Regulation of Gastric Acid Secretion by PKB/Akt2

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Key Words

PI3 kinase • PKB/Akt2 • Stomach • H⁺/K⁺ ATPase • K⁺ recycling • H⁺ secretion

Abstract

Pharmacological inhibition of phosphoinositol 3 kinase (PI3K) and partial deficiency of phosphoinositide dependent kinase PDK1 have previously been shown to enhance basal gastric acid secretion. PI3K/PDK1 dependent signaling involves activation of protein kinase B/Akt, which may thus be similarly involved in the regulation of gastric acid secretion. To test that hypothesis, gastric acid secretion was determined in isolated glands from gene targeted mice lacking functional Akt2 (akt2^{-/-}) or from their wild type littermates (akt2^{+/+}). According to BCECF-fluorescence cytosolic pH in isolated gastric glands was similar in akt2^{-/-} and akt2^{+/+} mice. Na⁺-independent pH recovery (Δ pH/min) following an ammonium pulse, a measure of H⁺/K⁺ ATPase activity, was, however, significantly faster in akt2^{-/-} than in akt2^{+/+} mice. In both genotypes, Δ pH/min was virtually abolished by H⁺/K⁺ ATPase inhibitor omeprazole (100 μ M). Increase of extracellular K⁺ concentrations to 35 mM (replacing Na⁺) increased Δ pH/min to a significantly larger extent

in akt2^{+/+} than in akt2^{-/-} mice and dissipated the differences between the genotypes. Similarly, treatment with 5 μ M forskolin enhanced Δ pH/min significantly only in akt2^{+/+} mice and abolished the differences between the genotypes. Conversely, protein kinase A inhibitor H89 (50 nM) decreased Δ pH/min to similarly low values in both genotypes. In conclusion, Akt2 suppresses gastric acid secretion and contributes to or even accounts for the inhibition of gastric acid secretion by PI3K.

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Introduction

Proton secretion in gastric glands is accomplished by the H⁺/K⁺ ATPase, which pumps H⁺ into the gastric lumen in exchange of K⁺ [1-4]. The luminal K⁺ is replenished by recirculation of K⁺ through luminal KCNQ1/KCNE K⁺ channels [5-7]. Accordingly, gastric acid secretion is abolished by genetic knockout of KCNQ1 [8]. The channels are activated by cAMP [9-12], which thus stimulates gastric acid secretion [2, 4, 13].

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Gastric acid secretion is further regulated by the phosphoinositol-3-kinase (PI3K) [14]. Pharmacological inhibition of PI3K in isolated gastric glands and parietal cells has previously been shown to result in a substantial increase of gastric acid secretion [14]. PI3K inhibition is effective through increase of cAMP formation [14]. PI3K leads to activation of phosphoinositide-dependent kinase PDK1 with subsequent activation of the SGK and PKB/Akt isoforms [15-21]. Accordingly, pharmacological PI3K inhibition abrogated the phosphorylation of PKB/Akt [14]. Moreover, gastric acid secretion was enhanced in PDK1 hypomorphic mice [22], i.e. mice expressing some 20% of normal PDK1 [23].

The present study explored, whether PKB/Akt2 contributes to the regulation of gastric acid secretion. To this end, gastric acid secretion was determined in gene targeted mice lacking functional PKB/Akt2 and their wild type littermates.

Materials and Methods

Animals

The PKB/Akt2 deficient mice were described earlier [24, 25]. Sex and age matched mice with age more than 3 months were used for the experiments. All animal experiments were conducted according to the German law for the care and use of laboratory animals and were approved by local authorities.

Treatments

Mice had free access to a standard mouse diet (C1310, Altromin, Lage, Germany) and tap water. Effects of omeprazole (100 μ M), forskolin (5 μ M) and H89 (50 nM) were determined by adding the substances directly to the perfusing solutions at the indicated final concentrations. The substances were obtained from Sigma (Taufkirchen, Germany). The stock solutions of omeprazole and forskolin were prepared in DMSO, whereas H89 was dissolved in distilled water. Sham experiments were performed by adding DMSO or plain distilled water to the perfusing solution at a final dilution of 0.1 %. Omeprazole was added to the luminal side of the parietal cells while incubating with BCECF.

Gastric H^+ secretion

For isolation of gastric glands animals were fasted for 16 hours prior to experiments on wire grids with free access to tap water. After sacrificing the animals the stomach was removed and cut longitudinally. After washing 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 40 x 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). Between 8 and 20 parietal cells were outlined and monitored during the course of the measurements. The results from each cell were averaged and taken for final analysis. Intensity ratio (490/440) data were converted into pH values using the high-K⁺/nigericin calibration technique [26]. To this end the glands were perfused at the end of each experiment for 5 minutes with standard high-K⁺/nigericin (10 μ g/ml) solution (pH 7.0). The intensity ratio data thus obtained were converted into pH values using the $r_{\max}$, $r_{\min}$, pK_a values previously generated from calibration experiments performed in isolated gastric glands to generate a standard nonlinear curve (pH range 5 to 8.5).

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⁺ [27]. The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power (β) of the cells [27]. Assuming that NH₄⁺ and NH₃ are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as NH₃:

$$\beta = \Delta[NH_4^+]/\Delta pH_i$$

where ΔpH_i is the decrease of cytosolic pH (pH_i) following ammonia removal and $\Delta[NH_4^+]_i$ 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 [28] and at an extracellular pH (pH₀) of 7.4 the NH₄⁺ concentration in extracellular fluid ([NH₄⁺]₀) is 19.37 [20/(1+10^{pH₀-pK})]. The intracellular NH₄⁺ concentration ([NH₄]_i) was calculated from:

$$[NH_4]_i = 19.37 \cdot 10^{pH_0 - pH_i}$$

The calculation of the buffer capacity required that NH₄⁺ 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 was no relevant further exit of NH₄⁺. The pH recovery during and following the NH₄⁺ pulse was determined in the absence of Na⁺ to prevent cellular realkalinization by the Na⁺/H⁺ exchanger.

To calculate the $\Delta pH/\min$ during re-alkalinization, a manual linear fit was placed over a narrow pH range (pH 6.7 to 6.9) which could be applied to all measured cells.

		Rate of pH recovery (delta pH units/minute)	Intracellular pH (units)	Buffer capacity (mM / pH unit)	Number of mice	Number of cells
Control	<i>akt2</i> ^{+/+}	0.031 ± 0.01	7.27 ± 0.06	40.4 ± 4.8	8	93
(Untreated)	<i>akt2</i> ^{-/-}	0.078 ± 0.009*	7.29 ± 0.04	37.1 ± 6.1	10	114
omp	<i>akt2</i> ^{+/+}	0.001 ± 0.005 [#]	7.27 ± 0.03	31.1 ± 1.7	4	72
	<i>akt2</i> ^{-/-}	0.006 ± 0.001 [#]	7.22 ± 0.03	35.5 ± 10.9	3	50
Sham	<i>akt2</i> ^{+/+}	0.031 ± 0.015	7.35 ± 0.07	41.0 ± 7.4	5	58
(for high K ⁺)	<i>akt2</i> ^{-/-}	0.093 ± 0.014*	7.28 ± 0.05	36.3 ± 8.1	5	64
high K ⁺	<i>akt2</i> ^{+/+}	0.254 ± 0.041 [#]	7.29 ± 0.06	32.3 ± 6.5	6	78
	<i>akt2</i> ^{-/-}	0.173 ± 0.024 [#]	7.29 ± 0.05	38.3 ± 7.6	7	61
Sham	<i>akt2</i> ^{+/+}	0.034 ± 0.007	7.26 ± 0.08	32.5 ± 9.1	4	37
(for FSK)	<i>akt2</i> ^{-/-}	0.120 ± 0.026*	7.29 ± 0.04	32.2 ± 2.5	4	38
FSK	<i>akt2</i> ^{+/+}	0.103 ± 0.014 [§]	7.29 ± 0.05	34.8 ± 5.5	7	75
	<i>akt2</i> ^{-/-}	0.132 ± 0.029	7.23 ± 0.03	34.1 ± 4.7	6	63
Sham	<i>akt2</i> ^{+/+}	0.040 ± 0.010	7.27 ± 0.09	40.2 ± 10.1	4	27
(for H89)	<i>akt2</i> ^{-/-}	0.093 ± 0.009*	7.25 ± 0.06	35.8 ± 3.7	4	48
H89	<i>akt2</i> ^{+/+}	0.030 ± 0.011	7.24 ± 0.03	39.7 ± 9.2	6	74
	<i>akt2</i> ^{-/-}	0.035 ± 0.014 [§]	7.19 ± 0.03	44.9 ± 4.6	7	64

Table 1. Gastric acid secretion in *akt2*^{-/-} mice and *akt2*^{+/+} mice under basal and stimulated conditions. * - indicates significant difference to respective wild type mice. # - indicates significant difference to respective control (untreated mice). § - indicates significant difference to respective sham.

The solutions were composed of (in mM): standard HEPES: 115 NaCl, 5 KCl, 1 CaCl₂, 1.2 MgSO₄, 2 NaH₂PO₄, 10 glucose, 32.2 HEPES; sodium free HEPES: 132.8 NMDG⁺Cl⁻, 3 KCl, 1 CaCl₂, 1.2 MgSO₄, 2 KH₂PO₄, 32.2 HEPES, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG⁺Cl⁻ and mannitol were replaced with 20 mM NH₄Cl); high K⁺ for calibration 105 KCl, 1 CaCl₂, 1.2 MgSO₄, 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 the K⁺ concentration was increased at the expense of Na⁺/NMDG to 35 mM.

Determination of pH, acid content and K⁺ concentration of the gastric lumen

The pH of the luminal stomach contents depends on the amount of acid secreted. Activation of gastric gland leads to decrease in luminal pH and conversely inhibition leads to increase in pH. The luminal pH of the overnight fasted mice therefore serves as a useful indicator of basal gastric acid secretion. To determine the basal intraluminal pH, a previously described method was slightly modified and used [29, 30]. Briefly, the mice were fasted overnight on wire grids with free access to tap water and sacrificed under slight ether anaesthesia. The whole stomach was then carefully removed and cut open and the luminal contents were collected by washing the stomach with 10 ml unbuffered isotonic sodium chloride solution (containing additionally 5 mM KCl and 10 mM glucose). The washings were then centrifuged at 150 g for 5 minutes to remove the particulate matter and the pH of the supernatant solution was measured by a standard pH meter (Knick, Germany). The acid content in the solution was measured by titrating the above obtained supernatant solution with 0.01 N NaOH to an endpoint of pH 7.0 by monitoring the pH with a pH meter and addition-

ally adding a universal pH indicator (Sigma, Taufkirchen, Germany) to the solution. The acid content thus obtained was then corrected by the acid content of the blank isotonic sodium chloride solution and used for further statistical analysis. The K⁺ ion concentrations in the luminal aspirates were measured by flame photometry (model AFM 5051; Eppendorf, Hamburg, Germany).

Determination of cAMP concentration

To determine the cAMP concentration of gastric tissue, mice were fasted overnight on wire grids with free access to tap water. After sacrificing the mice under slight ether anaesthesia, the mice were cut open and the gastric corpus was removed and quickly snap frozen until further analysis. The cAMP levels of the gastric tissue were then measured utilizing an ELISA kit (Calbiochem, Germany) according to manufacturers instructions. The obtained concentration of cAMP was then expressed in terms of pM/mg tissue taken.

Western blot analysis

Protein expression was examined by Western blotting. Mouse tissue was lysed in lysis buffer containing 50 mM Tris, 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxychlorate, 0.4% Beta-ME and 1 protease inhibitor tablet. Protein lysates were cleared of cellular debris by centrifugation for 10 min at 13.000 rpm at 4°C. The protein concentration was determined by the Bio-Rad Protein Assay System and equivalent amounts of proteins (20 μg) were separated on 10% SDS-PAGE under reducing conditions. Afterwards the proteins were transferred to nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA). The membranes were incubated with TBS containing 0.1% Tween 20 and 5% nonfat dry milk to block non-specific binding, then incubated with rabbit anti-PKA (1:1000), rabbit

anti-phospho-PKA (1:1000) or rabbit anti- β -actin (1:1000), rabbit anti akt2 (1:1000) or rabbit anti tubulin (1:1500) primary antibody overnight at 4°C and thereafter for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody (DakoCytomation, Hamburg). All the primary antibodies were purchased from 'Cell signalling', Frankfurt, Germany. Signals were visualized using the ECL chemiluminescence detection system (Amersham Biosciences).

Real-Time Reverse transcription-PCR (RT-PCR, Light Cycler)

Total RNA was extracted from mouse tissue in Trizol (Pqrlab, Erlangen, Germany) according to the manufacturer's instructions. After DNase digestion reverse transcription of total RNA was performed using random hexamers (Roche Diagnostics, Penzberg, Germany) and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Polymerase chain reaction (PCR) amplification of the respective genes were set up in a total volume of 20 μ l using 40 ng of cDNA, 500nM forward and reverse primer and SensiMixTM Lite Kit (Quantace Bioline GmbH, Luckenwalde, Germany) according to the manufacturer's protocol. Cycling conditions were chosen as follows: initial denaturation at 95°C for 5min, followed by 40 cycles of 95°C for 30sec, 55°C for 30 sec and 72°C for 30sec and terminal elongation at 72°C for 5min. For the amplification we used the following primers (5'→3' orientation): *Kcnq1*, fw GCC ACC ATC AAG GTC ATC AG rev TGA CAT CTC GCA CGT CGT AG; *Atp4b*, fw CCC AAT GGC ACC TTC AGT CT rev GGA GCT TGG CTG CCA CC; *Tbp*, fw CAC TCC TGC CAC ACC AGC TT rev TGG TCT TTA GGT CAA GTT TAC AGC C. Specificity of PCR products was confirmed by analysis of a melting curve. Real-time PCR amplifications were performed on a Light Cycler (Roche Diagnostics) and all experiments were done in doublets. Amplification of the house-keeping gene *Tbp* was performed to standardize the amount of sample RNA. Relative quantification of gene expression was performed using the Δ ct method as described earlier [31].

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

To isolate the parietal cells from gastric mucosa, a previously described protocol [32] was 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 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

Fig. 1. Akt2 expression in gastric tissue. Original Western blot illustrating Akt2 (upper panels) and tubulin (lower panels) protein abundance in the gastric tissue from akt2^{-/-} and akt2^{+/+} mice (n = 4 mice in each group).

Fig. 2. Body and stomach weight. Arithmetic means $\pm$ SEM (n = 5-6 mice each group) of a. Body weight and b. Empty stomach weights of akt2^{-/-} mice (closed bars) and akt2^{+/+} mice (open bars) * indicates significant difference between akt2^{-/-} mice and akt2^{+/+} mice.

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

Intracellular pH, pH recovery and buffer capacity of all parietal cells from one gland were averaged, and the respective mean values were used for further statistical analysis. Data are provided as arithmetic means $\pm$ 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.

Fig. 3. pH recovery in parietal cells following an ammonium pulse in the presence and absence of omeprazole. Alterations of cytosolic pH (Δ pH) in parietal cells following an ammonium pulse. To load the cells with H^+ , 20 mM NH_4Cl was added and Na^+ removed (replaced by NMDG, '0' Na^+) in a first step (see bars below each original tracing), NH_4Cl removed in a second step, Na^+ added in a third step and nigericin (pH_0 '7') applied in a fourth step to calibrate each individual experiment. a. Original tracings illustrating alterations of pH in typical experiments in $akt2^{+/+}$ mice (left panels) and $akt2^{-/-}$ mice (right panels) in the absence (upper panels) and presence (lower panels) of 100 μ M omeprazole. b. Arithmetic means $\pm$ SEM of Na^+ -independent alterations of cytosolic pH (Δ pH/min) in parietal cells from $akt2^{-/-}$ mice (closed bars) and $akt2^{+/+}$ mice (open bars) in the absence (n = 8-10 mice each group, left bars) and presence (n = 3-4 mice each group, right bars) of 100 μ M omeprazole. * indicates significant difference between $akt2^{-/-}$ mice and $akt2^{+/+}$ mice, # indicates significant difference between absence (control) and presence of omeprazole.

Results

Western blot analysis was performed in the gastric tissue to elucidate the expression of Akt2. As seen in Fig. 1, Akt2 protein was indeed expressed in the stomach tissue from $akt2^{+/+}$ mice and it was absent in the tissue from $akt2^{-/-}$ mice (Fig. 1). To study the gastric acid secretion, the mice were fasted overnight and the body weight and also the weight of empty stomach were recorded. The body weight was similar in $akt2^{-/-}$ and $akt2^{+/+}$ mice but the stomach weight was significantly larger in the $akt2^{-/-}$ mice (Fig. 2). According to BCECF fluorescence, the cytosolic pH was similar in isolated gastric glands from $akt2^{-/-}$ and from $akt2^{+/+}$ mice under basal conditions and was not significantly altered by the various experimental conditions (Table 1).

The buffer capacity was again similar in gastric gland cells from $akt2^{-/-}$ and $akt2^{+/+}$ mice and was again not significantly altered by the various experimental conditions (Table 1). Accordingly, a similar movement of H^+ across the cell membrane was expected to generate a similar change of cytosolic pH in gastric glands from $akt2^{-/-}$ and $akt2^{+/+}$ mice. Thus, alterations of cytosolic pH could be taken as a measure of the H^+ transport rate across the cell membrane.

Fig. 4. Intraluminal pH and acid content of stomach from overnight fasted mice. Arithmetic means $\pm$ SEM of (n = 7 mice each group) a. pH of the luminal contents and b. acid equivalents in the luminal contents of stomach from $akt2^{-/-}$ mice (closed bars) and $akt2^{+/+}$ mice (open bars) # indicates significant difference with respect to blank isotonic solution, * indicates significant difference between $akt2^{-/-}$ mice and $akt2^{+/+}$ mice.

The Na^+ -independent pH recovery following an ammonium pulse (Δ pH/min), a measure of H^+/K^+ ATPase activity, was significantly ($\sim$ 2-fold) faster in gastric glands from $akt2^{-/-}$ mice than in gastric glands from $akt2^{+/+}$ mice. In both genotypes, Δ pH/min was abolished in the presence of the H^+/K^+ ATPase inhibitor omeprazole (100 μ M) (Fig. 3).

Fig. 5. Influence of enhanced extracellular K^+ concentration on pH recovery in parietal cells. a. Original tracings illustrating alterations of pH in typical experiments in $akt2^{+/+}$ mice (left panels) and $akt2^{-/-}$ mice (right panels) at control (3 mM, upper panels) and at enhanced (35 mM, lower panels) extracellular K^+ concentration. b. Arithmetic means $\pm$ SEM ($n = 6-7$ mice each group) of Na^+ independent alterations of cytosolic pH ($\Delta pH/min$) at enhanced (35 mM) extracellular K^+ concentration in parietal cells from $akt2^{-/-}$ mice (closed bars) and $akt2^{+/+}$ mice (open bars). # indicates significant difference between high or low K^+ concentration.

Fig. 6. Effects of forskolin on pH recovery in parietal cells. a. Original tracings illustrating alterations of pH in typical experiments in $akt2^{+/+}$ mice (left panels) and $akt2^{-/-}$ mice (right panels) in the absence (upper panels) and presence (lower panels) of forskolin (FSK, 5 μM). b. Arithmetic means $\pm$ SEM ($n = 4-7$ mice each group) of Na^+ -independent alterations of cytosolic pH ($\Delta pH/min$) in parietal cells following an ammonium pulse in the absence (left bars) and presence (right bars) of forskolin (FSK, 5 μM) in gastric glands from $akt2^{-/-}$ mice (closed bars) and $akt2^{+/+}$ mice (open bars). * indicates significant difference between $akt2^{-/-}$ mice and $akt2^{+/+}$ mice, § indicates significant difference between absence (sham) and presence of forskolin.

To confirm the enhanced gastric acid secretion in $akt2^{-/-}$ mice, pH and the acid content of the gastric lumen were studied. The pH of the solution containing the luminal aspirates from both mice was significantly lower than the blank solution indicating the acidification of the solution (Fig. 4a). The luminal pH in the stomach from $akt2^{-/-}$ mice was significantly lower as compared to

that from $akt2^{+/+}$ mice. Accordingly the acid content was also significantly (~ 2 -fold) higher in the gastric lumen from $akt2^{-/-}$ mice (Fig. 4b), thus confirming the enhanced basal acid secretion in these mice.

An increase of the bath K^+ concentration to 35 mM (replacing $Na^+/NMDG$) increased $\Delta pH/min$ to similar values in gastric glands from $akt2^{-/-}$ and $akt2^{+/+}$ mice

Fig. 7. Parietal cell basal cAMP levels and phosphorylated PKA (P-PKA) levels. a. Arithmetic means $\pm$ SEM (n = 9-11 mice each group) of basal cAMP levels in the gastric tissue from *akt2*^{-/-} mice (closed bars) and *akt2*^{+/+} mice (open bars). * indicates significant difference between *akt2*^{-/-} mice and *akt2*^{+/+} mice. b. Original Western blot illustrating the phosphorylated PKA (upper panels), total PKA (middle panels) and β -actin (lower panels) in the gastric tissue from *akt2*^{-/-} and *akt2*^{+/+} mice (n = 3 mice in each group). c. Arithmetic means $\pm$ SEM of relative densities of P-PKA protein expression in stomach from *akt2*^{-/-} mice (closed bar) and *akt2*^{+/+} mice (open bar). * indicates significant difference between *akt2*^{-/-} and *akt2*^{+/+} mice.

(Fig. 5). The pH recovery tended to be higher in *akt2*^{+/+} mice than in *akt2*^{-/-} mice under conditions of enhanced K⁺ (Fig. 5). The difference did, however, not reach statistical significance. In any case, an increase of extracellular K⁺ concentration abolished the statistically significant difference of gastric acid secretion between *akt2*^{+/+} mice and *akt2*^{-/-} mice.

Treatment of gastric glands with 5 μ M forskolin significantly increased Δ pH/min in gastric glands from *akt2*^{+/+} mice but had no significant effect on Δ pH/min in gastric glands from *akt2*^{-/-} mice (Fig. 6). The difference between the genotypes was thus abolished in the presence of forskolin, suggesting a role of cAMP in the enhancement of gastric acid secretion in *akt2*^{-/-} mice.

Intracellular cAMP levels were significantly higher in the parietal cells from *akt2*^{-/-} mice (Fig. 7a) and accordingly Western blot analysis of the gastric tissue revealed increased phosphorylation of PKA in *akt2*^{-/-} mice (Fig. 7b, c).

A stimulation of gastric acid secretion could result from increased activity of KCNQ1 channels [32]. Therefore membrane expression of KCNQ1 was analyzed in isolated parietal cells using flow cytometry. Membrane abundance of KCNQ1 was significantly higher in parietal cells from *akt2*^{-/-} mice as compared to *akt2*^{+/+} mice (Fig. 8b). Increased activity of potassium channels (KCNQ1) would result in increased secretion of K⁺ ions into the lumen, which could result in elevated luminal K⁺ concentration. Flame photometric analysis indeed revealed a significantly higher K⁺ concentrations in the luminal aspirates of *akt2*^{-/-} mice, an observation again pointing to increased K⁺ channel activity (Fig 8c). Real time PCR analysis of KCNQ1 and the β -subunit of the H⁺/K⁺ ATPase were further done to explore, whether the continued activation of PKA in the *akt2*^{-/-} mice lead to any genomic upregulation of the channel and the pump. The expression of both the KCNQ1 channel and the β -subunit

Fig. 8. Expression of KCNQ1 and of the β -subunit of the H⁺/K⁺ ATPase in gastric tissue. a. Representative histogram of KCNQ1-dependent fluorescence intensity. b. Arithmetic means $\pm$ SEM (n = 4 mice each group) of the KCNQ1-dependent fluorescence intensity in parietal cells from *akt2*^{-/-} mice (closed bar) and *akt2*^{+/+} mice (open bar). c. Arithmetic means $\pm$ SEM (n = 4 mice each group) of K⁺ ion concentration in the gastric luminal aspirates from *akt2*^{-/-} mice (closed bar) and *akt2*^{+/+} mice (open bar). d. Arithmetic means $\pm$ SEM (n = 6-7 mice each group) of the relative expression of KCNQ1 mRNA in the gastric tissue from *akt2*^{-/-} mice (closed bars) and *akt2*^{+/+} mice (open bars). e. Arithmetic means $\pm$ SEM (n = 6-7 mice each group) of the relative expression of the H⁺/K⁺ ATPase β -subunit in gastric tissue from *akt2*^{-/-} mice (closed bars) and *akt2*^{+/+} mice (open bars). * indicates significant difference between *akt2*^{-/-} mice and *akt2*^{+/+} mice.

of the H⁺/K⁺ ATPase were significantly increased in the *akt2*^{-/-} mice (Fig. 8d, e).

Treatment of the gastric glands with the protein kinase A inhibitor H89 (50 nM) significantly decreased

Fig. 9. Effects of H89 on pH recovery in parietal cells. a. Original tracings illustrating alterations of pH in typical experiments in *akt2*^{+/+} mice (left panels) and *akt2*^{-/-} mice (right panels) in the absence (upper panels) and presence (lower panels) of PKA inhibitor H89 (H89, 50 nM). b. Arithmetic means $\pm$ SEM (n = 4-7 mice each group) of Na⁺-independent alterations of cytosolic pH (Δ pH/min) in parietal cells following an ammonium pulse in the presence of H89 (H89, 50 nM) in gastric glands from *akt2*^{-/-} mice (closed bars) and *akt2*^{+/+} mice (open bars). * indicates significant difference between *akt2*^{-/-} mice and *akt2*^{+/+} mice, § indicates significant difference between absence (sham) and presence of H89.

Δ pH/min in *akt2*^{-/-} mice but was without significant effect in *akt2*^{+/+} mice (Fig. 9). As a result, H89 dissipated the difference of Δ pH/min between the two genotypes thus clearly indicating the increased activity of cAMP activated PKA as a reason for enhanced gastric acid secretion in *akt2*^{-/-} mice.

Discussion

The present study reveals a role of the protein kinase isoform PKB/Akt2 in the regulation of gastric acid secretion. Apparently, PKB/Akt2 is mediating the inhibitory effects of phosphatidylinositol 3 kinase [14] and PDK1 [22] on basal gastric acid secretion. PI3K/PDK1 signaling leads to activation of the serum and glucocorticoid inducible kinase (SGK) and protein kinase B (PKB/Akt) isoforms [15-21, 33]. The observation that pharmacological PI3 kinase inhibition abolished the phosphorylation of PKB/Akt prompted the assumption that PKB/Akt could have been the downstream target of PI3 kinase signaling [14].

SGK1, on the other hand, does apparently not participate in the regulation of basal gastric acid secretion, as acid secretion in nonstimulated cells is similar in SGK1

knockout mice and their wild type littermates [34]. SGK1, however, contributes to or even accounts for the stimulation of gastric acid secretion by glucocorticoids [34]. Interestingly, stimulation of gastric acid secretion by dexamethasone was blunted by inhibition of PI3 kinase [35], an observation possibly reflecting PI3 kinase dependent activation of SGK1. Thus, the PI3 kinase may play a dual role in the regulation of gastric acid secretion. On the one hand, PI3 kinase signaling may downregulate basal gastric acid secretion by activating PKB and thus interfering with cAMP formation. On the other hand, PI3 kinase signaling may activate SGK1 and thus under the influence of glucocorticoids stimulate gastric acid secretion. At low expression levels of SGK1, as in the absence of glucocorticoids, the inhibitory effect appears to prevail. Beyond their effect on SGK1, glucocorticoids downregulate cyclooxygenase [36, 37], an effect similarly expected to enhance gastric acid secretion [36-39].

Akt2 sensitivity of gastric acid secretion apparently results from an influence of Akt2 on the availability of luminal K⁺ for the H⁺/K⁺ ATPase. Accordingly, increase of extracellular K⁺ concentration enhances gastric acid secretion to a greater extent in *akt2*^{+/+} mice than in *akt2*^{-/-} mice. The recycling of cellular K⁺ into the lumen is accomplished by K⁺ movement through KCNQ1

channels, which are critically important for gastric acid secretion [8, 40, 41]. The KCNQ1 channels are activated by SGK1 [42-44] and by protein kinase A [9-12].

The inhibitory effect of Akt2 on gastric acid secretion is apparently reversed by activation of adenylate cyclase with forskolin. Presumably, activation of Akt2 suppresses the formation of cAMP [14] and lack of Akt2 leads to unrestrained activation of gastric acid secretion. Accordingly, the protein kinase A inhibitor H89 substantially decreased the gastric acid secretion in akt2^{-/-} mice and again abolished the difference between the genotypes.

In conclusion, the gastric acid secretion is significantly enhanced in akt2^{-/-} mice as compared to akt2^{+/+} mice. Thus, Akt2 inhibits basal gastric acid secretion and thus contributes to or even accounts for the inhibitory effect of PI3 kinase and PDK1 on gastric acid secretion.

Abbreviations

BCECF (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester); DMSO (Dimethylsulfoxide); NMDG (N-methyl D-glucamine); HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); FSK (forskolin); omp (omeprazole); ATP4b (β -subunit of H⁺/K⁺ ATPase); Tbp (TATA box binding protein); SGK (serum and glucocorticoid inducible kinase); PDK (phosphoinositide-dependent kinase); PI3K (phosphoinositol 3 kinase); PKB/Akt (protein kinase B).

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