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Regulation of erythrocyte Na–K–2Cl cotransport by threonine phosphorylation

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Abstract

A method is described to measure threonine phosphorylation of the Na–K–2Cl cotransporter in ferret erythrocytes using readily available antibodies. We show that most, if not all, cotransporter in these cells is NKCC1, and this was immunoprecipitated with T4. Cotransport rate, measured as ^{86}Rb influx, correlates well with threonine phosphorylation of T4-immunoprecipitated protein. The cotransporter effects large fluxes and is significantly phosphorylated in cells under control conditions. Transport and phosphorylation increase 2.5- to 3-fold when cells are treated with calyculin A or Na^+ arsenite. Both fall to 60% control when cell [Mg^{2+}] is reduced below micromolar or when cells are treated with the kinase inhibitors, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine or staurosporine. Importantly, these latter interventions do not abolish either phosphorylation or transport suggesting that a phosphorylated form of the cotransporter is responsible for residual fluxes. Our experiments suggest protein phosphatase 1 (PrP-1) is extremely active in these cells and dephosphorylates key regulatory threonine residues on the cotransporter. Examination of the effects of kinase inhibition after cells have been treated with high concentrations of calyculin indicates that residual PrP-1 activity is capable of rapidly dephosphorylating the cotransporter. Experiments on cotransporter precipitation with microcystin sepharose suggest that PrP-1 binds to a phosphorylated form of the cotransporter.

Keywords: Sodium-potassium-chloride symporters; Erythrocytes; Threonine phosphorylation; Protein phosphatase 1; Calyculin A; Arsenite; PP1

1. Introduction

Na–K–2Cl cotransporters play key roles in regulating cell K^+ and Cl^- content and cell volume. In epithelia they enable transepithelial fluxes by providing entry routes for Na^+ and Cl^-, whereas in erythrocytes they are mainly involved in regulating cell, and perhaps, plasma K^+ concentration [1–5]. Their activity is regulated by a variety of stimuli including cell volume, hypoxia, hormones, growth factors and cell Cl^- concentration [1,2]. The ability of the transporter to respond to these signals is prevented by treating cells with inhibitors of protein kinases or phosphatases suggesting that changes in protein phosphorylation are involved in the signal transduction pathway [6–8]. It is now clear that phosphorylation of the cotransporter itself is a major determinant of transport rate [9–14]. Initial studies using ^{32}P indicated that 5 or 6 threonine and serine (but not tyrosine) residues are phosphorylated when the transporter is maximally activated [9,15]. More recent work has focused on the phosphorylation of three threonine residues (^{184}Thr, ^{189}Thr and ^{202}Thr) in the N-terminus of the shark cotransporter that are particularly important in regulating transport in response to changes in cell volume and Cl^- [14]. These three residues are well conserved in the cotransporter from a wide variety of animal species so their phosphorylation is likely to be a common theme in cotransporter regulation, with phosphorylation of ^{189}Thr (or its equivalent) being the main determinant of activity, and phosphorylation of ^{184}Thr and ^{202}Thr modulating the response [14].

Studies using inhibitors of protein phosphatase 1 (PrP-1) suggest that this plays a major role in dephosphorylating and thus inactivating the cotransporter, and the recent demon-

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stration that PrP-1 can bind to a motif on the cotransporter close to the key threonine residues in the N-terminus reinforces this idea [16]. However, the identity of the kinase, or kinases, that phosphorylate the cotransporter remains elusive [17–19].

Using ferret erythrocytes, a robust model for studying the properties of the mammalian cotransporter, we have explored the effects of inhibitors of kinases and phosphatases on transport [20,21]. Although the results indicate that transport is stimulated by phosphorylation, they are not consistent with the current view that cotransporter phosphorylation is regulated by a single kinase and phosphatase [15]. It seems more likely that the cotransporter is phosphorylated by several kinases and dephosphorylated by several phosphatases [3,5,22]. Transport is stimulated by calyculin A suggesting protein phosphatase 1 is one of the regulatory phosphatases [3,20]. It is also stimulated by Na^+ arsenite which is believed to activate a kinase [21]. However, treatment of cells in a variety of ways to inhibit protein kinases, including reduction of cell Mg^{2+} concentration to below 1 μM, inhibits only about 40% of the resting flux leaving a substantial residual flux [20]. This suggests that the cotransporter may exist in two forms, one that is constitutively active (it does not require to be phosphorylated) and one that is activated by phosphorylation in the conventional way, or, these procedures may trap the cotransporter in a partially phosphorylated, partially active state [3]. In order to resolve these issues and understand how cotransporter phosphorylation affects transport in ferret erythrocytes we describe a method that allows us to compare the extent to which the cotransporter is phosphorylated on threonine with transport rate under a variety of conditions. Some of these results have been published in abstract form [23,24].

2. Materials and methods

2.1. Solutions and chemicals

All solutions were prepared in double-glass distilled water with analytical grade reagents (BDH AnalaR, VWR International, Lutterworth, UK). Calyculin A, microcystin-LR and 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP1) were obtained from Alexis Biochemicals (Carlsbad, CA). Protease inhibitor cocktail set III, A23187 and the detergents: ASB-16 (an amidosulfobetaine) and CHAPS were obtained from Calbiochem (VWR International). EDTA, EGTA, HEPES, dithiothreitol (DTT), Triton X-100, Tween 20, Na^+ orthovanadate, Na^+ β-glycerophosphate, glycine, genistein, staurosporine and Tris base were obtained from Sigma (Poole, UK). Na^+ (meta)-arsenite was from Fluka Chemicals, (Gillingham, UK). Protein G sepharose (Amersham Biosciences, Little Chalfont, UK) was washed 3 times before use. Microcystin-Sepharose was from Upstate (Charlottesville, VA), recombinant N-glycosidase F from Roche Diagnostics (Lewes, UK), and anti-NKCC1 from Alpha Diagnostics International (San Antonio, TX). Bumetanide was a gift from Leo Laboratories Ltd. (Aylesbury, UK).

Ferret basic medium (FBM) comprised (mM): 150 NaCl, 5 KCl, 0.05 EGTA, 10 HEPES, pH 7.5 at 38 °C, adjusted with NaOH. Lysis buffer contained (mM): 10 HEPES, 2 EDTA, 2 Na^+ pyrophosphate, 2 NaF, 1 Na^+ β-glycerophosphate, 1 Na^+ vanadate, pH 7.6 at room temperature. Na^+ vanadate was added from a 200 mM stock just prior to use. The stock was prepared by dissolving Na^+ vanadate in water, adjusting pH to 10 with HCl and then boiling until colourless and then readjusting the pH to 10 [25]. It was frozen until used. Immunoprecipitation buffer (IPB) contained (mM): 150 NaCl, 20 HEPES, 1 EDTA, 2 NaF, 1 Na^+ β-glycerophosphate, pH adjusted to 7.6 at room temperature with NaOH. Tris-buffered saline (TBS) contained (mM): 136 NaCl, 20 Tris base, pH 7.6 adjusted with HCl at room temperature.

2.2. Preparation of erythrocyte suspensions and flux measurements

Blood was taken by cardiac puncture from adult ferrets terminally anaesthetised with Na^+ pentobarbitone (Sagatal, Rhône Mérieux, Harlow, UK, 120 mg kg^{-1}) according to guidance from the British Home Office. Cells were washed four times by centrifugation and resuspension in FBM, care being taken to remove all of the buffy coat. They were stored at 5 °C in FBM containing 22 mM glucose until used (normally within 3 days). All plastics used to handle cells were sterile and all solutions were passed through a 0.2-μm filter before use.

Washed erythrocytes at about 7% hematocrit were incubated at 38 °C for the indicated times in FBM containing 11 mM glucose and the following additions: control (no additions, 20 min), 20–50 nM calyculin A (15 min), 50 μM PP1 (30 min), 2 μM staurosporine (20 min), 0.3 mM genistein (20 min), 1 mM Na^+ arsenite (60 min), 0.008–0.03% gluteraldehyde (5 min), and various combinations of these. Where necessary cell [Mg^{2+}] was reduced to sub micromolar levels by incubating the cells in FBM containing 2 mM EDTA and 10 μM A23187 for 15 min [26].

Cotransporter fluxes were determined by adding ^{86}Rb to aliquots of these suspensions and then measuring the uptake of ^{86}Rb, with and without 10 μM bumetanide, a specific cotransporter inhibitor, as described previously [20].

2.3. Preparation of erythrocyte membranes

Cells were spun out of solution, cooled on ice and then injected into 20 volumes of ice-cold lysis buffer containing 1% protease inhibitor cocktail, and stirred for 10 min on ice. Membranes were washed 3 times by centrifugation (25,000×*g* for 10 min) and resuspension in ice-cold lysis

buffer. Membrane protein concentrations were measured with Coomassie Plus protein assay reagent (Pierce Biotechnology, Rockford, IL) or 2-D Quant (Amersham Biosciences). Samples were stored in liquid nitrogen after adding 2% protease inhibitor cocktail.

Membranes were deglycosylated by incubating them with 1% SDS at 60 °C for 20 min. Samples were then diluted 5-fold into deglycosylation buffer (in mM: 150 NaCl, 20 HEPES, 1 EDTA) containing 1% Triton X-100, 1% CHAPS and 2% protease inhibitor cocktail. Recombinant N-glycosidase F (80–160 units/ml) was added and the tubes were incubated at 37 °C overnight. Inclusion of 2% mercaptoethanol had no effect on results. Membranes were dephosphorylated by treating them with alkaline phosphatase (Promega, Madison, WI) for 1 h at 37 °C.

2.4. Immunoprecipitation of the cotransporter

Cotransporter was immunoprecipitated with T4 (Developmental Studies Hybridoma Bank, University of Iowa), a monoclonal antibody to the C-terminal of the cotransporter [27]. This antibody recognises both NKCC1 and NKCC2 isoforms of the cotransporter in the SDS-denatured state. Membranes were therefore heated to 60 °C in the presence of 1% SDS for 20 min and were then diluted 5-fold with IPB with the following additions: 1 mM Na^+ vanadate, 2% protease inhibitor cocktail, 1 μM microcystin L-R, 1% Triton X-100 and 0.5% ASB-16. The mixture was incubated for 1 h at 4 °C followed by centrifugation at 14,000×*g* for 10 min to remove any precipitate. The supernatant was pre-cleared with protein G sepharose, T4 ascites fluid was added and the tube was incubated at 4 °C for 60 min. The immune complex was precipitated with protein G sepharose and washed 3 times with IPB containing 1% Triton X-100 and 0.5% ASB-16. Cotransporter was eluted by heating the beads to 70 °C for 20 min in ×4 NuPAGE LDS sample buffer (Invitrogen Corporation, Carlsbad, CA) containing 166 mM DTT. The supernatant, diluted 4-fold and with [DTT] made up to 100 mM, was subjected to SDS-PAGE.

2.5. Microcystin pull down assay

Membranes, solubilised in IPB containing 1 mM Na^+ vanadate, 0.5% ASB-16, 1% Triton X-100 and 2% protease inhibitors, were incubated with Microcystin-Sepharose for 1 h at 4 °C. The beads were washed 3 times with IPB containing 0.5% ASB 16 and 0.1% Triton X-100. Proteins were eluted from the beads as described above.

2.6. Gel electrophoresis and Western blotting

We used either 3–8% Tris–acetate or 4–12% bis-tris NuPAGE gels (Invitrogen). Gels were calibrated against Mark 12 and HiMark unstained molecular weight markers (Invitrogen) and a biotinylated protein ladder (Cell Signaling). Membranes and protein samples were heated to 70 °C for 10 min in LDS sample buffer (Invitrogen) containing 100 mM DTT then and centrifuged at 14,000×*g* for 1 min prior to electrophoresis. Protein bands were stained with GelCode Blue, a colloidal Coomassie stain (Pierce).

Proteins were blotted to nitrocellulose in an Xcell II run at 30V for 90 min using a transfer buffer (in mM: 25 bicine, 25 bis-tris, 1 EDTA) containing 0.1% SDS and 10% methanol (Invitrogen). Blots were blocked with TBS containing 0.1% Tween 20 and either 5% dried milk powder, or 5% filtered (0.2 μm) bovine serum albumin (fraction V, ICN Biomedical, Costa Mesa, CA) when phospho-specific antibodies were used. They were then incubated at 4 °C with primary antibodies diluted in blocking buffer, T4 1:5000 for 1 h or rabbit anti-phosphothreonine (Cell Signaling Technology, Beverly MA) 1:2000 overnight. After washing and re-blocking, the blots were treated with horseradish peroxidase-conjugated secondary antibodies (1:10,000, Jackson ImmunoResearch Laboratories, West Grove, PA). Antibody binding was quantified using ECL reagents and Hyperfilm (Amersham Biosciences). Gels and blots were scanned and the images analysed with TotalLab (Non Linear Dynamics, Newcastle, UK).

Most data are expressed as means with standard errors and the significance of differences between means is assessed with two-tailed *t*-tests, the exact value of *P* being stated. Rate constants are given with standard deviations. Lines through data are fitted to specific models using non-linear regression analysis (Prism 4.03, GraphPad Software, San Diego, CA). All experiments were repeated with the blood from at least 3 ferrets. Representative blots are shown.

3. Results

3.1. Measurement of threonine phosphorylation of the cotransporter

Our first aim was to establish a simple robust assay to measure threonine phosphorylation of the cotransporter in ferret erythrocytes using readily available antibodies. Cotransporter was immunoprecipitated with the monoclonal antibody T4 [27]. The immunoprecipitate was then separated into components by SDS-PAGE. The proteins were blotted to nitrocellulose, care being taken to ensure that sufficient time was given for the complete transfer of large proteins, and the presence of phosphorylated threonine residues in the protein bands was detected with an anti-phosphothreonine antibody (Fig. 1). A prominent band covering molecular weights ranging from 140 to 160 kDa was observed in all samples, precisely the place where we would expect to find the cotransporter if its monomeric glycosylated molecular weight is about 150 kDa (see later). The relative intensity of this band depended on how the cells had been treated prior to the preparation of membranes. Treatments that activated cotransport, for instance, incuba-

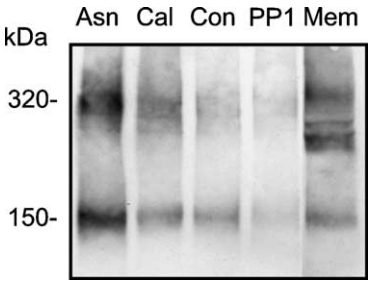

Fig. 1. Threonine phosphorylation of T4 immunoprecipitates. T4 immunoprecipitates were prepared from the membranes of ferret erythrocytes that had been incubated at 38 °C in FBM containing 11 mM glucose and the following additions: none for 30 min (Con), 50 μM PP1 for 30 min (PP1), 20 nM Calyculin A for 15 min (Cal) and 1 mM Na^+ arsenite for 60 min (Asn). Immunoprecipitates were run on a 3–8% Tris-acetate gel together with a sample of control membranes (Mem). The gel was blotted and probed with an antibody to phosphothreonine.

tion with calyculin A or Na^+ arsenite, increased the intensity compared to controls, whereas treatment with PP1, decreased the intensity (Fig. 1). This is consistent with the idea that calyculin or arsenite activate the cotransporter by causing its phosphorylation, whereas PP1 inhibits by reducing its phosphorylation. Usually a second band, covering the range 300–320 kDa (Fig. 1), was also seen in each lane. In some cases, the intensity of this band was similar to the low molecular weight band, though in most it was much lower, and it appeared to depend to some extent on how membrane samples were treated prior to electrophoresis (see later). Some faint bands at intermediate weights were also sometimes visible. Shown on the same blot (Fig. 1) is an example of a membrane sample, separated by SDS-PAGE, and probed with the anti-phosphothreonine antibody. Prominent bands were usually observed at 150, 250, 270 and 300 kDa.

In order to interpret these phosphorylation patterns, we need to know the identity of the proteins immunoprecipitated by T4 and the molecular weights of cotransporter forms found in the membrane or sample buffer.

3.2. Molecular weight of cotransporter complexes

In Western blots of ferret erythrocyte membranes the cotransporter is detected by a variety of cotransporter antibodies as a smear at 140–160 kDa together with high molecular weight complexes, comprising a prominent smear at about 300 kDa and often bands at 400–500 kDa (Fig. 2A). In addition, much sharper bands were seen at about 250 and 270 kDa (Fig. 2A). A similar pattern of bands is seen when T4-immunoprecipitates prepared from erythrocytes are run on SDS gels (Fig. 2B). There are prominent broad bands centred at 150 and 300 kDa, but the bands at 250 and 270 kDa are less conspicuous.

All high molecular weight species were seen despite heating the samples with SDS and reducing agents such as 100 mM DTT or 5 mM tris[2-carboxyethyl]phosphine (TCEP) prior to running gels. The proportion of T4 staining found at high molecular weights increased slowly with the length of time cells were stored (4 °C) before the preparation of membranes, and with the time that membranes were stored frozen (−20 °C) before electrophoresis (data not shown). A possible explanation for the bands at 300 and 500 kDa is that the cotransporter forms homodimers and trimers but we cannot rule out the possibility that it binds to other proteins, and the bands at 250 and 270 kDa may provide evidence for just such interactions. Importantly, SDS-resistant high molecular weight forms of the cotransporter assemble during storage, even in the cold. Variations were minimised by preparing membranes from cells within 3 days of blood collection, and by storing membranes in liquid nitrogen. Despite these precautions, bands at 250, 270 and 300 kDa could be detected even in the freshest samples, suggesting that these forms may normally be present in the membrane under physiological conditions. However, because some of these complexes form during storage or in sample buffer, it is difficult to assess the extent to which the cotransporter exists in these forms in the membrane. To address this issue, we treated cells with low concentrations of gluteraldehyde (0.008–0.03%) to cross-link proteins prior to preparation of membranes. The procedure, which has been used to fix erythrocyte ion transporters in their active or inactive states [28], greatly increased the proportion of transporter found in the 300-kDa form (Fig. 2C), supporting the idea that many cotransporter molecules in erythrocyte membranes are found in dimers, or bound to other proteins.

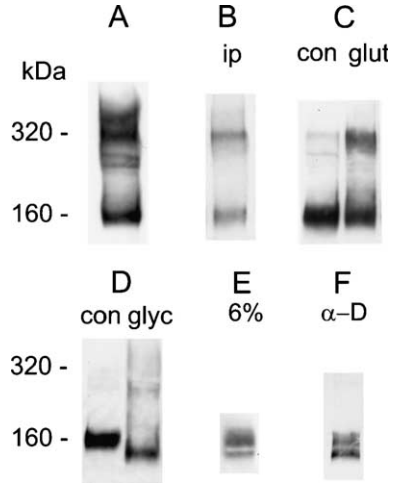

Fig. 2. Cotransporter bands in blots of erythrocyte membranes and T4-immunoprecipitates. Samples were run on: 3–8% Tris-acetate gels (A–D), 6% (E) or 9% (F) tris-glycine gels and probed with T4 (A–E) or an NKCC1-specific antibody from Alpha Diagnostics International (F). A: control membranes showing all the major cotransporter bands seen commonly, B: T4-immunoprecipitate prepared from control membranes. C: con—control membranes, glut—membranes from cells treated with 0.008% gluteraldehyde for 5 min, D: con—control membranes, glyc—membranes treated overnight at 37 °C with N-glycosidase F, E and F: cotransporter seen as clear doublets in control membranes.

The appearance of the cotransporter as a broad, smeared band has been reported for other cell types and species. This smearing is usually attributed to variable N-linked glycosylation of sites in the large extracellular loop of the cotransporter [27,29]. We examined this possibility by treating ferret erythrocyte membranes overnight with recombinant N-glycosidase F. Following treatment the apparent mean molecular weight of the transporter fell by about 25 kDa to 124±1 (n=4) kDa, close to the expected weight of the cotransporter based on its sequence. Unexpectedly, the band remained very smeared (Fig. 2D). Doubling the amount of glycanase did not cause any further change in molecular weight and nor did dephosphorylating the cotransporter with alkaline phosphatase (data not shown). Thus, variable glycosylation or phosphorylation does not explain the wide range of molecular weights the transporter displays. Densitometer scans of the 150-kDa band often showed two peaks suggesting that the band may comprise two components, an idea supported by the finding that it occasionally appeared as a distinct doublet with weights centred on 140 and 160 kDa (e.g. Fig. 2E and F). Doublets have also been observed in T4-immunoprecipitates from endothelial cells [30] and membranes from rat parotid gland [31]. In the latter case, the 140-kDa band was attributed to an unphosphorylated, immature form found in intracellular compartments. Such compartments do not exist in ferret erythrocytes, and the 140-kDa form resides in the plasma membrane like the 160-kDa form. In addition, dephosphorylation (with alkaline phosphatase) made no difference to the apparent molecular weight of the transporter in our studies (data not shown).

T4 interacts with both the NKCC1 and NKCC2 isoforms of the cotransporter [27] which have slightly different molecular weights [2], so a plausible explanation for the doublet at 140–160 kDa is that ferret erythrocytes express both isoforms. Membranes were therefore probed with a variety of antibodies that are specific for NKCC1. The results were similar to those with T4, and importantly, the Alpha Diagnostics antibody (developed to a peptide in the C-terminus) also revealed a doublet at 140–160 kDa (Fig. 2F). Antibodies specific for NKCC2 failed to identify proteins at molecular weights ranging from 120 to 160 kDa, the expected monomeric weight of this transporter (though occasionally there was staining at 240 kDa, data not shown). We also attempted to identify the proteins immunoprecipitated with T4 by MALDI-TOF mass spectroscopy following their digestion with trypsin. We were unable to identify the proteins in the prominent bands even at high yields. This is not an unusual problem with very hydrophobic membrane proteins especially if they have been treated with SDS [32,33] which is necessary when using T4. This technical problem is very disappointing as we had hoped to identify the components of the high molecular weight complexes by mass spectroscopy and thus establish whether the cotransporter dimerizes or binds to other proteins. Thus, although we cannot categorically rule out the possibility that some NKCC2 is expressed in these cells, we can conclude that the vast majority of bands detected by T4 contain NKCC1.

3.3. Relationship between threonine phosphorylation of the cotransporter and transport rate

Resting K^+ fluxes through the cotransporter are high, about 15 mmol l cell^{-1} h^{-1} when ferret erythrocytes are incubated under control conditions, and this is reduced to about 0.5 mmol l cell^{-1} h^{-1} in the presence of 10 μM bumetanide, indicating that over 95% of resting K^+ uptake is via the cotransporter [34]. Fig. 5 shows that fluxes are stimulated 2.6-fold by treating cells with calyculin A or with Na^+ arsenite, are reduced by about 40% when protein kinases are inhibited with PP1, staurosporine, genistein or by reducing cell Mg^{2+} content below 1 μM [20,21]. Figs. 1, 4 and 5 show that these changes in transport rate are paralleled by changes in phosphothreonine levels detected in T4-immunoprecipitates prepared from erythrocyte membranes.

It was necessary to establish a robust protocol to quantify cotransporter phosphorylation. We have seen that cotransporter molecules can be distributed amongst a variety of forms with different molecular weights and that heating samples with SDS and reducing agents do not cause these complexes to break up. It is thus important to include all relevant forms if cotransporter phosphorylation is to be measured accurately. We therefore analysed how normalised total phosphothreonine (all that found in a lane above 140 kDa normalised to the value found in the same region with control cells) correlated with the normalised amount of phosphothreonine found in the bands centred at 150 or 300 kDa under a variety of conditions (Fig. 3). The same relative changes in phosphorylation were found no matter which method of analysis was used. We therefore usually compared phosphorylation in the 150-kDa bands as these were the most prominent. Adding in phosphorylation from faint, ill-defined bands often simply increased the error on the measurements without affecting the ratios. It is also clear from these results that threonine phosphorylation of the cotransporter does not affect the propensity of the transporter to form or separate from the 300-kDa complexes.

Phosphothreonine was clearly detected in T4-immunoprecipitates prepared from all control membranes (e.g. Figs. 1, 4 and 6), consistent with the large resting fluxes found under control conditions. Figs. 1 and 5 show that calyculin increased cotransporter threonine phosphorylation 2.4-fold (±0.3, n=6 ferrets), consistent with PrP-1 being one of the cotransporter's regulatory phosphatases, and Na^+ arsenite increased it 3.1-fold (±0.7, n=5 ferrets). Kinase inhibition by drugs or Mg^{2+}-removal reduced it by about 40%, in line with the changes in transport rate (Figs. 4 and 5).

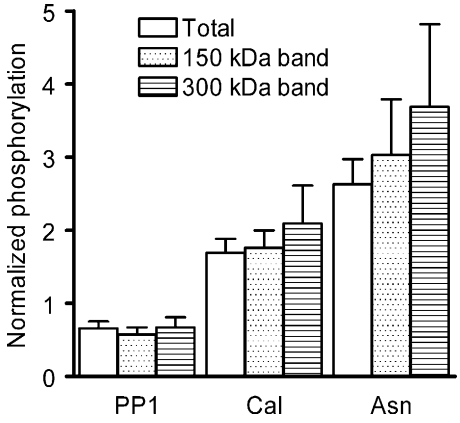

Fig. 3. Similar changes in threonine phosphorylation are seen in both the low and high molecular weight forms of the cotransporter. T4-immunoprecipitates were prepared from the membranes of erythrocytes that had been incubated at 38 °C in FBM containing 11 mM glucose and the following additions: 50 μM PP1 (PP1) for 30 min, 20 nM calyculin A (Cal) for 15 min, or 1 mM Na^+ arsenite (Asn) for 60 min. Control cells were incubated for 30 min in FBM with 11 mM glucose. Immunoprecipitates were run on 3–8% Tris-acetate gels, blotted and probed with an antibody to phosphothreonine. Signal intensity in the lanes at molecular weights above 140 kDa (Total), or in the bands centred on 150 kDa and 300 kDa were measured and expressed as a ratio of the level seen in the same regions on control lanes. Bars represent the mean ratios with their standard errors ($n=5$).

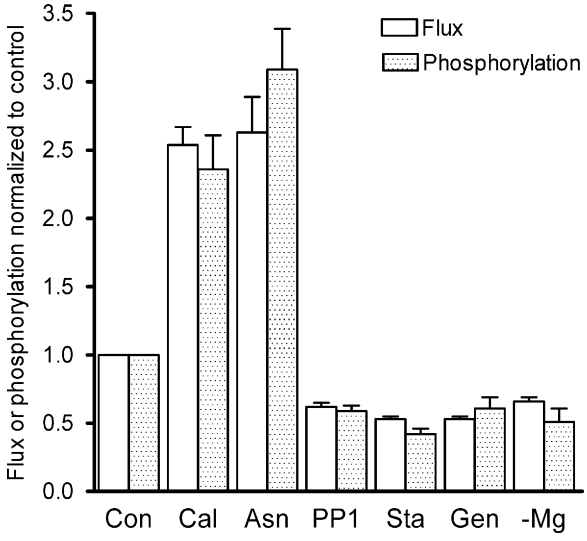

Fig. 5. Comparison of cotransporter threonine phosphorylation with fluxes. Threonine phosphorylation of the cotransporter was detected in T4-immunoprecipitates prepared from ferret erythrocytes that had been incubated at 38 °C in FBM containing 11 mM glucose and the following additions: none for 30 min (Con), 20–50 nM calyculin for 15 min (Cal), 1 mM Na^+ arsenite for 60 min (Asn), 50 μM PP1 for 30 min (PP1), 2 μM staurosporine for 20 min (Sta), 0.3 mM genistein for 20 min (Gen) or 2 mM EDTA and 10 μM A23187 for 15 min (−Mg). Cotransporter rate, measured as ^{86}Rb influx rate constant, was determined under the same conditions. Both phosphorylation and fluxes are normalized to the values seen in controls. Values are given as means with their standard errors.

Importantly, cotransporter threonine phosphorylation was not abolished by any of these procedures.

Stimulation of transport by calyculin A is prevented when cells are pre-treated with PP1, staurosporine, genistein or are Mg^{2+}-depleted [20]. The simplest explanation is that these treatments prevent phosphorylation of the cotransporter itself, though we could not rule out the possibility that they prevented the phosphorylation of a cofactor that is required for full activation of the transporter by protein–protein interactions. Experiments using T4-immunoprecipitates indicate these treatments inhibit transport by preventing phosphorylation of the transporter itself. Non-specific inhibition of kinases by Mg^{2+}-depletion completely prevented any rise in phosphorylation or stimulation of transport when calyculin was added (Fig. 6).

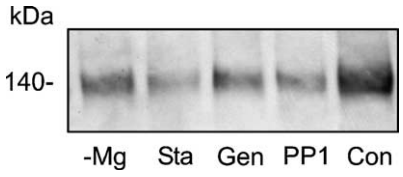

Fig. 4. Kinase inhibition reduces but does not abolish threonine phosphorylation of the cotransporter. T4-immunoprecipitates were prepared from the membranes of ferret erythrocytes that had been incubated at 38 °C in FBM containing 11 mM glucose and the following additions: none (Con), 50 μM PP1 for 30 min (PP1), 0.3 mM genistein for 20 min (Gen), 2 μM staurosporine for 20 min (Sta) or with 2 mM EDTA and 10 μM A23187 for 15 min (−Mg). Immunoprecipitates were run on 3–8% Tris-acetate gels, blotted and probed with an antibody to phosphothreonine.

Inhibition of Src kinases with PP1 also greatly reduced the stimulation of transport and the increase in phosphorylation, though did not completely prevent them (Fig. 6). In both cases, changes in transport parallel changes in phosphorylation.

3.4. Inhibition of transport and phosphorylation by PP1 or Mg^{2+}-removal after stimulation by calyculin

We have previously shown [20], and confirm here (Fig. 6) that Mg^{2+}-removal, or addition of PP1, after cells have been treated with calyculin partially inhibits transport over a 30-min period. Fig. 6 also shows that this reduction in transport is accompanied by a large reduction in threonine phosphorylation. Given the concentrations of calyculin used, this inhibition of transport and phosphorylation was surprising. The simplest explanation is that residual PrP-1 activity is sufficient to dephosphorylate the transporter when kinases are inhibited. If this were true both transport and phosphorylation should eventually fall back to the control level, at a rate that is a function of calyculin concentration, the higher the concentration, the less residual PrP-1 activity and the slower the rate. Fig. 7 shows that cotransport falls back to control at the same rate when PP1 or Mg^{2+}-removal is used to inhibit kinases (Fig. 7A). It also shows (Fig. 7B) that the rate of fall decreases with increasing calyculin concentration. Kinetic experiments on threonine phosphorylation are much harder to carry out,

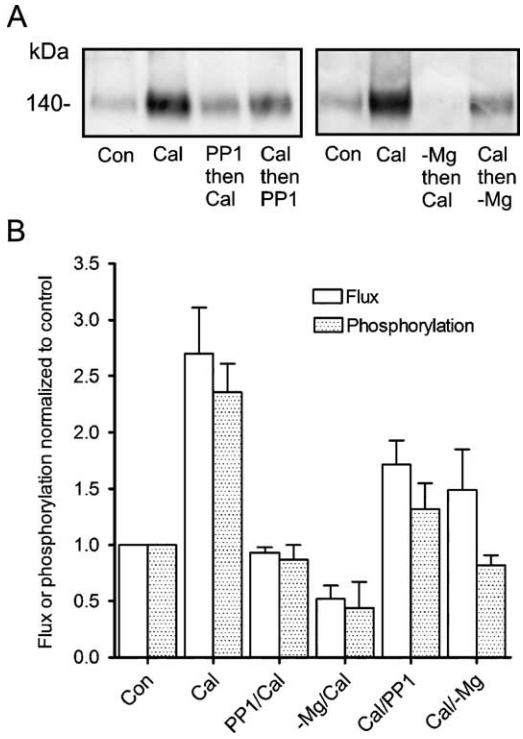

Fig. 6. PP1 or Mg^{2+}-depletion prevents and reverses cotransporter phosphorylation and fluxes stimulated by calyculin. A: T4-immunoprecipitates were prepared from the membranes of ferret erythrocytes that had been incubated at 38 °C in FBM containing 11 mM glucose and the following additions: none (Con), 50 nM calyculin for 15 min (Cal), 50 μM PP1 for 30 min followed by 50 nM calyculin for 15 min (PP1 then Cal), 50 nM calyculin for 15 min followed by 50 μM PP1 for 30 min (Cal then PP1), 2 mM EDTA with 10 μM A23187 for 15 min followed by 50 nM calyculin for 15 min (−Mg then Cal), 50 nM calyculin for 15 min followed by 2 mM EDTA with 10 μM A23187 (Cal then −Mg). Immunoprecipitates were run on 3–8% Tris-acetate gels, blotted and probed with an antibody to phosphothreonine. B: ^{86}Rb uptake was determined under the same conditions. Both phosphorylation and fluxes are normalized to the values seen in controls. Values are given as means with their standard errors.

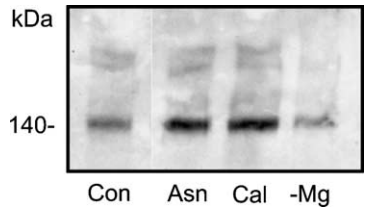

Fig. 8. Microcystin pulls down the cotransporter. Microcystin-Sepharose was used to precipitate proteins from the membranes of ferret erythrocytes that had been incubated at 38 °C in FBM containing 11 mM glucose and the following additions: none (Con), 1 mM Na^+ arsenite for 60 min (Asn), 50 nM calyculin A for 15 min (Cal), or with 2 mM EDTA and 10 μM A23187 for 15 min (−Mg). Eluates were run on 3–8% Tris-acetate gels, blotted and probed with T4.

however, phosphorylation had fallen below control levels in all samples taken 30 min after Mg^{2+} is removed (Fig. 6) and in one third of samples treated with PP1. In the other two thirds, it was about 50% above control, but very much lower that in the presence of calyculin alone.

3.5. Microcystin pull-down is more efficient with phosphorylated cotransporter

In view of the finding that PrP-1 may be one of the cotransporter's regulatory phosphatases we examined whether PrP-1 binds strongly to the cotransporter in ferret erythrocytes, as it does in other species [16]. If this were so, it should be possible to pull down the cotransporter with microcystin, a molecule that binds tightly to PrP-1. This was found to be the case (Fig. 8). Microcystin conjugated to Sepharose pulled down the cotransporter in both its low and high molecular weight forms. Interestingly, the amount of cotransporter pulled down in these assays closely parallels the phosphorylation state of the cotransporter. Thus, pre-treatment of cells with calyculin or arsenite increased the

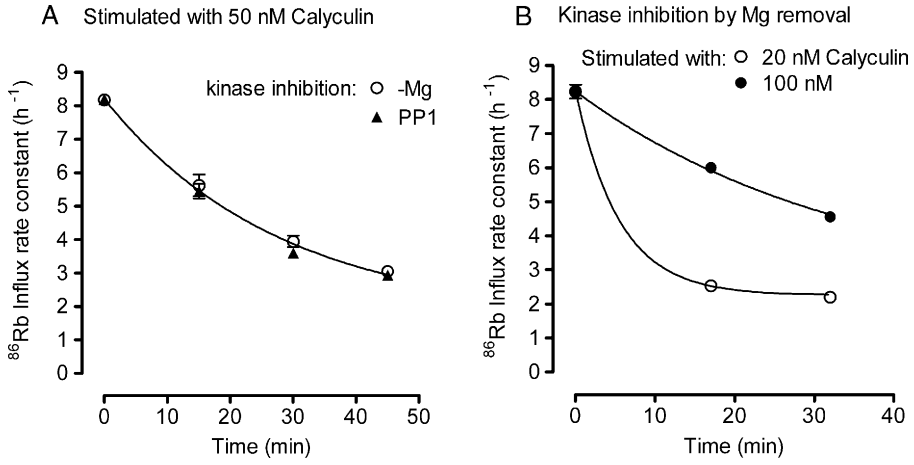

Fig. 7. Cotransport rate returns to normal when kinases are inhibited after calyculin treatment. Cells were incubated at 38 °C in FBM containing 11 mM glucose and the indicated calyculin concentration for 10 min. At this point, either 50 μM PP1 (PP1) or 10 μM A23187 with 2 mM EDTA (−Mg) were added. Samples of the suspension were transferred to separate tubes to which ^{86}Rb was added to measure fluxes over 4 min. Influx rate constants were determined and plotted at the mid point of the flux period timed from the addition of PP1 or A23187. Rate constants are shown with standard deviations if these are larger than point size. Lines are best fits to data, assuming that rate constants fall exponentially with time to the control level. A: comparison of the effects of PP1 or Mg^{2+}-removal on fluxes stimulated with 50 nM calyculin. B: comparison of the effects of Mg^{2+}-removal on fluxes stimulated with 20 or 100 nM calyculin.

amount of cotransporter (1.61±0.1 (n=2) and 2.66±0.84 (n=5) fold, respectively), whereas treatment of cells with PP1, Mg^{2+}-depletion, or genistein all reduced the amount of cotransporter pulled down (to 0.69±0.12 control, n=5).

4. Discussion

We describe a simple assay to measure threonine phosphorylation of the Na–K–2Cl cotransporter in ferret erythrocytes that has permitted us to address a number of ongoing questions about cotransport regulation. The assay is based on the immunoprecipitation of the cotransporter with T4 followed by measurement of threonine phosphorylation with an antibody. Although we cannot entirely rule out the possibility that some NKCC2 is expressed in ferret erythrocytes (though this would be highly unusual), we show that the material detected by T4 in these cells is consistent with it being, or containing, NKCC1. In order to make appropriate measurements of phosphorylation, it was necessary to understand the forms the cotransporter can adopt when isolated from cell membranes.

Ferret erythrocyte Na–K–2Cl cotransporter runs on SDS gels as a prominent broad band at 140–160 kDa. Often an additional broad band is seen at 300–320 kDa together with some sharp bands at intermediate weights. Some very high molecular weight complexes are occasionally seen (Fig. 2). Perhaps, up to half of the cotransporter molecules in the membrane are in high molecular weight complexes as indicated by the cross-linking study with gluteraldehyde (Fig. 2C), and these can survive and also form during sample preparation. The most likely explanation for the 300–320 kDa band is the formation of a dimer, as previously proposed on the basis of cross-linking studies in rat parotid glands [35], though binding to different proteins to form a 300 kDa complex cannot be excluded. All members of the cation-chloride-cotransporter (CCC) family (Na–K–2Cl, K–Cl, Na–Cl cotransporters, and CIP, a regulatory protein), are able to form complexes with themselves and with other members of the family, and the formation of these complexes can affect transport [36–40]. The cotransporter has also been shown to interact with unrelated proteins, for instance the anion-exchanger [41]. It would also appear that NKCC1 is one of the major threonine phosphorylated proteins (>100 kDa) in ferret erythrocyte membranes (Fig. 1).

Figs. 5 and 6 show there is a strong correlation between transport rate and cotransporter threonine phosphorylation in ferret erythrocytes. Calyculin and arsenite, compounds that maximally stimulate cotransport (2.6-fold, means not significantly different, P=0.72), also cause a large increase in threonine phosphorylation, 2.4-fold in the case of calyculin and 3.1-fold for arsenite. Although phosphorylation was almost always higher when cells from a particular ferret were treated with arsenite, this difference was not significant (P=0.09). On the other hand, inhibition of protein kinases with PP1, staurosporine, genistein or by removing intracellular Mg^{2+} reduced both transport and threonine phosphorylation to about 60% control. The correlation between transport and phosphorylation over this wide range of conditions, which includes the minimum and maximum rates of transport in these cells, is striking. However, we must be cautious if attempting to infer phosphorylation levels from transport rates. As discussed, phosphorylation may be higher than predicted when arsenite is used to stimulate transport, but most importantly, we have been unable to detect a significant rise in threonine phosphorylation when transport is maximally stimulated by deoxygenation [42]. We have yet to find a situation where the cotransporter is phosphorylated more than in resting cells without transport being stimulated in ferret erythrocytes.

Our results show that the cotransporter is partially threonine phosphorylated in ferret erythrocytes under resting conditions, and this may account for the high fluxes observed under these conditions. Importantly, we show that the transporter remains partially phosphorylated when cells are treated with kinase inhibitors or when their Mg^{2+} content is drastically reduced. This contradicts the hypothesis that some cotransporter molecules are active when not phosphorylated. It seems more likely that kinase inhibition leaves some cotransporter molecules phosphorylated and active. This may be because the sites are not accessible to phosphatases or that the appropriate phosphatases are not active. Experiments are under way to explore these possibilities.

The data in Fig. 7 show that PrP-1 is highly active in ferret erythrocytes and support the view that it dephosphorylates key threonine residues involved in regulating transport rate. They make less plausible the supposition that calyculin also activates a kinase that phosphorylates the transporter, an alternative explanation for the inhibition of transport by kinase inhibitors in the presence of calyculin. Thus, in the absence of kinase activity, residual PrP-1 can still rapidly dephosphorylate the transporter leading to a reduction in transport rate. With 20 nM calyculin, residual PrP-1 dephosphorylates the cotransporter so transport falls to within 5% of control in 16 min, with 100 nM calyculin this takes 100 min. They are also consistent with an EC_{50} for calyculin of 1–3 nM, values estimated from the amount of calyculin required to activate transport ([20] and Flatman, unpublished). The tight binding of PrP-1 to the cotransporter was exploited to precipitate the cotransporter from membranes with microcystin-sepharose. Unexpectedly, we found that the amount of cotransporter pulled down with microcystin parallels the level of cotransporter threonine phosphorylation. It appears that phosphorylated cotransporter binds PrP-1 more avidly.

In conclusion, the method described for assessing total threonine phosphorylation of the cotransporter provides important information on transporter function that complements that obtained using phospho-cotransporter specific

antibodies which examine the phosphorylation of one out of several threonine residues that can affect transport [31]. The combination of these methods would be particularly useful where it is not possible to make an antibody to a particular phosphorylated residue, as is the case with the cotransporter.

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