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Occlusion of K^+ in the $Na^+/K^+/2Cl^-$ cotransporter of Ehrlich ascites tumor cells

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Abstract

Proteins of n-octyl glucoside solubilized membrane vesicles derived from Ehrlich ascites tumor cells can occlude $^{86}Rb^+$. K^+ displaces $^{86}Rb^+$ and it is assumed that $^{86}Rb^+$ can be used as a tracer to measure K^+ occlusion. The following observations indicate that the $Na^+/K^+/2Cl^-$ cotransporter is responsible for this occlusion: (1) Na^+ does not compete for the K^+ binding site, but rather stimulates $^{86}Rb^+$ occlusion. (2) K^+ occlusion saturates with increasing $[Na^+]$ and $[K^+]$, the respective $K_{0.5}$ values being 50 ± 7 μM for Na^+ and 371 ± 63 μM for K^+. (3) Preincubation with 1 mM ouabain does not inhibit $^{86}Rb^+$ occlusion, arguing against the Na^+/K^+-ATPase as being responsible for the occlusion. This notion is supported by the $K_{0.5}$ value for K^+ being higher than reported for Na^+/K^+-ATPase and by the stimulatory effect of Na^+. (4) The K^+ occlusion is sensitive to $[Cl^-]$, and the occluded ion is protected by the presence of bumetanide during cation exchange chromatography. Our results suggest that occlusion measurements of substrate ions could be a profitable way to study the ion binding mechanism(s) of the $Na^+/K^+/2Cl^-$ cotransporter.

Keywords: Occlusion; Membrane vesicle; n-Octyl glucoside; Bumetanide; Cytochalasin B; $Na^+/K^+/2Cl^-$ cotransporter

1. Introduction

Since the existence of a coupled, electrically silent quaternary symport of Na^+, K^+ and two Cl^-, $Na^+/K^+/2Cl^-$ cotransport, was demonstrated across the membrane of Ehrlich ascites tumor cells [1], considerable research has been devoted to purification of this system and characterization of its structure and biological function (for reviews, see Refs. [2–5]). The $Na^+/K^+/2Cl^-$ cotransporter, which has a characteristic sensitivity to loop diuretics of the 5-sulfamoylbenzoic acid class such as bumetanide, has been identified in a variety of cells and tissues [2–4], and several isoforms have been cloned and sequenced [6–10]. In reabsorbtive and secretory epithelia, the cotransporter participates in transcellular transport of ions, followed by an osmotically obliged flux of water [11–15]. In nonepithelial single cells such as the Ehrlich cell [1,16–19] and the human erythrocyte [20], the activity of the cotransporter promotes cell volume maintenance.

The $Na^+/K^+/2Cl^-$ cotransporter has binding sites for one Na^+, one K^+ and two Cl^- ions, presumably alternating between the two faces of the membrane [2,4]. At the extracellular face, the ions bind in the order $Na^+ \rightarrow Cl^- \rightarrow K^+ \rightarrow Cl^-$ [3,4,21], and are transported with a 'first on, first of' mode of binding and release, known as glide symmetry ([22], see [4]). The two Cl^- binding sites appear to have different characteristics [23] in that the first and second binding sites have high and low Cl^- affinities, respectively (see [2]). In the process of transport, the four ions bound on one side of the membrane are translocated across the membrane followed by a release on the other side. We propose that during translocation, the cotransporter goes through a state in which the ions are trapped within the transmembrane domains of the protein, and are inaccessible from either side of the membrane. In this state, the ions are referred to as *occluded*. The term was coined by Post et al. [24] to describe a conformation of the Na^+/K^+-ATPase in which one Rb^+ ion was bound. The authors suggested that the cation first was released following a conformational change of the ATPase [24]. Direct evidence that Rb^+ could be occluded in the Na^+/K^+-ATPase was subsequently provided by the demonstration

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that the enzyme was capable of retaining labelled Rb^+ [25].

Studies on occlusion [25] have greatly enhanced understanding of the mechanism of cation transport in the Na^+/K^+-ATPase and the Ca^{2+}-ATPase from sarcoplasmic reticular membranes. Examples include: (i) a 19 kDa membrane spanning fragment of the α-chain of pig kidney Na^+/K^+-ATPase has been shown to contain a cation binding site [26]. This transmembrane fragment is essential for occlusion, and is separate from the ATP binding site on the enzyme [26–28]; (ii) the effect of covalent modifications of critical carboxyl residues by carbodiimides on Rb^+ and Na^+ occlusion has provided evidence that carboxyls are involved in the cation binding domain of Na^+/K^+-ATPase from pig kidney [29]. Using the same compounds, the study was extended to include the 19 kDa peptide of the α-chain of the Na^+/K^+-ATPase [30], mentioned above. A carboxyl group located in the K^+ (or Na^+) binding site of the 19 kDa fragment was identified, and it was suggested that different membrane spanning segments were donators of ligating groups to the cation occlusion cage [30]; (iii) site-directed mutagenesis followed by occlusion measurements has been carried out to identify amino acids involved in cation binding [31]. In rabbit muscle Ca^{2+}-ATPase, point mutations of selected amino acids abolished Ca^{2+} transport and Ca^{2+}-dependent phosphorylation [32]. From these results it was possible to pinpoint the residues involved in the Ca^{2+} binding site [32]; (iv) calculation of deocclusion rates of $^{86}Rb^+$ from an occluded state of the dog kidney Na^+/K^+-ATPase using a rapid filtration device with a time resolution of 10 ms has provided valuable information on the phosphorylation–dephosphorylation steps of the Na^+/K^+-ATPase transport cycle, the rate-limiting steps and the sensitivity of the Na^+/K^+-ATPase to ATP, ADP, Mg^{2+} and pH [33,34]; (v) detergents are also commonly applied in occlusion studies [35–37]. Occlusion of Na^+ and Rb^+ in solubilized monomeric $\alpha\beta$-units of the Na^+/K^+-ATPase provided evidence that Na^+ and Rb^+ are occluded within the structure of these units [37], suggesting that this monomeric $\alpha\beta$-unit defines the minimum protein unit required for active Na^+/K^+ transport [37]. For reviews of occluded cations in active transport, see Refs. [38,39].

Our laboratory has for more than a decade attempted to understand cellular volume control in Ehrlich cells (reviewed in [4,40]). Particular emphasis has been devoted to study of the structure, function and regulation of the $Na^+/K^+/2Cl^-$ cotransporter [18,19,41–48]. Information at the molecular level has not been available until the recent cloning and sequencing of the cDNA's encoding the $Na^+/K^+/2Cl^-$ cotransporter of the shark rectal gland [6], the rat kidney [7], the mouse kidney [8], the rabbit kidney [9], and the human colon [10]. Members of the family of $Na^+/K^+/2Cl^-$ cotransporters have 12 putative transmembrane helices and a variable number of PKC and PKA consensus phosphorylation sites ([10], see [3]). Details about the transport site (e.g. which residues are participating in ion binding) and the mechanisms underlying ion translocation still remain to be explored.

This paper addresses the question if ion occlusion occurs in the $Na^+/K^+/2Cl^-$ cotransporter of Ehrlich cells. Our goal was to develop an occlusion assay amenable to reveal details of the nature of the ion binding site(s) of this protein and of the ion translocation process, analogous to the ingenious studies on cation occlusion conducted on the Na^+/K^+-ATPase. Occlusion of ions in the $Na^+/K^+/2Cl^-$ cotransporter was previously suggested by Forbush and Haas [49]. In a study of the characteristics of [^{3}H]benzmetanide (a loop diuretic) binding to shark rectal gland membranes, it was discovered that the dissociation rate of [^{3}H]benzmetanide from the $Na^+/K^+/2Cl^-$ cotransporter was dependent upon the ions present when the inhibitor was bound, and, to a much lesser extent, upon the ions present in the dissociation medium. These results suggested that the substrate ions were occluded in the cotransporter [49]. No efforts to estimate binding affinities were made.

A preliminary report of our work was presented in abstract form [50].

2. Materials and methods

2.1. Cells and incubation media

Ehrlich ascites tumor cells (hyperdiploid strain) were maintained in female NMRI mice by weekly intraperitoneal transplantation, as described before [18,41,51]. In most experiments the tumor cells were harvested into a standard medium with the following composition (mM): Na^+, 150; K^+, 5; Mg^{2+}, 1; Ca^{2+}, 1; Cl^-, 150; SO_4^{2-}, 1; HPO_4^{2-}, 1; 3-(*N*-morpholino)propanesulfonic acid (Mops), 3.3; *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (Tes), 3.3; and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (Hepes), 5; 300 mosmol/kg H_20, pH 7.4; with heparin (2.5 IU/ml). The cells were washed [18,41], and incubated at 9% cytocrit for 15 min. The temperature of this and other incubations was, unless otherwise noted, 37°C. In experiments designed to examine the effect of Cl^- on K^+ occlusion, cells were harvested in a medium containing the same components as the standard medium, but with NO_3^- substituted for Cl^- in equimolar amounts (referred to as Cl^--free standard medium).

2.2. Preparation of membrane vesicles

Membrane vesicles were isolated from intact Ehrlich cells following a modified version [41] of a previously described method [52]. The isolation was at room temperature. Briefly, the cells were resuspended at 9% cytocrit in the standard medium/Cl^--free standard medium without

Ca^{2+}. Cytochalasin B, final concentration 42 μM, was added to induce formation of 'blebs' in the plasma membrane. The blebs, at this point continuous with the membrane, were sheared from the cells by mild homogenization in a Dounce homogenizer equipped with a loose fitting clearance pestle, resulting in the formation of large membrane vesicles (4 μm in diameter) [41], containing no cytoplasmic membranous structures [52]. The vesicles were isolated from the cell fraction by differential centrifugation [41] and subsequently washed twice in the standard medium/Cl^--free standard medium containing 1% bovine serum albumine (BSA). BSA binds cytochalasin B, thereby promoting removal of the drug from the vesicle preparation.

2.3. Solubilization

To remove contaminating ions prior to solubilization, the membrane vesicles (approx. 20 mg wet wt/ml) were lysed in an ice-cold hypotonic washing buffer, homogenized in a Dounce homogenizator equipped with a tight-fitting pestle, and subsequently centrifuged 100 000 × g for 30 min, 4°C. Depending on the type of experiment, one of three washing buffers were used. The three washing buffers all contained (mM): Hepes, 5; and phenylmethylsulfonyl fluoride (PMSF), 0.1; pH 7.8 (adjusted with Tris base). Washing buffer 1: for experiments on the effect of [K^+] or bumetanide on K^+ ($^{86}Rb^+$) occlusion, the washing buffer also contained (mM): Na_2EDTA, 0.1; and KCl, 0.05. Washing buffer 2: for experiments testing the effect of [Na^+] on K^+ occlusion, the washing buffer also contained (mM): H_2EDTA, 0.1; sodium gluconate, 0.01; and KCl, 0.05. Washing buffer 3: in experiments on the effect of [Cl^-] on K^+ occlusion, the washing buffer also contained (mM): Na_2EDTA, 0.1; potassium gluconate, 0.04; and KCl, 0.01.

The washed membrane vesicles were resuspended to 100 mg wet weight/ml in an ice-cold solubilization medium containing 1 mM ouabain to inhibit Na^+/K^+-ATPase and (mM): Hepes, 10; ATP, 1; Mg^{2+}, 1; PMSF, 0.1; pH 7.8 (Tris-base). One volume of the same medium including 4% n-octyl glucoside was added dropwise to a final concentration of 2% n-octyl glucoside (corresponding to 68.5 mM, i.e., about 2.7-times above the critical micellar concentration of the detergent, and 50 mg membrane vesicles wet weight/ml). n-Octyl glucoside was chosen because it is uncharged, convenient since the same detergent is present during the cation exchange chromatography of the occlusion assay. Further, n-octyl glucoside does not bind divalent cations, nor does it interfere with protein determination assays [53]. The membrane vesicles were solubilized by magnetic stirring for 90 min at 4°C.

2.4. K^+ ($^{86}Rb^+$) occlusion measurements

Upon solubilization, the mixture of n-octyl glucoside and more or less dissolved membrane vesicles was centrifuged to pellet insoluble material. Samples of 50 μl of the supernatant containing soluble proteins from the membrane vesicles, were removed for protein determination. The remainder of the supernatant was divided into 150-μl aliquots in 1.5 ml vials, which were stored on ice. K^+ occlusion was assayed using $^{86}Rb^+$ as tracer, and a modified version of the protocol of Shani et al. [54]: 150 μl supernatant (containing soluble proteins from the membrane vesicles) were warmed to room temperature in a waterbath. At zero time, 15 μl of experimental medium were added to the solubilized membrane vesicles. The experimental medium contained the same as the solubilization medium, i.e., (mM): Hepes, 10; ouabain, 1; ATP, 1; Mg^{2+}, 1; PMSF, 0.1; n-octyl glucoside, 2%; pH 7.8, and, in addition, concentrations of Na^+, K^+ and Cl^- as indicated for each experiment. The ionic strength was maintained constant by substituting $NMDG^+$ for Na^+ or K^+, and gluconate for Cl^-, where appropriate. The samples were incubated in an end-over-end mixer with slow rotation. At 0.5 min, 15 μl experimental medium with $^{86}RbCl$ (430 kBq/ml) were added. At 5.5 min, bumetanide was added to 100 μM because it increases the amount of occluded $^{86}Rb^+$ measured (see Fig. 2). At 6.5 min, the sample was cooled by centrifugation (15 000 × g, 1.5 min, 2°C), and 140 μl of the mixture was applied to a chilled cation exchange column (matrix: Dowex 50 W × 8, Tris-form) to separate free from bound isotope, as described before [18,42,54]. The sample was eluted from the column with 1.5 ml ice-cold eluating buffer containing (mM): sucrose, 250; Mops, 10; bumetanide (where appropriate), 0.1; n-octyl glucoside, 2%; and BSA, 1%; pH 7.8, and collected in counting vials. To determine specific activity of the isotope, the radioacitivities of the media were also measured. Prior to the experiments, binding of soluble proteins to the column matrix was prevented by equilibrating the Dowex resin with the BSA-containing eluating buffer, and by eluating a sample of the solubilized membrane vesicles through the column. Radioactivities of eluates and media were measured in a liquid scintillation spectrometer (Packard TriCarb 460 C Liquid Scintillation System), using 10 ml Ultima Gold™ (Packard) per counting vial as scintillation fluid. Net occlusion was determined from the difference between the amount of radioactivity emerging from the column after incubation in the experimental media and nonspecific binding, defined as the radioactivity measured in the presence of 5 mM Na^+, 10 mM K^+ and 5 mM Cl^-. It is assumed that 10 mM K^+, a 27-fold higher K^+ concentration than the $K_{0.5}$ value estimated for occlusion (this paper), has competitively displaced all the $^{86}Rb^+$ on the K^+ binding site of the $Na^+/K^+/2Cl^-$ cotransporters. The occlusion is presented as μmol K^+ (or $^{86}Rb^+$) occluded per g protein.

2.5. Protein determinations

Protein content of the solubilized membrane vesicle solutions was determined as previously described [41]. The

method is a version of the modified Lowry procedure of Peterson [55].

2.6. Data analysis

Curve fits were computed using the Marquardt–Levenberg algorithm (nonlinear least-squares iterative procedures) and SigmaPlot™, Jandel, Corte Madera, CA, USA (Figs. 3 and 4; see legends for details). Statistical significance was evaluated using a paired *t*-test (Figs. 1 and 2). Error bars show the S.E.M. values.

2.7. Materials

$^{86}Rb^+$ (as RbCl) was from Risø, Denmark. Bumetanide (stock solution: 10 mM in 96% ethanol) was a gift from Leo Pharmaceuticals, Denmark. Dowex 50 W × 8 was from Fluka AG, Switzerland. BSA and cytochalasin B (stock solution: 20.85 mM in 96% ethanol) were from Sigma, MO, USA. 1-*O*-n-Octyl β-D-glucopyranoside (n-octyl glucoside) was from Boehringer Mannheim, Germany. All other reagents were of analytic grade.

3. Results

3.1. Effect of Mg^{2+} and ATP on K^+ occlusion

Mg^{2+} and ATP have roles as regulators of the $Na^+/K^+/2Cl^-$ cotransporter. In ferret erythrocytes, increasing $[Mg^{2+}]_i$ stimulates cotransport [56]. In erythrocytes of ducks, 1.5 mM $[Mg^{2+}]_o$ increases the number of functioning cotransporters as measured by bumetanide binding [23]. In avian erythrocytes, the cotransporter is inhibited by depletion of either $[Mg^{2+}]_i$ or $[ATP]_i$ [57]. In squid giant axon, $[ATP]_i$ stimulates $Na^+/K^+/2Cl^-$ cotransport [58], although there is evidence that the nucleotide hydrolysis of ATP is not necessary [1]. To maximize K^+ ($^{86}Rb^+$) occlusion we included 1 mM of Mg^{2+} and ATP in the experimental media. Fig. 1 shows the effect on the occlusion of K^+ if either Mg^{2+} or ATP was omitted. The control value (with Mg^{2+} and ATP, open column), was set to 1. The nominal absence of Mg^{2+} (striped column) decreased K^+ occlusion to 0.82 ± 0.05 (not significant). However, the absence of ATP (cross-striped column) had a pronounced effect, decreasing K^+ occlusion significantly to 0.44 ± 0.07 ($P = 0.009$, $n = 3$).

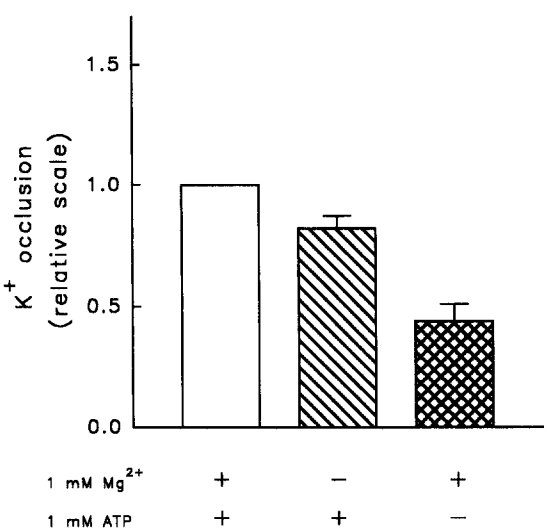

Fig. 1. Effect of Mg^{2+} and ATP on K^+ occlusion, measured as indicated in Section 2. Controls (open column): soluble proteins from the membrane vesicles were incubated with K^+ plus $^{86}Rb^+$, 0.35 mM, 5 mM NaCl, and 1 mM of Mg^{2+} and ATP. In parallel experimental groups either Mg^{2+} (striped column), or ATP (cross-striped column), were omitted from the media. Results are given relative to the respective controls in the same experiment. Mg^{2+}: after omission of Mg^{2+}, K^+ occlusion decreased to 0.82 ± 0.05 ($P = 0.084$, i.e., no significance at the 0.05 level, $n = 3$). Each experiment contained three or four individual determinations. ATP: occlusion of K^+ decreased to 0.44 ± 0.07 in absence of ATP ($P = 0.009$, $n = 3$). Each experiment contained two or three individual determinations. The control, set to 1.0, was (5.1 ± 0.1)·10^{-2} μmol occluded K^+ per g protein ($n = 6$).

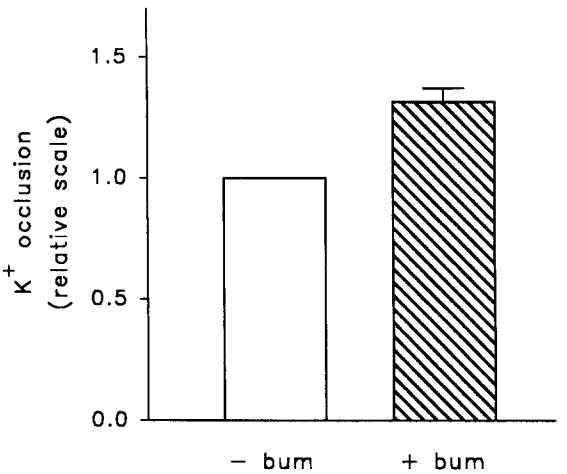

Fig. 2. Effect of bumetanide on K^+ occlusion. Following incubation of the soluble proteins from the membrane vesicles with K^+ plus $^{86}Rb^+$, 0.35 mM, and 5 mM NaCl, ethanol to the control (0.9% vol/vol, open column) or bumetanide (to 100 μM, striped column), were added 1 min before cooling and the subsequent cation exchange chromatography. Mean control was (4.0 ± 0.2)·10^{-2} μmol occluded K^+ per g protein. In presence of bumetanide, K^+ occlusion is stimulated by 1.31 ± 0.08 fold ($P = 0.025$, n = 3) relative to the controls (normalized to 1). Each experiment contained two or three determinations.

3.2. Bumetanide protects occluded K^+

Evidence is available that inhibition by bumetanide is a characteristic feature of the $Na^+/K/^+2Cl^-$ cotransporter, that bumetanide binding requires the simultaneous presence of Na^+, K^+ and Cl^-, and that binding is inhibited by high chloride concentrations [2,3]. Several reports suggest that the binding site for bumetanide (and related agents) is on the cotransporter itself [59–62], that the affinity of this binding site to bumetanide is Cl^- dependent [3,23], and that it may be identical to the low-affinity Cl^- site [63].

The latter however, is not universally accepted (see e.g. [64], and Section 4).

The effect of bumetanide on K^+ occlusion, shown in Fig. 2, was determined using the standard protocol in the absence (open column) or presence (striped column) of 100 μM bumetanide, added 5 min after addition of $^{86}Rb^+$, and 1 min before application of the sample to the cation exchange column. The results, normalized to the level of K^+ occlusion measured in absence of bumetanide, indicate that 100 μM bumetanide increased K^+ occlusion approx. 1.3-fold (1.31 ± 0.08, $P = 0.025$, $n = 3$). Therefore bumetanide (100 μM) was included in all subsequent experiments. It was not determined if a longer exposure to bumetanide would further enhance the level of occlusion.

3.3. Displacement of occluded $^{86}Rb^+$ by K^+

A series of experiments were carried out to evaluate the effect of varying K^+ concentrations on $^{86}Rb^+$ occlusion in presence of 5 mM NaCl, to determine if K^+ could compete with $^{86}Rb^+$. Fig. 3a demonstrates that the level of occluded $^{86}Rb^+$ decreases with increasing [K^+], indicating competition between $^{86}Rb^+$ and K^+ for the K^+ binding site. Assuming that Rb^+ and K^+ have equal affinity to the K^+ binding site of the $Na^+/K^+/2Cl^-$ cotransporter and that B_{max} (see below) for Rb^+ and K^+ are identical, the results were fitted to an equation (see legend to Fig. 3a) describing the relation between the binding of a ligand ($^{86}Rb^+$) and the concentration of a competitive inhibitor (K^+) [65]. The curve was generated from the means of the kinetic parameters for the experiment shown and from four other experiments of the same design. The mean values of the kinetic parameters K_i (the inhibition constant for K^+) and B_{max} (the maximal $^{86}Rb^+$ binding) were 363 ± 29 μM (K_i), and $(9.8 \pm 0.6) \cdot 10^{-2}$ μmol $^{86}Rb^+$ occluded per g protein (B_{max}) ($n = 5$).

3.4. Saturation of the K^+ occlusion

Fig. 3b, summarizing five experiments, shows that the level of occluded K^+ plotted against increasing K^+ concentrations results in a hyperbola. Curve fitting of the individual experiments gave estimates of the kinetic parameters B_{max}, the maximal K^+ binding, and the half-saturation constant $K_{0.5}$ (for details, see legend to Fig. 3b). B_{max} was $(10.9 \pm 0.9) \cdot 10^{-2}$ μmol K^+ occluded per g protein; $K_{0.5}$ was 371 ± 63 μM K^+.

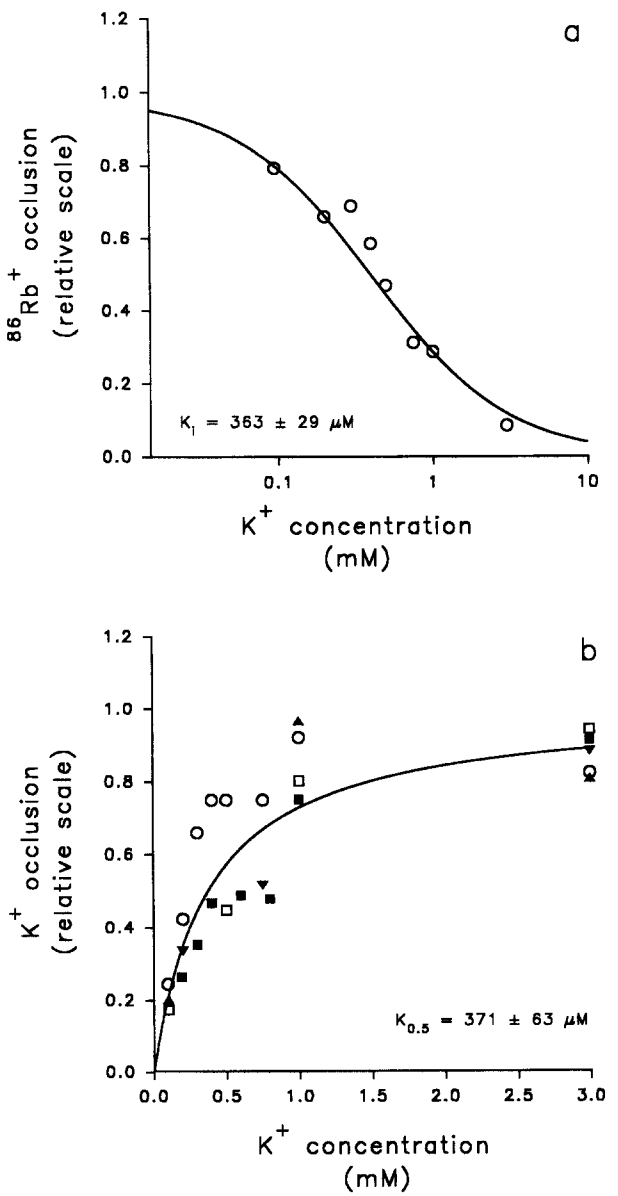

Fig. 3. Effect of [K^+] on $^{86}Rb^+$ and K^+ occlusion. The occlusion of $^{86}Rb^+$ and K^+ by the soluble proteins from the membrane vesicles was assayed with varying K^+ concentrations from 0.05 to 3 mM (incl. $^{86}Rb^+$), with NaCl kept constant at 5 mM. The data of both panels were normalized due to individual variation among the experiments. Panel a, the result of one experiment (open symbols) shows the displacement of occluded $^{86}Rb^+$ by increasing [K^+]. Assuming that Rb^+ and K^+ bind with equal affinity to the K^+ binding site on the $Na^+/K^+/2Cl^-$ cotransporter and that B_{max} (see below) for Rb^+ and K^+ are identical, the experimental data of this and four experiments of the same design were fitted individually to the equation $B = (B_{max} \cdot S)/(S + K_i(1 + I/K_i))$, where B = measured $^{86}Rb^+$ binding; B_{max} = maximal $^{86}Rb^+$ binding in the absence of K^+; $S = [^{86}Rb^+]$; $I = [K^+]$; and K_i = the inhibition constant. The equation gives the relationship between bound ligand ($^{86}Rb^+$) and concentration of the inhibitor, K^+ [65]. The computed fits gave estimates of B_{max} and K_i ($\pm$ asymptotic standard errors). Means of the estimates were: $B_{max} = (9.8 \pm 0.6) \cdot 10^{-2}$ μmol $^{86}Rb^+$ occluded per g protein; and $K_i = 363 \pm 29$ μM K^+ ($n = 5$). The curve was generated from the means of the kinetic parameters obtained in the five experiments. Panel b is based on the same experimental data as Panel a, but calculated as K^+ occlusion. The five experiments summarized show that a dose–response curve of K^+ occlusion as a function of [K^+] exhibits saturation kinetics. The curve was generated from the means of the kinetic parameters for the individual experiments, obtained by fits of the experimental data to the hyperbolic function: $B = (B_{max}/(1 + K_{0.5}/S)$, where B = measured K^+ binding; B_{max} = maximal K^+ binding; $K_{0.5} = K_{0.5}$ for K^+; and $S = [K^+]$. The fits gave estimates of B_{max} and $K_{0.5}$ ($\pm$ asymptotic standard errors). The mean estimates were $B_{max} = (10.9 \pm 0.9) \cdot 10^{-2}$ μmol K^+ occluded per g protein, and $K_{0.5} = 371 \pm 63$ μM K^+ ($n = 5$).

Note that the B_{max} value for $^{86}Rb^+$, $(9.8 \pm 0.6) \cdot 10^{-2}$ μmol $^{86}Rb^+$ occluded per g protein (Fig. 3a), is not significantly different from the B_{max} for K^+, $(10.9 \pm 0.9) \cdot 10^{-2}$ μmol K^+ occluded per g protein (Fig. 3b).

3.5. Effect of [Na^+] on K^+ occlusion

The working hypothesis was that the $Na^+/K^+/2Cl^-$ cotransporter may exhibit glide symmetry (first-ion-on = first-ion-off) as suggested by others [3,4,21], and that the experimental conditions of our occlusion assay may favour binding of the substrate ions to the outward-facing form of the cotransporter (see Section 4). If so, the ions bind in the order $Na^+ \rightarrow Cl_h^- \rightarrow K^+ \rightarrow Cl_l^-$ [3,4,21], i.e., K^+ ($^{86}Rb^+$) binds to a configuration of the cotransporter with one Na^+ and one Cl^- already bound (Cl_h and Cl_l refer to the high- and low-affinity Cl^- sites, respectively [2,23]). In transport studies in renal epithelial cells and in avian erythrocytes, $K_{0.5}$ values for the two Cl^- sites of 5 and 2–5 mM (Cl_h), and 55 and 112 mM (Cl_l), respectively, have been reported [2,66]. Consequently, K^+ ($^{86}Rb^+$) binding is dependent on the simultaneous presence of Na^+ and Cl^-.

Fig. 4a shows one experiment assessing whether Na^+ can displace $^{86}Rb^+$ in the solubilized membrane vesicles in the presence of K^+ plus $^{86}Rb^+$ (K^+, 198 μM; $^{86}Rb^+$, 152 μM; sum = 0.35 mM), and 5 mM Cl^-. It is seen that increasing [Na^+] does not displace, but rather stimulates $^{86}Rb^+$ binding in a hyperbolic fashion (Fig. 4a). Therefore Na^+ and Rb^+ are binding to separate positively interacting sites. Since the effect of Na^+ on occlusion is measured with respect to another ion ($^{86}Rb^+$), it may not be appropriate to deal with the kinetics for the effect of Na^+. Nevertheless the data fitted a hyperbolic function (detailed in the legend to Fig. 4) with a $K_{0.5}$ of 64 ± 14 μM for Na^+ promoting $^{86}Rb^+$ occlusion, and a maximal binding capacity, B'_{max}, of $(2.97 \pm 0.10) \cdot 10^{-2}$ μmol $^{86}Rb^+$ occluded per g protein (at a $^{86}Rb^+$ concentration of 152 μM). Two more experiments of the same design gave comparable results.

Plotting occluded K^+ against increasing [Na^+] also (and not surprisingly) results in a hyperbola (Fig. 4b). Fits of the individual experiments to the hyperbolic function described above, gave estimates of B'_{max} and $K_{0.5}$ (see the legend to Fig. 4b). Mean of the kinetic parameters obtained from the fits were a B'_{max} of $(6.3 \pm 0.5) \cdot 10^{-2}$ μmol K^+ occluded per g protein at a concentration of $^{86}Rb^+$ plus K^+ of 350 μM, and a $K_{0.5}$ of 50 ± 7 μM Na^+. Note that the concentration of $^{86}Rb^+$ plus K^+ used, 350 μM, is close to the $K_{0.5}$ for K^+, 371 ± 63 μM (Fig. 3b). Hence, a B'_{max} of $(6.3 \pm 0.5) \times 10^{-2}$ μmol K^+ oc-

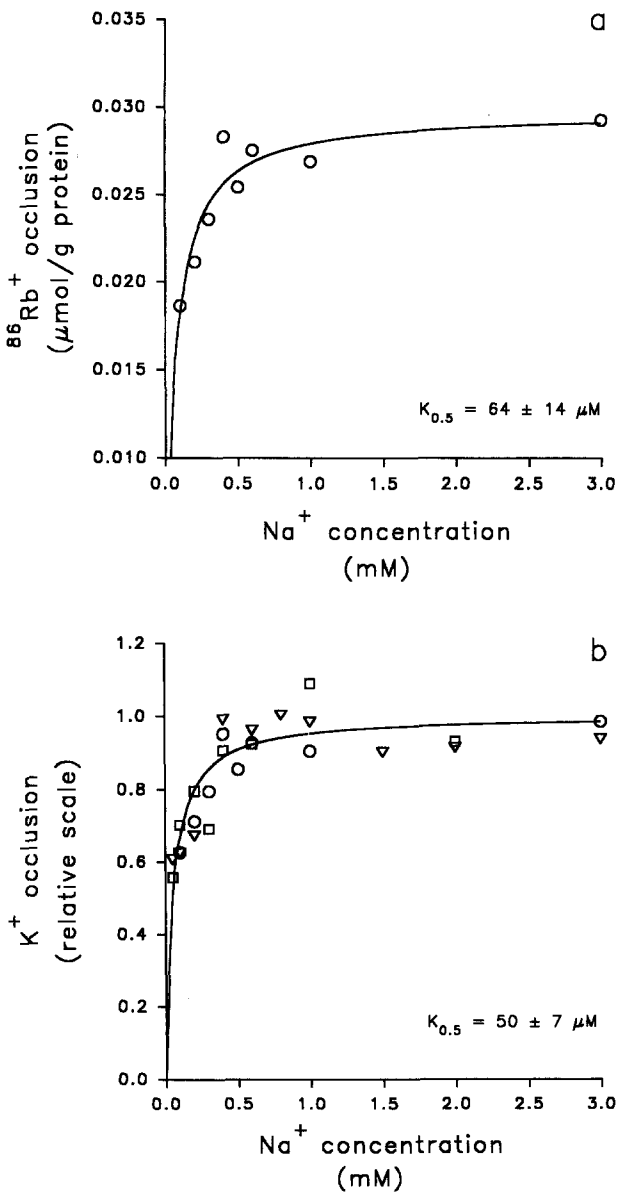

Fig. 4. Effect of [Na^+] on $^{86}Rb^+$ and K^+ occlusion. K^+ occlusion in the solubilized membrane vesicles was tested by varying the Na^+ concentration from 0.01 to 3 mM in the presence of 0.35 mM K^+ (incl. $^{86}Rb^+$), and 5 mM Cl^-. Panel a, results of one experiment, shows occlusion of $^{86}Rb^+$ (at a [$^{86}Rb^+$] of 152 μM) as a function of [Na^+]. The experimental data were fitted to the hyperbolic function: $B = (B'_{max}/(1 + K_{0.5}/S)$, where B = measured $^{86}Rb^+$ binding; B'_{max} = maximal $^{86}Rb^+$ binding (at 152 μM $^{86}Rb^+$ and 198 μM K^+); $K_{0.5}$ = $K_{0.5}$ for Na^+; and S = [Na^+]. The fit, represented by the solid line, gave the following estimates of the kinetic parameters (± asymptotic standard errors): $B'_{max} = (2.97 \pm 0.10) \cdot 10^{-2}$ μmol $^{86}Rb^+$ occluded per g protein, and an apparent $K_{0.5}$ for K^+ occlusion of 64 ± 14 μM Na^+. Two other experiments of the same design gave similar results. Panel b, based on the data of Panel a (open symbols) plus two more experiments (designated by other symbols), and calculated with respect to K^+ (assuming that $^{86}Rb^+$ and K^+ behave identically towards the binding site), shows that K^+ binding as a function of increasing [Na^+] also exhibits saturation kinetics. The experimental data (normalized in the panel due to individual variation among the experiments) were fitted individually to the hyperbolic function given above. The parameters $K_{0.5}$ and S were as described; B = measured K^+ binding; and B'_{max} = maximal K^+ binding (at a concentration of $^{86}Rb^+$ plus K^+ of 350 μM). Means of the estimates of the fits gave the following results: a $K_{0.5}$ for K^+ occlusion of 50 ± 7 μM Na^+, and a maximal binding capacity, B'_{max}, of $(6.3 \pm 0.5) \cdot 10^{-2}$ μmol K^+ occluded per g protein ($n = 3$). The solid line represent a fit to the normalized data, using the means of the kinetic parameters from the individual experiments.

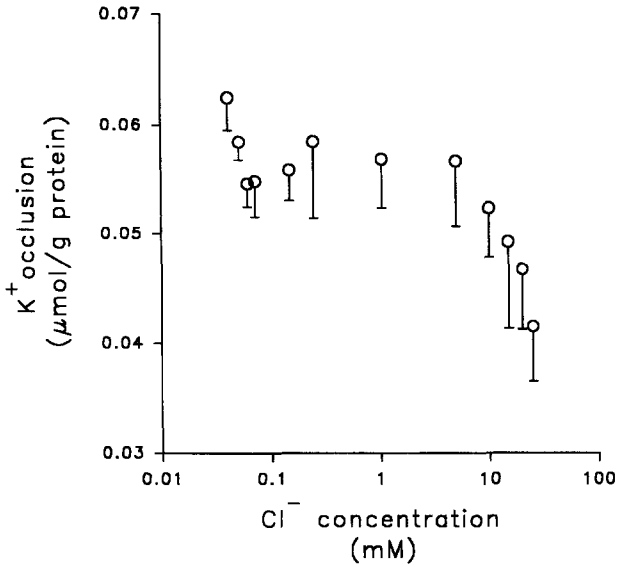

Fig. 5. Effect of $[Cl^-]$ on K^+ occlusion. To avoid contaminating Cl^-, harvesting of Ehrlich cells and preparation of membrane vesicles in these experiments were done in Cl^--free media, nitrate substituting chloride in equimolar amounts. The effect of Cl^- on K^+ occlusion was assessed by incubating solubilized membrane vesicles with 5 mM Na^+, K^+ plus $^{86}Rb^+$, 0.35 mM; and 41 μM–25 mM Cl^- (with gluconate added in appropriate concentrations to maintain constant ionic strength). Each symbol represents 3–5 independent experiments except two (at 41–52 μM, and 5 mM Cl^-, respectively), which were only done twice.

cluded per g protein is not surprising, since it is close to half the value for the B_{max} obtained when $[K^+]$ is not a limiting factor, $(10.9 \pm 0.9) \cdot 10^{-2}$ μmol K^+ occluded per g protein (Fig. 3b).

3.6. Effect of Cl^- on K^+ occlusion

If the $Na^+/K^+/2Cl^-$ cotransporter binds its substrate in the order $Na^+ \rightarrow Cl_h^- \rightarrow K^+ \rightarrow Cl_l^-$ [3,4,21], it follows that binding of K^+ ($^{86}Rb^+$) in the presence of Na^+ is inhibited if Cl_h is unoccupied by Cl^-. Therefore stimulation of K^+ ($^{86}Rb^+$) occlusion should be observed with increasing $[Cl^-]$ until the high-affinity Cl^- site is saturated. However, this could not be demonstrated in the range of Cl^- concentrations used in our experiments. Fig. 5 shows that the level of occlusion of K^+ in a medium containing Na^+ (5 mM), K^+ plus $^{86}Rb^+$ (sum = 0.35 mM), and Cl^- from 41 μM to 25 mM was highest at the lowest Cl^- concentration tested (41 μM) and *decreased* with increasing $[Cl^-]$. This observation suggests that the affinity of Cl^- for the high-affinity Cl^- site (Cl_h) is very high. It was not possible to obtain lower Cl^- concentrations since the isotope was purchased as $^{86}RbCl$; hence, a $[Cl^-]$ of 41–52 μM was unavoidable.

4. Discussion

A new approach to study the functional properties of the $Na^+/K^+/2Cl^-$ cotransporter is described: the measurement of occlusion of K^+ ($^{86}Rb^+$) by the cotransporter. Our results show that it is possible to measure K^+ ($^{86}Rb^+$) occlusion in proteins of detergent-solubilized membrane vesicles derived from Ehrlich cells, and suggest that the $Na^+/K^+/2Cl^-$ cotransporter is responsible for this occlusion. Support of this conclusion is that K^+ occlusion increases in a saturable manner with increasing $[K^+]$ (Fig. 3b) and $[Na^+]$ (Fig. 4b). A higher $K_{0.5}$ value for K^+ occlusion than the $K_{0.5}$ values reported for K^+ occlusion in solubilized Na^+/K^+-ATPase (approx. 10-times higher), and the insensitivity of K^+ ($^{86}Rb^+$) occlusion to 1 mM ouabain argue against the occlusion being due to the Na^+/K^+-ATPase. Further we have shown that the K^+ occlusion is protected by bumetanide (Fig. 2), consistent with the involvement of the $Na^+/K^+/2Cl^-$ cotransport system. Our result that K^+ ($^{86}Rb^+$) occlusion is *inhibited* by increasing $[Cl^-]$ (Fig. 5) thus at first sight seems contradictionary. An explanation is offered below.

4.1. Occlusion and transport

Occluded K^+ as a function of the $[K^+]$ describes a hyperbola (Fig. 3b). The same applies to occluded K^+ as a function of the $[Na^+]$ (Fig. 4b), suggesting that the binding sites for K^+ and Na^+ on the $Na^+/K^+/2Cl^-$ cotransporter are saturable, and that the binding of K^+ is dependent on a prior or simultaneous binding of Na^+. A saturation component was also observed in studies on the furosemide-sensitive K^+ uptake ($^{86}Rb^+$ was used as a tracer for K^+) in membrane vesicles of LLC-PK_1 cells (a renal cell line) as a function of $[Na^+]$ or $[K^+]$ ($K_{0.5}$ for $Na^+ = 0.42$ mM, and for K^+ 11.9 mM) [66], and in osteoblasts ($K_{0.5}$ for $Na^+ = 24.4$ mM, and for $K^+ = 2.9$ mM) [67]. Furosemide, like bumetanide, is a diagnostic inhibitor of the $Na^+/K^+/2Cl^-$ cotransporter although it is less specific, and at appropriate concentrations also inhibits e.g. K-Cl cotransport. In intact Ehrlich cells, bumetanide-sensitive Cl^- uptake has an apparent $K_{0.5}$ for K^+ of 3.3 mM [18].

A question arises, if the observed affinity for K^+ for occlusion in the $Na^+/K^+/2Cl^-$ cotransporter is within a reasonable range. Half maximal K^+ transport rates for the cotransport (estimated in influx studies) compared to the half-maximal affinity for K^+ during the occlusion (estimated in this paper), indicate that the affinity for K^+ during occlusion is higher than the affinity for K^+ during transport. In most tissues, $K_{0.5}$ for the transport rate of K^+ is in the millimolar range (winter flounder, 4.5 mM [14]; Ehrlich cells, 6.8 mM [17]; LLC-PK_1 cells, 11.9 mM [66]; TALH vesicles, 29.8 mM [68]) as opposed to $K_{0.5}$ for the affinity for K^+ during the occlusion in the $Na^+/K^+/2Cl^-$ cotransporter, approx. 371 μM in Ehrlich cells as demonstrated here (Fig. 3b). Transport activity obviously is not measurable in the soluble cotransport protein. Dealing with occlusion and binding affinities, it is important to note that occlusion of ions in a transport protein may not imply

anything about the affinity of the binding site for the ligand, but merely should be viewed as implying restricted access to a binding site area, as adduced for the Na^+/K^+-ATPase [33]. Thus that the $K_{0.5}$ for K^+ measured in the occlusion assay in this investigation is low compared to the $K_{0.5}$ values (for K^+) measured in influx experiments, may not be surprising.

In studies on Rb^+ occlusion in Na^+/K^+-ATPase, half-maximal occlusion was obtained at 30 μM Rb^+ in the salt glands of shark solubilized in octaethyleneglycol dodecyl monoether ($C_{12}E_8$) [69] and at approx. 40 μM Rb^+ in pig kidney Na^+/K^+-ATPase dissolved in the same detergent [37]. In membrane bound Na^+/K^+-ATPase, also from pig kidney, $K_{0.5}$ values of 47 μM Rb^+ [26] and 100–200 μM Rb^+ [54] have been reported. We have estimated a $K_{0.5}$ of approx. 371 μM K^+ in soluble $Na^+/K^+/2Cl^-$ cotransport proteins (Fig. 3b), which is approx. 9- and 12-times higher than the $K_{0.5}$ values for Rb^+ in soluble Na^+/K^+-ATPase of the pig kidney [37] and the shark rectal gland [69], respectively, and 2–8-times above the level found for the membrane bound Na^+/K^+-ATPase of pig kidney [26,54]. Thus, although the $K_{0.5}$ values for $^{86}Rb^+$ and for K^+ occlusion in the $Na^+/K^+/2Cl^-$ cotransport system (Fig. 3) are low compared to the $K_{0.5}$ values for K^+ estimated in previous influx experiments, they are still high compared to the $K_{0.5}$ values obtained for Rb^+ or K^+ occlusion in the Na^+/K^+-ATPase.

4.2. Occlusion is inhibited by Cl^-

$Na^+/K^+/2Cl^-$ cotransport requires the simultaneous presence of the three substrate ions [17]; therefore our results that increasing $[Cl^-]$ inhibited K^+ occlusion (Fig. 5) are difficult to interpret. Possible explanations may involve: (i) the high-affinity Cl^- site (Cl_h) of the $Na^+/K^+/2Cl^-$ cotransporter is occupied at 41 μM Cl^-, the lowest Cl^- concentration applied (Fig. 5). For comparison, Na^+/K^+-ATPase has one high- and one low-affinity K^+ binding site [54,70]; the high-affinity K^+ site is saturated at 10 μM Rb^+ [54]; (ii) if the inhibitory site for bumetanide is identical with the low-affinity Cl^- site [63], the off-rate of this molecule from the cotransport protein during passage of the cation exchange column may be slower than that of a Cl^- ion occupying the same site. According to the glide symmetry model, K^+, and therefore also $^{86}Rb^+$, only exchanges at the extracellular face of the membrane when Cl_l is unoccupied [4]. To maintain $^{86}Rb^+$ in the bound mode when the proteins passes through the cation exchange column, bumetanide on Cl_l, added after $^{86}Rb^+$, therefore may be preferable to a Cl^- ion, i.e., a higher level of $^{86}Rb^+$ occlusion is expected in the presence of bumetanide. The complex of the cotransporter then is on the form: {protein/$Na^+/Cl^-/^{86}Rb^+$/bumetanide}. This is supported by our results that indicate bumetanide has some protective effect on bound $^{86}Rb^+$ (Fig. 2). That increasing $[Cl^-]$ in the presence of bumetanide lowers the level of K^+ occlusion (Fig. 5) thus may reflect that bumetanide binding competitively is inhibited by high Cl^- concentrations. Examination of bumetanide binding to membranes of the rabbit parotid confirm that this type of binding competitively is inhibited by Cl^- [64]. Pertinent to this discussion are reports suggesting that bumetanide may bind to a site on the cotransporter different from Cl_l [23,41,64] (although the affinity of this site for bumetanide may be dependent on Cl^- [3,23]), and the compelling arguments provided by Moore et al. [71] that a high-affinity and a low-affinity anion site are associated with bumetanide binding. The first site was found to have a stimulatory, the latter an inhibitory effect on the bumetanide binding; but neither of these anion sites were found to be Cl^- binding sites [71].

Furthermore it has been established in a variety of cells that increasing $[Cl^-]_i$ inhibits $Na^+/K^+/2Cl^-$ cotransport [72,73], perhaps via an allosteric site or a regulatory protein. In Ehrlich cells, reduced $[Cl^-]_i$ seems to play a permissive role in the activation of the cotransporter [19]. Thus, such an inhibitory role for Cl^- on the $Na^+/K^+/Cl^-$ cotransport appears to be an alternative possible explanation of the data in Fig. 5.

4.3. Possible stoichiometry between bumetanide binding and K^+ occlusion

Using [^{3}H]bumetanide, our laboratory has previously established that membranes from Ehrlich cells bind 64 nmol bumetanide per g protein [43]. We have shown further that compared to intact cells, the membrane vesicles used in this study are enriched 3–4-fold with protein of the $Na^+/K^+/2Cl^-$ cotransporter [41]. Based on these numbers, an amount of bumetanide binding to the membrane vesicles in the order $(19–26) \cdot 10^{-2}$ μmol bumetanide per g protein, is expected. The predicted ratio between K^+ and bumetanide binding sites on the cotransporter is 1:1 [2,3]. Considering a maximal K^+ occlusion of $(10.9 \pm 0.9) \cdot 10^{-2}$ μmol per g protein (Fig. 3b), the amount of occluded K^+ is approx. 50% of the value expected, assuming one K^+ binding site per cotransport molecule [2,3], and a 100% recovery of functional cotransport proteins after the cation exchange chromatography. The minor discrepancy (per g protein) in the ratio between the amount of bumetanide binding to the Ehrlich cell membranes [43] and the maximal K^+ occlusion in the solubilized membrane vesicles (Fig. 3b) may reflect that some of the occluded $^{86}Rb^+$ is lost from the cotransport proteins by adsorbtion to the column matrix during the cation exchange chromatography. A longer time of exposure (> 1 min) of the solubilized proteins to bumetanide before cooling and application of the samples to the cation exchange columns could possible lead to a higher level of bumetanide binding and thereby to a better protection of the bound $^{86}Rb^+$ during passage of the column, resulting

in a higher level of $^{86}Rb^+$ occlusion. Alternatively it is indeed likely that the activity of some of the $Na^+/K^+/2Cl^-$ cotransporters is lost upon detergent solubilization. We have not been able to measure [^{3}H]bumetanide binding to the solubilized proteins after elution through the cation exchange columns. Thus, considering the differences in the methods for measuring [^{3}H]bumetanide binding and $^{86}Rb^+$ occlusion in mind, the two values seems to be in reasonable agreement.

4.4. Effect of Mg^{2+} and ATP on occlusion

Two major modes of action have been discussed for Mg^{2+} and ATP in Ref. [3,57] and [63]: (i) they bind directly to the $Na^+/K^+/2Cl^-$ cotransporter and act as allosteric modifiers; or (ii), they interact in a complex as the substrate for protein kinase(s) which in concert with protein phosphatase(s) maintain the cotransporter in a phosphorylated (active) or a dephosphorylated (inactive) state.

We have observed that the K^+ occlusion in proteins of the soluble membrane vesicles may decrease in the absence of Mg^{2+} (results not significant), and that the occlusion is halved in the absence of ATP (Fig. 1). These phenomena are currently under investigation.

4.5. Is Na^+/K^+-ATPase involved in the occlusion?

Na^+/K^+-ATPase, also capable of $^{86}Rb^+$ occlusion [26,29,33,34,37,54,69] is present in abundance in the membrane vesicles [52]. However, due to the experimental conditions, it seems unlikely that this enzyme contributes to the occlusion in our experiments. The arguments are three-fold: (i) $^{86}Rb^+$ occlusion in the Na^+/K^+-ATPase does not increase with increasing [Na^+] in the micromolar range (Professor P.L. Jørgensen, personal communication), as observed in Fig. 4a. (ii) The potent Na^+/K^+-ATPase inhibitor ouabain (1 mM) and Mg^{2+} were present in all solutions, always added previously to $^{86}Rb^+$. In presence of these two compounds, the Na^+/K^+-ATPase is not capable of occluding Rb^+ [54]. Glynn and Richards [74] point out that ouabain prevents Rb^+ occlusion in the Na^+/K^+-ATPase ($K_i \approx 20$ μM) if the cation is added after ouabain has complexed with the ATPase. If Rb^+ is already occluded when ouabain is added, the compound slows down the rate of release by several orders of magnitude [28]. Not surprisingly, ouabain also inhibits Rb^+ occlusion in the 19 kDa fragments of proteolytic digested Na^+/K^+-ATPase ($K_i \approx 400$ μM) [26]. (iii) The $K_{0.5}$ estimated for K^+ binding (~ 371 μM, Fig. 3b) is different from the $K_{0.5}$ values reported for the Na^+/K^+-ATPases (see above).

The results on Na^+/K^+-ATPase reveal that specific Rb^+ occlusion in this enzyme is 50–100-fold above the level of K^+ occlusion measured in the soluble $Na^+/K^+/2Cl^-$ cotransporter (compare our data to e.g. [33,37,54]). This is because the preparations of Na^+/K^+-ATPase used had a high degree of purity (yielding a high specific activity of enzyme), compared to our preparations made of solubilized membrane vesicles containing a number of other membrane proteins in addition to cotransport protein.

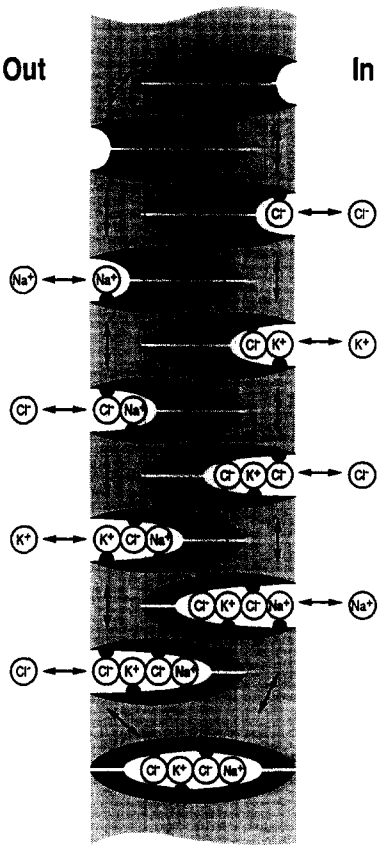

Fig. 6. Model of the ion translocation cycle of the $Na^+/K^+/2Cl^-$ cotransporter. The 'first-ion-on = first-ion-off' model for $Na^+/K^+/2Cl^-$ cotransport suggested by others [3,21], is illustrated in a new version including a putative occluded step in the translocation process, the fully loaded carrier, to which ions have no access from either side of the membrane. The model is modified from Ref. [4].

4.6. The 'first-ion-on = first-ion-off' model

A model of the translocation cycle of the $Na^+/K^+/2Cl^-$ cotransporter (see [3,4,21]) is shown in Fig. 6. The outward-facing cotransporter binds its four substrate ions so that the order of binding and release is identical: $Na^+ \rightarrow Cl^- \rightarrow K^+ \rightarrow Cl^-$. A putative 'occluding step' is included, showing the fully loaded carrier with no access of the substrate ions to the solution phase on either side of the membrane. Although we have no data demonstrating that the complete absence of Na^+ entirely eliminate K^+ occlusion (Fig. 4b), our results suggest that binding of substrate ions to the soluble $Na^+/K^+/2Cl^-$ cotransporters is ordered as if only the outward-facing configuration is accessible. In the intact membrane vesi-

cles, the flux through the cotransporter is inwardly directed, driven by the concentration gradients for Na^+ and Cl^- [18,41]. The cotransporters are also permanently activated [41], suggesting that after a translocation cycle (ending with dissociation of ions inside the membrane vesicles), the empty carrier returns to its outward-facing position. Solubilization of the intact membrane vesicles removes most of the native membrane lipids and associated proteins from the near vicinity of the cotransporter. This possibly fix the carrier in its outward-facing position, where bumetanide also binds (see [2,3]).

In summary, we have shown that proteins from n-octyl glucoside solubilized membrane vesicles derived from Ehrlich cells can occlude K^+ ($^{86}Rb^+$). The K^+ occlusion is saturable by increasing $[Na^+]$ and $[K^+]$; $^{86}Rb^+$ can be displaced from the occluded state by K^+, but not by Na^+; the K^+ occlusion is sensitive to Cl^- and bumetanide. Therefore we conclude that the occlusion is due to the $Na^+/K^+/2Cl^-$ cotransport system. If occlusion is a step in the ion translocating process, it follows that this step must be preserved in the solubilized protein.

Acknowledgements

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