Coupled Na/K/Cl Efflux

"Reverse" Unidirectional Fluxes in Squid Giant Axons

ANIBAL A. ALTAMIRANO and JOHN M. RUSSELL

From the Department of Physiology and Biophysics, The University of Texas Medical Branch, Galveston, Texas 77550, and the Marine Biological Laboratory, Woods Hole, Massachusetts 02543

ABSTRACT Studies of unidirectional Cl^-, Na^+, and K^+ effluxes were performed on isolated, internally dialyzed squid giant axons. The studies were designed to determine whether the coupled Na/K/Cl co-transporter previously identified as mediating influxes (Russell. 1983. *Journal of General Physiology.* 81:909–925) could also mediate the reverse fluxes (effluxes). We found that 10 μM bumetanide blocked 7–8 pmol/cm^2·s of Cl^- efflux from axons containing ATP, Na^+, and K^+. However, if any one of these solutes was removed from the internal dialysis fluid, Cl^- efflux was reduced by 7–8 pmol/cm^2·s and the remainder was insensitive to bumetanide. About 5 pmol/cm^2·s of Na^+ efflux was inhibited by 10 μM bumetanide in the continuous presence of 10^{-5} M ouabain and 10^{-7} M tetrodotoxin if Cl^-, K^+, and ATP were all present in the internal dialysis fluid. However, the omission of Cl^- or K^+ or ATP reduced the Na^+ efflux, leaving it bumetanide insensitive. K^+ efflux had to be studied under voltage-clamp conditions with the membrane potential held at −90 mV because the dominant pathway for K^+ efflux (the delayed rectifier) has a high degree of voltage sensitivity. Under this voltage-clamped condition, 1.8 pmol/cm^2·s of K^+ efflux could be inhibited by 10 μM bumetanide. All of these results are consistent with a tightly coupled Na/K/Cl co-transporting efflux mechanism. Furthermore, the requirements for *cis*-side co-ions and intracellular ATP are exactly like those previously described for the coupled Na/K/Cl influx process. We propose that the same transporter mediates both influx and efflux, hence demonstrating "reversibility," a necessary property for an ion-gradient–driven transport process.

INTRODUCTION

Anion/cation co-transport processes have now been described for a variety of cell types (Ellory et al., 1982), including the squid giant axon (Russell, 1979, 1983). It is generally considered that these transport mechanisms constitute

Address reprint requests to Dr. John M. Russell, Dept. of Physiology and Biophysics, University of Texas Medical Branch at Galveston, Galveston, TX 77550-2781.

J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/87/05/0669/18$1.00

Volume 89 May 1987 669–686

examples of secondary active transport. That is, they effect net ion fluxes by using the potential energy resident within transmembrane ion gradients, most notably that of Na^+. According to such models, the net direction of transport can be predicted from a knowledge of the relevant ion gradients. Such models predict reversal of net transport, if one can experimentally reverse ion gradients. Implicit in this prediction is that the responsible transport mechanism can mediate unidirectional fluxes in both directions across cell membranes unless other (e.g., metabolic or kinetic) factors prevent such bidirectional transport. In view of the fact that ATP is often a requirement for these co-transport processes, it is of considerable interest to test directly for reverse fluxes mediated by coupled anion/cation co-transporters.

Several technical problems may hamper such testing for most cell types or membrane vesicle preparations; for example, control of internal solute levels is difficult, particularly when such levels need to be far from those ordinarily encountered. In addition, the relevant fluxes may be small compared with other pathways for the ions, making it difficult to directly measure all three ionic fluxes mediated by the co-transporter. However, in regard to these problems, the squid giant axon is a particularly advantageous experimental subject. It is large enough to permit intracellular solute control by means of the technique of internal dialysis (Brinley and Mullins, 1967). The normal intracellular [Cl] is ~100–120 mM. This value is considerably higher than 35–40 mM, which equilibrium considerations would predict. Thus, an active Cl^- uptake process is indicated. Virtually all the unidirectional influx of Cl^- is via a specific influx mechanism. This process moves 3 Cl^-, 2 Na^+, and 1 K^+ into the axon by means of a tightly coupled co-transport process, which is electrically neutral (Russell, 1983, 1984). An important property of the co-transporter is that it not only requires the simultaneous presence of all three of the substrate ions in the extracellular fluid, but it also requires ATP in the internal fluid (Russell, 1976, 1979, 1983). Therefore, the question arises whether the transporter might use ATP to prevent or limit reversibility, in a manner similar to the Na pump. In partial answer to this question, we show in the present study that the coupled Na/K/Cl transport process of the squid axolemma does mediate "reverse" fluxes. That is, the undirectional effluxes of Cl^-, Na^+, and K^+ have been measured and shown to possess the same qualitative properties as previously demonstrated for unidirectional influxes. Thus, we now show that the effluxes of these ions are coupled to one another, are blocked by bumetanide in a dose-dependent manner, and require ATP. Although ATP is apparently required by this transporter to promote the net uptake by this mechanism, the ATP does not function to prevent the functional "backflux."

Some of these results were presented at the 29th annual meeting of the Biophysical Society (Russell, 1985).

METHODS

Axons

These experiments were performed at the Marine Biological Laboratory, Woods Hole, MA, during May and early June, 1984–1985. Live specimens of the squid, *Loligo pealei*,

were decapitated, and the first stellar nerves were removed and placed in cold, Woods Hole seawater. After careful cleaning, the giant axons were mounted horizontally in a dialysis chamber.

The temperature of the bath was maintained at 17°C by means of a coolant fluid circulating from a Lauda K2/RD cooler (Brinkmann Instruments Co., Westbury, NY) through the underside of the dialysis chamber; a thermistor (Fenwal Electronics, Framingham, MA) located just below the axon constantly monitored the bath temperature.

Solutions

The standard external solution that bathed the axon (squid seawater) had the following composition (mM): 425 Na^+, 10 K^+, 3 Ca^{++}, 65 Mg^{++}, 561 Cl^-, 10 EPPS [*N*(2-hydroxyethyl) piperazine-*N'*-3-propanesulfonic acid], 0.1 EDTA, 10^{-5} M ouabain, 10^{-7} M tetrodotoxin; pH 8.0; osmolality, 975 ± 5 mosmol/kg. Bumetanide was a gift of Hoffman-LaRoche (Nutley, NJ). It was made up as a 20-mM stock solution in ethanol and stored at 0°C.

A variety of internal dialysis fluids (DFs) was used in this study. Their compositions are listed in Table I. For ^{36}Cl-containing solutions, the contribution of the carrier Cl^- in the

TABLE I

Composition of Internal Dialysis Fluids

Solute	Standard DF	50 K/ 350 Na	0 K (DF-O)	0 K (DF-C)	15 K (DF-B)	0 Na (DF-E)	0 Na (DF-A)	0 Cl (DF-N)
				mM				
K	350	50	0	0	15	350	15	350
Na	50	350	50	400	385	0	0	50
NMDG	0	0	311	0	0	4.79	351.7	0
Glutamate	264	204	225	264	264	256.2	225	414
Glycine	212.2	212.2	241	212.2	212.2	225	190	208

All solutions contained (mM): 7.0 Mg, 150 Cl, 10 MOPS [3-(*N*-morpholino)propanesulfonic acid], 14.2 Tris, 2.0 EGTA, 0.5 phenol red, 3.0 ATP; pH 7.35; osmolality, 970 mosmol/kg.

isotope as supplied by New England Nuclear (Boston, MA) was taken into account. The final [Cl] for each solution was measured using a Buchler-Cotlove chloridometer (Nuclear-Chicago, Fort Lee, NJ). The specific activity of the ^{36}Cl-containing DFs was ~100 μCi/mmol. ^{22}Na was supplied by New England Nuclear as a carrier-free solution. It was added directly to the appropriate DF to attain a final specific activity of ~400 μCi/mmol. ^{42}K was obtained from New England Nuclear as the "very high specific activity nuclide." An aliquot was evaporated to dryness and the DF was added to attain a final specific activity (at zero decay time) of 3,500 μCi/mmol. ATP as the Tris salt was obtained vanadium-free from Sigma Chemical Co. (St. Louis, MO) and made up as a 400-mM stock solution. The pH was adjusted to 7.0 with Tris and the solution was stored frozen until it was added to the DF just before the DF was used in an experiment.

Voltage-Clamp Technique

For the K^+ efflux experiments, it was necessary to voltage-clamp the membrane resting potential. In order to accomplish this, a 25-μm platinized Pt/Ir wire was permanently placed within the lumen of the dialysis tube. The wire was then connected to a constant-current source (SD-9 DC Stimulator, Grass Instrument Co., Quincy, MA) in series with a 1-MΩ resistor. Current crossing the axolemma was picked up by an 18-gauge Pt wire inserted along the bottom of the chamber slot occupied by the axon. The current passing

back to the stimulator was measured by recording the voltage drop across a 5,100-Ω resistor in series with the return pathway. Membrane potential was constantly monitored using a 0.1 M KCl–filled micropipette inserted longitudinally next to the dialysis tube. The electrode was connected to a high-impedance electrometer. The feedback amplifier was a model JMR-1942 (T-M Products, Drumright, OK). The membrane potential and the current necessary to hold the membrane potential constant were recorded on an Omniscribe pen recorder (Houston Instruments, Austin, TX).

Dialysis

The technique of internal dialysis (Brinley and Mullins, 1967) was used in these experiments. The dialysis tube was a 12-cm length of hollow cellulose acetate tubing (140 μm o.d.; FRL, Inc., Dedham, MA) glued to a plastic T tube. For the present efflux experiments, the tube had a central region ~18 mm long that had been rendered porous by a 16–20-h soak in 0.1 N NaOH.

A 40–45-mm length of axon was cannulated at both ends, and then the dialysis tube was carefully guided through the axon until the porous region was positioned in the central portion of the axon. The axon was then lowered onto grease dams placed ~20 mm apart at either end of the central slot in the bath. The grease, a mixture of Vaseline and mineral oil, was also applied on top of the axon at the dam sites. Then greased plastic inserts were placed over the axon at these two points, isolating the central, dialyzed region of the axon from the cannulated ends.

Efflux Procedures

The axon was dialyzed with fluids containing the requisite radioactive isotope (e.g., ^{36}Cl, ^{22}Na, or ^{42}K) and solute composition. Isotopes that crossed the axolemma were collected by flowing squid seawater (SSW) over the axon at a constant rate of 2 ml/min. The SSW was then collected directly into scintillation vials for 4 min and 7 ml of 1:1 toluene–Triton X-100 counting cocktail containing 4 g/liter Omnifluor (New England Nuclear) was added. Each sample was counted to a counting error not greater than 5% using an LS-3801 scintillation counter (Beckman Instruments, Inc., Irvine, CA).

RESULTS

Previous studies on the Na/K/Cl co-transporter in the squid axon have dealt exclusively with the influxes of the three co-transported ions (Russell, 1979, 1983). These earlier studies revealed some specific characteristics of the transport process that "reverse" fluxes mediated by the same transporter might be expected to share. Among these characteristics, the following are most important: (*a*) inhibition of fluxes by bumetanide, (*b*) an absolute requirement for ATP, and (*c*) an absolute requirement for the simultaneous presence of all co-ions on the same side of the membrane. The design of the present study was to test for these characteristics using the unidirectional effluxes of Cl^-, Na^+, and K^+. This work focuses largely on Cl^- fluxes because Cl^- efflux via the co-transporter ought to be the largest and most accurately measured of the three, given the influx stoichiometry (Russell, 1983) of 3 Cl:2 Na:1 K. However, some data are presented on the effluxes of both Na^+ and K^+ and these data contribute to the overall picture of the Na/K/Cl transporter mediating fluxes in the reverse direction.

Cl^- Efflux Studies

Bumetanide-sensitive Cl^- efflux. The standard internal conditions used in the present study were as follows: 150 mM [Cl^-], 350 mM [K^+], 50 mM [Na^+], 3 mM

[ATP]. Under these conditions, when the external solution was SSW containing 10^{-5} M ouabain and 10^{-7} M tetrodotoxin (TTX), the average Cl^- efflux was 13.3 ± 0.9 pmol/cm^2·s (see Table II). Bumetanide (10^{-5} M) was applied externally to 17 axons under these control ionic conditions. The result was that about half the Cl^- efflux was inhibited. This inhibition was reversible, as can be seen in Fig. 1. It is important to note that the concentration of bumetanide used in these experiments (10 μM) was sufficient to completely inhibit the coupled transporter, even in the presence of the relatively high external [Cl^-] of SSW (561 mM) (see below, Fig. 4).

TABLE II

Bumetanide-sensitive Cl^- Efflux under Various Solute Conditions

	Control	10 μM Bumetanide	Bumetanide-sensitive	
		pmol/cm^2·s		
(*A*) Standard conditions: DF = 350 K/50 Na/150 Cl/3 ATP				
$\bar{x}$	13.3	6.4	6.8	($P < 0.01$)
SEM	0.9	0.4	0.6	
$n = 17$				
(*B*) ATP dependence: DF = 350 K/50 Na/150 Cl/0 ATP				
$\bar{x}$	5.8	5.4	0.5	
SEM	0.5	0.4	0.7	
$n = 7$				
(*C*) Intracellular K^+ dependence:				
(*i*) DF = 0 K/50 Na/150 Cl/3 ATP (DF-O)				
$\bar{x}$	4.8	5.3	−0.6	
SEM	1.1	1.2	0.9	
$n = 8$				
(*ii*) DF = 0 K/400 Na/150 Cl/3 ATP (DF-C)				
$\bar{x}$	4.6	3.7	1.2	($P < 0.05$)
SEM	0.8	1.0	0.4	
$n = 9$				
(*D*) Intracellular Na^+ dependence: DF = 350 K/0 Na/150 Cl/3 ATP (DF-E)				
$\bar{x}$	7.6	7.4	0.3	
SEM	0.8	0.7	0.4	
$n = 11$				

The demonstration of a bumetanide-sensitive Cl^- efflux provides the first piece of evidence for reverse fluxes occurring via the coupled Na/K/Cl transporter, which has heretofore been known in the squid axon as a transporter mediating the uptake of these ions. Further evidence to support the existence of an efflux mode of the co-transporter was sought by determining the ATP and co-ion requirements of the bumetanide-sensitive Cl^- cells.

ATP requirement for Cl^- efflux. The technique of intracellular dialysis permits us to reduce cellular ATP to levels that completely abolish the bumetanide-sensitive Cl^- influx or forward mode of the transporter (Russell, 1983). Fig. 2 demonstrates that total Cl^- efflux was lower in the absence of ATP than in its presence (cf. Table II, *A* and *B*). When bumetanide (10^{-5} M) was applied to the ATP-depleted axon, no effect was noted on the residual ATP-independent Cl^- efflux. In the continued presence of bumetanide, adding back ATP had no effect, but upon bumetanide removal, Cl^- efflux increased to ~11 pmol/cm^2·s.

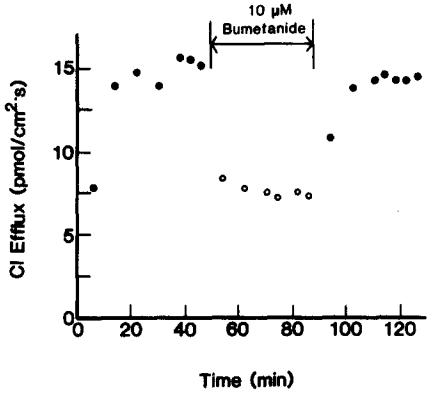

FIGURE 1. Reversible effect of 10 μM bumetanide on Cl^- efflux from an internally dialyzed squid axon. This axon was continuously dialyzed with standard DF (Table I) containing 350 mM K^+, 50 mM Na^+, and 150 mM Cl^-. Dialysis was begun 40 min before the zero time on the graph, at which time the DF was changed to one containing ^{36}Cl. Isotopic equilibrium was reached in 20 min. The axon was continually superfused with SSW containing 10^{-5} M ouabain and 10^{-7} M TTX. At 48 min, 10 μM bumetanide was added to the external fluid from a 20-mM stock solution in ethanol. Axon diameter, 440 μm; temperature, 17°C.

Thus, in the absence of ATP, there was no bumetanide-sensitive Cl^- efflux. Table II, part *B*, shows that there was essentially no effect on Cl^- efflux after treatment with 10 μM bumetanide in seven axons depleted of ATP. It is also of interest that the average Cl^- efflux from ATP-depleted axons (5.8 ± 0.5 pmol/cm^2·s) was essentially the same as the average Cl^- efflux from ATP-containing axons treated with 10 μM bumetanide (6.4 ± 0.4 pmol/cm^2·s). Thus, either removal of ATP or treatment with bumetanide inhibited Cl^- efflux to the same

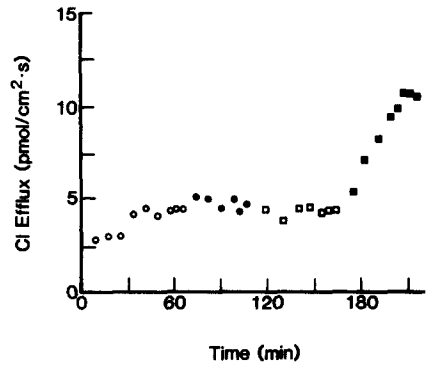

FIGURE 2. Lack of effect of 10 μM bumetanide on Cl^- efflux from an axon depleted of ATP. This axon was dialyzed for 65 min before zero time with ATP-free standard DF (Table I) while being continuously superfused with SSW containing 2 mM cyanide, 10^{-5} M ouabain, and 10^{-7} M TTX. 3 mM ATP was added back at 109 min. ○, 0 ATP; ●, 0 ATP plus 10 μM bumetanide; □, 3 ATP plus 10 μM bumetanide; ■, 3 ATP. Axon diameter, 498 μm; temperature, 17°C.

extent, as closely as can be determined. Such a result would be expected if both treatments blocked the same transport process.

Intracellular K^+ is required for bumetanide-sensitive Cl^- efflux. If the bumetanide- and ATP-sensitive Cl^- efflux is mediated by the Na/K/Cl transporter, then removal of cellular K^+ ought to reduce Cl^- efflux to ~5–6 pmol/cm^2·s, a residuum that ought not to be further reduced by bumetanide treatment. Fig. 3 illustrates the effect on Cl^- efflux of removing intracellular K^+. In this experiment, extracellular K^+ was first replaced with Na^+. This external modification had no discernible effect by itself, but permitted the next step in the experiment, namely the removal of intracellular K^+. In the experiment illustrated in Fig. 3, the intracellular K^+ was removed by substituting *N*-methyl-D-glucamine (NMDG;

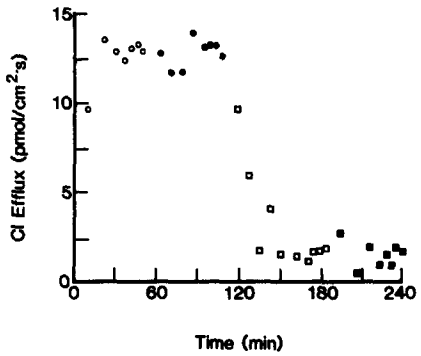

FIGURE 3. Intracellular K^+ is required for bumetanide-sensitive Cl^- efflux. This axon was dialyzed for 40 min before zero time, when ^{36}Cl was included in the standard DF (Table I). A steady Cl^- efflux of ~13 pmol/cm^2·s was reached within 20 min. At 52 min, the external superfusing solution was changed from SSW to K-free SSW (Na^+ replaced K^+). This removal of external K^+ had no effect upon Cl^- efflux. However, at 109 min, the DF was changed to a K-free fluid (DF-O, Table I). By 150 min, the Cl^- efflux decreased to 1.7 pmol/cm^2·s. The addition of 10 μM bumetanide had no further effect upon Cl^- efflux. Axon diameter, 430 μm; temperature, 17°C.

DF-O, Table I) leaving intracellular [Na^+] constant at 50 mM. Dialysis with K^+-free DF resulted in a significant decrease of Cl^- efflux (from ~12.5 to ~2 pmol/cm^2·s). Subsequent treatment with 10^{-5} bumetanide had no further inhibitory effect. The results of seven other identical experiments are collated in Table II, part *C* (*i*), which shows that after cellular K^+ depletion, Cl^- efflux could not be further reduced by bumetanide. Note that the average Cl^- efflux from K^+-depleted axons was essentially the same flux obtained by removal of ATP or treatment with 10 μM bumetanide.

In an attempt to determine whether intracellular Na^+ could, at least partially, substitute for K^+, another series of experiments was performed in which intracellular K^+ was replaced by Na^+. The results are presented in Table II, part *C* (*ii*). Unlike the case in which NMDG was substituted for K^+, in all nine axons in which Na^+ was substituted for K^+, treatment with 10 μM bumetanide resulted

in a measurable, small (but statistically significant; $P < 0.05$) decrease of Cl^- efflux. This result suggests that Na^+ may partially substitute for K^+ in activating Na/K/Cl co-transport. Another possibility is that this intracellular Na–dependent Cl^- efflux represents Na-Cl/Na-Cl exchange, as demonstrated in duck red cells (Lytle et al., 1986).

Intracellular Na^+ is required for bumetanide-sensitive Cl^- efflux. In an earlier report (Russell, 1979), we could find no evidence of an internal Na-dependent Cl^- efflux. However, in the present experiments, we noted that the total Cl^- efflux under control conditions was ~13 pmol/cm^2·s. In the former experiments, the Cl^- efflux was only ~5 pmol/cm^2·s. The latter value is close to that of the residual efflux after bumetanide treatment or ATP depletion. Therefore, we re-examined the question of the intracellular Na^+ dependence of a portion of Cl^- efflux. To test for an intracellular Na^+ requirement of the bumetanide-sensitive Cl^- efflux, we removed intracellular Na^+ by dialyzing with NMDG (DF-E, Table I). Table II, part *D*, presents the collated results from 11 axons dialyzed with Na^+-free DF for 90–120 min. The results demonstrate that the bumetanide-sensitive Cl^- efflux shares the co-ion requirement for Na^+ already demonstrated for the bumetanide-sensitive Cl^- influx. We have no explanation for our earlier failure to observe this Na^+ dependence.

Bumetanide dose-response relation. A quantitative assessment of the affinity of bumetanide for the Na/K/Cl transporter was important for the characterization of the transporter. In order to have as large a flux as possible to inhibit with bumetanide, we dialyzed axons with a fluid that was high in [Na^+] and somewhat low in [K^+]. In the absence of the relevant internal Na^+ and K^+ kinetic data, these values were arbitrarily set at 50 mM [K^+] and 350 mM [Na^+]. The external fluid was SSW that contained 561 mM Cl^- (see Methods). In 24 axons, the average Cl^- efflux before treatment with bumetanide was 12.0 ± 0.8 pmol/cm^2·s, and after 10 μM bumetanide, it was 4.4 ± 0.8 pmol/cm^2·s, giving a bumetanide-sensitive Cl^- efflux of 8.1 ± 0.6 pmol/cm^2·s. Thus, although this internal ionic arrangement did cause the bumetanide-sensitive Cl^- efflux to be slightly larger than the bumetanide-sensitive Cl^- efflux from axons dialyzed with 350 K/50 Na (6.8 pmol/cm^2·s; see Table II, part *A*), the difference is not large and is statistically significant only at the $P = 0.06$ level.

The bumetanide dose-response relationship was determined in 19 axons dialyzed with 50 K^+/350 Na^+ DF and bathed with SSW (which contained 561 mM Cl^-). The protocol was as follows. The Cl^- efflux was first determined in the absence of bumetanide. Then each axon was treated with 0.01, 0.05, 0.1, 1, or 5 μM bumetanide, and the efflux was measured again. Finally, every axon was treated with 10 μM bumetanide. Three axons were further treated with 50 μM bumetanide. In all three of the last experiments, exposure to 50 μM bumetanide after 10 μM resulted in a slight increase of Cl^- efflux. Presumably, the higher level of bumetanide (or ethanol) was somewhat toxic. The bumetanide was added to the external bathing solution from a 20-mM stock solution in ethanol. Control experiments ($n = 3$) showed that an ethanol concentration of 0.1% (which would be the resultant ethanol concentration at a final bumetanide concentration of 20 μM) caused no significant change in Cl^- efflux.

Fig. 4 shows the relation between Cl^- efflux and bumetanide concentration. These data show that inhibition was complete at a bumetanide concentration of 5 μM and was half-saturated at a bumetanide concentration of ~0.1 μM. These results validate the use of 10 μM bumetanide throughout the present work. It also is useful to re-emphasize explicitly that treatment with 10 μM bumetanide resulted in a Cl^- efflux that did not differ significantly from that observed after ATP depletion, intracellular K^+ removal, and intracellular Na^+ removal (cf. Table II). Thus, it seems that the bumetanide-sensitive Cl^- efflux is a quantitatively valid measure of the ATP-, K^+-, Na^+-dependent Cl^- efflux.

Dependence of bumetanide-sensitive Cl^- efflux on intracellular [Cl]. A series of experiments was performed in which the intracellular [Cl^-] was varied between

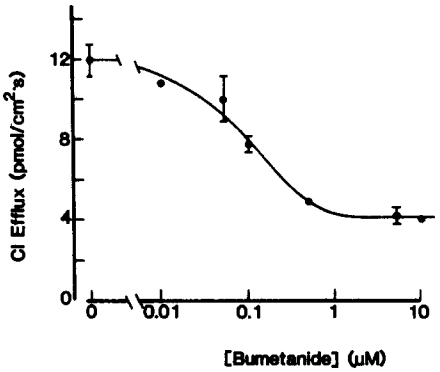

FIGURE 4. Dose-response relation between bumetanide concentration in the external bathing solution and Cl^- efflux from dialyzed squid axons. These data are collated from 19 axons dialyzed with 50 mM K^+, 350 mM Na^+, 150 mM Cl^- plus 3 mM ATP. Each symbol represents data from at least three axons. Where standard error bars are not shown, they are within the limits of the filled circle. The axons were superfused with SSW containing 10^{-5} M ouabain and 10^{-7} M TTX. Temperature, 17°C.

20 and 150 mM with 350 mM [K^+] and 50 mM [Na^+], and 3 mM ATP present at all times. The external fluid was SSW containing 10^{-5} M ouabain and 10^{-7} M TTX. Each axon was dialyzed for 90 min with the test [Cl^-], and then 10 μM bumetanide was applied. The concentrations of Cl^- in the axoplasm at the two lowest [Cl^-] values tested (20 and 30 mM) were verified in some parallel experiments using Cl^--sensitive liquid ion-exchanger microelectrodes. The results from a total of 23 axons are shown in Fig. 5. When fitted with the Hill equation, the bumetanide-sensitive Cl^- efflux exhibited a V_{max} of 10.1 pmol/$cm^2 \cdot s$, a half-saturation constant of 53 mM, and a Hill coefficient of 2.2.

Effect of trans-side ion removal on Cl^- efflux. Fig. 3 shows that Cl^- efflux was unaffected by the complete removal of external (*trans* side) K^+. This is of some interest in view of the fact that removal of external K^+ is known to inhibit Cl^- influx via the "forward" Na/K/Cl co-transporter (Russell, 1983). Thus, *trans*-side K^+ is not necessary for the transporter to be able to return to the "inward-facing" conformation.

A prominent feature of the coupled Na/K/Cl uptake process in the axon is the inverse relation between intracellular [Cl^-] and Cl^- influx (Russell, 1983). Thus, when intracellular [Cl^-] is nominally zero, Na/K/Cl-coupled influxes are at their maximum. We tested for a similar *trans*-side dependence of Cl^- efflux. In earlier work on Cl^- influx (Russell, 1979), we noted that replacing external Cl^- with methanesulfonate, sulfate, or gluconate gave the same result. In the present experiments, we used only methanesulfonate. In three axons, we noted a small increase of Cl^- efflux upon removal of external Cl^-. The average Cl^- efflux into normal SSW (561 mM [Cl^-]) was 11.7 ± 3.0 pmol/cm^2·s and into 0-Cl SSW it was 15.1 ± 4.1 pmol/cm^2·s. Fig. 6 illustrates this *trans*-side effect and its reversible nature. In one of these three axons, 10 μM bumetanide applied in

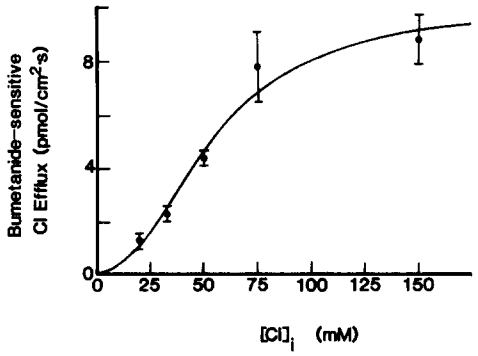

FIGURE 5. Intracellular [Cl^-] dependence of bumetanide-sensitive Cl^- efflux from dialyzed squid axons. 23 axons were dialyzed for at least 90 min with a DF containing a [Cl^-] varying from 20 to 150 mM. After a steady efflux level was attained, 10 μM bumetanide was applied. The average decrease of Cl^- efflux caused by bumetanide treatment for each Cl^- concentration tested is plotted against the Cl^- concentration. When these data were fitted to the Hill equation with a nonlinear least-squares curve-fitting program, the best-fit curve gave a V_{max} of 10.1 pmol/cm^2·s, a half-saturation constant of 53.1 mM, and a Hill coefficient of 2.2. The χ^2 goodness-of-fit was 0.111.

nominally 0-Cl^- SSW reduced Cl^- efflux to 8.3 pmol/cm^2·s. These results demonstrate that removal of *trans*-side Cl^- stimulated Cl^- efflux, but to a much lesser extent than previously observed for Cl^- influx. The extra Cl^- efflux appears to be inhibited by bumetanide. It is important to emphasize that such *trans*-side inhibition is the opposite effect from that expected for a Cl^-/Cl^- "isotopic" or self-exchange process. It also argues against the existence of Na-Cl/Na-Cl or K-Cl/K-Cl exchange modes in squid axon, such as have been reported for duck red cells (Lytle et al., 1986).

Na^+ Efflux Studies

Bumetanide-sensitive Na^+ efflux. If the bumetanide-sensitive Cl^- efflux was mediated by the coupled Na/K/Cl transporter, then one would expect to find a bumetanide-sensitive Na^+ efflux. Furthermore, if the stoichiometry of the efflux

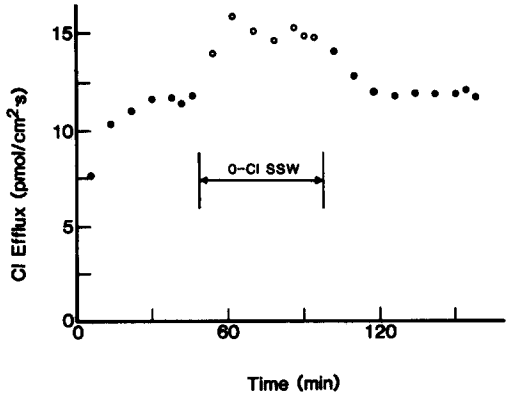

FIGURE 6. Effect of *trans*-side Cl^- removal on Cl^- efflux. The axon was dialyzed with standard DF (Table I). At 48 min, the external fluid was changed to one containing methanesulfonate instead of Cl^-. This resulted in a 3 pmol/cm^2·s increase of Cl^- efflux. This effect was fully reversible upon the return to Cl^--containing SSW. Axon diameter, 500 μm; temperature, 17°C.

was the same as the influx, one would expect, under standard conditions, to find a bumetanide-sensitive Na^+ efflux of $6.9 \times 2/3 = 4.6$ pmol/cm^2·s.

Six axons were dialyzed with standard DF while the external fluid was SSW plus 10^{-5} M ouabain and 10^{-7} M TTX. Fig. 7 shows the Na^+ efflux under these conditions and the effect of applying 10 μM bumetanide. In this axon, the Na^+ efflux in the presence of ouabain and TTX was 10.7 pmol/cm^2·s. Treatment with bumetanide reduced the Na^+ efflux to 5.8 pmol/cm^2·s. This response was typical when compared with the average results of all six axons (Table III, part *A*). Notice that the average bumetanide-sensitive Na^+ efflux was 4.8 pmol/cm^2·s, a value very close to that predicted above for a 3 Cl:2 Na:1 K stoichiometry.

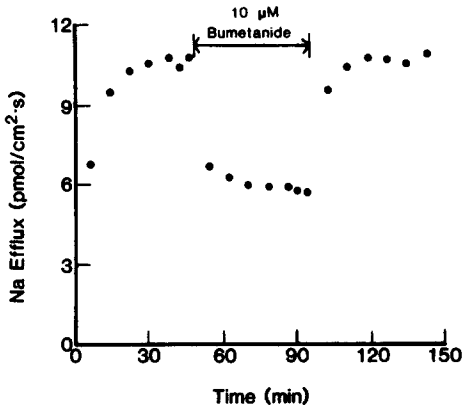

FIGURE 7. Reversible inhibition of Na^+ efflux by 10 μM bumetanide. The axon was dialyzed with standard DF (Table I) while being superfused with SSW containing 10^{-5} M ouabain and 10^{-7} M TTX. The application of bumetanide caused Na efflux to fall from 10.7 to 5.8 pmol/cm^2·s. Axon diameter, 510 μm; temperature, 17°C.

ATP dependence of Na^+ efflux. We tested for bumetanide-sensitive Na^+ efflux in five axons that were depleted of ATP by dialysis with ATP-free standard DF during treatment with 2 mM cyanide. 60–90 min of such treatment was sufficient to reduce ouabain- and TTX-resistant Na^+ efflux to very low values, averaging 1.4 pmol/cm^2·s (Table III, part *B*). The addition of 10 μM bumetanide was without further effect. Thus, the bumetanide-sensitive Na^+ efflux requires ATP. It is of interest that the Na^+ efflux in ATP-depleted axons was significantly lower than the flux from ATP-containing axons treated with bumetanide ($P < 0.05$). This result suggests the presence of an ATP-requiring Na^+ efflux that is resistant to treatment with ouabain, TTX, and bumetanide.

TABLE III

Bumetanide-sensitive Na^+ Efflux

	Control	10 μM Bumetanide	Bumetanide-sensitive
(*A*) Standard conditions: DF = 350 K/50 Na/150 Cl/3 ATP			
$\bar{x}$	10.3	5.4	4.8
SEM	0.8	0.5	0.4
$n = 6$			
(*B*) Dependence upon ATP: DF = 350 K/50 Na/150 Cl/0 ATP			
$\bar{x}$	1.4	1.4	0.04
SEM	1.0	1.0	0.1
$n = 5$			
(*C*) Intracellular Cl^- dependence: DF = 350 K/50 Na/0 Cl/3 ATP (DF-N)			
(*i*) Normal SSW ([Cl^-] = 561 mM)			
$\bar{x}$	5.8	4.2	1.6
SEM	0.9	1.0	0.5
$n = 5$			
(*ii*) 0-Cl SSW (gluconate replaced Cl)			
$\bar{x}$	5.1	4.6	0.5
SEM	1.2	1.1	0.5
$n = 5$			
(*D*) Intracellular K^+ dependence: DF = 0 K/50 Na/150 Cl/3 ATP (DF-0)			
$\bar{x}$	15.0	15.6	0.0
SEM	2.2	2.6	0.8
$n = 5$			

cis-side Cl^- dependence of Na efflux. Five axons were dialyzed with Cl^--free dialysis fluid (DF-N, Table I; glutamate replaced Cl) for at least 90 min, and then they were treated with 10 μM bumetanide. The results are shown in Table III, part *C* (*i*). Although the Na^+ efflux before bumetanide treatment was significantly lower than that found in axons dialyzed with Cl^--containing DF (see Table III, *A*), there was still a measurable amount of bumetanide-sensitive Na^+ efflux. These experiments were conducted in normal SSW (561 mM [Cl^-]) and we know that reducing intracellular [Cl^-] stimulates Cl^- influx (Russell, 1976, 1979, 1983). Thus, the most plausible explanation for the residual bumetanide-sensitive Na^+ efflux is that intracellular [Cl^-] was not truly zero when these flux values were obtained. This possibility was tested by superfusing five Cl^--depleted axons with Cl^--free SSW (gluconate replaced Cl^-). In these axons, there was no statistically significant bumetanide-sensitive Cl^- efflux (see Table III, *C*, *ii*).

Binding of radiolabeled bumetanide has been shown to require extracellular Cl^- (Forbush and Palfrey, 1983). Therefore, it is reasonable to ask whether the lack of bumetanide sensitivity simply reflects the inability of bumetanide to bind. However, we just showed above (see Fig. 6) that, in squid axon, bumetanide inhibition of Cl^- efflux can occur in the nominal absence of extracellular Cl^-. Thus, we believe these results are consistent with an intracellular Cl^-–dependent Na^+ efflux, which would be expected for an Na/K/Cl transporter in the "reverse" mode.

cis-side K^+ dependence of Na^+ efflux. Intracellular K^+ was reduced to zero by dialyzing with a K^+-free DF (DF-O, Table I) while the axon was superfused with K^+-free SSW (Na^+ replaced K^+), which contained 10^{-5} M ouabain and 10^{-7} M

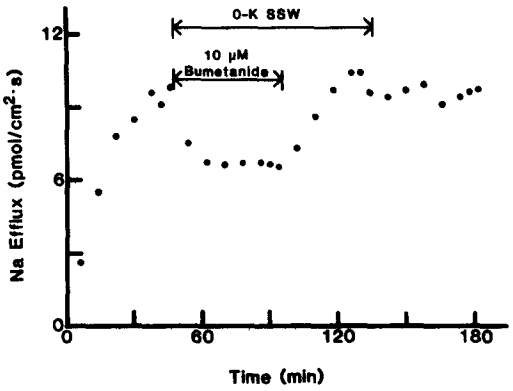

FIGURE 8. Demonstration that bumetanide can inhibit a portion of Na^+ efflux in the nominal absence of extracellular K^+. The axon was internally dialyzed with K^+-, Na^+-, Cl^--containing fluid (standard DF, Table I). These results show that only when bumetanide was added or removed from the external fluid was there an effect on Na^+ efflux. Axon diameter, 525 μm; temperature, 17°C.

TTX. Under these conditions, Na^+ efflux was relatively high, averaging 15 pmol/cm^2·s (see Table III, part *D*). The application of 10 μM bumetanide was without effect upon this Na^+ efflux. Thus, although Na^+ efflux was high under these conditions, it was apparently not occurring via Na/K/Cl co-transport, because it was unaffected by bumetanide (see below). Presumably, the high Na^+ efflux rates are the result of Na^+/Na^+ exchange activated by the depletion of cellular K^+ (not extracellular, see below) as has been noted by others (Smith, G., G. E. Breitwieser, and P. De Weer, personal communication).

Several laboratories have demonstrated that binding of labeled bumetanide (or *N*-methyl-furosemide) to cellular membranes is markedly reduced in the absence of extracellular K^+ (Hannafin et al., 1983; Forbush and Palfrey, 1983; Haas and Forbush, 1986). Thus, it was important for us to determine whether the lack of bumetanide-sensitive Na^+ efflux noted above was simply the result of an inability of bumetanide to bind to the Na/K/Cl transporter. Three axons dialyzed with standard DF (Table I) were tested for bumetanide-sensitive Na^+ efflux while being superfused with 0-K^+ SSW. The results from one of these axons are illustrated in Fig. 8. It can be seen that the simultaneous application

of bumetanide and K^+-free external fluid resulted in a reversible inhibition of Na^+ efflux by ~3.3 pmol/cm^2·s. As noted earlier for Cl^- efflux (Fig. 3), the 0-K^+ SSW alone had no significant effect, in this case, on Na^+ efflux. The average bumetanide-sensitive Na^+ efflux for all three axons with 0-K^+ SSW was 3.6 ± 0.3 pmol/cm^2·s. Although this value is slightly smaller than that reported for standard conditions (4.8 ± 0.4 pmol/cm^2·s; Table III, part *A*), the two sets of data are not statistically different. Thus, we conclude that, in the nominal absence of external K^+, bumetanide can inhibit Na/K/Cl co-transport in the squid axon.

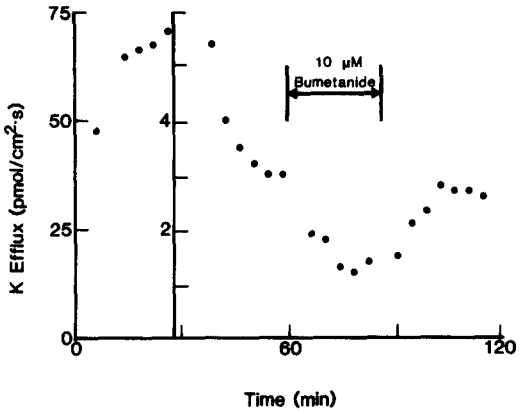

FIGURE 9. Reversible effect of 10 μM bumetanide on K^+ efflux from a voltage-clamped axon. This axon was dialyzed with 50 K^+/350 Na^+ DF (Table I). The axon was continuously superfused with SSW containing 10^{-5} M ouabain, 10^{-7} M TTX, and 1 mM DAP. For the first 28 min after the beginning of dialysis with ^{42}K-containing DF, the axon was not voltage-clamped and had a resting membrane potential of about −33 mV. The resultant K^+ efflux varied from 64.5 to 70.3 pmol/cm^2·s. Thereafter, the axon was voltage-clamped to −91 ± 1.0 mV (note new *y*-axis at 28 min when the axon was voltage-clamped). This resulted in a dramatic fall of K^+ efflux, to an average of 3.1 ± 0.08 pmol/cm^2·s. Treatment with 10 μM bumetanide reduced K^+ efflux further, to 1.3 ± 0.07 pmol/cm^2·s. After washing out the bumetanide, K^+ efflux recovered to 2.7 ± 0.7 pmol/cm^2·s. Thus, the bumetanide-sensitive K^+ efflux was 1.6 pmol/cm^2·s. The current required to maintain a −91-mV membrane potential varied from 4 to 8 μA. Axon diameter, 425 μm; temperature, 17°C.

K^+ Efflux Studies

Bumetanide-sensitive K^+ efflux. If the stoichiometry of the coupled Na/K/Cl efflux is the same as previously demonstrated for the coupled influx, the expected bumetanide-sensitive K^+ efflux would be ~2.2 pmol/cm^2·s. The presence of a large, voltage-sensitive K^+ efflux (Begenisich and De Weer, 1977; De Weer and Geduldig, 1978) makes the measurement of such a small K^+ efflux very difficult. In nine axons dialyzed with standard DF (350 K/50 Na/150 Cl/3 ATP), the average K^+ efflux was 54 pmol/cm^2·s and was very unstable, with even small changes of membrane resting potential causing larger changes in K^+ efflux than

that predicted for the bumetanide-sensitive component. Such a pronounced voltage sensitivity agrees quite well with the observations of De Weer and Geduldig (1978) that K^+ efflux trebled when the resting membrane potential was depolarized from −62 to −52 mV.

In order to reduce the background K^+ efflux and prevent spontaneous voltage changes, two steps were taken. First, intracellular [K^+] was reduced to 50 mM and 1 mM 2,4-diaminopyridine (DAP) was applied via the external solution. This agent is known to inhibit the voltage-sensitive K^+ channel in squid axons (Yeh et al., 1976). Because both of the treatments result in membrane depolarization and K^+ channels are activated by depolarization, it was further necessary to hyperpolarize the membrane potential in order to reduce K^+ efflux to a manageable level. Therefore, the second step was to voltage-clamp the resting membrane potential to a relatively negative value (−90 mV) and maintain it at this value throughout the experiment. In order to prevent possible confounding effects caused by pH changes resulting from passage of current, the DF used for these experiments contained 125 mM MOPS and 177.5 mM Tris buffer. The extra buffer osmotically replaced glycine. No obvious changes in the color of the phenol red–containing DF were noted while current was passed during these experiments. Presumably, the high buffer capacity and relatively low current used (4–8 μA) prevented any significant pH changes.

Fig. 9 illustrates one of five experiments performed using this voltage-clamp approach. In this axon, K^+ efflux before hyperpolarizing the membrane resting potential varied between 65 and 70 pmol/cm^2·s. We note that, upon hyperpolarization to −91 mV, K^+ efflux fell to 3.1 pmol/cm^2·s. Thus, voltage-clamping resulted in much lower and more stable flux values. In this axon, the subsequent application of 10 μM bumetanide reversibly reduced K^+ efflux by 1.6 pmol/cm^2·s. The average bumetanide-sensitive K^+ efflux in all five axons was 1.8 ± 0.7 pmol/cm^2·s, a value very close to that expected for the K^+ efflux carried by the Na/K/Cl transporter.

DISCUSSION

The results from the present study indicate that a coupled transport process mediates a portion of the unidirectional effluxes of Na^+, K^+, and Cl^-. Briefly, the evidence supporting this conclusion is as follows. The effluxes of all three ions have a bumetanide-sensitive component. The bumetanide-sensitive component of the Na^+ and Cl^- effluxes requires that ATP be present inside the axon. Furthermore, the bumetanide-sensitive efflux component of both Na^+ and Cl^- requires that the other two ions be present on the *cis* side as well. These are exactly the properties of the coupled Na/K/Cl uptake process previously identified in this preparation (Russell, 1979, 1983). Therefore, we ascribe the mechanism for the effluxes studied in the present report to the same co-transport process as that already identified as being responsible for coupled Na/K/Cl uptake.

The Na/K/Cl transport process is generally considered to be an example of a gradient-driven co-transport system (e.g., Geck and Pfeiffer, 1985). By definition, gradient-driven co-transport processes are based on coupling between net flows

of the involved solute species (e.g., Heinz, 1985). The idea that a transport process can be "fueled" by the energy inherent within the combined thermodynamic gradients of the transported solutes clearly implies that the transport process ought to be capable of effecting net transport in either direction. Presumably, such net fluxes represent the algebraic sum of unidirectional fluxes mediated by the coupled transport process. Thus, the transport process might be capable of effecting unidirectional fluxes in either direction unless one direction of movement was greatly favored by virtue of kinetic constraints placed on the unfavorable direction. In view of the fact that the Na/K/Cl transporter of the squid axon in the influx mode has an absolute requirement for ATP (Russell, 1979, 1983), it seemed possible that ATP might play a role of preventing "backflux," i.e., efflux by the transporter. Thus, ATP might reduce the transporter's affinity for the transported ions, causing these ions to be released intracellularly and allowing the transporter to return to an outward-facing mode, ready again to accept Cl^-, Na^+, and K^+.

The present finding clearly demonstrates that such an extreme model is not the case. The bumetanide-sensitive effluxes of Cl^- and Na^+ were shown to require the presence of cellular ATP, just as its presence was shown to be necessary for their influxes (Russell, 1979, 1983). Thus, ATP does not act to prevent reverse fluxes entirely. However, it is still possible that it acts to set kinetic parameters in such a way as to favor uptake. Careful kinetic studies will be required to evaluate this possibility.

The Na/K/Cl co-transport process in squid axon appears to share several important properties with those identified in a variety of other cells. It is inhibited by bumetanide with a $K_I \cong 0.1$ μM. The concentration dependence of Cl^- efflux upon intracellular [Cl^-] is consistent with the view that at least two Cl ions are required to activate transport (Hill coefficient, 2.2). Unless the cooperativity for substrate binding is very high, the Hill coefficient generally underestimates the actual binding stoichiometry (Segel, 1975; Piszkiewicz, 1977); thus, the present result is consistent with the directly measured influx stoichiometry of 3 Cl^- (Russell, 1983). The intracellular [Cl^-] at which the Cl^- efflux was half-saturated was 53 mM. This value is in good agreement with results from other preparations (e.g., Saier and Boyden, 1984; O'Grady et al., 1986). Similar quantitative kinetic data for squid axon are not yet available for K^+ and Na^+, but the fact that varying the intracellular [K^+]/[Na^+] ratio from 350/50 mM to 50/350 mM has only a minimal effect on the bumetanide-sensitive Cl^- efflux argues that the half-saturation constants for both cations are lower than 50 mM. This interpretation depends upon whether either cation can substitute for the other. Some evidence that Na^+ might partially substitute for K^+ (see Table II, part *C*, *ii*) places a limit on such speculation. Further experiments will be needed to answer this question.

A careful test of the stoichiometry was not performed in the present set of experiments; however, the bumetanide-sensitive Cl^- efflux with 350 mM [K^+] and 50 mM [Na^+] was 6.8 pmol/cm^2·s (Table II, part *A*). Under the same set of circumstances, the bumetanide-sensitive Na^+ efflux was 4.8 pmol/cm^2·s. This is very close to the 3 Cl^-:2 Na^+ stoichiometry previously demonstrated for the unidirectional influxes of these ions.

The coupled Na/K/Cl influx study previously reported was largely performed on axons dialyzed with Cl^--free and Na^+-free DF. Thus, under intracellular conditions that prevent "reverse" fluxes, i.e., efflux of Cl^- and Na^+ (see Tables II, part *D*, and III, part *C*), the "forward" flux still occurred (Russell, 1983). It follows that the transporter in the squid axon does not have an obligate requirement to return to the outward-facing state by "carrying" Na^+ or Cl^-. Thus, it appears that the transporter can revert to its original confirmation without having to transport either Na^+ or Cl^-. Whether or not it can do so in the absence of K^+ is less clear. Removing K^+ from the extracellular superfusing SSW was without effect upon Cl^- efflux (see Fig. 3) and Na^+ efflux (see Fig. 8); however, one might argue that the Schwann cell space permits a sufficiently high [K^+] in the immediate axolemmal environment to support return of the transporter. Indeed, K^+/K^+ exchange has been postulated to occur via the Na/K/Cl co-transporter in avian red blood cells (Haas and McManus, 1985) and renal medullary luminal membranes (Burnham et al., 1985). Since we have not directly tested the effect of removal of *trans*-side K^+ on either the bumetanide-sensitive K^+ influx or efflux in the squid axon, we cannot rule out the possibility of K^+/K^+ exchange via the Na/K/Cl co-transporter. However, a simple K^+/K^+ exchange seems unlikely in the squid axon since bumetanide-sensitive K^+ influx does not occur in the absence of either *cis* Na^+ or Cl^-. Nor does K-Cl/K-Cl exchange seem likely, because the removal of *trans*-side Cl^- markedly stimulates Cl^-, Na^+, and K^+ influx. Thus, we currently have no evidence that this transporter can effect "exchange" fluxes in the squid axon; however, further experiments are required to settle this issue. Despite the uncertainty about exchange modes, it seems clear that the transporter can return to its original conformation in the "unloaded" state.

We thank the director and staff of Marine Biological Laboratory, Woods Hole, MA, for the facilities placed at our disposal. We also wish to thank Mr. Eddie A. Fort for his excellent technical assistance during the performance of these studies.

This work was supported by Department of Health and Human Services grant NS-11946.

Original version received 31 July 1986 and accepted version received 22 December 1986.

REFERENCES

Begenisich, T., and P. De Weer. 1977. Ionic interactions in the potassium channel of squid giant axons. *Nature.* 269:710–711.

Boron, W. F., and J. M. Russell. 1983. Stoichiometry and ion dependencies of the intracellular-pH-regulating mechanism in squid giant axons. *Journal of General Physiology.* 81:373–399.

Brinley, F. J., Jr., and L. J. Mullins. 1967. Sodium extrusion by internally dialyzed squid axons. *Journal of General Physiology.* 50:2303–2332.

Burnham, C., S. J. D. Karlish, and P. L. Jorgensen. 1985. Identification and reconstitution of a $Na^+/K^+/Cl^-$ co-transporter and K^+ channel from luminal membranes of renal red outer medulla. *Biochimica et Biophysica Acta.* 821:461–469.

De Weer, P., and D. Geduldig. 1978. Contribution of sodium pump to resting potential of squid giant axon. *American Journal of Physiology.* 235:C55–C62.

Ellory, J. C., P. B. Dunham, P. J. Logue, and G. W. Stewart. 1982. Anion-dependent cation

transport in erythrocytes. *Philosophical Transactions of the Royal Society of London, Series B.* 299:483–495.

Forbush, B., III, and H. C. Palfrey. 1983. [^{3}H]bumetanide binding to membranes isolated from dog kidney outer medulla: relationship to the Na, K, Cl co-transport system. *Journal of Biological Chemistry.* 258:11787–11792.

Geck, P., and B. Pfeiffer. 1985. $Na^+ + K^+ + 2Cl^-$ co-transport in animal cells: its role in volume regulations. *Annals of the New York Academy of Sciences.* 456:166–182.

Haas, M., and B. Forbush III. 1986. [^{3}H]bumetanide binding to duck red cells: correlation with inhibition of ($Na^+K^+2Cl^-$) co-transport. *Journal of Biological Chemistry.* 261:8434–8441.

Haas, M., and T. J. McManus. 1985. Effect of norepinephrine on swelling-induced potassium transport in duck red cells. Evidence against a volume-regulatory decrease under physiological conditions. *Journal of General Physiology.* 85:649–668.

Hannafin, J., E. Kinne-Saffran, D. Friedman, and R. Kinne. 1983. Presences of a sodium-potassium chloride co-transport system in the rectal gland of *Squalus acanthias. Journal of Membrane Biology.* 75:73–83.

Heinz, E. 1985. Some basic aspects of coupling in secondary active transport. *Annals of the New York Academy of Sciences.* 456:1–9.

Lytle, C., M. Haas, and T. McManus. 1986. Chloride-dependent obligate cation exchange: a partial reaction of [$Na^+K^+2Cl^-$] co-transport. *Federation Proceedings.* 45:548. (Abstr.)

O'Grady, S. M., M. W. Musch, and M. Field. 1986. Stoichiometry and ion affinities of the Na-K-Cl co-transport system in the intestine of the winter flounder (*Pseudopleuronectes americanus*). *Journal of Membrane Biology.* 91:33–41.

Piszkiewicz, D. 1977. Kinetics of Chemical and Enzyme Catalyzed Reactions. Oxford University Press, New York. p. 160.

Russell, J. M. 1976. ATP-dependent chloride influx into internally dialyzed squid giant axons. *Journal of Membrane Biology.* 28:335–349.

Russell, J. M. 1979. Chloride and sodium influx: a coupled uptake mechanism in the squid giant axon. *Journal of General Physiology.* 73:801–818.

Russell, J. M. 1983. Cation-coupled chloride influx in squid axon: role of potassium and stoichiometry of the transport process. *Journal of General Physiology.* 81:909–925.

Russell, J. M. 1984. Chloride in the squid giant axon. *Current Topics in Membranes and Transport.* 22:177–193.

Russell, J. M. 1985. Reverse fluxes of Cl through the Na:K:Cl coupled transporter in squid axon. *Biophysical Journal.* 47:274*a*. (Abstr.)

Saier, M. H., and D. A. Boyden. 1984. Mechanism, regulation and physiological significance of the loop diuretic-sensitive NaCl/KCl symport system in animal cells. *Molecular and Cellular Biochemistry.* 59:11–32.

Segel, I. H. 1975. Enzyme Kinetics. Wiley-Interscience, New York. p. 361.

Yeh, J. Z., G. S. Oxford, C. H. Wu, and T. Narahashi. 1976. Dynamics of aminopyridine block of potassium channels in squid axon membrane. *Journal of General Physiology.* 68:517–536.