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The Na-K-2Cl Cotransporter Is in a Permanently Activated State in Cytoplasts from Ehrlich Ascites Tumor Cells

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Abstract. Brief incubation of Ehrlich ascites tumor cells with cytochalasin B causes the formation of blebs in the surface membrane. Gentle homogenization removes the blebs as intact cytoplasts which contain neither mitochondrian or nucleus, nor other cytoplasmic membranous organelles. The Na-K-2Cl cotransporter is present in the cytoplasts in a permanently activated state, whereas the Na-K-2Cl transport system in unperturbed intact cells is silent. Pretreatment of intact cells with cytochalasin B for 1 min stimulates the bumetanide-inhibitable K^+ influx ~fivefold. The influx into purified cytoplasts when expressed per g protein is three- to fourfold higher than the influx into cytochalasin B-treated intact cells. Thus, the membrane vesicles are enriched with the cotransporter, and the cotransporter is present in an activated state. The K influx into cytoplasts is inhibited about 40% by Na-free, Cl-free or bumetanide-containing media and to a similar extent by Fab fragments prepared from antiserum against purified proteins of the cotransporter. The K_I for bumetanide was 0.19 ± 0.06 μM for the cytoplasts as compared to 0.67 ± 0.11 μM for the intact cells. SDS gel electrophoresis of membrane proteins from the cytoplast membranes compared to the membranes of intact cells shows a reduced number of bands and a majority of bands showing reduced staining, whereas a few bands are stained more intensely. Particularly notable is a band at ~80 kD, which is similar to the molecular weight previously reported for the main membrane protein isolated from intact cells using a bumetanide-Sepharose affinity column. An immunoblot of the cytoplast preparation using antibodies against the purified bumetanide binding proteins showed strong immunodetection of the ~80 kD protein.

Key words: Membrane vesicles — Volume regulation — Bumetanide — Cytochalasin B

Introduction

The Na-K-2Cl cotransporter, a widely distributed membrane transport system, carries Na, K, and Cl into cells. The flux is electroneutral, usually with a stoichiometry of 1 Na: 1 K: 2 Cl. Transport is driven by the inwardly directed concentration gradients of Na and Cl. Though Cl is often near electrochemical equilibrium, it is far from chemical equilibrium. Because cotransport is electroneutral, the chemical concentration gradient of Cl contributes to the driving force independently of the membrane potential. Indeed, owing to the stoichiometry, the contribution of the Cl gradient (r_{Cl}) is larger (r_{Cl2}) than the contribution of the Na gradient provided they are of similar magnitudes. The transporter can promote solute entry into cells at rates sufficient to generate significant osmotic gradients which in turn cause cell swelling. Thereby Na-K-2Cl cotransport participates in regulation of cell volume. In like manner, it can participate in transcellular transport of salt and water in epithelia. Bumetanide is the diagnostic inhibitor of this transporter (for reviews, *see* O'Grady, Palfrey & Field, 1987; Haas, 1989; Parker & Dunham, 1989; Hoffmann & Simonsen, 1989; Hoffmann & Ussing, 1992). In Ehrlich ascites cells at constant volume in a physiological medium, the flux through the Na-K-2Cl cotrans-

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porter is relatively small, but osmotic shrinkage of the cells activates cotransport, which promotes osmotically obliged water influx and restoration of initial cell volume in a few minutes, at which time the cotransporter becomes inactivated again (Hoffmann, Sjøholm & Simonsen, 1983; Jensen, Jessen & Hoffmann, 1993).

Some of the unresolved questions about the mechanism of the Na-K-2Cl cotransporter and its regulation might be more productively addressed in model systems than in intact cells. There have been successful demonstrations of the cotransporter in vesicles derived from cell surface membranes of a variety of epithelial cell types (Eveloff et al., 1978; Eveloff & Kinne, 1983; Koenig, Ricapito & Kinne, 1983; Burnham, Karlish & Jørgensen, 1985; Kinne et al., 1985; Turner, George & Baum, 1986; Reeves et al., 1988; Ferrandi et al., 1990). There have also been preliminary results suggesting that it may be possible to reconstitute the cotransporter from solubilized membranes of epithelial cells into artificial membrane vesicles (Burnham et al., 1985; Corcelli & Turner, 1990; Ferri, Garnier & Corcelli, 1991). However, results obtained using these systems have thus far not led to significant new insights into the mechanism of cotransport and its regulation.

We report here the demonstration of Na-K-2Cl cotransport in large vesicles, called cytoplasts, derived from the surface membranes of Ehrlich cells. Brief incubation of the cells with cytochalasin B causes the formation of blebs in the surface membrane. Gentle homogenization removes the blebs as intact cytoplasts which contain no mitochondria, nuclei, or other cytoplasmic membranous organelles (Henius, Laris & Woodburn, 1979). The cytoplasts carry out glycolysis, and have functional Na/K pumps and Na-dependent α-aminoisobutyric acid transporters (Henius et al., 1979). We show that the Na-K-2Cl cotransporter is present in a permanently activated state in the cytoplasts, in contrast to intact cells in which activation of cotransport by cell shrinkage (or some other stimulus) is transient. Therefore, the cytoplasts may be a useful system for studying the mechanism of the cotransporter.

Materials and Methods

Cells

Ehrlich ascites tumor cells (hyperdiploid strain) were maintained in white female NMRI mice by weekly intraperitoneal transplant. Each third week the cells to be transplanted were washed in standard medium containing (mM): Na^+, 150; K^+, 5; Mg^{2+}, 1; Ca^{2+}, 1; Cl^-, 150; SO_4^{2+}, 1; HPO_4^{2-}, 1; MOPS, 3.3; TES, 3.3; HEPES, 5; pH 7.4. This wash reduced the probability of transfer of microbial pathogens with the tumor cells. Eight days after transplant, cells were harvested from sacrificed mice into the standard medium containing heparin (2.5 IU/ml). The cells were washed by centrifugation with the standard medium at 37°C (700 × *g* for 45 sec), resuspended at 8% cytocrit in the same medium, and incubated at 37°C for 15 min.

Cytoplasts

Cytoplasts were prepared from the Ehrlich cells essentially as described by Henius et al. (1979). Freshly harvested cells were centrifuged and resuspended at 9% cytocrit in the standard medium without Ca^{2+} and with cytochalasin B (cyto B, 42 μM) and incubated at 37°C for 1 min. Cyto B induces the formation of blebs in the surface membrane (*see* Fig. 1). Gentle homogenization (4 min) in a Dounce homogenizer using a loose fitting, large clearance pestle sheared the blebs from the cell surface. These blebs formed the cytoplasts, which are large, intact vesicles, ~4 μm in diameter. The remainder of the cells, the karyoplasts, contain the nuclei, mitochondria, and all other cytoplasmic membranous organelles (Henius et al., 1979). The mixture was diluted threefold with Ca-free standard medium. The karyoplasts were sedimented by centrifugation at 250 × *g* for 2 min. The supernatant was decanted and a crude preparation of the cytoplasts was collected by centrifugation (3,500 × *g* for 2 min). This suspension was washed free of cyto B by centrifugation and resuspension twice in standard medium containing 1% BSA (which binds cyto B) and suspended in standard medium at ~20 mg wet wt/ml. The suspension was purified further by centrifugation (250 × *g* for 2 min) to remove contaminating karyoplasts. The supernatant was saved, and the pellet, containing both cytoplasts and karyoplasts, was suspended once more in standard medium and centrifuged again (250 × *g* for 2 min). The two supernatants were pooled in a preweighed tube and centrifuged to collect the cytoplasts (3,500 × *g* for 2 min). The supernatant solution was removed by aspiration and tubes were weighed, giving the wet weight of the cytoplasts. The cytoplasts were suspended in standard medium at 20–30 mg wet wt/ml, kept at 0°C, and used in an experiment within 90 min. The homogenization and isolation were carried out at 25°C. When carried out at 4°C, there was poorer separation of cytoplasts and karyoplasts. [Henius et al. (1979) did not report the temperature of their isolation procedure.]

Membrane Preparation

Plasma membranes from the intact cells were prepared as previously described (Jessen et al., 1989). Membranes from the cytoplasts were prepared as follows: The cytoplasts were centrifuged at 100,000 × *g* max for 30 min, and the pellet was resuspended in a buffer containing 5 mM Tris, 1 mM Na_2EDTA and 0.1 mM PMSF, pH = 7.5. Homogenization of the cytoplasts was performed using a Dounce homogenizator with a tight fitting pestle. The membrane fraction was pelleted by centrifugation at 100,000 × *g* max for 30 min and the pellet resuspended in 4 ml, 5 mM Tris, 1 mM Na_2EDTA and 0.1 mM PMSF, pH = 7.5.

Unidirectional K^+ Influxes

These were measured in cells and cytoplasts using $^{86}Rb^+$ (Risø, Denmark) as a tracer. Cells were preincubated in standard medium at 37°C for 15 min. Cytoplasts were pelleted, suspended in the desired medium, and incubated at 37°C for 1 min. Fluxes were initiated by addition of one-third volume of the appropriate medium containing $^{86}Rb^+$ (350 kBq/ml) and, as required, bumetanide (final concentration, 30 μM) and/or ouabain (final concentration, 1 mM). The suspensions were incubated at 37°C, and samples were removed at intervals for separation of cells or cytoplasts from the medium, accomplished by ion exchange chromatography as described before (Jessen et al., 1989; Dunham, Jessen & Hoffmann, 1990; Jensen et al., 1993), a modification of earlier methods (Gasko et al., 1976; Garty, Rudy & Karlish, 1983). Samples (160 μl) of cell or cytoplast suspensions with

tracer were applied to chilled columns at selected times, and washed through with 2 × 750 µl of an ice-cold sucrose-BSA wash solution (250 mM sucrose, 10 mM MOPS, 1% BSA brought to pH 7.4 with Tris). The cells or vesicles emerged in the void volume in seconds, separated from bulk phase $^{86}Rb^+$. The samples (in 1.5 ml wash solution) were transferred to a counting cocktail, Ultima-Gold (Packard), and their radioactivities were determined by liquid scintillation counting. Influxes are expressed either as rate constants, as µmol/min/g wet wt, or as µmol/min/g protein. Rate constants were calculated by fitting the data to exponential functions. The curve-fitting procedure was an iterative nonlinear unweighted least squares analysis (Marquardt-Levensky algorithm, SigmaPlot, Jandel, Corte Madera, CA). Unidirectional Na^+ influxes were measured and calculated by the same procedures using $^{22}Na^+$ as a tracer.

Determination of $K_{0.5}$'s for Bumetanide Binding

The concentrations of bumetanide at half-maximal inhibition of bumetanide-inhibitable K^+ influx ($K_{0.5}$) were calculated by fitting influx data to a hyperbolic function. The curve-fitting procedure was the one just described for estimating rate constants. The hyperbolic function was

$$J = J_o - \{(J_o - J_{min})/(1 + K_{0.5}/[B])\}$$

where J = measured unidirectional K^+ influx, $[B]$ = bumetanide concentration, J_o = K^+ influx at zero $[B]$, J_{min} = bumetanide-insensitive K^+ influx, and $K_{0.5}$ = $[B]$ at half-maximal inhibition of bumetanide-inhibitable K^+ influx. For each experiment, three-parameter fits were made, giving estimates of J_o, J_{min}, and $K_{0.5}$ ± asymptotic standard errors. The $K_{0.5}$'s presented are the means so determined from several experiments for the three experimental conditions: (i) intact cells stimulated by cyto B; (ii) intact cells during RVI after cell shrinkage; and (iii) in cytoplasts.

Preparation of Fab Fragments

Antiserum against purified bumetanide-binding proteins from Ehrlich cell membranes had been raised (Dunham et al., 1990). Fab fragments were prepared from the antiserum and from nonimmune rabbit serum by mild treatment with papain (Hudson & Hay, 1980), as previously described (Dunham et al., 1990).

Electrophoretic Separation of Proteins

SDS-polyacrylamide gel electrophoresis was performed in slab gels (1.5 mm thick) using the discontinuous buffer system of Laemmli (1970) under reducing, denaturing conditions as modified slightly by us (Feit et al., 1988). The stacking and separation gels contained 4 and 10% acrylamide, respectively. For complete solubilization, the protein samples were heated at 90°C for 30 min in a buffer containing 2% SDS, 0.1 M dithiothreitol, 0.03 M Tris-HCl (pH 6.8), 8% glycerol, and 0.002% bromphenolblue. The following proteins were used as molecular weight standards: phosphorylase B (94 kD), bovine serum albumin (67 kD), glutamate dehydrogenase (53 kD), ovalbumin (43 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), and carbonic anhydrase (30 kD). After electrophoresis, the gel slabs were stained with 0.25% Coomassie Brilliant Blue R-250. The gels were destained and fixed by incubation in 2% glycerol, 50% methanol, 10% acetic acid for 24 hr and air-dried between cellophane sheets.

Western Blots

The proteins were transferred from gels to a PVDF-membrane (Immobilon, Millipore) by electroblotting, and immunodetected with antiserum as described in Dunham et al. (1990). The PVDF-membrane was blocked with 5% BSA (Sigma–A 4503) in (mM): Na^+, 162.5; PO_4^{2-}, 10; Cl^-, 145; and 0.01% (v/v) Antifoam A (Sigma–A 5633); pH 7.2. The PVDF-membrane was then treated with the antiserum against bumetanide-binding proteins (1:100 dilution). The secondary antibody was goat anti-rabbit IgG coupled to horseradish peroxidase (1:1000 dilution; Bio-Rad).

Protein Determinations

Protein contents of cell and cytoplast pellets were determined by a new modification of the Lowry method (Lowry et al., 1951). Kaplan and Pedersen (1989) have shown that lipids interfere strongly with the protein assay after deoxycholate-TCA precipitation of proteins and subsequent addition of SDS, as in the modified Lowry procedure of Peterson (1977). They also showed that the interference by lipids can be eliminated from a modified Amido Black 10B protein assay by precipitation with TCA (final concentration, 18%) in the presence of 0.7% SDS and 90 mM Tris at pH 7.5. We have substituted TCA precipitation for the deoxycholate-TCA precipitation step in the modified Lowry procedure of Peterson (1977). This results in an assay in which the interference by lipid is completely eliminated. The presence of 30 mg lipid in a sample containing as little as 10 µg protein has no significant effect on the assay, nor does the lipid affect the blank (no protein). BSA was used as a standard.

Materials

Dowex 50 W × 8 was from Fluka AG, Switzerland. Cyto B (stock solution: 20.8 mM in 96% ethanol), SDS, and DTT were from Sigma Chemical, St. Louis, MO. Molecular weight standards for electrophoresis: BSA (Sigma); phosphorylase B, glutamate dehydrogenase, ovalbumin, carbonic anhydrase, and glyceraldehyde-3-phosphate dehydrogenase (LKB). All conventional chemicals were of analytical grade.

Abbreviations

RVI: regulatory volume increase; BSA: bovine serum albumin; cyto B: cytochalasin B; DTT: D,L-dithiothreitol; HEPES: *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; MOPS: 3-(*N*-morpholino)propane sulfonic acid; PAGE: polyacrylamide gel electrophoresis; SDS: sodium dodecyl sulfate; TCA: trichloroacetic acid; NMDG: *N*-methyl-D-glucamine; Tris: tris(hydroxymethyl)aminomethane; TES: *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

Results

K^+ Influx into Intact Cells and Cytoplasts

Unidirectional K^+ influxes were compared in intact Ehrlich cells, intact cells pretreated with cyto B, and in cytoplasts, prepared by the use of cyto B. At the same time, influx was measured in a homogenate of cyto B-treated cells which contained the unpurified cytoplasts. Figure 2 shows the results of an experiment in which initial rates of bumetanide-inhibitable K^+ influxes (±30 µM bumetanide) were measured in these four prepara-

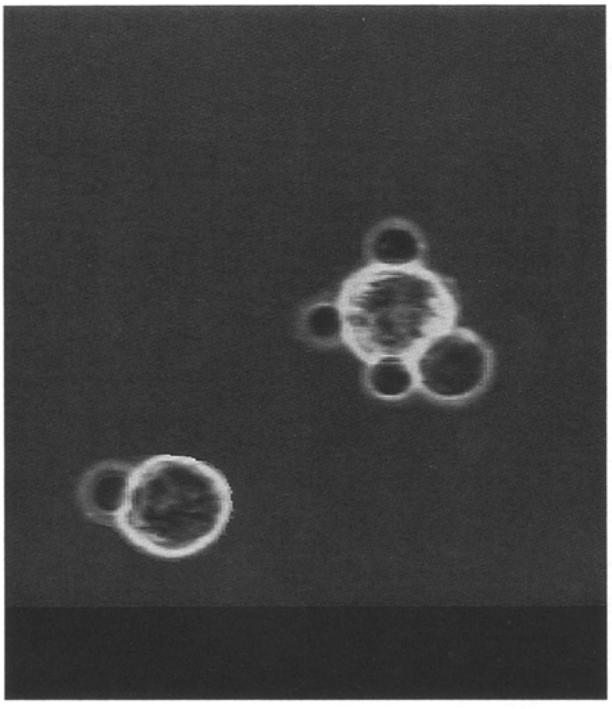

Fig. 1. Phase contrast micrograph of Ehrlich ascites tumor cells 1–3 min after addition of 42 μM Cytochalasin B. The magnification is 40× through an achrostigmat objective, 1.3 NA oil immersion. The picture is taken through a DAGE-MTI CCD camera.

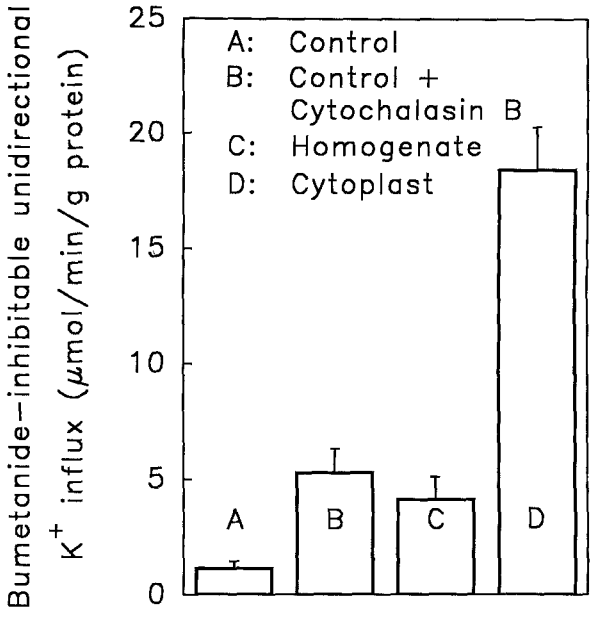

Fig. 2. Bumetanide-inhibitable K^+ influxes, measured using $^{86}Rb^+$ as a tracer, into: (*A*) intact Ehrlich ascites cells in isotonic medium, (*B*) intact cells after 1 min pretreatment with 42 μM cyto B, (*C*) homogenized preparation of cyto B-treated cells containing cytoplasts, and (*D*) cytoplasts purified as described in Materials and Methods. Values for tracer uptake were determined at 1, 1.5, 2, and 2.5 min, from which initial rates of influx were estimated by linear regression (*see* Materials and Methods). Bumetanide-inhibitable fluxes are the difference between influxes measured in the absence and presence of bumetanide (30 μM), added simultaneously with the tracer.

tions. In control cells, the bumetanide-inhibitable influx was 1.1 ± 0.3 μmol/min/g protein (n = 6), very similar to our recent result (Jensen et al., 1993). Pretreatment with cyto B for 1 min stimulated the bumetanide-inhibitable K^+ influx ~fivefold, as reported recently (Jessen & Hoffmann, 1992). The influx into a homogenate of the cells containing the cytoplasts was similar to that in intact cyto B-treated cells. Therefore, there is no diminution of cotransport activity associated with homogenization and formation of cytoplasts. The influx into purified cytoplasts was more than threefold higher than into intact cyto B-treated cells or the cell homogenate. Therefore, the membrane vesicles are enriched with the cotransporter in an activated state.

Time Courses of Unidirectional K^+ and Na^+ Influxes into Cytoplasts

Figure 3 shows the results of three experiments in which the time courses of uptake of $^{86}Rb^+$ and $^{22}Na^+$ into cytoplasts were measured. The curves were obtained by fitting the results to single exponentials as described in the figure legend. The time course of $^{86}Rb^+$ influx is nearly linear for 5 min. Therefore, samples taken prior to this time will provide good estimates of initial influx rate. The positive intercepts at time zero are due to medium containing tracer adherent to the surface of the cytoplasts. The ratio of the time zero intercept to a_∞ was considerably higher for Na^+ than for K^+, which can be explained as follows. The cytoplasts contain K^+ and Na^+ concentrations like intact cells (Henius et al., 1979), and [Na] in the adherent water layer as a fraction of [Na] in the cytoplasts is much greater than the corresponding fraction of [K] in the adherent water.

It was difficult to obtain consistently smooth curves for Na^+ influxes, so in most of the experiments only K^+ influxes were measured. In three other experiments on Na^+ influxes, bumetanide inhibited 26 ± 7% of total unidirectional Na^+ influx. The remainder of Na^+ influx is presumably through Na^+ channels, Na/H exchange, and Na-dependent HCO_3/Cl exchange.

Effect of Bumetanide and Ouabain on K^+ Influx

Figure 4 shows the effect of bumetanide on the time course of K^+ influx into cytoplasts. The bumetanide-insensitive influx was about 65% of the total, a typical result. Figure 5 shows the effects from a number of experiments of bumetanide and ouabain separately and together on unidirectional K^+ influxes. Bumetanide-in-

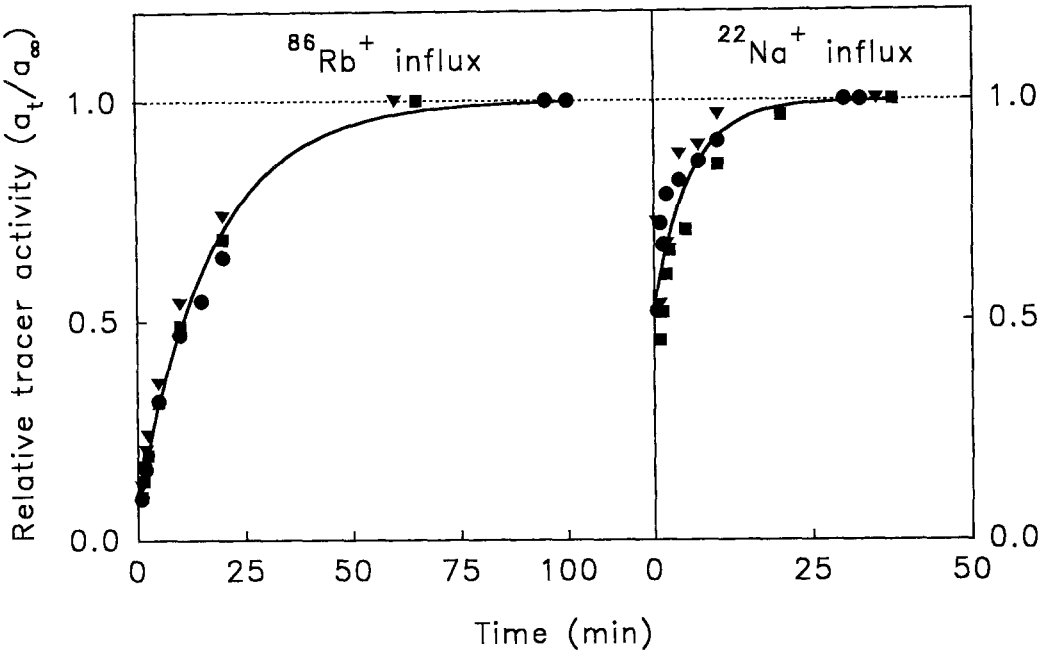

Fig. 3. Time courses of K^+ and Na^+ influxes into cytoplasts measured using $^{86}Rb^+$ and $^{22}Na^+$ as tracers. Shown are relative tracer activities in the cytoplasts, a_t/a_∞ (ratios of activities, time t/time ∞). The results are from three independent experiments, each represented by a different symbol. The curves were best fits of the data to a single exponential function obtained by iterative nonlinear least squares analyses (*see* Materials and Methods). The rate constants were 0.057 min^{-1} for K^+ influx and 0.178 min^{-1} for Na^+ influx.

hibitable and ouabain-inhibitable fluxes were, as fractions of total influx, 0.42 ± 0.03 (n = 13) and 0.46 ± 0.06 (n = 6), respectively. Bumetanide and ouabain together inhibited 0.80 ± 0.04 (n = 3) of the influx, so the effects of the two drugs were additive. Therefore, the cytoplasts contain functioning Na/K pumps, as shown by Henius et al. (1979), as well as activated Na-K-2Cl cotransporters. In one experiment, Ba^{2+} (5 mM) was without effect (*results not shown*), so the remaining 20% of K^+ influx is probably not through K^+ channels.

Dependence of K^+ Influx on Na^+ and Cl^-

Sensitivity to bumetanide is good evidence for Na-K-2Cl cotransport, but it is not conclusive. Ehrlich cells have K-Cl cotransport under some conditions (Thornhill & Laris, 1984) which may be partially inhibited by 30 μM bumetanide (Ellory et al., 1982). In an attempt to rule out a contribution of K-Cl cotransport to unidirectional K^+ influx in the cytoplasts, inhibition by bumetanide was compared to the effects of a Na-free medium. The results are shown in Fig. 6, along with the effect of a Cl-free medium. As in most experiments, bumetanide inhibited K^+ influx ~40%. K^+ influx was reduced to similar extents in both Na-free and Cl-free media, confirming that the bumetanide-inhibitable K^+ influx is mediated primarily by the Na-K-2Cl cotransporter. There was a suggestion that K^+ influx was lower in Cl-free than in Na-free medium, which may indicate a small component of K^+ influx mediated by K-Cl cotransport. This possibility was not investigated further.

Effect of FAB Fragments of Antibodies against Bumetanide-binding Proteins

In a further test for Na-K-2Cl cotransport in the cytoplasts, we used an antiserum we had raised against bumetanide-binding proteins purified from Ehrlich cell membranes (Dunham et al., 1990). There was strong evidence that the antiserum contains antibodies specific for the Na-K-2Cl cotransporter, and not for other transporters. It inhibited Na-K-2Cl cotransport in intact Ehrlich cells, but was without effect on K-Cl cotransport or K^+ channels (Dunham et al., 1990). Fab fragments inhibited nearly as well as intact immunoglobulin, so the inhibition by the antiserum could not be attributed to nonspecific crosslinking. Figure 7 shows that Fab fragments prepared from this antiserum inhibited K^+ influx ~30%, nearly as much as the inhibition by bumetanide in most experiments. This result is further support for a sizeable fraction of K^+ influx in the cytoplasts being mediated by Na-K-2Cl cotransport.

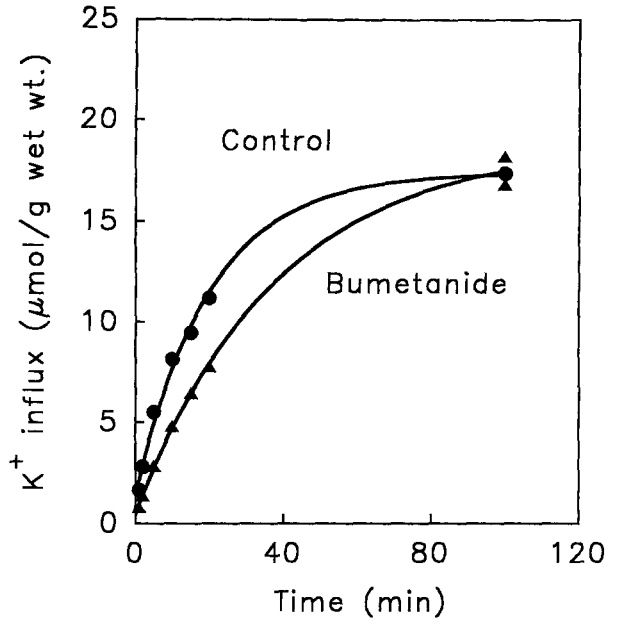

Fig. 4. Effects of bumetanide on K^+ influx into cytoplasts. Bumetanide (30 μM, final concentration) was added at time zero together with the tracer. The results are typical of three other experiments of the same design. The curves were calculated by fitting the data to a single exponential as in Fig. 3. The rate constants were 0.050 min^{-1} and 0.0266 min^{-1} for control and bumetanide, respectively.

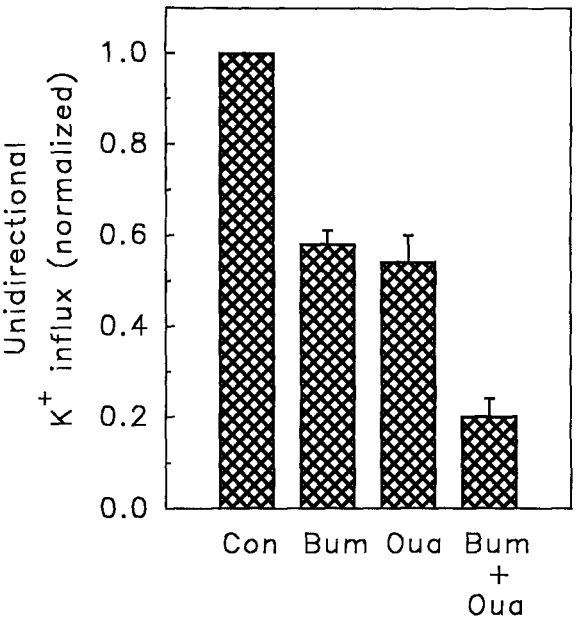

Fig. 5. Effects of bumetanide and ouabain on unidirectional K^+ influxes into cytoplasts. Influxes were measured in the absence of inhibitors (*Con*), with 30 μM bumetanide (*Bum*), with 1 mM ouabain (*Oua*), or with both ouabain and bumetanide at these concentrations (*Bum + Oua*). The drugs were added at time zero together with the tracer. Initial rates of K^+ influx in this and all subsequent figures were determined as in Fig. 2. The results were normalized by taking the ratio of each flux with drug(s) to the control measured on the same preparation. The mean control influx was 0.85 ± 0.09 μmol/g wet wt/min (± SEM, n = 14). The vertical bars represent SEM's from 13, 6, and 3 experiments with bumetanide, ouabain, and bumetanide + ouabain, respectively.

Comparison of Bumetanide Sensitivities of Cotransport in Cells and Cytoplasts

The apparent affinity of the cotransporter for bumetanide, the concentration of bumetanide at half-maximal inhibition of unidirectional K^+ influx ($K_{0.5}$), was determined from measurements of K^+ influxes over a wide range of bumetanide concentrations. $K_{0.5}$'s were determined for cytoplasts, for intact cells with the cotransporter stimulated by cyto B, and for intact cells during RVI with the cotransporter stimulated by osmotic shrinkage. Figure 8 shows the results of three experiments on cytoplasts. The mean $K_{0.5}$ was 0.19 μM ± 0.06, calculated by fitting all of the data simultaneously from the three experiments to the hyperbolic function (*see* Materials and Methods).

Figure 9 shows the results from experiments on cyto B-treated cells. The $K_{0.5}$ was estimated separately for three of the experiments and the mean $K_{0.5}$ was 0.21 μM ± 0.11 (n = 3). (The results indicated by (●) are from an experiment in which a fully inhibiting concentration of bumetanide was not included, and the results could not be fitted to the hyperbolic function.) The curve was generated by fitting all the data simultaneously. There was a large range, nearly threefold, in the control influxes in cyto B-treated cells, i.e., the influxes in the absence of bumetanide. To convey a better idea of the actual control influx in cyto B-treated cells, three additional control influx values (zero bumetanide) have been added to the graph, values obtained in an earlier study (Jessen & Hoffmann, 1992). These values are 26, 29, and 45 μmol/min/g protein.

Figure 10 shows the results of experiments on intact cells during RVI. The $K_{0.5}$ was estimated separately for each experiment; the mean $K_{0.5}$ was 0.67 μM ± 0.11 (n = 6). The curve in Fig. 10 was generated by fitting all of the values simultaneously; the $K_{0.5}$ from this procedure was 0.81 μM.

Comparison of Proteins of Intact Cells and Cytoplasts

Figure 11 shows an electrophoretic gel of membrane proteins from cells and cytoplasts. There are nearly as many proteins in the membranes of the cytoplasts as in the membranes of the whole cells. Some bands stained with similar intensity in the two preparations, for example, the intense band at ~40 kD. There are numerous bands in considerably reduced amounts in the cytoplasts, and a few which stained more intensely in cy-

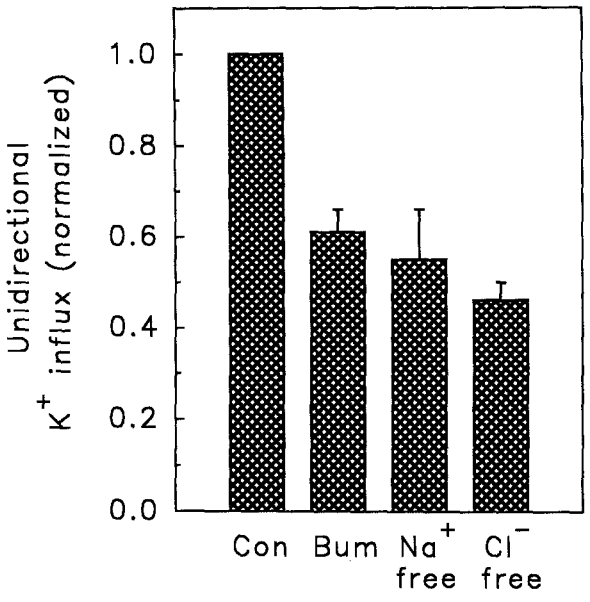

Fig. 6. Dependence of unidirectional K^+ influxes into cytoplasts on Na^+ and Cl^- in the medium. K^+ influxes were measured in normal medium (*Con*), in normal medium + 30 μM bumetanide (*Bum*), in Na-free medium (with NMDG substituted for Na^+), and in Cl-free medium (with gluconate substituted for Cl^-). The results were normalized as in Fig. 4. The cytoplasts were transferred to the appropriate media immediately prior to addition of $^{86}Rb^+$. The vertical bars represent SEM's from 5, 3, and 3 experiments with bumetanide, Na-free and Cl-free media, respectively.

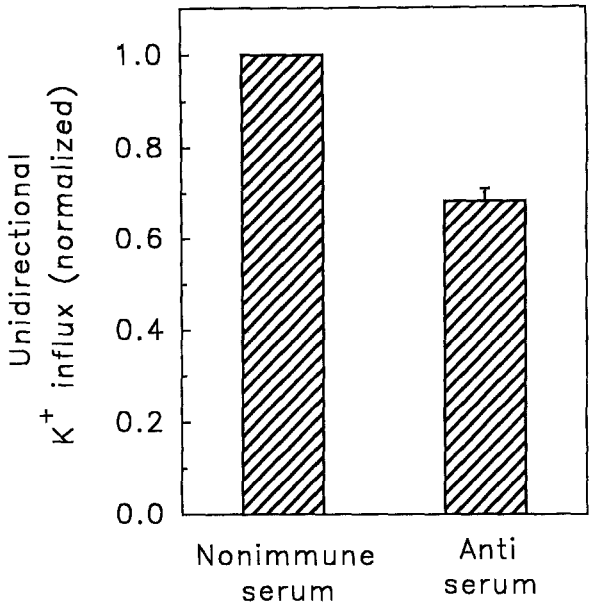

Fig. 7. Effect on unidirectional K^+ influx into cytoplasts of Fab fragments prepared from antibodies against purified bumetanide-binding proteins. In the control, Fab fragments prepared from nonimmune rabbit serum were used. Before measuring the fluxes, cytoplasts were collected by centrifugation (700 × *g*, 30 sec), resuspended in a normal medium containing a Fab preparation and incubated for 20 min at 37°C. Fab fragments were at concentrations equivalent to a 1:4 dilution of the sera. The control K^+ influx (nonimmune Fab fragments) were inhibited 32% ± 3 ($n = 3$) by Fab fragments prepared from the antiserum.

toplasts. Particularly notable is a band at ~80 kD, which is similar to the molecular weight previously reported (Feit et al., 1988) for the main membrane protein isolated using a bumetanide-Sepharose affinity column. The same membrane protein was reported identified by antibodies against purified bumetanide-binding proteins (Dunham et al., 1990).

Immunodetection of Cytoplast Proteins

To determine if the antibodies against the purified bumetanide-binding protein (Dunham et al., 1990) immunodetect any proteins in the cytoplast preparation, we separated the proteins by SDS/PAGE, and a Western blot was made from the gel. Figure 12 shows a blot from the gel treated with antiserum. The blot demonstrates strong immunodetection of a ~76 kD protein which is similar to the molecular weight previously reported (Feit et al., 1988) and to the band which is stained more intensely in cytoplasts than in total membranes (*see* Fig. 11).

Discussion

We show here that cytoplasts of Ehrlich ascites tumor cells, large vesicles derived from the cell surface membrane, have a permanently activated Na-K-2Cl cotransport system, and that the membrane vesicles are enriched with the cotransporter protein expressed per g membrane protein. This is the main finding of this study, providing an advantageous system for the study of the mechanism of the cotransporter. The evidence for the presence of the cotransporter is that a component of K^+ influx is inhibited by both bumetanide and an antibody specific for the cotransporter. Furthermore, the bumetanide-inhibitable component of K^+ influx requires both Na^+ and Cl^-. The cotransporter in the cytoplasts is in an activated state: bumetanide-inhibitable K^+ influx is several-fold higher in a homogenate of the cells containing the cytoplasts than it is in intact cells (Fig. 2). However, the system does not appear to be fully activated as the fluxes are lower by twofold or more than in intact cells during RVI.

The cytoplasts are prepared from blebs (*see* Fig. 1) induced in the cell surface by cyto B, an agent which also activates cotransport. Both of these effects of cyto B are most likely a consequence of modification of cytoskeletal proteins by cyto B, specifically disassembly of microfilaments (Stossel, 1989). Evidence has been obtained that disassembly of microfilaments is associated with activation of Na-K-2Cl cotransport in Ehrlich

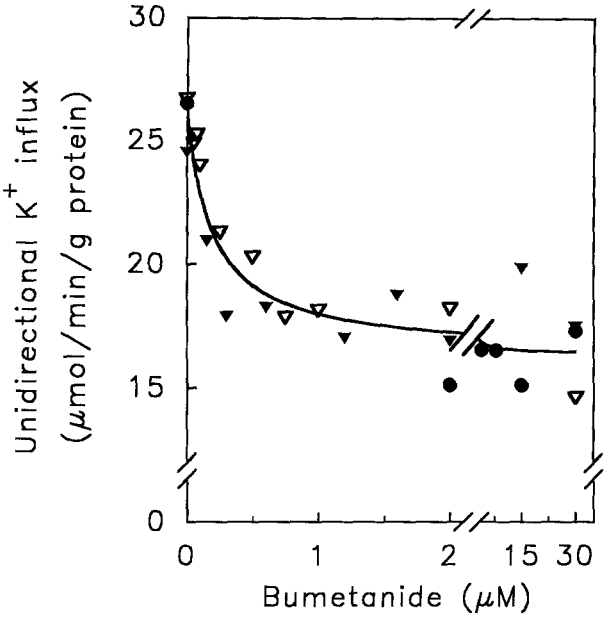

Fig. 8. Inhibition of unidirectional K^+ influxes by bumetanide in cytoplasts. Bumetanide was added to the concentrations indicated, along with $^{86}Rb^+$, at time zero. The curve was generated by fitting all of the data simultaneously to the hyperbolic function as described in Materials and Methods.

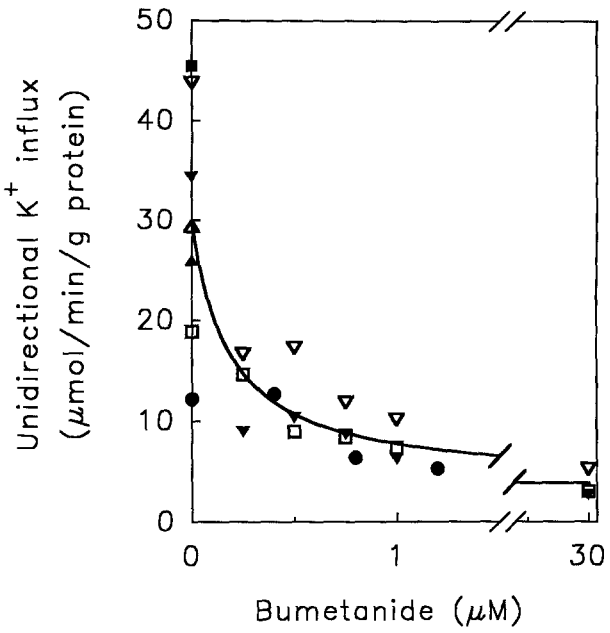

Fig. 9. Inhibition of K^+ influxes by bumetanide in intact cells with Na-K-2Cl cotransport stimulated by pretreatment with cyto B at 42 μM for 1 min. Bumetanide was added to the concentrations indicated, along with $^{86}Rb^+$, at time zero. The $K_{0.5}$ for bumetanide was estimated separately for each of three experiments; the mean $K_{0.5}$ was 0.21 μM ± 0.11. The curve was generated by fitting all the data simultaneously; the $K_{0.5}$ obtained from this procedure was 0.18 μM. Three additional control influx values have been added to the graph, values obtained in an earlier study (Jessen & Hoffmann, 1992). These values are 26, 29, and 45 μmol/min/g protein.

cells (Jessen & Hoffmann, 1992; Cornet, Lambert & Hoffmann, 1993). Undoubtedly it is the cyto B-treatment which is responsible for the activated state of the Na-K-2Cl cotransporter in the cytoplasts.

It was recently suggested that both Ca-calmodulin and protein kinase C are involved in the stimulation of Na-K-2Cl cotransport in Ehrlich cells (Jensen et al., 1993). Cotransport is not fully activated in cells stimulated with cyto B (Jessen & Hoffmann, 1992; *compare* Figs. 8 and 9). A similar modest activation of cotransport in intact Ehrlich cells is seen after disassembly of the microfilaments caused by influx of Ca^{2+} (Cornet et al., 1993) in the late phase of regulatory volume decrease (Jessen & Hoffmann, 1992). Only after cell shrinkage, where a significant increase in protein kinase C has been measured (A.K. Larsen, B.S. Jensen and E.K. Hoffmann, *unpublished results*) the cotransporter seems to be fully activated. Therefore, it is possible that the partial activation of the cotransporter in cytoplasts is a consequence of only one of the activating mechanisms, the Ca-calmodulin system.

The apparent affinity of the cotransporter for bumetanide in the cytoplasts and in cyto B-treated cells is more than twofold higher than in intact cells during RVI. Therefore, the higher affinity is attributable to cyto B. The apparent bumetanide affinity of the cotransporter of cyto B-treated intact cells, $K_{0.5}$ of ~0.7 μM, is similar to that of many cells (Haas, 1989).

It is worth noting that there is in the literature a wide range of $K_{0.5}$'s for bumetanide as an inhibitor of Na-K-2Cl cotransport. For most cell types it is 0.05–0.5 μM (Haas, 1989). There are exceptions: the $K_{0.5}$ is 9 μM for pig renal outer medulla (Jørgensen, Petersen & Rees, 1984), 5–10 μM for shark rectal gland (Haas, 1989), and 3 μM for rabbit parotid gland (Turner & George, 1988). By contrast, a particularly high affinity for bumetanide was reported for the cotransporter of another rabbit tissue, renal papillary epithelium, a $K_{0.5}$ of 5×10^{-5} μM (Sands, Knepper & Spring, 1986). It is unclear if this enormous range of apparent affinities is real or is related to differences in techniques.

If it is real, it may have significance. It has been argued that bumetanide binds to the cotransporter at one of the two Cl^- substrate sites (Haas & McManus, 1983). One might expect the enormous range of apparent affinities for bumetanide to be correlated with a corresponding range of affinities for Cl^-, but there is no evidence for a large range of Cl^- affinities in different cell systems (O'Grady et al., 1987). Therefore, bumetanide may bind to a site on the cotransporter other than a substrate site. Turner and George (1988) argued that bumetanide binds to a Cl^- binding site, but not to a site from which Cl^- is transported. A part of the

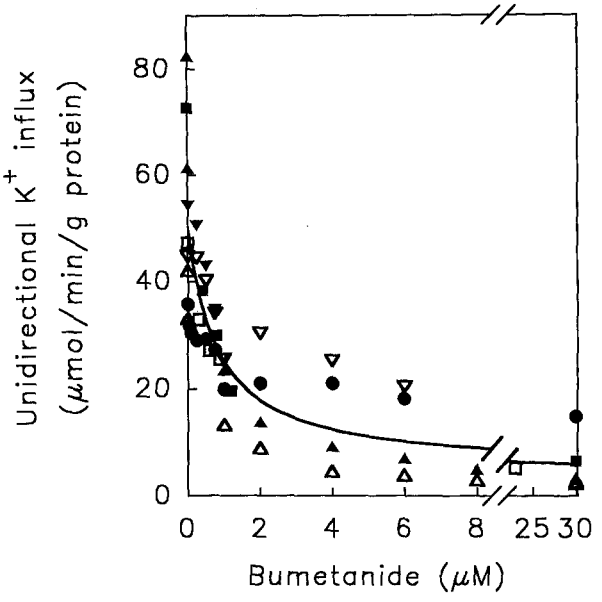

Fig. 10. Inhibition of K^+ influxes by bumetanide in intact cells during RVI. Na-K-2Cl cotransport and volume regulation had been activated by hypertonic challenge by exposing the cells to a medium with all inorganic ion concentrations 1.5 times those in standard medium, the method of Jensen et al. (1993). Bumetanide was added to the concentrations indicated, along with $^{86}Rb^+$, at time zero. The $K_{0.5}$ for bumetanide was estimated separately for each of three experiments; the mean $K_{0.5}$ was 67 μM ± 0.11. The curve was generated by fitting all the data simultaneously; the $K_{0.5}$ obtained from this procedure was 0.81 μM.

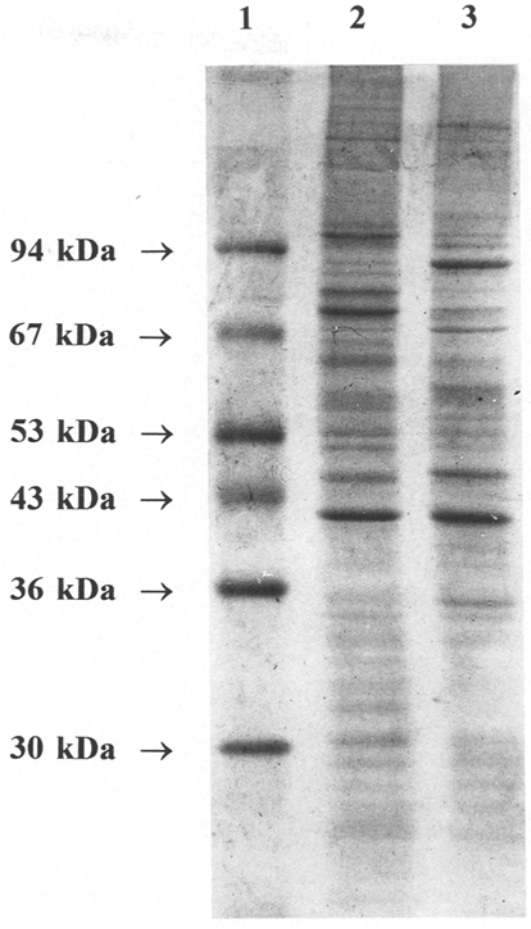

Fig. 11. Membrane proteins from intact Ehrlich cells (lane *2*) and membrane proteins from cytoplasts (lane *3*) separated by electrophoresis on a polyacrylamide gel under denaturing, reducing conditions. About 75 μg protein was loaded in lanes *2* and *3*. Molecular weight markers are in lane *1*. The molecular weights of the markers are 94, 67, 53, 43, 36 and 30 kD.

evidence was that SO_4^{-2} mimicked the inhibitory effect of Cl^- on bumetanide binding, but was without effect on Na^+ flux through the cotransporter. Conversely F^- stimulated Na^+ transport, presumably as an alternate substrate, but did not inhibit bumetanide binding. Similar results were recently presented from experiments on avian red cells (Hegde & Palfrey, 1992).

The increase in the affinity of this anion binding site for bumetanide caused by an agent, cyto B, which also activates cotransport (Fig. 1; Jessen & Hoffmann, 1992) suggests a regulatory function for this binding site. There have been several suggestions for a function of Cl^- in regulating Na-K-2Cl cotransport (Breitwieser, Altamirano & Russell, 1990; Levinson, 1990; Lytle & Forbush, 1992), but the Cl^- binding site, if it exists, is more likely intracellular. Because of the rapid time course of bumetanide binding in Ehrlich cells (equilibration in <1 min; Hoffmann et al., 1986), it is unlikely that bumetanide binds to an intracellular site in spite of it being a lipophilic anion. There is not sufficient data to suggest any relationship between the bumetanide binding site, the Cl^- binding regulatory site, and the effect of cyto B. At the same time, the results on cytoplasts show that this system will be a useful preparation for the study of the mechanism of the Na-K-2Cl cotransporter because of its relative abundance and permanent activation.

We reported earlier a $K_{0.5}$ for bumetanide for intact cells during RVI of ~6 μM (Hoffmann et al., 1986), a 10-fold lower apparent affinity than in the present report. The earlier value came from measurements of net Cl^- influx and from direct measurements of ^3H-bumetanide binding, both during RVI. There is no obvious explanation for the very different results. The possibility can be entertained that it is a consequence of the determination of apparent bumetanide affinity in the present study from unidirectional K^+ fluxes and that K-K exchange exceeds Cl-Cl exchange in Ehrlich cells (Jensen et al., 1993). This in turn would require that K-K exchange is a separate mode of operation of

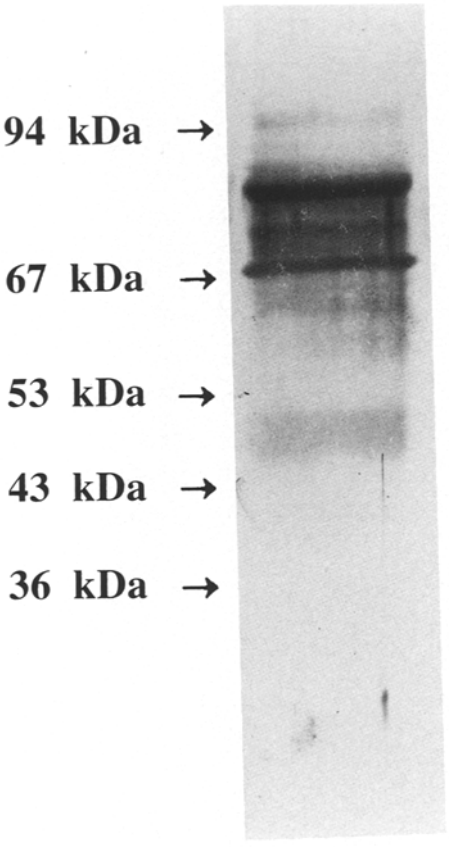

Fig. 12. An immunoblot (Western blot) of the cytoplast preparation using the antibodies against the purified bumetanide-binding proteins.

the cotransporter with a separate bumetanide-binding site. This seems unlikely. It is also possible that the cells have changed in the seven years since the lower bumetanide affinity was measured. This also seems unlikely. This question of the different apparent bumetanide affinities is currently under investigation.

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