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Review

The role of volume-sensitive ion transport systems in regulation of epithelial transport☆

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Abstract

This review focuses on using the knowledge on volume-sensitive transport systems in Ehrlich ascites tumour cells and NIH-3T3 cells to elucidate osmotic regulation of salt transport in epithelia. Using the intestine of the European eel (*Anguilla anguilla*) (an absorptive epithelium of the type described in the renal cortex thick ascending limb (cTAL)) we have focused on the role of swelling-activated K^+- and anion-conductive pathways in response to hypotonicity, and on the role of the apical (luminal) Na^+–K^+–$2Cl^-$ cotransporter (NKCC2) in the response to hypertonicity. The shrinkage-induced activation of NKCC2 involves an interaction between the cytoskeleton and protein phosphorylation events *via* PKC and myosin light chain kinase (MLCK). Killifish (*Fundulus heteroclitus*) opercular epithelium is a Cl^--secreting epithelium of the type described in exocrine glands, having a CFTR channel on the apical side and the Na^+/K^+ATPase, NKCC1 and a K^+ channel on the basolateral side. Osmotic control of Cl^- secretion across the operculum epithelium includes: (i) hyperosmotic shrinkage activation of NKCC1 *via* PKC, MLCK, p38, OSR1 and SPAK; (ii) deactivation of NKCC by hypotonic cell swelling and a protein phosphatase, and (iii) a protein tyrosine kinase acting on the focal adhesion kinase (FAK) to set levels of NKCC activity.
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Keywords: *Fundulus heteroclitus*; *Anguilla anguilla*; Opercular epithelium; Intestine; RVD; RVI; NKCC; Na^+, K^+, $2Cl^-$ cotransport; SPAK; Protein kinase; Ussing chamber

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Abbreviations: NKCC, Na^+, K^+, $2Cl^-$; RVI, volume regulatory increase; EATC, Ehrlich ascites tumour cells; SK, K channels having small conductance; IK, intermediate conductance; BK, large conductance; 2P-4TM, four transmembrane-spanning segments; TASK-2, acid sensitive potassium channel; LTD_4, leukotriene D4; EET, 5′,6′-epoxyeicosatrienoic acid; PGE_2, prostaglandin E_2; VRAC, volume regulated outward rectifying anion current; BAE, bovine aortic endothelial; PTPs, protein tyrosine phosphatases; PTKs, protein tyrosine kinases; PKA, protein kinase A; PKC, protein kinase C; CK2, casein kinase; MLCK, myosin light chain kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; Ste20, sterile 20; SPAK, sterile 20-related proline alanine-rich kinase; OSR1, oxidative stress response 1 kinase; ELA, Ehrlich Lettre Ascites; WNK, with no K (lysine) protein kinase; FW, freshwater; SW, seawater.

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1. Introduction

Swelling-activated K^+ and anion channels are important effectors during regulatory volume decrease (RVD after cell swelling, whereas a Na^+, K^+, $2Cl^-$ (NKCC) cotransporter and a Na^+/H^+ exchanger play major roles in the regulatory volume increase (RVI) following cell shrinkage. Transport pathways involved in RVD and RVI have been investigated in a wide variety of cell types (Hoffmann and Dunham, 1995; Lang et al., 1998; Wehner et al., 2003; Hoffmann and Pedersen, 2006). In the first part of this review we describe briefly the swelling activated channels and the shrinkage-activated NKCC and the signal transduction mechanisms involved in the activation of these transport systems by changes in cell volume. Other transport systems involved in rapid volume regulation and aspects of long-term adaptation to an anisosmotic environment are outside the scope of this review. In the second part of this review we will try to correlate this knowledge with the regulatory mechanisms involved in osmotic regulation of salt transport in epithelia using the killifish opercula epithelium and the eel intestinal epithelium as examples.

2. Volume-sensitive ion transport systems

2.1. Swelling-activated K^+ channels ($I_{K,vol}$)

Swelling activation of a K^+ leak pathway was initially established in lymphocytes (Roti Roti and Rothstein, 1973) and in Ehrlich ascites tumour cells (EATCs) (Hendil and Hoffmann, 1974). This swelling-activated increase in K^+ permeability has been established in different cell types to be related to a variety of swelling-activated K^+ channels including Ca^{2+}-activated channels of small conductance (SK), intermediate conductance (IK) or large conductance (BK); stretch-activated K^+ channels; voltage-dependent K^+ channels such as Kv 1.3 or Kv 1.5; KCNQ1/KCNE3 heterotetrameric channels and two-pore-regions, four-trans-membrane-spanning segment (2P-4TM) K^+ channels (Wehner et al., 2003; Stutzin and Hoffmann, 2006). The most likely candidate in EATCs is the 2P-4TM, acid-sensitive K^+ channel (TASK-2) (Niemeyer et al., 2000; 2001a,b). Many cloned K^+ channels have been found to be sensitive to cell volume changes when expressed in *Xenopus* oocytes or in HEK 293 cells including SK and IK channels, KCNQ1 and KCNQ4; HCN2 channels and TASK-2 channels (Calloe et al., 2005).

2.2. Activation and regulation of $I_{K,vol}$

Various eicosanoids seem to be involved in regulation of swelling-activated channels (Hoffmann, 2000; Stutzin and Hoffmann, 2006). In human platelets the 12-HPETE product, hepoxilin A activates $I_{K,vol}$ (Margalit and Livne, 1991, 1992) and in EATC, leukotriene D4 (LTD_4) activates $I_{K,vol}$ independent of any increase in cytosolic Ca^{2+} (Jørgensen et al., 1997; Hoffmann, 1999; Hougaard et al., 2000). A role for LTD_4 has also been shown in some other cell types but in several cell types the RVD response seems to be independent of LTD_4 (Stutzin and Hoffmann, 2006). The eicosanoids responsible for activation of RVD vary among cell types and the channels involved, but a common theme is that PLA_2 is activated during RVD, which releases arachidonic acid from the membrane phospholipids, as originally established in EATCs (Thoroed et al., 1997) and in IMCD cells (Tinel et al., 1997). In EATCs, activation of the 85-kDa Ca^{2+}-dependent $cPLA_2$ results in the release of arachidonic acid predominantly from the nuclear membrane (Pedersen et al., 2000). The

RVD response in EATCs is inhibited by tyrosine kinase inhibitors, and it has been suggested, that tyrosine kinases are implicated in the activation of $cPLA_2\alpha$ (Hoffmann, 2000). Whether tyrosin kinases are directly involed in activation of some or all of the swelling activated K^+ channels is still uncertain. Other eicosanoids of interest with RVD are 5′,6′-epoxyeicosatrienoic acid (EET), which activates TRPV4 (see Nilius et al., 2004; Cohen, 2005), and the prostaglandin E_2 (PGE_2), which increases Na^+ permeability in EATCs (Lambert, 1987).

2.3. Swelling-activated anion channels

Cl^- transport is for the most part carried out by an exchange-diffusion process with the conductive Cl^- permeability being very low in isotonic steady-state cells. This low permeability means that Cl^- conductance in many cells is much lower than the K^+ conductance. Cl^- channels activated by cell swelling are thus crucial elements in the RVD process. In EATCs it has been demonstrated that less than 5% of the Cl^- flux is a conductive leak flux (Hoffmann et al., 1975, 1979) and that the Cl^- conductance is much lower than the K^+ conductance (Hoffmann et al., 1979). In agreement with these observations is the finding that Cl^- flux increases during cell swelling in EATCs reflecting a swelling-activated increase in Cl^- conductance (Hoffmann, 1978, 1982). Swelling-activated Cl^- channels were thus first suggested in EATCs (Hoffmann, 1978, 1982) and soon after in lymphocytes (Grinstein et al., 1982) and have later been found in all cells investigated.

The biophysical features and pharmacology of the volume-regulated anion current (VRAC) are relatively similar in various cell types (Nilius et al., 1997). This will not be discussed here, but there are several excellent reviews of the subject (see Jentsch, 1996; Nilius et al., 1997; Jentsch et al., 2002; de Tassigny et al., 2003; Nilius and Droogmans, 2003; Stutzin and Hoffmann, 2006). Blockers of VRAC with high affinity and selectivity are unfortunately lacking but recently a new inhibitor of the family of acidic diaryl-ureas, NS3728, has been found to inhibit VRAC in HEK-293 cells and in EATC with an IC_{50} around 0.4 μM (Helix et al., 2003; Klausen et al., 2006). VRAC activity in bovine aortic endothelial (BAE) cells (Levitan et al., 2000; Romanenko et al., 2002, 2004) and in EATCs (Klausen et al., 2006) is modulated by changes in membrane cholesterol content that result in an increase in VRAC activity under modest osmotic shock, in cholesterol-depleted cells.

Tyrosine phosphatases (PTPs) blockers increase VRAC opening, and several inhibitors of protein tyrosine kinases (PTKs), including genistein, prevent VRAC activation, indicating that VRAC activation essentially relies on a strict phosphorylation/dephosphorylation balance (Tilly et al., 1993; Voets et al., 1998; Nilius et al., 2000; Du et al., 2004). There are indications that a member of the Src-family of PTKs is involved in VRAC activation (Lepple-Wienhues et al., 1998, 2000). It seems that the Rho pathway has a permissive effect in VRAC activation both in NIH3T3 cells (Pedersen et al., 2002) and in CPAE cells (Carton et al., 2002).

2.4. The NKCC cotransporters

Two distinct isoforms of the NKCC cotransporters have been cloned (NKCC1 and NKCC2). NKCC1 was first cloned from the shark (*Squalus acanthias*) rectal gland (Xu et al., 1994) and from the mouse inner medullary collecting duct (Delpire et al., 1994; Payne et al., 1995). NKCC1 is expressed ubiquitously and has a central role in keeping $[Cl^-]_I$ above electrochemical equilibrium and in cell volume regulation (Hoffmann and Dunham, 1995) and epithelial salt secretion (Haas and Forbush, 2000). NKCC1 is activated by cell shrinkage, as first demonstrated in avian erythrocytes and EATCs (Kregenow, 1981; Hoffmann et al., 1983), and later seen in most other cell types studied (see Russell, 1983; Hoffmann and Dunham, 1995; Flatman, 2002). NKCC1 has protein kinase A (PKA), protein kinase C (PKC) and casein kinase (CK2) consensus sites (Russell, 1983; Flatman, 2002), and its activation seems to be mediated by direct phosphorylation of several serine/threonine residues (Haas and Forbush, 2000). Activation could be *via* one specific NKCC1 kinase (Lytle, 1997) Three of the phosphoacceptors in the N terminus of NKCC1 have been identified (Darman and Forbush, 2002).

NKCC2 is a kidney-specific cotransporter, expressed on the apical membrane in the mammalian thick ascending limb of Henle's loop (Gamba et al., 1994; Payne and Forbush, 1994, 1995; Igarashi et al., 1995; Starremans et al., 2003) and in the shark kidney (Gagnon et al., 2002). An absorptive form of NKCC localized on the apical membrane has however also been found in the intestine of the Japanese eel (*Anguilla japonica*) (Ando and Utida, 1986), European eel (*Anguilla anguilla*) (Trischitta et al., 1992; Marvao et al., 1994) and winter flounder (*Pseudopleuronectes americanus*) (Palfrey and Rao, 1983; Suvitayavat et al., 1994), where it accounts for the active intestinal Cl^- absorption. A cDNA fragment coding for a homologue of renal NKCC2 was cloned from eel intestine and has a high similarity to cot2 (see Lionetto and Schettino, 2006). Both renal NKCC2 and the eel intestine luminal NKCC2 homologue are active under isotonic conditions and increase their activity following hypertonic stress (Gamba, 2005; Lionetto and Schettino, 2006).

2.4.1. Regulation of NKCC1

A number of ser/thr kinases seem to be important in NKCC1 regulation, although there is no evidence that any kinase directly phosphorylate NKCC1 in vivo. The three primary ser/thr kinases are PKC, myosin light chain kinase (MLCK) and PKA. PKC is activated after cell shrinkage in some cells (Larsen et al., 1994; Liedtke and Cole, 2002), but does not seem to be directly involved in activation of NKCC1 by cell shrinkage in most cells investigated (Pewitt et al., 1990; Panet and Atlan, 1990; Suvitayavat et al., 1994; Krarup et al., 1998). PKC-δ is however found to stimulate NKCC1 in rabbit tracheal epithelial cells during shrinkage (Liedtke and Cole, 2002). In a follow-up study the same group found that PKC-δ colocalizes with actin and

NKCC1 in the periphery of the cells and, that an interaction between PKC-δ and the cytoskeleton is necessary for NKCC1 activation (Liedtke et al., 2003; Smallwood et al., 2004). This PKC–NKCC complex could represent an osmosensing mechanism.

MLCK seems to play an important role: the MLCK inhibitor ML-7 inhibits shrinkage-induced activation of NKCC1 in various cell types, including Ehrlich cells (Krarup et al., 1998),vascular endothelial cells (O'Donnell et al., 1995; Klein and O'Neill, 1995), and kidney epithelial cells (Ciano-Oliveira et al., 2003) and under osmotic shrinkage phosphorylates MLC in some cell types (Klein and O'Neill, 1995; Takeda et al., 1993). These results could suggest a possible causal link between MLCK/MLC phosphorylation and the shrinkage activation of NKCC1. Nevertheless recent evidences (Ciano-Oliveira et al., 2005) indicate that ML-7 suppresses NKCC activity in kidney tubular LLC-PK1 cells independently of MLC phosphorylation. Thus, it seems that activation of NKCC1 by cell shrinkage is not dependent on MLC phosphorylation *per se* but rather on the redistribution of myosin to the cortical region (Ciano-Oliveira et al., 2005). In agreement with this hypothesis, myosin II is translocated to the cortical region in EATCs after cell shrinkage (Hoffmann et al., 2002; Pedersen and Hoffmann, 2002). PKA stimulates NKCC1 and increases its phosphorylation but indirectly *via* a phosphorylation of NKCC1 by PKA (Haas and Forbush, 2000). PKA does not seem to be involved in the shrinkage activation of NKCC1 (Pewitt et al., 1990; Krarup et al., 1998; Hoffmann and Mills, 1999).

Calyculin A, a ser/thr protein phosphatase inhibitor, stimulates NKCC1 in EATC (Krarup et al., 1998) and in many other cell types (Pewitt et al., 1990; Palfrey and Pewitt, 1993; Lytle, 1997; Krarup et al., 1998; Lionetto et al., 2002; Flatman, 2002) in agreement with the idea that regulation of NKCC1 is determined by phosphorylation/dephosphorylation. In shark rectal gland epithelium, a salt-secreting epithelium that shares many functional aspects with the secreting cells of the teleost gill and operculum membrane, there is a PP1 binding site in the amino terminus of NKCC1 at residues 107–122, and PP1 co-immunoprecipitates with NKCC1 (Darman et al., 2001). Thus, it seems that the activity of NKCC1 is regulated by a ser/thr kinase and its analogous phosphatase, both of which are again regulated by other upstream ser/thr kinases and phosphatases.

In addition to the ser/thr kinases discussed above several other kinases are somehow involved in regulation of NKCC1. P38 mitogen-activated protein kinase (MAPK) is activated by cell shrinkage in many cells (Roger et al., 1999; Gillis et al., 2001; Shen et al., 2002; Umenishi and Schrier, 2003; Bildin et al., 2003; Pedersen et al., 2002; Hoffmann et al., 2004; Friis et al., 2005; Marshall et al., 2005) but strangely enough p38 MAPK actually inhibits NKCC1 function in muscle cells (Gosmanov et al., 2002) apparently through an inhibition of ERK MAPK.

Another MAP kinase, c-Jun N-terminal kinase (JNK), is activated in endothelial cells after cell shrinkage, and JNK immuno-precipitated from shrunken cells, can directly phosphorylate NKCC1 fusion proteins, JNK immuno-precipitated from isotonic cells cannot do this (Klein et al., 1999). However, shrinkage activation of JNK is much too slow to account for the very fast activation of NKCC1, and shrinkage-induced JNK activation is not always seen in cells with shrinkage-induced NKCC1 activity (Liedtke and Cole, 2002).

The sterile 20 (Ste20)-related praline-alanine-rich kinase (SPAK) and the oxidative stress response 1 kinase (OSR1) bind to the N-terminal region of NKCC1 and NKCC2 (Piechotta et al., 2003; Delpire et al., 2003), and SPAK increases the activity of NKCC1 and is somehow involved in its activation after cell shrinkage (Delpire and Gagnon, 2006; Gagnon et al., 2006a,b). Additionally, NKCC1 and SPAK colocalize during hypertonic shrinkage in Ehrlich Lettre Ascites (ELA) cells (Darborg et al., 2005). Moreover, two upstream kinases WNK (with no lysine) 1 and WNK 4 are widely expressed SPAK-binding proteins and seem to act as direct activators of SPAK and OSR1 (Vitari et al., 2005) suggesting that WNK1, 4 and SPAK/OSR1 mediate the osmotic stress signal to the transporters.

Several investigations also suggest a role for cytoskeleton rearrangement in activation of NKCC1 (Hoffmann and Mills, 1999; Pedersen et al., 2001; Liedtke et al., 2003). Activation of NKCC1 by cell shrinkage is inhibited by cytochalasins but is unaffected by phalloidin (Jessen and Hoffmann, 1992; Matthews et al., 1998; Hoffmann and Mills, 1999). Meanwhile, cell shrinkage of most cells is associated with reinforcement of the cortical cytoskeleton (see Hoffmann and Mills, 1999; Pedersen et al., 2001). In EATCs, we have suggested a three-state model for NKCC: (i) a silent state under isotonic conditions dependent on the integrity of the actin cytoskeleton (State I) in which the rates of phosphorylation and dephosphorylation are similar; (ii) a partly activated and not further activatable state (State II) in which F-actin is depolymerized and there is a loss of myosin II from the cortical region and (iii) an entirely activated state (State III), which can only be obtained in the presence of an intact actin cytoskeleton. Cell shrinkage causes NKCC1 to enter State III as a result of the formation of a cortical F-actin/myosin II network to which the kinase(s) that are involved in shrinkage-induced NKCC1 activation may translocate (Hoffmann and Pedersen, 2006). As F-actin assembly increases, it is likely that a variety of proteins act as a scaffold to the actin network, thereby triggering the activation of the signaling cascade. For example, it is known that SPAK fails to interact with G-actin but binds to F-actin (Tsutsumi et al., 2000) thus, SPAK could be recruited and activated during F-actin assembly.

NKCC1 can also be activated by the addition of agonists, that induce inositol-phosphate-mediated Ca^{2+} signalling, resulting in KCl loss followed by cell shrinkage. This mechanism is well described in secreting epithelia but is also found in cells in suspension, such as EATCs (Jensen et al., 1993; Hoffmann et al., 1993). Whether cotransport activation could result from a drop in cellular Cl^- activity, or from cell shrinkage has been a matter of debate. The fact that both a drop in intracellular Cl^- activity and cell shrinkage influence activation of the cotransport system was first proposed by Ussing (1982); the idea that a reduced intracellular Cl^- concentration could play a permissive

role in the activation of the cotransporter was subsequently discussed for EATCs (Hoffmann et al., 1983) and thymocytes (Grinstein et al., 1983). In EATC it has been demonstrated that when cell shrinkage is prevented by use of a K^+ rich equilibrium salt solution (KCl substituted for NaCl in the medium), activation of the cotransporter essentially disappeared (Hoffmann, 1993). Thus, actual cell shrinkage (or a decrease in cellular Cl^- activity) is a prerequisite for a pronounced and persistent activation of NKCC1. However, that experiment did not distinguish between the drop in cellular Cl^- activity and cell shrinkage as effectors, a problem that also applies to secretory epithelia. The relative importance of cell shrinkage versus the decrease in cytosolic Cl^- activity in secretory epithelia is still unsolved. There is evidence that the key event in activation of NKCC1 is the drop in cytosolic Cl^- activity, rather than shrinkage (Lytle and Forbush, 1996; Hoffmann and Mills, 1999; Russell, 2000). Results for the killifish (*Fundulus heteroclitus*) epithelium (see below) and the shark rectal gland (Greger et al., 1999), however, point to an essential role for cell shrinkage thus, it seems that both cell shrinkage and a decrease in Cl^- concentration can increase NKCC1 activity.

3. Osmosensing chloride-secreting cells of killifish opercular epithelium

The euryhaline killifish (or mummichog) *Fundulus heteroclitus* is known for its exceptional euryhalinity and can tolerate salinities ranging from freshwater (FW) to seawater (SW) to hypersaline conditions, as well as having the capacity to adapt quickly to large changes in salinity, such as direct FW-to-SW and reverse transfers. The killifish opercular epithelium and the skin of other euryhaline species, such as the goby (*Gillichthys mirabilis*) are rich in chloride cells; they provide useful surrogate models for understanding branchial ion transport and an excellent system for examining the regulation of salt and water transport in Cl^- secreting epithelia (Karnaky et al., 1977; Marshall, 1977; Zadunaisky, 1984; Pequeux and Gilles, 1988; Marshall and Bryson, 1998). The opercular epithelium contains a high density of mitochondrion-rich, Cl^- secreting cells (4×10^5 cells/cm^2) and the short circuit current, measured in Ussing-style membrane chambers, equals the Cl^- net flux from the blood side (basolateral) to the SW side (apical) (Karnaky et al., 1977; Karnaky, 1992; Marshall et al., 1998). The opercular epithelium can secrete Cl^- even into full-strength saline (500 mM NaCl) bathing the mucosal side under these conditions, the open circuit potential rises to approximately +40 mV (Pequeux and Gilles, 1988) thus demonstrating than the *in vitro* system can operate in a manner similar to the whole animal.

Below, we review the osmosensing of the operculum epithelium when exposed to hypertonicity and hypotonicity, respectively. The major idea is that this osmosensing is dependent on the volume sensitivity of K^+ and Cl^- channels and on the integrin/FAK mechanosensor, in turn controlling the NKCC1 transporter as described in Section 1. In addition, we will briefly discuss the importance of cell volume changes for agonist induction of Cl^- secretion.

3.1. Low to high salinities (hypertonicity)

When FW killifish are transferred to SW, the plasma osmolarity increases initially to about 70 mOsm, and the complete return to normal plasma osmolarity takes a few days (Zadunaisky et al., 1995). The acclimation from low to high salinities involves a phase during which the secretion of Cl^- *via* chloride cells is rapidly increased in response to the initial increase in blood osmolarity. To imitate this increase in blood osmolarity, Zadunaisky et al. (1995) added either mannitol or NaCl to the basolateral side of the isolated operculum epithelium. They found that the signal for fast adaptation to higher salinities is in fact the increased tonicity of the plasma, resulting in cell shrinkage and activation of the NKCC cotransporter and the Na^+/H^+ exchanger (Zadunaisky et al., 1995). This finding is very similar to that observed for the RVI process seen in most cells studied (e.g. Hoffmann and Dunham, 1995). Here we focus only on the role of NKCC1.

The mitochondrion-rich cells in the operculum epithelium of the killifish express the NKCC1 isoform (Kunzelmann and Mall, 2002). Because NKCC1 is important for regulation of cell volume as well as for secretion of Cl^-, one likely hypothesis is that the volume sensitivity of NKCC1 is important for osmotic regulation of the whole animal through control of gill Cl^- secretion. Opercular membrane Cl^- secretion was indeed found to be sensitive to basolateral NKCC inhibitors like bumetanide, piretanide and furosemide and insensitive to a Cl^-/HCO_3^- inhibitor DIDS (Eriksson and Wistrand, 1986). In addition, Cl^- secretion is blocked in K^+-free (Marshall and Bryson, 1998) and Na^+-free salines, consistent with a role for a NKCC cotransporter in mediation of Cl^- transport. Potassium sensitivity of the NKCC-based ion secretion has also been demonstrated (Marshall and Bryson, 1998). As evidenced from Western blot analysis using anti-phosphoserine-antibodies, a heavily serine-phosphorylated protein of the molecular size of NKCC1 was seen in opercular epithelia from SW-adapted killifish, but was only weakly present in epithelia from FW-adapted fish (Hoffmann et al., 2002). Thus, adaptation to SW enhances expression of the NKCC1 protein and/or a ser/thr kinase, which subsequently can phosphorylate NKCC1. In addition, NKCC1 expression (measured by qRT-PCR) in the gills increased sequentially after transfer of killifish from brackish water to SW but not to FW (Scott et al., 2004). Expression of NKCC1 was found to be decreased after transfer in both gills and opercular epithelium (Hoffmann and Mills, 1999; Scott et al., 2005).

Various Cl^- channel and K^+ channel blockers added to the basolateral side of the killifish operculum epithelium all inhibited the steady-state Cl^- current as well as hypertonic (NaCl or mannitol) stimulation of the Cl^- secretion. The PKC inhibitor chelerythrine and the MLCK inhibitor ML-7 had strong inhibitory effects on the hypertonic activation of the Cl^- secretion as seen in Fig. 1, indicating that both MLCK and PKC are involved (Hoffmann et al., 2002). It was later shown that the PKCα and PKCβ1 inhibitor Gö6976 had little effect, suggesting that these subtypes are not involved (Marshall et al., 2005) thus, other isoforms of cPKC or the novel pPKCs like e.g. PKCδ must be involved. As mentioned in the Introduction,

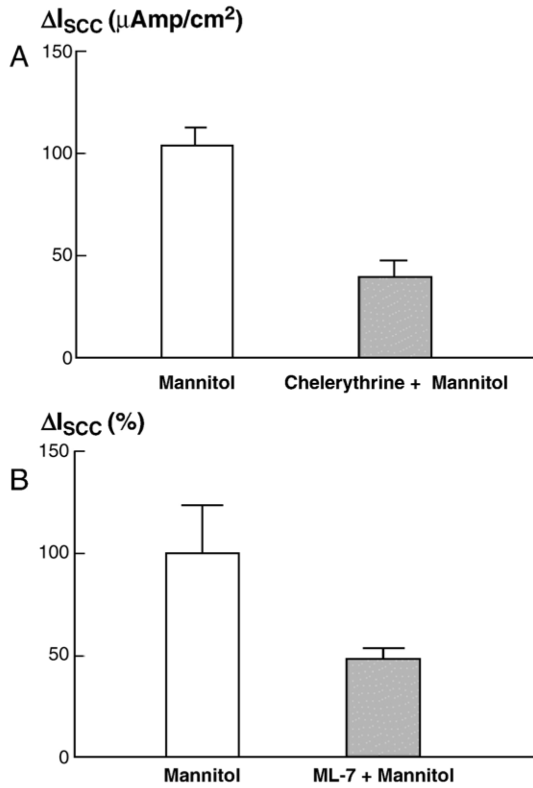

Fig. 1. (A) The response to hyperosmolarity obtained by addition of mannitol (100 mOsm) is significantly inhibited (p=0.014) by the protein kinase C inhibitor chelerythrine (40 μM) added to the basolateral side before addition of mannitol. Number of independent experiments: mannitol (n=13), chelerythrine +mannitol (n=3). (B) Effect of the MLCK inhibitor ML-7 (5 μM) on the response to mannitol (100 mOsm). ML-7 was added to the basolateral side before addition of mannitol. Number of independent experiments: mannitol (n=13), ML-7 plus mannitol (n=7). The inhibition is not significant on the 5% level (p=0.07). Redrawn from Hoffmann et al. (2002).

PKCδ is involved in the activation of NKCC1 in rabbit tracheal epithelial cells.

The two PKA inhibitors, H-89 and KT 5720, had no effect on the increase in current after mannitol addition, however, an isoproterenol stimulus was entirely blocked by H-89, signifying that the shrinkage activation of the NKCC cotransporter is independent of PKA activation, but that PKA is involved in activating the apical Cl^- channel *via* c-AMP {Hoffmann, 2002 2164 /id}. Steady-state Cl^- transport is stimulated by a PP-1 and PK2A ser/thr phosphatase inhibitor and inhibited by a PKC inhibitor. A PKA inhibitor had no effect thus, Cl^- transport seems to be controlled by constant phosphorylation and dephosphorylation involving PKC but not PKA (Hoffmann et al., 2002).

3.2. Hypotonicity

Exposure of the isolated opercular epithelium to a hypotonic environment is physiologically relevant because small estuarine fish like *Fundulus heteroclitus* are exposed regularly to FW microenvironments (~3% SW (1.0 g NaCl/l) or lower) when they feed in shallow water. Because the animals return to SW in between tides, only short-term regulatory mechanisms are needed rather than a permanent acclimation to FW (Marshall, 2003). There is a major drop in plasma osmolarity of approximately 60 mOsm/kg within the first 6 h after transfer of a killifish from SW to FW and the plasma osmolarity is restored within 24 h (Marshall et al., 2000).

To study the regulation of the hypotonic response, we exposed isolated opercular epithelia of killifish mounted in Ussing chambers to a hypotonic shock. Addition of hypotonic saline to the basolateral side of the operculum epithelium quickly inhibited Cl^- secretion (Fig. 2) in a dose-dependent fashion (Fig. 3), an effect that resulted from a change as small as

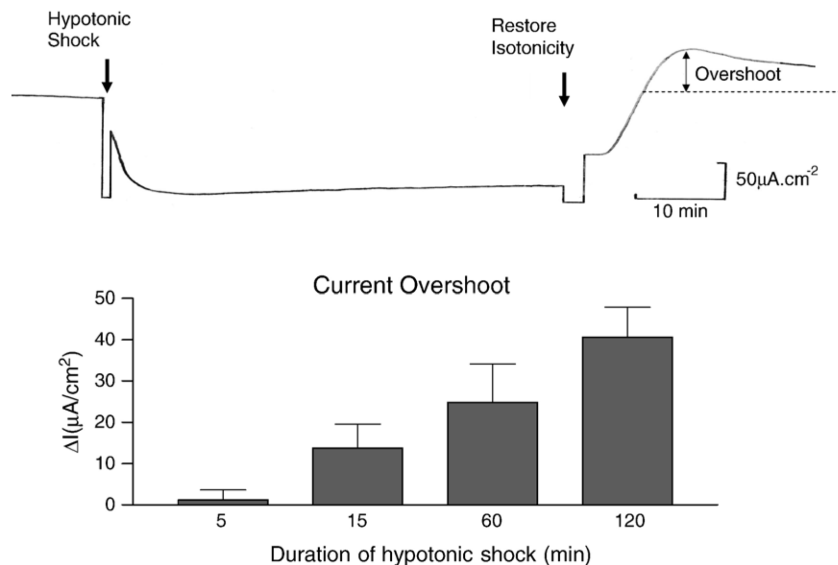

Fig. 2. Hypotonic shock (reduction of saline osmolarity by 60 mOsm/kg) sharply reduces I_{sc} to low, stable levels in isolated killifish opercular epithelia. Restoration of isotonic conditions produces an overshoot in I_{sc} (upper panel, second arrow), the size of which is a function of the overall time spent in hypotonic conditions (lower panel), suggestive of regulatory volume decrease compensation after hypotonic shock. Redrawn from Marshall et al. (2005).

20 mOsm/kg in osmolarity; thus it was not seen after addition of NaCl depleted isosmotic solutions (Zadunaisky et al., 1998; Marshall et al., 2000; Hoffmann et al., 2002). Restoration of osmolarity generated an overshoot in the Cl^- current that increased with the time of exposure to the hypotonic conditions (see Fig. 2) (Marshall et al., 2005). The decrease in current probably reflects a cell swelling and an RVD process. The overshoot in current after the return to isotonic medium was construed as an indication of a cell shrinkage after restoration of osmolarity resulting from the ion loss that had occurred during the RVD process which made the cells now hypotonic to a normal isoosmotic medium. There is preliminary evidence that inhibition of the Cl^- secretion under hypotonic conditions might partly result from swelling-activated Cl^- channels on the basolateral membrane (Zadunaisky et al., 1998) but for the most part, it seems that the basolateral cotransporter NKCC is the main player in the regulation. The hypothesis is that hypotonicity (cell swelling) by a series of steps dephosphorylates NKCC and decreases ion uptake, thereby inhibiting transepithelial Cl^- transport. Conversely, hypertonic shock would shrink cells, phosphorylate NKCC, induce ion uptake at the basolateral membrane, and augment transepithelial Cl^- transport, likely linked to RVI. Several kinases and phosphatases, discussed below, have been found to play a role in the regulation. Following discussion of these proteins, we present a preliminary model of this regulation involving these key players.

3.2.1. PKC and PKA

Chelerythrine, a rather general PKC inhibitor, significantly inhibited the resting membrane current Hoffmann et al., 2002, but had no effect on the current inhibition in hypotonic medium (Marshall et al., 2005). Chelerythrine blocked the recovery of the membrane current when isotonic solutions were restored (Marshall et al., 2005), indicating as discussed above the involvement of PKC in current stimulation (Hoffmann et al., 2002). Gö6976, which inhibits the PKCα,β subtypes, had essentially no effect, however, suggesting that PKCα and PKCβ1 are not involved (Marshall et al., 2005). H-89, which blocks PKA, did not interfere with hypotonic current inhibition but it did inhibit the recovery after restoration of isotonic conditions, indicating involvement of PKA in current recovery (Marshall et al., 2005).

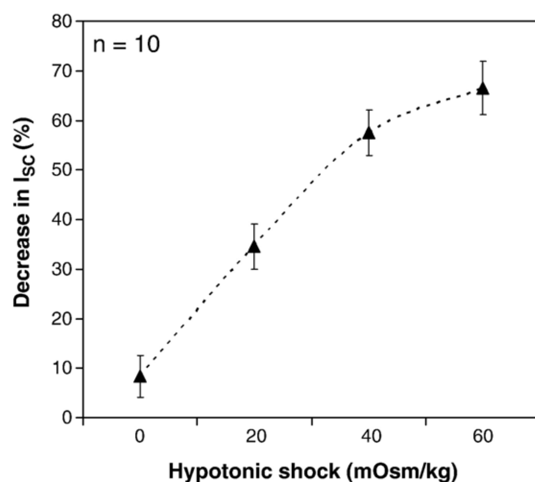

Fig. 3. The degree of I_{sc} inhibition in killifish opercular membranes is a function of the size of the hypotonic shock applied. Hypotonic shock as little as 20 mOsm/kg produced a significant decrease in I_{sc} (mean ± SEM, $n = 10$) and the inhibition increased with greater hypotonic shock, indicating that the system responds to very small osmotic stimuli. Redrawn from Marshall et al. (2000).

3.2.2. MAPKinases

In gill cells from *F. heteroclitus* that were exposed to osmotic shock, Kultz and Avila (2001) observed changes in activity and abundance of JNK and p38 MAPKs with a 5-fold increase in the expression of p38 after transfer from FW to SW. These findings point to the involvement of kinase cascades in the volume responses in the gill cells. JNK and p38 MAPK have both been implicated in volume responses of several cell types (see Section 1) thus, we examined MAP kinase phosphorylation after hypotonic stress and tested the effect of MAP kinase inhibitors on current response. Hypotonic stress rapidly and transiently increased the level of phosphorylated p38 MAPK (pp38) (measured by Western analysis) by 8-fold at 5 min, followed by a decrease and then a slower increase to 7 fold at 60 min (Marshall et al., 2005). The rapid activation of p38 with hypotonic shock (at 5 min) points to an involvement of this kinase in the swelling-induced inhibition of the Cl^- secretion that follows the same time course. The p38 kinase inhibitor SB 203580 significantly enhanced the decrease in current thus, it seems that p38 inhibits the hypotonic decrease in current (Marshall et al., 2005). The p38 inhibitor also inhibited current recovery when isotonic solution was restored, an outcome consistent with the idea that p38 stimulates an increase and inhibits a decrease in current. JNK was biphasically activated (i.e. phosphorylated) by both hypotonic and hypertonic shock with an increase of about 40–50% after 5 min. In hypertonic medium pJNK was still high after 30 min (Marshall et al., 2005).

3.2.3. SPAK and OSR

As discussed in Section 1, SPAK is a prime candidate for involvement in NKCC1 regulation. Immunoblot analysis showed that SPAK and OSR1 both were present in SW and FW acclimated fish with higher expression in FW. Immunocytochemical analysis showed that SPAK, OSR1 and phosphorylated focal adhesion kinase (pY^{407}-FAK) were colocalized with NKCC at the basolateral membrane (Marshall et al., 2005).

3.2.4. Protein phosphatases

The ser/thr protein phosphatases (PP1 and PP2A) inhibitor calyculin A augmented the steady-state current, and slowed the hypotonic current inhibition but significantly exacerbated the total decline in current (Hoffmann et al., 2002). The same outcome was seen with okadaic acid, and in addition, the recovery after restoration of osmolarity was completely blocked (Marshall et al., 2005). As discussed in Section 1, both the ser/thr protein phosphatases PP1 and PP2A bind directly to and regulate NKCC1 in shark rectal gland cells thus, it is likely that PP1 and/or PP2A may also be involved in dephosphorylation of NKCC1 in the killifish. This mechanism could explain the increase in steady-state current and the slow

down of the decrease in current in hypotonic medium but the fact that the total decease is potentiated and that the recovery is completely blocked points to a different role for a phosphatase.

3.2.5. Tyrosine kinases

The protein tyrosine kinase inhibitor genistein inhibited chloride secretion opercular epithelia from in SW-adapted fish in a way that mimicked the hypotonic response (Marshall et al., 2000, 2005). If the current was first inhibited with the α-adrenergic agonist clonidine then the tyrosine kinase inhibitor genistein instead increased the chloride current (Marshall et al., 2005). Thus, it seems that both a steady-state-stimulated and a steady-state-inhibited conditions are dependent on a tyrosine kinase. Genistein both inhibited the Cl^- secretion from high currents (Marshall et al., 2000) and dephosphorylated FAK at the Y^{407} tyrosine phosphorylation site, as detected by immunocytochemistry (Marshall et al., 2005). Hypotonic shock also caused rapid dephosphorylation of FAK at position Y^{407} (Marshall et al., 2005) and phosphorylated FAK was highly colocalized with NKCC and SPAK (Marshall et al., 2005). These findings are suggestive of an osmosensitive complex involving FAK and NKCC, but it is not known at this time whether any or all of the other phosphorylation sites on FAK are responsive to hypotonic shock.

3.2.6. A preliminary synthesis model

A preliminary model for rapid control of CFTR and NKCC in chloride cells has been presented (see Fig. 4) (Marshall et al., 2005) and includes, (1) activation of NKCC and CFTR *via* cAMP/PKA; (2) activation of NKCC during cell shrinkage by PKC, MLCK, p38, OSR1 and SPAK, (3) deactivation of NKCC by hypotonic cell swelling, Ca^{2+} and an as-yet-unidentified protein phosphatase; and (4) involvement of a protein tyrosine kinase acting on FAK to set levels of NKCC activity. As discussed above, prostaglandins are known to be involved in cellular osmotic responses and in EATCs, for example, it has been demonstrated that $cPLA_2$ is activated by hypotonic shock, resulting in an increase in arachidonic acid release and increased eicosanoid synthesis (Hoffmann, 2000). PGE_2 is known to rapidly inhibit Cl^- secretion in the opercular membrane of *Fundulus heteroclitus* (Vanpraag et al., 1987; Evans et al., 2004) and the tissue metabolizes several different eicosanoids, including prostaglandins, leukotrienes and hydroxyeicosatetraenoic acids (Vanpraag et al., 1987). Therefore a potential role for prostaglandins in the hypotonic response of the opercular epithelium was also examined. However, the cyclooxygenase inhibitor indomethacin had no effect, indicating that involvement of prostaglandins in the volume response is unlikely (Marshall et al., 2005).

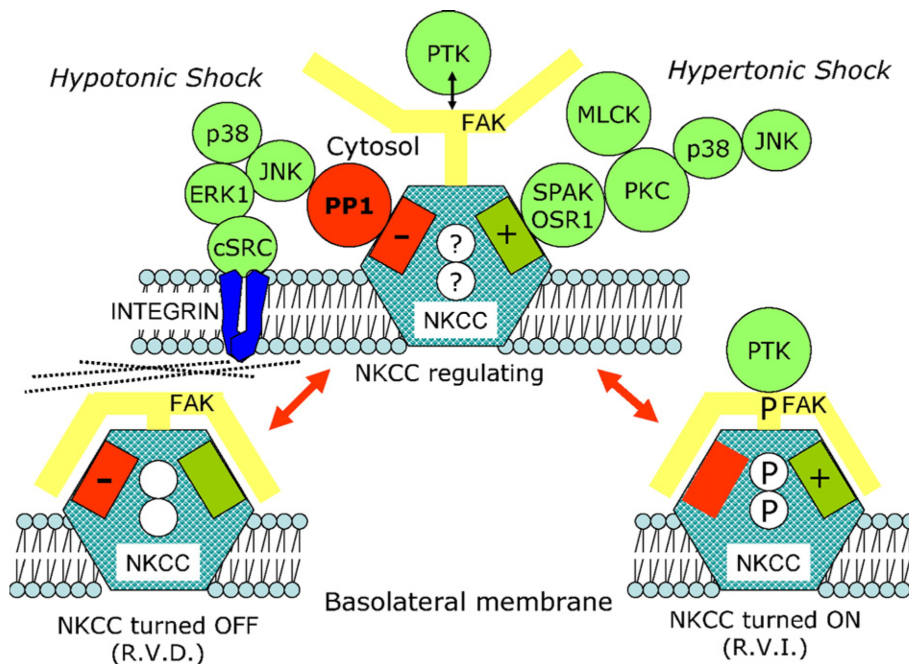

Fig. 4. A hypothetical model of the regulation of NKCC in the basolateral membrane of chloride secreting cells in isolated killifish opercular epithelia. Extracellular side is below; cytosolic components above. Right: the kinases that have been demonstrated to be near the basolateral membrane and that are activated by hypertonic stimuli and ultimate NKCC activation associated with regulatory volume increase (RVI), *viz.* JNK, MLCK, and P38 MAPK. Also included are kinases that are colocalized with NKCC (FAK, OSR1 and SPAK). Because SPAK and OSR1 coimmunoproecipitate with NKCC in other systems, they are placed nearest to NKCC. The order of the others in the cascade is unknown. Left: the inhibitory cascade terminating with PP1, a phosphatase that in other systems has been shown to coimmunoprecipate with NKCC. On this side are some of the known players (JNK and p38 MAPK), but also included is involvement of Integrin, cSRC and ERK, which in other systems are associated with detection and mediation of hypotonic stress. The model proposes three conditions: an unstable regulating phase that can result in NKCC becoming phosphorylated (i.e. a pseudo-stable phase of stimulation during RVI) or becoming dephosphorylated and entering a pseudo stable inactive state during RVD). The reason FAK is shown occluding phosphorylation sites is because of the unusual effect of the PTK genistein on the system, causing a decrease in I_{sc} when it is high as well as increasing I_{sc} when it is low. The PTK depicted separately from FAK could be FAK itself.

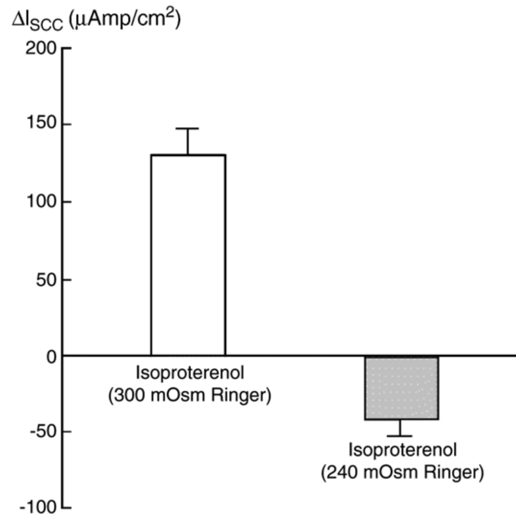

Fig. 5. Effect of isoproterenol (10^{-5} M) on the short-circuit current with or without simultaneous addition of 20% distilled water to avoid cell shrinkage induced by isoproterenol. The change in current is calculated as the difference between the current before addition of the agonist and the current after a new steady state is attained. The addition of isoproterenol to the 300 mOsm Ringer's solution stimulated the short-circuit current to 258 μA/cm^2, addition of isoproterenol in distilled water to a final osmolarity of 240 mOsm gave a significantly smaller increase in current ($p<0.01$). In fact the current was even lower than the steady-state current before stimulation. The number of independent experiments: isoproterenol in 300 mOsm Ringer's ($n=12$), isoproterenol in 240 mOsm Ringer's ($n=4$). Reproduced from Hoffmann et al. (2002).

3.3. Agonist induction

The β-agonist isoproterenol stimulates Cl^- secretion in standard saline solutions. We examined the Cl^- current activation in response to isoproterenol under conditions in which Cl^- activity drops with or without concomitant cell shrinkage, because cell volume in the latter case was associated with an increased value by simultaneous addition of water resulting in a weakly hypotonic solution (240 mOsm) (see Fig. 5) (Hoffmann et al., 2002). Under both conditions [Cl^-_i] decreased followed by cell shrinkage in the isotonic solution but without cell shrinkage in the hypotonic solution. In the latter situation all stimulation with isoproterenol disappeared. We could conclude that a key process for activation of the NKCC1 cotransporter after addition of isoproterenol is cell shrinkage (Hoffmann et al., 2002).

4. European eel intestine

The intestine of the euryhaline teleost European eel (*Anguilla anguilla*) is used as a model of a salt-absorbing epithelium; this epithelium is naturally, physiologically exposed to changes in extracellular osmolarity when the eel migrates from FW to SW and vice versa.

The ion transport model described for eel intestine is essentially identical to the model for the thick ascending limb (cTAL) of the mammalian renal cortex (Greger, 1985). NKCC2 in parallel with a K^+ conductance is localized on the apical membrane and accounts for the luminal uptake of Cl^-; it operates in series with a basolateral Cl^- conductance and presumably a basolateral electroneutral KCl cotransport. The Na^+–K^+–ATPase on the basolateral membrane generates an inwardly directed electrochemical gradient for Na^+, which provides the driving force for NKCC2 (Schettino and Lionetto, 2003). In the eel intestine, the transepithelial Cl^- active transport drives transepithelial Na^+ diffusive transport, directed by a transepithelial electrical potential of several millivolts (negative on the serosal side) and a measurable short circuit current (Schettino and Lionetto, 2003). Therefore, in the eel intestine, the absorptive NKCC2 can be functionally detected as bumetanide-sensitive, short-circuit current that represents about 90–95% of the overall short-circuit current of the epithelium.

4.1. Freshwater to seawater

When the eel moves from FW to SW, the intestinal epithelium is exposed to hyper-osmotic stress from the luminal side, the result of the SW that the fish drink (Maetz and Skadhauge, 1968), and from the basolateral side, from a rapid 40% increase in plasma osmolarity (about 137±34 mOsm). The plasma then slowly returns to normal plasma osmolarity (Lionetto et al., 2001). When isolated eel intestines from FW-adapted yellow eels are exposed on both sides by mannitol addition to a 25% increase in osmolarity, there is a biphasic cell shrinkage (measured as a decrease in epithelium height) amounting to 32% after 5 min exposure, followed by an RVI process (Lionetto et al., 2001). The rapid osmotic shrinkage reflects a high water permeability, the consequence of the presence of two aquaporin homologues (Martinez et al., 2005).

During such hypertonic stress (Ringer's osmolarity: 315 mOsm→375 mOsm with mannitol), there is a biphasic increase in short circuit current (I_{sc}) and transepithelial voltage (V_{te}). The initial phase which lasts about 10–15 min correlates with cellular shrinkage, while the second phase, which is maximal after 40–60 min, correlates with the RVI process (Fig. 6) (Lionetto et al., 2001; 2002). The initial transient phase is sensitive to Cl^- channel blockers and seems to represent a loss of Cl^- *via* basolateral Cl^- channels and a loss of K^+ *via* apical K^+ channels (Lionetto and Schettino, 2006).

4.1.1. Na^+–K^+–$2Cl^-$ in the hypertonic stress response

As shown in Fig. 6, the second phase of the V_{te} and I_{sc} response, which correlates with an RVI process, is completely inhibited by 10 μM bumetanide (added in the luminal bathing solution), revealing that the second phase is due to the shrinkage-induced stimulation of the cotransporter (Lionetto et al., 2001, 2002). Serosal addition of dimethyl-amiloride (100 μM), a specific inhibitor of NHE1 had no effect on the hyperosmotic response, excluding any role for the Na^+/H^+ antiporter in the response (Lionetto and Schettino, 2006). Luminal bumetanide not only can completely inhibit the second phase of the epithelium response to hypertonic stress but also can completely abolish epithelium RVI following hypertonic stress, as demonstrated by morphometrical analysis of epithelium height following osmotic stress (Fig. 6). The data show

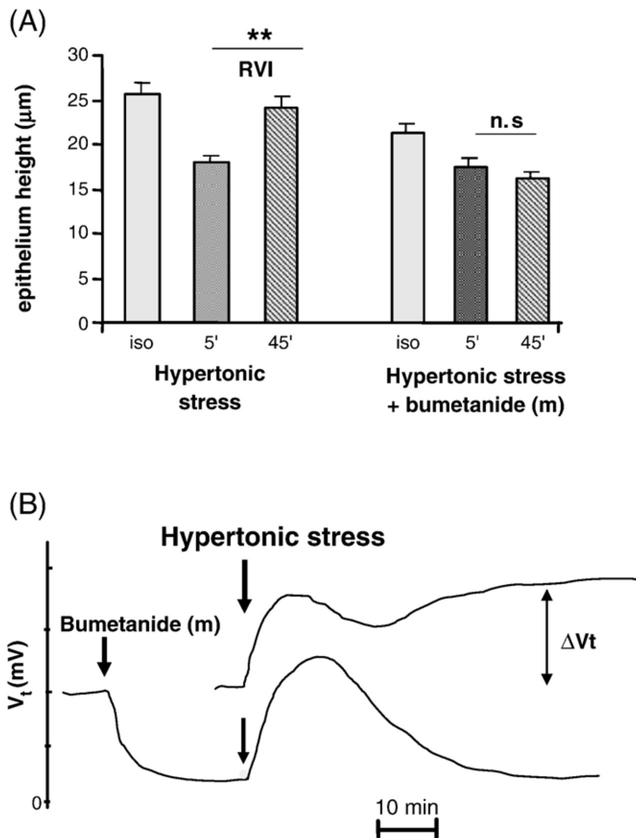

Fig. 6. Effect of bumetanide on RVI response (A) and electrophysiological response (B) to hypertonic stress in eel intestine. Redrawn from Lionetto and Schettino (2006).

that in the eel intestinal epithelium the hypertonicity of the external medium results in cell shrinkage which is a signal to increase NKCC2 activity. The high transepithelial Cl^- current is then carried through the luminal NKCC2 and the basolateral Cl^- channels. As discussed in the Introduction, a homologue of the renal NKCC2 is found in the luminal membrane of the eel intestine. Research with renal cells has shown that increased external osmolarity can increase NKCC2 activity (Gamba and Bobadilla, 2004). Similar to the situation with renal NKCC2, it seems that the eel intestine luminal NKCC2 also is active under basal isotonic conditions but dramatically increases its activity during hypertonic stress.

4.1.2. The role of cytoskeleton and of protein phosphorylation events

The actin-based cytoskeleton is implicated in volume-sensing and regulation in a wide range of cells and tissues (see e.g. Pedersen et al., 1999, 2001; Hoffmann and Mills, 1999) and as discussed in the Introduction, there is abundant evidence that shrinkage activation of NKCC is controlled by the actin-based cytoskeleton. It should be mentioned that most studies have focused on NKCC1, while little research has addressed NKCC2.

The eel enterocytes have a strong cortical F-actin cytoskeleton, specifically at the apical membrane (Lionetto et al., 2002). The bumetanide-sensitive response of V_{te} and I_{sc} to hypertonicity was potently inhibited by treatment with 20 µM cytochalasin D or 0.5 µM latrunculin A, known to induce F-actin depolymerization by different molecular mechanisms. The effectiveness of actin agents suggests that the activation of NKCC during hypertonic stress requires the integrity of the F-actin cytoskeleton (Lionetto et al., 2002). In contrast to the cytoskeleton involvement found in many other cells (see Introduction), no detectable modification in F-actin cytoskeleton was seen in the eel intestine after hypertonic stress exposure, and no significant changes in F-actin content were found using a quantitative F-actin assay (Lionetto et al., 2002). The results suggest that hypertonic activation of the cotransporter in eel intestine requires the integrity of the F-actin microfilament system but not a F-actin remodelling or an increase in polymerization (Lionetto et al., 2002).

4.1.3. PKC and MLCK

For NKCC, both PKC and MLCK have been assigned a role in shrinkage induced activation and both are known to interact with actin cytoskeleton, as discussed in the Introduction. Inhibitors of PKC and MLCK were therefore tested against the bumetanide-sensitive I_{sc} increase following hypertonic stress. Chelerythrine (the PKC inhibitor) blocked the bumetanide-sensitive I_{sc} increase, suggesting that PKC is involved in hypertonic NKCC2 stimulation (Lionetto et al., 2002). Which PKC isoform is involved has not been investigated, and neither has the question of whether the role of PKC in NKCC activation could result from a possible interaction with the cytoskeleton. The MLCK inhibitor ML-7 (20 µM) also abolished the hypertonic bumetanide-sensitive I_{sc} and V_{te} response in agreement with what has been seen in several cell types (see Section 1). As discussed in Section 1, a possible causal link between MLCK/MLC phosphorylation and the shrinkage activation of NKCC1 has been suggested, but other possibilities are also debated (Ciano-Oliveira et al., 2005), thus further studies are needed to clarify the role of MLCK in the stimulation of NKCC2 in eel intestine.

4.2. Seawater to freshwater

When the eel moves from SW to FW, the intestine is exposed to hypotonicity both on the basolateral side because of the initial ~20% decrease in plasma osmolarity (Lionetto et al., 2005) and on the apical side because the drinking behaviour of the eel can result in a very hypotonic medium (Maetz and Skadhauge, 1968). Eel intestinal epithelium mounted in an Ussing chamber responded to a hypotonic medium with a biphasic decrease in I_{sc} and V_{te}. The current changes were associated with an RVD response recorded by morphometrical measurement of epithelium height (Lionetto et al., 2005). As mentioned above, both membranes in the eel intestine have a high water permeability because of the presence of two aquaporin homologues (Martinez et al., 2005). Thus, it is likely that KCl efflux on both membranes is followed by osmotic water loss through aquaporins accounting for the RVD response. The possibility that the observed decrease of V_{te} and I_{sc} induced by hypotonicity might be the result of an inhibition of the luminal

NKCC cotransporter was ruled out by the observation that luminal blockage of NKCC2 by bumetanide did not alter the decrease in V_{te} and I_{sc} induced by external hypotonicity remained unaltered after (Lionetto and Schettino, 2006).

4.2.1. K^+ and Cl^- channels

As described in the Introduction an RVD response in epithelial and non-epithelial cells is predominantly correlated with the activation of K^+ and Cl^- efflux through independent swelling-activated K^+ and anion channels. The hypotonic response in V_{te} and I_{sc} in the eel intestine was found to be a result of activation of several anion and K^+-conductive pathways on both the apical and the basolateral membranes as shown in Fig. 7 which shows iberiotoxin-sensitive high conductance (BK) Ca-activated K^+ channels on the apical and basolateral membranes, apamin-sensitive small conductance (SK) Ca-activated K^+ channels mainly on the basolateral membrane and DIDS-sensitive anion channels on the apical membrane.

The hypotonicity-induced activation of large-conductance BK channels is reported to occur in other epithelial cell types such as the human bronchial epithelial cell line (Fernandez-Fernandez et al., 2002), rabbit kidney proximal tubule cells (Dubé et al., 1990), hepatocytes (Pon and Hill, 1997), rat collecting tubules (Stoner and Morley, 1995), and osteoblasts (Weskamp et al., 2000). SK channels have previously been reported to be involved in the RVD response in human liver cells (Roman et al., 2002). Involvement of both BK and SK swelling-activated channels in the same cell type has not been reported before although in human liver cells, an unidentified additional swelling-activated K^+ channel also seems to be involved (Roman et al., 2002). The Ba^{2+}-sensitive basal K^+ conductance is not involved in the response to hypotonicity in the eel intestine, as the electrogenic response was unaffected by serosal or mucosal application of Ba^{2+}. Apically added DIDS however significantly reduced the initial V_{te} response and the functional integrity of the basal Cl^- conductive pathway on the basolateral membrane is also required in the second phase of the hypotonicity response which was significantly inhibited by DIDS or NPPB added to the basolateral side˙ DIDS also affected on the basal electrophysiological parameters. Lionetto et al. (2005) concluded that hypotonic cell swelling in the eel intestine epithelium activates predominantly basolateral K^+ conductances and predominantly apical anion conductances.

4.2.2. Role of calcium

Chelation of intracellular Ca^{2+} by BAPTA-AM resulted in a significant inhibition of the hypotonic response; thus intracellular Ca^{2+} is important for the hypotonicity-induced decrease in V_{te} and I_{sc} (Lionetto et al., 2005). Depletion of the intracellular stores with thapsigargin had no effect, whereas the electrophysiological response to hypotonic stress was completely abolished in a Ca^{2+}-free Ringer's. It was suggested that a swelling-activated influx of Ca^{2+} increases local cytosolic Ca^{2+}-activating BK and SK channels (Lionetto et al., 2005). With respect to the increase in Cl^- conductance, with nonspecific inhibitors like DIDS and NPPB it is not possible to tell whether we are dealing with CACC or with VRAC, and this distinction needs to be further investigated.

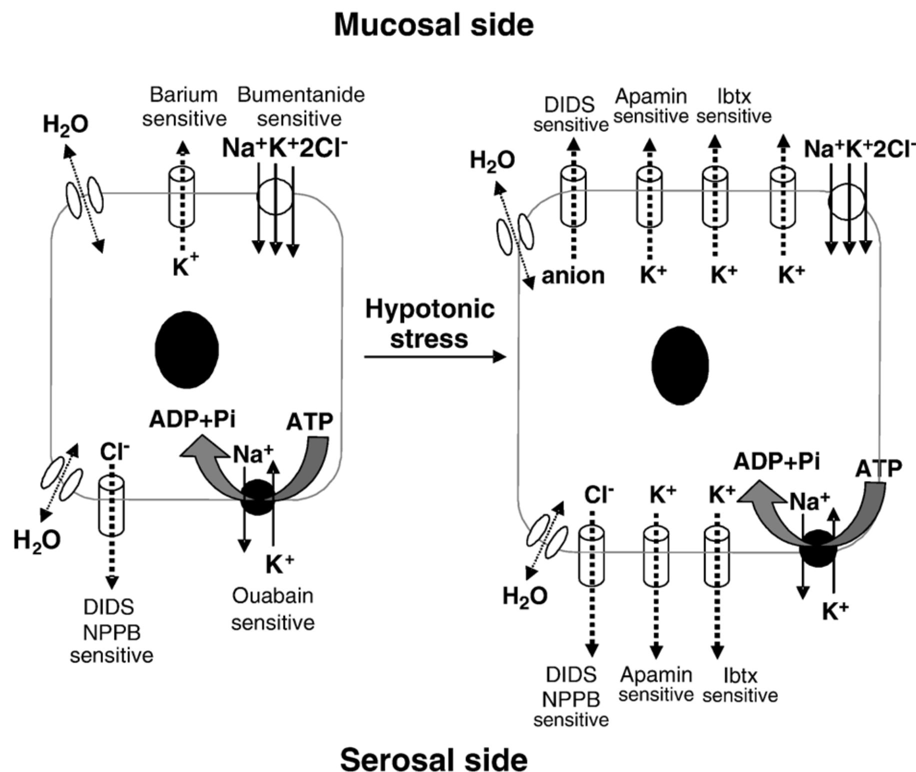

Fig. 7. Ionic conductances activated during RVD response in eel intestine. Redrawn from Lionetto et al. (2005).

5. Conclusions

It is well known that epithelial transport involving the entry or extrusion of osmotically active substances at the basolateral and apical cellular membranes represents a continuous challenge to epithelial cell volume regulation, because slight changes in the large apical or basolateral fluxes will lead to rapid changes in cell volume (Hoffmann and Ussing, 1992; Hoffmann and Dunham, 1995; Harvey, 1994; Beck et al., 1994). In addition, some epithelia can experience changes in extracellular osmolarity. Animals such as the killifish that naturally must adapt quickly to changes in external salinity *via* transport systems responsive to blood osmolarity are ideal models for such osmoresponsiveness. The response is easily measured and the target of the response is local, within the same cell. Thus the mitochondrion-rich cells of the killifish opercular membrane are a good model. Similarly, the intestine of the euryhaline teleost European eel is an excellent model of a salt-absorbing epithelium; in the eel, this epithelium is physiologically exposed to changes in extracellular osmolarity when the fish migrates from FW to SW and vice versa.

A diversity of cellular responses to changes in intracellular or extracellular osmolarity is emerging and at the effector level, it involves volume-sensitive channels ($I_{K,vol}$ VRAC and various non-selective channels including TRP channels) and shrinkage-activated transport systems like NKCC1 and NHE1. The picture of osmosensing or volume sensing specifically in epithelial cells is, however, still incomplete. The advent of phosphorylation- or site-specific antibodies for the well-conserved proteins that are in play may make systematic screening of existing models for the diversity of osmosensing mechanisms and of effector transport proteins routine. As such, the intestine system should be screened for upstream kinases like FAK and for candidates in the sensing system like FAK, cSRC and integrin. In addition the chloride cell system should be screened for totally unknown effectors involved after cell swelling, like direct volume sensitive channels. In this way, a clearer image of volume sensing in epithelia can be achieved.

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References

Ando, M., Utida, S., 1986. Effects of diuretics on sodium, potassium, chloride and water transport across the seawater eel intestine. Zool. Sci. 3, 605–612.

Beck, J.S., Laprade, R., Lapointe, J.Y., 1994. Coupling between transepithelial Na-transport and basolateral K-conductance in renal proximal tubule. Am. J. Physiol., Renal Physiol. 266, F517–F527.

Bildin, V.N., Wang, Z., Iserovich, P., Reinach, P.S., 2003. Hypertonicity-induced p38MAPK activation elicits recovery of corneal epithelial cell volume and layer integrity. J. Membr. Biol. 193, 1–13.

Calloe, K., Elmedyb, P., Olesen, S.P., Jorgensen, N.K., Grunnet, M., 2005. Hypoosmotic cell swelling as a novel mechanism for modulation of cloned HCN2 channels. Biophys. J. 89, 2159–2169.

Carton, I., Trouet, D., Hermans, D., Barth, H., Aktories, K., Droogmans, G., Jorgensen, N.K., Hoffmann, E.K., Nilius, B., Eggermont, J., 2002. RhoA exerts a permissive effect on volume-regulated anion channels in vascular endothelial cells. Am. J. Physiol., Cell Physiol. 283, C115–C125.

Ciano-Oliveira, C., Sirokmany, G., Szaszi, K., Arthur, W.T., Masszi, A., Peterson, M., Rotstein, O.D., Kapus, A., 2003. Hyperosmotic stress activates Rho: differential involvement in Rho kinase-dependent MLC phosphorylation and NKCC activation. Am. J. Physiol., Cell Physiol. 285, C555–C566.

Ciano-Oliveira, C., Lodyga, M., Fan, L.Z., Szaszi, K., Hosoya, H., Rotstein, O.D., Kapus, A., 2005. Is myosin light-chain phosphorylation a regulatory signal for the osmotic activation of the Na^+–K^+–2Cl(−) cotransporter? Am. J. Physiol., Cell Physiol. 289, C68–C81.

Cohen, D.M., 2005. TRPV4 and the mammalian kidney. Pflugers Arch. Eur. J. Physiol. 451, 168–175.

Darborg, B.V., Hoffmann, E.K., Pedersen, S.F., 2005. Causal relationship between NHE1, NKCC1, MAPKs and ERM proteins after osmotic shrinkage in Ehrlich Lettre mouse ascites tumor cells. FASEB J. 19, A1155.

Darman, R.B., Forbush, B., 2002. A regulatory locus of phosphorylation in the N terminus of the Na–K–Cl cotransporter, NKCC1. J. Biol. Chem. 277, 37542–37550.

Darman, R.B., Flemmer, A., Forbush, B., 2001. Modulation of ion transport by direct targeting of protein phosphatase type 1 to the Na–K–Cl cotransporter. J. Biol. Chem. 276, 34359–34362.

de Tassigny, A.D., Souktani, R., Ghaleh, B., Henry, P., Berdeaux, A., 2003. Structure and pharmacology of swelling-sensitive chloride channels, I-Cl, I-swell. Fundam. Clin. Pharmacol. 17, 539–553.

Delpire, E., Rauchman, M.I., Beier, D.R., Hebert, S.C., Gullans, S.R., 1994. Molecular cloning and chromosome localization of a putative basolateral Na^+–K^+–2Cl-cotransporter from mouse inner medullary collecting duct (mIMCD-3) cells. J. Biol. Chem. 269, 25677–25683.

Delpire, E., Gagnon, K.B., 2006. SPAK and OSR1, key kinases involved in the regulation of chloride transport. Acta. Physiol. 187, 103–115.

Delpire, E., England, R., Piechotta, K., 2003. Amino- and carboxyl-terminal determinants of NKCC1 function: a study of deletion mutants in *Xenopus laevis* oocytes. J. Gen. Physiol. 122, 16A.

Du, X.L., Gao, Z., Lau, C.P., Chiu, S.W., Tse, H.F., Baumgarten, C.A., Li, G.R., 2004. Differential effects of tyrosine kinase inhibitors on volume-sensitive chloride current in human atrial myocytes: evidence for dual regulation by Src and EGFR kinases. J. Gen. Physiol. 123, 427–439.

Dubé, L., Parent, L., Sauvé, R., 1990. Hypotonic shock activates a maxi K^+ channel in primary cultured proximal tubule cells. Am. J. Physiol., Renal Physiol. 259, F348–F356.

Eriksson, O., Wistrand, P.J., 1986. Chloride transport inhibition by various types of loop diuretics in fish opercular epithelium. Acta Physiol. Scand. 126, 93–101.

Evans, D.H., Rose, R.E., Roeser, J.M., Stidham, J.D., 2004. NaCl transport across the opercular epithelium of *Fundulus heteroclitus* is inhibited by an endothelin to NO, superoxide, and prostanoid signaling axis. Am. J. Physiol., Regul. Integr. Comp. Physiol. 286, R560–R568.

Fernandez-Fernandez, J.M., Nobles, M., Currid, A., Vazquez, E., Valverde, M. A., 2002. Maxi K^+ channel mediates regulatory volume decrease response in a human bronchial epithelial cell line. Am. J. Physiol., Cell Physiol. 283, C1705–C1714.

Flatman, P.W., 2002. Regulation of Na–K–2Cl cotransport by phosphorylation and protein–protein interactions. Biochim. Biophys. Acta, Biomembr. 1566, 140–151.

Friis, M.B., Friborg, C.R., Schneider, L., Nielsen, M.B., Lambert, I.H., Christensen, S.T., Hoffmann, E.K., 2005. Cell shrinkage as a signal to apoptosis in NIH 3T3 fibroblasts. J. Physiol. (Lond.) 567, 427–443.

Gagnon, E., Forbush, B., Flemmer, A.W., Gimenez, I., Caron, L., Isenring, P., 2002. Functional and molecular characterization of the shark renal Na–K–Cl cotransporter: novel aspects. Am. J. Physiol., Renal Physiol. 283, F1046–F1055.

Gagnon, K.B.E., England, R., Delpire, E., 2006a. Characterization of SPAK and OSR1, regulatory kinases of the Na–K–2Cl cotransporter. Mol. Cell. Biol. 26, 689–698.

Gagnon, K.B.E., England, R., Delpire, E., 2006b. Volume sensitivity of cation-Cl-cotransporters is modulated by the interaction of two kinases: Ste20-related proline-alanine-rich kinase and WNK4. Am. J. Physiol., Cell Physiol. 290, C134–C142.

Gamba, G., 2005. Molecular physiology and pathophysiology of electroneutral cation-chloride cotransporters. Physiol. Rev. 85, 423–493.

Gamba, G., Bobadilla, N.A., 2004. Molecular physiology of the renal Na^+–Cl- and Na^+–K^+–2Cl(−) cotransporters. Cell. Vol. Signal. 559, 55–65.

Gamba, G., Miyanoshita, A., Lombardi, M., Lytton, J., Lee, W.-S., Hediger, M. A., Hebert, S.C., 1994. Molecular cloning, primary structure, and characterization of two members of the mammalian electroneutral sodium-(potassium)-chloride cotransporter family expressed in kidney. J. Biol. Chem. 269, 17713–17722.

Gillis, D., Shrode, L.D., Krump, E., Howard, C.M., Rubie, E.A., Tibbles, L.A., Woodgett, J., Grinstein, S., 2001. Osmotic stimulation of the Na^+/H^+ exchanger NHE1: relationship to the activation of three MAPK pathways. J. Membr. Biol. 181, 205–214.

Gosmanov, A.R., Wong, J.A., Thomason, D.B., 2002. Duality of G protein-coupled mechanisms for beta-adrenergic activation of NKCC activity in skeletal muscle. Am. J. Physiol., Cell Physiol. 283, C1025–C1032.

Greger, R., 1985. Ion transport mechanisms in thick ascending limb of Henle's loop of mammalian nephron. Physiol. Rev. 65, 760–797.

Greger, R., Heitzmann, D., Hug, M.J., Hoffmann, E.K., Bleich, M., 1999. The Na^+2Cl–K^+ cotransporter in the rectal gland of *Squalus acanthias* is activated by cell shrinkage. Pflugers Arch. 438, 165–176.

Grinstein, S., Clarke, C.A., Rothstein, A., 1982. Increased anion permeability during volume regulation in human lymphocytes. Philos. Trans. R. Soc. Lond., B Biol. Sci. 299, 509–518.

Grinstein, S., Cohen, S., Sarkadi, B., Rothstein, A., 1983. Induction of 86^{Rb} fluxes by Ca^{2+} and volume changes in thymocytes and their isolated membranes. J. Cell. Physiol. 116, 352–362.

Haas, M., Forbush III, B., 2000. The Na–K–Cl cotransporter of secretory epithelia. Annu. Rev. Physiol. 62, 515–534.

Harvey, B.J., 1994. Crosstalk and epithelial ion transport. Curr. Opin. Nephrol. Hypertens. 3, 523–528.

Helix, N., Strobaek, D., Dahl, B.H., Christophersen, P., 2003. Inhibition of the endogenous volume-regulated anion channel (VRAC) in HEK293 cells by acidic di-aryl-ureas. J. Membr. Biol. 196, 83–94.

Hendil, K.B., Hoffmann, E.K., 1974. Cell volume regulation in Ehrlich ascites tumor cells. J. Cell. Physiol. 84, 115–125.

Hoffmann, E.K., 1978. Regulation of cell volume by selective changes in the leak permeabilities of Ehrlich ascites tumor cells. In: Jorgensen, C.B., Strange, K. (Eds.), Alfred Benzon Symposium IX. Osmotic and volume regulation. Munksgaard, Copenhagen, pp. 397–417.

Hoffmann, E.K., 1982. Anion exchange and anion–cation co-transport systems in mammalian cells. Philos. Trans. R. Soc. Lond., B Biol. Sci. 299, 519–535.

Hoffmann, E.K., 1993. Control of volume regulatory ion transport processes in a mammalian cell: signalling by secondary messengers. Alfred Benzon Symposium, Copenhagen, vol. 34, pp. 285–333.

Hoffmann, E.K., 1999. LTD4 activates charybdotoxin-sensitive and-insensitive K^+ channels in Ehrlich ascites tumor cells. Pflügers Arch. 438, 263–268.

Hoffmann, E.K., 2000. Intracellular signalling involved in volume regulatory decrease. Cell. Physiol. Biochem. 10, 273–288.

Hoffmann, E.K., Dunham, P.B., 1995. Membrane mechanisms and intracellular signalling in cell volume regulation. Int. Rev. Cyt. 161, 173–262.

Hoffmann, E.K., Mills, J.W., 1999. Membrane Events Involved in Volume Regulation. Current Topics In Membranes, vol. 48. Academic Press, USA, pp. 123–195.

Hoffmann, E.K., Pedersen, S.F., 2006. Sensors and signal transduction pathways in vertebrate cell volume regulation. Contrib. Nephrol. 152, 54–104.

Hoffmann, E.K., Ussing, H.H., 1992. Membrane mechanisms in volume regulation in vertebrate cells and epithelia. In: Giebisch, G.H., Schafer, J.A., Ussing, H.H., Kristensen, P. (Eds.), Membrane Transport in Biology, vol. 5, pp. 317–399.

Hoffmann, E.K., Simonsen, L.O., Sjöholm, C., 1975. Chloride fluxes and membrane potential in Ehrlich ascites tumor cells. 5th International Biophysical Congress, Copenhagen 4–9 August, p. 328.

Hoffmann, E.K., Simonsen, L.O., Sjöholm, C., 1979. Membrane potential, chloride exchange, and chloride conductance in Ehrlich mouse ascites tumour cells. J. Physiol. (Lond.) 296, 61–84.

Hoffmann, E.K., Sjöholm, C., Simonsen, L.O., 1983. Na^+, Cl^- cotransport in Ehrlich ascites tumor cells activated during volume regulation (regulatory volume increase). J. Membr. Biol. 76, 269–280.

Hoffmann, E.K., Simonsen, L.O., Lambert, I.H., 1993. Cell volume regulation. In: Lang, F., Haussinger, D. (Eds.), Interactions Cell Volume–Cell function, vol. 14. Springer Verlag, Berlin, Heidelberg, pp. 187–248.

Hoffmann, E.K., Hoffmann, E., Lang, F., Zadunaisky, J.A., 2002. Control of Cl-transport in the operculum epithelium of *Fundulus heteroclitus*: long- and short-term salinity adaptation. Biochim. Biophys. Acta 1566, 129–139.

Hoffmann, E.K., Nielsen, M., Christensen, S.T., 2004. Effects of osmotic stress on the activity of receptor tyrosine kinases and MAP kinases in HIH3T3-fibroblasts. Mol. Biol. Cell 15, 14A.

Hougaard, C., Niemeyer, M.I., Hoffmann, E.K., Sepúlveda, F.V., 2000. K^+ currents activated by leukotriene D4 or osmotic swelling in Ehrlich ascites tumour cells. Pflugers Arch. 440, 283–294.

Igarashi, P., Vanden-Heuvel, G.B., Payne, J.A., Forbush III, B., 1995. Cloning, embryonic expression, and alternative splicing of a murine kidney-specific Na–K–Cl cotransporter. Am. J. Physiol., Renal Physiol. 269, F405–F418.

Jensen, B.S., Jessen, F., Hoffmann, E.K., 1993. Na^+, K^+, Cl^- cotransport and its regulation in Ehrlich ascites tumor cells. Ca^{2+}/calmodulin and protein kinase C dependent pathways. J. Membr. Biol. 131, 161–178.

Jentsch, T.J., 1996. Chloride channels: a molecular perspective. Curr. Opin. Neurobiol. 6, 303–310.

Jentsch, T.J., Stein, V., Weinreich, F., Zdebik, A.A., 2002. Molecular structure and physiological function of chloride channels. Physiol. Rev. 82, 503–568.

Jessen, F., Hoffmann, E.K., 1992. Activation of the $Na^+/K^+/Cl^-$ cotransport system by reorganization of the actin filaments in Ehrlich ascites tumor cells. Biochim. Biophys. Acta 1110, 199–201.

Jørgensen, N.K., Christensen, S., Harbak, H., Brown, A.M., Lambert, I.H., Hoffmann, E.K., Simonsen, L.O., 1997. On the role of calcium in the regulatory volume decrease (RVD) response in Ehrlich mouse ascites tumor cells. J. Membr. Biol. 157, 281–299.

Karnaky Jr., K.J., 1992. Teleost osmoregulation: changes in the tight junction in response to salinity of the environment. Tight Junctions. CRC, Boca Raton, FL, pp. 175–201.

Karnaky Jr., K.J., Degnan, K.J., Zadunaisky, J.A., 1977. Chloride transport across isolated opercular epithelium of killifish: a membrane rich in chloride cells. Science 195, 203–205.

Klausen, T.K., Hougaard, C., Hoffmann, E.K., Pedersen, S.F., 2006. Cholesterol modulates the volume-regulated anion current in Ehrlich–Lettre ascites cells via effects on Rho and F-actin. Am. J. Physiol., Cell Physiol. 1 (4), C757–C771.

Klein, J.D., O'Neill, W.C., 1995. Volume-sensitive myosin phosphorylation in vascular endothelial cells: correlation with Na–K–2Cl cotransport. Am. J. Physiol., Cell Physiol. 269, C1524–C1531.

Klein, J.D., Lamitina, S.T., O'Neill, W.C., 1999. JNK is a volume-sensitive kinase that phosphorylates the Na–K–2Cl cotransporter in vitro. Am. J. Physiol., Cell Physiol. 277, C425–C431.

Krarup, T., Jakobsen, L.D., Jensen, B.S., Hoffmann, E.K., 1998. Na^+–K^+–$2Cl^-$ cotransport in Ehrlich cells: regulation by protein phosphatases and kinases. Am. J. Physiol., Cell Physiol. 275, C239–C250.

Kregenow, F.M., 1981. Osmoregulatory salt transporting mechanisms: control of cell volume in anisotonic media. Annu. Rev. Physiol. 43, 493–505.

Kultz, D., Avila, K., 2001. Mitogen-activated protein kinases are in vivo transducers of osmosensory signals in fish gill cells. Comp. Biochem. Physiol. B 129, 821–829.

Kunzelmann, K., Mall, M., 2002. Electrolyte transport in the mammalian colon: mechanisms and implications for disease. Physiol. Rev. 82, 245–289.

Lambert, I.H., 1987. Effect of arachidonic acid, fatty acids, prostaglandins, and leukotrienes on volume regulation in Ehrlich ascites tumor cells. J. Membr. Biol. 98, 207–221.

Lang, F., Busch, G.L., Ritter, M., Volkl, H., Waldegger, S., Gulbin, E., Haussinger, D., 1998. Functional significance of cell volume regulatory mechanisms. Physiol. Rev. 78, 247–306.

Larsen, A.K., Jensen, B.S., Hoffmann, E.K., 1994. Activation of protein kinase C during cell volume regulation in Ehrlich mouse ascites tumor cells. Biochim. Biophys. Acta 1222, 477–482.

Lepple-Wienhues, A., Szabó, I., Laun, T., Kaba, N.K., Gulbins, E., Lang, F., 1998. The tyrosine kinase $p56^{lck}$ mediates activation of swelling-induced chloride channels in lymphocytes. J. Cell Biol. 141, 281–286.

Lepple-Wienhues, A., Szabó, I., Wieland, U., Heil, L., Gulbins, E., Lang, F., 2000. Tyrosine kinases open lymphocyte chloride channels. Cell. Physiol. Biochem. 10, 307–312.

Levitan, I., Christian, A.E., Tulenko, T.N., Rothblat, G.H., 2000. Membrane cholesterol content modulates activation of volume-regulated anion current in bovine endothelial cells. J. Gen. Physiol. 115, 405–416.

Liedtke, C.M., Cole, T.S., 2002. Activation of NKCC1 by hyperosmotic stress in human tracheal epithelial cells involves PKC-delta and ERK. Biochim. Biophys. Acta, Mol. Cell Res. 1589, 77–88.

Liedtke, C.M., Hubbard, M., Wang, X.Y., 2003. Stability of actin cytoskeleton and PKC-delta binding to actin regulate NKCC1 function in airway epithelial cells. Am. J. Physiol., Cell Physiol. 284, C487–C496.

Lionetto, M.G., Schettino, T., 2006. The Na^+–K^+–2Cl-cotransporter and the osmotic stress response in a model salt transport epithelium. Acta Physiol. 187, 115–125.

Lionetto, M.G., Giordona, M.E., Nicolardi, G., Schettino, T., 2001. Hypertonicity stimulates Cl^- transport in the intestine of fresh water acclimated eel, *Anguilla anguilla*. Cell. Physiol. Biochem. 11, 41–54.

Lionetto, M.G., Pedersen, S.F., Hoffmann, E.K., Giordano, M.E., Schettino, T., 2002. Roles of the cytoskeleton and of protein phosphorylation events in the osmotic stress response in eel intestinal epithelium. Cell. Physiol. Biochem. 12, 163–178.

Lionetto, M.G., Giordano, M.E., De Nuccio, F., Nicolardi, G., Hoffmann, E.K., Schettino, T., 2005. Hypotonicity induced K^+ and anion conductive pathways activation in eel intestinal epithelium. J. Exp. Biol. 208, 749–760.

Lytle, C., 1997. Activation of the avian erythrocyte Na–K–Cl cotransport protein by cell shrinkage, cAMP, fluoride, and calyculin-A involves phosphorylation at common sites. J. Biol. Chem. 272, 15069–15077.

Lytle, C., Forbush, B., 1996. Regulatory phosphorylation of the secretory Na–K–Cl cotransporter: modulation by cytoplasmic Cl. Am. J. Physiol., Cell Biol. 270, C437–C448.

Maetz, J., Skadhauge, E., 1968. Drinking rates and gill ionic turnover in relation to external salinities in eel. Nature 217, 371–373.

Margalit, A., Livne, A.A., 1991. Lipoxygenase product controls the regulatory volume decrease of human platelets. Platelets 2, 207–214.

Margalit, A., Livne, A.A., 1992. Human platelets exposed to mechanical stresses express a potent lipoxygenase product. Thrombosis and Haemostasis. F.K. Schattauer Verlagsgesellschaft GmbH, Stuttgart, pp. 589–594.

Marshall, W.S., 1977. Transepithelial potential and short-circuit current across isolated skin of *Gillichthys mirabilis* (Teleostei–Gobiidae), acclimated to 5 percent and 100 percent seawater. J. Comp. Physiol. 114, 157–165.

Marshall, W.S., 2003. Rapid regulation of NaCl secretion by estuarine teleost fish: coping strategies for short-duration freshwater exposures. Biochim. Biophys. Acta, Biomembr. 1618, 95–105.

Marshall, W.S., Bryson, S.E., 1998. Transport mechanisms of seawater teleost chloride cells: an inclusive model of a multifunctional cell. Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 119, 97–106.

Marshall, W.S., Bryson, S.E., Luby, T., 2000. Control of epithelial Cl(−) secretion by basolateral osmolality in the euryhaline teleost *Fundulus heteroclitus*. J. Exp. Biol. 203, 1897–1905.

Marshall, W.S., Ossum, C.G., Hoffmann, E.K., 2005. Hypotonic shock mediation by p38 MAPK, JNK, PKC, FAK, OSR1 and SPAK in osmosensing chloride secreting cells of killifish opercular epithelium. J. Exp. Biol. 208, 1063–1077.

Martinez, A.S., Cutler, C.P., Wilson, G.D., Phillips, C., Hazon, N., Cramb, G., 2005. Cloning and expression of three aquaporin homologues from the European eel (*Anguilla anguilla*): effects of seawater acclimation and cortisol treatment on renal expression. Biol. Cell 97, 615–627.

Marvao, P., Emilio, M.G., Ferreira, K.G., Fernandes, P.L., Ferreira, H.G., 1994. Ion-transport in the intestine of *Anguilla anguilla* — gradients and translocators. J. Exp. Biol. 193, 97–117.

Matthews, J.B., Smith, J.A., Mun, E.C., Sicklick, J.K., 1998. Osmotic regulation of intestinal epithelial Na^+–K^+–Cl-cotransport: role of Cl^- and F-actin. Am. J. Physiol., Cell Physiol. 274, C697–C706.

Niemeyer, M.I., Hougaard, C., Hoffmann, E.K., Jorgensen, F., Stutzin, A., Sepulveda, F.V., 2000. Characterisation of a cell swelling-activated K^+-selective conductance of Ehrlich mouse ascites tumour cells. J. Physiol. (Lond.) 524, 757–767.

Niemeyer, M.I., Cid, L.P., Barros, L.F., Sepulveda, F.V., 2001a. Modulation of the two-pore domain acid-sensitive K^+ channel TASK-2 (KCNK5) by changes in cell volume. J. Biol. Chem. 276, 43166–43174.

Niemeyer, M.I., Cid, L.P., Sepulveda, F.V., 2001b. K^+ conductance activated during regulatory volume decrease. The channels in Ehrlich cells and their possible molecular counterpart. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 130, 565–575.

Nilius, B., Droogmans, G., 2003. Amazing chloride channels: an overview. Acta Physiol. Scand. 177, 119–147.

Nilius, B., Eggermont, J., Voets, T., Buyse, G., Manolopoulos, V., Droogmans, G., 1997. Properties of volume-regulated anion channels in mammalian cells. Prog. Biophys. Mol. Biol. 68, 69–119.

Nilius, B., Prenen, J., Walsh, M.P., Carton, I., Bollen, M., Droogmans, G., Eggermont, J., 2000. Myosin light chain phosphorylation-dependent modulation of volume-regulated anion channels in macrovascular endothelium. FEBS Lett. 466, 346–350.

Nilius, B., Vriens, J., Prenen, J., Droogmans, G., Voets, T., 2004. TRPV4 calcium entry channel: a paradigm for gating diversity. Am. J. Physiol., Cell Physiol. 286, C195–C205.

O'Donnell, M.E., Martinez, A., Sun, D., 1995. Endothelial Na–K–Cl cotransport regulation by tonicity and hormones: phosphorylation of cotransport protein. Am. J. Physiol., Cell Physiol. 269, C1513–C1523.

Palfrey, H.C., Pewitt, E.B., 1993. The ATP and Mg^{2+} dependence of Na^+–K^+–$2Cl^-$ cotransport reflects a requirement for protein phosphorylation: studies using calyculin A. Pflügers Arch. 425, 321–328.

Palfrey, H.C., Rao, M.C., 1983. Na/K/Cl co-transport and its regulation. J. Exp. Biol. 106, 43–54.

Panet, R., Atlan, H., 1990. Bumetanide-sensitive $Na^+/K^+/Cl^-$ transporter is stimulated by phorbol ester and different mitogens in quiescent human skin fibroblasts. J. Cell. Physiol. 145, 30–38.

Payne, J.A., Forbush III, B., 1994. Alternatively spliced isoforms of the putative renal Na–K–Cl cotransporter are differentially distributed within the rabbit kidney. Proc. Natl. Acad. Sci. U. S. A. 91, 4544–4548.

Payne, J.A., Forbush III, B., 1995. Molecular characterization of the epithelial Na–K–Cl cotransporter isoforms. Curr. Opin. Cell Biol. 7, 493–503.

Payne, J.A., Xu, J.-C., Haas, M., Lytle, C.Y., Ward, D., Forbush III, B., 1995. Primary structure, functional expression, and chromosomal localization of the bumetanide-sensitive Na–K–Cl cotransporter in human colon. J. Biol. Chem. 270, 17977–17985.

Pedersen, S.F., Hoffmann, E.K., 2002. Possible interrelationship between changes in F-actin and myosin II, protein phosphorylation, and cell volume regulation in Ehrlich ascites tumor cells. Exp. Cell Res. 277, 57–73.

Pedersen, S.F., Mills, J.W., Hoffmann, E.K., 1999. Role of the F-actin cytoskeleton in the RVD and RVI processes in Ehrlich ascites tumor cells. Exp. Cell Res. 252, 63–74.

Pedersen, S., Lambert, I.H., Thoroed, S.M., Hoffmann, E.K., 2000. Hypotonic cell swelling induces translocation of the a isoform of cytosolic phospholipase A_2 but not the g isoform in Ehrlich ascites tumor cells. Eur. J. Biochem. 267, 5531–5539.

Pedersen, S.F., Hoffmann, E.K., Mills, J.W., 2001. The cytoskeleton and cell volume regulation. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 130, 385–399.

Pedersen, S.F., Beisner, K.H., Hougaard, C., Willumsen, B.M., Lambert, I.H., Hoffmann, E.K., 2002. Rho family GTP binding proteins are involved in the regulatory volume decrease process in NIH3T3 mouse fibroblasts. J. Physiol. 541, 779–796.

Pedersen, S.F., Varming, C., Christensen, S.T., Hoffmann, E.K., 2002. Mechanisms of activation of NHE by cell shrinkage and by calyculin A in Ehrlich ascites tumor cells. J. Membr. Biol. 189, 67–81.

Pequeux, A., Gilles, R., 1988. The trans-epithelial potential difference of isolated perfused gills of the Chinese crab *Eriocheir sinensis* acclimated to fresh-water. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 89, 163–172.

Pewitt, E.B., Hegde, R.S., Haas, M., Palfrey, H.C., 1990. The regulation of Na/K/2Cl cotransport and bumetanide binding in avian erythrocytes by protein phosphorylation and dephosphorylation. Effects of kinase inhibitors and okadaic acid. J. Biol. Chem. 265, 20747–20756.

Piechotta, K., Garbarini, N., England, R., Delpire, E., 2003. Characterization of the interaction of the stress kinase SPAK with the Na^+–K^+–$2Cl^-$ cotransporter in the nervous system: evidence for a scaffolding role of the kinase. J. Biol. Chem. 278, 52848–52856.

Pon, D.C., Hill, C.E., 1997. Existence, properties, and functional expression of "Maxi-K"-type, Ca^{2+}-activated K^+ channels in short-term cultured hepatocytes. J. Cell. Physiol. 171, 87–94.

Roger, F., Martin, P.Y., Rousselot, M., Favre, H., Feraille, E., 1999. Cell shrinkage triggers the activation of mitogen-activated protein kinases by hypertonicity in the rat kidney medullary thick ascending limb of the Henle's loop. Requirement of p38 kinase for the regulatory volume increase response. J. Biol. Chem. 274, 34103–34110.

Roman, R., Feranchak, A.P., Troetsch, M., Dunkelberg, J.C., Kilic, G., Schlenker, T., Schaack, J., Fitz, J.G., 2002. Molecular characterization of volume-sensitive SKCa channels in human liver cell lines. Am. J. Physiol., Gastrointest. Liver Physiol. 282, G116–G122.

Romanenko, V.G., Rothblat, G.H., Levitan, I., 2002. Modulation of endothelial inward-rectifier K^+ current by optical isomers of cholesterol. Biophys. J. 83, 3211–3222.

Romanenko, V.G., Rothblat, G.H., Levitan, I., 2004. Sensitivity of volume-regulated anion current to cholesterol structural analogues. J. Gen. Physiol. 123, 77–87.

Roti Roti, L.W., Rothstein, A., 1973. Adaptation of mouse leukemic cells (L5178Y) to anisotonic media II. Cell growth. Exp. Cell Res. 79, 311–317.

Russell, J.M., 1983. Cation-coupled chloride influx in squid axon. Role of potassium and stoichiometry of the transport process. J. Gen. Physiol. 81, 909–925.

Russell, J.M., 2000. Sodium-potassium-chloride cotransport. Physiol. Rev. 80, 211–276.

Schettino, T., Lionetto, M.G., 2003. Cl-absorption in European eel intestine and its regulation. J. Exp. Zool., Part A Comp. Exp. Biol. 300A, 63–68.

Scott, G.R., Claiborne, J.B., Edwards, S.L., Schulte, P.M., Wood, C.M., 2005. Gene expression after freshwater transfer in gills and opercular epithelia of killifish: insight into divergent mechanisms of ion transport. J. Exp. Biol. 208, 2719–2729.

Scott, G.R., Richards, J.G., Forbush, B., Isenring, P., Schulte, P.M., 2004. Changes in gene expression in gills of the euryhaline killifish *Fundulus heteroclitus* after abrupt salinity transfer. Am. J. Physiol., Cell Physiol. 287, C300–C309.

Shen, M.R., Chou, C.Y., Hsu, K.F., Ellory, J.C., 2002. Osmotic shrinkage of human cervical cancer cells induces an extracellular Cl-dependent nonselective cation channel, which requires p38 MAPK. J. Biol. Chem. 277, 45776–45784.

Smallwood, N.D., Wang, X.Y., Hausman, B.S., Liedtke, C.M., 2004. Determination of binding site of actin on delta C2-like domain of protein kinase C (PKC) delta. FASEB J. 18, A321.

Starremans, P.G.J.F., Kersten, F.F.J., Van Den Heuvel, L.P.W.J., Knoers, N.V.A. M., Bindels, R.J.M., 2003. Dimeric architecture of the human bumetanide-sensitive Na–K–Cl co-transporter. J. Am. Soc. Nephrol. 14, 3039–3046.

Stoner, L.C., Morley, G.E., 1995. Effect of basolateral or apical hyposmolarity on apical maxi K channels of everted rat collecting tubule. Am. J. Physiol., Renal Physiol. 268, F569–F580.

Stutzin, A., Hoffmann, E.K., 2006. Swelling-activated ion channels; functional regulation in cell-swelling proliferation and apoptosis. Acta Physiol. 187, 27–42.

Suvitayavat, W., Dunham, P.B., Haas, M., Rao, M.C., 1994. Characterization of the proteins of the intestinal Na^+–K^+–2Cl-cotransporter. Am. J. Physiol. 267, C375–C384.

Takeda, M., Homma, T., Breyer, M.D., Horiba, N., Hoover, R.L., Kawamoto, S., Ichikawa, I., Kon, V., 1993. Volume and agonist-induced regulation of myosin light-chain phosphorylation in glomerular mesangial cells. Am. J. Physiol., Renal Physiol. 264, F421–F426.

Thoroed, S.M., Lauritzen, L., Lambert, I.H., Hansen, H.S., Hoffmann, E.K., 1997. Cell swelling activates phospholipase A_2 in Ehrlich ascites tumor cells. J. Membr. Biol. 160, 47–58.

Tilly, B.C., van den, B.N., Tertoolen, L.G., Edixhoven, M.J., de Jonge, H.R., 1993. Protein tyrosine phosphorylation is involved in osmoregulation of ionic conductances. J. Biol. Chem. 268, 19919–19922.

Tinel, H., Wehner, F., Kinne, R.K., 1997. Arachidonic acid as a second messenger for hypotonicity-induced calcium transients in rat IMCD cells. Pflügers Arch. 433, 245–253.

Trischitta, F., Denaro, M.G., Faggio, C., Schettino, T., 1992. An attempt to determine the mechanisms of Cl-exit across the basolateral membrane of eel intestine — use of different Cl-transport pathway inhibitors. J. Exp. Zool. 264, 11–18.

Tsutsumi, T., Ushiro, H., Kosaka, T., Kayahara, T., Nakano, K., 2000. Proline- and alanine-rich Ste20-related kinase associates with F-actin and translocates from the cytosol to cytoskeleton upon cellular stresses. J. Biol. Chem. 275, 9157–9162.

Umenishi, F., Schrier, R.W., 2003. Hypertonicity-induced aquaporin-1 (AQP1) expression is mediated by the activation of MAPK pathways and hypertonicity-responsive element in the AQP1 gene. J. Biol. Chem. 278, 15765–15770.

Ussing, H.H., 1982. Volume regulation of frog skin epithelium. Acta Physiol. Scand. 114, 363–369.

Vanpraag, D., Farber, S.J., Minkin, E., Primor, N., 1987. Production of eicosanoids by the killifish gills and opercular epithelia and their effect on active-transport of ions. Gen. Comp. Endocrinol. 67, 50–57.

Vitari, A.C., Deak, M., Morrice, N.A., Alessi, D.R., 2005. The WNK1 and WNK4 protein kinases that are mutated in Gordon's hypertension syndrome phosphorylate and activate SPAK and OSR1 protein kinases. Biochem. J. 391, 17–24.

Voets, T., Manolopoulos, V., Eggermont, J., Ellory, C., Droogmans, G., Nilius, B., 1998. Regulation of a swelling-activated chloride current in bovine endothelium by protein tyrosine phosphorylation and G proteins. J. Physiol. (Lond.) 506, 341–352.

Wehner, F., Olsen, H., Tinel, H., Kinne-Safran, E., Kinne, R.K.H., 2003. Cell volume regulation: osmolytes, osmolyte transport, and signal transduction. Rev. Physiol., Biochem. Pharmacol. 148, 1–80.

Weskamp, M., Seidl, W., Grissmer, S., 2000. Characterization of the increase in $[Ca^{2+}]_i$ during hypotonic shock and the involvement of Ca^{2+}-activated K^+ channels in the regulatory volume decrease in human osteoblast-like cells. J. Membr. Biol. 178, 11–20.

Xu, J.C., Lytle, C., Zhu, T.T., Payne, J.A., Benz Jr., E., Forbush, B., 1994. Molecular cloning and functional expression of the bumetanide-sensitive Na–K–2Cl cotransporter. Proc. Natl. Acad. Sci. U. S. A. 91, 2201–2205.

Zadunaisky, J.A., 1984. The chloride cell: the active transport of chloride and the paracellular pathways. Fish Physiol. XB, 129–176.

Zadunaisky, J.A., Cardona, S., Au, L., Roberts, D.M., Fisher, E., Lowenstein, B., Cragoe Jr., E., Spring, K.R., 1995. Chloride transport activation by plasma osmolarity during rapid adaptation to high salinity of *Fundulus heteroclitus*. J. Membr. Biol. 143, 207–217.

Zadunaisky, J.A., Hoffmann, E.K., Colón, E., Hofffmann, E., Einhorn, J., 1998. Volume activated chloride secretion in opercular epithelium of *Fundulus heteroclitus*. Bull. MDIBL 37, 74–75.