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Activation of Na^+,K^+,Cl^- cotransport in squid giant axon by extracellular ions: evidence for ordered binding

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Abstract

Activation of the influx mode of the Na^+,K^+,Cl^- cotransporter (NKCC) by extracellular Na^+, K^+ and Cl^- was studied using the internally dialyzed squid giant axon. Cooperative interactions among the three transported ions were assessed using ion activation of NKCC-mediated ^{36}Cl influx under two sets of experimental conditions. The first, or control condition, used high, non-limiting concentrations of two of the cotransported ions (the co-ions) while activating cotransport with the third ion. Under this non-limiting co-ion condition the calculated V_{max} of the cotransporter was between 57 and 60 pmol/cm^2/s. The apparent activation (K_{App}, or half-saturation) constants were: K^+, 9 mM; Na^+, 52 mM; and Cl^-, 146 mM. The second condition used limiting co-ion concentration conditions. In this case, activation by each ion was determined when one of the other two co-ions was present at or near its apparent half-saturation concentration as determined above. Under these limiting conditions, the K_{App} values for all three co-ions were significantly increased regardless of which co-ion was present at a limiting concentration. The effects on the apparent V_{max} were more complicated. When K^+ was the limiting co-ion, there was little effect on the V_{max} for Na^+ or Cl^- activation. In contrast, limiting concentrations of Na^+ or Cl^- both resulted in a large reduction of the apparent V_{max} when activating with the other two co-ions. These results are consistent with an ordered binding mechanism for the NKCC in which K^+ binds before Na^+ or Cl^-. Physiological implications for these results are discussed.

Keywords: Bumetanide; Na,K,Cl cotransport; Axon; Ion-dependent ion binding

1. Introduction

Obligatory coupled cotransport of Na^+, K^+, and Cl^- has been identified in a wide variety of cells since its initial identification in Ehrlich ascites tumor cells [1]. Two isoforms of the Na^+,K^+,Cl^-cotransporter (NKCC) have been cloned (NKCC1 and NKCC2; [2,3]. NKCC1 is, by far, the most wide-spread of the two, being found in nearly every animal cell in which it has been sought (e.g. [4]). On the other hand, NKCC2 has only been identified in salt-absorbing epithelia, such as the thick ascending limb of the loop of Henle in the renal nephron. The so-called 'loop diuretics', such as furosemide and bumetanide, completely block NKCC-mediated ion transport

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(mediated by either isoform) at relatively low concentrations (bumetanide $K_i = 0.1$ μM; [5]).

In addition to being inhibited by loop diuretics, the NKCC mechanism is characterized by an absolute requirement for the *cis*-side presence of all three of the cotransported ions (i.e. Na^+, K^+ and Cl^-). There is evidence that both binding and release of Na^+, K^+ and Cl^- by the NKCC is a highly ordered process [6–11]. By far the majority of transport studies on the NKCC have examined K^+ (Rb^+) influx. However, the unavoidable presence of *trans*-side (intracellular) K^+, Na^+ and Cl^- in these studies raises the possibility of isotopic exchange fluxes which have the potential to complicate all such studies on the cotransporter (e.g. [12,13]), including studies on binding order and cooperativity. In addition, evidence that cotransporter function is modulated by intracellular Na^+, K^+, and Cl^- has been steadily accumulating [8,14–20]. It is therefore clear that an assessment of the effects of external ions needs to be made under conditions which prevent or significantly reduce effects of intracellular ions.

We used the technique of intracellular dialysis to assess the activation by external ions of unidirectional influxes into the squid giant axon via the NKCC. By dialyzing with Na^+- and Cl^--free dialysis solutions, we achieved a nominally Na^+, and Cl^--free intracellular steady-state. We measured NKCC activity (defined as bumetanide-sensitive, unidirectional Cl^- influx) as a function of the extracellular concentration of each of the three cotransported ions in turn, while maintaining the extracellular concentration of the other two ions (the co-ions) constant. In control studies, we showed that the concentrations of the co-ions could be made high enough to be non-rate-limiting. A separate series of experiments for each of the cotransported ions was performed under conditions of limiting (low) concentration of each of the co-ions in turn. Under these latter conditions, we observed that the derived activation parameters (i.e. K_{app} and V_{max}) were significantly different from those obtained when the co-ion concentrations were not limiting. Such effects are expected with an ordered binding process. Furthermore, the pattern of effects on K_{App} and V_{max} fit a model of coupled cotransport developed by Stein [21] and suggest that K^+ binds before either Na^+ or Cl^-. These results have interesting implications for the normal functioning of the NKCC.

2. Materials and methods

Experiments were conducted at the Marine Biological Laboratory (Woods Hole, MA). 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.

2.1. Unidirectional flux measurements by intracellular dialysis

We used a modification of the technique of intracellular dialysis (first developed by Brinley and Mullins [22]). The details of this modification have been previously described (e.g. [15,16]). Briefly, a 35–40-mm length of axon was cannulated at both ends in a specially designed dialysis chamber. A dialysis tube was carefully guided through the axon until the porous region was positioned within the central portion of the axon. Once the dialysis tube was in position and the central, dialyzed region isolated from the cannulated ends (by grease seals), the appropriate dialysis fluid was pumped through it at the rate of 2 μl/min. All experiments were conducted at a temperature of 17°C.

2.1.1. Influx procedures

To measure unidirectional influx, the radionuclide ^{36}Cl was added to the external solution (40 μCi/mmol). The ^{36}Cl which crossed the axolemma and diffused through the axoplasm into the flowing internal dialysis fluid inside the dialysis tube was collected directly into scintillation vials over 5-min intervals. Seven ml of Aquasol cocktail (NEN, Life Sciences) were added, and each sample was counted to an error not greater than 4% in an LS-3801 scintillation counter (Beckman Instruments, Irvine, CA).

2.2. Experimental solutions

2.2.1. Intracellular fluid

The intracellular dialysis fluid (DF) was Na^+- and

Cl^--free and contained a relatively high $[K^+]$ so that raising $[K^+]_o$ to 100 mM (and in one set of experiments, to 200 mM) did not depolarize the membrane potential enough to significantly activate voltage-sensitive Cl^- channels [23]. The DF contained (in mmol/l): K^+, 400; Mg^{2+}, 8; glutamate, 416; glycine, 175; MOPS (3-[*N*-morpholino]-propanesulfonic acid), 25; Tris (tris(hydroxymethyl)amino methane), 19.3; EGTA (ethylene glycol-bis(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid), 2; phenol red, 0.1; ATP (adenosine 5′-triphosphate), 4; pH 7.30–7.35; osmolality, 968–972 mosmol/kg.

2.2.2. Extracellular fluids

The standard external solution had the following composition in mmol/l: K^+, 100; Na^+, 335; Mg^{2+}, 63; Ca^{2+}, 3; Cl^-, 561; EPPS (*N*-[2-hydroxyethyl]-piperazine-*N*′-[3-propane sulfonic acid]), 10; EDTA (ethylenediaminetetraacetic acid), 0.1; tetrodotoxin, 0.0001; ouabain, 0.01; pH, 8.00; osmolality, 973–978 mosmol/kg. $NMDG^+$ (*N*-methyl-D-glucamine) was the replacement cation when $[K^+]$ or $[Na^+]$ were varied. In early, pilot experiments, Cl^- was replaced either by gluconate, sulfamate, methanesulfonate or SO_4^{2-} with indistinguishable results. Therefore, the present study was conducted using sulfamate (and sometimes gluconate) as the Cl^--replacement anion.

2.3. Experimental protocol

Fig. 1 illustrates the general protocol we used. All axons in this study were dialyzed with the Na^+- and Cl^--free DF for at least 1 h before ^{36}Cl was added to the external solution. Thus, all the data we report came from axons that had been dialyzed for a total of at least 85–110 min with the Na^+- and Cl^--free DF. Experiments with Cl^--selective microelectrodes have shown that this is a sufficient period of dialysis to reduce $[Cl^-]_i$ to near 0 mM [24]. It is reasonable to assume that $[Na^+]_i$ would be similarly reduced by this period of dialysis. In the absence of intracellular Cl^-, there can be no bumetanide-sensitive self-exchange flux of Cl^- to complicate the interpretation of our results (e.g. [25]). Isotopic equilibrium was reached within 20 min of the addition of the ^{36}Cl to the external fluid. Control influx values were determined as the average of the last 4–5 data points obtained prior to application of bumetanide (denoted by the solid lines through data points in Fig. 1). Each axon was next exposed for 50 min to 10 μM bumetanide in the external solution. The difference between the control flux and that obtained 25–50 min after exposure to bumetanide (denoted by the dashed lines through the later data points in Fig. 1) was the bumetanide-sensitive influx. Thus, each axon provided one datum point of bumetanide-sensitive influx and the number in parentheses after each average presented in this paper represent the number of axons from which the averaged data were derived.

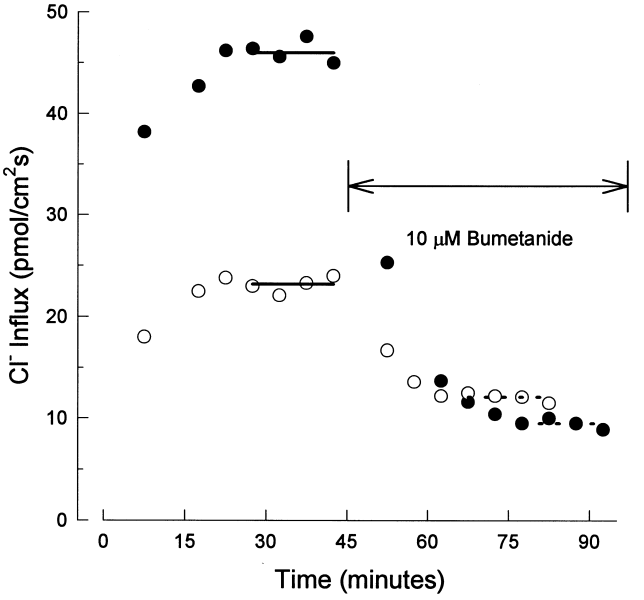

Fig. 1. Two representative Cl^- influx experiments under two different external ion conditions. At zero time, ^{36}Cl was added to the external solution. At that time, each axon had already been internally dialyzed with the dialysis fluid described in Section 2 for 60 min. Isotopic equilibrium was reached within 20–30 min following the addition of ^{36}Cl, permitting the measurement of the steady-state, unidirectional influx of Cl^-. Filled circles: this axon was bathed in an external fluid with $[K^+]$ = 10 mM and $[Na^+]$ = 425 mM. The steady-state influx prior to bumetanide treatment was 46 pmol/cm^2/s (represented by a solid line through the filled data points). After 10 μM bumetanide was applied externally, the influx decreased to 9.5 pmol/cm^2/s (represented by a dashed line through the filled data points), giving a bumetanide-sensitive influx of 36.5 pmol/cm^2/s. Open circles: this axon was bathed in an external fluid with $[K^+]$ = 10 mM as above, but with $[Na^+]$ = 50 mM. In this case, the steady-state influx prior to bumetanide treatment was 23.2 pmol/cm^2/s (solid line); after bumetanide treatment the influx was 12.1 pmol/cm^2/s (dashed line), giving a bumetanide-sensitive Cl^- influx of 11.1 pmol/cm^2/s.

2.4. Data analysis

The bumetanide-sensitive flux data were fitted with the following form of the Hill equation: $V = V_{max}[X]_o^n/([X]_o^n + K_{App}^n)$ where $[X]_o$ is the extracellular concentration of the activating co-ion, either K^+, Na^+ or Cl^-; n is the Hill coefficient and K_{App} is the apparent activation constant for ion X. The fitting program NFit (Island, Galveston, TX) was used. This program permits the independent determination of each of the variables (V_{max}, K_{App} and the Hill coefficient, n) for each series of data. Under conditions of non-limiting substrate ion concentrations, the program was allowed to obtain the best-fit for all three parameters. When one of the substrate ions was limiting, the Hill coefficient, n, was fixed at the value calculated for the non-limiting condition, since there is no reason to believe this parameter would be affected by changes of substrate concentration and fixing the value permitted a better fit under conditions of limiting substrate concentrations. Finally, although the lines through data points in Fig. 2 show averages ± S.E.M., the actual fitting to the Hill equation used the individual data points from all axons treated with any given protocol.

3. Results

The results of this study are presented in terms of the effects of reducing the concentration of each co-ion in turn on the activation parameters for each of the co-ions. As a practical matter, we first had to determine the optimal conditions for activation by each co-ion. We did this by determining activation constants (K_{App}) for each of the cotransported ions under conditions where the other two cotransported ions were present at the highest possible concentration that could be achieved without increasing the osmolarity of the solutions. This latter restriction was necessary given the osmo-sensitivity of the NKCC, not only in the squid axon [17], but in many other preparations as well (e.g. [5,26]). The results of this initial set of experiments served as our control condition for the rest of the study. Each individual control ion activation curve can be seen as the filled circles in each panel of Fig. 2. The K_{App} and V_{max} values determined in this set of control experiments are collated in Table 1A. The K_{App} values were approximated for use as the experimental concentration for each ion when it was the limiting co-ion.

3.1. Activation of NKCC-mediated cotransport influx by extracellular K^+

3.1.1. Effect of extracellular $[Na^+]$ on K_o^+ activation parameters

In order to assess the influence of varying $[Na^+]_o$ on activation of the NKCC by extracellular K^+, bumetanide-sensitive Cl^- influx was measured as a function of extracellular $[K^+]$ at two different $[Na^+]_o$ values: 335 and 50 mM. In both cases, $[Cl^-]_o = 561$ mM. This $[Cl^-]_o$ is the highest concentration obtainable without changing the osmolality or ionic strength of the external solutions. As seen in the filled circles in Fig. 2E, it is not rate-limiting. Fig. 2A (filled circles) shows that when both $[Na^+]_o$ and $[Cl^-]_o$ are high, activation of bumetanide-sensitive Cl^- influx by extracellular K^+ follows first order kinetics (Hill $n = 1.0$) with $K_{App} = 9$ mM and a V_{max} of 57 pmol/cm^2/s.

Reducing $[Na^+]_o$ to 50 mM strongly affected extracellular K^+ activation of the NKCC as seen in the second curve (open circles) of Fig. 2A. The K_{App} for K^+ was increased to about 27 mM. Thus, under the condition of a limiting extracellular concentration of Na^+ the K_{App} for extracellular K^+ was increased about three-fold. Furthermore, the apparent V_{max} of the cotransporter was reduced by about 35% to 37 pmol/cm^2/s.

3.1.2. Effect of extracellular $[Cl^-]$ on K_o^+ activation parameters

We next examined the effects of limiting external Cl^- concentration while determining the extracellular K^+ activation of NKCC-mediated Cl^- influx. For this series, we reduced $[Cl^-]_o$ from the control value of 561 to 180 mM, while maintaining $[Na^+]_o$ at 335 mM. The filled circles in Fig. 2B indicate the control condition and are re-drawn from Fig. 2A. The data fitted by the lower curve (open circles) in Fig. 2B show that reducing $[Cl^-]_o$ to 180 mM reduced the apparent V_{max} by about the same amount as did the equivalent reduction of $[Na^+]_o$ (to 39 pmol/cm^2/s; cf. Fig. 2A). However, the limiting $[Cl^-]_o$ had a much

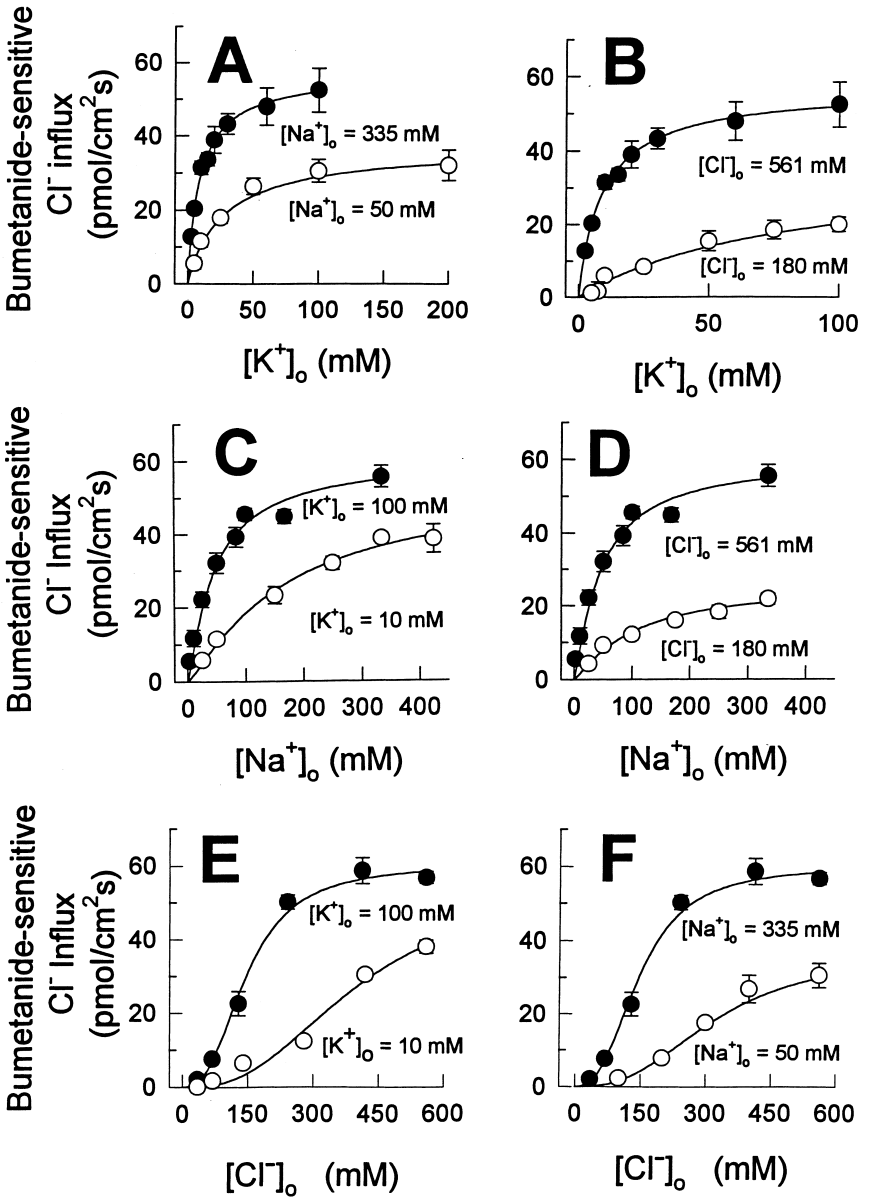

Fig. 2. Activation of bumetanide-sensitive Cl^- influx by extracellular ions: effects of reducing the concentrations of extracellular co-ions. Each data point represents the mean ± S.E.M. for 4–9 experiments similar to those illustrated in Fig. 1. The lines through the data points represent the best fit of the Hill equation ($v = V_{max} \cdot [K^+]^n / ([K^+]^n + K^n_{App})$). The best-fit parameters derived from this equation for all the curves are listed in Table 1. (A,B) Activation by external K^+. (A) Effect of reducing $[Na^+]_o$ from 335 mM (●) to 50 mM (○). The $[K^+]_o$ was varied by substituting K^+ with $NMDG^+$. $[Cl^-]_o$ was 561 mM throughout. (B) Effect of reducing $[Cl^-]_o$ from 561 mM (●) to 180 mM (○). $[Na^+]_o$ was 335 mM throughout. The $[K^+]_o$ was varied by substituting K^+ with $NMDG^+$. The data for $[Cl^-]_o = 561$ mM are the same as shown in A for $[Na^+]_o = 335$ mM. (C,D) Activation of bumetanide-sensitive Cl^- influx by extracellular Na^+. (C) Effect of reducing $[K^+]_o$ from 100 mM (●) to 10 mM (○). $[Cl^-]_o$ was 561 mM throughout. The $[Na^+]_o$ was varied by substituting Na^+ with $NMDG^+$. (D) Effect of reducing $[Cl^-]_o$ from 561 mM (●) to 180 mM (○). $[K^+]_o$ was 100 mM throughout. The $[Na^+]_o$ was varied by substituting Na^+ with $NMDG^+$. The data for $[Na^+]_o = 335$ mM are the same as shown in C for $[K^+]_o = 100$ mM. (E,F) Activation of bumetanide-sensitive Cl^- influx by extracellular Cl^-. (E) Effect of reducing $[K^+]_o$ from 100 mM (●) to 10 mM (○). $[Na^+]_o$ was 335 mM throughout. The $[Cl^-]_o$ was varied using $sulfamate^-$ or in some cases, $gluconate^-$. (F) Effect of reducing $[Na^+]_o$ from 335 mM (●) to 50 mM (○). $[K^+]_o$ was 100 mM throughout. The $[Cl^-]_o$ was varied using sulfamate or in some cases, gluconate. The data for the $[K^+]_o = 100$ mM are the same as shown in E for the $[Na^+]_o = 335$ mM.

greater effect on the apparent affinity, causing the K_{App} for K^+ to increase from 9 to 94 mM. Thus, the K_{App} of the cotransporter for external K^+ is much more sensitive to reduction of the external $[Cl^-]$ than it is to reduction of external $[Na^+]$.

3.2. Activation of NKCC-mediated cotransport influx by extracellular Na^+

3.2.1. Effect of extracellular $[K^+]$ on Na_o^+ activation parameters

Following the pattern of the studies on activation by extracellular K^+, we studied activation of cotransport by extracellular Na^+ at two different $[K^+]_o$ levels, 100 and 10 mM ($[Cl^-]_o$ was held constant at 561 mM). The upper curve (filled circles) of Fig. 2C represents the fitted data obtained when $[K^+]_o$ and $[Cl^-]_o$ were high and relatively non-limiting (100 and 561 mM, respectively). The V_{max} was 60 pmol/cm^2/s, which as expected, is essentially the same as that obtained when varying external $[K^+]$ at high concentrations of extracellular Na^+ and Cl^- (see Fig. 2A and Table 1A). The activation constant for external Na^+ under this control condition was about 52 mM. The best-fit Hill coefficient to the Na^+ activation data was 1.2.

The bottom curve (open circles) in Fig. 2C represents the external Na^+ activation under the condition of limiting $[K^+]_o$. Unlike the response of K^+ activation to reduced $[Na^+]_o$, reducing $[K^+]_o$ to 10 mM had little, if any, effect on the apparent V_{max} (= 55 pmol/cm^2/s) when activating with Na^+. However, the K_{App} for extracellular Na^+ was increased about 3-fold, from 60 to 176 mM.

3.2.2. Effect of extracellular $[Cl^-]$ on Na_o^+ activation parameters

The effects on activation by external Na^+ of reducing external $[Cl^-]$ to 180 mM followed a different pattern from the effects of reducing $[K^+]_o$. As seen in Fig. 2D, a reduction of $[Cl^-]_o$ increased the K_{App} for NKCC-mediated ^{36}Cl influx activated by extracellular Na^+ about 2-fold, from 52 to 103 mM. Under this same condition, the apparent V_{max} was reduced by more than half, from 60 to 26 pmol/cm^2/s.

3.3. Activation of NKCC-mediated cotransport influx by extracellular Cl^-

3.3.1. Effect of extracellular $[K^+]$ on Cl_o^- activation parameters

Activation of cotransport by extracellular Cl^- was studied at two different $[K^+]_o$ levels, 100 and 10 mM, while $[Na^-]_o$ was held constant at 335 mM. The upper curve (filled circles) of Fig. 2E represents the fit of data obtained when $[K^+]_o$ and $[Na^+]_o$ were high and relatively non-limiting (100 and 335 mM, respectively). In good agreement with activation by the other two co-ions (see Fig. 2A,C and Table 1A) under control, non-limiting conditions, the V_{max} for NKCC-mediated cotransport influx of Cl^- was 60 pmol/cm^2/s. The K_{App} for Cl^- under this optimal

Table 1
Ion activation parameters for the squid NKCC under (A) non-limiting and (B) limiting [co-ion] conditions

Conditions	K_{App} (mM)	V_{max} (pmol/cm^2/s)	Hill coefficient			
(A) Activating co-ion						
K^+ ($n=60$) ($[Na^+]=325$ mM; $[Cl^-]=561$ mM)	9.0 ± 1.6	57.3 ± 3.7	1.0 ± 0.			
Na^+ ($n=59$) ($[K^+]=100$ mM; $[Cl^-]=561$ mM)	51.7 ± 8.3	60.0 ± 4.4	1.2 ± 0.2			
Cl^- ($n=39$) ($[K^+]=100$ mM; $[Na^+]=325$ mM)	146.4 ± 9.1	60.1 ± 1.6	2.7 ± 0.6			
	Activating co-ion					
	K^+		Na^+		Cl^-	
	K_{App} (mM)	V_{max} (pmol/cm^2/s)	K_{App} (mM)	V_{max} (pmol/cm^2/s)	K_{App} (mM)	V_{max} (pmol/cm^2/s)
(B) Limiting [Co-ion]						
10 mM K^+	–	–	176 ± 30 (34)	55 ± 4 (34)	396 ± 32 (65)	54 ± 4 (65)
50 mM Na^+	27 ± 8 (37)	38 ± 3 (37)	–	–	324 ± 29 (32)	37 ± 3 (32)
180 mM Cl^-	94 ± 42 (34)	39 ± 9 (34)	103 ± 23 (46)	26 ± 2 (46)	–	–

control condition was about 146 mM. The best-fit Hill coefficient for the Cl^- activation data was 2.7.

The bottom curve (open circles) in Fig. 2E represents the external Cl^- activation under the condition of limiting $[K^+]_o$ (10 mM). Interestingly, making $[K^+]_o$ limiting yielded the same pattern of effects on external Cl^- activation parameters as noted above for external Na^+ activation parameters. The apparent V_{max} was little changed by reducing $[K^+]_o$ when activating with Cl^-, going from 60 to 54 pmol/cm^2/s. However, the K_{App} for Cl^- increased significantly, from 146 to 396 mM.

3.3.2. *Effect of extracellular [Na^+] on Cl_o^- activation parameters*

Fig. 2F illustrates the effects of activating NKCC influx with extracellular Cl^- when external $[Na^+]$ is limiting. It compares activation of NKCC-mediated Cl^- influx by extracellular Cl^- at $[Na^+]_o = 335$ (filled circles) and 50 mM (open circles) while maintaining $[K^+]_o$ at 100 mM. Unlike the effect of making external K^+ the limiting co-ion, when $[Na^+]_o$ was reduced from 335 to 50 mM, both the apparent V_{max} and the K_{App} were significantly affected. The V_{max} was reduced almost 40%, from 60 to 37 pmol/cm^2/s while the K_{App} for Cl^- increased from 146 to 324 pmol/cm^2/s.

4. Discussion

Previous studies have addressed the dependence of the Na^+,K^+,Cl^- cotransporter (NKCC) on the extracellular concentrations of K^+, Na^+ and Cl^- (e.g. [27,28]). These studies used preparations in which possible complications arising from concomitant changes of the intracellular ion composition were not considered, and in any case, could not be easily addressed or avoided. Such considerations may not be trivial, since we now know that intracellular ions can significantly modulate the behavior of the NKCC, even when studies are limited to the influx mode. Thus, intracellular Cl^- has profound effects on the behavior of the NKCC, in both the efflux and influx modes (e.g. [16,17,19,20,29]). In addition to the effects of intracellular Cl^-, a rise of $[Na^+]_i$ and/or $[K^+]_i$ has been reported to promote self-exchange fluxes [7,9] and to inhibit NKCC-mediated influxes ([19,20]). Also, it has been proposed that an internal site needs to be occupied by K^+ in order for cotransporter-mediated influx to occur [30]. Given these considerations and our still-incomplete understanding of the overall operation of this cotransporter in mechanistic terms, a re-examination of external ion activation of cotransporter-mediated influx in a preparation which permits control over the intracellular composition is necessary.

In the present study, we eliminated possible complicating effects of intracellular Cl^- and Na^+ by dialyzing the axoplasm with a Cl^-/Na^+-free fluid. It may also be important to recognize that these conditions permitted us to measure a *unidirectional* influx that was also a *net* influx. Furthermore, intracellular dialysis prevents any changes of intracellular pH or $[Ca^{2+}]$ which might occur in response to changes in extracellular ion concentrations and which have themselves been reported to affect NKCC fluxes (e.g. [31–33]).

4.1. *Relative affinities of the NKCC for the cotransported ions*

Table 1A shows that, for the squid giant axon under the condition of non-limiting co-ion concentrations, the sequence of the relative affinities ($1/K_{App}$) for the cotransported ions is: $K^+ > Na^+ > Cl^-$. This sequence agrees well with the results of several groups studying what is probably the NKCC1 isoform from a variety of cell types (e.g. [9,27,34]). These previous studies were all carried out in the presence of intracellular Na^+ and Cl^-, suggesting that these intracellular ions do not affect the *relative* external affinities of the cotransported ions.

4.2. *Binding of the cotransported ions to extracellular sites is an ordered process in the squid axon*

Evidence from several laboratories strongly indicates that binding of the cotransported ions to the NKCC is an ordered process (e.g. [6–9,11]). One of the goals of the present study was to ascertain whether similar evidence could be obtained in the internally dialyzed squid giant axon under ideal, unidirectional flux conditions. There are at least two reasons to address the ordered binding issue in the

squid axon. First, the squid axon cotransporter apparently binds and transports ions with a different stoichiometry ($Na^+:K^+:Cl^- = 2:1:3$; [16]) than has been reported for several vertebrate preparations ($Na^+:K^+:Cl^- = 1:1:2$; e.g. [5]). Secondly, the ability to internally dialyze the squid giant axon allows us to reduce the number of uncontrolled variables for what may be a very complicated and interrelated set of processes culminating in NKCC-mediated ion transport.

Our approach was to activate NKCC-mediated ^{36}Cl influx by varying the extracellular concentration of each one of the cotransported ions in turn. We studied each ion's ability to activate cotransporter-mediated influx under conditions in which each of the other two cotransported ions in turn was present either at high, relatively non-limiting concentrations or at low, limiting concentrations. The third co-ion was always present at a high concentration. As pointed out by Stein [21], this experimental design permits us to determine whether the ion binding processes are ordered and if so, to begin to determine that order. Given the potentially complicated nature of the cotransporter's operation in the squid axon (six ions must be bound and cotransported), our approach necessarily relies upon some simplifying assumptions. These assumptions are: (1) the ion binding to the NKCC followed rapid equilibration kinetics; (2) the multiple ion binding sites for Na^+ and Cl^- are equivalent; (3) the limiting step in transport is the translocation step across the membrane; (4) the third ionic species is not limiting for our measurements because it has been maintained at a high, non-limiting concentration; and (5) the ions used as replacements for cations or Cl^- do not interact significantly with the cotransporter.

In a system where the order of binding of the ions to the cotransporter is random, the activation coefficient (K_{App}) for the activating ion is unaffected by the concentration of the co-ions, whereas the apparent V_{max} is directly related to the concentration of the co-ions [21]. In other words, a random binding model predicts that when the concentration for any of the co-ions is made limiting, the K_{App} for the other two co-ions would be unaffected, but the V_{max} would decrease. Examination of our collated results in Table 1 shows that neither of these random binding/release model predictions were fulfilled. First, we have the obvious fact that, regardless of which co-ion we make limiting, reducing the concentration of one of the co-ions resulted in an increase in the K_{App} for the ion whose activation properties were being studied. Second, making external K^+ the limiting co-ion had little, if any, effect on the V_{max} when activating with either Na^+ or Cl^-. However, when either external Na^+ or Cl^- were the limiting co-ion, the apparent V_{max} was reduced by about 50%. Thus, our results are not compatible with a random order for binding. The strong interdependence of the activation parameters for each of the ions on the other two co-ions supports the view that external binding of the cotransported ions in the squid giant axon is an ordered process.

4.3. Order of ion binding to the NKCC in squid axon

Stein [21] has pointed out that in a system where the binding of ions is ordered, activating cotransport with the second ion to bind (ion B) at both high and low concentrations of the first ion to bind (ion A) would result in changes only in K^B_{App}. This pattern is referred to as K-type kinetics [21]. Conversely, in such a system, activating with the first ion to bind at both high and low concentrations of the second ion to bind will result in changes in both the V^A_{max} and K^A_{App}. This pattern is referred to as a combination of K- and V-type kinetics [21]. Table 1 shows that when activating cotransport with Na^+, only the K^{Na}_{App} changed, increasing from 52 to 176 mM when $[K^+]_o$ was reduced from 100 to 10 mM. Conversely, when activating with K^+ when $[Na^+]_o = 50$ mM resulted in an increase of K^K_{App} from 9 to 27 mM and a decrease in V^K_{max} (57 vs. 38 pmol/cm^2/s) when $[Na^+]_o$ was decreased from 335 to 50 mM. This result suggests that in the squid axon, K^+ binds before Na^+.

Applying the same analysis to the activation of cotransport by Cl^-, we see from Table 1 that when NKCC cotransport is activated by extracellular Cl^-, lowering $[K^+]_o$ from 100 to 10 mM caused a substantial increase in K^{Cl}_{App} (from 146 to 396 mM), but no significant change in V^{Cl}_{max} (60 vs. 54 pmol/cm^2/s). Conversely, when activating cotransport by extracellular K^+, reducing $[Cl^-]_o$ from 561 to 180 mM caused changes in both K^K_{App} (from 9 to 94 mM) and V^K_{max} (from 57 to 39 pmol/cm^2/s). This suggests that K^+ binds before Cl^-. However,

use of this analysis does not yield a clear answer as to whether Cl^- binds before or after Na^+ since activation with either ion in the presence of limiting concentrations of the other ion yields the combination of *V*- and *K*-type kinetics. Thus, our results suggest that in the squid axon NKCC, K^+ binds before either Na^+ or Cl^-. This order of binding differs from that reported by other workers (e.g. [8,9,11]. These latter studies indicate that Na^+ binds before K^+ or Cl^-. Although more work will be required to confirm this order of binding, it may indicate that the squid NKCC represents a different isoform than that in duck or human red cells or in HeLa cells. Since the stoichiometry of cotransport by the squid NKCC also differs from that reported for other tissues (e.g. [11,16], the existence of a unique squid isoform to explain these discrepancies is an attractive possibility.

4.4. Physiological implications of the apparent activation constants

4.4.1. At physiological ion concentrations, the NKCC operates below V_{max}

Table 1B shows that the apparent external affinities of the NKCC for each of the cotransported ions (K_{App}) are strongly affected by the external concentrations of the co-ions. The physiological extracellular ion concentrations for the squid giant axon are: $[K^+]_o = 18$ mM, $[Na^+]_o = 425$ mM, $[Cl^-]_o = 490$ mM [35,36]. Comparing these values with the K_{App} values in Table 1A shows that under normal, physiological conditions, the squid NKCC is apparently not saturated with respect to any of its external substrate ions. We cannot say precisely what the K_{App} values would be under physiological ion concentration conditions as we did not examine the K_{App} under this exact set of conditions. (In addition, it should be noted that we obtained the K_{App} values under the condition of 0 mM intracellular $[Cl^-]$ and $[Na^+]$.) However, we have previously shown that raising the intracellular concentrations of either Na^+ or Cl^- inhibits NKCC-mediated influx [20]. Thus, it is unlikely that raising their intracellular concentrations to more 'physiologic' values (e.g. $[Cl^-]_i = 120$ mM and $[Na^+]_i = 20$ mM [37]) would decrease the K_{App} for any of the cotransported ions. On the contrary, the combination of physiological $[K^+]_o$ being $\sim K_{App}$ and the high normal $[Cl^-]_i$ would suggest that under physiological ion conditions, the NKCC may have a very low affinity for external Na^+ and Cl^-.

In fact, the extracellular physiological condition is not far from our experimental condition of limiting $[K^+]_o$, i.e. $[K^+]_o = 10$ mM, $[Na^+]_o = 335$ mM, $[Cl^-]_o = 561$ mM (see Table 1B). Under this condition, the K_{App} for Na^+ increased 52 to 176 mM and that for Cl^- increased from 146 to 396 mM. When any one of the co-ions was made limiting the K_{App} for the other two ions increased. Since the normal physiological concentrations are limiting for all the co-ions, it seems reasonable to assume that the K_{App} values for all three ions could be even higher than they are under our 'non-limiting' condition. The implication is that in vivo, the cotransporter operates at rates well below its V_{max}. This raises the possibility that changes in the external concentration of any of the three cotransported ions could strongly influence the rate of cotransport. This effect would be in addition to already recognized modulators of NKCC activity, such as $[Cl^-]_i$, cell volume, phosphorylation state, etc. Each year squid migrate from deep ocean water (high salinity) to offshore water (lower salinity) and back. Since squid are osmoconformers, this means that significant changes of extracellular ion concentrations actually occur. It should be explicitly pointed out that subsaturation with respect to the ion substrates for the NKCC is unlikely to be unique to the squid. The apparent activation constant for the NKCC by K^+ in mammalian cells is in the range of 1–10 mM (e.g. [38]) and normal $[K^+]_o$ is around 4–5 mM. For Na^+, the reported activation constants range from 10 to 40 mM (e.g. [34,38]), while the normal $[Na^+]_o$ is around 135 mM. In the case of Cl^-, reported activation constants range between 50 and 80 mM, while normal $[Cl^-]_o$ is around 100 mM. Thus, particularly in the cases of K^+ and Cl^-, the mammalian NKCC is, like the squid system, operating under sub-saturated conditions with respect to its ion substrates.

The fact that the NKCC normally operates so far from its V_{max} raises the possibility that up- or down-regulation of cotransport in response to homeostatic signals may be the result of changes of the K_{App} of the NKCC for the cotransported ions. There is a good deal of evidence that, in order to perform ion cotransport, the NKCC protein must be phospho-

rylated (e.g. [39]). For the squid axon, we have shown that in the absence of cellular ATP there are no measurable NKCC-mediated ion fluxes. We have further shown that NKCC-mediated flux is a saturable function of [ATP] [14–16,25,40]. Whether phosphorylation acts to decrease the K_{App} values for the co-ions, or whether it permits the conformational changes necessary for transport remains to be determined.

4.4.2. Intracellular, but not extracellular, Cl^- has profound inhibitory effects on NKCC-mediated ion fluxes

We have previously demonstrated that intracellular Cl^- is necessary for NKCC-mediated efflux and, as expected, increases in $[Cl^-]_i$ up to about 125 mM result in a concentration-dependent increase of NKCC-mediated efflux [25]. However, further increases of intracellular $[Cl^-]$ beyond 125 mM inhibit both cotransport efflux and influx. Inhibition of both unidirectional cotransport fluxes is complete at $[Cl^-]_i = 300$ mM [20]. The mechanism of this profound inhibitory effect of intracellular Cl^- is uncertain at present.

One possible explanation arises from a corollary of an ordered binding/release process: increasing the *trans*-side concentration of any of the cotransported ions (relative to its intracellular K_{App}) would be expected to inhibit *cis*-to-*trans*-side cotransport fluxes by a kind of end-product inhibition (e.g. [20]). However, Breitwieser et al. [20] argued that this explanation was unlikely since high $[Cl^-]_i$ inhibits both influx and efflux and not just the influx. In contrast, while increasing intracellular $[Na^+]$ does somewhat inhibit NKCC-mediated influx, it does not inhibit efflux [20], consistent with a form of product inhibition. Taken together these results suggest that intracellular Cl^- inhibits NKCC transport by a different mechanism than does intracellular Na^+.

Unlike the inhibitory effect of elevated intracellular $[Cl^-]$ on cotransport efflux [20], our present results show that high extracellular $[Cl^-]$ does not inhibit cotransport influx (e.g. filled circles in Fig. 2E). Thus, the inhibitory effect of Cl^- on NKCC fluxes must be the result of an interaction between Cl^- and some unique intracellular site(s). It may be via an allosteric effect [41] or an effect on the phosphorylation state of the NKCC, perhaps via inhibition of a protein kinase responsible for activating the NKCC [40,42].

4.5. K_{App} for intracellular K^+ must be greater than that for extracellular K^+

As already discussed, our data support the view that ion cotransport via the NKCC involves a highly ordered ion binding and release process [7,9,11]. Furthermore, there is no evidence that net cotransport can occur for one ion without net transport of the others, i.e. there seems to be no 'slippage' in the cotransporter mechanism. This implies that only the fully loaded or completely unloaded form of the cotransporter can 'cross' the membrane. Therefore, in order to effect net ion uptake, the cotransporter must be able to release all of the bound ions into the cytoplasm. If one of the cotransported species is present on the inside at a concentration significantly higher than its apparent dissociation constant, the cotransporter will be unable to fully 'unload' and effect net uptake, i.e. it cannot return empty to the external face and it therefore will engage in exchange fluxes (e.g. [11]).

The $[K^+]$ in squid axoplasm is about 300 mM [37]. This value is more than 30-fold larger than the K^+ dissociation constant we report here for the external site (9 mM). The same is qualitatively true for all other cells, since $[K^+]_i$ is always much higher than the reported values for the extracellular activation coefficient for K^+. How, then, is K^+ released intracellularly? It seems highly likely that the apparent dissociation constant for K^+ at the intracellular NKCC binding site is much higher than the values reported for the extracellular site. This might explain why Kracke et al. [43] observed that an intracellular K^+ concentration of 40 mM was insufficient to activate coupled Na^+,K^+ transport via the NKCC in red blood cells.

Our present findings suggest a way the K_{App} for K^+ at the intracellular membrane face might be increased. Assume that the internal sites have ordered binding/release properties similar to those we have demonstrated for the external sites. If that is the case, then the much lower intracellular concentrations of Na^+ and Cl^- would result in a very significant increase of the K_{App} for intracellular K^+, permitting the release of K^+ into the axoplasm. As

Table 1B shows, reducing either $[Na^+]_o$ or $[Cl^-]_o$ alone to values that are still greater than actual intracellular concentrations of these two ions caused large increases in the K_{App} for extracellular K^+ (3- and 10-fold, respectively). We did not test the combination of both co-ions present at limiting concentrations, but it seems likely their effects would be additive, depending on the exact intracellular concentrations and K_{App} values.

4.6. The K_{App} for intracellular Na^+ may be less than for extracellular Na^+

Using two different approaches, we reported that under near-physiological intracellular ion conditions the K_{App} of the intracellular Na^+ binding sites of the NKCC is about 50 mM [20]. In agreement with our present findings for the *external* Na^+ binding sites, we found that the K_{App} for intracellular Na^+ was inversely dependent on the intracellular Cl^- concentration [20]. The fact that the apparent activation coefficient for the internal site ($\sim$50 mM) under near-physiological ion conditions is so near what we report here for the external site ($\sim$52 mM; Table 1A) under our control conditions is almost certainly fortuitous because the control *cis*-side co-ion conditions used in the present study for the external Na^+ site differ significantly from those used in the internal site study. In particular, the *cis*-side $[Cl^-]$ ($[Cl^-]_o$ = 561 mM) used in the present study on influx is much higher than that the *cis*-side $[Cl^-]$ used in the previous study ($[Cl^-]_i$ = 125 mM) for efflux. The combined results from these two studies make clear that under near-physiological conditions ($[K^+]_i$ = 200 mM, $[Cl^-]_i$ = 125 mM and $[K^+]_o$ = 10 mM and $[Cl^-]_o$ = 561 mM), the apparent activation coefficient of the intracellular Na^+ sites is lower ($\sim$50 mM) than the extracellular sites ($\sim$175 mM; Table 1B). However, given that the normal $[Na^+]_i$ is low ($\sim$20 mM; [37]), end-product inhibition by intracellular Na^+ ought not to present a serious impediment to net influx via cotransport.

4.7. Hill coefficients and stoichiometry of cotransport

The Hill coefficient values obtained from studies similar to the present study have routinely been used to estimate the stoichiometry of ion transport by the cotransporter. As seen in Table 1A, the Hill coefficients for activation of the squid axon NKCC by both K^+ and Na^+ are very near 1. This agrees with the results reported by several other groups, using vertebrate cells (e.g. [8,27,28]). But the directly measured stoichiometry for NKCC-mediated Na^+ influx by the squid NKCC is 2 [16]. In addition, the Hill coefficient for Cl^- for external Cl^- activation of the NKCC in the squid axon is very near 3.0, whereas other studies have reported values around 2.0. In the case of the squid axon, the directly measured transport stoichiometry for NKCC-mediated Cl^- transport is also 3 [16]. Thus, the use of Hill coefficients for estimating transport stoichiometry in the squid axon would result in an apparently electrogenic stoichiometry of 1:1:3 (K^+:Na^+:Cl^-). But, we have shown that the cotransporter in the squid axon is not electrogenic [36]. And, the directly measured stoichiometry of ion transport fluxes in the squid giant axon is: $1K^+$:$2Na^+$:$3Cl^-$ [16], a non-electrogenic stoichiometry. The fact that the values for the Na^+ stoichiometry and the Hill coefficient for activation of NKCC-mediated influx do not agree whereas those for Cl^- do agree should not be surprising since Hill coefficients are not a direct measure of transport stoichiometry, but rather are a measure of the degree of cooperativity of ion binding. This is emphasized by the fact that although 2 Na^+ are transported per NKCC transport cycle (in squid), the Hill coefficient for activation of cotransport is only slightly greater than one. The squid axon is thus far the only system in which stoichiometry has been determined by both unidirectional fluxes (under conditions where exchange fluxes were not possible) and ion activation (Hill) analysis. We have found the two approaches give different answers. This suggests caution should be exercised in other systems that do not permit a direct measure of stoichiometry, but which must rely only on a Hill-type analysis.

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