Rev Physiol Biochem Pharmacol (2003) 148:1–80
DOI 10.1007/s10254-003-0009-x

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Cell volume regulation: osmolytes, osmolyte transport, and signal transduction

Published online: 4 April 2003
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Abstract In recent years, it has become evident that the volume of a given cell is an important factor not only in defining its intracellular osmolality and its shape, but also in defining other cellular functions, such as transepithelial transport, cell migration, cell growth, cell death, and the regulation of intracellular metabolism. In addition, besides inorganic osmolytes, the existence of organic osmolytes in cells has been discovered. Osmolyte transport systems—channels and carriers alike—have been identified and characterized at a molecular level and also, to a certain extent, the intracellular signals regulating osmolyte movements across the plasma membrane. The current review reflects these developments and focuses on the contributions of inorganic and organic osmolytes and their transport systems in regulatory volume increase (RVI) and regulatory volume decrease (RVD) in a variety of cells. Furthermore, the current knowledge on signal transduction in volume regulation is compiled, revealing an astonishing diversity in transport systems, as well as of regulatory signals. The information available indicates the existence of intricate spatial and temporal networks that control cell volume and that we are just beginning to be able to investigate and to understand.

General introduction

For a number of years, cell volume regulation in mammalian cells has been considered of minor physiological importance since overall osmolality of the plasma is one of the very tightly regulated parameters of the body. In the meantime, however, it has been realized

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that quite a variety of tissues such as renal medullary cells and chondrocytes are exposed to anisotonic extracellular media and thus require volume regulatory mechanisms. Anisotonic conditions can also arise in pathological conditions such as hypo- or hypernatraemia when homeostatic functions of the body are insufficient (Law 1999; Law 1998; Verbalis et al. 1989; Verbalis 1994). Similarly, cells encased in a rigid surrounding, such as brain cells, depend on cell volume regulation for their proper function. Furthermore, even at normal extracellular osmolalities cells can generate transmembrane osmotic differences due to uptake of (organic) osmolytes or during transepithelial transport of solutes (McCarty and O'Neil 1991).

Cell volume changes also occur globally during maturation of erythrocytes, cell growth, differentiation, hypertrophy, and apoptosis. Locally, cell migration and shape changes require volume adaptations (Lang et al. 1998b). Finally, cell volume has been identified as a mechanism that regulates cell metabolism (Lang et al. 1989). This effect is particularly evident in the liver, where cell swelling increases protein and glycogen synthesis and cell shrinkage increases protein and glycogen breakdown (Häussinger 1996; Häussinger 1998). Thus, cell volume and its importance during the whole life cycle of a cell has become more and more evident and this area of physiology and pathophysiology has been attracting an increasing number of investigators.

During recent years, it also became more and more accepted that not only inorganic osmolytes such as K^+ and Cl^- are employed to restore osmotic equilibrium across the cell membranes but that also organic osmolytes play a significant role in cell volume homeostasis.

In most reviews, inorganic and organic osmolytes and their respective volume regulatory transporters are dealt with separately. The current review combines the two and discusses the question of a putative identity of some systems. This contribution also takes into account the progress made in identifying the molecular entities of the transporters by molecular biology techniques. Furthermore, the current state of knowledge with regard to the regulation and coordination of the various osmolyte transporters is reviewed in order to shed some light on the intriguing signal transduction networks used in cell volume regulation.

The osmometric behavior of cells

Any changes in cell volume of animal cells under anisotonic conditions are, in principle, based on a distinct permeability of the plasma membrane to water. In many instances, this permeability is increased by specialized membrane proteins, which mediate the facilitated diffusion of water, so-called water channels or aquaporins (Maunsbach et al. 1997; Marinelli and LaRusso 1997; Ma and Verkman 1999). Given a sizeable water permeability at its outer membrane, a cell then exhibits an "osmometric" behavior as its first passive response to anisotonicity, i.e., a movement of water will occur, which changes cell volume until the difference in osmotic pressure is equalized and a new chemical equilibrium of water across the plasma membrane is achieved. From a physicochemical point of view, however, a cell is not a *perfect* osmometer, which means that its volume will not change to the same extent as is predicted by the law of Boyle/van't Hoff. This means that apparently some 20%–40% of total cell volume usually comprises a phase that is not "cytosolic," which means that it is not osmotically active (Lucke and McCutcheon 1932). In practice, the osmotically active/inactive space of a living cell can be easily determined graphi-

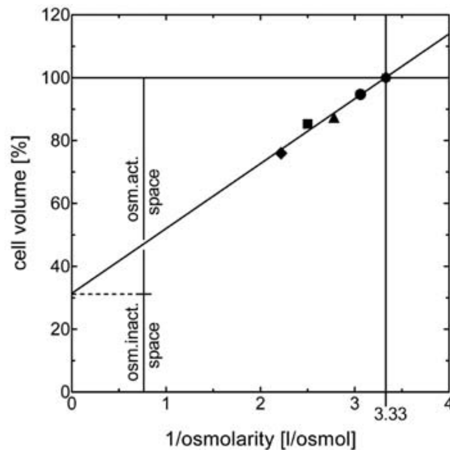

Fig. 1 With all Na^+ importers of RVI blocked (i.e., with 10^{-3} mol/l amiloride plus 10^{-4} mol/l furosemid; see text for details), the *passive* changes of rat hepatocyte volumes after 10 min exposure to hypertonicity (327, 360, 400, and 450 mOsm/l) are a linear function of the reciprocal of extracellular osmolarity. The value of 3.33 is equivalent to 300 mOsm/l where cell volumes equal 100% (control, ★). The intercept of the regression line with the volume axis is equivalent to the "osmotically inactive space" that amounts to 31.3% of total cell volumes (from Wehner and Tinel 2000)

cally by plotting its relative volume versus the reciprocal of extracellular osmolarity, which is equivalent to a Boyle/van't Hoff-Plot (see Fig. 1).

The intercept of the regression line with the Y-axis then gives the osmotically inactive space; its difference from the 100% mark is equivalent to the osmotically active part. This type of analysis, which deals with the passive osmotic behavior of a cell, however, is only valid if the active compensatory processes by which cells readjust their volume are not activated and/or are inhibited efficiently. In rat hepatocytes, for instance, this was achieved by blocking all Na^+ import mechanisms mediating regulatory volume increase (RVI) (namely Na^+ conductance, Na^+/H^+ antiport, and Na^+-K^+-$2Cl^-$ symport) by 10^{-3} Mol/l amiloride and 10^{-4} Mol/l furosemide and by then determining the actual changes of cell volume after 10 min of cell shrinkage, under four different hypertonic conditions. From these measurements, an osmotically inactive space could be determined that amounts to some 31% of total cell volume (Wehner and Tinel 2000).

Inorganic and organic osmolytes

For a number of years, research on volume regulation in mammalian cells has focused on the movement of the main intracellular inorganic osmolytes such as potassium and chloride. Compensatory changes in these osmolytes form indeed the backbone for volume regulation when only small perturbations of the intra- or extracellular osmolarity occur.

In recent years it has become, however, more and more evident that—similar to other phyla in nature—in mammalian cells also organic osmolytes are employed in volume regulation (Yancey et al. 1982; Hoffmann et al. 1988; Kinne 1993; Burg 1995; Burg 1996; Junankar and Kirk 2000).

Organic osmolytes comprise polyols such as sorbitol and myo-inositol, methylamines such as glycerophosphorylcholine and betaine, and amino acids such as taurine, glutamic acid, and ß-alanine amongst others (Yancey and Burg 1989; Yancey et al. 1982; Beck et al. 1985; Beck et al. 1998).

There are several hypotheses as to why in some cells organic solutes are used in addition to inorganic solutes. One hypothesis is the "compatible osmolyte hypothesis" which

is based on the observation that high concentrations of inorganic salts such as NaCl or KCl perturb the function of enzymes or other proteins (Noulin et al. 1999), whereas organic solutes do not (Yancey et al. 1982). Another hypothesis reflects the "counteracting osmolyte principle," which refers to the finding that methylamines attenuate the destabilizing effect of high concentrations of urea on protein function (Burg 1996). A third aspect is that several organic osmolytes are electroneutral and can replace inorganic osmolytes which, when released across the cell membrane, may change the membrane potential and thereby neuronal excitability in the brain (Iwasa et al. 1980) or driving forces for electrogenic sodium-cotransport systems. Again, the brain is particularly interesting because of the existence of reuptake systems for excitatory amino acids that are transported by sodium-cotransport systems (Curtis and Johnston 1974).

One of the major places for organic osmolyte accumulation is the renal medulla, where accumulation occurs because of a broad range of extracellular osmolalities exceeding normal osmolality (for review see Beck et al. 1985; Bagnasco et al. 1986; Yancey and Burg 1989; Garcia-Perez and Burg 1991; Kinne et al. 1993; Kinne 1993; Kinne et al. 1995; Kinne 1998; Grunewald and Kinne 1999; Kinne et al. 2001) in particular in the direction of hyperosmolality (Grunewald et al. 1993a; Grunewald et al. 1994; Handler and Kwon 2001). Also chondrocytes (de Angelis et al. 1999; Hall and Bush 2001) encounter hyperosmolality in the extracellular space because of the high concentration of fixed charges in the mucopolysaccharides in which they are embedded. Limited expandability in the brain (Strange 1992; Pasantes-Morales et al. 1994a; Pasantes-Morales et al. 1994b; Pasantes-Morales et al. 2000b) and regulation of cell transparency in the lens (lens fiber cells; Burg and Kador 1988; Cammarata et al. 2002), also require organic osmolytes.

Intracellular accumulation of organic osmolytes involves mainly two processes. The first represents uptake across the cell membrane by specific transport systems, the second intracellular generation of the osmolyte by metabolic reactions. Examples of the former are sodium-cotransport systems for myo-inositol, taurine, and betaine which use the sodium gradient across the plasma membrane as driving force. The general and specific properties of these cotransport systems are summarized in the section entitled "Organic osmolytes in RVI."

Metabolic reactions are primarily responsible for the generation of sorbitol from D-glucose and glycerophosphorylcholine (GPC). D-glucose taken up by the cells via uniport or symport systems can be readily converted into sorbitol by the enzyme aldose reductase using NADPH as cofactor (Gabbay 1973; Ohta et al. 1990). In an extensive study on renal inner medullary aldose reductase, the affinity for D-glucose was found to be about 370 mMol/l, the affinity for NADPH was 7.5 μMol/l. Thus, in the intact cell with a NADPH concentration of about 0.4 mMol/l the enzyme is saturated with NADPH, but only to a small extent with D-glucose. Due to the high K_m of the enzyme, in the intact cell sorbitol synthesis is characterized by the affinity of the cellular glucose uptake systems. For example, a value of about 60 mMol/l is obtained in intact IMCD cells, which reflects the K_m usually observed for glucose uniporters GLUT 1 or GLUT 2 (Grunewald et al. 1993b). Regulation of the synthesis occurs mainly by altering the V_{max} of the enzyme; a strong increase is observed in a variety of cells when they are exposed to hypertonic conditions in vivo or in vitro (Bedford et al. 1987; Bagnasco et al. 1988; Grunewald et al. 1998; Lang et al. 1998a). However, in disease states such as diabetes, the NADPH/NADP ratio is another important regulator of the rate of sorbitol synthesis (Grunewald et al. 1993b).

Sorbitol breakdown yielding fructose can occur via the sorbitol dehydrogenase pathway with NAD as the hydrogen acceptor. In some tissues that use sorbitol as organic osmolyte, these pathways are restricted to different, sometimes closely associated cells (Grunewald et al. 1995; Kinne et al. 1997). In general, the activity of sorbitol dehydrogenase is much lower than that of aldose reductase, thereby limiting the importance of this enzyme in osmotic adaptation (Grunewald et al. 1995; Kinne et al. 1997; Grunewald et al. 1998).

The major pathways involved in the metabolism of GPC in IMCD cells, for example, are the following. The precursor of GPC, choline, is taken up by sodium-independent transport systems. Choline is phosphorylated and then incorporated into the phospholipid phosphatidylcholine (PC). By a stepwise removal of the fatty acid residues (involving phospholipase A2), first lysophosphatidylcholine and then GPC are generated. Interestingly, this reaction seems to use a pool of PC different from that present in the plasma membrane. Breakdown of GPC is mediated by the GPC: choline diesterase reaction yielding choline and phosphoglycerol. The latter two reactions seem to be slow compared to the synthetic pathway. The predominant "osmosensitive" enzyme is the GPC: choline diesterase since studies in vivo and in vitro indicate that after longterm exposure of cells to hypertonic conditions, the rate of enzymatic breakdown is reduced and thus the overall concentration of the organic osmolyte is increased (Zablocki et al. 1991; Bauernschmitt and Kinne 1993).

The release of organic osmolytes from the cells involves channel-like proteins, in some instances organic and inorganic osmolytes share the same transporter. Thus, a complex picture emerges in which inorganic and organic osmolyte levels have to be controlled in a well-coordinated manner (Burg 1996). This coordination requires feedback systems that have to be elucidated and taken into account when considering volume regulatory processes in the cells.

Regulatory volume increase

Role of inorganic osmolytes in RVI

In order to achieve RVI, the intracellular osmolyte content has to be augmented rapidly. To this end, transport systems for inorganic osmolytes are activated as the first cellular response. The main osmolyte taken up by the cells is sodium since favorable driving forces for this cation exist across the plasma membrane. Sodium is subsequently exchanged against potassium by the action of the Na^+,K^+-ATPase, to restore the original sodium gradients and its electrochemical potential.

There are also mechanisms set in place that reduce loss of intracellular potassium and sometimes also chloride, depending on its electro-chemical equilibrium.

Na^+/H^+ antiport

Na^+/H^+ antiporters (NHEs) catalyze the secondary active and electroneutral exchange of H^+ against Na^+. With one exception, they are most important for the regulation of cell pH, but some NHEs are effective mediators of RVI. Six NHE isoforms have been cloned to date (NHE1–NHE6) which exhibit a common molecular organization (see for review

Orlowski and Grinstein 1997; Counillon and Pouyssegur 2000; Ritter et al. 2001). They consist of two main functional domains with amino acid identities of 45%–60% and 25%–35%, respectively. These are an N-terminal portion of 10–12 transmembrane regions (depending on the hydropathy algorithm used) and a large intracellular C-terminal part that is equipped with regulatory sites (see below). NHE1 to NHE5 are located in the plasma membrane, while NHE6 is sorted to the mitochondrial inner membrane. NHE1 and NHE6 are ubiquitously expressed whereas NHE2–NHE5 are restricted to specific tissues. In epithelia, NHE1 appears to be largely confined to the basolateral membrane (Coupaye-Gerard et al. 1996) but, in cell lines like OK (opossum kidney) or MDCK (Madin-Darby canine kidney), it may also occur apically (Noel et al. 1996). NHE2, NHE3, and NHE4 are found preferentially in the gastrointestinal tract and kidney where they appear to reside mainly in the apical membrane (Soleimani et al. 1994; Dudeja et al. 1996; Noel et al. 1996; Hoogerwerf et al. 1996; Sun et al. 1997; Bookstein et al. 1997). The presence of NHE2 in the kidney and NHE4 in the intestine, however, is discussed controversially (Bookstein et al. 1997). NHE5 was detected in nonepithelial tissues, preferentially in the brain, but also in spleen, testis, and skeletal muscle (Klanke et al. 1995). In many cells, several NHE isoforms are expressed and assigned to specific membrane domains so that (together with other transporters) they achieve a concerted cross-talk in the vectorial movement of ions; this especially holds for epithelia (Ritter et al. 2001).

Some NHEs are inhibited by submillimolar concentrations of amiloride and, more selectively, by its derivatives dimethyl-amiloride and ethyl-isopropyl-amiloride (Tse et al. 1994; Counillon and Pouyssegur 2000). There are significant differences in sensitivity to these blockers among the different isoforms, which (for amiloride) amount to almost four orders of magnitude. The amiloride sensitivity generally follows the order NHE1 > NHE2 >> NHE3 > NHE4 (Orlowski and Grinstein 1997; the latter two isoforms are therefore sometimes referred to as being amiloride-insensitive (Counillon and Pouyssegur 2000). The mitochondrial isoform NHE6 exhibits a rather low sensitivity to amiloride, but it is efficiently blocked by the analog, benzamil (Brierley et al. 1989).

With respect to their role in cell volume regulation, it was found that NHE1, NHE2, and NHE4 are activated under hypertonic conditions, whereas NHE3 is inhibited (Orlowski and Grinstein 1997; Hoffmann and Mills 1999; Ritter et al. 2001). NHE1 is clearly the Na^+/H^+ antiport most commonly employed in RVI, which is not surprising in regard to the ubiquitous expression of the protein. As a matter of fact, the shrinkage-induced activation of Na^+/H^+ antiport that had been reported in a variety of preparations prior to the molecular definition of NHE isoforms can now in many instances be attributed to NHE1 (Lang et al. 1998a; Lang et al. 1998b; Hoffmann and Mills 1999).

Concerning regulation, hypertonicity appears to shift the pH_i sensitivity of NHE1 to more alkaline values (Grinstein et al. 1985). Interestingly, although the C-terminal domain of NHE1 exhibits a number of phosphorylation sites (that are actually used for the activation by hormones and mitogens), no direct phosphorylation of the protein occurs upon cell shrinkage (Sardet et al. 1990; Sardet et al. 1991; Grinstein et al. 1992). Rather, there appears to be a significant hypertonicity-induced cross-talk between NHE1, stress fibers and the small GTPase Rho so that osmotically-induced changes in the actin texture (also involving integrins) are very likely to be part of the signaling cascade-mediating activation (Watson et al. 1992; Grinstein et al. 1993; Vexler et al. 1996; Hooley et al. 1996; Tominaga et al. 1998; Tominaga and Barber 1998). In addition, Ca^{2+} may participate in the stimulation because the C-terminal domain of NHE1 contains two calmodulin binding sites and deletion or mutation of the high affinity binding site significantly reduces the

osmotic sensitivity of the transporter (Bertrand et al. 1994). It is worth mentioning that NHE1 is also subject to transcriptional osmotic regulation (Ritter et al. 2001).

The hypertonic activation patterns of NHE2 are less well-defined. Proline-rich regions in the C-terminal portion of the protein appear to be involved which resemble SH3 binding domains and might reflect sites of interaction with the cytoskeleton and/or signaling molecules. Truncation experiments, however, revealed that these regions are probably involved in the proper targeting of NHE2 rather than its regulation (Chow et al. 1999). Besides this, NHE2 is subject to transcriptional control (Soleimani et al. 1994; Bai et al. 1999).

NHE3 is inhibited under hypertonic conditions. This effect is due to a reduction of the maximal transport velocity rather than to changes in substrate affinities (Nath et al. 1996). NHE3 is highly sensitive to the actin texture of cells (Kurashima et al. 1999; Szaszi et al. 2000). The protein associates with two regulatory factors, namely NHERF1 and NHERF2, and NHERF1 could be shown to bind to ezrin, an actin-binding protein of the ezrin-radixin-moesin (ERM) family that links membrane proteins to the cytoskeleton. Based on deletion experiments, it could also be shown that the same C-terminal region of NHE3 mediates actin sensitivity and NHERF binding (Kurashima et al. 1999).

Moreover, among the small GTPases of the Rho family controlling actin assembly, the inhibitory form of RhoA (but not Rac1 and Cdc42) greatly depressed NHE3 activity and comparable effects were observed upon a specific block of Rho kinase (ROK), a downstream effector of RhoA; furthermore, inhibition of ROK reduced the phosphorylation of myosin light chain (MLC). These data strongly suggest that the RhoA-ROK signalling pathway is a mechanism for the control of NHE3 activity, which is, at least in part, achieved by controlling the phosphorylation of MLC and, consequently, the organization of the actin cytoskeleton (Szászi et al. 2000).

Interestingly, considerable amounts of NHE3 are present in recycling endosomes (Ritter et al. 2001). This predominantly intracellular location may contribute to its paradoxical behavior during RVI.

NHE4 is clearly activated under hypertonic conditions; it exhibits a bell-shaped profile with maximal functionality close to 500 mOsm/l (Bookstein et al. 1994). This relatively low osmotic sensitivity is readily explained in terms of NHE4 tissue distribution with maximal expression found in the renal medulla (Bookstein et al. 1994; Ritter et al. 2001) where a high extracellular osmolality prevails.

Na^+-K^+-$2Cl^-$ symport

NKCC1 and NKCC2 are mediators of electroneutral Na^+, K^+, Cl^- cotransport across cell membranes at a stoichiometry of 1:1:2 (with very few exceptions; see Haas and Forbush, III 2000; Russell 2000 for reviews). On the molecular level, they share many similarities with other members of Cl^--dependent cation transporters, namely the Na^+-Cl^- symporter (NCC) and the four isoforms of K^+-Cl^- symporters (KCC1 to KCC4) cloned so far. These similarities include molecular weights in the range of 110 kD to 130 kD (deglycosylated), 12 predicted transmembrane regions, and large hydrophilic intracellular N- and C-terminal domains. The most conserved regions of these transporters are the transmembrane domains, as well as the putative intracellular loops connecting them, particularly the one between TM2 and TM3. NKCCs are blocked by the "loop" diuretics bumetanide and furosemide at micromolar concentrations; KCCs exhibit a lower affinity to these compounds, whereas a possible inhibition of NCC remains ambiguous (Russell 2000). NKCC1 exhibits

a broad tissue distribution and is found in many secretory epithelia (where it resides in the basolateral membrane), as well as in a variety of nonepithelial cells. In contrast, NKCC2 is only present (apically) in the thick ascending limb of Henle's loop and in the macula densa of the kidney (Haas and Forbush, III 2000; Russell 2000).

NKCC1 is one of the most routinely employed transporters of cell volume regulation (Russell 2000) during RVI (Lang et al. 1998a; Lang et al. 1998b; Hoffmann and Mills 1999; Haas and Forbush, III 2000). Activation of NKCC1 appears to involve phosphorylation at serine/threonine residues and cell shrinkage results in NKCC1 phosphorylation in a number of cells. In most instances, however, the actual kinase in charge remains to be identified (Hoffmann and Mills 1999; Haas and Forbush, III 2000). Recently, the phosphorylation of NKCC1 by c-Jun NH_2-terminal kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) family, was reported from bovine aortic endothelial cells that were shrunken under hypertonic or isotonic conditions (Klein et al. 1999). In rat hepatocytes, the hypertonic activation of Na^+-K^+-$2Cl^-$ symport (as well as that of Na^+ conductance) was inhibited by staurosporine, as well as by the PKC specific blocker bis-indolylmaleimide I (BIM; Heinzinger et al. 2001). In human tracheal epithelial cells, the hypertonic activation of NKCC1 appeared to be mediated by PKC-δ; this process is also likely to involve the (extracellular signal-regulated) kinase ERK (Liedtke and Cole 2002). In addition to phosphorylation and dephosphorylation, the activity of NKCC1 appears to be regulated by the state of the actin network, as well as by accessory proteins that remain to be characterized (Haas and Forbush, III 2000; Russell 2000).

Cation channels

Compared to the symport system discussed above, the rates of ion transport by channels are some 4–5 orders of magnitude higher. Accordingly, any modulation of channel activity in response to changes of cell volume will serve as a fast and very efficient regulatory mechanism.

If one considers the electrochemical driving forces for Na^+, K^+, and Cl^- transport across most cell membranes, the activation of Na^+-selective channels and conductive Na^+ entry would be a highly efficient mediator of RVI. The resultant depolarization of membrane voltage would favor a parallel conductive entry of Cl^-. Na^+ accumulating inside the cell would then be extruded via Na^+, K^+-ATPase so that constant driving forces for Na^+-coupled cotransporters are ensured. In sum, a net intracellular gain of K^+ and Cl^- and thus a rapid increase of cell volume would be achieved by these mechanisms.

There is an increasing number of systems from which the hypertonic activation of cation channels is reported. Two main classes of channels can be distinguished based on their sensitivity to amiloride. In the following, first the amiloride sensitive channels will be discussed.

The activation of conductive Na^+ entry as a mechanism of RVI was originally proposed by Hoffmann (Hoffmann 1978) and Okada (Okada and Hazama 1989) for Ehrlich ascites tumor cells and the Intestine 407 cell line, respectively. In 1995, a hypertonic stimulation of cell membrane Na^+ conductance was reported from current-clamp recordings on rat hepatocytes in confluent monolayer culture (Wehner et al. 1995; see Fig. 2).

Furthermore, in a quantitative study, it could be shown that the relative contribution of Na^+ conductance, Na^+/H^+ antiport, and Na^+-K^+-$2Cl^-$ symport to the initial uptake of Na^+ under hypertonic stress was approximately 4:1:1 (Wehner and Tinel 1998). This clearly

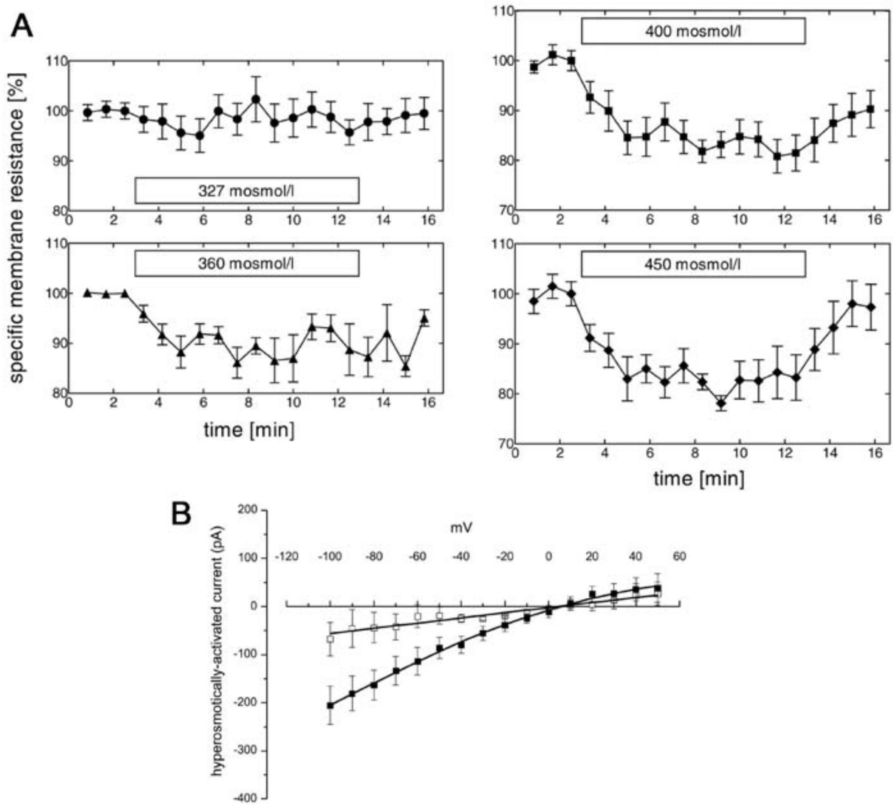

Fig. 2 **A** Cable analysis of specific membrane resistance, reflecting the hypertonic activation of Na^+ conductance in rat hepatocytes. Experiments were carried out in the continuous presence of 0.5 mM quinine; extracellular osmolarity was increased from 300 mOsm/l to the osmolarities indicated; means±SEM; *n*=4–5. **B** Current-voltage relationships of hypertonicity-induced membrane currents in rat hepatocytes obtained with (two-channel microelectrode) voltage-clamp techniques. The differences between currents obtained at 400 mOsm/l and 300 mOsm/l are depicted for cells injected with control-oligo-DNA (■) or anti-α-rENaC oligo DNA (□) (from Wehner and Tinel 2000; Böhmer and Wehner 2001)

renders Na^+ conductance the prominent mechanism of RVI in this system. The Na^+ conductance was inhibited by amiloride with an apparent K_i of 6 μMol/l and its overall sensitivity profile was EIPA > amiloride > benzamil (Wehner et al. 1997; Böhmer et al. 2000). Hence, at first sight, the hypertonicity-induced Na^+ conductance of rat hepatocytes may reflect a low (amiloride) affinity type rather than an epithelial Na^+ channel (ENaC); the latter typically exhibits K_i values in the upper nanomolar range and an inverse pharmacological profile (Garty and Palmer 1997; Fyfe et al. 1998). In voltage-clamp experiments on rat hepatocytes, a relatively low cation selectivity of the channel was detected with a P_{Na}/P_K of 1.4 (Böhmer and Wehner 2001). In patch-clamp experiments in the cell-attached configuration, hypertonicity-induced single channel events with a unitary conductance of 6 pS were recorded (P. Lawonn and F. Wehner, unpublished observation). With respect to intracellular signaling events, the hypertonic activation of Na^+ conductance (and Na^+-K^+-$2Cl^-$ symport, but not Na^+/H^+ antiport) is mediated by PKC (Heinzinger et al. 2001).

In the human hepatoma cell-line HepG2, hypertonic stress led to the activation of a nonselective cation conductance that was clearly sensitive to amiloride with 65% inhibition at 10^{-5} Mol/l (Wehner et al. 2002a). Interestingly, this hypertonicity-induced conductance was also partially inhibited by Gd^{3+} (10^{-4} Mol/l) and flufenamate (10^{-5} Mol/l) which are typical blockers of nonselective but amiloride-insensitive cation channels in a variety of systems (see below). Benzamil and EIPA (at 10^{-5} Mol/l each) had no effect on HepG2 cation conductance (Wehner et al. 2002a). In Ehrlich-Lettre ascites tumor cells, hypertonic conditions activated a cation conductance that did not discriminate between Na^+, K^+, and Li^+, but that was impermeable to NMDG and choline. In the range of 10^{-6} Mol/l to 10^{-3} Mol/l this cation conductance was blocked by the ion channel inhibitors benzamil > Gd^{3+} > amiloride > EIPA (in order of potency; Lawonn et al. 2003). In immortalized human nonpigmented ciliary epithelial cells, shrinkage upon return from hypo-osmotic to normosmotic conditions was followed by a partial (post-RVD) RVI (Civan et al. 1996) that was mediated by the parallel operation of Na^+/H^+ antiport, Na^+-Cl^- symport, Na^+-K^+-$2Cl^-$ symport, as well as by conductive Na^+ entry. In the range of 10^{-6} Mol/l and 10^{-5} Mol/l, this Na^+ conductance was significantly inhibited by amiloride and—even more efficiently—by benzamil, the most effective blocker of the ENaC (Civan et al. 1997; Garty and Palmer 1997; Fyfe et al. 1998). In principal cells of rat cortical collecting duct hypertonic stress led to a distinct depolarization of membrane voltage coinciding with an increase of cell Na^+ (Schlatter et al. 1997). These effects were partially inhibited by 10^{-5} Mol/l amiloride or Gd^{3+} and the effects were additive. This result was interpreted in terms of a parallel activation of Na^+ channels and nonselective cation channels.

In human red blood cell ghosts, hypertonic stress induced a cation conductance that appeared to be equally permeable to Na^+ and K^+ (but impermeable to NMDG) and that was partially inhibited by 10^{-4} Mol/l amiloride (Huber et al. 2001). These findings support earlier flux studies on lamprey erythrocytes in which hypertonic stress led to the activation of an amiloride-sensitive Na^+ transport that could not be attributed to Na^+/H^+ or Na^+/Na^+ antiport (Gusev and Sherstobitov 1996).

In U937 macrophages, hypertonic stress led to the activation of an inward current with a distinct selectivity for Na^+ over K^+ that was inhibited by amiloride with an apparent K_i close to 1 μMol/l. At the single channel level, this cation conductance appeared to be related to a 6 pS channel. Interestingly, these channels are upregulated by pretreatment of the cells with glucocorticoids, which are known to regulate macrophage function (Gamper et al. 2000).

The shrinkage-activated amiloride sensitive cation channel appears to be related to (α)ENaC. In rat hepatocytes, antisense nucleotides attenuated the activation of the channel (Böhmer and Wehner 2001; see Fig. 2B). In human ciliary body, α-ENaC mRNA was identified (Civan et al. 1997). Similarly, mRNA for α, β, and γ subunits was detected in rat hepatocytes and these subunits could also be identified on the protein level (Böhmer and Wehner 2001). Although the nonselectivity of hypertonicity-induced channels is quite in contrast to the high P_{Na}/P_K reported for α-, β-, γ-rENaC (which is as high as 40; Fyfe et al. 1998) this is not contradictory per se to a possible contribution of ENaCs because their biophysical properties (also including single-channel conductance) strongly depend on subunit composition (Garty and Palmer 1997) as well as their relative affinity to amiloride and its derivatives (Benos et al. 1997; Garty and Palmer 1997). Furthermore osmo-sensitivity of α-, β-, γ-ENaC was reported when the channel subunits were expressed in oocytes (Awayda and Subramanyam 1998). Since some of the results are, however, conflicting,

further studies are necessary to clarify the relation between ENaC and the amiloride-sensitive cation channels.

The second group of hypertonicity-induced cation channels is clearly *amiloride-insensitive* up to concentrations of 10^{-4} Mol/l. Quite typically, however, these channels are inhibited by the anti-inflammatory drug flufenamate (10^{-4} Mol/l), as well as by Gd^{3+} in the range of 10^{-5} to 10^{-3} Mol/l. They are expressed in human nasal epithelial cells (Chan and Nelson 1992), in the human colon cell-lines CaCo-2 and HT_{29} (Nelson et al. 1996; Koch and Korbmacher 1999), in the mouse cortical collecting duct cell line M1 (Volk et al. 1995), as well as in BSC-1 renal epithelial cells (derived from the African green monkey), A 10 vascular smooth muscle cells (established from rat embryonic aorta), and Neuro-2a cells (derived from mouse neuroblastoma; Koch and Korbmacher 1999). In general, these channels are rather nonselective with respect to monovalent cations, although there are certain peculiarities. In human nasal epithelium and in CaCo-2 cells, for instance, channels did not discriminate much between Na^+, K^+, and Cs^+, but exhibited a significantly lower permeability to Li^+ and, in the latter system, there was also a distinct permeability to NMDG (P_{Na}/P_{NMDG}=0.56; Chan and Nelson 1992; Nelson et al. 1996). In contrast, in the M1 cell-line the channel appeared to be equally permeable to Na^+, K^+, Cs^+, Li^+, and Rb^+ but virtually impermeable to NMDG (Korbmacher et al. 1995; Volk et al. 1995). In addition, there appear to be significant differences in the relative permeabilities of amiloride-insensitive channels to Cl^- with P_{Na}/P_{Cl} values in the range of 60 (Korbmacher et al. 1995) to 1.7 (Nelson et al. 1996). In some systems, the hypertonic activation of channels is Ca^{2+}-independent (Chan and Nelson 1992) whereas, in others, channel activity appears to require a minimum $(Ca^{2+})_i$ of 1 μMol/l (Korbmacher et al. 1995; Koch and Korbmacher 1999). Nevertheless, these channels do not appear to conduct significant amounts of divalent cations (Korbmacher et al. 1995; Koch and Korbmacher 2000). The unitary conductances of hypertonicity-induced but amiloride-insensitive channels generally is in the range of 15 pS to 27 pS (Korbmacher et al. 1995; Koch and Korbmacher 1999).

Most interestingly, at least some of these amiloride-insensitive nonselective cation channels are typically inhibited by cytosolic ATP concentrations in the millimolar range (Koch and Korbmacher 1999). This raises some concerns as to their actual role in RVI because, under most physiological conditions these channels will remain silent. Also of note in this respect, in a recent study on isolated rat colonic crypt cells, there was no detectable activation of nonselective cation channels under hypertonic stress (Weyand et al. 1998), whereas these cells are known to express Ca^{2+}-activated channels which become detectable in excized inside-out patches and which are very similar to those observed in HT29 cells (Bleich et al. 1996). Clearly, whereas nonselective and amiloride-insensitive cation channels appear to be ubiquitously expressed, their actual contribution to cell volume regulation remains to be elucidated.

In principle, the activation of cation conductances—either sensitive or insensitive to amiloride—that do not discriminate much between Na^+ and K^+ will lead to both Na^+ influx as well as K^+ efflux. Because of the inside negative membrane voltage, however, overall cation gain will significantly exceed overall cation loss equivalent to an increase of the overall intracellular osmotic activity.

K^+ channels

In most systems, the continuous channel-mediated K^+ leakage out of cells is equivalent to a significant loss of cellular osmolytes. This K^+ loss is commonly compensated for by the activation of Na^+, K^+-ATPase. Nevertheless, it is rather obvious that any inhibition of K^+ channels under hypertonic conditions will per se facilitate RVI and, in some cells, this mechanism appears to be an important mediator of volume regulation.

An inhibition of K^+ conductance was originally reported for the basolateral membrane of toad and rabbit urinary bladder (Lewis et al. 1985; Donaldson et al. 1989), as well as of frog skin (Costa et al. 1987; Leibowich et al. 1988) where cells were shrunken by extracellular Cl^- removal (leading to a loss of Cl^- because of the lower membrane permeability of the Cl^- substitute used when compared to Cl^- itself) and/or by increasing extracellular osmolarity. In addition, hypertonic stress reduced basolateral K^+ conductance in rabbit proximal tubule (Lapointe et al. 1990; Macri et al. 1997) and human nasal epithelium (Willumsen et al. 1994) and it decreased conductive K^+ loss in MDCK cells (Ritter et al. 1991). In mouse liver, a reduction of cell membrane K^+ conductance appeared to be the main mechanism of RVI (Graf et al. 1988; Wang and Wondergem 1991) and a transient inhibition of K^+ channels may contribute to the volume response of rat hepatocytes (Wehner et al. 1995; Wehner and Tinel 1998). Interestingly, whole-cell recordings on freshly isolated hippocampal neurons revealed a decrease of voltage-gated K^+ currents under hypertonic conditions (Huang and Somjen 1997). In dissociated rabbit corneal epithelial cells, a large conductance K^+ channel (167 pS in symmetrical 150 mMol/l KCl) could be identified that appeared to mediate the decrease in whole-cell K^+ conductance to 44% of control upon change to 130% extracellular osmolarity (Farrugia and Rae 1993). In a recent study on isolated rat colonic crypts, it was found that hypertonic stress (+50 mOsm/l and +100 mOsm/l) led to membrane depolarizations by 12 mV and 22 mV, respectively, coinciding with decreases in whole-cell conductance to 70% and 50% of control (Weyand et al. 1998). On the molecular level, these effects appeared to be correlated to a 16 pS K^+ channel that experienced decreases in activity ($N \cdot P_o$) to 47% and 44% when compared to isotonic conditions. Of note, this reduction of channel activity was most likely triggered by a hypertonicity-induced decrease of $(Ca^{2+})_i$. The channel was inhibited by (10^{-4} Mol/l) Ba^{2+} and (10^{-9} Mol/l) charybdotoxin (Bleich et al. 1996).

Anion channels

Inhibition of anion channels during RVI might directly contribute to the gain in osmolytes if the intracellular anion (Cl^- or HCO_3^-) activity is above electrochemical equilibrium. Indirectly, it restricts the movement of the counterion for K^+ flux across the membrane, thus impeding K^+ losses.

Decreases of Cl^- conductance under hypertonic conditions have been reported only from a limited number of preparations. In cultured human nasal epithelium, hypertonic stress reduced apical Cl^- conductance (Willumsen et al. 1994) whereas, in rabbit collapsed proximal convoluted tubules, the partial Cl^- conductance of the basolateral membrane was inhibited (Macri et al. 1997). In some instances, the decrease of Cl^- conductance under hypertonic conditions may reflect an inhibition of hypotonicity-induced Cl^- channel activation. In human vas deferens cells in primary culture, for example, with 290 mOsm/l solutions in the experimental bath, as well as in the patch pipette, there was a slowly devel-

oping increase of Cl^- conductance once the whole-cell configuration was achieved; this effect could be reversed by an increase of extracellular osmolarity (Winpenny et al. 1996). The increase of Cl^- conductance was interpreted to be due to the additional osmotic activity of intracellular macromolecules and the resultant swelling of cells.

Organic osmolytes in RVI

In most cells investigated, RVI after exposure to a hypertonic extracellular medium occurs within minutes and involves the net uptake of inorganic osmolytes. During this short-term regulation of cell volume, changes in organic osmolytes transport are generally not involved, although it has been observed that the plasma membrane permeability for organic osmolytes such as sorbitol (e.g., in IMCD cells grown at 600 mOsm/l and exposed to 900 mOsm/l medium) decreases (Bagnasco et al. 1988; Grunewald and Kinne 1989). Thus, leak pathways for organic osmolytes are downregulated to make the intracellular accumulation of organic osmolytes—by transporters or metabolic synthesis—more effective. Whether these leak pathways are identical to the ones activated during RVD remains to be investigated.

If exposure of cells to hypertonicity is extended to periods of hours or days, adaptive changes take place aimed to replace the augmented intracellular inorganic electrolytes by organic osmolytes. To this end, intracellular synthesis is increased (sorbitol and GPC) or intracellular breakdown is decreased (GPC; for review see Burg 1995; Burg et al. 1997). For taurine, betaine, and myo-inositol, the predominant effect is on the rate of uptake, which increases severalfold in a matter of hours (see Fig. 3).

Thus, strictly speaking, organic osmolytes are not directly involved in RVI; they serve, however, in the long run to maintain cellular electrolyte homeostasis. Therefore, the cellular uptake systems for organic osmolytes and their regulation are described in the following paragraphs.

Na^+-Cl^--taurine symport

Taurine, a β-amino acid with a sulfonic acid instead of a carboxylic acid as head group occurs in plasma at concentrations of 40 μMol/l. It has several major functions: as partner in the formation of taurocholate, as neurotransmitter (Jacobsen and Smith 1968; Curtis and Johnston 1974), and as an organic osmolyte. Although several tissues, including liver and astrocytes, can synthesize taurine from cysteine, the high intracellular concentration (e.g., 20 mMol/l in IMCD cells) can only be achieved by an active uptake into the cell (Ruhfus et al. 1998). The transporter mediating this uptake is a secondary active sodium-chloride taurine cotransport system in which up to three sodium ions are translocated with one chloride ion and one taurine molecule. Taurine transport with these characteristics has been found in renal (Chesney et al. 1985a; Kinne et al. 1998; Wolff and Kinne 1988; Zelikovic et al. 1989; Zelikovic and Budreau-Patters 1999) and intestinal brush border (O'Flaherty et al. 1997), in liver cells (Warskulat et al. 1997a, Warskulat et al. 1997b; Peters-Regehr et al. 1999), in H4IIE hepatoma cells (Warskulat et al. 1997b), Ehrlich ascites tumor cells (Hoffmann et al. 1988), astrocytes (Beetsch and Olson 1996), the retinal pigment epithelium (Handler and Kwon 2001), bovine aortic endothelial cells (Qian et al. 2000), renal IMCD cells, and MDCK cells (Uchida et al. 1991). The apparent affinity

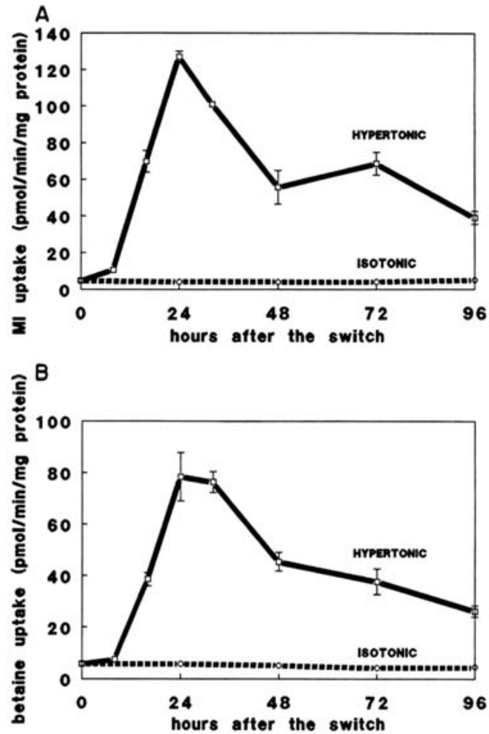

Fig. 3 Time course of basolateral *myo*-inositol (**A**) and betaine (**B**) uptake into MDCK cells switched into hypertonic medium. On day 0, cells cultured on filters in defined medium with 10% FBS were switched to same medium made hypertonic (500 mOsm/l) by addition of raffinose. Isotonic cells were maintained in isotonic defined medium with 10% FBS. Uptake was performed at 37°C for 30 min with 10 μMol/l *myo*-inositol and 10 μMol/l betaine. Results are means±SD to triplicate samples (from Yamauchi et al. 1991)

(K_m) for taurine is in the range of 10 μM to 100 μM (Uchida et al. 1991). The transporter also transports β-alanine and, with a lower affinity, γ-amino butyric acid (GABA). Molecular cloning from MDCK cells revealed that the transporter corresponds to a protein with 655 amino acids with a relative molecular mass of 74 kDa and probably has twelve transmembrane helices (Uchida et al. 1992). It shows significant amino acid sequence similarity to the MDCK cell betaine/GABA transporter and other Na^+, Cl^--dependent neurotransmitter transporters (Guastella et al. 1990). In dog tissues, the order of mRNA abundance for this transporter (dTAUT) was kidney cortex $\approx$ kidney medulla > ileal mucosa > brain > liver > heart > epididymis. Taurine transporters cloned from other sources such as LLC-PK1 cells from pig kidney, human retinal pigment epithelium, thyroid, placenta, and bovine aortic endothelial cells (Qian et al. 2000) were of similar size (621 and 620 amino acids, respectively) and have a high amino acid sequence similarity.

In a variety of cells and tissues such as MDCK cells (Uchida et al. 1991), human and bovine lens epithelial cells (Cammarata et al. 2002), bovine aortic endothelial cells (Qian et al. 2000), rat liver macrophages (Warskulat et al. 1997a), H4IIE hepatoma cells (Warskulat et al. 1997b), primary cultures of rat hepatocytes (Warskulat et al. 1997a), rat astrocyte cultures (Beetsch and Olson 1996), and human Caco-2 cells (Satsu et al. 1999), exposure to hyperosmotic media results in an increase in V_{max} of taurine transport and a concomitant increase in transporter mRNA (Burg et al. 1997; Handler and Kwon 2001; Cammarata et al. 2002).

High affinity taurine transport is inhibited by phosphorylation via PKC by decreasing affinity for taurine and sodium and reducing maximal velocity (Kulanthaivel et al. 1991;

Brandsch et al. 1993; Nakamura et al. 1996; Mollerup and Lambert 1998; Lima et al. 2000; Qian et al. 2000). Furthermore, taurine uptake adaptively decreases when taurine availability is augmented (Chesney et al. 1985b; Han et al. 1998). Although these regulatory processes appear not to be linked to the response to hypertonicity, they complicate the study of the molecular mechanisms of the latter. However, it can be assumed that the mechanisms involved in osmotic adaptation are similar to the ones described below for the betaine transport system, since both systems respond in a very comparable manner.

Na^+-Cl^--betaine symport

In medullary kidney cells and chondrocytes, which use betaine in volume regulation, intracellular concentrations of up to 50 mMol/l are found. Although cells usually contain choline dehydrogenase that catalyses the synthesis of betaine from choline, most of the betaine is taken up from the extracellular medium. The same holds for MDCK (Yamauchi et al. 1991) and PAP-HT25 cells (Ferraris et al. 1996). The uptake of betaine is dependent on the presence of both sodium and chloride and the transport system (Yamauchi et al. 1992; Moeckel et al. 1997) has been identified to belong to the family of brain GABA and noradrenaline transporters (Guastella et al. 1990).

Initial cloning of the transporter from MDCK cells revealed a protein of 614 amino acids with a molecular weight of 69 kDa. Its proposed membrane topology predicts 12 transmembrane helices and an intracellular NH_2 and COOH terminus (Yamauchi et al. 1992). When expressed in oocytes, the protein showed both betaine and γ-amino-n-butyric acid (GABA) transport activities that were chloride- as well as sodium-dependent (Matskevitch et al. 1999; Forlani et al. 2001). Because of these transport properties and the 43%–49% identity in the amino acid sequence (Rasola et al. 1995) with the rat brain GABA transporter and the human brain noradrenaline transporter, it was named betaine-GABA-transporter (BGT1).

Consecutive studies showed that the gene extends over 28 kilobases and consists of 18 exons. In addition, the 5′ end of the gene has three different first exons, thus a complex mixture of mRNAs exists. Three main types of mRNA (A, B, and C) can be distinguished that differ considerably in their 5′ untranslated sequences. Each type is expressed in a tissue-specific manner: kidney medulla (and MDCK cells) contain all three types, brain and kidney cortex, type B and C, and liver only type A (Takenaka et al. 1995; Burg et al. 1997).

Betaine transport shows apparent affinities (K_m) of 0.3 mMol/l–0.5 mMol/l in MDCK cells, and in oocytes that express BGT1, the affinity for GABA is at or below 0.1 mMol/l. At 1 mMol/l substrate, the current induced in BGT 1-expressing oocytes decreased in the following order: betaine > GABA > diaminobutyric acid = β-alanine > proline. At 1 mMol/l betaine, the affinity for sodium was 93.3 mMol/l and the affinity for chloride 76.1 mMol/l (Yamauchi et al. 1992).

In electrophysiological studies, a coupling ratio of Na/Cl/betaine of 3:2:1 was found. Furthermore, these studies revealed significant kinetic differences compared to the neuronal GABA-transporter GAT-1 (Matskevitch et al. 1999).

For the symport process, a transport model of ordered binding was proposed (Matskevitch et al. 1999; Forlani et al. 2001) in which betaine binds first to the extracellular side of a transporter. Sodium binding occurs thereafter, followed by chloride binding. Translocation of betaine and sodium is already observed in the absence of chloride, but chloride

augments the translocation. As for other cotransport systems, this model would predict that betaine facilitates sodium and chloride binding to the transporter; corresponding increases in the transporter affinities at higher betaine concentrations have been observed. The exact mechanism of chloride (and sodium and betaine) translocation are unknown to date. It should be mentioned, however, that in squid motor neurons betaine activates a large Cl^- selective current which is sodium-dependent (Petty and Lucero 1999). This observation raises the possibility that the betaine transporter might posses chloride channel properties.

Augmented extracellular osmolality increases betaine uptake into MDCK cells (Nakanishi et al. 1990; Yamauchi et al. 1991; Kempson 1998), rabbit PAP-HT 25 cells (renal papillary epithelium; Ferraris et al. 1996), and porcine chondrocytes (de Angelis et al. 1999) at the level of the transporter by increasing V_{max} but not K_m of betaine uptake (see Fig. 3). The increase in uptake is preceded by an enhanced transcription of BGT1 in MDCK cells followed by increases in BGT1-mRNA. As in the case of the sodium-myo-inositol transporter a "tonicity-responsive enhancer element" (designated TonE) of the gene is involved that spans −69/−50 of the sequence (Burg et al. 1997; Handler and Kwon 2001). In transgenic mice, the 5′-flanking region which contains TonE also mediates osmotic regulation of transcription in vivo (Handler and Kwon 2001). Osmoregulation of BGT1 mRNA has also been observed in renal medulla in the intact rat (Miyai et al. 1996; Moeckel et al. 1997), and in rat hepatic stellate cells (Peters-Regehr et al. 1999). Although the time course and extent of regulation of BGT-1 and SMIT by hypertonicity are quite similar, the regulatory pathways may be different at one point or another (Atta et al. 1999)

In addition to the relatively slow adaptation of betaine transport in vitro, more rapid changes have also been reported. For example, in mouse isolated perfused straight proximal tubules, basolateral betaine uptake (measured as substrate-dependent depolarization) is enhanced when the extracellular osmolality is increased (Völkl and Lang 2001). The speed of regulation suggests nongenomical processes. A similar rapid activation was observed in squid motor neurons (Petty and Lucero 1999). Whether these responses involve protein phosphatases that, potentially by dephosphorylation, could stimulate betaine transport is not clear.

Hypertonic activation of betaine uptake shows some more peculiarities. Under isotonic conditions betaine uptake into MDCK cells proceeds only across the basolateral membrane; after exposure to hypertonicity basal-lateral uptake is increased, but in addition, significant uptake across the apical membrane is observed (Yamauchi et al. 1991). This finding might be related to the existence of various isoforms of BGT1 (see above) that might differ in their cellular location.

Na^+-Myo-inositol symport

Physiological plasma concentrations of myo-inositol range in mammals from 4.5 mMol/l to 6.6 mMol/l, whereas intracellular concentrations up to 133 mMol/l can be found in rat glial (Strange et al. 1991) and renal medullary cells (Bagnasco et al. 1986; Nakanishi et al. 1988; Wirthensohn et al. 1989; Yancey and Burg 1989; Garcia-Perez and Burg 1991; Sizeland et al. 1993; Grunewald et al. 1995; Grunewald and Kinne 1999; Handler and Kwon 2001). This large concentration difference suggests active uptake of myo-inositol into these cells. Similarly, active myo-inositol uptake is observed in rat pancreatic islets, bovine lens epithelial cells, hamster small intestine, rat mesanglial cells, rat hepatocytes, crystalline lense, rabbit peripheral nerve, retinal pigment epithelial cells, rabbit ciliary

body, isolated rat Schwann cells, and endothelial cells (for references see Porcellati et al. 1999). This uptake process involves a sodium/myo-inositol symport system (SMIT) which as secondary active transport system employs the transmembrane electrochemical potential difference of sodium for intracellular accumulation of myo-inositol. Apparent K_m values for myo-inositol range from 30 μMol/l in MDCK cells (Kwon et al. 1992) to ~94 μMol/l in rabbit renal brush border (Hammerman et al. 1980) and 104 μMol/l in rabbit TALH cells (Yorek et al. 1999). Interestingly, also D-glucose is a substrate of this transporter (Hammerman et al. 1980; Hager et al. 1995). Phlorizin, the well-known inhibitor of the sodium-D-glucose cotransporter, also inhibits the sodium-myo-inositol transporter (K_i <60 μMol/l; Strange et al. 1991; Hager et al. 1995). Similarities between the two transporters were also detected at the DNA and protein level. There is 46% amino acid identity overall and apparently similar membrane topology of the two transporters; SMIT is a polypeptide of 718 amino acids with a relative molecular mass of 79.5 kDa (Kwon et al. 1992).Tissue abundance of mRNA in dog tissues is kidney medulla > kidney cortex > brain (Kwon et al. 1992). The SMIT cloned recently from bovine lens epithelial cells shows a 92% identity with the MDCK cell transporter (Zhou et al. 1994).

The sodium to myo-inositol stoichiometry of the transporter is 2:1 (Hager et al. 1995). The stoichiometry and electrogenicity of the transporter forms the basis for the extensive intracellular accumulation of myo-inositol. Kinetic and biochemical characteristics vary from tissue to tissue and cell to cell. These variations have recently been explained by the existence of alternate splicing, which generates isoforms that differ in their intracellular protein kinase A and protein kinase C phosphorylation sites at the carboxy terminus (Porcellati et al. 1999). In epithelial cells, the cellular location can also differ; thus, in the renal proximal tubule the Na^+/myo-inositol symport is present in the luminal brush border membrane, whereas in more distal renal cells the basolateral location prevails (see Grunewald and Kinne 1999).

SMIT is increased by augmentation of the extracellular osmolality in MDCK cells (Nakanishi et al. 1989; Yamauchi et al. 1991), glial cells (Strange et al. 1991; Kwon et al. 1992), neuronal cells (Yamashita et al. 1999), mesothelial cells (Matsuoka et al. 1999), bovine lens epithelial cells (Cammarata et al. 2002), and human retinal epithelial cells (Handler and Kwon 2001). In MDCK cells grown on filters, a 25-fold increase in uptake rate was observed within 24 h after exposure of the cells to hypertonic media (Yamauchi et al. 1991).

After restoring isotonicity, the transport rate returns to normal levels within 1 d. In both instances only the V_{max} of the transport is affected and K_m remains constant, suggesting a change in the number of transporters (Nakanishi et al. 1989). In similar studies in rat C6 glioma cells, myo-inositol uptake also increased after exposure of the cells to 440 mOsm/l solutions; however, the time course was slower and maximum uptake was obtained after 48 h (Strange et al. 1991). The increased uptake in response to hypertonicity is preceded by an increased abundance of mRNA for the transporter, which is the direct result of increased transcription of the gene (Yamauchi et al. 1993). Activation of transcription depends on an enhancer element named tonicity responsive enhancer (TonE). The SMIT-gene is regulated by multiple TonEs in its 5′-flanking region (Burg et al. 1997; Zhou and Cammarata 1999; Handler and Kwon 2001). An increase in SMIT m-RNA is also found in a TALH cell line derived from rabbit renal medulla (Yorek et al. 1999) and in mesothelial cells (Matsuoka et al. 1999). In the latter cell line, the first significant increase in mRNA could be observed already 1 h after exposure of the cells to 490 mOsm. The increase in transporter mRNA has also been observed under in vivo conditions, for example, in rat in-

ner medullary collecting duct cells when d DAVP was administered to chronically diuretic rats, a maneuver that rapidly increases the extracellular osmolality in the medulla (Burger-Kentischer et al. 1999). Injection of NaCl into rats increases SMIT-mRNA significantly within 1 h. Comparing the experiments in intact rats with cultured cells, the transporter mRNA seems to respond more rapidly in vivo than in vitro (Yamauchi et al. 1995).

Amino acid transport system A

Amino acid transport system A (System A), which mediates sodium-dependent uptake of neutral amino acids into mammalian cells, appears to be also subject to regulation by extracellular osmolality. In MDCK cells, as well as chondrocytes, an upregulation within 4–6 h of hypertonic exposure was observed, well before any change in BGT1 activity (Chen et al. 1996a; de Angelis et al. 1999; Horio et al. 1997; Kempson 1998). The increase in System A is blocked by inhibitors of RNA and protein synthesis, suggesting that an increase in the number of transporters is part of the mechanism (Kempson 1998). The adaptation of this system appears to be a relatively early response of cells exposed to hypertonicity; therefore, it deserves further investigation.

Regulatory volume decrease

Inorganic osmolytes in RVD

After cell swelling, transport systems are immediately activated that mediate the release of the major intracellular inorganic osmolytes potassium and chloride. Their transmembrane movement occurs either via separate pathways or directly coupled to each other.

K^+ channels

Due to the outwardly directed K^+ gradient in most animal cells, any increase of K^+ channel activity will augment the conductive exit of K^+. In addition, the increase of cell membrane K^+ conductance will hyperpolarize the cell membrane and (even if basal Cl^- conductance is not changing) this hyperpolarization will favor conductive Cl^- efflux. Likewise, if there is an initial increase of Cl^- conductance and if cell Cl^- is above electrochemical equilibrium (as it is in most systems) this will augment Cl^- exit and depolarize the cell membrane. Membrane depolarization in turn will facilitate conductive K^+ efflux. The most effective mechanism of RVD will, of course, be the parallel activation of K^+ and Cl^- channels. Because of the pronounced voltage-mediated coupling between both pathways conductive K^+ and Cl^- release may result in a quasielectroneutral mode of KCl export.

An increase of cell membrane K^+ conductance under hypotonic conditions and its significance for RVD have been reported from a variety of preparations (see Lang et al. 1998a; Lang et al. 1998b for comprehensive reviews). In many studies, these mechanisms were analyzed by means of $^{86}Rb^+$ (or $^{42}K^+$) fluxes, intracellular microelectrode recordings and rapid ion-substitutions, whole-cell patch-clamp measurements, and by use of specific K^+ channel blockers. Here, we will focus on data at the molecular level that were obtained

by means of single-channel recordings and by the cloning and expression of some of the volume-activated K^+ channels.

BK_{Ca} or maxi-K^+ channels exhibit large (*big*) unitary conductances in the range of some 100 pS to 250 pS and show under symmetrical high K^+ solutions a linear current-to-voltage relation (see Vergara et al. 1998 for review). In most instances, BK_{Ca} channels are inhibited by Ba^{++}, quinine, and TEA (tetraethylammonium), as well as by (the scorpion peptide) charybdotoxin. They are selectively blocked by (the scorpion toxin) iberiotoxin. BK_{Ca} channels are found in neurons, skeletal muscle, smooth muscle, and in epithelial cells, where they reside in the apical membrane. BK_{Ca} channels are activated by membrane depolarization and micromolar concentrations of cell Ca^{2+}. It is this Ca^{2+} sensitivity that might function as a link to a swelling-induced increase in intracellular calcium. Likewise, the voltage-dependence of BK_{Ca} channels may reflect the coupling mechanism to a hypotonicity-induced activation of Cl^- channels (see above).

BK_{Ca} channels consist of hetero-oligomeric complexes of the pore-forming α-subunits (first cloned from Drosophila as d*Slo* (Atkinson et al. 1991; Adelman et al. 1992) and the auxiliary β-subunits (Garcia-Calvo et al. 1994; Behrens et al. 2002). The α-subunits are expressed in multiple splice variants generating functional diversity among different cells, but they exhibit a high degree of homology among species. Their membrane topology is related to that of voltage-gated K^+ channels (see below; Toro et al. 1998; Vergara et al. 1998; Jensen et al. 2001). They contain seven transmembrane segments (so that the N-terminus is most likely extracellular), a positively charged TM4-segment (that is probably part of the voltage sensor), and four additional hydrophobic segments forming a unique secondary structure at the C-terminal cytosolic tail that appears to mediate Ca^{2+} binding (Schreiber and Salkoff 1997).

The β-subunit is likely to function as an additional modulator of channel characteristics, e.g., with respect to Ca^{2+} sensitivity, but it does not appear to be an obligatory component of all BK_{Ca} channels. Four β-subunits have been cloned that are mainly expressed in smooth muscle, endocrine cells, epithelia, and the central nervous system (Behrens et al. 2002). β-subunits have a proposed topology of spanning the membrane twice, with N- and C-termini inside the cell (Toro et al. 1998; Vergara et al. 1998).

The activation of BK_{Ca} channels under hypotonic conditions could be demonstrated in *Necturus* and rabbit proximal tubule cells (Dubé et al. 1990; Filipovic and Sackin 1991; Kawahara et al. 1991), in principal cells of rabbit and rat cortical collecting tubule (Ling et al. 1992; Schlatter 1993; Stoner and Morley 1995; Hirsch and Schlatter 1997), in clonal kidney (Vero) cells derived from African green monkey (Hafting and Sand 2000), in acinar cells of rat lacrimal gland (Park et al. 1994), in embryonic chick hepatocytes (Pon and Hill 1997), and in guinea pig jejunal villus enterocytes (MacLeod and Hamilton 1999b). In the A_3 cell line derived from rabbit medullary thick ascending limb, hypotonic stress, as well as negative pressure applied to the patch pipette (suction), significantly increased the open probability of BK_{Ca} channels (Taniguchi and Guggino 1989). Comparable effects were observed in intercalated cells of rat cortical collecting tubule (Pácha et al. 1991), in the human colonic cell line CaCo-2 (Bear 1991), and in vascular smooth muscle cells (Kirber et al. 1992).

These results suggest a direct effect of membrane stretch on the channel itself or on a closely associated component as a mode of channel regulation. Also of note is the distinct sensitivity of BK_{Ca} channels to extracellular ATP as it was observed in Vero cells (Hafting and Sand 2000). Thus, ATP release may well function as an autocrine (or paracrine) mech-

anism of hypotonic channel activation. In addition, a variety of protein kinases and phosphatases appear to have an impact on BK_{Ca} channel activity (Levitan 1994; Vergara et al. 1998; Hafting and Sand 2000) but their precise role in channel regulation remains to be elucidated.

IK_{Ca} channels are activated by cytosolic Ca^{2+} activities in the upper nanomolar range and exhibit an *intermediate conductance* that (at 0 mV) equals 20 pS–80 pS. They are voltage-independent but weakly inwardly rectifying (under symmetrical high K^+ conditions), (see Vergara et al. 1998; Jensen et al. 2001 for review). The apparently large range of unitary conductances reported for these channels is in part due to differences in experimental design and the very pronounced dependence of these channels on the actual (extracellular) K^+ activity. IK_{Ca} channels are weakly inhibited by quinine but efficiently blocked by charybdotoxin and (more selectively) by clotrimazole. The latter compound was found to have a high affinity to the "Gardós channel" (this channel is the IK_{Ca} of erythrocytes and actually the first Ca^{2+}-dependent K^+ transport described; Gardos 2002) and was therefore considered to be of potential use for the treatment of sickle cell anemia (Jensen et al. 2001). IK_{Ca} channels are virtually insensitive to the bee venom toxin apamin, which discriminates them from most isoforms of SK_{Ca} channels (see below; Jensen et al. 2001). Interestingly, IK_{Ca} channels are selectively activated by the channel modulator 1-EBIO (1-ethyl-benzimidazolinone; Jensen et al. 2001).

The first IK_{Ca} channel was cloned from human tissues (*KCNN4*; Ishii et al. 1997; Joiner et al. 1997; Logsdon et al. 1997; Jensen et al. 1998) and shortly thereafter the orthologues of mouse and rat followed (Warth et al. 1999; Vandorpe et al. 2002). The human IK_{Ca} channel is 88% and 90% identical to the mouse and rat channel, respectively, and 40% to 42% identical to SK_{Ca} channels. The short cytosolic N-terminus is followed by six transmembrane segments and a long intracellular C-terminus (Vergara et al. 1998; Jensen et al. 2001). The channel pore is most probably located in a hydrophobic pocket between TM5 and TM6 and the channel is likely to function as a homotetramer. The Ca^{2+} sensitivity of IK_{Ca} channels appears to be mediated by calmodulin binding to a C-terminal domain. Using Northern blot analyses, IK_{Ca} channels were mainly detected in tissues rich in epithelia and endothelia (Jensen et al. 2001). This is of note because of the known high rates of vectorial transport in such systems and the resultant need for an effective osmotic cell homoiostasis.

There are several reports which identify this type of channel as the mediator of conductive hypotonicity-induced K^+ release. In the human epithelial cell line Intestine 407, hypotonic swelling led to a significant increase of cell Ca^{2+}, thereby activating IK_{Ca} channels (Okada et al. 2001). Human T lymphocytes express a Ca^{2+}-activated K^+ conductance that was activated by hypotonic conditions (Khanna et al. 1999). The K^+ conductance was slightly inwardly rectifying and blocked by charybdotoxin as well as clotrimazole. In transformed Madin-Darby canine kidney (MDCK-F) cells, hypotonic stress increased cell Ca^{2+} and activated a 53 pS K^+ channel that was inwardly rectifying (Schwab et al. 1993) and blocked by charybdotoxin, Ba^{2+}, and TEA (Schwab and Oberleithner 1996; Schwab et al. 1999; Schneider et al. 2000). Because MDCK-F cells employ cell volume regulatory mechanisms for locomotion, cell migration could be shown to be inhibited with an identical pharmacological profile (Schwab and Oberleithner 1996). Moreover, the channel appeared to be activated by 1-EBIO (Schwab et al. 1999). In the distal nephron cell line A6 derived from *Xenopus* kidney, cell swelling activated a K^+ channel that (in symmetrical 130 mMol/l K^+) was slightly inwardly rectifying with a unitary conductance of 29 pS (at

0 mV) and inhibited by quinine (Nilius et al. 1995). It is tempting to speculate that the channel may be Ca^{2+}-sensitive because, in A6 cells, hypotonic stress was shown to elicit marked increases of cell Ca^{2+} that paralleled RVD and the activation of a TEA-sensitive K^+ conductance (Yu and Sokabe 1997; Urbach et al. 1999).

In some systems, the putative role of IK_{Ca} channels in the RVD process is not yet conclusively defined. In MDCK cells for instance, hypotonic stress was reported to transiently activate K^+ channels that were Ca^{2+}-sensitive and in cell-attached patches (with 150 mMol/l K^+ in the pipette); these channels were inwardly rectifying with a unitary conductance of 29 pS at 0 mV (Weiss and Lang 1992). From the same system, Banderali and Roy (Banderali and Roy 1992a) reported a Ca^{2+}-sensitive channel that showed a significant increase of open probability upon hypotonic cell swelling as well and that was blocked by quinine (Roy and Banderali 1994). In symmetrical 145 mMol/l K^+, however, the channel clearly exhibited a linear current-to-voltage relation with a unitary conductance of 24 pS (Banderali and Roy 1992a; Roy and Banderali 1994). In an early report on Ehrlich ascites tumor cells, RVD was found to involve the activation of an inwardly rectifying K^+ channel that (under symmetrical high K^+ conditions) exhibited unitary conductances of 40 pS and 15 pS in the negative and positive voltage range, respectively. The channel appeared to be Ca^{2+}-activated and its open probability did not depend on membrane voltage (Christensen and Hoffmann 1992). In a more recent study on the same preparation, however, Ca^{2+} signaling did not seem to play any significant role in RVD at all, and hypotonicity-induced K^+ release was found to be inhibited by Ba^{2+} but clearly not by charybdotoxin or clotrimazole (Jorgensen et al. 1997). In some other systems, cell swelling activates K^+ channels of intermediate conductance that apparently are not related to IK_{Ca}. In *Necturus* proximal tubule for instance, cell-attached patches on the basolateral membrane revealed the presence of a K^+ channel that could be activated by negative pressure as well as by a reduction in bath osmolarity (Sackin 1989). There was no detectable voltage dependence of channel gating and the channel did not appear to be Ca^{2+}-sensitive. Under symmetrical high K^+ conditions (70 mMol/l), the channel exhibited a linear current-to-voltage relation and a conductance of 36 pS (Filipovic and Sackin 1992). In cell-attached patches on *Xenopus* proximal tubule, a basolateral K^+ channel was observed that was activated by negative pressure and blocked by Ba^{2+} and was supposed to be involved in RVD (Kawahara 1990). With 100 mMol/l K^+ in the pipette, the channel showed little if any inward rectification with a unitary conductance of 28 pS and it did not appear to be Ca^{2+}-sensitive.

SK_{Ca} channels under symmetrical high K^+ conditions exhibit a *small conductance* of some 4 pS–18 pS (at 0 mV) and are activated by nanomolar concentrations of Ca^{2+}. They are inwardly rectifying and voltage-independent (see Vergara et al. 1998 for review). In electrically excitable cells, SK_{Ca} channels mediate the slow after-hyperpolarization following action potentials. SK_{Ca} channels have been recently cloned from—or identified in—rat brain and colon (rSK1–3 and rSK4, respectively), as well as human brain (hSK1) and placenta (hSK4; Köhler et al. 1996; Warth et al. 1999; Okada 1997; Joiner et al. 1997). The topology of SK_{Ca} channels is similar to that of voltage-gated K^+ channels with six transmembrane helices, an intracellular C- and N-terminus, positively charged residues in the TM4 segment, and the pore-forming region between TM5 and TM6. Primary sequences, however, are significantly different. The channels are likely to function as homotetramers, and gating of SK_{Ca} channels involves an interaction of calmodulin with C-terminal domains. SK_{Ca} isoforms differ markedly with respect to their sensitivity to apamin: SK2 is highly sensitive to the bee venom toxin (with a K_d of 60 pMol/l for rSK2) whereas SK1

and SK4 are not significantly inhibited in concentrations up to 100 nMol/l; SK3 exhibits an intermediate sensitivity (Vergara et al. 1998). The sensitivity to apamin is defined by a distinct group of amino acids in the deep pore of the channels, which also determine channel sensitivity to D-tubocurarine, an additional, quite selective blocker. In a recent study, a full-length 2.1 k-bp cDNA (hSK2) highly homologous to rat brain rSK2 was isolated from a human liver cDNA library and identical cDNAs were obtained from human primary hepatocytes, human HuH-7 hepatoma cells, and human Mz-ChA-1 cholangiocarcinoma cells (Roman et al. 2002). Stable transfection of CHO cells with hSK2 resulted in the expression of an apamin-sensitive K^+ conductance as revealed by whole-cell patch-clamp recordings. Thus far, there is only limited evidence for the involvement of SK_{Ca} in volume regulation. Although in Mz-ChA-1 cells hypotonic stress led to a prominent increase of K^+ conductance, and this effect as well as RVD was partially inhibited by apamin (at 50 nMol/l and 100 nMol/l, respectively), Ba^{2+} (at 5 mMol/l) exhibited a complete block of RVD, suggesting that additional K^+ channels contribute to this process (Roman et al. 2002).

Two members of *voltage-gated* K^+ channels could be defined as mediators of RVD, namely Kv1.3 and Kv1.5. They belong to the group of delayed rectifier channels that were first cloned from (electrically) excitable tissues (Stühmer et al. 1989; Tamkun et al. 1991) where they stabilize resting potential and mediate the rapid repolarization of action potentials. K^+ channels of the Kv group typically exhibit six transmembrane helices, an intracellular C- and N-terminus, and a positively charged TM4 segment that most likely is part of the gating machinery. Kv channels were found to be also expressed in electrically nonexcitable cells such as T-lymphocytes (Lewis and Cahalan 1995). There is increasing evidence suggesting that they play a role in cell volume regulation (Felipe et al. 1993; Lewis and Cahalan 1995). It was found, for instance, that a mouse T-lymphocyte cell-line (CTLL-2) that does not express voltage-dependent K^+ channels did not exhibit any significant RVD under hypotonic conditions. Transient transfection of these cells with Kv1.3, however, reconstituted their capability of an almost complete volume regulatory response and led to the generation of significant voltage-activated K^+ currents (Deutsch and Chen 1993). Moreover, the RVD was blunted in the presence of 50 nMol/l charybdotoxin. In a study on human T-lymphocytes both hSK2 (see above) and Kv1.3 were found to contribute to K^+ conductance and RVD (Khanna et al. 1999). In this investigation, the contribution of Kv1.3 was quantified in a differential approach by use of the specific blocker (scorpion toxin) margatoxin (applied at 5 nMol/l). Stable transfection of a mouse fibroblast cell line (Ltk^-) with Kv1.5 prevented isotonic cell swelling (that was elicited by the application of dexamethasone) and elicited sizeable voltage-activated K^+ currents (Felipe et al. 1993). Both effects of channel expression were blocked by 60 μMol/l quinine.

The I_{sK} protein was first cloned from rat kidney by use of the *Xenopus* oocyte expression system (Takumi et al. 1989). Its most remarkable feature is its *small size* of 126–130 amino acids (depending on the species) with a single transmembrane α–helical domain (Busch and Maylie 1993; Busch and Suessbrich 1997). In various systems, expression of I_{sK} elicited a K^+ conductance upon membrane depolarization that was very slowly activating with time constants of 10 s and more (Tai et al. 1997). I_{sK} functions as a regulatory subunit of K_vLQT1 (which exhibits the typical topology of voltage-dependent K^+ channels and a tissue distribution that is very similar to that of I_{sK}) thus forming the functional "minK" channels (Barhanin et al. 1996; Tai et al. 1997). Alternative partners for both K_vLQT1, as well as I_{sK}, however, may also exist.

In *Xenopus* oocytes that were injected with I_{sK} cRNA (and that were later found to endogenously express a K_vLQT1 analog; Sanguinetti et al. 1996), slowly developing voltage-activated K^+ currents were detected (Busch et al. 1992). Hypotonic stress led to a pronounced increase of these currents, to an accelerated activation, and to a shift in voltage dependence to more negative membrane voltages. The above effects were completely abolished in Ca^{2+}-free solutions (Busch et al. 1992). A significant contribution of minK channels to the RVD process was also reported from vestibular dark cells of gerbil inner ear (Shiga and Wangemann 1995; Wangemann et al. 1995) and from mouse tracheal epithelium (Lock and Valverde 2000). In the latter system, RVD was insensitive to Ba^{2+} and apamin and only weakly inhibited by TEA, which is to be expected for I_{sK}. In contrast, clofilium (a quaternary ammonium blocker, used at 100 μMol/l), which is a rather selective blocker of the K_vLQT/I_{sK} complex, potently inhibited RVD. Moreover, in tracheal epithelial cells, the I_{sK} (–/–) knockout mouse, RVD was no more detectable (Lock and Valverde 2000).

In a recent study, TASK-2 (KCNK5) was identified as a mediator of hypotonicity-induced conductive K^+ release in Ehrlich ascites tumor cells (Niemeyer et al. 2001). TASK, for TWIK (*T*andem of P domains in *W*eak *I*nward rectifier *K*$^+$ channels)-related acid-sensitive K^+ channels, belong to the group of two pore-domain K^+ channels (K_{2P}; Lesage and Lazdunski 2000). These channels have four transmembrane segments and they operate as dimers. There is a widespread distribution of K_{2P} channels in both (electrically) excitable and nonexcitable tissues. K_{2P} channels appear to define the background K^+ conductive properties of many cell membranes. They are insensitive to most classical K^+ channel blockers (Lesage and Lazdunski 2000). When expressed in HEK293 cells, TASK-2 elicited hypotonicity-induced currents that are very similar to those in Ehrlich cells, e.g., with respect to ion sensitivity ($K^+ > Rb^+ >> Cs^+ > NH_4^+ > Na^+ \cong Li^+$) and inhibition by clofilium, with an IC_{50} of 25 μMol/l. In addition, mTASK-2 (but not mTASK-1 and mTASK-3) could actually be identified in Ehrlich cells (Niemeyer et al. 2001).

In conclusion, hypotonicity-induced K^+ channels represent an effective mechanism for the selective release of K^+ during RVD. They differ considerably with respect to unitary conductance, gating mechanism, and molecular organization. On the other hand, the high degree of variability among the numerous proteins actually recruited for conductive K^+ release underlines the general importance of this mechanism for RVD (Wehner 1998).

Cl^- channels

The apparently most abundant form of hypotonicity-induced Cl^- channels belong to a group that has been named VSOR (volume-sensitive outwardly-rectifying) Cl^- channels (Okada 1997), VRAC (for "volume-regulated anion channels" (Nilius and Droogmans 2001), or VSOAC (volume-sensitive organic osmolyte-anion channels; Jackson et al. 1994). These channels are referred to as mediators of $I_{Cl,swell}$ (Nilius et al. 1999) or $I_{Cl,vol}$ (Hoffmann and Mills 1999). They appear to be ubiquitously expressed (see Strange et al. 1996; Okada 1997 for comprehensive reviews; see also Nilius and Droogmans 2001). An example for such a channel is given in Fig. 4.

Their significant outward rectification persists under symmetrical Cl^- conditions. An interesting feature of VSOR Cl^- channels is their pronounced inactivation when membrane voltage is stepped from very negative values to above +50 mV. The channels exhibit intermediate conductances in the range of 10 pS to 40 pS at negative and 40 pS to 80 pS at

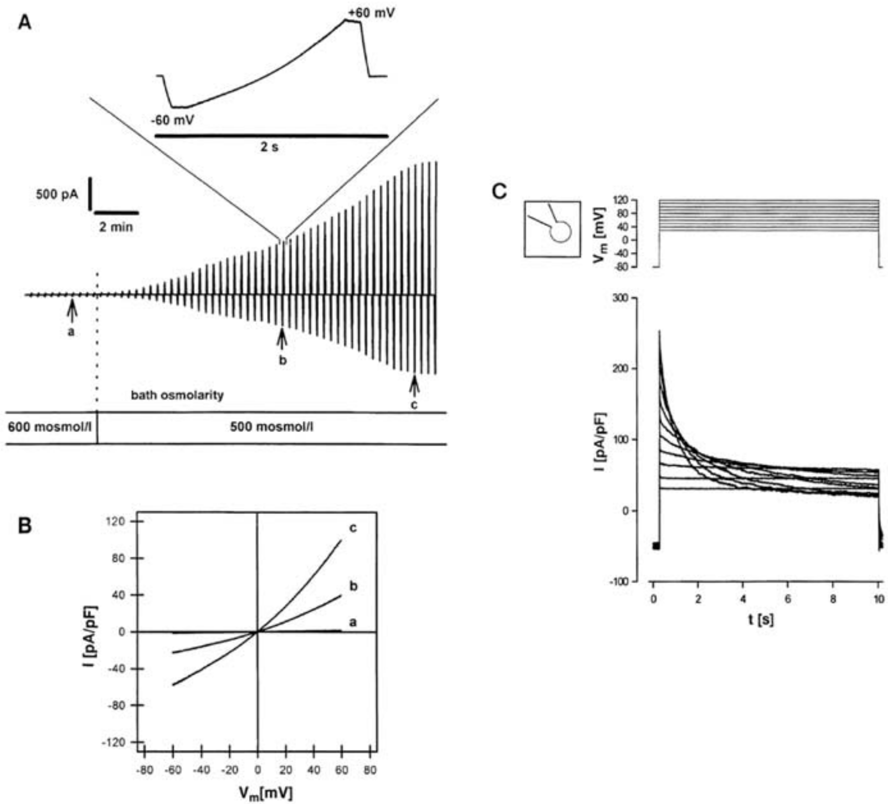

Fig. 4 **A** Activation of anion conductance in rat inner medullary collecting duct (IMCD) cells under hypotonic conditions. Whole-cell patch-clamp recording with symmetrical CsCl solutions; the osmolarity of the pipette solution was 600 mOsm/l. At the time indicated, osmolarity of the superfusate was reduced from 600 mOsm/l to 500 mOsm/l. Voltage ramps from −60 to +60 mV and 2 s duration were applied every 15 s. **B** Current-voltage relationship before (*line a*), at 6.5 min (*line b*), and at 11 min (*line c*) of hypotonicity; currents were referred to membrane capacitance. **C** Time-dependent inactivation of hypotonicity-induced Cl^- conductance at depolarizing voltages. Membrane voltage was stepped from −80 mV to positive values in the range of +30 to +120 mV with 10 mV increment after each pulse. Pulse duration and interval between pulses were 10 s and 15 s, respectively (**A** and **B** from Boese et al. 1996b; **C** from Boese et al. 1996a)

positive membrane voltages. The activation of VSOR Cl^- channels strictly depends on the nonhydrolytic binding of ATP at physiological intracellular concentrations (Jackson et al. 1994; Jackson et al. 1996; Meyer and Korbmacher 1996). Extracellular ATP in contrast, induces an open-channel block and it does so in its Mg^{2+}-free form (Nilius et al. 1994a; Jackson and Strange 1995a; Jackson et al. 1996; Tsumura et al. 1996). VSOR Cl^- channels exhibit the permeability sequence $SCN^- > I^- > NO_3^- > Br^- > Cl^- > HCO_3^- > F^-$ > isethionate > gluconate > glycine > taurine > aspartate, glutamate (Boese et al. 1996a; Strange et al. 1996; Okada 1997; Nilius and Droogmans 2001). The halide selectivity corresponds to Eisenman's sequence I, suggesting a relatively weak interaction of permeant ions with cationic sites inside the channel when compared to their dehydration energy (Wright and Diamond 1977). The physiological significance of the permeability of the channels to cer-

tain amino acids that function as organic osmolytes will be discussed below (see the section entitled “Swelling activated release”).

VSOR Cl^- channels are inhibited by submillimolar concentrations of SITS and DIDS. This inhibition is reversible and it is voltage-dependent so that outward currents are more sensitive than inward currents (which is supposed to reflect the preferred entrance of these compounds into the channel mouth at positive membrane voltages; Boese et al. 1996b; Okada 1997). In a voltage-independent fashion, the channel is also efficiently inhibited by NPPB and, in some preparations, by DPC and its derivatives flufenamate and niflumate. Interestingly in some systems, VSOR Cl^- channels were found to be blocked by inhibitors of p-glycoprotein (the product of the MDR1 gene), like tamoxifen, 1,9-dideoxy-forskolin, verapamil, and quinidine, which led to the hypothesis of a close molecular correlation between these membrane proteins. This notion appeared initially to be strongly supported by experiments in which p-glycoprotein was heterologously expressed in various cell systems (Gill et al. 1992; Valverde et al. 1992). Currently even a possible role of p-glycoprotein as a regulator of VSOR Cl^- channels is questioned (Okada 1997; Nilius and Droogmans 2001).

Despite the rather ubiquitous presence of VSOR Cl^- channels, their molecular entity still remains a mystery. One of the reasons for this is the absence of any specific inhibitor of VSOR Cl^- channels. A possible molecular candidate is I_{Cln}, a protein that was originally cloned from a Madin Darby canine kidney (MDCK) cell library and that elicited a nucleotide-sensitive (“n”), outwardly rectifying chloride current (with rapid inactivation at positive voltages) when expressed in *Xenopus* oocytes (Paulmichl et al. 1992). Because of the presence of VSOR Cl^- channels (or functionally related channels) in almost all cells, however, it was always difficult to decide whether the I_{Cln} protein reflects a channel regulator or the channel itself (Strange 1998; Fürst et al. 2000b). A possible role of I_{Cln} as a regulator has been inferred from the observation that, in most instances, it appears to be localized in the cytoplasm rather than in the plasma membrane (Krapivinsky et al. 1994; Buyse et al. 1997). On the other hand, a hypertonicity-induced translocation of the I_{Cln} protein from the cytosol to the plasma membrane has also been reported (Laich et al. 1997; Musch et al. 1997; Musch et al. 1998). This observation per se could, of course, equally well reflect the insertion of a channel or the movement of a regulatory factor. Hydrophobicity analysis indicated that I_{Cln} lacks transmembrane helices that are believed to form the pore of most vertebrate ion channels (Paulmichl et al. 1992). Accordingly, a putative model of an I_{Cln} channel was proposed consisting of a homodimer with each monomer containing four β-strands so that an eight-stranded, antiparallel β-barrel transmembrane pore is formed (Paulmichl et al. 1992; Fürst et al. 2000b). Interestingly, when purified I_{Cln} was reconstituted in lipid bilayers, single channel events could be detected that exhibited a significant rectification; ion substitutions, however, revealed that the channels observed were rather cation- than anion-selective (Li et al. 1998; Fürst et al. 2000a). In heart lipid bilayers, however, chloride selectivity was found (Garavaglia et al. 2002).

ClC-2 and ClC-3 were also considered as molecular candidates for VSOR Cl^- channels. The first member of this group of voltage-gated channels (ClC-0) was cloned from the electric ray *Torpedo* (Jentsch et al. 1990). Typically, ClCs exhibit ten to twelve transmembrane helices and the N- and C-termini are located in the cytosol. Most ClCs (except some prokaryotic isoforms) contain two hydrophobic domains close to the C-terminus that may be involved in their cellular sorting (Schmidt-Rose and Jentsch 1997; Jentsch et al. 1999). ClCs were supposed to function as dimers, with each monomer showing a separate pore.

This structure could recently be proven by X-ray analysis on two bacterial ClC isoforms with 3 Å resolution (Dutzler et al. 2002). ClC-2 is a broadly expressed Cl^- channel (Jentsch et al. 1999) that is activated by strong (nonphysiological) hyperpolarizations, acidic extracellular pH, and cell swelling (Gründer et al. 1992). However, it clearly differs from VSOR Cl^- channels with respect to its voltage dependence (it is *inwardly* rectifying) and its anion selectivity ($P_{Cl} > P_I$). An actual role of ClC-2 in cell volume regulation was a matter of debate (Jentsch et al. 1999), but recent evidence supports this notion. In *Xenopus* oocytes heterologously expressing ClC-2, for instance, hypotonic stress elicited a significant increase of cell membrane Cl^- conductance (Stegen et al. 2000). Likewise in Sf9 cells, expression of ClC-2 led to swelling-activated chloride currents that could be specifically inhibited by anti-ClC-2 antibodies (Xiong et al. 1999). In mouse atrial and ventricular myocytes, a Cl^- current was observed that was activated by hyperpolarization of membrane voltage and current activation was increased by cell swelling (and inhibited by cell shrinkage). The selectivity sequence of currents was $Cl^- > I^- >>$ aspartate, and RT-PCR and Northern blot analysis confirmed transcriptional expression of ClC-2 in both tissues (Duan et al. 2000). In rat HTC hepatoma cells, hypotonic stress elicited inwardly rectifying Cl^- currents that could be inhibited by anti-ClC-2 antibodies. Moreover, a cDNA closely homologous to rat brain ClC-2 was isolated and ClC-2 mRNA and membrane protein expression was demonstrated by in situ hybridization, immuno-cytochemistry, and Western blot analysis (Roman et al. 2001).

ClC-3 exhibits many similarities to VSOR Cl^- channels including outward rectification, unitary conductance, anion selectivity, voltage-dependent inactivation, and inhibition by extracellular nucleotides, stilbene derivatives, and tamoxifen (Strange 1998; Jentsch et al. 1999; Hume et al. 2000). There are some significant differences remaining, however. Among these is the enormous basal activity of ClC-3 when compared to VSOR Cl^- channels, the latter being undetectable under isotonic conditions. Moreover, ClC-3 appears to be inhibited by PKC, whereas PKC stimulates VSOR Cl^- channels in many preparations (Okada et al. 1998; Okada 1998; Strange 1998).

In most systems, hypotonic stress elicits a significant increase in cell Ca^{2+}. This in turn may lead to the activation of Ca^{2+}-dependent Cl^- channels (CaCCs) that may contribute significantly to the overall volume response (Jentsch 1996; Hoffmann and Mills 1999; Nilius and Droogmans 2001). In Ehrlich ascites tumor cells and in the mouse inner medullary collecting duct cell line mIMCD-K2, for instance, the contribution of both VSOR Cl^- channels and CaCCs to the hypotonicity-induced Cl^- release could be determined in a quantitative fashion (Pedersen et al. 1998; Boese et al. 2000). CaCCs activate slowly at positive and inactivate rapidly at negative voltages. They exhibit strong outward rectification with unitary conductances in the range of 3 pS to 10 pS and the ion permeability sequence is $I^- > Cl^- >$ gluconate (see Nilius and Droogmans 2001 for review). Channel activity is high at positive and low at negative voltages. CaCC currents are blocked by DIDS, NPPB, niflumate, and tamoxifen. The molecular correlate of CaCCs have not yet been unequivocally identified. The first member of a gene family of Ca^{2+} dependent Cl^- channels was cloned from bovine trachea (Cunningham et al. 1995) and since then a number of isoforms followed (Fuller and Benos 2000; Fuller et al. 2001). These "CLCA" are heterodimers of some 90 kDa and 35 kDa that are cleaved from a common precursor (at the inner loop between TM3 and TM4) and the most likely topology are five transmembrane regions with an extracellular N- and an intracellular C-terminus. There are a number of similarities between CLCAs and CaCCs but also significant differences, including uni-

tary conductances which appear to be higher in CLCAs. An actual role of CLCAs in cell volume regulation remains to be demonstrated (Nilius and Droogmans 2001; Papassotiriou et al. 2001).

BCl (or maxi Cl^-) channels that are activated under hypotonic conditions have been reported from cardiac myocytes of new-born rats (Coulombe and Coraboeuf 1992), mouse N1E115 neuroblastoma cells (Falke and Misler 1989), rat cortical astrocytes in primary culture (Jalonen 1993), the rabbit renal cortical collecting duct cell line RCCT-28A (Schwiebert et al. 1994), and primary cultures of bovine pigmented ciliary epithelium (Zhang and Jacob 1997). Typically, BCl channels exhibit unitary conductances in the range of 100 pS to 400 pS and a linear current-to-voltage relationship. In most instances, up to five identical subconductance levels were detected. BCl channels have a bell-shaped voltage dependence with very low activity outside the range of –40 mV to +40 mV. The anion-to-cation selectivity is higher than ten and the channels are inhibited by DIDS, NPPB, and DPC, but apparently not by tamoxifen. BCl channels may be activated by membrane stretch or by patch excision from nonswollen cells. Based on the effects of cytochalasins, a direct regulatory interaction with the actin cytoskeleton is assumed (Strange et al. 1996; Nilius et al. 1997). BCl channels may be related to the voltage-dependent anion channel (VDAC), a porin-like channel found in eukaryotic mitochondria or to the brain-derived voltage-dependent anion channel 1 (BR1-VDAC), which is also expressed in lymphocytes, kidney, heart, and skeletal muscle (Dermietzel et al. 1994; Thinnes et al. 2001).

Hypotonicity-induced SCl (or mini Cl^-) channels with unitary conductances in the range of 2 pS to 10 pS have been reported from single-channel patch-clamp studies on the ventricular membrane of choroid plexus (Christensen et al. 1989), Ehrlich ascites tumor cells (Christensen and Hoffmann 1992), and pigmented and nonpigmented bovine ciliary epithelium (Zhang and Jacob 1997). It is because of these low conductances that the biophysical properties and the actual contribution of SCl channels to macroscopic conductances were sometimes hard to define. In the human colon cell-line HT_{29}, for instance, the Cl^- channels activated by cell swelling appeared to be too small to be detected by use of single-channel patch-clamp techniques (Kubitz et al. 1992). Stationary noise-analysis of whole-cell currents was considered a way out and unitary currents in the range of 0.26 pS to 6 pS were determined by this method in mouse and human T and B lymphocytes (Lewis et al. 1993; Schumacher et al. 1995), human neutrophils (Stoddard et al. 1993), bovine chromaffin cells (Doroshenko and Neher 1992), and various endothelial, epithelial, fibroblast, and blood cells (Nilius et al. 1994b).

As was outlined in detail by Strange and coworkers (Jackson and Strange 1995b; Jackson et al. 1996), however, this approach may lead to a marked underestimation of unitary conductances (and to a considerable overestimation of the actual number of channel molecules involved) because some basic assumptions made—the number of channels remains constant and these channels respond to cell swelling with a gradual increase in open probability—may not be valid (see also Nilius et al. 1997). Instead, nonstationary noise-analysis may be more appropriate as could be shown for (at least some) VSOR Cl^- channels which respond to hypotonic stress with an abrupt shift of open probability from virtually zero to a value very close to unity so that the number of channels contributing to the overall membrane response is increasing, i.e., it is not constant (Jackson and Strange 1995b; Boese et al. 1996a). Accordingly, some of the above data obtained by noise-analysis may

need reevaluation, and the apparently low unitary conductances determined may actually be considerably higher.

Taken together, despite the enormous efforts undertaken in recent years to define the biophysical and molecular properties of hypotonicity-induced Cl^- channels, the overall image is still elusive and, in some instances, rather conflicting results were obtained. One of the most important steps to be achieved will be towards the molecular understanding of VSOR Cl^- channels, which appear to be the most commonly used mechanism of conductive Cl^- release during RVD.

K^+-Cl^- symport

Electroneutral KCl release appears to be one of the major features during RVD in many systems (Lang et al. 1998a; Lauf and Adragna 2000; Hoffmann and Mills 1999; Lang et al. 1998b).

K^+-Cl^- symport was first defined in erythrocytes as Cl^- dependent and ouabain-insensitive K^+ transport and, thereafter, detected in many other cell types (see Lauf and Adragna 2000 for review). Four isoforms of K^+-Cl^- symporters (KCC1–KCC4) have been cloned to date which exhibit significant molecular similarities to the Na^+-Cl^- symporter (NCC) and to the known mediators of Na^+-K^+-$2Cl^-$ symport (NKCC1 and NKCC2; see the section entitled "Na^+-K^+-$2Cl^-$ symport"). All together, they form the superfamily of cation-Cl^- cotransporters (CCCs) that appears to originate from an ancient short transport protein found in cyanobacteria (Gillen et al. 1996). CCCs contain twelve putative transmembrane domains with large N- and C-terminal regions in the cytosol. Typically, however, KCCs have a large extracellular loop between TM5 and TM6 that is variably N-glycosylated between the different isoforms (Gillen et al. 1996; Lauf and Adragna 2000). TM2 probably reflects the binding/transport site for K^+, whereas TM4 and TM7 are likely to be involved in Cl^- transport (Lauf and Adragna 2000). KCC1 appears to be ubiquitously expressed (Haas and Forbush III 2000; Lauf and Adragna 2000), while KCC2 was found exclusively in the brain (Payne 1997; Jarolimek et al. 1999). KCC3 was most prominent in heart and kidney, but also detectable in skeletal muscle, placenta, liver, lung, pancreas, endothelial cells, and in the central nervous system (Hiki et al. 1999; Mount et al. 1999; Pearson et al. 2001). KCC4 exhibited a rather restricted expression pattern with significant amounts of transcript only in muscle, lung, heart, and kidney (Mount et al. 1999). The likely function of KCC2 in the central nervous system is to decrease cell Cl^- below its electrochemical equilibrium and thus to facilitate the hyperpolarizing potency of GABA or glycine-gated Cl^- channels.

KCC1 (Gillen et al. 1996; Holtzman et al. 1998; Mount et al. 1999; Su et al. 1999; Mercado et al. 2000; Lauf et al. 2001) and KCC3 (Mount et al. 1999; Race et al. 1999), as well as KCC4 (Mercado et al. 2000), are clearly activated under hypotonic conditions (when heterologously expressed in HEK-293 cells or *Xenopus* oocytes). KCC2 was found to be insensitive to cell swelling (Payne 1997). With respect to the regulation of KCCs, *dephosphorylation* by specific phosphatases (e.g., PP1A) appears to be the trigger for stimulation (Lauf and Adragna 2000). This is an intriguing difference to NKCCs which are activated by *phosphorylation* and which are mediators of the opposite cellular volume response, namely RVI. Also certain protein kinases do participate in KCC regulation. In rat vascular smooth muscle cells, it was found that protein kinase G (PKG)-1 posttranscriptionally led to an acute upregulation of KCC3 mRNA (Di Fulvio et al. 2001b). Moreover, the NO/

cGMP signaling pathway participates in the mRNA regulation of KCC1, which is the major mediator of RVD in these cells (Di Fulvio et al. 2001a).

Cation channels

In some systems, hypotonic stress leads to the activation of nonselective cation channels. At first sight this may be surprising because, due to the steeper electrochemical Na^+ than K^+ gradient across the cell membrane, nonselective cation channels are expected to mediate a net uptake rather than a release of osmolytes. It has to be considered, however, that some of these channels exhibit a slightly higher permeability for K^+ when compared to Na^+. Moreover, certain nonselective cation channels are permeable to divalent cations so that they may play a role in Ca^{2+} signaling. Some nonselective cation channels are sensitive to membrane stretch.

A cation channel with a P_K/P_{Na} of 4.7 was reported from cultured rat mesangial cells (Craelius et al. 1993). In cell-attached patches, this channel could be activated by hypotonic stress and by negative pressure (suction) applied to the patch pipette. The channel exhibited unitary conductances of 76 pS and 40 pS with high K^+ and high Na^+ in the pipette, respectively. It was impermeable to Ca^{2+} and Ba^{2+} and its mean open time increased with membrane depolarization. In the rat pheochromocytoma cell-line PC12, cell swelling, as well as negative pressure, activated a cation channel of 46 pS with high K^+ and 27 pS with high Na^+ in the (cell-attached) pipette (Cornet et al. 1993). In ventricular myocytes of the rabbit, hypotonicity induced a cation conductance with a P_K/P_{Na} of 5.9 that was blocked by 10 μMol/l Gd^{3+} (Clemo and Baumgarten 1997).

A second group of cation channels does not discriminate significantly among small monovalent cations, but these channels exhibit a sizeable permeability to Ca^{2+} (and/or Ba^{2+}). Channels of this type were found in Ehrlich ascites tumor cells (Christensen and Hoffmann 1992), rat hepatocytes (Bear 1990), single proximal tubule cells of frog kidney (Robson and Hunter 1994), porcine cerebral capillaries (Popp et al. 1992; Popp et al. 1993), rat atrial cells (Kim and Fu 1993; Kim 1993), guinea-pig gastric smooth muscle cells (Yamamoto and Suzuki 1996), mouse N1E115 neuroblastoma cells (Falke and Misler 1989), as well as in *Rana* and *Xenopus* oocytes (Taglietti and Toselli 1988; Yang and Sachs 1989; Hurst and Hunter 1990; Schütt and Sackin 1997). Unitary conductances were in the range of 15 pS to 40 pS and, in most instances, channels were blocked by micromolar concentrations of Gd^{3+}.

Recently, first molecular correlates to hypotonicity-induced, nonselective, and Ca^{2+}-permeable cation channels could be identified in human, rat, mouse, and chicken. These are the "osmosensitive, transient receptor potential channel 4" (OTRPC4; Strotmann et al. 2000), TRP 12 (Wissenbach et al. 2000), and the "vanilloid receptor-related, osmotically activated channel" (VR-OAC; Liedtke et al. 2000). They belong to a family of gene products that are structurally related to the light-activated transient receptor potential (Trp) cation channel from *Drosophila melanogaster* (Montell and Rubin 1989). Typically, these osmo-sensitive channels have six putative membrane-spanning domains, a cytosolic N- and C-terminus, and a pore loop between TM5 and TM6; of note, there are three ankyrin-repeat domains close to the N-terminus (Liedtke et al. 2000). The channels appear to be abundantly expressed in kidney but also in lung, liver, spleen, fat tissue, heart, brain, testis, and endothelial cells. When expressed in HEK293 cells, OTRPC4 channels were activated under hypotonic conditions and exhibited outward rectification with unitary conductances

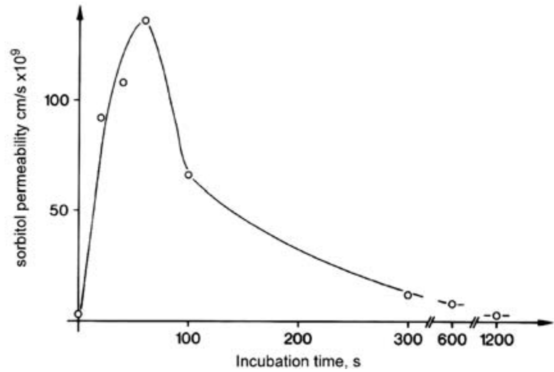

Fig. 5 Time course of activation of sorbitol efflux from IMCD cells during exposure to hypotonic solution. IMCD cells isolated at 600 mOsm/l were exposed at time 0 to a medium of 300 mOsm/l. Membrane permeability was calculated based on the difference between intracellular and extracellular concentration of sorbitol (from Czekay et al. 1994)

of 30 pS for inward and 90 pS for outward currents (Strotmann et al. 2000). P_{Ca}/P_{Na} was 0.8 and $P_K : P_{Cs} : P_{Na} : P_{Li}$ equaled 1.9:1.3:1:0:0.9 (Strotmann et al. 2000; Nilius et al. 2001). The cation channel was insensitive to membrane stretch. It was inhibited by Gd^{3+} (70%) and La^{3+} (30 %) at 100 μMol/l each and completely blocked by 10 μMol/l of the poly-cation ruthenium red, an effective inhibitor of the vanilloid receptor VR1 and the vanilloid receptor-like protein VRL-1 (Strotmann et al. 2000). Expression of VR-OAC in CHO cells led to hypotonicity-induced whole-cell currents with strong outward rectification (and with dual rectification in the absence of extracellular Ca^{2+}; Liedtke et al. 2000). In cell-attached measurements (at +80 mV), unitary current events corresponding to a conductance of 310 pS became detectable. VR-OAC channels were blocked by 500 μMol/l Gd^{3+}. Transient transfection of CHO and HEK293 cells with TRP 12 led to increased cytosolic Ca^{2+} levels when compared to nontransfected cells and, upon hypotonic stimulation, cell Ca^{2+} further increased (Wissenbach et al. 2000).

Swelling and stretch-activated channels that are nonselective for small monovalent cations and apparently impermeable to Ca^{2+} were found in the basolateral membrane of frog proximal tubule (Hunter 1990), in the mouse kidney cell line mIMCD-3 (Ono et al. 1994), and in human epididymal cells (Chan et al. 1994). Unitary conductances were in the range of 7 pS to 25 pS. In toad urinary bladder and in the A6 cell line derived from *Xenopus* kidney, cell swelling activated nonselective cation channels that were blocked by extracellular Ca^{2+} at millimolar concentrations (van Driessche and Erlij 1994; van Driessche et al. 1994). The actual role of these channels in the RVD process is completely unclear and remains to be elucidated.

Organic osmolytes in RVD

After exposure to hypotonic media, cells begin almost immediately to release organic osmolytes in addition to inorganic osmolytes. This release is not the consequence of a general increase of the membrane permeability to organic solutes, but shows a marked specificity (Grunewald and Kinne 1989; Furlong et al. 1991) and thus involves specific release pathways. These pathways are only briefly activated as exemplified for sorbitol efflux from IMCD cells in Fig. 5 (Kinne 1998).

Some of them have quite low osmotic thresholds. In some cells, organic osmolyte release accounts for about 50% of the total osmolytes released during RVD; thereby, its im-

portance in overall cell volume regulation becomes evident. It also becomes necessary for the cells to coordinate the various channel activities so that they can operate in concert at the proper activity. How this coordination is achieved is one of the enigmas that remain to be solved.

Swelling-activated taurine release

Taurine release induced by cell swelling is a well-studied phenomenon in Ehrlich Ascites tumor cells (Hoffmann et al. 1988), articular chondrocytes (Hall and Bush 2001), HeLa cells (Hall et al. 1996; Stutzin et al. 1999), glial cells (Jackson and Strange 1993; Roy 1995; Roy and Malo 1992), astrocytes (Pasantes-Morales et al. 1994b; Mongin et al. 1999; Olson 1999), liver cells (Warskulat et al. 1997a), endothelial cells (Weik et al. 1998; Nilius and Droogmans 2001), neurons (Pasantes-Morales et al. 1994a), and renal medullary cells (Banderali and Roy 1992b; Ruhfus and Kinne 1996; Sanchez-Olea et al. 1991). During RVD, 30% to 50% of the intracellular taurine leave the cell. Taurine efflux is passive and directed only by the concentration difference (Sanchez et al. 1991; Roy and Malo 1992), lacks saturability and transstimulation. In addition, taurine flux in a variety of cells is inhibited by a range of anion channel blockers (Goldstein and Davis 1994; Boese et al. 1996b; Goldstein et al. 1996; Hall et al. 1996; Kinne et al. 1996; Ruhfus and Kinne 1996; Junankar and Kirk 2000). These properties suggest that taurine efflux is mediated by a transport system which is more similar to a channel than to a transporter (Kirk 1997; Kirk and Strange 1998; Perlman and Goldstein 1999; Shen et al. 2002). The search for such channels has revealed the following candidates thus far (see also Pasantes-Morales et al. 1994b).

VSOR-Cl^- channel, VSOAC, or VRAC

As discussed in more detail above (see the section entitled “Cl^- channels”) these channels are outwardly rectifying (Boese et al. 1996a; Kirk and Strange 1998; Nilius and Droogmans 2001) and have significant permeabilities to anionic amino acids, including those with anionic side chains (glutamate and aspartate), as well as the anionic form of zwitterionic amino acids such as taurine. Attempts to further investigate the substrate specificity with regard to the organic osmolytes by measuring the effect of extracellular amino acids on RVD in glia cells (Pasantes-Morales et al. 1994b) or by electrophysiological studies revealed that the channel has a higher permeability to β-amino acids compared to α-amino acids. The anionic amino acids glutamate and aspartate appear to have a lower permeability than electroneutral amino acids of similar size (Pasantes-Morales et al. 1994b; Kirk 1997).

If compared to the chloride permeability of the channel, $P_{taurine}/P_{Cl^-}$ ranges from 0.15 to 0.25 in rat and human glial cells (Jackson and Strange 1993) and rat inner medullary collecting duct cells (Boese et al. 1996a), and to 0.49 in MDCK cells (Banderali and Roy 1992b). The size of the pore has been estimated to be between 5.6 Å and 11 Å (Jackson and Strange 1993; Nilius and Droogmans 2001). The channel therefore, seems to select the substrates both according to size and charge of the molecule.

Some electrophysiological studies also suggest that myo-inositol and sorbitol interact with the channel—since they are electroneutral molecules, no actual proof of translocation across the channel could be provided (Jackson and Strange 1993).

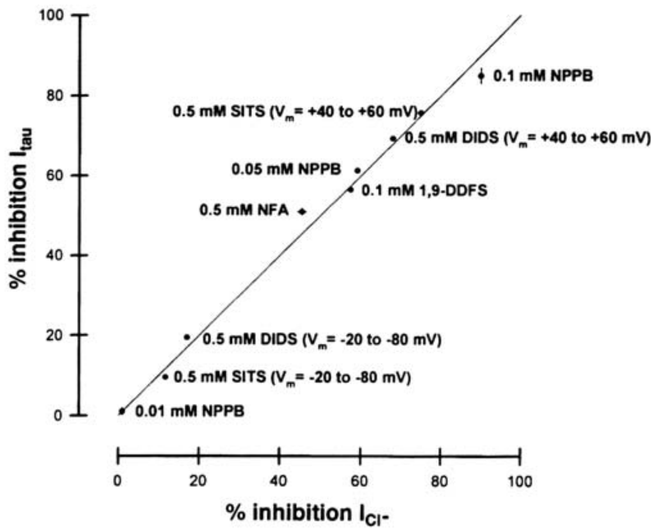

Fig. 6 Effects of various anion channel blockers on swelling-activated anion conductance in rat IMCD cells. Inhibition of volume-activated anion conductance under symmetrical taurine conditions (300 nM; pH 7.8; 31 nM charged; %inhibition I_{Tau}) plotted against inhibition of anion currents in symmetrical 140 mM CsCl conditions (%inhibition I_{Cl}–) by various anion channel blockers (unpaired experiments); n=5 for all experimental conditions. For most data points, SE values are smaller than the symbols used. *NFA* niflumic acid, *DDFS* 1,9-dideoxyforskolin, *NPPB* 5-nitro-2-(3-phenylpropylamino)benzoate, *DIDS* 4,4′-di-isothiocyanostilbene-2,2disulfonic acid, *SITS* 4-acetamino-4′-isothiocyanostilbene-2,2disulfonic acid (from Boese et al. 1996b)

In a variety of cells, VSOR-Cl^- channel and the taurine efflux pathways have an almost identical sensitivity to anion channel blockers. Figure 6 shows as an example the findings in rat IMCD cells. Because the rate of activation is also similar, these data suggest that chloride and taurine share the same release path.

Interestingly, VSOAC activity is regulated by the metabolic status of the cell. When intracellular ATP falls, the relative taurine efflux decreases. This has been observed in rat glial cells (Jackson et al. 1994), skate hepatocytes (Jackson et al. 1996), endothelial cells (Oike et al. 1994), and rat IMCD cells (Ruhfus and Kinne 1996). In patch clamp experiments, it was demonstrated that ATP or nonhydrolysable ATP derivatives have to be present at the cytoplasmic side of the membrane for activation of the channel by cell swelling (Jackson et al. 1994; Oiki et al. 1994; Oike et al. 1994; Jackson et al. 1996). These results point against a direct role of phosphorylation in the activation process. However, both phorbol esters and cAMP potentiate swelling induced organic osmolyte flux in C6 glioma cells, involving phosphorylation of VSOAC as a modifier of channel properties (Jackson and Strange 1993).

In agreement with findings of Motais (Motais et al. 1991) in trout red blood cells and in skate erythrocytes (Wittels et al. 2000) on swelling-activated taurine efflux, the activation of VSOAC is suppressed by high intracellular chloride concentrations (Jackson et al. 1996). The regulation by ATP and Cl^- has been interpreted to indicate that the cell very sparingly uses the metabolically expensive organic osmolytes only in situations where sufficient intracellular energy remains for a rapid recovery (Jackson et al. 1994; Jackson et al. 1996).

ICl_n protein

The ICl_n protein was cloned first from MDCK cells and exhibits a nucleotide-sensitive outwardly rectifying chloride current when expressed in oocytes (Krapivinsky et al. 1994; Fürst et al. 2000a). These properties are similar to those of swelling-activated chloride channels (see the section entitled "Cl^- channels"). When reconstituted in lipid bilayers (Garavaglia et al. 2002) and in oocytes (Stegen et al. 2000), ICl_n shows a significant permeability for taurine. Its proposed molecular structure composed of ß-barrels also bears similarities to porins invoked in organic solute transport in bacteria (Schirmer et al. 1995; Saier 2000). Furthermore, in neonatal myocytes, taurine efflux accompanies ICl_n transposition from the cytoplasm to the plasma membrane with a similar time course, suggesting, but not proving, that ICl_n is a regulator of the taurine channel or the channel itself. Knock down studies using antibodies or antisense oligonucleotides showed a significant reduction of RVD-related chloride currents (Gschwentner et al. 1995; Chen et al. 1999a); studies involving overexpression of ICl_n showed the reverse (Hubert et al. 2000). Unfortunately, no data on organic osmolyte fluxes are available from these experiments. Thus, the role of ICln as one of the swelling-activated taurine channels remains to be determined. The potential role of the Cl^-/HCO_3^- antiporter and of phospholemman as taurine channel is discussed in the section entitled "Cl^-/HCO_3^- antiport" and the section entitled "Phospholemman," respectively.

Finally, it should be noted that in several cells separate pathways for chloride and taurine have been described; thus, additional transporters seem to exist (Shennan et al. 1994; Lambert and Hoffmann 1994; Stutzin et al. 1999).

Myo-inositol release pathways

Flux studies in rat C6 glioma cells (Jackson and Strange 1993) and rat inner medullary collecting duct cells (Ruhfus and Kinne 1996; Ruhfus et al. 1998) revealed striking similarities between swelling-activated myo-inositol and taurine fluxes. In glioma cells and IMCD cells, the rate of activation, the extent of release, and the sensitivity to anion channel inhibitors were virtually identical (Jackson and Strange 1993; Ruhfus and Kinne 1996). In addition, dependence on intracellular ATP levels could be demonstrated in the two cell types for both fluxes. In IMCD cells, the majority of the taurine and myo-inositol occurs across the same membrane at the basal-lateral cell side (Kinne et al. 1996; Ruhfus et al. 1998). Furthermore, the activation pathways (Ca^{++}-independent and G-protein independent) are identical (Ruhfus and Kinne 1996). Electrophysiological investigations on the VSOAC in glia cells also showed that myo-inositol probably interacts with the channel (Jackson and Strange 1993).

Thus, it is very likely that myo-inositol efflux occurs via the same channel as taurine. Also, studies in skate erythrocyte suggest that taurine and myoinositol share a swelling-activated transport pathway (Goldstein and Davis 1994; Goldstein et al. 1996; Perlman and Goldstein 1999).

Sorbitol release pathways

Swelling-activated efflux of sorbitol has been observed in C6 glioma cells (Jackson and Strange 1993), a rabbit renal papillary cell line (Siebens and Spring 1989; Garty et al.

1991), HeLa Cells (Hall et al. 1996), rat IMCD cells (Grunewald and Kinne 1989; Ruhfus et al. 1998), rabbit kidney TALH cells (Kinne et al. 2000), and porcine urinary bladder cells (Mahler et al. 1998). In all instances tested, the flux, either measured as unidirectional sorbitol uptake (Jackson and Strange 1993; Hall et al. 1996; Kinne et al. 2000) or as solute efflux (Garty et al. 1991), lacked saturation kinetics. Furthermore, anion channel blockers such as NPPB, DIDS, 1,9-dideoxyforskolin, and cation channel blockers such as quinidine, inhibited sorbitol efflux in most systems, suggesting the involvement of a channel-like protein. With regard to the specificity towards the polyol, the length of the polyol chain and the orientation of the hydroxyl group at C6 appear to affect the permeability, although to a relatively small extent (Napathorn and Spring 1994).

In most cells, except IMCD cells, anions (chloride, iodide), potassium, choline, thymine, and taurine seem to be translocated via the same swelling-activated channel as sorbitol. This conclusion is based on the very similar inhibitory profile of various inhibitors on the fluxes (Hall et al. 1996), a similar lag phase before opening, and a similar dependence on the intracellular ATP level. In addition, in electrophysiological experiments in C6 cells sorbitol affected the volume-activated anion channel in such a way as to suggest an interaction with the anion binding site (Jackson and Strange 1993).

In rat IMCD cells, however, several differences between taurine/myo-inositol efflux and sorbitol efflux have been found. DIDS and SITS stimulate rather than inhibit sorbitol efflux; time of activation and closure of the two efflux pathways differ, as does the osmotic threshold for activation (Kinne 1998). Furthermore, activation of the sorbitol efflux pathway is calcium-dependent, probably involving activation of G-proteins (Ruhfus et al. 1996). Thus, for example, sorbitol efflux is reduced at low extracellular calcium and stimulated by arachidonic acid (Tinel et al. 1997; Kinne et al. 2000) and mastoparan under isotonic conditions (Ruhfus et al. 1996), whereas taurine (and myo-inositol) efflux is not affected by a change in overall calcium or by mastoparan, and inhibited by arachidonic acid (Kinne et al. 1996). However, local changes in the calcium concentration appear to affect swelling-induced Cl^- channels (Lemonnier et al. 2002).

It has to be noted, however, that in IMCD cells a considerable (about 50%) fraction of swelling-activated sorbitol efflux is not calcium dependent; thus, the existence of an additional channel—similar to the one proposed for other cells—cannot be excluded.

On the other hand, there is recent evidence for a highly selective sorbitol channel. Studies on malaria-induced pathways in chicken erythrocytes have shown that a solute channel is induced after infection with Plasmodium gallicaneum, which exhibits an about 17-fold higher permeability to sorbitol than to taurine and is relatively insensitive to DIDS and tamoxifen (Staines et al. 2002).

Other systems involved in volume regulation

Cl^-/HCO_3^- antiport

The superfamily of bicarbonate transporters (BTs) consists of two groups, namely, the AE family of anion exchangers (i.e., Cl^-/HCO_3^- antiporters) and the family of HCO_3^- transporters that are coupled to Na^+ (NBCs; see Boron 2001 for review). Whereas no direct contribution of NBCs to cell volume regulation has been reported so far, Cl^-/HCO_3^- antiport is of considerable importance for, both RVI as well as RVD. Four isoforms have been

cloned to date (AE1–AE4) starting with murine AE1 (erythrocyte band 3) as early as 1985 (Kopito and Lodish 1985). The erythrocyte AE1 is a 929 amino acid protein with two functional domains, a hydrophilic one that is related to its role as an anchor for the cytoskeleton, and a hydrophopic one reflecting its function as an anion exchanger with 12 transmembrane regions (Kopito and Lodish 1985). An N-terminal truncated isoform of AE1 is present in the kidney (Brosius III et al. 1989). AE2 is widely expressed in nonerythroid tissues, including the kidney, and AE3 appears to be mainly expressed in electrically excitable tissues. Recently, AE4 was cloned from kidney; it appears to represent the apical Cl^-/HCO_3^- antiporter in β-intercalated cells (Tsuganezawa et al. 2001).

One of the prime functions of Cl^-/HCO_3^- antiport in cell volume regulation is the HCO_3^--dependent uptake of Cl^- that is induced parallel to the hypertonic activation of Na^+/H^+ antiport. Both transporters are functionally coupled via cell pH and the net result of their activity is a quasi electroneutral uptake of Na^+ and Cl^-. Activation of Cl^-/HCO_3^- antiport parallel to that of Na^+/H^+ antiport has been reported from a variety of system, including mammalian hepatocytes (Graf and Häussinger 1996), human retinal pigment epithelial and nonpigmented ciliary epithelial cells (Civan et al. 1994b; Civan et al. 1996), C6 glioma cells (Mountian et al. 1996), flounder red blood cells (Weaver et al. 1999), as well as from mouse medullary thick ascending limb (Hebert and Sun 1988). In addition, a parallel hypertonic stimulation of endogenous NHE1 and of heterologously expressed AE2 was reported from *Xenopus* oocytes (Jiang et al. 1997). A concerted activation of Na^+/H^+ antiport and Cl^-/HCO_3^- antiport was also found in renal epithelial (MDCK-F) cells that employ these mechanisms of RVI for the purpose of locomotion (Klein et al. 2000).

Equally well, Cl^-/HCO_3^- antiport may be activated under hypotonic conditions. In skate and trout erythrocytes, and also when heterologously expressed in *Xenopus* oocytes, AE1 appears to mediate the hypotonicity-induced release of Cl^- (see the section entitled "Swelling activated taurine release;" Musch et al. 1994; Fievet et al. 1995; Motais et al. 1997; Guizouarn et al. 2001). Of note, it may do so in a channel-like fashion and, at the same time, it also appears to increase cell membrane Na^+ and K^+ permeabilities (Guizouam et al. 2001).

AE 1 is one of the most clearly established molecular identities involved in taurine transport from fish red blood cells that are nucleated (Motais et al. 1991; Motais et al. 1997; Guizouarn et al. 2001). In particular, for AE 1 derived from trout, it has been convincingly demonstrated that expression of the protein in oocytes elicits both anion conductance and taurine transport, which show the same inhibitor profile and are clearly distinguishable from the endogenous I_{Cl} swell of the oocytes (Fievet et al. 1995; Fievet et al. 1998; Koomoa et al. 2002).

The fact that in oocytes AE 1—contrary to intact trout red blood cells—cannot be activated by hypotonic stress and is not DIDS-sensitive might reflect different conformations of the protein in the two systems. Different conformations that change transport characteristics and sensitivity to modification have also been reported for ICl_n and phospholemman, and might be a general property of volume-regulated organic osmolyte channels. Another caveat remains: mouse AE 1 does not act as an osmolyte channel. In addition, in eel erythrocytes, significant differences between the AE 1-mediated SO_4 transport and swelling-activated taurine transport were observed (Lewis et al. 1996).Thus, a role of AE 1 in taurine release from mammalian cells remains to be demonstrated.

Phospholemman

Phospholemman (PLM) is a small membrane protein of 15 kDa which was first purified, cloned, and sequenced from dog heart (Palmer et al. 1991). The mature protein consists of 72 amino acids and has one single transmembrane domain. The name PLM denotes its high degree of phosphorylation, which (under most physiological conditions) appears to be mediated by protein kinases A and C (Palmer et al. 1991; Walaas et al. 1994; Mounsey et al. 1999). When expressed in *Xenopus* oocytes, PLM induces anion currents that are activated by large hyperpolarizations of membrane voltage and most likely it does so by inducing endogenous oocyte channels (Moorman et al. 1992; Mounsey et al. 1999). On the other hand, when reconstituted in lipid bilayers, PLM could be shown to form functional ion channels itself which exhibited rather slow gating kinetics and an enormous unitary conductance that is close to 700 pS (Moorman et al. 1995). It was also found that PLM (again in lipid bilayers) exhibits two different modes of operation, namely a cation- and an anion-selective one, and that the protein appears to spontaneously switch between these modes (Kowdley et al. 1997; Moorman and Jones 1998). PLM also exhibits a remarkably high permeability for taurine with a $P_{taurine}/P_{Cl}$ as high as 70 (Moorman et al. 1995) and a high selectivity with regard to the NH_2 group at the β-position (Moorman et al. 1995; Kowdley et al. 1997; Moorman and Jones 1998).

Overexpression of PLM in HEK293 cells significantly increased the membrane currents elicited by *hypotonic* stress (Moorman and Jones 1998) as well as the release of Cl^- (I^-) and taurine during RVD (Morales-Mulia et al. 2000); moreover, this osmolyte release could be markedly reduced by use of PLM-specific antisense oligonucleotides (Morán et al. 2001).

In solitary rat hepatocytes in primary culture, which could be shown to exhibit a distinct RVI (Kirschner et al. 1998) and which do express PLM, *hypertonic* conditions activate a channel that very much resembles PLM with respect to its unitary conductance, its gating pattern, and its nonselectivity for Na^+ over K^+. Moreover, in *Xenopus* oocytes expressing hepatocyte PLM, hypertonic stress induces a nonselective cation conductance and noise-analysis reveals the activation of a channel with characteristics that appear to be similar to those of PLM (Kirschner et al. 2003).

Taken together these results suggest a role of PLM in both RVD *and* RVI. This could mean that PLM functions as a last line of defense so that a cell under strong anisotonic conditions opens a large osmotic shunt just to survive. As an alternative view, given the regulatory role PLM appears to play in some systems (Moorman et al. 1992; Mounsey et al. 1999) and its high degree of phosphorylation per se (Palmer et al. 1991; Walaas et al. 1994; Mounsey et al. 1999) the protein may well be part of the signaling machinery that actually tunes the ion transporters responsible for cell volume regulation.

Na^+, K^+-ATPase

Cell volume homeostasis depends on the balance between osmolyte entry into the cell and removal of osmolytes from the cell interior. For cells that possess sodium entry pathways, such as sodium channels, Na^+/H^+ antiporter, Na^+, K^+, $2Cl^-$ symporter, or other sodium co-transporters, sodium entering the cells is removed by the primary active Na^+, K^+-ATPase. The activity of this enzyme, thereby plays an important role in maintaining the cell volume as indicated by the cell swelling observed in isotonic media when ouabain is present

in the incubation medium. This observation reflects the actual stoichiometry of Na^+, K^+-ATPase of 3 Na^+/2 K^+ which continuously leads to a net loss of cellular inorganic osmolytes. In this context, it is also of note that in some systems an *inhibition* of the enzyme appears to directly contribute to the RVI processes (see below).

Since the first description of the enzymatic activity in 1957 by Skou (Skou 1957), the transport cycle has been described in great detail, the two subunits α and β have been identified, and a cell-specific distribution of their isoforms has been described (for review see Blanco and Mercer 1998).

The α-subunit has a molecular weight of 110 kDa, includes ten predicted transmembrane domains, and is responsible for the catalytic activity of the enzyme. During the pump's catalytic cycle the α-unit is transiently phosphorylated on a residue of the large cytoplasmic loop between transmembrane domains 4 and 5. This protein also contains phosphorylation sites for PKA and PKC. Residues of the fourth transmembrane segments contribute to the cation selectivity of the enzyme (for review see Dunbar and Caplan 2000).

The β-subunit has a molecular mass of approximately 65 kDa and elicits only one transmembrane segment with a cytosolic N-terminus. The extracellular C-terminus is heavily glycosylated (for review, see Dunbar and Caplan 2000). Assembly between the α- and β-subunits is required for the enzyme complex to exit the ER.

In addition, a γ-subunit has been identified; there is still considerable debate as to whether this subunit is required for the enzyme activity and/or whether it represents a regulatory membrane protein, which in some cells is closely associated with the enzyme (for review see Therien and Blostein 2000). The variability of the expression of the different subunits, the composition of which changes the properties of the enzyme considerably, results in a large variation of the response of the enzyme to changes in extracellular osmolality. In the context of this chapter, therefore, only a few examples of typical responses will be discussed.

During RVI following the exposure of cells to shrinkage in hypertonic media, the Na^+, K^+-ATPase is particularly important in cells that primarily and predominantly activate regulatory osmolyte transport systems, which enhance the intracellular sodium content, such as the Na^+/H^+ antiporter, the Na^+, K^+, $2Cl^-$ symporter, or a sodium channel, as discussed above. In a study in which osmolyte and Na^+ transport balances of rat hepatocytes were quantified as a function of hypertonic stress (see Fig. 7), it could be demonstrated that ouabain-sensitive ^{86}Rb uptake, representing the transport activity of the Na^+, K^+-ATPase, increased almost fourfold in a saturable fashion with increasing extracellular osmolarity (Wehner and Tinel 2000).

This increase could be completely accounted for by the rise in intracellular sodium concentration and the apparent Michaelis-Menten constant for sodium of the enzyme of 12 mMol/l.

The regulation of transport activity by changes of intracellular sodium represents the most straightforward and well-known response of the Na^+, K^+-ATPase to an osmotic challenge (Wojnowski and Oberleithner 1991). There are, however, also instances where either no increase in transport activity or even a decrease during RVI is observed. No change in ouabain-sensitive $^{86}Rb^+$ uptake was, for example, observed in the human hepatoma cell line Hep G2 (Wehner et al. 2002a). In view of the already relatively high Na^+, K^+-ATPase transport activity in these cells under isotonic conditions, the authors argued that the enzyme might normally function at V_{max} level and therefore a further stimulation might not

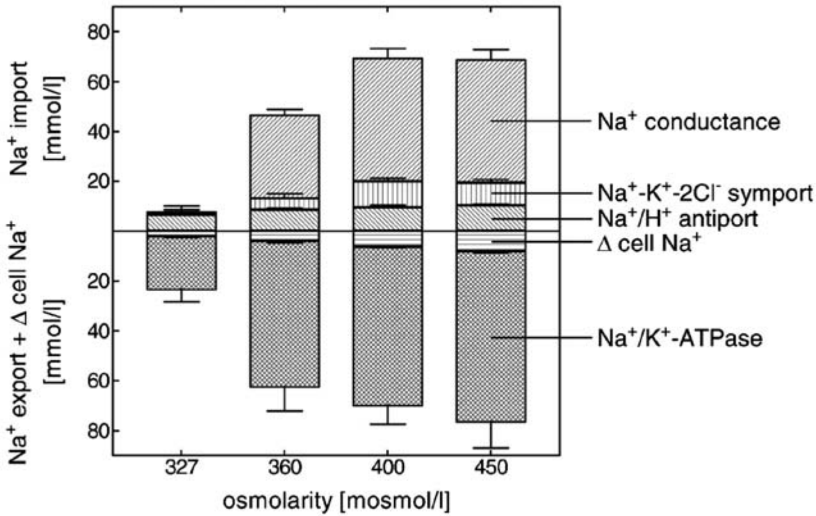

Fig. 7 Balance between Na^+ import and Na^+ export by the Na^+, K^+-ATPase plus the actual increase of cell Na^+. Rat hepatocytes in primary culture were exposed to increasing osmolarities. Note the saturability of the Na^+, K^+-ATPase pump rate (from Wehner and Tinel 2000)

be possible. In Malpighian tubule, an inhibition of Na^+, K^+-ATPase was observed during RVI. Here, the increase in intracellular adenosine, which inhibits the enzyme activity, was supposed to cause this effect (Caruso-Neves and Lopes 2000). In view of the fact that the Na^+, K^+-ATPase activity may also be altered by various kinases and phosphatases, as well as eicosanoids, intracellular ATP levels, intracellular pH (Goldstein and Davis 1994; Therien and Blostein 2000), rate of K^+ recycling across the membrane, and the membrane potential, the response of the enzyme during volume regulation can differ considerably depending on the cellular system being investigated.

It has to be noted that the alterations in Na^+, K^+-ATPase transport activity described above included regulation via phosphorylation, changes in transport rate, substrate, or inhibitor concentrations, or modifications in driving forces without changes in mRNA or enzyme content. Changes in the latter two parameters are observed when cells are exposed in vitro to hypertonic media for an extended time period. Thus, in mouse inner medullary collecting duct cells (Ohtaka et al. 1996), MDCK cells (Bowen 1992), human proximal tubule cells (Yordy and Bowen 1993), and vascular smooth muscle cells (Muto et al. 1998), changes in α-mRNA and β-mRNA are observed as early as one h after exposure of cells to a hypertonic medium. A maximum response is found between 8 h and 24 h of anisotonic incubation. The increase in mRNA leads to an augmentation of Na^+, K^+-ATPase abundance as demonstrated by a higher enzymatic activity in cell homogenates. An in vivo modulation of Na^+, K^+-ATPase expression was also observed in the renal papilla of water-deprived mice versus water-loaded mice, suggesting an osmoregulation of the enzyme in the intact animal (Capasso et al. 2001).

The mechanisms involved in the upregulation are still unclear; changes in the intracellular sodium concentration combined with an increased synthesis of intermediate regulatory proteins have been discussed (Yordy and Bowen 1993; Muto et al. 1998).

The physiological rationale for this long term adaptation is probably the same as for the short-term activation during RVI. In both instances, the cell aims to maintain a suitable sodium gradient across the plasma membrane. This mechanism assures the proper operation of sodium cotransport systems that include sodium osmolyte cotransport systems (see the section entitled "Organic osmolytes in RVI") and also systems regulating important intracellular parameters, such as intracellular pH and intracellular calcium. Thus, the activation of a net osmolyte export system during RVI, a reaction which at first sight appears to be counterproductive, fulfills an important function for general cell activity.

The effect of cell swelling on the Na^+, K^+-ATPase is difficult to estimate since a variety of stimulating and inhibiting parameters change simultaneously. Thus, cell swelling reduces intracellular sodium concentration, but activates K^+ channels (see the section entitled "K^+ channels") that facilitate K^+ recycling and thus could increase Na^+, K^+-ATPase activity. The increase in membrane potential would tend to decrease the transport rate of the electrogenic pump. The net effect of these—and other—regulatory changes differs from cell to cell. Nevertheless, there is evidence from various tissues that ouabain inhibits RVD, pointing to a significant role of the Na^+, K^+-ATPase in removing excess inorganic osmolytes from the cells (Fraser and Swanson 1994; Grunewald et al. 1994; Kanli and Norderhus 1998).

Signal transduction in volume regulation

Sensory systems and set points

Although the search for sensory mechanisms of cell volume changes has been carried out very intensively during recent years, a conclusive model of an osmosensor in mammalian cells has not yet been presented. The main cause for this gap lies in the nature of cellular signalling events. Usually, several signalling mechanisms are activated in parallel and some of them do influence each other mutually. This integrative character of signalling networks makes the assignment of signalling events to distinct pathways difficult. The concept that not one single osmosensor but rather a complex sensory system exists, has to be taken into account. Such a sensory system has to fulfill two major requirements. On the one hand, it has to be very sensitive to perceive even slight alterations of cell volume. On the other hand, the sensor must amplify and transfer the information to trigger signalling cascades leading to the activation of volume regulatory processes.

The mechanisms that have been reported to be implicated in sensing changes of cell hydration can be classified into three major groups, according to their cellular location. They can be (1) associated with the cytoskeleton perceiving changes in the interaction between extracellular matrix and plasma membrane receptors; (2) intracellular solute sensors sensitive to changes in macromolecular crowding and/or ionic strength and (3) membrane-based sensors which record alterations of cell membrane stretch.

Extracellular matrix and cytoskeleton

Cells adhere to the ambient extracellular matrix (ECM) via cellular integrins which are clustered to focal adhesions upon binding of ECM-ligands. Proteins that are associated with these adhesion sites may serve to maintain structural integrity, and equally well, they

may be involved in signal transduction. For instance, the focal adhesion kinase FAK is a tyrosine kinase that is involved in the transduction of integrin-mediated cellular responses (Burridge and Zhong 1997). The activity of both integrins and FAK is altered by focal adhesion clustering.

Focal adhesions and the intracellular actin cytoskeleton effect each other mutually in their structures. Interestingly, evidence has been provided which indicates that integrins could be part of a volume-sensing system. In rat skeletal muscle, for example, the ECM-integrin-mediated cell adhesion and the cytoskeleton are involved in mechanochemical transduction of cell volume changes to chemical signals modulating glutamine transport (Low and Taylor 1998). ECM-integrin binding also transduces changes in cell volume and/or shape into changes in endothelial permeability of cultured bovine aortic endothelia and frog mesenteric capillaries (Kajimura et al. 1997). At the frog neuromuscular junction, integrins contribute to the hypertonic enhancement of transmitter release (Kashani et al. 2001).

The finding that integrins may be involved in sensing cell volume changes is coincidental with the circumstance that in nearly all systems studied so far volume changes cause alterations of the cytoskeletal organization. In the majority of the systems examined, hypotonic cell swelling evokes disorganization of the F-actin cytoskeleton. This has been shown for Ehrlich Ascites tumor cells (Pedersen et al. 2001), rat IMCD cells (Czekay et al. 1994) shark rectal gland and C6 glial cells (reviewed in (Henson 1999; Papakonstanti et al. 2000; Pedersen et al. 2001). In contrast, hypotonic actin polymerization could be detected in rat hepatocytes (Theodoropoulos et al. 1992) and HL-60 cells (Hallows et al. 1991). Increasing extracellular tonicity enhances the relative F-actin content in Ehrlich Ascites tumor cells (Pedersen et al. 2001) and lowers it in HL-60 cells (Hallows et al. 1991). These examples illustrate how diverse the response of the actin cytoskeleton to cell volume changes may be.

The alteration of cell volume evokes changes of cell morphology which are paralleled by the development of mechanical tension. This tension impinges on both the cytoskeleton and the interaction between integrins and their ECM-ligands. In both cases, integrins are altered in their activity by conformational changes. They may be activated and clustered to focal adhesions or inactivated and dispersed from focal adhesions (Burridge and Chrzanowska-Wodnicka 1996; Ingber 1997; Schoenwaelder and Burridge 1999).

The assembly of integrins to focal adhesions can influence the cytoskeleton by two synergistic pathways, both of them involving activation of the small GTPase Rho. The contribution of Rho to the regulation of volume-sensitive transport mechanisms has been reported for several systems, (see Nilius et al. 1999; Okada 1997).

Activation of RhoA by integrin-mediated adhesion (Renshaw et al. 1996; Clark et al. 1998; Ren et al. 1999) and/or by isometric tension of cytoskeletal components (Burridge and Chrzanowska-Wodnicka 1996) induces the formation of stress fibers (Amano et al. 1997). This change is caused by influencing the state of contractile structures or by effecting the conformation of actin-associated proteins (Schoenwaelder and Burridge 1999). Rho activates Rho-kinase, which phosphorylates and thereby inhibits myosin phosphatase. This results in elevated phosphorylation of myosin light chain (MLC; Kimura et al. 1996). The consequence is that myosin filament assembly and actin-activated myosin ATPase activity (Burridge and Chrzanowska-Wodnicka 1996) are enhanced. This leads to the bundling of actin filaments and the assembly of stress fibers. Rho may also phosphorylate MLC directly (Amano et al. 1996).

The alteration of stress fiber assembly by actin-associated proteins occurs via lipid derived messengers. The activation of PI-5 kinase by RhoA elevates the intracellular level of phosphatidylinositol 4,5-bisphosphate (PIP_2; Chong et al. 1994). PIP_2 induces conformational changes of the actin-associated proteins vinculin (Weekes et al. 1996), ezrin-radixin-moesin (ERM; Niggli et al. 1995), and profilin. Thereby, binding sites for actin and other proteins are exposed. The resulting changes in stress fiber tension are then transduced to the integrins, which thereby are altered in their activity. This bidirectional signalling between the cytoskeleton and integrins allows the creation of a feedback-loop, by which the duration of volume-generated signalling can be determined (Burridge and Chrzanowska-Wodnicka 1996; Schoenwaelder and Burridge 1999).

In this context, another property of the sensor of volume changes, namely, the transduction of the perceived information to effectors, may be realized by three different mechanisms. Firstly, alterations in the cytoskeletal structure may affect the activity of signalling molecules by changing their intracellular localization. Secondly, the cytoskeleton may directly interact with and act upon volume regulatory transporters and, thirdly, vesicular transport, if involved in cell volume regulation, may be influenced, (see for example Czekay et al. 1994).

In many studies cited above, it is not yet clear whether the perturbations of the cytoskeleton observed are the cause or the consequence of volume changes and/or regulatory processes. Furthermore, a direct interaction with the target proteins and the consequences of this interaction have not been recorded in the intact cell. Thus, a higher spatial and temporal resolution of these processes is necessary to be able to conclusively judge upon the role of the cytoskeleton and the ECM in volume sensing.

As was outlined above, the alteration of cell adhesion effects FAK, as well as integrin signalling. For the activation of volume-sensitive Cl^- currents, it has been proposed that the stimulation of a tyrosine kinase is one of the early events in osmosensing (Sadoshima and Izumo 1993; Tilly et al. 1993). In most instances, however, tyrosine-phosphorylarions last only for a short period of time, which is mainly due to the presence of tyrosine-specific protein phosphatases. Accordingly, these tyrosine-phosphorylations, in most systems, are translated into longer lasting signalling events, namely, by the activation of serine/threonine phosphorylation cascades, such as the mitogen-activated protein kinase (MAP kinase) pathways. The sequential patterns by which MAP kinases are activated offer the opportunity of an amplification, stabilization, and integration of intracellular signalling events in response to extracellular stimuli at the same time. In fact, MAP kinases could be shown to be involved in the regulation of a variety of fundamental physiological processes such as cytoskeletal organization, nuclear transcription, protein translation, cell growth and differentiation, as well as cell volume homoeostasis.

Activation of ERK1/2 MAP kinases by cell volume changes appears to be a quite common phenomenon. A swelling-induced activation occurs in most mammalian cell types (van Der Wijk et al. 2000). In contrast, activation of ERK1/2 upon hypertonic cell shrinkage was observed, for instance, in MDCK cells (Itoh et al. 1994), H4IIE rat hepatoma cells (Schliess et al. 1996), and in bovine aortic endothelial cells (Duzgun et al. 2000). In these systems, hypertonic activation occurs within 10 min and the maximal induction is reached after 20 min–30 min.

The activity of p38 MAP kinase can be upregulated by hypertonicity as well as by hypotonicity (see Shrode et al. 1998). Hypertonic activation occurs, for example in U 937 and in IMCD-3 cells. In the latter system, the activation of p38 is achieved after approxi-

mately 10 min (Zhang and Cohen 1996; Shrode et al. 1997). Hypotonic activation of p38 has been observed in the perfused rat liver (Vom Dahl et al. 2001), in HTC rat liver hepatoma cells (Feranchak et al. 2001), and in intestine 407 cells (van Der Wijk et al. 1998; van Der Wijk et al. 2000). For the perfused rat liver and intestine 407 cells it could be demonstrated that p38 is activated rapidly and without a detectable lag phase. In IMCD-3 cells, the JNK MAP kinase is activated under hypertonic conditions (van Der Wijk et al. 1998). The swelling-induced activation of JNK is a slow process, occurring with a delay of some 10 min–20 min (van Der Wijk et al. 2000).

Though MAP kinases are activated by the anisotonic alteration of cell volume in many systems, a direct involvement in volume regulatory mechanisms has only been observed in a few systems. In human cervical cancer cells, for example, swelling-activated ERK1/2 is linked to the activation of Cl^- currents, K^+ currents and taurine transport (van Der Wijk et al. 2000; Shen et al. 2001). In contrast, even though Na^+/H^+ antiport can be activated in an ERK1/2-dependent manner, the osmotic activation of this antiporter does not involve ERK1/2 (or p38 MAP kinase) in Chinese hamster lung fibroblasts (Aharonovitz and Granot 1996; Bianchini et al. 1997; van Der Wijk et al. 2000). Moreover, Nilius et al. demonstrated that hypotonic activation of MAP kinases are not involved in the activation of volume-sensitive Cl^- currents (Nilius et al. 1997).

In most instances, the apparent absence of effects of MAP kinases on the (fast) regulatory movements of inorganic osmolytes can be explained in terms of their slower time course of activation that instead reflects that of organic osmolyte transport and metabolism. Accordingly, osmosensitive MAP kinases may be related to cell volume regulation by activating transcription factors, since the required upregulation of the corresponding metabolizing enzymes and organic osmolyte transporters is realized by enhancing their levels of transcription. An adjusted gene expression thus allows the cells to sustain cell volume disturbances for a prolonged period of osmotic stress.

Macromolecular crowding and ionic strength

Macromolecules occur in a high intracellular concentration; they occupy typically 20%–30% of the total cell volume (Ellis 2001). This noncytosolic and not osmotically active space (see the section entitled “The osmometric behavior of cells”) excludes molecules of low molecular weight. This phenomenon, known as macromolecular crowding, has fundamental effects on the rates and equilibria of biochemical reactions (Ellis 2001). Under conditions of increased macromolecular crowding, the reduction of excluded volume causes a decrease of total free energy of the solution. Therefore, the equilibrium constants of binding and association reactions that exclude the least volume to all the other macromolecules present are increased. Macromolecular crowding alters biochemical reaction rates by influencing both molecular diffusion rates and thermodynamic activities. While molecular diffusion rates will be reduced by increased crowding (Luby-Phelps 2000), their thermodynamic activity will be enhanced. The net result of these opposing effects depends on the nature of the rate-limiting step. If a reaction rate is limited by the encounter rate of the initial molecules, the rate will be decreased by crowding due to decreased diffusion rates. If the rate is limited by the activity of the transition complex, it will be increased by crowding due to enhanced thermodynamic activities (Ellis 2001).

Macromolecular crowding is reduced by increasing ionic strength, because proteins interact with the ambient electrolytes. The volume regulatory set points are thereby shifted

to smaller volumes (Parker et al. 1995). Since even slight changes in macromolecular crowding are sufficient to evoke large changes in enzymatic activity, macromolecular crowding has been discussed as a candidate for a cell volume sensory system. Indeed, the volume sensitive transporters Na^+/H^+ symporter (Parker et al. 1995), K^+-Cl^- symport (Minton et al. 1992; Minton 1994; Parker et al. 1995), VSOAC channel (Emma et al. 1997), and Cl^- channels (Hoffmann and Pedersen 1998; Nilius et al. 1998) are influenced by macromolecular crowding and ionic strength in various systems. A reduction of ionic strength activates some transport systems involved in RVD. An increase in ionic strength is supposed to mediate the induction of sodium organic osmolyte transporters and osmosensitive enzymes during prolonged exposure of cells to hypertonic media (see Burg 1995; and the section entitled "Organic osmolytes in RVI").

Macromolecular crowding and ionic strength are quite global parameters of idealized cells that can not yet be related to various cellular compartments and the place where signal generation and transduction occurs. Thus, their importance in localized reactions remains to be determined.

Cell membrane stretch

Cell swelling causes stretching of the plasma membrane, whereas shrinkage leads to a decrease in cell membrane stretch (Kinnunen 2000). These alterations of membrane tension may generate a signal perceived by stretch-sensitive channels. Among the stretch-sensitive channels, both stretch-activated and stretch-inactivated channels can be found. Stretch-activated channels can either be nonselective cation channels permeable for K^+, Na^+ and Ca^{2+}, or channels selective for Ca^{2+}, K^+ or Cl^- (Hoffmann and Dunham 1995; Lang et al. 1998a).

The activation of *nonselective* stretch-activated cation channels upon cell swelling was demonstrated in several cells (Christensen 1987; Lansman et al. 1987; Falke and Misler 1989; Bear 1990; Christensen and Hoffmann 1992). Activation of these channels contributes to RVD, probably mainly by the resulting Ca^{2+} current (Hoffmann and Dunham 1995; Lang et al. 1998a). The resulting (local) increase in intracellular Ca^{2+} then activates Ca^{2+}-sensitive K^+ channels (Christensen 1987; Ubl et al. 1988). The loss of K^+ is accompanied by osmotic water movement, leading to volume decrease (see the sections entitled "K^+ channels" and "Cation channels").

The activation of *selective* stretch-activated ion channels contributes directly to cell volume regulation. Stretch- and volume-sensitive K^+ channels were demonstrated for *Necturus* proximal tubule cells (Filipovic and Sackin 1992), gall bladder cells (Vanoye and Reuss 1999) and enterocytes (Dubinsky et al. 2000). Similarly, cell membrane stretch may also lead to the activation of stretch-sensitive maxi Cl^- channels, which were reported to be activated upon cell swelling in several systems (see the section entitled "Cl^- channels").

Stretch-inhibited channels are not as common as stretch-activated channels, and their relevance for cell volume regulation is usually regarded as negligible (Morris 1990; Hoffmann and Dunham 1995). Nevertheless, Suzuki et al. showed, that a cation channel cloned from rat kidney is inactivated by membrane stretch due to cell swelling and activated by cell shrinkage (Suzuki et al. 1999).

The mechanism by which stretch-sensitive channels are regulated by cell volume changes has not yet been fully elucidated. Signals modulating stretch-sensitive channels

could be mechanical tension, conveyed by the membrane or the cytoskeleton (Sokabe et al. 1991; Ingber 1997), or the release of stretch-sensitive messengers, such as fatty acids (Kirber et al. 1992), or ATP (Wang et al. 1996).

Though a variety of ion channels were reported to respond to membrane stretch caused by cell volume alterations, the physiological significance of this signal remains questionable, since the activation of these channels requires considerable stretch (Okada 1997). For example, it could be demonstrated that significant cell membrane stretch only arises when cell volume increases by 60%; volume regulatory mechanisms are, however, already activated when cell volume is altered by 5%–10% (Al Habori 2001). Thus, stretch-sensitive channels may only play a role in sensing cell volume changes when excessive cell swelling occurs and other signalling systems have failed (Lang et al. 1998a).

Lipid derived messengers

Phosphoinositides

Lipid signalling through phosphoinositides was reported to be involved in cell survival, cytoskeletal remodeling, metabolic control, and vesicular trafficking (Wymann and Pirola 1998). Since all these processes are related to cell volume regulation (Lang et al. 1998a; Hoffmann and Mills 1999), it seems not surprising that there is ample evidence for the involvement of lipid-derived messengers in processes maintaining osmolyte balance. Phosphoinositides can interfere at different levels with signalling cascades. As signalling intermediates, they can serve as substrate and cofactor for phospholipases or phosphatidylinositol kinases. They are also capable of regulating various enzymes like Rho GTPases and profilin and modulating ion channels and transporters.

For Ehrlich Ascites tumor cells, it could be shown that the level of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] responds to cell volume alterations. A decrease and an increase in cellular PI(4,5)P_2 levels occurs upon cell shrinkage and cell swelling, respectively (Hoffmann and Mills 1999). Similarly, a PI(4,5)P_2 decrease could be shown for rat hepatocytes (Baquet et al. 1991; Vom Dahl et al. 1991) upon cell shrinkage. In Swiss 3T3 mouse fibroblasts, hypertonic stress increases the levels of phosphatidylinositol 3,4-bisphosphate [PI(3,4)P_2] and phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3; Van der Kaay et al. 1999].

The effects of cell volume changes on PI(3,4,5)P_3 levels, phosphatidylinositol 3-kinase (PI3 K) and phosphatidylinositol 5-phosphatase activity, and the initiated signalling pathways are very variable. In rat skeletal muscle, hypotonicity as well as hypertonicity induce changes of glutamine uptake that are dependent on the activity of PI3 K (Low and Taylor 1998). The authors assume that PI3 K may serve to maintain an integrin-dependent mechanochemical transduction mechanism in an active state. The activity of PI3 K is also required to initiate the hypertonic activation of Na^+ conductance in rat hepatocytes (Wehner et al. 2002b).

In MDCK cells, hyperosmolality activates the serine-threonine-kinase Akt (also known as protein kinase B) in a PI3K-dependent manner (Terada et al. 2001). The product of PI3 K, PI(3,4,5)P_3, interacts with Akt itself as well as with 3-phosphoinositide-dependent kinases (PDK1 and PDK2). The actual activation of Akt occurs via Thr and Ser phosphorylation by PDK1 and PDK2 (Alessi et al. 1997; Stephens et al. 1998).

It was reported that the activation of Akt by hypertonicity prevents hyperosmolality-induced apoptotic changes in MDCK cells (Terada et al. 2001). Together with the finding that the hypotonic activation of PI3 K and Akt in HepG2 cells stimulates proliferation (Kim et al. 2001) ,this suggests that both enzymes influence the osmo-sensitive balance between cell survival and apoptosis.

In Swiss 3T3 mouse fibroblasts, hypertonicity-activated PI3 K elevates PI(3,4,5)P_3 levels without enhancing Akt activity, due to negative regulation of Akt by osmotic stress (Van der Kaay et al. 1999). This is coincident with the hypertonic, PI(3,4,5)P_3-dependent inhibition of PKBα in HEK-293 cells caused by inhibition of phosphorylation and activation of dephosphorylation of Akt, probably via PP2A (Meier et al. 1998). The inhibition of Akt seems to be triggered by PI(3,4,5)P_3 levels, independent of whether these occur by activation of PI3 K (Van der Kaay et al. 1999) or by inhibition of phosphatidylinositol 5-phosphatase (Meier et al. 1998).

Hypertonic accumulation of PI(3,4,5)P_3 activates p70 S6 kinase in Swiss 3T3 cells (Van der Kaay et al. 1999). This activation of p70 S6 kinase could be related to cell volume regulation on the basis of alterations in glycogen metabolism, since p70 S6 kinase activity is required for osmotically triggered changes in glycogen synthesis in rat skeletal muscle; during RVI, glycogen synthesis is inhibited, whereas it is stimulated during RVD (Low et al. 1996). The effect of cell volume changes on glycogen metabolism has also been demonstrated for rat hepatocytes (Häussinger et al. 1991). In this system, hypotonicity activates both PI3 K and p70 S6 kinase, but only PI3 K is involved in the hypotonic regulation of glycogen synthase (Krause et al. 1996).

The transcription of the serum and glucocorticoid dependent kinase (sgk) is clearly sensitive to cell volume changes. In the human hepatoma cell line HepG2, upregulation of sgk occurs upon cell shrinkage, whereas swelling reduces sgk transcript levels (Waldegger et al. 1997). Furthermore, sgk, like Akt and p70 S6, is a downstream target of PI3 K (Kobayashi and Cohen 1999). Most interestingly, stimulation of sgk results in the activation of ENaC in A6 cells (Chen et al. 1999b), rabbit cortical collecting duct cells (Naray-Fejes-Toth et al. 1999), and in *Xenopus* oocytes (Böhmer et al. 2000). For the latter system, it could also be demonstrated that sgk increases the abundance of the ENaC-protein in the plasma membrane (Alvarez et al. 1999; Wagner et al. 2001). In addition, sgk stimulates the activity of the neuronal K^+ channel Kv 1.3 as well as that of Na^+-K^+-ATPase and may thereby contribute to cell volume regulation (Setiawan et al. 2002; Wärntges et al. 2002).

The phosphorylation sites at which Akt and other protein kinases, such as p70 S6 kinase, PKCδ, and SGK are activated exhibit high similarity. This suggests that PDK1 and PDK2 may contribute equally well to the PI3K-dependent activation of these enzymes (Kobayashi and Cohen 1999).

Moreover, in rat HTC hepatoma cells (Feranchak et al. 1998) and in Mz-Cha-1 human cholangiocarcinoma cells (Feranchak et al. 1999), PI3 K contributes to cell volume regulation by the activation of Cl^- secretion via ATP release. The hypotonic activation of Na^+/H^+ exchange and HCO_3^- absorption in rat renal medullary thick ascending limb (MTAL) requires PI3 K activity as well (Good et al. 2000). The fact, that PI3 K is activated by *hypotonicity* as well as by *hypertonicity* in several systems reflects the general role of PI3 K as a key enzyme in signalling cascades. The actual result of this activation is then defined by its downstream targets that may vary considerably between different cells.

Arachidonic acid and eicosanoids

Arachidonic acid (AA) can be directly released from phosphatidylcholine and phosphatidylethanolamine by the activity of phospholipase A_2 (PLA_2). The cleavage of phosphatidylinositol by phospholipase C yields arachidonic acid out of diacylglycerol (DAG). Phospholipase D produces phosphatic acid, which can be transferred to arachidonic acid by the action of diglyceride lipase. Phosphatic acid may also be hydrolysed by PLA_2 into AA and lysophosphatic acid (LPA).

An increase in the intracellular concentration of arachidonic acid and its derivates upon hypotonic cell swelling has been shown for rat IMCD cells (Tinel et al. 1997), human neuroblastoma cells (Basavappa et al. 1998), and Ehrlich Ascites tumor cells (Thoroed et al. 1997). This release of AA seems to be mainly caused by the activation of PLA_2, as shown for Ehrlich Ascites tumor cells (Thoroed et al. 1997), human platelets (Margalit et al. 1993a), and human neuroblastoma cells (Basavappa et al. 1998).

In agreement with these findings, inhibition of PLA_2 prevents the activation of hypotonically activated events like Cl^- current in bovine pigmented ciliary epithelial cells (Mitchell et al. 1997) and iodide efflux in rat brain endothelial cells (von Weikersthal et al. 1997).

Influence on volume regulatory mechanism can be carried out by AA itself or by eicosanoids made from AA. AA is able to directly inhibit volume-sensitive Cl^- currents in guinea pig antral gastric myocytes (Xu et al. 1997), rat osteoblast-like cells (Gosling et al. 1996), Ehrlich Ascites tumor cells (Hoffmann and Lambert 1994), human neuroblastoma cells (Basavappa et al. 1998), and rat hepatocytes (Sakai et al. 1996). The activation of C1C-2 Cl^-channels by AA has been reported for the human lung epithelial cell lines Calu-3, A549, and BEAS-2B (Cuppoletti et al. 2001), and for HEK-293 cells (Tewari et al. 2000). AA is capable of activating K^+ channels in the colonic secretory cell line T84 (Devor and Frizzell 1998), in rat neuronal cells (Kim et al. 1995), and in rabbit cortical collecting tubule (CCT) cells (Ling et al. 1992).

Via alteration of cytosolic Ca^{2+} (Tinel et al. 1997; Jorgensen et al. 1999), AA can influence Ca^{2+}-sensitive Cl^- and K^+ currents, as well as sorbitol efflux in IMCD cells (see the section entitled “Sorbitol release pathways”).

The variety of the response of organic osmolyte release pathways in IMCD cells to AA is illustrated in Fig. 8. Here, isotonic taurine and *myo*-inositol efflux is inhibited, whereas AA can mimic the hypotonic response for sorbitol, betaine, and GPC.

AA can be metabolized in several pathways; two of them have been reported to be involved in mediating mechanisms of cell volume regulation. The lipoxygenase pathway leads to formation of leukotrienes and prostanoids, whereas prostaglandins and thromboxanes are formed in the cyclooxygenase pathway by the activity of cyclooxygenase and peroxidase.

Products of the lipoxygenase pathway seem to be involved mainly in the activation of RVD. The 5-LIP metabolite LTD_4 is able to activate hypotonic Cl^- and K^+ currents in Ehrlich Ascites tumor cells (Jorgensen et al. 1996); the isotonic cell shrinkage induced by LTD_4 occurs by the activation of the same mechanisms (Lambert 1989). In addition, LTD_4 acts as a second messenger for the taurine leak pathway and thereby contributes to RVD in this system (Lambert and Hoffmann 1993). An alteration of K^+ channels by 5-LIP product has also been shown for mudpuppy red blood cells (Light et al. 1997). In human skin fibroblasts, the activity of 5-LIP is required for the activation of swelling-induced Cl^-

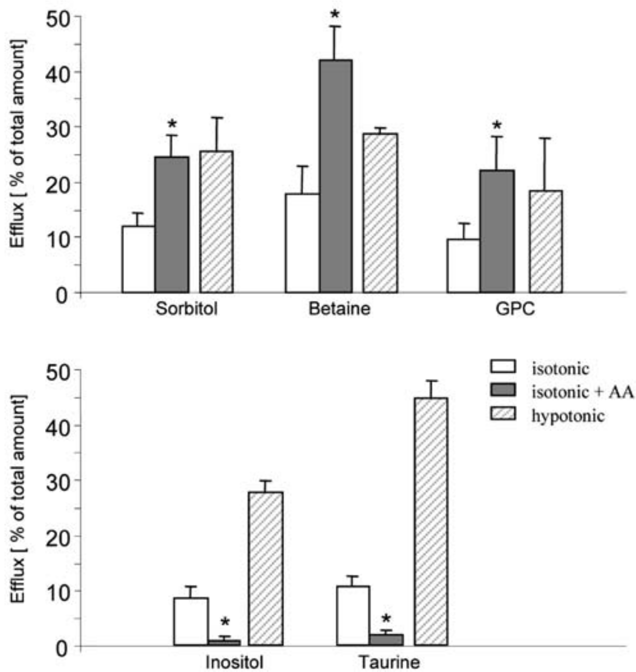

Fig. 8 Effect of arachidonic acid (*AA*) and hypotonicity on osmolyte efflux in rat inner medullary collecting duct (IMCD) cells. Efflux of osmolytes (in % of total amount; ±SE/10 min) was determined after 10 min exposure to 10 μM AA at isotonicity or after a decrease of extracellular osmolarity (600→300 mOsm/l) by means of HPLC; n=4 for all experimental conditions (B. Ruhfus and R.K. Kinne, unpublished data)

efflux (Mastrocola et al. 1993). The 12-LIP product hepoxilin A_3 is involved in the hypertonic activation of K^+ channels in human platelets (Margalit et al. 1993b).

In Ehrlich Ascites tumor cells, the stimulation of leukotriene synthesis is concomitant with an inhibition of prostaglandin synthesis (Lambert et al. 1987). The acceleration of RVD by LTD_4 or AA and the deceleration by prostaglandin E_2 (PGE_2) in mouse colonic crypts (Mignen et al. 1999) confirms that the reciprocal alteration of lipoxygenase and cyclooxygenase pathway affect synergistically volume recovery in these systems. In Ehrlich Ascites tumor cells, PGE_2 has no effect on the swelling-induced Cl^- permeability or K^+ permeability (Lambert et al. 1987). However, since PGE_2 is able to activate Na^+ channels, a decrease in PGE_2 synthesis may contribute to volume decrease by a reduction in the passive membrane conductance for Na^+ (Hoffmann 1978). In MDCK cells (Steidl et al. 1991) and human erythrocytes (Li et al. 1996), PGE2 has been reported to activate K^+ channels resulting in a decrease of cell volume. Cl^- channels can be activated by cyclooxygenase-dependent PGE_2 synthesis in the hepatoma cell line HTC (Kilic and Fitz 2002). For human amnion-derived WISH cells, it could be shown that COX-2 gene expression is upregulated by hypotonic stress; as a consequence thereof, PGE_2 release is increased (Lundgren et al. 1997). Of note, there is no evidence for the involvement of PLA_2-signalling in cell volume restoration upon hypertonic stress.

An involvement of phospholipase C (PLC) in cell volume regulation has been reported after hypotonic cell swelling, as well as after hypertonic cell shrinkage. PLC can influence

volume regulatory processes by different mechanisms, depending on the products of the reaction.

Ca^{2+} oscillations can be IP_3– as AA-sensitive as well (Ling et al. 1992; Tinel et al. 1997); both messengers result from PLC activity. Involvement of PLC in hypotonic Ca^{2+} oscillations has been reported for rat IMCD cells (Tinel et al. 1997), rat caudal artery endothelial cells (Shinozuka et al. 2001), rabbit proximale tubule cells (O'Neil and Leng 1997), and bovine aortic endothelial cells (Oike et al. 2000). The relevance of Ca^{2+} signalling for cell volume regulation is discussed in detail in the section entitled "Intracellular calcium."

In pigmented ciliary epithelial cells, volume-sensitive Cl^- currents are dependent on the activity of both PLA_2 and PLC. The contribution of PLC may be the synthesis of AA or enhancement of PLA_2 activity via a PKC-dependent phosphorylation of PLA_2 (Mitchell et al. 1997).

Phosphatic acid and lysophosphatic acid

Phospholipase D (PLD) generates the lipid second messengers phosphatic acid (PA) and choline by the cleavage of phosphatidylcholine. Only few examples exist for the involvement of PLD in volume-regulatory signalling. In skate erythrocytes, hypotonic treatment increases the turnover of phosphatidylcholine and the formation of PA due to the activation of PLD (Musch and Goldstein 1990). Sorbitol-induced osmotic stress enhances activity of PLD and PA release in rat adipocytes, leading to activation of aPKCs and translocation of GLUT4 transporters (Sajan et al. 2002). The PIP_2-synthesizing enzyme PIP 5-kinase can be regulated by PA; therefore, PLD may play a signalling role in PIP_2 sensitive events (Cockcroft 2001). Since alterations of PIP_2 levels are related to cell volume regulation, PLD may contribute to volume-dependent phosphoinositide signalling.

PA can be metabolized to AA by the action of PLD, but the relevance of PLD for volume-induced AA metabolism seems to be minor. In Ehrlich Ascites tumor cells, the contribution of PLD to the hypotonic AA release could be excluded (Thoroed et al. 1997).

Enzymatic conversion of PA by extracellular PLA_2 yields lysophosphatic acid (LPA); the initialization of intracellular signalling cascades occurs by binding of LPA to G protein coupled receptors (Goetzl and An 1998). For Ehrlich Ascites tumor cells, it could be revealed that extracellular application of LPA caused cell shrinkage by evoking Ca^{2+} oscillations and activation of Ca^{2+}-sensitive K^+ and Cl^- currents. As a consequence of this volume decrease, the Na^+/H^+ exchanger is activated (Pedersen et al. 2000). On the basis of the similarity between cell signalling after hypertonic cell shrinkage and after LPA treatment, the authors assume an interrelation in the regulation of both events.

Diacylglycerol

In erythrocytes, the intracellular level of diacylglycerol (DAG) is increased by hypotonicity (Musch and Goldstein 1990). DAG is a product of PLC and able to activate protein kinase C (PKC). PKC is a serine/threonine kinase of central importance in intracellular signalling cascades; hence, it is not astonishing that PKC is activated upon hypotonic cell swelling as well as upon hypertonic cell shrinkage in various systems.

Contribution of PLC to RVI after hypertonic stress has been reported for NIH/3T3 mouse fibroblasts (Zhuang et al. 2000) and Ehrlich Ascites tumor cells (Jensen et al. 1993); in both systems, PLC acts via PKC. In NIH/3T3 cells, the hypertonic phosphorylation of ERK 1/2 is due to DAG-dependent activation of classical and novel PKCs (Zhuang et al. 2000). Hypertonic activation of PLC enhances $Na^+/K^+/2Cl^-$ cotransport activity in Ehrlich Ascites tumor cells via Ca^{2+}-dependent PKC (Jensen et al. 1993).

In Ehrlich Ascites tumor cells, hypertonicity-activated PKC contributes to RVI by activating NHE and NKCC, and by inhibiting volume-sensitive Cl^- currents (Larsen et al. 1994; Hoffmann and Dunham 1995; O'Donnell et al. 1995; Pedersen et al. 1996). Inhibition of volume-sensitive Cl^- currents by PKC has also been reported for human nonpigmented ciliary epithelial cells (Coca-Prados et al. 1996), for cardiac ClC-3 expressed in NIH/3T3 cells (Duan et al. 1997), and HeLa cells (Hardy et al. 1995); however, in other systems, PKC may either stimulate chloride currents or leave them unaffected (reviewed in Pasantes-Morales et al. 2000a). The contribution of PKC to shrinkage-induced activation of NKCC is also cell-specific. While NKCC is downregulated by PKC in sweat glands (Toyomoto et al. 1997), NKCC remains unaffected in bovine endothelial cells (O'Donnell et al. 1995). PKC-dependent activation of NKCC has been demonstrated for rat hepatocytes; in this system, hypertonic Na^+ currents are activated by PKC, while hypertonicity activated NHE is not regulated by PKC (Heinzinger et al. 2001). In NIH/3T3 cells, hyperosmolality enhances the activity of c and n PKC, leading to activation of ERK1/2 (Zhuang et al. 2000).

After osmotic cell swelling, PKC is activated in the late phase of RVD in Ehrlich Ascites tumor cells; inhibition of VSCC by PKC terminates the volume response (Larsen et al. 1994). In contrast, hypotonic Cl^- membrane permeability is enhanced by PKC in HTC rat hepatoma and Mz-ChA-1 human cholangiocarcinoma cells (Roman et al. 1998).

The swelling-induced release of organic osmolytes is also influenced by the hypertonic activation of PKC. While inhibition of PKC prevents the hypertonic taurine release in rat cortical cup model (Estevez et al. 1999), the osmo-sensitive taurine release in cerebellar granule neurons is only prevented after chronic downregulation of PKC (Morales-Mulia et al. 2001). Thus, lipid-derived messengers are certainly involved in the cellular response to osmotic challenges. The complexity of the networks they are part of precludes, however, prediction of the outcome of the experiments aimed at elucidating their physiological role. Apparently, various cells have developed different responses; the reason for this phenomenon may lie in their differentiation into functionally diverse entities.

Cyclic nucleotides

The intracellular amounts of cAMP, as well as cAMP-dependent phosphorylation, have been demonstrated to be unaffected by osmotic cell shrinkage in duck and turkey erythrocytes (Kregenow et al. 1976; Alper et al. 1980). Initiation of the cAMP pathway is more likely to inhibit RVI, since cAMP-dependent activation of PKA can prevent the uptake of the organic osmolytes betaine and myo-inositol, as shown for MDCK cells (Preston et al. 1995). The hypertonic increase of intracellular cAMP suppresses the activation of Erk and p38; both MAP-kinases are known to be activated after hypertonic cell shrinkage in various systems (Orlic et al. 2002). However, cAMP is able to evoke cell swelling in sweat glands (Ohtsuyama et al. 1993). Osmotic cell swelling has been found to increase intracel-

lular cAMP levels in various systems and cAMP leads to cell volume decrease (reviewed in Lang et al. 1998a).

In rectal gland tubular cells from *Squalus acanthias*, cAMP-mediated cell shrinkage occurs by stimulation of NaCl excretion. A PKA-dependent phosphorylation of CFTR-type Cl^- channels is followed by activation of Na^+-K^+-$2Cl^-$ cotransporter (Greger 1996). In the human hematopoietic myeloblastic leukemia cell line ML-1, outward-rectified Cl^- channels are activated by PKA (Xu and Lu 1994). The activation of volume-sensitive Cl^- currents is inhibited by cAMP in myocardial cells, indicating that activation of these channels requires dephosphorylation of a cAMP-dependent phosphorylation site (Hall et al. 1995; Nagasaki et al. 2000).

Although hypotonicity increases intracellular cAMP levels in S49 mouse lymphoma cells, cAMP is not involved in triggering volume regulatory processes (Watson et al. 1991). As shown for the hypotonic myo-inositol efflux in human NT2-N neurons, PKA does not contribute to the osmotic regulation of this transporter (Novak et al. 2000). The efflux of organic osmolytes can be inhibited by PKA, since inhibition of PKA enhances the speed of the hypotonic activation of anionic amino acid conductance in *Leishmania major* promastigotes (Vieira et al. 1997).

Intracellular calcium

An involvement of calcium in volume regulation has been found in many cellular systems. In particular, the regulation of cell swelling seems to often be accompanied by calcium signals. In the case of hypertonic stress and cell shrinkage, other intracellular signal transduction processes seem to be responsible for the recovery of the cell volume and thus the reports about an involvement of calcium in RVI are very rare (Marchenko and Sage 2000; Erickson et al. 2001). In this overview, we will focus, therefore, on the role of calcium during RVD.

The core process of volume regulation after cell swelling is a release of osmolytes, resulting in reduction of cellular water content. Many different transport processes involved in RVD have been found to be calcium-dependent. Calcium-regulated potassium conductance via BK_{Ca}, IK_{Ca}, and SK_{Ca} have been found in many cells (see the section entitled "K^+ channels"). Also chloride conductances induced by volume changes have been observed to be regulated by calcium/calmodulin-dependent processes (see the section entitled "Cl^- channels"). In guinea pig jejunal villus epithelial cells, a calcium/calmodulin kinase II-mediated phosphorylation is a critical determinant of the volume regulation (MacLeod and Hamilton 1999a). In some cells, the calcium action on the same transport system seems to be transmitted via different effector proteins; for example, in human cervical cancer cells, myosin light-chain kinase and protein kinase C regulate a volume-sensitive chloride channel (Chou et al. 1998; Shen et al. 2002). In astrocytes, chloride conductance as well as taurine release are processes regulated by calcium and calmodulin (Li et al. 2002). In rat IMCD cells, calcium is involved in the release of sorbitol (Ruhfus et al. 1996). Recently, a coupling between arginine vasopressin-induced intracellular calcium mobilization and apical exocytosis was investigated in isolated perfused rat IMCD segments (Yip 2002). Vasopressin induced a rapid increase of the intracellular calcium followed by sustained calcium oscillations and changes in cell volume. Apical exocytosis accompanied by an increase of osmotic water permeability could be prevented by an inhibition of intracellular calcium release. The study shows that in IMCD cells vasopressin, via

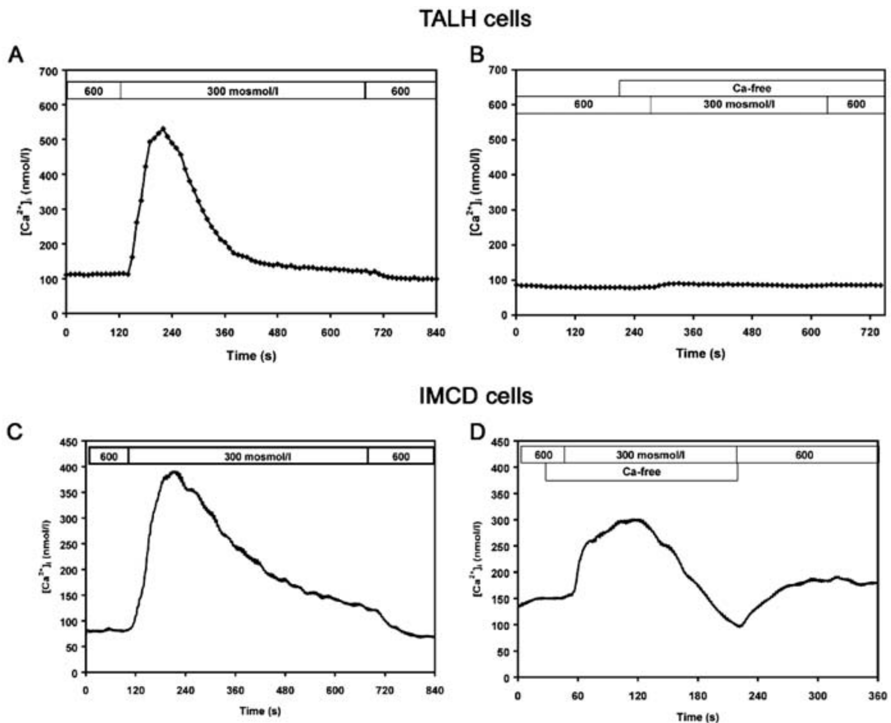

Fig. 9 Changes in the intracellular calcium concentration of TALH cells (**A**, **B**) and IMCD cells (**C**, **D**). At time indicated osmolarity was reduced from 600 mOsm/l to 300 mOsm/l by omission of sucrose. The experiments in calcium-containing solutions show a sustained calcium increase in both TALH (**A**) and IMCD (**C**) cells. In low-calcium solutions there was a calcium increase in the IMCD-cells due to a calcium release from intracellular stores (**D**). In TALH cells (**B**) no changes of the calcium concentration could be observed in calcium-free solutions as a prevention of the calcium influx made the calcium-induced calcium release impossible (**A** and **B** from Tinel et al. 2002; **C** and **D** from Tinel et al. 1994)

the V_2 receptor, triggers calcium oscillations which are required for exocytotic insertion of aquaporin-2 (Yip 2002). Activation of the sorbitol channel in rabbit TALH cells involves phosphorylation by a calcium/calmodulin kinase (Kinne-Saffran and Kinne 1997).

The source of the calcium involved in the volume regulation seems to be cell type-dependent. In several cells, RVD strictly depends on calcium entry across the plasma membrane. In these systems, the volume response can be inhibited by extracellular calcium removal or by addition of calcium channel blockers. A swelling-induced rise in intracellular calcium in rabbit medullary thick ascending limb cells was absent in low-calcium solutions (Montrose-Rafizadeh et al. 1991; see Fig. 9).

Similarly, strict dependence on calcium entry was found in, e.g., osteosarcoma cells (Yamaguchi et al. 1989), rabbit proximal tubule cells (Suzuki et al. 1990), intestine 407 cells (Hazama and Okada 1988), and cultured mouse primary sensory neurons (Viana et al. 2001). The diversity of calcium channels which are responsible for the calcium influx during hypotonic stress in different cell types is very high. Cell swelling induces stretch of the plasma membrane; thus, it is possible that stretch-activated calcium channels are activated by this process. Indeed, there are many reports showing that Gd^{3+}, a nonspecific in-

hibitor of stretch-activated channels, can block the calcium increase during hypotonic stress (Chen et al. 1996b; Urbach et al. 1999; Miyauchi et al. 2000; Viana et al. 2001).

Stretch-activated calcium influx might, in some cells, be mediated by voltage-sensitive calcium pathways, as it has been shown in osteocytes (Miyauchi et al. 2000). In these cells, antisense oligodeoxynucleotides against the α 1C subunit of voltage-operated L-type calcium channels abolished the calcium increase during hypotonic stress. The expression of the α 1C subunit of voltage-operated L-type calcium channels has also been shown in UMR-106 cells (Kizer et al. 1999). The existence of a voltage-activated calcium L-type channel which may participate in the volume regulation has also been identified in the apical membrane of cultured rabbit proximal tubule cells using the patch-clamp technique (Zhang and O'Neil 1996). In many other cell types the calcium increase during hypotonic stress was sensitive to blockers of voltage-activated calcium channels (Montrose-Rafizadeh and Guggino 1991; Bender et al. 1994; Mignen et al. 1999). In inner medullary collecting duct cells (IMCD), diltiazem, nifedipine, and verapamil partly reduced the calcium increase (Tinel et al. 2000). However, the concentration of the blocker was quite high (40 µMol/l). Thus, calcium influx seems to occur via calcium channels which possess pharmacological characteristics different from those of classical voltage-gated channel. The fact that an inhibitor of receptor-mediated channels (SKF 96365) also reduced the calcium influx in IMCD cells supports the idea (Tinel et al. 2000). Channels involved in hypotonic calcium influx in thick ascending limb of Henle's loop (TALH) cells could be inhibited by nifedipine, but not diltiazem or verapamil (Tinel et al. 2002). These results can be correlated with the existence of homologous genes for different types of subunits and several types of auxiliary subunits of voltage-dependent calcium channels (Isom et al. 1994). It can also explain the difference compared to the characteristics of the calcium influx observed in TALH cells, which were not adapted to higher osmolarity (Montrose-Rafizadeh and Guggino 1991). In these cells, hypotonicity-induced calcium entry occurred via a nifedipine- and verapamil-sensitive pathway.

A complete inhibition of the calcium increase by calcium channel blockers or lack of the calcium signal in calcium-free solution has often been interpreted as proof that the intracellular calcium stores are not involved in the calcium rise during hypotonic stress. The assumption would be correct only if no ryanodine-sensitive calcium stores exist in the cell. Using caffeine, it has been shown that, e.g., TALH cells possess calcium stores that, although at first sight not involved in the volume regulation, can be mobilized experimentally in isotonic solution (Tinel et al. 2002). Caffeine causes calcium release from ryanodine-sensitive stores. The ryanodine receptor in the membrane of these stores is sensitive to calcium, which in micromolar concentration stimulates the receptor and triggers a calcium release (Tinel et al. 2000). This phenomenon is referred to as calcium-induced calcium release (CICR), a mechanism responsible for, e.g., heart muscle contraction. In TALH cells, the existence of CICR during hypotonic stimulation has been shown using manganese quenching experiments (see Figs. 9, 10).

Calcium release from intracellular stores followed the calcium influx during hypotonic stress (Tinel et al. 2002). The involvement of CICR in volume regulation has already been described in a human intestinal epithelial cell line (Intestine 407; Hazama and Okada 1990). For the volume regulation of these cells, the calcium influx is necessary, but not sufficient, unless it elicits CICR. In rabbit corneal epithelial cell, hypotonic stress has been found to induce a calcium release from ryanodine-sensitive stores followed by activation of a calcium influx (Wu et al. 1997). The authors describe the mechanism as CICR; how-

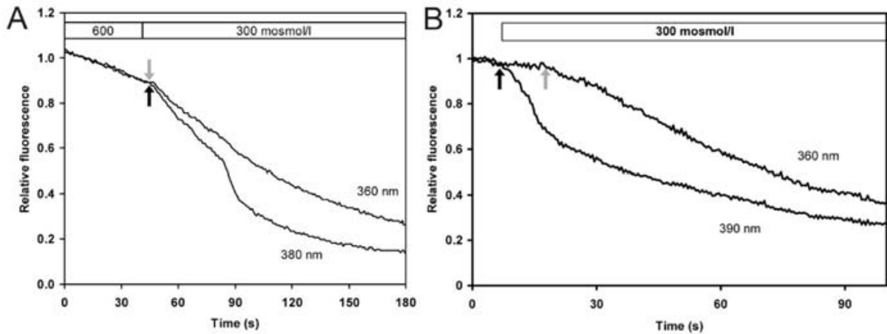

Fig. 10 Quench of fura-2 fluorescence by manganese under hypotonic conditions. The osmolarity was reduced from 600 mOsm/l to 300 mOsm/l by omission of sucrose. **A** In TALH cells, the hypotonic stress in calcium-containing solutions induced a monoexponential decay of the fluorescence intensity at 360 nm, reflecting a constant calcium influx. At 380 nm, the fluorescence decay was biphasic. During the first 40 s, both manganese and calcium entered the cell and induced a reduction of the fluorescence intensity. The additional fast decay was due to calcium-induced calcium release. **B** In IMCD cells hypotonic stress in calcium-free solutions induced a fast decay of the fluorescence at 390 nm due to calcium release from intracellular stores followed by an opening of a calcium influx pathway visible as a decrease in the fluorescence at 360 nm. → start of increase in intracellular calcium, ⇒start of calcium-entry from the extracellular medium (**A** from Tinel et al. 2002; **B** from Tinel et al. 1994)

ever, it corresponds more to the store-operated calcium influx. The RVD in these cells was also strictly dependent on the calcium rise. In TALH cells, the volume regulation was only partly calcium-dependent; thus, the calcium release from intracellular stores probably works as an amplifier of the signal. CICR is very important for the generation of calcium oscillations and calcium waves (Tinel et al. 2000). In some cells, there are initiation sites where the signal appears first before spreading through the cell as a wave (Tinel et al. 1999). Such local calcium events during the volume regulation of TALH cells could control membrane recycling, as has been described to occur in IMCD cells (Czekay et al. 1994). During exposure of IMCD cells to hypotonicity, the submembranal actin web rapidly disintegrates, and reserve vesicles, probably containing a sorbitol transporter, move to and fuse with the basolateral plasma membrane. The fusion causes a rapid increase in sorbitol permeability (see Fig. 4 above). These membrane areas are recovered by internalization, and the transport systems for sorbitol are concomitantly retrieved (Czekay et al. 1994).

The majority of studies concerning the role of calcium in volume regulation describe a biphasic calcium rise consisting of a calcium influx followed by calcium release from intracellular stores which are not ryanodine-sensitive. Manoeuvres leading to depletion of the intracellular stores or inhibiting the calcium release abolish the calcium increase in these cells during hypotonic stress (Hoffmann et al. 1984; McCarty and O'Neil 1991; Bender et al. 1994; Civan et al. 1994a; Mignen et al. 1999). Intracellular stores seem to participate in the shaping of the calcium signal when the changes of the cell volume occur in isotonic solution, e.g., in Ehrlich ascites tumor cells after stimulation with lysophosphatidic acid (Pedersen et al. 2000), or in pneumocytes after pretreatment with insulin (Marunaka et al. 1999). In some of the reports, the calcium stores are described as being inositol-trisphosphate (IP_3)-sensitive. An increase of the intracellular concentration of IP_3 has been measured during isotonic swelling of hepatocytes (Baquet et al. 1991), and during volume

regulation of rabbit proximal tubule cells (Suzuki et al. 1990) and astrocytes (Bender et al. 1993).

Although in IMCD cells the calcium rise could be blocked by inhibitors of the calcium release from IP_3-sensitive stores, the signal transduction pathways leading to the calcium mobilization seem to be different (Tinel et al. 1994). In these cells, cell swelling in hypotonic solution is paralleled by an increase in intracellular calcium concentration typically appearing in two distinct phases. Whereas the first phase of the calcium response reflects predominantly calcium release from intracellular stores, the second sustained component is due to an increase in calcium entry from the extracellular compartment (Mooren and Kinne 1994; Tinel et al. 1994; see Figs. 9 and 10). However, measurement of intracellular IP_3 revealed no significant changes in the concentration of this second messenger (Tinel et al. 1997). Instead, inhibition of the arachidonic acid metabolism led to a considerable reduction of calcium release, as well as RVD. Thus, in these cells the calcium mobilization during RVD may be initiated by arachidonic acid or by its metabolites. Under isotonic conditions, an application of arachidonic acid induces a considerable increase in the intracellular calcium concentration in IMCD cells, both in high and in low calcium solutions, indicating that IMCD cells posses arachidonic acid-sensitive calcium stores. Extracellular ATP induces in these cells an increase in the intracellular IP_3 concentration and calcium rise, which reveals the existence of functionally active IP_3-sensitive stores. Thus, IMCD-cells possess arachidonic acid-sensitive as well as IP_3-sensitive calcium stores. These stores may be physically distinct; alternatively, the arachidonic acid-sensitive stores may be a part of the IP_3-sensitive pool. This is not contradictory to the finding that stimulation of calcium release by IP_3 during volume regulation apparently does not occur in IMCD cells. Arachidonic acid could change the affinity of the IP_3-receptor for IP_3 so that the IP_3 concentration already present in the cytosol of nonstimulated cells might induce a calcium release. It is possible that cooperation of different calcium stores control the volume regulation of these cells.

A combination of two different calcium-releasing mechanisms in the regulation of the cell volume has also been found in astrocytes (Bender and Norenberg 1994). In these cells, intracellular calcium release from CICR-stores and from IP_3-sensitive intracellular calcium stores play a role in the mechanism of potassium release under hyposmotic conditions. In submandibular gland acinar cells, the calcium increase is a result of a calcium release from agonist-sensitive stores and from mitochondria (Park 2002). Mitochondria are known to participate in modulating calcium signals in other cells (Hoffmann et al. 1984), and it is possible that they fulfill this function also during volume regulation, but this question remains to be elucidated.

The signal transduction processes leading to generation of the second messengers and to the calcium release (IP_3 or arachidonic acid) involves probably the action of phospholipases. Inhibitors of the phospholipase A_2 impaired the volume regulation of, e.g., human neuroblastoma cells (Basavappa et al. 1998) and human platelets (Margalit et al. 1993a). In cultured rabbit principal cells, apical application of the phospholipase A_2 agonist increased single-channel open probability of a volume-sensitive potassium channel (Ling et al. 1992). In IMCD cells, the activation of the arachidonic acid cascade during RVD occurs via activation of phospholipase C and probably phospholipase A_2 (Mooren and Kinne 1994; see also the section entitled “Arachidonic acid and eicosanoids”).

Usually phospholipases are activated by G-proteins. In IMCD cells, G-proteins are involved in the generation of the calcium signal (Mooren and Kinne 1994). Involvement of

G-proteins in the regulation of RVD has also been reported to occur in human platelets (Margalit et al. 1993a), and inhibition of RVD by pertussis toxin has been described in proximal tubule cells (Suzuki et al. 1990).

How G-proteins are actually activated during osmotic swelling remains obscure. A mechanoreceptor which could serve as a volume sensor has only been described for neurons (Oliet and Bourque 1993). A mechanical-biochemical coupling involving G-proteins and phospholipase A_2 during cell swelling has been postulated for platelets (Margalit et al. 1993a). OTRPC4, a nonselective calcium-permeable channel which belongs to the TRP channel family, has been proposed to be a candidate for a molecular sensor that confers osmosensitivity in mammalian cells (Strotmann et al. 2000). It exhibits spontaneous activity in isotonic media and is rapidly activated by a decrease (and is inhibited by an increase) of the extracellular osmolarity. Changes in osmolarity of as little as 10% result in significant changes in intracellular calcium concentration in HEK293 cells expressing the channel. This increase resulted exclusively from calcium influx from the extracellular space, with no involvement of calcium release from intracellular stores. In contrast to the members of the classical TRP channel family that are activated secondary to the activation of phospholipase C-coupled receptors, the OTRPCs have been proposed to be involved in the transduction of physical and chemical stimuli. However, OTRPC4 activity was unaffected by pressure-induced stretching of the membrane. The authors propose the channel as a universal sensor in mammalian cells, but the results from other studies where calcium signals could be abolished by inhibition of different steps of the signal transduction contradict this assumption.

In some cells, an autocrine (or paracrine) action of ATP has been found to initiate the calcium signalling during the volume regulation. During osmotic swelling, intracellular ATP is released to the extracellular space in a number of cells. In the immediate vicinity of the cell surface, released ATP has been shown to reach a concentration high enough to simulate P_2-purinergic receptors in a human epithelial cell line, intestine 407 (Dezaki et al. 2000). RVD in these cells is facilitated by ATP by augmenting intracellular calcium rise via the stimulation of purinergic P_{2Y2} receptors. Similar observations were made in endothelial cells from the rat caudal artery (Shinozuka et al. 2001). In hepatocytes, the signalling pathways seem to be more complicated. Ferenchak et al. have found a constitutive and volume-dependent ATP release, which was a critical determinant of membrane chloride permeability. P_2 receptor antagonist suramin abolished the volume recovery after exposure of hepatocytes to hypotonic buffer (Feranchak et al. 2000). However, Roe et al. have shown that the swelling-induced calcium signal in hepatocytes was unaffected by either extracellular ATP depletion or blockade of P_2 receptors (Roe et al. 2001). The swelling elicited an increase of the intracellular calcium, which was essential for ion channel activation and volume recovery, but this increase did not stem from activation of volume-sensitive P_2 receptors. Taken together, the results from both groups indicate that in hepatocytes two different pathways initiate the calcium signalling during hypotonic stress: a purinergic-dependent and a purinergic-independent pathway.

The mechanisms guaranteeing the interplay between the calcium release and calcium influx are not very well understood. In IMCD cells, the calcium release from the stores is a prerequisite for the activation of the calcium influx (Tinel et al. 1994). The opening of the plasma membrane ion channels happens possibly to prolong the calcium response beyond the point where intracellular stores are depleted and/or to support the refilling of these stores after calcium depletion. If the calcium release takes place in calcium-free iso-

tonic solutions, a readdition of calcium to the extracellular medium induces a sustained increase of the intracellular calcium concentration. This indicates that the IMCD cells possess a capacitative calcium influx mechanism. The activation of this pathway is not dependent on an increase of the intracellular calcium concentration. Thus, there has to be another signal activating the calcium channels in the plasma membrane. In IMCD cells, the cytoskeleton could forward the message from the empty stores to the plasma membrane as Mooren and Kinne have observed a complete inhibition of the hypotonic calcium increase by cytochalasin B (Mooren and Kinne 1994). The signal transduction from the stores to the plasma membrane takes some 17 s, as could be estimated using the manganese quenching technique (Tinel et al. 1994; see Fig. 10).

In proximal straight tubule, a temporary dependence of the RVD on calcium has been observed (McCarty and O'Neil 1991). There is a short period of time (about 60 s), during which extracellular calcium is required. Outside of this "calcium window," RVD would inactivate and could not be reactivated by subsequent addition of calcium. It was found that the calcium permeability did not inactivate over several minutes, indicating that the temporary dependence of RVD on extracellular calcium is not due to the transient activation of calcium entry pathway (McCarty and O'Neil 1991).

A very interesting new aspect of the interaction between calcium stores and channels has been found by Lemonnier et al. in human prostate cancer epithelial cells (Lemonnier et al. 2002). In these cells, although variations of both intracellular and extracellular calcium concentrations had no visible effects on a volume-regulated anion channel, calcium influx via store-operated channels strongly modulates it. The authors suggest that interaction between the volume-regulated anion channel and calcium occurs in the confined compartments at the inner surface of the membrane that are not accessible to changes of global intracellular calcium. These domains can be readily reached by calcium entering the cell via plasma membrane, especially through the store-operated calcium channels. This preferred access of store-operated calcium channels to the volume-regulated anion channels suggests colocalization of these channels in the cell membrane (Lemonnier et al. 2002).

This finding belongs to the rare results illustrating the importance of the spatio-temporal aspect of calcium signalling. The intensive work during the last few years showed that, e.g., in secretory cells, the global calcium rise observed in many studies is mainly due to experimental manipulations and that local calcium events (sparks, oscillations, waves) reflect what actually happens under physiological conditions (Tinel et al. 1999; Tinel et al 2000). Future work concerning calcium signalling during volume regulation should concentrate much more on the local calcium events. It would be very important to recognise the mechanisms confining the signals in time and space to allow regulation of specific volume-related processes without influencing other cellular processes influenced by calcium.

Conclusions and perspectives

The data presented above document once more the complexity of events that ultimately result in the maintenance of cell volume and its restoration after disturbance by an osmotic difference between intra- and extracellular space. This complexity, although difficult to decipher, provides different cells with various mechanisms that are adapted to protect themselves and at the same time to fulfill their overall role in the organism. Within one

particular cell, the presence of multiple systems might open multiple possibilities to achieve a graded response to osmotic stimuli.

To obtain further insight into this complexity several steps at different levels of resolution are necessary. At the level of the single molecules, proper molecular and pharmacological identification, proper biophysical characterization, and proper analysis of biochemical alterations have to be continued and intensified. At the cellular level, the simultaneous measurement of the activities of the most important osmolyte transporters and the concentration of the various osmolytes at a higher temporal and spatial resolution have to be performed. The same holds for the determination of the localization and activity of the various elements of the signal transduction network.

Only then will it be possible to describe the dynamic nature of cell volume regulation and its underlying mechanisms in a quantitative way based on causal relationships.

Acknowledgements. We thank Leon Goldstein, Rene Notais, Bernd Nilius and Markus Paulmichl for providing us with unpublished results of their current work. Stefan H. Boese was kind enough to comment on the chapters relating to chloride channels.

The expert and speedy secretarial help of Petra Jurczynski, the outstanding editorial assistance of Natascha Kist, the knowledgeable advice of Rosemarie Hübner and the professional creation of the artwork by Gesine Schulte are gratefully acknowledged.

Dedication: This chapter is dedicated to the many successful years of collaboration of the authors and to the excellent Ph.D. students and Postdocs, who shared our excitement.

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