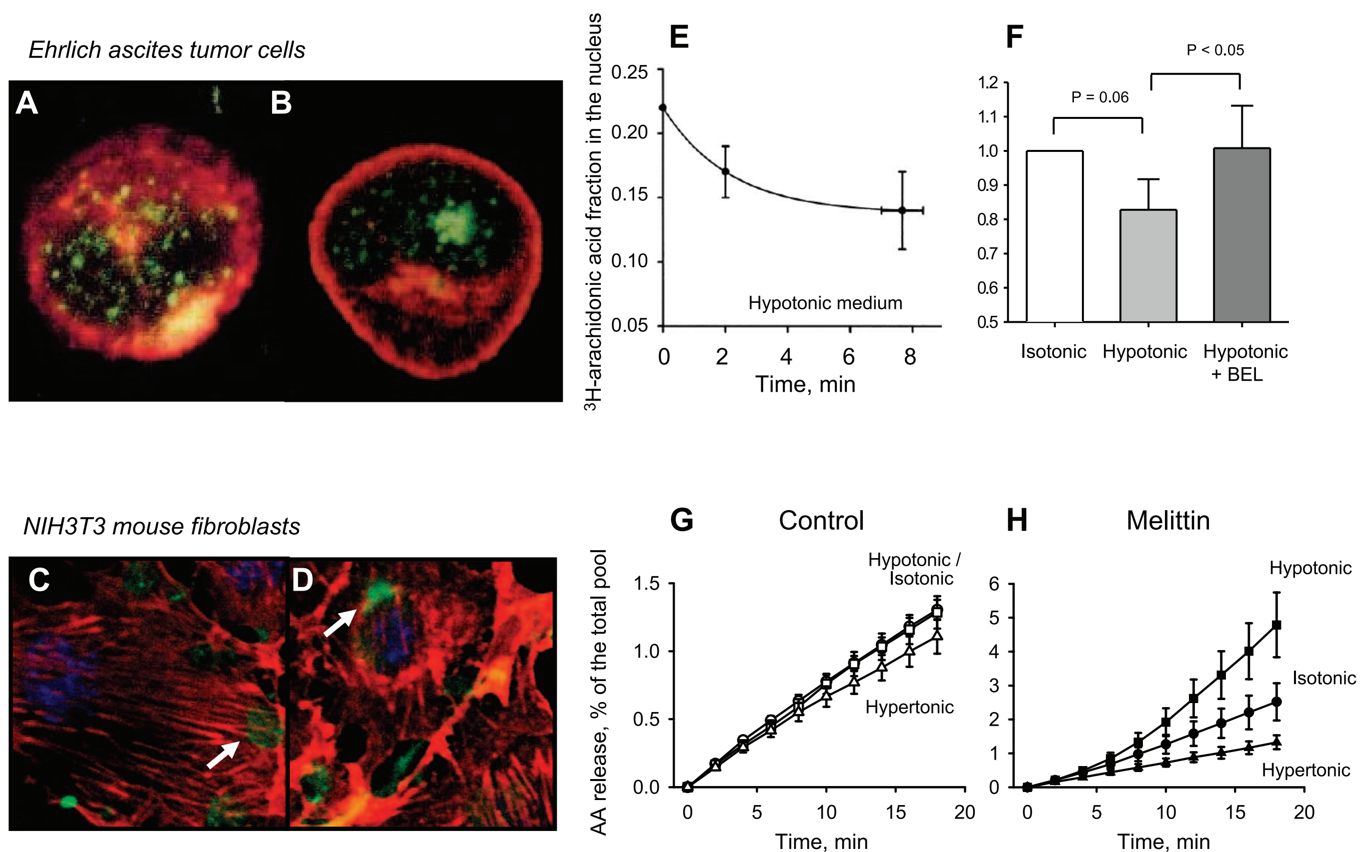

FIG. 5. Translocation of PLA_2 and release of arachidonic acid following cell swelling. *A* and *B*: EAT cells were exposed to isotonic (*A*, 300 mosM) or hypotonic solution (*B*, 150 mosM) for 1 min and fixed for confocal laser scanning microscopy (CLSM). The cells were labeled with rabbit anti-$cPLA_{2\alpha}$ and visualized using a FITC-conjugated antibody (green). The plasma membrane was visualized using tetramethylrhodamine-conjugated agglutinin (red). *C* and *D*: NIH3T3 cells were exposed to isotonic (*C*) or hypotonic solution (*D*) for 5 min before fixation for CLSM. The Ca^{2+}-independent $iPLA_2$, F-actin, and the nucleus were visalized by rabbit anti-$iPLA_{2\beta}$/FITC-conjugated anti-rabbit (green), rhodamine-conjugated phalloidin (red), and 4,6-diamidino-2-phenylindole (blue), respectively. *E*: release of [^{3}H]arachidonic acid (AA) from the nuclei from EAT cells following cell swelling. EAT cells were preloaded for 2 h with [^{3}H]AA, washed, and exposed to isosmotic or hypotonic (150 mosM) solutions. The fraction of [^{3}H]AA in the nucleus was estimated as ^{3}H activity in the nuclear fraction divided by the activity in the nuclear plus cytosolic fraction. *F*: $iPLA_2$-mediated release of [^{3}H]AA from the nuclei from NIH3T3 cells following cell swelling. Cells were loaded with ^{3}H-labeled AA (24 h) before exposure for 5 min to isotonic or hypotonic media with or without the $iPLA_2$ inhibitor BEL. The nuclei were purified (Sigma NUC-201 nuclei isolation kit) for estimation of ^{3}H-labeled AA. The nuclear content was estimated as the fraction of ^{3}H activity present in the nuclei per milligram protein. Values are presented relative to the isotonic value. *G* and *H*: AA release to the extracellular compartment was followed with time under isotonic (300 mosM), hypotonic (200 mosM), or hypertonic (600 mosM) conditions from NIH3T3 cells preloaded with ^{3}H-labeled AA using tracer technique. AA release is given as the fraction (in %) of the total ^{3}H-labeled AA pool. Melittin (1 μg/ml, *H*) was added at the time of the shift in osmolarity to potentiate PLA_2 activity. [Data in *A*, *B*, and *E* from Pedersen et al. (782); data in *C*, *D*, and *F*–*H* from Pedersen et al. (800).]

that exhibits a great similarity to that inducing swelling-induced taurine loss (492). Substantiating the notion that PLA_2 activity is volume sensitive, the effect of melittin on arachidonic release and taurine efflux is increased in swollen, and abolished in shrunken, NIH3T3 cells, respectively (see Fig. 5, *G* and *H*, and Ref. 800). Similarly, H_2O_2 potentiates melittin-induced taurine release under isotonic conditions, yet not under hypertonic conditions (503). Thus further stimulation of arachidonic acid release by mellitin and H_2O_2 requires that a PLA_2 is already active, which is not the case in shrunken cells. PLA_2 activation was, accordingly, proposed to be an initial, upstream event in swelling-induced osmolyte release in, e.g., NIH3T3 cells (800).

It seems highly probable that several PLA_2 isoforms are involved in cell volume regulation. Pharmacological evidence indicates that the swelling-induced arachidonic acid release and RVD involves $cPLA_2$ in EAT cells (490, 989) and CHP-100 neuroblastoma cells (47), $iPLA_2$ and $sPLA_2$ in NIH3T3 cells (492, 503, 800), and a Ca^{2+}-dependent PLA_2 activity in rat inner medullar collecting duct cells (996). The mechanism(s) underlying the regulation of PLA_2 activity by osmotic stress remains incompletely elucidated. Direct activation of snake venom $sPLA_2$ by osmotic swelling/membrane stretch has been demonstrated in artifical lipid vesicles (536), but as noted in section III*A*, the extent to which this phenomenon is relevant in intact animal cells is not clear. In EAT cells, $cPLA_2$-IVA is diffusely distributed under isotonic conditions and translocates to the nucleus within minutes following hypotonic exposure (Fig. 5, *A* and *B*). Immunostaining of $iPLA_2$-VIA in NIH3T3 cells reveals a punctuate labeling in lamellipodia-like and endoplasmatic reticulum-like structures under isotonic conditions, and a more pronounced perinuclear localization following hypotonic exposure (Fig. 5, *C* and *D*). Both $cPLA_2$ and $iPLA_2$ translocation to the nucleus following hypotonic exposure is accompanied by arachidonic acid release from the nuclear membranes (Fig. 5, *E* and *F*). $cPLA_2$-IVA translocates to the nucleus following increases in $[Ca^{2+}]_i$ in, e.g., rat alveolar cells (809) and EAT cells (782), and the nuclear translocation of $cPLA_2$ is generally assumed to be Ca^{2+} dependent. However, in EAT cells, there is no detectable increase in $[Ca^{2+}]_i$ following hypotonic exposure (416). The swelling-induced translocation of $cPLA_2$ to the nuclear membrane is also unaffected by inhibitors of ERK1/2 and p38 MAPK (782). The actin-based cytoskeleton is modulated by cell volume perturbations (sect. IV*D*); however, swelling-induced $cPLA_2$-IVA translocation in EAT cells to the nuclear membrane is unaffected by disruption of F-actin (782), and swelling- and melittin-induced arachidonic acid release and taurine efflux were almost unaffected by F-actin disruption in NIH3T3 cells (800).

2. *Phosphoinositide kinases and phospholipases C and D*

We will limit the discussion here to PtdIns(4,5)P_2, one of the two most abundant phosphoinositides in mammalian cells (the other being PtdIns4P; see, e.g., Ref. 537), and the one for which the volume-dependent changes are most widely documented. Thus the cellular PtdIns(4,5)P_2 level has been shown to increase rapidly after osmotic cell shrinkage in a variety of cell types (695, 706, 1122), and conversely, several studies have demonstrated a decrease in the cellular PtdIns(4,5)P_2 level after osmotic swelling (135, 694, 706). However, there are also reports describing osmotically induced changes in the cellular levels of other phosphoinositides (639, 695, 889, 890, 1023). When interpreting the data discussed below, it should be kept in mind that the measurement and manipulation of PtdIns(4,5)P_2 are subject to considerable technical difficulty, the question of whether there are large local differences in the concentration of PtdIns(4,5)P_2 in the membrane being particularly contentious (for a discussion, see Ref. 266).

A) MECHANISMS REGULATING THE CELLULAR PTDINS(4,5)P_2 LEVEL AFTER CELL VOLUME PERTURBATIONS. The main regulators of cellular phosphatidylinositol, or phosphoinositide, homeostasis are the phosphoinositol phosphate kinases (PIPKs), PPI phosphatases, and phospholipase C (PLC) (754, 975, 1130). Theoretically, an increase in the PtdIns(4,5)P_2 level in a given plasma membrane location can result from, e.g., *1*) increased activity of PIP5KI, which phosphorylates the D5 hydroxyl of the inositol ring of phosphoinositides, or decreased activity of PI3K; *2*) inactivation of the relevant PPI phosphatases; *3*) inactivation of a PLC; or *4*) local changes in sequestering by PtdIns(4,5)P_2 binding proteins such as myristoylated alanine-rich C kinase substrate (MARCKS) (see Refs. 915, 1130). Conversely, the opposites of these scenarios could in principle mediate the decrease in cellular PtdIns(4,5)P_2 level in swollen cells.

Some of these mechanisms have been experimentally verified. Recent studies in EAT cells established that it was not the volume change per se, but the change in ionic strength, which was required for the changes in PtdIns(4,5)P_2 levels after osmotic shrinkage and swelling (706). In HeLa cells, the shrinkage-induced increase in PtdIns(4,5)P_2 was proposed to be mediated through increased recruitment and activity of PIP5KIβ in a manner dependent on Ser/Thr dephosphorylation (1122). The Rho family small G proteins, Rho, Rac, and Cdc42 are all important regulators of PIP5KIβ (754), yet the shrinkage-induced PIP5KIβ activation in HeLa cells was independent of Rho kinase (1122). In congruence with this, we recently found that the shrinkage-induced increase in PtdIns(4,5)P_2 appears to be required for cortical recruitment and subsequent activation of ERM pro-

teins, which in turn are involved in Rho activation (837). This implies a role for PtdIns(4,5)P_2 upstream of Rho activation, which also is most consistent with the notion that Rho is not the major regulator of PIP5KIβ in shrunken cells. The potent stimulatory effect on PIP5KIβ of phosphatidic acid (PA), which can be produced either downstream from PtdIns(4,5)P_2 hydrolysis or via PLD-mediated hydrolysis of, e.g., phosphatidylcholine (754), could also be of potential relevance after osmotic stress. However, PLD appears to be activated by swelling rather than by shrinkage (686, 997). Finally, osmotic shrinkage may exert an inhibitory effect on at least some of the phosphoinositide phosphatases (639).

PLC-γ2 is activated by hypotonic swelling of WEHI-231 cells (694) and of mouse B lymphocytes (577), and this was proposed to underlie the swelling-induced decrease in PtdIns(4,5)P_2 in these cells (577, 694). In apparent contrast, in EAT cells, where a marked decrease in PtdIns(4,5)P_2 after swelling was also demonstrated, there was no accompanying increase in either inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] or Ins(1,3,4)P_3, arguing against a role for PLC activation (706). A swelling-induced increase in the activity of PI3K, as reported in some cells (473), could also contribute to PtdIns(4,5)P_2 depletion under these conditions; however, this has yet to be directly demonstrated.

B) POSSIBLE ROLES OF PTDINS(4,5)P_2 IN VOLUME-SENSITIVE SIGNALING AND REGULATION OF OSMOLYTE TRANSPORT. By what mechanisms might the rapid increase and decrease, respectively, in PtdIns(4,5)P_2 levels after shrinkage and swelling be relevant for the subsequent cellular events? A rapidly growing body of evidence testifies to the major role of phospholipids, and PtdIns(4,5)P_2 in particular, in the regulation of multiple ion channels and transporters (350, 380, 965, 1043); a summary of those known or likely to be of relevance for cell volume regulation are given in Table 1. Here, we will limit the discussion to the acute effects of PtdIns(4,5)P_2 on volume-dependent membrane transport processes, and specifically, effects involving *1*) direct interactions with membrane transport proteins and *2*) PLC-mediated Ins(1,4,5)P_3 and diacylglycerol (DAG) production. However, the involvement of PtdIns(4,5)P_2 in multiple other events elicited by cell volume perturbations seems highly probable. For instance, the pivotal role for PtdIns(4,5)P_2 in cytoskeletal reorganization and the functional consequences thereof are discussed in section IV*D*. Other possible mechanisms, which have to our knowledge not yet been directly addressed with respect to cell volume, include effects of PtdIns(4,5)P_2 on receptor function (see Ref. 281), intracellular signaling events (see Ref. 965), endo-/exocytosis (399), or transcriptional effects (884).

Several studies have demonstrated a direct, regulatory interaction of PtdIns(4,5)P_2 with transport proteins relevant for volume regulation (Table 1). The interactions between the transport proteins and the highly negatively charged PtdIns(4,5)P_2 appear to involve interactions with either cationic and hydrophobic residues as described for the Na^+/H^+ exchanger NHE1 (8), or with pleckstrin homology (PH) domains, as shown for some TRP channels (1043). The interaction can be inhibitory, as proposed for large-conductance background K^+ channels (694) and for TRPV1 under the special conditions of weak stimulation by, e.g., capsaicin [the fully activated current is potentiated by PtdIns(4,5)P_2] (591). It can also be stimulatory, as shown for several other TRP channels (1043), TREK1 two-pore K^+ channels (121), NHE1 (8), and

TABLE 1. *Proposed effects of PtdIns(4,5)P_2 on volume-sensitive transporters and channels*

Transporter	Demonstrated or Proposed Volume Sensitivity of Transporter	Effect of PtdIns(4,5)P_2	Proposed Mechanism	Reference Nos.
TRPV1	Shrinkage activated	Stimulatory (indirect inhibition at, e.g., low capsaicin concentration)	Direct stimulatory effect; indirect inhibitory effect	591, 796; see 561*
TRPC6	Swelling/stretch activated	Inhibitory	Increased membrane curvature upon PtdIns(4,5)P_2 hydrolysis	943
TRPM7	Swelling and/or stretch activated	Stimulatory	Interaction of channel with PLC resulting in highly local changes in PtdIns(4,5)P_2	869, 730*
TREK-1	Swelling/stretch activated	Stimulatory	Direct interaction	121
LK_{bg}	Swelling/stretch activated	Inhibitory	Lipid raft/caveolae? (βMCD sensitive)	694
NHE1 (and likely also NHE2-5)	Shrinkage activated (NHE1)	Stimulatory	Direct interaction	7
NCX	Shrinkage activated	Stimulatory	Direct interaction	349, 1106*
VRAC	Swelling activated	No effect	NA	456
ENaC	Shrinkage activated	Stimulatory	Direct interaction	817*, 860,1132

Unless otherwise noted, references refer to the demonstration of regulation by phospholipids. References marked by an asterisk refer to the volume sensitivity of the transporter; for further references on this, see the text section on the properties of the relevant transporter. PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; βMCD, methyl-β-cyclodextrin, used to deplete the membrane of cholesterol; NA, not applicable.

several other transporters and channels of known or postulated relevance for osmotically perturbed cells (Table 1). To what extent are these effects of PtdIns(4,5)P_2 likely to be relevant for the regulation of volume-sensitive osmolyte transport? The positive regulation of NHE1 by PtdIns(4,5)P_2 would be consistent with the activation of NHE1 by cell shrinkage (sect. VI*A*). TRPV1 is generally activated by PtdIns(4,5)P_2 (591) and appears to be shrinkage-activated in some cell types (sect. IV*B*), which would also be consistent with a role for PtdIns(4,5)P_2 in TRPV1 volume sensitivity. However, whether PtdIns(4,5)P_2 plays a role in the shrinkage activation of NHE1 or TRPV1 has, to our knowledge, yet to be investigated. The Na^+/Ca^{2+} exchanger NCX is also activated both by shrinkage and by increases in PtdIns(4,5)P_2 (1106; see Ref. 350), yet is not an RVI effector; conversely, NCX contributes to steady-state net osmolyte extrusion in some cell types (768, 976).

After cell swelling, the activation of PLC and consequent reduction in PtdIns(4,5)P_2 could play a role in the activation of channels normally under tonic negative regulation by PtdIns(4,5)P_2. Such a scenario was proposed for background K^+ channels in mouse B cells, where the K^+ channels presumably provided the driving force for Ca^{2+} influx (694). On the other hand, the TRPs most likely to be activated by hypotonic swelling in most cell types studied are reported to be either stimulated or unaffected by PtdIns(4,5)P_2, and could thus in principle be upstream from a Ca^{2+}-dependent activation of PLC, by providing the Ca^{2+} influx pathway (see Ref. 1043). Similarly, the stimulation of TREK-1 by PtdIns(4,5)P_2 (121) is not reconcilable with a role for PtdIns(4,5)P_2 in the swelling-induced activation of this channel (774). Finally, we found no evidence for regulation of VRAC in ELA cells by PtdIns(4,5)P_2 (456). Thus, clearly, although they may be very important for the control of individual transport proteins (and, as noted above, for many other consequences of cell volume perturbation), the global changes in cellular PtdIns(4,5)P_2 upon cell volume perturbation are unlikely to be global upstream regulators of volume-sensitive transport. It has recently been suggested that rather than regulating the transport protein directly, PtdIns(4,5)P_2 may serve as an upstream regulator of the volume set point (706). The corresponding increase in the cellular level of Ins(1,4,5)P_3 has long been assigned roles in the swelling-activated increase in $[Ca^{2+}]_i$ in cells where such an increase occurs, through Ins(1,4,5)P_3 receptor-mediated Ca^{2+} release from intracellular stores (577, 666; see also sect. V*B*). On the other hand, this is not the only mechanism, as the swelling-induced increase in $[Ca^{2+}]_i$ is independent of PLC in some cell types (237).

D. The Cytoskeleton and Small GTP-Binding Proteins

The cytoskeleton has long been assigned important roles in mechanosensing and transduction (see, e.g., Refs. 9, 396) and has been the subject of much interest as a player in the events initiated by cell volume perturbations. Extensive reorganization of the cytoskeleton occurs rapidly after osmotic volume perturbations in the great majority of cell types studied. In addition to playing a role in control of volume recovery in at least some cell types, this reorganization may exert protective roles, e.g., by cortical reinforcement, and contribute to volume-dependent regulation of gene transcription. Demonstrating the importance of these phenomena, several (patho)physiological effects of volume perturbations have been shown to be mediated by the cytoskeleton, including shrinkage-induced inhibition of neutrophil function (852) and stimulation of glucose transport (307, 308), the protective effect of hypertonic preconditioning prior to ischemia (750), and the swelling-induced inhibition of autophagic proteolysis (1048, 1051). Below, we discuss pertinent aspects of the current knowledge on the role of the cytoskeleton in volume-dependent signaling events.

1. *Patterns of volume-dependent cytoskeletal reorganization*

Of the three major categories of cytoskeletal filaments (F-actin, intermediate filaments, and microtubules), the response to cell shrinkage is by far best understood for F-actin. Most commonly, osmotic shrinkage has been found to be associated with a net increase, and osmotic swelling with a net decrease, in actin polymerization. This pattern is most characteristic in nonadherent cells, in which the majority of polymerized actin is found as a cortical ring (216, 220, 319, 320, 543, 795, 852). In adherent fibroblasts and epithelial cells, osmotically induced changes in the net cellular content of polymerized actin are often small (e.g., Ref. 418, 786) or absent (837). At least in some epithelial cells, this reflects that the shrinkage-induced increase in cortical F-actin is accompanied by a decrease in stress fiber content (for an excellent discussion, see Ref. 139). Another characteristic effect in some cell types is an increase in the number and/or length of F-actin containing protrusions in shrunken cells, and their disappearance in swollen cells, respectively (155, 164, 354, 837) (Figs. 1 and 6*A*). However, osmotic actin reorganization not consistent with the above patterns has also been noted in several cell types (116, 346, 681, 977, 992). A number of actin-associated cytoskeletal proteins have been shown to be affected by osmotic

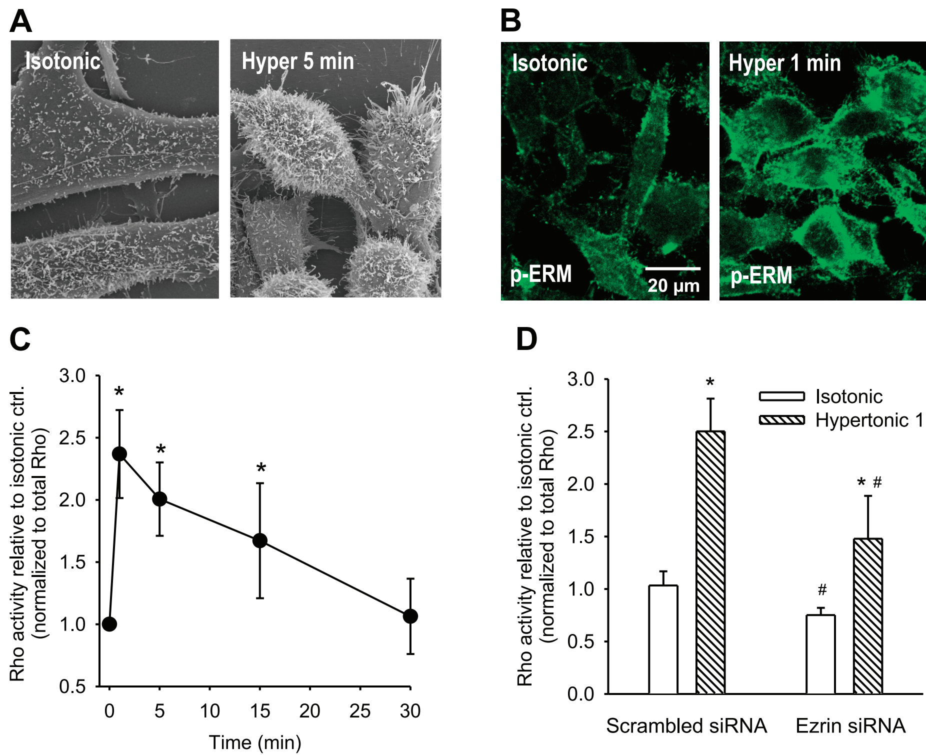

FIG. 6. Cortical reorganization, ezrin/radixin/moesin (ERM) protein activation, and Rho activity after osmotic shrinkage of ELA cells. *A*: scanning electron micrographs of ELA cells under isotonic conditions and after 5 min of osmotic shrinkage induced by doubling of extracellular osmolarity with NaCl. Note the marked increase in the number and length of microvillus-like projections. *B*: confocal images of ELA cells labeled with an antibody against phosphorylated ERM proteins, after isotonic conditions or 1 min of hypertonicity as in *A*. *C*: Rho activity in ELA cells after a hypertonic challenge induced as in *A*, measured by GST pull-down assay. *D*: Rho activity in ELA cells under isotonic conditions and after 1 min of hypertonicity as in *A*, in control cells, and after siRNA-mediated knockdown of ezrin. See text for details. [From Rasmussen et al. (837).]

perturbations.[1] The light chain of nonmuscle myosin II (myosin light chain, MLC) is phosphorylated upon osmotic shrinkage in a variety of cell types (137, 459, 734). In EAT cells, myosin II rapidly translocated to the cortical region upon hypertonic shrinkage, and to the Golgi/perinuclear region upon hypotonic swelling (790). In arterial endothelial cells, hypertonic shrinkage elicited the formation of striated actin-myosin II structures which later reorganized into polygonal actin structures (613). Cortactin is a ubiquitous actin binding protein localizing to regions of rapid actin polymerization. In addition to F-actin, cortactin interacts with the Arp2/3 complex, Wiscott-Aldrich protein (WASP), dynamin, and MLC kinase (MLCK) (156). Upon hypertonic shrinkage of fibroblasts and epithelial cells, cortactin is tyrosine phosphorylated and translocates (albeit independently of this phosphorylation) to the cortical region, where it forms a complex with Arp2/3 (191, 433). Ezrin/radixin/moesin (ERM) proteins belong to the band 4.1 family of plasma membrane-cytoskeleton linker proteins, which share a COOH-terminal F-actin binding site and an NH_2-terminal domain through which they, directly or indirectly, interact with a wide range of integral membrane proteins (82). Osmotic shrinkage elicits rapid (<1 min) activation and cytosol-to-cortex translocation of ERM proteins, and specifically ezrin, in a variety of cell types (837, 1108) (Fig. 6*B*). Another ERM protein, moesin, was recently shown to be activated upon swelling of collecting duct principal cells (977).

Much less is known regarding osmotic effects on the two other types of filaments, microtubules (MTs) and intermediate filaments (IFs). No changes in MT organization after hyposmotic stress could be detected in PC12 cells (155) or in shark rectal gland cells (346). A very rapid, transient MT depolymerization after hypotonicity in combination with urea was observed in keratinocytes (164). In contrast, an apparent stabilization of MTs after hypotonic exposure was described in isolated rat hepatocytes; however, only time points ≥20 min were studied (337). To our knowledge, no studies have yet addressed the effects of hypertonicity on MT organization. Considering the known role of the MT-based cytoskeleton in F-actin reorganization (48), it would seem that the effect of cell volume perturbations on the MT-based cytoskeleton deserves to be revisited.

In shark rectal gland cells (346) and PC12 cells (155), hypotonic swelling was reported to not detectably affect IF organization, whereas in opossum kidney cells, hypotonicity resulted in vimentin reorganization into short fragments (171). In the above-mentioned study in keratinocytes, hypotonicity in the presence of urea did not detectably affect the organization of wild-type keratin, but

[1] The classification of a given actin-associated protein as a regulator or a cytoskeletal protein *per se* is to some extent arbitrary. For the purpose of the present review, we have grouped myosin II, cortactin, and ERM proteins with the F-actin binding cytoskeletal proteins. However, obviously these proteins also impact profoundly on F-actin organization, and in this capacity, they will be discussed in section D2.

did disrupt the weaker, mutant keratins from epidermolysis bullosa simplex patients (164). Finally, altered keratin phosphorylation in response to hypotonic challenges has been observed in HT29 cells (980).

2. *Mechanisms of volume-dependent cytoskeletal reorganization*

Given the limited information regarding MT and IF regulation by cell volume, we shall limit this section to the actin cytoskeleton. An overview of some of the mechanisms likely to be involved in the reorganization of the actin cytoskeleton in response to cell shrinkage is given in Figure 7. Since nucleation of new filaments is the limiting factor in actin polymerization, changes in the cellular content of polymerized actin generally involve changes in either *1*) de novo nucleation, involving nucleation factors such as the Arp2/3 complex; *2*) F-actin severing, involving severing proteins such as cofilin or gelsolin; or *3*) capping/uncapping of F-actin, involving changes in the activity of capping proteins, to which gelsolin also belongs (as it caps the filament following severing), as does the small heat shock protein, HSP25/27 (see Refs. 150, 508). Below, we discuss how the Rho family small GTP binding (G) proteins, PtdIns(4,5)P_2, changes in $[Ca^{2+}]_i$, and protein phosphorylation/dephosphorylation may impact on the Arp2/3 complex, cofilin, and gelsolin to mediate cytoskeletal reorganization in osmotically perturbed cells.

The Rho family small G proteins, Rho, Rac, and Cdc42, are widely studied in their capacity as pivotal regulators of actin organization (315, 974). Rho family G proteins are highly sensitive to cell volume changes, and there is solid evidence pointing to their involvement in at least some of the cytoskeletal rearrangement under these conditions. Rho activity was rapidly increased after hypertonic cell shrinkage in kidney epithelial cells, ELA cells (Fig. 6*C*), and hepatocytes (138, 748, 837), and was, conversely, decreased in osmotically swollen ELA cells (456). Nonetheless, there does not appear to be a straightforward relation between cell volume and Rho family G protein activity. The earliest report proposing a role for Rho in volume-dependent actin reorganization indicated (albeit Rho activity was not directly measured) that Rho was activated by cell swelling in Intestine 407 cells (992), and a lack of effect of cell swelling on Rho activity was reported in endothelial cells (117). Rac and Cdc42 activity were similarly shown to be increased by shrinkage in a variety of cell types (191, 546, 1014), yet was also increased by hypotonic swelling of Rat-2 fibroblasts (116), and decreased after hypertonicity in hepatocytes (748).

The most upstream elements leading to Rho protein regulation by cell volume are still elusive; candidates are integrins and growth factor receptors, which have been implicated in volume sensing and are known activators of Rho family proteins (sect. IV*A*). Interestingly, both a decrease in cell volume in the absence of an increase in ionic strength, and an increase in ionic strength in the absence of a change in cell volume, activated Rho (138) and Rac (546). Other than that, the events between the volume decrease/ionic strength increase and Rho protein activation remain enigmatic, at least in part due to the daunting number of upstream regulators of these proteins: the guanine nucleotide exchange factors (GEFs), the GTPase activating proteins (GAPs), and the guanine nucleotide dissociation inhibitors (GDIs) (974). Interest-

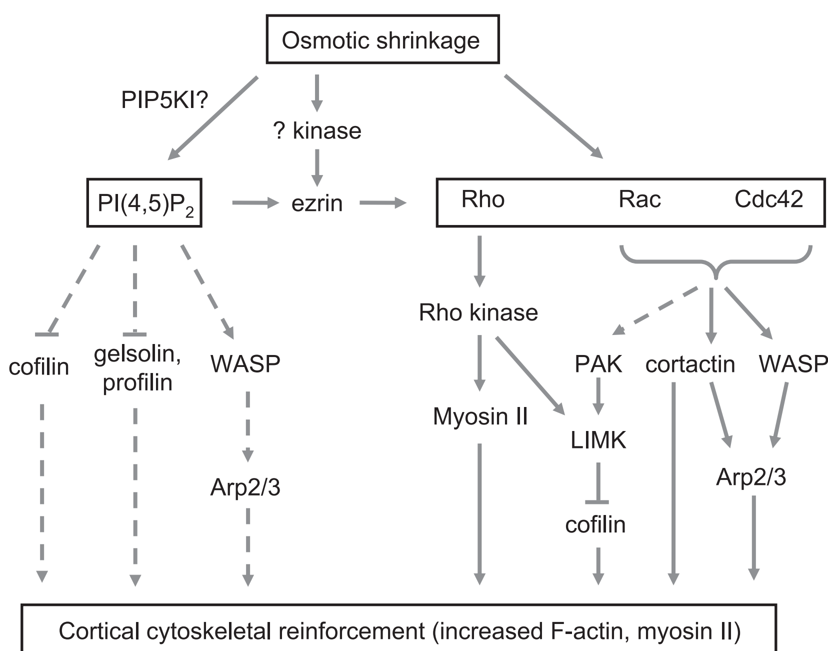

FIG. 7. Mechanisms of reorganization of the cortical actin-based cytoskeleton after osmotic shrinkage. The figure illustrates major, established and yet hypothetical, events leading to the reorganization of the cortical, actin-based cytoskeleton after osmotic cell shrinkage. Connections shown by dotted lines have been demonstrated in other contexts, but evidence of a link to volume regulation is still lacking. It may also be noted that the role of the Rho-ROCK-LIMK-cofilin pathway is still incompletely elucidated (434). See text for details. LIMK, LIM kinase; PAK, p21-activated kinase; PIP5K, phosphatidylinositol 4-phosphate 5-kinase; WASP, Wiscott-Aldrich protein.

ingly, ERM proteins which, as described above, are rapidly activated by osmotic shrinkage in several cell types, regulate both Rho-GDI (973) and the GEF Dbl, leading to activation of Rho and Cdc42, respectively (824). In accordance with such a scheme, we recently reported that siRNA-mediated knockdown of ezrin decreases the shrinkage activation of Rho in ELA cells by over 50% (837) (Fig. 6*D*). To our knowledge, this was the first study to identify a mechanism of Rho activation by osmotic shrinkage. Future studies should address the possible roles of ERM-mediated regulation of GDI and/or GEF function in control of the activity of Rho family G proteins after cell volume perturbations. In turn, the Rho family proteins have been assigned roles in several of the volume-dependent changes in the actin-based cytoskeleton, including the shrinkage- (191, 546, 613) and swelling-induced (116) F-actin rearrangements per se, the shrinkage-induced MLC phosphorylation (138), cortactin and Arp2/3 translocation (191), LIM kinase (LIMK) activation, and cofilin phosphorylation (434). While not further discussed here, it should also be noted that the Rho family G proteins have a multitude of effectors, not all of which exert their effects via the cytoskeleton (e.g., Ref. 61). Such mechanisms most probably also play important roles in volume-dependent signaling, as demonstrated for, e.g., FAK activation by shrinkage (595, 596; sect. V*F*).

As described in section IV*C2*, the cellular level of PtdIns(4,5)P_2, a major regulator of actin organization (404), rapidly increases after osmotic shrinkage and decreases after osmotic swelling. A central role for PtdIns(4,5)P_2 in shrinkage-induced actin remodeling was recently proposed in HeLa cells (1122). In congruence with this, in ELA cells, PtdIns(4,5)P_2 was necessary for the cortical translocation and activation of ezrin, which in turn was important for F-actin reorganization (837). PtdIns(4,5)P_2 also activates WASP, which will similarly favor actin polymerization via the Arp2/3 complex (404), and PtdIns (4,5)P_2 inhibits proteins that favor net F-actin depolymerization or severing, such as the capping protein gelsolin, the monomer binding protein profilin, and the severing protein cofilin (404). In accordance with a role for cofilin inhibition in this process, reduced F-actin severing and cofilin phosphorylation (indicative of inhibition of its severing activity) were observed after osmotic shrinkage in kidney tubular cells (434). Conversely, an increase in $[Ca^{2+}]_i$, as it occurs in some, albeit not all, swollen cells (sect. V*B*) will activate gelsolin, leading to F-actin capping and thus reduced F-actin levels, a scheme assigned a role in the swelling-induced decrease in cortical F-actin in chondrocytes (220).

There is also evidence for, and against, the involvement of both Ser/Thr and tyrosine protein kinases in the osmotic F-actin reorganization. In EAT cells, the shrinkage-induced increase in cortical F-actin was unaffected by pharmacological inhibition of Rho kinase, MLCK, PKC, and p38 MAPK (790). There is, however, evidence for roles for MAPKs in volume-sensitive regulation of the actin cytoskeleton in other cell types, as discussed in section V*C2*. In contrast, the swelling-induced decrease in F-actin may be regulated by MLCK, yet apparently in a cell type-specific manner (786, 790).

Rho kinase appears to be a major mediator of both phosphorylation and translocation of myosin II after shrinkage (138, 790). In addition, a number of Rho family-regulated Ser/Thr kinases central to regulation of actin organization, including p21-activated kinase (PAK) and LIMK, have been shown to be activated by osmotic shrinkage (145, 434, 855). Specifically, in kidney epithelial cells, LIMK was activated downstream of Rho-Rho kinase and appeared to play a role in the ensuing actin reorganization by mediating the phosphorylation, and hence inhibition, of cofilin (434). Finally, Ser/Thr protein phosphatases 1 and 2A are also important regulators of actin organization (e.g., Ref. 161) and are regulated by cell volume perturbations (sect. V*E*), yet their role in volume-dependent actin reorganization has, to our knowledge, never been addressed.

With respect to tyrosine phosphorylation, Src family kinases and FAK have been widely shown to be activated by cell volume perturbations (sect. V*F*) and are important regulators of F-actin organization (245). In mouse fibroblasts, cortactin is phosphorylated by the Src kinase Fyn, downstream from shrinkage-induced activation of the tyrosine kinase FER (431). Nonetheless, Src family kinases did not appear to play a role in the shrinkage-induced increase in cortical F-actin in neutrophils (546), nor was the tyrosine phosphorylation of cortactin necessary for its shrinkage-induced translocation (191). Swelling-induced actin reorganization in human umbilical vein endothelial cells was proposed to be dependent on FAK activity (352), and a similar scheme was tentatively suggested after hypertonic activation of FAK in epithelial cells and fibroblasts (595).

Collectively, it may be concluded that PtdIns(4,5)P_2 and Rho family G proteins appear to be of widespread importance for the actin reorganization in osmotically perturbed cells; that there is evidence to suggest roles for changes in de novo nucleation involving the Arp2/3 complex, and for changes in F-actin severing involving cofilin, in the volume-dependent actin reorganization; and that a role for changes in F-actin capping, e.g., downstream from changes in gelsolin activity is possible, yet remains to be directly addressed.

3. *The actin cytoskeleton as a regulator of ion transport in osmotically perturbed cells*

Given the cell-type dependence of actin organization and effects of volume perturbations on actin organization, it is not surprising that also the downstream effects of

actin in the regulation of volume-sensitive transport differ widely between cell types. Studies in a wide variety of cell types have demonstrated that disruption of the actin-based cytoskeleton impacts on the RVD and RVI processes. Generally, in these studies, F-actin disrupting agents such as cytochalasins and latrunculin were found to inhibit the RVD (204, 216, 243, 795) and/or RVI response (216, 573, 795), although in a few cases, acceleration of RVD (441) and RVI (442) by F-actin disruption was reported. On the other hand, in a number of studies, disruption of the actin cytoskeleton was without detectable effect on RVD (319, 320) and RVI (243, 320). Similarly, MT depolymerization by colchicine or stabilization by taxol (paclitaxel) has been shown to inhibit (573, 921) or have no effect on (216, 825) the RVD and RVI responses. A single study suggests a role for IFs in RVD (195).

Although there is little doubt that the cytoskeleton is involved at multiple levels in the events occurring after cell volume perturbations, such data must be interpreted with caution. First, in many studies in which the effects of cytoskeleton-disrupting agents on volume-sensitive transporters and channels was addressed, it was not determined whether osmotic perturbation in fact had an effect on the cytoskeletal component in question, precluding distinction between a permissive or a causal role for these structures. Second, it was not always verified whether the treatment in fact had the expected effect on cytoskeletal integrity. Third, at least some of the compounds used, including, e.g., several of the cytochalasins, have effects not related to their effects on cytoskeletal integrity (e.g., Refs. 647, 795).

That said, nearly all the transporters and channels mediating RVD or RVI in various cell types have been suggested to be regulated by the actin-based cytoskeleton.[2] This includes swelling-activated Cl^- (456, 543, 636, 912, 921, 1141) and K^+-channels (109, 418, 812), and after shrinkage, Na^+ channels (748) and both NKCC1 and NKCC2 (367, 412, 634) (see sect. VI*B*) as well as NHE4 (69). In contrast, shrinkage activation of NHE1 is probably not dependent on the actin cytoskeleton (69; see also sect. VI*A*), although in contrast, the hypotonicity-induced inhibition of this transporter may be actin dependent (219).

What are the mechanisms by which the cytoskeleton might regulate or modulate volume regulation? A number of scenarios have been supported experimentally. First, a given transport protein might be regulated by direct interaction with the cytoskeleton, as proposed for the ClC-3 channel (636). Second, the cytoskeleton might regulate signaling events controlling the transport protein. One example of this is the swelling-induced increase in $[Ca^{2+}]_i$, which is inhibited by cytochalasins in many cell types (59, 317), an effect which may reflect regulation of TRP channels or (other) SA channels by the actin cytoskeleton (663; see Ref. 322). Third, cell shrinkage inhibits, and cell swelling stimulates, exocytosis (and vesicle recycling in general), in a manner which is, not surprisingly, dependent on the cytoskeleton (747, 852, 1024, 1034). Changes in vesicle recycling may regulate the transporters/channels both through signaling events, exemplified by the vesicle recycling-dependent, swelling-induced release of ATP from intestine 407 cells (1024) and through plasma membrane insertion/retrieval of the transport proteins. Possible examples are NKCC2 in the eel intestine (575), the swelling-activated taurine channel in *Raja erinacea* RBCs (825), and the swelling-activated Cl^- current in nonpigmented ciliary epithelial cells (1034).

4. *Other effects of osmotically induced cytoskeletal reorganization*

As discussed in section IX, cell volume perturbations elicit a wide array of signaling events impacting on cell migration, survival, and proliferation, events which are also known to be heavily dependent on F-actin (287, 315, 844). Little evidence is yet available that directly demonstrates roles for the cytoskeleton in regulation of these processes during cell volume perturbations, and this constitutes an important area for future research. For instance, the actin cytoskeleton has been implicated in the hypertonic activation of JNK (sect. V*C1*). There is also evidence to suggest that the actin cytoskeleton is important in volume-dependent control of gene transcription. The role of the cytoskeleton in regulation of the tonicity-responsive enhancer binding protein, TonEBP, is described in section VIII*A*. Another important example is the swelling-induced activation of the transcription factor AP1, which has been shown to be disrupted by cytochalasin-induced loss of F-actin integrity (447). Lending further credence to the notion of volume-dependent F-actin reorganization as an important mechanism of transcriptional regulation, Rho activation, an important consequence of osmotic shrinkage in many cell types (sect. IV*D2*), was shown to regulate the transcription factors serum response factor (SRF), and myocardin-related transcription factor (MRTF) by mechanisms involving MLC (after disruption of intercellular contacts; Refs. 223, 650), or the LIMK-cofilin pathway (after mechanical force application; Refs. 274, 1146).

V. SIGNAL TRANSDUCTION IN RESPONSE TO CELL VOLUME PERTURBATIONS

A. Arachidonic Acid Metabolites

In unperturbed cells, the concentration of free arachidonic acid is low because fatty acids released from the

[2] Where relevant, the regulation of specific transporters by the cytoskeleton is discussed in more detail in section VI and VII.

sn-2 position of glycerophospholipids by PLA_2 are either rapidly reincorporated into the phospholipids by CoA-dependent acyltransferases or converted to hydroxy fatty acids, leukotrienes, and lipoxins by lipoxygenases; to prostaglandins, prostacyclins, and thromboxanes by cyclooxygenases; to epoxides by cytochrom *P*-450; or nonenzymatically to isoprostanes (see Fig. 8) (142). Effects of arachidonic acid per se (sect. IV*C1*) require micromolar concentrations and only seem relevant under conditions of excessive mobilization of fatty acids. Prostaglandins and thromboxanes, produced via the cyclooxygenases (COX1/COX2), act through GTP protein-coupled receptors (GPCR) and their intracellular signaling elements include Ca^{2+}, cAMP/PKA, and MAPKs (79, 142). The leukotrienes LTB_4, LTC_4, LTD_4, and LTE_4 are potent biological substances involved in, e.g., immune cell chemokinesis, chemotaxis, adherence, and aggregation as well as in lysosomal degradation, vaso- and bronchoconstriction, and stimulation of pulmonary mucus secretion (142, 429, 684, 703). 5-Lipoxygenase (5-LO) plays a role in the swelling-induced activation of osmolyte transporting systems as evidenced by the ability of various 5-LO inhibitors (redox inhibitors, substrate inhibitors, direct inhibitors, functional inhibitors) to inhibit the restoration of the cell volume following osmotic cell swelling (490, 632) and the concomitant efflux of the organic osmolyte taurine, Cl^-, and K^+ (357, 491, 632). The 5-LO is a monomeric enzyme with a small NH_2-terminal domain which interacts with Ca^{2+} and membranes, and a large COOH-terminal, catalytic domain, which contains the catalytic Fe^{2+} (829). Fatty acids with double bonds at carbons 5 and 8, e.g., arachidonic acid (5,8,11,14-eicosatetraenoic acid), EPA (5,8,11,14,17-eicosapentaenoic acid), 5,8,11-eicosatrienoic acid, and 5,8-eicosadienoic acid are considered good 5-LO substrates (944). The 5-LO catalyzes the oxidation of arachidonic acid into 5-hydroperoxyeicosatetraenoic acid (5-HPETE) as well as the subsequent dehydration of the 5-HPETE to the unstable LTA_4 (see also Fig. 8). LTC_4 is

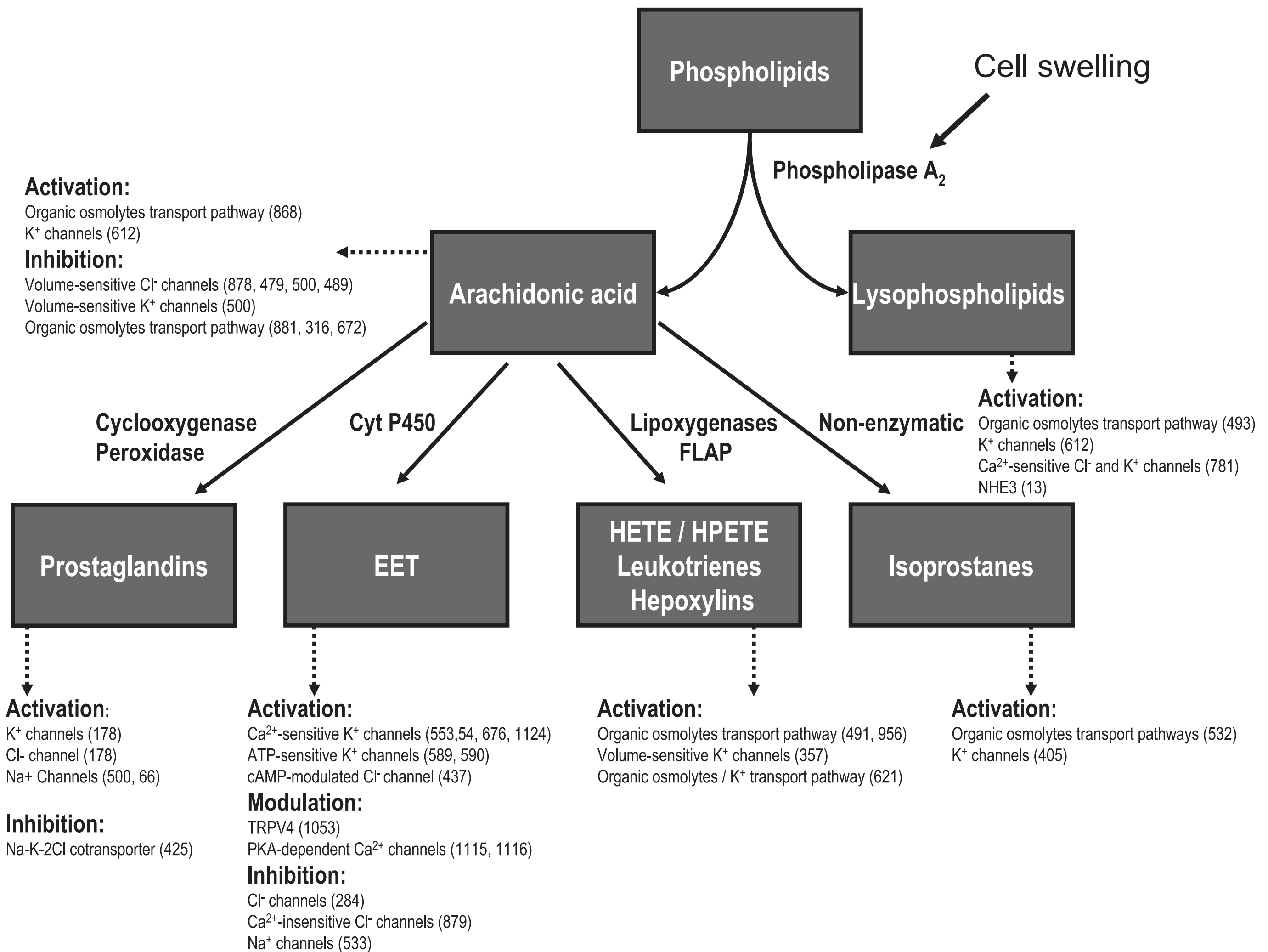

FIG. 8. PLA_2 metabolism and the eicosanoids that regulate transporters and channels potentially involved in volume regulation. See text for details.

formed from LTA_4 by a glutathione *S*-transferase (LTC_4 synthase)-mediated conjugation of glutathione (γ-glutamyl-cysteinyl-glycine), and LTD_4 and LTE_4 are subsequently formed from LTC_4 by sequential loss of the glutamic acid and the glycine residues (703). Activation of 5-LO involves its translocation to the perinuclear membrane, substrate transfer by the membrane-bound 5-LO activating protein (FLAP), oxidation of the non-heme iron in the active site of 5-LO ($Fe^{2+} \rightarrow Fe^{3+}$, H_2O_2 and hydroperoxide mediated), and, at some stage, ATP-mediated stabilization and phosphorylation of the 5-LO (see Refs. 142, 403, 830). Binding of Ca^{2+} to the 5-LO increases its hydrophobicity and promotes association between 5-LO and FLAP at the membrane (831). EAT cells synthesize a range of 5-LO products (490), and their leukotriene synthesis is increased dramatically within the initial 2 min following hypotonic exposure, at the expense of the prostaglandin synthesis (Fig. 9*A*). The affinity of 5-LO towards Ca^{2+} is low (K_D ~6 μM; Ref. 323) and taking the lack of any detectable increase in $[Ca^{2+}]_i$ in EAT cells following hypotonic cell swelling (416) into consideration, it seems unlikely that an increased 5-LO activity under hypotonic conditions is induced by an increase in $[Ca^{2+}]_i$. Ca^{2+} promotes 5-LO association with synthetic phosphatidylcholine (PC) liposomes (728), and it has been suggested that a PC selectivity of the 5-LO could account for 5-LO targeting to the nuclear membrane (481). Membrane binding per se may, however, not confer 5-LO activity, and although Ca^{2+} is required for 5-LO activity, 5-LO binds to, e.g., cationic phospholipids in the absence of Ca^{2+} (761).

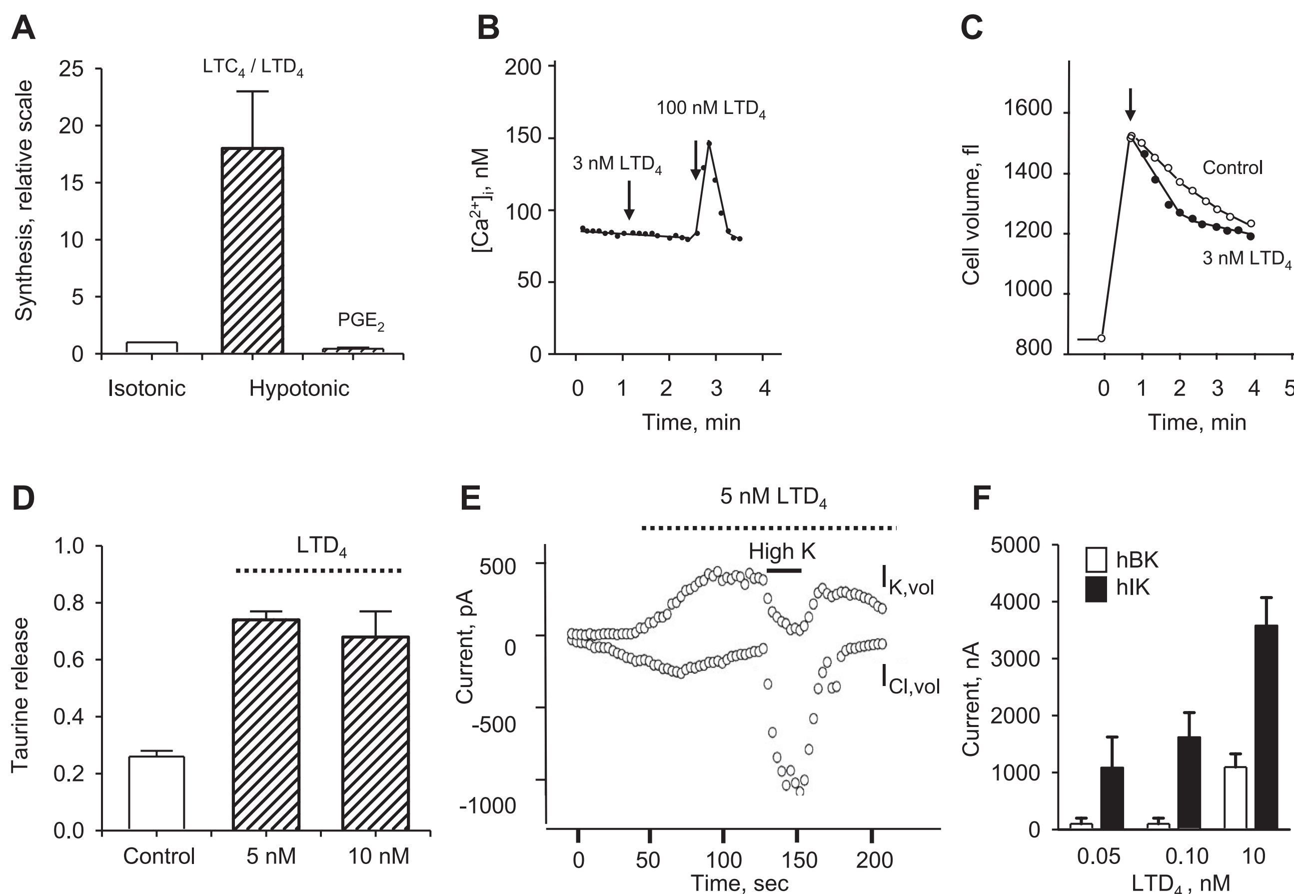

FIG. 9. LTD_4 plays a role as a second messenger involved in the swelling-induced activation of K^+ channels and the taurine efflux pathway. *A*: rate of LTD_4/LTC_4 and PGE_2 synthesis was measured in EAT cells within the first minutes following isotonic (300 mosM) or hypotonic (150 mosM) exposure. The eicosanoid concentration was estimated in the extracellular compartment by radioimmunoassay and given per milligram cell dry weight. [Values, presented relative to the value in isotonic medium, are from Lambert et al. (500).] *B*: effect of a low (3 nM) and high (100 nM) dose of LTD_4 on $[Ca^{2+}]_i$ in EAT cells was investigated by the fura 2-fluorescent technique. [From Jorgensen et al. (417).] *C*: effect of a low dose of LTD_4 (3 nM) on RVD in EAT cells was assessed by the Coulter technique as outlined in Fig. 1. LTD_4 was added at the time of maximal cell swelling as indicated by the arrow. [From Jorgensen et al. (417).] *D*: effect of LTD_4 (5 nM, 10 nM) on taurine release from EAT cells under isotonic conditions was assessed by tracer technique. Values are given as rate constants for the initial taurine release ($min^{-1} \times$ g cell water/g cell dry wt). [Data from Lambert (491).] *E*: K^+ current (I_K) activated by a low LTD_4 concentration (5 nM) was measured under isotonic conditions in EAT cells using whole cell patch-clamp technique, a holding potential of −30 mV, and pulsing to the equilibrium portentials (*E*): +5 mV (E_{Cl}) or −83 mV (E_K) with a fast ramp protocol. The currents measured at +5 mV and −83 mV represent $I_{K,Vol}$ (TASK-2) and $I_{Cl,Vol}$, respectively. [Data from Hougaard et al. (385).] *F*: comparison of the dose-dependent activation of the hIK and hBK by LTD_4 in *Xenopus* ooxytes coexpressing the LTD_4 receptor ($mCysLT_1$) with hIK or hBK channels. Current was measured using a two-electrode clamp technique. [Data from Wulff et al. (1112).]

The 5-LO is subject to phosphorylation by the MAPKs ERK1/2 and p38 MAPK (598, 1087, 1089), as well as by tyrosine kinases (540). It has been suggested that binding of polyunsaturated fatty acids to putative regulatory fatty acid binding sites on the 5-LO could promote phosphorylation via the ERK2 (1086) and p38 MAPK/ MAPKAPK 2/3 pathways (876). The ERK- and MAPKAPK-mediated phosphorylation of 5-LO leads to its activation, whereas PKA-mediated 5-LO phosphorylation suppresses catalysis and prevents its nuclear localization (597, 598). Oxidative stress (H_2O_2) and inhibition of tyrosine phosphatases stimulates p38 MAPK activity in BL41 cells; hence, under these conditions, 5-LO may be stimulated either via promotion of the above-mentioned $Fe^{2+} \rightarrow Fe^{3+}$ conversion or by phosphorylation (1088). As noted elsewhere, $cPLA_2$ and $iPLA_2$ translocate to the nucleus and mobilize arachidonic acid from the nuclear membrane in EAT cells and NIH3T3 cells following hypotonic exposure (sect. IV*C1*, Fig. 5), in accordance with the notion that PLA_2, 5-LO, FLAP, and LTC_4 synthase colocalize at the nuclear membrane during leukotriene biosynthesis (84).

The synthesis and release of LTC_4/LTD_4 in EAT cells is increased within the first minutes following hypotonic exposure (Fig. 9*A*), and exogenous addition of LTD_4, at a concentration that does not provoke Ca^{2+} mobilization (Fig. 9*B*), accelerates the RVD response (Fig. 9*C*) and stimulates efflux of taurine (Fig. 9*D*) and K^+ (Fig. 9*E*; Ref. 357) from EAT cells under isotonic conditions. The effect of LTD_4 is impaired in the presence of LTD_4 receptor antagonists (417, 488), and it has accordingly been suggested that swelling-induced activation of taurine and K^+ efflux in EAT cells involves the following signaling cascade: swelling-induced translocation of $cPLA_2$ to the nuclear membrane, arachidonic acid mobilization, oxidation of arachidonic acid to LTD_4 via the 5-LO and LTC_4 synthase, release of LTD_4, and finally, binding of LTD_4 to a G protein-coupled LTD_4 receptor (358). Coexpression in *Xenopus* oocytes of the $hLTD_4$ receptor together with the Ca^{2+}-sensitive K^+ channels hIK and hBK revealed that 0.1 nM LTD_4 activates the IK channel but not the highly Ca^{2+}-sensitive BK channel. This indicates that no increase in $[Ca^{2+}]_i$ has occurred and that there seems to be a direct coupling between the LTD_4 receptor and IK channel (Fig. 9*F*) (1112). Leukotrienes have also been assigned a role in RVD in rat colon (193, 646), and in release of inositol in glia cells (956). Finally, 12-LO metabolites were found to be involved in TRPV1 activation (395) and in RVD in human platelets (621).

B. The Free, Intracellular Ca^{2+} Concentration

The effect of cell swelling on $[Ca^{2+}]_i$ differs widely between cell types, as summarized in Table 2. An involvement of Ca^{2+} in the RVD response following cell swelling has been found in many cell systems, particularly those of epithelial origin. Swelling of such cells appears to induce either a single increase in $[Ca^{2+}]_i$, or a biphasic increase, in which both phases depend on Ca^{2+} influx from the extracellular compartment, whereas the sec-

TABLE 2. *Calcium signaling during RVD*

Cell Type	Swelling-Induced Increase in $[Ca^{2+}]_i$	Source of Ca^{2+}	RVD Dependence of Extracellular Ca^{2+}	Reference Nos.
Rat C6 glioma cells	Not detectable			956
H4IIE hepatoma cells	Not detectable			896
EAT	Not detectable		No	416
Rat sympathetic neurons	Not detectable			531
HeLa cells	Not detectable			222
NIH3T3	Not detectable		Not investigated	786
Eccrine clear cells	Yes (220 → 435 nM)	Influx	Yes	880
Intestinal epithelial cells	Yes (135 → 170 nM)	Influx	Yes	602
Human osteoblast-like cells	Yes (150 → 1300 nM)	Influx	Not investigated	1090
Proliferating prostate cancer spheroids	Yes	Influx and release from intracellular stores	Yes	888
RINm5F insulinoma cells	Yes	Influx	Yes	914
TALH cells: rabbit renal thick ascending limb of Henle's loop	Yes (100 → 650 nM)	Influx	Yes	664, 995
Rabbit corneal epithelial cells	Yes (100 → 320 nM)	Influx and release from intracellular stores	Yes	1111
SiHa cells: human cervical cancer cells	Yes (120 → 1,050 nM)	Influx and release from intracellular stores	Yes	920
Crypts of mouse distal colon	Yes (175 → 450 nM)	Influx and release from intracellular stores	Yes	646
Rat astrocytes	Yes (150 → 580 nM)	Release and release from intracellular stores	Yes	733

$[Ca^{2+}]_i$ values are given for isotonic and hypotonic conditions, the precise degree of which varies between studies, and can be found in the references provided.

ond phase represents Ca^{2+}-induced release of Ca^{2+} from internal stores (340). In EAT cells, <10% of the cell population showed an increase in $[Ca^{2+}]_i$ following hypotonic exposure in Ca^{2+}-containing media, and no increase in $[Ca^{2+}]_i$ was detected in the absence of extracellular Ca^{2+} (416). Similarly, in many other nonepithelial cells, no detectable increase in $[Ca^{2+}]_i$ is observed following osmotic cell swelling. A Ca^{2+} dependence of RVD often appears to reflect the involvement of Ca^{2+}-activated K^+ channels (602, 646, 880, 1090). In contrast, RVD in EAT cells is even slightly improved following chelation of extracellular Ca^{2+}, due to an increased activity of a latent KCl cotransporter (416, 470, 498). (For further references on the role of Ca^{2+} in cell volume regulation see Refs. 360, 366, 493, 510, 737, 770, 1081.) Several channels have been suggested to be responsible for swelling-induced Ca^{2+} entry, including nonselective SA channels and TRP channels (sect. IV*B*) and voltage-dependent L-type Ca^{2+} channels (see Ref. 1081). In rat cortical collecting duct cells, expression of AQP2 was critical both for the increase in $[Ca^{2+}]_i$ and the activation of nonselective SA channels after cell swelling (264).

1. Ca^{2+}/calmodulin

The suppression of RVD and the concomitant loss of ions and organic osmolytes by exposure to calmodulin (CaM) antagonists has pointed to a role of Ca^{2+}/CaM in these processes (106, 222, 294, 295, 365, 371, 451, 604), although it should be noted that many CaM antagonists exhibit relatively poor specificity (863). Inhibitors of the CaM/CaM kinase (CaMK) system reduced volume-sensitive K^+ currents in jejunal enterocytes (602) and Cl^- efflux in HeLa cells (451). Swelling-induced taurine release in EAT cells (499) and HeLa cells (451) was also impaired in the presence of CaM antagonists. In contrast, in NIH3T3 cells, CaM antagonists significantly potentiated swelling-induced taurine release, likely because the CaM antagonists lift a tonic inhibition of $iPLA_2$ activity (492).

2. Requirement for a permissive intracellular Ca^{2+} concentration

Although an increase in $[Ca^{2+}]_i$ is not required for the RVD response in most cells studied (see Table 2), Ca^{2+} was found to play a permissive role, e.g., for RVD in rabbit medulla thick ascending limb cells and neuroblastoma cells (14, 664), for swelling-induced activation of VRAC in vascular endothelial cells (971), prostate cancer epithelial cells (538) and mouse cholangiocyte cells (122), and of Ca^{2+}-activated K^+ channels (hIK, rSK3) expressed in *Xenopus* oocytes (305). A reduction of taurine release following removal of extracellular Ca^{2+} and depletion of intracellular thapsigargin-sensitive Ca^{2+} stores has also been reported (46, 746). On the other hand, strong buffering of cellular Ca^{2+} ($[Ca^{2+}]_i$ <10 nM) had no effect on the RVD response (416) or on the activation of VRAC and $I_{K,vol}$ channel in EAT cells (385). Similarly, RVD in lymphocytes was unaffected by chelation or depletion of intracellular Ca^{2+} (297, 298).

4. Ca^{2+} mobilizing messengers

Increasing $[Ca^{2+}]_i$ by addition of Ca^{2+} mobilizing agonists (ATP, LPA, LTD_4, bradykinin, thrombin) or Ca^{2+} ionophores (A23187, ionomycin) has been demonstrated to accelerate the RVD response and induce significant cell shrinkage under isotonic conditions in, e.g., EAT cells (365, 416, 417, 780, 781, 799, 848). The rate of RVD is limited by the K^+ permeability, and the accelerated RVD and improved KCl loss after an increase in $[Ca^{2+}]_i$ are often taken to reflect activation of Ca^{2+}-sensitive K^+ and most probably also Cl^- channels. The CaM sensitivity of RVD has been proposed to be tightly coupled to the Ca^{2+}-sensitive K^+ channels (see Ref. 877); however, the Ca^{2+}-mediated potentiation of VRAC is also reported to involve CaMKII (112, 661). The swelling-induced taurine release is potentiated following addition of Ca^{2+}-mobilizing agonists in many (248, 263, 492), although not all (81, 240), cell types, i.e., an increase in $[Ca^{2+}]_i$ does not necessarily elicit activation of taurine efflux (222, 288). Ca^{2+} agonist-induced potentiation of taurine release under hypotonic conditions is mediated by a novel PKC isoform, which is presumed to be activated by the agonist-induced PLC activation and DAG mobilization (222). The inactivation of the volume-sensitive taurine efflux pathway following osmotic swelling is also accelerated in the presence of Ca^{2+}-mobilizing agonists; however, this reflects the acceleration of volume recovery under these conditions (222).

5. ATP as an autocrine messenger inducing Ca^{2+} influx

In several cell types, mechanical stress has been shown to induce release of ATP. In EAT cells, this elicited Ca^{2+} influx and activation of an outwardly rectifying whole cell current (783). ATP has been assigned a role as an autocrine messenger during RVD in rat hepatoma cells, such that ATP released during osmotic swelling binds to a purinergic receptor (subtype $P2Y_1$), eliciting a transient increase in $[Ca^{2+}]_i$ and consequently activation of Ca^{2+}-sensitive K^+ channels (189, 420, 421, 1068). ATP is also released by osmotic cell swelling from pancreatic β-cells (338), ocular ciliary epithelial cells (653), airway epithelial cells (685), and a number of other cell types (for a review, see Ref. 872). It should be emphasized, however, that autocrine ATP release cannot ubiquitously account for the activation of swelling-activated K^+ currents. For instance, in EAT cells, addition of ATP elicits an increase in $[Ca^{2+}]_i$ (799), yet does not activate the swelling-activated K^+ channels, but rather a Ca^{2+}-activated, IK-like, charyb-

dotoxin-sensitive channel (357). The nature of the ATP efflux pathway is a controversial issue; however, swelling-induced ATP release from C127i cells was proposed to be mediated by a voltage-dependent large-conductance anion channel, and to be mediated by neither cystic fibrosis transmembrane regulator (CFTR) nor VRAC (341, 871). Similarly, swelling-induced ATP release from kidney macula densa cells (51) and cardiomyocytes (215) was proposed to be mediated by voltage-dependent large-conductance anion channels.

C. MAPKs

MAPKs are highly conserved mediators of a wide variety of mitogenic and stress-activated signals (see Refs. 486, 1093, 1114). The core module mediating the activation of MAPKs is a three-tiered phospho-relay system: the first component is a MAPK kinase kinase, to which belong the MAPK-ERK kinase kinases (MEKKs), the mixed lineage kinases (MLKs), apoptosis-stimulating kinase (ASK1), transforming growth factor-β-activated kinase 1 (TAK1), and a number of others (see Refs. 163, 486, 1013, 1093). The MAPK kinase kinase phosphorylates and activates the second component, a MAPK kinase (MEK or MKK), which again activates the MAPK by dual phosphorylation on threonine and tyrosine residues. Further upstream, the activation of the MAPK kinase kinases in response to mitogenic or stress stimuli can occur through multiple pathways involving a plethora of signaling events, common components in which are small G proteins and Ste20-related protein kinases (466, 1013; see Ref. 486). Another important feature of the MAPK pathways is their regulation through endogenous scaffold proteins and inhibitors such as kinase suppressor of Ras (KSR), JNK-interacting protein (JIP), and the Sprouty (Spry)/Spred proteins (190, 311, 466, 679). At least seven subfamilies of MAPKs have been identified to date, of which the three most well-described in mammalian cells are extracellular signal-regulated kinase 1/2 (ERK1/2), the p38 MAPKs, and c-Jun NH_2-terminal kinases (JNK1,2) (26, 159). As detailed below, all of these MAPK families play central roles in osmotic stress-induced signaling events. Most commonly, the MAPK kinases MEK1/2, MKK3/6, and MKK4/7 activate ERK1/2, p38 MAPK, and JNK, respectively; however, substantial cross-talk exists (1013; see Ref. 486). Especially at the MAPK kinase kinase level, regulation of ERK1/2, p38 MAPK, and JNK exhibits a large degree of promiscuity (1013; see Ref. 486). A fourth subfamily relevant to osmotic stress (see below) is ERK5, which is regulated in a more stringent manner by a MEKK2-MEK5 pathway (689). The MAPKs are pivotal regulators of essential cell functions including proliferation and death/survival balance, generally such that ERK activity favors proliferation and survival, whereas the opposite is true for p38 MAPK (136, 1114). The effect of JNK on proliferation and survival is less straightforward, at least in some cases due to differential roles of the JNK isoforms 1 and 2 in these processes (576, see Ref. 917).

Regulation of MAPKs by osmotic stress has been widely studied in yeast, in which the p38 MAPK ortholog HOG1 plays a major role in the response to osmotic shrinkage (see Ref. 375). The MAPKs are also strongly affected by cell volume perturbations in vertebrate cells (Fig. 10): the activity of ERK1/2, p38 MAPK, JNK, and ERK5 has been shown to be modulated by osmotic stress in a wide variety of cell types (67, 249, 279, 446, 558, 788, 803, 854, 874; see Refs. 177, 708, 1067). The specific pattern of MAPK modulation is highly cell type and condition dependent. This is especially true for ERK1/2 activity, which in some cells is increased (255, 483, 1007, 1133), and in others decreased (249, 708, 788) by cell shrinkage, whereas cell swelling is generally associated with ERK1/2 stimulation (e.g., Refs. 446, 708, 874) (Fig. 10*B*). In contrast, cell shrinkage nearly ubiquitously stimulates the activity of the stress-activated MAPKs, p38 MAPK (67, 249, 279, 367, 803, 854), and JNK (279) (Fig. 10, *A* and *B*), but the time course of activation varies widely between cell types. Moreover, there are several examples of swelling-induced stimulation of JNK and p38 MAPK activity (e.g., Refs. 624, 713, 759, 993, 1049), raising the question of to which extent changes in MAPK activity are part of the volume response per se, as opposed to being general stress responses activated by cell volume perturbations. Finally, a recent study demonstrated that also ERK5 is activated by hyperosmotic stress in fibroblasts and appears to promote cell survival under these conditions by downregulating Fas ligand expression (1067).

1. *Mechanisms of regulation of MAPK activity by osmotic stress*

It is well established that the activation of the yeast p38 MAPK homolog HOG1 by hyperosmotic stress is mediated through a complex consisting of the small G protein Cdc42, the scaffold protein Ste50, Ste11, and Pbs2 (see Refs. 375, 981). In higher eukaryotes, the mechanisms by which cell volume perturbations modulate MAPK activity are still incompletely understood, but several parallels to the yeast pathway have been identified in recent years. Thus evidence from a range of cell types points to important roles for Cdc42 and/or Rac in shrinkage-induced activation of p38 MAPK (249, 1014, 1091; see also Fig. 19). Importantly, a complex of Rac, the scaffolding protein OSM, MEKK3, and MKK3 was found to be responsible for shrinkage-induced p38 MAPK activation in mammalian cells, by analogy with the HOG1 signaling cascade in yeast (1014). The signal initiating the formation of this scaffold is unknown, but a role for the actin cytoskeleton as part of the osmosensing system was pro-

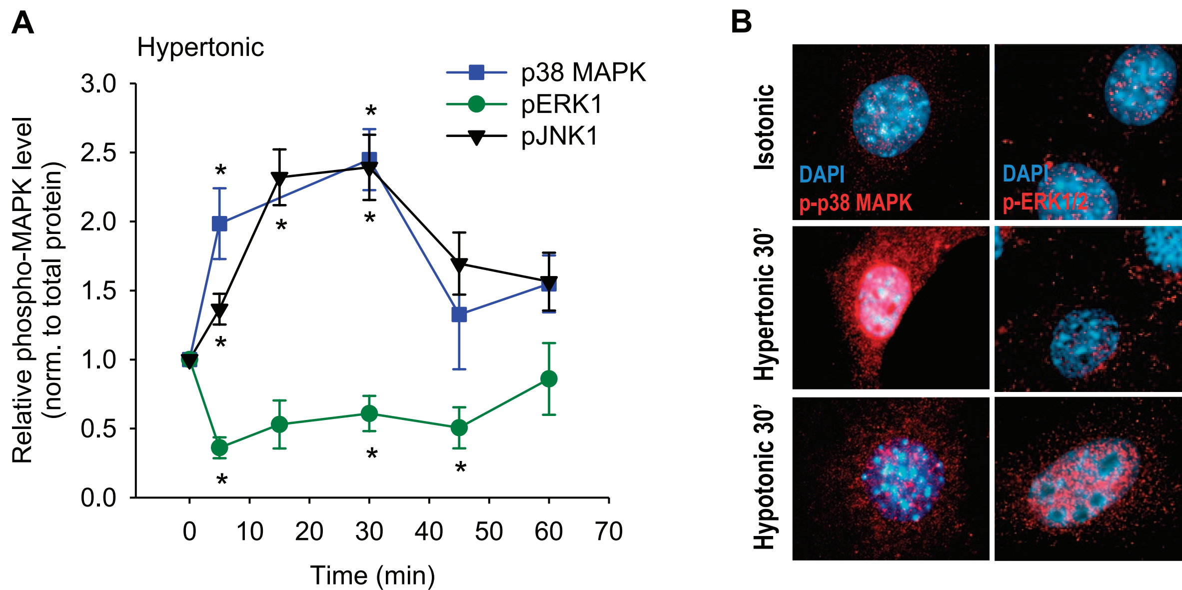

FIG. 10. Activation of mitogen-activated protein kinases (MAPKs) by cell volume perturbations. *A*: inhibition of ERK1, and activation of JNK1, and p38 MAPK, in interphase ELA cells after of osmotic shrinkage induced by doubling of extracellular osmolarity with NaCl. Kinase activity was evaluated by Western blotting against the phosphorylated, active forms of the proteins, followed by normalization to total protein and subsequent quantification. [Values from Pedersen et al. (788).] *B*: immunofluorescence images demonstrating the translocation of p38 MAPK and ERK1/2 to the nucleus after hyper- and hyposmotic challenge, respectively, in NIH3T3 cells in the absence of serum. [From Nielsen et al. (708).]

posed (1014). On the other hand, in other cell types, endogenous MKK3/6 activity was not detectably increased by shrinkage in spite of robust p38 MAPK activation, possibly due to negative autoregulation (757, 788).

In ELA cells in interphase (which exhibit a high basal ERK1/2 activity) (788) and in NIH3T3 cells (249), ERK was strongly inhibited within 5 min of osmotic shrinkage and recovered to normal levels within ~1 h. In ELA cells, the inhibitory effect of shrinkage appeared to occur at or upstream of the level of Raf, since both MEK1/2 (788) and Raf (802) activity were also rapidly inhibited under these conditions. Notably, shrinkage-induced inhibition of ERK1/2 was strongly dependent on NHE1 activity (788), in a manner which was at least in part upstream of or at the level of Raf (802). This points to a major role for NHE1 in regulation of ERK activity under these conditions, in accordance with the role of this transporter in regulation of MAPK activity after a variety of other physiological and pathophysiological stimuli (789). In some cell types, the shrinkage-induced decrease in ERK1/2 activity is dependent on the concomitant increase in p38 MAPK activity (249, 1139). This was also the case in ELA cells, pointing to a necessary role of both NHE1 and p38 MAPK (788). It is interesting to note that the suppressors of ERK signaling, Sprouty (Spry) and Spred, are targeted to the plasma membrane by PtdIns(4,5)P_2 after Rac activation and that this is essential for their inhibition of ERK signaling (569). It is thus tempting to speculate that the increases in PtdIns(4,5)P_2 level and Rac activity in shrunken cells (sect. IV, *C2* and *D*, respectively) may contribute to ERK inhibition under these conditions by inducing Spry recruitment.

ERK1/2 activation by hypertonicity in embryonic stem cells was at least partially dependent on MEKK1, and partially on Raf (1133). Recently, the ERK scaffold KSR, which is generally required for Raf-mediated ERK1/2 activation, was also found to be involved in ERK1/2 activation by hyperosmolarity, in a manner involving direct interaction between KSR and MEKK1 (255). In conjunction with the above-mentioned hypertonic induction of an Rac-OSM-MEKK3-MKK3-p38 MAPK axis (1014), this may point to an important role of scaffolding events in MAPK regulation by osmotic stress (for a discussion, see Ref. 348). Counter to the proposed role of the cytoskeleton in the Rac-OSM-MEKK3-MKK3-p38 MAPK axis (1014) however, the relative ERK1/2 inhibition by shrinkage in ELA cells was unaffected by disruption of the actin cytoskeleton, although this treatment strongly increased basal ERK1/2 activity (788).

Interestingly, the MAPK kinase kinase TAK1 was found to be strongly activated by osmotic shrinkage, and this was essential for shrinkage-induced activation of JNK, but not of p38 MAPK, in mouse embryonic fibroblasts (389). On the other hand, MEKK1 ablation has also been reported to abolish shrinkage-activation of JNK (1133); hence, either the activation pathway is cell type specific, or both pathways are necessary, yet neither is sufficient. In ELA cells, NHE1 contributed to the shrinkage-induced stimulation of JNK, and in marked contrast to the above-mentioned inhibitory effect on ERK1/2,

which was not intracellular pH (pH_i) mediated, JNK stimulation appeared entirely attributable to the robust NHE1-mediated increase in pH_i (788). JNK activation by hypertonicity has also been reported to be strongly dependent on cytoskeletal integrity (1133).

Finally, MAPK activity is also modulated by the activity of MAPK phosphatases (MKPs), and there is evidence to suggest that cell volume-dependent regulation of MKPs may be involved in regulation of MAPK activity (1107). Further upstream, effects of cell volume perturbations on growth factor receptors appear to play a role in modulation of MAPK activity in some cell types (sect. IV*A2*). However, swelling-induced activation of ERK1/2 appears to occur independent of changes in PDGFRβ activity (708), in spite of the fact that ligand-dependent activation of PDGFRβ is strongly cell volume dependent (sect. IV*A*).

2. *Roles of MAPKs in the response to osmotic volume perturbations*

The most widely studied consequences of MAPK activation after cell volume perturbations are effects on long-term adaptive changes in osmolyte transporter expression, likely predominantly through p38 MAPK-mediated effects on TonEBP (see Ref. 917 and sect. VIII*A*). The ERK pathway may also link shrinkage to TonEBP activation. Thus shrinkage activation of ERK1/2 was reported to regulate the transcription of the active taurine transporter TauT and Hsp70 in invertebrate disc cells, presumably through transactivation of TonEBP (1007). In ELA cells in interphase, osmotic shrinkage strongly inhibited the activity of p90RSK downstream from inhibition of ERK (802), suggesting that transcription factors that are p90RSK targets, such as SRF and c-FOS, may also be strongly affected under these conditions. Finally, shrinkage activation of p38 MAPK was recently demonstrated to lead to sumoylation of STAT1, suggesting that osmotic stress also regulates STAT1 targets in this manner (1026).

Although the roles of MAPKs in osmotically perturbed cells are thus by far best understood at the level of regulation of gene transcription, the temporal sequence of MAPK activation and volume regulation in some, albeit clearly not all, cell types is consistent with a causal role for the MAPKs in the regulation of volume regulatory osmolyte transport. Indeed, there is evidence to suggest the involvement of ERK, p38 MAPK, and JNK in control of RVI (285, 558, 803, 854) and RVD (759, 1049) in at least some cell types. MAPK activation may also contribute to the volume-dependent reorganization of the actin cytoskeleton (sect. IV*D*). Thus activation of either ERK1/2 or ERK5 can elicit disruption of the actin cytoskeleton (44), and shrinkage-induced p38 MAPK activation was directly shown to be upstream of HSP27 phosphorylation in the hippocampus (726). Moreover, MEKK4, which is upstream of JNK and p38 MAPK activation by many stimuli, was shown to be important for actin recovery after osmotic shock in mammalian cells (57).

As further described in section IX, *C* and *D*, the volume sensitivity of MAPKs also plays a central role in the effects of cell volume perturbations on cell proliferation and programmed cell death (PCD). Briefly, consistent with their general roles in these processes, p38 MAPK and JNK tend to counteract cell proliferation and induce PCD in osmotically perturbed cells, while the opposite is true for ERK (136, 249, 788, 1114).

In spite of the demonstrated regulation of ERK5 by cell volume perturbation (1067 and above), nothing is to our knowledge known regarding the possible role of ERK5 in the ensuing response. However, it should be noted that the compounds PD98059 and UO126, which are classically used to inhibit MEK1 and hence its downstream target ERK1/2, also inhibit ERK5 activity (428), which means that most of the proposed roles of ERK1/2 in osmotically perturbed cells may need to be reevaluated for contributions from ERK5.

D. With-No-Lysine Kinases and Ste-20-Related Kinases

The existence of a volume-sensitive protein kinase, which is activated by cell shrinkage and inhibited by cell swelling, and in turn activates the shrinkage-activated transport proteins and inhibits those activated by swelling, has been hypothesized since relaxation kinetic studies in RBCs in the early 1990s pointed to such a scheme (406, 407, 767). Also in accordance with this notion, inhibitors of Ser/Thr protein phosphatases almost ubiquitously stimulate transport proteins activated by shrinkage (see Fig. 2, *C* and *D*; sect. VI) and inhibit those activated by swelling (sect. VII). A one-kinase-one-phosphatase scenario, in which phosphorylation activates, and dephosphorylation inhibits, the shrinkage-activated transport protein, and vice versa for those activated by swelling, is doubtlessly too simplistic (e.g., Ref. 212). However, the notion of an at least partially reciprocal system of protein kinase-dependent regulation of volume-sensitive transporters and channels has recently received substantial support from the discovery of the two kinase families discussed in this section: the with-no-lysine kinases (WNKs) and the Ste20-related kinases.

1. *WNKs*

The WNKs are a family of Ser/Thr protein kinases comprising four mammalian isoforms, WNK1-4 (424, 806, 1033). They derive their name from a unique substitution in the catalytic core, a cysteine replacing a lysine which is invariant in essentially all other protein kinases (1033). WNK1 and WNK4 became the subject of intense research

interest when they were found to be mutated in pseudohypoaldosteronism type II (PHA II) or Gordon's syndrome, a rare form of congenital hypertension with hyperkalaemia and metabolic acidosis (1096; see Refs. 424, 806). Wild-type WNK4 inhibits NCC in the distal nephron by reducing the number of transport proteins at the plasma membrane, whereas wild-type WNK1 appears to indirectly regulate NCC by inhibiting the effect of WNK4 on the transporter (1096; see Refs. 424, 806). The WNK mutations associated with PHAII lead to increased WNK1 expression and decreased WNK4 expression, respectively, and the resulting excessive NCC activity is a major factor in the pathophysiology of PHAII (see Refs. 424, 806).

Following these seminal studies, it was found that the WNKs regulate the activity of a wide variety of transport proteins. It is characteristic that the specific effects of WNKs differ widely between the transporter targets. For instance, WNK-mediated regulation can lead to transport protein up- or downregulation and may or may not be dependent on *1*) WNK catalytic activity, *2*) endocytosis/recycling (see Ref. 806), and *3*) the additional involvement of GCK-VI family kinases SPAK and/or OSR1 (see below). The transport proteins demonstrated to be regulated by WNKs include the SLC12A family of cation-chloride cotransporters (sects. VI*B* and VII*C*, respectively), the renal outer medulla K^+ channel (ROMK1), the epithelial Na^+ channel (ENaC), CFTR, and TRPV4 and TRPV5 (see Ref. 806).

WNK1 has been shown to be activated by hypertonicity in a variety of cell types (539, 1117, 1135). In some (539) yet not in all (1135) cell types, hypotonicity was also reported to activate WNK1, although to a lesser degree. The mechanism(s) by which WNKs are regulated by cell volume perturbations are incompletely elucidated. A recent study in HEK293 and HeLa cells (1135) showed that WNK1 was activated both by high salt and sorbitol-induced hypertonicity. Intriguingly, the rapid (within 0.5 min) activation of WNK1 by hypertonicity was associated with a dramatic translocation of WNK1, from the cytosol to intracellular vesicles, possibly the *trans*-Golgi network/recycling endosomes (1135). Moreover, the shrinkage activation of WNK1 appeared to be critically dependent on phosphorylation of a highly conserved serine residue, Ser^{382}, which is present in all WNK isoforms (1135). WNK1 is activated downstream of PKB (1037), yet PKB signaling is often (153, 639), although not always (984), found to be inhibited by osmotic shrinkage. As discussed elsewhere (183), it is also possible that the actin-based cytoskeleton plays a role in regulation of WNK-SPAK signaling by osmotic shrinkage; however, this has yet to be directly investigated. The activity of WNKs is closely associated with that of the serum- and glucocorticoid-dependent kinase (SGK1), which is regulated by hypertonic stress and in turn has been shown to regulate plasma membrane insertion of NKCC1. Thus WNK1 activates SGK1, SGK1 phosphorylates WNK4 leading to abrogation of WNK4 effects on ENaC and ROMK, and the three kinases physically interact (847; see Ref. 806). The possible relevance of this to cell volume regulation is, however, not straightforward, as SGK1 transcription is increased, yet the activity of the expressed protein decreased, by hypertonic stress (1059; see Ref. 233).

2. Ste20-related kinases

The Ste20-related Ser/Thr kinases are a family of evolutionarily conserved Ser/Thr kinases with important roles in control of pivotal cellular processes including proliferation, PCD, and a variety of stress responses (168, 954). The Ste20-related kinases are divided into two subfamilies on the basis of their structural organization: the germinal center kinases (GCKs) and the p21-activated kinases (PAKs) (168, 954). The PAKs are shrinkage-activated kinases with major roles in cytoskeletal organization (sect. IV*D*). The GCKs are further divided into group I-VIII kinases. Group IV includes the Nck-interacting kinase (NIK), which is involved in the activation of NHE1 by PDGF (1126); however, the possible role of NIK in shrinkage activation of NHE1 is still unknown. Group VI includes Ste20-related proline-alanine rich kinase (SPAK), oxidative stress response kinase-1 (OSR1), and the single *C. elegans* GCK-VI family kinase GCK-3 (954). As outlined below and described in more detail for the individual transporters (sects. VI*B* and VII*C*), the regulation of some members of the SLC12A family by WNKs is dependent on GCK-VI family kinases. Substantiating the notion of WNKs and GCK-VI family Ste20-related kinases as central and conserved regulators of volume-regulatory processes, it was recently reported that interactions between the *C. elegans* WNK-1 and GCK-3 are essential for whole animal volume regulation and survival after hypertonic stress (132), and that GCK-3 binds and regulates the volume-sensitive *C. elegans* Cl^- channel CLH-3b (186).

3. Interplay between WNKs and Ste20-related kinases in cell volume regulation

The main evidence placing SPAK/OSR1 as intermediates in shrinkage- and WNK-mediated NKCC regulation is as follows. First, WNK1 and WNK4 bind, phosphorylate, and activate SPAK and OSR1 (259, 675, 1038). Second, SPAK and OSR1 have been shown to be activated by hypertonicity and low Cl^- conditions (126, 258, 675, 1135), in a manner abrogated by genetic ablation of WNK1 or WNK4 kinase activity (1039). Third, SPAK phosphorylates NKCC1 on specific Thr residues required for its activation by shrinkage (260). Interestingly, the requirement for SPAK/OSR1 is not ubiquitous, as WNK3 stimulates NKCC1 in the absence of SPAK/OSR1 coexpression in *Xenopus* oocytes (423). Similarly, in most cases, SPAK/OSR1 seem to play no or minor roles in the WNK-depen-

dent regulation of the KCC branch of the SLC12A family (176, 271) (sect. VIIC). Finally, similar to WNK1 (539), SPAK/OSR1 may also at least in some tissues be activated by hypotonic stress (675). Further complexity is added by the fact that WNK1 phosphorylates both WNK2 and WNK4 and appears to be a negative regulator of WNK4-mediated effects (539, 1128), yet both kinases seem to activate SPAK/OSR1 (1038). Thus the mechanisms by which WNK-SPAK-SLC12A interactions regulate ion transport in osmotically perturbed cells are complex, and in spite of the massive progress in recent years, many open questions remain.

E. Other Ser/Thr Protein Kinases and Phosphatases

In addition to the MAPK (sect. VC), and WNKs/Ste20 (sect. VD) pathways discussed above, a long list of other Ser/Thr protein kinases have been demonstrated or proposed to be regulated by osmotic volume perturbations in various cell types. These include LIMK and PAK (145, 434, 855), PKB (153, 639, 984), PKC (519, 580), SGK (1059), and PKA (751). Most of these have been mentioned in their relevant contexts in other sections and will not be further discussed here. For a recent review, see also Reference 10. Finally, although evidence in this regard is still relatively scarce, there are also studies pointing to the regulation of Ser/Thr protein phosphatases (1 and 2A) by osmotic volume perturbations (64, 980).

F. Tyrosine Kinases: Src Family Kinases and FAK

1. *Src family kinases*

The Src family of cytosolic tyrosine kinases consists of nine subfamilies (Src, Fyn, Yes, Lck, Hck, Blk, Fgr, Lyn, and Yrk) (152, 816), which regulate multiple cellular processes. Observations in a wide variety of cell types suggest that Src kinases could play a central role in volume-sensing signaling (for an excellent review, see Ref. 146). Multiple Src family kinases are activated in response to both cell shrinkage (433, 478, 838) and swelling (39, 541, 1118), by mechanisms which remain to be fully elucidated. It was recently demonstrated that Src is part of the signaling cascade from integrin activation to downstream elements such as MAPKs after osmotic swelling of liver cells (1050). As discussed in the relevant sections on these transporters, Src modulates the activity of, e.g., the swelling-activated $K_v1.3$ and $K_v1.5$ K^+ channels (151, 188, 727), the BK channels (570), VRAC (541), and organic osmolyte pathways (see Refs. 493, 771). The role of Src in modulation of VRAC is controversial. In endothelial cells, Nilius and co-workers (1004) demonstrated a Src-mediated inhibition of VRAC which required tageting af Src to caveolae. In a brain model, VRAC activation was, accordingly, inhibited by the Src inhibitor PP2 (331), whereas in cardiac myocytes, PP2 conversely augmented VRAC (1060). This apparent difference may, however, in part reflect that PP2 inhibits a number of other protein kinases than Src with relatively high potency, including p38 MAPK and casein kinase 1 (30).

2. *Focal adhesion kinase*

Focal adhesion kinase (FAK) is an evolutionarily conserved nonreceptor protein tyrosine kinase localizing to focal adhesions (FAs) (see Ref. 769). FAK is both a scaffold protein recruiting signaling molecules to the FA, and a mediator of tyrosine phosphorylation of several substrates (see Ref. 149). FAK undergoes autophosphorylation at Tyr^{397} in response to integrin clustering and growth factor receptor stimulation, in a β-integrin-dependent manner (149, 321). Other important phosphorylation sites on FAK are Tyr^{577} in the kinase-activating loop, and Tyr^{861} which is located COOH-terminally between the kinase domain and the FA targeting domain (149), which is known to recruit scaffolding proteins involed in regulation of the F-actin cytoskeleton (595).

Hyperosmotic stress stimulates phosphorylation of specific tyrosine residues on FAK in a variety of cell types (187, 595, 596, 1143). In Swiss 3T3 cells, hyperosmotic stress rapidly stimulated FAK phosphorylation at Tyr^{397} via a Cdc42-dependent pathway independent of Src, Rho, Rho kinase, PI3K, and p38 MAPK, and at Tyr^{577} via a Src-dependent pathway (596). The mechanism of Cdc42-dependent FAK phosphorylation is unknown, but was tentatively suggested to be caused by FAK aggregation and dimerization during cell shrinkage (see Ref. 596). Exposure to hyperosmotic stress in epithelial cells increased phosphorylation of FAK on Tyr^{861} and minor phosphorylation at Tyr^{397} and Tyr^{576} (595). Finally, in NIH3T3 cells, hyperosmotic stress induced FAK phosphorylation at Tyr^{576}, Tyr^{397}, and Tyr^{861} (B. Jørgensen, S. F. Pedersen, and E. K. Hoffmann, unpublished data). Whereas a protective effect of FAK against shrinkage-induced PCD has been described in several cell types (595, 596, 1143), no studies have, to our knowledge, yet addressed the possible role of FAK in RVI.

Phosphorylation of FAK in response to hyposmolarity has also been reported in several cell types (175, 352, 447, 549), in a manner found to be dependent on Rho family G proteins (352), or on an ErbB4-Src-PI3K signaling cascade (549). Studies directly evaluating the role of FAK on RVD have, to our knowledge, not been carried out; however, a role of FAK in regulation of VRAC has been suggested in several cell types (87, 992). As described in section IVA, a model coupling integrin stretch to activation of VRAC by a series of events involving activation of FAK and Src, transactivation of the EGF receptor and activation of PI3K has been proposed (86). On the other hand, Src inhibition increased VRAC in cardiomyo-

cytes (1060), and in endothelial cells there seems to be no involvement of FAK in VRAC activation (970).

G. Reactive Oxygen Species

Reactive oxygen species (ROS; see Table 3) have for many years been regarded as toxic by-products of oxidative metabolism, mediators in immune defense, promoters of nonspecific oxidative damage, and accelerators of cell death (see Refs. 205, 1020). However, within recent years, it has been found that nonphagocytes produce ROS in a tightly regulated process, although in lesser amounts compared with phagocytes, and that ROS function as useful biomolecules through the selective oxidation of a limited spectrum of target molecules, e.g., receptors, enzymes, and transcription factors (205, 493, 556, 1020, 1031). Superoxides are produced from O_2 by absorption of ionizing radiation in water, slip of electrons from the mitochondrial electron transport chain, and oxidative reactions. The lipid-permeable hydrogen peroxide (H_2O_2) originates from mitochondrial or cytosolic dismutation of superoxide, whereas the hydroxyl radicals are generated by peroxidation from superoxides and H_2O_2 (see Table 3). Lipid peroxidation is a self-propagating process that involves the formation of a carbon-centered radical in an unsaturated fatty acid, which reacts with molecular oxygen and forms a peroxy radical. This peroxy radical is in itself able to remove hydrogen from an unsaturated fatty

TABLE 3. *Reactive oxygen species*

Reactive Oxygen Species		
Radicals	Superoxide anion*	$O_2^{\bullet-}$
	Hydroxyl radical	$\bullet OH$
	Peroxyl radical	$RO_2^{\bullet}$
	Alkoxyl radical	$RO^{\bullet}$
	Hydroperoxyl	$O_2^{\bullet-} + H^+ \leftrightarrow HO_2^{\bullet}$ ($pK_a = 4.3$)
	Nitrix Oxide*	$NO^{\bullet}$
Nonradicals	Hydrogen peroxide	H_2O_2
	Lipid peroxide	ROOH & ROOR
	Hypochlorous acid	HOCl
	Peroxynitrite*	$ONOO^-$
	Taurine Chloramine*	$SO^-{}_3(CH)_2NHCl$
Generation of radicals	Heat or ultraviolet light activation	$H\text{-}O\text{-}O\text{-}H \rightarrow HO^{\bullet} + {}^{\bullet}OH$
		$R\text{-}O\text{-}O\text{-}H \rightarrow R\text{-}O^{\bullet} + {}^{\bullet}OH$
	Membranes	NADPH oxidase $\rightarrow O_2^{\bullet-}$
	Mitochondria	Slip in the respiration: complex I/III
		$O_2 + e^- \rightarrow O_2^{\bullet-}$
		$O_2^{\bullet-} + e^- + 2H^+ \rightarrow H_2O_2$
		$H_2O_2 + e^- + H^+ \rightarrow OH^{\bullet} + H_2O$
	Peroxisomes	Glycolate oxidases, D-amino acid oxidase, acyl-CoA oxidase
	Cytosol	Xanthine/urate oxidase, D-amino acid oxidase
	Fenton reaction	*1*) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$
		2) $Fe^{3+} + O_2^{\bullet-} \rightarrow Fe^{2+} + O_2$
	Haber-Weiss reaction	*1*) and *2*) $O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^{\bullet} + OH^-$
Lipid peroxidation	Initiation	Formation of carbon-centered lipid (L) radicals $LH + X^{\bullet} \rightarrow L^{\bullet} + XH$
	Propagation	$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$
		$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$
	Termination	$LOO^{\bullet} + LOO^{\bullet} \rightarrow$ nonradical product
		$LOO^{\bullet}$ + vitamin E $\rightarrow$ vitamin E$\bullet$ + LOOH
	Smooth ER	*P*-450
	Cytosol	Lipoxygenase and cyclooxygenase
Termination (enzymatic, antioxidative systems)	Superoxide dismutase	$2H^+ + 2O_2^{\bullet-} \rightarrow H_2O_2 + O_2$
	Catalase	$H_2O_2 + H_2O_2 \rightarrow 2H_2O + O_2$
	Peroxidase	$H_2O_2 + RH_2 \rightarrow 2H_2O + R$
	Glutathione peroxidase	$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$
Termination (nonenzymatic, antioxidative systems)	Vitamins	Vitamins C and E: work synergistically
	Carotenoids/polyphenols	
	Glutathione	GSSG (oxidized) $\leftrightarrow$ GSH (reduced)
	Thioredoxin	$TRX_{ox} \leftrightarrow TRX_{red}$

Reactive oxygen species include nonradical oxygen species (H_2O_2) and oxygen-centred radicals, which contain one or more unpaired electrons. *These compounds are also entitled reactive nitrogen species (RNS). References for the information provided in this table are as follows: 205, 224, 317a, 505, 967, 986, 1020.

acid, resulting in the formation of a lipid hydroperoxide and a carbon-centered radical (see Table 3). Enzymatic lipid peroxidation involves the cyclooxygenases and the lipoxygenases, and it has recently turned out that also secondary aldehydes, i.e., isoketals, generated downstream from isoprostane via covalent modification of signaling molecules, can mediate ROS-induced cellular damage (665). Accumulation of polar products from lipid peroxidation in the membrane interior will cause a shift in the biophysical properties of the membrane and thus affect the state/activity of membrane-bound receptors, transport proteins, and enzymes. Cytoplasmatic enzymes might also be affected by lipid peroxidation, as shown, e.g., for creatine kinase (467). Cells possess efficient antioxidative systems, including dismutases, catalases, peroxidases, as well as dietary, water- and lipid-phase antioxidants, that limit the availability and action of free radicals (see Table 3). In the context of cell volume regulation, it is interesting to note that the organic osmolyte taurine and its precursors, cysteine and hypotaurine, have been implicated in cellular defense against oxidative stress (118, 394), and taurine supplementation is reported to reduce lipid peroxidation (835). Through chlorination of taurine with the myeloperoxidase product HOCl (300, 1085) neutrophils form taurine chloramine (TauCl) which is considered to be a weaker oxidant than HOCl. As the cellular concentration of taurine in, e.g., EAT cells is reduced from 55 to 7 mM following hypotonic exposure (364), the swelling-induced net loss of taurine could result in loss of cellular antioxidative capacity.

ROS exert their effects by altering the ratio between the reduced and the oxidized state of the intracellular redox equivalents ($NADPH/NADP^+$) and redox systems (gluthatione, thioredoxin) or by oxidative modification of proteins. Glutathione is the major soluble antioxidant in mammalian cells (630) and functions not only as an efficient ROS scavenger but also as substrate in the synthesis of lipid-derived second messengers (leukotrienes). Gluthatione appears in a reduced form (GSH) and in two oxidized forms, i.e., gluthatione disulfide (GSSG) and *S*-glutathionylated (protein-S-S-G) derivatives (234). Glutathione is highly abundant in the cytosol, nucleus, and mitochondria. It is estimated that the concentration of reduced glutathione under nonstressed conditions is ~10- to 100-fold higher than that of the oxidized form. Thioredoxin ($TRX_{ox} \leftrightarrow TRX_{red}$) is a small multifunctional protein with two redox-active cysteines in its active center, which reduces ROS directly and refolds oxidized proteins (Table 3). Oxidative modification of proteins often implies oxidation of the sulfhydryl group of cysteine residues ($\text{-SH} \rightarrow \text{-SOH} \rightarrow \text{-SO}_2\text{H} \rightarrow \text{-SO}_3\text{H}$) and dityrosine formation. Caspases and tyrosine phosphatases contain cysteine residues in their active site, and their enzymatic activity is regulated by the redox state of the cell, including the ROS level (205, 1012). Notably, transient, ROS-mediated inactivation of tyrosine phosphatases in swollen cells would exacerbate and/or prolong tyrosine kinase-mediated events under these conditions. The formation of dityrosines may also be relevant to the osmotic stress response: dityrosine formation leads to protein cross-linkage, which in turn has been shown to modulate gene expression and growth stimulatory signals, and promote inflammatory and apoptotic signal transduction (986).

1. *Effect of cell volume perturbations on cellular ROS production*

ROS production increases within the first minutes following hypotonic exposure in, e.g., NIH3T3 fibroblasts (492), skeletal muscle (753), and hepatocytes (1031). The swelling-induced ROS production in HTC cells is reduced in the presence of the NADPH oxidase inhibitor diphenyl iodonium (DPI). ROS production in NIH3T3 cells is also reduced by DPI (250) as well as by the $iPLA_2$ inhibitor BEL (492), in accordance with the view that ROS are generated by an $iPLA_2$-stimulated NADPH oxidase. In this context, it is notable that $cPLA_2$ and the NADPH oxidases colocalize in plasma membranes of stimulated phagocytes (930) and that ROS generation through a PLA_2-lipoxygenase pathway was demonstrated in rat fibroblasts following stimulation with TNF-α (1101) and in skeletal muscle cells during heat stress (1148). The NADPH oxidase system in nonphagocytic cells, designated NOX, comprises one of the catalytic, membrane-integrated $gp91^{phox}$ analogs (NOX1, 3–5, DUOX1,2) associated with the subunit $p22^{phox}$. Some of these catalytic subunits are regulated by a subset of regulatory units, i.e., the small GTPase Rac1/2, $p40^{phox}/p47^{phox}/p67^{phox}$ or NOXO1/NOXA1 (see Refs. 506, 966). The NOX group is of particular interest in the context of cell volume because their enzymatic activity is stimulated via receptors for interleukins, growth factors, tumor necrosis factor (TNF)-α, thrombin, and by mechanical force and osmotic cell swelling (205, 493, 986, 1031). c-Src stimulates NOX-driven ROS generation in a process that seems to involve PI3K/PKB, PKC, p21-activated kinases, Raf-1, tyrosine phosphorylation, and subsequent translocation of the regulatory unit $p47^{phox}$ (1001). Consistent with this model, the nonspecific phosphatase inhibitor vanadate stimulates ROS production in NIH3T3 cells and ELA cells under hypotonic conditions (494).

The superoxides in nonphagocytic cells are produced mainly intracellularly (see Ref. 1020), and it has been suggested that the NADPH oxidase complex may be preassembled at intracellular membranes (1, 552). NOX components have specific subcellular localizations that could point to the existence of tightly controlled local ROS production and signaling (see Ref. 506). NOX2 ($\approx gp91^{phox}$) are targeted to FAs in lamellipodia/membrane ruffles in a process involving the scaffold proteins TRAF4/Hic-5 (1109, 1110), and to lipid rafts, where ROS production increases following Fas ligand stimulation (1137). NOX4 seems to act independently

of any of the regulatory components (15, 629), whereas NOX5 activity is stimulated by Ca^{2+} (35).

2. Roles of ROS in the regulation of volume regulatory ion transport

ROS have been shown to activate or potentiate several swelling-activated transport systems in various cell types (3, 445, 492, 928, 1031). Where relevant, these effects will be discussed in the sections describing the individual channels and transporters.

VI. REGULATORY VOLUME INCREASE

Figure 11 provides an overview of the main effectors in the early phases of RVI in most cell types studied. In the following, we discuss these membrane transport proteins and the mechanisms by which they are regulated by cell volume perturbations. The upregulation of the expression of osmolyte transport and synthesis upon long-term volume perturbations is discussed in section VIII, and the physiological and pathophysiological implications of their activity in section IX, respectively.

A. The Na^+/H^+ Exchangers

Na^+/H^+ exchangers (NHEs) are found in essentially all organisms studied. The pro- and eukaryotic NHEs collectively belong to the monovalent cation proton antiporter (CPA1) group of transporters (www.tcdb.org; Ref. 875). Here, we are only concerned with the vertebrate NHE isoforms, their sensitivity to cell volume perturbations, and their roles in the response to such perturbations. Other aspects of NHE evolution, structure, regulation, and function have recently been reviewed elsewhere (83, 614, 640, 752, 785, 797).

1. Fundamental properties, localization, and pharmacology of the SLC9A family

The SLC9A family of mammalian NHEs comprises 10 cloned and at least partially characterized members.[3] NHEs are complexly regulated, multifunctional proteins

[3] An additional gene product was assigned the name SLC9A11 (http://www.gene.ucl.ac.uk/cgi-bin/nomenclature/), but is, to our knowledge, not yet proven to be a functional Na^+/H^+ exchanger.

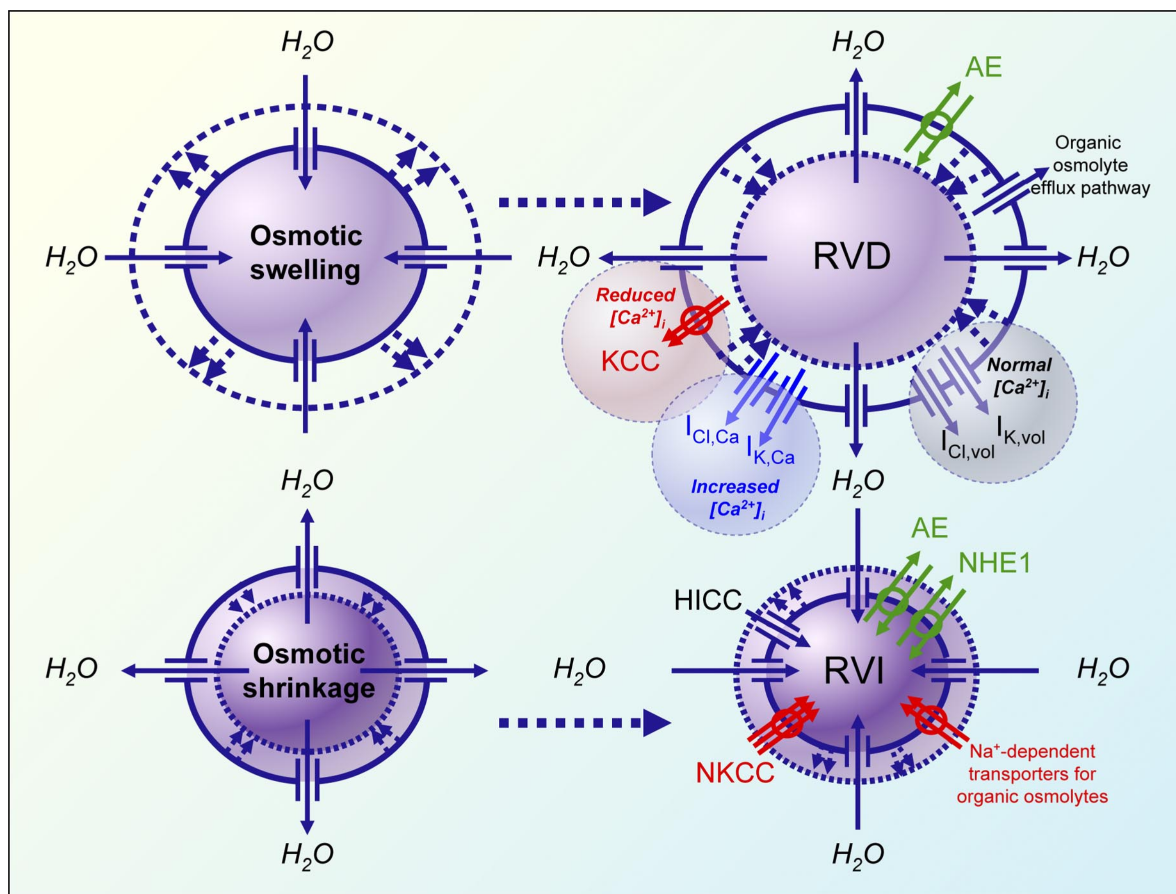

FIG. 11. Effectors involved in RVD and RVI. See text for details.

mediating (with the exception of the organellar isoforms which may also mediate K^+/H^+ exchange; Ref. 83). The 1:1 exchange of Na^+ for H^+ across the membranes in which they reside. NHEs are central to the homeostasis and regulation of cellular, organellar, and cell surface pH, cell and systemic volume, and vectorial ion transport (12, 93, 752). Consequently, many pivotal cellular functions, including cell migration, vesicle trafficking, growth/proliferation, and death/survival balance are dependent on NHEs, and their absence or dysfunction has been linked to multiple pathological conditions including cancer, diabetes, hypertension, epilepsy, congenital secretory diarrhea, and ischemia/reperfusion damage (see Refs. 752, 797). An overview of the SLC9A family members and their relation to cell volume regulation is provided in Table 4. NHE1–5 are found in the plasma membrane. NHE3 and NHE5, yet not NHE1, NHE2, and NHE4, further exhibit regulated recycling between the plasma membrane and endosomal compartments (165, 969). Of these transporters, NHE1 is essentially ubiquitous and localizes to the basolateral membrane of polarized cells; NHE2 and -3 are apical isoforms restricted to various types of epithelial cells; NHE4 is basolateral and found mainly in stomach, kidney, and brain; and NHE5 is a neuronal isoform (752). NHE6, -7, and -9 are very broadly distributed and have been proposed to be mainly organellar transporters (690), although recycling and transient localization of NHE6 and NHE9 to the plasma membrane were recently demonstrated (351). NHE8 is also broadly expressed, and while first thought to be intracellular, has now been shown to also localize to the apical membrane in kidney epithelial cells, where it participates in Na^+/H^+ exchange after acid loading (1140). The latest NHE characterized is a sperm-specific isoform, sNHE or NHE10 (1061, 1061) (Table 4). Several homologs of the mammalian NHEs have been found in other vertebrate species. In the first vertebrates, the fishes, NHE1 homologs play an important role in the control of RBC volume, a crucial factor regulating blood oxygen transport capacity (see, e.g., Refs. 410, 787). The NHE protein in salmonid RBCs is denoted βNHE for its activation by β-adrenergic stimuli (71), yet in spite of this difference in regulation compared with the mammalian NHE1, sequence comparison clearly groups the NHEs of teleost RBCs as NHE1 homologs (793; see Ref. 787).

The predicted membrane topology of all the NHEs is an NH_2-terminal region with 12 predicted TM domains (~500 amino acids) and a COOH-terminal cytoplasmic tail region (~300 amino acids) containing the residues involved in exchanger regulation and interactions with binding partners (931, 1057). Recent high-resolution structures of the distantly related bacterial NHE, NhaA, support this model, and also lend credence to the notion that TM4 is critical for ion transport (393).

A number of pharmacological inhibitors are currently used to inhibit NHEs. The first compound used to inhibit NHE1 was the K^+-sparing diuretic amiloride, which, however, is a relatively poor tool in this regard, as it also inhibits ENaC and NCX (see Ref. 631). Most newer drugs exhibit much greater specificity for NHE1 and are predominantly derivatives of either amiloride, e.g., ethylisopropylamiloride (EIPA) and dimethylamiloride (DMA), or benzoylguanidines, e.g., HOE-642 (cariporide), HOE-694, and eniporide (see Ref. 631). The order of sensitivity of these drugs is generally NHE1 > NHE2 > NHE5 > NHE3 (631). The interaction of NHE1 with both amiloride- and benzoylguanidine-based inhibitors involves multiple noncontiguous regions in the transmembrane domain. These include a crucial two-amino acid motif in TM4 which is lacking in NHE3, causing this isoform to be especially insensitive to many of the compounds targeting NHE1 (160, 443, 792). NHE3-selective compounds, S1611 and S3226, are, however, also available (910).

The NHEs exhibit striking differences in their sensitivity to cell volume perturbations (Table 4). In the following, we will discuss those NHE isoforms that exhibit volume sensitivity and, where known, the mechanisms and consequences of their regulation by cell volume. The

TABLE 4. *Overview of the SLC9A family members and their relation to cell volume perturbations*

Family Member	Accession No.	Tissue and Subcellular Distribution	Cell Volume Sensitivity
SLC9A1/NHE1	NM 003047	Ubiquitous; basolateral plasma membrane	Shrinkage activated/swelling inhibited
SLC9A2/NHE2	NM 003048	Stomach/gastrointestinal system, muscle, many epithelia; apical plasma membrane	Shrinkage activated*
SLC9A3/NHE3	NM 004174	Stomach/gastrointestinal system, kidney, gall bladder, many epithelia; apical plasma membrane/recycling	Swelling activated/shrinkage inhibited+
SLC9A4/NHE4	NP 001011552	Mainly stomach; basolateral plasma membrane	Shrinkage activated
SLC9A5/NHE5	NM 004594	Neuronal; plasma membrane/recycling	Shrinkage inhibited
SLC9A6/NHE6	NM 006359	Ubiquitous; organellar/recycling	?
SLC9A7/NHE7	NM 032591	Ubiquitous; organellar	?
SLC9A8/NHE8	NP 056081	Ubiquitous; plasma membrane/organellar	?
SLC9A9/NHE9	NP 775924	Ubiquitous; organellar/recycling	?
SLC9A10/NHE10 (sNHE)	AY 368685	Sperm specific	?

* See, however, Refs. 696, 964 for a different view. +See, however, Refs. 432, 964 for a different view.

majority of the discussion is devoted to the ubiquitous, shrinkage-activated NHE1.

2. SLC9A1/NHE1

Although we focus here on NHE1 as a volume-regulatory transporter, it is important to note that NHE1 is activated by a wide variety of other stimuli and is a pivotal player in many other physiological processes than cell volume regulation (for reviews, see Refs. 410, 752, 785, 787, 826, 894). Some of these roles of NHE1 are discussed in section IX, including its roles in cell migration (952), proliferation/survival balance (see Refs. 785, 933), and transepithelial ion transport processes (244). Pathophysiologically, increased NHE1 activity at steady-state volume is involved in the detrimental swelling of, e.g., astrocytes and cardiomyocytes after ischemic challenges (see Ref. 797), and in the increased invasive/metastatic properties and growth capacity of many cancer cells (see Ref. 114).

A) NHE1 AS PART OF A REGULATED MACROMOLECULAR COMPLEX. Similar to many other transport proteins, NHE1 (as well as NHE3, and likely many, if not all, other NHE isoforms) interacts with multiple binding partners (Fig. 12). This subject was recently reviewed (640) and will only be briefly covered here. While much is yet to be understood regarding their functional importance, it is interesting to note that these interactions appear to not only regulate NHE1 function and localization, but may also in turn regulate cell morphology via cytoskeletal anchoring, and cause NHE1 to act as platform for scaffolding and regulation of cellular signaling processes (184, 185, 788; see Ref. 640). Proteins with identified binding sites on NHE1 are, to date, as follows: three Ca^{2+} binding proteins, calcineurin homologous protein (CHP) (762), CaM (56), and tescalcin (609); the F-actin-plasma membrane linker proteins of the ezrin/radixin/moesin (ERM) family (184, 185); the Ste20-related kinase NIK; carbonic anhydrase II (CAII; Ref. 557); and 14-3-3 protein when NHE1 is phosphorylated on S^{703} (human NHE1 numbering) (Fig. 12). Heat shock protein 70 (HSP70) (934), Src homology 2 domain-containing protein tyrosine phosphatase (SHP-2) (1120), and 6-phosphofructokinase (PFK-1) (640) have also been shown to interact with the NHE1 tail, and binding of protein phosphatase 1 has been suggested (652); however, for these proteins, the binding sites remain to be determined. In addition, NHE1 interacts directly with PtdIns(4,5)P_2 via the most proximal part of the COOH-terminal tail region (8) (Fig. 12). The distal end of the NHE1 tail contains a number of consensus sites for phosphorylation by Ser/Thr kinases (see, e.g., Refs. 614, 752).

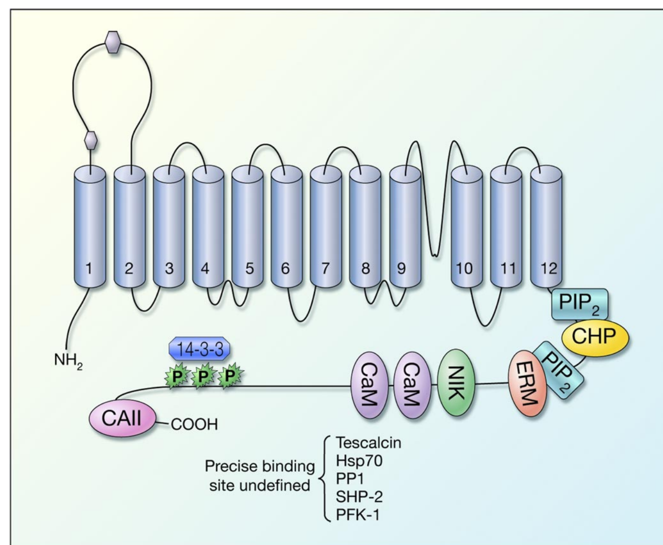

FIG. 12. NHE1 topology and binding partners. Figure illustrates the proposed membrane topology of NHE1 and the approximate locations of direct interaction with identified binding partners. See text for details. CAII, carbonic anhydrase II; CHP, calcineurin homolog protein; ERM, ezrin/radixin/moesin; Hsp70, heat shock protein 70; NIK, Nck-interacting kinase; PIP_2, phosphatidylinositol 4,5-bisphosphate; PP1, protein phosphatase-1; SHP-1, Src homology 2 domain-containing protein tyrosine phosphatase (SHP-2); PFK, phosphofructokinase.

B) NHE1 AS A MECHANISM OF RVI. Volume-sensitive Na^+/H^+ exchanger as a mechanism of RVI was first demonstrated by Cala in the early 1980s, in studies of volume regulation in RBCs from the giant salamander, *Amphiuma tridactylum*. This was seminal work at a time when cell volume regulation was still thought to depend exclusively on conductive transport or anion/cation cotransport (105, 474, 477; for a discussion, see Ref. 787). Cala proposed that RVI in *Amphiuma* RBCs is mediated by a Na^+/H^+ exchanger operating in parallel with a Cl^-/HCO_3^- exchanger, resulting in the net uptake of NaCl and osmotically obliged water (105). The *Amphiuma* exchanger was since cloned (638) and shown to be highly similar to the human NHE1 (886). In accordance with its ubiquitous distribution, and exquisite volume sensitivity, NHE1 has subsequently been found to be the isoform mediating RVI in the great majority of cell types studied, and volume sensitivity is by far best understood for this isoform (see also Ref. 12).

C) POSSIBLE MECHANISMS OF NHE1 REGULATION BY CELL VOLUME PERTURBATIONS. NHE1 is not only activated by cell shrinkage, but also inhibited by cell swelling (219, 432; Table 4), and as such perfectly suited as a volume-homeostatic mechanism. The regulation of NHE1 by cell volume is still incompletely understood. Fundamentally, the current knowledge in this regard consists of some leads, often cell-type specific, and a substantial list of events which do not mediate this regulation, as will be summarized below. What seems clear is that it is the decrease in cell volume, rather than the increase in intracellular ionic strength, which is determinant for NHE1 activity (478). Furthermore, although $[Cl^-]_i$ may regulate NHE1, NHE1 activation by shrinkage is not secondary to the increase in $[Cl^-]_i$ under these conditions (6). Finally, it is also clear that the

shrinkage-induced NHE1 activation does not reflect recruitment of additional NHE1 proteins to the plasma membrane (58, 196).

The activation of NHE1 by shrinkage is generally thought to be associated with an increase in the affinity for H^+ at an intracellular allosteric site, causing the exchanger to be activated at physiological pH_i (296). Recent findings in dog RBCs moreover suggested that shrinkage reduces the Na^+ affinity at an inhibitory extracellular binding site, thereby relieving inhibition by extracellular Na^+ and activating the exchanger (211). The mechanism by which these changes occur, and (if the findings in dog RBCs can be extended to other cell types), the possible relationship between these two sites are, however, still elusive.

Phosphorylation of residues in the COOH-terminal tail of NHE1 is important in the activation of NHE1 by, e.g., growth factors and hormones (see, e.g., Refs. 614, 752). However, net phosphorylation analyses and phosphopeptide mapping indicate that the phosphorylation of NHE1 is not altered by cell shrinkage (299, 378). This is generally taken to indicate that shrinkage activation of NHE1 occurs independently of direct phosphorylation of the NHE1 protein. In congruence with this, shrinkage activation is not prevented by deletion of the major NHE1 phosphorylation sites (58). Protein phosphorylation events do, however, seem to play a role in regulation of NHE1 by volume perturbations, and it is tempting to speculate that this might reflect a phosphorylation-dependent regulation of interactions between NHE1 and its binding partners. Thus, in CHO-K1 cells, NHE1 activation by shrinkage was proposed to reflect an increase in the activity of the tyrosine kinase Jak2, followed by Jak2-dependent phosphorylation of CaM, and subsequent recruitment of CaM to NHE1 (270). However, the Jak inhibitor AG490, which essentially abolishes the shrinkage-induced increase in CaM-NHE1 interaction, only reduced shrinkage-induced NHE1 activity by 50% (270). Hence, even not taking into account the fact that AG490 may not be specific for Jak (804), this pathway cannot alone account for the NHE1 activation. A role for CaM would, however, be in accordance with the earlier finding that deletion of the CaM binding region constitutively activates Na^+/H^+ and renders it largely insensitive to cell shrinkage (suggesting that the CaM binding site is part of an autoinhibitory region, and that CaM binding relieves this inhibition) (56, 1056). In some cell types, PKC and p38 MAPK have been shown to play partial roles in shrinkage-induced NHE1 activation (794, 803), and a role for JNK was proposed for the *Xenopus* NHE (285). Studies in other cell types, however, have argued against roles for at least conventional PKCs (378, 932) and for either of the MAPKs (279). MLCK was assigned a role in NHE1 activation by shrinkage in primary rat astrocytes (932). However, this was based on concentrations of the inhibitor ML-7, which would also have inhibited PKA and to some extent PKC (932). Accordingly, in other cell types, no evidence could be found for a role for MLCK (378, 803).

Although the actin cytoskeleton, to which NHE1 is linked via ERM proteins (see above), seems an attractive candidate for mediating NHE1 activation by cell shrinkage, there is little evidence to support such a scheme. In most cell types studied, F-actin disruption does not inhibit shrinkage-induced NHE1 activation (69, 256, 1134), although there are reports to the contrary in rat osteoclasts (172) and trout hepatocytes (216). Furthermore, siRNA-mediated deletion of ezrin augmented rather than inhibited the shrinkage-induced activation of NHE1 in Cos-7 and ELA cells, by a mechanisms that remains to be elucidated (837).

The possible role of plasma membrane shape changes and/or specific lipids in NHE1 volume sensitivity has also been considered. An interesting study by Hilgeman's group established that in CHO cells, NHE1 was sensitive to the lipid composition of the plasma membrane such that cholesterol, and other agents thickening the membrane, activated NHE1 (256). However, this was not the case when NHE1 was expressed in AP1 cells, in which NHE1 is nonetheless volume sensitive, arguing against a primary role of plasma membrane physical properties in NHE1 volume sensitivity (256). With respect to specific phospholipids, the PtdIns(4,5)P_2 level increases in shrunken cells (sect. IV*C2*), and given the requirement for this phospholipid for full NHE1 function in other contexts (7, 256), a role for PtdIns(4,5)P_2 in shrinkage activation of NHE1 seems an attractive hypothesis, but has, to our knowledge, yet to be tested.

Thus the mechanism(s) by which NHE1 is activated by shrinkage remains to be fully elucidated. A number of candidates stand out. For instance, it will be important to verify the possible role for PtdIns(4,5)P_2. Given the major roles of Ste20-related kinases in regulation of the cation cotransporters by cell volume (sect. V*D*), the possible role of NIK should also be addressed. It may also be of interest that NHE1 has been proposed to localize to caveolae (92), which have been implicated in volume sensing (see sect. III).

3. *Other volume-sensitive vertebrate Na^+/H^+ exchangers*

Given its essentially ubiquitous distribution and robust shrinkage activation, NHE1 is likely the only or by far the most important mechanism of NHE-mediated RVI in most cells. However, in cell types in which these exchangers are expressed at significant levels, NHE2 and NHE4 may also contribute, and indeed may have special functions in the RVI response. Again pointing to the involvement of regulatory cofactors, the volume sensitivity of NHE2 seems to be cell-type specific (Table 4). In PS120

cells, Su et al. (964) found that replacing the first extracellular loop with that of NHE1 rendered NHE2 volume sensitive, suggesting that in NHE2, yet not in NHE1, this region plays an inhibitory role in shrinkage-activation. NHE4 is robustly activated by cell shrinkage in a manner dependent on the actin cytoskeleton and has been suggested to play a special role in RVI in tissues exposed to very high osmolarity, such as the kidney medulla (69, 70).

In contrast to NHE1, -2, and -4, NHE3 has been found to be inhibited by osmotic shrinkage in a variety of epithelial cells (283, 432, 696, 938, 1077), and to conversely be activated by cell swelling in most cells studied, including rat thick ascending limb (283, 1077) and MDCK cells (13) (Table 4). However, divergent views have also been reported (432, 964), likely indicating that the volume sensitivity of NHE3 may be dependent on the cell type in which it is expressed. This may reflect the requirement for a cell type-specific regulatory intermediate, in accordance with the substantial number of cell type-specific NHE3 regulators (see Ref. 199); however, the extent to which these regulators play a role in volume-sensitive NHE3 regulation is to our knowledge unelucidated. Notably, recent studies in MDCK cells indicated that the activation of NHE3 by cell swelling was dependent on the membrane deformation under these conditions, whereas it was not due to either insertion of additional NHE3 proteins in the plasma membrane, or altered association of NHE3 with the cytoskeleton (13).

In contrast to the regulation of cell AR volume by NHE1, the volume sensitivity of NHE3 seems to play a major role in control of systemic volume and hence blood pressure. This notion is supported by the finding that NHE3 knockout mice exhibit significantly reduced blood pressure compared with wild-type animals (273, 902; see Ref. 12). Presumably, NHE3 stimulation in the proximal tubule when the filtrate is hypotonic will increase Na^+ reabsorption and hence result in decreased Na^+ delivery to the distal tubule, and vice versa for a hypertonic filtrate which inhibits NHE3, and this will regulate plasma tonicity towards the normal levels (see Ref. 12).

Finally, NHE5 is also inhibited by cell shrinkage, in accordance with the fact that this isoform exhibits the closest sequence homology to NHE3 (25).

B. The Na^+-K^+-$2Cl^-$ Cotransporters

The NKCCs belong to the cation-chloride cotransporter (CCC) superfamily. The vertebrate NKCCs, together with the Na^+- Cl^- cotransporters (NCCs) and K^+-Cl^- cotransporters (KCCs, sect. VII*C*) constitute the SLC12A family (Table 5). Similar to the NHEs, the NKCCs play major roles in a wide variety of important physiological processes beyond cell volume regulation. For instance, NKCC1 is a central effector in epithelial secretory processes (sect. IX*A*), as well as in glial K^+ buffering and regulation of neuronal $[Cl^-]$ and consequently of GABAergic transmission. NKCC1 has also been implicated in the control of cellular metabolism (894) and growth (414). For comprehensive reviews on NKCC1, see References 182, 312, 342, and 870. Pathophysiologically, a large body of evidence indicates that at least in the brain, NKCC1-mediated cell swelling plays a major role in edema and necrosis induced by ischemic challenges (for a discussion, see Ref. 797).

1. *Fundamental properties, localization, and pharmacology of the NKCC1 and -2*

Two vertebrate NKCC isoforms have been cloned and characterized: NKCC1 (SLC12A2) is ubiquitously expressed and, in epithelial cells, generally localizes to the basolateral membrane (see Refs. 265, 312, 870).[4] In mammals, NKCC2 (SLC12A1), which exhibits ~60% homology to NKCC1, is mainly expressed in the thick ascending limb of the kidney, where it localizes to the apical membrane and mediates ion reabsorption (870). NKCC2 homologs are also found in tissues from nonmammalian vertebrates, including shark kidney (257) and eel intestine

[4] In the choroid plexus epithelium and brain microvascular endothelial cells, NKCC1 has been found to localize to the apical membrane (735, 819).

TABLE 5. *Overview of the SLC12A family members and their relation to cell volume perturbations*

Family Member	Accession No.	Tissue and Subcellular Distribution	Cell Volume Sensitivity
SLC12A1/NKCC2	NM000338	Kidney specific; apical plasma membrane	Shrinkage activated
SLC12A2/NKCC1	NM001046	Ubiquitous; basolateral plasma membrane	Shrinkage activated
SLC12A3/NCC	NM000339	Kidney specific; apical plasma membrane	Shrinkage activated? (227)
SLC12A4/KCC1	NM005072	Ubiquitous	Swelling activated
SLC12A5/KCC2	AF 208159	Neuronal; basolateral plasma membrane (?)	Swelling activated (see, however, Ref. 778)
SLC12A6/KCC3	AF 314956	Broadly expressed	Swelling activated
SLC12A7/KCC4	NM006598	Broadly expressed; basolateral plasma membrane (?)	Swelling activated
SLC12A8/CCC9	AF 319951	Broadly expressed	?
SLC12A9/CIP	NM020246	Broadly expressed	?

(575). NKCC1 and -2 have apparent molecular masses of ~130 and 120 kDa, respectively.[5] The NKCCs share a proposed membrane topology of 12 central TM domains, a short cytosolic NH_2-terminal region. and a long cytosolic COOH-terminal region (see Refs. 265, 870). Both isoforms are inhibited by bumetanide, furosemide, and related loop diuretics (265, 870).

Both NKCC1 and NKCC2 are activated by osmotic cell shrinkage; however, given the scarcity of evidence available for NKCC2, and the limited distribution of this isoform, the great majority of this section will focus on NKCC1.

2. *SLC12A2/NKCC1*

A) NKCC1 AS PART OF A MACROMOLECULAR COMPLEX? The identification by Delpire's group in 2002 that the Ste20-related kinases SPAK and OSR1 interact directly with this transporter (814, 815) was a substantial step forward in the understanding of NKCC1 regulation by cell volume perturbations, as described below. Other NKCC1 binding partners include the Ser/Thr protein phosphatases 1 (170) and -2A (560). The identified protein partners of NKCC1, with SPAK as a major example (see below), also interact in a regulated manner with a wide array of other proteins. Hence, the number of proteins associating directly or indirectly with NKCC1 at any given time is likely to be substantial; however, the dynamics of these interactions are only beginning to be unraveled.

B) NKCC1 AS A MECHANISM OF RVI. Coupled anion-cation transport in RVI was first demonstrated in avian RBCs (475, 476) and shortly after in mammalian cells (32, 355). Similar to NHE1, NKCC1 is a major mediator of RVI in a wide variety of cell types studied. In a few cell types studied, including RBCs from teleost fishes and amphibians, NKCC1 does not appear to be expressed and RVI is mediated by NHE1 in parallel with AE, whereas in a few others, NHE1 expression is absent or negligible, and NKCC1 appears to be the sole mediator of RVI (see Ref. 797). More commonly, however, NHE1 and NKCC1 are coexpressed and may both contribute significantly to RVI (e.g., Refs. 732, 794, 1083; see Ref. 360, 797). The driving force for transport of ions into the cell by NKCC1 is generally much less robust than that for inward Na^+ transport by NHE1, and consequently, the extent to which the activation of NKCC1 actually contributes to RVI even if the transporter is robustly activated, is dependent on the cell type and mode of shrinkage (408, 862; see Ref. 870). Finally, it is noteworthy that NKCC1, similar to NHE1, may serve scaffolding roles in osmotically stressed cells. Thus NKCC1, SPAK, and p38 MAPK appear to form a dynamic complex, which has been proposed to function as a stress relay system modulating the activity of p38 MAPK (814).

C) MECHANISMS OF NKCC1 REGULATION BY CELL VOLUME PERTURBATIONS. It has been clearly established that the NKCC1 protein is directly phosphorylated in response to osmotic shrinkage. Studies from a wide range of cell types have demonstrated that shrinkage activation of NKCC1 occurs at least in part through phosphorylation of specific threonine residues in the NH_2-terminal region of the cotransporter protein (260, 599, 734; see Refs. 312, 870). Similar residues are phosphorylated in response to other stimuli activating the transporter, including reductions in $[Cl^-]_i$, and it was therefore suggested early on that the candidate kinase would be activated by both cell volume and reductions in $[Cl^-]_i$ (see Refs. 265, 870). Burgeoning evidence indicates that two families of protein kinases, the WNKs and the Ste20-related kinases SPAK and OSR1, fulfill these criteria and play major roles in the regulation of both the shrinkage- and swelling-activated members of the SLC12A family. The upstream elements of the WNK-SPAK/ORS1 pathways are discussed in more detail in section V*D*, the regulation of KCCs are discussed in section VII*C*, and we focus here specifically on the relationship between WNKs, SPAK/OSR1, and NKCC1.

A role for SPAK in activation of NKCC1 by low $[Cl^-]_i$ was shown first (203), followed by the demonstration of a role for SPAK in NKCC1 activation by osmotic shrinkage (259). In mass spectroscopy analyses of shark NKCC1, Alessi's group (1039) showed that SPAK and OSR1 directly phosphorylate NKCC1 on three specific threonine residues, which are also phosphorylated upon osmotic shrinkage. However, a more recent study in which mouse NKCC1 mutants were expressed in *Xenopus* oocytes showed that only one of these residues, as well as another threonine not identified by the Alessi group (Thr^{206} and Thr^{211} numbered according to the mouse sequence), was of importance for SPAK-mediated NKCC1 regulation (260). NKCC1 contains two SPAK binding motifs in its NH_2-terminal cytosolic region, and it was demonstrated that physical docking of SPAK to one of these motifs was necessary for its basal and shrinkage-induced activation (260). On its own, WNK4 inhibited human NKCC1 activity in *Xenopus* oocyte expression studies (422). However, after identifying WNK4 as a SPAK binding partner in a yeast two-hybrid screen (814), Gagnon and co-workers found that when WNK4 and SPAK are both coexpressed with NKCC1, the transporter is activated and cannot be further activated by shrinkage, consistent with a WNK4-SPAK-NKCC1 activation pathway (259). It seems likely that several WNK isoforms may be upstream activators of NKCC1 in shrunken cells. Thus WNK1 is activated by hypertonicity (1117, 1135) and was shown to be upstream of SPAK- and OSR1-mediated phosphorylation of human NKCC1 (675). Moreover, in HeLa cells, a WNK1-OSR1 pathway was found to regulate NKCC1 function, and in

[5] Alternatively spliced forms exist for both isoforms, yet will not be further discussed here (see Ref. 265).

these cells, WNK1 appeared to be a more potent activator of SPAK and OSR1 than did WNK4 (17). On the other hand, WNK1 is a powerful regulator of other WNK isoforms (539), which might have affected these findings. Further complicating the picture, NKCC1 is also activated downstream of WNK3 after coexpression in *Xenopus* oocytes, in a manner associated with both increased NKCC1 phosphorylation and surface expression, yet not dependent on coexpression with SPAK/OSR1 (423). Thus it appears that different WNK isoforms may regulate NKCC1 by somewhat differing mechanisms. In the case of WNK4, the data are consistent with a pathway in which WNK4 is activated by hypertonicity, upon which it phosphorylates and activates SPAK, which then docks to and directly phosphorylates NKCC1, leading to its activation (259, 260, 1039). Although WNK4 and SPAK also interact physically, there is as yet no evidence of the existence of a complex containing all three proteins (for a discussion, see Ref. 260). Intriguingly, recent findings suggest that the regulation of NKCC1 by SPAK is interrelated to its regulation by the Ser/Thr phosphatase 1. Thus, on the one hand, protein phosphatase 1 and SPAK may compete for binding to the second SPAK site, which overlaps with the binding site for protein phosphatase 1. On the other hand, the NKCC1 NH_2 terminus may bind SPAK at the first, and protein phosphatase 1 at the second site, thus acting as a scaffold for protein phosphatase 1-mediated dephosphorylation of SPAK (260, 261). A recently identified SPAK binding partner, the apoptosis-associated tyrosine kinase (AATYK), may similarly scaffold SPAK and protein phosphatase 1. The expression of this kinase attenuated isotonic NKCC1 activity in a manner counteracted by mutation of either the SPAK or protein phosphatase 1 binding sites on the kinases; however, the possible relevance of AATYK to NKCC1 regulation in shrunken cells is unknown (261).

Beyond SPAK/OSR1, a number of other Ser/Thr protein kinases have been implicated in shrinkage-induced NKCC1 activation, including MLCK (459, 472, 734), JNK (458), PKC (343, 408, 558), FAK (see Ref. 368), and also tyrosine phosphorylation events (1145). Conversely, inhibitors of Ser/Thr protein phosphatases 1 and 2A activate NKCC1 and potentiate its activation by osmotic shrinkage (239, 472, 687, 734). Of these, the possible role of MLCK is by far the most studied and will be discussed below together with the roles of myosin II and F-actin, with which it is intimately linked.

It is well established that the actin-based cytoskeleton plays an important role in regulation of NKCC1 (367, 412, 559, 633–635; see Ref. 791). Interestingly, it appears that an intact F-actin cytoskeleton is required both for maintaining NKCC1 silent under isotonic conditions and for its shrinkage-induced activation (367, 412, 633, 634; see Ref. 791). One consequence of this is that in contrast to NHE1, which is inhibited by cell swelling, NKCC1 is moderately activated by swelling (412), presumably because of the reorganization of, and net decrease in, F-actin in swollen cells (for a discussion, see Refs. 367, 791).

The light chain of the actin-associated protein myosin (MLC) has been shown to be phosphorylated upon cell shrinkage in endothelial cells (459, 734) (sect. IV*D*). This, in conjunction with the inhibitory effect of the MLCK inhibitor ML-7 on shrinkage-induced NKCC1 activity observed in several cell types (459, 472, 734), has led to a proposed role for MLCK-mediated MLC phosphorylation in shrinkage activation of NKCC1. However, findings in kidney epithelial cells were not in agreement with a model based on simple coupling of MLC phosphorylation and NKCC1 activation (137, 138). In these cells, shrinkage-induced MLC phosphorylation was dependent on Rho kinase and was not blocked by ML-7, whereas NKCC1 activation was blocked by ML-7 and insensitive to Rho kinase inhibition (137, 138). On the other hand, myosin activity did appear to be required, since shrinkage-induced NKCC1 activity could be inhibited by blebbistatin, which inhibits the myosin ATPase function (137, 138). Thus, at least in kidney epithelial cells, the effect of ML-7 on the cotransporter does not reflect a decrease in MLC phosphorylation. Then what is the mechanism? Studies in mammalian RBCs suggested that high concentrations (IC_{50} ~20 μM) of ML-7 may affect a volume-sensitive kinase other than MLCK (438), and it is therefore possible that the role of MLCK in some of the above studies has to be reevaluated. On the other hand, targeting of such a kinase does not explain the potent inhibition by ML-7 of shrinkage-induced NKCC1 activity in EAT cells, in which the IC_{50} is in the nanomolar range (see Fig. 13*D*), similar to that reported for inhibition of MLCK (472). In these cells, osmotic shrinkage is associated with reinforcement of the cortical F-actin network and translocation of nonmuscle myosin II to the cell periphery (790, 795; sect. IV*D*). In isolated EAT membrane vesicles (Fig. 13*A*), which are devoid of myosin II (Fig. 13*E*), and in which cortical F-actin is perturbed, NKCC1 expression per milligram protein is increased compared with that in the intact cells (Fig. 13*C*), and NKCC1 is moderately active under unstimulated conditions (Fig. 13*B*) (363). We recently demonstrated that in these vesicles, NKCC1 can no longer be shrinkage-activated and is insensitive to ML-7 (367; Fig. 13*B*). In contrast, the transporter is still responsive to other stimuli such as the protein phosphatase 1 and 2A inhibitor calyculin A, suggesting that shrinkage-activation is specifically lost under these conditions (367). These findings are most compatible with a model in which F-actin and myosin, and presumably MLCK-mediated MLC phosphorylation, are important for shrinkage-induced NKCC1 activation (367). Interestingly, the marked translocation of SPAK to the cell periphery observed upon shrinkage of intact EAT cells was absent in the vesicles (Fig. 13*F*), which might suggest that the link between NKCC1 regulation by SPAK and by F-actin/myosin

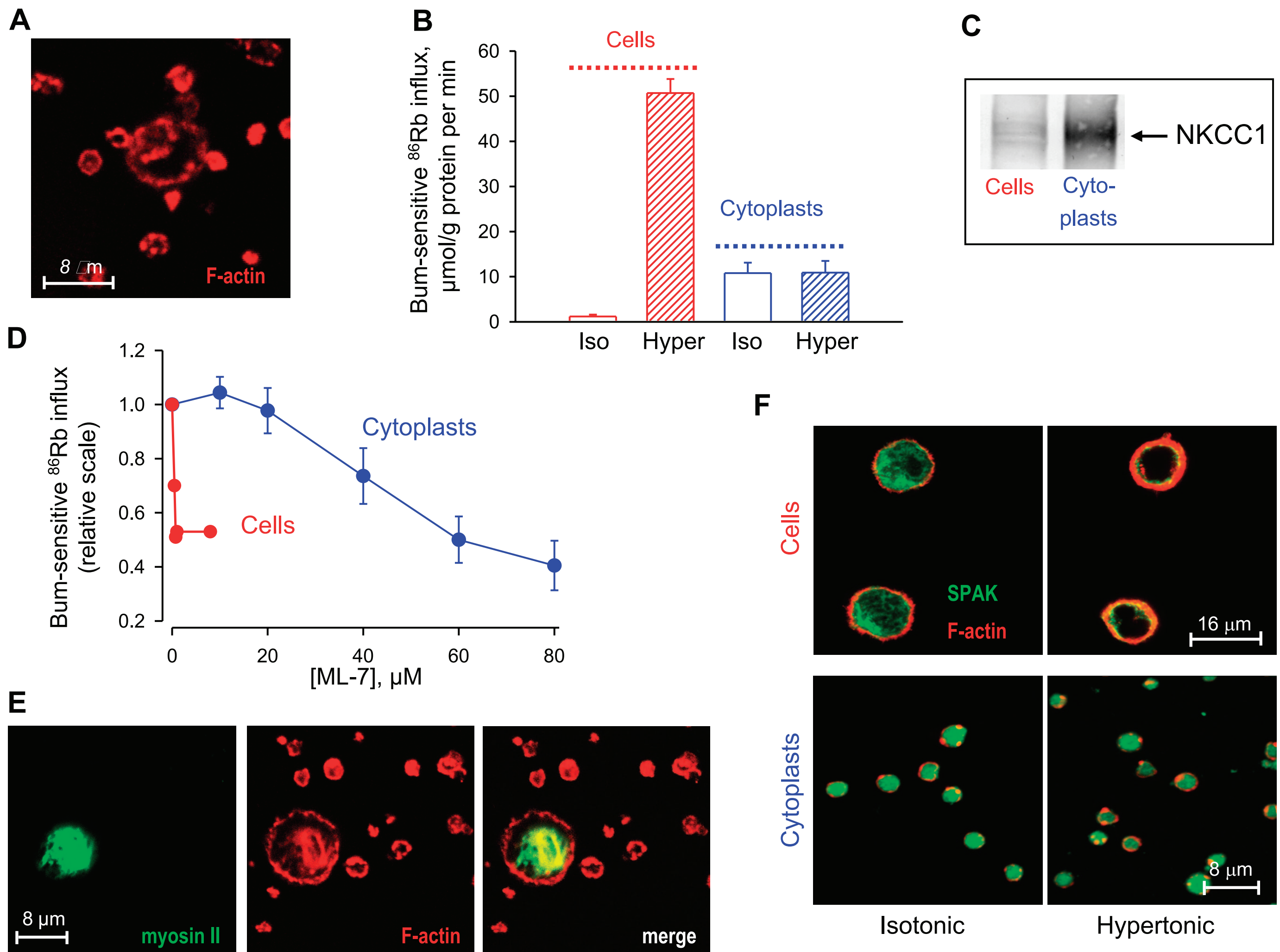

FIG. 13. NKCC1 activation by hypertonic challenge: roles for MLCK, myosin II, and SPAK? *A*: preparation of cytoplasts, isolated membrane vesicles, from EAT cells. [From Hoffmann and Pedersen (367).] *B*: NKCC1 in cytoplasts is constitutively partially active and cannot be further activated by hypertonic shrinkage. NKCC1 activity was measured in intact cells (red) and cytoplasts (blue) as the unidirectional bumetanide-sensitive ^{86}Rb influx within the time period 10 s to 3 min after osmotic shrinkage by salt addition to double extracellular osmolarity. [Values from Hoffmann and Pedersen (367), Hoffmann et al. (373), and Jensen et al. (408).] *C*: loss of shrinkage-activation does not reflect loss of NKCC1 protein, the concentration of which is increased in the cytoplasts compared with that in intact cells. [From Hoffmann and Pedersen (367).] *D*: in contrast to the potent inhibition in intact cells (IC_{50} ~0.4 μM), NKCC1 activity (measured as bumetanide-sensitive ^{86}Rb influx) in cytoplasts is insensitive to the MLCK inhibitor ML-7 (IC_{50} ~60 μM). [Values from Hoffmann and Pedersen (367) and Krarup et al. (472).] *E*: myosin II, which rapidly translocates to the cortical region in intact EAT cells after osmotic shrinkage, is seen in the intact cells, yet entirely missing from the cytoplasts. [From Hoffmann and Pedersen (367).] *F*: after osmotic shrinkage, the Ste20-related kinase SPAK translocates to the cortical region in intact EAT cells, yet not in cytoplasts. [From Hoffmann and Pedersen (367).]

may involve an F-actin/myosin-dependent recruitment of SPAK to the membrane, required for its interaction with NKCC1. In congruence with this notion, SPAK has been shown to bind to F-actin, yet not to G-actin (1008). On the other hand, studies in bovine aortic endothelial cells have suggested that the inhibitory effect of ML-7 on NKCC1 is not associated with a decrease in the phosphorylation of the NKCC1 protein (459, 734). This may suggest that while, at least in some cells, MLCK is necessary, its activity is not sufficient for full NKCC1 activation.

Recent studies demonstrated the regulation of NKCC1 by recruitment to the plasma membrane after activation of PKC-ϵ in intestine (180) and after Ca^{2+} increases in human colonic crypts (842). To our knowledge, no studies have directly addressed the possible role of NKCC1 trafficking in its regulation by cell volume perturbations. Apparently arguing against NKCC1 trafficking in its activation by shrinkage, however, in EAT cells, unstimulated cells in isotonic medium, with negligible bumetanide-inhibitable fluxes, have the same number of bumetanide binding sites as cells undergoing volume regulation (369). Another important challenge for future studies will be to identify possible points of convergence between WNK-SPAK, MLCK/myosin/F-actin, and other proposed regulators of NKCC1 volume sensitivity.

3. *SLC12A1/NKCC2*

NKCC2 is also stimulated by osmotic cell shrinkage, and its activation is associated with phosphorylation of the same three threonine residues shown to be important for shrinkage-induced NKCC1 activation (280). Relatively little is known about the mechanism of activation of NKCC2 by cell shrinkage. However, as NKCC2 shares both the first SPAK binding site and the sites of SPAK-mediated phosphorylation with NKCC1, it seems likely that similar mechanisms may be involved (814, 815; see Ref. 261). Moreover, similar to what was found for NKCC1, WNK3 has been shown to activate NKCC2 after expression in *Xenopus* oocytes, in a manner associated with increased NKCC2 phosphorylation and plasma membrane expression and not requiring coexpression with SPAK/OSR1 (846). NKCC2 lacks, however, the protein phosphatase 1 binding site found in NKCC1, suggesting that the mechanisms of regulation are not identical (see Ref. 261).

The roles of the cytoskeleton and of other protein kinases in NKCC2 regulation have been studied for the NKCC2 homolog found in the apical membrane of the intestinal epithelial cells of the European eel, *Anguilla anguilla* (573, 575). On the basis of pharmacological evidence, both PKC and MLCK were found to contribute to the activation of NKCC2 by hypertonic stress. Moreover, in this tissue, the hypertonic activation of the apical cotransporter was strongly dependent on the integrity of both F-actin and microtubules (573, 575). The latter was tentatively suggested to reflect a role of NKCC2 trafficking in its volume sensitivity; however, this remains to be experimentally verified.

C. Organic Osmolyte Uptake Systems: TauT

A shift in the cellular content of organic osmolytes such as taurine is an important feature of the readjustment of the cell volume following osmotic perturbation. The cellular taurine content is a balance between active, Na^+/Cl^--dependent uptake via the carrier denoted TauT, and passive release via a volume-sensitive, yet unidentified transport protein. TauT is regulated both at the posttranscriptional level by acute cell volume perturbations, and at the expression level after long-term osmotic stress. The former is dealt with below, and the latter in section VIII*B3*.

TauT has a molecular mass in the range 65–74 kDa and is predicted to contain 12 TM domains and several intracellular phosphorylation motifs (413, 578, 1009, 1035). TauT shares a high sequence homology with the transporters for creatine and for the neurotransmitters GABA, norepinephrine, dopamine, and serotonin (567). Several TauT isoforms are recognized by commercial TauT antibodies (823, 1052, 1119), in accordance with the notion that neurotransmitter transporters function as homodimers or homo-oligomers (332, 381, 897). TauT knockout mice exhibit increased photoreceptor apoptosis and structural and functional abnormalities in the olfactory epithelium (1073).

In many cell types, TauT-mediated taurine transport is attenuated acutely by reduction in the extracellular NaCl concentration, acidification, depolarization of the plasma membrane, and following protein kinase-mediated phosphorylation of TauT or a putative regulator of TauT (see Ref. 493). The Na^+:Cl^-:taurine stoichiometry for active taurine transport has been estimated at 2–3:1:1, and it appears that the taurine transport cycle is initiated by binding of one Na^+ ion to TauT, altering its tertiary structure before binding of the taurine molecule takes place. It is moreover proposed that Cl^- facilitates binding of the second Na^+ to the carrier (see Ref. 493). Taurine uptake was stimulated in brush-border membrane vesicles by a negative potential on the *trans*-side of the membrane (41, 1099, 1136), and in EAT cells after hyperpolarization of V_m (498). The plasma membrane of EAT cells is hyperpolarized following extracellular alkalinization, and with the isoelectric point of TauT (5.98) taken into account, it has been suggested that the increased taurine uptake at alkaline pH reflects an increased availability of unloaded, negatively charged carrier at the outer face of the plasma membrane (498). Thus the reduced taurine uptake in EAT cells following acute reduction in extracellular osmolarity (364) could well reflect both V_m depolarization (501) and cytoplasmic acidification (581).

Regulation of TauT involves PKC, PKA, CK1 and -2, as well as protein phosphatases, and TauT activation is characterized by reduction in the transport capacity (V_{max}) and affinity towards taurine (increase in K_m) (see, e.g., Refs. 326, 400a, 660, 983). In EAT cells, PKA normally stimulates, whereas activation of PKC reduces, taurine uptake and at the same time renders the cells insensitive to PKA-mediated stimulation (660). PKC-mediated impairment of TauT-mediated taurine uptake involves a serine residue which in MDCK cells is located at the fourth intracellular segment of TauT (326). TauT has a conserved putative CK2 phosphorylation site in the NH_2-terminal domain and in NIH3T3 cells inhibition of the constitutively active CK2 increases the Na^+ affinity and increases the maximal taurine uptake (400a).

D. Hypertonicity-Induced Cation Channels

Shrinkage-induced activation of cation channels can be demonstrated in many cell types, and hypertonicity-induced cation channels (HICCs) have proven to be a major mechanism of RVI in some cells (817, 860, 1079, 1082, 1083). Pharmacologically, three types of HICCs can be distinguished: *1*) amiloride-sensitive nonselective cat-

ion channels (68, 390, 1079, 1080), at least some of which have been suggested to be related to ENaC, and which are in general insensitive to Gd^{3+} and flufenamate (see Ref. 1079). With the use of anti-α-, anti-β-, and anti-γ-rENaC siRNA, it was recently shown that all three subunits of ENaC are functionally related to HICC activation in hepatocytes (817). In human glioma cells, it was demonstrated that an amiloride-blockable, psalmotoxin-sensitive cation channel, a member of the DEG/ENaC superfamily, is important in RVI (860). Based on pharmacological studies in rat hepatocytes, the hypertonic activation of these HICCs was proposed to involve PKC (343), PLC, G proteins, and tyrosine kinases (343, 1080).

2) Amiloride-insensitive nonselective cation channels have a conductance of 15–27 pS (1082), are blocked by flufenamate and Gd^{3+}, and are expressed in a variety of systems (119, 1046, 1082). These HICCs are inhibited by cytosolic ATP in the lower millimolar range and thus are expected to be closed under most physiological conditions (464). This raises some concern as for their actual role in RVI. Nonetheless, such a contribution, which exceeded that of NHE1, was reported in HeLa cells (1082), although an explanation for this apparent paradox was not offered. It was, furthermore, suggested that the channel might be a TRP channel (1082). *3*) HICC that are both Gd^{3+} and amiloride sensitive, and thus may reflect a molecular link between the two other groups, have been reported in ELA cells (529) and human hepatocytes (555). In ELA cells, the single-channel conductance was 14 pS in cell-attached patches, and the relative blocking efficiency of amiloride and its congeners was very similar to that for ENaC (529).

VII. REGULATORY VOLUME DECREASE

Figure 1 shows a typical RVD response, and Figure 11 provides an overview of the main effectors in the early phases of RVD in most cell types studied. In the following, we discuss these membrane transport proteins and the mechanisms by which they are regulated by osmotic volume perturbations. The physiological and pathophysiological relevance of these transporters is further discussed in section IX.

A. Swelling-Activated Cl^- Channels

Increases in K^+ conductance (g_K) during RVD will hyperpolarize V_m and result in conductive Cl^- efflux as long as the basal cellular Cl^- conductance (g_{Cl}) is of a reasonable size. Due to the substantial exchange diffusion of Cl^- across cell membranes, it was initially assumed that the basal g_{Cl} was very high, and thus that an increase in g_{Cl} was not necessary for RVD (for a discussion, see Ref. 359). However, in EAT cells, <5% of the unidirectional Cl^- flux under isotonic conditions was found to be conductive leak flux, with the rest being anion exchange (AE; Fig. 14*A*). Thus g_{Cl} was much lower than g_K (372, 501), meaning that the swelling-activated K^+ efflux would be limited by V_m unless there was a simultaneous increase in g_{Cl}, and indeed, such an increase was subsequently demonstrated (354; see also Fig. 14*A*). This was soon after also found in lymphocytes (293) and later demonstrated in essentially all cell types investigated. RVD in the presence of the K^+ ionophore gramicidin is rate-limited by the Cl^- permeability, which the volume change in the presence of the ionophore has consequently been used to monitor (365). From Figure 14*B*, it is seen that Cl^- permeability increases abruptly when the cells are swollen and decreases again within 10 min following hypotonic exposure. Direct electrophysiological measurements of the swelling-activated Cl^- current ($I_{Cl,vol}$) were first performed in intestine 407 cells (339). The channel mediating $I_{Cl,vol}$ has, in addition to the term VRAC used in this review, been denoted volume-sensitive organic osmolyte and anion channel (VSOAC) and volume-sensitive outwardly rectifying anion channel (VSOR) (714, 716, 741, 955). In cases where the swelling-activated Cl^- flux has not been directly demonstrated to exhibit the biophysical characteristics of VRAC, we will denote the current $I_{Cl,vol}$. The biophysical and pharmacological characteristics of $I_{Cl,vol}$ are similar in most cell types (see Refs. 714, 716, 741, 955). Figure 14*C* shows a typical current-voltage curve for VRAC. The current exhibits moderate outward rectification, with a varying degree of inactivation at larger positive potentials (see, e.g., Refs. 400, 541, 801) which in EAT cells is not very strong. The permeability sequence for VRAC is generally found to be $SCN^- > I^- > NO_3^- > Br^- > Cl^- > F^- >$ gluconate, corresponding to low-field ion selectivity or Eisenman's sequence I (21, 225, 400, 801). VRAC is also permeable to HCO_3^- (719). HCO_3^- is present in cells at significant concentrations, and loss of cellular HCO_3^- will be compensated for by CO_2 hydration and H^+ generation; thus the importance of HCO_3^- will depend on the buffering capacity and carbonic anhydrase activity of the cell. If the buffer capacity is high, HCO_3 may be a significant factor as a companying anion to K^+, particularly in cells with low $[Cl^-]_i$ such as skeletal muscle and nerve (see, e.g., Ref. 370). A controversial issue has been whether VRAC mediates the swelling-activated taurine efflux; this is discussed in section VII*D*.

In many cell types, VRAC is inhibited by classical Cl^- channel blockers such as DIDS and NPPB, whereas in others, it is relative insensitive to DIDS, which inhibits VRAC exclusively at positive potentials (e.g., Ref. 801). VRAC is also commonly inhibited by the estrogen receptor antagonist tamoxifen (for VRAC pharmacology, see Refs. 225, 411, 716, 723). Specific inhibitors of VRAC are lacking, which limits the progress in trying to identify these channels at the molecular level (see Ref. 411). The

most selective inhibitors are an indanone compound, DCPIB (179) and an acidic di-aryl-urea, NS3728, which inhibits VRAC in HEK-293 cells and in EAT cells with an IC_{50} around 0.4 μM (344, 456). VRAC activity depends on cytosolic free ATP, but not on ATP hydrolysis, suggesting a modulatory binding of ATP to a channel component (400, 740; see Ref. 741). Furthermore, depolarization induces blocking of the channel by extracellular anionic ATP (ATP^{4-}), suggesting that the outer vestibule is larger, yet the pore slightly smaller, than the radius of ATP^{4-} (0.6–0.7 nm) (985).

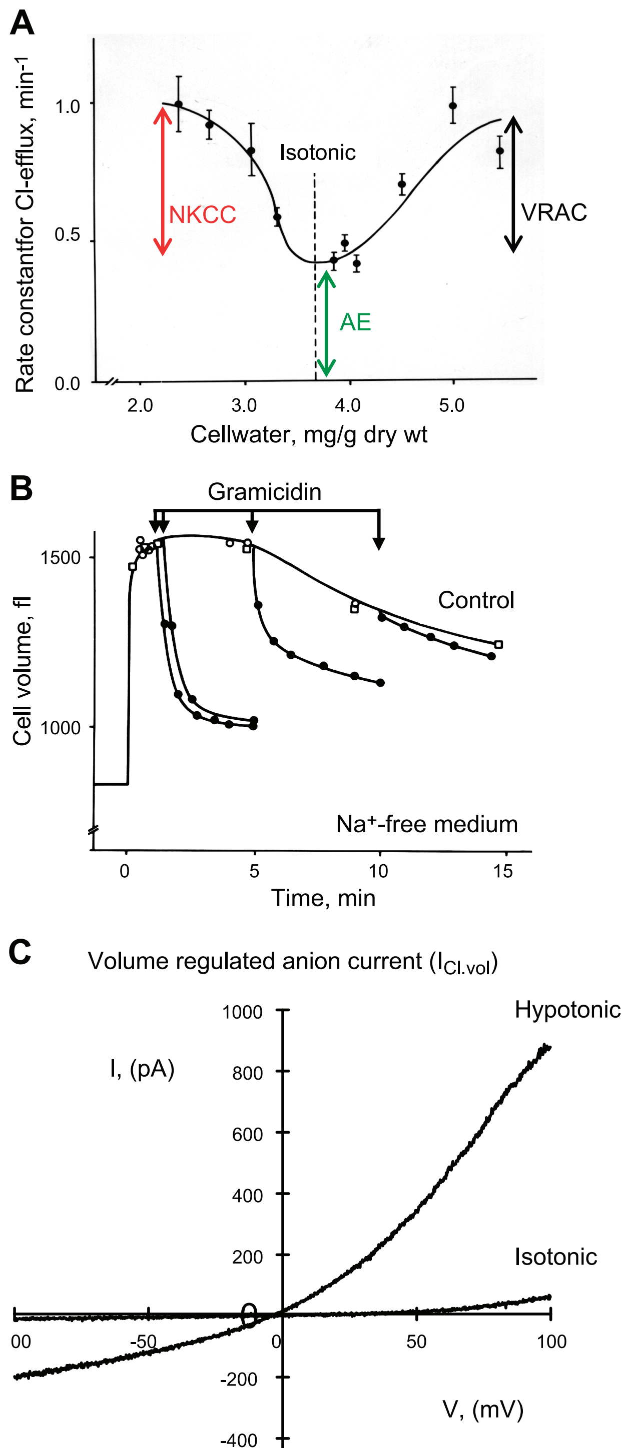

FIG. 14. Volume-sensitive Cl^- permeability. *A*: volume dependence of the rate constant for ^{36}Cl efflux measured unidirectional steady-state efflux. 95% of the efflux under isotonic conditions was determined as an electroneutral anion exchange (AE). The remaining efflux constituted conductive Cl^- flux which increased significantly after cell swelling. This current is taken to represent VRAC activity. The anion/cation cotransporter, being quiescent under isotonic conditions, accounts for the increased Cl^- efflux in shrunken cells. This cation-dependent Cl^- efflux is taken to represent NKCC. [Values from Hoffmann (355) and Hoffmann et al. (372).] *B*: transient increase in the Cl^- permeability in EAT cells in Na^+-free medium containing a K^+ channel inhibitor (quinine). Gramicidin (0.5 μM) was added to ensure a high cation permeability, i.e., the Cl^- permeability is rate limiting the cell shrinkage after gramicidin. [From Hoffmann et al. (365).] *C*: *I-V* relationship obtained by whole cell patch-clamp recordings (−140 mV to +80 mV, using a fast ramp protocol) of the Cl^- current in EAT cells under isotonic and hypotonic (27% decrease) conditions. [Data from Pedersen et al. (801).]

1. Molecular identity of VRAC

The molecular identity of VRAC is undefined, despite a wealth of effort and multiple proposed candidates (for a discussion, see, e.g., Ref. 715). P-glycoprotein is withdrawn as a candidate for the channel per se (see Refs. 716, 741), but several groups have reported evidence for P-glycoprotein being a regulator of VRAC (655, 1022, 1027). With respect to the possible role of I_{Cln}, the discussion can be followed in References 99, 267, 306, 550, 777, and 1040. ClC-2 is activated by cell swelling (302), but the current carried by this channel differs pharmacologically and biophysically from those generally described for VRAC (716). ClC-3 was also proposed to mediate $I_{Cl,vol}$ (207, 1125), but has been challenged by the finding of VRAC activity in cells from CLC-3 knockout mice (20, 282, 949). Phospholemman, a protein with a single membrane-spanning domain, has also been proposed as a candidate, but its biophysical properties diverge from the properties described for VRAC (716). Bestrophins are a newly identified family of Cl^- channels which in *Drosophila* are both Ca^{2+} and volume sensitive. Knock-down of *dBest1* abolishes the volume-regulated Cl^- current in *Drosophila* and significantly reduces RVD (130). Bestrophins are, however, not obvious VRAC candidates in vertebrates, since the kinetics and rectification are distinctly different (e.g., linear current-voltage relationships) from those of VRAC, and since bestrophins are rather insensitive to hyposmolality, although strongly inhibited by hyperosmotic solutions (238). Notably, however, VRAC was ~10-fold greater in hBest1-transfected HEK 293, HeLa, and ARPE-19 cells compared with wild-type cells (238). On this basis, one might be tempted to speculate that hBest1 could be a component of VRAC.

2. *Mechanisms of activation of VRAC by osmotic swelling*

The mechanisms involved in VRAC activation are still poorly understood, although several important players have been identified (see, e.g., Refs. 715, 741). In the following, we outline the most important of these mechanisms and signaling cascades.

A) IONIC STRENGTH. A reduction in intracellular ionic strength was shown to activate VRAC in, e.g., trout RBCs (309), Chinese hamster ovary (CHO) cells (110), and endothelial cells (719, 1041). Similarly, single-channel recodings in permeabilized cells showed that a reduction in intracellular ionic strength activates VRAC, directly or via a volume sensor (873).

B) MEMBRANE LIPID COMPOSITION. A decrease in membrane cholesterol content potentiates VRAC in several cell types including bovine aortic endothelial (BAE) cells (544, 857) and in EAT cells (456)(Fig. 4). Furthermore, cholesterol depletion activates VRAC even in nonswollen cells (456, 857), suggesting that it modulates the volume-sensing mechanism/volume set point. Modulation of VRAC by cholesterol was proposed to involve a change in the physical properties of the membrane, including disruption of lipid rafts and caveolae. However, as discussed in section III*B*, cholesterol depletion also alters, e.g., F-actin organization, and in fact, modulation of VRAC by cholesterol depletion appears to at least in part reflect changes in the F-actin cytoskeleton (100, 101, 456). A role for caveolae disruption in VRAC activation would contrast with the finding that VRAC stimulation by cholesterol depletion is seen also in caveolin-deficient Caco-2 cells and after sphingomyelinase treatment (568). Swelling-induced activation of VRAC is, in contrast, significantly impaired in caveolin-1-deficient Caco-2 cells and restored by overexpression of this protein (1006). Cholesterol modulates membrane deformation energy (see sect. III*B*), which is important for opening of at least some ion channels (594). Pointing to a role for membrane deformation energy in modulation of VRAC, an increase in the membrane content of polyunsaturated fatty acids, which also decreases the membrane deformation energy, increased the swelling-activated Cl^- permeability and the rate of RVD in ELA cells (527).

C) LIPID MEDIATORS. Arachidonic acid directly inhibits VRAC in most cell types (sect. IV*C1*). A specific $cPLA_2$ inhibitor nevertheless blocked the activation of VRAC by swelling (47), implying that this effect is likely to be dependent on an arachidonic acid metabolite. With respect to the possible role of leukotrienes in VRAC regulation, lipoxygenase inhibitors exert either weak (479, 717) or strong (201, 225, 632) inhibitory effects on VRAC in various cell types (225, 632). Adding to the uncertainty is the fact that some of the LO inhibitors can block VRAC directly (955). Addition of LTD_4 activates both Ca^{2+}-dependent and -independent anion conductances in rat hepatocytes. However, addition of 100 nM LTD_4 to neuroblastoma cells (45), or 5 nM LTD_4 to EAT cells (385), failed to elicit Cl^- efflux/currents, in agreement with findings from several other cell types (2, 225). Thus a general role for LTD_4 in activation of VRAC seems unlikely. Finally, although PtdIns(4,5)P_2 levels rapidly decrease in swollen ELA cells, studies in these cells were inconsistent with a role for PtdIns(4,5)P_2 in modulation of VRAC activity (456; sect. IV*C*).

D) CYTOSKELETON. In some cell types, $I_{Cl,vol}$ is stimulated by disruption of the F-actin cytoskeleton; in others, it is unaffected or even inhibited (see Refs. 366, 791). In most cases, however, it seems that there is a stimulatory effect of F-actin disruption on $I_{Cl,vol}$ (543, 677, 912), and it was suggested that unfolding of membrane invaginations (see also Fig. 1) is important for $I_{Cl,vol}$ activation by swelling, and that the F-actin cytoskeleton modulates the volume sensitivity of the channel by conferring resistance to this process (677, 741). In ELA cells, isotonic VRAC activity was stimulated, and swelling-induced activity partially inhibited, by latrunculin B-induced F-actin disruption, and it was suggested that (different parts of the) actin cytoskeleton may play a dual role, keeping $I_{Cl,vol}$ silent under isotonic conditions, yet being involved in its swelling-induced stimulation (456). Accordingly, it was recently proposed that disruption of peripheral actin is necessary for VRAC activation but that the integrity of perinuclear F-actin is required for the signal transduction events upstream of VRAC activation (1062). Also providing a link to the cytoskeleton, recent findings point to a role of integrins in VRAC activation (sect. IV*A1*).

E) GTP BINDING PROTEINS. Exposure of cells to toxins that specifically inactivate Rho GTPases significantly reduces VRAC activation by cell swelling or by intracellular GTP (724), and intracellular application of guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) has been shown to activate VRAC in the absence of cell swelling (200, 201, 654, 717), suggesting roles for both Rho family and heterotrimeric G proteins in VRAC regulation. Accordingly, expression of constitutively active Rho in NIH3T3 cells augmented the RVD response (Fig. 15*A*) and potentiated the increase in VRAC after a 7.5%, yet not after a 30%, reduction in extracellular osmolarity (Fig. 15*C*; Ref. 786). Moreover, in both CPAE cells (724) and NIH3T3 cells (Fig. 15*B*), VRAC was inhibited by the Rho kinase inhibitor Y-27632. Hence, it seems that RhoA, although not the volume sensor per se, is an important upstream modulator of VRAC in NHI3T3 cells (786). Consistent with this, a permissive effect of RhoA in VRAC activation was proposed in CPAE cells (117).

F) $[Ca^{2+}]_i$. Ca^{2+} dependence of $I_{Cl,vol}$ has been proposed in a few cell types (45, 923), but in most cells, activation of $I_{Cl,vol}$ does not require a measurable increase in $[Ca^{2+}]_i$ and is seen also in the presence of

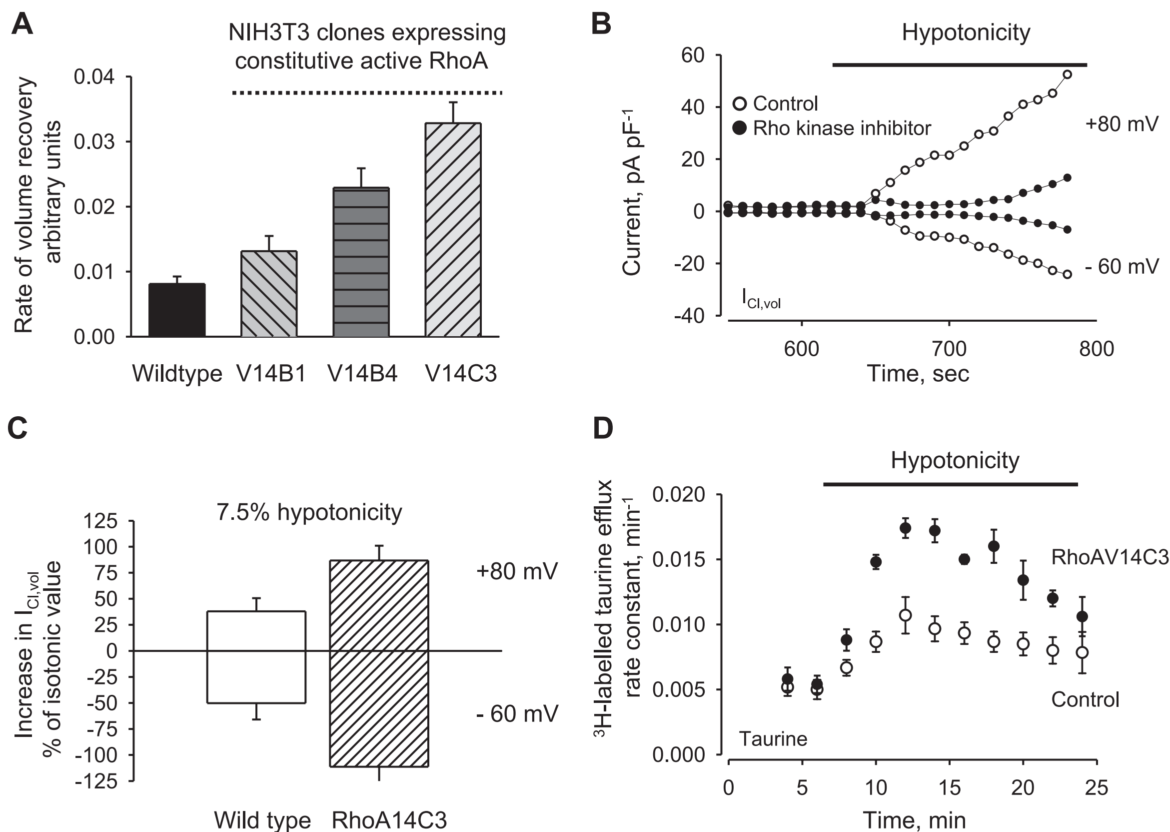

FIG. 15. Role of RhoA expression on the RVD rate, volume-set point, $I_{Cl,Vol}$, and taurine release. *A*: rate of RVD in wild type and three clones expressing constitutively active RhoA (V14B1, V14B4, V14C3) were calculated from light scatter experiments. *B*: effect of the Rho kinase inhibitor Y-27632 (10 μM) on $I_{Cl,Vol}$ in wild-type NIH3T3 cells was estimated by whole cell patch-clamp technique at −60 and +80 mV, using a fast ramp protocol. *C*: effect of RhoA overexpression on $I_{Cl,Vol}$ after a small (7.5%) reduction in the extracellular tonicity. Current was estimated as indicated under *B*. Hypotonic values are given relative to isotonic values. *D*: effect of RhoA on overexpression on swelling-induced taurine efflux after hypotonic exposure (200 mosM). Rate constant for the swelling-induced taurine release was recorded as described in Fig. 4. [From Pedersen et al. (786).]

strong $[Ca^{2+}]_i$ buffering (201, 801, 970). Moreover, in, e.g., ELA cells, the biophysical properties of $I_{Cl,vol}$ are clearly different from those of the current activated by an increase in $[Ca^{2+}]_i$ ($I_{Cl,Ca}$) (801), in congruence with the fact that no increase in $[Ca^{2+}]_i$ is detected after swelling in these cells (416).

In general, $I_{Cl,vol}$ does not appear to be activated by membrane stretch (134; see Ref. 741), indicating firstly, that the channel itself is not directly stretch activated, and second, that it is not functionally coupled with nonselective SA channels (e.g., via a local increase in $[Ca^{2+}]_i$). Similar to what is true for $I_{K,vol}$, a contribution from Ca^{2+}-activated Cl^- channels is, however, to be expected in cell types in which RVD is associated with an increase in $[Ca^{2+}]_i$, such as many epithelial cells (for a discussion, see sect. V*B*).

G) PROTEIN PHOSPHORYLATION AND DEPHOSPHORYLATION. The role of Rho kinase was discussed above (see also Fig. 15 and Refs. 721, 724, 786). For MLCK, divergent findings have been reported: whereas VRAC was attenuated after inhibition of MLCK in CPAE cells (721), it was strongly potentiated by MLCK inhibition in NIH3T3 cells (786). While there is no reason to doubt either finding, both of which were based on both small molecule inhibitors and specific peptide inhibitors of MLCK, it may be noted that a role for MLCK in activation of VRAC seems difficult to reconcile with the marked dependence also of some shrinkage-activated transport proteins on MLCK activity (sect. VI*B*).

Several inhibitors of tyrosine kinases, such as genistein, tyrphostin B46, and tyrphostin A25, attenuate, and tyrosine phosphatase inhibitors such as vanadate and dephosphatin stimulate, $I_{Cl,vol}$ in several cell types (206, 541, 992, 994, 1042). Many of these compounds are, however, relatively promiscuous, which may contribute to the lack of a clear picture of which tyrosine kinases and phosphatases may regulate VRAC, although cell-type specific differences are also likely to play a role. Thus the lymphocyte-specific Src family kinase p56lck mediates VRAC activation in Jurkat cells (541), whereas in human atrial myocytes, Src family kinases were reported to negatively regulate VRAC (206).

H) ROS. ROS, which are released in response to osmotic cell swelling in at least some cell types (sect. VG), seem to be important regulators of $I_{Cl,vol}$ (85, 415, 928, 1031). In HTC and HeLa cells, H_2O_2 elicits VRAC activation under isotonic conditions in a manner inhibited by osmotic shrinkage (928, 1030, 1031), indicating that ROS are not the volume signal per se, but downstream modulators of channel function. In HTC cells, the effect of ROS on VRAC appears to be mediated via activation of PLCγ and subsequent Ca^{2+} mobilization (1030). A role for ROS in VRAC activation was also proposed in rabbit ventricular myocytes, by a mechanism involving β1-integrin activation and downstream production of superoxide anions by the NADPH oxidase (85), and roles for ROS-induced phosphorylation-dependent events have also been suggested (928, 1031).

B. Swelling-Activated K^+ Channels

Activation of K^+-selective currents ($I_{K,vol}$) after cell swelling has been reported in a great variety of cell types, and a wide variety of K^+ channels have been shown to be volume sensitive (Fig. 16; see, e.g., Refs. 960, 1078, 1081). That RVD involves a swelling-activated conductive K^+ efflux pathway was first shown in lymphocytes and EAT cells (345, 861), and later in many other cell types (see Ref. 360). As discussed in section VIIA, it was originally presumed that this increase in g_K could elicit RVD in the absence of an increase in g_{Cl}, but it was later shown that K^+ and Cl^- conductive pathways are activated in parallel, resulting in an almost electroneutral KCl efflux.

It should be emphasized here that the finding that a given K^+ channel is volume sensitive in a given cell type obviously does not prove that the channel is responsible for the dominating swelling-activated K^+ current in that cell type. For instance, the intermediate-conductance K^+ channel shown to be volume sensitive in EAT cells (134), and after expression in *Xenopus* oocytes (418) only contributes insignificantly to the swelling-activated K^+ current in EAT cells, which is dominated by a TASK channel (385, 848) (see below).

Large-conductance or maxi (BK) K^+ channels exhibit a unitary conductance in the range 100–300 pS, are activated by membrane depolarization and micromolar $[Ca^{2+}]_i$, and are relatively selectively inhibited by charybdotoxin and iberiotoxin. Swelling activation of BK channels has been shown, e.g., in cells from rabbit kidney proximal tubule, thick ascending limb, and collecting duct (208, 953, 979); pig jejunal enterocytes (603); and human bronchial epithelial cells (228), pituitary tumor cells (402), and osteoblast-like cells (1090). Expression studies in oocytes indicated that BK channels were only modestly volume sensitive (305) and that the volume sensitivity seems in many cases to be secondary to an increase in $[Ca^{2+}]_i$ during cell swelling (107, 228). In eel intestinal epithelium, it was recently shown that swelling activation of BK channels was dependent on an increase in $[Ca^{2+}]_i$, but that the resulting current was larger than accounted for by the increased $[Ca^{2+}]_i$ (574), ostensibly due to BK modulation by protein phosphorylation events (107). In some cases, BK channels have been found to be directly stretch-activated (450), which in principle could also account for their swelling activation (see, however, sect. IIIA). Intermediate-conductance K^+ (IK) channels are swelling activated in many cell types, as, e.g., shown in osteosarcoma cells (1121), EAT cells (134), renal A6 cells

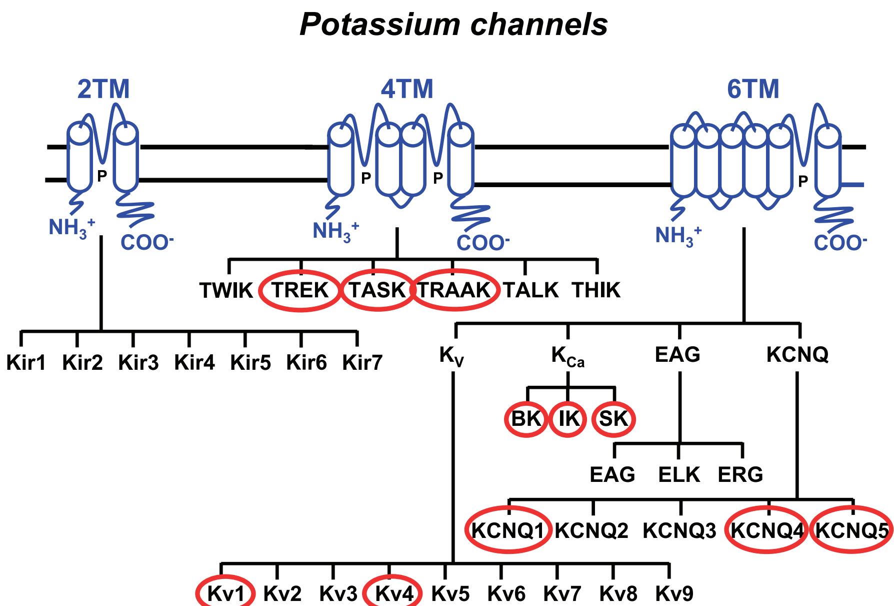

FIG. 16. Volume-sensitive K^+ channels. K^+ channels reported to be volume sensitive are marked by a red circle. See text for details.

(1017), human T lymphocytes (444), mouse proximal tubule cells (43), intestine 407 cells (1064), hepatocytes (40), and human lens epithelial cells (525). Swelling activation has also been demonstrated for cloned IK channels expressed in *Xenopus* oocytes (418). Cell-attached patch-clamp recordings from EAT cells showed concurrent swelling activation in the same patches of IK channels and Ca^{2+}-permeable, nonselective cation channels, and a role for a local increase in $[Ca^{2+}]_i$ in IK activation by swelling was suggested (134). In intestine 407 cells and in kidney A6 cells, it seems that swelling-induced release of ATP and consequent stimulation of purinoreceptors contribute to IK activation (723, 744). Finally, Ca^{2+}-activated K^+ channels of small conductance (SK) amounting to 4–18 pS are only reported to be volume sensitive in human liver cells (856) and in human cholangiocarcinoma cells (856) or expressed in *Xenopus* oocytes (418).

Voltage-dependent K^+ channels (Kv) consist of 12 subfamilies (Kv1–12) with a unitary conductance in the range 2–25 pS. In many cells, Kv channels contribute to RVD, as shown, e.g., for Kv1.3 or Kv1.5 in lymphocytes (188, 226, 547), Kv4.2 and Kv4.3 in mouse myocytes (1062), and Kv1.5 and Kv1.3 in murine spermatozoa (38). Some *KCNQ* channels also contribute to cell volume regulation. Expression of KCNQ1, KCNQ4, and KCNQ5 in *Xenopus* oocytes yields voltage-dependent K^+ currents that are very sensitive to changes in cell volume, with KCNQ5 as the most sensitive (304, 384). A role for KCNQ1 in RVD was also proposed in MCF-7 human mammary epithelial cells (1029) and in rat hepatocytes (507). A contribution of the putative KCNQ1 β-subunit KCNE1 (MinK) to RVD has been suggested in cells from the inner ear (1070), and in mouse tracheal epithelium where a KCNQ1/MinK complex was proposed to form a heterotetrameric channel (582). Finally, members of the family of two-pore-domain K^+ (K_{2P}) channels with four TM helixes (2P-4TM) are involved in volume regulation in EAT cells (711), kidney cells (42), and murine spermatozoa (38). The volume-sensitive members include the 2P-4TM, acid-sensitive K^+ channel (TASK-2/KCNK5) (42, 709–711), TREK-1/KCNK2, and TRAAK/KCNK4 (610–612, 774, 774). $I_{K,vol}$ in EAT cells has been identified as a TASK channel (711); it is resistant to most classical K^+ channel inhibitors (416, 848), blocked by clofilium (711), independent of intracellular Ca^{2+}, and lacks intrinsic voltage dependence (711). The permeability sequence is $K^+ > Rb^+ >> Cs^+$, NH_4^+, Na^+, Li^+ (710, 848), and the conductance is decreased at acidic pH and increased by alkalinization (383) in agreement with the known pH dependence of the RVD response in EAT cells (470). Expression of TASK-2 was demonstrated in EAT cells, and when expressed in HEK293 cells, TASK-2 elicits a swelling-induced current very similar to $I_{K,vol}$ in EAT cells (709), supporting the conclusion that TASK-2 is the swelling-activated channel in EAT cells.

1. *Mechanisms of activation of* $I_{K,vol}$ *by cell swelling*

A) MEMBRANE STRETCH. Membrane stretch is not a universal mechanism of activation of $I_{K,vol}$, since, e.g., in EAT cells, this channel cannot be activated directly by membrane stretch (134). However, several K^+ channels, including some BK channels (272), and some K_{2P} channels (379, 542, 610, 611, 774) are activated both directly by membrane stretch and by cell swelling, and it seems that in some cases, stretch-activated K^+ channels can account for $I_{K,vol}$ (1028).

B) CYTOSKELETON. A role for microtubules in $I_{K,vol}$ activation has been suggested in rat colonic epithelium (843). More commonly, however, the actin cytoskeleton has been implicated. In *Xenopus* oocytes expressing KCNQ1, cytochalasin D treatment inhibited the swelling-induced activation of the channel (304), and the same was found for $I_{K,vol}$ in hepatocytes (507), and for IK channels expressed in HEK cells (418). Grunnet and co-workers found that NH_2-terminal truncation of the KCNQ1 channel and treatment with cytochalasin D both abolished volume sensitivity of KCNQ1 expressed in *Xenopus* oocytes and proposed that this region is involved in the interaction of the channel with the cytoskeleton (304). The cortical actin cross-linking protein filamin was found to be required for swelling-induced K^+ channel activation and RVD in melanoma cells (111).

On the other hand, F-actin integrity is not ubiquitously required for $I_{K,vol}$: in a study in trigeminal ganglion neurons, swelling activation of voltage-gated K^+ channels was actually stimulated by cytochalasin treatment (812).

C) $[Ca^{2+}]_i$. As discussed in section V*B*, in many epithelial cells, a rise in $[Ca^{2+}]_i$ is important for the RVD response, but in many other cells, neither activation of $I_{K,vol}$ (385) nor RVD (14, 244, 416; see Ref. 360) is dependent on an increase in $[Ca^{2+}]_i$. Interestingly, even swelling activation of Ca^{2+}-activated K^+ channels in several cell types can occur independently of a detectable increase in $[Ca^{2+}]_i$ (272, 305, 418). Extracellular ATP released to the extracellular medium appears to contribute to activation of Ca^{2+}-sensitive K^+ channels after swelling in several cell types (189, 420; see also sect. V*B*). However, as noted above, in many cell types, $I_{K,vol}$ is mediated by Ca^{2+}-insensitive channels. For instance, in EAT cells, addition of ATP elicits an increase in $[Ca^{2+}]_i$ (799), yet does not activate $I_{K,vol}$ (which, as described above, is dominated by TASK-2 in these cells), but rather an IK-type channel (357).

D) PROTEIN KINASES AND PHOSPHATASES. PKC seems to be involved in modulation of $I_{K,vol}$ in many cells. Thus stimulation of PKC by DAG analogs potentiated RVD and mimicked the effect of swelling on K^+ efflux in salivary ductal cells (671) and in isolated perfused liver (507), and inhibitors of PKC attenuate RVD (507, 565). The mechanism(s) involved is unknown; cytoskeletal integrity was

reported to be required for the effect of PKC on $I_{K,vol}$ (565). PKC may also be involved in the closing of channels mediating $I_{K,vol}$, as suggested by the fact that the PKC inhibitor chelerythrine (10 μM) decreased, and the Ser/Thr phosphatase inhibitor calyculin A (100 nM) increased, the volume set point after an RVD response (358). In agreement with this, PKC is activated in the late phases of RVD in EAT cells (519). Chelerythrine may, however, not be a good inhibitor of PKC activity (173).

Activation of Src tyrosine kinases during RVD has been demonstrated in a range of cell types (sect. V*F1*), but definitive evidence linking the activation of tyrosine kinases to activation of $I_{K,vol}$ is lacking. Several K^+ channels known to be involved in RVD are also affected by tyrosine phosphorylation/dephosphorylation; however, both dephosphorylation (376, 377) and phosphorylation (570, 571) have been reported to activate the channel. In EAT cells, the relatively unspecific tyrosine kinase inhibitor genistein (370 μM) inhibited the RVD response by ~90%, and the tyrosine phosphatase inhibitor mpv (pic) (10 μM) significantly decreased the volume set point after the RVD response (Fig. 2, *A* and *B*). Thus tyrosine phosphorylation appears to play a role in RVD in EAT cells, possibly as a part of the volume-sensing mechanism. In apparent contrast, in villus epithelial cells, genistein (100 μM) had no effect on RVD (602).

E) LIPID MEDIATORS. PtdIns(4,5)P_2, the cellular level of which is decreased upon cell swelling (sect. IV*C2*), inhibits several K_{2P} channels (584). Arachidonic acid, which is released upon swelling in many cell types (sect. V*A*), directly stimulates TRAAK at micromolar concentrations (235), yet inhibits TASK-2 (711). Several of the eicosanoids resulting from arachidonic acid metabolism (see Fig. 8) play a role in activation of $I_{K,vol}$ (Fig. 11*E*) (see Ref. 358). BK channels have been shown to be activated by 3,15-di-HETE and 3-HETE (140). The 12-HPETE product hepoxylin A activated $I_{K,vol}$ in human platelets (622), but not in EAT cells (358). In EAT cells, LTD_4 is synthesized during RVD (sect. V*A*) and is involved in the activation of the RVD response by stimulating $I_{K,vol}$ (500), in a manner independent of an increase in $[Ca^{2+}]_i$ (357, 385, 416). Thus addition of low concentrations of LTD_4 (<5 nM) to EAT cells results in a K^+ efflux which, similar to $I_{K,vol}$, is ChTX insensitive, whereas higher concentrations of LTD_4 activate a ChTX-sensitive Ca^{2+}-activated K^+ efflux (357). Accordingly, 5 nM LTD_4 activates a whole cell K^+ current with a similar conductance, current-voltage relation, and pharmacological profile as $I_{K,vol}$ (385). LTD_4 binds to $CysLT_1$ receptors, a murine isoform of which has been cloned (659). Binding of LTD_4 to the receptor results in an increase in $[Ca^{2+}]_i$ in several cell types including EAT cells (80, 417, 780, 988), and consequent activation of Ca^{2+}-activated K^+ channels. LTD_4 activates pertussis toxin (PTX)-insensitive as well as -sensitive G proteins in intestinal epithelial cells, yet only the latter are involved in Ca^{2+} signaling (705). Furthermore, $I_{K,vol}$ in EAT cell is under the control of one or more G proteins (712). Hence, it is possible that PTX-insensitive G proteins are involved in stimulation of Ca^{2+}-independent $I_{K,vol}$ in EAT cells.

C. The K^+-Cl^- Cotransporters

The K^+-Cl^- cotransporters (KCCs) constitute one branch of the SLC12A family, the other branch being the shrinkage-activated NKCCs described above (sect. VI*B*). The four KCC isoforms, KCC1-4, are encoded by the genes *SLC12A4-7*, respectively (Table 5) (265, 342). The KCCs play important roles in cell volume regulation, as well as in transepithelial ion transport, vascular smooth muscle relaxation, $[Cl^-]_i$ regulation, extracellular K^+ buffering in the brain, and even pH_i regulation, due to the ability of the KCCs to carry NH_4^+ (see Refs. 265, 342). In addition to osmotic swelling, a number of other activators of KCC in various cell types have been extensively characterized, including urea, high oxygen pressure, low extracellular pH, the thiol-alkylating agent *N*-ethylmaleimide (NEM), and various stimuli regulating protein phosphatase 1, such as PDGFR activation and increases in cellular nitric oxide (NO) levels. These are not the subject of the present review, but have been described elsewhere (see Refs. 4, 5, 265, 342).

1. *Fundamental properties, localization, and pharmacology of the KCCs*

The KCCs have apparent molecular masses of 120–130 kDa and share a predicted topology of 12 TM regions and a short NH_2-terminal and long COOH-terminal cytoplasmic domains (265, 342). Bumetanide, furosemide, and other loop diuretics inhibit the KCCs, although with much lower affinities than for inhibition of the NKCCs (642, 778; see Ref. 265). KCC1 is ubiquitously expressed and seems to have cell volume regulation as its main physiological function (342). KCC2 is expressed in neurons, where its main roles seem to be the regulation of $[Cl^-]_i$ and buffering of extracellular K^+ (778; see Ref. 342). KCC3 (which exhibits the closest homology with KCC1) and KCC4 (which is most similar to KCC2) are relatively broadly distributed, and at least in kidney epithelial cells appear to localize to the basolateral membrane (642, 643, 680). These isoforms have been assigned roles in, e.g., transepithelial transport, vascular smooth muscle relaxation, and as described below, cell volume regulation (see Refs. 4, 265, 342). The contribution of KCCs to vascular disease has recently been reviewed (5). Due to alternative splicing, a short and a long isoform of KCC3, KCC3a and KCC3b, respectively, exist, with KCC3b being most abundant in the kidney (see Ref. 265).

2. Regulation of the KCCs by cell volume perturbations

A) ACTIVATION OF KCC AS A MECHANISM OF RVD. Long before the cloning of the KCCs, swelling-activated, coupled K^+, Cl^- efflux was described in RBCs as a mechanism of volume regulation after osmotic swelling (213, 342, 526), and KCl cotransport has since been shown to be a mechanism of RVD in a wide variety of cell types (309, 470; see Refs. 265, 342). All four KCCs (including both KCC3a and KCC3b) are activated by cell swelling (259, 642, 680, 828, 957), although apparently not when expressed in HEK293 cells (778), possibly reflecting the lack of a critical cofactor in the expression system. It is to our knowledge still not fully elucidated which KCC isoform(s) mediate the transport in RBCs; however, based on cation affinities and anion selectivity sequence, it is unlikely to be only KCC1 (342) and may involve KCC3 and/or a yet unidentified isoform (642).

B) MECHANISMS IN CELL VOLUME DEPENDENT REGULATION OF KCC ACTIVITY. It was demonstrated already in the early 1990s, and substantiated by a host of later studies, that Ser/Thr protein phosphatases play a major role in control of KCC activity, such that phosphatase activation stimulates, and inhibition attenuates, KCC, respectively (63, 65, 406, 407, 426, 471, 642, 767; see Ref. 265). The protein phosphatase 1/2A inhibitor calyculin A blocks the swelling activation of all four KCC isoforms (642, 643, 939), implicating one or both of these protein phosphatases in KCC regulation. Moreover, the protein phosphatase 2B inhibitor cyclosporin A was found to inhibit KCC activation by dominant negative WNK3 and WNK4 (176, 271), pointing to a possible role for this isoform in the volume-dependent regulation of KCC activity.

The specific molecular mechanisms of KCC regulation are not fully elucidated, and both two- and three-state models for KCC regulation have been proposed (212, 406). While volume-dependent regulation of the phosphatases may also play a role (64), most studies addressing the issue have been most compatible with a model in which KCC regulation by cell volume perturbation involves a volume-sensitive kinase, which is inhibited by swelling, and activated by shrinkage (406). As discussed in section V*D*, the WNKs have emerged as candidates for kinases with these properties. Similar to NKCC1 and NKCC2 (see sect. VI*B*), KCC3, yet not KCC1 and KCC4 (and, although not tested, presumably not KCC2, since this isoform lacks the minimal SPAK binding motif, see Ref. 265), was found to interact directly with SPAK via an (R/K)FX(V/I) motif in the NH_2 terminus of the cotransporter (815). Consistent with the early studies in RBCs, indicating that the shrinkage- and swelling-activated transporters are reciprocally related, such that phosphorylation is required for activation of the shrinkage-activated transporters (NHE1, NKCCs), and dephosphorylation for activation of the swelling-activated KCCs (406, 407, 767), the effects of the WNKs on the NKCCs and KCCs tend to be opposite. Thus, whereas the NKCCs are activated by active WNKs (although in the case of WNK4, this requires the additional presence of SPAK; see below and sect. VI*B*), the KCCs are inhibited. This has been convincingly shown for all four KCC isforms after coexpression with WNK3 (176) or WNK4 (271) in *Xenopus* oocytes. The inhibitory effects of WNK3 and WNK4 on the KCCs were found not to be dependent on coexpression with SPAK (176, 271). Nonetheless, at least for KCC2 and KCC3, there does appear to be an interaction between WNK4 and SPAK in cotransporter regulation (259, 271). Interestingly, catalytically inactive WNK3 activated all four KCC isoforms under isotonic conditions (176). In contrast, catalytically inactive WNK4 activated KCC2 and KCC3, yet not KCC1 and KCC4, under isotonic conditions (271), substantiating the notion that the WNKs do not regulate all KCCs by fully identical mechanisms. The activating effect of catalytically inactive WNK3 and WNK4 on KCC activity could be counteracted by Ser/Thr protein phosphatase inhibitors, leading to the suggestion that the WNKs may act as regulators of a KCC-regulatory phosphatase activity (176, 271).

While there is, thus, substantial evidence for regulation of KCCs by protein phosphorylation/dephosphorylation events, the molecular mechanism is not as well understood as for NKCC1 (see sect. VI*B*). Consensus sites for phosphorylation by both Ser/Thr and tyrosine kinases are found in the COOH-terminal domain of the KCCs (1142; see Ref. 265). However, direct phosphorylation/dephosphorylation of KCCs after cell volume perturbations has, to our knowledge, yet to be demonstrated, although such changes were recently demonstrated for KCC2 after other stimuli (1058, 1095). Findings by Strange et al. (957) were inconsistent with a role for tyrosine phosphorylation in KCC2 regulation, although a COOH-terminal tyrosine residue was found to be important for the function of the cotransporter, possibly due to a role in protein conformation or protein-protein interactions (957). Finally, in light of the fact that the MLCK inhibitor ML-7 has an inhibitory effect on the shrinkage-activated NKCC1 (sect. VI*B*), it may be noteworthy that ML-7 was found to stimulate KCC activity in mammalian RBCs (438).

D. Organic Osmolyte Efflux Pathways: Taurine Release

The role of taurine in cell volume regulation was first acknowledged in invertebrates (278) and later also in vertebrates including mammals (252, 361). Taurine release from, e.g., NIH3T3 cells is not only increased by osmotic swelling, but also decreased by osmotic shrinkage, favoring preservation of cellular osmolyte content and thereby cell volume after cell shrinkage (800).

1. *Molecular identity of the taurine efflux pathway*

The Na^+-dependent taurine transporter TauT (sect. VI*C*) seems to contribute to taurine release under isotonic conditions (498), during ischemia (885), and following depolarization of the membrane (36), whereas its contribution to the swelling-induced taurine and thus restoration of the cell volume appears limited. Furthermore, the volume-sensitive taurine efflux pathway does not respond to diet-induced adaptive regulation as does TauT, supporting the notion that the swelling-induced taurine efflux pathway is different from TauT (1052). The swelling-induced taurine efflux increases exponentially with reduction of extracellular tonicity (364, 492, 772, 786, 868), and it has been estimated that a 20% decrease in the osmolarity is required for activation of taurine efflux in, e.g., NIH3T3 cells (786). The volume-sensitive taurine efflux pathway has the properties of a diffusion pathway permeable to various organic osmolytes (taurine > sorbitol > choline > thymidine >> sucrose) (316) and is sensitive to an array of inhibitor compounds (DIDS, 5-nitro-2-(3-phenylpropylamino)-benzoic acid, 1,9-dideoxyforskolin, tamoxifen, polyunsaturated fatty acids) (see Table 6 and Refs. 452, 493). The molecular identity of the pathway is not known. I_{Cln} is permeable to taurine (947) but is more likely to function as a regulator of diverse cellular functions, e.g., ion permeation, cytoskeletal organization, and RNA processing (254) than as a taurine efflux pathway (see also sect. VII*A*). *Xenopus* oocytes expressing ClC-3 exhibit volume-sensitive taurine efflux (947), but the role of ClC-3 in cell volume regulation is questionable (see sect. VII*A*). Phospholemman has a high preference for taurine compared with Cl^- (667) and is still considered as a candidate for the swelling-induced taurine efflux pathway, since its overexpression potentiates taurine release (668, 669), whereas a reduction in the expression reduces the taurine release (673). The band 3 anion exchanger (AE1 isoform) has also been reported to contribute to the taurine permeability (232, 625, 779), and it has been demonstrated that AE1 in RBCs from the little skate (*Raja erinacea*) conducts taurine and moves from an intracellular association with lipid rafts into the plasma membrane in a process that involves protein tyrosine phosphorylation (779). On the other hand, the anion exchanger is not involved in the swelling-induced taurine release from EAT cells (498).

TABLE 6. *Differences between VRAC/$I_{Cl,vol}$ and the volume-sensitive taurine channel in EAT cells*

	VRAC/$I_{Cl,vol}$	Volume-Sensitive Taurine Channel
Pharmacological profile		
Arachidonic acid (10–50 μM)	Strong inhibition	Stimulation
DIDS (300 μM)	Very weak inhibition	Strong inhibition
Tamoxifen (10 μM)	Strong inhibition	Very weak inhibition
Niflumic acid (100 μM)	No effect	Strong inhibition
Activators		
LTD_4 (5 nM)	No effect	Activation

VRAC, volume-regulated anion channel; $I_{Cl,vol}$, swelling-activated Cl^- current. Table is modified from Hoffmann (358). For details, see section VII*D1*.

A) IS VRAC THE SWELLING-ACTIVATED TAURINE CHANNEL? The possible role for VRAC (sect. VII*A*) as a mediator of swelling-induced taurine efflux is a contentious issue (see Refs. 34, 715, 955). In our view, conclusive evidence for a significant role for VRAC in taurine release is still lacking. A significant permeability of VRAC to large organic anions and zwitterions, such as taurine, gluconate, and aspartate, has been demonstrated in whole cell patch-clamp experiments (716, 955). On the other hand, in EAT cells, exogenous addition of a low dose LTD_4 (5 nM), that activates the swelling-induced taurine efflux pathway (Fig. 9, *D* and *E*), fails to activate a Cl^- conductance (358, 491). Moreover, the swelling-induced taurine and Cl^- efflux pathways exhibit different pharmacological profiles (Table 6). This indicates that the great majority of the taurine and Cl^- efflux are mediated by separate channels and that the taurine channel is different from VRAC. Further support for this view is that: *1*) swelling-induced taurine release in the absence of Cl^- channel activity has been demonstrated in *Xenopus* oocytes (947) and in mammary tissue explants (924); *2*) the taurine and anion efflux pathways differ with respect to their time course of activity following hypotonic exposure (672, 773, 961), *3*) the two pathways have different sensitivity towards DIDS and arachidonic acid (192, 499, 672) (for EAT cells see Table 6), and *4*) regulation by RhoA (786). It is noted that, in fact, the taurine leak pathway accounts for 5% of the total g_{Cl} in EAT cells (50% reduction in osmolarity; Ref. 499) and for a larger fraction of the g_K in rat hepatoma cells (421).

2. *Mechanisms of activation of the taurine efflux pathway*

Activation of PLA_2 and oxidation of arachidonic acid via the 5-LO system are permissive elements for volume-sensitive taurine loss in, e.g., EAT cells, HeLa cells, C2C12 myotubes, and NIH3T3 cells (492, 498, 502, 504; see also sect. V*A*). As will be described in this section, swelling-induced taurine release is modulated by lipid mediators, Ca^{2+}/CaM, protein kinases/phosphatases, and ROS. Lysophospholipids are produced, e.g., as a consequence of PLA_2 activity (Fig. 8). The lysophospholipid LPC in micromolar concentrations induces taurine loss under isotonic conditions, but taurine loss induced by LPC and osmotic swelling differ with respect to sensitivity towards serum starvation, and exposure to cholesterol, 5-LO inhibitors, and chan-

nel blockers (495, 496, 502, 504, 753). As the LPC concentration after osmotic swelling furthermore is less than that required for activation of taurine loss (495), it is assumed that LPC does not act as a second messenger in activation of taurine loss following osmotic swelling. On the other hand, nanomolar concentrations of the Ca^{2+}-mobilizing lysophospholipid LPA (see sect. v*B*) potentiate ROS production and concomitant taurine release in NIH3T3 cells under hypotonic conditions (250). In some cell types, activation of swelling-induced taurine efflux requires extracellular Ca^{2+} (514, 1036), whereas in others, it occurs (674, 733), or is even improved (435, 498), in the absence of extracellular Ca^{2+} (see also sect. v*B*). Addition of Ca^{2+}-mobilizing agonists does not elicit taurine release under isotonic conditions but potentiates the swelling-induced taurine efflux from a variety of cells presumably via pathways involving CaM and CaMKII, PI3K, and PKC (112, 222, 451, 551, 661). In contrast, a concentration (3 nM) of LTD_4 too low to elicit detectable Ca^{2+} mobilization induces taurine release under isotonic conditions (see Fig. 9). LTD_4 has, accordingly, been assigned a role as a second messenger in the activation of taurine efflux during RVD in EAT cells. Swelling-induced osmolyte transport is impaired in the presence of ROS scavengers or inhibitors of the NOX system (492, 494, 1031). ROS, by analogy with Ca^{2+}, only potentiate taurine efflux under hypotonic conditions, and their effect has often been linked to inhibition of protein tyrosine phosphatase activity (492, 494). Interestingly, the increased ROS production following hypotonic exposure in, e.g., NIH3T3 cells appears to both potentiate the signaling cascade leading to the activation of the efflux pathway, and delay the inactivation of the pathway (494). A role for tyrosine phosphorylation events in modulation of swelling-induced taurine release is suggested by the inhibitory effect of tyrosine kinase blockers (genistein, tyrphostins) and potentiating effect of tyrosine phosphatase inhibitors on the efflux (492, 662, 670, 920). Pharmacological evidence indicates that the tyrosine kinases regulating the taurine efflux pathway include receptor tyrosine kinases (247, 492), FAK, and members of the Src family (391, 492).

VIII. ADAPTION TO LONG-TERM CELL VOLUME PERTURBATIONS

Long-term exposure to hypertonic conditions results in altered transcription of a number of osmoregulatory genes, most of which are involved in uptake and synthesis of compatible (nonionic) osmolytes including methylamines (e.g., betaine), polyalcohols (e.g., sorbitol and inositol), and amino acids and their derivatives (97). Such organic osmolytes are particularly important in the renal medulla, where extracellular osmolarity can reach 4–5 times that of isotonicity. Whereas ions have a destabilizing effect on macromolecules, organic osmolytes have a stabilizing effect (1127). In renal medullary cells, the five major osmolytes are sorbitol, *myo*-inositol, betaine, taurine, and glycerophosphocholine (GPC) (27, 691, 1010; for excellent reviews, see Refs. 96, 268). In almost every case, transcription of the osmoregulatory genes involves the transcription factor TonEBP, which will thus be described first.

A. Tonicity-Responsive Enhancer Binding Protein (TonEBP/OREBP/NFAT5)

Tonicity-responsive enhancer binding protein (TonEBP/OREBP/NFAT5) is a transcription factor that binds to certain genes involved in the osmoregulatory response (for an excellent review, see Ref. 229). TonEBP plays a very important role in hypertonicity-induced transcription (656, 845). TonEBP was identified simultaneously by several groups and is accordingly referred to as either TonEBP, OREBP, or NFAT5 (463, 656, 758, 1002). TonEBP is a 200-kDa protein that binds to DNA as a preformed homodimer, forming a ring around DNA where Arg^{217}, Arg^{226}, Glu^{223}, Tyr^{220}, and Gln^{364} mediate the sequence-specific DNA binding (959). Each TonEBP target gene contains a DNA consensus motif called tonicity responsive enhancer (TonE) (see Ref. 229). The affinity of TonEBP for the TonE sites is low, with a K_D around 50 nM. TonEBP seems to be part of a large complex including hsp90 and poly(ADP ribose) polymerase (PARP-1) (127). TonEBP is activated after increases in external osmolarity by upregulation at the mRNA level, and, conversely, is downregulated under hypotonic conditions or after accumulation of organic osmolytes (103, 1102). We recently demonstrated that TonEBP was strongly activated after 4 and 16 h in hypertonic medium, but not after isotonic shrinkage, indicating that increased cellular ionic strength rather than cell shrinkage per se activates TonEBP (853). TonEBP is also phosphorylated during hypertonic incubation (166, 586), but the role of the phosphorylation, as well as the kinases involved, remains to be fully elucidated. Phosphorylation of TonEBP is not necessary for its binding to TonE, but seems to be important for its nuclear localization (166; see Ref. 96). Of particular interest is the PI3K-related kinase ATM (ataxia telangiectasia mutated), a Ser/Thr protein kinase activated at strong hypertonicity as a consequence of changes in chromatin structure (31). In NIH3T3 fibroblasts, early-stage PCD was associated with DNA-damage-independent ATM phosphorylation paralleled by chromatin decondensation (901). ATM kinase is necessary for full activation of TonEBP, but it is not clear whether ATM phosphorylates TonEBP (397). It seems that ATM is involved in the trans-

location of TonEBP to the nucleus during hypertonic stress (1144). Furthermore, several studies have shown that p38 MAPK, which is potently activated by hypertonic shrinkage (sect. VC), plays an important role in TonEBP activation (461, 688). Ko et al. (461) found that coactivation of p38 MAPK and the tyrosine kinase Fyn, which is also activated by hypertonic shrinkage, activates TonEBP. Also of note, the volume-sensitive FER/Fyn tyrosine kinase pathway, which, as noted in section IVD, leads to cortactin phosphorylation in shrunken cells, was proposed to be a major pathway for regulation TonEBP (461). A relation between small G proteins and TonEBP activation is also possible. Pointing to such a scheme, a TonEBP-like protein in *Drosophila* shows strong interaction with Ras (387), and NIH3T3 cells expressing constitutively active Ras exhibit increased basal TonEBP activity and an augmented response of TonEBP to osmotic stress (K. B. Schou, J. D. Ferraris, E. K. Hoffmann, and M. B. Burg, unpublished data). In agreement with this, it was recently reported that kinases activated downstream of Ras, such as ERK1/2, also regulate the activity of TonEBP (1007).

B. Organic Osmolytes: Transport and Synthesis

Accumulation of organic osmolytes after long-term cell shrinkage is dependent on increased expression of the Na^+/*myo*-inositol cotransporter (SMIT), the Na^+/Cl^-/betaine cotransporter (BGT), and TauT (sect. VIC), as well as of aldose reductase (AR), which catalyzes the conversion of glucose to sorbitol. The transcriptional regulation of these proteins was shown by Miyakawa and co-workers (656, 657) to be facilitated by TonE/TonEBP. $TonEBP^{-/-}$ mice exhibit severe degeneration of the renal medulla, caused by low levels of SMIT, AR, and TauT expression (585). Furthermore, TonEBP activation leads to increased expression of HSP70 (sect. VIIIC), which protects cells from urea-induced damage (701, 1103). Below, we briefly review pertinent studies regarding regulation of these proteins in osmotically perturbed cells.

1. SMIT

The important organic osmolyte *myo*-inositol is accumulated by the the Na^+-coupled *myo*-inositol transport system SMIT, which is located in the basolateral membrane of renal medulla cells (485). Hypertonic salt stress, but not urea stress, results in increased transcription of the gene coding for SMIT and increased uptake of *myo*-inositol in the kidney cells (485, 692, 1123) and liver cells (1075). There are five TonE sites on the SMIT gene, and their deletion results in a dramatic decrease in SMIT-mediated transport during hypertonic stress (845).

2. BGT1

The Na^+-coupled transporter BGT1 can accumulate betaine 1,000-fold in medulla cells (268). Hypertonic stress, but not urea stress, stimulates the transcription of the BGT1 gene, the amount of mRNA for BGT1, and the uptake of betaine in kidney (1011) and liver (1138) cells. There are two TonE-sites upstream of the BGT1 gene, both of which are essential for the increased expression during hypertonic treatment (657). BGT1 is regulated not only at the transcriptional level but also by increased insertion in the plasma membrane (439).

3. TauT

The acute regulation of TauT by hyperosmolarity was described in section VID. TauT is, however, also subject to transcriptional regulation after long-term shrinkage. Han and co-workers (324, 326) have characterized the promoter region of the TauT gene from rat kidney and demonstrated that the tumor suppressor p53 represses TauT expression, whereas the protooncogenes WT1, c-Jun, and c-*myc* transactivate TauT expression (see Ref. 326). Long-term hypertonic exposure increases the synthesis of TauT mRNA as well as uptake of taurine uptake (882, 927, 1009), in a signaling process that involves phosphorylation and translocation of TonEBP (166, 657, 688, 1102). The TauT gene is also a target for adaptive regulation by dietary taurine availability. Exposing mammalian cells to high extracellular taurine concentration results in a reduction of TauT mRNA and protein levels as well as of taurine uptake (62, 325, 927, 1052). Mice overexpressing p53 exhibit renal hypoplasia and renal insufficiency, and taurine-deficient kittens suffer from renal developmental abnormalities, and it has been proposed that TauT is the target coupling p53 to renal development and PCD (see 324).

4. Enzymes involved in sorbitol and betaine synthesis

Sorbitol is a polyol synthesized from glucose by AR. Hypertonic stress results in increased AR activity (28) and increased transcription of AR mRNA (269, 303, 937). TonEBP also regulates AR: the gene for AR contains several TonE sites (230, 231, 867) without which sorbitol accumulation during hypertonic treatment is dramatically decreased (462). Betaine is metabolized by the betaine homocysteine *S*-methyltransferase (BHMT; Refs. 97, 1094). Expression of *Bhmt* mRNA, BHMT protein, and activity in H4IIE rat hepatoma cells are suppressed under hypertonic and upregulated under hypotonic conditions, in a manner dependent on cell shrinkage rather than on increased ionic strength and osmolarity (891). Furthermore, the osmosensitivity of Bhmt mRNA expression appeared to involve tyrosine kinases and cyclic nucleotide-dependent kinases (891). Increased metabolism of organic osmolytes under

hypotonic conditions has previously been demonstrated in EAT cells (497), further supporting the notion that altered elimination of betaine could be a part of the volume regulatory response.

C. Other Proteins Regulated at the Transcriptional Level After Cell Volume Perturbations

In a recent review, Burg et al. (96) list ~200 proteins, the expression of which is altered with increased osmolarity. In addition to the above-mentioned osmolyte transporters (sect. VIII*B*), transporters and channels reported to be regulated in this manner include several of the aquaporins, NKCC1, ROMK channels, NHE1 and -2, and the Na^+-K^+-ATPase. Microarray screening has also been applied, showing that ~12,000 transcripts are downregulated, and 4,000 upregulated (990, 991), and the expression of tonicity-sensitive proteins has been studied by proteomics approaches (1021). The expression of the two-pore K^+ channel TASK-2, which is important in RVD in EAT cells (see Ref. 960 and sect. VII*B*) is 1.7 times upregulated in hypertonic medium and 2.7 times downregulated in a hypotonic medium, which correlates with measurements of the maximal swelling-activated TASK-2 current in the anisotonicity-adapted cells (T. Wulff, H. Eriksen, T. Litman, and E. K. Hoffmann, unpublished data). In liver cells, hyperosmolarity increased the expression of, e.g., tyrosine aminotransferase (1074), the Ser/Thr kinase SGK1 (1059), the glycine transporter Glyt, the multispecific organic osmolyte transporter Oatp1, as well as the insulin growth factor binding protein (IGFP) (891).

1. *Heat shock proteins*

Heat shock proteins are a group of highly conserved proteins induced by cellular stresses such as, e.g., heat, anoxia, ultraviolet light, and osmotic stress. A large number of heat shock proteins have been identified and classified according to their apparent molecular mass, e.g., as heat shock protein 70 (Hsp70), etc (23). High NaCl increases expression of a variety of heat shock proteins in renal medullary cells, and this in turn protects the cell from the damaging effect of high salt and urea (see Refs. 50, 72). While many types of stress induce both the hsp70.1 and the hsp70.3 gene in murine cells, hypertonic stress appears to induce hsp70.1 selectively, in a manner dependent on TonEs in the 5′-flanking region (534, 1102). The human genome contains two inducible hsp70 genes, hsp70-1 and hsp70-2, which are counterparts of murine hsp70.3 and hsp70.1, respectively. For these proteins, it was reported that only hsp70-2, which contains three TonE sites, is induced by hypertonic stress (102, 347). Finally, embryonic fibroblasts from hsp $70.1^{-/-}$ mice exhibited strongly reduced tolerance to high salt stress compared with wild-type controls (926). A linear increase in osmolarity increases Hsp70 expression more than a step increase (102), correlating with better survival after hyperosmotic stress (102). On the other hand, although increased Hsp70 expression strongly protected WEHI cells and murine embryonic fibroblasts from shrinkage-induced PCD, it did not improve volume regulation after acute hypertonic stress (732), arguing against a role for Hsp70 in the acute regulation of the shrinkage-activated transporters (NHE1 and NKCC1 in these cells).

IX. CELL VOLUME AS A SIGNAL

As noted in section II*B*, a cell does not have one given "volume set point"; rather, cell volume is modulated in response to specific stimuli and conditions. These changes in cell volume in turn serve as signals initiating or modulating a wide variety of downstream responses, including, but far from limited to, proliferation, migration, cell death, transepithelial transport, pathogen-host interactions, and hormone and transmitter release. An overview of these processes is provided in Figure 17. Below, we consider four selected examples: the involvement of cell volume in control of transepithelial transport, cell migration, PCD, and cell proliferation.

A. Transepithelial Transport

Epithelial transport represents a continuous challenge to epithelial cell volume regulation, because slight changes in the large apical or basolateral fluxes associated with transport will lead to rapid changes in cell

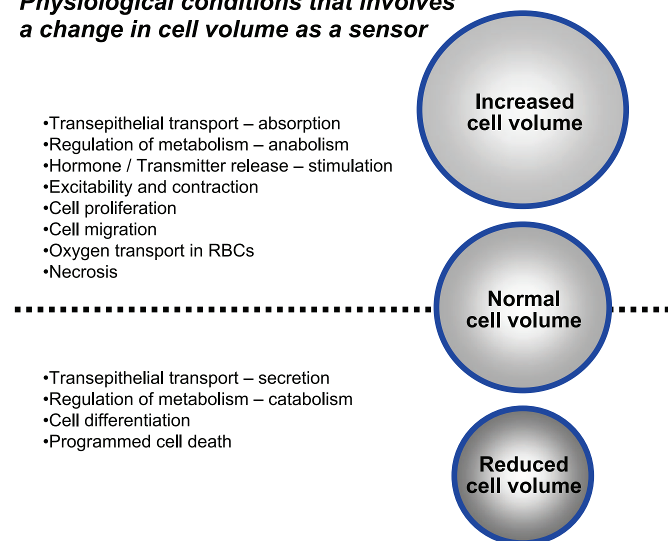

FIG. 17. Examples of physiological conditions in which cell volume changes play a major role in the sensing and signalling events.

volume (524, 605). Normally, the volume regulatory mechanisms will, as described in the first part of this review, maintain cell volume within a narrow range. However, as will be discussed below, it seems that epithelial volume is not always precisely regulated and that changes in epithelial cell volume (water content) probably are an integral part of the mechanisms of epithelial absorption (see Refs. 510, 903), secretion (242, 290, 362, 374, 811), and isosmotic transport (see Ref. 523).

1. Importance of cell swelling for epithelial absorption

In several epithelia, luminal Na^+-coupled uptake of glucose and amino acids elicits cell swelling, leading to activation of basolateral K^+ channels (e.g., Refs. 515, 903, 1028; see Ref. 157), which in turn maintain the electrical driving force for the uptake process (158). However, the role of cell volume in this process is a controversial issue, and other parameters, such as pH_i and $[Ca^{2+}]_i$, could also be responsible for this "cross-talk" between apical and basolateral membranes (330).

2. Importance of cell shrinkage for secretion

The first step in stimulation of exocrine glands is opening of K^+ and Cl^- channels followed by increased NKCC1 (244, 289, 291, 374, 810) and NHE1 (616) activity. NKCC1 activation after stimulation of secretion appears to be dependent both on cell shrinkage and on reduced $[Cl^-]_i$. Cell shrinkage has been shown to occur, at least transiently, during stimulated secretion in secretory epithelia (241, 244, 697) and in colonic secretory cells (615). However, there is also strong evidence that reduced $[Cl]_i$, rather than cell shrinkage, is the major stimulus for basolateral NKCC activation in secretagogue-treated secretory epithelia (see Refs. 312, 600). In contrast, stimulation of Cl^- transport in killifish opercular epithelium by β-adrenergic agonists such as isoproterenol (10^{-5} M) is abolished in weakly hypotonic medium (362). Under these conditions, the cytosolic Cl^- activity drops, but the cells do not shrink, suggesting that a certain degree of shrinkage is necessary to activate NKCC1. Similarly, in perfused shark rectal gland, the primary process triggering NKCC activation was found to be transient cell shrinkage (290).

3. Isosmotic transport

The term *isosmotic transport* refers to the conditions when net fluid uptake takes place at transepithelial osmotic equilibrium, i.e., with no external driving force for water, and with the transported fluid being in osmotic equilibrium with the external solution. This raises the question of how the epithelium accomplishes to keep the transported fluid isosmotic with the external solutions. The simple and ingenious idea first suggested by Ussing (1019) is that the regulated "recycling" of the actively pumped ion (Na^+) controls the tonicity of the transported fluid, a theory later described as the "Na^+ recirculation theory" (520, 523, 699). It is tempting to suggest that this is a situation in which changes in cell volume could play an important physiological role. Hoffmann and Ussing (374) presented the hypothesis that the secretion is kept isotonic because the recirculation of Na^+ is regulated by the osmolarity of the secretion via its effect on the cell volume, and thus on volume-sensitive Na^+ transporters and channels. In other words, the epithelial cells are osmotic sensors, and their swelling and shrinkage in response to hypo- and hypertonic challenges, respectively, constitute the initial signals for adjusting ion recirculation. If the secreted fluid becomes too hypotonic, the cells swell and less Na^+ is recycled. Thus more NaCl is secreted, increasing the tonicity of the secreted fluid. If the secreted fluid becomes too hypertonic, the cell shrinks, shrinkage activated Na^+ transport is activated, and a greater fraction of the Na^+ recirculates. As a consequence, less NaCl is excreted, decreasing the tonicity of the transported fluid. Patch-clamp studies on the apical membrane of exocrine frog skin glands verified the coexpression of a CFTR Cl^- channel with a maxi K^+ channel and a small 5-pS highly selective Na^+ channel, all of which were activated during secretion. The function of the Na^+ channel is as yet unknown, but it would be a likely candidate as recirculation pathway (940, 941). For elegant descriptions of the Na^+ recirculation theory applied to absorbing epithelia, see References 521–523.

4. Osmotic regulation of salt transport

Another important role of volume changes and of volume-sensitive transporters and channels is found in the osmotic regulation of salt transport in salt-absorbing or salt-excreting epithelia that experience large changes in extracellular osmolarity, and have to adapt the Cl^- transport accordingly. Well-studied examples are the intestine of the European eel (an absorptive epithelium of the type described in the renal cortex thick ascending limb, cTAL) and the killifish opercular epithelium (a Cl^- secreting epithelium of the type described in exocrine glands). In the eel intestinal epithelium, transepithelial Cl^- absorption is sustained by the operation of an apical NKCC2 in series with a basolateral g_{Cl} and in parallel with an apical g_K. The Na^+-K^+-ATPase on the basolateral membrane provides the driving force for NKCC2, a model similar to that proposed for the mammalian cTAL. It has been shown that swelling-activated K^+ and anion-conductive pathways are important in the response to hypotonicity in the eel intestine (572), and that conversely, shrinkage activation of NKCC2 plays a central role in the response to hypertonicity (573; see Refs. 368, 575; see also sect. VIB). Moreover, pharmacological evidence indicates that apical and basolateral BK channels also play impor-

tant roles in the RVD response of the eel intestinal epithelium (574). In the killifish opercular epithelium, osmotic control of Cl^- secretion across the operculum epithelium was proposed, albeit based on colocalization and pharmacological evidence only, to involve the following events: *1*) hyperosmotic activation of NKCC1 on the basolateral membrane via PKC, MLCK (362), p38 MAPK, OSR1, and SPAK (624); *2*) deactivation of NKCC by hypotonic cell swelling and a protein phosphatase (624); and *3*) a protein tyrosine kinase acting on FAK, which seems to have an important, not yet defined function in regulating NKCC activity (624).

B. Cell Migration and Invasion

Osmotic stress has a major impact on the migratory properties of a range of cell types. Neutrophil migration is strongly inhibited by hypertonic stress (851, 858), and cell swelling has been shown to be necessary for fMLP-mediated neutrophil migration (849). In transformed renal epithelial cells, both hypo- and hypertonicity inhibited migration (907, 908). Furthermore, cell adhesion, which plays a central role in migratory capacity (see, e.g., Ref. 53), also appears to be modulated by volume-dependent events. Thus hypertonic cell shrinkage inhibited tumor cell-endothelial cell adhesion (925), and inhibition of NHE1 further reduced adhesion in osmotically shrunken cells (987). As described below, there is evidence to suggest that the integrated activity of several volume-sensitive transporters and channels is pivotal to control of cell migration, acting in concert with cytoskeletal reorganization (e.g., 844).

1. Volume-sensitive transport proteins involved in cell migration

The evidence for the involvement of specific volume-regulatory ion transporters and channels in cell migration has been reviewed elsewhere (see Refs. 401, 637, 904–906) and is summarized in Figure 18. The shrinkage-activated Na^+/H^+ exchanger, NHE1 (sect. VI*A*), is located in the leading edge of migrating cells and has been shown to play a major role in migration and chemotaxis in a wide range of transformed and nontransformed cell types (129, 184, 487, 841, 950, 951, 963). Although too extensive a subject to cover here, a major pathophysiological consequence is that the upregulation of NHE1 in tumor cells contributes importantly to the invasive/metastatic capacity of these cells (for a review, see Ref. 114).

Activation of the swelling-activated Cl^- current, VRAC (sect. VII*A*), has also been demonstrated to facilitate cell migration (619, 834, 942). Notably, the increased migratory capacity in H-Ras-transformed NIH3T3 fibroblasts was found to reflect an increased volume sensitivity of VRAC (898). Thus small increases in cell volume were

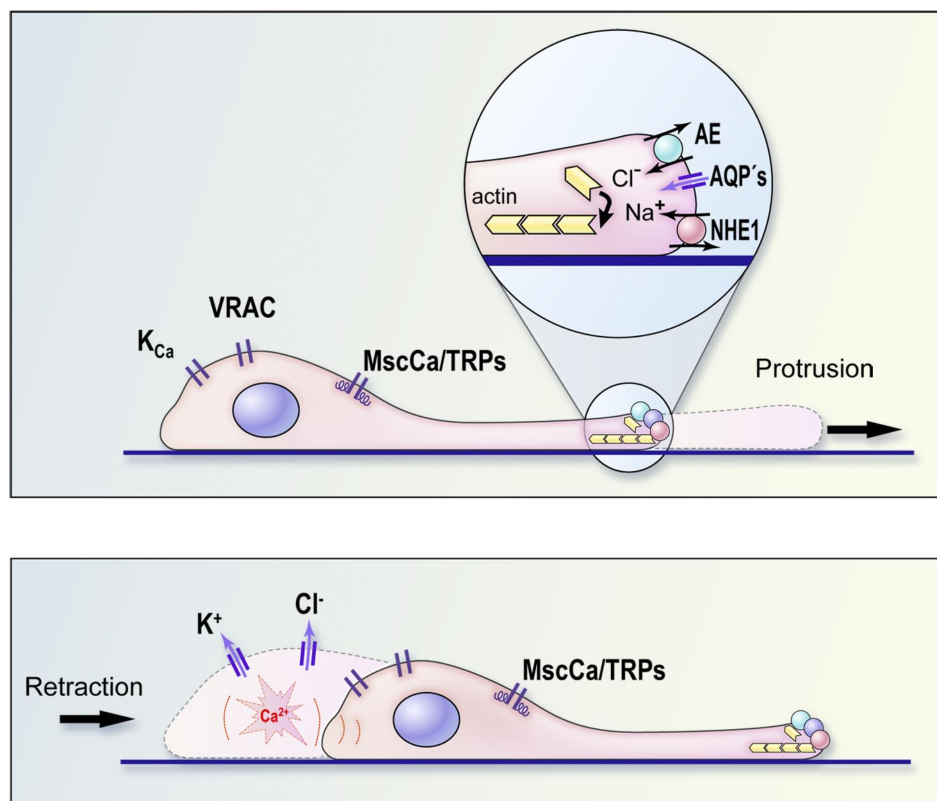

FIG. 18. Proposed model for the involvement of ion transport proteins in cell migration. See section IX*B* for details. [Model based on Schneider et al. (898), Schwab (904), and Schwab et al. (906).]

associated with severalfold greater VRAC activity in H-Ras-transformed fibroblasts compared with wild-type fibroblasts, and the Cl^- channel inhibitor NS3728, which potently blocks VRAC activity (344, 456), inhibited migration much more strongly in H-Ras-transformed cells compared with wild-type cells (898).

IK channels, which are also activated by cell swelling (sect. VII*B)*, have been shown to be important players in regulation of migration in a variety of cell types (907, 908), as have several other types of Ca^{2+}-activated K^+ channels (see Refs. 637, 906). TRPV1, and to a lesser extent TRPV4, both of which are implicated in osmosensing (see sect. IV*B*), were recently shown to contribute to the migratory properties of HepG2 cells (1071), and other TRP channels have also been shown to modulate cell migration (see Ref. 906). Finally, several aquaporins have been assigned a role in cell migration, ostensibly by facilitating osmotic water flow in the membrane protrusions mediating forward movement (386, 763).

2. *Mechanisms of activation and effect of volume-sensitive transport proteins in migration*

These and similar findings led Schwab and co-workers to formulate the general hypothesis illustrated in Figure 18: that shrinkage-activated transporters are found at the leading edge, and swelling-activated channels at the lagging edge of migrating cells, and that the resulting cell swelling at the leading edge and shrinkage at the lagging edge facilitate protrusion and retraction, respectively (see Refs. 904–906). The intracellular "valve" restricting volume changes either to the front or to the rear part of migrating cells could be represented by the poroelastic properties of the cytosol (120). Many migrating cells exhibit a $[Ca^{2+}]_i$ gradient from the lamellipodium to the rear part of the cell, such that $[Ca^{2+}]_i$ is consistently highest at the rear end of the cell (e.g., Ref. 89). TRPV1-mediated Ca^{2+} influx may contribute to setting up such a $[Ca^{2+}]_i$ gradient (1071), although it remains to be directly demonstrated whether the subcellular localization of TRPV1 in migrating cells is in accordance with this view. The activation of the TRP channels in migrating cells could involve both mechano- and osmosensitivity of these channels, and to our knowledge, this issue has yet to be directly addressed. In addition to favoring actin depolymerization, the higher $[Ca^{2+}]_i$ in the rear part of the cell is thought to increase the activity of Ca^{2+}-activated Cl^- and K^+ channels in this end of the cell, leading to shrinkage and hence facilitating rear end retraction. Consistent with this notion, IK channel activity (yet not localization) in migrating cells restricts RVD to the rear end of the cell (900, 909). In contrast, activation of NHE1 at the leading edge mediates net osmolyte influx, and likely contributes to the formation of a local osmotic gradient which in turn triggers local aquaporin-mediated water influx (see Refs. 904–906). The mechanisms involved in the activation of NHE1 at the leading edge have been addressed in detail in cancer cells, in which it was shown to involve a cAMP-PKA-Rho-ROCK-p38 MAPK pathway of NHE1 activation specifically induced by the tumor cell environment (113, 841).

Importantly, while it is clear that cell volume perturbations affect migration, and that the above-mentioned volume regulatory transporters and channels play important roles in migration, it does not necessarily follow that the role of these transporters in migration is only changes in cell volume. For instance, there is strong evidence that NHE1-mediated changes in extracellular pH may be crucial for its role in cell migration/invasion (950, 951, 963), and NHE1-mediated cytoskeletal reorganization may also play a role, either through NHE1 interaction with ERM proteins (129, 184), or, although this has yet to be directly demonstrated, through effects on strongly pH_i-dependent cytoskeletal regulators such as cofilin (945; see also sect. IV*D*). NHE1 may be of particular importance for directional/stimulated migration. Thus *1*) NHE1 activity was required for fMLP-mediated, yet not for basal, neutrophil migration (849); *2*) in *Dictyostelium*, a developmentally regulated NHE1 homolog was required for chemotaxis (776); and *3*) we recently demonstrated a pivotal role for NHE1 in directional migration after activation of the PDGF receptor α (PDGFRα) in the primary cilium, leading us to propose a model in which the cilium acts as a "cellular GPS," signaling to NHE1 at the leading edge, which in turn stimulates directional migration (899).

C. Programmed Cell Death

One of the morphological hallmarks of programmed cell death (PCD), a subset of which is known as apoptosis, is a marked cell shrinkage (440), later denoted "apoptotic volume decrease," or AVD (606). AVD is an isosmotic cell shrinkage (77) which is seen early after apoptotic stimuli and seems to be a prerequisite for PCD (78, 606, 744). AVD results from a loss of KCl via K^+ and Cl^- channels, and concomitant loss of water (74, 511, 742–744). During AVD, RVI is inhibited by yet not fully resolved mechanisms (742). VRAC seems to be the anion channel involved in AVD in HeLa cells (928), human endothelial ECV304 cells (821), rabbit ventricular myocytes (702), and mouse ventricular myocytes (745). Accordingly, the PCD-inducing agent cisplatin activated VRAC in human epidermis cancer cells (398). Various K^+ channels appear to be involved in AVD, depending on the cell type or stimulus used (see Ref. 77). Among these are the two-pore K^+ channels that have been implicated in RVD in multiple cell types (sect. VII*B*; see also Refs. 774, 1003). Inhibitors of swelling-activated K^+ and Cl^- channels attenuate AVD, and several groups have suggested that the

same channels are involved in RVD and in AVD (218, 607, 743). Moreover, apoptotic cells exhibit an augmented RVD response that could reflect that volume-sensitive channels are more sensitive to cell swelling (606, 744). Whether the sensor and trigger mechanism for RVD and AVD have identical components, only with a different set point, is still under discussion (742). In most cells (with RBCs and muscle cells as exceptions), g_{Cl} is significantly lower than g_K under steady-state. Consequently, an increase in g_K alone results in K^+ loss and Na^+ uptake and not in KCl loss and cell shrinkage, as demonstrated, e.g., in EAT cells, in which g_{Cl} is 15 times lower than g_K (501). This means that activation of VRAC is a necessity for initiation of AVD, and in congruence with this, depolarization of V_m during AVD has been demonstrated in several cell types (214, 617, 618, 729). In addition to reflecting a greater increase in g_{Cl} than in g_K, this may, however, also reflect an increase in g_{Na} (729, 1072).

1. Role of ion and taurine loss in PCD

In some cells, a decrease not only in the cellular K^+ content, but also in the cellular K^+ concentration ($[K^+]_i$), during AVD has been reported (37, 392), and several studies point to the importance of the decrease in $[K^+]_i$ in the activation of PCD (78, 167, 606, 1055, 1131). In the cases where a decrease in K^+ concentration is seen during AVD, this seems to reflect an inhibition of the Na^+-K^+-ATPase (760). Accordingly, ouabain is found to enhance Fas-induced PCD in Jurkat cells (75). A decrease in $[K^+]_i$ augments chromatin condensation, DNA fragmentation, and caspase activation (392). RVD (581) as well as AVD (839) are accompanied by cellular acidification in several cell types, and this acidification may be partly responsible for activation of endonucleases and chromatin breakdown during PCD (968). Taurine release is also triggered during PCD (516), and erythrocytes from the taurine transporter knockout mouse are more resistant to apoptosis (513). This might be related to the fact that TauCl, which is the chlorinated product of taurine, causes PCD through direct damage to the mitochondria (454), i.e., net loss of taurine could result in a reduction in TauCl and thus a reduced risk for PCD.

2. Role of shrinkage as a signal in PCD

AVD in several cell types can be separated into an early and a late phase, and the early AVD was found to occur prior to caspase activation (606, 608), DNA laddering (78, 148), cytochrome *c* release (606), and translocation of phosphatidylserine (218). On the basis of this, Okada and Maeno (743) suggested that cell shrinkage during early AVD is a triggering step for the apoptotic process. On the other hand, there are examples where PCD has been shown to occur whithout a previous AVD (76, 246, 382).

To address whether cell shrinkage per se can be the trigger for PCD, several groups have investigated the effect of hypertonic shrinkage in the absence of a decrease in $[K^+]_i$ or cellular acidification. Bortner and Cidlowski (73) showed already in 1996 that hypertonicity could induce PCD in some cell types, yet not in others (73), and they suggested that the extent to which cells underwent PCD after hypertonic stress correlated inversely with their capacity for RVI. Confirming this, subsequent studies in a wide range of cell types have demonstrated that long-term hypertonic shrinkage always results in PCD (e.g., Refs. 217, 249, 517, 606, 645, 732, 744, 788, 984). As an example, Chinese hamster ovary (CHO) cells lacking NHE1 and thus deficient in RVI are more susceptible to PCD than cells reconstituted with NHE1 (608). Furthermore, in hepatocytes, hypertonic conditions were shown to traffic the death receptor CD95 to the plasma membrane, sensitizing cells to CD95L-induced PCD (840). Thus cell shrinkage seems to be a trigger of PCD independent of a reduction in $[K^+]_i$ and cellular acidification.

3. Signaling events from cell shrinkage to PCD

In NIH3T3 cells, the sequential signaling events from cell shrinkage leading to PCD were shown to involve the Rac, p38 MAPK, p53, and caspase 3 (see Fig. 19). Furthermore, growth factor receptors have been shown to be less sensitive under the hypertonic conditions (153, 708), resulting in decreased activity of the PI3K-PKB survival pathway (708). Rac and p38 MAPK are activated by cell shrinkage in a variety of cell types (sects. VI*D* and V*C*), and Rac has been proposed to be an upstream activator of p38 MAPK after osmotic shrinkage (1014; sect. V*C*). Overexpression of constitutively active Rac potentiated shrinkage-induced activation of p38 MAPK and caspase-3, and p38 MAPK exerted its effect upstream of p53 and caspase-3 (249). Simultaneously with the activation of p38 MAPK, phosphorylation of ERK1/2 was transiently decreased in NIH3T3 cells (249) and ELA (788) cells after osmotic shrinkage (sect. V*C*). Inhibition of ERK1/2I enhances induction of PCD in hepatocytes (827), and in PC12 cells, the balance between survival and PCD was found to be controlled by the relative activities of p38 MAPK and ERK1/2 (1114). It is therefore likely that also the inhibition of ERK might be important for the shrinkage-induced PCD. Furthermore, MEKK1, a potent activator of MKK4, which in turn mediates JNK activation, was recently shown to induce the ubiquitination and consequent downregulation of the JNK target transcription factor c-Jun after osmotic shrinkage, an effect which appeared to promote shrinkage-induced PCD (1113).

In spite of the many indications of a role for p38 MAPK in PCD (e.g., Refs. 249, 822, 887, 978), the precise role of p38 MAPK is still incompletely understood and

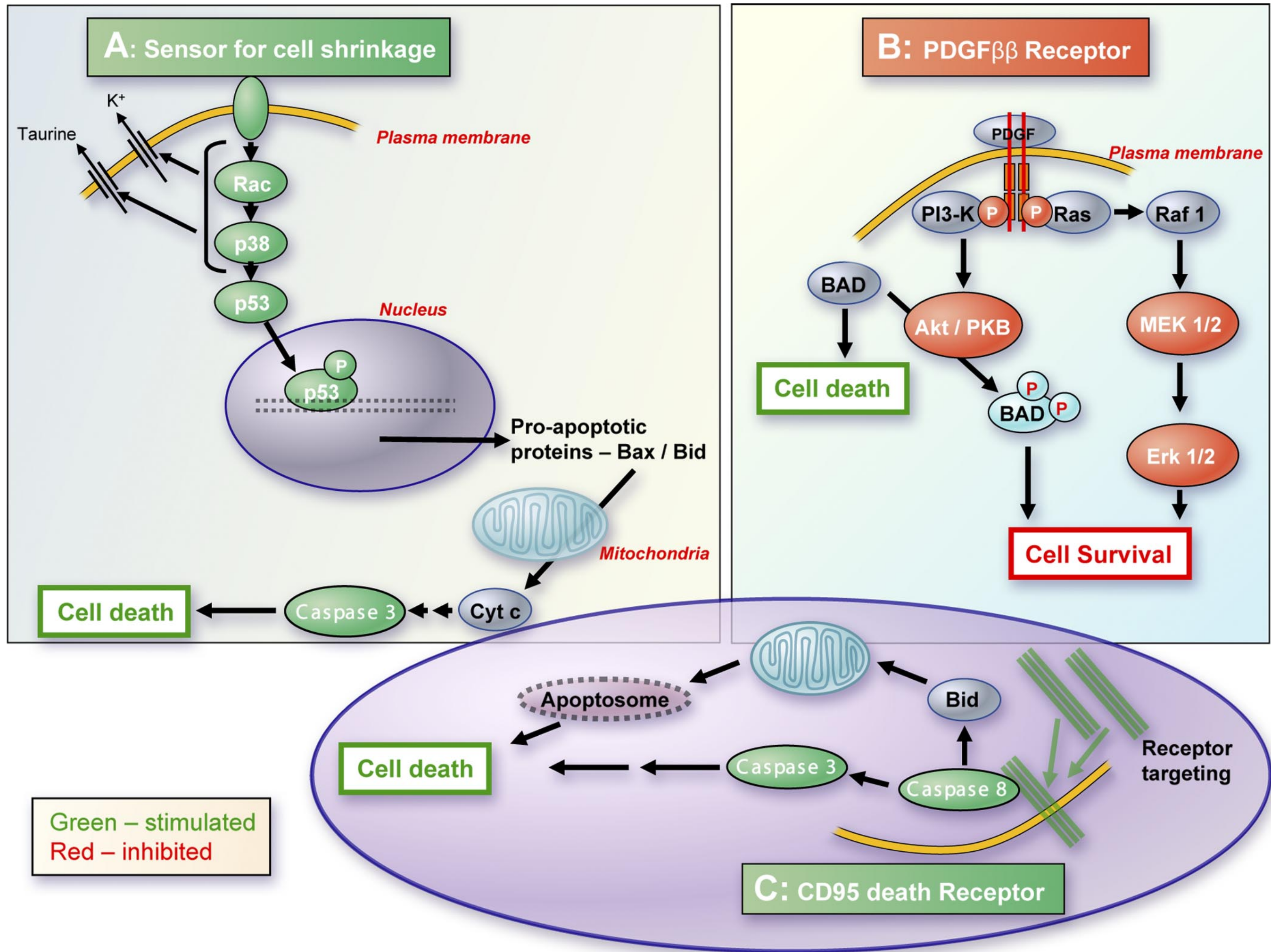

FIG. 19. Proposed signaling events in cell shrinkage-induced programmed cell death. *A*: cell shrinkage detected by a volume sensor (see sect. IV) activates the monomeric GTP binding protein Rac, and p38 MAPK, followed by phosphorylation and nuclear translocation of p53. This is proposed to result in the transcription of proapoptotic proteins and found to result in caspase-3 activation. Green indicates stimulatory pathways supported experimentally. [From Friis et al. (249).] *B*: cell shrinkage inhibits $PDGF_{\beta\beta}$ receptor-mediated signaling. Red indicates inhibitory pathways supported experimentally. Reduced Akt/PKB activity results in reduced BAD phosphorylation and hence an increase in BAD-mediated programmed cell death. Reduced MEK1/2 and ERK 1/2 activity leads to reduced cell survival. [Model based on Nielsen et al. (708).] *C*: death receptor CD95 normally shows a predominant intracellular localization. Hyperosmotic exposure induces CD95 trafficking to the plasma membrane, followed by activation of caspase-3 and -8 and sensitization of the cells towards CD95.[Model based on Reinehr and Haussinger (839) and Reinehr et al. (840).]

might vary between cell types (484). One way in which p38 MAPK can influence PCD is via interaction with p53. That p38 MAPK can activate/phosphorylate p53 also after osmotic stress has actually been found in several cell types (e.g., Refs. 91, 249, 453, 883), and a central role of p53 in PCD is well described since 1993 (509). p53 is involved in the upregulation of multiple proapoptotic genes, the products of which are involved in both the intrinsic mitochondrial (e.g., BCl-2 family proteins) and the extrinsic, receptor-mediated apoptotic pathway (e.g., Fas) (328, 333; see also Ref. 1084). In addition, p53 has nontranscriptional effects on PCD, including inhibitory interactions with BCl-2 and direct activation of Bax in the cytoplasm (131). Hyperosmotic stress elicits p53 phosphorylation on Ser^{15} in mouse renal inner medullary collecting duct cells, renal tubular cells, and NIH3T3 fibroblasts (197, 249, 984; see Ref. 95). In both renal tubular cells and NIH3T3 fibroblasts, p53 phosphorylation was maximal at 600 mosM, decreasing again at higher osmolarities (197, 249; K. Schou, S. T. Christensen, and E. K. Hoffmann, unpublished results). Notably, the increase in p53 activity at moderate osmolarities was in fact protective against PCD, probably by arresting the cell in G_0 and restricting DNA replication (197, 198).

D. Cell Proliferation

Since during proliferation a parent cell generates two daughter cells of the same size as the parent cell, it is obvious that a cell volume increase occurs at some point during the cell cycle. As described in the following, several lines of evidence indicate that cell volume is a major component in the regulation of cell cycle progression. Although the mechanism is not well described, cell proliferation is often stimulated by osmotic swelling and inhibited by shrinkage (e.g., Refs. 16, 209, 866; see Refs. 95, 516). Similarly, cell division depends on an increase in cell volume (see Refs. 510, 864) and is delayed in shrunken cells (737). In miMCD3 cells, cell shrinkage increased the length of the S and G_2/M phases (645), and in fibroblasts, cell volume increased in parallel to G_1-S transition (805). In CNE-2Z cells, cell volume was found to change during the cell cycle, being greatest in M phase and smallest in G_1 phase, and the rate of RVD changed accordingly, the fastest RVD occuring in G_1. Ras oncogene expression in fibroblasts is paralleled by enhanced NHE1 and NKCC1 activity, leading to an increase in cell volume which is required for the stimulation of cell proliferation by Ras (850). In glioma cells, it was recently demonstrated that a dramatic volume decrease occurs as cells progress through the M phase, such that they all reach one common preferred volume at division. This volume decrease was functionally linked to the rate of cell division and to the condensation of chromatin in the M phase (313). The mechanisms by which changes in cell volume impact on cell cycle control are still unelucidated, but mechanisms involving tyrosine kinase receptors and MAPKs could play important roles. Thus cell swelling by hyposmotic stress in general stimulates ERK1/2 (sect. v*C*), a major player in control of cell cycle progression (see, e.g., Ref. 641). Consistent with such a scheme, inhibition of ERK1/2 decreases both osmosensitive taurine release and cell proliferation in glioma cells (52).

Which ions and ion transport proteins may be important for control of the cell cycle? It was proposed early on that $[Ca^{2+}]_i$ and specifically $[Ca^{2+}]_i$ oscillations play a critical role in control of the cell cycle (55). It was subsequently suggested that such oscillations elicit the activation of NKCC1 and NHE1, eliciting a cell volume increase that favors proliferation (see Ref. 516). The $[Ca^{2+}]_i$ oscillations translate into oscillation of the activity of Ca^{2+}-activated K^+ channels, and thus of V_m, as shown in Ras oncogene-expressing cells (512). A variety of K^+ channels have been implicated in the regulation of proliferation (972, 1069) and cell cycle progression (1066). Accordingly, tumor cells often show enhanced K^+ channel activity (775, 1069). Cl^- channels have also been widely reported to be involved in control of cell proliferation. Thus Cl^- channel blockers are found to inhibit cell proliferation (124, 455, 764, 865, 922, 1044, 1100), and VRAC is differentially expressed during the cell cycle (202, 455, 922, 1031). Also ClC-3 expression varies through the cell cycle (1065), and ClC-3 deficiency prevents proliferation of rat aortic smooth muscle cells (1063). Finally, in nasopharyngeal carcinoma cells, $I_{Cl,vol}$ activity was found to be important in control of passage through the G_1 restriction point (124). For a recent review on the roles of cell volume regulatory ion channels in control of proliferation, see Reference 511.

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All authors contributed equally to this work and are listed in alphabetical order on the title page.

We apologize to those whose relevant work we could not cite due to space restrictions.

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