Subtractive hybridization unravels a role for the ion cotransporter NKCC1 in the murine intestinal pacemaker

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IN THE GASTROINTESTINAL (GI) tract, the spatiotemporal organization of contractile activity is essential for efficient propulsion of luminal content. Besides the enteric nervous system, the role of the interstitial cells of Cajal (ICC) in the coordination of contractile activity has been established in recent years (18, 41, 48, 66), and diabetic gastroparesis (17). Functional alterations of ICC have to be considered in other conditions where ICC distribution appears unaffected, and targeting the SW might be an innovative way to new therapeutic developments in GI motility disorders (46).

Whereas the electrophysiology of SW has been extensively investigated in tissues and dissociated ICC, the molecular mechanism underlying the pacemaking currents in ICC remains poorly understood. By using suppression subtractive hybridization (SSH), the present study aimed to identify transcripts expressed in the muscularis propria of the jejunum in wild type (WT) but lacking in ICC-deficient mice and to unravel their function. Here, we report that Na+-K+-2Cl cotransporter NKCC1 is expressed in ICC-MP and is involved in the SW mechanism in the jejunal muscularis.

MATERIALS AND METHODS

Animals. Mice were maintained and experiments performed in accordance with the local ethics committees for animal well-being of the Faculty of Medicine, Université Libre de Bruxelles, Brussels, Belgium, the University of Antwerp, Antwerp, Belgium and of Johnson and Johnson, Pharmaceutical Research and Development, a subdivision of Janssen Pharmaceutica, Beerse, Belgium.
Heterozygous Sl/+ and Sld/+ mice were obtained from Jackson Laboratory (Bar Harbor, ME). Wv/+ and WvLacZ/Wv mice were a generous gift from J. J. Panthier (Ecole Nationale Veterinaire, Maisons-Alfort, France). The product of the WvLacZ transgene lacks kinase activity and is functionally equivalent to the spontaneous null allele W (4). Heterozygous mice were bred to obtain viable Sl/Sld, WvLacZ/Wv, and WT littermates.

Disruption of the mouse Slc12a2/NKCC1 gene has been previously reported. Heterozygous couples were bred and litters were genotyped as described. Homozygous NKCC1 knockout (KO) animals were readily distinguished by their small size and shaker/waltzer behavior (8). WT littermates served as controls.

One-month-old mice were killed by cervical dislocation. The jejunum was quickly dissected out, flushed with ice-cold Krebs solution, and pinned onto a Petri dish cooled at 4°C. The mesentery was carefully removed under a binocular.

PCR suppression SSH. For poly A+ and total RNA isolation, the muscle layers were peeled off the mucosa and submucosa by blunt dissection with fine tweezers, and the mucosa was discarded. Muscle strips were immediately transferred into cryotubes, frozen in liquid nitrogen, and stored at −80°C until use. Total RNA was extracted using the Rneasy fibrous tissue mini kit (Westbur, Leusden, the Netherlands) and treated with DNase I (On Column Dnase, Westburg) according to the manufacturer’s instructions to avoid genomic DNA contamination.

Poly A+ RNA was isolated from total RNA using Oligotex mRNA spin columns (Westburg). The mRNA was precipitated (1/10 V 0.3 M NaAc, 2.5 V 100% EtOH) overnight at −80°C, spun at 4°C during 30 min, washed with 70% EtOH, and diluted to 1 μg/μl in Rnase-free water.

PCR select cDNA subtraction was performed starting from 2 μg poly A+ RNA according to the manufacturer’s instructions (Clontech, Palo Alto, CA). Two populations of mRNA isolated from jejunum muscle layers of WT vs. ICC-deficient mice were compared by SSH to enrich for differentially expressed transcripts. The subtraction was carried out in two ways: 1) the forward subtraction, WT minus ICC deficient, contained cDNA enriched for ICC-related genes, whereas 2) the reverse subtraction, ICC deficient minus WT, served as negative control. The subtraction procedure was performed on both models of ICC-deficient mice, Sl/Sld and WvLacZ/Wv. To minimize the influence of the different genetic background of Sl/Sld and WvLacZ/Wv animals, only clones differentially expressed in both models were further considered.

The forward subtracted cDNA library was cloned in pGem-t-easy (Promega, Madison, WI) and chemically transformed in one-shot cells (N.V. Invitrogen, Merialbeke, Belgium). Four thousand bacterial colonies were randomly selected and amplified. Amplification of the PCR product was confirmed on a 1% agarose gel. The NKCC1 full-length clone (Rapid amplification of cDNA ends (RACE)) was confirmed on a 1% agarose gel. After the DNA was extracted from the same litter. Total RNA isolation from jejunum muscle layers was performed as described in the previous paragraph. Reverse transcription was performed with 25 pmol/μl oligo(dT)15 primer using Powerscript reverse transcriptase (BD Biosciences, Erembodegem, Belgium) according to the manufacturer’s instructions.

Amplification reactions were performed in triplicates with 1 × SybrGreen PCR master mix (Applied Biosystems), 200 nM of gene specific primers and 25 ng sample DNA in a 50-μl final volume. The primers were designed with Primer Express 1.5 according to the manufacturer’s instructions (Table 1), and qPCR was performed on an ABI Prism 1700 Sequence detector. Identical thermal profile conditions (95°C for 10 min, 45 cycles of 95°C for 15 s, and 60°C for 1 min) were used for all primer sets. Emitted fluorescence was measured during the annealing/extension phase, and amplification plots were generated using the sequence detection system (Applied Biosystems). To differentiate specific amplicons from nonspecific products, a DNA dissociation curve was generated after each reaction.

All mRNAs were quantified relative to GAPDH mRNA using the comparative threshold cycle number (Ct) method. The Ct difference (ΔCt = Ctgene − CtGAPDH) was taken as a relative quantity of the transcript. To ascertain the validity of the ΔΔCt calculation (ΔΔCt = ΔCtICC deficient − ΔCtWT), the amplification efficiency was checked and found to be identical for all the genes measured. Statistical analysis was performed with the unpaired t-test, and a P value <0.05 was considered to represent a statistically significant difference.

Rapid amplification of cDNA ends. The NKCC1 full-length clone was identified by the SMART-Rapid amplification of cDNA ends (RACE) cDNA amplification kit (Clontech) according to the manufacturer’s specifications (primer in Table 1). DNA sequencing was performed using the ABI PRISM BigDye Terminators v3.0 Cycling Sequencing Kit according to the instructions of the supplier (Applied Biosystems, Foster City, CA) except for the amount of dyes used, which was reduced to 1 μl for a 20-μl reaction. The software used for sequence assembly was Sequencher version 4.0.5 from Gene Codes (Ann Arbor, MI).

Immunohistochemistry. For morphological experiments, small pieces of jejunum were harvested, fixed overnight at 4°C in fresh 4% paraformaldehyde solution in 0.1 M PBS, cryopreserved in graded solutions of sucrose (10, 20, and 30%; overnight each), oriented transversally, embedded in Tissue-Tek optimum cutting temperature compound (Miles, Elkhart, IN), snap-frozen in 2-methyl butane that had been cooled on dry ice and stored at −80°C until sectioning. Twelve-micrometer sections were cut on a cryostat and mounted on slides coated with 0.1% poly-L-lysine (Sigma, St. Louis, MO), air dried for 20 min, and stored at −20°C until use.

Besides mouse jejunum, two specimens of normal human jejunum from our tissue collection (58) were used. The use of human tissues

| Table 1. Sequence of primers used for RT-qPCR and for SMART-RACE |
|-----------------|-----------------|-----------------|
| **Sequence Oligo (5′-3′)** | **Sequence Oligo (3′-5′)** |
| RT-qPCR c-Kit | TGGAGAAGCTCTTCATGCAAAGAAAAGACGCAACAATAATATCTGAT |
| NKCC1 | CATCCTGGTGGTTGAAAGG |
| GAPDH | TGTTGCTGGGTTGACTCTGA |
| SMART-RACE | DCAAAAGCCAGGACGCAAGGCACAAATAATATCTGAT |

RT-qPCR, real-time quantitative PCR; RACE, rapid amplification of cDNA ends.
was approved by the Medical Institutional Ethics Committees of the Hôpital Universitaire des Enfants Reine Fabiola and Faculté de Médecine, Université Libre de Bruxelles, Brussels, Belgium.

Sections were rinsed three times in 10 mM Tris in 0.15 M sodium chloride, pH 7.4 [Tris-buffered saline (TBS)], containing 0.1% (vol/vol) Triton X-100 (TBS-TX), incubated for 1 h in 10% normal horse serum (NHS; Hormonologie Laboratoire, Marloie, Belgium) in TBS-TX to reduce background staining, and incubated overnight with the primary antisera diluted in TBS-TX containing 2% NHS. The primary antibodies used were a goat anti-mouse polyclonal IgG, raised against KIT(M14) (1/900; Santa Cruz Biotechnology, Santa Cruz, CA), and a rabbit anti-rat NKCC1 polyclonal antibody (1/500; Gentaur, Belgium). The slides were rinsed in TBS, incubated in the dark for 2 h at room temperature in TBS containing the secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) coupled respectively to FITC and Cy3, rinsed in TBS, and mounted with Slow-fade Light Anti-fade (Molecular Probes, Eugene, OR). The optimal working dilution had been determined empirically for each antibody. Omission of one of the primary or of one of the secondary antibodies resulted in the absence of immunoreactivity. The protocol used for double immunofluorescence staining did not modify the distribution or the intensity of each individual labeling observed in corresponding single procedures.

Preparations were observed using a Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany) at excitation wavelengths 488 and 543 nm. The images were analyzed with Zeiss LSM 510 Image Examiner.

Extracellular recording of SW activity in vitro. SW were recorded from a 4 cm-long segment of proximal jejunum of WT and NKCC1 KO mice. The segment was opened and placed with the serosal surface facing upward in a 150-ml recording chamber continuously perfused at 50 ml/min with prewarmed (37°C) oxygenated (95% O2:5% CO2) Tyrode’s solution of the following composition (in mM): 118 NaCl, 2.70 KCl, 1.8 CaCl2, 2H2O, 1.04 MgCl2-6H2O, 11.9 NaHCO3, 0.42 NaH2PO4-H2O, 136.9 NaCl, and 5.55 glucose, pH 7.4. Tissue was allowed to equilibrate for 10 min. The extracellular electrical activity was recorded using a 16-channel Teflon-coated silver electrode array designed by W. Lammers (Al Ain, United Arab Emirates (31–33) (http://www.smoothmap.org)) gently positioned on the serosal surface using a micromanipulator. Electrical signals were recorded using an NI-SCX-1000 data-acquisition system (National Instruments, Austin, TX) for 1 min before the addition of bumetanide (Burinex, LEO Pharmaceutical Products, Ballerup, Denmark), 10 min after addition of bumetanide, and after 10 min of washout. The signals were sampled at 200 Hz and digitally stored on disc using Labview 7.0 software (National Instruments). Signals were filtered using a 50-Hz notch filter and a five-point smoothing for display and analysis purposes. Data are expressed as means ± SE. Differences were evaluated using a paired t-test.

Intracellular recording of SW activity in vitro. SW were recorded from the smooth muscle cells of proximal jejunum of WT Swiss mice and NKCC1 KO mice with a standard microelectrode technique. A segment of intestine was opened along the mesenteric border, and mucosal and submucosal layers were removed under stereomicroscopic visualization. The muscle strip (15 × 6 mm) was then pinned, serosal side down, to the Sylgard (Dow-Corning Europe, Belgium) floor of a recording chamber placed on the stage of an inverted microscope (Diaphot, Nikon, Tokyo, Japan). The tissue was continuously superfused (10 ml/min; temperature 36.5–37°C) with oxygenated Krebs-Ringer solution of the following composition (in mM): 118 NaCl, 4.75 KCl, 2.54 CaCl2-2H2O, 1.2 MgSO4-7H2O, 1 NaH2PO4-2H2O, 25 NaHCO3, and 11.1 glucose. All experiments were performed in the presence of the L-type Ca2+ blocker nicardipine (3 μM, Sigma) to reduce mechanical activity. Smooth muscle cells were impaled with a borosilicate glass microelectrode (1-mm outer diameter; Clarc Electromedical Instruments, Reading, UK) pulled on a P-97 Brown-Flaming micropipette puller (Sutter Instruments, Novato, CA). The electrodes were back filled with 1 M KCl (resistence 50–70 MΩ). Potentials were recorded with an electrometer amplifier (Axoclamp 2A; Axon Instruments, Foster City, CA). After amplification and low-pass filtering (500 Hz), the signal was digitized at a sample rate of 1 kHz using a Labmaster TL-1 DMA Interface (Axon Instruments) and displayed and stored on a personal computer. Data were obtained from the same cell before and after addition of bumetanide (Sigma) to the superfusion solution. Data analysis was performed using pClamp 6.02 (Axon Instruments) and InStat 3.05 (GraphPad Software, San Diego, CA) software. Data were expressed as means ± SE. Differences were evaluated using a paired t-test.

RESULTS

**NKCC1 is downregulated in the jejunal muscularis propria of ICC-deficient mice.** Four thousand transcripts from the forward subtracted library were screened, from which 148 and 139 clones hybridized at least three times more to the ICC-enriched probes from Sl/Sld and WlacZ/Wv, respectively. After SSH, 56 of these clones appeared downregulated in both ICC-deficient models (Fig. 1). These 56 candidate genes were then assessed by RT-qPCR. A threelfold greater expression in WT than in at least one of the ICC-deficient mouse models was arbitrarily set as cut off level. KIT (Y00864) and NKCC1 (U13174) emerged as significantly downregulated in ICC-deficient compared with WT mice. KIT expression was 5.3 ± 0.3 (n = 6, P = 0.001) and 4.2 ± 0.3 (n = 6, P = 0.001) times higher in WT than in ICC-deficient Sl/Sld and WlacZ/Wv, respectively. NKCC1 expression was 3.5 ± 0.2 (n = 6, P = 0.013) and 1.9 ± 0.3 (n = 6, P = 0.005)-fold higher in WT than in ICC-deficient Sl/Sld and WlacZ/Wv, respectively.

Because SSH identified only fragments of transcripts of 300–500 bp, RACE was performed to obtain the full-length clone of NKCC1. The isolated clone fully matched the published sequence of the Slc12a2 gene product (68). Exon 21, which encompass the unique PKA consensus site primarily

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**Fig. 1. Identification of downregulated genes using Southern blot analysis.** Four thousand clones of the forward subtraction, wild-type (WT) minus Sl/Sld (A) and WT minus WlacZ/Wv (B), were spotted in 96-well configurations on Hybond N+ membranes. The membranes were prehybridized at 42°C in 50% formamide in 6× SSPE, 5× Denhardt’s solution, 0.1% SDS, and 0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin, and then hybridized at 42°C in the same buffer containing the 32P-labeled probe. After hybridization, the membranes were washed sequentially in 2× SSPE, 0.1% SDS, 2× SSPE, 0.1% SDS, and 0.1× SSPE, 0.1% SDS. The membranes were exposed to X-ray film at −70°C. Bands were excised and the DNA bands were then quantitated using a phosphorimager.
lacking in the brain NKCC1 isoform (44) was present, and no splice variant was detected (35).

NKCC1 is expressed by the pacemaking ICC-MP in the mouse jejunum. In the jejunum of WT mice, NKCC1 immunoreactivity (ir) was observed in the region between the circular and longitudinal muscle layers. Kit-ir ICC-MP surrounding the myenteric ganglia exhibited strong NKCC1-ir, whereas myenteric neurons exhibited a fainter NKCC1-ir. No NKCC1-ir was observed in smooth muscle cells of both layers nor in KIT-ir ICC-DMP (Fig. 2, A-C). In the jejunum of ICC-deficient SI/Sld mice, which lack KIT-ir ICC-MP, NKCC1-ir was present only in the myenteric ganglia (Fig. 2, D-F). Furthermore, in the jejunum of both WT and ICC-deficient animals, strong NKCC1-ir was present in the epithelial cells lining the mucosal crypts.

The NKCC1-ir staining pattern was similar in the human jejunum (Fig. 2, G-I). Kit-ir mast cells occasionally found in the circular muscle layer were NKCC1 negative (Fig. 2, G-I). 

NKCC1 is involved in the SW activity of the mouse jejunum. Extracellular recordings showed spontaneous SW in WT small intestine with amplitudes of 400–600 μV and a frequency of 36.1 ± 2.1 SW/min (n = 8; Fig. 3). SW remained stable during the entire observation period (data not shown). In WT, application of 0.4 μM bumetanide had no effect on the SW (n = 5, Fig. 3A). However, 10 min after the addition of 4 μM bumetanide, the shape of the SW became irregular, and the amplitude decreased to 100–150 μV (n = 4, P < 0.0001; Fig. 3B), whereas the change in frequency was not statistically significant (P = 0.36). After the addition of 40 μM bumetanide (n = 8, Fig. 3C), the shape of SW became more irregular, the
amplitude decreased to 50–100 μV (P < 0.0001), and the frequency decreased from 36.1 ± 2.1 to 27.4 ± 1.3 (P < 0.0001). After washout, SW recovered completely after 4 μM and partially after 40 μM bumetanide treatment.

The small intestine of NKCC1 KO animals displayed slow-wave activity with amplitudes of 50–100 μV and a frequency of 26.0 ± 4.7 SW/min (n = 4). Addition of 4 and 40 μM (Fig. 3D) bumetanide (n = 4) had no effect on the amplitude or frequency of the SW.

When recording SW in control conditions using microelectrodes, SW were characterized by a steep depolarization followed by a plateau phase and a slower repolarization (Fig. 4, A–C, top). In WT animals, the addition of 0.4 μM bumetanide in the superfusion solution had no significant effect on the SW amplitude and shape (n = 5), whereas there was a small, but significant, decrease in SW frequency (Fig. 4A and Table 2). The addition of 4 μM (n = 10; Fig. 4B and Table 2) and 40 μM bumetanide (n = 9; Fig. 4C and Table 2) further significantly decreased the frequency of the SW and altered the shape of the SW within a few minutes. The amplitude decreased very significantly (P ≤ 0.0005, paired t-test) and, as can be seen in the figures (3, B and C), there was a slower depolarization and a marked slower repolarization compared with the control situation. Furthermore, cells depolarized with both concentrations of bumetanide. In three of nine cases, the SW disappeared completely after the addition of 40 μM bumetanide. After washout, SW recovered, albeit slowly and not always completely during the recording period (Fig. 4C).

Also, in the jejunal of NKCC1 KO animals, SW had a lower amplitude of 14 ± 2 mV and a frequency of 26.6 ± 1.7 SW/min (n = 14). The smooth muscle cells had a resting membrane potential of −46 ± 2 mV (n = 14). The addition of 4 μM (n = 7; Fig. 5A and Table 3) and 40 μM bumetanide (n = 7; Fig. 5B and Table 3) had no further effect on the shape and frequency of the SW nor on the resting membrane potential.

DISCUSSION

Identification of genes expressed in the GI muscle layers of WT animals but lacking in ICC-deficient animals may lead to a better understanding of the mechanisms generating pacemaker currents in the GI tract. Various methods have been developed for analyzing differences in gene expression, including mRNA differential display (34), serial analyses of gene expression (SAGE) (60), cDNA microarray (7), and SSH (10). mRNA differential display is a fairly complex and labor-intensive procedure, whereas both SAGE and cDNA microarray require genomic information and are thus limited to already identified sequences. The SSH technique combines equalization of abundantly expressed cDNAs and over a 1,000-fold enrichment of rare sequences (10). However, a high false-positive rate has been reported, resulting in the identification of <20% of putative clones and confirmed differential expression of merely 2% of the candidates (45). In our hands, using SSH, 56 candidates of 4,000 clones analyzed (1.4%) were identified. Of these 56, 2 genes, namely KIT and NKCC1, were confirmed by RT-qPCR to be significantly downregulated two- to fivefold in ICC-deficient models. The diversity of cell types in the GI musculature and the low abundance of ICC, combined with the complex genetic background of the models used may explain this fairly low success rate.
The protein tyrosine kinase receptor KIT has been extensively used to reveal the networks of ICC in the GI tract by immunohistochemistry (50, 65). Although KIT is apparently not directly involved in the generation of pacemaker currents (13), the KIT-SCF signaling pathway is vital for the differentiation and maintenance of several populations of ICC, including the pacemaking ICC in the jejunum, which are lacking in the W1224/Wv and Sl/Sld mouse models (4, 50, 65, 67). The identification of KIT as a gene downregulated in WT vs. ICC-deficient mice established the specificity of the SSH method.

The other gene product identified here by SSH, NKCC1, is a bumetanide-sensitive sodium-potassium-chloride cotransporter encoded by the Slc12a2 gene. It belongs to the family of Na-K-Cl cotransporters, which encompass NKCC1, a widely expressed basolateral secretory cotransporter, and NKCC2, an apical absorptive cotransporter specific to the vertebrate kidney (42). NKCC cotransporters are characterized by their specific and reversible inhibition by bumetanide and other loop diuretics (47).

NKCC1 is expressed by sensory neurons, where it maintains the intracellular chloride concentration above electrochemical equilibrium, affecting the postsynaptic responses to presynaptic stimuli (2, 24, 38, 44, 51, 52, 55, 61). The cotransporter also regulates the vascular smooth muscle tone (1, 26, 40). In salivary and airway epithelia, NKCC1 is involved in chloride secretion, whereas in the gastric epithelium, basolateral NKCC1 raises the intracellular chloride concentration of the acid secreting cells above electrochemical equilibrium, providing the driving force for Cl\(^-\) to exit the cell on the apical side (47, 53). In situ hybridization had revealed strong NKCC1 expression in mucosal crypts, but was not previously reported in the muscularis propria of the GI tract (14). We have shown here that, besides a very strong NKCC1 expression in mucosal crypts, NKCC1-ir was expressed in myenteric neurons and in KIT-ir ICC-MP, the ICC population that generates the pacemaking activity. Conversely, ICC-DMP, the other population of KIT-ir ICC in the muscularis propria of the jejunum, and smooth muscle cells were NKCC1 negative. The selective expression of NKCC1 in the pacemaking ICC-MP, and its downregulation in ICC-deficient models identified by SSH, raised the hypothesis that NKCC1 may be involved in the electrical SW activity, which is independent of neuronal activity (65).

In WT small intestine, SW are characterized by a steep depolarization followed by a plateau phase and a slower repolarization (29). The SW frequencies recorded in this study (37.3 ± 1.3 and 36.1 ± 2.1 SW/min, intracellular and extracellular recordings, respectively) were in line with published data (22, 65). Using both intracellular and extracellular methods, marked alterations of the SW amplitude, shape, and frequency were observed on pharmacological blockade of NKCC1 with 4 \(\mu\)M bumetanide, in line with the reported \(K_i\) value for bumetanide in the micromolar range (47). Bumetanide (40 \(\mu\)M) resulted in an even greater reduction of the SW amplitude and frequency. Reduction of the SW frequency by bumetanide has previously been reported in the guinea pig gastric antrum (56). The effect of bumetanide was reversible after washout, although recovery was slow and incomplete during the recording period with the 40 \(\mu\)M dose.
Bumetanide, even at the 40 μM dose, had no effect in NKCC1-KO animals, establishing the specificity of bumetanide for NKCC1 in the range of concentrations used. The constitutive lack of NKCC1 in KO animals resulted in a decrease of the frequency and amplitude of SW in line with the constitutive lack of NKCC1 in KO animals, establishing the specificity of bumetanide for NKCC1 in the range of concentrations used. The major effect of NKCC1 inhibition appears to be the decrease of the frequency and amplitude of SW in line with the effects of acute inhibition of NKCC1 by bumetanide.

Disturbed GI motility has been reported in mice deficient in pacemaking ICC (9, 21, 37, 65), and lack of ICC has been reported in a broad range of human GI motility disorders (46). Noteworthy, in NKCC1 KO mice, morbidity related to various severe GI dysfunctions, including hemorrhage, intussusception, and fecal impaction, has been reported around the weaning period (12). Another strain of NKCC1 KO appeared, however, essentially unaffected (14). The NKCC1 KO mice used in this study tend to be short lived, although no necropsy was performed. In NKCC1 KO mice, the intestinal ion and fluid flux are markedly altered but to a much lesser extent than in CFTR-KO mice, a model for cystic fibrosis in which severe impairment of the epithelial transport is blamed for the severe disturbance of GI transit. An unspecified “defect in the circulatory system” has been blamed for the GI morbidity observed in NKCC1 KO mice (12). Our observations on the role of NKCC1 in the pacemaker mechanism raises the possibility that the GI disturbances observed in NKCC1 KO mice may also be due, at least in part, to alterations of the electrical activity in the muscularis propria. Additional factors may be responsible for the variable phenotype observed (12, 14).

NKCC cotransporters are electroneutral, with a transport stoichiometry of 1Na⁺:1K⁺:2Cl⁻. The driving force for net transport is determined solely by the chemical gradients of these three ions. Under normal physiological conditions, NKCC cotransporters usually mediate net inward ion movement. The major effect of NKCC1 inhibition appears to be the disturbance of the intracellular Cl⁻ equilibrium (47). Inhibition of Cl⁻ currents leads to a decrease of SW amplitude and plateau component (19), in line with our results (25). NKCC1 inhibition also affects Na⁺ and K⁺ homeostasis and hence may influence inward Na⁺ and K⁺ currents. Noteworthy, a decrease of SW amplitude and an immediate hyperpolarization have been reported after removal of extracellular Na⁺ (54), whereas others reported a decrease of the SW amplitude and an accompanying depolarization (30). Furthermore, K⁺ currents also influence the resting membrane potential and excitability of ICC (15, 19, 28). The specific contribution of NKCC1 to the homeostasis of the different ions involved in the pacemaker mechanism needs to be further examined.

Our results suggest that NKCC1, which is expressed only in KIT-ir ICC-MP and myenteric neurons but not in ICC-DMP, participates to the complex mechanisms underlying the membrane potential of the smooth muscle cells and the amplitude and frequency of SW. Although NKCC1 is clearly not solely involved, it nevertheless appears to be one critical component.

### Table 2. Effect of bumetanide on the smooth muscle cells of wild-type animals

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Bumetanide (0.4 μM)</th>
<th>Control</th>
<th>Bumetanide (4 μM)</th>
<th>Control</th>
<th>Bumetanide (40 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential, mV</td>
<td>55±3</td>
<td>54±3</td>
<td>-58±2</td>
<td>-53±3*</td>
<td>-56±2</td>
<td>-46±2*</td>
</tr>
<tr>
<td>Amplitude of the SW, mV</td>
<td>22±2</td>
<td>21±3</td>
<td>27±2</td>
<td>14±2†</td>
<td>25±2</td>
<td>12±2†</td>
</tr>
<tr>
<td>t_{1/2} of the SW, s</td>
<td>0.76±0.03</td>
<td>0.73±0.05</td>
<td>0.77±0.03</td>
<td>0.75±0.03</td>
<td>0.88±0.05</td>
<td>0.76±0.07*</td>
</tr>
<tr>
<td>SW frequency, SW/min</td>
<td>38.4±4.4</td>
<td>35.6±4.2*</td>
<td>38.1±1.1</td>
<td>34.4±1.2*</td>
<td>35.8±2.5</td>
<td>32.3±1.7*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. †In 3 cases the slow wave disappeared completely. ‡t_{1/2}, duration of the slow wave (sw), measured halfway to maximal amplitude. *P ≤ 0.05 (paired t-test), †P ≤ 0.0005 (paired t-test).

### Table 3. Effect of bumetanide on the smooth muscle cells of NKCC1 knockout mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Bumetanide (4 μM)</th>
<th>Control</th>
<th>Bumetanide (40 μM)</th>
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</thead>
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<tr>
<td>Resting membrane potential, mV</td>
<td>-47±2</td>
<td>-48±3</td>
<td>-45±4</td>
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<tr>
<td>Amplitude of the SW, mV</td>
<td>15±3</td>
<td>15±3</td>
<td>13±4</td>
<td>12±4</td>
</tr>
<tr>
<td>t_{1/2} of the SW, s</td>
<td>0.65±0.09</td>
<td>0.72±0.13</td>
<td>0.94±0.17</td>
<td>0.82±0.16</td>
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<tr>
<td>SW frequency, SW/min</td>
<td>28.6±2.1</td>
<td>29.0±2.4</td>
<td>24.6±2.6</td>
<td>24.6±2.5</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. None of the data are significantly different (paired t-test).
because adaptive mechanisms, if they exist, are unable to counterbalance the lack of NKCC1 in KO animals.

In conclusion, expression of the cotransporter NKCC1 is downregulated in the muscle layers of the jejunum in ICC-deficient mice, and NKCC1 is selectively expressed in the pacemaking ICC-MP in the mouse and human jejunum. Pharmacological inhibition in vitro and gene knockout of NKCC1 markedly altered the SW properties, indicating that NKCC1 is one of the components of the complex pacemaking mechanism in the jejunum. Further insight into the molecular mechanism of the GI pacemaker and its role in intestinal motor function may lead to new therapeutic approaches in GI motility disorders.

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