Secretagogue stimulation enhances NBCe1 (electrogenic Na\(^+\)/HCO\(_3\)^− cotransporter) surface expression in murine colonic crypts

Haoyang Yu,1 Brigitte Riederer,1 Nicole Stieger,1 Walter F. Boron,2 Gary E. Shull,3 Michael P. Manns,1 Ursula E. Seidler,1 and Oliver Bachmann1

1Department of Gastroenterology, Hepatology, and Endocrinology, Hannover Medical School, Hannover, Germany; 2Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, Ohio; 3Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, Ohio

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Yu H, Riederer B, Stieger N, Boron WF, Shull GE, Manns MP, Seidler UE, Bachmann O. Secretagogue stimulation enhances NBCe1 (electrogenic Na\(^+\)/HCO\(_3\)^− cotransporter) surface expression in murine colonic crypts. Am J Physiol Gastrointest Liver Physiol 297: G1223–G1231, 2009. First published September 24, 2009; doi:10.1152/ajpgi.00157.2009.—A Na\(^+\)/HCO\(_3\)^− cotransporter (NBC) is located in the basolateral membrane of the gastrointestinal epithelium, where it imports HCO\(_3\)^− during stimulated anion secretion. Having previously demonstrated secretagogue activation of NBC in murine colonic crypts, we now asked whether vesicle traffic and exocytosis are involved in this process. Electrogenic NBCe1-B was expressed at significantly higher levels than electroneutral NBCn1 in colonic crypts as determined by QRT-PCR. In cell surface biotinylation experiments, a time-dependent increase in biotinylated NBCe1 was observed, which occurred with a peak of +54.8% after 20 min with forskolin (P < 0.05) and more rapidly with a peak of +59.8% after 10 min with carbachol (P < 0.05) and which corresponded well with the time course of secretagogue-stimulated colonic bicarbonate secretion in Ussing chamber experiments. Accordingly, in isolated colonic crypts pretreated with forskolin and carbachol for 10 min, respectively, and subjected to immunohistochemistry, the NBCe1 signal showed a markedly stronger colocalization with the E-cadherin signal, which was used as a membrane marker, compared with the untreated control. Cytochalasin D did not change the observed increase in membrane abundance, whereas colchicine alone enhanced NBCe1 membrane expression without an additional increase after carbachol or forskolin, and LY294002 had a marked inhibitory effect. Taken together, our results demonstrate a secretagogue-induced increase of NBCe1 membrane expression. Vesicle traffic and exocytosis might thus represent a novel mechanism of intestinal NBC activation by secretagogues.

The colonic epithelium actively secretes HCO\(_3\)^− against an electrochemical gradient (13, 15). HCO\(_3\)^− is extruded across the apical membrane both electrogenically as well as electroneutrally in exchange for Cl− or short-chain fatty acids (49). During secretory diarrhea, excessive HCO\(_3\)^− secretion in the small and large intestine propagates metabolic acidosis (6).

The rate-limiting step for intestinal and pancreatic HCO\(_3\)^− secretion is intracellular supply by CO\(_2\) hydration or basolateral uptake (24, 25). Na\(^+\)/HCO\(_3\)^− cotransport (NBC) (8), which was initially cloned from the kidney (36), is found in the basolateral membrane of the colonic epithelium and is believed to mediate the uptake of HCO\(_3\)^− destined for secretion. This concept is supported by experiments demonstrating that colonic HCO\(_3\)^− secretion is dependent on serosal CO\(_2\) (47, 49) and inhibited by basolateral addition of stilbenes (20). Furthermore, duodenal NBC together with basolateral anion exchange was suggested to be an alternative Cl− uptake pathway (50), and the proximal colon of the NBCe1-deficient mouse exhibits defective anion secretion (21).

We previously showed that NBC in murine colonic crypts can be directly activated by cAMP-dependent and cholinergic secretagogues (4, 5), a process probably involving phosphorylation of the electrogenic NBC subtype NBCe1-B (22) and Ca\(^{2+}\)/calmodulin- and PKC-dependent signaling (4), respectively. Notably, however, Ussing chamber experiments using murine duodenum (50) and colon (14) showed a rapid increase in short-circuit current (Isc) as a measure of net total anion secretion, whereas duodenal HCO\(_3\)^− secretion, as measured by the pH-stat technique, increased rather slowly (44, 50). This might indicate that phosphorylation of constitutively expressed NBC is not the only mechanism of its activation but that other pathways explaining a delayed onset of intestinal HCO\(_3\)^− secretion are involved.

Recently, Perry et al. (32) provided evidence for NBCe1 endocytosis into cytoplasmic vesicles occurring after cholinergic stimulation in a salivary gland cell line, which was interpreted as part of the transition to a new steady-state during stimulated secretion and which indicates that vesicle traffic plays some role in NBCe1 regulation. It is still unclear, however, whether an increase in NBCe1 membrane abundance occurs during the early phase of secretagogue stimulation, which precedes the delayed endocytosis observed in salivary glands and which could represent a novel mechanism of NBC activation in the colon. In this study, we therefore addressed the question whether vesicle traffic and exocytosis of NBC is involved in the activation of NBC by secretagogues in murine colonic crypts.

MATERIALS AND METHODS

Materials. In this study, we used the polyclonal anti-NBCe1 antibody K1A (43), which recognizes the cytoplasmic COOH terminus common to the NBCe1-A and NBCe1-B subtypes. The anti-E-cadherin antibody, Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 633 goat anti-rat IgG, Nigericin, and 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein (BCECF/AM) were from Invitrogen (Karlsruhe, Germany), and the anti-β-actin and anti-villin antibody were from Abcam (Cambridge, UK). Forskolin, carbachol, cytochalasin D, and colchicine were from Sigma (Deisenhofen, Germany). All other chemicals were either obtained from Sigma or from Merck (Darmstadt, Germany) at tissue culture grade or the highest grade available.

Address for reprint requests and other correspondence: O. Bachmann, Hannover Medical School, Dept. of Gastroenterology, Hepatology, and Endocrinology, Carl-Neuberg-Str. 1, 30625 Hannover, Germany, (e-mail: bachmann.oliver@mh-hannover.de).

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Animals. C57BL/6 mice aged 3 mo were used for all experiments except the antibody testing, for which NBCe1-deficient mice [previously characterized (21) and grown on a mixed 129S6/SvEv and Black Swiss background] and NBCn1-deficient mice [generated in the laboratory of Christian Aukjer, Aarhus University, Denmark (7) and grown on a mixed 129S6/SvEv and Black Swiss background] and their littermates were utilized. Because the NBCe1-deficient animals die at a very early age attributable to metabolic disturbances, the colony of NBCe1+/− and NBCe1−/− mice aged 14 days was prepared. Genotyping was carried out as previously described (21). All animals were kept in the animal facility of the Medical School of Hannover under standardized light and climate conditions and had access to water and chow ad libitum. Animal experiments followed approved protocols at the Medical School of Hannover and the local authorities for the regulation of animal welfare (Niedersächsisches Landesministerium für Verbraucherschutz und Lebensmittelsicherheit).

Preparation of colonic crypts. Murine colonic crypts were isolated exactly as previously described (4) from a 3–4-cm proximal colonic segment filled with and immersed in EDTA-containing solution (in mM: 127 NaCl, 5 KCl, 1 MgCl2, 5 sodium pyruvate, 25 glucose, 1% EDTA, and 1% BSA, pH 7.4, gassed with 100% O2, 37°C, 15 min), excised after CO2 narcosis and cervical dislocation, and stored in buffer A (in mM: 120 NaCl, 14 HEPES, 7 Tris, 3 K2HPO4, 2 K2HPO4, 1.2 MgSO4, 1.2 calcium gluconate, and 20 glucose, pH 7.4, gassed with 100% O2) on ice until use.

**RT-PCR.** RT-PCR for NBCe1-B and NBCe1-A was performed for 30 cycles (denature 94°C, 30 s; anneal 58°C, 30 s; extend 72°C, 180 s) using the previously published primers (3) 5′-ATGGTGTGTGATGAAAGAGAAGTAAGAG-3′ and 5′-CAGCTGAAGATGGAAGGAGAAGAC-3′, respectively, as well as the common antisense primer 5′-GACGGAGGGTTTTTCC-3′ and the primer of the PCR products was verified by restriction enzyme digest.

**Quantitative real-time PCR.** PCR primers were designed using the “Primer Express” software (Applied Biosystems, Foster City, CA). Primer sequences were as follows: NBCe1 for: 5′-CTCACTCTTCTGTGTTTGCTC-3′, NBCe1 rev: 5′-GGTGTGAGGAAATATGAC-3′, PCR-product length 298 bp, NBCn1, solute carrier family 4, member 7, (NBCn1, solute carrier family 4, member 7, Slc4a7, 103 bp, (NBCn1, solute carrier family 4, member 7, Slc4a7, 103 bp), and the colon homogenates from NBCe1 knockout and wild-type mice were size fractionated as described by Hillesheim et al. (23). Blots were probed with anti-NBCe1 antibody (K1A, 1:1,000 in TBS-Tween), anti-β-actin antibody (1:15,000) and anti-villin antibody (1:10,000) for 1 h at room temperature. The antigen-antibody complexes on the PVDF membranes were visualized by chemiluminescence (ECL Western blotting detection reagents, Amersham Pharmacia Biotech), and the image was captured on light-sensitive imaging film (Hyper film ECL, Amersham Biosciences). Antibody specificity testing was done with the use of homologous tissues from NBCe1−/− and wild-type littermates (Fig. 3A).

**Immunoblotting.** The protein samples obtained after biotinylation and the colon homogenates from NBCe1 knockout and wild-type mice were size fractionated as described by Hillesheim et al. (23). Blots were probed with anti-NBCe1 antibody (K1A, 1:1,000 in TBS-Tween), anti-β-actin antibody (1:15,000), or anti-villin antibody (1:10,000) and incubated overnight at 4°C. The secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase, KPL) was diluted (1:10,000 for anti-NBCe1, 1:20,000 for anti-β-actin and antivillin) in TBS-Tween and incubated for 1 h at room temperature. The antigen-antibody complexes on the PVDF membranes were visualized by chemiluminescence (ECL Western blotting detection reagents, Amersham Pharmacia Biotech), and the image was captured on light-sensitive imaging film (Hyper film ECL, Amersham Biosciences). Antibody specificity testing was done with the use of homologous tissues from NBCe1−/− mice and their wild-type littermates (Fig. 3A).

**Immunohistochemistry.** For the experiments involving NBCe1−/−/ NBCn1−/− mice and their littermates (Fig. 3D), the colon was prepared as described in Ref. 23. To study NBCe1 trafficking, isolated murine colonic crypts were immediately fixed in 4% paraformaldehyde for 15 min, washed with PBS, and attached to poly-l-lysine-coated cover slides. After permeabilization with 0.1% Triton X-100 for 30 min, the cover slides were treated with blocking solution (10% goat serum, 1% BSA in PBS) at room temperature for 2 h and washed with wash buffer (50 mM NH4Cl in PBS). Crypts or cryosections were then incubated 24 h at 4°C with the anti-NBCe1 (diluted 1:500) and anti-E-cadherin (only ckey preparations, diluted 1:500) antibodies in antibody diluent (1% goat serum, 0.1% BSA in PBS). Subsequently, the cover slides were incubated with the secondary antibodies, Alexa Fluor 488 goat-anti-rabbit IgG (diluted 1:2,000 in antibody diluent) and Alexa Fluor 633 goat anti-rat IgG (only ckey preparations, diluted 1:1,000 in antibody diluent), at room temperature for
ANoVA was used for multiple comparisons (ANoVA for correlated unpaired form, where appropriate, was used for pair-wise tests, and of quantitative PCR data was carried out using the efficiency-cali-

Microfluorometry. Measurement of intracellular pH (pH_i) was carried out using a video imaging system exactly as previously described (4). Briefly, after being loaded with 5 μM BCECF for 20 min, crypts were fixed between a glass coverslip and a polycarbonate membrane in a custom-made perfusion chamber, mounted onto the heated stage of an inverted microscope (Zeiss Axiovert 200; Carl Zeiss, Jena, Germany), and perfused continuously following the appropriate protocol (in buffer C, 40 mM NaCl of buffer B was replaced by 40 mM NH_4Cl; in buffer D, NaCl and NaHCO_3 of buffer B were replaced by tetramethylammonium chloride and choline-

RESULTS

We first sought to clarify which NBC isoform is the predominant one in the colonic epithelium. Expression of the electrogenic NBC splice variant NBCe1-B has previously been demonstrated by RT-PCR and in situ hybridization (5), and expression of electroneutral NBCn1 was recently shown via β-galactosidase staining in an NBCn1 transgenic mouse model (7) in this location. Quantitative PCR with cytokeratin 18 as an internal control revealed a significantly stronger NBCe1 than NBCn1 expression in mouse crypts (Fig. 1A), similar to what we had found in a previous study using semiquantitative PCR (5). Even considering the difficulty of comparing the expression levels of two mRNA species (37), this result is in agreement with a prominent role of NBCe1-B in murine colonic epithelium. Next, we investigated the distribution of NBCe1 splice variants in isolated colonic crypts and renal tissue and performed PCR experiments using NBCe1-A and NBCe1-B-specific primers (Fig. 1B). As in other species (3, 38), NBCe1-A was exclusively found in the kidney, whereas NBCe1-B was found in both colon, where a stronger signal was detected, and in the kidney. This experiment confirms that NBCe1-B is the only NBCe1 subtype expressed in colonic crypts.

Because NBC is believed to participate in intestinal epithelial anion secretion, we performed Ussing chamber experiments with epithelial sheets from murine colon to characterize the time course of colonic epithelial I_sc and HCO_3^- secretion. Although forskolin and carbachol elicited a prompt I_sc response, which was sustained in the case of forskolin but transient in the case of carbachol, the increase in HCO_3^- secretion was gradual in both instances and reached its maximum after 5–10 min (Fig. 2).

In search for further regulatory pathways potentially explaining this delayed onset of colonic HCO_3^- secretion, we studied NBCe1 plasma membrane expression by cell surface biotinylation during secretagogue stimulation in murine colonic crypts after having excluded cross reactivity of the NBCe1 antibody with other epitopes (e.g., NBCn1) using NBCe1 and NBCn1 knockout mouse tissue (Materials and Methods, Figs. 3, A and D). Crypts were incubated with forskolin or carbachol for different time points ranging from 0 to 20 min. Indeed, the amount of NBCe1 attached to streptavidin beads in relation to whole cell lysate and detected by Western blot increased significantly with time (Fig. 4). This increase reached its maximum after 10 min with forskolin (Fig. 4A) and after 5 min with carbachol (Fig. 4B).

To substantiate this finding further, immunohistochemical studies were carried out using isolated colonic crypts stained with anti-NBCe1 and anti-E-cadherin (as a basolateral membrane marker, Ref. 32). Having established in the biotinylation
results of these experiments that significant NBCe1 exocytosis occurs after 10 min with both secretagogues, crypts were preincubated with vehicle, forskolin, or carbachol for this time span before fixation and permeabilization. NBCe1 staining followed a basolateral pattern (Fig. 5, A, D, and G), which was already observed in the tissue sections from NBCe1 knockout mice for antibody specificity testing (see MATERIALS AND METHODS, Fig. 3D). However, closer examination shows that, under control conditions, the NBCe1 staining is present, not only over the E-cadherin staining, but also deeper in the cell (Fig. 5, A–C). What is particularly interesting is that each secretagogue causes the NBC pattern to overlap with that of E-cadherin (Fig. 5, D–I). Analysis of the integrated fluorescence intensity in the normalized intensity traces revealed that the NBCe1 signal is distributed significantly more widely within the cell than the E-cadherin signal under control (Fig. 5C) but not under stimulated conditions (Fig. 5, F and I).

Cytoskeletal elements are involved in vesicle trafficking of various electrolyte transporters (17, 19, 30, 41, 45), including renal NBC (19). We therefore investigated the effect of agents interfering with the cytoskeleton and endo/exocytosis on the secretagogue-dependent increase in NBCe1 membrane abundance in biotinylation experiments (Fig. 6). After a 10-min incubation with the inhibitor of actin polymerization cytochalasin D, crypts were treated for an additional 10-min interval with either vehicle, forskolin, or carbachol. However, cytochalasin D neither changed baseline membrane expression significantly nor prevented the stimulatory effect of secretagogues (Fig. 6A). To verify whether short-time incubation with cytochalasin D causes actin filament disruption in isolated crypts, phalloidin staining with and without exposure to cytochalasin D for 10 min was analyzed. Whereas the control tissue showed a strong membrane signal, phalloidin staining was overall markedly weaker with a patchy intracellular pattern after cytochalasin D treatment (Fig. 6B). As opposed to cytochalasin D, the microtubule inhibitor colchicine significantly increased baseline NBCe1 membrane abundance to the level that otherwise would have been seen in the presence of a secretagogue. Moreover, in the presence of colchicine, neither forskolin nor carbachol had an additional effect (Fig. 6C). Because colchicine has been shown to inhibit endocytosis in other experimental systems (18, 28, 42), we tested the endocytosis inhibitor chlorpromazine, which caused a time-dependent increase in NBCe1 membrane abundance (Fig. 6D). The phosphatidylinositol 3 (PI3)-kinase inhibitor LY294002, which has been shown to mediate trafficking events (29), significantly reduced NBCe1 membrane, with a still significant, but probably diminished, increase after secretagogues (Fig. 6E).

Next, we carried out functional studies to quantify NBC activity with and without colchicine in isolated murine colonic crypts. In microfluorometric NH4+ prepulse experiments (9) with the pH-sensitive dye BCECF (35) and in the presence of CO2/HCO3− as well as 500 μM DMA, which completely inhibits Na+/H+ exchanger (NHE) activity (4), proton flux rates were comparable to the values we had measured previously. However, colchicine did not significantly alter acid-activated NBC activity (Fig. 7) compared with the untreated control. Thus, despite increased membrane expression, colchicine does not lead to an increase in acid-activated NBC transport rates.

DISCUSSION

Recent studies have revealed that the NBCe1 Na+/HCO3− cotransporter is an important component in the complex process of intestinal epithelial anion secretion (21, 25) and can be directly activated by secretagogues (4, 5, 22). In this study, we were able to demonstrate NBCe1 translocation into the plasma membrane of murine colonic crypts during stimulation with forskolin and carbachol, which represents a novel regulatory mechanism for colonic NBC.

Although the presence of the NBCe1-B subtype of the electrogenic isoform NBCe1 in the colonic epithelium has been established some time ago (1, 5), the possible relevance of other NBCs is unclear, one candidate being electroneutral NBCn1. On a transcriptional level, however, NBCn1 mRNA abundance in colonic crypts was very low compared with NBCe1-B, which might translate into a higher functional relevance of electrogenic NBC. Consistent with these findings is the anion secretory defect found in the proximal colon of the NBCe1-deficient mouse (21).
Vesicle traffic and exo-/endocytosis is an increasingly recognized regulatory mechanism for intestinal ion transporters and has been demonstrated for the apical isoform NHE3 (17), the cystic fibrosis transmembrane conductance regulator (26), the epithelial Na+/H+ channel (11), and the basolateral Na+/H+/K+2Cl− cotransporter 1 (16, 34). Whether vesicle traffic and endo-/exocytosis are also relevant for the NBC of intestinal epithelial cells has so far been unresolved. It is known that a cytoplasmic motif within the NBCe1 structure is necessary for correct basolateral targeting (27). The renal NBCe1 subtype NBCe1-A, which has been studied in more detail, is redistributed to the surface of renal cells during CO2 exposure to counteract acidification (19). Furthermore, NBCe1-A and NBCe1-B were shown to be adaptively redistributed in response to chronic alkalosis in an immortalized rat proximal tubular cell line to favor decreased HCO3− absorption (10). Very recently, regulation of endogenous NBCe1-B by subcellular redistribution in response to carbachol was reported in the rat parotid acinar cell line ParC5 (32). Cholinergic stimulation is known to stimulate salivary gland HCO3− secretion, and NBC is expressed in the basolateral membrane of salivary gland cells (10, 38), where it presumably imports Na+ and

Fig. 3. Antibody specificity testing and validation of cell surface biotinylation in native murine colonic crypts. A: anti-NBCe1 antibody K1A detected a band of the expected size in the Western blot only in protein preparations from the colon of suckling NBCe1+/+ mice but not of NBCe1−/− mice. B: to test whether most of the biotinylated NBCe1 protein had been pulled down after incubating the lysate twice with streptavidin beads, all fractions were individually subjected to Western blot (L: lysate; B1, first beads fraction; B2, second beads fraction; S: supernatant, pipetted volume in μl). Clearly, the S2 fraction contained only a very small amount of NBCe1, indicating that, after two incubations with streptavidin beads, no significant amounts of biotinylated NBCe1 are left in the sample. C: in addition to viability testing by Trypan blue exclusion (not shown), cell integrity of isolated crypts was tested by probing lysate and bead samples with anti-β-actin and villin as an intracellular marker proteins. At the incubation times used in the subsequent experiments, no β-actin or villin was detected in the bead fraction, indicating that the tested intracellular proteins are not biotinylated during the procedure. D: to test antibody specificity for immunohistochemistry, frozen sections of the colon of NBCe1+/+ and NBCe1−/− mice and their healthy littermates were stained with anti-NBCe1 (green, NBCe1; red, propidium iodide). In NBCe1+/+ tissue, but not NBCe1−/− tissue, an NBCe1 signal with a basolateral staining pattern was detected and was unchanged in the absence of NBCn1.

Fig. 4. NBCe1 surface expression in response to secretagogues. A: immediately after preparation and microscopic evaluation, 4 different fractions were incubated either 20 min with vehicle (DMSO for the forskolin experiment, H2O for the carbachol experiment, 1st column), 15 min with vehicle followed by 5 min with forskolin (10−5 M, 2nd column), 10 min with vehicle followed by 10 min with forskolin (3rd column), or 20 min with forskolin (4th column). Relative surface expression was calculated after the biotinylation protocol (see MATERIALS AND METHODS) by dividing the integrated optical density (ODI) of the “beads” band by the ODI of the respective “lysate” band. The initial value was set to 100%. Relative NBCe1 content in the beads fraction increased over time and was significantly higher than the basal value at 10 and 20 min of forskolin. B: in another set of experiments, carbachol (10−5 M) was used instead of forskolin. The increase in surface NBCe1 occurred faster than with forskolin and appeared somewhat more transient although the latter observation did not reach statistical significance (n = 5 preparations from separate mice in each group, *P < 0.05, ANOVA for correlated samples followed by Tukey’s honestly significant difference test, HSD).
HCO₃⁻. After 15 or 30 min of carbachol, NBCe1 had moved from the cell surface into early endosomes, as assessed by biotinylation and immunohistochemistry (32). This endocytosis of NBCe1 was interpreted as part of the transition to a sustained phase of fluid secretion following secretagogue stimulation. NBCN1, on the other hand, was found to be constitutively expressed.

Although there are thus clues that vesicle traffic is involved in NBC regulation in renal and salivary gland cells (19, 32), it is unclear whether NBCe1 translocates to the basolateral membrane from a cytoplasmic compartment in the colonic epithelium during the early phase of stimulation with secretagogues. In this context, it has to be kept in mind that 1) the renal and pancreatic/intestinal NBCs largely differ in their primary structure, function (1, 22), and regulation (4, 5, 39, 40) and that 2) NBCe1-B regulation is strongly dependent on the expressing cell type (3, 22). To clarify whether secretagogue stimulation increases colonic NBC surface expression as a potential correlate for cAMP-dependent and cholinergic activation of NBC function and/or HCO₃⁻ secretion, we therefore studied NBCe1-B membrane abundance in a native crypt preparation from murine colon. To this end, we employed the method of cell surface biotinylation, which is routinely used with cell lines (19, 32), with isolated crypts, in which the basolateral membrane containing NBCe1-B is supposedly freely accessible to biotinylation buffer. Because isolated colonic crypts tend to aggregate with time, we did not use this method to study long-term events (32).

To further elucidate the process of NBC exocytosis, we first employed compounds interfering with cytoskeleton assembly because cytoskeletal structures have been shown to be involved in vesicle traffic during regulation of various ion transport pathways (17, 19, 46). Inhibition of the actin cytoskeleton with cytochalasin D did not influence the increase in membrane NBCe1 abundance after secretagogues, which matches a previous observation on CO₂-mediated exocytosis of renal NBC (19). Next, we used the microtubule assembly inhibitor colchicine because this compound has been shown to inhibit vesicle endocytosis in various experimental systems (18, 28, 42). This approach led to an increase in NBCe1 membrane abundance, without a further increase during secretagogue incubation. The explanation for this effect might lie in NBCe1 accumulation in the cell membrane attributable to endocytosis inhibition during vesicle recycling, and the endocytosis inhibitor chlorpromazine increased NBCe1 membrane content. Given the role of PI3 kinase in agonist-induced processes such as regulated exocytosis, which has been demonstrated in mast cell degranulation (2), insulin-induced membrane translocation of GLUT4 glucose transporters (48), and neurosecretory granule exocytosis (31), we investigated the role of this pathway during NBCe1

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**Fig. 5.** Immunohistochemical staining and quantification of the fluorescence signal for NBCe1 and E-cadherin (ECad) (used as a basolateral membrane marker) under control conditions (A–C), after forskolin (D–F), and after carbachol incubation (G–I; see MATERIALS AND METHODS for details). A, D, and G: immunohistochemical images (green, NBCe1; red, ECad; blue, DAPI). Top, left: NBCe1 only. Top, right: ECad only. Bottom, left: NBCe1 and E-cadherin. Bottom, right: NBCe1, E-cadherin, and DAPI. B, E, and H: plot profiles over the mid-crypt basolateral membrane for the NBCe1 (green) and ECad (red) signals over 20 μm; the ECad peak intensity was centered, and both peak intensities normalized to 100%. Although significant cytoplasmic staining for NBCe1 is visible under control conditions, which is distinct from the ECad staining (A and B), short-term incubation with forskolin or carbachol led to predominant NBCe1 membrane localization (D and G) and almost complete overlap of the NBCe1 and ECad signal intensity traces (E and H). The integrated fluorescence intensities of the NBCe1 and ECad signals were only different for the control group (C) but not for the forskolin and carbachol groups (F and I). Representative images (A, D, G) and plot profiles (B, E, H); scale bar = 25 μm. C, F, and I: n = 8 regions of interest from 5 slides/preparations in each group, *P < 0.05 (Student’s t-test for paired samples, NS: not significant).
membrane trafficking. Indeed, PI3 kinase inhibition with LY294002, which is more stable than wortmannin (29), diminished membrane-associated NBCe1, whereas secretagogues still led to a modest increase, indicating that PI3-kinase is important for NBCe1 baseline activity and not the only mechanism mediating secretagogue activation. To test whether increased membrane abundance caused by colchicine is correlated with enhanced NBCe1 activity, we performed microfluorometric experiments, which did not show a significantly different NBC activity with and without colchicine. This indicates that an increase in NBC membrane abundance alone is not sufficient for activation of NBC, but that other steps, e.g., phosphorylation, have to take place. Alternatively, colchicine might have a direct inhibitory effect on the transporter, which prevents its stimulation by secretagogues. Furthermore, it has to be noted that the incubation times for the utilized compounds were relatively short compared with those in previously published experiments with cell lines, attributable to the limited viability of the crypts, and that we cannot exclude that additional effects might occur after longer incubation. Our findings therefore lead to the hypothesis that HCO₃⁻ secretion is activated in a complex fashion involving multiple steps, including NBC vesicle traffic and exocytosis, and additional signaling by second messenger pathways such as the action of protein kinases and intracellular Ca²⁺, as well as the activation of other transport pathways such as basolateral K⁺ channels.

Fig. 6. NBCe1 surface expression in response to secretagogues after incubation with inhibitors of cytoskeleton assembly. A: to assess the effect of the actin polymerization inhibitor cytochalasin D (Cyto D) (10⁻⁵ M), crypts were either incubated without active compound (1st column), for 20 min with cytochalasin D (2nd column), for 10 min with cytochalasin D followed by 10 min of cytochalasin D and carbachol (10⁻⁴ M, 3rd column), or for 10 min with cytochalasin D followed by 10 min of cytochalasin D and forskolin (10⁻⁵ M, 4th column). Cytochalasin D alone had no effect, and subsequent incubation with secretagogues caused an increase in NBCe1 membrane expression that was comparable to the experiment without inhibitor (see Fig. 3). B: immunohistochemistry with phalloidin to visualize the actin cytoskeleton in isolated colonic crypts after incubation with cytochalasin D for 10 min vs. vehicle. C: in another set of experiments, the microtubule inhibitor colchicine (10⁻⁶ M, Colch) was used instead of cytochalasin D. Interestingly, colchicine by itself caused a significant increase in NBCe1 surface expression, whereas forskolin or carbachol did not have an additional effect. D: because the effect of colchicine may be due to endocytosis inhibition, the effect of the endocytosis inhibitor chlorpromazine (20 µg/ml) was studied. This compound significantly increased NBCe1 membrane content after 5 and 10 min. E: phosphatidylinositide (PI3) kinase is important for exocytosis in different experimental systems, and the PI3 kinase inhibitor LY294002 (LY, 20 µM) significantly reduced NBCe1 membrane expression. Subsequent stimulation with secretagogues caused a modest increase (n = 5 preparations from separate mice in each group, *P < 0.05 vs. 1st column, #P < 0.05 vs. 2nd column, ANOVA for correlated samples followed by Tukey’s HSD).

Fig. 7. Acid-induced proton flux rates in the absence and presence of colchicine and in the presence of dimethylamiloride (DMA) and CO₂/HCO₃⁻ in isolated murine colonic crypts. A: representative traces. After an equilibration phase, crypts were subjected to an NH₄⁺ prepulse protocol. After NH₄Cl (40 mM), pH increased abruptly, followed by a slow reacidification. NH₄Cl and Na⁺ withdrawal caused a marked acidification. After pH stabilization, colchicine (10⁻⁶ M) and DMA (500 µM) were added to the perfusate for 10 min. Subsequently, NaCl was reintroduced, which caused intracellular alkalization. B: multiplication with the intracellular buffering capacity yielded proton flux rates of 7.40 ± 0.83 mM/min for the control group and 6.13 ± 0.71 mM/min for the colchicine group (n = 6–7 experiments from 3–5 mice, Student’s t-test; NS, not significant).
for forskolin- and carbachol-induced colonic epithelial HCO₃⁻ secretion, and which is likely involved in the NBC activation we had previously found after short-term incubation of colonic crypts with Secretagogues. We thus propose vesicle traffic and exocytosis as a new mode of NBC activation in murine colonic epithelium.

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