The Na-K-Cl cotransporter (NKCC) mediates the coupled movement of ions into most animal cells, playing important roles in maintenance of cell volume and in epithelial Cl transport. Two forms of NKCC have been described: NKCC1, the “housekeeping” isoform that is also responsible for Cl accumulation in secretory epithelial cells, and NKCC2, which mediates apical Na+K+Cl entry into renal epithelial cells. Here we examine the kinetic properties of NKCC1, NKCC2, and the endogenous HEK-293 cell cotransporter. Stable expression of rabbit NKCC2A was obtained in HEK-293 cells utilizing a chimera (h1r2A0.7) in which the 5’-untranslated region and cDNA encoding 104 amino acids of the N terminus are replaced by the corresponding sequence of NKCC1. h1r2A0.7 exhibits Na and Cl affinities near those of NKCC1, but it has a 4-fold lower Rb affinity, and a 3-fold higher affinity for the inhibitor bumetanide. The activity of h1r2A0.7 is increased on incubation in low [Cl] media as is NKCC1, but the resting level of activity is higher in h1r2A0.7 and activation is more rapid. h1r2A0.7 exhibits an appropriate volume response, unlike NKCC1 for which concomitant changes in [Cl] appear to be the overriding factor. These results support a model in which apical NKCC2 activity is matched to basolateral Cl exit through changes in [Cl]. Reverse transcriptase-polymerase chain reaction of HEK-293 cell mRNA is positive with NKCC1 primers and negative with NKCC2 primers. Surprisingly, we found that the behavior of the endogenous HEK cell Na-K-Cl cotransporter is unlike either of the two forms which have been described: compared with NKCC1, HEK cell cotransporter has a 2.5-fold lower Na affinity, an 8-fold lower Rb affinity, and a 4-fold higher bumetanide affinity. These results suggest the presence of a novel isoform of NKCC in HEK-293 cells.

The Na-K-Cl cotransporter (NKCC or BSC) mediates the coupled movement of Na, K, and Cl ions across the plasma membrane of animal cells. The transporter plays an important role in electrolyte movement across polarized epithelia and is also thought to be involved in regulation of intracellular volume and intracellular [Cl] (1, 2). NKCC is a member of the Na-coupled group of cation-chloride cotransporters (CCCs) (1, 3), a family which also includes K-Cl cotransporters (KCC) (3, 4). Three Na-coupled cation-chloride cotransporters have been described to date. 1) The “secretory” (or “housekeeping” or “basolateral”) Na-K-Cl cotransporter, NKCC1 (or BSC), is widely distributed in mammalian tissues (5, 6) and is especially prominent in the basolateral membranes of secretory epithelial cells; within the kidney, NKCC1 is found in epithelial cells in the collecting duct and in the glomerulus (7, 8). 2) The “renal” or “apical” Na-K-Cl cotransporter, NKCC2 (or BSC1) (9, 10), is found only in the apical membrane of epithelial cells in the thick ascending limb of the loop of Henle (TAL) (11–15). Three splice variants of NKCC2 (A, B, and F), differing in the sequence of the second predicted transmembrane domain, are differentially distributed along the nephron (9, 11). 3) The Na-Cl cotransporter, NCC (or TSC) (16), is restricted to the apical membrane of the distal tubule in the mammalian kidney.

The activity of the Na-K-Cl cotransporter is increased in most cells in response to cell shrinkage, leading to a regulatory increase in cell volume (1). Additionally, in secretory epithelia, cotransporter activity is strongly regulated as part of the process controlling fluid secretion: it appears that a decrease in intracellular [Cl] is the message which triggers an increase in transport mediated by NKCC2 utilizing a chimera (h1r2A0.7) in which 104 amino acids of the N terminus are replaced by...
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The Chimera h_r2A0.7—

EXPERIMENTAL PROCEDURES

The Chimera h_r2A0.7—

The final construct was analyzed by automated sequencing and restriction analysis.

Corresponding residues of NKCC1. Apparently, translation or processing efficiency is higher with the NKCC1 5'-UTR and N terminus. Most of this region is very poorly conserved from one species to another and from one isoform to another, both for NKCC and KCC (3, 24). Since our previous experiments demonstrate that neither the N nor the C terminus contributes to the differences in ion affinities between sNKCC1 and hNKCC1 (21), we do not anticipate that the N-terminal change in h_r2A0.7 significantly alters the function of NKCC2.

The HEK-293 cell line used for expression of NKCC in this and previous studies is derived from human embryonic kidney, immortalized by adenovirus transformation (25). HEK cells have a rather low level of endogenous ion fluxes, including Na-K-Cl cotransport. The de-differentiated line does not exhibit epithelial characteristics, and it is therefore not possible to predict which isoform of Na-K-Cl cotransporter might be present.

In this study, we compare the kinetic and regulatory behavior of NKCC1, h_r2A0.7, and the endogenous HEK cell cotransporter. We find that NKCC1 and NKCC2 (as h_r2A0.7) are different from one another in ion and bumetanide affinities as well as in the relative sensitivities to cell volume and [Cl]. Surprisingly, we find also that the endogenous Na-K-Cl cotransporter in HEK cells exhibits unique functional features, its behavior being different from that of both NKCC1 and NKCC2. Part of this work has been previously reported in abstract form (26).

Cell Lines—Control HEK cells, mock-transfected cells, and lines stably expressing sNKCC1 and hNKCC1 were the same as in Ref. 21. h_r2A0.7 DNA was transfected into HEK cells by calcium phosphate precipitation, and stable lines were isolated by G-418 resistance, as described previously (6, 21). T_n4 cells, obtained from J. Madara, were as in Ref. 6. NIH-3T3 cells and the E12a mutant (28) were from T. G. O’Brien. All lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and G-418 (for transfected cells), as described previously (21). We have studied two lines of h_r2A0.7 from separate transfections; the results for the two lines were indistinguishable from one another, and we have combined the data as reported here. Similarly, several experiments with hNKCC1 were performed with a different line reported in Ref. 6, with indistinguishable results. All experiments with sNKCC1 were carried out with the cell line described in Ref. 21. This line appears to be functionally the same as a line described in Ref. 22 except that the cotransport flux is somewhat higher.

Binding and Flux Studies—86Rb influx in HEK cells was determined as described previously (6, 21). Briefly, cells were grown to confluence (6–8 days at 37 °C) in polylysine-coated 96-well plates. In most experiments, cells were preincubated in hypotonic low Cl medium (regular flux medium diluted 1:2 with water and with gluconate replacing all but 2.5 mM Cl) to activate the cotransporter (6, 22). Fluxes were carried out for 1 min at room temperature (22 °C) in “regular flux medium” that contained 135 mM NaCl, 5 mM BaCl_2 (2 μM/ml 86Rb), 1 mM CaCl_2, 1 mM MgCl_2, 1 mM Na_2HPO_4, 1 mM Na_2SO_4, 15 mM Na-HEPES, pH 7.4 and, when used for the flux assay but not for preincubations, 0.1 mM ouabain. Each concentration curve was carried out in a single row of the 96-well plate, and in each experiment there were 2–6 replicate rows. Counts of the 1-min 86Rb influx were normalized to the value at the highest ion concentration, or to the longest time point in an activation time course, or to the value of uninhibited flux in inhibition studies. In previous experiments, we determined that at confluence, the coefficient of standard variation of protein content in several wells is quite small, approximately 10% of the mean. This coefficient is similar to that calculated for absolute counts in several wells under the same conditions, and therefore, it has not been useful to routinely determine protein on a well-by-well basis. Rows with obvious rogue values were omitted from averages, which in all cases was less than one row in 10. Data are expressed as means ± S.E. among all rows in several experiments (on average 20–30 rows in 5–8 experiments). Similarly, K_m and K_i values were obtained on a per row basis by non-linear least squares curve fitting using the Simplex algorithm (program PLOT, B. Forbush). Where error bars are not visible, they are smaller than the symbols. We have not attempted to correct fluxes in transfected cells for a potential background contribution from HEK cell cotransporter because we have evidence that expression of exogenous cotransporter suppresses endogenous cotransporter.

RNA Extraction and Reverse-transcriptase-PCR—Poly(A)-selected RNA was isolated from T_n4 cells, 3T3 fibroblasts, E12a cells, and HEK cells. Confluent cells from 10-cm dishes were homogenized and digested for 1 h in 200 μg/ml proteinase K, 0.5% SDS, 100 mM NaCl, 20 mM Tris-Cl and 1 mM EDTA, at pH 8.0 and at 37 °C. After adjusting NaCl concentration to 400 mM, the cell lysates were incubated with oligo(dT)-cellulose for 4 h at room temperature. Poly(A) RNA was eluted in 1 mM EDTA, 0.05% SDS, pH 8.0, and concentrated by ethanol precipitation. Poly(A) RNA was primed with a gene-specific antisense oligonucleotide derived from a conserved region in the third transmembrane domain of either NKCC1 (gaatcagctgttaattgctag; base pair 1422) or NKCC2 (ctcagcagttggcaacacag; bp 1487). The primers were extended with

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The enzyme AMV in an appropriate reaction buffer with 0.4 μM dNTPs. For PCR, a sense oligonucleotide derived from another conserved sequence in the N terminus of NKCC1 (ggccaacctgggaccaagcag; bp 784) or NKCC2 (cgggtggttagcagatgct; bp 1041) was added to the first cDNA strand, along with the DNA polymerases Taq and Fwo.

The NKCC1 primers correspond to regions that are identical between human and mouse NKCC1; NKCC2 primers correspond to a region identical in mNKCC2 and rNKCC2 and with 1 and 2 mismatches in hNKCC2. The NKCC1 target sequences are less than 20% identical to corresponding NKCC2 sequences, and NKCC2 target sequences are, respectively, less than 20 and 50% identical to corresponding NKCC1 regions.

The region of interest (630 bp in NKCC1 and 440 bp in NKCC2) was amplified through 40 cycles of PCR with denaturation steps of 40 s at 95 °C, annealing 50 s at 50 °C, and extension 1 min at 70 °C. To test for amplification yields, 0.1 ng of plasmid DNA (either hNKCC1/Bluescript SK or rNKCC2A/Bluescript SK) was processed in the same manner as the poly(A) RNA, including an incubation period with reverse transcriptase. Samples of PCR reactions were analyzed on a 1.2% agarose gel stained with ethidium bromide (10 μg/ml).

RESULTS

Functional Expression of NKCC2—In previous efforts we have been unable to measure ion fluxes of rNKCC2 transfected in mammalian cell lines (COS cells, HEK, 3T3, Madin-Darby canine kidney (MDCK)), apparently because of low levels of expression or poor cell surface delivery (9). Also, truncation and modification of the 5' UTR of rNKCC2 did not increase functional expression. We have successfully approached the problem using a chimera, h1r2A0.7, in which the 5'-UTR and cDNA encoding the first 104 amino acids of rNKCC2A were replaced with the corresponding region from hNKCC1. The N terminus of the cation-chloride cotransporters is very poorly conserved across isoforms and species (3, 24), and we have shown that it does not play a role in sNKCC1/hNKCC1 ion affinity differences (21). When stimulated by preincubation in low [Cl] medium (see below), h1r2A0.7-transfected cell lines were found to transport 86Rb about 2.5-fold faster than control HEK cells (data not shown). This is a lower level of transport than obtained on transfection of sNKCC1 (4–6-fold above control) or hNKCC1 (7–9-fold above control), but as will be seen below, the properties of h1r2A0.7 and HEK-cell cotransporter are readily distinguished.

Kinetic Behavior of Cotransporters—To compare the transport behavior of NKCC isoforms, we measured the dependence of 86Rb influx on Na, Rb, Cl, and bumetanide concentration for sNKCC1, hNKCC1, h1r2A0.7, as well as for HEK cells. The results are illustrated in Figs. 2 and 4, and the K_m values for Na, Rb, and Cl and the K_i for bumetanide are summarized in Fig. 3. As has been consistently noted for the Na-K-Cl cotransporter, Na, Rb, and bumetanide dependences of 86Rb influx fit a model of ligand binding at a single site (Fig. 2), whereas the relation between [Cl] and 86Rb influx is sigmoidal, consistent with two binding and translocation sites for Cl (Fig. 4). The data generally agree well with values that have been previously reported by this laboratory in separate studies (6, 21, 22).

The kinetic data demonstrate that NKCC2A (as h1r2A0.7) presents distinct ligand binding characteristics. The K_m of h1r2A0.7 for Na is 40% lower than that of hNKCC1, and the K_m for Rb is 4-fold higher compared with hNKCC1. In comparison to previous measurements of K_m values for ion transport in mouse kidney cortical TAL (29) and in a mouse medullary TAL cell line (30), K_m(Cl) is in the same range (34 compared with ~50 and 67 mM, respectively), K_m(Rb) is significantly greater (8 compared with 1–2 and 1.3 mM), and K_m(Na) is similar to that reported for the TAL line (10 versus 7 mM) but greater than recorded in the cortical TAL (2–3 mM). While some of these differences may be attributable to methodology, it is also possible that they reflect differences in ion affinities of the three splice variants of NKCC2 that are differentially distributed along the TAL (9, 11).

In examining the kinetic behavior of endogenous HEK cell cotransport, we were surprised to find that it was significantly different both from NKCC1 (noted previously in Ref. 6) and from h1r2A0.7. In particular, Na and Rb affinities are much

Fig. 2. 86Rb influx as a function of Na, Cl, or Rb concentration for hNKCC1, h1r2A0.7 and untransfected HEK-293 cells. After preincubation in low Cl hypotonic medium, 86Rb influx was determined in regular flux medium in which Na or Rb was replaced with N-methylglucamine, or Cl was replaced with gluconate to achieve the indicated ionic concentrations. The data are shown as averages of 24–52 flux rows in five to nine experiments for Na, 24–54 flux rows in four to seven experiments for Rb, and 24–35 flux rows in four to eight experiments for Cl. For Na and Rb, the points are fit by the Michaelis-Menten equation, and for Cl by the Hill equation with n = 2. Where error bars are not visible, they are smaller than the symbols.

Fig. 3. K_m for Na, Rb, and Cl. Values were obtained from fits of the data in Fig. 2 and from similar data for sNKCC1 (18–29 rows from three to five experiments) and mock-transfected HEK cells (29–41 rows from five to eight experiments). The data for individual flux rows were analyzed by non-linear least squares fits, means ± S.E. of the K_m and K_i values are shown.

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lower in the untransfected HEK cell compared with cells transfected with NKCC1 or h1r2A0.7; the bumetanide affinity is similar to that of h1r2A0.7 and 3-fold higher than that of hNKCC1. As illustrated in Fig. 3, there was no difference between untransfected and mock-transfected HEK-cells, demonstrating that the kinetic properties of the transporter are not affected by the transfection process, by the maintenance of foreign DNA in the cell line, or by expression of the G-418-resistance gene product. These data suggest that the endogenous Na-K-Cl cotransporter in HEK cells is a distinct form.

Inhibition of Cotransport Activity by Hg—Inorganic mercury has been shown to inhibit electrolyte secretion in the spiny dogfish rectal gland in a manner consistent with binding of Hg to sNKCC1 (31). Fig. 5, A and B, shows the effect of Hg on cotransport activity in transfected and untransfected HEK-cells. The shark rectal gland cotransporter, sNKCC1, is seen to lose Cl 6 times faster than hNKCC1-transfected cells. The shark rectal gland cotransporter, sNKCC1, is seen to lose Cl 6 times faster than hNKCC1-transfected cells. There is little reason to accept this alternative explanation since it is very unlikely that untransfected HEK cells would lose Cl 6 times faster than hNKCC1-transfected cells.

In Fig. 6A, it may be noted that the level of cotransporter activity in resting cells (that is with no pre-stimulation) varies from one form to another; this resting level is plotted in the bar graph in Fig. 6B. Importantly, the renal cotransporter NKCC2 (as h12A0.7) has substantial activity under resting conditions, approximately 22% of maximal. On the other hand, hNKCC1 activity is almost undetectable in the resting condition, approximately 2% of the maximal level.

The Na-K-Cl cotransporter is known to be activated by cell shrinkage in a wide variety of cell types (1). The effect of pre-incubation in media of different osmolalities is illustrated in Fig. 6C. Surprisingly, neither untransfected nor NKCC1-transfected HEK cells demonstrate the expected response to cell volume. Rather, there is significant decrease in transport activity at elevated tonicity, both in normal ionic conditions and following activation by incubation in low [Cl]. We suggest that this effect is due to the concentrative effect of cell shrinkage that increases cell [Cl] and thereby inactivates the cotransporter, overriding a weak or non-existent volume response of the normal type. This explanation has been previously offered for paradoxical shrinkage-activation of the shark rectal gland cotransporter (32).

The NKCC2 chimera does exhibit a modest volume response,
increasing in activity by about 50% with a 2-fold increase in
tonicity (Fig. 6, right panel). This demonstrates that HEK
cells do have volume-response machinery. It is possible that
the appropriate response is seen with h1r2A0.7 but not with
NKCC1 because the counteracting effect of increased [Cl]i is a
stronger modulator of NKCC1.

mRNA Encoding NKCC—To determine if the HEK cell co-
transporter is closely related to either of the two known NKCC
isoforms, we used RT-PCR to amplify a 630-bp sequence from
NKCC1 and a 440-bp sequence from NKCC2. As illustrated in
Fig. 7, the NKCC1 oligonucleotides (top panel) yielded a PCR
product of the expected size for all the cell lines tested, whereas
the result with NKCC2 oligonucleotides (bottom panel) was
negative for each line. The high amplification yields in HEK
cells demonstrate either that hNKCC1 is present in HEK cells
or that the endogenous cotransporter is very similar to NKCC1
in the regions of the PCR primers. On the other hand, it is
apparent that the HEK cell does not express an appreciable
level of hNKCC2 mRNA.

In this experiment we also analyzed a line of mouse 3T3
fibroblasts (E12a) that has been shown to be deficient in Na-
K-Cl cotransport activity (28). As seen in Fig. 7, NKCC1 is
expressed in the E12a line as well as in the control 3T3 cells
(Fig. 7). This result indicates that the mutation in E12a results
in a defect that arises beyond the point of transcription, i.e.
decreased translation of NKCC mRNA or a defective cotrans-
port protein.

DISCUSSION

The results presented here provide a comparison of kinetic
characteristics of hNKCC1, a NKCC2A chimera, and the na-
tive HEK cell cotransporter. These kinetic parameters are
summarized in Table I. As considered above, the characteristics
of ion and bumetanide binding as well as the characteristics
of regulation are different for each of the cotransporter
forms.
NKCC1, NKCC2A was also found to have severalfold greater sensitivity to the loop diuretic drug bumetanide, a difference that may be advantageous from the standpoint of drug efficacy. We have used a chimeric construct in order to obtain sufficient expression to measure NKCC2-mediated flux. In addition to changes in intracellular Cl (see Fig. 6). Together, the results of native NKCC2A.

A significant finding of this report is the activation of h1r2A0.7 in response to a decrease in [Cl]. This suggests that NKCC2 is modulated by [Cl], in the renal epithelial cell, providing a way for the apical cotransporter to respond to changes in the rate of Cl exit across the basolateral membrane. In many species vasopressin regulates NaCl and water reabsorption in the medullary thick ascending limb via a CAM-dependent mechanism. By demonstrating a route for basolateral → apical communication, the present result supports a model in which the initial point of CAM regulation is at the basolateral exit pathways (33), although it does not rule out a model in which the cotransporter is the primary site of regulation (34).

Compared with NKCC1, h1r2A0.7 displays a higher level of constitutive activity and exhibits a faster and smaller response to changes in intracellular Cl (see Fig. 6). Together, the results suggest that the [Cl] set point is higher for NKCC2. In addition, the h1r2A0.7 response to [Cl] appears to be more easily overridden by a response to a change in cell volume.

Our previous results with sNKCC1/hNKCC1 chimeras demonstrate that the N- and C termini of NKCC are not important in determining ion and bumetanide affinity differences, and it is reasonable to expect that the chimera h1r2A0.7 is fully representative of NKCC2A with regard to these properties. On the other hand, the N terminus of sNKCC1 has been shown to be involved in regulation of transport by a mechanism that involves phosphorylation of T194 and T195 (17, 35). The 15-residue region surrounding these phosphoacceptors is well conserved, with a single amino acid change in the h1r2A0.7 chimera (Q92NKCC2 → R92hNKCC1). We do not know if the region upstream of the phosphoacceptors plays a role in regulation and, if so, whether the introduction of NKCC1 residues in this region makes the regulatory behavior of h1r2A0.7 different from that of native NKCC2A.

The HEK cell cotransporter has functional characteristics that are quite different from both NKCC1 and h1r2A0.7. In particular, the affinity of the endogenous transporter for Rb is 8- and 2.5-fold lower than that of NKCC1 and h1r2A0.7, respectively, and the affinity for Na is >1.5-fold lower than that of both of the described isoforms. The results of RT-PCR experiments demonstrate that the HEK cell does not express a detectable message for NKCC2 and that NKCC1, or a form homologous to NKCC1, is present (Fig. 7).

We consider three possible explanations for the uniqueness of the HEK cell cotransporter. (a) Assuming that the endogenous HEK cell cotransporter is in fact hNKCC1, why is the kinetic and regulatory behavior different from hNKCC1? One possibility is that the HEK cell cotransporter contains an accessory subunit that is not available in the amounts necessary to accompany overexpressed hNKCC1 in transfected cells. A related idea is that the hNKCC1 protein may be post-translationally modified and that the modification machinery is inadequate for the overexpressed protein. (b) It is possible that the HEK cell cotransporter is a splice variant of hNKCC1, with functional characteristics that are different from the transporter encoded by the cDNA isolated from T-84 cells (6). We have reported three splice variants of NKCC2 that differ in the sequence of the predicted second transmembrane domain. Although the hypothesis has not yet been tested, alternative splicing of this region might be expected to result in transporters with different ion affinities (9). In a search for splice variants of NKCC1, Delpire and co-workers (36) found no evidence of alternative splicing in this region but did report that 16 residues in the C terminus are sometimes removed by splicing. In light of our recent finding that sNKCC1/hNKCC1 ion affinity differences are due only to differences within the central hydrophobic domain (21), it seems unlikely that this alteration would result in the discrepancy between HEK cell affinities and those of hNKCC1. (c) It is possible that the HEK cell cotransporter is an isoform of NKCC that has not yet been identified. At present there is no direct evidence for or against this hypothesis. However, in a general sense, the possibility of additional isoforms is supported by the broad distribution of the Na-K-Cl cotransporter in cells with very different physiological function. In epithelial tissues, the function of the Na-K-Cl cotransporter is transepithelial transport, and regulation of the transporter is presumably optimized for hormonal control and/or apical-basolateral communication. In non-polarized tissues, the Na-K-Cl cotransporter plays a role in regulation of intracellular volume (1) and may also be important in its effect on extracellular [K] (37). Thus, we propose that the HEK cell cotransporter may represent a unique NKCC isoform that operates with low cation affinities.

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