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Inhibition of Na–K–Cl cotransport in Ehrlich ascites cells by antiserum against purified proteins of the cotransporter

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ABSTRACT **Two proteins were purified earlier from solubilized membranes of Ehrlich ascites cells by using a bumetanide-Sepharose affinity column. These proteins were proposed to be constituents of the Na–K–Cl cotransporter. However, the specificity of binding of bumetanide to the cotransporter was insufficient evidence for this proposal. We now have direct evidence that the purified protein contains components of the cotransporter. Antiserum raised against the bumetanide-binding proteins strongly inhibits Na–K–Cl cotransport measured by two independent methods. Cotransport was induced by hypertonic challenge and was measured as the bumetanide-sensitive portion of unidirectional Cl influx and as regulatory cell volume increase. In both assays, cotransport was strongly inhibited by the antiserum. Fab fragments of the antibodies inhibited cotransport to a similar extent.**

Na–K–Cl cotransport is widely distributed and plays central roles in regulation of cell volume and in transepithelial transport of salt and water (1–3). The structure of the cotransporter has not been characterized. By contrast the complete primary structures have been determined for several other Na-coupled transporters, including the Na/K pump (4, 5), the Na–glucose cotransporter (6), and the Na/H exchanger (7). An antibody against the Na–K–Cl cotransporter will facilitate the eventual determination of its structure.

The Na–K–Cl cotransporter in Ehrlich ascites tumor cells mediates substantial fluxes in K-depleted cells (8) or during regulatory volume increase (RVI) (9). Transport responsible for RVI was originally envisioned as Na–Cl cotransport (9). The two pathways are distinguishable and may be alternate modes of the same transporter (10, 11). We showed (12) that Na–K–Cl cotransport, not Na–Cl cotransport, is responsible for RVI in Ehrlich cells. The evidence was that Cl influx during RVI required K. In addition there was a bumetanide-inhibitable K influx during RVI dependent on Na and Cl. RVI in Ehrlich cells does not occur unless the cells are preincubated in a hypotonic medium prior to the hypertonic challenge (9). This property, shared with kidney cells (13) among others, has not been explained but may reflect a requirement for the reduction of a critical intracellular solute, perhaps Cl (14).

To isolate proteins of the cotransporter, we employed bumetanide, a sulfamoylbenzoic acid diuretic, which inhibits Na–K–Cl cotransport with high affinity (15). Inhibition of cotransport in Ehrlich cells also has high specificity, the evidence being a 1:1 relationship between [^{3}H]bumetanide binding and inhibition (16). To purify candidate proteins of the cotransporter, we used a bumetanide-Sepharose affinity column (17). Isolated plasma membranes from Ehrlich cells were solubilized and applied to the column. Two main proteins retarded by the column were eluted by bumetanide (17).

We present herein evidence that this purified protein preparation contains constituents of the Na–K–Cl cotransporter: antiserum raised against the bumetanide-binding proteins strongly inhibited bumetanide-sensitive Cl influx and RVI in Ehrlich cells (18). Western blots demonstrated that the antiserum contained antibodies against the purified proteins.

MATERIALS AND METHODS

Cells and Membranes. Ehrlich ascites tumor cells were maintained in white mice by biweekly intraperitoneal transfer (11). Cells were harvested from sacrificed mice and washed by centrifugation as described (16). Plasma membranes from cells disrupted by nitrogen cavitation were isolated by differential centrifugation and were solubilized in *n*-octyl glucoside as described (17).

Solutions and Antisera. Washed cells were suspended at 8% cytocrit in isotonic saline (300 mosmol/kg) containing 150 mM Na; 5 mM K, 1 mM Mg, 1 mM Ca, 150 mM Cl, 1 mM SO_4, 3.3 mM Mops, 3.3 mM Tes, and 5 mM Hepes (pH 7.4). After incubation in isotonic saline for 20–60 min at 37°C, cells were centrifuged and resuspended in a hypotonic medium at ≈50% isotonic osmolarity. Hypotonic medium was made by mixing 1:1 (vol/vol) an isotonic medium and a solution of Mops, Tes, and Hepes at their concentrations in the isotonic saline. Isotonic medium was either the saline described above or rabbit serum (nonimmune serum, antiserum, or preimmune sera from the rabbits to be immunized), assumed to be isotonic and diluted as indicated with the isotonic saline.

Antisera were raised in two rabbits immunized by injection of 0.25 mg of purified bumetanide-binding protein into the popliteal lymph node; second immunizations were made after 2 weeks with 0.1 mg of protein (carried out at the Pocono Rabbit Farm and Laboratory, Canadensis, PA).

Fab fragments of the antibody were produced by mild treatment with papain (19) of either the antiserum or IgG purified from the antiserum by $(NH_4)_2SO_4$ precipitation and anion-exchange chromatography (DEAE-cellulose; Whatman) (19). Equal volumes of antiserum or purified IgG and 0.5 M sodium phosphate (pH 8.0) were mixed, and cysteine and EDTA were added to final concentrations of 10 mM and 2 mM, respectively. Papain (Sigma) was added to a final concentration of 50 μg/ml, and the mixture was incubated for 4 hr at 37°C. The digest was dialyzed for 48 hr at 4°C against 10 mM sodium acetate (pH 5.5). The preparation made directly from antiserum was adjusted to pH 7.4 and to 50% isotonic osmolarity to give a 1:5 dilution of the Fab fragments relative to the initial antiserum. Fab fragments prepared from purified IgG were separated from the papain and Fc fragments by cation-exchange chromatography (CM-cellulose; Whatman) (19). Fab fragments were concentrated by dialysis

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Abbreviations: RVI, regulatory volume increase; RVD, regulatory volume decrease; NEM, *N*-ethylmaleimide; mosmol, milliosmole(s).

against dry polyethylene glycol (20 kDa) followed by gel filtration on a Sephadex G-25 column with 50% isotonic medium.

Purification of Bumetanide-Binding Proteins. A bumetanide-Sepharose affinity column was constructed as described (17). Briefly, 4′-azidobumetanide was bound to activated thiol-Sepharose by photolysis. Solubilized membranes were applied to the column, and retarded proteins were eluted with bumetanide (1 mM). The bumetanide-binding proteins were purified further by rechromatography in the presence of bumetanide, during which bumetanide-binding proteins were not retarded but contaminating proteins were. The proteins were concentrated by trichloroacetic acid precipitation, redissolved in Laemmli buffer (20), and separated by SDS/PAGE (17, 20, 21). Gels were stained with silver. Six high molecular mass standards were used (SDS-6H; Sigma), 29–205 kDa.

Western Blots. The proteins were transferred from gels to nitrocellulose by electroblotting and immunodetected with antiserum by standard techniques (22, 23). The nitrocellulose was blocked with gelatin [3% (wt/vol) gelatin (EIA grade; Bio-Rad) in 0.5 M NaCl/20 mM Tris·HCl, pH 7.5]. The nitrocellulose 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 (1:300 dilution; Bio-Rad).

Unidirectional Cl Influxes. These were measured using ^{36}Cl after a hypertonic challenge. Cells were preincubated 20 min in a 50% hypotonic medium, and then a hypertonic challenge was administered by restoring tonicity to 300 mosmol/kg by addition of an appropriate volume of 2× saline (all salts except the buffers were at 2× their concentration in the isotonic saline); the 2× saline contained ^{36}Cl. Some samples received bumetanide at the same time (final concentration, 25 μM). The suspensions were incubated at 37°C, and samples were removed at intervals for separation of cells from the medium by ion-exchange chromatography (21, 24, 25). An anion-exchange column (Dowex 2, mesh 50–100, Sigma) was prepared in a Pasteur pipet (void volume, 700 μl). The resin was converted in bulk first to the OH form and then to the Mops form by incubation overnight in a Mops solution near saturation (25). In the columns, the resin was washed with 250 mM sucrose/10 mM Mops/1% bovine serum albumin (fraction V; Sigma) brought to pH 7.4 with Tris. Cl readily exchanges with Mops, thereby enabling rapid removal of ^{36}Cl from the cell suspensions. Bovine serum albumin reduces binding of the cells to the column. To abolish binding of cells, columns were preloaded with cells by washing a sample of cells through the column just prior to the experiment; this sample had the same volume and cytocrit as the experimental samples. Samples (160 μl) of the cell suspensions with tracer were applied to chilled columns at selected times and washed through with 1.5 ml of sucrose/bovine serum albumin. The cells emerged in the void volume in seconds, separated from bulk-phase ^{36}Cl (when medium containing ^{36}Cl, but lacking cells, was applied to columns followed by washing, all radioactivity was retained). Radioactivity of cell lysates was determined by liquid scintillation counting in Pico Fluor 15 (Packard). Unidirectional Cl influxes are presented as nmol/mg of cell protein, calculated from radioactivities of cell lysates, specific activities of the medium, and cell protein content of the suspensions (26).

To estimate extracellular ^{36}Cl carried by the cells through the columns in a surface layer, fluxes were measured in each experiment at 4°C. There was a slow influx; extrapolation to time zero gave a value assumed to be extracellular tracer. This "background," ≈50% of the radioactivity of cell samples taken at 1 min at 37°C, was subtracted from all values in each experiment. The backgrounds were determined for cells preincubated in both nonimmune serum and antiserum.

Measurements of Cell Volumes. These were carried out as described (27). Cells were preincubated as described above in hypotonic medium made from the isotonic saline, preimmune serum, nonimmune serum, or antiserum. Samples (200 μl) of these suspensions were added to 100 ml of hypotonic medium for triplicate determinations of swollen-cell volumes prior to the hypertonic challenge. Volumes were determined using a Coulter counter (model ZB with Coulter Channelyzer model C1000, 100-μm tube orifice, calibrated with 13.5-μm diameter latex beads). The hypertonic challenge was administered by delivery of 200 μl of a hypotonic suspension into 100 ml of isotonic saline. Measurements were made at intervals for ≈5 min. Results are expressed as median cell volumes in fl.

RESULTS

Immunodetection of Bumetanide-Binding Proteins. Bumetanide-binding proteins were isolated, and antisera against them were raised. To determine if the antiserum contained antibodies against these proteins, the purified protein preparation was separated by SDS/PAGE, and a Western blot was made from the gel. Fig. 1 shows the silver-stained gel and a blot from the gel treated with antiserum. The gel shows the main bumetanide-binding proteins isolated by affinity chromatography of ≈82 and ≈39 kDa. Earlier estimates of the molecular masses of the major bumetanide-binding proteins were similar, ≈76 and ≈38 kDa (17). The blot demonstrates strong immunodetection of the 82-kDa protein; detection of the 39-kDa band is less clear. The multiple bands in the blot >39 kDa are probably artifacts of the transfer. In other blots of the same protein preparation, there was clear detection of the 39-kDa band. The protein preparation in this experiment was the one used to immunize the rabbits.

Inhibition of Na–K–Cl Cotransport. We tested the antiserum on Na–K–Cl cotransport induced by a hypertonic challenge as described above and earlier (11). The Na–K–Cl influx in turn induces a RVI. We measured the effect of antiserum on cotransport during RVI by two methods, measurements of (*i*) bumetanide-sensitive unidirectional Cl influxes and (*ii*) cell volume increases.

Cl Influx. Cells were exposed to antiserum or nonimmune serum, at 1:6 dilutions, during the hypotonic preincubation. Fig. 2 shows the unidirectional Cl influxes in a representative experiment during RVI, alone or with bumetanide (25 μM) added at the hypertonic challenge. Initial rates of influx were calculated by linear regression from the values at 0.5, 1.0, and 1.5 min. Mean influxes from this experiment and two others like it are presented in Table 1. Antiserum inhibited ≈50% of the initial influx measured in nonimmune serum. Bumetanide inhibited less than antiserum did, and antiserum plus bumetanide inhibited more than antiserum alone; however, neither of these differences was significant. Thus the results indicate that the antiserum inhibits bumetanide-inhibitable Cl influx and essentially only this component of Cl influx; the antiserum appears to be specific for Na–K–Cl cotransport and obviously inhibits cotransport by binding extracellularly.

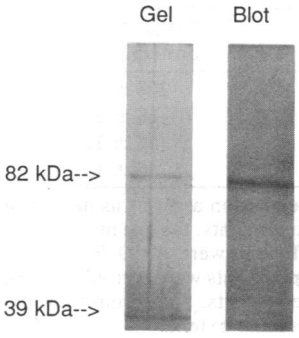

FIG. 1. SDS/polyacrylamide electrophoretic gel (silver-stained) of purified bumetanide-binding proteins and a Western blot of the proteins was immunodetected with the antiserum against the purified proteins.

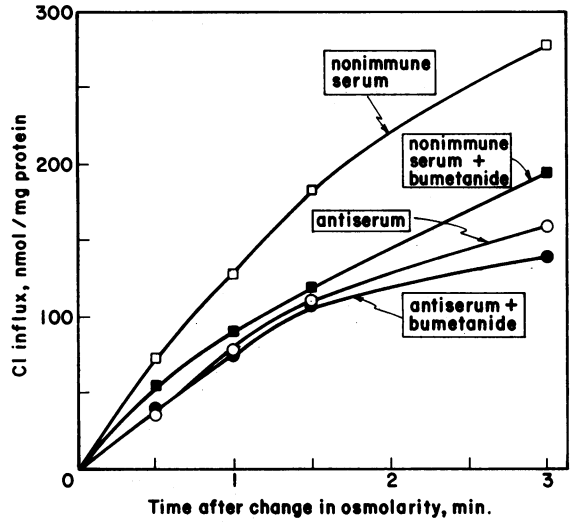

FIG. 2. Effect of antiserum to bumetanide-binding proteins on unidirectional Cl influx in Ehrlich ascites cells during RVI after a hypertonic challenge. The antiserum and nonimmune serum were at 1:6 dilutions during a 20-min preincubation in the hypotonic medium. ^{36}Cl was added simultaneously with the hypertonic challenge, as was bumetanide (to 25 μM) in some samples.

The steady-state level of Cl achieved should be inhibited as well as the initial rate because bumetanide reduces extent of cell volume increase from a hypertonic challenge (11). This was observed in the experiment in Fig. 2 and two others: the steady level of ^{36}Cl at 5 min was inhibited to the same extent (40–50%) by antiserum, bumetanide, and antiserum plus bumetanide (results not shown).

An experiment on unidirectional K influx (^{86}Rb as tracer) was carried out similarly (extracellular tracer separated from cells in a cation-exchange column). Antiserum inhibited most of bumetanide-inhibitable K influx, strengthening the conclusion that the antiserum contains antibodies against the Na–K–Cl cotransporter (B. S. Jensen, F.J., P.B.D., and E.K.H., unpublished results).

RVI. The second measure of Na–K–Cl cotransport was the increase in cell volume during RVI. Fig. 3 shows the results of an experiment in which cells had been preincubated in hypotonic medium with either preimmune serum or antiserum. Cells in hypotonic preimmune serum shrank from 1169 fl to 720 fl after the hypertonic challenge and then increased their volumes 110 fl in 5 min to ≈830 fl (the volume of "fresh" cells; ref. 27). Control cells preincubated without serum regulated their volumes upward by 117 fl after the hypertonic challenge (data not shown). RVI was inhibited ≈98% in cells pretreated with antiserum, which supports the conclusion that antibodies in the antiserum bind to the cotransporter and inhibit its function.

Table 1. Effects of antibodies to bumetanide-binding proteins on initial rates of Cl influx in Ehrlich ascites cells during RVI

Addition(s)	Cl influx, nmol per mg of protein per min
Nonimmune serum	108 ± 13
Nonimmune serum + bumetanide	68 ± 8
Antiserum	55 ± 11
Antiserum + bumetanide	46 ± 13

Fluxes were calculated by linear regression analysis as described in the text from the results of three experiments, the one in Fig. 2 and two others like it. All correlation coefficients were ≥0.99. Results are mean ± SEM (n = 3). These three experiments were carried out with antiserum from one of the immunized rabbits. Antiserum from the other rabbit inhibited Cl influx to a similar extent.

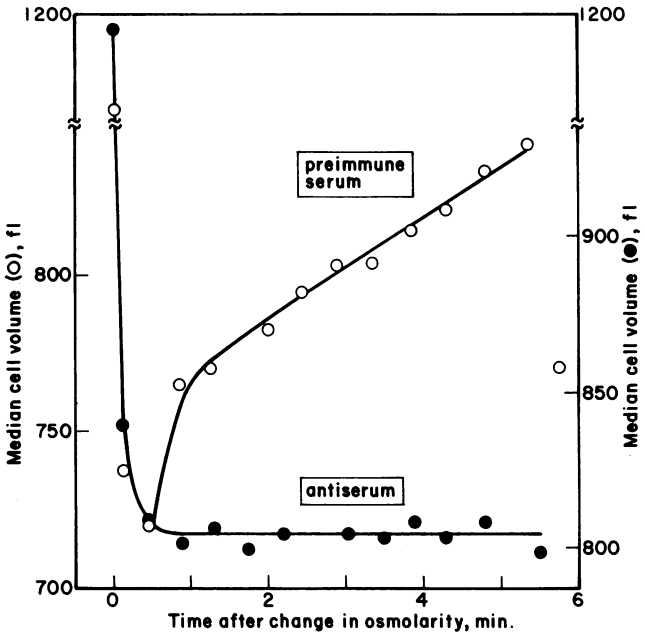

FIG. 3. Effect of antiserum to bumetanide-binding proteins on RVI of Ehrlich ascites cells measured after a hypertonic challenge administered at time zero. Cells had been preincubated in hypotonic medium with preimmune serum or antiserum (1:2 dilutions), after which mean cell volumes were 1169 and 1196 fl for preimmune and antiserum, respectively. The y-axes for cells in preimmune serum (○) and antiserum (●) are labeled differently. In the same experiment bumetanide added with the hypertonic challenge completely inhibited cell volume increase (results not shown). Similar results were obtained in four other experiments of the same design, including one in which antiserum from the other immunized rabbit also completely inhibited cotransport.

The extent of cell shrinkage after the hypertonic challenge was reduced 12% in the antiserum-treated cells (to 800 fl) compared to preimmune serum-treated cells (to 720 fl). The failure to regulate volume in antiserum was not due to this reduced shrinkage. RVI in cells that had shrunk 18% less than controls (by a hypertonic challenge with a medium at ≈85% isotonic osmolality; 255 mosmol/kg) was 70% of the RVI in control cells (300 mosmol/kg) (results not shown).

Therefore, the reduced shrinkage *per se* cannot account for the inhibition of volume regulation. The puzzling inhibition by antiserum of the extent of shrinkage might have been a consequence of crosslinking of surface proteins by immunoglobulins. If so inhibition of cotransport by the antiserum might be an indirect, though perhaps specific, effect of the crosslinking. To test this, Fab fragments of the immunoglobulins were made and tested on RVI. As shown in Fig. 4, Fab fragments inhibited RVI when compared with the volume increase in cells preincubated with nonimmune serum. The inhibition was 95% by Fab fragments prepared directly from the antiserum and diluted 1:5 (Fig. 4*A*). Fab fragments prepared from purified immunoglobulins that were more dilute with respect to the original serum inhibited 50% or more (Fig. 4*B*). Furthermore, Fab fragments did not inhibit the extent of cell shrinkage. Intact antibody tested in the experiment in Fig. 4*A* inhibited RVI completely and also inhibited the extent of cell shrinkage (results not shown). In cells preincubated with Fab fragments, bumetanide inhibited RVI completely (Fig. 4*A*). The observations in Fig. 4 strengthen our conclusion that the antibody binds specifically to the cotransporter. The inhibition by Fab fragments in Fig. 4*A* was not due to the presence of papain: RVI was not inhibited in cells preincubated for 20 min with papain (at 10 μg/ml, the highest concentrations to which cells were exposed; results not shown). The Fab preparation used in Fig.

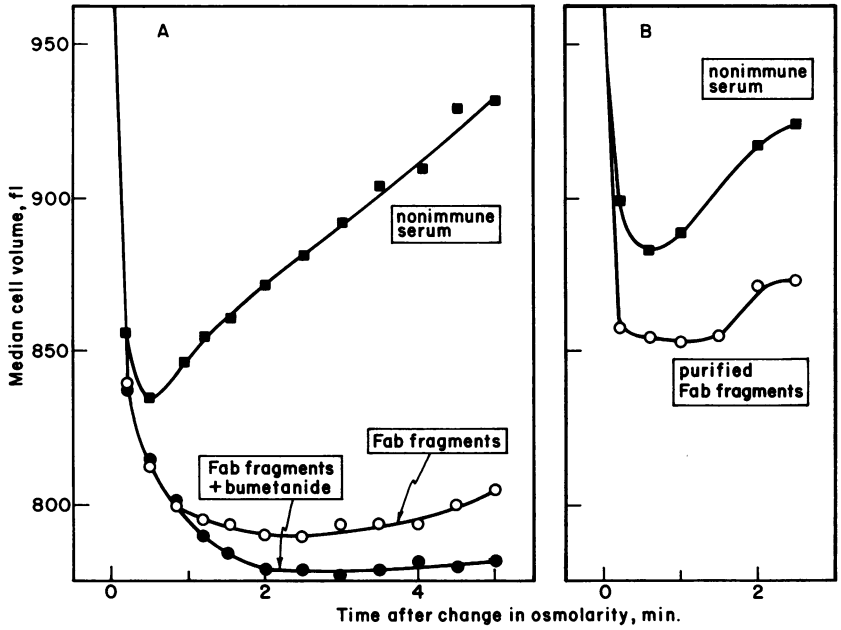

FIG. 4. Effect on RVI of Fab fragments of the antibodies against bumetanide-binding proteins. Fab fragments were prepared either from the antiserum directly (*A*) or from immunoglobulins purified from the antiserum (*B*). Cells were preincubated 20 min in hypotonic medium containing nonimmune serum (diluted 1:5) or a Fab fragment preparation. Fab fragments were at concentrations equivalent to 1:5 (*A*) or ≈1:12 (*B*) dilutions of the antiserum. The median cell volumes in hypotonic media were as follows: 1423 fl for nonimmune serum and 1291 fl for Fab fragments (*A*) and 1421 fl for nonimmune serum and 1360 fl for Fab fragments (*B*). At time zero with the hypertonic challenge, one sample in *A* received bumetanide (25 μM). Results similar to those in *B* were obtained in three other experiments of similar design, including one with a different Fab fragment preparation. Purified Fab fragments inhibited the rate of volume increase to a fraction of 0.60 ± 0.07 of the control (mean ± SEM; $n = 4$). Fab fragments prepared from immunoglobulins purified from nonimmune serum had no effect on RVI.

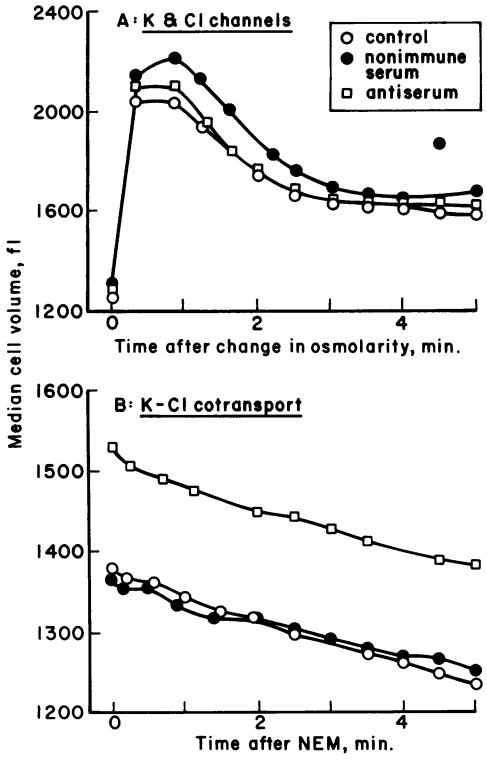

FIG. 5. Effects of antiserum to bumetanide-binding proteins on KCl efflux by two pathways. (*A*) Conductive K and Cl channels during RVD. (*B*) K–Cl cotransport induced by NEM. Measurements of changes in median cell volumes were taken as estimates of KCl efflux (18). (*A*) Cells were preincubated in isotonic medium (control) or isotonic medium plus antiserum or nonimmune serum (1:5 dilutions of sera). At time zero measurements were made of median cell volume, cells were transferred to hypotonic medium (50% of isotonic osmolarity, pH 8.0), and additional measurements of cell volumes were made. (*B*) Cells were incubated in isotonic medium as in *A*. At time zero cell volumes were measured, and NEM was added to a final concentration of 0.1 mM. In the same preparations of cells used in the experiment in *A*, antiserum inhibited RVI nearly completely (results not shown). Each experiment on KCl efflux was repeated on another preparation of cells with identical results.

4*A* was not tested for intact immunoglobulin; the failure to inhibit shrinkage shows that the level of intact antibody was low.

It is possible that antibodies are present that bind to other transporters. Specifically the increased volume of cells exposed to antiserum after 20 min in hypotonic medium might be due to inhibition of regulatory volume decrease (RVD). There are two pathways for the KCl efflux promoting RVD in Ehrlich cells, K and Cl channels and K–Cl cotransport (28). For cells in slightly alkaline medium containing Ca, the channels are the predominant pathway. In slightly acidic and/or Ca-free medium, cotransport is a substantial fraction of KCl efflux; K–Cl cotransport can also be induced by *N*-ethylmaleimide (NEM) (28). We tested the antiserum on both KCl efflux pathways. The results are shown in Fig. 5. To induce efflux through the channels, cells were preincubated in isotonic medium containing antiserum, nonimmune serum, or no serum (control). At time zero, cells were swollen in hypotonic medium (pH 8.0). The results (Fig. 5*A*) show that the cells swelled and then RVD ensued immediately. There was no effect of antiserum on RVD.

To investigate K–Cl cotransport, cells were preincubated 10 min in isotonic medium containing sera or no addition as in Fig. 5*A*. At time zero, NEM was added, inducing K–Cl cotransport and cell shrinkage. Antiserum pretreatment had caused cell swelling but did not inhibit K–Cl cotransport (Fig. 5*B*). The results in Fig. 5 indicate that the antiserum does not inhibit RVD by either KCl efflux mechanism. The swelling induced by antiserum in some experiments, but not all (note Fig. 5*A*), has some other explanation.

DISCUSSION

We report here the production of antiserum containing antibodies against bumetanide-binding proteins purified from membranes of Ehrlich cells. This antiserum strongly inhibits Na–K–Cl cotransport in Ehrlich cells, demonstrated by two measurements of cotransport: (*i*) inhibition of bumetanide-inhibitable Cl influx during RVI or (*ii*) inhibition of the increase in cell volume promoted by cotransport. Therefore, antibodies bind to an extracellular domain of the cotransporter and inhibit its function; one or more of the bumetanide-binding proteins is a constituent of the cotransporter.

A photoactive compound of the bumetanide series, ^{3}H-labeled 4-benzoyl-5-sulfamoyl-3-(3-thenyloxy)benzoic acid, binds to a 150-kDa protein in dog kidney membranes (29) and duck erythrocytes (30). A similar (≈160 kDa) bumetanide-binding protein was partially purified from rabbit parotid gland (31). These proteins may be dimers containing a protein related to the 82-kDa protein that we have identified as a possible constituent of the Na–K–Cl cotransporter in Ehrlich cells. This possibility is supported by our results in a non-denaturing nonreducing cholate gel that indicated that the 82-kDa protein can exist as a dimer (17). Radiation inactivation studies and target-size analyses on rabbit kidney outer medulla have attributed Na–K–Cl cotransport to a molecular mass of 83 kDa (32).

An antibody that inhibits transport in intact cells is not typical. Most antibodies to transporters either do not bind extracellularly or bind without altering function. Examples include antibodies to the Na/K pump (33, 34), the Ca pump (35), and the amiloride-sensitive Na channel (36). In contrast an antibody inhibits Cl channels in *Necturus* gall bladder (37). That antibodies to the Na–K–Cl cotransporter inhibit transport in intact Ehrlich cells may be instructive about the structure of the transport protein. The extracellular domain of the Na–K–Cl cotransporter may be larger than in other transport proteins or may be more immunogenic.

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