APC Sensitive Gastric Acid Secretion

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Key Words

H⁺ secretion • APC • *Apc*^{Min/+} • Stomach • Omeprazole
• Gastrin • Potassium • Forskolin

Abstract

Adenomatous polyposis coli (APC) is a tumor suppressor gene inactivated in familial adenomatous polyposis and sporadic colorectal cancer. Mice carrying a loss-of-function mutation in the *apc* gene (*apc*^{Min/+}) spontaneously develop gastrointestinal tumors. APC fosters degradation of β -catenin, which in turn upregulates the serum- and glucocorticoid-inducible kinase SGK1. SGK1 stimulates KCNQ1, which is required for luminal K⁺ recycling and thus for gastric acid secretion. BCECF-fluorescence was utilized to determine gastric acid secretion in isolated gastric glands from *apc*^{Min/+} mice and their wild type littermates (*apc*^{+/+}). Western blotting was employed to analyse β -catenin and SGK1 expression and immunohistochemistry to determine KCNQ1 protein abundance. β -catenin and SGK1 expression were enhanced in *apc*^{Min/+} mice. Cytosolic pH was similar in *apc*^{Min/+} mice and *apc*^{+/+} mice. Na⁺-independent pH recovery following an ammonium pulse (Δ pH/min), which reflects H⁺/K⁺ ATPase activity, was, however, significantly faster in *apc*^{Min/+} mice than in *apc*^{+/+} mice.

In both genotypes Δ pH/min was abolished in the presence of H⁺/K⁺ ATPase inhibitor omeprazole (100 μ M). Treatment of *apc*^{Min/+} and *apc*^{+/+} mice with 5 μ M forskolin 15 minutes prior to the experiment or increase in local K⁺-concentrations to 35 mM (replacing Na⁺/NMDG) significantly increased Δ pH/min and abrogated the differences between genotypes. The increase of Δ pH/min in *apc*^{Min/+} mice required SGK1, as it was abolished by additional knockout of SGK1 (*apc*^{Min/+}/*sgk1*^{-/-}). In conclusion, basal gastric acid secretion is significantly enhanced in *apc*^{Min/+} mice, pointing to a role of APC in the regulation of gastric acid secretion. The effect of APC requires H⁺/K⁺ ATPase activity and is at least partially due to SGK1-dependent upregulation of KCNQ1.

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Introduction

Inactivating mutations of the adenomatous polyposis coli (APC) tumor suppressor gene lead to the development of multiple colonic adenomatous polyps eventually resulting in colon carcinoma [1, 2]. APC fosters the degradation of cytosolic β -catenin and thus interferes with the Wnt/ β -catenin pathway [3-9]. Lack of APC leads

to accumulation of β -catenin, which travels into the nucleus and triggers the expression of several genes important for cell proliferation including Myc and Cyclin D1 [10-12]. Genes regulated by β -catenin include the serum- and glucocorticoid-inducible kinase SGK1 [13, 14]. Conversely, SGK1 phosphorylates the glycogen synthase kinase 3β (GSK3 β) [15], which has in turn been shown to be involved in APC-dependent regulation of cell function [16-18].

SGK1 is a potent stimulator of the K⁺ channel KCNQ1 [19], which participates in the cellular mechanisms involved in gastric acid secretion [20-22]. In the apical cell membrane of the parietal cell, H⁺ is extruded by the H⁺/K⁺-ATPase in exchange for K⁺. The continued function of the H⁺/K⁺-ATPase thus depends on the availability of K⁺ in the lumen. Luminal K⁺ is provided by recycling of K⁺ from the cell into the lumen though KCNQ1 channels in the apical cell membrane [20-22]. Glucocorticoids stimulate gastric acid secretion at least partially by upregulation of SGK1 and subsequent activation of KCNQ1 channels [23].

Mice carrying a mutation in the *apc* gene (*apc*^{Min/+}) leading to truncation of the gene product at amino acid 850 develop multiple intestinal tumors [24]. Moreover, *apc*^{Min/+} mice develop gastric tumors [25], which may similarly be associated with enhanced nuclear β -catenin accumulation [26-28].

The present study has thus been performed to elucidate whether gastric acid secretion is altered in *apc*^{Min/+} mice. To this end, gastric H⁺ transport was studied in isolated gastric glands from *apc*^{Min/+} mice and their wild type littermates (*apc*^{+/+}). The results provide evidence for enhanced acid secretion in gastric glands of *apc*^{Min/+} mice.

Materials and Methods

Animals

Mice with mutated *apc* (*apc*^{Min/+}) and their wild type littermates (*apc*^{+/+}) with a mixed (C57/Bl-6-Sv129J) background were generated by breeding of male *apc*^{Min/+} mice initially obtained from the Jackson Laboratory. Where indicated, additional mice were generated by crossbreeding *apc*^{Min/+} with gene targeted mice lacking functional SGK1 (*sgk1*^{-/-}) [29] to generate mice carrying the mutant APC and simultaneously lacking SGK1 (*apc*^{Min/+}/*sgk1*^{-/-}). Sex- and age-matched mice of more than 4 months age were used for the experiments. All animal experiments were conducted according to the guidelines of the American Physiological Society and were approved by local authorities according to the German law for the care and use of laboratory animals.

Treatments

Omeprazole (100 μ M) and forskolin (5 μ M) were added directly to the perfusing solutions. Sham experiments were carried out by adding DMSO to the perfusing solutions at a final concentration of 0.1%.

Gastric H⁺ secretion

For isolation of gastric glands animals were fasted for 16 hours prior to the experiments on wire grids with free access to tap water. After sacrificing the animals by cervical dislocation under slight ether anesthesia, the stomach was removed and cut longitudinally. The stomach was then washed with standard HEPES solution. The fundic and pyloric regions were discarded, and the gastric corpus was sliced into 0.3 cm² sections. The tissues were transferred onto the cooled stage of a dissecting microscope, and individual glands were carefully detached from the gastric wall by snapping off the tissue using sharpened microdissection tweezers. Care was taken not to touch the apical part of the glands. The glands were attached to a glass coverslip precoated with Cell-Tak adhesive (BD Biosciences).

The solutions, flow lines and perfusion chamber were maintained at 37°C by a thermostatically controlled heating system. The volume of the perfusion chamber was 600 μ l, and the flow rate was 4 ml/min for all solutions. For digital imaging of cytosolic pH_i isolated individual glands were incubated in a HEPES-buffered Ringer solution containing 10 μ M BCECF-AM (Molecular Probes, Leiden, The Netherlands) for 15 min at 37°C. After loading, the chamber was flushed for 5 min with Ringer solution to remove any deesterified dye sticking to the outside of the glands. The perfusion chamber was mounted on the stage of an inverted microscope (Zeiss Axiovert 135), which was used in the epifluorescence mode with a 40x oil immersion objective (Zeiss Neoplan, Germany). BCECF was successively excited at 490/10 and 440/10 nm, and the resultant fluorescent signal was monitored at 535/10 nm using an intensified charge-coupled device camera (Proxitronic, Germany) and specialized computer software (Metafluor, USA). 8 - 20 parietal cells were outlined and monitored during the course of the measurements. The results from each cell were averaged and taken for the final analysis. Intensity ratio data (490/440) were converted into pH values using the high-K⁺/nigericin calibration technique [30].

For acid loading, cells were transiently exposed to a solution containing 20 mM NH₄Cl leading to marked initial alkalization of cytosolic pH (pH_i) due to entry of NH₃ and binding of H⁺ to form NH₄⁺ [31]. The acidification of cytosolic pH upon removal of ammonia allowed the calculation of the mean intrinsic buffering power of the cells [31]. Assuming that NH₄⁺ and NH₃ are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH₃:

$$\beta = \Delta[\text{NH}_4^+]_i / \Delta\text{pH}_i$$

where ΔpH_i is the decrease of cytosolic pH (pH_i) following ammonia removal and $\Delta[\text{NH}_4^+]_i$ is the decrease of cytosolic NH₄⁺ concentration, which is identical to the concentration of [NH₄⁺]_i immediately before the removal of ammonia. The pK for NH₄⁺/NH₃ is 8.9 [32], and at an extracellular pH (pH_o) of 7.4 the NH₄⁺ concentration in extracellular fluid ([NH₄⁺]_o) is 19.37 [20/(1+10^{pH_o-pK})]. The intracellular NH₄⁺ concentration ([NH₄⁺]_i) was calculated from:

$$[\text{NH}_4^+]_i = 19.37 \cdot 10^{\text{pH}_0 - \text{pH}_i}$$

The calculation of buffer capacity requires that NH_4^+ exits completely. After the initial decline, pH_i indeed showed little further change in the absence of Na^+ and presence of omeprazole, indicating that there is no relevant further exit of NH_4^+ . To calculate the $\Delta\text{pH}/\text{min}$ during re-alkalinization, a manual linear fit was placed over a narrow pH range which could be applied to all measured cells. The solutions were composed of (in mM): standard Hepes: 115 NaCl, 5 KCl, 1 CaCl_2 , 1.2 MgSO_4 , 2 NaH_2PO_4 , 10 glucose, 32.2 Hepes; sodium free Hepes: 132.8 NMDG, 3 KCl, 1 CaCl_2 , 1.2 MgSO_4 , 2 KH_2PO_4 , 32 Hepes, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM NH_4Cl); high K^+ for calibration: 105 KCl, 1 CaCl_2 , 1.2 MgSO_4 , 32.2 Hepes, 10 mannitol, 5 μM nigericin. The pH of the solutions was titrated to 7.4 or 7.0 with HCl/NaOH, HCl/NMDG and HCl/KOH, respectively, at 37°C. Where indicated, 100 μM omeprazole (Astra-Zeneca, Planckstadt, Germany) or 5 μM forskolin (Sigma, Deisenhofen, Germany) were added to the BCECF incubation medium and standard Hepes solution or K^+ concentration increased at the expense of Na^+ /NMDG to 35 mM.

Determination of serum gastrin

To determine the serum gastrin levels, the animals were fasted overnight for 16 hours with free access to tap water. About 150 μl of blood specimens were drawn into heparinized hematocrit capillaries by retro-orbital puncture during light anesthesia with diethylether (Roth, Karlsruhe, Germany). Total serum gastrin concentrations were determined by radio immuno assay (RIA) kit according to the manufacturer's (DiaSorin, Dietzenbach, Germany) instructions.

Western blotting

Mice were sacrificed by cervical dislocation under ether anaesthesia, and the abdomen was cut open. The stomach was then longitudinally cut, and the lumen was cleaned with PBS. 2 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 1 mM NaF, 1 mM Na_3VO_4 , β -mercaptoethanol) containing protease inhibitor cocktail (Sigma) were added to the tissue and homogenized for 2 min on ice. Samples were centrifuged at 10,000 g for 30 min, and supernatants were collected. After measurement of the total protein concentration (Biorad Protein kit), 100 μg of protein were solubilized in Laemmli sample buffer at 65°C for 5 min and resolved by 12% SDS-PAGE. For immunoblotting proteins were electro-transferred onto a nitrocellulose membrane and blocked in 5% nonfat milk in PBS-0.10% Tween 20 for one hour at room temperature. The membrane was incubated with either affinity purified rabbit anti-mouse β -catenin antibody (1:2000; 92 kDa, Cell Signalling, USA), or with a polyclonal monospecific antibody against the SGK1 protein, raised by a commercial antibody service (1:100; 55 kDa, Dr. Pineda, Berlin, Germany) as previously described [33], or with a mouse monoclonal anti-GAPDH antibody (1:1000; 42 kDa, Sigma) overnight at 4°C. After washing and subsequent blocking, blots were incubated with secondary antibodies conjugated with alkaline phosphatase or horse radish peroxidase (goat anti-rabbit 1:2000 and donkey anti-mouse 1:10000, Promega, USA), for 1 h at

room temperature. Antibody binding was detected with the Immobilon Western kit (Millipore, Billerica, MA, USA) with the Diana III chemiluminescence detection system. Bands were quantified with the Aida Image Analyzer software (Raytest, Germany).

Immunofluorescence

For immunofluorescence analysis of KCNQ1 protein abundance in the cell membrane of gastric glands the animals were sacrificed by cervical dislocation under ether anesthesia, and the corpus regions of the stomach was isolated and fixed in 4% paraformaldehyde/0.1 M sodium phosphate buffer (pH 7.4) for two hours and then stored in PBS. After immersion in 30% sucrose for 24 h, 15 μm cryostat sections were taken. After a brief rinse, the sections were incubated for 1 h in normal goat serum with 0.3% Triton X-100. All the following washes and incubations were performed in PBS containing 0.3% Triton X-100 and 1% dimethyl sulfoxide. Without rinsing, primary polyclonal goat anti-KCNQ1 antibody (C20, Santa Cruz Biotechnologies), diluted 1:100, was applied to the sections for 12 h at 4°C. After three washes at 10 min, the sections were incubated with donkey antigoat Alexa488 secondary antibody (Invitrogen/Molecular Probes) for 1.5 h. The slides were analyzed on a Zeiss LSM 510 confocal microscope equipped with a 40x oil immersion lens (NA 1.3).

Quantitative real-time polymerase chain reaction measurements

To determine the transcript levels of KCNQ1 and SGK1, gastric tissue was quickly removed and frozen in liquid nitrogen. Automated disruption and homogenization of frozen tissue was achieved using the MagNa Lyser Instrument™ (Roche Diagnostics, Mannheim, Germany). For each sample, oneway special tubes were filled with ceramic beads, 20–30 mg of frozen tissue and 600 μl of RLT-buffer (Qiagen, Hilden, Germany). Cleared cell lysate was transferred for further RNA purification (RNAeasy Mini Kit, Qiagen, Hilden, Germany). Subsequently, 1 μg of total RNA was reverse transcribed to complementary DNA (cDNA) utilizing the reverse transcription system (Bioscience, USA) with oligo(dT) primers according to the manufacturer's protocol. To determine mRNA levels, quantitative real-time polymerase chain reaction (PCR) with the LightCycler System™ (Roche Diagnostics, Mannheim, Germany) was established. PCR reactions for KCNQ1 and SGK1 were performed in a final volume of 20 μl containing 2 μl cDNA, 2.4 μl MgCl_2 (3 μM), 1 μl primermix (0.5 μM of both primers), 2 μl cDNA Master SybrGreen I mix (Roche Molecular Biochemicals, Mannheim, Germany), and 12.6 μl diethyl pyrocarbonate (DEPC)-treated water. The transcript levels of the housekeeping gene mGAPDH were determined in each sample using a commercial primer kit (Search LC, Heidelberg, Germany). PCR reactions for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed in a final volume of 20 μl containing 2 μl cDNA, 2 μl primer mix (Search LC, Heidelberg, Germany), 2 μl cDNA Master Sybr Green I mix (Roche Molecular Biochemicals, Mannheim, Germany), and 14 μl DEPC-treated water. The target DNA was amplified during 35 cycles of 95°C for 10 s, 68°C for 10 s, and 72°C for 16 s, each with a temperature transition rate of 20°C/s

Fig. 1. pH recovery in parietal cells following an ammonium pulse in the absence and presence of omeprazole. Alterations of cytosolic pH (Δ pH) in parietal cells following an ammonium pulse. To load the cells with H⁺ 20 mM NH₄Cl were added and Na⁺ removed (replaced by NMDG) in a first step (see bars below each original tracing), NH₄Cl removed in a second step, Na⁺ added in a third step and nigericin applied in a fourth step to calibrate each individual experiment. **a.** Original tracings illustrating alterations of pH in typical experiments in *apc*^{Min/+} mice (right panels) and *apc*^{+/+} mice (left panels) in the absence (upper panels) and presence (lower panels) of 100 μ M omeprazole (OMP). **b.** Arithmetic means $\pm$ SEM of Na⁺-independent alterations of cytosolic pH (Δ pH/min) in parietal cells from *apc*^{Min/+} mice (closed bars) and *apc*^{+/+} mice (open bars) in the absence (left bars) and presence (right bars) of 100 μ M omeprazole. * indicates significant difference from *apc*^{+/+} mice, # indicates significant difference from absence of omeprazole (control) ($p < 0.05$).

and a secondary target temperature of 58°C with a step size of 0.5°C. Melting curve analysis was performed at 95°C 0 s, 58°C 10 s, and 95°C 0 s to determine melting temperatures of primer dimers and the specific PCR products. Melting curve analysis confirmed the amplified product, which was then separated on 1.5% agarose gels to confirm the expected size (218 bp for KCNQ1 and 416 bp for sgk1). Finally, results were calculated as a ratio of the target vs. house keeping gene transcripts. The following primers were used:

KCNQ1 forward 5' CCA TCA TTG ACC TCA TCGTG 3'
 KCNQ1 reverse 5' CCA GAA AGC CAA TGT ACAGG 3'
 SGK1 forward: 5'-TGT CTT GGG GCT GTC CTG TAT G-3'
 SGK1 reverse: 5'-GCT TCT GCT GCT TCC TTC ACAC-3'.

Sequence analysis confirmed the amplification of PCR products of the expected gene for KCNQ1 (accession number NM_008434) and SGK1 (NM_011361).

Flow cytometric analysis (FACS) of KCNQ1 expression in the isolated parietal cells

To isolate the parietal cells from gastric mucosa, a protocol described by Jewell et al. [34] was slightly modified used. Briefly, the mice were overnight-fasted prior to experiments on wire grids with free access to tap water. The mice were then sacrificed, and the stomach was cut open longitudinally. The fundic and the pyloric regions were quickly discarded and the gastric corpus was taken onto ice-cold standard HEPES buffer. The entire isolation procedure was carried out on ice (4°C). The tissue was then cleaned with standard HEPES buffer and mucus removed. After taking the tissue into fresh standard HEPES buffer, the muscular layer was then separated from the mucosal layer by scraping with a glass slide. The scrapings were then cut into very small pieces with a stainless steel razor and the isolated cells were separated from the tissue debris by

Fig. 2. Effects of forskolin on pH recovery in parietal cells. a. Original tracings illustrating alterations of pH in typical experiments in *apc*^{Min/+} mice (right panels) and *apc*^{+/+} mice (left panels) in the absence (upper panels) and presence (lower panels) of forskolin (FSK, 5 μM). b. Arithmetic means ± SEM of Na⁺-independent alterations of cytosolic pH (ΔpH/min) in parietal cells following an ammonium pulse (see Fig. 1) in the absence (sham) and presence of forskolin (FSK, 5 μM) in *apc*^{Min/+} (closed bars) and *apc*^{+/+} mice (open bars). § indicates significant difference from respective sham treatment, * indicates significant difference from *apc*^{+/+} mice (p<0.05).

gradient centrifugation. The obtained cell pellet was resuspended again in standard HEPES buffer, and the suspension was passed through a stainless steel needle (35 gauge). The cells thus obtained were washed once with standard HEPES buffer and then incubated at 37°C for 30 min first with primary polyclonal anti-goat KCNQ1 antibody, 1:100 dilution (C20, Santa Cruz Biotechnologies) and then with anti-goat FITC secondary antibody, 1:1000 dilution (Santa Cruz Biotechnologies) in an atmosphere with 5% CO₂. The cells were washed twice and resuspended in Ringer solution containing 5 mM CaCl₂ for flow cytometric analysis ((FACS-Calibur from Becton Dickinson; Heidelberg, Germany). Cells were analysed by forward scatter, and FITC-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Statistics

The intracellular pH, pH recovery and buffer capacity values of all parietal cells from one gland were averaged, and the respective mean value was used for further statistical analysis. Data are provided as arithmetic means ± SEM, n

represents the number of mice used. All data were tested for significance using Student's t-test with Welch's correction or ANOVA (Dunnets test), where applicable, and only results with P < 0.05 were considered statistically significant.

Results

The stomach was morphologically similar in heterozygous mice with defective APC (*apc*^{Min/+}) and their wild type littermates (*apc*^{+/+}). Accordingly, the stomach weight was similar in both genotypes (0.22 ± 0.01 g, n = 5 in *apc*^{Min/+} mice and 0.25 ± 0.02 g, n = 8 in *apc*^{+/+} mice).

BCECF fluorescence was employed to determine cytosolic pH in gastric glands from *apc*^{Min/+} and *apc*^{+/+} mice. The cytosolic pH in freshly isolated gastric glands (Fig. 1) was similar in untreated *apc*^{+/+} mice (7.13 ± 0.02, n = 10) and *apc*^{Min/+} mice (7.14 ± 0.01, n = 10).

Fig. 3. Influence of enhanced extracellular K^+ concentration on pH recovery in parietal cells. a. Original tracings illustrating alterations of pH at enhanced extracellular K^+ concentration (35 mM) in $apc^{Min/+}$ mice (right panels) and $apc^{+/+}$ mice (left panels). b. Arithmetic means $\pm$ SEM of Na^+ -independent ΔpH in parietal cells from $apc^{Min/+}$ mice (closed bar) and $apc^{+/+}$ mice (open bar) at high (35 mM K^+) extracellular K^+ concentration. # indicates significant difference from normal K^+ concentration (3 mM K^+ , control, see Fig. 1) ($p < 0.05$).

Ammonium pulse experiments were utilized to load the cells with H^+ [31]. The superfusion with 20 mM NH_4Cl and simultaneous removal of Na^+ (replacement with NMDG $^+$) was followed by slight alkalization (Fig. 1, upper trace). Na^+ was removed from the extracellular bath solution in order to suppress Na^+/H^+ exchange-dependent pH recovery after an acid load. The subsequent replacement of NH_4Cl with NMDG-Cl in the continued absence of Na^+ led to a sharp acidification due to exit of NH_3 and retention of H^+ within the cells. The acidification allowed the calculation of an apparent buffer capacity which was again similar in untreated $apc^{+/+}$ mice (37.7 ± 3.8 mM/pH, $n = 10$) and $apc^{Min/+}$ mice (39.4 ± 5.2 mM/pH, $n = 10$).

As illustrated in Fig. 1b, pH recovery following an ammonium pulse in the absence of Na^+ was significantly (~ 2 -fold) higher in $apc^{Min/+}$ mice (0.076 ± 0.010 $\Delta pH/min$, $n = 10$) than in $apc^{+/+}$ mice (0.034 ± 0.008 $\Delta pH/min$, $n = 10$). Addition of 100 μM omeprazole decreased the Na^+ -independent pH recovery to 0.006 ± 0.006 $\Delta pH/min$ ($n = 5$) in $apc^{Min/+}$ mice and to 0.006 ± 0.005 $\Delta pH/min$ ($n = 4$) in $apc^{+/+}$ mice. Thus, in both genotypes, Na^+ -independent pH recovery was abolished following pharmacological inhibition of H^+/K^+ -ATPase activity (Fig. 1, lower trace).

As illustrated in Fig. 2, forskolin (5 μM) led to a strong stimulation of gastric acid secretion in untreated $apc^{+/+}$ mice (0.209 ± 0.028 $\Delta pH/min$, $n = 7$) and $apc^{Min/+}$ mice (0.198 ± 0.016 $\Delta pH/min$, $n = 6$). Notably, forskolin treatment increased the Na^+ -independent acidification ($\Delta pH/min$) to similar values in both genotypes. Thus, following treatment with forskolin there was no significant difference between $apc^{+/+}$ and $apc^{Min/+}$ mice.

An increase in the extracellular K^+ concentration to 35 mM by replacing Na^+ led to a significant increase in Na^+ -independent pH recovery to 0.259 ± 0.048 $\Delta pH/min$ ($n = 6$) in $apc^{Min/+}$ mice and to 0.218 ± 0.039 $\Delta pH/min$, ($n = 5$) in $apc^{+/+}$ mice (Fig. 3). Again, following increase in the extracellular K^+ concentration pH recovery ($\Delta pH/min$) increased to similar values in both genotypes. At increased extracellular K^+ concentration the differences in H^+ secretion between $apc^{+/+}$ and $apc^{Min/+}$ mice were not statistically significant.

Total serum gastrin concentrations were measured to explore, whether the observed differences between $apc^{+/+}$ and $apc^{Min/+}$ parietal cells were due to differences in gastrin levels. Serum gastrin levels were, however, similar in $apc^{Min/+}$ mice (35.6 ± 2.4 pM/L, $n = 8$) and $apc^{+/+}$ mice (36.3 ± 1.8 pM/L, $n = 15$).

Western blotting was performed to elucidate

Fig. 4. β -catenin and SGK1 protein abundance in stomach. a. Original Western Blot illustrating the β -catenin (upper panel) and GAPDH (lower panel) protein abundance in $apc^{Min/+}$ mice and $apc^{+/+}$ mice. b. Arithmetic means $\pm$ SEM of relative densities of β -catenin protein expression in stomach from $apc^{Min/+}$ mice (closed bar) and $apc^{+/+}$ mice (open bar). * indicates significant difference between $apc^{Min/+}$ and $apc^{+/+}$ mice ($p < 0.05$). c. Original Western Blot illustrating the SGK1 (upper panel) and GAPDH (lower panel) protein abundance in $apc^{Min/+}$ mice and $apc^{+/+}$ mice. d. Arithmetic means $\pm$ SEM of relative densities of SGK1 protein expression in stomach from $apc^{Min/+}$ mice (closed bar) and $apc^{+/+}$ mice (open bar). * indicates significant difference between $apc^{Min/+}$ and $apc^{+/+}$ mice ($p < 0.05$).

Fig. 5. Cell membrane KCNQ1 protein expression in parietal cells. a. Immunofluorescence of KCNQ1 expression in parietal cells from $apc^{Min/+}$ mice (right panels) and $apc^{+/+}$ mice (left panels). Antibodies were directed against KCNQ1 (green fluorescence). Three different microscopic fields are shown for each genotype. b. KCNQ1 mRNA expression in the stomach from $apc^{Min/+}$ mice (closed bars) and $apc^{+/+}$ mice (open bars).

whether the functional differences between $apc^{+/+}$ and $apc^{Min/+}$ parietal cells could have been related to differences in protein abundance of β -catenin and/or SGK1. As illustrated in Fig. 4, the protein abundance for both, β -catenin and SGK1 was indeed significantly larger in $apc^{Min/+}$ than in $apc^{+/+}$ mice. Quantitative real time PCR analysis further revealed significantly higher expression of SGK1 in $apc^{Min/+}$ mice (50.0 ± 6.0 copies/GAPDH X 1000, $n = 9$) as compared to $apc^{+/+}$ mice (31.7 ± 4.6 copies/GAPDH X 1000, $n = 11$).

Immunohistochemistry was performed to elucidate whether the enhanced expression of β -catenin and SGK1 influenced KCNQ1 transcription, expression or protein abundance in the cell membranes of $apc^{Min/+}$ mice. According to real time PCR, KCNQ1 transcript levels in the gastric glands were not significantly different between $apc^{+/+}$ (2.7 ± 0.5 copies/GAPDH X 1000, $n = 9$) and $apc^{Min/+}$ (3.3 ± 0.4 copies/GAPDH X 1000, $n = 9$) mice. As shown in Fig. 5, the total KCNQ1 protein abundance was again similar in $apc^{+/+}$ and $apc^{Min/+}$ parietal cells. The localization of the protein was however, visibly different between the genotypes. In the parietal cells from $apc^{+/+}$ KCNQ1 was diffusely distributed throughout the cytoplasm, whereas in the parietal cells from $apc^{Min/+}$ the protein was localized at the plasma membrane.

Fig. 6. Flow cytometric (FACS) analysis of the membrane KCNQ1 expression in isolated parietal cells. a. Representative histogram of KCNQ1-dependent fluorescence intensity b. Arithmetic means $\pm$ SEM of the percentage of gated cells (corrected to control) from *apc*^{Min/+} mice (closed bar) and *apc*^{+/+} mice (open bar). * indicates significant difference between *apc*^{Min/+} and *apc*^{+/+} mice ($p < 0.05$).

Fig. 7. SGK1 requirement of accelerated pH recovery in parietal cells in APC-deficient mice. a. Original tracings illustrating alterations of pH in typical experiments in *apc*^{Min/+}/*sgk1*^{+/+} mice (left panel) and *apc*^{Min/+}/*sgk1*^{-/-} mice (right panel). b. Arithmetic means $\pm$ SEM of Na⁺-independent alterations of cytosolic pH (Δ pH/min) in parietal cells from *apc*^{+/+}/*sgk1*^{+/+} mice (open bar) *apc*^{Min/+}/*sgk1*^{+/+} mice (closed bar) and *apc*^{Min/+}/*sgk1*^{-/-} mice (hatched bar). * indicates significant difference from *apc*^{+/+}/*sgk1*^{+/+} mice, # indicates significant difference from *apc*^{Min/+}/*sgk1*^{+/+} mice.

To quantitatively analyze the membrane abundance of KCNQ1, the parietal cells were isolated, stained and analyzed in FACS. As shown in the Fig. 6, the KCNQ1 abundance in the cell membrane was significantly larger in *apc*^{Min/+} than in *apc*^{+/+} parietal cells.

To explore, whether the stimulation of H⁺ secretion in *apc*^{Min/+} mice indeed required the presence of SGK1, additional experiments were performed in mice carrying the mutant APC and at the same time lacking functional SGK1 (*apc*^{Min/+}/*sgk1*^{-/-}). Comparison was made to both, *apc*^{+/+}/*sgk1*^{+/+} and *apc*^{Min/+}/*sgk1*^{+/+} littermates. As illustrated in Fig. 7, the enhanced H⁺ secretion in *apc*^{Min/+} mice was absent in mice lacking SGK1 (*apc*^{Min/+}/*sgk1*^{-/-}). The rate of pH recovery in *apc*^{Min/+}/*sgk1*^{-/-} mice (0.043 $\pm$ 0.008 Δ pH/min, $n = 10$) was not significantly different from that of *apc*^{+/+}/*sgk1*^{+/+} mice (0.034 $\pm$ 0.008 Δ pH/min, $n = 10$), but significantly smaller than that of *apc*^{Min/+}/*sgk1*^{+/+} (0.076 $\pm$ 0.009 Δ pH/min, $n = 10$) mice.

Discussion

The present observations provide evidence for an involvement of APC in the regulation of gastric acid secretion. Na⁺-independent omeprazole-sensitive realkalinization of gastric gland cells following an ammonium pulse was significantly more rapid in heterozygous mice with defective APC (*apc*^{Min/+}) than in their wild type littermates (*apc*^{+/+}). Accordingly, in the absence of stimulation, H⁺/K⁺-ATPase activity was about two fold as fast in *apc*^{Min/+} mice as in *apc*^{+/+} mice. Notably, the marked difference is observed between heterozygous and wild type mice. Thus, the moderate decrease of APC expression in heterozygous mice is sufficient to substantially modify gastric acid secretion. Thus, APC is obviously a powerful regulator of gastric acid secretion.

APC deficiency could enhance H⁺ secretion by enhancing K⁺ recycling through KCNQ1 channels [20, 21]. The K⁺ recycling from the cell into the lumen provides

the H⁺/K⁺-ATPase with luminal K⁺ and is thus critically important for gastric H⁺ secretion [20, 21]. Accordingly, an increase in the extracellular K⁺ concentration leads to a marked increase in H⁺/K⁺-ATPase activity. The increase in the extracellular K⁺ concentration presumably enhances the luminal K⁺ concentration by depolarization of the basolateral cell membrane and through a circular current of the apical cell membrane with subsequent activation of apical KCNQ1 activity [23]. Under those conditions, the extracellular K⁺ concentration is no more limiting for H⁺/K⁺ ATPase activity. The difference of H⁺ extrusion between *apc*^{Min/+} mice and *apc*^{+/+} mice is not statistically significant at enhanced extracellular K⁺ concentration, an observation supporting an effect of APC on K⁺ recycling. However, the results do not rule out a direct stimulation of H⁺/K⁺-ATPase activity by SGK1.

Previously, glucocorticoids have been shown to stimulate gastric acid secretion by a mechanism similarly sensitive to extracellular K⁺ concentration [23]. The glucocorticoid was shown to be effective through SGK1, which has previously been shown to stimulate KCNE1/KCNQ1 trafficking and activity [19, 35, 36]. SGK1 stimulates the expression of further channels [37-45]. The kinase is at least in part effective through increase of channel protein abundance in the cell membrane [41]. Western blotting disclosed higher SGK1 expression levels in gastric glands from *apc*^{Min/+} mice than in those from *apc*^{+/+} mice. Most recently, SGK1 expression has been shown to be stimulated by β -catenin through binding of

TCF/ β -catenin complexes to the SGK1 promoter [13]. As a matter of fact β -catenin expression is similarly higher in *apc*^{Min/+} than in *apc*^{+/+} gastric glands. Most importantly, immunochemistry reveals enhanced KCNQ1 protein abundance in the plasma membrane of parietal cells.

The difference between *apc*^{Min/+} mice and *apc*^{+/+} mice was further dissipated by prior treatment with forskolin, which leads to stimulation of protein kinase A (PKA) and, similar to SGK1, to upregulation of KCNQ1 activity [18, 46, 47]. Apparently, similar to SGK1, protein kinase A phosphorylates KCNQ1 and increases KCNQ1 trafficking to the cell membrane.

In conclusion, basal gastric acid secretion is markedly increased in mice carrying the defective APC gene. Thus, APC is a powerful regulator of gastric acid secretion.

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