J. Membrane Biol. 131, 161–178 (1993)

The Journal of
Membrane Biology


# $Na^+$, $K^+$, $Cl^-$ Cotransport and its Regulation in Ehrlich Ascites Tumor Cells. $Ca^{2+}$/Calmodulin and Protein Kinase C Dependent Pathways

Bo Skaaning Jensen†, Flemming Jessen‡, and Else K. Hoffmann†

†Institute of Biological Chemistry A, The August Krogh Institute, University of Copenhagen, DK-2100 Copenhagen Ø, Denmark, and ‡Technological Laboratory, Ministry of Fisheries, DK-2800 Lyngby, Denmark

**Summary.** Net $Cl^-$ uptake as well as unidirectional $^{36}Cl$ influx during regulatory volume increase (RVI) require external $K^+$. Half-maximal rate of bumetanide-sensitive $^{36}Cl$ uptake is attained at about 3.3 mM external $K^+$. The bumetanide-sensitive $K^+$ influx found during RVI is strongly dependent on both $Na^+$ and $Cl^-$. The bumetanide-sensitive unidirectional $Na^+$ influx during RVI is dependent on $K^+$ as well as on $Cl^-$. The cotransporter activated during RVI in Ehrlich cells, therefore, seems to transport $Na^+$, $K^+$ and $Cl^-$. In the presence of ouabain and $Ba^+$ the stoichiometry of the bumetanide-sensitive net fluxes can be measured at 1.0 $Na^+$, 0.8 $K^+$, 2.0 $Cl^-$ or approximately 1 : Na, 1 : K, 2 : Cl. Under these circumstances the $K^+$ and $Cl^-$ flux ratios (influx/efflux) for the bumetanide-sensitive component were estimated at 1.34 ± 0.08 and 1.82 ± 0.15 which should be compared to the gradient for the $Na^+$, $K^+$, $2Cl^-$ cotransport system at 1.75 ± 0.24.

Addition of sucrose to hypertonicity causes the Ehrlich cells to shrink with no signs of RVI, whereas shrinkage with hypertonic standard medium (all extracellular ion concentrations increased) results in a RVI response towards the original cell volume. Under both conditions a bumetanide-sensitive unidirectional $K^+$ influx is activated. During hypotonic conditions a small bumetanide-sensitive $K^+$ influx is observed, indicating that the cotransport system is already activated.

The cotransport is activated 10–15 fold by bradykinin, an agonist which stimulates phospholipase C resulting in release of internal $Ca^{2+}$ and activation of protein kinase C.

The anti-calmodulin drug pimozide inhibits most of the bumetanide-sensitive $K^+$ influx during RVI. The cotransporter can be activated by the phorbol ester TPA. These results indicate that the stimulation of the $Na^+$, $K^+$, $Cl^-$ cotransport involves both $Ca^{2+}$/calmodulin and protein kinase C.

**Key Words** $Na^+$, $K^+$, $Cl^-$ cotransport · cell volume regulation · bradykinin · $Ca^{2+}$/calmodulin · protein kinase C · Ehrlich ascites tumor cells

## Introduction

Following shrinkage in hypertonic (high NaCl) medium, a regulatory volume increase (RVI) can be observed in some tissues and cell types [e.g., *Amphiuma* red blood cells (Cala, 1980)]. Other cells, such as Ehrlich ascites tumor cells, simply shrink as osmometers with no sign of volume recovery after addition of sucrose (*see* Hoffmann & Simonsen, 1989) or NaCl (Hempling, 1960; Levinson, 1991b). The same kind of behavior is shown by lymphocytes (Grinstein et al., 1984; *see* Rotin, Mason & Grinstein, 1991) and frog skin epithelial cells (Ussing, 1986) after addition of double-strength Ringer's solution. Nearly all cells, however, show a volume recovery when hypotonic pretreatment resulting in a net loss of KCl (the RVD response) is followed by restoration of tonicity. After the initial osmotic shrinkage the cells recover their volume with an associated KCl uptake. It is not clear why some cells show an RVI response only when the "RVD after RVI" protocol is applied. This conditioned RVI response has been called by Lewis and Donaldson (1990) "Pseudo-Regulatory Volume Increase." The main features of the RVD and the RVI response in Ehrlich ascites tumor cells have recently been reviewed (Hoffmann, Lambert & Simonsen, 1988; Hoffmann & Simonsen, 1989; Hoffmann, Simonsen & Lambert, 1992).

It was previously demonstrated that the primary process during the RVI response in Ehrlich cells is an activation of an otherwise quiescent bumetanide-sensitive $Na^+$, $Cl^-$ and/or $Na^+$, $K^+$, $Cl^-$ cotransport system, with subsequent replacement of $Na^+$ by $K^+$ via the $Na^+/K^+$ pump, stimulated by the $Na^+$ influx (Hoffmann, Sjøholm & Simonsen, 1983). The available evidence suggested involvement of $Na^+$, $Cl^-$ cotransport rather than $Na^+$, $K^+$, $Cl^-$ cotransport.

In this investigation we present evidence that the $Na^+$, $K^+$, $Cl^-$ cotransporter is involved in the RVI process in Ehrlich cells rather than $Na^+$, $Cl^-$ cotransport.

The actual mechanism of activation of the RVI response in osmotically shrunken cells is unknown.

In frog skin epithelial cells Ussing (1982) has proposed that reduced internal $Cl^-$ may play a permissive role and modulate the response to cell shrinkage. A similar role has been suggested to be played by low internal $Cl^-$ in the activation of the RVI response in lymphocytes (Grinstein, Clarke & Rothstein, 1983). In squid giant axons cell shrinkage at high external osmolarity has recently been reported to stimulate influx via the $Na^+$, $K^+$, $Cl^-$ cotransporter by shifting the relationship between internal $Cl^-$ concentration and influx via the cotransport system, and hence reduce inhibition of the cotransporter by internal $Cl^-$ (Breitweiser, Altamirano & Russell, 1990).

In avian red cells cell shrinkage may influence the operation of the $Na^+$, $K^+$, $Cl^-$ cotransport system by inducing a conformational change similar to that induced by cAMP (*see* Palfrey & Rao, 1983; Siebens, 1985), although pharmacologic evidence suggests that phosphorylation stimulated by shrinkage and by cAMP involve different protein kinases (Pewitt et al., 1990).

The cAMP level does not seem to play any role in the volume-sensitive cotransport in Ehrlich cells (Geck & Pfeiffer, 1985). Theophylline and other alkylxanthines have been reported to inhibit the furosemide-sensitive cotransport in Ehrlich cells, presumably by reducing the critical cell volume above which the cotransport system becomes quiescent. The cotransport inhibition by these drugs is not mediated by cAMP or cGMP (Geck & Pfeiffer, 1985).

The role of cAMP in the regulation of the $Na^+$, $K^+$, $Cl^-$ cotransport system is ambiguous. Apart from avian red cells (*see above*) cAMP stimulates the cotransport system in cultured myocardiac cells from chicken (Liu et al., 1989) and in epithelia such as shark rectal gland (Silva et al., 1984), canine airway epithelia (Haas, Johnson & Boucher, 1990), and intestinal epithelial cell lines (Mandel, Dharmsathaphorn & McRoberts, 1986; Kim et al., 1988), whereas cAMP inhibits the cotransport system in vascular smooth muscle cells (Smith & Smith, 1987), vascular endothelial cells (Brock et al., 1986), fibroblasts (Owen & Prastein, 1985), human erythrocytes (Garay, 1982), and ferret red cells (Mercer & Hoffman, 1985). As discussed previously, there is evidence that cAMP may convert $K^+$-independent $Na^+$, $Cl^-$ cotransport into $K^+$-dependent $Na^+$, $K^+$, $Cl^-$ cotransport in mouse medullary thick ascending limb cells (Sun et al., 1991).

cGMP has also been implicated in the regulation of the $Na^+$, $K^+$, $Cl^-$ cotransporter. The cotransporter is stimulated by cGMP in vascular smooth muscle cells (O'Donnell & Owen, 1986), but inhibited in endothelial cells (O'Donnell, 1989) and in HeLa cells (Kort & Koch, 1990).

$Ca^{2+}$/calmodulin and protein kinase C have also been assigned a role in the activation of the $Na^+$, $K^+$, $Cl^-$ cotransporter (for review, *see e.g.*, Haas, 1989). In the present investigation the involvement of these two regulatory systems in the activation of the $Na^+$, $K^+$, $Cl^-$ cotransport will be elucidated. Whether the signal transduction pathway by which hypertonicity stimulates the $Na^+$, $K^+$, $Cl^-$ cotransporter may occur via a $Ca^{2+}$/calmodulin-dependent mechanism and/or via protein kinase C is also investigated. Some of the present data have been presented in abstract form (Jensen & Hoffmann, 1990; Jensen, Jessen & Hoffmann, 1992).

## Materials and Methods

### Cell Suspension

Ehrlich ascites tumor cells (hyperdiploid strain) were maintained in white NMRI mice by weekly intraperitoneal transplantation. Each third week the cells to be transplanted were washed in standard incubation medium prior to implantation. This wash prevented transfer of an eventual infection together with the tumor cells. Cells were harvested from sacrificed mice and washed with standard medium by centrifugation as described previously (*see* Hoffmann, Lambert & Simonsen, 1986). The washed cells were suspended at 8% cytocrit in standard medium, which contained (mM): 150 $Na^+$, 5 $K^+$, 1 $Mg^{2+}$, 1 $Ca^{2+}$, 150 $Cl^-$, 1 $SO_4^{2-}$, 1 $HPO_4^{2-}$, 3.3 MOPS (3-(N-morpholino) propane sulfonic acid), 3.3 TES (N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid), 5 HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid).

### Incubation Media

The following media were used: (A) Standard medium 300 mOsm (*see above*); (B) Hypotonic 225 mOsm and 150 mOsm saline solutions prepared by diluting the standard medium with distilled water containing MOPS, TES and HEPES in concentrations as in solution A, in order to lower both ion concentrations and total osmolarity; (C) Double-strength saline solution with double ion concentrations compared to solution A, but with the same buffer concentration; (D) Hypotonic or double-strength nitrate media were prepared by substituting the $Na^+$, $K^+$ and $Ca^{2+}$ salts of $NO_3^-$ for NaCl, KCl and $CaCl_2$, respectively; (E) Hypotonic or double-strength N-methyl-D-glucamineCl media (NMDGCl) where N-methyl-D-glucammonium was substituted for $Na^+$. NMDGCl was prepared from a N-methyl-D-glucamine stock solution titrated with the equimolar amount of HCl; (F) Double-strength media, where $NMDG^+$ was substituted for some or all of the $K^+$, contained (mM): 219 $Na^+$ 0–90 $K^+$, 2 $Mg^{2+}$, 2 $Ca^{2+}$, 315 $Cl^-$, 2 $SO_4^{2-}$, 2 $HPO_4^{2-}$, 11–101 $NMDG^+$, 3.3 MOPS, 3.3 TES, 5 HEPES; (G) Double-strength and hypotonic media, where NaCl was substituted for KCl; (H) 600 mOsm sucrose.

### Reagents

All reagents were analytical grade. Staurosporine, bradykinin, Fura-2-AM and 12-O-tetradecanoylphorbol 13-acetate (TPA) were obtained from Sigma, St. Louis, MO. Pimozide was a gift

from Lundbeck & Co., Copenhagen. Bumetanide was a gift from Dr. P. W. Feit. Bradykinin was added to the cell suspension from a 500 $\mu$M stock solution in ethanol, TPA from a 25 $\mu$M stock solution in ethanol, staurosporine from a 500 $\mu$M stock solution in dimethylsulfoxide (DMSO), pimozide from a 5 mM stock solution in ethanol and bumetanide from a 5 mM stock solution in ethanol. Fura-2-AM was added from a 1 mM stock solution in DMSO.

## Measurements of Ion Content

Cells (1 ml cell suspension) were separated from the medium by centrifugation (20,000 × $g$, 60 sec.) in preweighed vials. An aliquot of the supernatant was removed for determination of medium concentrations of $Na^+$, $K^+$, and $Cl^-$. The pellet was lysed with distilled water, deproteinized with perchloric acid (PCA, final concentration 7%) and subsequently centrifuged (20,000 × $g$, 10 min). The supernatant was used for determination of cellular $Na^+$, $K^+$, and $Cl^-$ after correction for trapped extracellular medium, defined as $^3$H-inulin space as described previously (Hoffmann et al., 1983). The pellet was used for determination of cell dry weight after correction for the PCA content in the pellet. It was determined in separate experiments that the actual cell dry weight is 77% of the measured dry weight of the PCA precipitate (Lambert, Hoffmann & Jørgensen, 1989). Potassium and sodium were assessed by atomic absorption spectrophotometry (Perkin Elmer atomic absorption spectrophotometer, model 2380). $Cl^-$ was determined by coulometric titration (CMT 10 Chloride titrator, Radiometer, Denmark).

The cellular ion concentrations are given as the concentration in cell water after correction for trapped volume ([$^3$H] inulin space) in the cell pellets.

## Measurements of Cell Volume

Cells were incubated in the standard incubation medium (cytocrit 8%). Samples (200 $\mu$l) of this suspension were diluted into 50 ml of standard medium, whereupon 50 ml experimental medium was added for determination of cell volume using a Coulter counter (model ZB equipped with a Coulter channelyzer, model C-1000). The tube orifice was 100 $\mu$m. Calibration was carried out with latex beads (13.5 $\mu$m, Coulter Electronics, England).

## Potassium Influx Measurements

### 1. Protocol

The unidirectional $K^+$ influx was measured using $^{86}$Rb (Risø, Denmark) as a tracer. Cells were preincubated 15–40 min in the actual incubation medium, except for cells that were pretreated with staurosporine (100 nM) which were preincubated for 40 min. The flux was initiated by mixing the cell suspension (cytocrit 8%) with 1/3 volume of experimental medium containing either $^{86}$Rb (50,000 Bq/ml) or $^{86}$Rb plus bumetanide (final concentration, 30 $\mu$M). Some aliquots received pimozide (120 $\mu$M) just prior to addition of experimental medium, whereas others received bradykinin (1 $\mu$M) or 12-O-tetradecanoylphorbol 13-acetate (TPA, 100 nM) with the experimental medium. The effect of staurosporine (100 nM) was examined after 40 min of preincubation before addition of the experimental medium. The cell suspensions were incubated at 37°C, and samples were removed at intervals for separation of cells from the medium by ion exchange chromatography (Gasko et al., 1976, Garty, Rudy & Karlish, 1983, Jessen et al., 1989). Briefly: cation exchange columns (Dowex 50, mesh 50–100, Fluka) were prepared in Pasteur pipettes (void volume, 700 $\mu$l). The resin was converted in bulk to the Tris form by washing the resin in distilled water followed by incubation overnight in a Tris solution near saturation. In the columns, the resin was washed with a solution containing sucrose (250 mM), MOPS (10 mM) and bovine serum albumin (BSA, fraction V; 1%, Sigma) brought to pH 7.4 with Tris. $K^+$ and $Rb^+$ readily exchanges with Tris, thereby enabling rapid removal of $^{86}$Rb from the cell suspensions. BSA reduces binding of the cells to the column. Binding of cells was also prevented by preloading the columns with cells by flushing the column with a sample of the cell suspension just prior to the experiment. Samples (160 $\mu$l) of the cell suspension with tracer were applied to ice-cold columns at selected times and subsequently flushed with 1.5 ml of the sucrose/BSA solution. The cells emerged in the void volume in seconds, separated from bulk-phase $^{86}$Rb. (When a sample containing $^{86}$Rb without cells was applied to a column as described above, all radioactivity was retained in the column.) Unidirectional $K^+$ influxes are presented as $\mu$mol/g cell dry weight, calculated from the radioactivities of the cell lysates, the specific activities of the medium, and dry weight of the cell suspensions.

### 2. Protocol

The unidirectional $^{42}$K (Risø, Denmark) during regulatory volume increase presented in Fig. 6 and Table 4 was measured by adding $^{42}$K to the external medium and monitoring the cell $^{42}$K activity with time in parallel with measurement of the ion content (*see above*).

## Chloride Influx Measurements

### 1. Protocol

The unidirectional influx of $Cl^-$ was assessed by ion exchange chromatography using $^{36}$Cl (Amersham, England) as a tracer. The procedure was essentially as described for unidirectional $K^+$ influx measurements except that the activity of $^{36}$Cl was 40,000 Bq/ml and the resin was Dowex 2 (mesh 50–100, Sigma). The resin was first converted to the $OH^-$ form with 1 N NaOH and then to the MOPS form by incubation overnight in a nearly saturated MOPS solution. Unidirectional $Cl^-$ influxes are presented as $\mu$mol/g protein, calculated from the radioactivities of the cell lysates, the specific activities of the medium, and cell protein content of the suspensions (Peterson, 1977).

### 2. Protocol

The unidirectional $^{36}$Cl influx during regulatory volume increase presented in Fig. 6 and Table 4 was measured by adding $^{36}$Cl to the external medium and monitoring the cell $^{36}$Cl activity with time in parallel with measurement of the ion content (*see above*).

## Sodium Influx Measurements

The unidirectional influx of $^{22}$Na was assessed by ion exchange chromatography using $^{22}$Na (Amersham, England) as a tracer. The procedure was essentially as described for unidirectional $K^+$

influx measurements except that the activity of $^{22}Na$ was 40,000 Bq/ml.

### Fluorometric Measurement of Intracellular $Ca^{2+}$

For intracellular $Ca^{2+}$ concentrations 40 ml cell suspension (cytocrit 4%) in standard medium with 0.2% BSA was loaded with 40 $\mu$l 1 mM Fura-2-AM for 20 min at 37°C. The cells were washed once with standard medium and finally resuspended at a cytocrit of 5% in standard medium. The Fura-2 fluorescence measurements were performed on 3 ml suspension with a 0.5% cytocrit. Fluorometric measurements were performed in polystyrene cuvettes on a Perkin Elmer R 100A recorder and a Perkin Elmer CP 100 graphic printer. The temperature of the cuvette was thermostatically controlled and the cell suspension was continuously stirred by use of a Teflon-coated magnet, driven by a motor attached to the cuvette house.

The measurements were obtained by rapidly shifting the excitation monochrometer between 340 nm and 380 nm, and measuring the emission constantly at 510 nm (*see* Grynkiewicz, Poenie & Tsien, 1985). At the end of each experiment the cell suspension is centrifuged and fluorescence of the extracellular medium is measured. This value is subtracted from all measurements. The free $Ca^{2+}$ concentration was calculated from the measurements of the ratio of fluorescence intensities according to the equation:

$$[Ca^{2+}] = K_d \cdot ((R - R_{min})/(R_{max} - R) \cdot S_{f380}/S_{b380}$$

where $K_d$ is the dissociation constant (0.135 $\mu$M, *see* Grynkiewicz et al., 1985) and $R$ is the fluorescence ratio of 340 nm and 380 nm excitation. $R_{max}$ and $R_{min}$ are the equivalent fluorescence ratios of Fura-2 after addition of digitonin at saturating $Ca^{2+}$ concentrations, and in nominally $Ca^{2+}$-free medium (with 1 mM EGTA), respectively. $S_{f380}$ and $S_{b380}$ are proportionality coefficients, measured from the fluorescence intensity at 380 nm excitation using calibration solutions containing low concentrations of free $Ca^{2+}$ and $Ca^{2+}$ concentrations where the dye is saturated.

### Measurements of Radioactivity

Radioactivity of cell lysates was determined by liquid scintillation counting (Packard TriCarb 460 C Liquid Scintillation System) using Ultima Gold™ (Packard) as scintillation fluid.

## Results

### Regulatory Volume Increase

When Ehrlich ascites tumor cells are preincubated in a hypotonic medium for 15–40 min to reduce their intracellular ion content and then resuspended in an isotonic medium, the initial osmotic cell shrinkage is followed by a rapid volume increase back towards the original cell volume. This involves a net uptake of KCl followed by a concomitant water uptake (Regulatory Volume Increase, RVI) (Hoffmann et al., 1983). Table 1 shows that the Ehrlich ascites tumor cells are unable to perform such RVI response in the absence of either $Na^+$, $K^+$ or $Cl^-$. Table 1 shows further that Ehrlich cells, even after the initial osmotic shrinkage (time 0 to 0.5 min), continue to shrink in a $Na^+$-free, $K^+$-free, or $Cl^-$-free medium.

**Table 1.** Dependence on $Na^+$, $K^+$, and $Cl^-$ of the "Regulatory Volume Increase" after hypertonic challenge

| | Cell volume increase after hypertonic shock fl/min |
|---|---|
| Control | 39 ± 2 (8) |
| Sodium free | −13 (2) |
| Potassium free | −49 (2) |
| Chloride free | −28 (2) |

The cells were preincubated for 15–30 minutes in a hypotonic medium (150 mOsm). At time zero a sample of the cell suspension was diluted 500- to 1,500-fold in standard incubation medium and the cell volume was followed with time using a Coulter counter. Choline was substituted for $Na^+$ in the $Na^+$-free medium, $NMDG^+$ was substituted for $K^+$ in the $K^+$-free medium and nitrate was substituted for $Cl^-$ in the $Cl^-$-free medium. The changes in cell volume are calculated from the values 0.5 and 4.5 min after the hypertonic challenge. The values are given as mean ± SEM with the number of experiments given in parentheses.

The net uptake of $Cl^-$ during RVI has previously been demonstrated to be strongly dependent on extracellular $Na^+$, since no cellular $Cl^-$ uptake could be demonstrated during RVI after substitution of extracellular $Na^+$ with choline (Hoffmann et al., 1983).

The $K^+$ dependence of the $Cl^-$ uptake during RVI is investigated by two independent methods: (i) the $K^+$ dependence of the net uptake of $Cl^-$, and (ii) the $K^+$ dependence of bumetanide-sensitive unidirectional $Cl^-$ influx.

Figure 1 shows the net $Cl^-$ uptake during RVI at different extracellular $K^+$ concentrations as a function of time. It is seen that at low concentrations of extracellular $K^+$ (less than 2 mM) no net $Cl^-$ uptake occurs during the first 4–6 min after hypertonic shock. On the contrary, Fig. 1 also demonstrates that the cellular $Cl^-$ uptake is stimulated at high extracellular $K^+$ concentrations (12 mM) as compared to the cellular $Cl^-$ uptake at normal extracellular $K^+$ concentrations (5 mM).

Figure 2 shows the unidirectional $Cl^-$ uptake during RVI at 10 mM $K^+$ in the presence and absence of bumetanide as a function of time. Bumetanide is known to inhibit cotransport systems in Ehrlich ascites tumor cells (Geck et al., 1978; Geck et al., 1980; Aull, 1981; Hoffmann et al., 1983) and is used

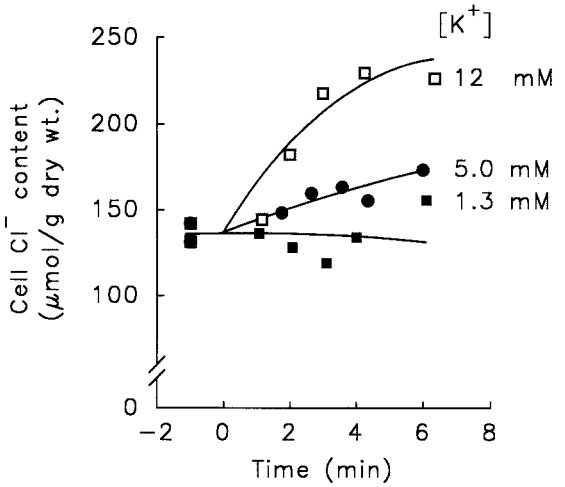


**Fig. 1.** Dependence on extracellular $K^+$ of the net $Cl^-$ influx during Regulatory Volume Increase. The cells were pretreated by exposure to low external osmolarity (225 mOsm) for 20 to 40 min (*see* Materials and Methods). At zero time a tonicity of 300 mOsm was restored by addition of ¼ volume of a double-strength saline solution and cell $Cl^-$ content was followed with time. The presumable impermeable cation N-methyl-D-glucamine was used as a substitute for $K^+$ in the low external $K^+$ experiments. The figure is representative of three separate experiments. The lines were fitted by eye. The large solid squares show $Cl^-$ content just prior to hypertonic challenge.

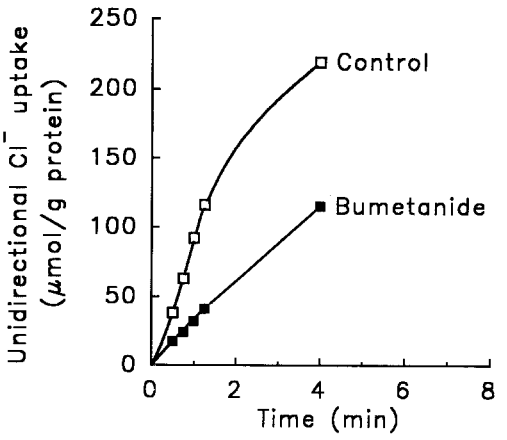


**Fig. 2.** Bumetanide-sensitivity of the unidirectional $Cl^-$ uptake during RVI. The unidirectional $Cl^-$ uptake was measured using $^{36}Cl$ (50,000 Bq/ml). The cells were preincubated for 20 to 40 min in a $K^+$ free hypotonic medium (150 mOsm). At time zero a hypertonic challenge was administered by restoring tonicity to 300 mOsm by addition of an appropriate volume of double-strength saline solution containing $K^+$ as well as the $^{36}Cl$. The final $[K^+]_0$ was 10 mM. Some aliquots received bumetanide at the same time (final concentration, 30 μM).

at a concentration six times the $K_i$ for inhibition of the $Cl^-$-dependent cotransport system (Hoffmann, Schiødt & Dunham, 1986).

Figure 3 shows the bumetanide-sensitive unidirectional $Cl^-$ influx during RVI as a function of extracellular $K^+$. The unidirectional $Cl^-$ influx (initial rates of $Cl^-$ uptake were calculated by linear regression from the values of 0.5, 0.75, 1.00 and 1.25 min) was determined with or without bumetanide (30 μM). The maximal $Cl^-$ influx under these conditions was calculated to be 51.7 μmol · g protein$^{-1}$ · min$^{-1}$ with an apparent $K_m$ of 3.3 mM $K^+$ (*see* Figure 3).

Thus, the activated bumetanide-sensitive unidirectional $Cl^-$ influx as well as the net $Cl^-$ uptake during RVI are $K^+$ dependent. The $K^+$ dependence in Fig. 3 (tracer fluxes) reflects the kinetics of $K^+$ binding to the external site on the cotransporter, whereas the $K^+$ dependence in Fig. 1 (net fluxes) reflects in addition an increase in the driving force with increasing external $K^+$.

To investigate whether the $K^+$ ion is transported together with $Cl^-$ or just acts as a cofactor for the $Cl^-$ uptake during RVI, the bumetanide sensitivity of the $K^+$ influx during RVI was investigated. Figure 4 shows the $K^+$ uptake (using $^{86}Rb$ as tracer) as a function of time after hypertonic shock, alone or in the presence of $Ba^{2+}$ (5 mM), ouabain (1 mM) or bumetanide (30 μM). $Ba^{2+}$ inhibited less than 5% of the total unidirectional K influx during RVI, whereas ouabain inhibited the $K^+$ influx 19%. Bumetanide inhibited approximately 75% of the total $K^+$ influx. The $K^+$ uptake during RVI is mainly via a bumetanide-sensitive transport system. As seen in Table 2, the bumetanide-sensitive unidirectional $K^+$ influx during physiological steady state is negligible (1.2 μmol · g protein$^{-1}$ · min$^{-1}$), compared to the flux (50.7 μmol · g protein$^{-1}$ · min$^{-1}$) found during RVI. This demonstrates a large activation of the bumetanide-sensitive unidirectional $K^+$ influx during RVI.

Table 2 demonstrates that the bumetanide-sensitive $K^+$ influx is dependent on $Na^+$ as well as on $Cl^-$, being reduced by 85% and 99% respectively in the absence of extracellular $Na^+$ or $Cl^-$. The 15% bumetanide-sensitive $K^+$ influx remaining in $Na^+$-free medium might represent a bumetanide-sensitive KCl cotransporter or $K^+/K^+$ exchange via the $Na^+$, $K^+$, $Cl^-$ cotransporter. This would also explain why ouabain does not inhibit 40% of the bumetanide-sensitive $K^+$ influx as expected (*see* legend to Table 2).

Two proteins were purified earlier from solubilized membranes from Ehrlich ascites tumor cells by using a bumetanide-Sepharose affinity column (Feit et al., 1988). Antiserum raised against the bumetanide-binding proteins strongly inhibited the $Na^+$, $K^+$, $Cl^-$ cotransporter measured by two independent methods (Dunham, Jessen & Hoffmann, 1990). Cotransport was induced by hypertonic challenge and was measured as the bumetanide-sensitive portion

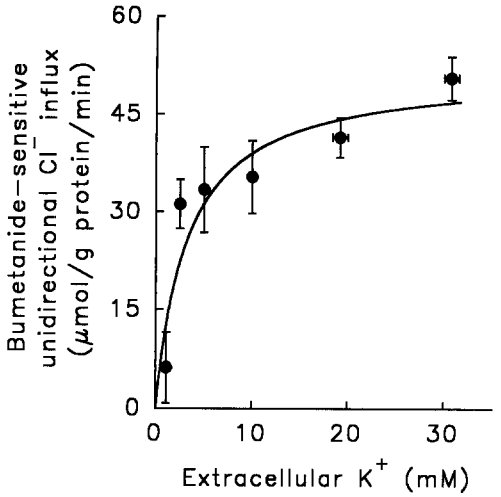


**Fig. 3.** Dependence on extracellular $K^+$ of the activated unidirectional $Cl^-$ influx after a hypertonic challenge. The unidirectional $Cl^-$ influx was measured using $^{36}Cl$ (50,000 Bq/ml). The cells were preincubated for 20 to 40 min in a $K^+$ free hypotonic medium (150 mOsm). At time zero a hypertonic challenge was administered by restoring tonicity to 300 mOsm by addition of an appropriate volume of double-strength saline solution containing the $^{36}Cl$. Some aliquots received bumetanide at the same time (final concentration, 30 $\mu$M). The presumable impermeable cation N-methyl-D-glucamine was used as a substitute for $K^+$. The suspensions were incubated at 37°C, and at desired intervals samples were removed for separation of cells from the medium as described in Materials and Methods. The initial rates of influx were calculated by linear regression from the values at 0.5, 0.75, 1.0 and 1.25 min. All correlation coefficients were $\geq 0.99$. Finally, the fluxes from the aliquots containing bumetanide were subtracted from their controls to give the bumetanide-sensitive $Cl^-$ fluxes. The values are given as mean ± SEM. ($n$ = 3–6) experiments. The curve was fitted to the Michaelis-Menten equation by a fitting program using the Marquardt-Levenberg algorithm.

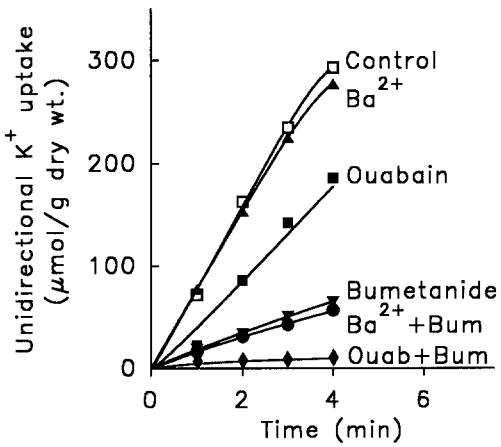


**Fig. 4.** Dependence on barium, ouabain and bumetanide of the $K^+$ influx during Regulatory Volume Increase. Experimental protocol as in Fig. 2, except that $^{86}Rb$ was used as a tracer for $K^+$. Ouabain was used at a final concentration of 1 mM, the concentration of barium was 5 mM and the bumetanide concentration was 30 $\mu$M. Two additional experiments gave similar results.

**Table 2.** Dependence on extracellular $Na^+$ and $Cl^-$ of the activated unidirectional $K^+$ influx during Regulatory Volume Increase

| | Bumetanide-sensitive, unidirectional $K^+$ influx | |
|---|---|---|
| | Physiological steady state | RVI |
| | $\mu$mol · g protein$^{-1}$ · min$^{-1}$ | |
| Control | 1.2 ± 0.4 (5) | 50.7 ± 3.1 (5)[a,b] |
| Sodium free | — | 7.8 ± 2.8 (3) |
| Chloride free | — | −0.3 ± 1.7 (3) |

Experimental protocol as in Fig. 2. $^{86}Rb$ was used as tracer for $K^+$. It should be noted that the final $[K^+]_o$ = 10 mM. The initial rates of influx were calculated from the values at 0.5, 0.75, 1.00 and 1.25 min. All correlation coefficients were $\geq 0.99$. The values are given as mean (± SEM) with the number of experiments given in parentheses.

[a] The total bumetanide-sensitive $K^+$ influx during RVI is a composite comprising $K^+$ influx via the $Na^+$, $K^+$, $Cl^-$ cotransport system plus some $K^+$ influx via the $Na^+$, $K^+$-pump, in exchange for $Na^+$ taken up via the cotransport system. If the $Na^+/K^+$ ATPase is a $3Na^+/2K^+$ exchange pump and all $Na^+$ is immediately exchanged for $K^+$, ouabain will inhibit 40% of the bumetanide-sensitive $K^+$ influx. In three experiments in the presence of ouabain the bumetanide-sensitive $K^+$ influx was reduced by about 15% (*see* Fig. 4).

[b] This value can be converted to $\mu$mol · g dry wt$^{-1}$ · min$^{-1}$ by multiplication by 0.56 giving 28.4 ± 1.7 $\mu$mol · g dry wt$^{-1}$ · min$^{-1}$.

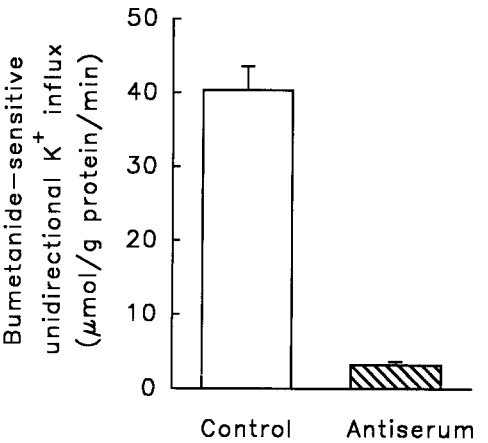


**Fig. 5.** Effect of antiserum to bumetanide-binding proteins on bumetanide-sensitive unidirectional $K^+$ influx in Ehrlich ascites tumor cells during RVI. The antiserum and nonimmune serum (control) were at 1:6 dilutions during a 20-min preincubation in the hypotonic medium. $^{86}Rb$ was added simultaneously with the hypertonic challenge as was bumetanide (30 $\mu$M) in some samples. The values are given as mean ± SEM ($n$ = 3) where $n$ is the number of independent experiments.

of the unidirectional $Cl^-$ influx and as regulatory cell volume increase. In both assays, cotransport was strongly inhibited by the antiserum. These antibodies inhibit the bumetanide-sensitive unidirectional $K^+$ influx as well. This is seen in Fig. 5 which shows

**Table 3.** Dependence on extracellular $Cl^-$ and $K^+$ of the activated unidirectional $Na^+$ influx during Regulatory Volume Increase

| | Bumetanide-sensitive, unidirectional $Na^+$ influx | |
|---|---|---|
| | Physiological steady state | RVI |
| | Relative scale | |
| Control | 0.10 ± 0.25 (3) | 1.00 ± 0.03 (3) |
| Potassium free | — | −0.10 (2) |
| Chloride free | — | 0.48 (2) |

Experimental protocol as in Fig. 2, except that $^{22}Na$ was used as a tracer for $Na^+$. The initial rates of influx were calculated from the values at 0.5, 1.0, 1.5, 2.0 and 2.5 min. All correlation coefficients were ≥ 0.97. All influx-rates are given relative to the bumetanide-sensitive $Na^+$ influx during RVI in standard incubation medium (Control), which is 10.5 ± 0.3 μmol/g dry wt/min. The values are given as mean (± SEM) with the number of experiments given in parentheses.

the bumetanide-sensitive unidirectional $K^+$ influx in the presence of nonimmune serum or antiserum.

Table 3 shows that the $Na^+$ influx during RVI is bumetanide-sensitive and that this bumetanide-sensitive $Na^+$ influx is dependent on extracellular $Cl^-$ and $K^+$. The bumetanide-sensitive $Na^+$ influx is only partly inhibited in the absence of $Cl^-$. Furthermore, Table 3 shows that the bumetanide-sensitive $Na^+$ influx during physiological steady-state conditions is negligible, but is activated during RVI. No bumetanide-sensitive uptake of $Na^+$, $K^+$, or $Cl^-$ can be demonstrated during RVI unless all three ions are present.

Conclusively, the $Cl^-$-dependent cotransport system activated during RVI transports $Na^+$, $K^+$, and $Cl^-$.

## Stoichiometry of the $Na^+$, $K^+$, $Cl^-$ Cotransport System and Flux Ratio Analysis

To assess the stoichiometry of transport during RVI via the cotransport system, the net ion uptake during regulatory volume increase is measured under conditions where the $Na^+/K^+$ pump is inhibited with 1 mM ouabain. From Hoffmann et al. (1983), it is known that no net $K^+$ uptake is seen under these circumstances. This might be caused by a fast recycling of $K^+$ through $K^+$ channels. To avoid this, the present experiments are performed in the presence of 2 mM $Ba^{2+}$ in order to block the $K^+$ channels. The bumetanide-sensitive part of the fluxes are taken to represent the cotransporter.

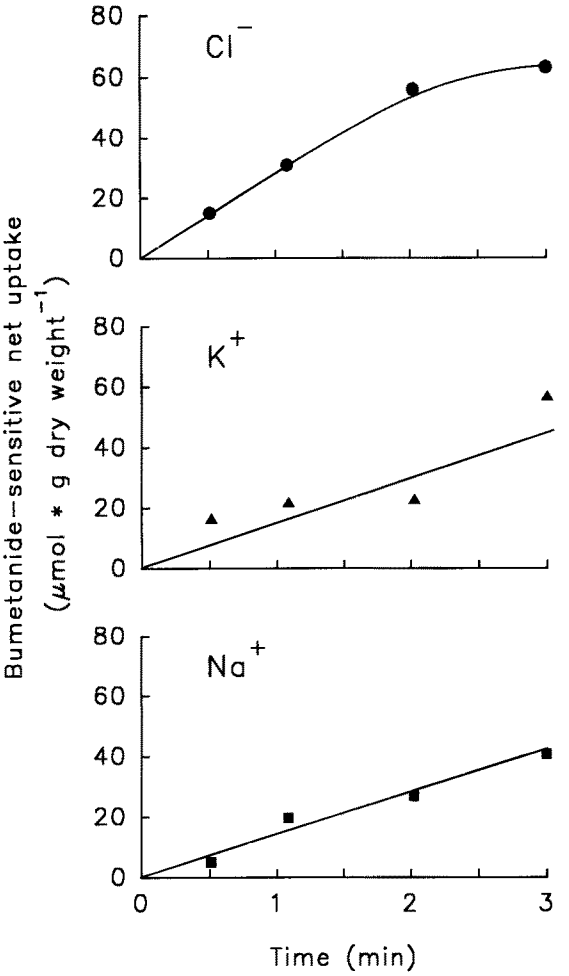


**Fig. 6.** Bumetanide-sensitive components of the net ion uptake in the presence of ouabain and barium during Regulatory Volume Increase in Ehrlich cells. The cells were pretreated by exposure to low external osmolarity (150 mOsm) for 20 to 40 min (*see* Materials and Methods). At zero time a tonicity of 300 mOsm was restored by addition of ⅓ volume of a double-strength saline solution containing 1 mM ouabain and 2 mM $Ba^{2+}$ or 1 mM ouabain, 2 mM $Ba^{2+}$ and 30 μM bumetanide, whereupon cell $Na^+$ content, cell $K^+$ content and cell $Cl^-$ content were followed with time. The figure shows the bumetanide-sensitive component of the net ion uptake measured as the difference between the net uptakes in parallel groups with and without 30 μM bumetanide. Shown are the mean values from three independent experiments, except the $Na^+$ uptake 2 min after hypertonic treatment, which is the mean of two independent experiments.

Figure 6 shows that a bumetanide-sensitive net uptake of $Na^+$, $K^+$, and $Cl^-$ during regulatory volume increase could be demonstrated in ouabain-poisoned cells in the presence of barium. It should be noted that the measured net uptake of $K^+$ in the absence of bumetanide is smaller than the bumetanide-sensitive net uptake shown in figure. This is caused by the fact that the cells lost some $K^+$ in the presence of bumetanide even though $Ba^{2+}$ was

**Table 4.** Flux ratio analysis of unidirectional chloride and potassium fluxes during Regulatory Volume Increase in Ehrlich cells

| Bumetanide-sensitive component | $Cl^-$ | $K^+$ | $Na^+$ |
|---|---|---|---|
| | $\mu$mol · g dry $wt^{-1}$ · $min^{-1}$ | | |
| Net flux | 29.0 ± 3.8 | 11.5 ± 2.6 | 14.9 ± 1.9 |
| Unidirectional influx | 65.7 ± 6.1 | 45.9 ± 2.7 | — |
| Unidirectional efflux | 36.7 ± 4.1 | 34.4 ± 1.4 | — |
| Flux ratio, Influx/efflux[a] | 1.82 ± 0.15 | 1.34 ± 0.08 | — |
| Ion concentration ratio: $\frac{[Na]_o[K]_o[Cl]_o^2}{[Na]_i[K]_i[Cl]_i^2}$ | | 1.75 ± 0.24 | |
| Stoichiometry of net fluxes: $[Cl^-]:[K^+]:[Na^+]$ | 2.0 | 0.8 | 1.0 |

The values are given as mean ± SEM for five sets of individual experiments, with concurrent measurements of unidirectional $^{42}K$ and $^{36}Cl$ influx and net ion uptake. Experimental protocol as in Fig. 6. The mean value of the measured internal ion concentrations were (mmol/liter cell water): $K^+$, 182 ± 13 (5), $Na^+$, 20 ± 1 (5), $Cl^-$, 67 ± 6 (5). The mean value of the measured external ion concentrations were (mM): $K^+$, 5.9 ± 0.2 (5), $Na^+$, 184 ± 8 (5), $Cl^-$, 156 ± 6 (5).
The bumetanide-sensitive components were determined as the difference between the fluxes in parallel groups with and without 30 $\mu$M bumetanide.
[a] The values are given as the mean ± SEM of the flux ratios calculated in each individual experiment.

present. Very recent results from Levinson (1991*a*) confirm this as he demonstrated a $K^+$ loss during RVI in the presence of ouabain and bumetanide. The bumetanide-sensitive $Na^+$, $K^+$, and $Cl^-$ net fluxes are found to have the stoichiometry 14.9 ± 1.9 $\mu$mol · g dry $wt^{-1}$ · $min^{-1}$ $Na^+$, 11.5 ± 2.6 $\mu$mol · g dry $wt^{-1}$ · $min^{-1}$ $K^+$ and 29.0 ± 3.8 $\mu$mol · g dry $wt^{-1}$ · $min^{-1}$ $Cl^-$, or approximately 1 : $Na^+$, 1 : $K^+$, 2 : $Cl^-$ (*see* Table 4). These findings provide evidence for the involvement of a $Na^+$, $K^+$, $2Cl^-$ cotransport system during regulatory volume increase. It is only the high $K^+$ leak which prevents a net uptake of $K^+$ during RVI in the presence of ouabain.

In the experiments presented in Table 4 the unidirectional $^{42}K$ and $^{36}Cl$ influxes are measured concurrently with the net $K^+$ and $Cl^-$ uptake during RVI in the presence or absence of 30 $\mu$M bumetanide. The bumetanide-sensitive components are determined from the differences in paired controls. The unidirectional $K^+$ and $Cl^-$ effluxes are calculated as the differences between the simultaneously measured unidirectional $^{42}K$ and $^{36}Cl$ influxes and the net $K^+$ and $Cl^-$ fluxes, and the flux ratio was calculated as the ratio between unidirectional influx and efflux. The fluxes are measured as initial rate of uptake after restoration of tonicity, and consequently the calculated flux ratio is extrapolated to zero time. The flux ratio can be analyzed also under non-steady-state conditions, and the extrapolated value should represent the true flux ratio at that time (Sten-Knudsen & Ussing, 1981). As seen in Table 4, the bumetanide-sensitive component, which essentially accounts for the net uptake induced during regulatory volume increase has a flux ratio of 1.34 ± 0.08 for $K^+$ and 1.82 ± 0.15 for $Cl^-$. This means that for the transport system involved in regulatory volume increase, the minimum ratio between the driving forces for influx and efflux must be 1.82. This can be compared to the value 1.85 obtained by Hoffmann et al., 1983. The ratio between the driving forces for unidirectional influx and efflux for the above transport system is calculated from the internal and external ion concentrations measured at the first data point after restoration of tonicity (taken at 0.5 min). In these calculations the activity coefficients are assumed to be equal in cell water and in the extracellular medium. The ratio of the driving forces for a $Na^+$, $K^+$, $2Cl^-$ cotransport system (1.75 ± 0.24) can account for the observed flux ratio.

## Regulation of the $Na^+$, $K^+$, $Cl^-$ Cotransport System

Ehrlich ascites tumor cells regulate their volume back towards their original steady-state cell volume when a hypertonic shock follows the hypotonic pretreatment. This is the traditional RVI after RVD

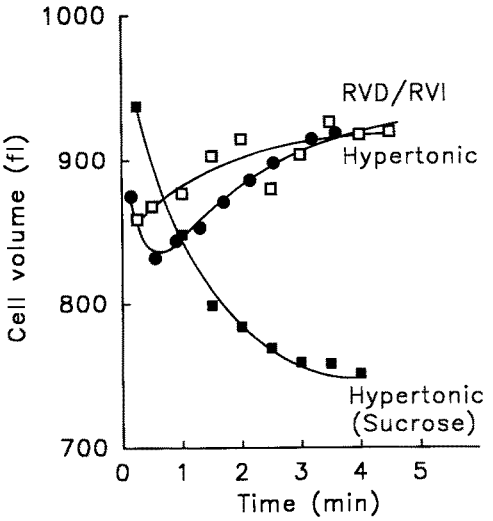


**Fig. 7.** Cell volume after hypertonic challenge following different preincubation procedures. *RVD/RVI* (●): The cells were preincubated for 20 min in a hypotonic medium (150 mOsm). At zero time a sample of the cell suspension was diluted 500-fold in an isotonic standard incubation medium (300 mOsm) and the cell volume was followed with time using a Coulter counter. The cell volume in the hypotonic medium was measured at 1,346 fl. *Sucrose* (■)/*Standard hypertonic medium* (□): The cells were preincubated for 20–40 min in standard incubation medium (300 mOsm). At time zero sucrose (600 mOsm) or hypertonic standard medium (600 mOsm) was added to the cell suspension to give a final osmolarity of 450 mOsm. Cell volume was followed with time using a Coulter counter. The cell volume in the isotonic medium was measured at 1,128 fl. Similar results were obtained in three other experiments of the same design.

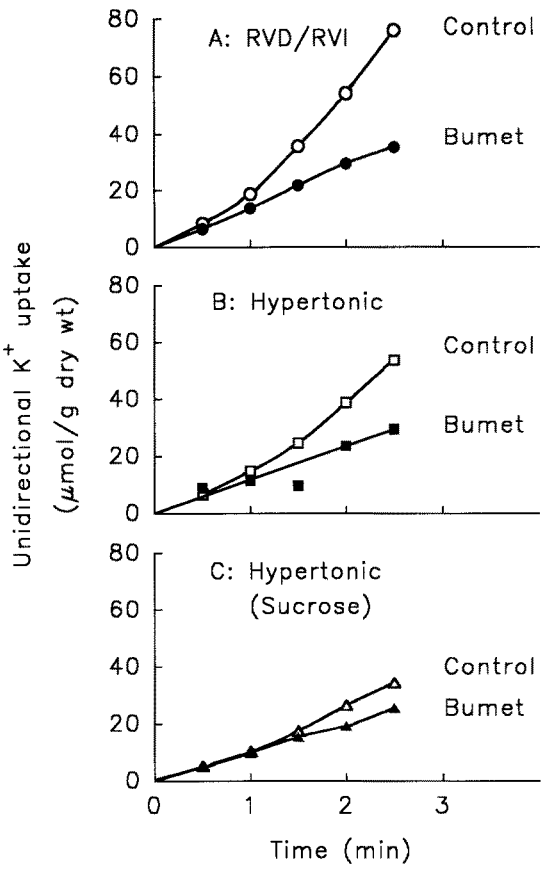


**Fig. 8.** Activation of bumetanide-sensitive $K^+$ influx following hypertonic shock. (*A*) (*RVD/RVI*; ○ ●): Experimental procedures as in Fig. 4. (*B*) (*Hypertonic, standard medium*; □ ■): The cells were preincubated in isotonic standard medium. At time zero hypertonic standard medium was added to give a final medium osmolarity of 400 mOsm. Some aliquots received bumetanide (final concentration, 30 μM) together with the isotope. (*C*) (*Hypertonic, sucrose*; △ ▲): The cells were preincubated in isotonic standard medium. At time zero sucrose containing $^{86}Rb$ (50,000 Bq/ml) was added to give a final medium osmolarity of 400 mOsm. Some of the aliquots received bumetanide (final concentration, 30 μM) together with the isotope.

The bumetanide-sensitive $K^+$ influx after cell shrinkage with sucrose is 7.6 ± 2.1 μmol · g dry $wt^{-1}$ · $min^{-1}$ (calculated by linear regression from the values at 0.5, 1.0, 1.5, 2.0 and 2.5 min, $n$ = 4), whereas the bumetanide-sensitive $K^+$ influx after shrinkage with hypertonic standard medium is 19.2 ± 3.0 μmol · g dry $wt^{-1}$ · $min^{-1}$ (calculated by linear regression from the values at 1.0, 1.5, 2.0 and 2.5 min, $n$ = 4, in the period where the cotransporter is fully activated). Finally, the bumetanide-sensitive $K^+$ influx during the RVI/RVD protocol is 24.1 ± 1.9 μmol · g dry $wt^{-1}$ · $min^{-1}$ (calculated by linear regression from the values at 1.0, 1.5, 2.0, and 2.5 min, $n$ = 11, in the period where the cotransporter is fully activated).

protocol. Figure 7 shows the cell volume following three different experimental protocols. If hyperosmotic sucrose is added to the cells in isotonic steady state, the cells shrink with no signs of volume regulation. If hypertonic standard medium ($[K^+]$ = 10 mM) is added, the cells initially shrink and then they regulate their volume back towards their original volume. A hypotonic preincubation is thus not necessary for a regulatory volume increase. It should be noted that all extracellular ions are increased in the hypertonic standard medium with the final $[K^+]_o$ equal to 6.5 mM.

If this regulatory volume increase is also mediated by the $Na^+$, $K^+$, $Cl^-$ cotransport system, then the unidirectional $K^+$ influx should be bumetanide-sensitive. Figure 8 shows the $K^+$ uptake after the traditional RVD/RVI protocol (Fig. 8*A*), after shrinkage with hypertonic standard medium (Fig. 8*B*) and after shrinkage with sucrose (Fig. 8*C*) as a function of time. The $K^+$ uptake is found to be partially bumetanide-sensitive under all three conditions.

The bumetanide-sensitive $K^+$ influx after cell shrinkage with sucrose is 7.6 ± 2.1 μmol · g dry $wt^{-1}$ · $min^{-1}$ ($n$ = 4), whereas the bumetanide-sensitive $K^+$ influx after shrinkage with hypertonic standard medium is 19.2 ± 3.0 μmol · g dry $wt^{-1}$ · $min^{-1}$ ($n$ = 4). Finally, the bumetanide-sensitive $K^+$ influx during the RVI/RVD protocol is 24.1 ± 1.9 μmol · g dry $wt^{-1}$ · $min^{-1}$ ($n$ = 11) (*see* Table 5 and Fig. 8).

**Table 5.** Ion concentration ratios for $Na^+$, $K^+$, $2Cl^-$ cotransport system after the RVD/RVI protocol, after addition of hypertonic standard medium and after addition of sucrose to hypertonicity

| | RVD/RVI | Hypertonic standard medium | Hypertonic sucrose medium |
|---|---|---|---|
| $\frac{[Na]_o[K]_o[Cl]_o^2}{[Na]_i[K]_i[Cl]_i^2}$ | 1.5 ± 0.13 (8)[a] | 1.43 ± 0.10 (3)[b] | 0.13 ± 0.03 (3)[b] |
| Bumetanide-sensitive $K^+$ influx ($\mu$mol/g dry wt/min) | 24.1 ± 1.9 (11)[b] | 19.2 ± 3.0 (4)[b] | 7.6 ± 2.1 (4)[b] |
| $[K]_o$ | 5.6 ± 0.2 (8)[a] | 6.0 ± 0.2 (3) | 3.7 ± 0.2 (3) |
| $[Na]_o$ | 150 ± 2.9 (8)[a] | 173.5 ± 2.2 (3) | 101.5 ± 0.8 (3) |
| $[Cl]_o$ | 150 ± 2.2 (8)[a] | 200.0 ± 1.5 (3) | 99.7 ± 1.1 (3) |

The ion concentration ratios were calculated for three sets of individual experiments, where the ion concentrations are measured 0.5 min after hypertonic treatment. The mean value of the measured internal ion concentrations were (mmol/liter cell water):
RVD/RVI: $K^+$, 183 ± 7 (8), $Na^+$, 22 ± 2 (8), $Cl^-$, 59 ± 2 (8); Hypertonic standard medium: $K^+$, 208 ± 2 (3), $Na^+$ 39 ± 1 (3), $Cl^-$, 59 ± 2 (3); Hypertonic sucrose medium: $K^+$, 206 ± 14 (3), $Na^+$, 40 ± 8 (3), $Cl^-$, 63 ± 2 (3).
[a] The values presented here are from Hoffmann et al., 1983.
[b] The values presented here have been calculated from Fig. 8. The values are given as mean (+/− SEM) with the number of independent experiments in parentheses.

Cell shrinkage activates the $Cl^-$-dependent cotransport system independent of whether the shrinkage is caused by sucrose or hypertonic standard medium. Table 5 gives the gradient for a $Na^+$, $K^+$, $2Cl^-$ cotransport system at the time of maximal cell shrinkage (0.5 min) in the three situations. It is seen that the reason why the cells don't regulate their cell volume after shrinkage with sucrose is the absence of an inwardly directed gradient for the $Cl^-$-dependent cotransport system. This is yet another argument for the transport system being a $Na^+$, $K^+$, $Cl^-$ cotransport system rather than a $Na^+$, $Cl^-$ cotransport system, since the gradient for a $Na^+$, $Cl^-$ cotransport system would be inward after shrinkage with sucrose as well as NaCl.

The bumetanide-sensitive $K^+$ influx after the traditional "RVD after RVI" protocol is of the same magnitude as that seen after cell shrinkage with hypertonic standard medium. The major difference between the two situations is that $Na_o$ is higher in the hypertonic standard medium (*see* Table 5). To test whether the activation of the cotransporter is influenced by $Na_o$ and/or $Cl_i$ two sets of experiments were performed as illustrated in Table 6, i.e., extracellular $Na^+$ was varied from 50–300 mM substituting $Na^+$ with $NMDG^+$ or all intracellular $Cl^-$ was replaced by $NO_3^-$. As seen from Table 6 doubling extracellular $Na^+$ did not influence the activation of the cotransporter. The bumetanide-sensitive $K^+$ influx in "$Cl^-$-free" cells is reduced by 54% compared to the control situation (*see* Table 6), so while intracellular $Cl^-$ is not essential for activation, it may play some role. Perhaps the decrease in unidirectional $K^+$ influx seen upon complete removal of intracellular $Cl^-$ is due to complete inhibition of the

**Table 6.** Dependence on intracellular $Cl^-$ and extracellular $Na^+$ of the activated unidirectional $K^+$ influx during Regulatory Volume Increase

| Bumetanide-sensitive, unidirectional $K^+$ influx | |
|---|---|
| $[Na^+]_o$, mM | $\mu$mol · g dry $wt^{-1}$ · $min^{-1}$ |
| 300 | 16.1 ± 1.1 (3) |
| 150 | 16.4 ± 1.4 (3) |
| "$Cl^-$ free" cells[a] | 7.4 ± 1.0 (3) |

Experimental protocol as in Fig. 2. $^{86}Rb$ was used as tracer for $K^+$. It should be noted, that the final $[K^+]_o$ = 10 mM. The initial rates of influx were calculated from the values at 0.5, 0.75, 1.00 and 1.25 min. All correlation coefficients were ≥ 0.97.
[a] "$Cl^-$ free" cells were washed twice in nitrate medium and then preincubated for 30 min in nitrate medium in order to reduce intracellular $Cl^-$. At time zero the cells were retransferred to $Cl^-$ medium. Note that $[Na^+]_o$ = 300 mM.
The values are given as mean (± SEM) with the number of experiments given in parentheses.

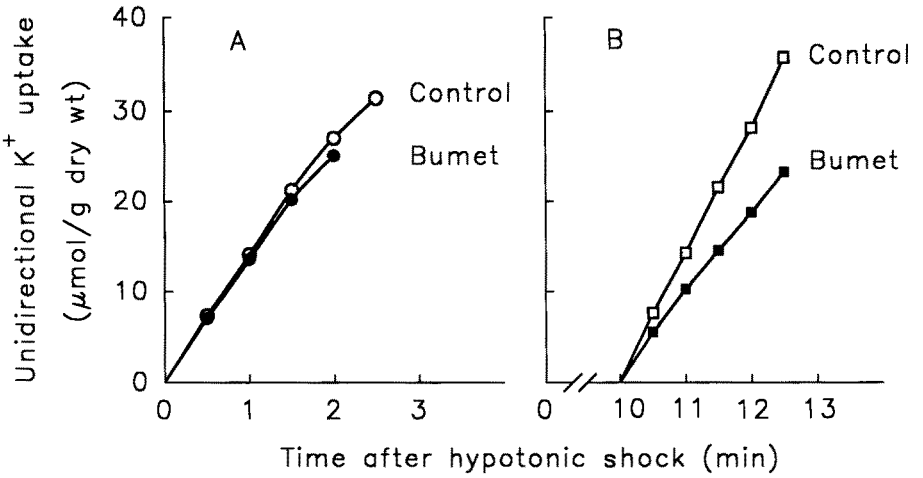


**Fig. 9.** Activation of a bumetanide-sensitive unidirectional $K^+$ uptake during Regulatory Volume Decrease. (*A*) (○, ●): The cells were preincubated in standard medium for 20- 40 min. At time zero $H_2O$ (containing $^{86}Rb$ (50,000 Bq/ml)) buffered with MOPS, TES and HEPES was added to give a final medium osmolarity of 225 mOsm. The final concentration of bumetanide was 30 μM. The $K^+$ uptake was followed as described in Materials and Methods. (*B*) (□, ■): The cells were preincubated in a hypotonic medium (225 mOsm) for 10 min. At time 10 min containing $^{86}Rb$ (50,000 Bq/ml) was added. Bumetanide was used in a final concentration of 30 μM. Two further experiments gave similar results.

$K^+/K^+$ exchange mode of the cotransporter, as the current model for $K^+/K^+$ exchange via the $Na^+$, $K^+$, $2Cl^-$ cotransport system suggests that this mode requires the presence of intracellular $Cl^-$ (*see* Duhm, 1987). To test if the $Na^+$, $K^+$, $Cl^-$ cotransport system is activated already during RVD the $K^+$ influx was measured immediately after the hypotonic shock and after 10 min of hypotonic incubation. Figure 9 shows the $K^+$ uptake as a function of time after hypotonic shock. No bumetanide-sensitive $K^+$ influx can be demonstrated immediately after hypotonic shock (Fig. 9, panel *A*), whereas Fig. 9, panel *B*, shows that after hypotonic incubation for 10 min the cells have a significant bumetanide-sensitive $K^+$ influx amounting to 5 μmol · g dry $wt^{-1}$ · $min^{-1}$ (calculated from the values after 0.5, 1.0, 1.5, 2.0 and 2.5 min, $n = 3$). It should be noted that the cells are still in hypotonic medium when the flux is measured. In the absence of extracellular $Na^+$ no bumetanide-sensitive $K^+$ influx can be demonstrated after 10 min of hypotonic incubation (*data not shown*). The $Na^+$ dependence suggests that the flux is not KCl cotransport and that the $Na^+$, $K^+$, $Cl^-$ cotransport system is activated during the first 10 min of hypotonic incubation. The flux obtained during RVD should be compared with the much larger flux obtained after the RVD/RVI protocol amounting to 24.1 ± 1.9 μmol · g dry $wt^{-1}$ · $min^{-1}$ (*see* Table 5).

The $Na^+$, $K^+$, $Cl^-$ cotransport system is active during RVI and partly active during RVD, but inactive under physiological steady-state conditions (*see* Table 2). This suggests one or more regulatory mechanisms. The simplest hypothesis is that the same mechanism is involved in the regulation during RVD and during RVI.

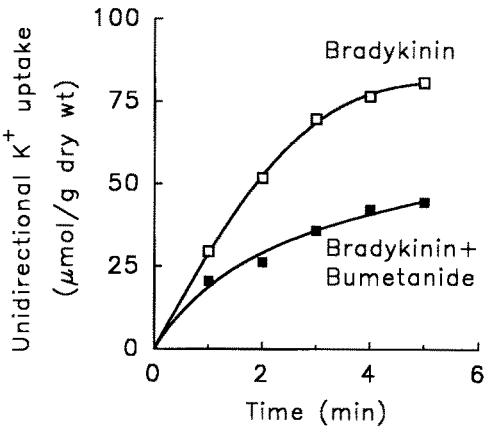


**Fig. 10.** $K^+$ uptake after bradykinin stimulation. The cells were preincubated in standard medium for 15–30 min. At time zero $^{86}Rb$ (50,000 Bq/ml) and bradykinin (final concentration 1 μM) were added. Bumetanide was added to give a final concentration of 30 μM. The figure represents nine individual experiments.

The $Ca^{2+}$ mobilizing agent bradykinin (a nonpeptide) mimics the RVD-response in Ehrlich ascites tumor cells (Simonsen et al., 1990; *see* Hoffmann & Kolb, 1991). It was therefore examined whether addition of bradykinin can activate the $Na^+$, $K^+$, $Cl^-$ cotransport system. Figure 10 shows the $K^+$ uptake as a function of time after addition of bradykinin to Ehrlich ascites tumor cells in physiological steady state. As shown in Fig. 10 bradykinin activates a bumetanide-sensitive $K^+$ uptake. As

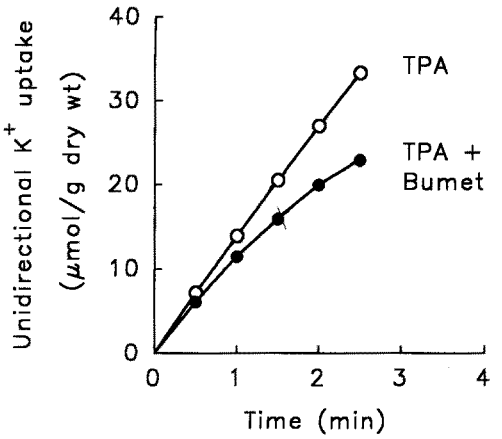


**Fig. 11.** Effect of phorbol ester TPA on the bumetanide-sensitive $K^+$ uptake during steady state. The cells were preincubated in standard medium for 20–40 min. At time zero $^{86}Rb$ (50,000 Bq/ml) and TPA (final concentration, 100 nM) were added. The $K^+$ uptake was followed as described previously. Bumetanide was used in a final concentration of 30 μM. The figure represents three experiments giving identical results.

**Table 7.** Activation mechanisms for the $Na^+$, $K^+$, $Cl^-$ cotransport system in Ehrlich ascites tumor cells[a]

| | Bumetanide-sensitive, unidirectional $K^+$ influx | |
|---|---|---|
| | Physiological steady state | RVI |
| | μmol · g dry $wt^{-1}$ · $min^{-1}$ | |
| Control | 1.2 ± 0.4 (5) | 19.2 ± 3.0 (4) |
| Control (1‰ DMSO) | — | 11.0 ± 0.3 (3) |
| Staurosporine (100 nM) | — | 11.3 ± 0.6 (3) |
| Pimozide (120 μM) | — | 5.1 ± 2.3 (3) |
| Bradykinin (1 μM)[b] | 18.1 ± 3.0 (9) | — |
| TPA (100 nM) | 4.5 ± 0.4 (3) | — |

[a] Data from Figs. 10, 11 and 12.
[b] In some of the experiments with bradykinin, $Ba^{2+}$ (2 mM) was added in an attempt to block $K^+$ channels.
The values are given as mean (± SEM) with the number of independent experiments given in parentheses.

mentioned above, bradykinin causes a release of $Ca^{2+}$, which in turn opens $K^+$ and $Cl^-$ channels leading to a cell shrinkage. In six experiments $Ba^{2+}$ (2 mM) was present (sulphate-free medium) in an attempt to block $K^+$ channels. The initial bradykinin stimulation was not significantly different with and without $Ba^{2+}$ (2 mM). It seems reasonable to conclude that bradykinin activates the $Na^+$, $K^+$, $Cl^-$ cotransport system either via Ins (1, 4, 5) $P_3$ and subsequent release of $Ca^{2+}$ or via DAG and subsequent activation of protein kinase C or both. The relation between this and the concomitant cell shrinkage in the activation of the cotransporter is under investigation.

The phorbol ester TPA is a well-known protein kinase C activator (Nishizuka, 1984). Figure 11 shows the effect of TPA on the $K^+$ uptake during physiological steady-state conditions. After addition of TPA (100 nM) to the cells a small bumetanide-sensitive $K^+$ influx is found. This bumetanide-sensitive $K^+$ influx (4.5 ± 0.4 μmol · g dry $wt^{-1}$ · $min^{-1}$, $n$ = 3) (*see* Table 7) is in the same range as the bumetanide-sensitive $K^+$ influx found during hypotonic shock. Thus, stimulation of protein kinase C and hypotonic cell swelling both result in a partial activation of the $Na^+$, $K^+$, $Cl^-$ cotransport system. It should be noted that the effects of TPA are known to be very dependent on concentration and duration of exposure. We can therefore not be certain that we actually have stimulated protein kinase C maximally in the present experiment.

To test whether $Ca^{2+}$/calmodulin or protein kinase C or both are responsible for the activation during RVI we tested inhibitors of the $Ca^{2+}$/calmodulin complex and protein kinase C, respectively, and furthermore measured intracellular [$Ca^{2+}$] during the RVD/RVI protocol.

Intracellular [$Ca^{2+}$] was followed with time in two independent experiments during the hypotonic preincubation and after restoration of tonicity, i.e., RVI. During the first 10 min of hypotonic preincubation internal $Ca^{2+}$ is increased from 60 nM to 90 nM, whereas restoration of tonicity increases internal $Ca^{2+}$ with another 10 nM.

Figure 12 shows the $K^+$ uptake after a hypertonic shock in the presence and absence of bumetanide and the anti-calmodulin drug pimozide. In the presence of pimozide (120 μM) the bumetanide-sensitive $K^+$ influx is reduced to 5.1 ± 2.3 μmol · g dry $wt^{-1}$ · $min^{-1}$ ($n$ = 3). This is a 73% reduction compared to the control situation. We have tried to add pimozide (120 μM) 1 min after addition of the hypertonic standard medium in order to determine if the effect was solely on the activation step. Under these circumstances pimozide still inhibited 44%. Thus, it is likely that $Ca^{2+}$/calmodulin is involved in the activation of the $Na^+$, $K^+$, $Cl^-$ cotransporter during RVI.

Staurosporine (100 nM), a potent protein kinase C inhibitor (Tamaoki et al., 1986), was examined. Staurosporine had no effect on the bumetanide-sensitive unidirectional $K^+$ influx during RVI. The control flux in the presence of DMSO (1‰) was 11.0 ± 0.3 μmol · g dry $wt^{-1}$ · $min^{-1}$ ($n$ = 3) and the flux in the presence of staurosporine was 11.3 ± 0.6 μmol · g dry $wt^{-1}$ · $min^{-1}$ ($n$ = 3). This indicates that protein kinase C does not play any major role in

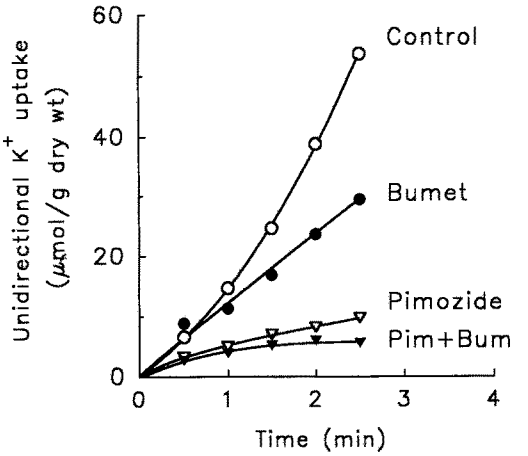


**Fig. 12.** Effect of pimozide on the activated $K^+$ influx after hypertonic shock. The cells were preincubated in standard medium for 20–40 min. Control (○, ●): At time zero hypertonic medium containing $^{86}Rb$ (50,000 Bq/ml) was added to give a final osmolarity of 400 mOsm. The $K^+$ uptake was followed as described previously. Bumetanide was used in a final concentration of 30 μM. Pimozide (▽, ▼): Pimozide in a final concentration of 120 μM was added 30 sec before the hypertonic challenge. At time zero hypertonic medium containing $^{86}Rb$ (50,000 Bq/ml) was added to give a final osmolarity of 400 mOsm, and the $K^+$ uptake was followed with time. Bumetanide was used in a final concentration of 30 μM. The figure represents three experiments giving identical results.

the activation of the cotransporter during regulatory volume increase after addition of hyperosmotic salt solution. It should be noted that DMSO (1‰) alone inhibits the bumetanide-sensitive $K^+$ influx during RVI (*see* Table 7).

The above results (summarized in Table 7) indicate the involvement of $Ca^{2+}$/calmodulin as well as protein kinase C in the activation of the $Na^+$, $K^+$, $Cl^-$ cotransport system. During RVI the activation seems predominantly to be via $Ca^{2+}$/calmodulin-dependent processes.

## Discussion

It was previously demonstrated that the primary process during RVI was an activation of a normally silent bumetanide-sensitive cation/anion cotransport driven by the $Na^+$ and $Cl^-$ gradients with subsequent replacement of $Na^+$ by $K^+$ via the $Na^+$, $K^+$ pump stimulated by the $Na^+$ influx (Hoffmann et al., 1983).

The available evidence suggested the involvement of $Na^+$, $Cl^-$ cotransport in RVI rather than $Na^+$, $K^+$, $Cl^-$ cotransport. This conclusion was based on the findings:

(i) Under conditions where the $Na^+$, $K^+$ pump was inhibited with ouabain, an uptake of $Na^+$ and $Cl^-$ in about equimolar amounts could be demonstrated, whereas the uptake of $K^+$ was negligible. The uptake of $Na^+$ and $Cl^-$ was inhibited by bumetanide in these experiments, though a recycling of $K^+$ could conceivably have masked a $K^+$ uptake via the cotransport system.

(ii) Flux ratio analysis of the bumetanide-sensitive $Cl^-$ flux during RVI indicated that the ratio of the driving forces for $Na^+$, $K^+$, $Cl^-$ cotransport was hardly sufficient to account for the observed magnitude of the ratio of influx/efflux although the significance of the difference between these values is only marginal. Moreover, a net uptake of KCl could be demonstrated even at low external $K^+$ (3.5–4 mM), under conditions where the calculated driving force for a $Na^+$, $K^+$, $2Cl^-$ cotransport system is in the outward direction. In this calculation it was assumed that the activity coefficients for the ions in the medium and in the cytosol are equal. Whether differences in activity coefficients could be big enough to make the energy deficit vanish, as suggested by Geck (1990), is still an open question.

In the present study we demonstrate that RVI in Ehrlich cells is due to the activation of a $Na^+$, $K^+$, $Cl^-$ cotransport system. The arguments for this can be summarized as follows:

(i) The net $Cl^-$ uptake during RVI is dependent on extracellular $Na^+$ and $K^+$ (Fig. 1), and no net $Cl^-$ uptake can be demonstrated at low concentrations of extracellular concentrations of $K^+$ (less than 2 mM).

(ii) The bumetanide-sensitive unidirectional $Cl^-$ influx during RVI is strongly dependent on extracellular $K^+$ with half maximal rate at 3.3 mM external $K^+$ (Fig. 3).

(iii) During RVI the unidirectional $K^+$ influx is bumetanide-sensitive and this part of the $K^+$ influx is strongly dependent on both extracellular $Na^+$ and $Cl^-$ (Table 2).

(iv) The unidirectional $Na^+$ influx during RVI is bumetanide-sensitive. This part of the $Na^+$ influx is dependent on extracellular $Cl^-$ and after replacement of extracellular $K^+$ with $Na^+$ no bumetanide-sensitive unidirectional $Na^+$ influx can be demonstrated at all (Table 3).

(v) In the presence of ouabain and $Ba^{2+}$ a bumetanide-sensitive net uptake of $Na^+$, $K^+$, and $Cl^-$ can be demonstrated (Table 4 and Fig. 6). The stoichiometry of the bumetanide-sensitive net fluxes is found to be 1.0 $Na^+$ : 0.8 $K^+$ : 2.0 $Cl^-$ or approximately 1 $Na^+$ : 1 $K^+$ : 2 $Cl^-$. Under these circumstances the $K^+$ and $Cl^-$ flux ratios (influx/efflux) for the bumetanide-sensitive component were estimated at 1.34 ± 0.08 and 1.82 ± 0.15 which should be compared to the gradient for the $Na^+$, $K^+$, $2Cl^-$

cotransport system at 1.75 ± 0.24. Whereas the $Cl^-$ flux ratio fits extremely well with the gradient, $K^+$ tends to have a lower flux ratio, which might indicate a $K^+/K^+$ exchange via the cotransporter.

Levinson (1991*a*) has also presented evidence that the RVI response in Ehrlich cells is mediated by the $Na^+$, $K^+$, $Cl^-$ cotransport system. Heretofore it was possible that $Na^+$, $Cl^-$ and $Na^+$, $K^+$, $Cl^-$ cotransport coexisted in Ehrlich cells. The two transport systems are distinguishable, but may represent alternate modes of operation of the same cotransporter. Recently, Sun et al. (1991) reported that $Na^+$, $Cl^-$ cotransport systems may be converted into $Na^+$, $K^+$, $Cl^-$ cotransport systems by hormonal control. The addition of antidiuretic hormone (ADH) to mouse medullary thick ascending limb cells converts the $K^+$-independent $Na^+$, $Cl^-$ cotransporter to a $Na^+$, $K^+$, $Cl^-$ cotransporter.

It is not possible to measure a stoichiometry for the $Na^+$, $K^+$, $Cl^-$ cotransport system on the basis of the unidirectional influxes presented here. The reason for this is that all flux measurements are unidirectional influxes measured under conditions where all three ions had the opportunity to recycle and to undergo self-exchange via the cotransporter. To explain the differences in the unidirectional $Na^+$ and $K^+$ influxes, we have to suggest that the $K^+/K^+$ exchange through the cotransporter exceeds the $Na^+/Na^+$ exchange. This is also supported by the fact that the flux ratio for $K^+$ (influx/efflux) is lower than predicted from the gradient (*see above*) during RVI. This question is currently under investigation. Bumetanide-sensitive self-exchange of both $Na^+$, $K^+$ and $Cl^-$ via the cotransport system is a well-known phenomenon, and in some cells this one-for-one exchange is seen parallel to the net transport via the cotransporter (Duhm, 1987; Lauf et al., 1987; Flatman, 1989).

The problem of measuring the stoichiometry was solved by measuring bumetanide-sensitive net fluxes in the presence of $Ba^{2+}$ and ouabain. The stoichiometry found on the bumetanide-sensitive net fluxes of $Na^+$, $K^+$, and $Cl^-$ in ouabain-poisoned cells in the presence of $Ba^{2+}$ is 1.0 $Na^+$ : 0.8 $K^+$ : 2.0 $Cl^-$.

Geck et al. (1980) showed $Na^+$, $K^+$, $Cl^-$ cotransport in Ehrlich cells, but the cells were not studied under physiological conditions, since they had been depleted of $K^+$.

### Activation of $Na^+$, $K^+$, $Cl^-$ Cotransport after Cell Swelling and Cell Shrinkage

Until now it has been assumed that the $Na^+$, $K^+$, $Cl^-$ cotransport system in Ehrlich cells is activated only following the RVD/RVI protocol. According to this protocol the cells are preincubated in a hypotonic medium to reduce their content of ions, whereupon the cells are exposed to isotonic medium (Hoffmann et al., 1983). A similar effect can be achieved by addition of the calcium ionophore A23187 to cells in steady state at isotonicity. The cells will shrink as a consequence of the loss of KCl and the following regulatory volume increase is mediated by the $Na^+$, $K^+$, $Cl^-$ cotransport system (Hoffmann, Simonsen & Lambert, 1984). The role of $Ca^{2+}$ and/or cell shrinkage in this activation is under investigation.

Hempling (1960) showed that Ehrlich cells after hypertonic treatment with NaCl are unable to perform regulatory volume increase. This has recently been confirmed by Levinson (1991*b*), though Levinson (1991*b*) demonstrated that the $Na^+$, $K^+$, $Cl^-$ cotransport system was activated. This was explained as an inhibitory effect of the cellular $Cl^-$ concentration on the net uptake of ions via the cotransporter. In the present study we demonstrated that Ehrlich cells actually are able to perform RVI after addition of hypertonic standard medium. Furthermore, reducing intracellular $Cl^-$ by complete substitution with $NO_3^-$ did not result in a more effective activation of the cotransporter (*see* Table 6). The difference between the experiments of Levinson (1991*b*) and the present experiments is that external $K^+$ in the present investigation is raised together with $Na^+$ and $Cl^-$ resulting in an inward driving force, $[Na^+]_o[K^+]_o[Cl^-]_o^2/[Na^+]_i[K^+]_i[Cl^-]_i^2$, higher than 1 (*see* Table 5) where the driving force obtained in the experiments by Levinson (1991*b*) was 0.6.

MDCK cells (Roy & Sauvé, 1987), Chinese hamster ovary cells (Sarkardi et al., 1985), HeLa cells (Tivey, Simmons & Aiton, 1985) and C6 glioma cells (Chassande et al., 1988) are unable to perform RVI after shrinkage with sucrose, but they do not shrink further after the initial cell shrinkage. This is in contradiction to Ehrlich cells which initially shrink, and then continue to shrink for as long as 25 min (Hendil & Hoffmann, 1974; Levinson, 1991*b*). The $Na^+$, $K^+$, $Cl^-$ cotransport system is activated after cell shrinkage following addition of sucrose, but no volume regulation is seen. The gradient for the $Na^+$, $K^+$, $Cl^-$ cotransport system is in the outward direction after addition of sucrose (*see* Table 5), and the continued shrinkage seen after the initial cell shrinkage can be explained as a net efflux of ions mediated by the cotransporter. The gradients after the RVD/RVI protocol and after addition of hypertonic standard medium are almost identical (*see* Table 5) and the external $K^+$ is higher in the hypertonic standard medium. A role for intracellular $Cl^-$ in regulating net cotransport activity is of course a possibility and has been suggested in a variety of cells including Ehrlich cells (Levinson, 1990) and squid giant axons (Breit-

weiser et al., 1990). However, as seen from Table 6, a complete exchange of intracellular $NO_3^-$ for $Cl^-$ does not increase transport activity, making this possibility less attractive.

Thus, events occurring during RVD seem to be important. We show here that $Na^+$, $K^+$, $Cl^-$ cotransport in Ehrlich cells is also activated after cell swelling. This is surprising since $Na^+$, $K^+$, $Cl^-$ cotransport in duck erythrocytes (Haas & McManus, 1985) is inhibited by cell swelling. Cell swelling activated transport systems include among others $K^+$ and $Cl^-$ channels in Ehrlich cells (Hoffmann et al., 1984), KCl cotransport in mammalian erythrocytes (Kaji, 1989; Kracke & Dunham, 1990) and in Ehrlich cells at low pH and after $Ca^{2+}$ depletion (Kramhøft et al., 1986), as well as coupled exchange of $K^+$ for $H^+$ and $Cl^-$ for $HCO_3^-$ in *Amphiuma* erythrocytes (Cala, 1980). In Ehrlich cells a nonselective cation channel is activated by both cell shrinkage and cell swelling (Christensen & Hoffmann, 1992). To the knowledge of the authors no other $Na^+$, $K^+$, $Cl^-$ cotransport system has been reported to be activated by cell swelling as well as cell shrinkage. Recently, however, preliminary reports of a similar phenomenon were reported in vascular endothelial cells (Klein, Bell & O'Neill, 1990) and in shark rectal gland (Lytle, Lear & Forbush, 1990). Lytle and coworkers suggest that the activation after cell swelling is related to the decrease in internal $Cl^-$. This seems not to be the case in the present experiments since internal $Cl^-$ is decreased at 1 min after the hypotonic shock, whereas the activation was not apparent.

It has recently been demonstrated that there is a disassembly of F-actin during RVD in the period 1–10 min after the hypotonic shock (Cornet, Lambert & Hoffmann, 1993). Disassembly of F-actin seems to activate the $Na^+$, $K^+$, $Cl^-$ cotransporter in Ehrlich cells (Jessen & Hoffmann, 1992). It is likely that this is the explanation of the activation of the $Na^+$, $K^+$, $Cl^-$ cotransport system seen during RVD. Disassembly and reassembly of F-actin filaments also appear to be an important event in cAMP-stimulated net $Cl^-$ secretion by T84 colonic epithelial cells, in which $Na^+$, $K^+$, $Cl^-$ cotransport as well as $Cl^-$ channels are vital participants (Sharipo et al., 1991).

## Regulation of the $Na^+$, $K^+$, $Cl^-$ Cotransport System

$Ca^{2+}$/calmodulin and protein kinase C have been assigned roles in the activation of the $Na^+$, $K^+$, $Cl^-$ cotransporter (for review, *see e.g.*, Haas, 1989 and Hoffmann et al., 1992). In accord with this, the cotransporter can be activated by several agonists which stimulate phospholipase C, resulting in both a release of internal $Ca^{2+}$ and an activation of protein kinase C. This is illustrated by the bradykinin-stimulation of the $Na^+$, $K^+$, $Cl^-$ cotransport system in Ehrlich cells (*see* Fig. 10 and Table 7). Similar results have been reported in several cell types after stimulation by either bradykinin, thrombin, vasopressin or angiotensin II (*see* Brock et al., 1986; Paris & Pouysségur, 1986; Homma, Burns & Harris, 1990; Klein & O'Neill, 1990; Smith & Smith, 1987; O'Donnell, 1989; Owen & Ridge, 1989; O'Donnell, 1991). The stimulation by the above agonists could conceivably be mediated by an increase in cytosolic $Ca^{2+}$ and/or activation of protein kinase C.

Paris and Pouysségur (1986) have suggested that the stimulation by $\alpha$-thrombin of the $Na^+$, $K^+$, $Cl^-$ cotransport system in hamster fibroblasts is mediated via protein kinase C, since the cotransporter can also be activated by the phorbol ester TPA. TPA also stimulates the $Na^+$, $K^+$, $Cl^-$ cotransport system in Ehrlich cells (*see* Fig. 11 and Table 7). Protein kinase C is also reported to activate the $Na^+$, $K^+$, $Cl^-$ cotransporter in human fibroblasts (Panet & Atlan, 1990), whereas the cotransporter is inhibited by TPA in vascular smooth muscle (Owen, 1985). There is some evidence that protein kinase C is also involved in a negative feedback control of the cotransporter (Paris & Pouysségur, 1986; O'Donnell, 1991).

The stimulation of the $Na^+$, $K^+$, $Cl^-$ cotransporter by thrombin and angiotensin II in rat glomerular mesangial cells has been shown to involve both protein kinase C and $Ca^{2+}$/calmodulin (Homma et al., 1990).

$Ca^{2+}$ has been reported to be involved in the activation of the cotransporter in vascular smooth muscle cells (Smith & Smith, 1987), in endothelial cells (O'Donnell, 1989), and in rabbit tracheal epithelial cells (Liedtke, 1990). In vascular smooth muscle the stimulation of the $Na^+$, $K^+$, $Cl^-$ cotransport system by angiotensin II is mediated by an increase in free cytosolic $Ca^{2+}$ involving both $Ca^{2+}$ entry via receptor-operated $Ca^{2+}$ channels and $Ca^{2+}$ release from internal stores (Owen & Ridge, 1989; O'Donnell, 1991).

The activation of the $Na^+$, $K^+$, $Cl^-$ cotransporter, during RVI is inhibited 73% by pimozide in Ehrlich cells (*see* Fig. 11 and Table 7), suggesting the involvement of $Ca^{2+}$/calmodulin in the activation of the cotransporter in these cells. It should be noted, however, that pimozide added 1 min after the hypertonic medium still inhibited about 44% indicating either that $Ca^{2+}$/calmodulin are involved in keeping the cotransporter activated or that pimozide also directly inhibits the cotransporter. There is an increase in free $Ca_i^{2+}$ in the period 1–10 min after hypotonic shock of 30 nM and a further increase of

10 nM after restoration of tonicity. Whether this increase in intracellular $Ca^{2+}$ activity is sufficient to account for the activation of the $Na^+$, $K^+$, $Cl^-$ cotransport system seen after the RVD/RVI protocol is uncertain. The microfilament reorganization during RVD (Cornet et al., 1993) is only seen in the presence of external $Ca^{2+}$. Thus, the 30 nM increase in $Ca^{2+}$ may be important for this reorganization. Since microfilament reassembly affects the cotransporter (Jessen & Hoffmann, 1992), the increase of 30 nM might be of importance. Measurements of $Ca^{2+}$ transients at the single cell level are needed to approach this question. There are contradictory results obtained using agents which modify protein kinase C. As discussed above the stimulation of the cotransporter by TPA (*see* Fig. 12 and Table 7) indicates that protein kinase C may also be involved in the activation. Since staurosporine (*see above*) had no effect on the activation of the $Na^+$, $K^+$, $Cl^-$ cotransport during RVI it seems, however, that protein kinase C may only play a minor role during RVI.

The authors want to thank Dr. P.B. Dunham, University of Syracuse, for helpful discussion and critical reading of the manuscript. We are grateful to Marianne Schiødt and Birgit Jørgensen for expert technical assistance.

## References

Aull, F. 1981. Potassium chloride cotransport in steady-state ascites tumor cells. Does bumetanide inhibit? *Biochim. Biophys. Acta* **643**:339–345

Breitweiser, G.E., Altamirano, A.A., Russell, J.M. 1990. Osmotic stimulation of $Na^+$-$K^+$-$Cl^-$ cotransport in squid giant axon is $[Cl^-]_i$ dependent. *Am. J. Physiol.* **258**:C749–C753

Brock, T.A., Brugnara, C., Canessa, M., Gimbrone, M.A. 1986. Bradykinin and vasopressin stimulate $Na^+$, $K^+$, $Cl^-$ cotransport in cultured endothelial cells. *Am. J. Physiol.* **250**:C888–C895

Cala, P.M. 1980. Volume regulation by *Amphiuma* red blood cells. The membrane potential and its implications regarding the nature of the ion-flux pathways. *J. Gen. Physiol.* **76**:683–708

Chassande, O., Frelin, C., Farahifar, D., Jean, T., Lazdunski, M. 1988. The $Na^+$, $K^+$, $Cl^-$ cotransport in C6 glioma cells. Properties and role in volume regulation. *Eur. J. Biochem.* **171**:425–433

Christensen, O., Hoffmann, E.K. 1992. Cell swelling activaties $K^+$ and $Cl^-$ channels as well as nonselective stretch-activated cation channels in Ehrlich ascites tumor cells. *J. Membrane Biol.* **129**:13–36

Cornet, M., Lambert, I.H., Hoffmann, E.K. 1993. Relation between cytoskeleton, hypo-osmotic treatment and volume regulation in Ehrlich ascites tumor cells. *J. Membrane Biol.* **131**:55–66

Duhm, J. 1987. Furosemide-sensitive $K^+(Rb^+)$ transport in human erythrocytes: Modes of operation, dependence on extracellular and intracellular $Na^+$, kinetics, pH dependency and the effect of cell volume and N-ethylmalemide. *J. Membrane Biol.* **98**:15–32

Dunham, P.B., Jessen, F., Hoffmann, E.K. 1990. Inhibition of Na-K-Cl cotransport in Ehrlich ascites cells by antiserum against purified proteins of the cotransporter. *Proc. Natl. Acad. Sci. USA* **87**:6828-6832

Feit, P.W., Hoffmann, E.K., Schiødt, M., Kristensen, P., Jessen, F., Dunham, P.B. 1988. Purification of proteins of the Na/Cl cotransporter from membranes of Ehrlich ascites cells using a bumetanide-sepharose affinity column. *J. Membrane Biol.* **103**:135–147

Flatman, P.W. 1989. Stoichiometry of net sodium and potassium fluxes mediated by the Na-K-Cl co-transport system in ferret red cells. *Q. J. Exp. Physiol.* **74**:939–41

Garay, R.P. 1982. Inhibition of the $Na^+/K^+$ cotransport system by cyclic AMP and intracellular $Ca^{2+}$ in human red cells. *Biochim. Biophys. Acta* **688**:786–792

Garty, H., Rudy, B., Karlish, S.J.D. 1983. A simple and sensitive procedure for measuring isotope fluxes through ion-specific channels in heterogenous populations of membrane vesicles. *J. Biol. Chem.* **258**:13094–13099

Gasko, O.D., Knowles, A.F., Shertzer, H.G., Suolinna, E.-M., Racker, E. 1976. The use of ion-exchange resins for studying ion transport in biological systems. *Anal. Biochem.* **72**:57–67

Geck, P. 1990. Volume regulation in Ehrlich cells. *In:* Cell Volume Regulation. K.W. Beyenbach, editor. *Comp. Physiol.* **4**:26–58

Geck, P., Heinz, E., Pietrzyk, C., Pfeiffer, B. 1978. The effect of furosemide on ouabain-insensitive $K^+$ and $Cl^-$ movement in Ehrlich cells. *In:* Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach. pp. 301–307. R.W. Straub and L. Bolis, editors. Raven, New York

Geck, P., Pfeiffer, B. 1985. $Na^+$, $K^+$, $2Cl^-$ cotransport in animal cells—Its role in volume regulation. *In:* Membrane Transport Driven by Ion Gradients. G. Semenza and R. Kinne, editors. *Ann. NY Acad. Sci.* **456**:166–182

Geck, P., Pietrzyk, C. Burckhardt, B.-C., Pfeiffer, B., Heinz, E. 1980. Electrically silent cotransport of $Na^+$, $K^+$ and $Cl^-$ in Ehrlich cells. *Biochim. Biophys. Acta* **600**:432–447

Grinstein, S., Clarke, C.A., Rothstein, A. 1983. Activation of $Na^+/H^+$ exchange in lymphocytes by osmotically induced volume changes and by cytoplasmic acidification. *J. Gen. Physiol.* **82**:619–638

Grinstein, S., Rothstein, A., Sarkadi, B., Gelfand, E.W. 1984. Responses of lymphocytes to anisotonic media: volume-regulating behavior. *Am. J. Physiol.* **246**:C204–C215

Grynkiewicz, G., Poenie, M., Tsien, R.Y. 1985. A new generation of $Ca^{2+}$ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **260**:3440–3450

Haas, M. 1989. Properties and diversity of (Na-K-Cl) cotransporters. *Annu. Rev. Physiol.* **51**:443–457

Haas, M., Johnson, L.G., Boucher, R.C. 1990. Regulation of Na-K-Cl cotransport in cultured canine airway epithelia: a $^3$[H]bumetanide binding study. *Am. J. Physiol.* **259**:C557–C569

Haas, M., McManus, T.J. 1982. Bumetanide inhibition of (Na + K + 2Cl) cotransport and K/Rb exchange at a chloride site in duck red cells: modulation by external cations. *Biophys. J.* **37**:214*a*

Haas, M., McManus, T.J. 1985. Effect of norepinephrine on swelling-induced potassium transport in duck red cells. Evidence against a volume-regulatory decrease under physiological conditions. *J. Gen. Physiol.* **85**:649–667

Hempling, H.G. 1960. Permeability of the Ehrlich ascites tumor cell to water. *J. Gen. Physiol.* **44:**365–379

Hendil, K.B., Hoffmann, E.K. 1974. Cell volume regulation in Ehrlich ascites tumor cells. *J. Cell. Physiol.* **84:**115–125

Hoffmann, E.K., Kolb, A. 1992. The mechanisms of activation of regulatory volume responses after cell swelling. *In:* Volume and Osmolality Control in Animal Cells. R. Gilles, L. Bolio and E. Hoffmann, editors. ACEP Series. Springer, Heidelberg

Hoffmann, E.K., Lambert, I.H., Simonsen, L.O. 1986. Separate, $Ca^{2+}$ activated $K^+$ and $Cl^-$ transport pathways in Ehrlich ascites tumor cells. *J. Membrane Biol.* **91:**227–244

Hoffmann, E.K., Lambert, I.H., Simonsen L.O. 1988. Mechanisms in volume regulation in Ehrlich ascites tumor cells. *Renal Physiol. Biochem.* **11:**221–247

Hoffmann, E.K., Schiødt, M., Dunham, P.B. 1986. The number of chloride-cation cotransport sites on Ehrlich ascites cells measured with $^3H$ bumetanide. *Am. J. Physiol.* **250:**C688-C693

Hoffmann, E.K., Simonsen, L.O. 1989. Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol. Rev.* **69:**315–382

Hoffmann, E.K., Simonsen, L.O., Lambert, I.H. 1984. Volume-induced increase of $K^+$ and $Cl^-$ permeabilities in Ehrlich ascites tumor cells. Role of internal $Ca^{2+}$. *J. Membrane Biol.* **78:**211–222

Hoffmann, E.K., Simonsen, L.O., Lambert, I.H. 1992. Cell volume regulation: Intracellular transmission. *In:* Interaction cell volume-cell function. ACEP Series. Springer, Heidelberg (*in press*)

Hoffmann, E.K., Sjøholm, C., Simonsen, L.O. 1983. $Na^+$, $Cl^-$ cotransport in Ehrlich ascites tumor cells activated during volume regulation (regulatory volume increase). *J. Membrane Biol.* **76:**269–280

Homma, T., Burns, K.D., Harris, R.C. 1990. Agonist stimulation of $Na^+/K^+/Cl^-$ cotransport in rat glomerular mesangial cells. Evidence for protein kinase C-dependent and $Ca^{2+}$/calmodulin-dependent pathways. *J. Biol. Chem.* **265:**17613-17620

Jensen, B.S., Hoffmann, E.K. 1990. On the K-dependence of the cation-anion cotransport system in Ehrlich ascites tumor cells. *Acta Physiol. Scand* **140:34***A*

Jensen, B.S., Jessen, F., Hoffmann, E.K. 1992. Activation of $Na^+$, $K^+$, $Cl^-$ cotransport in Ehrlich ascites tumor cells. *Acta Physiol. Scand.* **146(suppl. 608):**P3, 48

Jessen, F., Cherksey, B.D., Zeuthen, T., Hoffmann, E.K. 1989. Isolation and reconstitution of furosemide-binding proteins from Ehrlich ascites tumor cells. *J. Membrane Biol.* **108:**139–151

Jessen, F., Hoffmann, E.K. 1992. Activation of the Na-K-Cl cotransport system by disassembly of the actin filaments in Ehrlich ascites tumor cells. *Biochim. Biophys. Acta* **1110:**199–201

Kaji, D.M. 1989. Kinetics of volume sensitive K transport in human erythrocytes: Evidence for asymmetry. *Am. J. Physiol.* **256:**C1214–C1223

Kim, H.D., Tsai, Y.-S., Franklin, C.C., Turner, J.T. 1988. Characterization of $Na^+/K^+/Cl^-$ cotransport in cultured HT29 human colonic adenocarcinoma cells. *Biochim. Biophys. Acta* **946(2):**397–404

Klein, J.D., O'Neill, W.C. 1990. Effect of bradykinin on Na-K-2Cl cotransport and bumetanide binding in aortic endothelial cells. *J. Biol. Chem.* **265:**22238-22242

Klein, J.D. Bell, J.M., O'Neill, W.C. 1990. *J. Am. Soc. Nephrol.* **1:**722 (Abstr.)

Kort, J.J., Koch, G. 1990. The $Na^+$, $K^+$, $2Cl^-$-cotransport system in HeLa cells: Aspect of its physiological regulation. *J. Cell. Phys.* **145:**253–261

Kracke, G.R., Dunham, P.B. 1990. Volume-sensitive K-Cl cotransport in inside-out vesicles made from erythrocytes membranes from sheep of low-K phenotype. *Proc. Natl. Acad. Sci. USA* **87:**8575-8579

Kramhøft, B., Lambert, I.H., Hoffmann, E.K., Jørgensen, F. 1986. Activation of $Cl^-$ dependent K transport in Ehrlich ascites tumor cells. *Am. J. Physiol.* **251(20):**C369–C379

Lambert, I.H., Hoffmann, E.K., Jørgensen, F. 1989. Membrane potential, anion and cation conductances in Ehrlich ascites tumor cells. *J. Membrane Biol.* **111:**113–132

Lauf, P.K., McManus, T.J., Haas, M., Forbush, B., Duhm, J., Flatman, P.W., Saier, M.H., Russell, J.M. 1987. Physiology and biophysics of chloride and cation cotransport across cell membranes. *Fed. Proc.* **46(7):**2377–2394

Levinson, C. 1990. Regulatory volume increase in Ehrlich ascites tumor cells. *Biochim. Biophys. Acta* **1021:**1–8

Levinson, C. 1991*a*. Regulatory volume increase in Ehrlich ascites tumor cells is mediated by the 2Cl : Na : K cotransport system. *FASEB J.* **4:**A564

Levinson, C. 1991*b*. Inability of Ehrlich ascites tumor cells to volume regulate following a hyperosmotic challenge. *J. Membrane Biol.* **121:**279–288

Lewis, S.A., Donaldson, P. 1990. Ion channels and cell volume regulation: Chaos in an organized system. *News. Physiol. Sci.* **5:**112–119

Liedtke, C.M. 1990. Calcium and $\alpha$-adrenergic regulation of Na-Cl(K) cotransport in rabbit tracheal epithelial cells. *Am. J. Physiol.* **259:**L66–L72

Liu, S., Jacob, R., Piwnica-Worms, D., Lieberman, M. 1989. Interaction of (Na + K + 2Cl) cotransport and the Na/K pump in cultured chick cardiac myocytes. *Mol. Cell. Biochem.* **89:**147–150

Lytle, C., Lear, S., Forbush, B. 1990. Regulation of the Na, K, Cl-cotransporter in tubules isolated from shark rectal gland. *Biophys. J.* **57:**183*a*

Mandel, K.G., Dharmsathaphorn, K., McRoberts, J.A. 1986. Characterization of a cyclic AMP activated $Cl^-$ transport pathway in the apical membrane of a human colonic cell line. *J. Biol. Chem.* **261(2):**704–712

Mercer, R.W., Hoffmann, J.F. 1985. Bumetanide-sensitive Na/K cotransport in ferret red blood cells. *Biophys. J.* **47:**157*a*

Nishizuka, Y. 1984. Studies and perspectives of protein kinase C. *Nature* **308:**693–698

O'Donnell, M.E. 1989. Regulation of Na-K-Cl cotransport in endothelial cells by atrial natriuretic factor. *Am. J. Physiol.* **257:**C36–C44

O'Donnell, M.E. 1991. Endothelial cell sodium-potassium-chloride cotransport. Evidence of regulation by $Ca^{2+}$ and protein kinase C. *J. Biol. Chem.* **266:**11559–11566

O'Donnell, M.E., Owen, N.E. 1986. Role of cyclic GMP in atrial natriuretic factor stimulation of $Na^+$, $K^+$, $Cl^-$ cotransport in vascular smooth cells. *J. Biol. Chem.* **261:**15461–15466

Owen, N.E. 1985. Effect of TPA on ion fluxes and DNA synthesis in vascular smooth muscle cells. *J. Cell Biol.* **101:**454–459

Owen, N.E., Prastein, M.L. 1985. Na/K/Cl cotransport in cultured human fibroblasts. *J. Biol. Chem.* **260:**1445-1451

Owen, N.E., Ridge, K.M. 1989. Mechanism of angiotensin II stimulation of Na-KCl cotransport of vascular smooth muscle cells. *Am. J. Physiol.* **257:**C629–C636

Palfrey, H.C., Rao, M.C. 1983. Na/K/Cl co-transport and its regulation. *J. Exp. Biol.* **106:**43–54

Panet, R., Atlan, H. 1990. Bumetanide-sensitive $Na^+/K^+/Cl^-$

transporter is stimulated by phorbol ester and different mitogens in quiescent human skin fibroblasts. *J. Cell. Phys.* **145:**30–38

Paris, S., Pouysségur, J. 1986. Growth factors activate the bumetanide-sensitive $Na^+/K^+/Cl^-$ cotransport in hamster fibroblasts. *J. Biol. Chem.* **261(14):**6177-6183

Peterson, G.L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83:**346–56

Pewitt, E.B., Hegde, R.S., Haas, M., Palfrey, H.C. 1990. The regulation of Na/K/2Cl cotransport and bumetanide binding in avian erythrocytes by protein phosphorylation and dephosphorylation. *J. Biol. Chem.* **265:**20747–20756

Rotin, D., Mason, M.J., Grinstein, S. 1991. Channels, antiports, and regulation of cell volume in lymphoid cells. *Advances in Comparative and Environmental Physiology*. Vol. 9, chap. 5. Springer-Verlag, Berlin, Heidelberg

Roy, G., Sauvé, R. 1987. Effect of anisotonic media on volume, ion and amino-acid content and membrane potential of kidney cells (MDCK) in culture. *J. Membrane Biol.* **100:**83–96

Sarkardi, B., Cheung, R., Mack, E., Grinstein, S., Gelfand, E.W., Rothstein, A. 1985. Cation and anion pathways in volume regulatory response of human lymphocytes to hypoosmotic media. *Am. J. Physiol.* **248:**C480–C487

Sharipo, M., Matthews, J., Hecht, G., Delp, C., Madara, J.L. 1991. *J. Clin. Invest.* **87:**1903–1909

Siebens, A.W. 1985. Cellular volume control. *In:* The Kidney: Physiology and Pathophysiology. D.W. Seldin and G. Giebisch, editors. pp. 91–115. Raven, New York

Silva, P., Spokes, K., Epstein, J.A., Stevens, A., Epstein, F.H. 1984. Cyclic AMP stimulates ouabain-insensitive ion-movement in shark [*Squalus acanthias*] rectal gland. *J. Comp. Physiol. B* **154:**139–144

Simonsen, L.O., Brown, A.M., Christensen, S., Harbak, H., Svane, P.C., Hoffmann, E.K. 1990. Thrombin and bradykinin mimic the volume response induced by cell swelling in Ehrlich mouse ascites tumour cells. *Renal. Physiol. Biochem.* **13:**176

Smith, J.B., Smith, L. 1987. $Na^+/K^+/Cl^-$ cotransport in cultured vascular smooth muscle cells: stimulation by angiotensin II and calcium ionophores, inhibition by cyclic AMP and calmodulin antagonists. *J. Membrane Biol.* **99:**51–63

Sten-Knudsen, O., Ussing, H.H. 1981. The flux ratio equation under nonstationary conditions. *J. Membrane Biol.* **63:**233–242

Sun, A., Grossman, E.B., Lombard, M., Hebert, S.C. 1991. Vasopressin alters the mechanism of apical $Cl^-$ entry from $Na^+$, $Cl^-$ to $Na^+$, $K^+$, $2Cl^-$ cotransport in mouse medullary thick ascending limb. *J. Membrane Biol.* **120:**83–94

Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., Tomita, F. 1986. Staurosporine, a potent inhibitor of phospholipid/$Ca^{++}$ dependent protein kinase. *Biochem. Biophys. Res. Comm.* **135(2):**397–402

Tivey, D.R. Simmons, N.L., Aiton, J.F. 1985. Role of passive potassium fluxes in cell volume regulation in cultured HeLa cells. *J. Membrane Biol.* **87:**93–105

Ussing, H.H. 1982. Volume regulation of frog skin epithelium. *Acta Physiol. Scand.* **114:**363–369

Ussing, H.H. 1986. Epithelial cell volume regulation illustrated by experiments in frog skin. *Renal Physiol.* **9:**38–46

Received 13 February 1992; revised 5 June 1992