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ROLE OF ION TRANSPORTERS IN THE BILE ACID-INDUCED ESOPHAGEAL INJURY

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Running title: Effect of bile acids on the esophageal epithelium

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Abbreviations: BAC**:** bile acid cocktail; BE: Barrett's esophagus; $[Ca^{2+}]_i$ intracellular Ca^{2+} concentration; CBE: Cl^-/HCO_3^- exchanger; EECs: esophageal epithelial cells; GERD: gastroesophageal reflux disease; pH_i: intracellular pH, NHE: Na^+/H^+ exchanger; NBC: Na^+/HCO_3^- cotransporter; PAT-1: putative anion transporter-1; SE: squamous epithelium

ABSTRACT

Barrett's esophagus (BE) is considered to be the most severe complication of gastro-esophageal reflux disease (GERD), in which the prolonged, repetitive episodes of combined acidic and biliary reflux result in the replacement of the squamous esophageal lining by columnar epithelium. Therefore, acid extruding mechanisms of esophageal epithelial cells (EECs) may play an important role in the defence. Our aim was to identify the presence of acid/base transporters on EECs and to investigate the effect of bile acids on their expressions and functions. Human EEC lines (CP-A and CP-D) was acutely exposed to bile acid cocktail (BAC) and the changes in intracellular pH (pH_i) and Ca^{2+} concentration ($[Ca^{2+}]_i$) were measured by microfluorometry. mRNA and protein expression of ion transporters were investigated by RT-PCR, Western Blot and immunohistochemistry. We have identified the presence of Na^+/H^+ exchanger (NHE), Na^+/HCO_3^- cotransporter (NBC) and a Cl^- dependent HCO_3^- secretory mechanism in CP-A and CP-D cells. Acute administration of BAC stimulated HCO_3^- secretion in both cell lines and the NHE activity in CP-D cells by an IP_3-dependent calcium release. Chronic administration of BAC to EECs increased the expression of ion transporters compared to non-treated cells. Similar expression pattern was observed in biopsy samples from BE compared to normal epithelium. We have shown that acute administration of bile acids differently alters ion transport mechanisms of EECs, whereas chronic exposure to bile acids increases the expression of acid/base transporters. We speculate that these adaptive processes of EECs, represent an important mucosal defence against the bile acid-induced epithelial injury.

Keywords: esophagus, epithelium, bile acids, ion transporters.

INTRODUCTION

Barrett's esophagus (BE) is a premalignant condition of esophageal adenocarcinoma, characterized by the replacement of the normal squamous epithelium (SE) with a columnar, specialized intestinal type mucosa.(50) It is considered to be the most severe complication of gastro-esophageal reflux disease (GERD),(7, 20) in which the prolonged, long term, repetitive episodes of combined acidic and biliary reflux are thought to induce the development of a metaplastic mucosal lining in the esophagus.(44) By definition, esophageal columnar metaplasia is present if columnar lining can be observed above the esophagogastric junction (top of the gastric folds or distal end of esophageal palisade veins) during endoscopy. These metaplastic areas, however have a significant histological diversity. Although, specialized intestinal metaplasia is accepted most widely as the premalignant condition for esophageal adenocarcinoma, other histological structures – such as gastric, pancreatic or even ciliated metaplasias – are commonly present and subsequently they may also have a role in the timeline of the metaplasia – dysplasia – carcinoma sequence according to a rencent hypothesis.(36) Furthermore, both British and Montreal defintion of BE pay attention to the non-intestinal type esophageal metaplasias, dispite they have far less – if any – pontential for malignant transformation compared to specialized intestinal metaplasia.(64)

Several studies have established the harmful effects of both gastric and bile acids on the esophageal mucosa. (13, 40, 41, 47, 58, 63) Since they were also shown to promote cell differentiation and proliferation, their role in the development of columnar metaplasia and later esophageal adenocarcinoma is widely accepted. (10, 14, 22, 24, 30, 44, 45) However, the underlying mechanism by which metaplastic columnar epithelium then dysplasia and finally invasive cancer develops, is not completely understood yet.

Several defensive mechanisms exist in esophageal epithelial cells (EECs) against the reflux-induced esophageal injury. One of the most important is the esophageal epithelial

resistance.(22, 38) It consists of functional and structural components such as, (i) surface mucus and unstirred water layers with HCO_3^- in it, which provides an alkaline environment, (ii) cell junctions (tight junctions) and transport proteins at the apical and basolateral membranes, which prevent the diffusion of H^+ into the intercellular space and into the cell, respectively and (iii) intracellular buffering systems, such as HCO_3^- or phosphates buffering systems.(38, 39)

The transport proteins on the apical and basolateral membranes of EECs play an important role in the epithelial defense mechanisms.(38, 39) At the apical membrane of EECs only a non-selective cation channel has been identified so far.(2) This channel is present in the SE of rabbits and has been shown equally permeable to Na^+, Li^+, K^+ or even H^+. The physiological role of this channel in esophageal epithelial function is poorly understood. Tobey et al. have shown that acidic pH inhibits channel activity so H^+ can not enter the cell through this channel and therefore may represent a protective mechanism against luminal acidity.(57) Others suggest that this cation channel plays role in cell differentiation. Blockade of this channel by acidic pH may inhibit the replenishment of polarized epithelial cells from undifferentiated basal cells.(2)

In contrast, at the basolateral membrane of SE several ion transporters have been identified. Tobey et al. have shown the presence of a Na^+-dependent and Na^+–independent, disulfonic stilbene-sensitive, Cl^-/HCO_3^- exchangers (CBE) on cultured rabbit SE.(60, 61) The Na^+-independent CBE mediates the efflux of HCO_3^- into the lumen, which results in the acidification of the intracellular pH (pH_i). In contrast, the Na^+-dependent CBE operates in a reverse mode and promotes the influx of HCO_3^- in exhange for intracellular Cl^- and therefore contributes to the alkalisation of the cell.(60, 61) Beside the CBEs an amiloride-sensitive, Na^+/H^+ exchanger (NHE) has also been identified on the basolateral membrane of rat, rabbit and human SE.(29, 48, 59) Among the 9, known NHE isoforms, NHE1 has been shown to

present on EECs using reverse-transcription PCR (RT-PCR) and western-blot. The major role of NHE1 in the esophagus, is the regulation of pH_i by the electroneutral exchange of intracellular H^+ to extracellular Na^+. In addition, NHE1 is also important in several defensive mechanisms such as cell volume regulation, proliferation and cell survival.(6, 10, 70)

These studies have been performed on normal esophageal epithelium; however the activity or expression of these ion transporters in the columnar epithelia or under pathophysiological conditions is less characterized. Goldman et. al has recently shown that acute administration of bile acids dose-dependently decreases the pH_i of human EECs derived from normal mucosa and BE.(15) This effect of bile acids is due to the activation of nitric oxide synthase which cause increased nitric oxide production that leads to the inhibition of NHE1 activity. Blockage of NHE1 results in extensive intracellular acidification and therefore DNA damage. Combination of bile acids at acidic pH caused a further decrease in pH_i and resulted in a higher degree of DNA damage. It has also been shown that NHE1 is expressed at higher level in BE than in normal epithelium.(15) The DNA damaging effect of bile and acid have also been shown in normal esophageal cell line (HET1-A) which may participate in the development and progression of BE.(24)

Ion transport processes highly contribute to luminal acid clearence mechanisms as well as esophageal tissue resistance, therefore the understanding of esophageal epithelial ion transport processes under physiological and pathophysiological conditions is of crucial importance. Ion transporters have been well characterized in SE but less in columnar epithelial cells; however, columnar epithelial cells play an essential role in the protection of the esophagus against further reflux-induced esophageal injury by the action of acid/base transporters. Therefore, our aims in this study were (i) to identify transport mechanisms in columnar epithelial cells derived from Barrett's metaplasia (ii) to characterize the effect of main internal risk factors (such as HCl, bile acids) on the acid/base transporters and (iii) to

compare the mRNA and protein expression profile of acid/base transporters in human squamous and columnar epithelial cells obtained from normal esophageal mucosa and BE.

MATERIALS AND METHODS

Cell line

CP-A human, non-dysplastic Barrett's esophageal cell line was obtained from American Type Culture Collection. CP-D human, dysplastic Barrett's cell line was kindly provided by Peter Rabinovich (University of Washington). Cells were maintained in MCDB-153 medium supplemented with 5% fetal bovine serum, 4 mM L-glutamine, 0.4 μg/ml hydrocortisone, 20 mg/L adenine, 20 ng/ml recombinant human Epidermal Growth Factor, 8.4 μg/L cholera toxin, 140 μg/ml Bovine Pituitary Extract, 1x ITS Supplement [5 μg/ml Insulin; 5 μg /ml Transferrin; 5 ng/ml Sodium Selenite]. Medium was replaced in every 2 days and cells were seeded at 100% confluency. Cultures were continually incubated at 37 °C and gassed with the mixture of 5% CO2 and 95% air. Passage numbers between 20-30 were used in all experiment.

Patients

Fourteen patients with endoscopic evidence of esophageal metaplasia were enrolled in the First Department of Medicine, University of Szeged. Endoscopic procedures were carried out by standard, high resolution, white-light endoscopes (Olympus GIF-Q165) and the Prague C&M criteria were applied for the description of esophageal metaplasia.(49)

Four biopsy samples were obtained from the macroscopically visible metaplastic columnar epithelium of the esophagus and an other four from the normal squamous lining. Two of each samples were formalin-fixed and submitted for histological evaluation including

174 immunohistochemistry. The remaining two samples were immediately placed and stored in
175 RNA-later solution for real-time PCR analysis at -20°C. The patient details are shown in
176 Table 1. All procedures were performed with informed patient consent and under approved
177 human subject's protocols from University of Szeged (No.: 2348).
178
179 **Chemicals and solutions**
180 General laboratory chemicals and bile acid salts were obtained from Sigma-Aldrich
181 (Budapest, Hungary). 2,7-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester
182 (BCECF-AM), 2-(6-(bis(carboxymethyl)amino)-5-(2-(2-(bis(carboxymethyl)amino)-5-
183 methylphenoxy)ethoxy)-2-benzofuranyl)-5-oxazolecarboxylic acetoxymethyl ester (Fura-2
184 AM), 1,2-bis(o-aminophenoxy)ethane-N,N,N9,N9-tetraacetic acid (BAPTA-AM), 4,4'-
185 diisothiocyanatodihydrostilbene-2,2'-disulfonic acid, disodium salt (H_2DIDS) were from
186 Molecular Probes Inc (Eugene, OR). BCECF-AM (2 µmol/l) and BAPTA-AM (40 µmol/l)
187 were prepared in dimethyl sulfoxide (DMSO), whereas FURA-2-AM (5 µmol/l) was
188 dissolved in DMSO containing 20% pluronic acid. 4-isopropyl-3-methylsulphonylbenzoyl-
189 guanidin methanesulphonate (HOE-642) was provided by Sanofi Aventis (Frankfurt,
190 Germany) and was dissolved in DMSO. Nigericin (10 mM) was prepared in ethanol and
191 stored at -20 ºC.
192 The compositions of the solutions used are shown in Table 2. Standard HEPES-
193 buffered solutions were gassed with 100% O_2 and their pH was set to 7.4 with NaOH.
194 Standard HCO_3-/CO_2-buffered solutions were gassed with 95% O_2/5% CO_2 to set pH to 7.4.
195 All experiments were performed at 37 ºC.
196
197 **Measurment of intracellular pH and Ca^{2+} with microfluorimetry**

150.000-250.000 cells were seeded to 24 mm cover slips which were mounted on the stage of an inverted fluorescence microscope linked to an Xcellence imaging system (Olympus, Budapest, Hungary). Cells were bathed with different solutions at 37^{o}C at the perfusion rate of 5-6 ml/min. 6-7 cells/region of interests (ROIs) were examined in each experiments and one measurement per second was obtained. In order to estimate pH_i cells were loaded with the pH-sensitive fluorescent dye, BCECF-AM for 20-30 min at room temperature. Cells were excited with 490 and 440 nm wavelengths, and the 490/440 fluorescence emission ratio was measured at 535 nm. The calibration of the fluorescent emission ratio to pH_i was performed with the high-K^+-nigericin technique, as previously described.(19, 56) To determine the changes of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) cells were incubated with FURA2-AM and pluronic acid for 50-60 min. For excitation, 340 and 380 nm filters were used, and the changes in $[Ca^{2+}]_i$ were calculated from the 340/380 fluorescence ratio measured at 510 nm.

Determination of buffering capacity and base efflux

The total buffering capacity (β_{total}) of cells was estimated according to the NH_4^+ prepulse technique, as previously described.(18, 69) Briefly, EECs were exposed to various concentrations of NH_4Cl in a Na^+- and HCO_3^--free solutions. The total buffering capacity of the cells was calculated using the following equation: $\beta_{total} = \beta_i + \beta_{HCO3-} = \beta_i + 2.3 \times [HCO_3^-]_i$, where β_i refers to the ability of intrinsic cellular components to buffer changes of pH_i and was estimated by the Henderson–Hasselbach equation. β_{HCO3-} is the buffering capacity of the HCO_3^-/CO_2 system. The measured rates of pH_i change ($\Delta pH/\Delta t$) were converted to transmembrane base flux $J(B^-)$ using the equation: $J(B^-)=\Delta pH/\Delta t \times \beta_{total}$. The β_{total} value at the start point pH_i was used for the calculation of $J(B^-)$. We denote base influx as $J(B)$ and base efflux (secretion) as $-J(B^-)$.

Measurment of the activity of Na^+/H^+ exchanger, Na^+/HCO_3^- cotransporter and Cl^-/HCO_3^- anion exchanger

In order to estimate the activity of NHEs, the Na^+/HCO_3^- cotransporter (NBC) and CBE the NH_4Cl prepulse technique was used. Briefly, exposure of esophageal cells to 20 mM NH_4Cl for 3 min induced an immediate rise in pH_i due to the rapid entry of lipophilic, basic NH_3 into the cells. After the removal of NH_4Cl, pH_i rapidly decreased. This acidification is caused by the dissociation of intracellular NH_4^+ to H^+ and NH_3, followed by the diffusion of NH_3 out of the cell. In standard Hepes-buffered solution the initial rate of pH_i ($\Delta pH/\Delta t$) recovery from the acid load **(**over the first 60 sec) reflects the activities of NHEs, whereas in HCO_3-/CO_2-buffered solutions represents the activities of both NHEs and NBC.(18)

Two independent methods have been performed in order to estimate CBE activity . Using the NH_4Cl prepulse technique the initial rate of pH_i recovery from alkalosis in HCO_3-/CO_2-buffered solutions was analyzed.(18) Previous data have indicated that under these conditions the recovery over the first 30 seconds reflects the activity of CBE.(18) The Cl^- withdrawal technique was also applied, where removal of Cl^- from the external solution causes an immediate and reversible alkalisation of the pH_i due to the reverse operation of CBE under these conditions. Previous data have shown that the initial rate of alkalisation over the first 60 seconds reflects the activity of CBE.(66)

In order to evaluate transmembrane base flux ($J(B^-)$) the following equation was used: $J(B^-)= \Delta pH/\Delta t \times \beta_{total}$, where $\Delta pH/\Delta t$ was calculated by linear regression analysis, whereas the total buffering capacity (β_{total}) was estimated by the Henderson–Hasselbach equation using the following formula: $\beta_{total} = \beta_i + \beta_{HCO3-} = \beta_i + 2.3x[HCO_3^-]_i$. We denote base influx as $J(B)$ and base efflux (secretion) as $-J(B^-)$.(18, 69)

Bile acid treatments

In order to mimic the chronic bile acid exposure in GERD *in vitro*, cells were treated with bile acid cocktail (BAC) at pH 7.5 and 5.5. Two days prior to bile acids treatment, cells were seeded at 10^6 cells/75 cm^2 tissue culture flasks and were grown to 70-80% of confluence. On the second day, after the seeding, cells were treated with bile acids for 10 min pulses, 3 times a day up to 7 days.(12) The compostion of BAC was: 170 μM glycocholic acid (GC), 125 μM glycochenodeoxycholic acid (GCDC), 100 μM deoxycholic acid (DC), 50 μM glycodeoxycholic acid (GDC), 25 μM taurocholic acid (TC), 25 μM taurochenodeoxycholic acid (TCDC) and 8 μM taurodeoxycholic acid (TDC). The composition and concentration of BAC mimics the bile acid profile of GERD.(12, 26, 32)

Quantitative real time PCR analysis

Total RNA was purified from individual cell culture and biopsy samples using the RNA isolation kit of Macherey-Nagel (Nucleospin RNA II kit, Macherey-Nagel, Düren, Germany). All the preparation steps were carried out according to the manufacturer's instructions. RNA samples were stored at –80^{o}C in the presence 30 U of Prime RNAse inhibitor (Fermentas, Lithuania) for further analysis. The quantity of isolated RNA samples was checked by spectrophotometry (NanoDrop 3.1.0, Rockland, DE, USA).

In order to monitor gene expression, quantitative real-time PCR (QRT-PCR) was performed on a RotorGene 3000 instrument (Corbett Research, Sydney, Australia) using the TaqMan probe sets of NHE1, NHE2, NBC and SLC26A6 genes (Applied Biosystems Foster City, CA, USA). 3 μg of total RNA was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems Foster City, CA, USA) according to the manufacturer's instructions in final volume of 30 μL. The temperature profile of the reverse transcription was the following: 10 min at room temperature, 2 hours at 37 ^{0}C, 5 min on ice and finally 10 min at 75 ^{0}C for enzyme inactivation. These steps were carried out in a Thermal Cycler machine

(MJ Research Waltham, MA, USA). After dilution with 30 μL of water, 1 μL of the diluted reaction mix was used as template in the QRT- PCR. For all the reactions TaqMan Universal Master Mix (Applied Biosystems Foster City, CA, USA) were used according to the manufacturer's instructions. Each reaction mixture (final volume: 20 μL) contained 1 μL of primer-TaqMan probe mix. The QRT-PCR reactions were carried out under the following conditions: 15 min at 95^{o}C and 45 cycles of 95^{o}C for 15 sec, 60^{o}C for 1 min. Fluorescein dye (FAM) intensity was detected after each cycle. All of the samples were run in triplicates and non-template control sample was used for each PCR run to check the primer-dimer formation. The average C_T value was calculated for each of the target genes (NHE1, NHE2, NBC and SLC26A6) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) and the ΔC_T was determined as the mean C_T of the gene of interest minus the mean C_T of HPRT.

In the case of cell lines, the relative changes in gene expression were determined using the $\Delta\Delta C_T$ method as described in Applied Biosystems User Bulletin No. 2 (P/N 4303859). $\Delta\Delta C_T$ was calculated using the following formula: $\Delta\Delta C_T = \Delta C_T$ of treated cells – ΔC_T of control, non-treated cells. The N-fold differential expression in the target gene was expressed as $2^{-\Delta\Delta CT}$. Genes with expression values less than or equal to 0.5 were considered to be down-regulated, whereas values higher than or equal to 2 were considered to be up-regulated. Values ranging from 0.51 to 1.99 were not considered to be significant.

In the case of biopsy samples, the relative expression values of NHE1, NHE2, NBC and SLC26A6 in normal and BE samples was used to create box plots. In order to compare the expression of genes between normal and BE samples, Wilcoxon test was used.

Western Blot analysis

Whole cell lysates were prepared as described previously.(25) Protein concentration of samples and bovine serum albumin standard was determined using the Bradford protein assay

(Bio-Rad Laboratories, Hercules, CA, USA). 30 μg of denatured protein was fractionated on a NuPAGE Bis-Tris 4–12% gel (Life Technologies, Carlsbad, CA, USA). Following electrotransfer, Immobilon-P membranes (Millipore, Billerica, MA, USA) were blocked with PBST containing 5% milk, followed by overnight incubation with the following primary antibodies: rabbit anti-NHE1 and -NHE2 (1:200, Alomone Laboratories, Jerusalem, Israel), rabbit anti-NBC (1:500, Abcam Cambridge, MA, USA), goat anti-Slc26a6 (1:200, Santa Cruz, Dallas, TX, USA) at 4 C. Mouse anti-GAPDH (1:10000 Merck Millipore) was used as an internal control. The secondary antibodies were all from Sigma-Aldrich and used at 1:10000. Targeted proteins were visualized using a chemiluminescence detection system (Amersham ECL or ECL Prime; GE Healthcare Life Sciences, Pittsburgh, PA, USA)

Immunohistochemistry

Immunohistochemical analysis of NHE1 and NHE2 expressions was performed on 4% buffered, formalin-fixed sections of human esophageal biopsy samples (n=14) embedded in paraffin. The 5 μm thick sections were stained in an automated system (Autostain, Dako, Glostrup, Denmark). Briefly, the slides were deparaffinised, and endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 (10 min). Antigenic sites were disclosed by applying citrate buffer in a pressure cooker (120 ºC, 3 min). To minimise non-specific background staining, the sections were then pre-incubated with milk (30 min). Subsequently, the sections were incubated with a mouse monoclonal anti-NHE1 (1:100 dilution, Abcam, Cambridge, UK) or chicken anti-NHE2 (1:50 dilution, Chemicon, Temecula, CA, USA). Primary antibodies exposed to LSAB2 labelling (Dako, Glostrup, Denmark) for 2 X 10 min. The immunoreactivity was visualised with 3,3'-diaminobenzidine (10 min); then the sections were dehydrated, mounted and examined. NHE1 and NHE2-containing cells were identified

by the presence of a dark-red/brown chromogen. The specificity of the primary antibodies was assessed by using mouse IgG1 or chicken IgY isotype controls.

Statistical analysis

Results are expressed as means (SEM) (n=6-7 cells/20–25 ROIs). Statistical analyses were performed using analysis of variance (ANOVA). p values ≤ 0.05 were accepted as significant.

RESULTS

pH regulatory mechanisms of human EECs

In the first series of experiments, the resting pH_i was determined. Cells were exposed to standard HEPES solution (pH 7.4), followed by a 5-minute exposure to a high K^+/nigericin-Hepes solution at pH 7.28, 7.4 and 7.6. The classical linear model was used to determine the resting pH_i of the cells.(19, 56) The resting pH_i levels of CP-A and CP-D were 7.32±0.03 and 7.31±0.03, respectively (data not shown). The resting pH_i did not differ significantly among the pH experiments.

In the next step, the major acid/base transporters of Barrett's derived cells (CP-A and CP-D) was identified. NHE is an electroneutral transporter which mediates the efflux of H^+ and influx of Na^+ across the plasma membrane via the electrochemical Na^+ gradient. Removal of Na^+ from the standard Hepes-buffered solution resulted in a rapid intracellular acidification (Fig. 1A) in CP-A cells which is likely due to the blockade of NHE. The NH_4Cl prepulse technique was also used to confirm the presence of NHE. Fig. 1B shows that administration of 20 mM NH_4Cl (3 min) in standard Hepes-buffered solution causes an immediate intracellular alkalization due to the rapid influx of NH_3 into the cells. After the removal of NH_4Cl from the

external solution the pH_i dramatically decreases (due to the dissociation of NH_4^+) then returns to the baseline level. When Na^+ was removed from the external solution the restoration of pH_i was completely abolished. (Fig. 1B) Similar results were found in CP-D cells, which indicate that these cells also express functionally active NHE. So far, 9 NHE isoforms have been identified, all of which show different regulation and expression pattern in the human body. Functional measurements were performed to identify which isoforms are present in CP-A and CP-D cells. HOE-642 is a dose dependent isoform-selective inhibitor of NHE. At 1 μM, HOE-642 inhibits only NHE1, whereas at 50 μM inhibits both NHE1 and NHE2. Using the NH_4Cl prepulse technique it was shown that 1 μM HOE-642 inhibited the recovery from acid load by 77.3 ± 3.0 % in CP-A and 70.0 ± 0.3 % in CP-D cells, whereas in the presence of 50 μM HOE-642, the recovery was completely abolished in both cell lines. (Fig. 1C and D)

NBC also plays a crucial role in pH regulation in several types of epithelial cells.(5, 53, 62) NBC is an electrogenic transporter which mediates the influx of Na^+ and HCO_3^- into the cells with a 1:2, Na^+/HCO_3^- stoichiometry. In standard HCO_3^-/CO_2-buffered extracellular solution, the pH_i of CP-A cells rapidly decreased by the quick diffusion of CO_2 into the cytoplasm. (Fig. 2A) A low level of pH_i recovery was found after acidosis, which is probably due to the influx of HCO_3^- into the cells through NBC. Removal of Na^+ resulted in the same level of acidification as in the standard Hepes-buffered solution. (Fig. 2A) In order to further confirm the presence of NBC, the effect of H_2DIDS on the recovery from CO_2-induced acidosis was investigated. H_2DIDS is an inhibitor of both NBC and CBE. As seen on Fig. 2B, 500 μM H_2DIDS completely inhibited the regeneration from acidosis. However, after the removal of H_2DIDS from the external solution, the pH_i completely recovered. Since CBE did not affect the recovery from acidosis (see Fig. 2A), we hypothesize that a functionally active NBC is present in CP-A cells. Using the same experimental protocol, the presence of NBC was also confirmed in CP-D cells.

In order to estimate the activities of NHE and NBC, the effect of H_2DIDS (500 μM) and HOE-642 (50 μM) on the recovery from acid load was tested separately and together. Both H_2DIDS and HOE-642 equally reduced the recovery from acidosis, whereas combined administration of these two agents completely abolished it. (Fig. 2C and D)

Next we attempted to identify functionally active CBE. The activity of CBE was investigated by the Cl^- removal technique in the presence and absence of HCO_3^-/CO_2. In the absence of HCO_3^-, Cl^- removal caused a very low level and reversible alkalization. (Fig. 3A) However, in standard HCO_3^-/CO_2-buffered solution, significantly higher alkalization was observed, indicating the presence of a functionally active CBE on CP-A cells. (Fig. 3B) In case of CP-D cells, a marked alkalization was also observed after the removal of external Cl^- in the presence of HCO_3^-/CO_2, suggesting that these cells also possess CBE.

Bile acids induce an intracellular acidification in CP-A cells

In order to mimic the pathophysiological conditions in GERD, BAC was prepared using a mixture of 7 bile acids, as described in Materials and Methods.(12) The effect of BAC on the pH_i of CP-A cells was tested under acidic (pH 5.5) and neutral (pH 7.5) conditions. At pH 7.5, 100 and 300 μM BAC had no effect on pH_i, whereas at higher concentration (500 μM), BAC caused a small acidification in CP-A cells.(0.1 ± 0.03; Fig. 4A) In contrast, at pH 5.5, bile acids resulted in a dose-dependent, robust decrease in pH_i (Fig. 4B). The pH_i recovered to a variable degree during continued exposure to bile acids, whereas completely returned to the basal level after the removal of bile acids from the external solution. (Fig. 4A and B). In order to examine whether the effect of bile acids at pH 5.5 is a specific effect or only due to the low pH, the effect of acidic pH by itself on pH_i was observed. Administration of Hepes-buffered solution at pH 5.5 induced a slight, reversible decrease in pH_i (from 7.32 ± 0.01 to 7.26 ± 0.01; Fig. 4D) indicating that although acid alone is able to decrease the pH_i of

CP-A cells, in combination with bile acids induce a more robust intracellular acidification. The maximal pH_i changes (ΔpH_{max}) are summarized on Fig. 4C and D. We have also investigated the rate ($-J(B^-)$) at bile acids get into the cells (Fig. 4E and F). $-J(B^-)$ was calculated from the $\Delta pH/\Delta t$ obtained by linear regression analysis of pH_i measurements made over the first 60 s after bile acid administration. Our results have shown that $-J(B^-)$ was much higher at pH 5.5 than pH 7.5.

The effect of individual bile acids (100 μM each) on pH_i was also tested. Administration of the non-conjugated DC resulted in the greatest pH_i decrease compared to the other bile acids. (Fig. 4G) The effect of DC was twice as high under acidic than under neutral conditions. In contrast, conjugated bile acids had only a slight effect at pH 7.5, whereas induced a more pronounced acidification at pH 5.5 (Fig. 4G).

Bile acids cause an IP_3-mediated calcium signaling in CP-A cells

Since bile acids have ionophore properties,(33, 37) their effect on $[Ca^{2+}]_i$ was investigated both under neutral and acidic conditions. At 100 and 300 μM concentrations, BAC had only a slight or no effect on $[Ca^{2+}]_i$. (Fig. 5A and B) Administration of acid by itself, also had only a marginal effect on $[Ca^{2+}]_i$. (Fig. 5D) In contrast, at 500 μM concentration bile acids induced a reversible increase in $[Ca^{2+}]_i$ at pH 7.5, which was more pronounced at pH 5.5. (Fig. 5A and B).

Next the source of calcium release was identified. The effect of BAC on $[Ca^{2+}]_i$ was examined either in the absence of external Ca^{2+} or in the presence of different inhibitors. Removal of Ca^{2+} from the extracellular solution slightly decreased the level of $[Ca^{2+}]_i$ due to a certain degree of $[Ca^{2+}]_i$ depletion. Under these conditions administration of 500 μM BAC, caused a slight increase in $[Ca^{2+}]_i$ indicating that BAC induces calcium signalling from intracellular sources. (Fig. 5E) Next we attempted to identify the intracellular organellum

422 from which the calcium releases. Ruthenium red (RR) and caffeine are specific inhibitors of
423 ryanodine (Ry) and inositol triphosphate (IP_3) receptors, respectively, which mediate the
424 release of calcium from endoplasmic reticulum (ER). 10 μM RR had no effect on BAC-
425 induced calcium release in calcium-free external solution. However, 20 mM caffeine
426 completely blocked the effect of BAC on calcium signalling. The effect of BAC in the
427 presence of gadolinium (Gd^{3+}, 1μM), a plasma membrane Ca^{2+} channel inhibitor was also
428 investigated. Administration of Gd^{3+} decreased the effect of 500 μM BAC on $[Ca^{2+}]_i$ by 58.83
429 ± 1.3 % (Fig. 5E), indicating that beside the release of Ca^{2+} from intracellular sources, bile
430 acids also induce the entry of extracellular Ca^{2+}.
431
432 **Acute effect of bile acids on the activity of ion transporters in EECs**
433 Next, the effect of BAC on the activity of acid/base transporters was examined using
434 the NH_4Cl prepulse technique. Administration of BAC dose-dependently decreased the
435 recovery from acidosis in Hepes-buffered solution (Fig. 6A and B), indicating that bile acids
436 inhibit the activity of NHE in CP-A cells. In order to determine which NHE isoform is
437 involved in the inhibitory effect of bile acids, the effect of BAC was tested in the presence of
438 the isoform-specific NHE inhibitor, HOE-642. 1 μM HOE-642 decreased the recovery from
439 acidosis from 7.68 ± 1.11 to 1.78 ± 0.2. Administration of 500 μM BAC, in the continuous
440 presence of 1 μM HOE-642, further decreased the acid recovery to 0.56 ± 0.09 (Fig. 6C)
441 Since 500 μM BAC inhibited acid recovery by 77.15 ± 3.2%, and nearly 77% of the total
442 NHE activity is due to NHE1, these results indicate that BAC remarkably inhibits NHE1
443 however it also blocks NHE2 activity. 50 μM HOE-642 completely blocked the recovery
444 from acidosis that was not affected by bile acids.
445 In HCO_3^-/CO_2-buffered external solution, where both NHE and NBC are active, BAC
446 caused a slighter decrease (42.56 ± 2.8% at 100 μM BAC, 47.09 ± 2.6% at 300 μM BAC and

50 ± 4.2% at 500 μM BAC; Fig. 6D and E) in CP-A cells, compared to Hepes-buffered solution. In order to evaluate the effects of bile acids on NBC alone NHE activity was completely blocked by the administration of 50 μM HOE-642. The NHE inhibitor decreased the acid recovery from 18.9 ± 2.47 to 7.85 ± 1.44 therefore the remaining recovery is due to NBC. Administration of 500 μM BAC in the continuous presence of HOE-642 increased the recovery to 14.88 ± 1.42 (Fig. 6F) suggesting that bile acids enhance the activity of NBC.

We have previously shown that the initial rate of recovery from alkalosis reflects the activity of CBE in the presence of HCO_3^-/CO_2. Treatment of CP-A cells with BAC dose-dependently increased the recovery from alkalosis (Fig. 6D and G), indicating that bile acids stimulate the HCO_3^- secretion of these cells.

The effect of BAC was also evaluated on CP-D cells. Bile acid treatment significantly increased the rate of acid recovery in Hepes-buffered solution (Fig. 6B) and the rate of acid and alkali recoveries in HCO_3^-/CO_2-buffered solution (Fig. 6E and G), indicating that the activities of the major ion transporters are increased due to bile acid treatment.

Ca^{2+} plays an essential role in the function of several intracellular processes, therefore we examined whether the inhibitory/stimulatory effect of BAC on acid/base transporters is mediated by Ca^{2+}. Pretreatment of the cells with the Ca^{2+} chelator BAPTA-AM, a significant decrease was obtained both in the inhibitory and stimulatory effects of 500 μM BAC on the ion transporters, indicating that the effects of bile acids on the acid/base transporters are calcium-dependent (data not shown).

Chronic exposure of EECs to bile acids increase the expression of acid/base transporters

In the next step, the long term effect of bile acids was assessed under neutral and acidic conditions. CP-A and CP-D cells were grown to 70-80% confluency and treated with 100 and 500 μM BAC at pH 7.5 and pH 5.5 as described in Materials and Methods. 7-days

treatment with BAC significantly increased the expression of NHE1, NHE2, NBC and an electrogenic CBE, putative anion transporter-1 (PAT-1 also known Slc26a6) compared to non-treated control cells at pH 7.5 in CP-A cells. (Fig. 7A) The expression of these ion transporters also increased in CP-D cells, however, significant changes were only detected in the case of NHE1 and NBC.(Fig. 7B) We have also performed these experiments under acidic (pH 5.5) conditions. In CP-A cells, at acidic pH alone or in combination with bile acids the expression levels of ion transporters did not change significantly (Fig. 7C) and a decrease in cell number was observed compared to the control groups. In contrast, CP-D cells displayed a significant increase in NHE1 levels after bile acid treatment at pH 5.5.(Fig. 7D) We have also shown that the enhanced mRNA levels of NHE1 were associated with significantly increased protein expression. (Fig. 7E) The Slc26a6 transporter expression also increased in CP-A cells at neutral pH (data not shown). These data are in accordance with our PCR results. However, in the case of NHE2 and NBC, there were no significant difference in the protein expression, between the control and the bile acid-treated group, at neutral pH (data not shown).

mRNA expression pattern of ion transporters was investigated in 14 pairs of normal squamous and BE biopsy samples obtained from patients with known BE. (Table 1) Using QRT-PCR, increased mRNA expressions of NHE1, NHE2, NBC and PAT-1 in BE were found both in intestinal (Fig. 8A) and non-intestinal (Fig. 8B) metaplasia compared to normal mucosa. The protein expression of NHE1 and NHE2 were also investigated by immunohistochemistry. Biopsy samples from both intestinal and non-intestinal metaplastic columnar mucosa but not from normal mucosa displayed strong membrane stainings against NHE1 and NHE2 antibodies (Fig. 8C).

DISCUSSION

Epithelial cells of the esophagus form a defensive wall against the toxic components of the refluxate. These cells reside in either stratified squamous or single lined columnar epithelium and protect the underlying tissue layers by various mechanisms. EECs provide esophageal epithelial resistance by the action of acid/base transporters which play an essential role in the maintenance of normal function of epithelial cells and therefore in the protection of the esophageal mucosa.

In this study, we have characterized the presence of ion transporters in Barrett's specialized columnar epithelial cells and investigated the effects of the major component of the refluxate on the activity and expression of these ion transporters. Using functional and molecular biological techniques we have comfirmed the presence of two acid-extruding ion transporters, NHE and NBC and one acid-loading transporter, PAT-1 in EECs. The predominant NHE isoforms were NHE1 and NHE2 although the acid-extruding mechanism is rather attributable to NHE1. Furthermore, we have demonstrated that NHEs and NBC are equally involved in the alkalisation of EECs. We have provided evidence that Barrett's cells possess PAT-1, a Cl^-/HCO_3^- transporter which mediates the exchange of intracellular HCO_3^- to extracellular Cl^- and therefore plays an important role in the acidification of the cells, however, other Cl^-/HCO_3^- exchangers may also be involved in the pH regulation of these cells.

The major toxic factors in the refluxate are gastric acid and bile.(17, 23, 27, 31, 45, 52) We have demonstrated that bile acids induce intracellular acidosis in EECs and their effect was more pronounced under acidic condition, in accordance with previous findings in Barrett's-derived and normal esophageal cell lines.(15) The administered mixture of bile acids was designed to mimic the bile acid composition of the refluxate under pathophysiological

conditions.(12, 26, 32), In accordance with the previous observations on mouse EECs only DC was shown to induce acidification at neutral conditions, and had the greatest effect at acidic pH among the seven bile acids investigated.(71) The solubility and therefore the toxicity of bile acids are mainly determined by their pKa value. The pKa value of non-conjugated bile acids, such as DC, is between 5.2-6.2 therefore at neutral pH (7.5) they are mainly in a protonated, unsoluable form. However, at pH 5.5, unconjugated bile acids are less ionised, they can penetrate through the cell membrane and influence intracellular pathways. In contrast, conjugated bile acids have a lower pKa values: taurine conjugated bile acids have a pKa between 1.8 and 1.9 and glycine conjugated bile acids have a pKa between 4.3 and 5.2.(34, 51) Therefore, at pH 5.5 and 7.5 most of these bile acids are still in ionised, inactive form which suggest that conjugated bile acids have smaller effect on cells than their non-conjugated counterparts, under these conditions. Nevertheless, not only pKa value determines the effect of bile acids. Due to their detergent properties, bile acids are able to increase the permeability of the cell membrane to various ions which also contributes to their damaging effect.(23, 46) In addition, the acidic pH also promote the disruption of the plasma membrane, which further facilitates the entry of bile acids into the cells.(21)

Bile acids also induced a dose-dependent increase in $[Ca^{2+}]_i$. Similarly to the pH measurements, the effect of bile acids was more robust under acidic conditions. These findings were in agreement with observations of other laboratories that demonstrated that exposure to DC or acidic media induced intracellular Ca^{2+} elevation in CP-A cells (14, 30) and mouse EECs.(71) It has also been shown that caffeine, an inhibitor of IP_3-mediated Ca^{2+} responses, completely inhibited the bile acid-induced Ca^{2+} signalling in the absence of extracellular Ca^{2+}, suggesting the involvement of IP_3 receptors in the bile acid-induced calcium release. Similar mechanisms have been described in colonic crypt, hepatocytes or pancreatic duct and acini. (3, 8, 11, 42, 66, 67) Gadolinium, a known inhibitor of plasma

membrane Ca^{2+} entry channels, strongly blocked the bile acid induced Ca^{2+} signalling indicating that bile acids also promote the influx of extracellular calcium. The exact mechanism by which bile acids induce the entry of extracellular Ca^{2+} is not known. In rat hepatocytes, bile acids directly stimulate store-operated Ca^{2+} channels on the plasma membrane;(1) however further investigations are necessary to identify those Ca^{2+} channels that contribute to the effect of bile acids on the esophagus.

Acute effect of bile acids. Since the protective role of columnar epithelial cells highly depends on the normal function of acid/base transporters, we investigated, the effects of bile acids on the activity of the previously characterized ion transporters. Administration of BAC, dose-dependently decreased the activity of NHEs (both NHE1 and NHE2), whereas stimulated the activities of NBC and PAT-1 in CP-A cells. Inhibition of NHEs probably contributes to the acidification of the CP-A cells. In contrast, the acidification and consequently the cell death can be prevented by the increased activity of the HCO_3^- import system through the NBC. In addition, the efflux of HCO_3^- through the Cl^-/HCO_3^- exchanger, PAT-1 also plays an important role in the protection of the cells, by the neutralization of the cell environment in the surface mucus layer. The increased activities of NBC and PAT-1 probably compensate the decreased NHE activity and therefore try to maintain the acid/base equilibrium of the cell.

Interestingly, we found that NHE activity was stimulated in CP-D cells after bile acid treatment. This difference to CP-A cells can be explained by the advanced stage of CP-D cells. It has been described earlier that dysplastic Barrett's mucosa has more severe and prolonged acidic and biliary reflux exposure.(41) Furthermore, it has also been observed that CP-D cells are more resistant to GERD-like stimuli compared to CP-A cells.(28) The mechanism for this alteration and its potential physiological role cannot be explained by the present studies and is an area of focus for future work.

The underlying mechanism by which bile acids exert their stimulatory/inhibitory effects has also been investigated. Previous studies have demonstrated that the effect of bile acids on ion transporters is mediated by transient elevation of $[Ca^{2+}]_i$.(42, 66) Our results have shown that chelation of $[Ca^{2+}]_i$ by BAPTA-AM almost completely abolished both the inhibitory and stimulatory effect of BAC on ion transporters. Although, we have not studied the mechanism by which Ca^{2+} mediate the effect of bile acids on CP-A cells, we propose that the activation of PAT-1 is connected to the activation of Ca^{2+}-activated ion channels, such as Ca^{2+}-activated Cl^- channels or K^+ channels, as demonstrated in other epithelia.(35, 65, 72) In contrast to PAT-1, high levels of $[Ca^{2+}]_i$ strongly inhibited NHE activity. Previous studies on rabbit ileal brush-border membrane and renal NHE containing proteoliposomes have demonstrated that the phosphorylation of specific proteins by the Ca^{2+}/calmodulin cascade results in a robust blockade of NHE.(9, 68) Taken together these data indicate that the increased levels of Ca^{2+} probably do not directly modulates the activity of ion transporters; however further investigations are needed to identify those intracellular signalling pathways or molecules which are involved in this process.

Chronic effect of bile acids. Beside the investigation of the acute effect of bile acids, we also studied the expression profile of ion transporters after chronic exposure to bile acids. 7-days treatment with bile acids increased the mRNA expression of all of the investigated transporters in CP-A cells and the mRNA expression of NHE1 and NBC in CP-D cells, at neutral pH. In contrast, the expression of the transporters did not change significantly under acidic conditions in CP-A cells, moreover, the cell number dramatically decreased. In contrast, the expression of NHE1 significantly increased in CP-D cells at pH 5.5. We could also confirm the increased expression of NHE1 at protein level. We speculate that the overexpression of ion transporters is probably a defensive or adaptive mechanism by which the cells try to compensate the toxic, acidifying effect of bile acids.

In order to extend our study, we also investigated the mRNA expression of ion transporters in biopsy samples obtained from normal squamous and different types of columnar metaplastic mucosa. In Europe, BE is characterized by the presence of macroscopically visible metaplastic columnar epithelium.(55) In contrast, in the USA only the intestinal type of metaplasia is considered to be BE.(43) Thus, we divided our samples into intestinal and non- intestinal groups and analyzed them separately. NHEs, NBC and PAT-1 displayed higher mRNA levels in both intestinal and non-intestinal metaplasia compared to normal tissue. Increased protein expression of NHE1 and NHE2 were also confirmed in BE. These results are consistent with the report by Goldman et al., that demonstrated upregulation of NHE-1 in BE compared to normal epithelium both at mRNA and protein levels in biopsy samples and cell lines.(15) Similarly to findings of other laboratories in various tissues, we observed strong apical staining for NHE-2.(4, 16, 54)

Our data indicate that the metaplastic columnar tissue is adapted better to the acidic environment, compared to the normal epithelium. Firstly, BE has a much higher capacity for HCO_3^- secretion through the luminal CBE. Since HCO_3^- effectively neutralizes the acidic chyme, which arises during the backward diffusion of gastric content, metaplastic tissue is more resistant against the injurious agents. Secondly, the proton extruding (NHEs) and HCO_3^- loading (NBC) transporters are highly up-regulated in BE which also present an effective, protective mechanism against cellular acidification.

Taken together, we have shown that exposure of columnar esophageal cells to bile acids induce a cellular acidification. Although, we did not investigate the exact mechanism, we speculate that the Ca^{2+} dependent inhibition of NHE is likely to contribute in this process. Prolonged exposure of columnar cells to bile acids increases the expression of acid/base transporters and we showed that bile acids by themselves are less toxic than in combination with acid. Moreover, we found that the major acid/base transporters are over expressed in BE

tissue, indicating that the metaplastic tissue is adapted better to the injurious environment providing more effective protection to the underlying layers.

We suspect that altered activities and expression of ion transporters after bile acid exposure are part of an early adaptive process of EECs. Our findings may help to better understand the esophageal response to injury and the role of ion transporters in this process. We believe that pharmacological activation of ion transporters increases epithelial resistance in acidic environment and therefore may protect the esophageal mucosa against the injurious bile acids.

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DISCLOSURES

The authors hereby declare that there are no conflict of interests to disclose.

AUTHOR CONTRIBUTIONS

András Rosztóczy, Richárd Róka and Tibor Wittmann were involved in patient selection and sample collection. Zoltán Rakonczay Jr., Péter Hegyi and László Tiszlavicz were involved in molecular biology experiments. Máté Katona and Klaudia Birkás performed microfluorimetric experiments and were involved in tissue culturing. Dorottya Laczkó was

involved in all of the above mentioned experiments. Dorottya Laczkó and András Rosztóczy edited and revised the manuscript. Viktória Venglovecz designed and supervised the project and drafted the manuscript.

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913 **FIGURE LEGENDS**

914

915 **Figure 1. Investigation of Na^+/H^+ exchanger (NHE) activity on esophageal epithelial cells**
916 **(EECs).** (**A**) Removal of Na^+ from the standard Hepes solution caused a rapid and marked
917 intracellular acidosis in CP-A cells which confirms the presence of a Na^+-dependent H^+ efflux
918 mechanism. (**B**) Recovery from acid load reflects the activity of NHE in standard Hepes-
919 buffered solution. In the case of the second NH_4Cl pulse, Na^+ was removed from the external
920 solution 10 min before the pulse started, during the NH_4Cl pulse and 10 min after the pulse.
921 (**C**) Representative pH_i curve shows the recovery from acid load in the presence of 1 and 50
922 μM HOE-642. (**D**) Summary data of the calculated activities of the different NHE isoforms in
923 the presence of isoform selective NHE inhibitor, HOE-642. The rate of acid recovery ($J(B^-)$)
924 was calculated from the $\Delta pH/\Delta t$ obtained by linear regression analysis of pH_i measurments
925 made over the first 60 s of recovery from the lowest pH_i level (start point pH_i). The buffering
926 capacity at the start point pH_i was used for the calculation of $J(B^-)$. Data are presented as
927 means ± SEM. * : $p \leq 0.05$ vs. Control. n=15-25. N.D.: not detected.

928

929 **Figure 2. Investigation of Na^+/HCO_3^- cotransporter (NBC) activity on esophageal**
930 **epithelial cells (EECs).** (**A**) Representative pH_i curve showing the effect of Na^+ removal on
931 CP-A cells in HCO_3^-/CO_2-buffered solution. (**B**) Administration of 500 μM H_2DIDS
932 completely abolished the recovery from acidosis in CP-A cells. **(C)** Representative pH_i traces
933 showing the effect of H_2DIDS (500 μM) and HOE-642 (1 μM) on the recovery from acidosis
934 in HCO_3^-/CO_2-buffered solution. CP-A cells were acid loaded twice. The first NH_4Cl pulse
935 was the control and the second was the test. H_2DIDS/HOE-642 was added 1 min before the
936 end of NH_4Cl pulse and further 2 min after the pulse. (**D**) Summary data of the calculated
937 NHE and NBC activities. The rate of acid recovery ($J(B^-)$) was calculated as described in

Figure 1. * : p≤ 0.05 vs. Control. Data are presented as means ± SEM. n=15-25. N.D.: not detected.

Figure 3. Investigation of Cl^-/HCO_3^- exchanger (CBE) activity on CP-A cells. The activity of CBE was investigated by the Cl^- removal technique in the presence and absence of HCO_3^-/CO_2. In standard Hepes-buffered solution (**A**), removal of Cl^- (5 min) had no significant effect on pH_i. However, in standard HCO_3^-/CO_2 solution (**B**), the steady state pH_i in the absence of Cl^- significantly increased, indicating the presence of a functionally active CBE on CP-A cells.

Figure 4. Effect of bile acids on the intracellular pH (pH_i) of CP-A cells. CP-A cells were exposed to 100, 300 and 500 μM bile acid cocktail (BAC) for 5 min at (**A**) pH 7.5 and (**B**) pH 5.5. Summary data for the maximal pH_i change (ΔpH_{max}) at pH 7.5 (**C**) and pH 5.5 (**D**) and the calculated base flux ($-J(B^-)$ induced by BAC (**E** and **F**). $-J(B^-)$ was calculated from the $\Delta pH/\Delta t$ obtained by linear regression analysis of pH_i measurements made over the first 60 s after bile acid administration. The start point pH_i for the measurment of $\Delta pH/\Delta t$ was the pH_i immediately before exposure to bile acids. The buffering capacity at the start point pH_i was used for the calculation of $-J(B^-)$. (**G**) Effect of individual bile acids (100 μM each) on ΔpH_{max} at at pH 7.5 (black column) and pH 5.5 (empty column). Data are presented as means ± SEM. n=15-25. N.D.: not detected. DC: deoxycholic acid, TC: taurocholic acid, TDC: taurodeoxycholic acid, GC: glycocholic acid, GDC: glycodeoxycholic acid, GCDC: glycochenodeoxycholic acid, TCDC: taurochenodeoxycholic acid.

Figure 5. Effect of bile acids on intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ of CP-A cells. Representative experimental traces showing the effect of 100, 300 and 500 μM bile acid

963 cocktail (BAC) at pH 7.5 (**A**) and pH 5.5 (**B**) on $[Ca^{2+}]_i$. Summary data of the bile acids-
964 induced $[Ca^{2+}]_i$ changes at pH 7.5 (**C**) and pH 5.5 (**D**). Values are expressed as percent of
965 basal $[Ca^{2+}]_i$. (**E**) Effect of extracellular Ca^{2+} removal, caffeine (20 mM), ruthenium red (10
966 μM) and gadolinium (1μM) on the rise in $[Ca^{2+}]_i$ induced by 500 μM BAC. All experiments
967 were performed in HEPES-buffered solution. Data are presented as means ± SEM. * : $p \leq 0.05$
968 vs. 500 μM BAC. n = 10-21.
969
970 **Figure 6. Effects of bile acids on acid/base transporters on esophageal epithelial cells**
971 **(EECs). (A)** Representative pH_i traces show the effect of 100, 300 and 500 μM bile acid
972 cocktail (BAC) in Hepes-buffered solution on the CP-A cell line. Cells were treated with bile
973 acids 3 min before the pulse started, during the NH_4Cl pulse and 3 min after the pulse. (**B**)
974 Summary of the calculated Na^+/H^+ exchanger (NHE) activity in CP-A and CP-D cells. The
975 rate of acid recovery ($J(B^-)$) was calculated as described in Figure 1. (**C**) Summary of the
976 effect of 500 μM BAC on the activities of different NHE isoforms in CP-A cells in the
977 presence of 1 and 50 μM HOE-642. (**D**) Representative pH_i traces show the effect of 100, 300
978 and 500 μM bile acid cocktail (BAC) in HCO_3^-/CO_2 -buffered solution on the CP-A cell line.
979 Cells were treated with bile acids 3 min before the pulse started, during the NH_4Cl pulse and 3
980 min after the pulse. (**E**) Summary of the calculated NHE and Na^+/HCO_3^- cotransporter (NBC)
981 activities in CP-A and CP-D cells. The rate of acid recovery ($J(B^-)$) was calculated as
982 described in Figure 1. (**F**) Summary data of the calculated rates of pH_i recovery from acid
983 load in HCO_3^-/CO_2^- buffered solution in CP-A cells. The effect of bile acids on NBC activity
984 was evaluated in the presence of 50 μM HOE-642. The rate of acid recovery ($J(B^-)$) was
985 calculated as described in Figure 1. (**G**) Summary of the calculated Cl^-/HCO_3^- exchanger
986 activity in CP-A and CP-D cells. The rate of alkali recovery ($-J(B^-)$) was calculated from the
987 $\Delta pH/\Delta t$ obtained by linear regression analysis of pH_i measurements made over the first 30 s

of recovery from the highest pH_i level (start point pH_i). The buffering capacity at the start point pH_i was used for the calculation of $J(B^-)$. Data are presented as means ± SEM.*: $p \leq 0.05$ vs. Control. n=15-25.

Figure 7. Expression of ion transporters in Barrett's cell lines. CP-A and CP-D cells were treated with different bile acids for 7 days at pH 7.5 (**A** and **B**) and 5.5 (**C** and **D**) and the relative mRNA expressions of NHE1, NHE2, NBC and SLC26A6 were investigated by real-time PCR. Data are presented as means ± SEM. (E) Western Blot analysis for NHE1 protein expression after 100 and 500 μM bile acid treatments. BAC: bile acid cocktail.

Figure 8. Expression of ion transporters in human esophageal biopsy samples. Box plots are showing the relative expression of NHE1, NHE2, NBC and SLC26A6 in biopsy samples derived from intestinal (**A**) and non-intestinal (**B**) metaplasia. Median values are shown as a horizontal black bar within each box. *:$p \leq 0.05$ vs. normal squamous epithelium. n=7. (**C**) Representative pictures show immunohistochemical staining of NHE1 and NHE2 in normal esophageal squamous mucosa and intestinal metaplastic tissue specimens. Isotype negative control (NC) was also included to assess non-specific staining. Scale bar represent 50 μm. SQ: squamous mucosa, M: metaplasia. Arrows pointing toward NHE-1 and NHE-2 staining.

Table 1. **Patient details**. The lengths of metaplasia are given according to the Prague C&M criteria.

Table 2. **Composition of solutions**. Values are in mM.

Figure 1

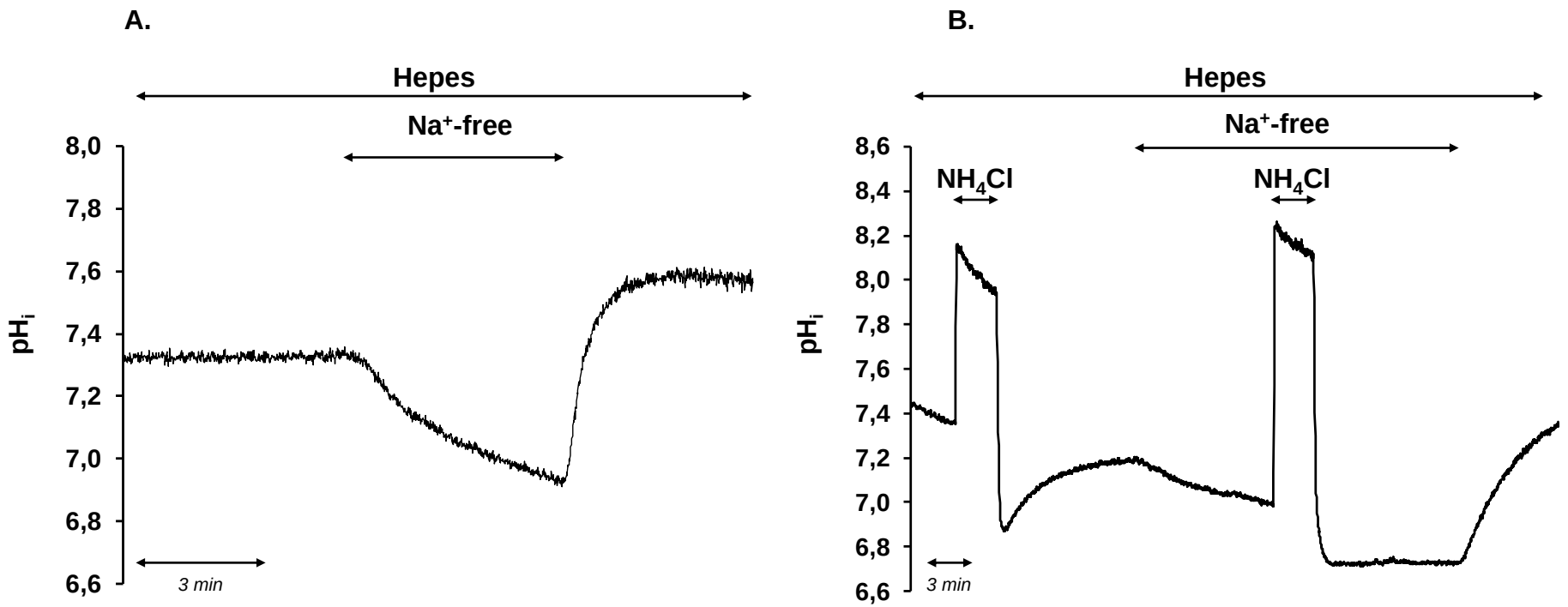

A.
Hepes
Na+-free
8,0
7,8
7,6
7,4
7,2
7,0
6,8
6,6
pHi
3 min
B.
Hepes
Na+-free
NH4Cl
NH4Cl
8,6
8,4
8,2
8,0
7,8
7,6
7,4
7,2
7,0
6,8
6,6
pHi
3 min

Figure 1

C.

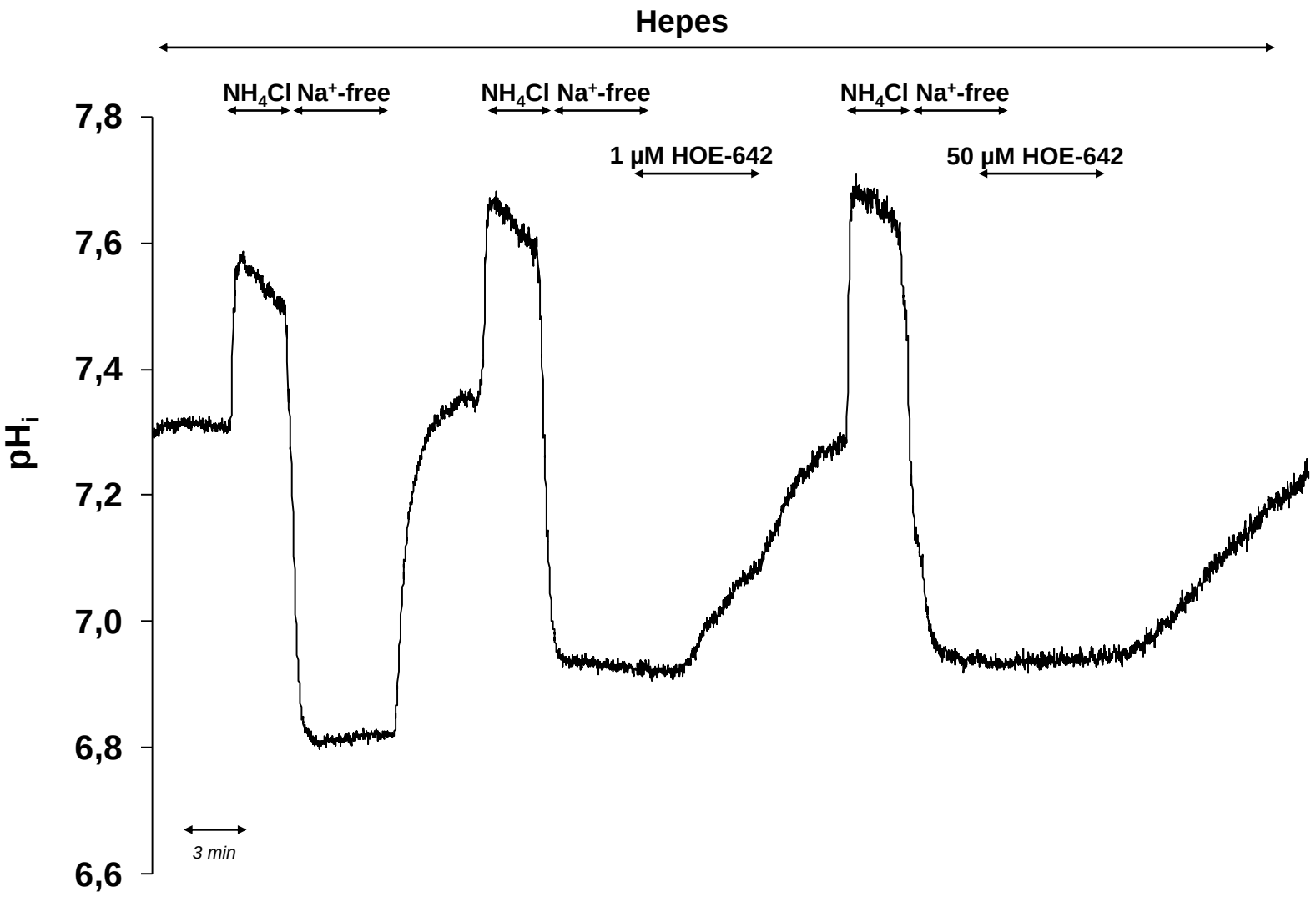

Hepes
NH4Cl Na+-free
NH4Cl Na+-free
NH4Cl Na+-free
1 µM HOE-642
50 µM HOE-642
7,8
7,6
7,4
7,2
7,0
6,8
6,6
pHi
3 min

Figure 1

D.

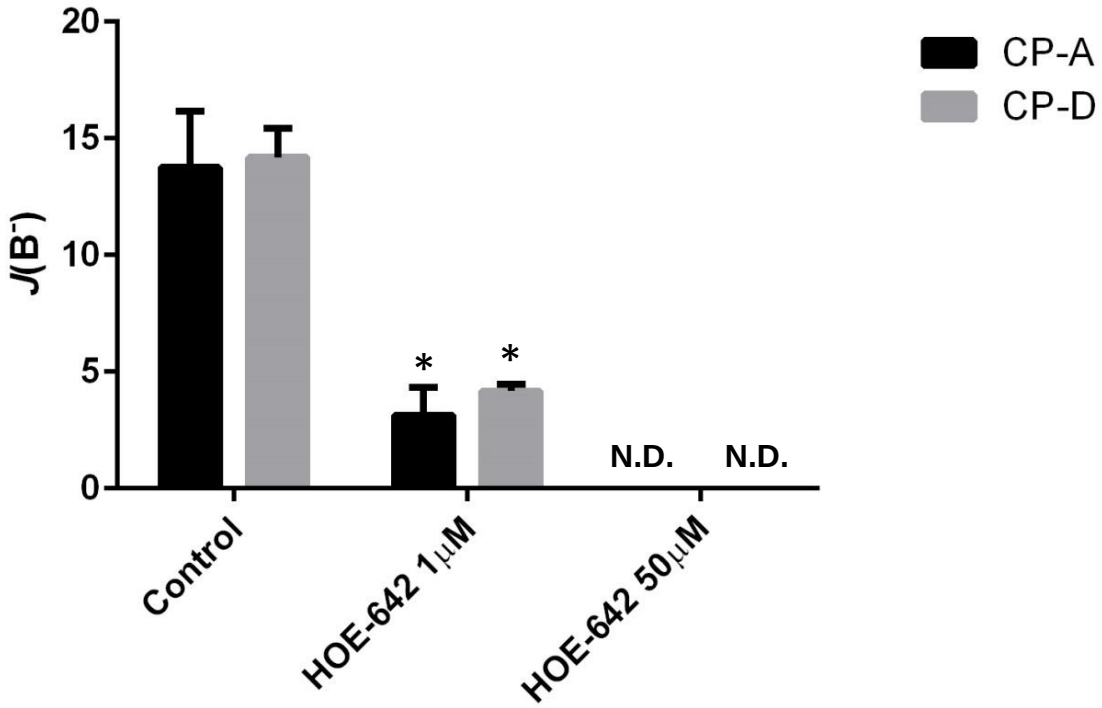


Figure 2

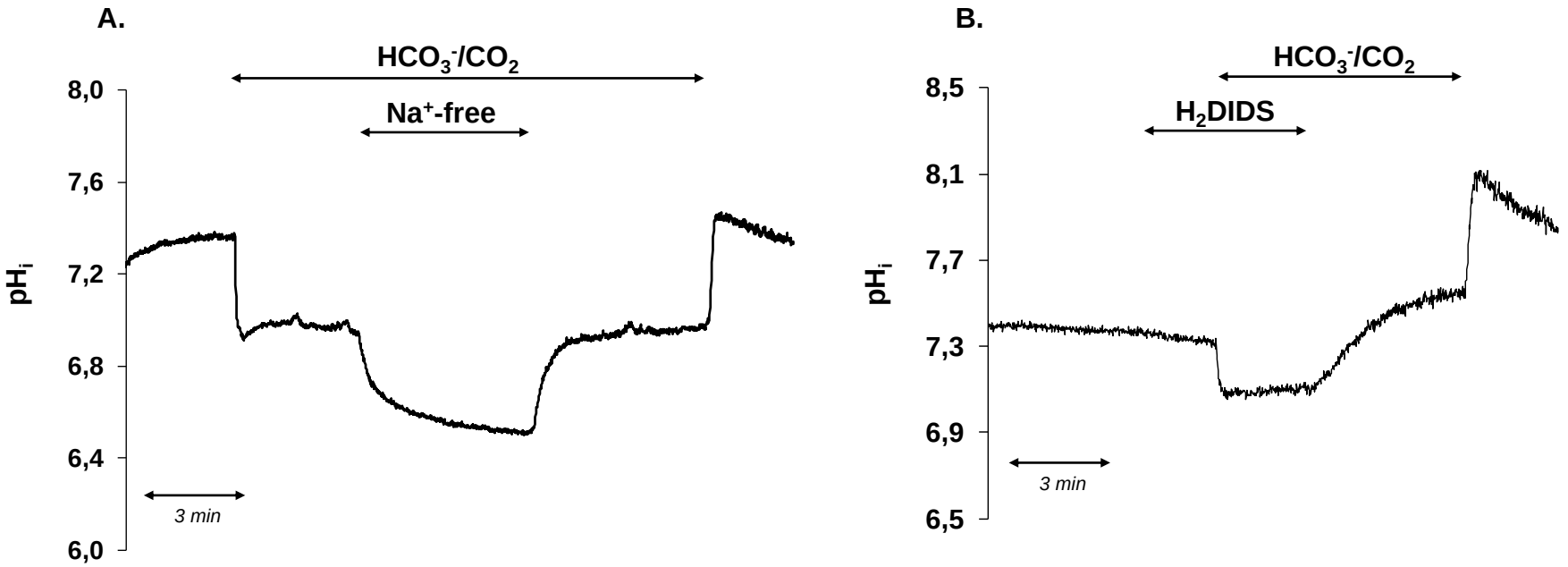

A.
HCO3-/CO2
Na+-free
8,0
7,6
7,2
6,8
6,4
6,0
pHi
3 min
B.
HCO3-/CO2
H2DIDS
8,5
8,1
7,7
7,3
6,9
6,5
pHi
3 min

Figure 2

C.

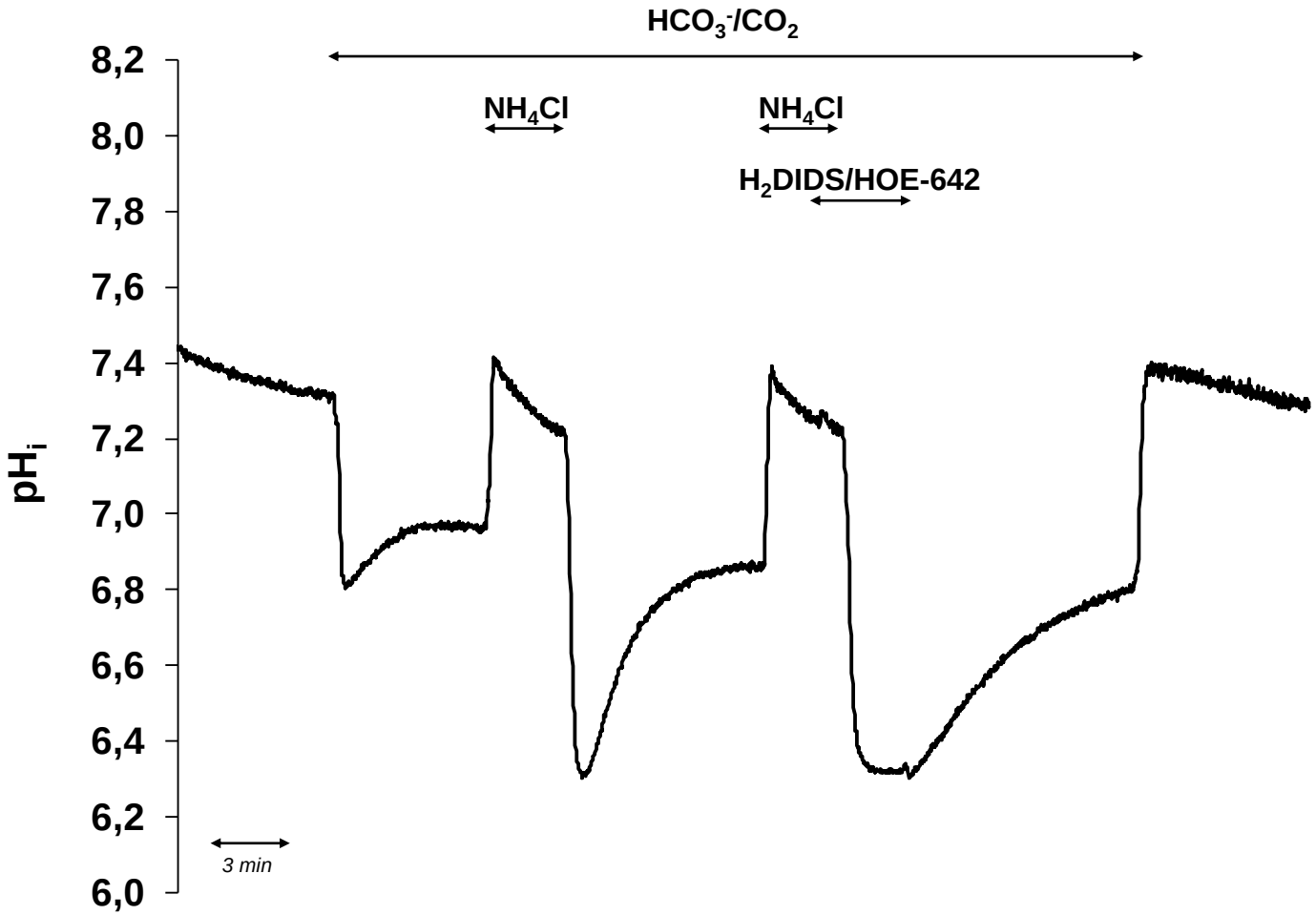

HCO3-/CO2
NH4Cl
NH4Cl
H2DIDS/HOE-642
8,2
8,0
7,8
7,6
7,4
7,2
7,0
6,8
6,6
6,4
6,2
6,0
pHi
3 min

Figure 2

D.

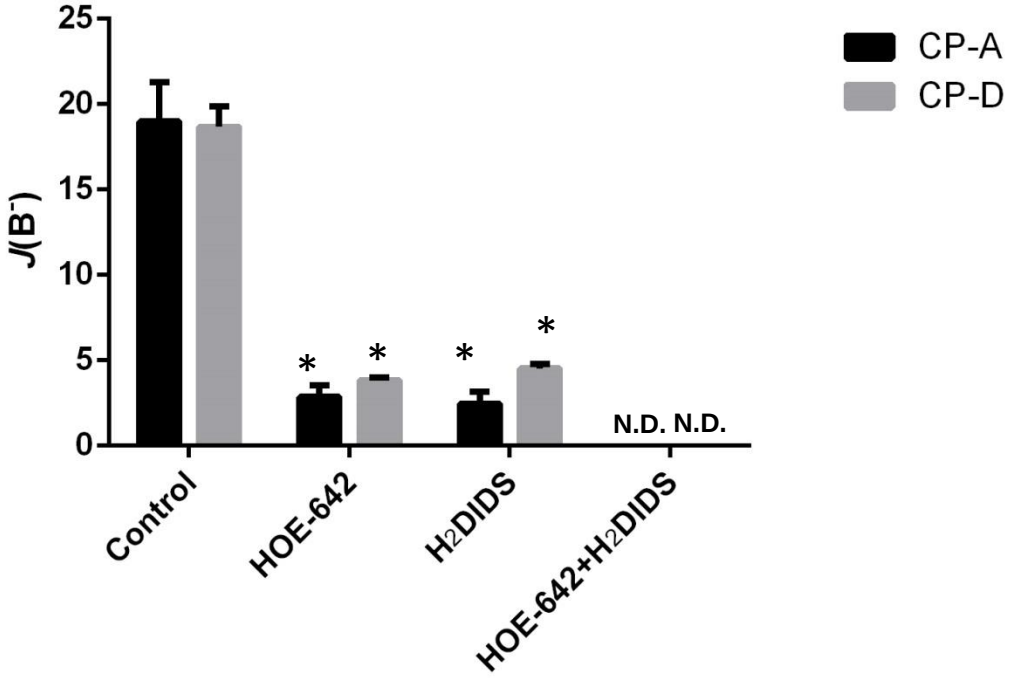

25
20
15
10
5
0
J(B-)
CP-A
CP-D
*
*
*
*
N.D. N.D.
Control
HOE-642
H2DIDS
HOE-642+H2DIDS

Figure 3

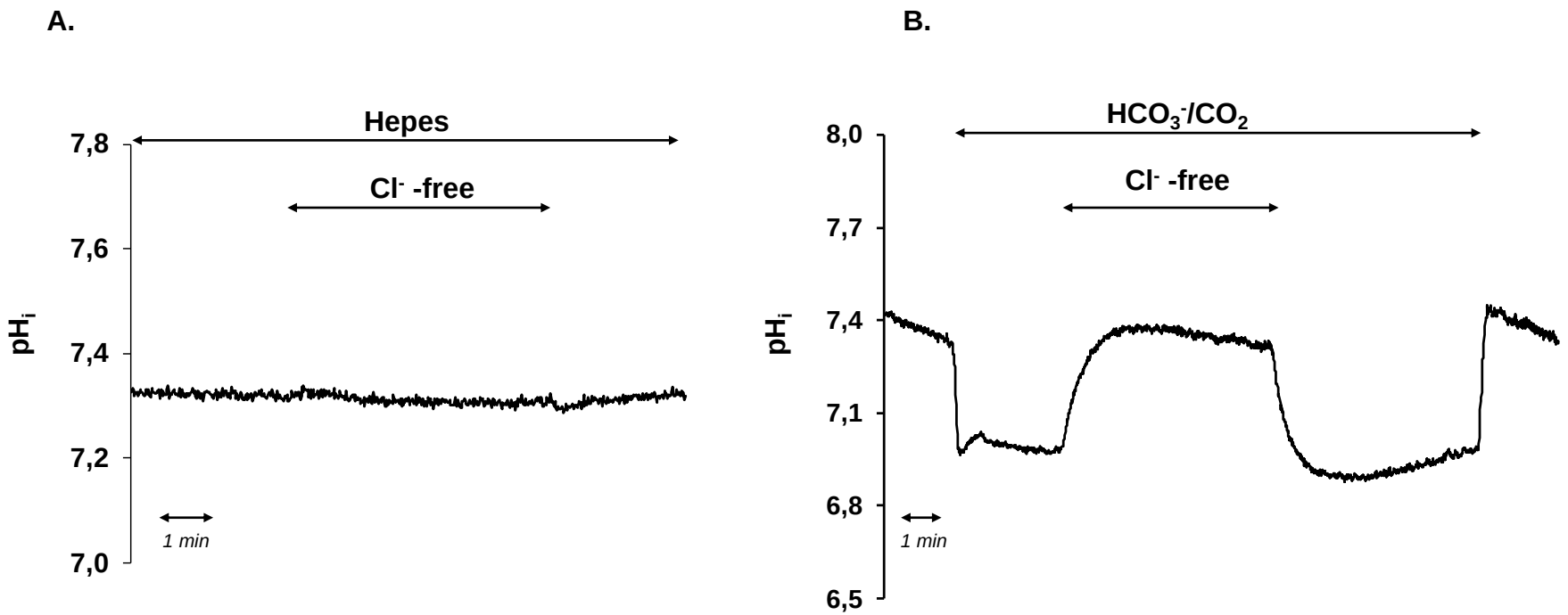

A.
Hepes
Cl- -free
7,8
7,6
7,4
7,2
7,0
pHi
1 min
B.
HCO3-/CO2
Cl- -free
8,0
7,7
7,4
7,1
6,8
6,5
pHi
1 min

Figure 4

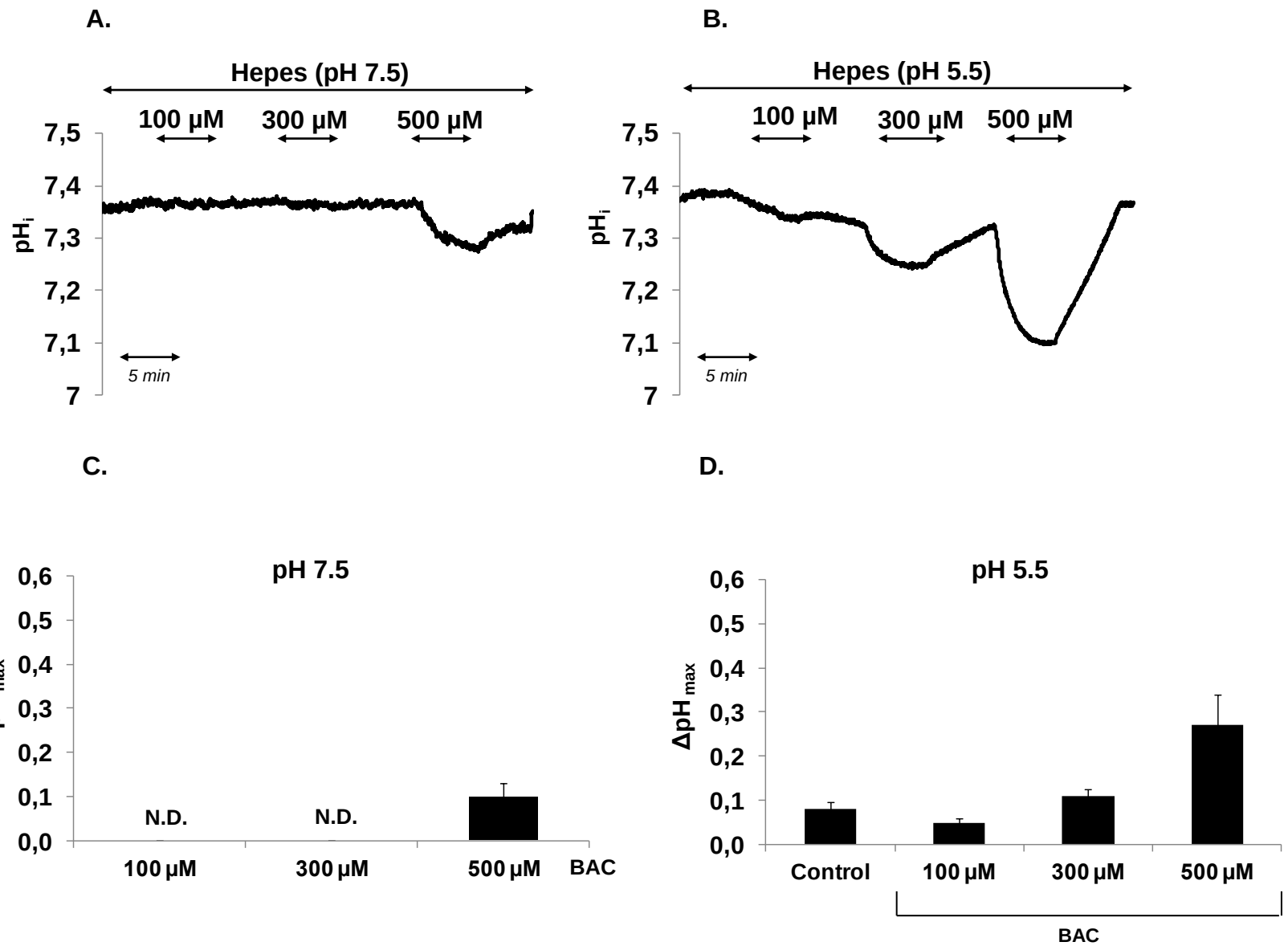
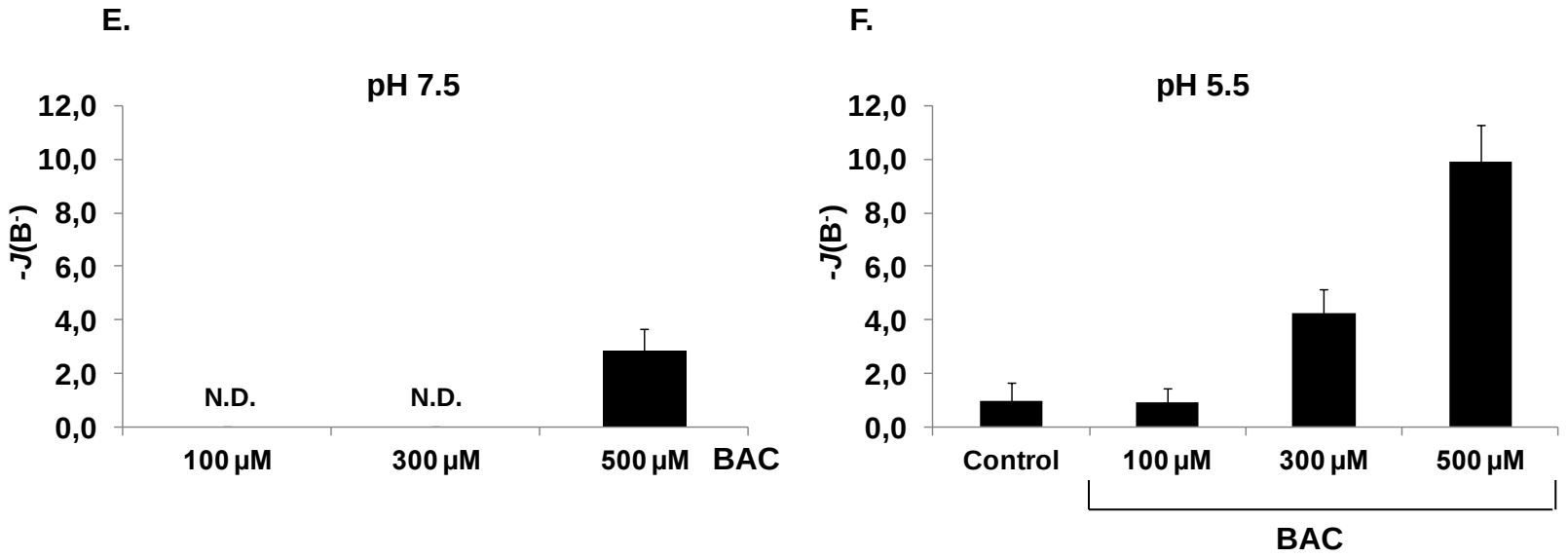

A.
Hepes (pH 7.5)
100 µM
300 µM
500 µM
7,5
7,4
7,3
7,2
7,1
7
pHi
5 min
B.
Hepes (pH 5.5)
100 µM
300 µM
500 µM
7,5
7,4
7,3
7,2
7,1
7
pHi
5 min
C.
pH 7.5
ΔpHmax
0,6
0,5
0,4
0,3
0,2
0,1
0,0
N.D.
N.D.
100 µM
300 µM
500 µM
BAC
D.
pH 5.5
ΔpHmax
0,6
0,5
0,4
0,3
0,2
0,1
0,0
Control
100 µM
300 µM
500 µM
BAC

Figure 4


E.
pH 7.5
12,0
10,0
8,0
6,0
4,0
2,0
0,0
-J(B·)
N.D.
N.D.
100 µM
300 µM
500 µM
BAC
F.
pH 5.5
12,0
10,0
8,0
6,0
4,0
2,0
0,0
-J(B·)
Control
100 µM
300 µM
500 µM
BAC

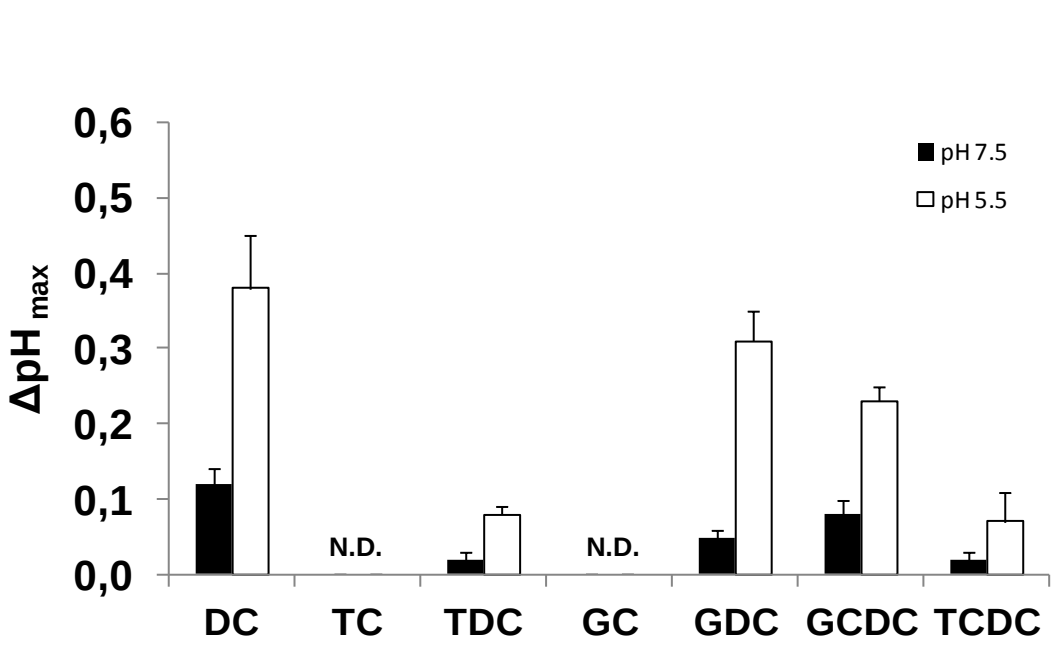

G.
0,6
0,5
0,4
0,3
0,2
0,1
0,0
ΔpH max
pH 7.5
pH 5.5
N.D.
N.D.
DC
TC
TDC
GC
GDC
GCDC
TCDC

Figure 5

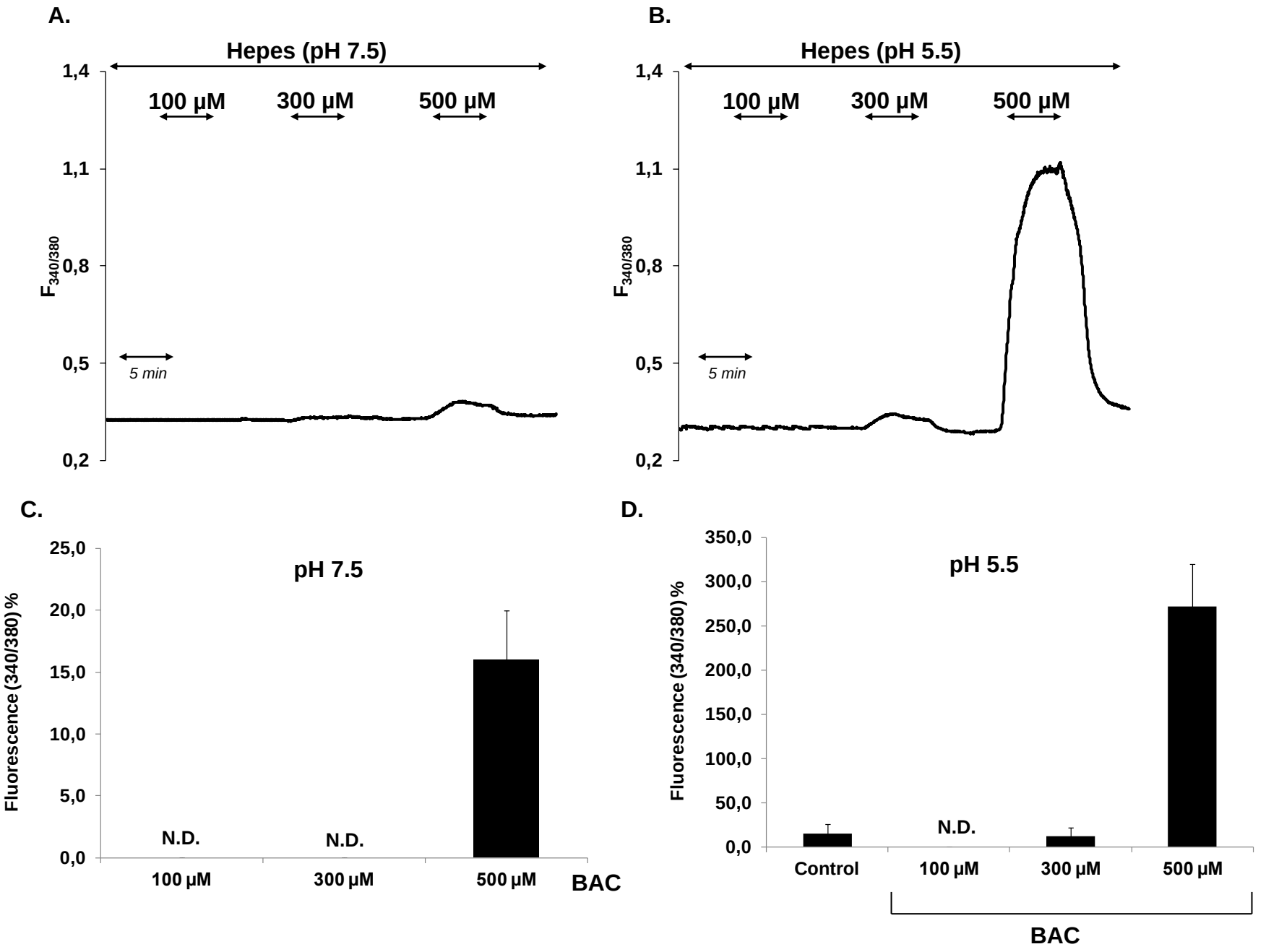

A.
Hepes (pH 7.5)
100 µM
300 µM
500 µM
1,4
1,1
0,8
0,5
0,2
F340/380
5 min
B.
Hepes (pH 5.5)
100 µM
300 µM
500 µM
1,4
1,1
0,8
0,5
0,2
F340/380
5 min
C.
pH 7.5
25,0
20,0
15,0
10,0
5,0
0,0
Fluorescence (340/380) %
N.D.
N.D.
100 µM
300 µM
500 µM
BAC
D.
pH 5.5
350,0
300,0
250,0
200,0
150,0
100,0
50,0
0,0
Fluorescence (340/380) %
N.D.
Control
100 µM
300 µM
500 µM
BAC

Figure 5

E.

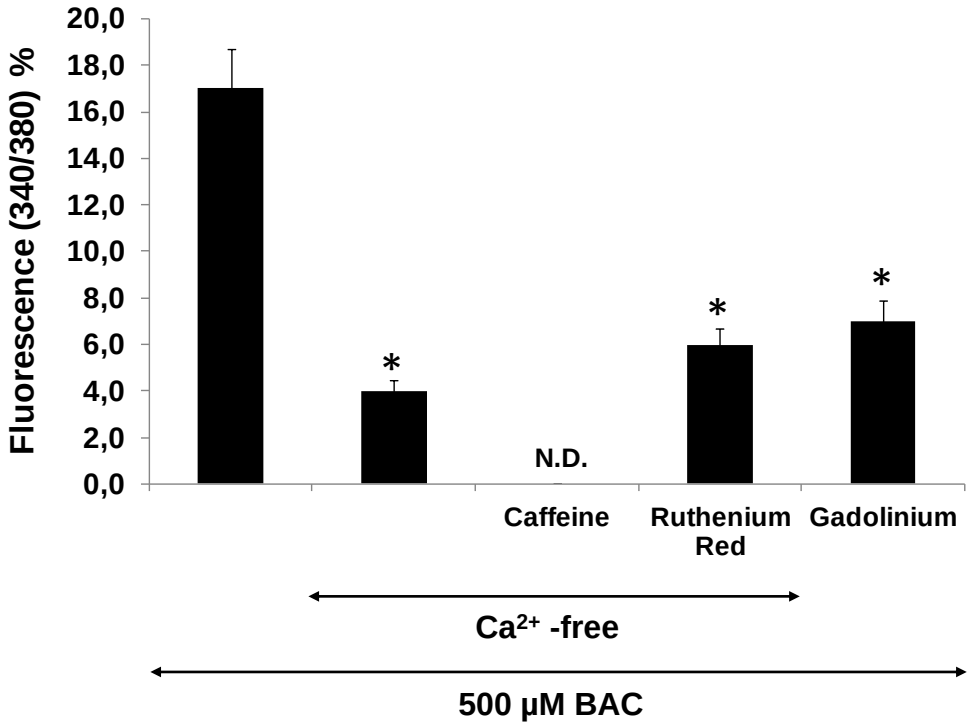

Fluorescence (340/380) %
20,0
18,0
16,0
14,0
12,0
10,0
8,0
6,0
4,0
2,0
0,0
*
*
*
N.D.
Caffeine
Ruthenium Red
Gadolinium
Ca2+ -free
500 µM BAC

Figure 6

A.

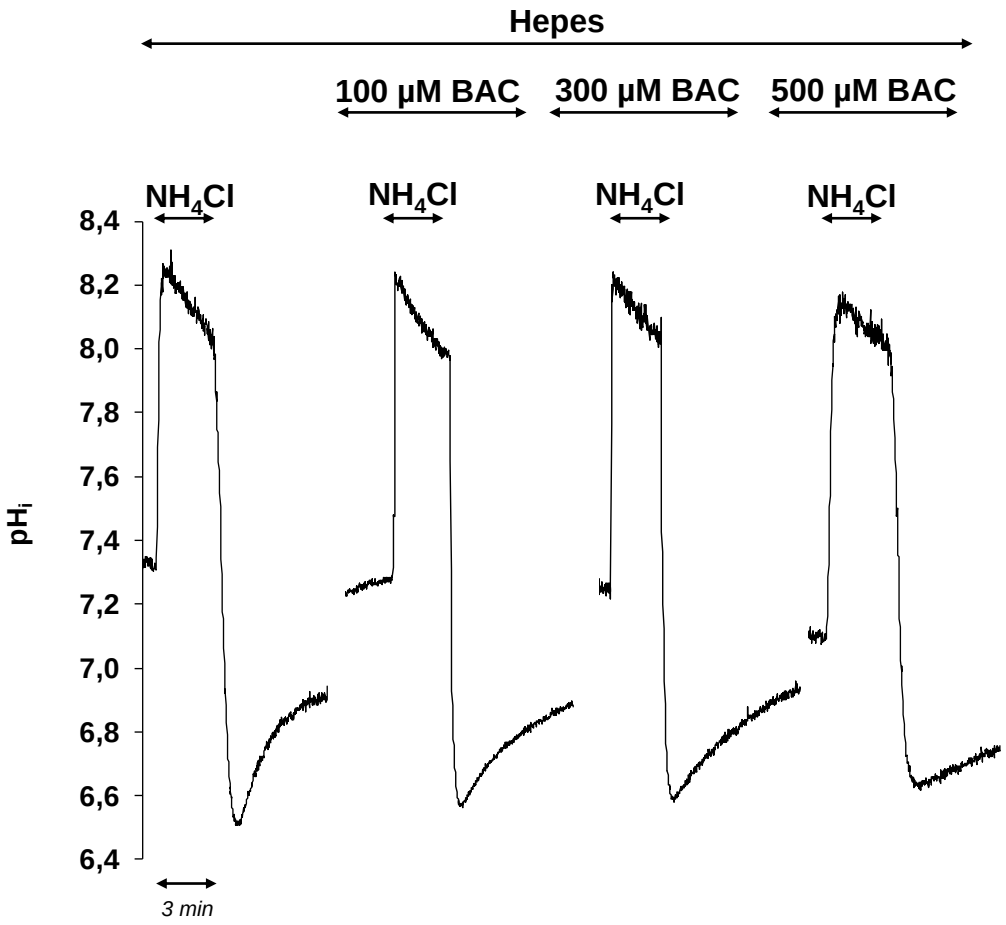
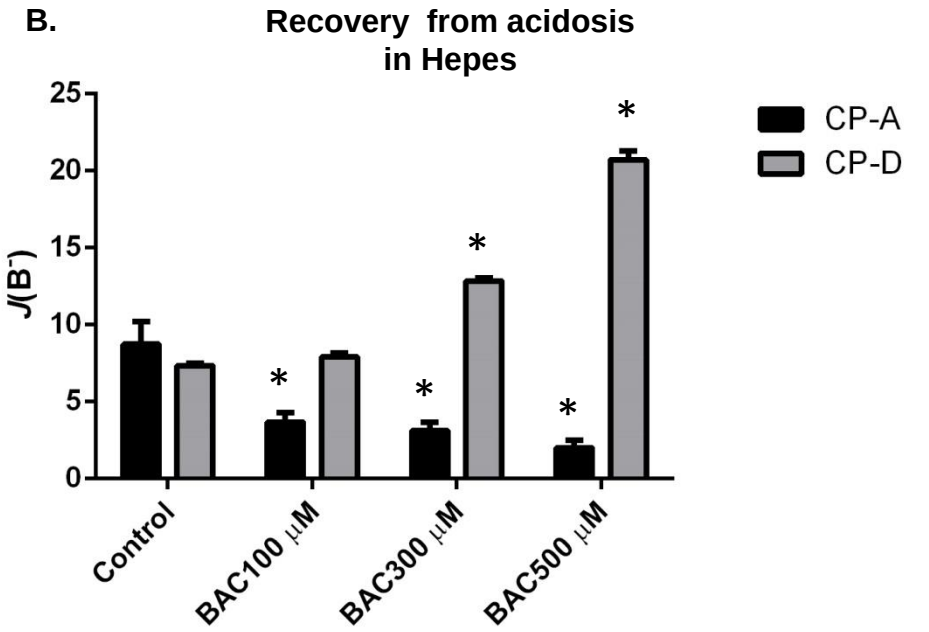

Hepes
100 µM BAC
300 µM BAC
500 µM BAC
NH4Cl
NH4Cl
NH4Cl
NH4Cl
8,4
8,2
8,0
7,8
7,6
7,4
7,2
7,0
6,8
6,6
6,4
pHi
3 min

Figure 6


B.
Recovery from acidosis in Hepes
25
20
15
10
5
0
J(B-)
CP-A
CP-D
*
Control
BAC100 µM
BAC300 µM
BAC500 µM

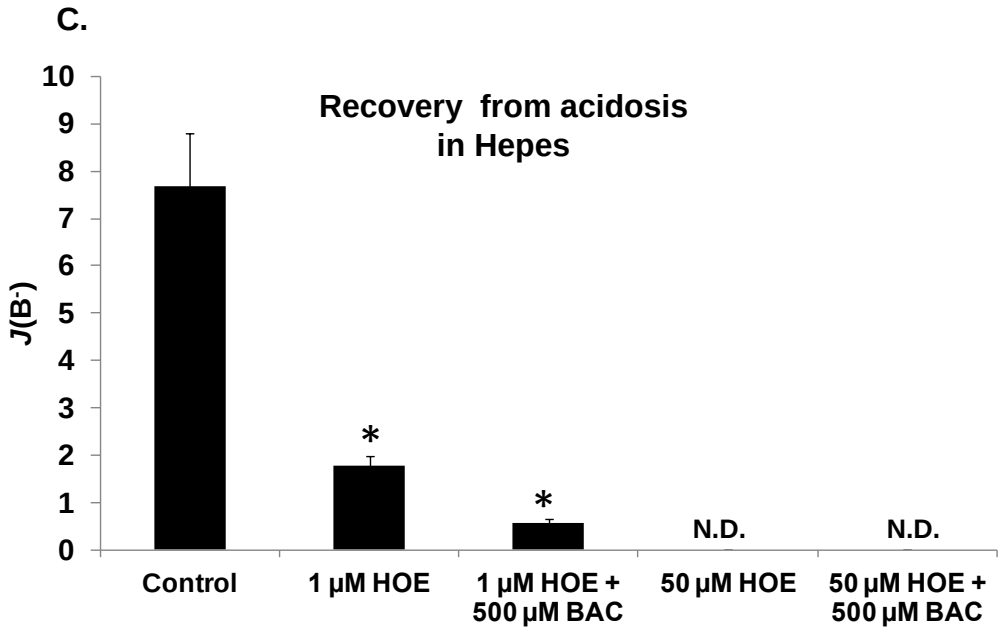

C.
Recovery from acidosis in Hepes
10
9
8
7
6
5
4
3
2
1
0
J(B-)
*
N.D.
Control
1 µM HOE
1 µM HOE + 500 µM BAC
50 µM HOE
50 µM HOE + 500 µM BAC

Figure 6

D.

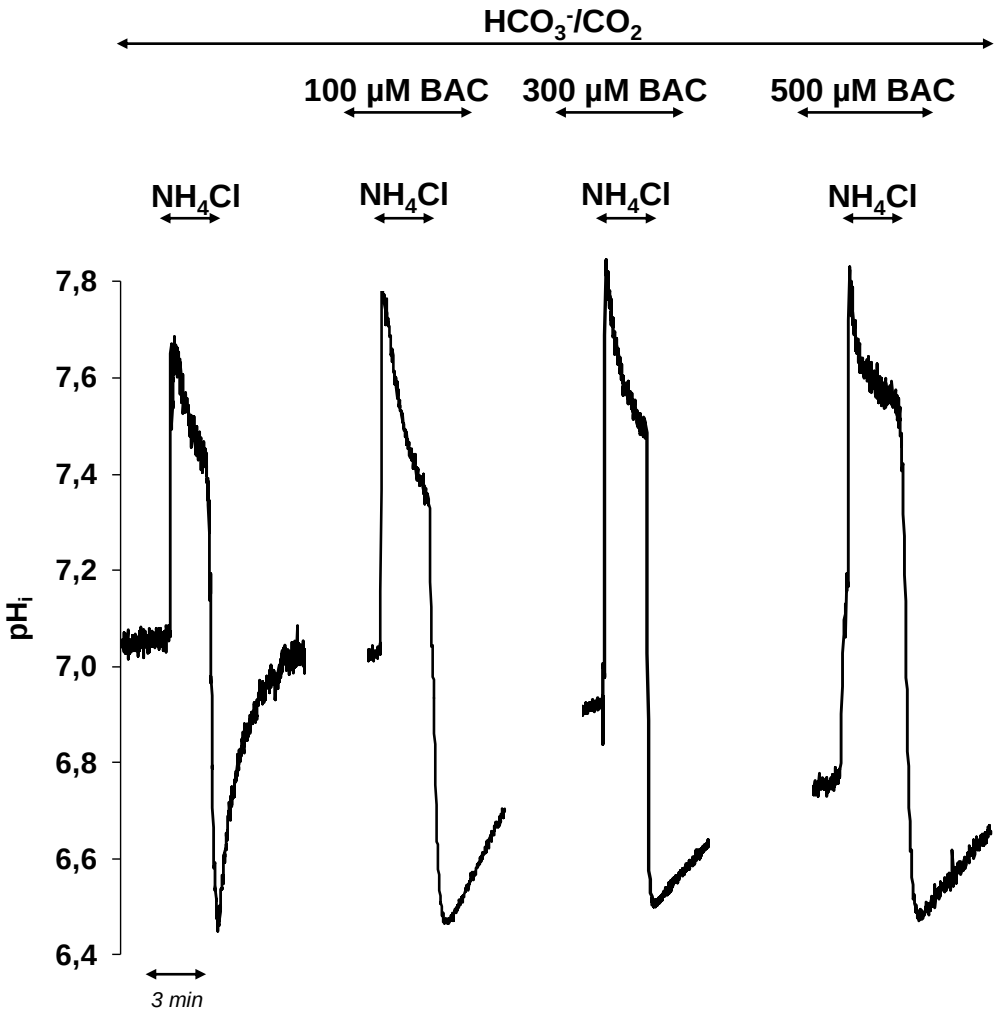
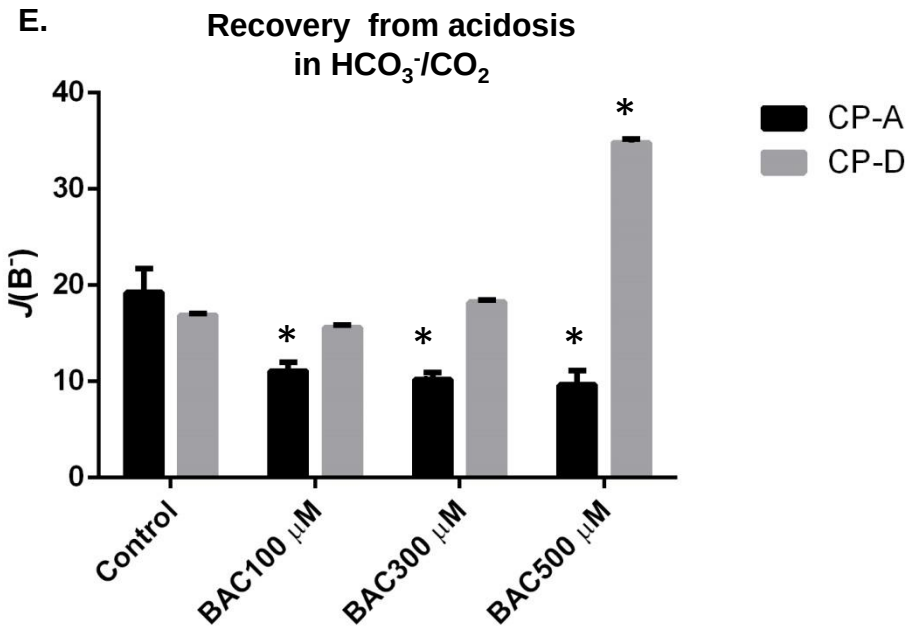
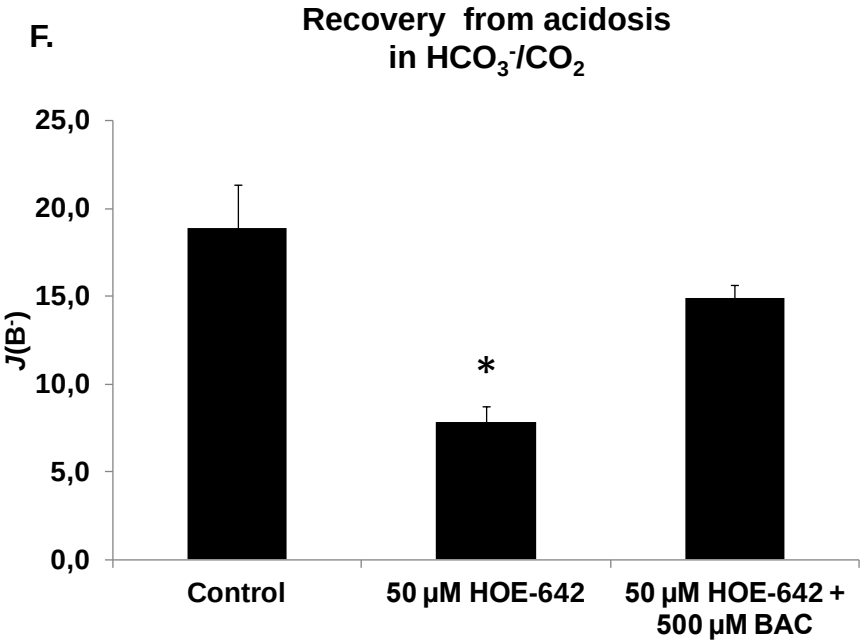

HCO3-/CO2
100 µM BAC
300 µM BAC
500 µM BAC
NH4Cl
NH4Cl
NH4Cl
NH4Cl
7,8
7,6
7,4
7,2
7,0
6,8
6,6
6,4
pHi
3 min

Figure 6


E.
Recovery from acidosis in HCO_3^-/CO_2
$J(B^-)$
40
30
20
10
0
Control
BAC100 µM
BAC300 µM
BAC500 µM
CP-A
CP-D


F.
Recovery from acidosis in HCO_3^-/CO_2
$J(B^-)$
25,0
20,0
15,0
10,0
5,0
0,0
Control
50 µM HOE-642
50 µM HOE-642 + 500 µM BAC

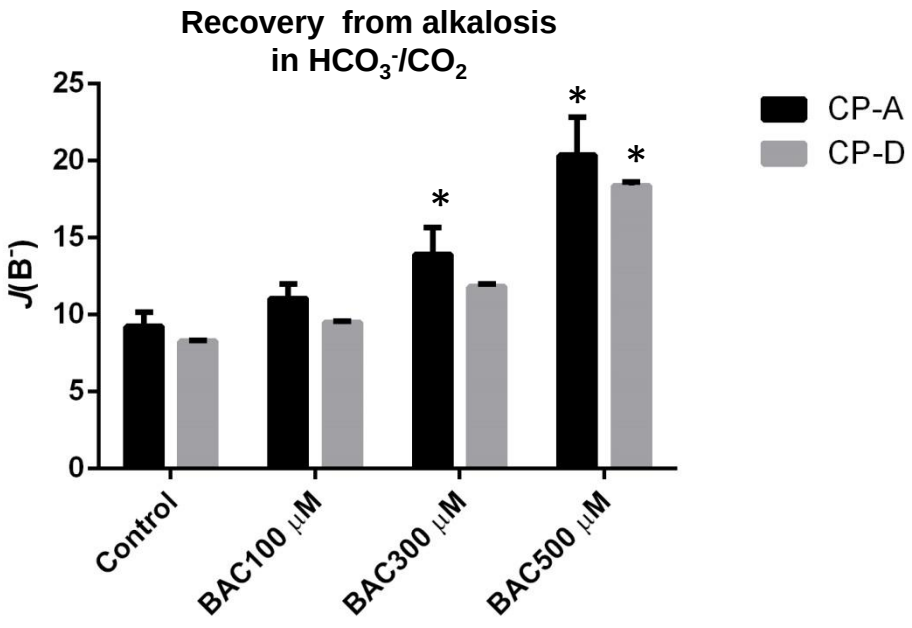

G.
Recovery from alkalosis in HCO_3^-/CO_2
$J(B^-)$
25
20
15
10
5
0
Control
BAC100 µM
BAC300 µM
BAC500 µM
CP-A
CP-D

Figure 7

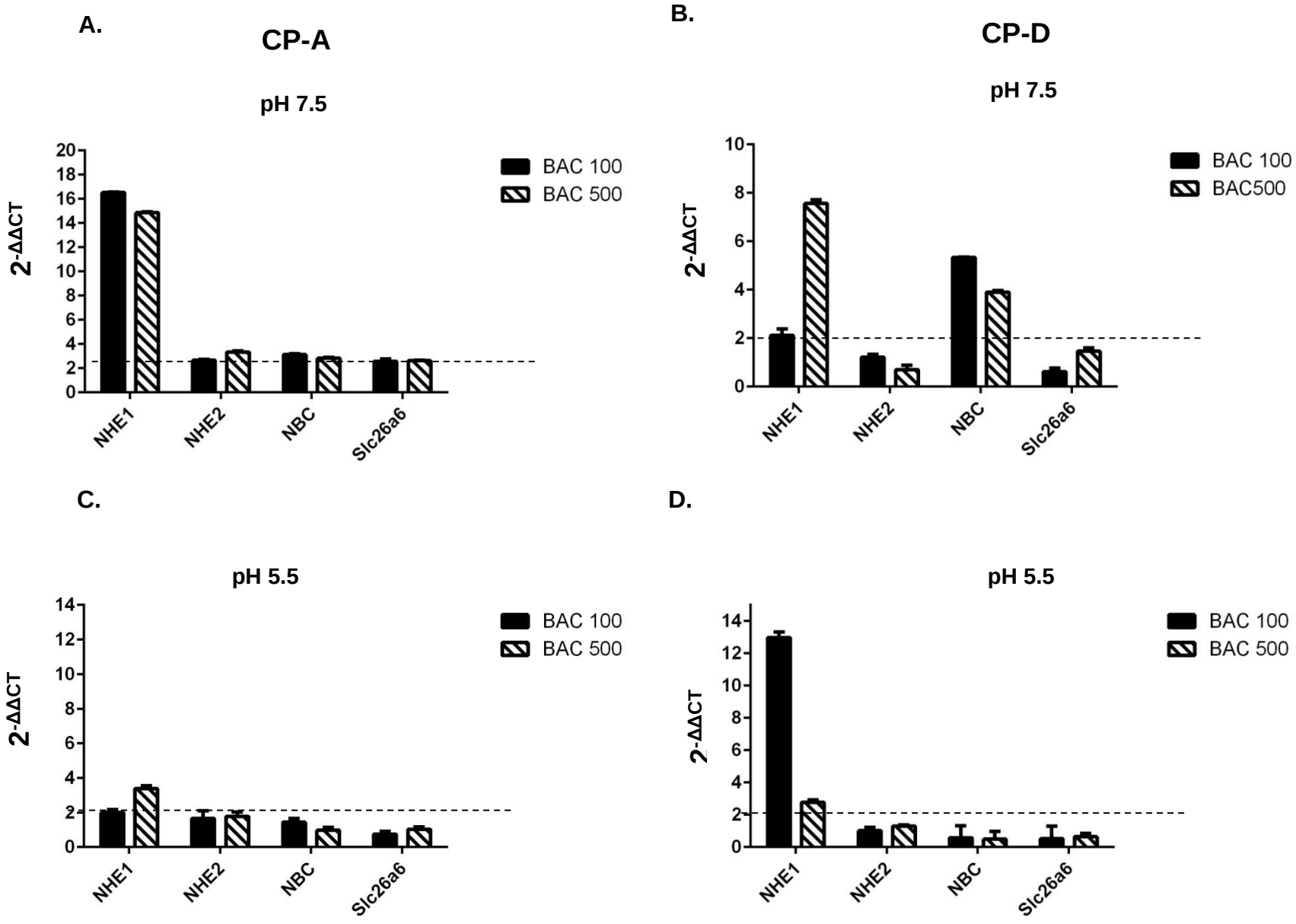

A.
CP-A
pH 7.5
2-ΔΔCT
BAC 100
BAC 500
NHE1
NHE2
NBC
Slc26a6
B.
CP-D
pH 7.5
2-ΔΔCT
BAC 100
BAC500
NHE1
NHE2
NBC
Slc26a6
C.
pH 5.5
2-ΔΔCT
BAC 100
BAC 500
NHE1
NHE2
NBC
Slc26a6
D.
pH 5.5
2-ΔΔCT
BAC 100
BAC 500
NHE1
NHE2
NBC
Slc26a6

Figure 7

E.

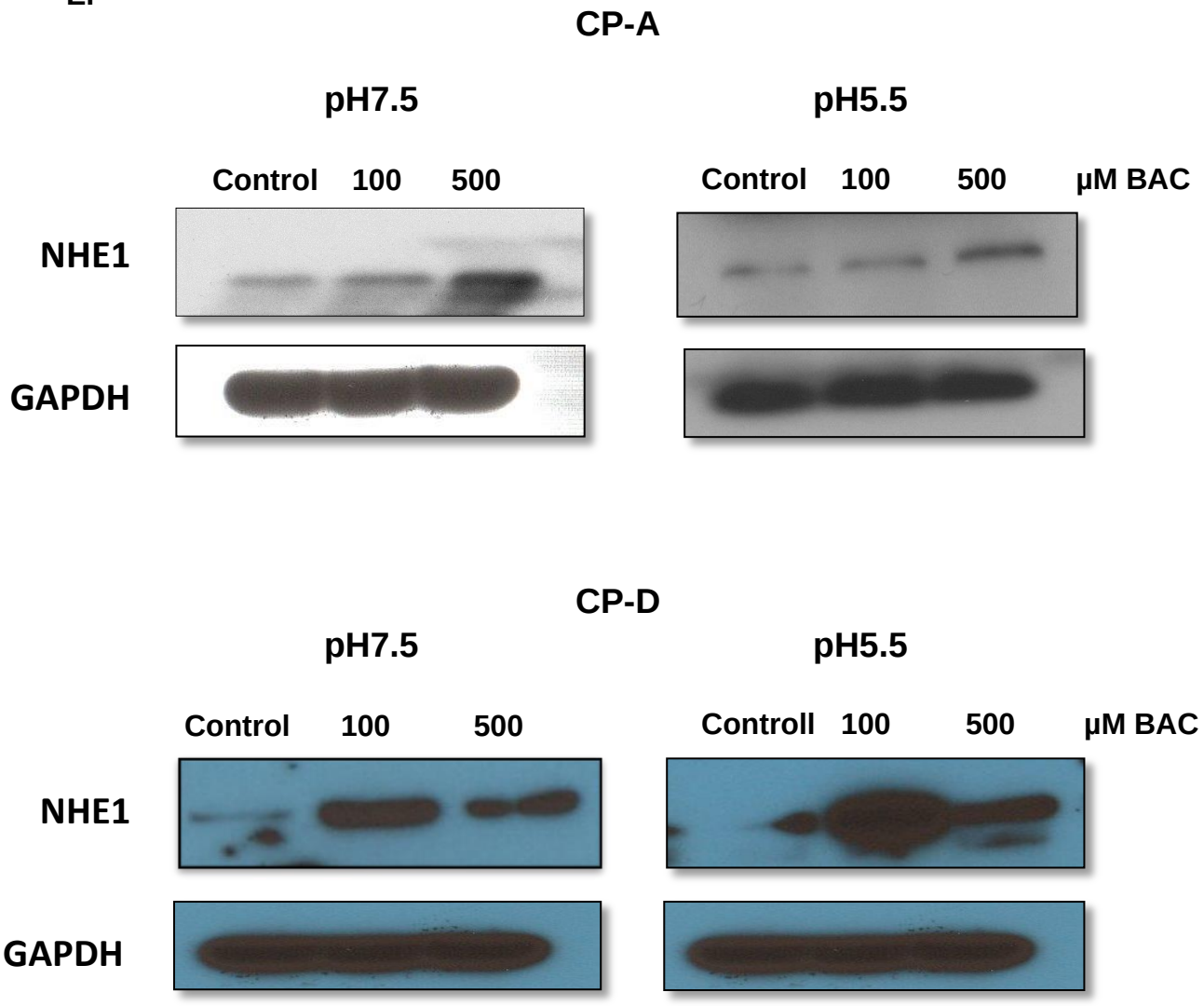

CP-A
pH7.5
pH5.5
Control
100
500
Control
100
500
μM BAC
NHE1
GAPDH
CP-D
pH7.5
pH5.5
Control
100
500
Controll
100
500
μM BAC
NHE1
GAPDH

Figure 8

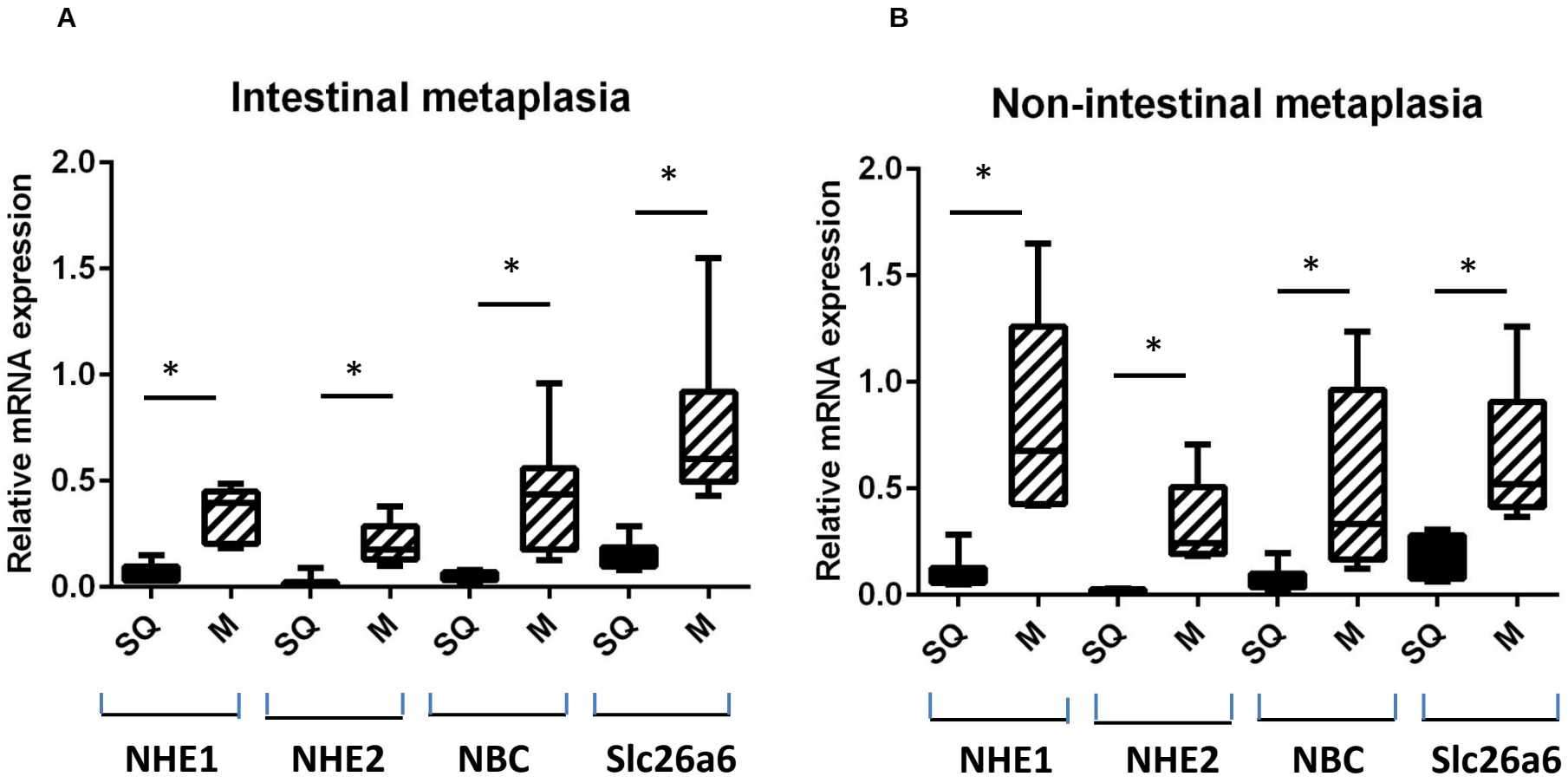

A
Intestinal metaplasia
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
*
*
*
*
SQ
M
SQ
M
SQ
M
SQ
M
NHE1
NHE2
NBC
Slc26a6
B
Non-intestinal metaplasia
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
*
*
*
*
SQ
M
SQ
M
SQ
M
SQ
M
NHE1
NHE2
NBC
Slc26a6

Figure 8

C

NHE1

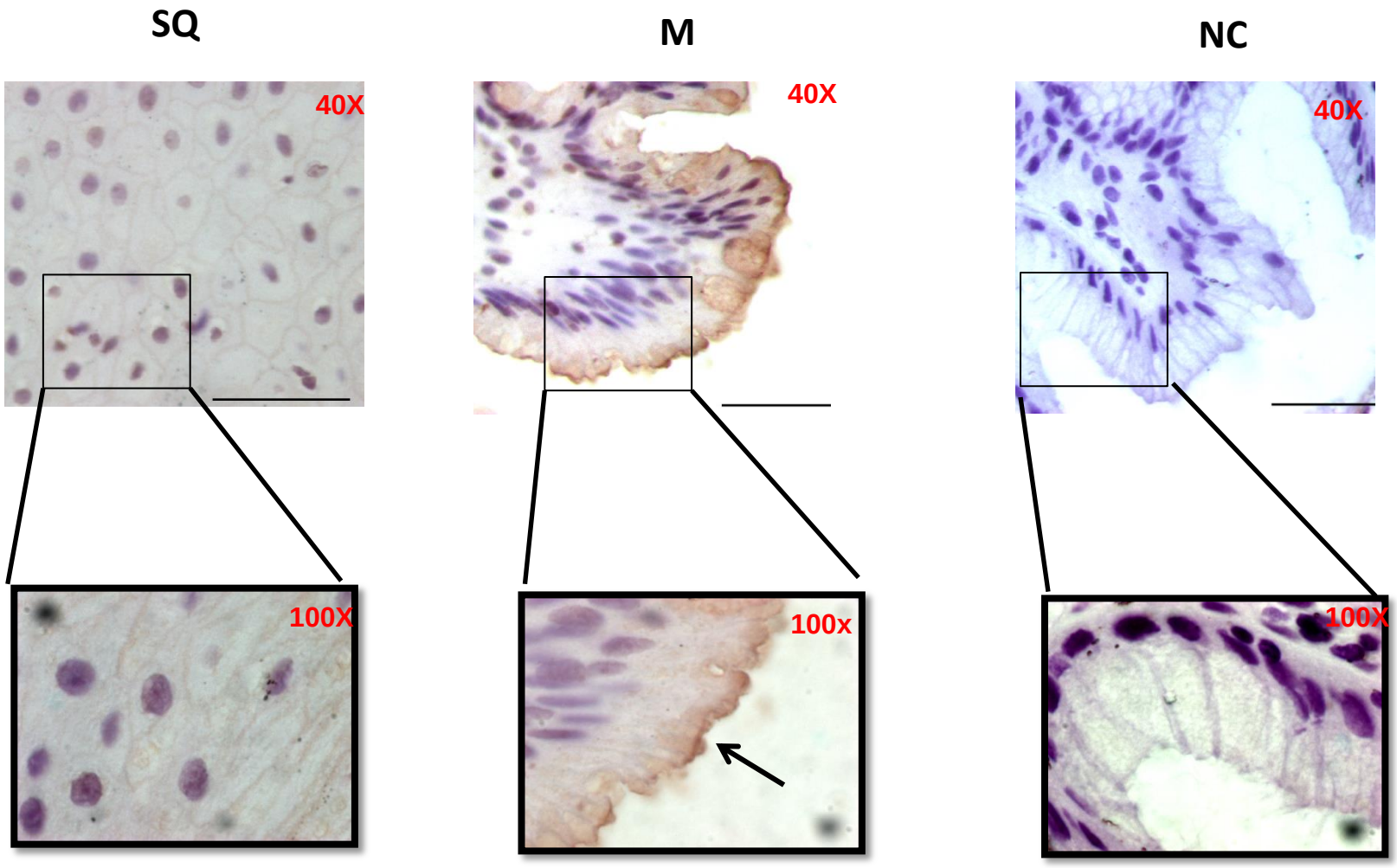


Figure 8

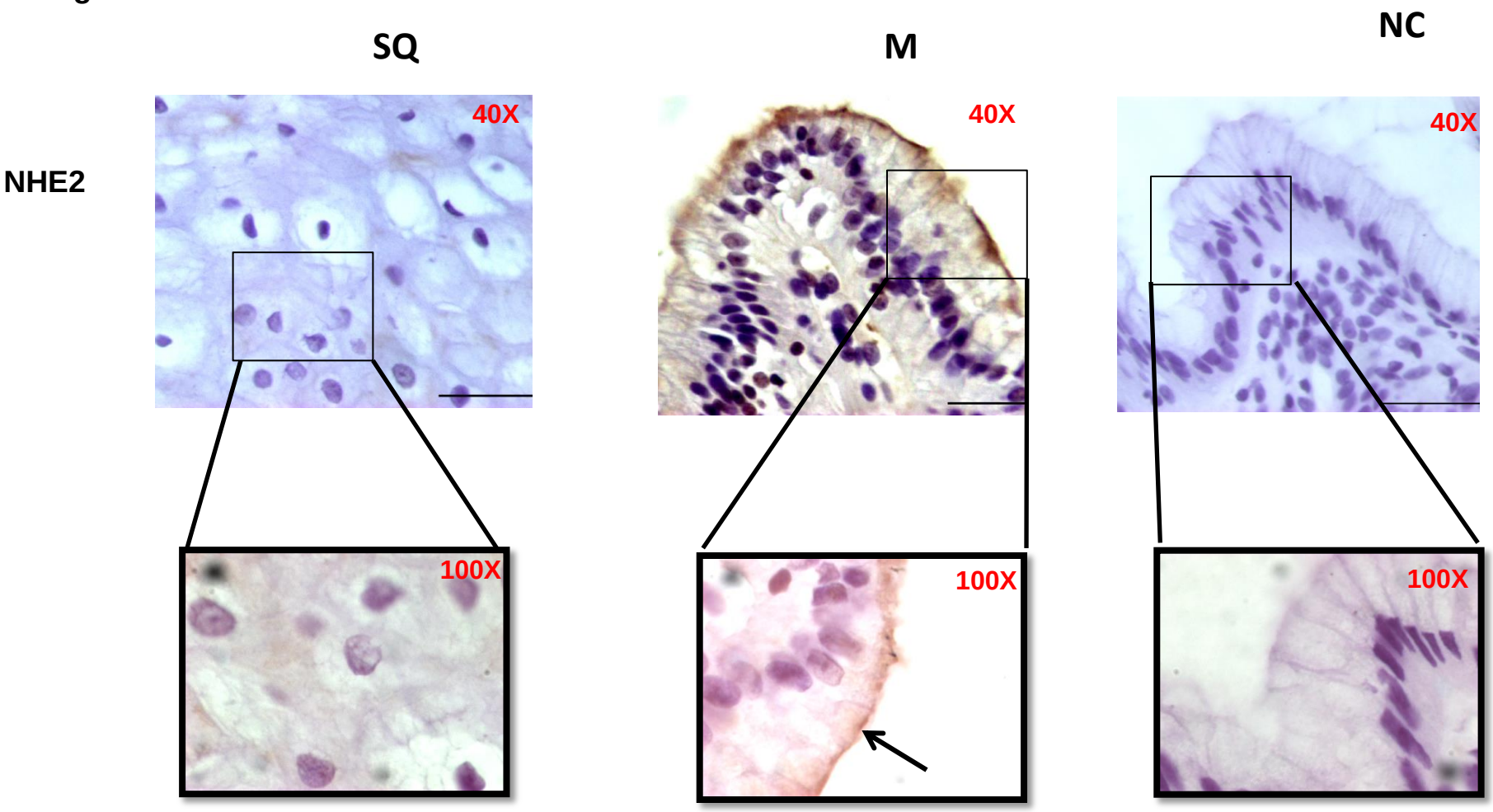


Table 1.

Patient No.	Gender	Age	Type of metaplasia	Length of metaplasia
1.	Male	82	Intestinal	c0m3
2.	Male	76	Intestinal	c3m4
3.	Male	57	Intestinal	c2m4
4.	Male	49	Intestinal	c3m4
5.	Female	70	Intestinal	c1.5m5
6.	Female	65	Intestinal	c8m10
7.	Female	62	Intestinal	c1m3
8.	Male	47	Non-intestinal	c1m1
9.	Female	81	Non-intestinal	c1m2
10.	Female	61	Non-intestinal	c0m1.5
11.	Female	58	Non-intestinal	c0m1
12.	Female	56	Non-intestinal	c0m1
13.	Female	55	Non-intestinal	c0m1
14.	Female	50	Non-intestinal	c0m0.5

Table 2.

	Standard Hepes	Standard HCO_3^-	NH_4Cl Hepes	NH_4Cl HCO_3^-	Na^+-free Hepes	Cl^--free Hepes	Cl^--free HCO_3^-
NaCl	130	115	110	95			
KCl	5	5	5	5	5		
$MgCl_2$	1	1	1	1	1		
$CaCl_2$	1	1	1	1	1		
Na-Hepes	10		10				
Glucose	10	10	10	10	10	10	10
$NaHCO_3$		25		25			25
NH_4Cl			20	20			
Hepes						10	
NMDG-Cl					10		
Na-gluconate					140	140	115
Mg-gluconate						1	1
Ca-gluconate						6	6
K-sulfate						5	2.5