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pH Regulation and Bicarbonate Transport of Isolated Porcine Submucosal Glands

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Summary

We have previously demonstrated that the airway serous cell line Calu-3 employs a number of pH regulatory mechanisms required for bicarbonate secretion by these cells [1]. The aim of the present study was to investigate the pH regulatory mechanisms of serous cells of freshly isolated submucosal glands (SMG). Porcine SMG were dissected out of pig tracheas obtained from a local slaughterhouse. Single glands were transferred into the chamber of an inverted microscope, immobilized by two holding pipettes and the serous cells loaded with the fluorescent pH probe 2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein (BCECF). Fluorescence was monitored from small areas consisting of up to 20 cells. The fluorescence ratio of the emission after excitation at 488 nm and 436 nm respectively was used to estimate cytosolic pH (pH_i). Resting pH_i of SMG cells in the absence of HCO_3^-/CO_2 was 7.1±0.16 (n=24). Addition of a solution buffered with HCO_3^-/CO_2 to the bath transiently acidified the cells by 0.18±0.03 (n=18). pH_i rapidly recovered to a slightly more alkaline value than baseline pH_i. Removal of the HCO_3^-/CO_2 buffer strongly alkalinized SMG cells by 0.2±0.03 (n=18). To challenge pH regulatory mechanisms we exposed the cells to 20 mmol/L NH_4^+ in the absence and presence of HCO_3^-/CO_2. In both cases we observed a rapid increase in pH_i followed by a slight recovery. Washout of NH_4^+ strongly acidified the cells. Realkalinization of pH_i could only be observed in the presence of Na^+. This effect was inhibited by the addition of the specific Na^+/H^+ exchanger isoform 1 (NHE1) blocker 3-methylsulfonyl-4-piperidinobenzoyl guanidine hydrochloride (HOE 694, 10–100 µmol/L) with an halfmaximal inhibitory concentration (IC_{50}) of approximately 20 µmol/L. Full recovery of pH_i in the presence of HOE 694 was observed when the cells were bathed in HCO_3^-/CO_2 solution. Addition of forskolin (5 µmol/L) in the presence of HCO_3^-/CO_2 did not significantly alter pH_i or change pH_i recovery after acid loading. We conclude that SMG cells possess both HCO_3^- dependent and HCO_3^- independent pH_i; regulatory mechanisms that require the presence of extracellular Na^+. Further studies are required to understand whether bicarbonate is only transported to regulate pH_i or whether this transport determines the overall secretory capacity of SMG serous cells.

Airway submucosal glands (SMG) produce serous and mucous secretions that contribute to the composition of the airway surface liquid [2]. The number and morphology of these glands varies from species to species. SMG are numerous in humans, cats and pigs while they can hardly be found in rodents. Human and

porcine SMG consist of four distinctive subunits, namely a ciliated duct, emptying into the airway lumen, a nonciliated collecting duct, mucus tubules and finally the serous tubules. Myoepithelial cells are believed to line the tubules possibly facilitating the propagation of the gland secretions into the airways. Immunohistochemical studies have demonstrated a high expression level of the cystic fibrosis transmembrane regulator (CFTR) protein in the serous cells of SMG [3]. In fact, one of the striking observations in patients with cystic fibrosis are SMG ducts plugged with inspissated mucus which is accompanied by a hyperplasia of the serous tubules [4]. Due to the large number of glands in pig trachea and their high resemblance of human glands the pig has long been favored to study the properties of the bronchial mucous membrane [5]. The pioneering work of Ballard and coworkers [6] has demonstrated the importance of SMG for the fluid and electrolyte secretion of porcine distal airways. Recent experiments on Calu-3 cells, a human cell line of serous cell origin [7, 8], have indicated that serous cells also secrete bicarbonate ions upon stimulation with agonists increasing cAMP. A companion report [9] in this issue emphasizes the functional importance of apical CFTR activity and basolateral HCO_3^- uptake mechanisms for the secretion of Calu-3 cells. Ballard *et al.* have recently demonstrated the impact of CFTR function on bicarbonate transport in isolated pig bronchi [10]. Secretion of bicarbonate requires the concerted action of pH regulatory mechanisms to avoid pH excesses due to changes in the cytosolic buffer capacity. Aim of the present study was to investigate the H^+ and HCO_3^- transporters involved in the pH_i-regulation of porcine SMG.

Pig tracheas were obtained from the local slaughterhouse and immediately transferred into cold HCO_3^-/CO_2 buffered solution. Tracheas could be stored for up to 3 days without a significant loss in gland viability. For the preparation of glands, mucosal sheets were carefully removed from the cartilage and placed under a dissection microscope facing the muscular layer upwards. The latter was gently dissected apart giving access to the mucosal and submucosal layers. Glands were identified at 20-100 x magnification under dark field optics (Stemi, Zeiss, Oberkochen, Germany). Single glands were dissected using sharpened forceps. Isolated glands were transferred into a bath chamber on the stage of an inverted microscope (Axiovert 10, Zeiss, Oberkochen, Germany) and immobilized using two suction pipettes. For the experiments described here only serous portions of the glands were used. Figure 1 depicts a cluster of serous tubules shortly after dissection.

The glands were incubated at room temperature with the pH sensitive fluorophore BCECF-AM (5 µmol/L, Molecular Probes, Eugene, OR, USA) dissolved in phosphate buffered Ringers solution (PBR). After 20-40 minutes incubation excess dye was removed and the bath chamber was continuously perfused at a rate of 10 mL/min ensuring a bath exchange of

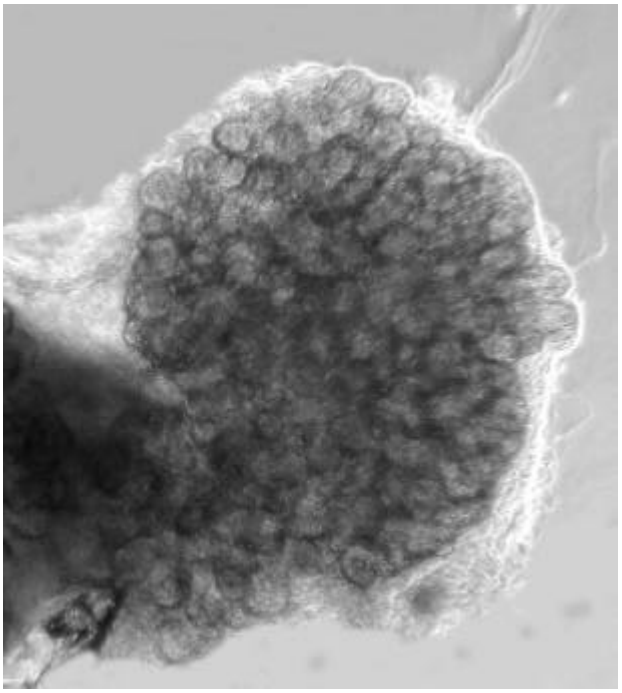

Figure 1. Differential interference contrast (DIC) image of dissected porcine SMG at 50x magnification. The cluster of serous tubules is held in place from the left by a glass capillary.

approximately 1 Hz. Measurement of pH_i was performed using a ratiometric technique as described earlier [11]. Briefly, dye was excited using the light of a Xenon lamp (Zeiss, Oberkochen, Germany) passing through a rotating filter wheel (Physiologisches Institut Freiburg, Freiburg Germany) at 436 nm and 488 nm filter bandwith respectively. Emitted fluorescence of 10-20 cells was long-pass filtered and collected using a photodetector (Hamamatsu, Tokyo, Japan). Fluorescence intensity was recorded with an analog-digital (AD) interface build into a personal computer. Custom made software allowed for online analysis and storage of the recorded intensities. Calibrations were performed using the K^+/H^+ exchanger nigericin in KCl solutions adjusted to the respective pH as published previously [12]. Data are given as absolute pH values or as rate of pH change (ΔpH_i/min).

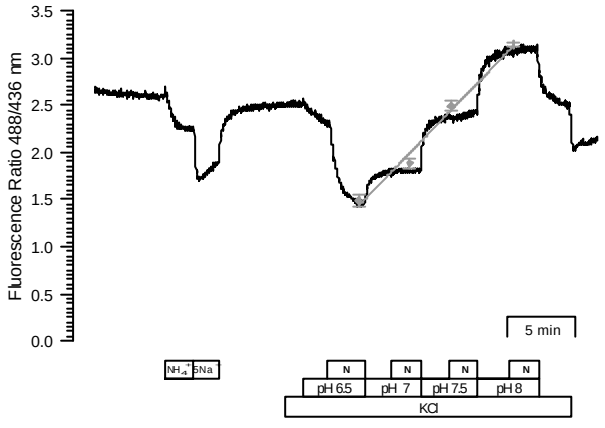

Figure 2. Original recording of an experiment. The BCECF fluorescence ratio is plotted against time. Application of 20 mmol/L NH_4Cl only slightly alkalinized the cytosol followed by an acidification in the presence of the buffer. A strong acidification is observed once NH_4Cl is removed and the bath solution is switched to low Na^+ (5 mmol/L Na^+, $5Na^+$). The second half of the trace depicts a typical calibration procedure. Bath solution is changed to a solution containing 140 mmol/L K^+ (KCl) to clamp membrane potential to nominally 0 mV. Under this condition extracellular pH (pH_e) is changed in the range from pH 6.5–8. Note that changing the pH_e *per se* does not exert a strong effect on pH_i. Only after application of the K^+/H^+ exchanger nigericin (N, 1 µmol/L) pH_i did approach pH_e. Superimposed on the original recording are the fitted results of 5 similar experiments. The fit equation was then used to translate ratio values into absolute pH values.

Results from a typical calibration experiment are shown in Figure 2. It can easily be recognized that baseline pH of the cells in this very experiment was around 7.5. However taken all experiments together we estimated a resting pH_i of porcine SMG after incubation of 7.1±0.16 (n=24).

Also shown in Figure 2 is the effect of the addition of 20 mmol/L NH_4Cl to the bath solution. NH_4Cl rapidly alkalinized the SMG by 0.1±0.1. Cells usually acidified in the presence of NH_4^+ to a value generally lower than baseline indicating a high rate of NH_4^+ transport into the cytosol. Removal of extracellular NH_4^+ and replacement of the bulk of Na^+ in the bath solution by N-methyl D-glucamine ($NMDG^+$) ($5Na^+$) strongly acidified the cells. Little to no pH_i recovery was observed in the nominal absence of Na^+. Readdition of Na^+ to the bath realkalinized the cells at a rate of 0.36/min.

These observations indicated the presence of a Na^+ dependent H^+ exporter. Most cells possess a Na^+/H^+ exchanger (NHE) isoform to extrude excess H^+ ions. The most ubiquitously expressed isoform is NHE1. To pharmacologically characterize the NHE present in porcine SMG we used the compound HOE 694, a selective blocker of NHE1 [13]. Addition of HOE 694 resulted in a slight acidification of baseline pH. A more dramatic effect of HOE 694 was observed when the compound was given during acid load experiments using the above described $NH_4^+/5Na^+$ protocol. A representative recording is shown in Figure 3. HOE 694 given at concentrations of 10 µmol/L and 50 µmol/L respectively reduced pH_i recovery after acid loading in a concentration dependent manner. From a total of five experiments we estimated an apparent IC 50 of 20-30 µmol/L. At concentrations lower than 100 µmol/L the effect of HOE 694 was fully reversible.

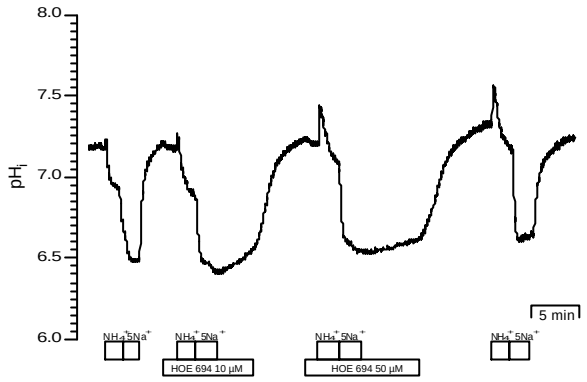

Figure 3. Effect of the NHE1 blocker HOE 694 given at 10 and 50 µmol/L as indicated. Application of the compound at a concentration of 50 µmol/L almost completely blocked pH_i recovery.

The concentration response relationship established here is in good agreement with data published previously on other epithelial and non-epithelial cells [12, 14] and suggests that NHE1 is the important pH_i regulator for porcine SMG in the absence of HCO_3^-/CO_2. To this end we can not rule out the possibility that other NHE isoforms (NHE2, NHE3) play a role in maintaining pH_i. Further experiments are required to clarify this point. Since we were also interested in the HCO_3^- transport properties of these cells we performed experiments during which the phosphate buffered bath solution was replaced by a solution containing 25 mmol/L HCO_3^- equilibrated with 5% CO_2 to maintain a pH of 7.4. A representative recording is depicted in Figure 4. HCO_3^-/CO_2 induced a rapid decrease in pH_i followed by a realkalinization slightly above the previous baseline pH_i. If we performed the $NH_4^+/5Na^+$ pulse protocol in the presence of HCO_3^- / CO_2 the rate of pH_i recovery was about 23% faster as compared to control (PBR) solution. Little to no pH_i recovery could be observed when the Na^+ in the bath solution was replaced by the membrane impermeable cation $NMDG^+$ again indicative for a Na^+ dependency of this process. To dissect the effects of HCO_3^- and H^+ transporters we added HOE 694 in the presence of HCO_3^-/CO_2. As evident in Figure 4, the NHE1 blocker failed to inhibit pH_i recovery in the presence of the HCO_3^- buffer.

We therefore conclude that porcine submucosal cells possess a HOE 694 sensitive Na^+/H^+ exchanger and a HOE 694 insensitive, Na^+ dependent HCO_3^- importer. A number of electrogenic and electroneutral Na^+ dependent HCO_3^- transporters (NBC) have been identified recently [15, 16, 17, 18]. Two recently cloned electrogenic NBC isoforms raised our attention since they were highly expressed in kidney, pancreas and lung. RT-PCR studies demonstrated mRNA for both isoforms in the serous cell line Calu-3. Antibodies against either isoform bind to human, canine, and porcine SMGs. It therefore seems prudent to conclude that NBC contributes to the HCO_3^- import and thus supports the HCO_3^- secretion by these cells.

The data presented here correspond nicely with data obtained from the serous cell line Calu-3 [1]. However, we have previously reported that forskolin, an activator of the adenylyl cyclase *per se* leads to slight acidification of Calu-3 cells when HCO_3^-/CO_2 is present. For this effect we offer two explanations: 1) The cAMP induced activation of CFTR facilitates HCO_3^- extrusion in a direct or indirect fashion. Recent

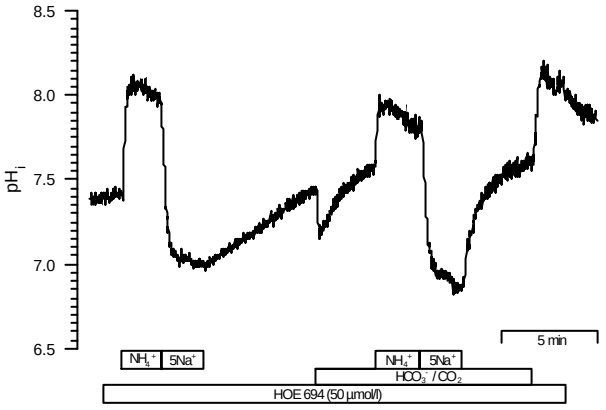

Figure 4. Effect of HOE 694 in the absence and presence of HCO_3^-/CO_2 . The application of the HCO_3^- buffer itself induced a transient acidification presumably due to the high permeability of CO_2. *Vice versa*, removal of HCO_3^-/CO_2 strongly alkalinized the cells. The recovery from the alkaline load had a much slower time course than the recovery from acid load.

reports have emphasized the fact that activated CFTR increases the rate of Cl^-/HCO_3^- exchangers (AE) [19, 20]. AE have been detected in Calu-3 cells [21] and could thus permit HCO_3^- exit from the cytosol. However we favor the idea that CFTR itself is the HCO_3^- conductor. 2) The acidification could be due to inhibition of NHE. To this end we would rather discard this explanation since the acidification induced by forskolin was only observed in the presence of HCO_3^-/CO_2 and also present during inhibition of NHE1 with HOE 694. We have also reported that forskolin increases the pH_i recovery rate after acid load. This effect might be explained by the fact that the forskolin-induced depolarization of the membrane potential would possibly increase the driving force for an electrogenic HCO_3^- importer with a stoechiometry of 2 HCO_3^- : 1 Na^+ or 3 HCO_3^- : 1 Na^+, respectively. Prompted by our previous results using Calu-3 cells we investigated the effect of forskolin on porcine SMGs. To this end we have not been able to observe a significant effect of forskolin on resting pH_i. We could also not detect any significant change in pH_i recovery after acid load, an effect that would suggest a similar action of forskolin in SMG as detected in Calu-3 cells. The seeming inability of forskolin to alter pH_i does not generally imply that agonists raising cAMP are without effects on HCO_3^- and fluid secretion of porcine SMG at all. One possible explanation would be that the serous SMG cells are already prestimulated be it through the process of gland preparation or due to a constitutive activation of the cAMP pathway. It is also possible that these cells closely balance HCO_3^- import and export during secretion thus rendering it impossible for us to detect changes in cytosolic buffer concentration. Further studies aimed to assess the regulation of gland function will certainly shed more light on this issue.

Key words Cytophotometry; Ion Transport; Sodium-Hydrogen Antiporter

Abbreviations AE: anion exchanger; BCECF: 2',7'-bis-(2-carboxyethyl)-5,6-carboxyfluorescein; CFTR: cystic fibrosis transmembrane conductance regulator; DIC: differential interference contrast; $IC_{50:}$ halfmaximal inhibitory concentration; HOE 694: 3-methylsulfonyl-4-piperidinobenzoyl guanidine hydrochloride; NBC: Na^+ bicarbonate cotransporter; NHE Na^+/H^+ exchanger; NMDG: N-methyl D-glucamine; PBR: phosphate buffered Ringers saline; pH_i: cytosolic pH; SMG: submucosal glands

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