Elevated $[Cl^-]_i$ and $[Na^+]_i$ Inhibit Na^+,K^+,Cl^- Cotransport by Different Mechanisms in Squid Giant Axons

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ABSTRACT   Bumetanide-sensitive (BS) unidirectional fluxes of $^{36}Cl^-$ or $^{22}Na^+$ were measured in internally dialyzed squid giant axons while varying the intra- or extracellular concentrations of Na^+ and/or Cl^-. Raising either $[Cl^-]_i$ or $[Na^+]_i$ resulted in a concentration-dependent reduction of the BS influx of both $^{36}Cl^-$ and $^{22}Na^+$. Raising $[Cl^-]_i$ above 200 mM completely blocked BS influxes. However, raising $[Na^+]_i$ to 290 mM resulted in saturable but incomplete inhibition of both BS Na^+ influx and BS Cl^- influx. The consequences of varying intracellular Cl^- on cotransporter effluxes were complex. At lower $[Cl^-]_i$ values (below 100 mM) intracellular Cl^- activated cotransporter effluxes. Surprisingly, however, raising $[Cl^-]_i$ levels >125 mM resulted in a $[Cl^-]_i$- dependent inhibition of BS effluxes of both Na^+ and Cl^-. On the other hand, raising $[Na^+]_i$ resulted only in the activation of the BS Na^+ efflux; intracellular Na^+ did not inhibit BS efflux even at 290 mM. The inhibitory effects of intracellular Na^+ on cotransporter-mediated influxes, and lack of inhibitory effects on BS effluxes, are consistent with the *trans*-side inhibition expected for an ordered binding/release model of cotransporter operation. However, the inhibitory effects of intracellular Cl^- on both influxes and effluxes are not explained by such a model. These data suggest that Cl^- may interact with an intracellular site (or sites), which does not mediate Cl^- transport, but does modulate the transport activity of the Na^+, K^+,Cl^- cotransporter.

INTRODUCTION

Na^+,K^+,Cl^- cotransport has been identified in a wide variety of cells since its initial identification in Ehrlich ascites tumor cells (Geck et al., 1980). It behaves as a tightly coupled transport process having an absolute requirement for the *cis*-side presence of all three cotransported ions. The loop diuretics such as furosemide and bumetanide block Na^+,K^+,Cl^- cotransport at relatively low concentrations (in the low μM range; e.g., Russell, 1983, Palfrey and O'Donnell, 1992).

Although considerable attention has been directed toward understanding the regulation of Na^+,K^+,Cl^- cotransport by second-messengers (e.g., Palfrey and Greengard, 1981; Brock et al., 1986; O'Donnell and Owen, 1986), the possibility of "intrinsic" regulation by the cotransported ions themselves has only recently been generally recognized (Palfrey and O'Donnell,

1992). The ability of high intracellular $[Cl^-]$ to profoundly reduce unidirectional influxes via the cotransporter in the squid giant axon has been known for some time (Russell, 1976, 1979, 1983; Altamirano et al., 1989; Breitwieser et al., 1990). More recently, evidence from other preparations has been forthcoming for similar inhibitory effects of intracellular Cl^- (Levinson, 1990; Forbush et al., 1992; Lytle and Forbush, 1992; O'Neill and Klein, 1992; Haas and McBrayer, 1994) as well as for intracellular Na^+ and K^+ (Whisenant et al., 1992) on cotransporter-related activities.

The mechanism(s) for these effects of the intracellular ions is/are unknown. One possibility is that these effects represent kinetic consequences inherent in a highly ordered ion binding/release mechanism. There is good evidence that Na^+, K^+, and Cl^- bind to the cotransporter in a highly ordered and cooperative manner and specific models of ion binding and release have been suggested (Lytle and McManus, 1986; Miyamoto et al., 1986; Duhm, 1987; Lauf et al., 1987). With such models, one can make specific predictions regarding the effects of raising the intracellular concentrations of each of the cotransported ions on unidirectional fluxes.

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In the present study, a systematic examination of the effects of changes of intracellular [Cl⁻] and [Na⁺] on cotransporter-mediated, unidirectional fluxes has been performed. The effects of intracellular Na⁺ are consistent with a highly ordered binding model, whereas some of the effects of intracellular Cl⁻ suggest an additional effect independent of binding to, and release from, an anion transport site on the cotransporter.

Some of these results have been presented in abstract form (Altamirano et al., 1989; Breitwieser et al., 1992, 1993).

METHODS

Experiments were conducted at the Marine Biological Laboratory (Woods Hole, MA) in May and June of 1989–1995. Live specimens of the squid *Loligo pealei* were decapitated and the first stellar nerve removed from the mantle. The giant axon was carefully dissected from this nerve and mounted horizontally in the dialysis chamber by cannulating at either end (e.g., Russell, 1983).

Unidirectional Flux Measurements by Intracellular Dialysis

The technique of intracellular dialysis (Brinley and Mullins, 1967) was used as previously described (Russell, 1979, 1983; Breitwieser et al., 1990). Briefly, a 35–40 mm length of axon was cannulated at both ends in a specially designed dialysis chamber, and then the dialysis tube was carefully guided longitudinally through the axon until the porous region was positioned in the central portion of the axon. The axon was then dialyzed through this tube at a rate of 2 μl/min. The temperature of the dialysis chamber bath was maintained at 17°C.

Unidirectional flux procedures. To measure unidirectional fluxes, fluids contained the requisite radionuclide, either ³⁶Cl (40 μCi/mmol for influx or 100 μCi/mmol for efflux) or ²²Na (50 μCi/mmol for influx or 100 μCi/mmol for efflux). In the case of ³⁶Cl, the carrier Cl⁻ was taken into account when determining the final [Cl⁻]. Radioisotope which crossed the axolemma was picked up either in the flowing internal dialysis fluid or the flowing external seawater which was collected directly into scintillation vials for 5-min sample periods. Each sample was counted to an error not >4% using an LS-3801 scintillation counter (Beckman Instruments, Inc., Irvine, CA).

Experimental Solutions

Table I gives the compositions of the internal dialysis fluids (DF) and the squid seawaters (SSW) used in these experiments.

General Experimental Conditions

For the influx studies, the control conditions were: external fluid (squid seawater), [K⁺]ₒ = 100 mM, [Na⁺]ₒ = 335 mM, and [Cl⁻]ₒ = 561 mM; internal fluid (dialysis fluid), [K⁺]ᵢ = 200 mM, [Na⁺]ᵢ = 0 mM and [Cl⁻]ᵢ = 0 mM. We will refer to this condition as follows: [100 K,335 Na,561 Cl]ₒ/[200 K,0 Na,0 Cl]ᵢ. The external ion concentrations were set at optimal levels based on our studies of external ion activation of the cotransporter (Altamirano, A.A., G.E. Breitwieser, and J.M. Russell, unpublished observations). The [K⁺]ᵢ was 200 mM to permit us to vary [Na⁺]ᵢ between 0 and 290 mM while minimizing depolarization of the

TABLE I
Composition of Experimental Fluids

	Dialysis Fluids	Squid Seawater
	mmol/liter	
K⁺	200	100
Na⁺	0–290	0–335
NMDG⁺	0–290	0–335
Ca²⁺	0	3
Mg²⁺	8	63
Cl⁻	0–300	0–561
Glutamate⁻	206–506	—
Sulfamate⁻	—	0–561
EGTA	2	—
EDTA	—	0.1
EPPS	—	10
MOPS	25	—
TRIS	19.3	—
ATP	4	—
Phenol red	0.5	0.2
Ouabain	—	0.01
TTX	—	0.0001
pH	7.30–7.35	8.0
Osmolality	970	975

NMDG⁺ = *N*-methyl-D-glucammonium; EGTA = ethylene glycol-bis (β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid; EDTA = ethylenediaminetetraacetic acid; EPPS = *N*-[2-hydroxyethyl]-piperazine-*N'*-[3-propane sulfonic acid]; MOPS = 3-[*N*-morpholino]-propane sulfonic acid; Tris = tris(hydroxymethyl)amino methane; ATP = adenosine-5'-triphosphate; TTX = tetrodotoxin.

axon. Axonal depolarization to 0 mV or more positive values activates voltage-sensitive Na⁺ and Cl⁻ channels resulting in large, noncotransporter-mediated ion fluxes which obscure cotransporter-mediated fluxes of these same ions (unpublished observations). Unless otherwise noted, when [Cl⁻]ᵢ was varied, [Na⁺]ᵢ was 0 mM and likewise, when [Na⁺]ᵢ was varied, [Cl⁻]ᵢ = 0 mM.

RESULTS

Effects of Intracellular Anions on Cotransport-mediated Fluxes

Cl⁻ influx. As previously mentioned, high intracellular [Cl⁻] has long been known to inhibit the influx of all three cotransported ions in the squid axon (Russell, 1976, 1979, 1983; Altamirano et al., 1989; Breitwieser et al., 1990). We have already shown that cotransporter-mediated Cl⁻ influx is half-inhibited when [Cl⁻]ᵢ = 90–100 mM and completely inhibited when [Cl⁻]ᵢ = 250–300 mM (Breitwieser et al., 1990).

In the present study, we examined the effects of several other intracellular anions on cotransporter-mediated Cl⁻ influx. Intracellular Cl⁻ was first dialyzed out, and then 150 mM of the test anion dialyzed in. Fig. 1 compares the inhibitory effects on BS Cl⁻ influx of 150 mM intracellular Cl⁻ as well as several other test anions (SCN⁻, Br⁻, I⁻, NO₃⁻, and SO₄²⁻).
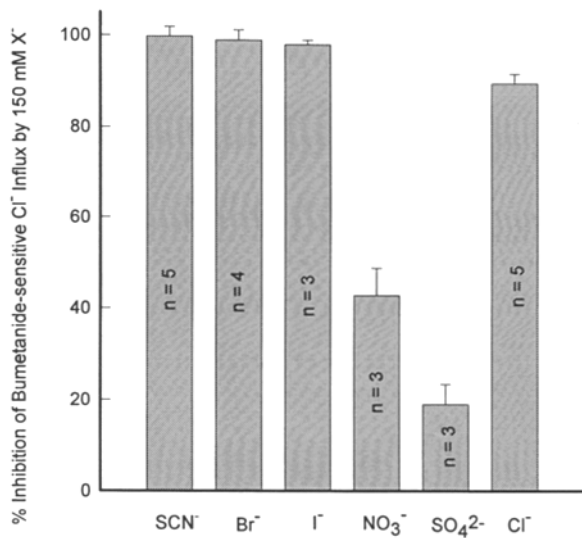
FIGURE 1. Effect on bumetanide-sensitive Cl^- influx of various anions in the intracellular fluid. Axons were dialyzed for 60 min with a Cl^--free/Na^+-free dialysis fluid (DF) before ^{36}Cl was added to the external fluid to begin the influx measurements. After a 45-min period to assess the control Cl^- influx (in the absence of intracellular Cl^-), the DF was changed to one containing 150 mM of the anion being tested. Samples collected 45–75 min after the test anion was added were used to calculate the Cl^- influx under the condition of 150 mM $[X^-]_i$. Finally, 10 μM bumetanide was added to the external fluid. The control bumetanide-sensitive (BS) influx was calculated as the difference between the control influx and the influx in the presence of bumetanide; the test BS influx was the difference between the influx with 150 mM X^- and the influx in the presence of bumetanide. The difference between control influx and test influx divided by the control influx gives the percent of inhibition. Bars represent the mean ± SEM of the results from the number of axons indicated within each bar.

FIGURE 2. Dose–response relation for inhibition of bumetanide-sensitive Cl^- influx by intracellular Cl^-, SCN^- and Br^-. The external fluid contained 100 mM $[K^+]$, 335 mM $[Na^+]$ and 561 mM $[Cl^-]$ and the internal dialysis fluid contained 200 mM $[K^+]$, 0 mM $[Na^+]$ and variable $[X^-]$ as indicated. Axons were dialyzed free of intracellular Cl^- and a control influx was obtained. Then the test anion was dialyzed into the axon at the various concentrations (one concentration per axon). Finally, each axon was treated with 10 μM bumetanide to determine the bumetanide-sensitive Cl^- influx. Each point represents the mean of at least three axons. The smooth curves represent fits to the Hill equation: $(V = V_{max} \cdot [X^-]^n/[X^-]^n + K_{app}^n)$. The best-fit parameters for each ion are as follows: Cl^-, $K^i_{0.5} = 91.7 \pm 3.0$ mM, Hill $n = 4.0 \pm 0.4$; SCN^-, $K^i_{0.5} = 17.9 \pm 2.3$ mM, Hill $n = 1.6 \pm 0.3$; Br^-, $K^i_{0.5} = 31.9 \pm 2.4$ mM, Hill $n = 2.0 \pm 0.3$.

Although all the anions tested caused inhibition of cotransporter-mediated Cl^- influx, SCN^-, Br^-, Cl^- and I^- were particularly effective. Fig. 2 compares the concentration-dependent inhibitory effects of Cl^-, SCN^- and Br^- on cotransport-mediated Cl^- influx. The half-inhibitory constants $(K^i_{0.5})$ were as follows: $SCN^- = 18$ mM; $Br^- = 32$ mM; $Cl^- = 92$ mM. Others have reported that SCN^- is unable to replace Cl^- in supporting cotransport (Owen and Prastein, 1986, Miyamoto et al., 1986, but see Kinne et al., 1986). We have confirmed this for the squid axon. In axons dialyzed with Cl^--free fluid, addition of intracellular SCN^- (over the range 2–50 mM) did not activate a bumetanide-sensitive Na^+ efflux (data not shown).

Cl^- and Na^+ effluxes. We previously examined activation of cotransporter-mediated efflux as a function of $[Cl^-]_i$ up to $[Cl^-]_i = 150$ mM (Altamirano and Russell, 1987). Since this is in the range where intracellular Cl^- begins to inhibit cotransporter-mediated influx, we reexamined the effect of changing $[Cl^-]_i$ on BS Cl^- ef-
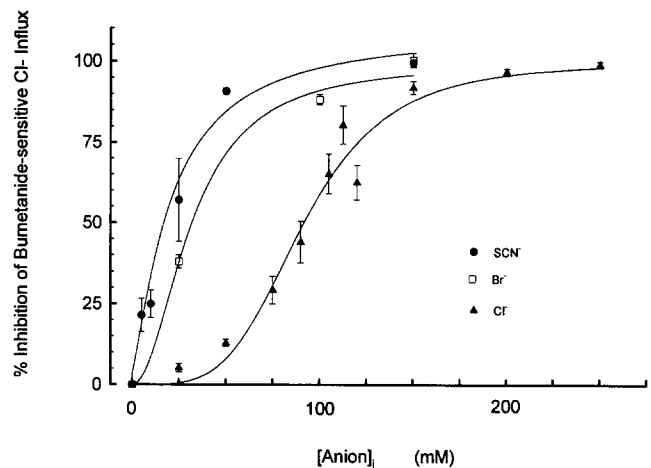
flux by extending the range of $[Cl^-]_i$ values studied (between $[Cl^-]_i = 50$ and 300 mM). For these ^{36}Cl efflux studies the ionic conditions were as follows: [100 K,335 Na,561 $Cl]_o$/[200 K,200 Na,Δ $Cl]_i$. Fig. 3 shows that a biphasic relationship exists between $[Cl^-]_i$ and the BS efflux of Cl^-. At lower $[Cl^-]_i$ values (0–125 mM), the cotransporter-mediated Cl^- efflux rises as function of $[Cl^-]_i$ along a sigmoidal curve. This is as expected for a substrate of the cotransporter which has multiple Cl^- binding sites. However, further increases of $[Cl^-]_i$ above 125–150 mM, reveal profound inhibitory effects culminating in complete inhibition of BS Cl^- efflux at $[Cl^-]_i = 300$ mM. Thus, addition of intracellular Cl^- can result in either a net activation (at lower $[Cl^-]_i$ levels) or a net inhibition of the cotransporter (at high $[Cl^-]_i$ levels), making it difficult to determine the apparent affinities of the intracellular Cl^- sites mediating transport versus those mediating inhibition of cotransport.

Bumetanide-sensitive Na^+ efflux requires the presence of intracellular Cl^- (e.g., Altamirano and Russell, 1987). However, under the following set of ionic conditions, [100 K,335 Na,561 $Cl]_o$/[200 K,75 Na,Δ $Cl]_i$, raising $[Cl^-]_i$ from 125 to 300 mM abolished the BS Na^+

TABLE II

Effects of Increased [Cl]ᵢ on Bumetanide-sensitive Effluxes of Na⁺ and Cl⁻

[Cl]ᵢ	Cl⁻ efflux	Na⁺ efflux
mm	*pmol/cm²s*	*pmol/cm²s*
125	13.3 ± 1.4	7.0 ± 0.4
	(n = 5)	(n = 4)
300	−0.2 ± 0.3	0.14 ± 0.07
	(*n* = 3)	(*n* = 3)

efflux (Table II) as we have observed for BS Cl⁻ efflux. Thus, just as is the case for cotransporter-mediated influxes, high levels of intracellular Cl⁻ reduce the cotransporter-mediated effluxes of both Cl⁻ and Na⁺.

Effect of extracellular Cl⁻ on cotransporter-mediated efflux. Intracellular Cl⁻ clearly has a profound inhibitory effect on both cotransporter-mediated influx and efflux. If these effects are due entirely, or in part, to interaction of Cl⁻ with transport sites, one might expect similar effects of extracellular Cl⁻ on Cl⁻ efflux (see Altamirano and Russell, 1987). We therefore tested the effects of removing extracellular Cl⁻ using the following set of ionic conditions; [100 K,335 Na,0 or 561 Cl]ₒ/[200 K,200 Na,125 Cl]ᵢ. Fig. 4 *A* is representative of results obtained in four axons in which extracellular Cl⁻ was replaced by sulfamate⁻. In each of these axons, reducing [Cl⁻]ₒ from 561 to 0 mM increased total Cl⁻ efflux

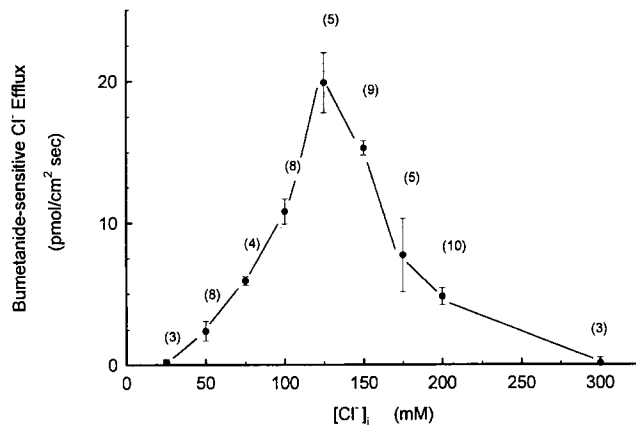FIGURE 3. Biphasic dependence of bumetanide-sensitive Cl⁻ efflux on [Cl⁻]ᵢ. [K]ᵢ = 200 mM; [Na⁺]ᵢ = 200 mM throughout these experiments. Intracellular Cl⁻ was replaced by glutamate⁻ in this study. The external seawater contained 100 mM [K⁺], 335 mM [Na⁺] and 561 mM [Cl⁻]. Each axon was dialyzed with the appropriate [Cl⁻] for 50 min before adding ³⁶Cl to the dialysis fluid. After 50 min of measuring efflux, 10 μM bumetanide was added to the external seawater and the efflux measured for another 45 min. The effect of bumetanide was usually complete within 15 min after it was added (e.g., see Figs. 4 and 5). Numbers in parentheses denote number of axons represented in each data point. Smooth line was drawn by eye to fit the data.
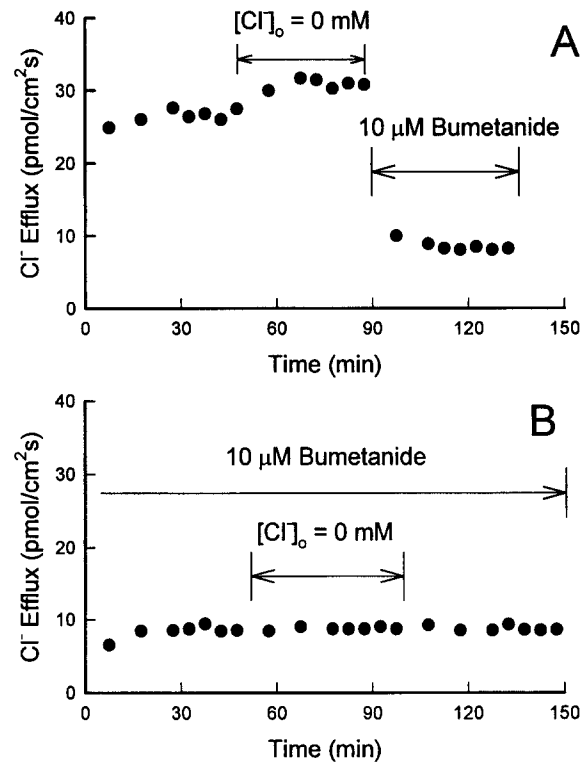

FIGURE 4. Effect of removal of extracellular Cl⁻ (replaced with sulfamate) on Cl⁻ efflux. The composition of the intracellular fluid was: [K⁺] = 200 mM, [Na⁺] = 200 mM, [Cl⁻] = 125 mM. The external seawater contained [K⁺] = 100 mM, [Na⁺] = 335 mM, [Cl⁻] = 0 or 561 mM. (*A*) In this experiment the average Cl⁻ efflux when [Cl⁻]ₒ = 561 mM was 26.9 pmol/cm²s; when [Cl⁻]ₒ = 0 mM, 30.4 pmol/cm²s; after bumetanide, 8.4 pmol/cm²s. (*B*) In this experiment, the axon was superfused throughout with seawater containing 10 μM bumetanide. The average Cl⁻ efflux before changing to 0-Cl⁻ seawater (sulfamate replacement) was 9.3 pmol/cm²s, during the 0-Cl⁻ treatment the average efflux was 9.6 pmol/cm²s, after return to 561 mM [Cl⁻]ₒ, the average efflux was 9.3 pmol/cm²s.

from 24.5 ± 5.4 to 30.6 ± 4.7 p/cs. The average increase was 6.1 ± 2.1 p/cs (*p* = 0.03; paired students *t* test). Subsequent application of bumetanide reduced the Cl⁻ efflux by 16 p/cs (to 14.3 ± 6.1 p/cs). When bumetanide was present throughout the experiment (Fig. 4 *B*), decreasing [Cl⁻]ₒ had no effect on Cl⁻ efflux, suggesting that all of the observed effects of reducing [Cl⁻]ₒ were due to modulation of cotransporter-mediated efflux.

The BS Cl⁻ efflux into 561 mM [Cl⁻]ₒ averages 80% of that measured in the presence of 0 mM [Cl⁻]ₒ suggesting a *trans*-side inhibitory effect of extracellular Cl⁻. This *trans*-side effect on efflux is qualitatively similar to that observed for influx. However, cotransporter-mediated Cl⁻ efflux is quantitatively much less sensitive to *trans*-side Cl⁻ (extracellular Cl⁻) than is BS Cl⁻ influx. Influx is completely inhibited by 300 mM [Cl⁻]ᵢ

whereas 561 mM $[Cl^-]_o$ only causes a 20% inhibition. Another significant difference between the effects of intra- and extra-cellular Cl^- is that extracellular Cl^- does not inhibit Cl^- influx (no cis-side inhibition). When BS Cl^- influx was measured as a function of $[Cl^-]_o$, the result was a sigmoid-shaped, saturable, activation of cotransport (Altamirano, A.A., G.A. Breitwieser, and J.M. Russell, manuscript in preparation); no inhibition of BS Cl^- influx at higher $[Cl^-]_o$ levels (up to 561 mM; Altamirano et al., 1987) was observed in striking contrast to the inhibitory effects of increasing intracellular $[Cl^-]$ on Cl^- efflux. Thus, the effects of extracellular Cl^- on unidirectional cotransporter fluxes differ both quantitatively and qualitatively from those of intracellular Cl^-.

Effects of Intracellular Na^+ on Cotransporter-mediated Fluxes

Effects of intracellular Na^+ on BS Cl^- influx; $[Cl^-]_i = 0$ mM. An ordered binding/release model predicts that raising the intracellular concentration of any of the three cotransported ions could have inhibitory effects on influx via the cotransporter. The effect of a given ion will depend not only on its concentration but also on its relative order of release compared to that of the cotransported ions whose influx is being measured (see Discussion). The effect on Cl^- influx of varying intracellular $[Na^+]$ from 0 to 200 mM was studied under the following set of ionic conditions: $[100 \text{ K},335 \text{ Na},561 \text{ Cl}]_o/[200 \text{ K},\Delta \text{ Na},0 \text{ Cl}]_i$. Fig. 5 shows an example in which a 60% reduction of BS Cl^- influx occurred when $[Na^+]_i$ was increased from 0 to 200 mM. Controls in

which bumetanide was present throughout the experiment verified that raising $[Na^+]_i$ from 0 to 200 mM had no effect on bumetanide-insensitive Cl^- influx.

The same protocol and ionic conditions shown in Fig. 5 were used as $[Na^+]_i$ was systematically varied between 0–290 mM. Collated data in Fig. 6 A show that BS Cl^- influx was inhibited as $[Na^+]_i$ was increased. However, in contrast to the effect of raising $[Cl^-]_i$ (cf. Fig. 2), complete inhibition of cotransport influx was never achieved, even at $[Na^+]_i$ as high as 290 mM. In Fig. 6 A, the influx data have been normalized as percent inhibition of BS Cl^- influx (0% inhibition at 0 mM $[Na^+]_i$) and then fitted according to a single-site binding model. This fit yields an apparent inhibitory constant $(K^i_{0.5})$ for intracellular Na^+ of 138 mM, and an apparent maximal inhibition of BS Cl^- influx of 80%.

Effects of intracellular Na^+ on BS Cl^- influx: $[Cl^-]_i = 125$ mM. To determine whether the presence of intracellular Cl^- modifies inhibition of cotransporter influx by intracellular Na^+, we varied $[Na^+]_i$ in the presence of 125 mM $[Cl^-]_i$ (ionic conditions: $[100 \text{ K},335 \text{ Na},561 \text{ Cl}]_o/[200 \text{ K},\Delta \text{ Na},125 \text{ Cl}]_i$). (The reader will recall that at this $[Cl^-]_i$, cotransport-mediated *efflux* was maximal [when $[Na^+]_i = 200$ mM; see Fig. 3].) Fig. 6 B shows
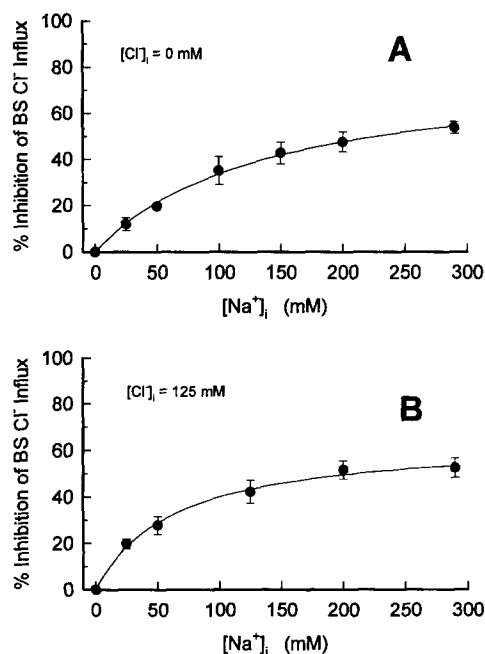
FIGURE 6. Relationship between the bumetanide-sensitive (BS) Cl^- influx and intracellular $[Na^+]$ ($[K^+]_i = 200$ mM, $[Cl^-]_i = 0$ or 125 mM). Each symbol represents the mean ± SEM of data from at least three axons. The data are plotted as percent of inhibition and fitted to the Michaelis-Menten equation as shown by the smooth line. (A) Relationship between the BS Cl^- influx and intracellular $[Na^+]_i$ in the absence of intracellular Cl^- (Cl^- replaced by glutamate$^-$): $K^i_{0.5} = 138 ± 14$ mM and maximal inhibition = 80 ± 3.6%. (B) Relationship in the presence of 125 mM $[Cl^-]_i$: $K^i_{0.5} = 59 ± 8.6$ mM and maximal inhibition = 63 ± 2.9%.
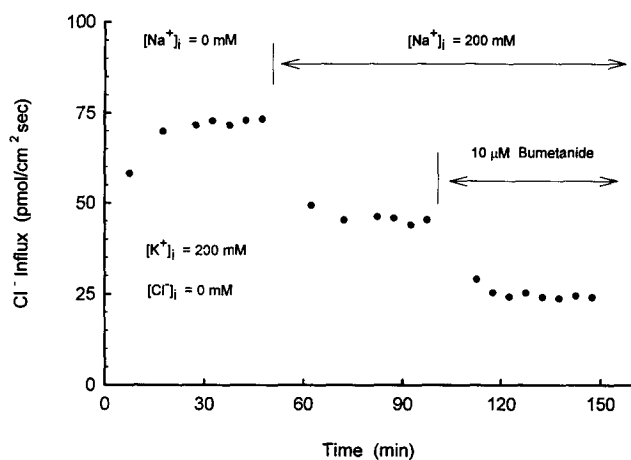
FIGURE 5. Effect of raising $[Na^+]_i$ on Cl^- influx in an internally dialyzed squid axon. For 50 min before zero time, this axon was dialyzed with a fluid that contained 200 mM K^+, 0 mM Na^+ and 0 mM Cl^-. At zero time the axon was exposed to squid seawater ($[K^+] = 100$ mM, $[Na^+] = 335$ mM and $[Cl^-] = 561$ mM) that contained ^{36}Cl and influx measurements begun. After 50 min, the internal dialysis fluid was changed to one containing 200 mM Na^+. This resulted in a fall of Cl^- influx from ∼71 pmol/cm²s to ∼43 pmol/cm²s. At 100 min, 10 μM bumetanide was added to the seawater and the Cl^- influx declined further to ∼25 pmol/cm²s.

that under this condition the apparent inhibitory constant for intracellular Na^+ inhibition of BS Cl^- influx was 59 mM, about half that observed in the absence of intracellular Cl^-. However, even in the presence of intracellular Cl^-, raising $[Na^+]_i$ as high as 290 mM did not completely inhibit cotransport influx; the apparent maximal inhibition of the BS Cl^- influx was ~63% (Fig. 6 B). Thus, raising $[Cl^-]_i$ acted to increase the apparent inhibitory affinity for Na^+ but did not result in a complete inhibition of cotransport by intracellular Na^+.

Effects of intracellular Na^+ on BS Na^+ influx: $[Cl^-]_i$ = 0 mM. To determine whether the effects of intracellular Na^+ were on cotransport functions in general, or limited to cotransport of Cl^-, we also studied the effects of varying $[Na^+]_i$ on BS Na^+ influx in the absence or presence of intracellular Cl^-. For the first series of these studies, the ionic conditions were: [100 K, 335 Na, 561 $Cl]_o$/[200 K,Δ Na,0 $Cl]_i$. Each axon was dialyzed with a single $[Na^+]$ and the BS Na^+ influx measured. Fig. 7 A shows the results from a series of such experiments. The effects on BS Na^+ influx of raising $[Na^+]_i$ in the absence of intracellular Cl^- were similar to those on BS Cl^- influx (Fig. 6 A; $[Cl^-]_i$ = 0 mM), that is, intracellular Na^+ inhibited BS Na^+ influx with

an apparent inhibitory constant of 114 mM (cf. 138 mM for inhibition of BS Cl^- influx under identical conditions, Fig. 6 A). Just as was the case for Cl^- influx, intracellular Na^+ up to 290 mM did not completely inhibit the BS Na^+ influx; the apparent maximal inhibition was ~50%.

Effect of intracellular Na^+ on BS Na^+ influx: $[Cl^-]_i$ = 125 mM. The influence of intracellular Cl^- on the forgoing relationship was studied under the following set of ionic conditions: [100 K,335 Na,561 $Cl]_o$/[200 K, Δ Na,125 $Cl]_i$. Fig. 7 B shows that at $[Cl^-]_i$ = 125 mM the apparent inhibitory constant for the intracellular Na^+ is reduced from 114 mM (see Fig. 7 A) to 45 mM without significantly affecting the apparent maximal inhibition caused by raising $[Na^+]_i$. Thus, intracellular Cl^- increases the apparent affinity of the putative Na^+ binding site mediating the partial inhibition of Na^+ influx. This agrees with the results noted when studying the effects of varying the intracellular $[Na^+]$ and $[Cl^-]$ on BS Cl^- influx.

Activation of bumetanide-sensitive Na^+ efflux by intracellular Na^+. To further examine the potential interactions of intracellular Na^+ with the cotransporter, we determined the relationship between $[Na^+]_i$ and BS Na^+ efflux. For these studies, the ionic conditions were: [100 K,0 Na,561 $Cl]_o$/[200 K, Δ Na,125 $Cl]_i$. $[Cl^-]_i$ was set at the optimal level for efflux, 125 mM (see Fig. 3), and the axons were superfused with a Na^+-free media to prevent Na^+/Na^+ exchange fluxes. The activation of BS Na^+ efflux by intracellular Na^+ is illustrated in Fig. 8. In sharp contrast to the relationship between Cl^- efflux and intracellular $[Cl^-]$ (cf. Fig. 3), raising intracellular Na^+ monotonically activates BS Na^+ efflux with-
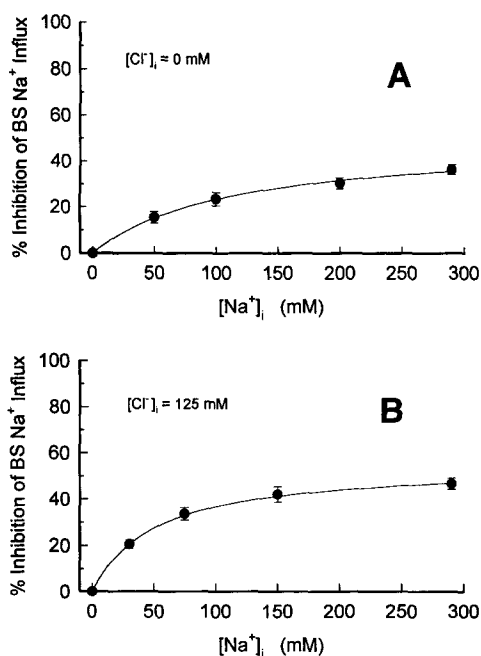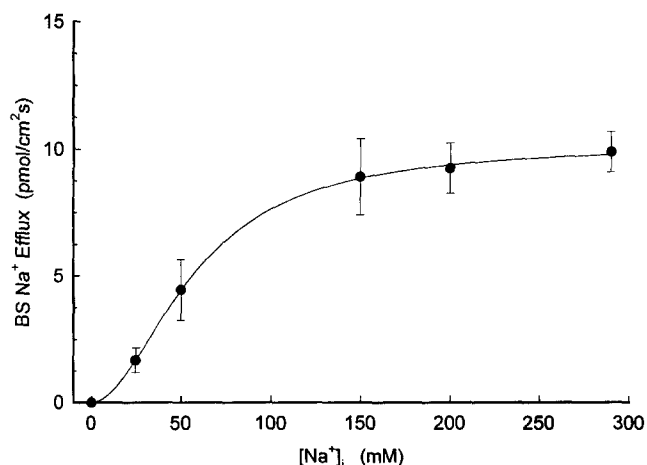

FIGURE 7. Relationship between bumetanide-sensitive Na^+ influx and $[Na^+]_i$. ($[K^+]_i$ = 200 mM). Each symbol represents the mean ± SEM of data from at least five axons. The data are plotted as percent inhibition and fitted with the Michaelis-Menten equation as shown by the smooth line. (A) $[Cl^-]_i$ = 0 mM (Cl^- replaced by glutamate$^-$): $K^i_{0.5}$ = 114 ± 14 mM; maximal inhibition = 49.6 ± 2.5%. (B) $[Cl^-]_i$ = 125 mM: $K^i_{0.5}$ = 45.3 ± 2.2 mM; maximal inhibition = 54.3 ± 0.6%.

FIGURE 8. Activation of bumetanide-sensitive Na^+ efflux by intracellular Na^+ ($[K^+]_i$ = 200 mM; $[Cl^-]_i$ = 125 mM). Each symbol represents the mean ± SEM of data from at least four axons. The smooth line is a fit of the data to the Hill equation which gave the following parameters: $K_{0.5}$ = 57 ± 1.5 mM; V_{max} = 10.2 ± 0.1 pmol/cm²s; Hill n = 1.98 ± 0.08.

out any indication of inhibition at high $[Na^+]_i$ (up to 290 mM). Note that the apparent affinities for internal Na^+ for both activation of cotransport Na^+ efflux (57 mM; at $[Cl^-]_i = 125$ mM) as well as for inhibition of Na^+ and Cl^- influxes are very similar (59 mM, Fig. 6 B and 45 mM, Fig. 7 B; at 125 mM $[Cl^-]_i$).

DISCUSSION

In this study, we systematically explored the effects of intracellular Cl^- and Na^+ on Na^+,K^+,Cl^- cotransport-mediated unidirectional fluxes of Cl^- and Na^+. The present results coupled with previously published observations (Russell, 1979; 1983; Breitwieser et al., 1990) show that both intracellular ions substantially affect cotransporter-mediated, unidirectional influxes as well as effluxes. However, the patterns of effects on unidirectional fluxes are quite different for the two ions. For example, intracellular Cl^- can completely inhibit the cotransporter-mediated influxes and effluxes of all three cotransported ions, whereas intracellular Na^+ only partially inhibits the cotransporter-mediated influxes of Na^+ and Cl^- and does not inhibit effluxes at all. The central question is whether these intracellular effects by cotransported ions on cotransporter-mediated fluxes represent effects mediated by hitherto unidentified modifier sites (either as discrete sites or via allosteric modifications) or are simply predictable consequences of a cotransporter process possessing a tightly-coupled, highly ordered binding and release mechanism for the cotransported ions.

An Ordered Binding Model for Na^+,K^+,Cl^- Cotransport: Some General Predictions

Evidence from several laboratories (Lytle and Mc-Manus, 1986; Lauf et al., 1987; Miyamoto et al., 1986; Duhm, 1987) including our own (Altamirano, A.A., G.A. Breitwieser, and J.M. Russell, manuscript in preparation) supports the view that the cotransporter binds and releases the transported ions in a tightly coupled and highly ordered manner. An inherent property of a highly ordered binding/release mechanism is that raising the concentration of any of the *trans*-side ions might be expected, by mass action, to reduce unidirectional, cotransporter-mediated fluxes. This follows from the fact that the binding or release of any given ion depends upon the binding or release of the ion preceding it in a highly ordered process. Thus, by raising $[Na^+]_i$, occupancy of an intracellular Na^+ site (or sites) is (are) favored and this makes the internal release of all subsequent ions in the release order less likely. Implicit in this description of transport by an ordered binding/release model is the assumption that the cotransporter can only "cross" the membrane in either the fully loaded or the fully unloaded forms.

Evidence for an Intracellular Cl^- Modulatory Site

We have shown here and in previous publications (Russell, 1983; Breitwieser et al., 1990) that intracellular Cl^- can completely inhibit all the cotransporter-mediated, unidirectional influxes of Na^+, K^+ and Cl^-. A significant new finding of this study is that high $[Cl^-]_i$ also completely inhibits the BS effluxes of both Cl^- (see Fig. 3) and Na^+ (Table II). It is not obvious how this latter observation could be explained as a mass action effect on an ordered binding/release mechanism. The simplest explanation for these observations is that, in addition to interacting with intracellular transport sites, intracellular Cl^- interacts with a site(s) or region(s) which is/are distinct from the cotransport binding/release sites and which mediate inhibition of transport. It is possible that intracellular SCN^- binds to, or interacts with, this putative site(s) since SCN^-, in the absence of intracellular Cl^-, is a very effective inhibitor of cotransport influx (see Figs. 1 and 2), but does not support cotransport efflux. Alternatively, SCN^- may bind to a transport site, but not permit transport to occur.

Effects of Extracellular Cl^- Are Consistent with an Ordered Binding/Release Model

Two observations suggest that the effects of extracellular Cl^- are mediated by a different mechanism than the effects of intracellular Cl^-. First, raising $[Cl^-]_o$ to 561 mM only reduced BS Cl^- efflux by $\sim$20%, in contrast to the total inhibition of BS Cl^- influx observed when $[Cl^-]_i$ was raised to 200–300 mM (see Fig. 2). This difference might simply reflect substantially different affinities, but the same general mechansim, on the two sides of the membrane. A second, and perhaps more persuasive reason, is that raising $[Cl^-]_o$ to 561 mM does not inhibit Cl^- influx, i.e., external Cl^- does not inhibit cotransport influx, unlike intracellular Cl^-, which inhibits cotransport efflux (Altamirano et al., 1987; Altamirano et al., manuscript in preparation).

Effects of Elevated $[Na^+]_i$ May Be Explained by an Ordered Binding/Release Model

Although raising $[Na^+]_i$ inhibited the bumetanide-sensitive influxes of both Cl^- and Na^+, we saw no evidence that such inhibition could be complete (e.g., Fig. 6, A and B; Fig. 7, A and B). In addition, intracellular Na^+ did not inhibit bumetanide-sensitive Na^+ efflux. Thus, there are important quantitative and qualitative differences between the effects of intracellular Na^+ and those of intracellular Cl^- on the cotransporter.

We have calculated apparent binding constants for Na^+ binding to three functionally distinct intracellular sites, one involved with activation of cotransporter-mediated efflux and the other two involved with inhibition of cotransporter-mediated influxes: (a) an intra-

cellular site or sites by which Na^+ activates cotransport efflux (in the presence of intracellular Cl^-) with an apparent activation constant of 57 mM, (Fig. 8); (*b*) an intracellular site at which Na^+ mediates inhibition of Cl^- influx in the presence of intracellular Cl^- with an apparent inhibitory constant of 59 mM (Fig. 6 *B*); and (*c*) an intracellular site at which Na^+ mediates inhibition of Na^+ influx when intracellular Cl^- is present, with an apparent inhibitory constant of 45 mM, (Fig. 7 *B*). The apparent binding constants for these three functionally-defined sites fall within the relatively narrow range of 45–65 mM. The most parsimonious interpretation of these independent results is that the three functionally-defined sites are, in fact, the same, i.e., they are Na^+ cotransport binding/release sites.

In the absence of intracellular Cl^-, the apparent inhibitory constant for intracellular Na^+ was higher (114 mM; Fig. 7 *A*) than that noted in the presence of 125 mM intracellular Cl^- (45 mM; Fig. 7 *B*). Thus, the presence of intracellular Cl^- increased the apparent affinity of the "inhibitory" site for Na^+. This is expected if Cl^- must be released before Na^+ release. In an ordered binding/release model, the presence of high $[Cl^-]_i$ would facilitate occupancy of the Cl^- site making the subsequent release of Na^+ less likely; thus increasing the apparent affinity of the Na^+ site. This effect of intracellular Cl^- on inhibition of influx is consistent with the fact that intracellular Cl^- is necessary for cotransport efflux of Na^+ (Altamirano and Russell, 1987). Thus, all our observations are consistent with the effects of intracellular Na^+ being mediated via a transport site participating in an ordered binding/release mechanism.

Physiological Consequences of Inhibition by Intracellular Ions

Regardless of the mechanism of intracellular ion-mediated inhibition, such inhibition has physiologically important consequences. For the squid axon, under normal intracellular ion conditions, $([Cl^-]_i = 125$ mM; $[Na^+]_i = 50$ mM) influx and efflux are relatively small and about equal (being 6-7.5 and 4-5 picomol/cm^2.s for Cl^- and Na^+, respectively) despite the fact that the net thermodynamic driving force greatly favors net influx.

Thus, a low, steady state cotransporter-mediated influx, coupled with leak flux, prevents the cotransporter from raising $[Cl^-]_i$ to the level predicted from a thermodynamic consideration of the ion gradients which serve as the energy source of this cotransporter (Russell, 1983). In principle, this "excess" thermodynamic energy favoring influx could be accessed by adjusting the inhibitory constants for intracellular Cl^-. In fact, we have demonstrated that shrinkage of the squid axon stimulates cotransport influx by shifting the intracellu-

lar Cl^- inhibitory relationship towards higher $[Cl^-]_i$ (Breitwieser et al., 1990). Another obvious consequence of maintaining a relatively low cotransporter-mediated influx would be to lessen the intracellular "Na^+ load" which must be cleared out by the sodium pump.

Intracellular [Cl⁻] as a Modulator in Other Systems

The present results strongly suggest an "intrinsic" regulatory role for intracellular Cl^- with respect to the Na^+,K^+,Cl^- cotransporter. In fact, numerous recent reports suggest that intracellular Cl^- may be an important modulator of a surprisingly wide variety of cellular functions including ion transporters, ion channels and other cellular and/or membrane-localized proteins such as G-proteins (Higashijima, et al., 1987; Nakajima et al., 1992). Nucleotide receptors (Middleton et al., 1993) and ubiquinol oxidase (Orii et al., 1995) are two recent additions linked to changes of $[Cl^-]_i$.

Intracellular Cl^- as a modulator of ion transporters. Intracellular Cl^- has been suggested (in the absence of direct $[Cl^-]_i$ measurements) to exert a modulatory effect on a variety of ion transport mechanisms, supporting earlier findings in the internally dialyzed squid giant axon (Russell, 1976, 1979, 1983; Breitwieser et al., 1990, 1992*a*, *b*). In shark rectal gland, [3H]-benzmetanide binding (used as a marker for Na^+,K^+,Cl^- cotransport activity) can be stimulated by perfusing the gland with a low $[Cl^-]$ Ringer solution (Lytle and Forbush, 1992; Forbush et al., 1992). O'Neill and Klein (1992) reported that preincubation of vascular endothelial cells in Na^+ or K^+-free media (which presumably lowers $[Cl^-]_i$) leads to a stimulation of cotransport influx. Haas and McBrayer (1994) reported that nystatin-permeabilized tracheal epithelial cells bathed in low $[Cl^-]$ solutions (NO_3^- replaced Cl^-) exhibited higher Cl^- flux than those bathed in high $[Cl^-]$ solutions. Muscarinic stimulation of both the Na^+/H^+ exchanger and the Na^+,K^+,Cl^- cotransporter activity in salivary acinar cells is reported to be mediated via a reduction of $[Cl^-]_i$ (Robertson and Foskett, 1994). However, shrinkage activation of the Na^+/H^+ exchanger in internally dialyzed barnacle muscle fibers requires the presence of intracellular Cl^- (Davis et al., 1994). These latter two results suggest that Na^+/H^+ exchange may have an optimal $[Cl^-]_i$ level, as we have shown for the efflux mode of the Na^+,K^+,Cl^- cotransporter.

Intracellular Cl^- as a modulator of ion channels. In addition to effects on ion transporters, intracellular $[Cl^-]$ has also been implicated in the regulation of several ion conductance mechanisms. For instance, intracellular anions, including Cl^-, appear to slow down the gating of delayed rectifier K^+ channels in squid giant axons (Adams and Oxford, 1983). A Ca^{2+}-activated, non-selective cation channel in lung epithelium has an

increased open probability when $[Cl^-]_i$ is decreased (Tohda et al., 1994). In intralobular duct cells of mouse salivary glands, increases of $[Cl^-]_i$ result in an increase in Na^+ conductance and a decrease in Cl^- conductance (Dinudom, et al., 1993). A stimulatory effect of intracellular Cl^- has been observed for CFTR activation, independent of its regulation by cAMP (Wang et al., 1993). Cell shrinkage can also activate a Cl^--dependent cation conductance (Chan and Nelson, 1992).

How Does Intracellular Cl^- Exert Modulatory Effects?

The molecular mechanism(s) by which intracellular Cl^- exerts modulatory effects is currently unknown, but two recent studies suggest rather different possibilities. Perutz and co-workers (1994), studying Cl^--mediated reduction in oxygen affinity of hemoglobin, have demonstrated a kind of allosteric effect which does not depend on binding of Cl^-, but rather seems to be due to the neutralization of electrostatic repulsion of positive charges in the hemoglobin binding cavity (Shih and Perutz, 1987; Perutz et al., 1994). This is a variation of the mechanism suggested for high intracellular Cl^--

mediated activation of CFTR, since other halides, including I^-, mimic the effect (Wang et al., 1993).

Another recent study suggests a somewhat more direct effect of Cl^-. This study used an apically-enriched membrane preparation from lung epithelium to show that phosphorylation of a 37-kD protein was biphasically dependent on $[Cl^-]$. Maximal phosphorylation was observed at $[Cl^-] = 40$ mM. Higher (or lower) $[Cl^-]$ caused reduced phosphorylation (Treharne et al., 1994). Note that this is the same qualitative pattern of modulation we observe for the effect of intracellular Cl^- on the Na^+,K^+,Cl^- cotransporter in the efflux mode (e.g., Fig. 3), but not the influx mode. These observations are particularly interesting because Lytle and Forbush (1992) have suggested that intracellular Cl^- might exert its inhibitory effect on cotransport by reducing phosphorylation of the cotransport protein. Since we have recently provided evidence against a stimulatory effect of intracellular Cl^- on protein phosphatases (Altamirano et al., 1996), it is possible that elevated $[Cl^-]_i$ inhibits cotransport by reducing protein kinase activity.

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