J. Membrane Biol. 169, 111–122 (1999)

The Journal of
Membrane
Biology

Surface pH at the Basolateral Membrane of the Caecal Mucosa of Guinea Pig

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Received: 17 December 1998/Revised: 24 February 1999

Abstract. Since the major mechanisms responsible for regulation of intracellular pH of enterocytes are located in the basolateral membrane, respective effects may be expected on pH in the compartment near the basolateral membrane. A method was established to estimate the pH at the basolateral membrane (pH_b) of isolated caecal epithelia of guinea pig using pH-sensitive fluorescein attached to lectin (*lens culinaris*). In the presence of bicarbonate and a perfusion solution-pH of 7.4, pH_b was 7.70 ± 0.15. In the absence of bicarbonate or chloride as well as by inhibition of the basolateral Cl^--HCO_3^- exchange with H_2-DIDS, pH_b was reduced near to solution-pH. Inhibition of the basolateral Na^+-H^+ exchanger by adding a sodium- and bicarbonate-free, low-buffered solution increased pH_b. Decrease of pH of serosal perfusion solution to 6.4 provoked a similar decrease of pH_b to solution pH. Short-chain fatty acids (SCFA) added to the mucosal solution caused a slight decrease of pH_b. SCFA added to the serosal side alkalized pH_b. However, in the presence of bicarbonate pH_b returned quickly to the initial pH_b, and after removal of SCFA a transient acidification of pH_b was seen. These responses could not be inhibited by MIA or H_2-DIDS. We conclude that no constant pH-microclimate exists at the basolateral side. The regulation of the intracellular pH of enterocytes reflects pH_b. The slightly alkaline pH_b is due to the bicarbonate efflux. Data support the presence of an $SCFA^-$-HCO_3^- exchange.

Key words: Surface pH — Caecum — Guinea pig — Basolateral membrane — Na-H exchange — Cl-HCO_3 exchange

Introduction

Short-chain fatty acids (SCFA), such as acetate, propionate and butyrate, are the major metabolites of anerobic microbial fermentation of polysaccharides in the hind gut. They represent important nutrients for the caecal and colonic epithelial cells, and they contribute in considerable amounts to the overall energy metabolism of herbivores [1, 38, 43]. In most of the mammals studied so far, concentrations of SCFA in the caecal and colonic contents are about 100 mmol · 1^{-1}, which implies a large transepithelial SCFA gradient [4, 18]. SCFA are rapidly absorbed [18] in the undissociated (protonated) form as well as in the ionized form [20, 22]. However, observations concerning the extent of absorption in these ways are very conflicting. In a number of recent studies SCFA absorption is believed to occur mainly by diffusion in the protonated form [9, 12, 41]. Other investigators postulate a predominantely ionized transport of SCFA [25, 30, 36]. Obviously major segmental and species differences exist in respect to the transport in the protonated and in the ionized form [19, 21].

The absorption of SCFA in the protonated form as well as the flux of bicarbonate in exchange with SCFA-anions implies a considerable proton load for the enterocytes of the large intestine. It was therefore not unexpected that the addition of SCFA cause an acidification of the intracellular pH (pH_i) [5, 14, 15]. SCFA also cause pH changes at the apical membrane of epithelia [11, 23]. However, effective mechanisms responsible for the regulation of pH_i are mainly located in the basolateral membrane of the enterocytes [5, 6, 7, 8, 29, 41, 45]. We therefore expected relevant changes in the extracellular pH at the basolateral membrane (pH_b) during pH_i-regulation. Changes in pH_b could have considerable effect on the transepithelial transport of weak electrolytes [33]. Furthermore, it was not known whether at the basolateral side a pH-microclimate exists similar to that at the luminal side of intestinal mucosa [23, 31, 34].

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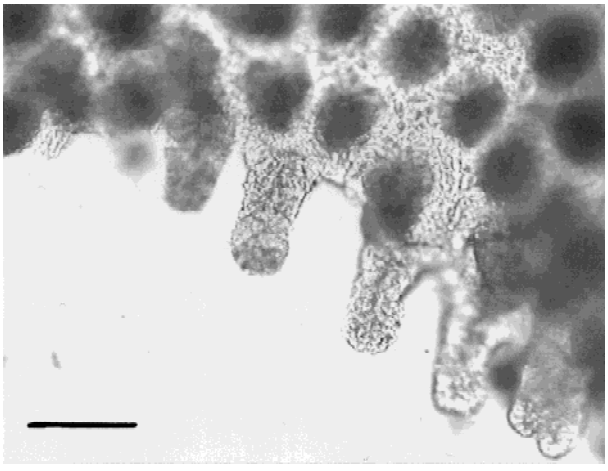

Fig. 1. Caecal epithelium after separation from the submucosal tissue. For the demonstration of the serosal side, the tissue sheet was folded so that the mucosal side is on the mucosal side, and the serosal side is outwardly directed. The picture was taken at the edge of the folded sheet. Single crypts come out fingerlike. The dark spots are crypts not in the focal plane. Transmission light microscopy (10× objective). The bar represents 50 μm.

We were interested in factors affecting pH_b (e.g., SCFA) and in mechanisms and processes that are involved in changes of pH_b. Recently the pH in the subepithelial tissue [11, 12] and in the lateral intercellular space of monolayers [10, 17, 26] have been described. An adequate method for the estimation of the pH at the basolateral membrane of epithelia (pH_b) is not available so far. It was therefore our aim to establish such a method for pH_b-measurements using a pH-sensitive fluorochrome attached to lectin (*lens culinaris*).

Materials and Methods

Tissue Preparation

Male guinea pigs (body weight of 300–500 g) fed a standard diet (Altromin No. 3122, Altromin, Lage, Germany) with water *ad libitum* were killed by decapitation between 8:00 and 9:00 A.M. The caecum was excised and flushed with ice-cold Krebs-Ringer solution to remove the luminal contents. Mucosal sheets were kept on ice in Krebs-Ringer solution gassed with 95% O_2 and 5% CO_2 until use. For experiments the epithelium was separated from the submucosal tissue using a glass slide. The separation procedure removes almost all the submucosal tissue including the lamina propria of the caecal epithelium of the guinea pig. Submucosal cells are virtually absent in the tissue preparation (*see* Fig. 1). Due to the low density of crypts in the guinea pig caecum such a preparation is possible. Afterwards the epithelial layer was slightly stretched and fixed by a supporting silicone ring with Histoacryl© [23] (Braun Melsungen, Melsungen).

Perfusion Chamber

The silicone ring with the stabilized epithelium was mounted in a small perfusion chamber [6, 23]. The chamber allows separate superfusion of the apical and of the basolateral side of the epithelium by two silicone half chambers having a fluid volume of 20 μl on each side. A nylon net (mesh-size: 200 μm, Polymon, Switzerland) placed on the inferior basolateral side and a silk net (mesh-size: 100 μm) placed on the upper apical side served as a mechanical support. The microperfusion chamber was mounted on a heatable and motordriven stage of an inverted microscope (Axiovert 35M, Zeiss, Oberkochen). The serosal side of the epithelium was directed towards the objective. The perfusion was driven by gravity at a rate of about 400 $\mu l \cdot min^{-1}$. Solutions on both side could be changed using two miniature eight-port valves (Hamilton, Bonaduz, Switzerland). For connections isoverinic-tubing with a low permeability for CO_2, internal diameter 0.5 mm (Amicon, Langenfeld), had been used. If solutions had been in the tubing for longer periods tubing were flushed with the fresh solutions prior to the recorded experiments. The solutions were kept at 37°C and were gassed as described below.

Perfusate Solutions and Chemicals

Krebs-Ringer solution buffered with bicarbonate contained (in $mmol \cdot l^{-1}$): 113.6 NaCl, 5.4 KCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 0.6 NaH_2PO_4, 2.4 Na_2HPO_4, 10.0 Glucose, 21 $NaHCO_3$, gassed with 95% O_2/5%CO_2 (carbogen) (*solution 1*). Bicarbonate-free Krebs-Ringer solution buffered with N-2-hydroxyethylpiperazine-N′-2-ethane-sulfonic acid (HEPES) contained 21.0 $mmol \cdot l^{-1}$ NaHEPES (Serva, Heidelberg, Germany) instead of $NaHCO_3$ and was equilibrated with 100% O_2 (*solution 2*). Low-buffered, bicarbonate-free solution contained 5 $mmol \cdot l^{-1}$ NaHEPES instead of 21 $mmol \cdot l^{-1}$ and was also equilibrated with 100% O_2 (*solution 3*). In SCFA media the NaCl was replaced by equimolar (113.6 $mmol \cdot l^{-1}$) sodium *n*-butyrate whereby the concentration of chloride was reduced to 15.3 $mmol \cdot l^{-1}$ in bicarbonate-containing solution (*solution 4*) and to 10.4 $mmol \cdot l^{-1}$ in bicarbonate-free solution (*solution 5*). In propionate-containing solution (*solution 6*) NaCl was also replaced by equimolar (113.6 $mmol \cdot l^{-1}$) Na-propionate resulting in a low concentration of chloride (10.4 $mmol \cdot l^{-1}$). In sodium-free and bicarbonate-free, low-buffered media sodium was replaced with N-Methyl-D-Glucamine and the concentration of HEPES was reduced to 5 $mmol \cdot l^{-1}$ (*solution 7*). The solution with a reduced chloride concentration contains 11.4 $mmol \cdot l^{-1}$ chloride while 113.6 $mmol \cdot l^{-1}$ chloride were substituted by gluconate (*solution 8*). The solution with a reduced pH was Krebs-Ringer solution titrated to a pH of 6.4 by HCl (*solution 9*). All the other solutions were titrated to a pH of 7.4 and adjusted to a final osmolarity of 300 ± 2 $mosmol \cdot l^{-1}$ with mannitol. The osmolarity was determined by freezing point depression (Roebling Osmometer, Berlin, Germany).

To investigate the influence of different basolateral ion transport mechanisms on pH_b, several drugs inhibiting these transporters were added to the perfusion solution. 5×10^{-5} $mol \cdot l^{-1}$ of 5-N-methyl-N-isobutyl-amiloride (MIA, Research Biochemical International, Köln, Germany) presolved in dimethyl-sulfoxide, (DMSO, SIGMA chemicals, Deisenhofen, Germany) in a concentration of 10^{-1} $mol \cdot l^{-1}$ were added to the perfusate to inhibit the Na^+-H^+ exchanger. 2×10^{-4} $mol \cdot l^{-1}$ dihydro-4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (H_2-DIDS, Molecular Probes, Eugene, OR), also dissolved in DMSO to a stock-solution of 2×10^{-1} $mol \cdot l^{-1}$, was used for inhibition of the Cl^--HCO_3^- exchanger. The dihydrogenated form of DIDS does not fluoresce, in contrast to the nonhydrogenated form. All other chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany).

Labeling with FITC-Lectin

The basolateral pH (pH_b) was estimated with lectin (*lens culinaris*) labeled with the pH-sensitive fluorescent dye fluorescein-

isothiocyanate (FITC) (Molecular Probes, Eugene, OR). As the lectin binds to sugar residues (mainly mannosyl- and glycosyl-residues) of membrane proteins, the fluorescein stays at the basolateral membrane during the perfusion. Dye loading was carried out on ice by applying 10 μl of 20 μmol · l^{-1} FITC-labeled lectin to the serosal side of the epithelium for 12 min. Afterwards any unattached dye was washed away, and the epithelium was mounted in the microperfusion chamber.

Microspectrofluorometry

The fluorescence intensity at the basolateral side of the caecal epithelium was determined using a Zeiss MPM 200 system (Zeiss, Oberkochen, Germany) built around an inverted microscope equipped with a 75 W xenon lamp, a high speed shutter and a rotating filter wheel equipped with two bandpass filters of 436 ± 20 nm and 485 ± 10 nm. In pilot studies an excitation spectrum of FITC-labeled lectins was measured in solutions of different pH (pH 5.0 to pH 8.0) with the excitation wavelength varying from 400–510 nm. In this way, the excitation maxima and the isobestic point could be estimated. The isobestic point, where all curves run through a single point, was situated at 420 nm, while the excitation-maxima were achieved at 490 nm. These results fit to the wavelength range of the two bandpass filters (436 ± 20 nm, 485 ± 10 nm) used in the further measurements. The excitation beam was reflected by a 510 nm dichroic mirror and directed through a 40× long distance objective (LD40, Zeiss, Oberkochen, Germany). To achieve measurements at both wavelengths with the same gain setting of the photomultiplier the excitation intensity at 485 nm was reduced to 25% by a neutral density filter. To minimize photobleaching, the intensity at both wavelengths was further reduced to 10% by a neutral density filter, and the excitation time was restricted to 200 msec for each time point using a high-speed shutter. The fluorescence emission was collected by a photomultiplier (RS928, Hamamatsu, Herrsching, Germany) in the range of 515–530 nm. Measurements were taken looking at surface cells. Results from the crypt base are not presented in this paper. The emission from an area defined by a pin hole corresponds to 4–6 cells. The high-speed shutter, filter wheel and photomultiplier unit were driven by a MSP21 controller (Zeiss, Oberkochen, Germany). The whole system was connected to an IBM-compatible personal computer, that controlled the time intervals, collected the fluorescence signals and performed online calculations of signal ratios from the two wavelengths continuously [6, 23].

Calibration of the Fluorescence Signal

The ratios of the fluorescence signals were calibrated by the K^+/nigericin method [46]. Cells were superfused on both sides with calibration buffer containing 133.4 mmol · l^{-1} K^+, which reflected approximately the intracellular concentration of K^+, and 10^{-5} mol · l^{-1} nigericin. The pH of these calibration buffers was tritrated to pH values between 6.0 and 8.0 (*see* Fig. 2). Since nigericin is a K^+/H^+-ionophore, intracellular H^+-concentrations equilibrate to extracellular concentrations [6]. By this method potential pH gradients produced by active proton transport mechanisms should be diminished.

Confocal Laser-scanning Microscopy

The position of FITC after dye staining of the epithelium was investigated by a confocal laser-scanning microscope (Bio-Rad, MRC 600, Hemel Hampstead, England). The FITC-labeled epithelium was focused through in the z-direction in micrometer-steps. The fluorescence intensity of the layers was collected by a photomultiplier.

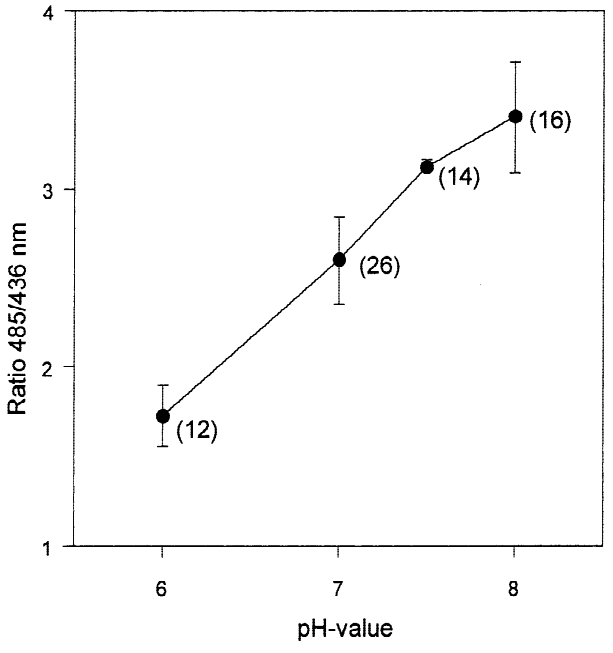

Fig. 2. Comparison of the ratio of calibration-experiments with caecal epithelia. The calibration-buffers contained 10^{-5} mol · l^{-1} nigericin and had pH-values between 6 and 8. The number of experiments is shown in brackets.

Quenching of the Fluorescence Intensity with Anti-fluorescein Antibody

For quenching the fluorescence intensity of a FITC lectin-labeled epithelium, a monoclonal IgG antibody against free and bound fluorescein (Boehringer Mannheim GmbH, Germany) was used. After mounting the stripped epithelium of the caecum of guinea pig into the microperfusion chamber and after staining it with FITC lectin, the fluorescence intensity was measured near the isosbestic point. 25 μl of the antibody solution containing 7.7 μg of anti-fluorescein antibody were added to the serosal side of the epithelium. After 45 min of incubation with the antibody the fluorescence intensity was measured again.

Ussing Chamber Experiments

Transepithelial conductance (g_t) and short-circuit current (I_{SC}) of stripped mucosal sheets of the caecal epithelium were measured in Ussing-chamber experiments after superfusion with a solution containing FITC-labeled lectin (*lens culinaris*) (3.3 μmol · l^{-1}, 30 min) and without addition of lectin (control). The inner diameter of the chamber was 1.13 cm^2. To both sides of the epithelium 10 ml of Krebs-Ringer solution (pH 7.4; 37°C, *solution 1*, containing 10^{-6} mol · l^{-1} indomethacin) was added and gassed by a gas lift system with carbogen. Each chamber was connected with an automatic computer-controlled voltage clamp amplifier (AC copy, Aachen, Germany). Details have been described recently [20, 22].

Statistics

Results are given as mean ± SD; *n* designates the number of experiments. Differences were evaluated by paired and unpaired *t*-tests where appropriate with $P < 0.05$ considered as significant.

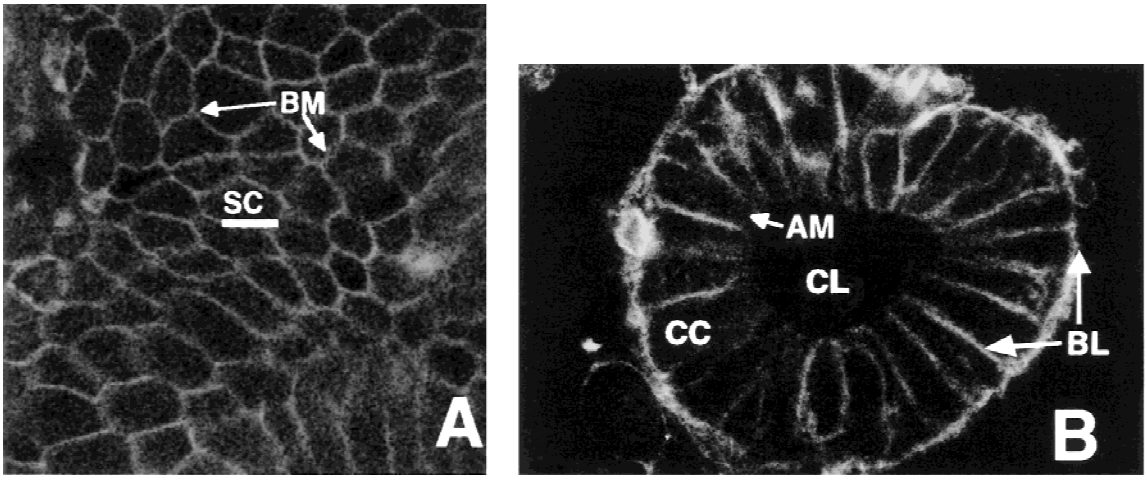

Fig. 3. FITC-lectin was added to the serosal side of the caecal epithelium. The location of the dye was checked by confocal fluorescence microscopy. (*A*) The white cell margins represent the fluorescence intensity at the basolateral membrane (BM) in the intercellular space of surface cells (SC) (magnitude 100×). (*B*) Cross-section of a FITC-lectin-labeled crypt (magnitude 400×). The white margins of the crypt cells (CC) represent intercellular spaces and the basolateral membrane near the basal lamina stained by the fluorochrome. Only the basolateral membranes (BM) are labeled, not the apical membranes (AM), or the crypt lumen (CL).

Results

Validity of the Fluorometric Method for Estimation of the Surface pH at the Basolateral Membrane

Localization of the Dye Molecular

The localization of the lectin molecule was checked by confocal laser scanning microscopy to ensure that after labeling the serosal side of the epithelium with FITC the lectin is located and remained at the basolateral membrane. In Fig. 3*A* the fluorescence intensity of the FITC-lectin is seen only in the intercellular space of caecal surface epithelial cells, and not inside the cells. In Fig. 3*B* the localization of the dye in a crypt is in the intercellular space and close to the basal lamina at the basolateral membrane, however, not in the crypt lumen and not at the apical surface. We conclude that FITC-lectin does not pass the tight junctions and is attached to the basolateral membrane after serosal addition.

Quenching of the Fluorescence of the Epithelium Labeled with FITC-Lectin by Anti-Fluorescein Antibodies

To check whether the attached FITC-lectin in Fig. 3 is solely located extracellularly we quenched the fluorescence of the lectin labeled epithelium with a anti-fluorescein antibody added to the serosal side of the epithelium. As shown in Fig. 4, the basolateral fluorescence caused by FITC-labeled lectin could be reduced by 85%

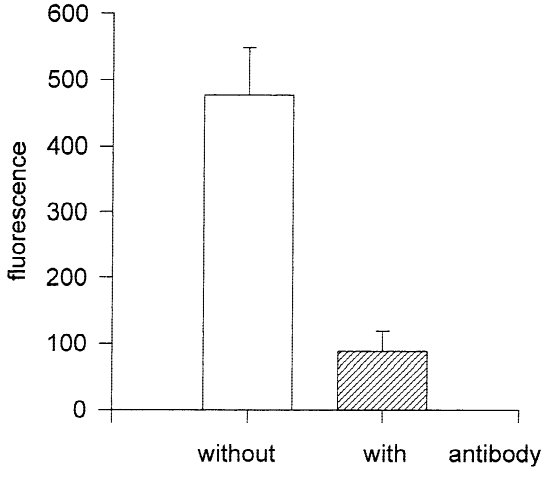

Fig. 4. Quenching of the fluorescein-molecule of the FITC-labeled lectins attached to the basolateral membrane by a anti-fluorescein antibody. After the incubation of the guinea pig caecal epithelium with FITC-lectin from the serosal side 7.7 μg of the lyophilized antibody solution was added to the serosal solution (*solute 1*) for 45 min. The fluorescence intensity was measured in the epithelia with and without the antibody (standard deviations of 20 measurements).

with the fluorescein-specific antibody. The antibody may not completely reach all binding sides of the fluorescein due to the steric conformation of the lectin, and this might explain the remaining fluorescence of 15%.

Do Lectins Affect Electrophysiological Parameters of the Caecal Epithelium?

Transepithelial conductance (g_t) and short-circuit current (I_{SC}) as electric parameters for the viability of the dye-

Table. Transepithelial conductance g_t ($mS \cdot cm^{-2} \cdot hr^{-1}$) and short-circuit current I_{SC} ($\mu equiv \cdot cm^{-2} \cdot hr^{-1}$) of guinea pig caecal epithelia in Ussing-chambers without (control) and after incubation with 3.3 $\mu mol \cdot l^{-1}$ FITC-labeled lectins for 30 min (number of chambers $n = 12$). Results are given as mean ± SD. Neither the transepithelial conductance nor the short-circuit current of the control epithelia are significantly different from the FITC-lectin-labeled epithelia.

	Control-epithelia	Epithelia incubated with FITC-labeled lectins
g_t	5.23 ± 1.02	5.48 ± 0.54
I_{SC}	2.28 ± 0.25	2.54 ± 0.31

labeled membrane were measured in Ussing-chamber experiments. Neither the short-circuit current nor the transepithelial conductance were changed significantly by the addition of lectin to the serosal solution in comparison to control experiments without addition of lectin (Table).

Surface-pH at the Basolateral Membrane (pH_b) of Caecal Epithelia of Guinea Pigs

pH_b in Bicarbonate-containing and Bicarbonate-free Solutions

For the estimation of the basic surface pH_b, the isolated caecal epithelium was superfused with standard Krebs-Henseleit solution (*solution 1*) containing 21 $mmol \cdot l^{-1}$ bicarbonate at pH 7.4 at both sides of the tissue. Under these conditions a basic pH_b of 7.70 ± 0.15 ($n = 86$) was measured, which is significantly above the pH of the perfusion solution. Under bicarbonate-free conditions (*solution 2*), when bicarbonate was replaced by 21 $mmol \cdot l^{-1}$ HEPES, pH_b was 7.50 ± 0.14 ($n = 26$) and did not differ significantly from solution-pH.

Exchange Mechanisms Influencing pH_b

Some regulation mechanisms like the Na^+-H^+ exchanger and the Cl^--HCO_3^- exchanger [6, 16, 29] have been described at the basolateral membrane. To find out whether bicarbonate-dependent mechanisms are involved in the alkaline pH_b in bicarbonate-containing solution, the basolateral Cl^--HCO_3^- exchanger was inhibited with 2×10^{-4} $mol \cdot l^{-1}$ H_2-DIDS added to the serosal solution. As shown in Fig. 5 the inhibition of the Cl^--HCO_3^- exchanger caused a decrease of pH_b by 0.08 ± 0.04 pH-units ($n = 15$; $P < 0.0001$). In further experiments the chloride concentration was reduced in the perfusion solution at the serosal side (*solution 8*) from 124 $mmol \cdot l^{-1}$ to 11.4 $mmol \cdot l^{-1}$, at the mucosal side the chloride concentration remained high (*solution 1*). This low serosal chloride concentration caused a decrease of pH_b by 0.14 ± 0.09 pH units ($n = 14$; $P < 0.001$).

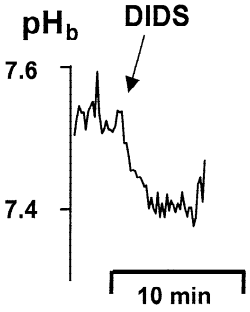

Fig. 5. pH_b before and after the inhibition of the basolateral Cl^--HCO_3^- exchanger by addition of H_2-DIDS (2×10^{-4} $mol \cdot l^{-1}$) to the serosal solution (*solution 1*).

We expected that also the Na^+-H^+ exchanger at the basolateral membrane affects pH_b. To test this hypothesis the epithelium was superfused on the serosal side with sodium-free, bicarbonate-free 21 $mmol \cdot l^{-1}$ HEPES solution (*solution 2*). However, no significant changes of pH_b were seen ($n = 5$; $P = 0.902$). On the other hand, an effect was achieved with sodium- and bicarbonate-free, low-buffered solutions (*solutions 3 and 7*). As shown in Fig. 6 the removal of the sodium gradient as the driving force for the basolateral Na^+-H^+ exchanger led to an increase of pH_b by 0.21 ± 0.09 pH-units ($n = 17$; $P < 0.0001$). Furthermore, the activity of the Na^+-H^+ exchanger could be shown in an experiment with an ammonium prepulse in low-buffered, bicarbonate-free perfusion solution (Fig. 7). It is well documented that the intracellular pH of enterocytes acidifies markedly when ammonia is added and then removed from the bathing solution [3, 32]. After the addition of the low-buffered, bicarbonate-free solution (*solution 7*), pH_b had decreased by 0.12 ± 0.04 pH-units ($n = 6$; $P < 0.001$). Addition of ammonia increased pH_b slightly by 0.10 pH-units ($n = 6$; $P = 0.03$; Wilcoxon Signed Rank Test was performed). Upon removal of sodium an additional increase of pH_b was observed (initial increase of pH 0.22 ± 0.10 pH-units; $n = 6$; $P < 0.01$; after 10 min 0.09 ± 0.08 pH-units; $n = 6$; $P < 0.05$). After readdition of sodium, pH_b acidified dramatically (max. change of pH_b 0.52 ± 0.02 pH-units; $n = 6$; $P < 0.0001$) with a recovery back to the starting values within 10 min. In the presence of 5×10^5 $mol \cdot l^{-1}$ MIA the primary acidifying peak was not as impressive as in the absence of MIA (max. pH_b-change 0.15 ± 0.04 pH-units; $n = 6$; $P < 0.005$). These findings emphasize that, in the presence of physiological concentrations of bicarbonate in the perfusion solution, proton efflux via the basolateral Na^+-H^+ does not change pH_b due to the high buffer capacity. In the absence of bicarbonate and in a low-buffered medium, the activity of the Na^+-H^+ exchanger can be demonstrated by using

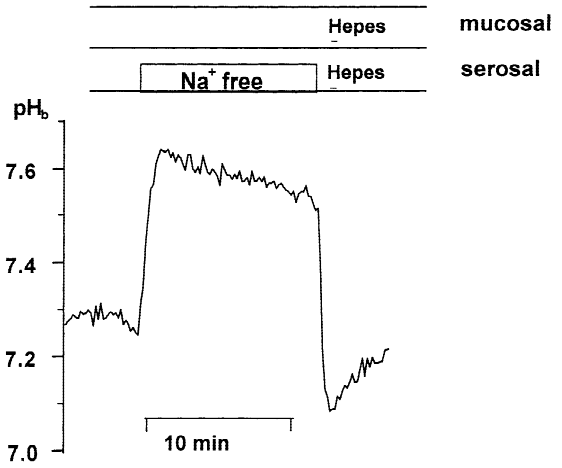

Fig. 6. pH_b before and after the inhibition of the basolateral Na^+-H^+ exchanger by serosal removal of sodium in bicarbonate-free, low-buffered (5 mmol · l^{-1} HEPES) solution (*solutions 3 and 7*).

sodium-containing and sodium-free solution. The inhibition with MIA had an effect on pH_b only after acidification of the enterocytes by means of an ammonia-pulse. Without acidification, the addition of MIA to bicarbonate-free, low-buffered solution did not cause pH_b-changes ($n = 18$).

Effects of a Decreased pH of the Perfusion Solutions on pH_b

Because basolateral Cl^--HCO_3^- exchanger and the Na^+-H^+ exchanger can influence pH_b, we may expect that a constant microclimate is present at the basolateral side, similar to that at the apical side of the hind gut mucosa [23, 24]. For this reason, the basolateral side and the apical sides of the epithelium were superfused alternately with solutions of low pH (*solution 9*) (Fig. 8). Lowering the pH of the luminal solution by one pH-unit from pH 7.4 to pH 6.4 led only to a slight decrease of pH_b by 0.14 ± 0.06 pH-units from pH 7.70 to pH 7.56 ($n = 8$; $P < 0.001$). However, the decrease of the serosal bulk-pH reduced pH_b to pH values near to that of the perfusion solution; pH_b declined by 1.29 ± 0.28 pH-units from pH 7.69 to the solution pH of 6.4 ($n = 8$; $P < 0.0001$).

Effects of Short-chain Fatty Acids on pH_b

SCFA Added to the Mucosal Solution. If the transport of SCFA from the lumen into the blood across the basolateral membrane occurs either in the protonated form or in exchange with extracellular HCO_3^-, we should expect a decrease of pH_b. Therefore 113.6 mmol · l^{-1} Na-butyrate was added to the mucosal side to either a bicarbonate-containing solution (*solution 4*) or to a bicarbonate-free solution (*solution 5*) on both sides of the mucosa. The addition of butyrate to the mucosal solution caused only a slight decrease of pH_b, in bicarbonate-containing solution (0.06 ± 0.04 pH-units; $n = 20$; $P < 0.0001$) and also in bicarbonate-free solution (0.06 ± 0.07 pH-units; $n = 16$; $P < 0.01$). The pH-changes in bicarbonate-containing and in bicarbonate-free solution did not differ significantly. The addition of another short-chain fatty acid, of 113.6 mmol · l^{-1} propionate, added to the luminal solution (*solution 6*) caused a comparable slight decrease in pH_b (0.09 ± 0.05 pH-units; $n = 5$; $P < 0.01$); findings did not differ significantly from those with butyrate.

SCFA Added to the Serosal Solution. If the transport of SCFA across the basolateral membrane from the serosal side into the caecal enterocyte would occur either in the protonized form or in exchange with intracellular bicarbonate we should expect an increase of pH_b. The serosal addition of 113.6 mmol · l^{-1} butyrate in bicarbonate-free solution (*solution 5*) caused an increase of pH_b (0.15 ± 0.08 pH-units; $n = 26$; $P < 0.0001$). pH_b returned to the original values after the removal of butyrate (Fig. 9). However, in bicarbonate-containing solution only a transient alkalinization of pH_b (0.19 ± 0.09 pH-units; $n = 69$; $P < 0.0001$) was seen after serosal addition of butyrate. Although butyrate always was present, pH_b quickly returned back to the baseline pH_b in the presence of butyrate (Fig. 10). After removal of butyrate, a transient acidification of pH_b (0.20 ± 0.07; $n = 69$; $P \leq 0.0001$) with a rapid return back to the baseline pH_b was recorded. The serosal addition of 113.6 mmol · l^{-1} propionate in bicarbonate-containing solution (*solution 6*) also caused an initial increase of pH_b (0.19 ± 0.03 pH-units; $n = 5$; $P < 0.001$) with a quick recovery to baseline pH. After removal of propionate, an acidifying overshoot of pH_b was measured (0.18 ± 0.05; $n = 5$; $P = 0.0005$). The addition of propionate to the serosal solution caused similar pH_b changes as the addition of butyrate.

In further experiments the Na^+-H^+ exchanger and the Cl^--HCO_3^- exchanger were inhibited before the addition of butyrate. After inhibition of the basolateral Na^+-H^+ exchanger with 5×10^{-5} mol · l^{-1} MIA ($n = 11$) or after inhibition of the basolateral Cl^--HCO_3^- exchanger with 2×10^{-4} mol · l^{-1} H_2-DIDS ($n = 21$) the transient pH_b changes after addition of butyrate were very similar to those without the inhibitors. In these experiments (Fig. 11) either the Na^+-H^+ exchanger or the Cl^--HCO_3^- exchanger might have regulated pH_b during butyrate application. We, therefore, inhibited both exchangers with 5×10^{-5} mol · l^{-1} MIA and with 2×10^{-4} mol · l^{-1} H_2-DIDS before 113.6 mmol · l^{-1} of butyrate in bicarbonate-containing solution was added (*solution 4*). As seen in Fig. 12, inhibition of both exchangers resulted in similar pH_b changes compared to those seen without the inhibitors ($n = 4$).

Without the application of the drugs the addition of

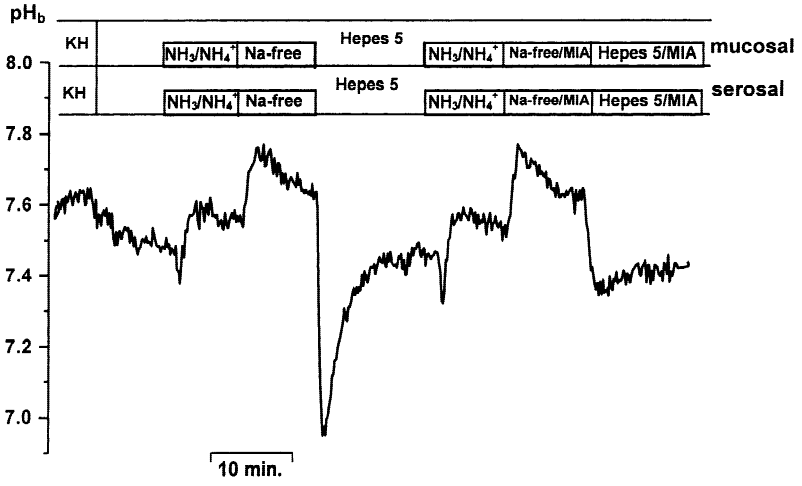

Fig. 7. Documentation of the participation of the Na^+-H^+ exchanger in pH_b-regulation. The experiment was done in bicarbonate-free, low-buffered solution (*solution 3*). After addition of 25 mmol · l^{-1} NH_3/NH_4^+ to both sides of the epithelium the perfusate was replaced by a sodium-free solution (*solution 7*). Finally both chambers were perfused again with the bicarbonate-free, low-buffered solution. The same experimental sequence was repeated with the Na^+-H^+ exchanger being inhibited by bilateral addition of 5×10^{-5} mmol · l^{-1} MIA during and after the replacement of sodium.

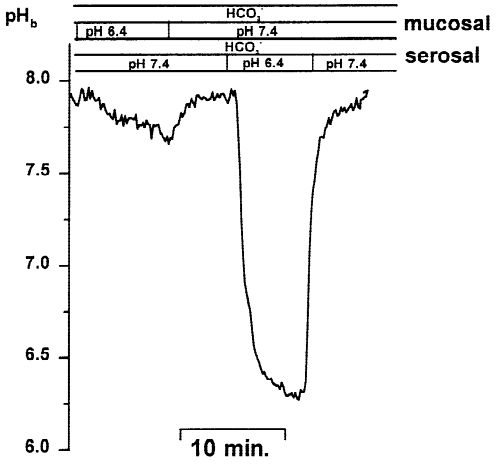

Fig. 8. pH_b during a decrease of the pH in the bulk solution (*solution 9*) at the apical or at the basolateral side. The bicarbonate-containing solutions had been titrated to pH 7.4 (*solution 1*).

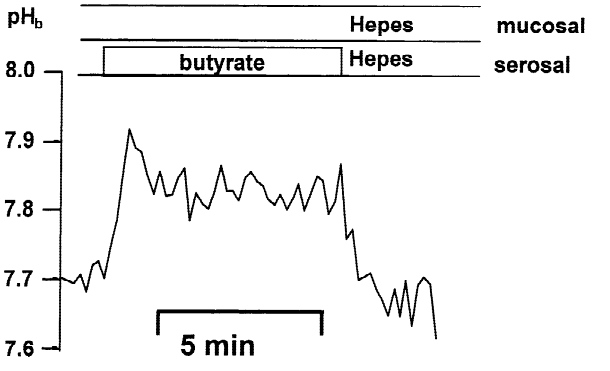

Fig. 9. Effect of serosal addition of 113.6 mmol · l^{-1} butyrate on pH_b in bicarbonate-free solution on both sides of the epithelium (*solutions 2 and 5*).

butyrate caused a transient increase of pH_b of 0.19 ± 0.09 and upon removal a transient decrease of 0.20 ± 0.07 (n = 69). In the presence of MIA these values are 0.18 ± 0.07 and 0.18 ± 0.09 (n = 11) units, respectively. In the presence of DIDS these values are 0.21 ± 0.08 and 0.21 ± 0.09 (n = 25), respectively. There had been no difference between values with and without the drugs.

Discussion

Considerable amounts of SCFA are produced and absorbed in the large intestine [4]. The absorption of the protonized form [9, 20, 41, 42] as well as in exchange with bicarbonate [22, 30, 35, 36] represents a substantial displacement of protons across the cell membrane of the enterocytes. An effective pH regulation of the enterocytes is, therefore, of considerable importance. Recent studies have shown that the regulation of intracellular pH is achieved mainly by the Na^+-H^+ exchanger and the Cl^--HCO_3^- exchanger in the basolateral membrane of the enterocytes in the large intestine [5, 6, 7, 8, 29, 39, 45]. We, thus, may expect that a proton load from the luminal side of the enterocytes should result in an acidification of the compartment near the basolateral membrane of the hind gut mucosa. In contrast, a flux of protons from the serosal side into the enterocyte might cause an alkalization of pH_b. To test this hypothesis it was first necessary to develop a method that allows continual measurement of the pH at the basolateral membrane.

A New Method for the Estimation of the pH at the Surface of the Basolateral Membrane of the Hind Gut Epithelium

It is difficult, if not impossible, to control with sufficient accuracy the position of the tip of a pH-sensitive microelectrode for pH measurements at the serosal side of the hind gut epithelium. Moreover, during perfusion and

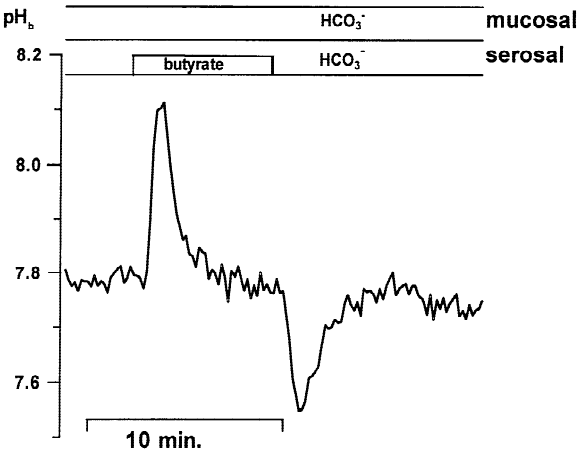

Fig. 10. Effect of serosal addition of 113.6 mmol · l^{-1} butyrate on pH$_b$ in bicarbonate-containing solution (*solutions 1 and 4*) on both sides of the epithelium.

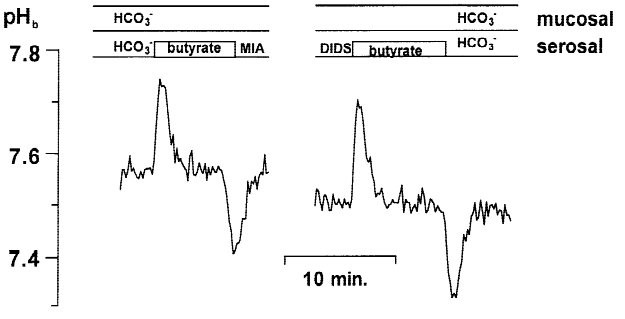

Fig. 11. Effects of basolateral addition of 113.6 mmol · l^{-1} butyrate in bicarbonate-containing solution (*solutions 1 and 4*) on pH$_b$ when the basolateral Na^+-H^+ exchanger was inhibited with 5×10^{-5} mol · l^{-1} MIA (left side) or the basolateral Cl^--HCO_3^- exchanger with 2×10^{-4} mol · l^{-1} H_2-DIDS (right side).

changes of the perfusion solutions, slight movements of the epithelium would cause unpredictable alterations of the tip position. Chu et al. [12, 13] recently studied the pH in the subepithelial compartment by confocal measurements with the pH-sensitive fluorescence dye carboxy-SNARF-1 in the serosal and in the luminal solution. This is an elegant approach to study pH gradients in the mucosa, however, as mentioned above, this method is not appropriate for estimations in the narrow intercellular lateral space and directly at the basolateral membrane. Another group [10, 17, 26] estimated the pH in the lateral intercellular space of cultured renal (MDCK) cell monolayers with the fluorochrome BCECF. After incorporation of the fluorescence-dye in the cells BCECF was secreted by these cells across the basolateral membrane in the lateral intercellular space. The basolateral support of the monolayers was impermeable for BCECF, and this allowed estimations of the pH

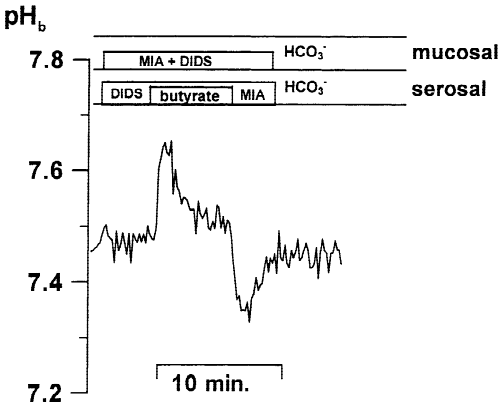

Fig. 12. Effect of serosal perfusion with 113.6 mmol · l^{-1} butyrate in bicarbonate-containing solution (*solutions 1 and 4*) on pH$_b$ when the basolateral Na^+-H^+ exchanger as well as the basolateral Cl^--HCO_3^- exchanger had been inhibited with 5×10^{-5} mol · l^{-1} MIA and 2×10^{-4} mol · l^{-1} H_2-DIDS, respectively.

in the lateral intercellular space. This method is limited to special studies in polarized cell cultures. Due to the need for an impermeable support for BCECF cells were grown on coverslips [26] or on supports with a reduced permeability [10, 17]. The compartment below the basolateral membrane does not exchange or may have a reduced exchange with the serosal perfusion solution.

In a recent study, we had used 5-N-hexadecanoylaminofluorescein (Molecular Probes) (HAF) for studies of the pH in the microclimate at the apical membrane of the colonic epithelium [23]. Unfortunately it was not possible to use this method for successful measurements at the basolateral membrane. Pilot studies indicate that HAF mainly binds unspecifically to the remaining connective tissue in the propria mucosa and to the basal lamina. Reliable attachment of HAF to the basolateral membrane could not be achieved.

It turned out that a lectin (*lens culinaris*) labeled with fluorescein (FITC) is a suitable tool for measurement of the pH at the basolateral membrane. It could be shown with confocal microscopy that FITC-labeled lectin of *lens culinaris* is attached at the basolateral membrane of the serosal side of the caecal epithelium and also in the paracellular pathway. Due to the low density of crypts in the guinea pig caecum a preparation of the epithelium can be achieved in which cells from the lamina propia are virtually absent (Fig. 1). The FITC-labeled lectin does not enter the enterocytes and does not pass the tight junctions after addition to the serosal side (Fig. 3). The extracellular localization of fluorescein could be demonstrated by adding anti-fluorescein antibody (Fig. 4). The intracellularly located fluorescein would not have been quenched by the large antibody molecule, which is not expected to enter the cells. Furthermore Ussing-chamber experiments have proved that

neither the short-circuit current nor the transepithelial resistance of the caecal mucosa are affected by FITC-labeled lectin (Table). That indicates that FITC-labeled lectin did not cause electrogenic ion fluxes. In contrast to results with concanavalin A in alveolar macrophages [2] and lymphocytes [24] in our studies lectin from *lens culinaris* had no influence on the basolateral Na^+-H^+ exchange as demonstrated by measurements of the intracellular pH with and without the attached lectin (*data not shown*). The FITC-labeled lectin remains attached to the basolateral membrane for longer periods in sufficient concentrations. The concentration of the attached dye had been estimated by repeated determination of the fluorescence intensity after excitation at 436 nm (the isosbestic point, where the fluorescence intensity depends only on the concentration of the dye). The average loss of fluorescence intensity of 14 arbitrarily chosen experiments was 19.2% after 2 hr. This loss of fluorescence might be explained partly by the loss of dead cells with membrane-bound dye or by washout.

No Constant pH Microclimate at the Surface of the Basolateral Membrane of the Caecal Mucosa

At the luminal surface of the hind gut mucosa, a rather constant pH microclimate near pH 7.0 has been shown by a number of investigators. This pH microclimate is rather independent of changes of the pH in the luminal solution as shown for guinea pig, rat and human [23, 27, 31, 34]. At the surface of the basolateral membrane of the caecal mucosa we could not find such a constant pH microclimate. The pH_b followed the pH of the perfusion solution (Fig. 8). The observation is different from those reported by the group of Spring [10, 17, 26] who found a pH in the lateral space of cultured renal MDCK cells of 0.4 below the basolateral perfusion solution or Chu & Montrose [12] where the observed pH in the submucosal compartment was 0.2 units below the perfusion solution. These differences might be due to the support or the submucosal tissue, respectively. We had observed differences between the calibration in vivo and in vitro in our studies with FITC-lectin and also with HAF [23]. The vivo calibration curve was shifted to a more alkaline pH as compared to the in vitro calibration (approximately 0.4 pH units).

Transport Mechanisms in the Basolateral Membrane Affect Basolateral Surface pH

The Cl^--HCO_3^- exchanger in the basolateral membrane of enterocytes in the mucosa of the large intestine is described as one of the major mechanisms in the regulation of pH_i [5, 6, 44]. The basic pH_b is slightly more alkaline in bicarbonate-containing solution at a pH of the serosal perfusion solution of pH 7.4. We explain this by an efflux of bicarbonate across the basolateral membrane. A higher gradient exists for chloride (approximately 18 times higher in the extracellular fluid) as compared to bicarbonate (approximately 2 times higher in the extracellular fluid). Bicarbonate-efflux across the basolateral membrane can be assumed similar to the bicarbonate-efflux at the apical membrane [22]. We suggest that after an inhibition of the basolateral Cl^--HCO_3^- exchanger by 2×10^{-4} mol $\cdot$ l^{-1} H_2-DIDS as well as by perfusion with bicarbonate-free solutions, bicarbonate efflux ceased and therefore pH_b returned close to the pH of the serosal perfusion solution of pH 7.4 (Figs. 5–7). Similarly the reduction of chloride in the serosal perfusion solution decreased pH_b. This is explained by the diminished chloride gradient and thereby a reduced bicarbonate efflux.

The Na^+-H^+ exchanger at the basolateral side is of considerable importance for pH_i regulation [5, 16, 29, 45]. It is activated by intracellular acidification [5, 6, 28]. Under basic conditions the activity of the basolateral Na^+-H^+ exchanger is not reflected in changes of pH_b as the proton secretion is masked by the effective bicarbonate buffer system as well as by bicarbonate efflux. However, in bicarbonate-free and low-buffered perfusion solution the involvement of the Na^+-H^+ exchanger could be shown when the Na^+-H^+ exchanger was inhibited by withdrawal of sodium from the serosal solution, this resulted in an alkalization of pH_b (Fig. 6). That indicates that protons were no longer transported from the cytoplasm of the enterocytes across the basolateral membrane. After an ammonia prepulse, that provokes an intracellular acidification [3, 32], a marked increase of pH_b was seen in bicarbonate-free and low-buffered perfusion solution by removal of sodium from the serosal side (Fig. 7). This rapid increase of pH_b most likely is due to a rapid efflux of NH_3 from the enterocytes across the basolateral membrane. The readdition of sodium is accompanied by a potent transient decrease of pH_b due to the activity of the Na^+-H^+ exchanger followed by a pH-recovery to the initial control value within a few minutes (Fig. 7). This is evidence that in the presence of sodium the Na^+-H^+ exchange is markedly involved in regulation of pH_i, and this is reflected by changes in pH_b under these experimental conditions. The pronounced reduction of the respective pH_b overshoot after sodium readdition in the presence of MIA is in agreement with an inhibition of this regulation. We have, however, no convincing explanation for the rapid drop of pH_b after this readdition of sodium in the presence of MIA; the Na^+-H^+ exchanger might not have been inhibited totally by MIA. In the presence of bicarbonate and without an intracellular acidification of the enterocytes the inhibition of the Na^+-H^+ exchanger at the basolateral membrane with MIA had no significant effect on pH_b indicating that the

Na^+-H^+ exchange was masked by the bicarbonate buffer system.

Fluxes of Short-Chain Fatty Acids Affect pH_b

SCFA are absorbed in the large intestine in the protonated and in the ionized form. Thereby considerable amounts of protons are moved across the enterocytes. SCFA absorption therefore require an effective regulation of pH_i. The intracellular pH regulation of enterocytes in response to SCFA exposure had been studied recently [5, 14, 12, 15, 41]. Most of these findings confirmed that the major pH_i regulation is achieved by mechanisms in the basolateral membrane. We therefore expected changes in pH_b after addition of SCFA to the mucosal or to the serosal side of the caecal epithelium. After addition of butyrate to the luminal side of the epithelium pH_b decreased only slightly. This is consistent with findings of Chu and Montrose [12] who observed also a decrease of pH in the serosal tissue surrounding colonic crypts upon mucosal addition of butyrate. It is interesting to note that in our experiments the slight decrease of pH_b after mucosal addition of butyrate did not depend on the presence of bicarbonate. We interpret the observed decrease of pH_b to be the consequence of a diffusion of SCFA in the protonated form from the cytoplasm across the basolateral membrane of the caecal enterocytes.

To study the effect of SCFA transport at the basolateral side of the epithelium in more detail we applied butyrate also to the serosal side. The addition of butyrate or propionate to the serosal perfusion solution caused a persisting increase of pH_b in bicarbonate-free solution (Fig. 9) and a transient increase in the presence of bicarbonate (Fig. 10). This is in accordance with findings by Chu and Montrose [12] who also observed a continuous alkalinization in the subepithelial tissue after serosal addition of butyrate in bicarbonate-free solution. After removal of butyrate the pH at the basolateral side of the colonic epithelium returned to basic pH values. We interpret these results as the portion of butyrate being transported in the nonionized form across the basolateral membrane. This is in agreement with the decrease of pH_i after addition of butyrate to the serosal solution as shown in studies under the same experimental conditions [5].

We, first of all, assumed that the transient changes of pH_b after addition of butyrate to the serosal bicarbonate-containing solution were caused by regulation mechanisms in the basolateral membrane, the Cl^--HCO_3^- exchange and the Na^+-H^+ exchange. It was, therefore, unexpected that after inhibition of the basolateral Na^+-H^+ exchanger and the Cl^--HCO_3^- exchanger separately or simultaneously, the addition of butyrate to the serosal perfusion solution caused similar pH_b changes to those observed in the experiments without inhibition (Figs. 10–12). However, after the addition of butyrate in bicarbonate-free media a regulation of pH_b after the alkalinization was not seen (Fig. 9). These findings support the presence of a $SCFA^-$-HCO_3^- exchanger in the basolateral membrane. From studies with isolated membrane vesicles it was also supposed that a $SCFA^-$-HCO_3^- exchange mechanism exists in the basolateral [35] and also in the apical [30] membrane of the rat distal colon, as well as in the apical membrane of the human colon [25]. Such a $SCFA^-$-HCO_3^- exchanger seems to be responsible for the predominant portion of SCFA-absorption in the caecum and in the proximal colon of guinea pig [18, 22]. Measurements of the HCO_3^--permeability also support the existence of a $SCFA^-$-HCO_3^- exchanger in the apical membrane of the proximal colon of the guinea pig. In the distal colon, the exchanger seems to be present to a lesser extent [E. Gros, *personal communication*]. A monocarboxylate transporter isoform typ 1 (MCT1) that may be involved in SCFA transport in the apical membrane had been recently identified [37].

We conclude that it has been shown with a newly established method that at the surface of the basolateral membrane of the caecal epithelium a pH-microclimate does not exist in contrast to the pH-microclimate at the luminal surface. Mostly, pH_b is similar or close to the pH of the serosal perfusion solution. The slightly more alkaline pH_b at pH 7.4 of the perfusion solution is maintained by the Cl^--HCO_3^- exchanger. An influence of the activity of the basolateral Na^+-H^+-exchanger on pH_b seems to be masked under physiological conditions by the capacity of the serosal bicarbonate buffer system. Findings after addition of butyrate or propionate to the serosal perfusion solutions offer further support for the presence of a DIDS- and MIA-insensitive $SCFA^-$-HCO_3^- exchange in the basolateral membrane of the epithelium of the large intestine.

We thank Gerhild Becker, Marion Burmester and Kathrin Hansen for excellent technical assistance, Prof. W.G. Forssmann, Niedersächsisches Institut für Peptidforschung (IPF), Hannover, for the possibility to use the confocal microscope.

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 280: "Gastrointestinale Barrier") and by the Graduiertenkolleg "Zell- und Molekularbiologie in der Tiermedizin."

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