In chloride-secretory epithelia, the basolateral Na-K-2Cl cotransporter (NKCC1) is thought to play a major role in transepithelial Cl\(^-\) fluid transport. Similarly, in marginal cells of the inner ear, NKCC1 has been proposed as a component of the entry pathway for K\(^+\) that is secreted into the endolymph, thus playing a critical role in hearing. To test these hypotheses, we generated Nkcc1 knockout mice and analyzed them. Homozygous mutants (Nkcc1\(^{-/-}\)) mice exhibited growth retardation, a 28% incidence of death around the time of weaning, and mild difficulties in maintaining their balance. Mean arterial blood pressure was significantly reduced in both heterozygous and homozygous mutants, indicating an important function for NKCC1 in the maintenance of arterial blood pressure. cAMP-induced short circuit currents, consistent with a critical role for NKCC1 in transepithelial Cl\(^-\) movements involved in generation of the K\(^+\)-rich endolymph and the endocochlear potential.

NKCC1, the basolateral or secretory isoform of the Na-K-2Cl cotransporter (1, 2), is a member of the cation-coupled chloride transporter family, such as the apical Na-K-2Cl cotransporter of the renal thick ascending limb (3, 5), the Na-K-2Cl cotransporter of the renal distal convoluted tubule (6), and four KCl cotransporters (7–9). NKCC1 mediates the coupled electroneutral transport of 1 Na\(^+\), 1 K\(^+\), and 2 Cl\(^-\) ions across the plasma membrane (4), driven by the inwardly directed Na\(^+\) and Cl\(^-\) gradients that occur under physiological conditions. It has a broad tissue distribution (1, 2) and is expressed in both nonepithelial and epithelial cell-types. Regulation of cell volume is a major function of NKCC1 (3), and in vascular endothelial cells, it may play an important role in controlling cytosolic concentrations of Cl\(^-\) and K\(^+\) during agonist stimulation (10). In epithelial cells, it is restricted to basolateral membranes (11, 12), and there is strong pharmacological evidence that it provides an entry pathway for ions that are secreted across the apical membrane.

Studies of tissue samples of wild-type and CFTR-deficient mice have shown that cAMP-induced Cl\(^-\) secretion in intestinal and nasal epithelial cells is sharply inhibited by treatment with bumetanide, an inhibitor of NKCC1 (13). This observation suggests that basolateral Cl\(^-\) uptake via NKCC1 is important in maintaining CFTR-mediated Cl\(^-\) secretion across the apical membrane. There is recent evidence that NKCC1 might also provide a basolateral Cl\(^-\) entry mechanism that is required for the maintenance of high levels of HCl secretion by gastric parietal cells (14), which is generally thought to be dependent on Cl\(^-\)/HCO\(_3\)^- exchange. In the inner ear, NKCC1 is expressed at high levels on the basolateral membrane of marginal cells of the stria vascularis (15, 16). K\(^+\) secretion by marginal cells is responsible for the high concentrations of K\(^+\) in the endolymph and for the endocochlear potential, which is eliminated by peripheral or systemic application of bumetanide or furosemide (17–19). These observations and the ototoxicity of loop diuretics (20) suggest that NKCC1 contributes to the high rates of K\(^+\) uptake needed to maintain K\(^+\) currents across the apical membrane of the marginal cell and that NKCC1 plays a critical role in hearing.

Many of the conclusions regarding the functions of NKCC1 in secretory epithelial tissues are based on studies in which loop diuretics have been used as inhibitors. However, these compounds inhibit other members of the cation-coupled cotransporter family, such as the apical Na-K-2Cl cotransporter of the thick ascending limb and isoform 1 of the KCl cotransporter (7), and the existence of additional members of this family is a distinct possibility (21). To obtain a better understanding of the physiological functions of NKCC1, we have prepared a mouse line carrying a null mutation in the nkec1 gene (locus Slc12a2) and have performed an initial analysis of the phenotype.
Generation of Mice that lack NKCC1—Body weight was measured at 3, 5, 10, and 30 days of age. In addition, the growth curves were determined by measuring villus tip enterocytes for the height of the enterocytes. The height of the enterocytes was attenuated in the Nkcc1−/− mice, which was similar to that shown in Fig. 1A. In addition, the growth curve of the Nkcc1−/− mice was attenuated 5–8-week-old Nkcc1−/−, Nkcc1+/−, and Nkcc1+/+ mice were recorded as described previously (28) using three subcutaneous electrodes, one inserted under each ear and one at the top of the skull. The mice were positioned between high frequency transducers in a sound-attenuated chamber, and sound stimuli were directed into the ear by tubing. The ABR was brain-averaged (time-locked with onset of 128–1024 stimuli, at 20/s) out of the continuous electroencephalographic and electrocardiographic activity. The ABR peak amplitudes were averaged in the 0.5–8.0 μV range; the electroencephalographic and electrocardiographic activity ranged to >30 μV but averaged <0.5 μV due to the randomness with regard to onset of the acoustic stimulus. All electroencephalographic and electrocardiographic activity that exceeded 30 μV was rejected from the averaging technique. The sound stimuli were used as a 0.1-ms broad band click and 3-ms pure tones of 8, 16, and 32 kHz at 20–100 dB. The threshold of hearing was defined as the minimum sound intensity, in dB, required to elicit a characteristic wave form.

Generation of NKCC1−/− Mice and Gross Phenotype—To disrupt the gene encoding NKCC1, we used a construct that contained the neo+ gene cassette inserted into exon 6 (Fig. 1A). Targeted ES cells were identified by Southern blot analysis similar to that shown in Fig. 1B and used for blastocyst-mediated transgenesis. Chimeric mice were generated as described previously (24).

Staining Mouse Intestinal Section Assay—Escherichia coli heat-stable enterotoxin, STa, was reconstituted to 10 μg/ml in sterile saline, with 1% Evan’s Blue dye added as a tracer. 1 μg of STa was administered to 4-day-old suckling mice by intragastric injection (25). Mice were allowed to suckle until toxin administration, but not afterward. After 3 h incubation at room temperature, mice were euthanized, and the entire intestinal tract from proximal duodenum to distal colon was excised. The wet weights of the fluid-filled intestine and remaining carcass were measured, and the gut weight to carcass weight ratio was determined. Animals into which STa was injected successfully were identified by their blue-stained stomachs.
expression of the 6.5-kb Nkcc1 mRNA was reduced in Nkcc1+/− tissues and eliminated in Nkcc1−/− tissues.

Genotypy analysis of 560 offspring from heterozygous matings showed that wild-type, heterozygous, and homozygous mutant offspring were born in approximately a 1:2:1 Mendelian ratio (30% +/+; 46% +/-; 24% −/−). Relative to both wild-type and heterozygous mice, Nkcc1−/− mice experienced growth retardation (Fig. 2) that was readily apparent within 1 week of birth and particularly severe during the third week of life. At weaning on day 21, Nkcc1−/− mice (5.5 ± 0.3 g) were significantly smaller than both wild-type (12.8 ± 0.3 g) and heterozygous (12.8 ± 0.2 g) mice. Homozygous mutants gained weight well after weaning; however, they remained smaller as adults (~80% of wild-type mice) and, in general, had less body fat. Extrapolation of the growth patterns to their day of birth suggested that there was little, if any, growth retardation of Nkcc1−/− mice in utero. To further test for the possibility of in utero growth retardation, 12 offspring from a heterozygous mating were removed by Caesarian section on postcoital day 17 and weighed. No significant difference in body weight was observed in this small sample of mice (Nkcc1+/− and Nkcc1−/− mice, 0.64 ± 0.02 g, n = 8; Nkcc1−/− mice, 0.65 ± 0.01 g, n = 4).

Nkcc1−/− mice frequently exhibited unusual head posterior, in which the head was tilted to one side or tilted upward and backward with the nose held high. The mutants also exhibited circling behavior accompanied by a tendency to engage in rapid spinning, which persisted throughout the life of the animal. In some instances, Nkcc1−/− mice engaged in repetitive circling in a vertical direction, in which they climbed the underside of the downward sloping end of the cage top, where food was placed, moved upside down along the underside of the cage top, dropped to the floor and ran back to the sloping end of the cage top to continue the process. While spinning, mutant mice sometimes lost their balance, but quickly righted themselves. Loss of balance occurred occasionally when the mutants were walking in a straight line.

A histological survey of sections from brain, heart, lung, liver, spleen, salivary gland, skeletal muscle, eye, and pancreas of 15–17-day-old Nkcc1+/+, Nkcc1+/−, and Nkcc1−/− mice revealed no significant lesions, with the exception of a single homozygous mutant in which cells of the choroid plexus were vacuolated and swollen. However, the choroid plexus of another young mutant appeared normal, and no abnormalities were observed in the choroid plexus of two adult mutant mice that were examined. Although gross histological abnormalities were not observed in the intestinal tract of young mutant mice that appeared relatively healthy, abnormal intestinal histopathology was observed among homozygous mutants that sickened or died around the time of weaning (discussed below). Also, in enterocytes of one of the sick homozygous mutant mice, there appeared to be very little cytoplasm between the nucleus and the basal surface of the cell when compared with enterocytes of an age-matched wild-type mouse. To assess the significance of this observation, morphometry of the intestinal epithelium of
viable young (21 days of age or less) and adult (8 weeks old) Nkcc1+/+ and Nkcc1−/− mice was performed. For the adult mice, there were no significant differences between the two genotypes in any of the measurements (described under “Experimental Procedures”). For the young mice, there were significant differences in total epithelial cell height (Nkcc1−/−, 21.2 ± 1.4 μm, n = 8; Nkcc1+/+, 25.3 ± 1.2 μm, n = 11; p < 0.03) and in the height of the basal cytoplasm (Nkcc1−/−, 3.59 ± 0.39 μm, n = 8; Nkcc1+/+, 5.01 ± 0.33 μm, n = 11; p < 0.01).

Increased Incidence of Death among Null Mutants—Almost 30% of Nkcc1−/− mice died just before or just after weaning at 21 days of age (Fig. 3). The animals were active and seemed fully viable one day and then were found dead in their cages the next day, suggesting a rapid onset of morbidity and then death. We were able to identify and examine seven Nkcc1−/− mice between the ages of 18 and 26 days that had hunched backs, were relatively immobile, and seemed to be near death. All of the mice showed evidence of bleeding in the intestine, and one mouse had a fecal blockage of the large intestine. Fig. 4 shows a 23-day-old mutant that exhibited bleeding in the cecum and a severe blockage of the colon (Fig. 4, A and B) and a 26-day-old mouse that exhibited bleeding in the small intestine and cecum (Fig. 4C). The cecum of Nkcc1−/− mice often had a worm-like appearance (Fig. 4C) similar to that described for CFTR-deficient mice (29).

We also identified a 13-month-old null mutant mouse that had seemed healthy throughout its life but was found dead in its cage one morning. It had a worm-like extension of its cecum (Fig. 4D) and exhibited a severe blockage of both its cecum (Fig. 4D) and colon (Fig. 4E) by feces. A 21-day-old heterozygous mouse was found dead with blood in the lumen of its cecum, a blocked colon, and enteric intussusception, a condition in which part of the small intestine prolapses into the lumen of an adjacent region (Fig. 4F). Like homozygous mutants, its cecum had an extension with a coiled, worm-like appearance (see Fig. 4F). Fig. 5 shows the intestinal tract and sections through the cecum of an 18-day-old null mutant that was identified while still alive but in a morbid state; it died naturally just before it was examined. Bleeding throughout the small intestine was observed, and a large focal hemorrhage was visible in the intact cecum (Fig. 5A). Blood was within both the lumen of the cecum (Fig. 5B) and the interstitium (Fig. 5C and D), and in some regions, there were pools of blood (Fig. 5D). There was no evidence of a significant inflammatory response. These observations raise the possibility that a defect in the circulatory system might have contributed to the increased death rate of null mutants.

Mean Arterial Blood Pressure Is Reduced in NKCC1-deficient Mice—NKCC1 has been found in both endothelial cells (10) and vascular smooth muscle (30), consistent with the possibility that NKCC1 deficiency might cause a vascular defect. Blood pressures of anesthetized mice were measured using a femoral artery catheter (Fig. 6). Relative to that of wild-type mice (91.8 ± 3.9 mm Hg, n = 5), mean arterial pressure was significantly reduced in both Nkcc1−/− (77.7 ± 4.1 mm Hg, n = 6) and Nkcc1−/− (68.6 ± 2.8 mm Hg, n = 6) mice.

Analysis of Secretory Function of NKCC1-deficient Epithelial Tissues—As an initial test of secretory function in the intestinal tract, the suckling mouse secretion assay was performed to determine whether cGMP-mediated secretion was impaired in the intestine of Nkcc1−/− mice. Injection of E. coli heat-stable enterotoxin STA into the stomachs of 4–5-day-old mice normally induces fluid accumulation in the intestinal lumen, yielding an increased gut weight/carcass weight (G/C) ratio. As shown in Fig. 7, comparisons of the G/C ratios between maximally stimulated and unstimulated mice of all three genotypes indicated that Nkcc1−/− mice (G/C ratio = 0.117 ± 0.007) were able to secrete fluid as well as Nkcc1+/+ (G/C ratio = 0.122 ±

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![Figure 3](image1.png)

**Fig. 3.** Survival rate for Nkcc1+/+ and Nkcc1−/− mice. The percentage of survival among Nkcc1−/− and Nkcc1+/+ mice is plotted against age. After 28 days of age, Nkcc1−/− mice did not exhibit an increased incidence of death relative to Nkcc1+/+ mice.

![Figure 4](image2.png)

**Fig. 4.** Gross pathology of intestinal tract. A and B, intestinal tract of a 23-day-old Nkcc1−/− mouse with blood in the cecum (A) and a severe blockage of the colon (A and B). In these and subsequent panels, the single arrow indicates the cecum, double arrows indicate the colon, and arrowheads indicate the small intestine. C, small intestine, cecum, and colon of Nkcc1−/− mouse that died at 26 days of age. Note blood in the small intestine and in the wormlike cecum. D and E, intestinal tract from 13-month-old Nkcc1−/− mouse found dead in its cage. Note the wormlike extension (asterisk in D) of the cecum and severe obstructions of the cecum (D) and colon (E) by feces. F, intestinal tract of 21-day-old heterozygous mutant found dead in its cage; it had blood in the lumen of its cecum, obstruction of both cecum and colon by feces, intussusception of the small intestine (at the site marked by the arrowhead), and a wormlike extension (asterisk) of its cecum. It should be noted that obstructions were observed in only 5 of 31 (16%) of the dead or dying mice.
Short circuit currents were also measured in cultured tracheal epithelial cells (Fig. 9). Basal ISC was significantly lower in tracheal cell monolayers from Nkcc1+/− mice. Treatment with amiloride to inhibit the epithelial Na+ channel caused a significant reduction of ISC in Nkcc1+/+ cells but not in Nkcc1−/− cells. Stimulation with forskolin caused ISC to increase in both sets of samples, but CAMP-stimulated ISC was significantly lower in Nkcc1−/− cells than in Nkcc1+/+ cells. Treatment with bumetanide reduced ISC significantly in wild-type cells but did not reduce ISC in Nkcc1−/− cells. Short circuit currents in Nkcc1+/+ and Nkcc1−/− tracheal cells that had been treated with amiloride, forskolin, and bumetanide were both significantly elevated relative to those recorded after treatment with amiloride alone.

There is evidence that NKCC1 plays a major role in the secretion of HCl in amphibian gastric mucosa (14). To determine whether the lack of NKCC1 in the mouse impairs gastric acid secretion, wild-type and homozygous mutant mice were treated with histamine, and the pH of the stomach contents was measured. As shown in Fig. 10A, Nkcc1−/− mice were able to acidify their stomach contents as well as Nkcc1+/+ mice (pH = 2.85 ± 0.15 in Nkcc1−/−; 3.44 ± 0.30 in Nkcc1+/+). These data are consistent with the results of histological analyses of stomach sections and ultrastructural analyses of gastric parietal cells. When examined at high magnification by light microscopy (Fig. 10B, left panels), parietal cells of homozygous mutants were abundant and appeared normal. Electron microscopy revealed a well developed secretory canaliculus (Fig. 10B, right panels) and no apparent ultrastructural abnormalities that might be indicative of reduced secretion of acid or impaired viability of the parietal cell.

**Northern Blot Analysis of Ion Transporters Involved in Secretion and Absorption in the Gastrointestinal Tract**—It was of interest to determine whether the lack of NKCC1 might lead to alterations in mRNAs encoding other transporters involved in secretion or absorption in the gastrointestinal tract. As shown in Fig. 11, mRNA encoding NHE3 was reduced in both small intestine and colon of Nkcc1−/− mice (63 and 50% of wild-type, respectively, when measured by phosphorimager analysis). mRNAs encoding CFTR and AE2 appeared to be slightly reduced in colon of mutant mice, but not in small intestine or stomach.

**Auditory Brainstem Responses**—Nkcc1−/− mice did not give an ear twitch in response to a hand clap (Preyer’s reflex), indicating that their hearing might be impaired. To test the possibility and the extent of any hearing loss, ABR measurements were performed. As shown in Fig. 12A, Nkcc1−/− mice exhibited the characteristic ABR waveform at sound pressure
was measured using Ussing chambers under basal conditions (Basal experiments with jejunum (Bumet) and bumetanide (Bumet) and after sequential additions of amiloride (Amil), forskolin (Forsk), and bumetanide-resistant Isc (Bumet). *, p < 0.05 versus basal Isc of same genotype; †, p < 0.05 versus cAMP-stimulated Isc of Nkcc1±/± mice; ‡, p < 0.05 versus Nkcc1±/± of same treatment group.

Fig. 8. Short-circuit currents in jejunum and cecum of wild-type and mutant mice. Isc was measured using voltage-clamped Ussing chambers under basal conditions and during sequential addition of forskolin and bumetanide. A, typical Isc recordings from the cecum of Nkcc1±/± (left) and Nkcc1±/± (right) mice. Current deflections result from repetitive voltage spikes used to measure tissue resistance. B and C, summary of experiments with jejunum (B) and cecum (C) of Nkcc1±/± (n = 6) and Nkcc1±/± (n = 6) mice showing basal Isc (Basal), forskolin-stimulated Isc (Forsk) (forskolin is a cAMP-mediated secretagogue), and bumetanide-resistant Isc (Bumet). *, p < 0.05 versus basal Isc for same genotype; †, p < 0.05 versus same treatment group.

Histology of the Inner Ear—In wild-type ears (Fig. 13A), the lumen of the cochlear duct (scala media), containing the K⁺-rich endolymph generated by the stria vascularis, was separated by Reissner’s membrane from the scala vestibuli, which contains perilymph with the typically low K⁺ concentrations of most extracellular fluid. In contrast, in most sections of Nkcc1±/± ears, the lumen of the cochlear duct was collapsed, and Reissner’s membrane was lying against the interdental cells of the spiral limbus, the tectorial membrane, and the stria vascularis (Fig. 13B), rather than being in its usual position separating a patent lumen of the scala media from the scala vestibuli. Although complete collapse of the cochlear duct was a common feature, some exceptions were observed. One mutant had a relatively wide scala media in both ears. In another mutant, the scala media of the right ear was collapsed, but the scala media of the left ear was wide. The left ear of this mutant was found to have a ruptured Reissner’s membrane in deeper serial sections (Fig. 13C). Such a torn membrane could allow influx of perilymph from the scala vestibuli and might account for the wider lumen in other regions of the cochlear duct. In sections of mutant ears with a wide scala media lumen, Reissner’s membrane was collapsed partially, lying against the spiral limbus region, the tectorial membrane, and only the uppermost portion of the stria vascularis.

The organ of Corti from a wild-type mouse is shown in Fig. 13D. Well developed pillar cells define the tunnel of Corti, and outer and inner hair cells and their support cells are visible on either side of the tunnel. Efferent nerve fibers can be seen passing through the tunnel en route toward the outer hair cells, and Rosenthal’s canal was filled with Schwann cells and myelinated nerve fibers. The tectorial membrane overlies the organ of Corti and contacts the stereocilia of the hair cells. The structure of the organ of Corti in Nkcc1±/± mice was perturbed in several ways. Our analysis included the evaluation of sections from calciﬁed (e.g., inner ears that had not been decalciﬁed in EDTA) and decalciﬁed tissues. Sections of calciﬁed Nkcc1±/± ears (Fig. 13E) revealed an accumulation of calciﬁed crystalline material at the tectorial membrane. In some sections from decalciﬁed mutant ears (Fig. 13F), the tectorial membrane had densely stained thick ﬁbers at the periphery. Neither the calciﬁed tectorial membrane-associated material nor the densely stained thick ﬁbers were observed in sections of nondecalciﬁed or decalciﬁed Nkcc1±/± ears, respectively. Although pillar cells could be identiﬁed in most sections of Nkcc1±/± ears, the tunnel of Corti was absent often, and inner hair cells and particularly outer hair cells could be identiﬁed only infrequently. Neuronal cell bodies in the spiral ganglion were reduced in numbers in some sections of Nkcc1±/± ears (compare panels A and B of Fig. 13). Rosenthal’s canals were ﬁlled with myelinated nerve processes and Schwann cells in ears of both wild-type and mutant mice.

In wild-type ears (Fig. 13A–C and Fig. 14) is a specialized region of the epithelial lining of the cochlear duct that is essential for the production of endolymph, and it consists of marginal cells, intermediate cells, basal cells, and capillaries. In the stria vascularis of wild-type mice (Fig. 14A), marginal cells, which normally secrete K⁺ and form a continuous sheet in contact with the endolymph, are columnar and have elaborate extensions of the basal region of the cell that results in an extensive plasmalemma surface area. In the stria vascularis of homozygous mutants (Fig. 14B), the marginal cells lacked the numerous basal processes seen in Nkcc1±/± marginal cells and were separated from basal and intermediate cells by intercellular spaces that were considerably wider than those observed in wild-type mice (compare panels A–C of Fig. 13 and panels A and B of Fig. 14). Reissner’s membrane is composed of a squamous epithelium on the scala media side that is backed by an even thinner squamous mesothelium on the scala vestibuli side, and it normally separates the endolymph in the cochlear duct from the perilymph in the scala vestibuli. In regions of Nkcc1±/± ears that had virtually no scala media lumen, the collapsed Reissner’s membrane was often in direct contact with the marginal cell layer of the stria vascularis (Fig. 14B).

The vestibular organs include the semicircular ducts, the sacculle, and the utricle, each of which has an epithelial lining
with specialized regions of innervated sensory epithelium. In the ampulla of each of the three semicircular ducts, this innervated area is termed the crista ampullaris, and in both the saccule and utricle, it is termed a macula. In the semicircular ducts of wild-type mice, the membranous labyrinth was open, consistent with its having been filled with endolymph (Fig. 15A). Associated with the crista ampullaris was a tall gelatinous cupula that extended toward the epithelial lining opposite the sensory epithelium. In the ampullae of Nkcc1−/− semicircular ducts, the membranous labyrinth was collapsed upon the cupula (Fig. 15B), and the perilymphatic space was expanded. The saccule (Fig. 15C) and utricle (data not shown) of Nkcc1−/− mice appeared normal, with the otolithic membrane carrying calcium carbonate crystals, the otoconia, on its luminal surface and contacting the stereocilia of the sensory hair cells in the underlying maculae. The membranous labyrinth in both saccule and utricle of Nkcc1−/− mice was open. In contrast, the membranous labyrinth of the saccule (Fig. 15D) and the utricle (data not shown) of Nkcc1−/− mice was irregular and generally collapsed so that the epithelial lining opposing the macula was lying against the otolithic membrane, somewhat encapsulating the otoconia. In optimal planes of section of the Nkcc1−/− maculae and cristae (Fig. 15, B and D), hair cells with their tufts of stereocilia could be found.

**DISCUSSION**

Our major objective was to develop an NKCC1-deficient mouse that could be used to assess the role of this transporter in the secretion of ions and fluid by epithelial tissues. Northern blot analysis demonstrated that the wild-type mRNA was expressed at reduced levels in heterozygotes and was absent in homozygous mutants, confirming that disruption of exon 6 had produced a null mutation. Offspring of heterozygous matings were born in a normal Mendelian ratio and exhibited no evidence of growth retardation in utero or structural malformations in...
Corti is abnormal in mutant mice (panels A, C, E, and F). In a mutant cochlea, the spiral ligament (SL) is seen in wild-type mice (panel A). In a mutant cochlea with collapsed lumen, the outer hair cells (OHC) are absent (panel B). The typical architecture of the organ of Corti is seen in wild-type mice (panel D), with the tunnel of Corti (T) formed by pillar cells (P) and flanked by outer (OHC) and inner (IHC) hair cells. A nerve (N) can be seen traversing the tunnel of Corti. The organ of Corti is abnormal in mutant mice (panels E and F), although pillar cells and occasional hair cells can be seen. Calcification (Ca++) at the tectorial membrane was observed in sections of mutant ears that had not been decalcified during processing of tissues (panel E), and a dense fibrous material was observed on the tectorial membrane of mutant ears that had been decalcified (panel F). Myelinated nerve fibers and Schwann cells fill Rosenthal’s canals (R). The light staining of nerves in Rosenthal’s canal in E is due to incomplete osmication during sample preparation. Spiral ganglion (G) neuronal cell bodies were reduced in number in some mutants (panel B). The scala tympani (ST) is a perilymph channel below the flexible basilar membrane, on which the organ of Corti rests. Scale bar, 100 μm in A-C, 50 μm in D-F.

**FIG. 13.** Histology of the auditory system. Sections of the cochlea of Nkcc1+/+ (panels A and D) and Nkcc1+/− (panels B, C, E, and F) mice. Reissner’s membrane (RM) separates the scala media (SM) and the scala vestibuli (SV) in wild-type cochlea (panel A). In a mutant cochlea with collapsed lumen, Reissner’s membrane is pressed against the spiral limbus (L), tectorial membrane (TM), and stria vascularis (S) (panels B, E, and F), or may be ruptured (panel C) as seen in a mutant ear with a wide scala media lumen. Intercellular spaces in the stria vascularis of mutant mice (panels B and C) are greater than those of wild-type mice (panel A). The typical architecture of the organ of Corti is seen in wild-type mice (panel D), with the tunnel of Corti (T) formed by pillar cells (P) and flanked by outer (OHC) and inner (IHC) hair cells. A nerve (N) can be seen traversing the tunnel of Corti. The organ of Corti is abnormal in mutant mice (panels E and F), although pillar cells and occasional hair cells can be seen. Calcification (Ca++) at the tectorial membrane was observed in sections of mutant ears that had not been decalcified during processing of tissues (panel E), and a dense fibrous material was observed on the tectorial membrane of mutant ears that had been decalcified (panel F). Myelinated nerve fibers and Schwann cells fill Rosenthal’s canals (R). The light staining of nerves in Rosenthal’s canal in E is due to incomplete osmication during sample preparation. Spiral ganglion (G) neuronal cell bodies were reduced in number in some mutants (panel B). The scala tympani (ST) is a perilymph channel below the flexible basilar membrane, on which the organ of Corti rests. Scale bar, 100 μm in A-C, 50 μm in D-F.

**FIG. 14.** Histology of the stria vascularis. Sections of the stria vascularis of Nkcc1+/+ (panel A) and Nkcc1−/− (panel B) mice. In the wild-type, marginal cells (M) are in contact with the endolymph of the scala media (SM). In the mutant, Reissner’s membrane (RM), bordered on the left by the scala vestibuli (SV), is pressed against the marginal cell layer. Nkcc1−/− marginal cells have fewer extensions of their basal surfaces than those of wild-type mice. The intercellular spaces between the marginal cell layer and deeper layers of the stria vascularis are wider in mutant than in wild-type ears. Basal cells (B) are present in both genotypes. Scale bar, 25 μm.

**FIG. 15.** Histology of the vestibular system. In the ampulla of the semicircular ducts of Nkcc1+/+ mice (panel A), the gelatinous cupula (C) overlying the crista ampullaris (CA) sensory epithelium is bathed in endolymphatic fluid. In the Nkcc1−/− ampulla (panel B), the epithelial lining that normally encloses the endolymph is collapsed upon the cupula. Remnants of trabeculae, strands of connective tissue cells that normally attach the epithelial lining of the semicircular duct to the bone, can be seen on both the basal surface of the epithelial lining and on the bone (panel B, upper left). In panel C, the Nkcc1+/− sacule otoconia (O), embedded in the gelatinous otoconial membrane (OM) that overlies the neuroepithelium of the macula (M), are bathed in endolymph. In the Nkcc1−/− sacule (panel D), the epithelial lining of the sacule has collapsed onto the otoconia. Scale bars: A, 80 μm; B, 100 μm; C and D, 50 μm.

Nkcc1−/− mice, it is conceivable that a further deficit in blood pressure, for example, from hypovolemia occurring as a result of insufficient consumption of milk, might lead to circulatory shock.
Additional studies will be needed to test this hypothesis.

Earlier studies demonstrated that cAMP-stimulated, CFTR-mediated short circuit currents in jejunum, cecum, and trachea are bumetanide-sensitive (13) and that treatment of T84 cells with forskolin increases bumetanide-sensitive $^{86}$Rb-uptake activity (32). This suggests that Cl$^{-}$ uptake via NKCC1 contributes to cAMP-stimulated Cl$^{-}$ secretion. Our studies showing that cAMP-stimulated $I_{sc}$ in jejunum, cecum, and tracheal epithelium of adult Nkcc1$^{-/-}$ mice was limited to $50\%$ of the levels observed in Nkcc1$^{+/+}$ tissues provide direct evidence that NKCC1 serves as an entry pathway for Cl$^{-}$ secreted by the CFTR. Nevertheless, cAMP-stimulated short circuit currents in jejunum, cecum, and tracheal epithelium of NKCC1-deficient mice were significantly elevated relative to basal levels, indicating that other basolateral transport mechanisms contribute to ion uptake needed for maximally stimulated secretion. It is possible that much of the remaining cAMP-stimulated $I_{sc}$ in Nkcc1$^{-/-}$ tissues is due to HCO$_3$$^{-}$ secretion, as recent studies have shown that a portion of the agonist-stimulated $I_{sc}$ is due to CFTR-dependent HCO$_3$$^{-}$ secretion (34–36).

In contrast to the results obtained in Ussing chamber studies of isolated adult tissues, a deficit in STa-stimulated secretion was not observed in the intestines of 4–5-day-old NKCC1-deficient mice. In adult tissues, anion secretion in response to STa or to guanylin or uroguanylin, the endogenous ligands for the receptor that is activated by STa, has been shown to be dependent on CFTR activity (33–37) and to be inhibited by bumetanide (35). It is possible that the differences observed between the Ussing chamber and suckling mouse assays are related to the age of the animals, with NKCC1 contributing little to electrolyte uptake needed for stimulated secretion in the 4–5-day-old mouse but contributing significantly to this process in the adult. If this is the case, then other basolateral transport mechanisms must mediate uptake of anions and Na$^{+}$, an important role in volume regulation, it seemed possible that the viability of the parietal cell, it is critical that cellular volume homeostasis be maintained. Because NKCC1 is known to play an important role in volume regulation, it seemed possible that the viability of Nkcc1$^{-/-}$ parietal cells might be perturbed. However, there was no evidence of a loss of gastric parietal cells in null mutants, and ultrastructural analysis revealed an apparently normal secretory canaliculus and no evidence of impaired viability such as that observed in mice lacking the NHE2 Na$^{+}$/H$^{+}$ exchanger (23).

Previous studies have shown that high levels of NKCC1 (15, 16) and the Na$^{+}$,K$^{+}$-ATPase (46, 47) are present on the basolateral membrane of marginal cells of the stria vascularis, which are primarily responsible for generating the K$^{+}$-rich endolymph present in the scala media. The high K$^{+}$ concentrations of the endolymph and the endocochlear potential are necessary for functional activity of the sensory hair cells of the auditory and vestibular systems (48). Inhibition of NKCC1 with bumetanide and other loop diuretics (17–19, 49) or the Na$^{+}$,K$^{+}$-ATPase with ouabain (50) reduces the endocochlear potential, and treatment with loop diuretics causes ototoxicity (20, 51). These previous observations raised the possibility that the combined activities of NKCC1 and the Na$^{+}$,K$^{+}$-ATPase on the basolateral membrane may be involved in maintaining the high rate of K$^{+}$ uptake needed for secretion of K$^{+}$ into the endolymph.

The profound deafness revealed by ABR measurements demonstrated that the auditory system of NKCC1-deficient mice was severely perturbed, and the circling behavior and mild balance problems indicated that the vestibular system also was impaired. The collapse of the membranous labyrinth in the auditory and vestibular systems of Nkcc1$^{-/-}$ mice provided a clear histological correlate of the hearing and balance defects and demonstrated that NKCC1 plays an essential role in the generation of the endolymph. The degree of the collapse varied. In the most severely affected sections of the cochlea, Reissner’s membrane was pressed tightly against the stria vascularis and the organ of Corti; however, in no ear was there a total collapse of the entire membranous labyrinth. In some planes of section of a cochlea with a severe collapse, small areas of Reissner’s
membrane were separated from the epithelium against which it had collapsed. The lumen of the entire vestibular system also was never collapsed completely. For example, the saccule shown in Fig. 15D exhibited a severe collapse, but the utricle of the same ear was more open. Thus, some fluid must be present within the membranous labyrinth of NKCC1-deficient mice, but the nature of the fluid has not been determined. However, given the complete loss of hearing in \( \text{Nkcc1}^{-/-} \) mice and the critical dependence of sensory transduction by cochlear hair cells on the high \( K^+ \) concentrations of the endolymph and the endocochlear potential, it is likely that the composition and electrical potential of the relatively limited amount of fluid within the cochlear duct is altered. Interestingly, the balance defect was much less severe than the hearing defect, demonstrating that the sensory epithelium of the vestibular system functions to some degree despite the perturbations of the endolymph and the histological changes in the vestibular organs.

The apparent decrease in the volume of the cochlear endolymph was more severe than anticipated, as one might have predicted that the \( Na^+\,K^+\text{-ATPase} \), in the absence of NKCC1, would mediate sufficient \( K^+ \) uptake to support a substantial level of \( K^+ \) and fluid secretion. The expansion of the intracellular space between marginal cells and basal cells of the stria vascularis and the collapse of the membranous labyrinth of \( \text{Nkcc1}^{-/-} \) mice suggest that ions and fluid are being sequestered into the intrastrial space but are not being cleared by the marginal cells. Our results indicate that the activities of NKCC1 and the \( Na^+\,K^+\text{-ATPase} \) on the basolateral membrane of the marginal cell are tightly coupled and that alternate \( Na^+ \) leak pathways are unable to drive sufficient \( Na^+\,K^+\text{-ATPase} \) activity to support high levels of \( K^+ \) and fluid secretion into the scala media. With NKCC1 providing the predominant leak pathway for \( Na^+ \), one turnover of the \( Na^+\,K^+\text{-ATPase} \) would be coupled with three turnovers of NKCC1, for a net uptake of 5 \( K^+ \). Thus, the operation of the two transporters in concert would provide a highly efficient system for \( K^+ \) uptake.

An interesting observation in ears of \( \text{Nkcc1}^{-/-} \) mice that had not been decalcified with EDTA was the presence of calcium crystals on the tectorial membrane. Calcification of the tectorial membrane has been observed in deaf Dalmatian dogs in which atrophy of the stria vasculare and collapse of Reissner’s membrane also had occurred (52). Because of the high positive endocochlear potential, the \( Ca^{2+} \) concentration of the cochlear endolymph is very low (\( \sim 30 \mu M \)) relative to normal extracellular fluid (53). The calcification of the tectorial membrane may be due to an increase in the \( Ca^{2+} \) concentration of the endolymph, which has been shown to occur when the endocochlear potential is reduced (53).

The development of the NKCC1-deficient mouse has allowed a direct assessment of the role of this transporter in \( Cl^- \) and \( K^+ \) secretion in epithelial tissues and of the in vivo physiological consequences of the loss of its activity. Our results provide direct evidence in support of previous studies indicating that NKCC1 plays an important role in \( Cl^- \) secretion in the intestine and \( K^+ \) secretion in the inner ear and suggest that NKCC1 functions in the maintenance of arterial blood pressure. Major questions remain regarding the nature of the basolateral transport mechanisms that allow a substantial level of secretion in the intestine of \( \text{Nkcc1}^{-/-} \) mice, the effect of NKCC1 deficiency on the endocochlear potential and ionic composition of the endolymph, and the mechanism by which NKCC1 deficiency affects blood pressure. These and other questions concerning the physiological functions of NKCC1 can be addressed using the \( \text{Nkcc1}^{-/-} \) mice.

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