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Authors

McDaniel, Nichole
Pace, Amy J
Spiegel, Stefanie
et al.

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Role of Na-K-2Cl cotransporter-1 in gastric secretion of nonacidic fluid and pepsinogen

Nichole McDaniel,¹ Amy J. Pace,² Stefanie Spiegel,³ Regina Engelhardt,³
Beverly H. Koller,² Ursula Seidler,³ and Christian Lytle¹

¹Division of Biomedical Sciences, University of California, Riverside, California; ²Department of Medicine, University of North Carolina, Chapel Hill, North Carolina; and ³Department of Gastroenterology, Hepatology and Endocrinology, Medical School of Hannover, Hannover, Germany

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McDaniel, Nichole, Amy J. Pace, Stefanie Spiegel, Regina Engelhardt, Beverly H. Koller, Ursula Seidler, and Christian Lytle. Role of Na-K-2Cl cotransporter-1 in gastric secretion of nonacidic fluid and pepsinogen. *Am J Physiol Gastrointest Liver Physiol* 289: G550–G560, 2005; doi:10.1152/ajpgi.00095.2005.—Na-K-2Cl cotransporter-1 (NKCC) has been detected at exceptionally high levels in the gastric mucosa of several species, prompting speculation that it plays important roles in gastric secretion. To investigate this possibility, we 1) immunolocalized NKCC protein in the mouse gastric mucosa, 2) compared the volume and composition of gastric fluid from NKCC-deficient mice and their normal littermates, and 3) measured acid secretion and electrogenic ion transport by chambered mouse gastric mucosa. NKCC was localized to the basolateral margin of parietal cells, mucous neck cells, and antral base cells. In NKCC-deficient mice, gastric secretions of Na⁺, K⁺, Cl[−], fluid, and pepsinogen were markedly impaired, whereas secretion of acid was normal. After stimulation with forskolin or 8-bromo-cAMP, chambered corpus mucosa vigorously secreted acid, and this was accompanied by an increase in transmucosal electrical current. Inhibition of NKCC with bumetanide reduced current to resting levels but had no effect on acid output. Although prominent pathways for basolateral Cl[−] uptake (NKCC) and apical Cl[−] exit [cystic fibrosis transmembrane conductance regulator (CFTR)] were found in antral base cells, no impairment in gastric secretion was detected in CFTR-deficient mice. Our results establish that NKCC contributes importantly to secretions of Na⁺, K⁺, Cl[−], fluid, and pepsinogen by the gastric mucosa through a process that is electrogenic in character and independent of acid secretion. The probable source of the NKCC-dependent nonacidic electrogenic fluid secretion is the parietal cell. The observed dependence of pepsinogen secretion on NKCC supports the concept that a nonacidic secretory stream elaborated from parietal cells facilitates flushing of the proenzyme from the gastric gland lumen.

mouse; NKCC1; cystic fibrosis transmembrane conductance regulator; CFTR; pepsin

THE STOMACH ACTIVELY SECRETES Cl[−] in substantial excess of H⁺ (12, 19, 21, 47, 72). Although the mechanistic details of HCl secretion and its regulation are well defined (32), comparatively little is known about the cellular source, molecular mechanism, and control of “nonacidic” Cl[−] secretion.

The undisputed origin of gastric acid is the parietal cell (32). When stimulated, the parietal cell secretes HCl through a concerted operation of apical membrane H⁺/K⁺ exchange pumps, a Cl[−] exit pathway (possibly CIC-2), and a K⁺ recycling pathway (probably Kir4.1, Kir2.1, and/or KCNQ1), to-

gether with basolateral mechanisms for Cl[−] uptake and H⁺ uptake (HCO₃[−] export). It is generally accepted that functional coupling between apical HCl exit and basolateral HCl uptake is accomplished, largely if not wholly, by a DIDS-sensitive Cl[−]/HCO₃[−] exchange process (54, 59) that displays a high transport capacity (74) and strong allosteric activation by intracellular alkalinity (68). This process appears to be mediated by anion exchanger-2 (AE2), the main anion exchanger isoform in the stomach (37, 64, 73). In the mouse, AE2 is essential not only for gastric acid secretion but also for normal parietal cell development (22). The nonacidic component of Cl[−] secretion has been observed in a variety of gastric preparations as a transmucosal movement of Cl[−] in excess of H⁺ or a short-circuit current (*I*_{sc}) that is stimulated by histamine and inhibited by serosal replacement of Na⁺ or by addition of serosal barium (7, 12, 19, 47, 52, 66). Recent experiments on the perfused rat stomach suggested that the nonacidic component of Cl[−] secretion is independent of proton pumping and is stimulated, together with HCO₃[−] secretion, by PGE₂ and luminal acidification (12).

Na-K-2Cl cotransporter-1 (NKCC) is a membrane glycoprotein that harnesses the chemical gradients of Na⁺ and Cl[−] to electroneutrally move salt and osmotically obliged water into the cell. In many fluid-secreting epithelial cells, basolaterally situated NKCC mediates salt uptake in concert with Na⁺-K⁺-ATPase and K⁺ channels for the purpose of transepithelial Cl[−] secretion (45, 65). Exceptionally high levels of NKCC have been found in the gastric mucosa of a variety of animal species (18, 40, 49, 60, 71). In a previous study (49) of the rat stomach, we established that NKCC is localized to parietal cells inhabiting the base of glands in the gastric corpus and to mucous gland cells forming the extreme base of antral glands.

The breadth of functions that Na-K-2Cl cotransport might play in gastric secretion has been poorly understood. Previous studies of amphibian (71) and guinea pig (5) gastric mucosa concluded that NKCC is involved in acid secretion based on evidence that a major component of HCl output is dependent on serosal Na⁺ and is blocked by NKCC inhibitors such as bumetanide or furosemide. However, a recent study with NKCC-deficient mice indicated that Na-K-2Cl cotransport is not required for the stomach to acidify its contents after stimulation with histamine (18). In addition, studies on chambered gastric mucosa isolated from the eel (76) and bullfrog (28) and on isolated rabbit gastric glands

Address for reprint requests and other correspondence: C. Lytle, Div. of Biomedical Sciences, 2226 Webber Hall, Univ. of California, Riverside, CA 92521 (e-mail: christian.lytle@ucr.edu).

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(50) have found no inhibitory effect of bumetanide on gastric acid secretion.

We have proposed that gastric NKCC might instead contribute importantly to nonacidic electrolyte secretion (49). To explore this hypothesis, we compared gastric H^+ , Na^+ , K^+ , Cl^- , pepsinogen, and fluid secretions in NKCC-deficient mice and their normal littermates using the pylorus ligation technique. To assess the electrogenic character of NKCC-dependent ion flow, we measured the effect of bumetanide on I_{sc} and H^+ secretion by chambered mucosal sheets isolated from the mouse gastric corpus. Our results indicate that NKCC contributes importantly to both nonacidic electrogenic ion secretion and pepsinogen secretion in the mouse stomach.

MATERIALS AND METHODS

Materials. Reagent-grade salts, porcine pepsinogen, forskolin, carbachol, 8-bromo-cAMP (8-Br-cAMP), amiloride, bumetanide, DIDS, and histological stains were obtained from Sigma. Secondary antibodies, ABC kits, and substrates were from Vector Laboratories. Pentagastrin (Bachem) was dissolved in DMSO at 7.5 mg/ml and diluted 1,000-fold in sterile PBS just before use.

Mouse lines. The NKCC-deficient mice used in this study were generated by targeted gene disruption as described previously (57). This *Slc12a2* ^{Δ 506–621} mutant manifests several developmental and physiological defects; in addition to impairments in intestinal (26) and airway (24, 27) Cl^- secretion, these mice exhibit growth retardation, deafness, ataxia, abnormal spermatogenesis (57), and inner ear histopathology (58). Studies on a different NKCC-deficient mouse line (18) have revealed similar phenotypic alterations, along with reduced mean arterial pressure (18, 53) and impaired saliva secretion (17). Measurements were performed on litter-matched male and female adult (2–8 mo of age) mice bred on a heterogeneous strain background (C57Bl/6J + DBA/2J). Mice were housed with a 12:12-h light-dark cycle with access to standard mouse chow and water ad libitum and euthanized by CO_2 asphyxiation. All animal studies were approved by the University of California Riverside Institutional Animal Care and Use Committee.

We also compared gastric secretion in cystic fibrosis transmembrane conductance regulator (CFTR)-null (*CFTR*^{tm1UNC}) mice with their heterozygous (*CFTR*^{+/-}) littermates. *CFTR*^{tm1UNC} mice were bred in a B6D2/129 genetic background as described previously (70). Both *CFTR*^{-/-} and control *CFTR*^{+/-} mice were maintained on standard chow and Colyte solution (Schwarz Pharma) ad libitum. Colyte, an osmotic laxative composed of 6% polyethylene glycol and electrolytes, prevents intestinal impaction in CFTR-deficient mice maintained on a solid diet without altering intestinal histomorphology (11).

Antibodies. For immunolocalization of NKCC, we employed affinity-purified rabbit antibodies directed against the carboxy-terminal domain (N1c) or the amino-terminal domain (NT) of human NKCC. Pepsinogen was labeled with a rabbit antiserum directed against porcine pepsinogen. For immunolocalization of AE2, we employed a previously characterized (73) affinity-purified rabbit antibody (SA6), which was kindly supplied by Drs. A. Stuart-Tilley and S. Alper (Beth Israel-Deaconess Medical Center). A rabbit antibody (R3195) raised against the 13-amino acid carboxy terminus of rodent CFTR (20) was provided by Dr. C. Marino (University of Tennessee). Parietal cells were identified by labeling with mouse antibody 2G11 against H^+ - K^+ -ATPase β -subunit (Affinity Bioreagents) or with rhodamine-conjugated *Dolichos biflorus* lectin (Vector Laboratories).

Gastric gland isolation. Glands were isolated from mouse gastric corpus for confocal immunocytochemistry by a modification of the collagenase method originally described by Berglinth and Obrink (9). Stomachs were excised, opened along the greater curvature, and pinned to a wax dissection plate on ice. The muscle layers overlying

the corpus region of the stomach were separated from the mucosa by a blistering technique (19). We injected oxygen-saturated gland medium (HCO_3^- -free minimal essential medium supplemented with 20 mM HEPES, pH 7.4) at multiple sites just below the mucosa using a 27-gauge needle; the muscularis externa was then dissected away with fine scissors. The mucosal sheet was minced into small pieces (~ 2 mm³) with a razor blade, washed with ice-cold gland medium, and transferred to a 50-ml flask containing 15 ml gland medium, 5 mg collagenase type-1A (Sigma C9891), and 15 mg BSA. The flask was incubated at 37°C with orbital mixing and continuous oxygenation. After 20–30 min, the suspension was diluted 1:1 with gland medium and the mucosal fragments were dispersed by gentle pipetting. Glands were attached to polylysine-coated glass slides and circumscribed with a thick hydrophobic ring (ImmEdge pen, Vector). The ring was filled with 0.3 ml of medium and incubated on a thermostated (37°C) aluminum block mounted inside an oxygenated humidified chamber atop an oscillating table (Thermolyne Roto-Mix, 30 rpm). After 20–30 min, the slide was submerged in neutral-buffered formalin for 30 min, rinsed in PBS containing azide, and stored at 4°C for up to 3 wk.

Tissue preparation. NKCC-deficient mice and their wild-type littermates were given free access to food and water. After CO_2 asphyxiation, the stomach was isolated, rinsed with PBS, and immersed in ice-cold PLP fixative (2% paraformaldehyde, 75 mM lysine, 10 mM sodium periodate, and 45 mM sodium phosphate, pH 7.4) (48). One hour later, the tissue was transferred to fresh fixative and incubated for an additional 3 h on ice. The tissue was stored for up to 3 mo in cryoprotectant (30% sucrose in PBS). For sectioning, tissues were embedded in freezing medium (Triangle Biomedical Sciences) and frozen at $-25^\circ C$. Sections of 5 μm thickness were cut on a cryostat microtome (HM 500 OM, Microm Lamborgeraete) and mounted on glass slides (Superfrost Plus, Fisher). Every tenth consecutive slide was stained with hematoxylin and eosin for histological assessment.

Immunoperoxidase staining. After antigen retrieval (1% SDS in K^+ -free PBS for 10 min), sections were incubated sequentially with blocking solution (PBS containing 20% goat serum, 0.2% BSA, and 25 mM glycine, pH 7.4), biotin blocker (Vector, 10 min), avidin blocker (Vector, 10 min), peroxidase inhibitors (PBS containing 1% H_2O_2 and 0.05% sodium azide, 15 min), primary antibody (2 h), and biotinylated secondary antibody. Bound antibody was detected with a peroxidase-conjugated streptavidin system (Vectastain Elite ABC kit, Vector Laboratories). Vector-SG or Vector-DAB were employed as substrate with eosin or hematoxylin counterstaining, respectively.

Immunofluorescence labeling was performed on 5- μm cryosections of PLP-fixed tissues as described previously (49) or on formalin-fixed glands attached to glass slides. Tissue was exposed successively to antigen retrieval solution (1% SDS in K^+ -free PBS, 10 min), blocking solution (5% normal goat serum, 5% BSA, 25 mM glycine, and 0.2% Triton X-100, in PBS, pH 7.4, 40 min), primary antibodies (diluted in blocking solution, 1 h), and fluorophore-conjugated secondary antibodies (diluted in PBS), with three rinses between each step. Coverslips were mounted over Vectashield medium (Vector). Fluorescence images were captured with a Zeiss LSM-510 confocal microscope and assembled with Adobe Photoshop software.

Pylorus ligation was performed as described previously (55, 69). Mice were fasted for 15 h in wire-mesh cages (to prevent caprophagia) with free access to tap water. After mice were anesthetized with 2% isoflurane vapor in oxygen (Vasco), gastric secretion was stimulated by administration of 16 $\mu g/kg$ iv pentagastrin in sterile saline or 2 mg ip histamine in 200 μl of sterile saline. After laparotomy, the pylorus was ligated with 3-0 silk. To counter postsurgical dehydration, the peritoneal cavity was infused with 1 ml ($\sim 8\%$ of total body water) of a sterile physiological salt solution, and the abdominal incision was repaired with 3-0 silk sutures. Two hours later, the mice were anesthetized, the esophagus was ligated, and the stomach was removed. Its contents were collected in preweighed tubes, weighed,

and centrifuged (5 min, 12,000 g). Occasionally, samples were discolored with small amounts of blood, and these data were discarded. The volume of supernatant was determined gravimetrically, and its pH was measured with a miniature spear-tip electrode (Orion). Acid equivalents were measured by back titration to pH 7.0 with 10 mM NaOH. K^+ and Na^+ were measured by flame photometry (Beckman Kline-Flame) and Cl^- by coulometric titration (Buchler-Cotlove). The pellet, consisting mainly of insoluble mucus, was weighed before and after drying for 12 h at 60°C. The volume of gastric fluid was calculated from the sum of the supernatant mass and pellet volatile mass.

The NKCC^{-/-} mice used in this study were ~33% smaller than their sibling controls, like those used in previous studies (26, 57, 58); however, their empty stomachs averaged only ~15% smaller (34.9 ± 2.2 mg for NKCC^{-/-} mice compared with 40.9 ± 2.5 mg for age-matched NKCC^{+/+} mice; $n = 16$). To compensate for these size differences, secretion data were normalized to stomach dry mass, determined by weighing the empty stomach after drying for 12 h at 60°C.

We measured pepsin activity using a standard hemoglobin digestion method (3). Briefly, 100 μ l of 10-fold diluted gastric juice were mixed with 400 μ l of 2.5% hemoglobin and 100 μ l of 0.3 N HCl and incubated at 37°C. Under these acidic conditions, all secreted pepsinogen is converted to pepsin. After 10 min, 1 ml of 0.3 N TCA was added, and the samples were mixed and filtered using Whatman no. 3 paper. The absorbance of the filtrate was measured at 280 nm (Lambda 3A UV/VIS spectrophotometer, Perkin-Elmer), and the pepsin activity was calculated from a standard curve obtained with 100 μ l of 5, 10, or 20 μ g/ml purified porcine pepsin in 10 mM HCl. The quantity of pepsin accumulated in the gastric juice 2 h after pylorus ligation was expressed as micrograms of pepsin per milligram of dry empty stomach.

Hydration measurements. Total body water was measured gravimetrically as described previously (10). NKCC-deficient mice and their sibling controls were allowed free access to food and water. After mice were euthanized with pentobarbital sodium, a midline incision was made through the abdominal wall and chest cavity. Samples of blood and urine were collected from the exposed heart and bladder with syringe needles. The gastrointestinal tract was removed, cleared of its contents by rinsing with PBS, and returned to the carcass. The carcass was then weighed, dried to a constant mass (60°C for 7 days), and weighed again. The ratio of volatile mass to total mass was recorded as fractional body water. Plasma and urine osmolalities were measured with a vapor pressure osmometer (Wescor 5500).

Electrophysiology and H^+ transport measurements. The mucosal layer was dissected from the mouse gastric corpus under a stereomicroscope and mounted between two Lucite half chambers of a water-jacketed Ussing system equipped with a gas-lift system. The exposed surface area was 0.625 cm². The serosal solution contained (in mM) 108 NaCl, 22 NaHCO₃, 3 KCl, 1.3 MgSO₄, 2 CaCl₂, 1.5 KH₂PO₄, 8.9 glucose, and 10 sodium pyruvate and was gassed with 95% O₂-5% CO₂, pH 7.4. Indomethacin (1 μ M) and tetrodotoxin (0.1 μ M) were included in the serosal solution to minimize variation due to intrinsic prostanoid and neural tone, respectively. The mucosal solution (140 mM NaCl) was gassed with 100% O₂ and maintained at pH 7.4 by the controlled addition (in 0.1- μ l steps) of dilute (1 mM) NaOH using a pH-Stat titration system (Radiometer, Copenhagen, Denmark). Trans-epithelial voltage (V_t) and resistance (R_t) and acid secretion were monitored under open-circuit conditions with series resistance compensation using a current-voltage clamp device (World Precision Instruments, Sarasota, FL). V_t was measured with Ag/AgCl electrodes connected to the chambers via 3 M KCl bridges and referenced to the serosal side of the epithelium. R_t was measured by recording voltage deflections resulting from periodic current pulses (25 μ A, 200 ms) passed across the tissue. Equivalent I_{sc} was calculated from Ohm's law ($I_{sc} = V_t/R_t$). Acid secretion, measured under open-circuit conditions, was calculated from the amount of NaOH needed to maintain

the mucosal bath at a constant pH of 7.4. Unlike many previously described in vitro mammalian preparations, mouse gastric mucosa prepared in this manner remained stable and highly responsive to secretory stimuli for several hours, with acid secretion rates ($\sim 7 \mu\text{mol} \cdot \text{cm}^{-2} \cdot \text{h}^{-1}$) comparable with those observed in the stimulated lumen-perfused mouse.

Statistics. Data are presented as means $\pm$ SE. The significance of the difference between means of paired data was assessed by Student's *t*-test. Differences were regarded as significant at $P < 0.05$.

RESULTS

Sites of NKCC in the mouse stomach. Immunofluorescent labeling of gastric glands isolated from the central zymogenic region (corpus) of the mouse stomach revealed exceptionally high levels of NKCC along the basolateral margin of parietal cells (Fig. 1). NKCC was also expressed by mucous cells in the gland neck but not by their zymogenic descendants in the gland base. Similar patterns of staining were obtained with three other independently generated NKCC antibodies: rabbit N1c and TEFS1 and mouse monoclonal T4 (not shown). As noted in the rat (6), most of the glands isolated from the mouse stomach were single units (Fig. 1, left), although some were bifurcated (Fig. 1, right).

Immunoperoxidase staining of mucosal sections revealed that surface epithelial cells express little or no NKCC (Fig. 2c). As expected, no specific labeling was observed in sections obtained from NKCC knockout mice (Fig. 2g). As noted in our group's previous study (49) of the rat, the parietal cells above the isthmus were generally larger in size and expressed more basolateral membrane AE2 than those below (Fig. 2d). This gradient of AE2 expression along the gland axis was found to be much more prominent in the region of corpus nearest the esophagus (where the glands are longest, $\sim 400 \mu\text{m}$) than in the distal corpus and greater curvature (where the glands are shortest, $\sim 175 \mu\text{m}$) (51). The overall distribution of AE2 in normal mice and NKCC-deficient mice was similar (Fig. 2, d and h), indicating that the developmental absence of one Cl^- entry pathway (NKCC) is not associated with a compensatory overexpression of another (AE2) in parietal cells.

As noted for another line of NKCC knockout mouse (18), ablation of NKCC had no overt effects on the morphology of the gastric mucosa (e.g., Fig. 2, a, b, e, and f). Histological assessment of the entire corpus revealed no apparent mucosal hyperplasia and no significant change in cell (parietal, mucous, chief) census or density of gastric units within a unit cross-section of mucosa (15 ± 2 U/400 μm in NKCC^{+/+} mice vs. 16 ± 3 U/400 μm in NKCC^{-/-} mice). This contrasts with the striking mucosal alterations observed in various mutant mice in which acid secretion is impaired (67).

The only other region of the mouse stomach in which very high NKCC expression was detected was the antrum (Fig. 3). Labeling was exceptionally strong in the mucous cells (44) that form the extreme base of the antral gland. These same cells contained, in addition to a basolateral pathway for rapid Cl^- entry (NKCC), apical pathways for Cl^- exit (CFTR) and for water movement [aquaporin-5 (AQP5)] (Fig. 3). Another apical constituent of these cells was pepsinogen (Fig. 3). Genetic ablation of NKCC produced no conspicuous alterations in antral histology, CFTR expression, or pepsinogen expression by antral mucous gland cells (not shown).

Fig. 1. Immunofluorescence localization of Na-K-2Cl cotransporter-1 (NKCC) in mouse gastric glands. Isolated glands were stained with rabbit NT antibody (against NKCC) and mouse 2G11 antibody (against $H^+-K^+-ATPase$ β -subunit; right) or DBA lectin (left) to visualize NKCC (green signal) and parietal cells (red signal). Confocal images of glands extending from isthmus (top) to base (bottom) were taken along the plane of the central lumen. NKCC labeling is most prominent along the basolateral margin of parietal cells. NKCC was also detected in mucous neck cells (solid arrow) but not in their zymogenic descendents in the gland base (open arrow). Paired images of glands exposed to secondary antibodies alone were essentially blank (not shown).

Role of NKCC in gastric electrolyte secretion. The unusual abundance of NKCC protein in both the corpus (parietal cells) and antrum (base mucous cells) of the stomach suggested that Na-K-2Cl cotransport might contribute importantly to gastric electrolyte secretion. To evaluate this possibility, we measured the volume and composition of gastric juice in normal and NKCC-deficient mice using the pylorus ligation technique (55). We chose not to obstruct esophageal drainage because salivary inflow, a potential complication, contributes negligibly to the gastric fluid volume absent parasympathetic stimulation (17) and because ligation of the distal esophagus is known to blunt the acid secretory responses of the rodent stomach (29).

In a series of early experiments, we found that peritoneal administration of histamine (3 mg) elevated acid secretion from 0.3 ± 0.1 to $1.6 \pm 0.4 \mu\text{mol} \cdot \text{mg}^{-1} \cdot 2 \text{ h}^{-1}$ and fluid secretion from 10.5 ± 1.9 to $19 \pm 2.7 \mu\text{l} \cdot \text{mg}^{-1} \cdot 2 \text{ h}^{-1}$ ($n = 5$). Comparable responses were observed after intravenous injection with pentagastrin (16 $\mu\text{g}/\text{kg}$): acid secretion increased to $1.1 \pm 0.3 \mu\text{mol} \cdot \text{mg}^{-1} \cdot 2 \text{ h}^{-1}$ and fluid secretion increased to $21 \mu\text{l} \cdot \text{mg}^{-1} \cdot 2 \text{ h}^{-1}$; thus pentagastrin was used in all subsequent *in vivo* experiments as a standard gastric secretagogue. Basal secretory tone, i.e., in the absence of any external stimulus, was not routinely measured, because pylorus ligation itself is known to elevate gastric acid secretion through a gastrin-independent vagovagal reflex (1, 29).

Whereas genetic ablation of NKCC had no effect on the secretion of proton equivalents, other components of gastric secretion were markedly impaired: NKCC-deficient mice secreted 38% less Na^+ , 70% less K^+ , 27% less Cl^- , and 32% less fluid over a 2-h interval than their sibling controls (Fig. 4). Measurements of the major cations ($\text{H}^+ + \text{Na}^+ + \text{K}^+$) and the main anion (Cl^-) in gastric fluid indicated that these cations

amounted to $\sim 78\%$ of the Cl^- in both control and NKCC-deficient mice. The source of this small "cation gap" is not known. The amount of Cl^- secreted in excess of H^+ , a component customarily designated "nonacidic" Cl^- secretion, was substantially reduced in NKCC-deficient mice (Fig. 4). Thus, although NKCC is not required for HCl secretion, it plays a critical role in the secretion of Na^+ , K^+ , and "nonacidic" Cl^- , along with the fluid osmotically associated with these ion flows. Gastric juice collected from pentagastrin-treated mice 2 h after pylorus ligation was slightly hyposmotic to plasma in control mice ($290 \pm 6.6 \text{ mosmol}/\text{kgH}_2\text{O}$, $n = 10$) yet nearly isosmotic in NKCC-deficient mice ($319 \pm 6.3 \text{ mosmol}/\text{kgH}_2\text{O}$, $n = 7$). The osmolality of plasma collected from control mice ($332 \pm 3 \text{ mosmol}/\text{kgH}_2\text{O}$, $n = 5$) and from NKCC-deficient mice ($333 \pm 12 \text{ mosmol}/\text{kgH}_2\text{O}$, $n = 5$) did not differ.

Role of antral CFTR in gastric electrolyte secretion. One source of NKCC-dependent electrolyte secretion could be the antrum. In Fig. 3, mucous cells that comprise the base of antral glands seem specially configured for rapid Cl^- secretion, with prominent pathways for basolateral Cl^- entry (NKCC), apical Cl^- exit (CFTR), and osmosis (AQP5). To evaluate the contribution of these cells and CFTR to gastric secretion, we compared the volume and composition of gastric juice in CFTR-deficient mice and their heterozygous littermates using the pylorus ligation technique. We reasoned that this component of Cl^- secretion might be selectively absent from CFTR-deficient mice, given that antral base cells are the only detectable site of CFTR expression in the mouse stomach and that CFTR function is essential for active Cl^- secretion in similarly configured intestinal crypt cells (25). We found, however, that CFTR deletion had no significant effect on any measured

Fig. 2. Histological comparison of gastric mucosa of normal and NKCC-deficient mice. Sections of gastric corpus of normal adult mice (*a–d*) and their NKCC^{-/-} littermates (*e–h*) were stained with hematoxylin-eosin (H&E; *a* and *e*) or with periodic acid-Schiff reagent-alcian blue (PAS; *b* and *f*) to reveal acidic mucins in the pit (purple) and zymogenic cells in the base (pale blue). Overt differences in gastric unit organization or pit/neck/parietal cell census were not apparent in NKCC-deficient mice. Immunoperoxidase staining (black-gray vector-SG) with antibody N1c revealed exceptionally high levels of NKCC in parietal cells in normal mice (*c*) but not in NKCC^{-/-} mice (*g*). Staining with antibody SA6 indicated that anion exchanger-2 (AE2) is most prominent in parietal cells above the neck (*d*). A similar gradient of AE2 expression was found in NKCC-deficient mice (*h*). Bar = 50 μ m.

component of gastric ion secretion in pentagastrin-stimulated mice (Fig. 5).

Role of NKCC and CFTR in gastric pepsin secretion. Our group (49) has postulated that the NKCC-dependent secretion of neutral fluid might serve to flush pepsinogen from its diffusionally confined site of secretion (zymogenic cells at the base of gastric units in the corpus and antrum) to its ultimate site of action (stomach lumen). To evaluate this hypothesis, we measured the amount of pepsinogen conveyed to gastric juice over a 2-h interval in pentagastrin-stimulated mice. Ablation of NKCC reduced accumulation of luminal pepsin by 36%, whereas ablation of CFTR was without effect (Fig. 6). These results support the idea that fluid elaborated from parietal cells in the gastric gland base facilitates bulk transport of pepsinogen through the narrow and convoluted gland lumen. Although impaired clearance of pepsin from the acidic gland lumen might be expected to produce focal injury, no evidence for mucosal damage and inflammation was apparent in the NKCC^{-/-} mouse stomach (Fig. 2).

Hydration. When fed a normal diet and given free access to water, NKCC-deficient mice and their normal littermates did not differ significantly in total body water content ($49 \pm 3\%$ and $49 \pm 4\%$, respectively; $n = 5$). Plasma Na⁺ concentration and osmolality were also similar, confirming recent findings with other NKCC-deficient mice (53), as were urine Na⁺ and K⁺ concentrations (not shown). These results, combined with evidence that the concentration of serum aldosterone in NKCC knockout mice is normal (53), make it unlikely that the impaired gastric fluid secretion observed in these mice reflects a defensive response to dehydration.

Electrogenic transport and acid secretion by isolated gastric mucosa. The gastric corpus of most animal species actively secretes Cl⁻ in substantial excess of H⁺ and absorbs Na⁺ through electrogenic mechanisms that give rise to a lumen-negative V_i (16, 21, 46, 66). To characterize these processes in the mouse model and their dependence on NKCC activity, we isolated the mucosal layer from the corpus region (to circumvent possible antral contributions) and monitored electrical

Fig. 3. Antral gland base cells express pathways for Cl^- and water secretion. Antral mucosa from normal adult mice were stained with H&E or with antibodies against NKCC, aquaporin-5 (AQP5), cystic fibrosis transmembrane conductance regulator (CFTR), or pepsinogen. Immunolabeling was visualized with Alexa fluor 488 conjugated to anti-rabbit IgG (NKCC, CFTR, AQP5) or with immunoperoxidase deposition of diaminobenzidine substrate and hematoxylin counterstain [CFTR or pepsinogen (Pep); bottom]. Specific immunolabeling was largely confined to the apical margin (AQP5, CFTR, pepsinogen) or basolateral margin (NKCC) of cells comprising the terminal segment of the antral gland. Adjacent sections exposed to secondary antibodies alone were essentially blank (bottom left). Bars = 25 μm .

parameters and acid secretion in an Ussing chamber (Figs. 7 and 8).

Two types of experiments were carried out. In the first set (Fig. 7), we waited 45–90 min until the V_t rose to a steady plateau of approximately -7 mV. The emergence of this lumen-negative voltage was attributable to the appearance of an amiloride-sensitive cation conductance, as observed in other animal models (43, 46). Stimulation with 8-Br-cAMP evoked four responses: 1) a rapid and transient increase in V_t , 2) a subsequent decrease in V_t to -4 mV, 3) a drop in mucosal resistance, and 4) an increase in acid secretion that peaked after 20 min. At this 20-min time point, I_{sc} was similar to that before stimulation, despite underlying alterations in multiple ion transport processes after cAMP stimulation. As in other animal species, transition to the acid-secreting state was accompanied by a measurable decrease in mucosal resistance, presumably associated with an expansion of the apical secretory surface. Application of mucosal amiloride (10 μM) reduced both I_{sc} and V_t by $\sim 50\%$ but did not alter acid output, indicating that a major source of current in the corpus epithelium is active amiloride-sensitive Na^+ absorption. In other experiments, we

Fig. 4. Gastric fluid and electrolyte secretion in pentagastrin-stimulated normal and NKCC-deficient mice. Data represent means $\pm$ SE of measurements on 12 pairs of age-matched NKCC $^{+/+}$ and NKCC $^{-/-}$ mice after administration of pentagastrin (16 $\mu\text{g}/\text{kg}$ iv). Net secretion is expressed as the quantity of H^+ or fluid accumulated in the gastric lumen 2 h after pylorus ligation, normalized to the dry weight of the emptied stomach. Deletion of NKCC significantly impaired secretion of Na^+ , K^+ , Cl^- , and fluid, but not of H^+ . Significantly different from NKCC $^{+/+}$: * $P < 0.01$, ** $P < 0.001$.

Fig. 5. CFTR deletion does not affect pentagastrin-stimulated gastric fluid and electrolyte secretion. Data represent means $\pm$ SE of measurements on 11 heterozygous CFTR mice (CFTR^{+/+}) and 9 homozygous mutant CFTR littermates (CFTR^{-/-}). All mice were fed standard chow and water containing Colyte laxative. Secretion was measured by the pylorus ligation technique as described in Fig. 4. None of the measured parameters was significantly affected by CFTR knockout.

noted that the amiloride-sensitive component of I_{sc} became progressively smaller after 8-Br-cAMP stimulation (data not shown), suggesting that electrogenic Na⁺ absorption is down-regulated under acid-secreting conditions, consistent with previous observations with piglet gastric mucosa (19). Addition of a concentration of bumetanide that fully inhibits Na-K-2Cl cotransport (100 μ M) to the serosal bath decreased V_t and I_{sc} further without affecting acid secretion. Finally, addition of DIDS reduced both V_t and I_{sc} further and abolished acid secretion. It should be noted that the concentration of DIDS added (1 mM) would be sufficient to fully inhibit the three members of the SLC4 anion exchanger family (AE1, AE2, and AE3) (34, 41) known to exist in the rodent gastric mucosa (78) as well as SLC26A7, a Cl⁻/HCO₃⁻ exchanger (62) or channel (39) found in the basolateral membrane of parietal cells.

A second series of experiments focused on the bumetanide-sensitive component of I_{sc} (Fig. 8). Rather than waiting for the Na⁺ conductance to develop fully, we stimulated secretion as soon as a steady low rate of acid secretion was observed. Addition of the cAMP-dependent secretagogue forskolin evoked concomitant increases in acid output (Fig. 8A) and I_{sc} (Fig. 8B). As in the previous series of experiments, inhibition of NKCC with serosal bumetanide reduced electrogenic ion flow to near basal levels without affecting acid secretion.

Fig. 6. Gastric secretion of pepsinogen is impaired in NKCC-deficient mice but normal in CFTR-deficient mice. Gastric samples collected in the pyloric ligation experiments presented in Figs. 4 and 5 were analyzed for pepsin activity by a proteolysis assay using acid-denatured hemoglobin as substrate (see MATERIALS AND METHODS). Data were normalized to the dry weight of the emptied stomach. NKCC-deficient mice secreted significantly less pepsin than normal mice (* P < 0.01).

Fig. 7. Electrophysiological responses of isolated mouse gastric mucosa to 8-bromo-cAMP (8-Br-cAMP) and to ion transport inhibitors. After dissection from the mouse corpus, the mucosal layer was mounted in an Ussing chamber and allowed to equilibrate for at least 30 min in the presence of indomethacin and tetrodotoxin. Electrical parameters and acid secretory rates were recorded 20 min after addition of 1 mM 8-Br-cAMP (serosal), then 10 μ M amiloride (luminal), then 100 μ M bumetanide (serosal), and finally 1 mM DIDS. V_t , transepithelial voltage; R_t , transepithelial resistance; I_{sc} , short-circuit current.

Together, these results suggest that, in the mouse corpus, nonacidic Cl⁻ secretion is an electrogenic process that involves Na-K-2Cl cotransport, whereas H⁺-linked Cl⁻ secretion is a macroscopically electroneutral process that does not.

We conducted a limited set of preliminary experiments to assess whether the NKCC-mediated Cl⁻ transport also contributes to V_t and I_{sc} in the resting state. Exposing the unstimulated mouse corpus to luminal amiloride (10 μ M) reduced both V_t and I_{sc} significantly (data not shown). Serosal bumetanide (100 μ M) also reduced V_t and I_{sc} , although to a lesser extent. Thus NKCC-dependent Cl⁻ secretion appears to be active in resting mucosa and further activated by cAMP-dependent stimulation.

DISCUSSION

The findings presented here establish that NKCC contributes importantly to nonacidic electrogenic ion secretion in the mouse stomach. This conclusion is supported by two lines of evidence: 1) in mice genetically devoid of NKCC, gastric

Fig. 8. Electrophysiological responses of isolated mouse gastric mucosa to forskolin and NKCC inhibition. Mucosal sheets were isolated from mouse gastric corpus and mounted in Ussing chambers. A: acid secretory rate measured at successive 2.5-min intervals in response to $10 \mu\text{M}$ forskolin added to the serosal bath at 25 min. At 65 min, $50 \mu\text{M}$ bumetanide was (●) or was not (○) added to the serosal bath. Inhibition of NKCC was without significant effect on acid output. B: equivalent I_{sc} monitored at 5-min intervals ($n = 6$).

secretions of Na^+ , K^+ , Cl^- , water, and pepsinogen are markedly impaired, whereas secretion of HCl is normal; and 2) in mucosae isolated from the gastric corpus, inhibition of Na-K-2Cl cotransport with bumetanide substantially reduces cAMP-stimulated I_{sc} but not acid secretion.

The most probable source of this nonacidic electrogenic ion secretion is parietal cells. These cells, individually and collectively, are the most prominent site of NKCC expression in the gastric mucosa of most animal species thus far examined, including the mouse (Figs. 1 and 2), rat (49), and rabbit and human (C. Lytle, unpublished observations). The concept that NKCC functions as a dominant basolateral pathway for Cl^- uptake in parietal cells has recently been substantiated by

preliminary experiments on isolated rat gastric glands (42). Although mucous neck cells could be a second source of NKCC-dependent ion secretion in the mouse stomach, their contribution is probably of lesser importance because parietal cells outnumber mucous neck cells 5:1 (33); in some animal species, including the rat and rabbit, mucous neck cells are devoid of NKCC (Ref. 49 and unpublished observations).

Our finding that Cl^- taken up by NKCC is not utilized for the purpose of acid secretion (HCl) corroborates earlier observations that gastric acidification is not impaired in NKCC knockout mice (18) and that histamine-stimulated aminopyrine accumulation by isolated rabbit gastric glands (50) and acid secretion by chambered eel gastric mucosa (76) are not inhibited by doses of bumetanide that abolish Na-K-2Cl cotransport. Contrasting results have been obtained in two previous studies with different animal models. First, in chambered mucosa isolated from the guinea pig stomach, serosal application of 1 mM furosemide inhibited acid secretion along with transmucosal current and Cl^- secretion (5). This effect, however, seems unrelated to Na-K-2Cl cotransport because we have been unable to detect NKCC protein in guinea pig parietal cells (unpublished observations). Second, in gastric mucosa isolated from the amphibian *Necturus*, serosal application of $50 \mu\text{M}$ bumetanide blocked acid secretion (71). In *Necturus*, both acidic and nonacidic Cl^- secretions appear to originate from the oxyntopeptic cell (21). Thus, unlike its mammalian counterpart, the amphibian oxyntopeptic cell appears to utilize Na-K-2Cl cotransport, rather than $\text{Cl}^-/\text{HCO}_3^-$ exchange, to import Cl^- destined for secretion with H^+ . Evidently, this is not a feature of all amphibian oxyntopeptic cells, because acid secretion by bullfrog gastric mucosa was found to be insensitive to bumetanide (28).

When the corpus of the mouse stomach is separated from the antrum, stripped of its serosal layer, and incubated in an Ussing chamber, it generates a mucosal negative potential difference and an I_{sc} comprising two major components: one inhibited by mucosal amiloride and the other by serosal bumetanide (Figs. 7 and 8). The amiloride-sensitive component, now recognized as electrogenic Na^+ absorption, has been observed in gastric preparations from the rat (15), pig (19), monkey (75), and lizard (31). This absorptive process appears to be a property of mucus-secreting surface epithelial cells that line the corpus and antrum; the strongest evidence for spatial segregation of Na^+ absorptive and Cl^- secretory functions has come from electrophysiological studies of amphibian (*Necturus*) gastric mucosa (16). The second component of I_{sc} largely reflects nonacidic Cl^- secretion driven by Na-K-2Cl cotransport, as discussed below.

Our results support earlier speculation (38, 47, 49, 52, 76) that parietal cells function in two operationally distinct modes of Cl^- secretion: one coupled to H^+ secretion and the other nonacidic and electrogenic. What remains uncertain is whether both modes coexist in all parietal cells or whether they occur in spatially or functionally distinct populations. We (49) have proposed that HCl secretion predominates in parietal cells that migrate from the isthmus toward the gastric pit (which express abundant AE2 but no NKCC), whereas nonacidic Cl^- transport predominates in parietal cells that migrate into the gland base (which express abundant NKCC but diminished levels of AE2). The concept that parietal cells nearest the surface are far more active in HCl secretion than those deep in the gland fits

evidence that base parietal cells are morphologically unresponsive to acid secretagogues (35, 36, 38) with $H^+-K^+-ATPase$ units that appear to be catalytically dormant (13) and refractory to physiological stimulation (14). This concept does not preclude the possibility that NKCC-expressing parietal cells in the neck and base retain some capacity, albeit diminished, to secrete acid. Indeed, acidification of the secretory canaliculus and the adjacent luminal compartment can be visualized with fluorescent dyes (2, 4, 8, 23, 61) in parietal cells inhabiting all segments of the rabbit gastric gland after stimulation with histamine. On the other hand, in preliminary experiments on isolated rabbit gastric glands, we have noted spatial and temporal differences in the onset, rate, and extent of histamine-stimulated acidification consistent with the idea that acid secretion in the gland base is generally weaker. Further experiments are needed to test the possibility that base parietal cells operate in two modes of Cl^- secretion: acidic and nonacidic.

Another site of NKCC-dependent electrolyte and pepsinogen secretion might be the gland cells that inhabit the base of each antral mucous unit (44). Because they are richly equipped with pathways for basolateral Cl^- entry (NKCC), apical Cl^- exit (CFTR), and osmosis (AQP5), it seems likely that their principal function is electrogenic Cl^- secretion. We reasoned that this component of Cl^- secretion might be selectively absent from CFTR-deficient mice, given that antral base cells are the only detectable site of CFTR expression in the mouse stomach and that CFTR function is required for active Cl^- secretion in similarly configured intestinal crypt cells (25). Indeed, a severe impairment in nonacidic gastric fluid secretion has been reported in patients with cystic fibrosis (30). However, we found no difference in the character of gastric secretion between mice with or without CFTR function. Evidently, antral contributions to gastric electrolyte secretion in the mouse are either negligible or not dependent on CFTR.

Our observation that pepsinogen secretion depends partly on NKCC is especially intriguing because chief cells do not themselves express the cotransporter. Evidently, the observed dependency involves events beyond the formation and exocytotic release of pepsinogen by the zymogenic cells that inhabit the neck and base of the gastric glands. We (49) have postulated that the NKCC-dependent secretion of neutral fluid by base parietal cells serves to facilitate bulk transport of pepsinogen from its site of secretion (base chief cells) to its site of action (stomach lumen). Thus the nonacidic secretory stream would not only flush pepsinogen through the narrow gland lumen but also delay its activation until the proenzyme reaches the highly acidic environment adjoining superficial parietal cells. This delayed activation would presumably safeguard deeper gland cells (which lack a protective apical mucous coating) from proteolytic attack. It follows from the "flushing" concept that impairment of the secretory stream would impede pepsinogen clearance from the gland lumen. As predicted, gastric pepsinogen secretion was reduced in NKCC-deficient mice. Impairment of pepsinogen clearance, in turn, might allow premature activation of pepsinogen followed by local proteolytic injury. However, no focal damage or inflammation was evident in NKCC-deficient mice. An alternative possibility is that the NKCC-dependent component of pepsinogen secretion originates from antral base cells, rather than chief cells, through processes that do not depend on CFTR.

If parietal cells situated deep in the gland base are in fact a significant source of gastric acid, the NKCC-dependent secretory stream might also serve to flush this acid from the gland lumen. However, elimination of this stream by genetic ablation or inhibition of NKCC did not impair acid secretion (Figs. 4, 7, and 8). This result suggests that luminal convection/diffusion does not limit the rate of H^+ secretion, as surmised previously (63), and is compatible with the notion that acid is mainly secreted by superficial parietal cells in close proximity to the gastric pit.

Early studies on the gastric secretory responses to histamine and acetylcholine in humans and dogs showed that acid secretion is accompanied by a marked increase in K^+ movement to the gastric fluid (77). The mechanisms and cellular sources of this K^+ secretion remain poorly understood (21). It might reflect "slippage" in apical K^+ recycling by parietal cells, i.e., imperfect functional coupling between the H^+/K^+ exchange pump and adjacent K^+ channels. Our results indicate that in the mouse, gastric K^+ secretion depends almost entirely on NKCC (Fig. 4). This implicates parietal cells or antral base cells as potential sources of K^+ secretion. It has been suggested that diffusional restrictions within the gastric gland lumen limit the rate of K^+ (but not H^+) flux between the epithelial cells and the stomach lumen (63). If this proves correct, the impairment of bulk K^+ secretion that we observed in NKCC-deficient mice could reflect a loss of NKCC-dependent convective flushing of the gland lumen.

Although NKCC-dependent Cl^- secretion is not associated with H^+ secretion, it is likewise stimulated by cAMP-dependent secretagogues such as histamine (76) and forskolin (Fig. 8). There is some evidence that it may be subject to regulation by other signals: in the rat gastric corpus, for example, peripheral-type benzodiazepine receptor agonists have been found to stimulate bumetanide-sensitive electrogenic Cl^- secretion without influencing acid secretion (56). This mitochondrial receptor is expressed only in surface epithelial cells and basilar parietal cells of the rat stomach (56); the coexistence of this receptor and NKCC only in basilar parietal cells supports the notion that bumetanide-sensitive electrogenic Cl^- secretion originates from this subset of parietal cells.

Together, our results suggest that parietal cells somehow selectively utilize the inward flow of Cl^- via Na-K-2Cl cotransport (or the outward electrochemical Cl^- gradient generated by this transporter) for the purpose of nonacidic electrogenic ion secretion.

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