Factors Affecting the Microclimate pH of the Rat Jejunum in Ringer Bicarbonate Buffer

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General characteristics of the microclimate layer on the mucosal surface of the rat jejunum incubated in bicarbonate buffers were investigated in vitro using pH sensitive flat membrane microelectrode. Jejunal surface microclimate (JSM) pH changed from 7.30±0.03 to 5.83±0.04 when the incubation buffer pH decreased from 8.03±0.02 to 6.12±0.01. Treatment of the mucosal side with mucolytic substances l-cysteine (1% (wt/v)) and 1,4-dithio-1,1-threitol (2 mM) significantly (p<0.01) increased JSM pH. Respiratory chain inhibitor, sodium azide (10 mM) also significantly (p<0.05) increased JSM pH. D-Glucose (10 mM) at the mucosal side markedly (p<0.05) decreased JSM pH, which was attenuated by Na+/H+ exchange inhibitor, amiloride (1 mM). Amiloride had no effect on JSM pH when D-glucose was not present at the mucosal side. In contrast to previous observations using bicarbonate free incubation buffers, we have demonstrated that JSM pH is not a constant value, but is dependent on pH of the incubation buffer. Additionally, Na+/H+ exchanger does not contribute to acidic properties of JSM, when there is no D-glucose in the bicarbonate incubation buffer at the mucosal side of the tissue. In conclusion, we suggest that the bicarbonate buffers which are more close to in vivo situation than bicarbonate free buffers should be preferable incubation media when examining JSM.

Key words microclimate pH; small intestine; mucus; sodium/proton exchanger; peptide transporter

Small intestine is the major site of nutrient and drug absorption. There are many different transporters in the brush-border membrane i.e. H+/oligopeptide cotransporter (PepT1), H+/lactic acid cotransporter, H+/nicotinic acid cotransporter and folate/OH- antiporter, which are dependent on the acidic microclimate pH on the intestinal surface.1) The microclimate pH has therefore an evident physiological role in absorption and secretion of many nutrients and drugs. The general characteristics of the small intestinal acidic microclimate layer (i.e. the role of mucus and Na+/H+ exchanger, the influence of bulk-phase pH, the effect of D-glucose and metabolic inhibitors) have been well studied in vitro2-5) and in vivo6,7) using incubation buffers not containing bicarbonate. On the other hand, there is only limited literature data about the factors, which are important for maintaining acidic pH on the small intestinal surface in bicarbonate buffers, although the existence of acidic microclimate was first demonstrated in vitro using rat small intestine incubated in bicarbonate buffers.8,9)

Bicarbonate, which originates from the bile (up to 60—70 mM) and pancreatic secretions (70—130 mM), is an important anion in the intestinal juice and plays a fundamental role in regulating buffer pH in the intestinal lumen.10,11) Therefore, one can expect different characteristics of the small intestinal acidic microclimate layer in bicarbonate buffers compared to those observed in incubation buffers without bicarbonate.

In the present study we examined the characteristics of rat jejunal surface microclimate (JSM) in bicarbonate incubation buffers and compared the results with already mentioned literature data, obtained in non-bicarbonate buffers.

MATERIALS AND METHODS

Chemicals Amiloride hydrochloride and l-cysteine were from Sigma Aldrich Chemie (Deisenhofen, Germany). 1,4-Dithio-1,1-threitol (DTT) was purchased from Fluka (Deisenhofen, Germany). Sodium azide was obtained from Riedel-de Haen AG Seelze, (Hanover, Germany). All chemicals used in this study were of analytical grade.

Tissue Preparation The experiments conform to the European convention for the protection of vertebrate animals used for Experimental and other Scientific Purposes (Council of Europe No 123, Strasbourg 1986).

Male Wistar rats (250—320 g), which had been starved for 18 h before the experiments, were stunned by a blow on the head and subsequently killed by exsanguination. Small intestine was immediately excised and placed into the ice-cold bubbled (carbogen, 95:5 O2/CO2) 10 mM solution of D-glucose in standard Ringer buffer containing (mM): 140.6 Na+, 5 K+, 1.2 Ca2+, 1.2 Mg2+, 121.8 Cl-, 25 HCO3-, 0.4 HPO42-, 1.6 H2PO4-. The tissue was rinsed with ice-cold standard Ringer buffer to remove luminal content. Jejunum 25—60 cm distally from the piloric sphincter was used in the experiments. The 3 cm long intestinal segments were opened along the mesenteric border, stretched onto a special insert and placed between the two side-by-side diffusion chambers with the exposed tissue area of 1 cm² and volume 2.5 ml in each compartment (Physiologic Instruments, San Diego, CA, U.S.A.). During the incubation period the intestinal segments were bathed on both sides with the appropriate Ringer buffer solution supplemented with 10 mM D-glucose at the serosal and 10 mM mannitol at the mucosal side, except in the experiments with D-glucose, where 10 mM D-glucose was present on both sides. The bathing solution was continuously gassed with carbogen and kept at 37±0.3 °C. Different pH values (the same pH was at the mucosal and serosal side) of the Ringer buffer were achieved by changing the amount of HCO3-, H2PO4-, HPO42- and Pco2 (14 mM (for pH 6 and 8) and 35 mM (for pH 7.5, 7 and 6.5); this corresponds to 2% and 5% of CO2 in the carbogen mixture, respectively). The osmolarity of all buffers was 307 mOsm/kg. The concentration of HCO3 was calculated using the Henderson-Hasselbalch equation. The pH and CO2 solubility were 6.115
and 0.0306 [mV/mmHg], respectively.

The tissue viability and integrity were checked by monitoring transepithelial potential difference (PD), short circuit current ($I_{sc}$) and tissue electrical resistance (TER) by a multichannel voltage-current clamp (model VCC MC6, Physiologic Instruments). TER was determined according to the Ohm’s law. $I_{sc}$ and TER were corrected for fluid resistance before mounting the tissue in the diffusion chamber system. Typically, PD and $I_{sc}$ values were between $1-2$ mV and $30-90 \mu A/cm^2$ for experiments without D-glucose at the mucosal side, or $2-5$ mV and $100-200 \mu A/cm^2$ for experiments with D-glucose at the mucosal side. TER values were between $20-35 \Omega \cdot cm^2$.

**Determination of Jejunal Surface Microclimate (JSM)**

**pH**

pH measurements were performed with a flat membrane pH microelectrode MI-406 with a tip diameter $1.5\ mm$ and response time less than $15\ s$ (Microelectrodes, Inc., Bedford, NH, U.S.A.). The reference electrode, MI-402 (Microelectrodes, Inc.) was dipped into the same incubation medium as the pH microelectrode. Both electrodes were connected to a digital pH meter (model MA-5736, Iskra, Ljubljana, Slovenia). After incubation in side-by-side diffusion chambers ($50-60\ min$) under the appropriate experimental conditions [different pH, DTT (2 mM, mucosal side), l-cysteine (1% (wt/v), mucosal side), amiloride (1 mM, mucosal side), D-glucose (10 mM, both sides) or sodium azide (10 mM, both sides)] the insert with the mounted tissue was immediately placed into a thermostated bath ($37^\circ C$, gassed with carbogen) with the mucosal surface upwards. The flow ($4\ mL/min$) through the bath was maintained with the same solution as used at the mucosal side of the tissue in the diffusion chambers. The pH measurements of JSM began with pH determination of the bathing solution. The pH microelectrode was then advanced to the epithelial surface by a micromanipulator until the tip of the electrode touched the mucus layer; this was noticed as a change in pH. Afterwards the electrode was lowered down for additional $0.5\ mm$. Stable pH reading was achieved within $3-4\ min$. After pH determinations the insert with the mounted tissue was placed again into the diffusion chambers for evaluating the tissue viability. The procedure of the pH determination did not affect the tissue viability; this was checked by comparison of the tissue electrical parameters (PD, $I_{sc}$ and TER) before and after pH measurements.

**Statistics**

Results are expressed as means±S.E.M. Two-group comparisons were analysed by unpaired two-tailed $t$-test or paired Student’s two-tailed $t$-test. In the case of unpaired $t$-test, $F$-test for variances was first applied. If the variances were equal, the standard Student’s $t$-test was performed otherwise Behrens–Fisher test was used.

**RESULTS**

As shown in Table 1, JSM pH is significantly lower than the incubation buffer pH, demonstrating the acidic properties of JSM. The biggest ($\Delta pH=0.73$ or JSM [H$^+$]/buffer [H$^+$]=5.37) and the smallest difference ($\Delta pH=0.29$ or JSM [H$^+$]/buffer [H$^+$]=1.95) between the buffer and JSM pH was observed at buffer with the highest (pH=8.03) and the lowest pH (pH=6.12) values, respectively. The results in Table 1 also show that JSM pH was dependent on the changes in the buffer pH and changed in the same direction as the buffer pH, although not for the same amount; i.e. when the buffer pH was lowered for 1.91 the JSM pH decreased only for 1.47.

When the mucosal side of the intestine, incubated in the buffer of pH 7.50±0.01 ($n=12$), was treated with mucolytic (disulphide bond reducing agent) compound, l-cysteine (1% (wt/v)) JSM pH increased from 7.13±0.04 ($n=6$) to 7.28±0.02 ($n=6$), ($p<0.01$, paired $t$-test). Similarly, the treatment of the mucosal side with another disulphide bond reducing agent, DTT (2 mM) also increased JSM pH from 7.08±0.02 ($n=6$) to 7.21±0.02 ($n=6$), ($p<0.01$, paired $t$-test). These results demonstrate the importance of intact mucus layer for maintaining low pH of JSM.

The results presented in Fig. 1 show that the treatment of the mucosal side with Na$^+/H^+$ exchange inhibitor, amiloride (1 mM) had no effect on JSM pH when D-glucose was absent from the mucosal side. The addition of D-glucose (10 mM) to the mucosal side of the intestine markedly decreased JSM pH (Fig. 1). This decrease of JSM pH was attenuated by concomitant administration of amiloride (1 mM) to the mucosal side (Fig. 1).

The addition of respiratory chain inhibitor, sodium azide (10 mM) to the mucosal and serosal side of the intestine significantly increased JSM pH (Fig. 2). The effect of ATP-depletion could also be noticed as the abolishment of PD and $I_{sc}$ in 10 min after the addition of sodium azide to the incubation buffer. This shows that the intact intracellular metabolism plays an important role in creating acidic microclimate on the mucosal surface of the rat jejunum in vitro.

**DISCUSSION**

Previous pH measurements of the jejunal surface microclimate (JSM) have been performed mainly in the non bicarbonate buffered incubation media, although the bicarbonate anion is an important ion in the intestinal lumen in vivo. The main difficulty in experiments using bicarbonate buffers is that the pH of the buffer becomes alkaline if it is not continuously gassed with CO$_2$. In this case, the inconstancy of bicarbonate buffer pH could cause difficulties at JSM pH determinations, especially if longer period of tissue incubation is required. Therefore, more stable bicarbonate free incubation buffers were used in such experiments.

In our study, the tissue in side-by-side diffusion cells was incubated in bicarbonate buffer solution, which was continuously gassed with carbogen (CO$_2$/O$_2$, 5/95 or 2/98). This system maintained constant pH of the bicarbonate buffer during the incubation period and consequently allowed the use of more
physiological bicarbonate buffers instead of bicarbonate free media at JSM pH measurements. It is also important to stress that the tissue electrical parameters were measured before and after pH determinations providing us with the necessary data about the tissue viability.

Our results clearly demonstrate the existence of an acidic microclimate layer on the mucosal surface of the rat jejunum incubated in the bicarbonate buffers. JSM pH is dependent on the incubation buffer pH (Table 1). This is not in agreement with the observations in the different non bicarbonate buffered media (phosphate buffer, citrate–phosphate–borate buffer, TES buffer) using different types of pH microelectrodes in vitro\(^2,3\) and in situ\(^6,7\). Their studies demonstrated only negligible influence of the incubation buffer pH (between pH 6 and pH 8) on the JSM pH. Possible explanation for this discrepancy is Cl\(^-\)/HCO\(_3\)\(^-\) exchange property of jejunal mucus.\(^12\) Carbonic anhydrase (CA) type IV is a membrane-associated isoenzyme localised at the apical membrane of the jejunal enterocytes.\(^13\) This enzyme catalyzes the hydration of CO\(_2\), producing HCO\(_3\)\(^-\) and H\(^+\) in the mucus layer. Bicarbonate is then exchanged for Cl\(^-\) from the incubation buffer, while the remaining proton contributes to the acidic properties of JSM. With decreasing pH of the incubation buffer the concentration of bicarbonate in the buffer decreases (from 35 mM at pH 8.03 to 0.4 mM at pH 6.12), which facilitates HCO\(_3\)\(^-\)/Cl\(^-\) exchange between mucus and incubation buffer. Consequently, lower pH of JSM can be generated. Similar to our observations, rat JSM pH in vivo was also dependent on pH of the bicarbonate incubation buffer.\(^14\)

Additional factors which might influence the observed relationship between incubation buffer pH and JSM pH are the time of the tissue incubation\(^9\) and stirring conditions.\(^2\) These factors appear to be more important for JSM studies in the bicarbonate incubation buffers, because anion exchange activity of jejunal mucus is time dependent and reaches its maximum activity in approximately 20—30 min after the exposure to the buffer.\(^12\) If the pH determinations of JSM are performed immediately (or very soon) after the exposure of the intestine to buffer containing bicarbonate, HCO\(_3\)\(^-\)/Cl\(^-\) exchange activity of jejunal mucus could not reach its max-

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Fig. 1. Effects of D-Glucose (10 mM, Mucosal Side) and Amiloride (1 mM, Mucosal Side) on the Jejunal Surface Microclimate (JSM) pH at Two Different pH of the Incubation Buffer

Results are means±S.E.M. Effect of D-glucose was evaluated by unpaired t-test, effect of amiloride was evaluated by paired t-test. *p<0.05; **p<0.001; n.s., not significant (p>0.05).

Fig. 2. Effect of Sodium Azide (10 mM, Mucosal and Serosal Side) on the Jejunal Surface Microclimate (JSM) pH at Three Different pH of the Incubation Buffer

Results are means±S.E.M. *p<0.05, **p<0.01, ***p<0.001 (paired t-test).
mum. This can explain smaller (compared to our results) influence of bicarbonate buffer pH on the rat JSM pH in vitro observed by the others. 15)

Besides the ability to exchange anions with the luminal solution small intestinal mucus also retards H+ diffusion due to its viscoelastic properties. 12,16) This additionally contributes to maintenance of the acidic JSM pH. Compounds with thiol group, such as l-cysteine and DTT, disrupt disulphide linkages between glycoprotein subunits in the mucus structure, which leads to the reduction of viscoelastic properties of mucus. 17,18) Consequently, the ability of mucus to retard H+ diffusion is deteriorated, resulting in the increment of JSM pH. Similar effect of disulphide bond reducing agents on the rat JSM pH was observed also by the investigators using incubation buffers without bicarbonate in situ 3—5 and in vitro. 3—5

There are at least three isoforms (NHE1-basolateral, NHE2-apical and basolateral and NHE3-apical) of the Na+/H+ exchanger present in the small intestine. 19) The primary role of the apical Na+/H+ exchangers is optimisation of the small intestinal absorptive function, while basolateral Na+/H+ exchange is primarily a homeostatic mechanism controlling bulk cytosolic pH and cell volume. 20) Our results demonstrate that apical Na+/H+ exchange significantly contribute to acidic properties of JSM only when D-glucose was present in the incubation buffer at the mucosal side of the tissue (Fig. 1). This is in agreement with the observation that the absorption of D-glucose selectively activates NHE3. 21) After a meal, the intestinal lumen contains a mixed complement of substrates, such as di- and tripeptides, amino acids, vitamins and sugars. NHE3 activation by D-glucose transport provides high H+ gradient across the brush border membrane, which enables optimal absorption of di-, tripeptides and some vitamins (i.e. nicotinic acid) by H+-dependent transporters. This effect of D-glucose is of great importance for sufficient dietary proteins absorption, which are absorbed as di- and tripeptides by PepT1 rather than as free amino acids. 22) PepT1 also transports numerous peptide-mimetic therapeutic compounds including angiotensin converting enzyme inhibitors, β-lactam antibiotics and valacyclovir. 22—25) Therefore, D-glucose in the intestinal lumen might also enhance the absorption of peptide-mimetic drugs.

Similar influence of D-glucose on the rat JSM pH in vitro was observed by Lucas et al. 19 who used bicarbonate incubation buffer and also by the investigators who used bicarbonate free buffers. 3—5,15) It was also observed that the effect of D-glucose on JSM pH was partially inhibited by amiloride in bicarbonate free incubation medium. 30) In contrast to our observations (Fig. 1), studies using bicarbonate free incubation medium showed that amiloride (1 mM) increases JSM pH also in the absence of D-glucose at the mucosal side of the tissue. 4,5) This suggests that the incubation of the tissue in bicarbonate free buffers activates the apical Na+/H+ exchange, even if there is no D-glucose in the intestinal lumen. This could explain in general lower JSM pH values, obtained in in vitro experiments with bicarbonate free incubation buffers, 3—5 compared to our experiments.

ATP, released from the intracellular compartment, is believed to play a fundamental role in the regulation of mucus secretion from airway goblet cells via the apical P2Y2 receptors. 26) Similar mechanism of mucus secretion could exist also in the small intestine, since ATP can be released also from the intestinal epithelia. 27) This might explain the increment of JSM pH caused by ATP-depleter, sodium azide (Fig. 2), since the secretion of mucus is most probably deteriorated under the ATP-depleted conditions. The increase of JSM pH caused by metabolic inhibitors (dinitrophenol and iodoacetate) was observed also in the non-bicarbonate buffered incubation medium. 31)

In conclusion, we have demonstrated that some characteristics of JSM in bicarbonate incubation buffers are different from those determined previously in bicarbonate free buffers. 2—7) We have showed that JSM pH is not a constant value, but it is influenced by changes of the incubation buffer pH, and that the apical Na+/H+ exchange does not influence JSM pH when there is no D-glucose in the intestinal lumen. Therefore, we recommend that the bicarbonate buffers which are closer to in vivo situation than bicarbonate free media should be used when examining the intestinal physiology, especially when JSM is the main object of the investigation.

REFERENCES