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BILE ACIDS INHIBIT Na+/H+ EXCHANGER AND Cl-/HCO3- EXCHANGER ACTIVITIES VIA CELLULAR ENERGY BREAKDOWN AND Ca2+ OVERLOAD IN HUMAN COLONIC CRYPTS

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Abstract:	Bile acids play important physiological role in the solubilisation and absorption of dietary lipids. However, under pathophysiological conditions, such as short bowel syndrome, they can reach the colon in high concentrations inducing diarrhea. In this study our aim was to characterise the cellular pathomechanism of bile-induced diarrhea using human samples. Colonic crypts were isolated from biopsies of patients (controls with negative colonoscopic findings) and of cholecystectomised/ileum-resected patients with or without diarrhoea. In vitro measurement of the transporter activities revealed impaired Na+/H+ exchanger (NHE) and Cl-/HCO3- exchanger (CBE) activities in cholecystectomised/ileum-resected patients suffering from diarrhea, compared to control patients. Acute treatment of colonic crypts with 0.3mM chenodeoxycholate caused dose-dependent intracellular acidosis; moreover, the activities of acid/base transporters (NHE and CBE) were strongly impaired. This concentration of chenodeoxycholate did not cause morphological changes in colonic epithelial cells, although significantly reduced the intracellular ATP level, decreased

	mitochondrial transmembrane potential and caused sustained intracellular Ca2+ elevation. We also showed that chenodeoxycholate induced Ca2+ release from the endoplasmic reticulum and extracellular Ca2+ influx contributing to the Ca2+ elevation. Importantly, our results suggest that the chenodeoxycholate induced inhibition of NHE activities was ATP-dependent, whereas the inhibition of CBE activity was mediated by the sustained Ca2+ elevation. We suggest that bile acids inhibit the function of ion transporters via cellular energy breakdown and Ca2+ overload in human colonic epithelial cells, which can reduce fluid and electrolyte absorption in the colon and promote the development of diarrhea. Keywords: bile acids, colonic epithelial cells, ion transporters, intracellular Ca2+
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BILE ACIDS INHIBIT Na^+/H^+ EXCHANGER AND Cl^-/HCO_3^- EXCHANGER ACTIVITIES VIA CELLULAR ENERGY BREAKDOWN AND Ca^{2+} OVERLOAD IN HUMAN COLONIC CRYPTS

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Abbreviations used in this paper: BA, bile acids; BAM, bile acid malabsorption; BAPTA-AM, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CBE, Cl^-/HCO_3^- exchanger; CCCP, carbonyl cyanide m-chlorophenyl hydrazone; CDC, chenodeoxycholate; ER, endoplasmic reticulum; GCDC, glycochenodeoxycholate; H_2DIDS, dihydro-4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; HOE-642, 4-isopropyl-3-methylsulphonylbenzoyl-guanidin methanesulphonate; IP_3R, inositol-triphosphate receptor; $(\Delta\Psi)_m$; mitochondrial transmembrane potential; NHE, Na^+/H^+ exchanger; pH_i, intracellular pH; RR, ruthenium red; RyR, ryanodin receptor; SERCA, sarcoplasmic/endoplasmic reticulum calcium ATPase; SLC26, solute carrier family 26; TEM, transmission electron microscopy; Tg, Thapsigargin;

Abstract

Bile acids play important physiological role in the solubilisation and absorption of dietary lipids. However, under pathophysiological conditions, such as short bowel syndrome, they can reach the colon in high concentrations inducing diarrhea. In this study our aim was to characterise the cellular pathomechanism of bile-induced diarrhea using human samples. Colonic crypts were isolated from biopsies of patients (controls with negative colonoscopic findings) and of cholecystectomised/ileum-resected patients with or without diarrhoea. *In vitro* measurement of the transporter activities revealed impaired Na^+/H^+ exchanger (NHE) and Cl^-/HCO_3^- exchanger (CBE) activities in cholecystectomised/ileum-resected patients suffering from diarrhea, compared to control patients. Acute treatment of colonic crypts with 0.3mM chenodeoxycholate caused dose-dependent intracellular acidosis; moreover, the activities of acid/base transporters (NHE and CBE) were strongly impaired. This concentration of chenodeoxycholate did not cause morphological changes in colonic epithelial cells, although significantly reduced the intracellular ATP level, decreased mitochondrial transmembrane potential and caused sustained intracellular Ca^{2+} elevation. We also showed that chenodeoxycholate induced Ca^{2+} release from the endoplasmic reticulum and extracellular Ca^{2+} influx contributing to the Ca^{2+} elevation. Importantly, our results suggest that the chenodeoxycholate induced inhibition of NHE activities was ATP-dependent, whereas the inhibition of CBE activity was mediated by the sustained Ca^{2+} elevation.

We suggest that bile acids inhibit the function of ion transporters via cellular energy breakdown and Ca^{2+} overload in human colonic epithelial cells, which can reduce fluid and electrolyte absorption in the colon and promote the development of diarrhea.

Keywords: bile acids, colonic epithelial cells, ion transporters, intracellular Ca^{2+}

Introduction

One of the main non-motor functions of the colon is to absorb about 90% of the fluid (~1.5 to 1.9 L), arriving daily from the small intestine [41], which is mediated by the polarised epithelial cell layer. The colonic epithelial cells express numerous ion channels, pumps and transporters located either on the luminal or the basolateral membrane, allowing highly efficient transport of water and ions, especially Na^+ and Cl^- [25]. The adequate activity of these transporters is essential to keep the precise balance between absorption and secretion. The electroneutral NaCl absorption in the colon is most likely mediated via the coupled activity of Na^+/H^+ exchangers (NHEs) and the SLC26 Cl^-/HCO_3^- exchangers (CBE) [30,45].

Bile acids (BA) are natural detergents excreted into the small intestine, and most of them (90-95%) are reclaimed in the distal ileum then returned to the liver. If this precisely regulated enterohepatic circulation impairs, bile acid malabsorption (BAM) occurs, thus BA can enter the colon in higher concentrations and can induce diarrhoea through a yet unidentified mechanism. BAM and diarrhoea are well-known clinical complications following ileal resection or cholecystectomy [1,11,37]. The lack of proper diagnostic approaches and limited number of clinical guidlines on the appropriate management of patients with BAM making the disease underrecognized and great challenge for gastroenterologists [46]. Further complicates the situation that cholestyramine therapy (a bile acid sequestrant) does not solve the problem in every case [10,34,48,3]. Moreover, cholestyramine treatment can reduce the bioavailability of co-administered drugs as well. These observations suggest the neccessity of developing new therapeutical approaches, therefore it is crucial to understand to pathogenesis of bile acid induced diarrhoea.

In this study we provide strong evidence that BA impair the ion transport mechanisms of human colonic epithelial cells which could play an important role in the development of BAM associated diarrhoea. We demonstrate that chronic exposure of the colon to high concentrations of BA results in decreased activities of acid/base transporters, responsible for NaCl absorption. In addition, the non-conjugated BA chenodeoxycholate inhibits the activities of NHEs and CBE via $(ATP)_i$ depletion and sustained intracellular Ca^{2+} elevation in isolated human colonic epithelial cells. These intracellular changes could reduce fluid and electrolyte absorption in the colon and promote the development diarrhoea. Thus, our results might contribute to the development of new therapeutic approaches in the treatment of bile-induced diarrhea.

Materials and methods

Human subjects involved in the study

The patients involved in the study were divided into three groups. The first group contained patients having diarrhoea after ileum-resection/cholecystectomy (Diarrhoea). In the second group ileum-resected/cholecystectomised patients were involved who did not develop diarrhoea (NON-Diarrhoea). The control group included patients without any surgical intervention in the gastrointestinal tract. The patients enrolled in this study were between the age of 25-55 years. Informed consent was obtained prior to endoscopy. Protocols of the study were approved by the regional ethical committee at the University of Szeged, Szeged, Hungary. 3-6 colonic biopsies were obtained from the proximal colon (cecum, colon ascendens) from each patient undergoing colonoscopy at the First Department of Medicine. In none of the patients were macroscopic (by endoscopy) or microscopic (by histology) signs of the presence of inflammation in the colon. Patients with normal endoscopic findings were examined because of colorectal cancer screening or different abdominal complaints.

Materials and solutions for the experiment

The compositions of the solutions used are shown in Table 1. The pH of HEPES-buffered solutions was set to 7.4 with NaOH at 37^{o}C. HCO_3^--buffered solutions were gassed with 95% O_2/5% CO_2 to set the pH to 7.4 at 37^{o}C.

General laboratory chemicals were obtained from Sigma-Aldrich. Collagenase A was obtained from Roche Diagnostic (Mannheim, Germany). HOE-642 (4-isopropyl-3-methylsulphonylbenzoyl-guanidin methanesulphonate) was provided by Sanofi Aventis (Frankfurt, Germany) and was dissolved in dimethyl sulfoxide (DMSO). BCECF-AM (20,70-biscarboxyethyl-5(6)-carboxyfluorescein-acetoxymethylester), FURA-2-AM (2-(6-(bis(carboxymethyl)amino)-5-(2-(2-(bis(carboxymethyl)amino)-5-methylphenoxy)-ethoxy)-2-benzofuranyl)-5-oxazolecarboxylic acetoxymethyl ester), BAPTA-AM (1,2-bis(o-aminophenoxy)ethane-N,N,N9,N9-tetraacetic acid), Magnesium-green-AM and TMRM (tetramethylrhodamine methyl ester) were obtained from Invitrogen (Eugene, OR); cell and tissue adhesive from Becton Dickinson Bioscience (Cell Tak, Bedford, MA). BCECF-AM, BAPTA-AM and TMRM were dissolved in DMSO, FURA-2-AM, and Magnesium-green-AM were dissolved in pluronic acid and DMSO. Thapsigargin was obtained from Merck (Darmstadt, Germany) and it was dissolved in DMSO.

Isolation of colonic crypts

Colonic crypts were isolated from three human biopsy specimens obtained from the proximal (cecum or colon ascendens) part of the large intestine. Only one segment of the colon was investigated in each patient. The tissue samples were placed immediately in ice-cold $NaHCO_3$ containing Hank's balanced salt solution (HBSS). The samples were washed three times with HBSS, cut into small pieces with a razor blade and incubated in 1mM dithiothreitol (DTT) in HBSS for 15 minutes followed by 2x30 minutes enzymatic digestion with 0.38mg/mL collagenase A at 37°C and continuously gassed with 5% CO_2/95% O_2. The small fragments were mixed with a Pasteur pipette, the large fragments were allowed to settle down to the bottom of the flask under gravity for 35–40 seconds, and the supernatant removed and visualised under a Nikon stereo microscope (Jencons-PLS, Grinstead, UK). The crypts (200–300 crypts/isolation) were aspirated into a micropipette and transferred into a Petri dish. For fluorescent measurements the crypts were kept in a culture solution for 3 hours at 4°C before the experiments. The culture solution contained Dulbecco's Modified Eagle's Medium (DMEM), 10% fetal bovine serum (FBS; Sigma-Aldrich, Budapest, Hungary), 2mM L-glutamine, 100U/mL penicillin, and 100µg streptomycin.

Measurement of intracellular pH (pH_i), Ca^{2+} concentration ($[Ca^{2+}]_i$) and ATP level [$(ATP)_i$] by microfluorometry

Colonic crypts were attached to 24mm glass coverslips covered with CellTak 3h after isolation and placed in a perfusion chamber mounted on the stage of an inverted fluorescent microscope linked to an excellence imaging system (Olympus, Budapest, Hungary).

During the microfluorometry experiments, colonic crypts were incubated in standard HEPES solution at 37°C and loaded with the appropriate fluorescent dye. Crypts were continuously perfused with different solutions at a rate of 9-10ml/min. Two to three small areas (region of interest (ROIs)) from the surface of each crypt were investigated (Fig. 1).

pH_i was estimated with the pH-sensitive fluorescent dye BCECF-AM. After incubating with BCECF-AM (2µmol/l) for 20-30 minutes, colonic crypts were excited with light at wavelengths of 495nm and 440nm and the 495/440 fluorescence emission ratio were measured at 535nm [17,42].

For the measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) the cells were loaded with the Ca^{2+} sensitive fluorescent dye FURA-2-AM (5µmol/l) for 60 min. For excitation, 340 and 380nm filters were used, and the changes in $[Ca^{2+}]_i$ were calculated from the fluorescence ratio (F340/F380) measured at 510nm.

To determine changes of $(ATP)_i$ concentrations the fluorescent dye Mg-green-AM was used, which has been shown to indirectly reflect the changes in $(ATP)_i$. Colonic crypts were incubated with Mg-Green (4µmol/L) for 60 minutes than were excited with 476nm light and emission was detected at 500–550nm. Because ATP has a 10-fold greater affinity for Mg^{2+} than ADP, and most intracellular Mg^{2+} is present as Mg-ATP [22,27], the ADP:ATP ratio can be monitored. The elevation of fluorescence intensity caused by the increase in free intracellular Mg^{2+} concentration suggests a reduction of $(ATP)_i$ [7]. The $(ATP)_i$ measurements were performed in standard HEPES-buffered solution.

Measurement of NHE activities

During the measurement of pH_i, in order to characterize NHE activity NH_4Cl pulse technique was used in HEPES-buffered solution. Exposure of colonic crypts for 3 minutes to 20mM NH_4Cl induced an immediate rise in pH_i due to the rapid entry of lipophilic base 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. Under these conditions, the initial rate of pH_i recovery from the acid load reflects the activities of NHEs. $-J(B^-)$ was calculated from the first 60 sec of pH_i recovery from acidification.

Further experiments were done to investigate the activities of the different NHE isoforms. The crypts were acid loaded by exposure to a 3-min-pulse of 20mM NH_4Cl in HEPES solution followed by a 10-min-exposure of Na^+-free HEPES solution. Due to the blocked acid/base transporters (neither sodium nor bicarbonate are present in the solution); the pH_i is set to a stable acidic level. NHE activity was switched on by re-addition of extracellular sodium and the activities of NHEs were determined by measuring the initial rate of pH_i recovery over the first 60 sec. The activities of the different NHE isoforms are extracted by using the isoform selective NHE inhibitor HOE-642. The isoform selectivity of HOE-642 is dose-dependent, 1µM HOE642 inhibits NHE1 whereas 50µM HOE642 inhibits both NHE1 and 2 but not NHE3 [2,6]. The activities (A) of NHE isoforms can be calculated from the recoveries (R) as follows:

$$A_{NHE1} = R_{0\mu M\ HOE\text{-}642} - R_{1\mu M\ HOE\text{-}642}$$

$$A_{NHE2} = R_{1\mu M\ HOE\text{-}642} - R_{50\mu M\ HOE\text{-}642}$$

$$A_{NHE3} = R_{50\mu M\ HOE\text{-}642}$$

Determination of buffering capacity and base efflux

The total buffering capacity (β_{total}) of colonic epithelial cells was estimated according to the NH_4^+ pre-pulse technique [16,47]. Colonic epithelial cells were exposed to various concentrations of NH_4Cl in a Na^+- and HCO_3^--free solution. β_i (which refers to the ability of intrinsic cellular components to buffer changes of pH_i) was estimated by the Henderson–Hasselbach equation. β_{total} was calculated from: $\beta_{total} = \beta_i + \beta_{HCO3-} = \beta_i + 2.3x[HCO_3^-]_i$, where β_{HCO3-} is the buffering capacity of the HCO_3^-/CO_2 system. The measured rates of pH_i change (dpH/dt) were converted to transmembrane base flux $J(B^-)$ using the equation: $J(B^-)$=dpH/dtx β_{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 $-J(B^-)$.

Measurement of CBE activity

Cl^- withdrawal technique was used to investigate the activity of CBE [43]. Removing Cl^- from the standard HCO_3^-/CO_2 buffered solution caused alkalization due to the reversed activity of the Cl^-/HCO_3^- exchanger. The activity of the exchanger was determined by measuring the initial rate of alkalization over the first 30 sec.

Electron microscopy

Morphological changes of the different cell organelles of the colonic epithelial cells were evaluated by transmission electron microscopy (TEM). Biopsy samples were fixed in 2% glutarldehyde (in PBS) overnight at 4°C degree. Samples were cut into small pieces (1X1 mm) than were infiltrated with 2% gelatin (PBS) and the small cubes were made, which were than embedded to Embed 812 (EMS, USA) using a routine TEM embedding protocol. After the semithin sections (1µm), the thin (70nm) sections were cut for TEM examination.

Measurement of mitochondrial transmembrane potential ($(\Delta\psi)_m$)

Changes of $(\Delta\psi)_m$ were assessed by loading cells with 100nmol/L Tetramethylrhodamine, methyl ester (TMRM) for 30 minutes at 37°C to measure fluorescence in the perigranular mitochondrial region. Depolarisation of the mitochondria results in redistribution of TMRM from the mitochondria to the cytosol, causing a decrease in mitochondrial fluorescence [44]. Excitation 488nm, emission was detected at >550nm with Olympus Fluoview FV10i confocal system [7].

Statistical analysis

Values are means ± SE. Statistical analyses were performed using analysis of variance (ANOVA) with the post-hoc test Dunnett or Bonferroni. $P \leq 0.05$ was accepted as significant.

Results

Chronic exposure of the colon to bile acids impair the activities of NHEs and CBE of isolated human colonic epithelial cells

Colonic crypts were isolated from patients whose colon is probably exposed to high concentrations of BA. Ileum-resected or cholecystectomised patients were divided into two groups depending on the presence (Diarrhoea; D) or absence (NON-Diarrhoea; NON-D) of diarrhoea after the surgical intervention. The calculated acid/base transporter activities were compared to those measured in control patients to determine the effects of BA on the epithelial ion transport.

Representative curves of the pH_i traces and the summary data of the calculated NHE activities are shown in Fig. 2a, b. The activities of the different NHE isoforms are extracted by using the isoform selective NHE inhibitor HOE-642. 1µM HOE642 inhibits NHE1 whereas 50µM HOE642 inhibits both NHE1 and 2 but not NHE3 [2,6]. The functions of all examined NHE isoforms were significantly reduced in patients in group D compared to control patients.

The function of CBE was investigated using the Cl^- withdrawal technique (Fig. 2c). Removal of Cl^- from the standard HCO_3^-/CO_2 bath solution caused a marked alkalization in colonic crypt cells suggesting the presence of a functionally active anion exchange mechanism. The activity of the CBE was significantly impaired in D group compared to control patients (Fig. 2d). In colonic crypt cells, isolated from NON-D patients, the activities of the examined acid/base transporters were not changed significantly, compared to the control group, suggesting the significant role of ion transporters in bile-induced diarrhoea.

Bile acid administration dose-dependently reduce the pH_i of isolated human colonic epithelial cells

Our next aim was to characterise the acute effects of BA on colonic epithelial cells. For these experiments, colonic crypts were isolated from control patients. The administration of chenodeoxycholate (CDC) resulted in a dose-dependent reduction of the pH_i of perfused colonic epithelial cells (Fig. 3). The characteristic response was a rapid decrease in pH_i which than slowly recovered to a variable degree during continuous exposure to BA (Fig. 3a-d). In HEPES-buffered solution, the ΔpH_{max} was more prominent during CDC administration compared to those observed in HCO_3^--containing solution, which can be explained by the increased buffering capacity of the colonic epithelial cells in the presence of HCO_3^-/CO_2.

Acute exposure to bile acid inhibits the activities of acid/base transporters of isolated human colonic epithelial cells

Impaired NHE and CBE activities were observed in patients suffering from diarrhoea, whose colon is probably continuously exposed to an elevated BA concentration, therefore we wanted to characterize the effects of acute BA administration on the ion transport mechanisms of human colonic epithelial cells. Colonic crypts isolated from control patients were used during these series of experiments. In order to investigate the effects of BA on the activities of NHEs, we analysed the pH_i recovery from an acid load induced by the removal of NH_4Cl. The representative pH_i traces (Fig. 4a) and the summary data of the calculated NHE activities (Fig. 4b) show that 10 min treatment with 0.1mM CDC had no effects on the NHE activities. When the colonic crypts were perfused with 0.3 mM CDC, an inhibition of the activities of NHEs was observed. To identify the exact NHE isoforms, which were inhibited by 0.3mM CDC, we used the ammonium pulse technique with the isoform-selective NHE inhibitor HOE-642 during the continuously perfusion with 0.3mM CDC. According to our results, all of the NHE isoforms were significantly inhibited by 0.3mM CDC (Fig. 4c).

We also tested the effects of higher BA concentrations, although when 20mM NH_4Cl was applied at the same time with 1 mM CDC, the fluorescent intensities at 440 and 495nm rapidly decreased causing an immediate elevation of the 440/495 ratio. This might be explained by the loss of BCECF and reflects the permeation of the plasma membrane, or by the lysis of the cells.

The Cl^--withdrawal technique was applied to examine the activity of CBE as well. The apical Cl^- removal from the extracellular solution increased the pH_i of the cells by driving HCO_3^- into the cell via the apical CBE, whereas, re-addition of Cl^- decreased pH_i inducing secretion of HCO_3^- via the CBE. Treating the crypts with 0.3mM CDC resulted in a strong inhibition in the activity of CBE (Fig 4.d,e).

Besides the non-conjugated BA we also investigated the effects of the conjugated BA on the ion transport activities of the colonic epithelial cells. We tested the effects of glycochenodeoxycholate in different concentrations (0.1, 0.3, 1mM) on the activities of NHE and CBE; however no changes were observed (data not shown).

High concentration of bile acid induces severe mitochondrial damage

Our next aim was to explore the intracellular mechanisms by which BA inhibit acid/base transporters. Since previous works have reported that BA can disrupt intracellular organelles (mitochondria, or Golgi) [4,29,31], we first analysed the structure of the cell compartments of human colonic epithelial cells following

incubation with BA. Transmission electron microscopy (TEM) revealed that administration of low concentrations of CDC (0.1mM or 0.3mM) for 1-10 min had no effect on the structure of intracellular organelles (Fig. 5a). On the other hand, 10 min exposure of the human colonic epithelial cells to high concentration (1mM) of CDC strongly damaged all of the mitochondria. The mitochondria swelled up and the inner membrane structures were disrupted. We did not observe such alteration in other intracellular organelles, such as ER, Golgi or nuclei. For positive control experiments, the mitochondrial toxin carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 100μM) was applied. The mitochondrial injury was similar to those seen after 1mM CDC treatment.

Bile acid treatment decreases $(ATP)_i$ and $(\Delta\psi)_m$ of isolated human colonic epithelial cells

Since TEM experiments didn't reveal the inhibitory mechanism of 0.3mM CDC completely, we tried to dissect the mechanism at a functional level. Therefore, in the next step we aimed to find out whether CDC has any influence on the $(ATP)_i$ level of the human colonic epithelial cells. Using the Mg-Green fluorescent probe, which is indirectly sensitive to $(ATP)_i$ (see Methods) we showed, that 0.3mM CDC significantly, but reversibly depleted $(ATP)_i$ of isolated human colonic epithelial cells (Fig. 5b, c). Following administration of 0.1mM CDC the $(ATP)_i$ was not affected (data not shown). In case of 1mM CDC, not only the structural impairement of the mitochondria was evident but a significant and irreversible decrease of the $(ATP)_i$ level was perceptible as well. For positive control, the mitochondrial toxin CCCP was applied similarly to the morphological studies. The fact that high concentration of CDC caused more prominent $(ATP)_i$ depletion than CCCP, suggests that BA have additional effects, which further decreases $(ATP)_i$. To investigate the effects of BA on glicolytic $(ATP)_i$ production the combination of deoxyglucose (DOG)/idoacetamide (IAA) was used, which inhibit the glycolytic metabolism of colonic epithelial cells. 10mM DOG + 5mM IAA significantly and irreversibly depleted $(ATP)_i$. CCCP and DOG+IAA together mimicked the effect of high conenctrations of CDC on the $(ATP)_i$ of isolated human colonic epithelial cells.

Alterations of $(\Delta\psi)_m$ were also examined. Representative traces in Fig. 5d demonstrate that administration of 0.1mM or 0.3mM CDC induced a significant decrease in TMRM fluorescence, which indicates the loss of $(\Delta\psi)_m$. This effect was reversible, $(\Delta\psi)_m$ returned to basal level following removal of CDC. Furthermore 1mM CDC or 100μM CCCP caused a marked and irreversible reduction of $(\Delta\psi)_m$.

Bile acid treatment dose dependently increases $[Ca^{2+}]_i$ via endoplasmic reticulum (ER) Ca^{2+} release and extracellular Ca^{2+} influx

To further investigate the intracellular effects of BA, the changes of $[Ca^{2+}]_i$ in isolated human colonic epithelial cells was measured during CDC-treatment. In our experiments, administration of CDC caused a dose-dependent increase in $[Ca^{2+}]_i$ (Fig. 6a-c). The increase in $[Ca^{2+}]_i$ was sustained, plateau-like pathophysiological signal. The removal of Ca^{2+} from the extracellular solution significantly decreased this effect. Pretreatment of the colonic crypts with BAPTA-AM (40μM), a fast chelator of $[Ca^{2+}]_i$ abolished the effect of 0.1 and 0.3mM CDC, however, a moderate increase of $[Ca^{2+}]_i$ was still observable when 1mM CDC was applied (Fig. 6d).

We made attempts to identify the source of Ca^{2+}, released during CDC-treatment. Caffeine (20mM) and/or Ruthenium red (RR, 10μM) were utilised in order to antagonise inositol triphosphate receptor (IP_3R) and ryanodin receptor (RyR), which can mediate Ca^{2+} release from the endoplasmic reticulum (ER). Representative curves and the summary bar chart (Fig. 8a, b) demonstrate that the application of caffeine significantly inhibited the increase in $[Ca^{2+}]_i$ generated by 0.3mM CDC, while the administration of RR had no effect on the Ca^{2+} release. The rate of $[Ca^{2+}]_i$ increase was significantly diminished as well during the administration of caffeine (Fig. 8c). In the next step gadolinium (Gd^{3+}, 1μM) was applied to block plasma membrane Ca^{2+} entry channels. Gd^{3+} alone was not able to decrease the elevation of $[Ca^{2+}]_i$ induced by 0.3mM CDC, while the simultaneous administration of Gd^{3+}, caffeine and RR significantly reduced it. To further characterize the CDC-induced increase of $[Ca^{2+}]_i$ in colonic epithelial cells, thapsigargin (Tg), the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA pump) inhibitor was applied. In Ca^{2+}-free solution, Tg (2μM) induced Ca^{2+} store depletion with consequent $[Ca^{2+}]_i$ elevation (Fig. 8d). This elevation was markedly decreased when Tg was administered after 0.3 mM CDC (Fig. 8e). Tg, when administered during 0.3mM CDC, further induced a slight increase in $[Ca^{2+}]_i$ (Fig 8). These observations suggest that beside the extracellular Ca^{2+} influx, CDC deplete ER Ca^{2+}-stores via IP_3R mediated processes.

The BA induced inhibition of Na^+/H^+ exchange activities is ATP-dependent, whereas the inhibition of CBE activity is Ca^{2+}-dependent in isolated human colonic epithelial cells

Next we examined the potential connection between the intracellular effects of 0.3mM CDC ($(ATP)_i$ depletion and $[Ca^{2+}]_i$ elevation) and the decreased function of acid/base transporters following treatment with the BA. Ammonium pulse technique (Fig. 9a) showed that chelation of intracellular Ca^{2+} with BAPTA-AM did not influence the inhibitory effect of 0.3mM CDC on the NHE activity. In contrast, when the glycolysis inhitbitors DOG+IAA were applied the same inhibition of NHEs was perceptible (Fig. 9b, d), which suggests that CDC

inhibits NHE via $(ATP)_i$ depletion. This result confirms the observation of other workgroups that the NHEs are ATP-dependent transporters [5,38,39].

We also investigated the inhibitory effect of 0.3mM CDC on CBE in more details. We tested the effects of $[Ca^{2+}]_i$ chelation and $(ATP)_i$ depletion during the Cl^--removal technique in isolated human colonic epithelial cells. We showed that the intracellular Ca^{2+}-chelator BAPTA-AM completely abolished the inhibitory effect of 0.3mM CDC on the activity of CBE. In contrast, depletion of $(ATP)_i$ with DOG+IAA was not able to reduce CBE activity. These observations suggest that bile acids inhibit CBE activity via toxic $[Ca^{2+}]_i$ elevation unlike NHE activity, which is inhibited $(ATP)_i$ depletion.

Discussion

BA are amphipathic molecules participating in fat solubilisation and lipid digestion [23]. Besides the physiological functions, BAs are also known to cause bile-induced diarrhoea, a common feature of BAM, which often develops after small bowel resection or post-cholecystectomy [1,37,40]. Although bile-induced diarrhoea is a frequent complication affecting high number of patients, its pathogenesis is not completely undertood yet. In this study we provide strong human evidence, that bile acids impair the activity of the acid/base transporters (NHE and CBE) via different mechanisms in colonic epithelial cells. The impaired activity of NHE and CBE can decrease the fluid and electrolyte absorption in the colon and promote the development of diarrhoea.

The colonic epithelial cells express different ion channels and transporters, which transport ions and water. NHE1 is constitutively expressed on the basolateral membrane of the epithelial cells. Although it does not play a role in the Na^+ absorption, it fulfils housekeeping functions, regulating cell volume and pH_i [24]. In addition the presence of NHE2 and NHE3 on the apical membrane of colonic epithelial cells has been confirmed [20]. NHE3 knock out mice develope diarrhoea, which supports the idea that this might be the dominant NHE isoform, responsible for Na^+ uptake in the intestine [12,35,49]. Together with NHE3, the SLC26A3 Cl^-/HCO_3^- exchanger (or downregulated in adenoma; DRA), a member of the SLC26 gene family, maintains the absorption of NaCl in the colon. Mutation of this transporter results in congenital chloride-losing diarrhoea [19], moreover, similar conditions develop in SLC26A3-deficient mice [36]. The fact that impaired activities of these transporters were observed in diarrhoea-associated diseases, such as ulcerative colitis and secretory diarrhoea [8,28,50], further underlies the importance of the NHE3 and SLC26A3 in colonic electrolyte and water absorption.

BA can induce diarrhoea when they are present in the colon at high concentrations; therefore we recruited ileum-resected or cholecystectomised patients and divided them into two groups depending on having (D) or not having diarrhea (NON-D) after the surgical intervention. The measurement of the ion transporter activites revealed that in the D group the activities of NHE1-3 isoforms were markedly lower, compared to the control group. This inhibitory pattern is clearly different from our previous observations in ulcerative colitis patients, where the activity of NHE1 was increased, whereas the activity of NHE3 was significantly decreased in colonic epithelial cell [9]. Moreover the activity of CBE was significantly diminished in patients in group D as well. Importantly, the function of the acid/base transporters was unaltered in colonic epithelial cells isolated from NON-D patients. These data suggest that the reduced absorptive function of the colon is probably due to the continuous presence of non-physiological concentration of BA.

In the next step we aimed to characterise the acute effects of BA on human colonic epithelial cells. Hamilton et al. showed, that the physiological concentration of total BA – including conjugated and non-conjugated forms - in the proximal colon (cecum) reaches 1mM [15]. In addition, in the large intestine BAs are metabolized, mainly deconjugated, by resident bacteria [18,21,33] further elevating the concentration of the non-conjugated BA. Based on these observations, we used 0.1mM CDC as low, physiological and 0.3-1mM CDC as high, non-physiological concentrations. In our experiments, administration of CDC caused an immediate, dose-dependent and reversible decrease of the pH_i in human colonic epithelial cells. Moreover we also provided evidence that administration of high concentration (0.3mM) of CDC resulted in a significant inhibition of NHEs and CBE of human colonic epithelial cells, suggesting the possible toxic effects of high doses of non-conjugated BA. Using the isoform-specific NHE inhibitor HOE-642 we also showed that the functions of NHE1-3 isoforms were reduced in response to 0.3mM CDC. Similarly, high concentration of CDC strongly inhibited the acid/base transporters of guinea pig pancreatic ductal epithelial cells [43].

To dissect the intracellular mechanisms of the inhibitory effects of BA on colonic epithelial cells, first we investigated the effects of CDC on the morphology of intracellular organelles. Previous studies demonstrated that BA can perturb intracellular organelles and induce Golgi fragmentation and disruption of the mitochondria in cultured colonic epithelial cells and in pancreatic ductal cells [4,29,31]. In our experiments, 10 minute exposure of the colonic epithelial cells to 0.3mM CDC did not induce any visible alteration in the morphology of the cell compartments. Nevertheless, 1mM CDC caused a severe damage in all of the mitochondria. The mitochondria swelled up and the structure of the inner membranes was disrupted, whereas other intracellular organelles seemed to remain intact. Although 0.3mM CDC inhibited the activities of acid/base transporters, it did not induce alteration in the structure of intracellular organelles, therefore we investigated whether mitochondrial function is altered by BA. Administration of 0.3mM CDC significantly but reversibly depleted the $(ATP)_i$ and decreased $(\Delta\psi)_m$ of isolated human colonic epithelial cells. The toxic effects of BAs were previously demonstrated on isolated rodent pancreatic acinar [44].

Next, we investigated another potential intracellular target of BA, the Ca^{2+} signalisation, a well-known mediator of numerous cellular processes. It was shown previously that the Cl^-/HCO_3^- exchanger SLC26A3 and NHE3 are inhibited by the pathological increase of intracellular Ca^{2+} [6,26]. In our experiments, CDC induced a dose-dependent sustained increase in $[Ca^{2+}]_i$. The removal of Ca^{2+} from the extracellular solution significatnly decreased, while preincubation of the colonic crypts with BAPTA-AM (40μM), a fast intracellular Ca^{2+}chelator, almost completely abolished the effect of 0.3mM CDC. If elevation of $[Ca^{2+}]_i$ occurs, the source of Ca^{2+} could be

the release from the ER via IP_3R and/or RyR or Ca^{2+} entry from the extracellular space [32,14,13]. In our experiments, the IP_3R antagonist caffeine, but not the RyR-blocker RR or the plasma membrane Ca^{2+} channel inhibitor Gd^{3+}, reduced the CDC induced $[Ca^{2+}]_i$ elevation. These observations suggest that BA mobilizes stored Ca^{2+} from the ER via IP_3R. However, since the inhibition of IP_3R did not completely abolished the effect of CDC, proposes that extracellular Ca^{2+} influx must play a role as well. This process is most probably mediated by a Gd^{3+} insensitive Ca^{2+} channel, Na^+/Ca^{2+} exchangersor non-specific cation channels. These ion channels have large Na^+, Ca^{2+} and/or K^+ conductance but since specific inhibitors are lacking, it is difficult to distinguish their functions. Beside, the SERCA inhibitor thapsigargin induced a further elevation of the $[Ca^{2+}]_i$ after or during CDC administration suggests that CDC does not completely empty the ER Ca^{2+} store. These observations further confirmed our hypothesis that CDC mobilizes Ca^{2+} from the ER and promotes the influx of external Ca^{2+}. The fact that the increase in $[Ca^{2+}]_i$ in response to CDC was reversible argues the possibility that it was caused by the detergent property of the BA.

Finally, we examined whether there is a connection between the inhibitory effect of 0.3mM CDC on the activities of acid/base transporters and its effects on $(ATP)_i$ or $[Ca^{2+}]_i$. We showed that the depletion of $(ATP)_i$ resulted in a similar decrease of NHE activities as it was observed following the administration of 0.3mM CDC. In contrast, pretreatment of the colonic epithelial cells with the $[Ca^{2+}]_i$ chelator BAPTA-AM did not prevent the toxic effect of 0.3mM CDC on the activities of NHEs. These results indicate that 0.3mM CDC inhibits the functions of NHEs rather via depleting $(ATP)_i$. This is in agreement with previous observations that NHEs are ATP-dependent transporters [5,38,39]. Beside the Na^+ transport, the Cl^- absorptive capacity via the CBE of the colonic epithelial cells was also tested. $(ATP)_i$ depletion caused by the glycolysis inhibitors did not have any influence on the activity of CBE. However, preincubation of the colonic crypts with the $[Ca^{2+}]_i$ chelator BAPTA-AM restored the diminished activity of CBE caused by 0.3mM CDC. This observation further supports the hypothesis that CBE is inhibited by the non-physiological elevation of $[Ca^{2+}]_i$ [26].

Taken together using isolated human colonic crypts we showed that non-conjugated bile acids, when they are present in the colon at a relative high concentration, can enter the colonic epithelial cells. Via impaired cellular ATP production and toxic $[Ca^{2+}]_i$ elevation bile acids can decrease the activities of acid/base transporters, responsible for NaCl absorption, which can reduce fluid and electrolyte absorption in the colon promoting the development of diarrhoea. Stimulating either the sodium uptake via NHE or the chloride uptake via CBE shall offer new therapeutical approaches in the treatment of bile-induced diarrhea.

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Figure 1. Phase contrast (A) and fluorescent (B) pictures of an isolated human colonic crypt. Crypts were fixed on glass coverglass. Three regions of interests (ROIs) of each crypt were excited with lights at different wavelengths and the fluorescence emissions were measured. B: base, M: middle, S: surface, L: lumen of the crypt, BL: basolateral membrane.

Figure 2. The activities of NHE and CBE were decreased in colonic epithelial cells isolated from ileum-resected/cholecystectomised patients suffering from diarrhea. (A) The activities of different NHE isoforms were determined by NH_4Cl pulse technique with the isoform-specific NHE inhibitor HOE-642 as described in materials and methods. **(B)** Summary data of the calculated NHE activities. The activities of NHE1-3 were significantly impaired in patients suffering from diarrhea. **(C)** Representative pH_i traces showing the effect of the Cl^- removal on the pH_i of the colonic epithelial cells. **(D)** Summary data of the calculated CBE activities, which were significantly decreased in patients suffering from diarrhea. Groups of patients were C: control; D: ileum-resected/cholecystectomised with diarrhea and NON-D: ileum-resected/cholecystectomised without diarrhea. Data are presented as means ± SEM. n=5-6 patients/16-24 crypts/32-48 ROIs, *p<0.05 vs. control

Figure 3. Bile acids induce dose-dependent acidosis in isolated human colonic epithelial cells. Representative pH_i traces demonstrating the effect of non-conjugated CDC (0.1, 0.3 and 1mM) administered in HEPES- **(A)** or in HCO_3^-/CO_2-buffered **(C)** solution. Summary data of the calculated base flux ($J(B^-/min)$) **(B)** and the maximal pH_i change (ΔpH_{max}) **(D)** induced by bile acids. Bile acids dose-dependently reduced the pH_i of colonic epithelial cells. Data are presented as means ± SEM. n= 4-6 patients/14-18 crypts/28-36 ROIs. *p<0.05 vs. CDC, #p<0.05 vs. HEPES. ND: not detectable.

Figure 4. Administration of 0.3mM CDC significantly inhibited the activities of acid/base transporters in isolated human colonic crypts. (A) Representative pH_i curves showing the effects of CDC (0.1 and 0.3mM) on the recovery from an acid load induced by removal of 20mM NH_4Cl. **(B)** Summary data of the initial rate of pH_i recovery from acid load. **(C)** Summary data of the effect of 0.3mM CDC on the calculated activities of the different NHE isoforms. The isoform-selective NHE inhibitor HOE-642 revealed that all NHE isoforms were inhibited by 0.3mM CDC. **(D)** Representative pH_i traces showing the effect of CDC (0.1 and 0.3mM) during the removal of extracellular Cl^-. **(E)** Summary data of the initial rate of pH_i elevation after Cl^- withdrawal. **A** and **B** were in HEPES-buffered, **C** and **D** were performed in HCO_3^--buffered solution. High concentration of CDC

significantly decreased the activity of CBE. Data are presented as means ± SEM. n=3-6 patients/6-12 crypts/12-40 ROIs, *p<0.05 vs. control.

Figure 5. Treatment with 0.3mM CDC does not alter the structure of intracellular organelles but it significantly decreases $(ATP)_i$ and disturbs $(\Delta\psi)_m$. (A) Representative transmission electron microscopy pictures showing the effects of CDC (0.3mM and 1mM) on the mitochondria of human colonic epithelial cells. L: crypt lumen, arrow: mitochondria. 1 mM but not 0.3mM CDC induced severe mitochondrial damage. **(B)** Representative curves of the Mg-green fluorescent experiments. Elevation of fluorescent intensity represents depletion in $(ATP)_i$. **(C)** Summary data for the maximal fluorescent intensity changes. High concentrations of CDC significantly decreased $(ATP)_i$. **(D)** Representative traces of the $(\Delta\psi)_m$ measurements. Decrease of fluorescent intensity represents loss of mitochondrial transmembrane potential. **(E)** Summary data for the maximal fluorescent intensity changes. High concentrations of CDC significantly decreased $(\Delta\psi)_m$. All experiments were performed in HEPES-containing solution. Data are presented as means ± SEM. n=3 patients/5-6 crypts/13-15 ROIs. *p<0.05 vs. 1mM CDC.

Figure 6. CDC dose-dependently increased the $[Ca^{2+}]_i$ of isolated human colonic epithelial cells. (A-C) Representative curves showing the effect of CDC (0.1, 0.3 and 1mM) on $[Ca^{2+}]_i$ of isolated human colonic crypts in Ca^{2+}-free or 1mM Ca^{2+}-containing solution with/without pretreatment with the $[Ca^{2+}]_i$ chelator BAPTA-AM. **(D)** Summary data for the maximal fluorescent intensity changes. All experiments were performed in HEPES-buffered solution. Data are presented as means ± SEM. n=2-4 patients/5-10 crypts/10-21 ROIs.

Figure 7. CDC released Ca^{2+} from the ER and induced extracellular Ca^{2+} influx in isolated human colonic epithelial cells. (A) Representative curves showing the effect of the IP_3R inhibitor caffeine (20mM) and RyR inhibitor Ruthenium Red (10μM) on the increase of $[Ca^{2+}]_i$ induced by 0.3mM CDC. **(B)** Summary data of the maximal fluorescent intensity changes. The inhibition of IP_3R, but not RyR decreased the CDC induced Ca^{2+} release. **(C)** Representative curves showing the effect of the plasma membrane Ca^{2+} channel inhibitor Gd^{3+} (1μM). **(D)** Summary data of the maximal fluorescent intensity changes. All experiment were performed in HEPES-buffered solution. Data are presented as means ± SEM. n=2-4 patients/5-8 crypts/10-18 ROIs. *p<0.05 vs. CDC in 0 mM Ca^{2+} containing solution, #p<0.05 vs. CDC in 1mM Ca^{2+} containing solution. **(E)** Representative traces showing the effects of SERCA-inhibitor Tg (2μM) administered alone and following or

during 0.3mM CDC on the $[Ca^{2+}]_i$. **(F)** Summary data of the maximal fluorescent intensity changes. All experiments were performed in Ca^{2+}-free HEPES-buffered solution. Data are presented as means ± SEM. n=2-3 patients/5-6 crypts/12-14 ROIs. #p<0.05, ##p<0.01 vs. Tg alone.

Figure 8. 0.3mM CDC induced $(ATP)_i$-dependent inhibition of NHEs and Ca^{2+}-dependent decrease in the activity of CBE in isolated human colonic epithelial cells. (A) Representative pH_i curves showing the effect of 0.3mM CDC, $(ATP)_i$ depletion or pretreatment with the $[Ca^{2+}]_i$ chelator BAPTA-AM on the initial rate of pH_i recovery from an acid load induced by the removal of 20mM NH_4Cl. **(B)** Summary data of the initial rate of pH_i recovery from an acid load in HCO_3^--containing solution. The depletion of $(ATP)_i$, mimicked, but the prevention of Ca^{2+} elevation did not change the effect of CDC on the NHE activities. **(C)** Representative pH_i curves demonstrating the effect of 0.3mM CDC, $(ATP)_i$ depletion or pretreatment with the $[Ca^{2+}]_i$ chelator BAPTA-AM on the removal of extracellular Cl^-. **(D)** Summary data of the initial rate of pH_i elevation after Cl^- withdraw. The chelation of the $[Ca^{2+}]_i$ elevation abolished the inhibitory effect of CDC. **A** and **B** were in HEPES-, **C** and **D** were performed in HCO_3^--containing solution. Data are presented as means ± SEM. n=3-6 patients/5-12 crypts/8-40 ROIs. *p<0.05 vs. control.

Table 1. Composition of solutions for *in vitro* studies. Values are concentrations in mmol/L.

Figure1
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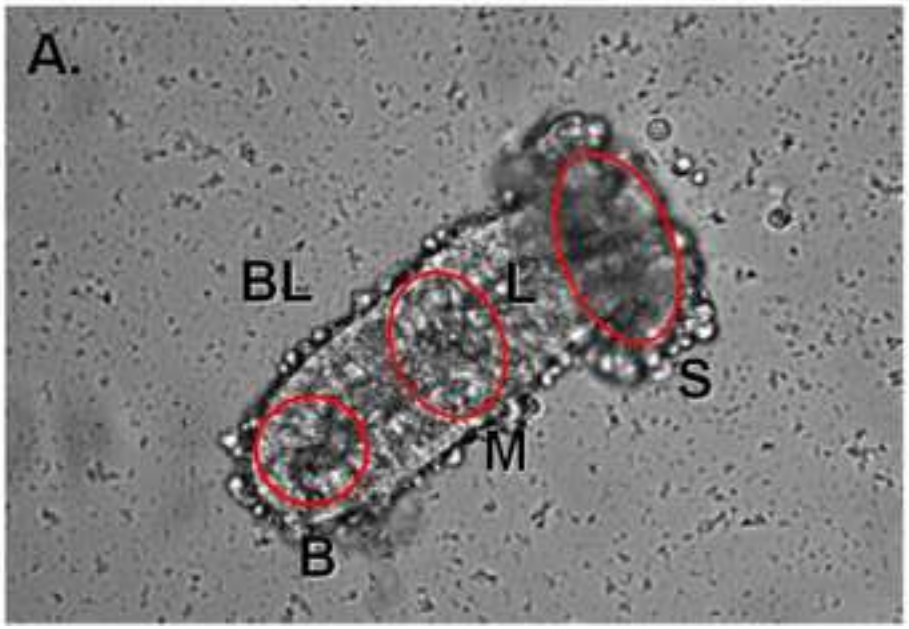

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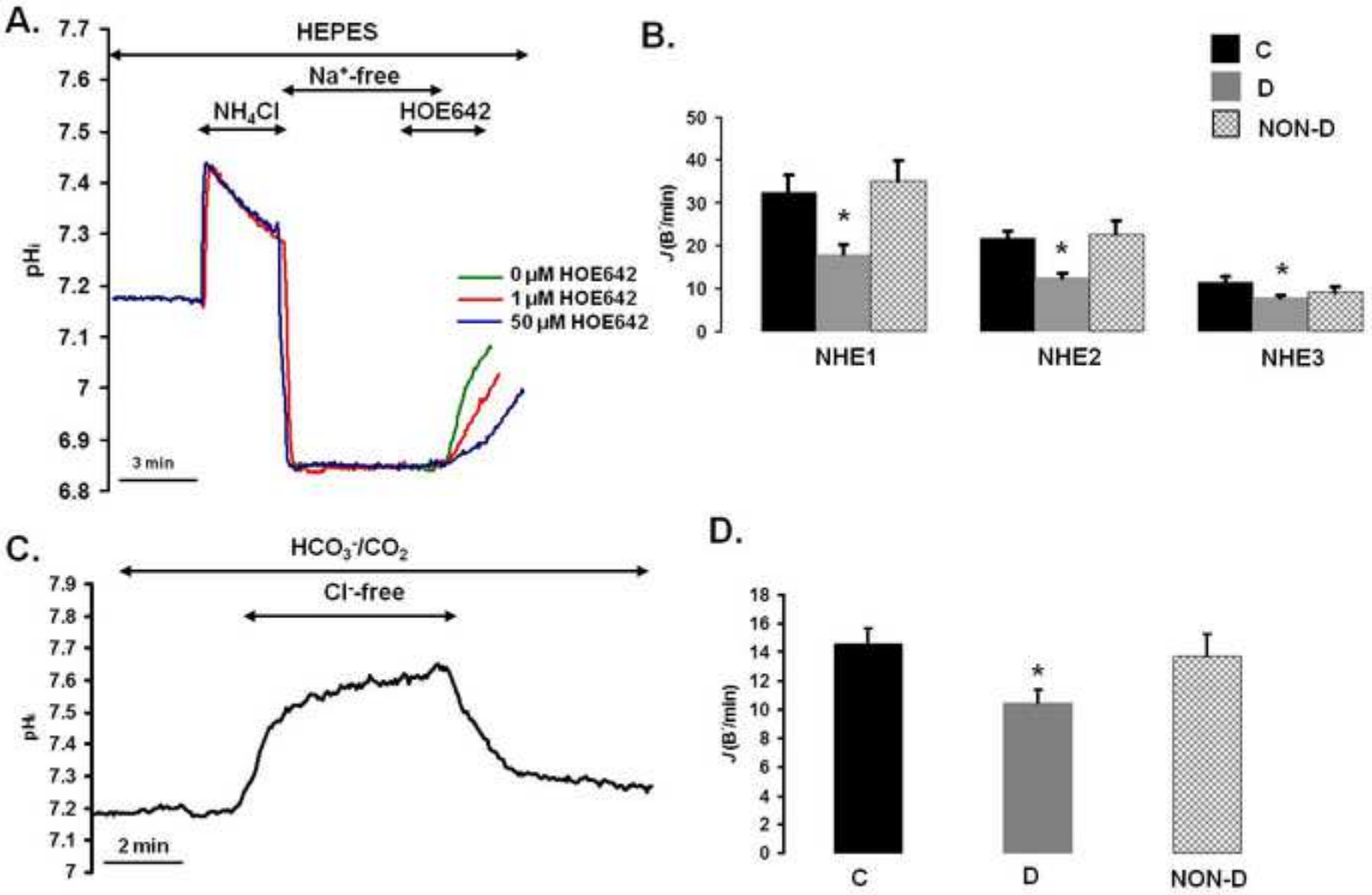

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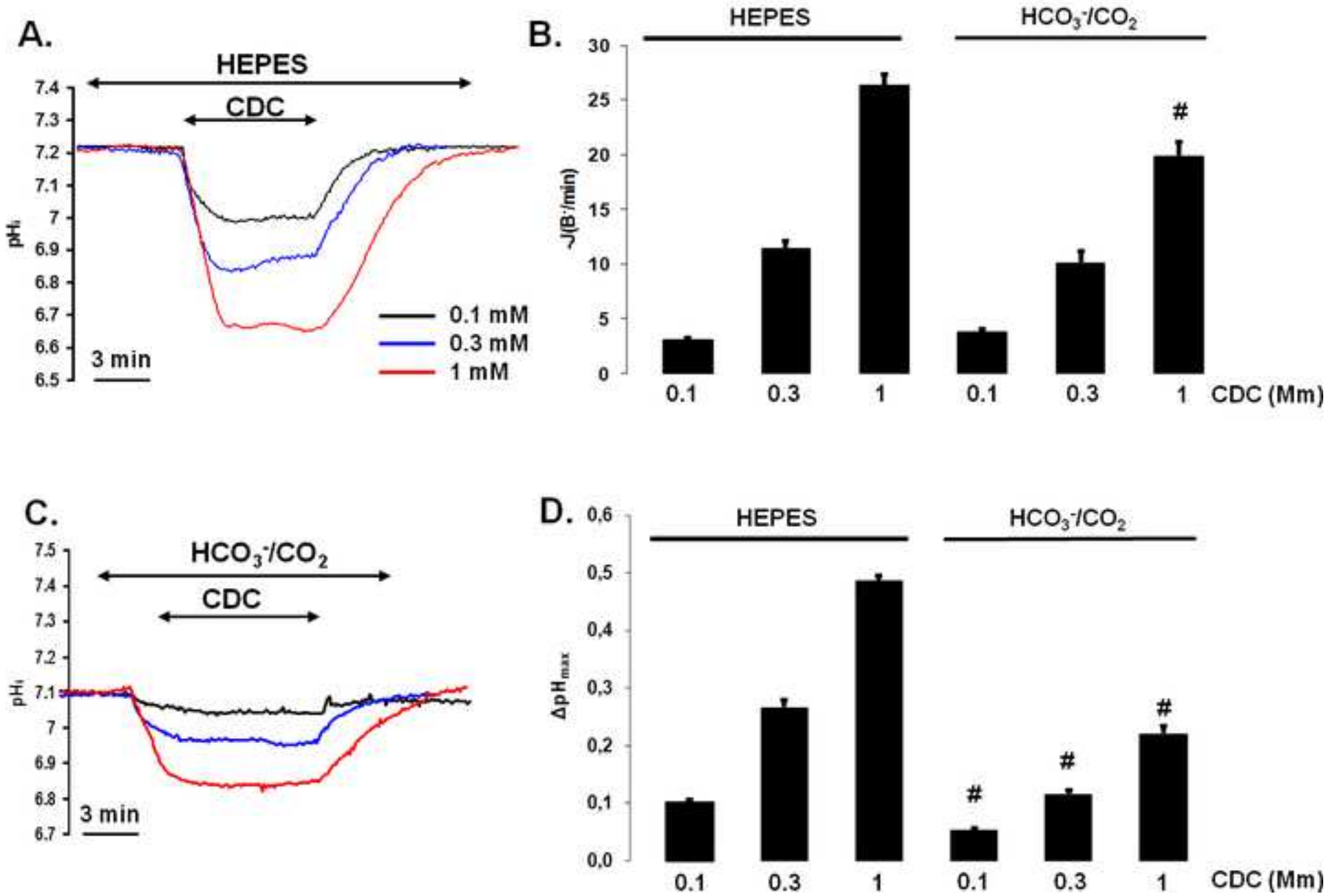

Figure4

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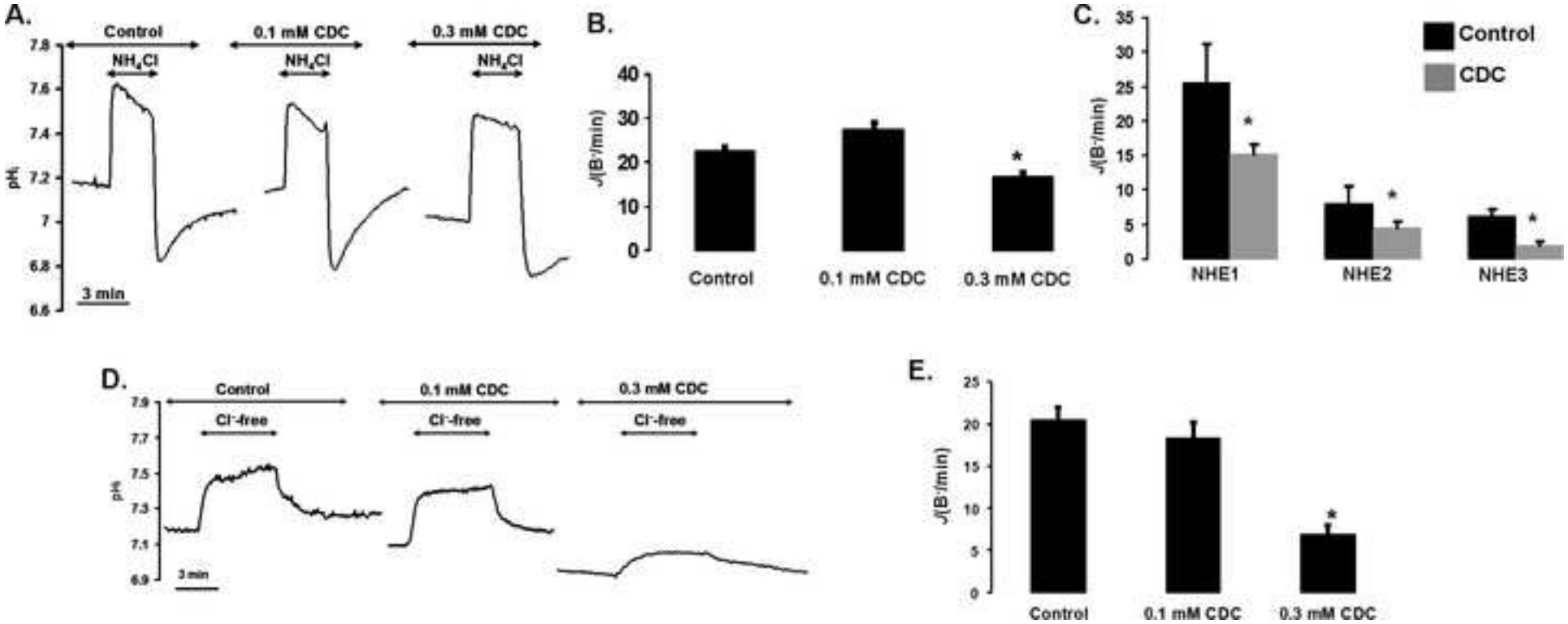

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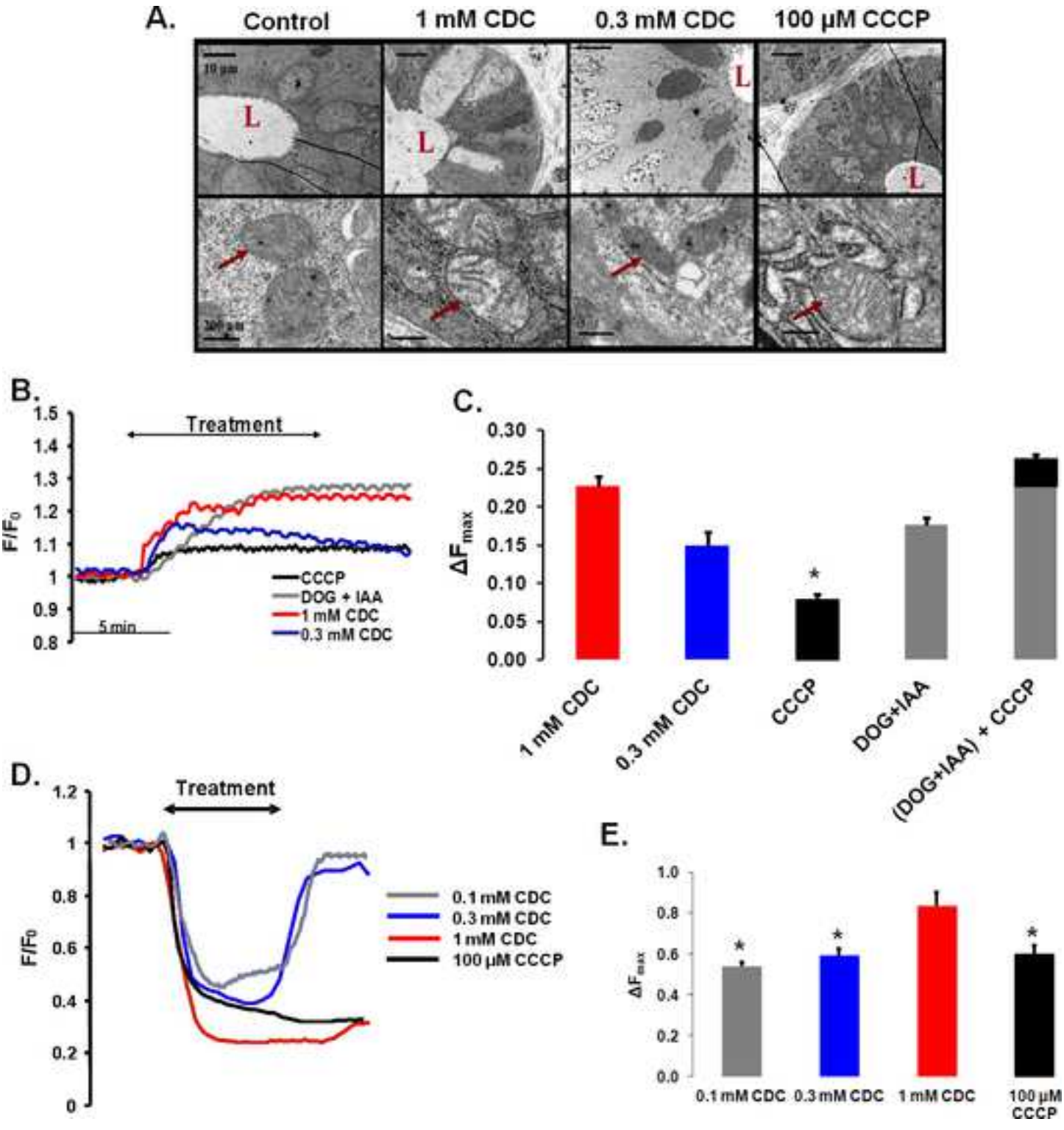

Figure6
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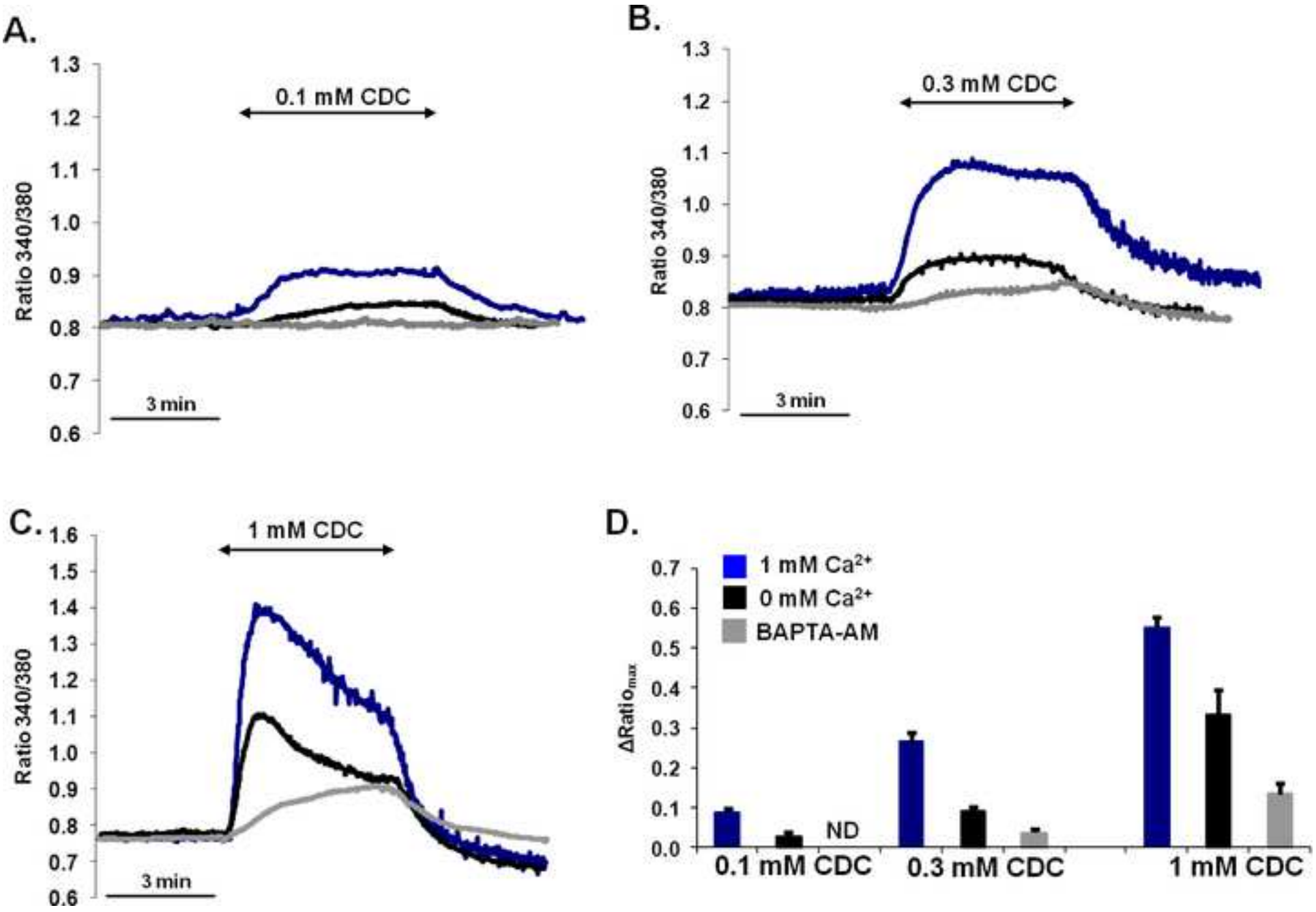

Figure7
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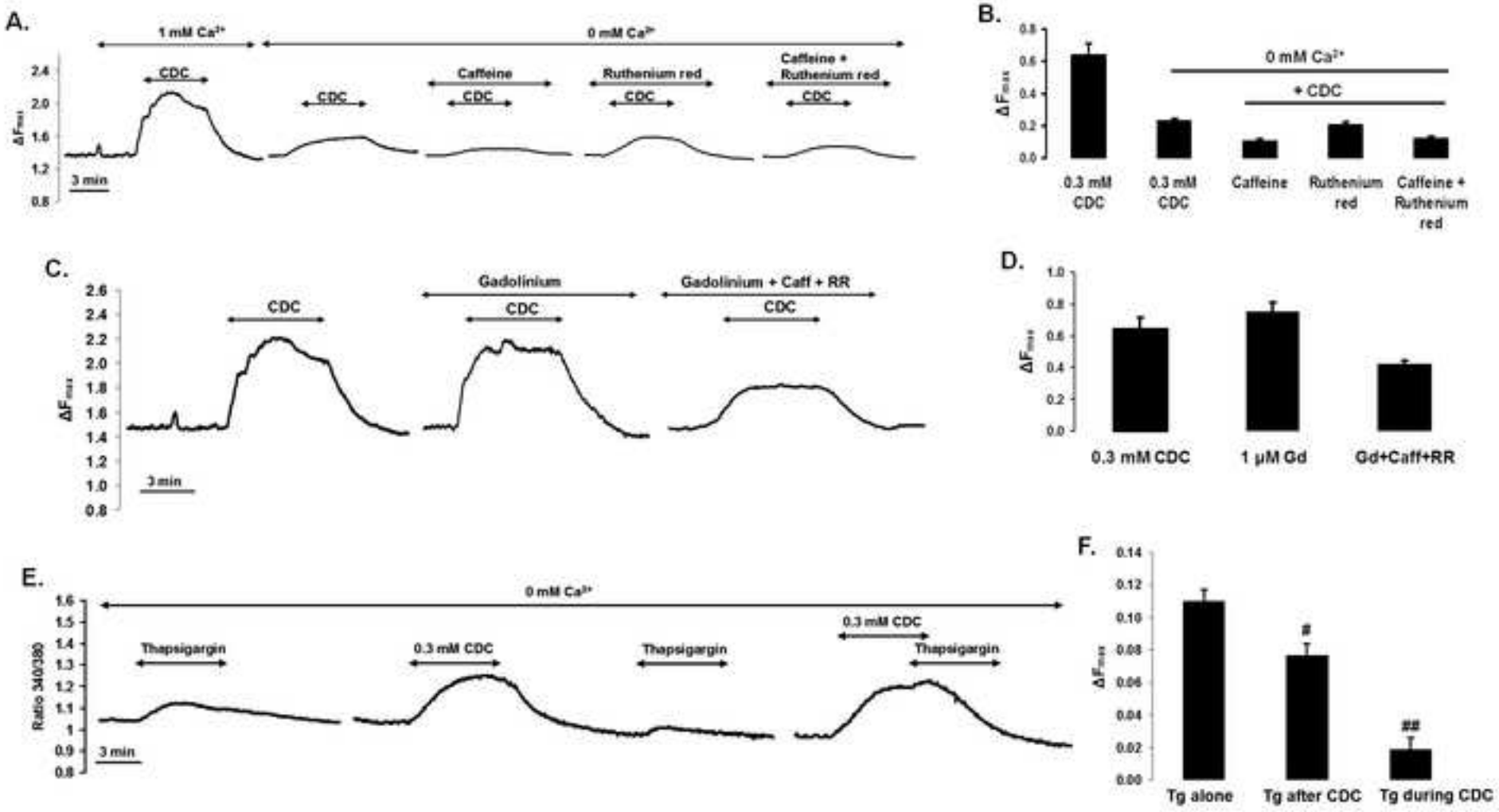

Figure8
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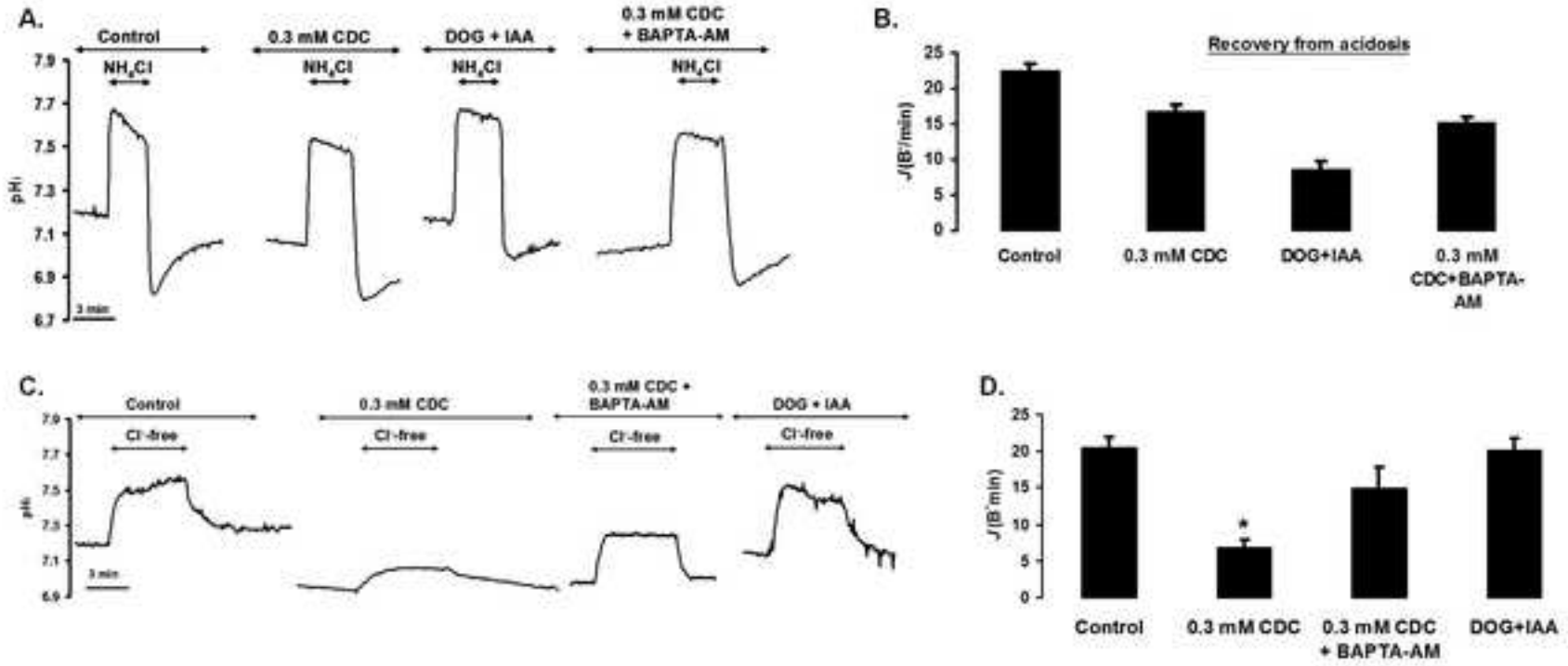

Table1

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	Standard HEPES	Standard HCO_3^-	Cl^--free HCO_3^-	Ca^{2+}-free HEPES
NaCl	130	115		132
KCl	5	5		5
$MgCl_2$	1	1		1
$CaCl_2$	1	1		
Na-HEPES	10			10
Glucose	10	10	10	10
$NaHCO_3$		25	25	
Na-gluconate			115	
Mg-gluconate			1	
Ca-gluconate			6	
K_2-sulfate			2.5	
EGTA				0.1