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Biochimica et Biophysica Acta 1566 (2002) 140–151

BIOCHIMICA ET BIOPHYSICA ACTA
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Review

Regulation of Na–K–2Cl cotransport by phosphorylation and protein–protein interactions

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Received 29 May 2002; accepted 6 August 2002

Abstract

The Na–K–2Cl cotransporter plays important roles in cell ion homeostasis and volume control and is particularly important in mediating the movement of ions and thus water across epithelia. In addition to being affected by the concentration of the transported ions, cotransport is affected by cell volume, hormones, growth factors, oxygen tension, and intracellular ionized Mg^{2+} concentration. These probably influence transport through three main routes acting in parallel: cotransporter phosphorylation, protein–protein interactions and cell Cl^- concentration. Many effects are mediated, at least in part, by changes in protein phosphorylation, and are disrupted by kinase and phosphatase inhibitors, and manoeuvres that reduce cell ATP content. In some cases, phosphorylation of the cotransporter itself on serine and threonine (but not tyrosine) is associated with changes in transport rate, in others, phosphorylation of associated proteins has more influence. Analysis of the stimulation of cotransport by calyculin A, arsenite and deoxygenation suggests that the cotransporter is phosphorylated by several kinases and dephosphorylated by several phosphatases. These kinases and phosphatases may themselves be regulated by phosphorylation of residues including tyrosine, with Src kinases possibly playing an important role. Protein–protein interactions also influence cotransport activity. Cotransporter molecules bind to each other to form high molecular weight complexes, they also bind to other members of the cation–chloride cotransport family, to a variety of cytoskeletal proteins, and to enzymes that are part of regulatory cascades. Many of these interactions affect transport and may override the effects of cotransporter phosphorylation. Cell Cl^- may also directly affect the way the cotransporter functions independently of its role as substrate.

Keywords: Na–K–2Cl cotransport; Protein phosphorylation; Cytoskeleton; Protein–protein interaction; Red cell (erythrocyte); Epithelia

1. Introduction

The Na–K–2Cl cotransporter plays important roles in cell ion homeostasis and volume control. It is particularly important in mediating the movement of ions and thus water across epithelia. In these cells, the transport of ions across the basolateral and apical membranes must be very carefully controlled and coordinated to prevent massive changes in ion concentration and cell volume during secretion and reabsorption. The primary control of both processes appears to be directed at changing the activity of Cl^- and K^+ channels. These channels rapidly mediate the movement of large numbers of ions into or out of epithelial cells, and these fluxes must be balanced by an equivalent flux involving appropriate carriers and pumps. The Na–K–2Cl cotransporters are important providers of these balancing ion fluxes. Ussing [1] was among the first to suggest the existence of cotransport in epithelia and also suggested that the transporter is regulated by volume and cell Cl^- concentration.

Several excellent reviews deal with the discovery, properties and regulation of the cotransporter [2–5]. In this paper, I will briefly review the properties of the cotransporter and will then examine how phosphorylation affects transporter function and explore the possibility that the transporter forms high molecular weight complexes that are significant in its regulation. To do this, I will draw on information from the study of red cells, a model system that has long provided insight into epithelial function [6].

2. Na–K–2Cl cotransporter isoforms

Isoforms of the Na–K–2Cl cotransporter are widely distributed among animal cells. Most cells contain NKCC1 (or BSC2) and this is often referred to as the housekeeping

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0005-2736/02/$ - see front matter
PII: S0005-2736(02)00586-2

or secretory isoform. A separate gene product, NKCC2 (or BSC1), is found mainly in the mammalian kidney where it is involved in the reabsorption of Na^+, K^+ and Cl^- from the fluid in the thick ascending limb of Henle's loop (TAL). It is often referred to as the reabsorptive isoform.

The gene (Slc12a2) for the human form of NKCC1 (on chromosome 5q23.2) encodes for a 1212 amino acid protein with a predicted molecular weight of about 132 kDa. The protein is glycosylated and runs as a smear with an apparent molecular weight of 160–170 kDa on sodium dodecyl sulfate polyacyrlamide gels (SDS-PAGE) [7]. A similar picture is found for NKCC1 in other species, though the protein may have a slightly higher apparent molecular weight (up to 190 kDa) [8–12]. Deglycosylation with N-glycanase reduces the apparent molecular weight of NKCC1 to about 135 kDa, close to the weight predicted from cDNA [13]. A splice variant of NKCC1 has been observed in the brain [14]. It lacks a short peptide from the carboxy-terminal that codes for a protein kinase A (PKA) consensus site.

The gene for human NKCC2 (Slc12a1 on chromosome 15q15–q21.1) encodes for a shorter, 1099 residue protein, with a predicted molecular weight of 121 kDa [15]. This runs at an apparent weight of 150–160 kDa on SDS gels due to glycosylation [16]. A total of six splice variants of NKCC2 have been identified. There are three splice variants of full-length NKCC2 expressed in different parts of the TAL [15,17]. The proteins differ in a small region including part of transmembrane domain (TM) 2 that is believed to be involved in determining the Na^+, K^+ and Cl^- binding affinities of the transporter. Variant F is found in cells in the outer medullary region of the TAL and has the lowest affinities for Na^+, K^+ and Cl^- (and possibly the highest turnover rate), variant A is found in cells both in the outer medullary and cortical TAL, and has higher affinities for the transported ions. Variant B is found in the macula densa region of the kidney and has the highest Cl^- affinity. Carboxy-terminal-truncated versions of splice variants A, B and F have been identified in mouse kidney (770 residues), and these have an apparent molecular weight of about 120 kDa [18].

3. The cation–chloride cotransporter family

The Na–K–2Cl cotransporters are part of a large superfamily of transporters—the cation–chloride cotransporter (CCC) family [19–21]. This family includes the K^+-independent Na–Cl cotransporter (NCC or TSC) found in the distal convoluted tubule of the kidney, and which is responsible for the electroneutral reabsorption of Na^+ and Cl^-. This transporter is not affected by bumetanide but is inhibited by thiazide diuretics. Four Na^+-independent, K–Cl cotransporters have been described (KCC1, KCC2, KCC3 and KCC4). These transporters produce an electroneutral loss of K^+ and Cl^- from cells, can play an important role in regulatory volume decrease, and are inhibited by furosemide and by high doses of bumetanide. Recently a new CCC family member has been identified. This protein does not appear to transport ions itself but inhibits NKCC with which it associates. It is called the cation–chloride cotransporter-interacting protein (CIP1) [20].

4. General properties of Na–K–2Cl cotransporters

The cotransporter usually moves 1 Na^+, 1 K^+ and 2 Cl^- ions across cell membranes in an electroneutral fashion, so that operation of the transporter neither generates a current nor is affected by membrane potential [2,4,22–24]. There are some possible exceptions to this stoichiometry. NKCC1 from squid axons appears to transport $2Na^+$:$1K^+$:$3Cl^-$ [4,25], and a truncated form of NKCC2 may transport $1Na^+$:$1Cl^-$ independently of K^+ [26]. Ions bind to the cotransporter in a strictly ordered sequence, so that from the outside of the cell, binding of Na^+ is followed by Cl^-, then K^+ and finally Cl^- [24]. At the inside surface, the ions are again released in an ordered sequence, following the dictum, *first on*, *first off*, also known as glide symmetry [24]. As access to the ion binding sites changes from the exterior cell surface to the internal surface, the ions become temporarily occluded within the transporter [27]. A key diagnostic feature of Na–K–2Cl cotransporters is their very high sensitivity to inhibition by bumetanide and other loop diuretics. NKCC2 is more sensitive to inhibition by bumetanide than NKCC1 [4,28]. The transporter can move ions in both directions across the membrane but the net direction of transport is governed by the combined chemical gradients for Na^+, K^+ and $(Cl^-)^2$. Thus, when the transporter is activated, it tends to cause the entry of these ions into cells, which is followed by the movement of water causing cell volume to increase. Activation of the cotransporter can thus play an important role in the regulatory volume increase (RVI) that occurs when some cells are shrunk. Much effort has been directed at trying to identify how cells detect the change in volume and produce an appropriate change in cotransport rate [29–32].

Although the transporter is not a primary active transporter and does not consume ATP [22], it is inhibited in cells whose ATP content has been reduced [33–35]. This need for ATP reflects an important role for phosphorylation in regulating transport [36]. However, the transporter is capable of secondary active transport, for instance moving Cl^- or K^+ into cells using the energy in the Na^+ gradient. An important step in establishing that the cotransporter could transport Cl^- in red cells, and was not simply Cl^--dependent, was the finding that Cl^- moving down its electrochemical gradient could drive Na^+ up its gradient [23]. The transporter thus plays an important role in regulating cell Cl^- and K^+ content, and cells can lose K^+ and Cl^- when treated with loop diuretics.

The cotransporter plays a particularly important role in the movement of ions across epithelia. NKCC1 is usually found in the basolateral membranes and causes the uptake of Na^+, K^+ and Cl^- from the serosal solution into the cells. The Na^+ and Cl^- can then be secreted by a variety of mechanisms across the apical membrane. NKCC2 is found in the apical membranes of TAL cells, and is responsible for reabsorbing Na^+, K^+ and Cl^- from the urine. The K^+ is recycled back into the urine through K^+ channels, thus making the urine electrically positive with respect to the serosal side. This potential then drives the reabsorption of cations, including Mg^{2+}, through the tight junctions. In the TAL, we see how the use of splice variants allows matching of transporter ion affinities (and possibly transport rates) to the decreasing ion concentrations in the urine as it flows from medulla to cortex [17]. NKCC2 splice variant B is thought to play an important role in the macula densa's ability to sense the Na^+ and Cl^- concentrations in the distal urine and send information back to the afferent arteriole (tubulo-glomerular feedback).

5. Cotransporter structure

Sequence and hydropathy analysis of NKCC1, together with information from antibody accessibility studies suggest that NKCC1 has 12 transmembrane domains with large cytoplasmic carboxy- and amino-termini, and has an extracellular domain between TM7 and 8 that is glycosylated [7,8,13,37]. This picture has been recently confirmed by studying the way putative transmembrane domains are expressed in an in vitro translation system [38]. Most of our knowledge about which parts of the Na–K–2Cl cotransporter are involved in ion and bumetanide binding comes from studies of NKCC1 from shark rectal gland and human colon cells, and in particular by studying the properties of chimeric proteins formed by joining sections of the human and shark transporters, and also from site-directed mutagenesis [28,39–42]. Regions in TM2, 4 and 7 determine the Na^+, K^+ and Cl^- affinities of the transporter while these same regions, together with regions in TM11 and 12, determine bumetanide binding affinity. This latter finding brings into question the idea that bumetanide inhibits the cotransporter by simply binding to the Cl^- site [43], clearly other regions of the transporter are involved too.

Experiments with ^{32}P show that the cotransporter itself is phosphorylated at multiple sites when transport is activated by a variety of different stimuli [11,36,44–47]. Analysis of the primary sequence of the transporter suggests that there are many potential targets for protein kinases, depending on the isoform and the species. However, as we will see later, it has proved extremely difficult to identify the kinases and phosphatases that are responsible for regulating cotransporter phosphorylation. An interesting recent development is the identification a highly conserved sequence in the cytoplasmic amino-terminal domain of the cotransporter that binds the catalytic subunit of protein phosphatase 1 [48]. The cotransporter appears to function as its own scaffolding to bring together regulatory enzymes and their targets.

6. Regulation of transport

Until their binding sites are saturated, transport rate will depend on the concentrations of the transported ions. In addition, a number of other factors influence transport rate. Cotransport is usually stimulated by cell shrinkage, and in a few cases by cell swelling [2,49], it is stimulated by hormones such as noradrenaline and vasopressin [2], growth factors including serum [2,50], reduced oxygen tension [51,52], increased intracellular Mg^{2+} concentration [53], and reduced intracellular Cl^- acting at a site separate from the transport site [54,55]. These factors influence transport through three main routes acting in parallel: cotransporter phosphorylation, protein–protein interactions and probably a direct effect of cell Cl^- concentration itself. Inhibition of protein kinases and phosphatases, and reduction in cell ATP content interfere with the cell's ability to respond appropriately to many of these stimuli, suggesting that changes in protein phosphorylation are, at least partly, involved in detection, signal transduction and response to these stimuli [34,35,51,52,56–61]. As phosphorylation of the transporter itself has been shown to correlate with transport rate under a number of conditions, it is tempting to speculate that phosphorylation of the cotransporter is the final common pathway through which transport is regulated [9–11,36,44,45]. However, a number of careful studies have shown that transport may be influenced by protein–protein interactions either within the membrane or with elements in the cytoskeleton. Conditions can be found where transport rate changes independently of cotransporter phosphorylation [10] (see Section 9).

Analysis of the effects of cell volume on cotransport are complicated by the difficulty in separating direct effects of cell volume, perhaps mediated by changes in the structure of the cytoskeleton [62], from secondary effects due to subtle changes in cell Cl^- content. For instance, the activation of secretion may start with the opening of apical Cl^- channels and the loss of cell Cl^- and water. The question is whether the activation of cotransport that follows is due to the reduction of Cl^- or of cell volume. Evidence can be found supporting a primary role for cell Cl^- [5,55] or cell volume [63].

7. Role of phosphorylation

The first indication that protein phosphorylation might be important in regulating cotransport came from studies on the effects of starvation, metabolic depletion and internal perfusion of cells [33,34,64–68]. On the basis of

these studies, it was suggested that the cotransporter itself or an associated regulatory protein might be activated by phosphorylation [69–72]. As described above, it is now known that the cotransporter is phosphorylated under a variety of conditions that stimulate cotransport. Key studies revealed that it is phosphorylated on at least five sites which include serine and threonine residues in the cytoplasmic amino and carboxy-termini. There is no evidence for regulatory phosphorylation of tyrosine [36,45]. Importantly, several different means of stimulating transport were shown to produce identical patterns of phosphorylation as judged by phosphopeptide mapping [45]. This finding gave credence to the idea that regulatory phosphorylation of the cotransporter is carried out by a single kinase. However, it still needs to be established whether phosphorylation at the different sites have equivalent effects on transport, whether there are additive or inhibitory effects, and importantly whether phosphorylation is necessary for transport.

7.1. Experiments on ferret red cells

In order to address some of these issues, we have been examining the properties of Na–K–2Cl cotransport in ferret red cells. These cells are a useful model in which to study the properties of the mammalian Na–K–2Cl cotransporter [59,67,73]. They have a very high resting cotransport rate and more than 90% of K^+ uptake is though the transporter. Measurement of bumetanide-sensitive ^{86}Rb (tracer for K^+) uptake provides a robust assay for cotransporter activity. In addition, there is little contamination of the fluxes by K^+–K^+ exchange as these cells have a low K^+ content [24,74].

7.2. A one-kinase/one-phosphatase model

The simplest model of cotransporter regulation by phosphorylation assumes the transporter is phosphorylated (at multiple sites) and dephosphorylated by one kinase (CT-kinase) and one phosphatase (CT-phosphatase). Regulation of transport would then focus on the regulation of these two enzymes. If this were so, it should be possible to inhibit all cotransport activity by inhibiting the kinase (assuming that the transporter must be phosphorylated to be active), and it should be possible to demonstrate maximal stimulation by inhibiting the phosphatase (see Fig. 1).

7.3. Calyculin A maximally stimulates cotransport

Cotransport in ferret red cells is maximally activated within a few minutes by the potent protein phosphatase inhibitor, calyculin A, and this activity is then maintained [75]. This implies that the cells contain a highly active CT-kinase, the activity of which is revealed by the addition of calyculin. Thus, steady-state levels of phosphorylated and dephosphorylated cotransporter are maintained by a highly active CT-kinase and phosphatase. Studies of the potencies

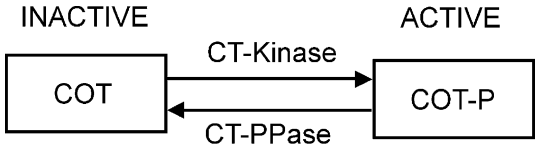

Fig. 1. One-kinase/one-phosphatase model for the regulation of cotransport. In this model, only phosphorylated cotransporter (COT-P) is active. The cotransporter (COT) is phosphorylated at multiple sites by a single cotransporter kinase (CT-kinase) and dephosphorylated by a single cotransporter phosphatase (CT-PPase).

of a range of phosphatase inhibitors on transport in intact cells, and more compellingly, on NKCC1 dephosphorylation in membranes, suggest that calyculin stimulates cotransport by inhibiting protein phosphatase 1 rather than protein phosphatase 2A [48,55,58]. Treatment of ferret red cells with cyclosporin A causes only minor transport stimulation suggesting that protein phosphatase 2B plays a minor role in cotransport regulation in these cells, and is consistent with this transporter's insensitivity to changes in Ca^{2+} concentration [75,76].

In red cells, calyculin A causes maximal cotransport stimulation that is stable over long periods [45,58,75]. However, in Ehrlich ascites cells, stimulation is transient, perhaps being offset by events caused by the gradual cell swelling also caused by calyculin [61]. This inactivation can be delayed by shrinking the cells. These findings suggest either, that another phosphatase becomes active, or that interactions with the cytoskeleton, or cell Cl^- (see Sections 8 and 9), override the effects of transporter phosphorylation.

7.4. Non-specific inhibition of kinases by removing cell Mg^{2+} reveals significant residual cotransport

In order to inhibit kinases non-specifically in a cell extract it is normal practice to reduce Mg^{2+} to sub-micromolar levels by adding a chelator such as EDTA. This can also be achieved in intact red cells by treating them with the ionophore A23187 in the presence of extracellular EDTA [77]. Under these conditions, the cotransporter continues to function at about 15–20% of its maximal rate (equivalent to about 50% resting rate) [53] despite the fact that all kinases should be inactive. The fact that cotransport continues at a significant level suggests that, either some cotransporter molecules can operate when dephosphorylated, or that some of the cotransporter is trapped in a partially phosphorylated, partially active state, in the absence of Mg^{2+}. If the latter is true, then at least some of the phosphorylated cotransporter molecules are probably dephosphorylated by a Mg^{2+}-sensitive protein phosphatase, otherwise, residual phosphatase activity should lead to total dephosphorylation and total loss of activity. The alternative possibility, that two forms of the cotransporter may exist, is supported by the finding of doublets (approximately 140 and 160 kDa) in Western blots of membranes probed with anti-cotransporter antibodies (T4, see Ref. [78]) [50,79].

7.5. A specific kinase inhibitor that totally inhibits cotransport has yet to be found

We have not been able to find a kinase inhibitor that inhibits all the cotransport activity in ferret red cells [75,80]. Inhibitors of PKA, PKC, PKG and casein kinase II have little effect on transport. Of the kinase inhibitors tested, staurosporine, genistein and PP1 (4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimadine), were the most effective at inhibiting cotransport (inhibit about 40–50% resting activity), but were no more effective than Mg^{2+}-removal. In addition, the use of these inhibitors in combination with each other, or with Mg^{2+}-removal, was no more effective than the agents alone [75]. Again, the simplest explanation for these findings is that some cotransporter molecules do not need to be phosphorylated to be active. Clearly, it is important to establish whether this is so. In order to assess this, the cotransporter was immunoprecipitated from ferret red cells under a variety of conditions using antibody T4. Analysis of the immunoprecipitate's incorporation of ^{32}P and reactivity to phospho-specific antibodies suggests that the cotransporter is phosphorylated under resting conditions and that this increases when cells are treated with calyculin. Phosphorylation is reduced when cells are treated with PP1, but is still easily detectable (Flatman and Matskevich, unpublished observations). This suggests that the residual transport seen in cells treated with PP1 (and the same probably applies to those treated with staurosporine or Mg^{2+}-removal) is associated with a phosphorylated rather than with an unphosphorylated form of the cotransporter. Residual phosphorylation of the cotransporter in the presence of staurosporine has also been seen in salivary glands [47].

If we make the assumption that only phosphorylated cotransporter is active and that one kinase and phosphatase are responsible for regulatory phosphorylation, it follows that a potent CT-kinase inhibitor has not yet been found, and an alternative explanation must be found for the partial inhibition of transport by staurosporine, genistein and PP1. One possibility is that these compounds inhibit a kinase that phosphorylates and inhibits the CT-phosphatase (Fig. 2). Thus, in the presence of staurosporine or PP1, the CT-phosphatase becomes more active, and more, but not all, of the cotransporter becomes dephosphorylated and inactive.

It is interesting to note that the kinase inhibitors that are most effective in reducing transport all inhibit tyrosine kinases [81–83]. Mg^{2+}-removal is the least selective way of inhibiting kinases, whereas PP1 is probably the most selective, and inhibits Src kinases [83], perhaps suggesting an important role for Src kinases in the regulation of cotransport. Yet all these agents reduce transport to about the same extent. Transport rate correlates with changes in serine/threonine phosphorylation of the cotransporter and not with tyrosine phosphorylation [36,45]. This supports the idea that these inhibitors are acting indirectly perhaps by preventing phosphorylation of a CT-kinase or phosphatase. Interestingly, tyrosine phosphatase inhibitors like vanadate and bpV(phen) have little effect on transport in ferret red cells [35,75].

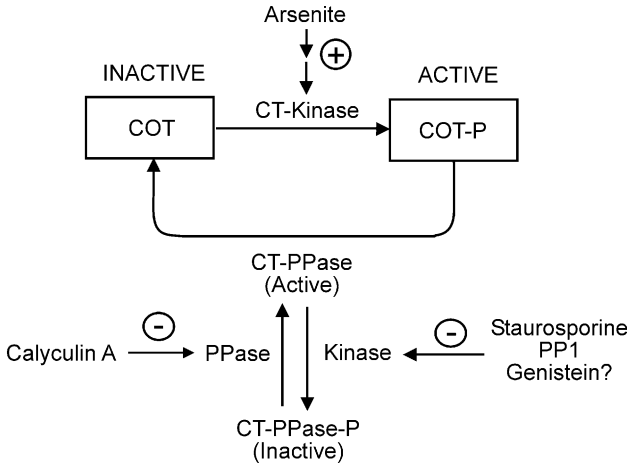

Fig. 2. A one-kinase/one-phosphatase model to account for the effects of calyculin, arsenite and kinase inhibitors [80]. If only phosphorylated cotransporter is active, the kinase inhibitors, staurosporine, PP1 and genistein inhibit a kinase that phosphorylates and inhibits CT-phosphatase (to form inactive CT-PPase-P). Calyculin A inhibits a phosphatase (PPase) that dephosphorylates and activates CT-PPase. Arsenite indirectly activates CT-kinase. Other abbreviations as in Fig. 1.

Analysis of the actions of genistein, particularly in association with calyculin suggests that this agent may be acting as a direct inhibitor of the Na–K–2Cl cotransporter (like bumetanide) as well as a tyrosine kinase inhibitor [75,80].

7.6. Combined use of kinase and phosphatase inhibitors

Examination of the effects of the sequential treatment of cells with kinase and phosphatase inhibitors on ^{86}Rb uptake provides insight into the mechanisms regulating phosphorylation. Treatment of cells with PP1, staurosporine or Mg^{2+}-removal before calyculin almost completely prevents stimulation of transport, whereas addition of these agents after calyculin causes a small (about 30%) reduction in the stimulated rate [75]. Similar results for the interactions between calyculin and staurosporine have been observed in duck and turkey red cells [45,51]. Assuming that only phosphorylated cotransporter is active, and that there is a single kinase and phosphatase, this implies that calyculin inhibits a phosphatase that dephosphorylates the CT-phosphatase rather than inhibiting the CT-phosphatase itself (Fig. 2). In addition, the CT-phosphatase must be Mg^{2+}-sensitive in order to explain continued transport in the absence of Mg^{2+}.

The suggestion that calyculin does not directly inhibit the CT-phosphatase is controversial. In support of direct inhibition is the recent finding that NKCC1 has a binding site for the catalytic subunit of protein phosphatase 1 [48]. The

presumption here is that protein phosphatase 1 binds to the cotransporter to improve access to phosphate groups on the cotransporter itself. However, it is possible that the binding of the phosphatase to the cotransporter allows it to dephosphorylate another phosphatase that is part of the regulatory cascade. Evidence in favour of this latter view may come from work on membranes prepared from salivary glands [47]. If protein phosphatase 1 forms a tight complex with the cotransporter and is capable of dephosphorylating the cotransporter, we would expect the addition of calyculin to these membranes to maintain the cotransporter in a phosphorylated state. However, calyculin did not prevent dephosphorylation of the transporter, whereas vanadate did.

7.7. Stimulation by arsenite

Cotransport in ferret red cells is also maximally stimulated by arsenite ions [80]. In this case, staurosporine, PP1 and Mg^{2+}-removal both prevent stimulation when added before arsenite and reverse the stimulation when added after arsenite. Treatment of cells with a combination of arsenite and calyculin produce maximal stimulation even in cells that have been stored for a few days (a time when stimulation by either calyculin or arsenite alone has started to wane). The kinase inhibitor PP1 does not prevent nor reverse the stimulation caused by the combination of calyculin and arsenite, and staurosporine only has a minor effect. On the other hand, Mg^{2+}-removal completely prevents stimulation by this combination, and causes a small amount of inhibition when added later. In order to explain the effects of arsenite, both alone, and in combination with calyculin, in terms of the one-kinase/one-phosphatase model, it is necessary to propose that arsenite stimulates the CT-kinase, probably indirectly (Fig. 2) [80].

The finding that cotransport activity returns to resting level in cells that have been stimulated with arsenite and are then Mg^{2+}-depleted is hard to explain with the proposed model. Mg^{2+}-removal would be expected to leave transport rates high as the phosphatase is probably more sensitive to Mg^{2+}-removal than the kinase. A more plausible explanation is that the cotransporter is dephosphorylated by more than one phosphatase. The same suggestion has been made to explain the response of the cotransporter to a range of pharmacological manipulations in Ehrlich cells [61].

7.8. Cotransporter phosphorylation may involve multiple kinases and phosphatases

More problems for the one-kinase/one-phosphatase model arise when attempting to analyse the effects of deoxygenation on the cotransporter. Deoxygenation maximally stimulates Na–K–2Cl cotransport [51,52], and this stimulation is both prevented and reversed by PP1, staurosporine and Mg^{2+}-removal, just as was seen with arsenite [52]. However, the effects of arsenite can be distinguished from those of deoxygenation. If deoxygenation and arsenite affect cotransport in identical ways, PP1 should have little effect on transport stimulated by the combined effects of deoxygenation and calyculin (compare with its effect on the combined action of arsenite and calyculin). When the experiment was carried out, PP1 was found to reduce transport by more than 30%. Also if deoxygenation acts like arsenite, the combined effects of these treatments should be indistinguishable from either alone. In this case, experiments revealed that addition of PP1 to cells after transport had been stimulated by the combination of arsenite and deoxygenation, caused only a minor reduction in transport rate (Flatman, unpublished results).

In order to encompass all these findings, it is probably necessary to abandon the idea that cotransporter phosphorylation is maintained by a single kinase and phosphatase. The data are more consistent with the idea that deoxygenation either activates a kinase that is quiescent in oxygenated cells, or causes a conformational change that allows a kinase access to appropriate sites on the cotransporter. There is evidence that deoxygenation causes profound changes in both membrane protein phosphorylation, and in the association of structural proteins and enzymes with integral membrane proteins [84–87]. The data also suggest that PP1 and staurosporine inhibit (indirectly) the kinase activated by deoxygenation.

Models for transport regulation involving multiple kinases and phosphatases can be complex (Fig. 3). It is not clear whether phosphorylation by one of the CT-kinases is reversed by one particular phosphatase or whether it is reversed by the action of several phosphatases, and vice versa. Yet despite these problems some conclusions can be made. For instance, deoxygenation probably promotes phosphorylation by a kinase that is distinct from the kinase active at rest and revealed by treating cells with calyculin. Once we abandon the one-kinase/one-phosphatase model, it is possible to attribute some of the actions of PP1 and staurosporine to inhibition (probably indirect) of a CT-kinase active at rest and a CT-kinase activated by deoxygenation. We can also propose that protein phosphatase 1 directly dephosphorylates some cotransporter molecules, in which case the corresponding kinase is probably inhibited by staurosporine and PP1. On the other hand, if some cotransporter molecules are dephosphorylated by a Mg^{2+}-sensitive protein phosphatase (2C? see Ref. [88]), these are probably phosphorylated by a kinase insensitive to staurosporine and PP1. This would explain how the cotransporter retains similar partial activity in the presence of kinase inhibitors and in the absence of Mg^{2+} (and would correspond to activity associated with COT-P3 in Fig. 3).

Multiple kinase models also explain why it has been so difficult to find a specific kinase inhibitor that prevents phosphorylation of the transporter in response to all stimuli. There is no master CT-kinase. As discussed above, inhibitors of PKA, PKC, PKG and casein kinase II have little effect on resting cotransport activity, suggesting that none of

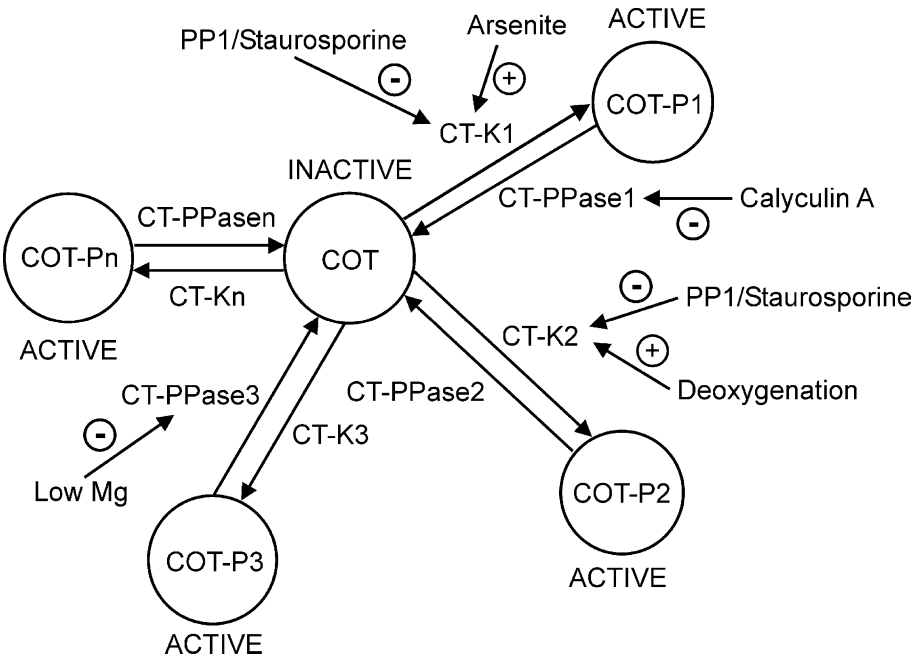

Fig. 3. Multiple kinase and phosphatase model for cotransporter regulation. The cotransporter can be phosphorylated (COT-P1, COT-P2, COT-P3, COT-Pn) and activated by several kinases (CT-K1, CT-K2, CT-K3, CT-Kn) and dephosphorylated by several phosphatases (CT-PPase1, CT-PPase2, CT-PPase3, CT-PPasen). Possible sites for the actions of PP1, staurosporine, arsenite, calyculin A, and deoxygenation are shown. CT-PPase1 may be protein phosphatase 1, and CT-PPase3 protein phosphatase 2C (inhibition by low Mg^{2+} concentration is indicated) [88]. Other abbreviations as in Figs. 1 and 2.

these kinases are the major CT-kinase active at rest in ferret red cells. The properties of a volume-sensitive CT-kinase, regulated by phosphorylation, have been described in duck red cells [60]. In shrunken endothelial cells, c-*Jun* NH_2-terminal kinase (JNK) becomes activated and this kinase has been shown to phosphorylate fusion proteins made of the amino and carboxy cytoplasmic domains of NKCC1 in vitro [89]. Thus JNK could be the CT-kinase that phosphorylates and activates the cotransporter in response to cell shrinkage. However, it has still to be established whether JNK can phosphorylate the cotransporter in the cell. Further evidence that JNK plays a role in the cell's response to volume changes is the finding that hypertonic activation of Na/H exchange in *Xenopus* oocytes involves activation of JNK [90]. In mammalian cells, hypertonicity has been shown to activate three MAP kinase pathways, JNK, ERK and p38 [91,92] causing changes in solute transport. Interestingly, SB203580, an inhibitor of p38 MAP kinase, and PD98059, an inhibitor of p42/p44 MAP kinase, have no effects on resting or arsenite-stimulated Na–K–2Cl cotransport in ferret red cells [80].

8. Regulation by intracellular Cl

Epithelial fluxes mediated by Na–K–2Cl cotransporters can be matched to those through channels by the concentrations of Na^+ and Cl^- inside the cell, and by their thermodynamic gradients across the membrane. Intracellular Cl^- was suggested to play a particularly important role in the regulation of cotransport in these tissues [1] and had been found to affect Cl^- transport in squid axons [4,93]. In epithelia, secretion may be initiated by the opening of apical Cl^- channels. Cell Cl^- concentration falls and directly enhances Cl^- entry on the Na–K–2Cl cotransporter as the inward Cl^- gradient across the basolateral membrane increases. However, changes in intracellular Cl^- concentration have additional effects on the transporter, some of which are mediated by changes in transporter phosphorylation. This is important as it means that cotransporter activity does not blindly follow Cl^- level, but is coordinated with other regulatory inputs.

Cotransporter stimulation by apically applied UTP is secondary to a fall in cell Cl^- concentration in dog tracheal epithelia [54,94]. UTP binds to a P_2-purinergic receptor to activate apical Cl^- channels and Cl^- leaves the epithelium. The fall in Cl^- activates basolateral Na–K–2Cl cotransporters mainly through an increase in their phosphorylation measured by ^{32}P incorporation into immunoprecipitated NKCC1. Cell Cl^- reduction alone (without UTP), achieved by incubating cells in media containing nystatin and low Cl^-, also causes parallel changes in cotransporter activation and phosphorylation. Similar observations have been made in shark rectal gland [55]. These glands secrete in response to secretogogues that raise cAMP levels (e.g. forskolin), and this secretion is accompanied by increased cotransporter activity and phosphorylation. An obvious explanation is that the cotransporter is phosphorylated by PKA, however, shark NKCC1 lacks a consensus site for phosphorylation by PKA [13].

Further investigation showed that the cotransporter was neither phosphorylated nor activated under conditions that prevented changes in cell Cl^- content in response to secretogogues, suggesting that intracellular Cl^- is the key intermediary and works mainly by affecting cotransporter phosphorylation.

Further evidence for the importance of intracellular Cl^- in regulating the cotransporter comes from studies on the expression of the K–Cl cotransporter (KCC1) in HEK-293 cells [95]. Overexpression of KCC1 leads to a depletion of cell Cl^- and this activates endogenous Na–K–2Cl cotransporters. A steep relationship between cell Cl^- concentration and Na–K–2Cl cotransporter rate was found over the physiological range.

In endothelial cells, although hypertonic shrinkage activates unidirectional (possibly exchange) fluxes through the cotransporter, net fluxes are not produced [31,96]. This has led to the suggestion that intracellular Cl^- (concentration rises on hypertonic shrinkage) slows the rate at which ion binding sites on the fully unloaded transporter gain access to the external medium, thus inhibiting net influx but still permitting exchange fluxes (measured with ^{86}Rb) [31,50].

The cotransporter in cells from the trabecular meshwork of the human eye is also very sensitive to changes in cell Cl^- concentration (stimulated by a fall in cell Cl^-) and volume [97]. These two stimuli appear to act independently of each other.

The mechanisms described above attempt to clamp cell Cl^- at a particular value, an appropriate response for a mature cell. However, during growth and development, there must be mechanisms that allow uptake of ions with less inhibition from intracellular Cl^-. Growth factors, for instance serum, stimulate the Na–K–2Cl cotransporter to increase cell ion content. However, this stimulation is not accompanied by changes in NKCC1 phosphorylation, nor by a fall of cell Cl^- content (but rather by a slight increase) [50]. It was suggested that serum might alter the Cl^- set point of the transporter so that it is less sensitive to inhibition by intracellular Cl^-. How this change in set point is brought about is not clear. One possibility is that cells contain a Cl^- sensing protein, the activity of which is altered by serum. This protein interacts with the cotransporter so that it is disinhibited from mediating a net inward movement of ions.

9. Interactions with the cytoskeleton

It has long been speculated that the actin-based cytoskeleton might be involved in cotransporter regulation [71]. An early observation was that a 230 kDa protein, goblin, was phosphorylated when cotransport was stimulated in turkey red cells by isoprenaline [98]. The time courses of activation of cotransport and phosphorylation were similar, and both responded in similar ways to pharmacological interventions. It later transpired that goblin is similar to the mammalian cytoskeletal protein ankyrin [71]. This clearly showed that phosphorylation of a cytoskeletal protein affects cotransporter function.

Studies on gut epithelial cells suggest that there are two main pathways that regulate cotransport in response to secretogogues which increase cAMP levels (e.g. forskolin). Their relative influence varies with cell type. One route involves direct effects of PKA and the other relies on a fall of cell Cl^- concentration that causes both phosphorylation of the cotransporter and a reorganization of the cytoskeleton [49,94,99,100]. Secretogogues that increase cell cAMP levels usually activate apical Cl^- channels in gut epithelia and also activate basolateral NKCC1 and cause remodelling of basolateral F-actin. Agents that stabilize F-actin (e.g. phalloidin and jasplakinolide) reduce cAMP-mediated stimulation of Na–K–2Cl cotransport [99–101]. On the other hand, cytochalasin D, which promotes disassembly of the cytoskeleton, activates the cotransporter whereas latrunculin A, which binds to G-actin preventing polymerisation and thus keeping actin in a monomeric form, has no effect on transport suggesting that short filaments of actin activate the cotransporter [101]. By comparing the responses of HT29 cell lines that respond to cAMP with a fall in cell Cl^- concentration with those in which Cl^- does not change, it was concluded that stabilization of the cytoskeleton prevents cotransport stimulation by the Cl^--dependent route, but not by the cAMP-dependent route [100]. However, in cells that respond to cAMP with a fall in Cl^- concentration, phalloidin did not prevent a concomitant increase in bumetanide binding. Such an increase may indicate either an increase in the number of cotransporter molecules in the membrane, or an increase in cotransporter phosphorylation [36]. This suggests that interactions with the actin cytoskeleton can override the influence of either increased transporter phosphorylation or cotransporter number.

In T84 cells, forskolin does not affect the number of cotransporters in the cell membrane, judged either by antibody or bumetanide binding, but it does substantially increase the surface expression of two proteins (130 and 160 kDa) which co-precipitate with the cotransporter [12]. Preloading cells with phalloidin reduces the expression of these proteins.

Phalloidin has little effect on cotransport stimulated in T84 cells by hypertonicity but does markedly inhibit cotransport stimulated by hypotonicity (fall of cell Cl^- concentration?) [49]. In both cases, increased basolateral cotransport is not accompanied by changes in apical Cl^- secretion indicating that apical and basolateral transporters can be regulated independently of each other. It also shows that cotransporter activation per se is not sufficient to activate secretion. A similar conclusion was reached in studies of cotransport stimulation by calyculin in shark rectal gland [55].

In vascular endothelial cells, inhibition of myosin light chain kinase (MLCK) with ML-7 prevents both phosphory-

lation of myosin light chains and activation of cotransport on cell shrinkage [10]. However, ML-7 has no effect on shrinkage-induced cotransporter phosphorylation. Again, interactions with the cytoskeleton override the effects of cotransporter phosphorylation. ML-7 also inhibits the stimulation of cotransport caused by shrinking Ehrlich ascites cells suggesting a role for MLCK in the regulation of NKCC1 during RVI [61]. Brief treatment of these cells with cytochalasin B produces membrane blebs that lack actin and myosin and in which the cotransporter is permanently and partially activated under isotonic conditions [62,102,103]. It is not possible to activate the transporter further by shrinking the blebs nor does ML-7 have any effect on transport [62].

Similar interactions with myosin have been observed in T84 cells [104]. In this case, inhibition of MLCK with ML-7, or inhibition of myosin ATPase with butanedione monoxime, prevents the stimulation of cotransport by cAMP, suggesting that contraction of actin-myosin is required for cotransporter activation under these conditions. Recently, it has been demonstrated that the contractile state of vascular smooth muscle affects cotransport [105]. Phenylephrine causes both contraction of the muscle and activation of cotransport. However, cotransport activation is prevented if the muscle is stretched by more than 10% and prevented from shortening. Nitrovasodilators inhibit cotransport and reduce NKCC1 phosphorylation [106].

10. Does oligomerization of the cotransporter play a role in transport regulation?

The Na–K–2Cl cotransporter and other members of the CCC family appear readily to form stable complexes with themselves and other proteins. These complexes may be important in regulating transport. Phosphorylation of elements of the cytoskeleton can affect cotransport activity independently of the cotransporter's own phosphorylation state, suggesting that protein–protein interactions are important in cotransport regulation. Expression of inactive Na–K–2Cl cotransporter mutants in translation systems often suppresses the activity of the endogenous NKCC [39], again suggesting protein–protein interactions, and the cotransporter forms complexes with one of its own regulatory enzymes, protein phosphatase 1 [48]. Studies using reversible chemical cross-linking of proteins indicate that NKCC1 probably exists as a homodimer in membranes from salivary glands and HEK293 cells [107]. These homodimers are stable in mild detergents like 0.3% Triton X-100 and 20 mM deoxycholate but are disrupted by SDS. Early studies on furosemide binding proteins (including CCC family members) isolated from Ehrlich ascites cells, analysed on non-reducing gels and following chemical cross-linking, also suggest that these proteins normally exist as homodimers [108].

High molecular weight complexes have been observed when proteins from ferret red cells membranes are separated by SDS-PAGE and on non-denaturing gels (blue-native-gel electrophoresis). They are also seen when proteins, immunoprecipitated from ferret red cell membranes with cotransporter antibody T4, are separated by SDS-PAGE [79]. These complexes, whether in membranes or immunoprecipitates, are very stable, surviving boiling in SDS and reducing agents. Their molecular weights, 340, 470 and 600 kDa are consistent with the cotransporter forming dimers, trimers and possibly tetramers, though it is possible that other proteins are involved.

Formation of complexes by members of the CCC family may alter transport properties. The newly discovered CIP1 does not transport ions itself but associates with NKCC1 to inhibit transport [20]. It does not inhibit transport by NKCC2 or KCC1. Carboxy-terminal-truncated splice variants of NKCC2 associate with full-length NKCC2 to inhibit transport [18], and this inhibition is reduced if the proteins are phosphorylated by PKA [109]. One of these truncated variants transports Na^+ and Cl^- but not K^+ under hypotonic conditions [26]. Thus, cAMP switches the transport properties of TAL cells: at low levels, the truncated variant is active, and cells reabsorb Na^+ and Cl^- independently of K^+, whereas at higher levels, NKCC2 becomes active, and K^+ is reabsorbed along with Na^+ and Cl^-.

K–Cl cotransporters also exist in membranes as multimers [110]. Truncation of the amino end of K–Cl cotransporters leads to loss of transport function, and these proteins inhibit transport in co-expressed wild-type KCCs. Further truncation of the carboxy-terminal of these mutants prevents the inhibitory effect. Truncation of the carboxy-terminal alone leads to loss of function but these proteins do not inhibit neighbouring KCCs.

11. Conclusions and perspectives

Na–K–2Cl cotransport is influenced by a wide range of factors. These probably influence activity through at least three main routes operating in parallel: cotransporter phosphorylation, protein–protein interactions and cell Cl^- concentration. It seems unlikely that there is any one final common pathway for control, and even with phosphorylation, it appears that the transporter can be phosphorylated in several different ways. Protein–protein interactions can override the effects of phosphorylation in several situations and cell Cl^- concentration may directly affect transport behaviour, possibly switching it between futile self-exchange and productive net transport. Important tasks for future studies are to establish how the patterns of both cotransporter phosphorylation and oligomerization affect transport. Finally, with the development of techniques such as mass spectrometry, it should be possible to assess how other post-translational changes to cotransporter structure affect function.

Acknowledgements

I should like to thank the Wellcome Trust for supporting work in my laboratory.

References

[1] H.H. Ussing, Volume regulation of frog skin epithelium, Acta Physiol. Scand. 114 (1982) 363–369.
[2] M. Haas, Properties and diversity of (Na–K–Cl) cotransporters, Annu. Rev. Physiol. 51 (1989) 443–457.
[3] M. Haas, B. Forbush III, The Na–K–Cl cotransporters, J. Bioenerg. Biomembranes 30 (1998) 161–172.
[4] J.M. Russell, Sodium–potassium–chloride cotransport, Physiol. Rev. 80 (2000) 211–276.
[5] M. Haas, B. Forbush III, The Na–K–Cl cotransporter of secretory epithelia, Annu. Rev. Physiol. 62 (2000) 515–534.
[6] H.C. Palfrey, P. Greengard, Hormone-sensitive ion transport systems in erythrocytes as models for epithelial ion pathways, Ann. N.Y. Acad. Sci. 372 (1981) 291–307.
[7] J.A. Payne, J.-C. Xu, M. Haas, C.Y. Lytle, D. Ward, B. Forbush III, Primary structure, functional expression, and chromosomal localization of the bumetanide-sensitive Na–K–Cl cotransporter in human colon, J. Biol. Chem. 270 (1995) 17977–17985.
[8] C. Lytle, J.-C. Xu, D. Biemesderfer, M. Haas, B. Forbush III, The Na–K–Cl cotransport protein of shark rectal gland: I. Development of monoclonal antibodies, immunoaffinity purification, and the partial biochemical characterization, J. Biol. Chem. 267 (1992) 25428–25437.
[9] J. Torchia, C. Lytle, D.J. Pon, B. Forbush III, A.K. Sen, The Na–K–Cl cotransporter of avian salt gland. Phosphorylation in response to cAMP-dependent and calcium-dependent secretogogues, J. Biol. Chem. 267 (1992) 25444–25450.
[10] J.D. Klein, W.C. O'Neill, Volume-sensitive myosin phosphorylation in vascular endothelial cells: correlation with Na–K–2Cl cotransport, Am. J. Physiol. 269 (1995) C1524–C1531.
[11] A. Tanimura, K. Kurihara, S.J. Reshkin, R.J. Turner, Involvement of direct phosphorylation in the regulation of the rat parotid Na^+–K^+–$2Cl^-$ cotransporter, J. Biol. Chem. 270 (1995) 25252–25258.
[12] L. D'Andrea, C. Lytle, J.B. Matthews, P. Hofman, B. Forbush III, J.L. Madara, Na:K:2Cl cotransporter (NKCC) of intestinal epithelial cells, J. Biol. Chem. 271 (1996) 28969–28976.
[13] J.-C. Xu, C. Lytle, T.T. Zhu, J.A. Payne, E. Benz Jr., B. Forbush III, Molecular cloning and functional expression of the bumetanide-sensitive Na–K–Cl cotransporter, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 2201–2205.
[14] J. Randall, T. Thorne, E. Delpire, Partial cloning abd characterization of Slc12a2: the gene encoding the secretory Na^+–K^+–$2Cl^-$ cotransporter, Am. J. Physiol. 273 (1997) C1267–C1277.
[15] J.A. Payne, B. Forbush III, 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 (1994) 4544–4548.
[16] C.A. Ecelbarger, J. Terris, J.R. Hoyer, S. Nielsen, J.B. Wade, M.A. Knepper, Localization and regulation of the rat renal Na^+–K^+–$2Cl^-$ cotransporter, BSC-1, Am. J. Physiol. 271 (1996) F619–F628.
[17] I. Giménez, P. Isenring, B. Forbush, Spatially distributed alternative splice variants of the renal Na–K–Cl cotransporter exhibit dramatically different affinities for the transported ions, J. Biol. Chem. 277 (2002) 8767–8770.
[18] D.B. Mount, A. Baekgaard, A.E. Hall, C. Plata, J. Xu, D.R. Beier, G. Gamba, S.C. Hebert, Isoforms of the Na–K–2Cl cotransporter in murine TAL I. Molecular characterization and intrarenal localization, Am. J. Physiol. 276 (1999) F347–F358.
[19] D.B. Mount, E. Delpire, G. Gamba, A.E. Hall, E. Poch, R.S. Hoover Jr., S.C. Hebert, The electroneutral cation–chloride cotransporters, J. Exp. Biol. 201 (1998) 2091–2102.
[20] L. Caron, F. Rousseau, E. Gagnon, P. Isenring, Cloning and functional characterization of a cation–Cl^- cotransporter-interacting protein, J. Biol. Chem. 275 (2000) 32027–32036.
[21] E. Delpire, D.B. Mount, Human and murine phenotypes associated with defects in cation–chloride cotransport, Annu. Rev. Physiol. 64 (2002) 803–843.
[22] P. Geck, C. Pietrzyk, B.-C. Burckhardt, B. Pfeiffer, E. Heinz, Electrically silent cotransport of Na^+, K^+ and Cl^- in Ehrlich cells, Biochim. Biophys. Acta 600 (1980) 432–447.
[23] M. Haas, W.F. Schmidt III, T.J. McManus, Catecholamine-stimulated ion transport in duck red cells. Gradient effects in electrically neutral [Na+K+2Cl] co-transport, J. Gen. Physiol. 80 (1982) 125–147.
[24] C. Lytle, T.J. McManus, M. Haas, A model of Na–K–2Cl cotransport based on ordered ion binding and glide symmetry, Am. J. Physiol. 274 (1998) C299–C309.
[25] J.M. Russell, Cation-coupled chloride influx in squid axon, J. Gen. Physiol. 81 (1983) 909–925.
[26] C. Plata, P. Meade, A. Hall, R.C. Welch, N. Vázquez, S.C. Hebert, G. Gamba, Alternatively spliced isoform of apical Na^+–K^+–Cl^- cotransporter gene encodes a furosemide-sensitive Na^+–Cl^- cotransporter, Am. J. Physiol., Renal Fluid Electrolyte Physiol. 280 (2001) F574–F582.
[27] T. Krarup, B.S. Jensen, E.K. Hoffmann, Occlusion of K^+ in the Na^+/K^+/$2Cl^-$ cotransporter of Ehrlich ascites tumor cells, Biochim. Biophys. Acta 1284 (1996) 97–108.
[28] P. Isenring, S.C. Jacoby, J.A. Payne, B. Forbush III, Comparison of Na–K–Cl cotransporters, J. Biol. Chem. 273 (1998) 11295–11301.
[29] J.C. Parker, In defense of cell volume? Am. J. Physiol. 265 (1993) C1191–C1200.
[30] F. Lang, G.L. Busch, M. Ritter, H. Völkl, S. Waldegger, E. Gulbins, D. Häussinger, Functional significance of cell volume regulatory mechanisms, Physiol. Rev. 78 (1998) 247–306.
[31] W.C. O'Neill, Physiological significance of volume-regulatory transporters, Am. J. Physiol. 276 (1999) C995–C1011.
[32] E.K. Hoffmann, J.W. Mills, Membrane events involved in volume regulation, Curr. Top. Membr. 48 (1999) 123–196.
[33] N.C. Adragna, C.M. Perkins, P.K. Lauf, Furosemide-sensitive Na^+–K^+ cotransport and cellular metabolism in human erythrocytes, Biochim. Biophys. Acta 812 (1985) 293–296.
[34] A.A. Altamirano, G.A. Breitwieser, J.M. Russell, Vanadate and fluoride effects on Na^+–K^+–Cl^- cotransport in squid giant axon, Am. J. Physiol. 254 (1988) C582–C586.
[35] P.W. Flatman, The effects of metabolism on Na^+–K^+–Cl^- co-transport in ferret red cells, J. Physiol. (Lond) 437 (1991) 495–510.
[36] C. Lytle, B. Forbush III, The Na–K–Cl cotransport protein of shark rectal gland: II. Regulation by direct phosphorylation, J. Biol. Chem. 267 (1992) 25438–25443.
[37] M.L. Moore-Hoon, R.J. Turner, Molecular and topological characterization of the rat parotid Na^+–K^+–$2Cl^-$ cotransporter, Biochim. Biophys. Acta 1373 (1998) 261–269.
[38] T. Gerelsaikhan, R.J. Turner, Transmembrane topology of the secretory Na^+–K^+–$2Cl^-$ cotransporter NKCC1 studied by in vitro translation, J. Biol. Chem. 275 (2000) 40471–40477.
[39] P. Isenring, B. Forbush III, Ion and bumetanide binding by the Na–K–Cl cotransporter, J. Biol. Chem. 272 (1997) 24556–24562.
[40] P. Isenring, S.C. Jacoby, J. Chang, B. Forbush III, Mutagenic mapping of the Na–K–Cl cotransporter for domains involved in ion transport and bumetanide binding, J. Gen. Physiol. 112 (1998) 549–558.
[41] P. Isenring, S.C. Jacoby, B. Forbush III, The role of transmembrane domain 2 in cation transport by the Na–K–Cl cotransporter, Proc. Natl. Acad. Sci. U. S. A. 95 (1998) 7179–7184.
[42] P. Isenring, B. Forbush, Ion transport and ligand binding by the Na–

K–Cl cotransporter, structure–function studies, Comp. Biochem. Physiol. 130A (2001) 487–497.
[43] M. Haas, T.J. McManus, Bumetanide inhibits (Na+K+2Cl) cotransport at a chloride site, Am. J. Physiol. 245 (1983) C235–C240.
[44] M.E. O'Donnell, A. Martinez, D. Sun, Endothelial Na–K–Cl cotransport regulation by tonicity and hormones: phosphorylation of cotransport protein, Am. J. Physiol. 269 (1995) C1513–C1523.
[45] C. Lytle, 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 (1997) 15069–15077.
[46] K. Kurihara, M.L. Moore-Hoon, M. Saitoh, R.J. Turner, Characterization of a phosphorylation event resulting in upregulation of the salivary Na^+–K^+–$2Cl^-$ cotransporter, Am. J. Physiol. 277 (1999) C1184–C1193.
[47] K. Kurihara, N. Nakanishi, M.L. Moore-Hoon, R.J. Turner, Phosphorylation of the salivary Na^+–K^+–$2Cl^-$ cotransporter, Am. J. Physiol., Cell Physiol. 282 (2002) C817–C823.
[48] R.B. Darman, A. Flemmer, B. Forbush, Modulation of ion transport by direct targeting of protein phosphatase type I to the Na–K–Cl cotransporter, J. Biol. Chem. 276 (2001) 34359–34362.
[49] J.B. Matthews, J.A. Smith, E.C. Mun, J.K. Sicklick, Osmotic regulation of intestinal epithelial Na^+–K^+–Cl^- cotransport: role of Cl^- and F-actin, Am. J. Physiol. 274 (1998) C697–C706.
[50] G. Jiang, J.D. Klein, W.C. O'Neill, Growth factors stimulate the Na–K–2Cl cotransporter NKCC1 through a novel Cl^--dependent mechanism, Am. J. Physiol., Cell Physiol. 281 (2001) C1948–C1953.
[51] M.C. Muzyamba, A.R. Cossins, J.S. Gibson, Regulation of Na^+–K^+–$2Cl^-$ cotransport in turkey red cells: the role of oxygen tension and protein phosphorylation, J. Physiol. (Lond) 517 (1999) 421–429.
[52] P.W. Flatman, Deoxygenation stimulates Na^+–K^+–$2Cl^-$ cotransport in ferret erythrocytes, J. Physiol. (Lond) 531 (2001) 122P–123P.
[53] P.W. Flatman, The effects of magnesium on potassium transport in ferret red cells, J. Physiol. (Lond) 397 (1988) 471–487.
[54] M. Haas, D. McBrayer, C. Lytle, $[Cl^-]_i$-dependent phosphorylation of the Na–K–Cl cotransport protein of dog tracheal epithelial cells, J. Biol. Chem. 270 (1995) 28955–28961.
[55] C. Lytle, B. Forbush III, Regulatory phosphorylation of the secretory Na–K–Cl cotransporter: modulation by cytoplasmic Cl, Am. J. Physiol. 270 (1996) C437–C448.
[56] E.B. Pewitt, R.S. Hegde, M. Haas, H.C. Palfrey, 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 (1990) 20747–20756.
[57] J.D. Klein, P.B. Perry, W.C. O'Neill, Regulation by cell volume of Na^+–K^+–$2Cl^-$ cotransport in vascular endothelial cells: role of protein phosphorylation, J. Membr. Biol. 132 (1993) 243–252.
[58] H.C. Palfrey, E.B. Pewitt, The ATP and Mg^{2+} dependence of Na^+–K^+–$2Cl^-$ cotransport reflects a requirement for protein phosphorylation: studies using calyculin A, Pflugers Arch. 425 (1993) 321–328.
[59] H. Mairbäurl, C. Herth, Na^+–K^+–$2Cl^-$ cotransport, Na^+/H^+ exchange, and cell volume in ferret erythrocytes, Am. J. Physiol. 271 (1996) C1603–C1611.
[60] C. Lytle, A volume-sensitive protein kinase regulates the Na–K–2Cl cotransporter in duck red blood cells, Am. J. Physiol. 274 (1998) C1002–C1010.
[61] T. Krarup, L.D. Jakobsen, B.S. Jensen, E.K. Hoffmann, Na^+–K^+–$2Cl^-$ cotransport in Ehrlich cells: regulation by protein phosphatases and kinases, Am. J. Physiol. 275 (1998) C239–C250.
[62] S.F. Pedersen, E.K. Hoffmann, J.W. Mills, The cytoskeleton and cell volume regulation, Comp. Biochem. Physiol. 130A (2001) 385–399.
[63] R. Greger, D. Heitzmann, M.J. Hug, E.K. Hoffmann, M. Bleich, The $Na^+2Cl^-K^+$ cotransporter in the rectal gland of *Squalus acanthias* is activated by cell shrinkage, Pflugers Arch. 438 (1999) 165–176.
[64] M.J. Rindler, J.A. McRoberts, M.H. Saier Jr., (Na^+,K^+)-cotransport in the Madin–Darby canine kidney cell line, J. Biol. Chem. 257 (1982) 2254–2259.
[65] G. Dagher, C. Brugnara, M. Canessa, Effect of metabolic depletion on the furosemide-sensitive Na and K fluxes in human red cells, J. Membr. Biol. 86 (1985) 145–155.
[66] S. Ueberschär, T. Bakker-Grunwald, Effects of ATP and cyclic AMP on the ($Na^+K^+2Cl^-$)-cotransport system in turkey erythrocytes, Biochim. Biophys. Acta 818 (1985) 260–266.
[67] A.C. Hall, J.C. Ellory, Measurement and stoichiometry of bumetanide-sensitive (2Na:1K:3Cl) cotransport in ferret red cells, J. Membr. Biol. 85 (1985) 205–213.
[68] T. Ikehara, H. Yamaguchi, K. Hosokawa, H. Miyamoto, Kinetic mechanism of ATP action in Na^+–K^+–Cl^- cotransport of HeLa cells determined by Rb^+ influx studies, Am. J. Physiol. 258 (1990) C599–C609.
[69] H.C. Palfrey, M.C. Rao, Na/K/Cl co-transport and its regulation, J. Exp. Biol. 106 (1983) 43–54.
[70] M.H. Saier Jr., D.A. Boyden, Mechanism, regulation and physiological significance of the loop diuretic-sensitive NaCl/KCl symport system in animal cells, Mol. Cell. Biochem. 59 (1984) 11–32.
[71] S.M. O'Grady, H.C. Palfrey, M. Field, Characteristics and functions of Na–K–Cl cotransport in epithelial tissues, Am. J. Physiol. 253 (1987) C177–C192.
[72] M. Haas, The Na–K–Cl cotransporters, Am. J. Physiol. 267 (1994) C869–C885.
[73] P.W. Flatman, P.L.R. Andrews, Cation and ATP content of ferret red cells, Comp. Biochem. Physiol. 74A (1983) 939–943.
[74] P.W. Flatman, Stoichiometry of net sodium and potassium fluxes mediated by the Na–K–Cl co-transport system in ferret red cells, Q. J. Exp. Physiol. 74 (1989) 939–941.
[75] P.W. Flatman, J. Creanor, Regulation of Na^+–K^+–$2Cl^-$ cotransport by protein phosphorylation in ferret erythrocytes, J. Physiol. (Lond) 517 (1999) 699–708.
[76] P.W. Flatman, The effects of calcium on potassium transport in ferret red cells, J. Physiol. (Lond) 386 (1987) 407–423.
[77] P.W. Flatman, V.L. Lew, Magnesium buffering in intact human red blood cells measured using the ionophore A23177, J. Physiol. (Lond) 305 (1980) 13–30.
[78] C. Lytle, J.-C. Xu, D. Biemesderfer, B. Forbush III, Distribution and diversity of Na–K–Cl cotransport proteins: a study with monoclonal antibodies, Am. J. Physiol. 269 (1995) C1496–C1505.
[79] I. Matskevich, D.K. Apps, P.W. Flatman, The Na–K–2Cl cotransporter forms high molecular weight complexes in ferret red blood cell membranes, Pflugers Arch. 443 (2002) S186.
[80] P.W. Flatman, J. Creanor, Stimulation of Na^+–K^+–$2Cl^-$ cotransport by arsenite in ferret erythrocytes, J. Physiol. (Lond) 519 (1999) 143–152.
[81] T. Akiyama, H. Ogawara, Use and specificity of genistein as inhibitor of protein-tyrosine kinases, Methods Enzymol. 201 (1991) 362–370.
[82] T. Tamaoki, Use and specificity of staurosporine, UCN-01, and calphostin C as protein kinase inhibitors, Methods Enzymol. 201 (1991) 340–347.
[83] J.H. Hanke, J.P. Gardner, R.L. Dow, P.S. Changelian, W.H. Brissette, E.J. Weringer, B.A. Pollok, P.A. Connelly, Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor, J. Biol. Chem. 271 (1996) 695–701.
[84] H. Fathallah, E. Coezy, R.-S. de Neef, M.-D. Hardy-Dessources, F. Giraud, Inhibition of deoxygenation-induced membrane protein dephosphorylation and cell dehydration by phorbol esters and okadaic acid in sickle cells, Blood 86 (1995) 1999–2007.
[85] A. Barbul, Y. Zipser, A. Nachles, R. Korenstein, Deoxygenation and elevation of intracellular magnesium induce tyrosine phosphorylation of band 3 in human erythrocytes, FEBS Lett. 455 (1999) 87–91.
[86] G. Chétrite, R. Cassoly, Affinity of hemoglobin for the cytoplasmic fragment of human erythrocyte membrane band 3, J. Mol. Biol. 185 (1985) 639–644.

[87] I. Messana, M. Orlando, L. Cassiano, L. Pennacchietti, C. Zuppi, M. Castagnola, B. Giardina, Human erythrocyte metabolism is modulated by O_2-linked transition of hemoglobin, FEBS Lett. 390 (1996) 25–28.
[88] S. Herzig, J. Neumann, Effects of serine/threonine protein phosphatases on ion channels in excitable membranes, Physiol. Rev. 80 (2000) 173–210.
[89] J.D. Klein, S.T. Lamitina, W.C. O'Neill, JNK is a volume-sensitive kinase that phosphorylates the Na–K–2Cl cotransporter in vitro, Am. J. Physiol. 277 (1999) C425–C431.
[90] G.G. Goss, L. Jiang, D.H. Vandorpe, D. Kieller, M.N. Chernova, M. Robertson, S.L. Alper, Role of JNK in hypertonic activation of Cl^--dependent Na^+/H^+ exchange in *Xenopus* oocytes, Am. J. Physiol., Cell Physiol. 281 (2001) C1978–C1990.
[91] D. Sheikh-Hamad, J. Di Mari, W.N. Suki, R. Safirstein, B.A. Watts III, D. Rouse, p38 Kinase activity is essential for osmotic induction of mRNAs for HSP70 and transporter for organic solute betaine in Madin–Darby canine kidney cells, J. Biol. Chem. 273 (1998) 1832–1837.
[92] F. Roger, P.-Y. Martin, M. Rousselot, H. Favre, E. Féraille, Cell shrinkage triggers the activation of mitogen-activated protein kinases by hypertonicity in the rat medullary thick ascending limb of the Henle's loop, J. Biol. Chem. 274 (1999) 34103–34110.
[93] J.M. Russell, ATP-dependent chloride influx into internally dialyzed squid giant axons, J. Membr. Biol. 28 (1976) 335–349.
[94] M. Haas, D.G. McBrayer, J.R. Yankaskas, Dual mechanisms for Na–K–Cl cotransport regulation in airway epithelial cells, Am. J. Physiol. 264 (1993) C189–C200.
[95] C.M. Gillen, B. Forbush III, Functional interaction of the K–Cl cotransporter (KCC1) with the Na–K–Cl cotransporter in HEK-293 cells, Am. J. Physiol. 276 (1999) C328–C336.
[96] W.C. O'Neill, J.D. Klein, Regulation of vascular endothelial cell volume by Na–K–2Cl cotransport, Am. J. Physiol. 262 (1992) C436–C444.
[97] L.K. Putney, C.R.T. Vibat, M.E. O'Donnell, Intracellular Cl regulates Na–K–Cl cotransport activity in human trabecular meshwork cells, Am. J. Physiol. 277 (1999) C373–C383.
[98] S.L. Alper, K.G. Beam, P. Greengard, Hormonal control of Na^+–K^+ co-transport in turkey erythrocytes, J. Biol. Chem. 255 (1980) 4864–4871.
[99] J.B. Matthews, C.S. Awtrey, J.L. Madara, Microfilament-dependent activation of $Na^+/K^+/2Cl^-$ cotransport by cAMP in intestinal epithelial monolayers, J. Clin. Invest. 90 (1992) 1608–1613.
[100] J.B. Matthews, J.A. Smith, K.J. Tally, C.S. Awtrey, H. Nguyen, J. Rich, J.L. Madara, Na–K–2Cl cotransport in intestinal epithelial cells, J. Biol. Chem. 269 (1994) 15703–15709.
[101] J.B. Matthews, J.A. Smith, B.J. Hrnjez, Effects of F-actin stabilization or disassembly on epithelial Cl^- secretion and Na–K–2Cl cotransport, Am. J. Physiol. 272 (1997) C254–C262.
[102] F. Jessen, E.K. Hoffmann, Activation of the $Na^+/K^+/Cl^-$ cotransport system by reorganization of the actin filaments in Ehrlich ascites tumor cells, Biochim. Biophys. Acta 1110 (1992) 199–201.
[103] E.K. Hoffmann, F. Jessen, P.B. Dunham, The Na–K–2Cl cotransporter is in a permanently activated state in cytoplasts from Ehrlich ascites tumor cells, J. Membr. Biol. 138 (1994) 229–239.
[104] G. Hecht, A. Koutsouris, Myosin regulation of NKCC1: effects on cAMP-mediated Cl^- secretion in intestinal epithelia, Am. J. Physiol. 277 (1999) C441–C447.
[105] F. Akar, G. Jiang, R.J. Paul, W.C. O'Neill, Contractile regulation of the Na^+–K^+–$2Cl^-$ cotransporter in vascular smooth muscle, Am. J. Physiol., Cell Physiol. 281 (2001) C579–C584.
[106] F. Akar, E. Skinner, J.D. Klein, M. Jena, R.J. Paul, W.C. O'Neill, Vasoconstrictors and nitrovasodilators reciprocally regulate the Na^+–K^+–$2Cl^-$ cotransporter in rat aorta, Am. J. Physiol. 276 (1999) C1383–C1390.
[107] M.L. Moore-Hoon, R.J. Turner, The structural unit of the secretory Na^+–K^+–$2Cl^-$ cotransporter (NKCC1) is a homodimer, Biochemistry 39 (2000) 3718–3724.
[108] F. Jessen, B.D. Cherksey, T. Zeuthen, E.K. Hoffmann, Isolation and reconstitution of furosemide-binding proteins from Ehrlich ascites tumor cells, J. Membr. Biol. 108 (1989) 139–151.
[109] C. Plata, D.B. Mount, V. Rubio, S.C. Hebert, G. Gamba, Isoforms of the Na–K–2Cl cotransporter in murine TAL: II. Functional characterization and activation by cAMP, Am. J. Physiol. 276 (1999) F359–F366.
[110] S. Casula, B.E. Shmukler, S. Wilhelm, A.K. Stuart-Tilley, W. Su, M.N. Chernova, C. Brugnara, S.L. Alper, A dominant negative mutant of the KCC1 K–Cl cotransporter, J. Biol. Chem. 276 (2001) 41870–41878.