

# Phosphate transport in the duodenum and jejunum of goats and its adaptation by dietary phosphate and calcium

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**Huber, Korinna, Christina Walter, Bernd Schröder, and Gerhard Breves.** Phosphate transport in the duodenum and jejunum of goats and its adaptation by dietary phosphate and calcium. *Am J Physiol Regulatory Integrative Comp Physiol* 283: R296–R302, 2002; 10.1152/ajpregu.00760.2001.—Endogenous  $P_i$  recycling is a characteristic feature of the P homeostasis in ruminants. A pronounced salivary  $P_i$  secretion into the rumen is balanced by a high intestinal  $P_i$  absorption and an almost complete renal  $P_i$  reabsorption. In monogastric animals, the major  $P_i$  transport mechanism across the apical membrane of the enterocyte is an  $Na^+$ -dependent transport mediated by NaPi cotransporter type IIb. In ruminants, an  $Na^+$ -, as well as an  $H^+$ -dependent,  $P_i$  transport system seems to exist in the small intestines. Therefore, morphological localization, type of ionic dependence, and ability to adapt to dietary P or Ca restriction of duodenal and jejunal  $P_i$  transport were characterized in goats. In the duodenum, there was an  $H^+$ -dependent,  $Na^+$ -sensitive  $P_i$  transport system that did not belong to the NaPi type II family and was not influenced by dietary P or Ca restriction. In contrast, in the jejunum, there was an  $Na^+$ -dependent,  $H^+$ -sensitive  $P_i$  transport mainly mediated by NaPi IIb. P restriction stimulated the NaPi IIb protein expression, resulting in higher  $P_i$  transport capacity.

NaPi cotransporter type IIb; proton-sensitive phosphate transport; ruminants

IN RUMINANTS, PHOSPHATE ( $P_i$ ) is essential for both the host animal and the rumen microbes. A substantial proportion of microbial requirement is met by a substantial endogenous recycling of  $P_i$ , which is maintained by salivary  $P_i$  secretion and respective intestinal  $P_i$  absorption. In addition, an almost complete renal  $P_i$  reabsorption prevents urinary  $P_i$  loss.

In the animal kingdom, membrane transport systems for  $P_i$  are key elements in maintaining  $P_i$  homeostasis in the organisms. Two  $Na^+$ - $P_i$  cotransporter families are well described in their functional and structural properties for mammals (16).  $P_i$  absorption in the small intestines of monogastric animals is mainly achieved by a secondary active  $Na^+$ -coupled  $P_i$  cotransporter located in the brush-border membrane of enterocytes. This apical  $P_i$  transport is stimulated by dietary P restriction and calcitriol (3). In ruminants, the mechanism of intestinal  $P_i$  transport is obviously

more complex than in monogastric species. In the duodenum of sheep, an  $Na^+$ -independent, proton-driven  $P_i$  transport has been described (13). Destruction of the inwardly directed  $H^+$  gradient into duodenal brush-border membrane vesicles (BBMV) inhibited  $P_i$  uptake, suggesting the presence of an  $H^+$ - $P_i$  cotransport system. On the other hand, for sheep and goat jejunum, an  $Na^+$ -dependent  $P_i$  transport system has been reported (14, 15). Stoichiometry of this  $Na^+$ - $P_i$  transport suggested an interaction of two or more Na ions with one inorganic P ion at pH 7.4. The structural identification of the jejunal  $Na^+$ - $P_i$  transporter in goats was achieved by RT-PCR cloning (5) with primers homologous to the NaPi IIb nucleotide sequence cloned from mouse duodenum (4). In the jejunum of goats, an  $Na^+$ - $P_i$  cotransporter of type IIb was expressed with high homology to murine intestinal NaPi IIb, because mouse-specific NaPi IIb antibody was able to detect goat NaPi IIb protein in jejunal brush-border membranes (5). Goat transporter affinity for  $P_i$  ( $K_m$   $0.03 \pm 0.08$  mM) was in the same range as that described for murine NaPi IIb ( $K_m$  0.05 mM), but the affinity for  $Na^+$  was quite different:  $K_m$   $5.9 \pm 0.7$  mM in goat NaPi IIb and  $\sim 30$  mM in mouse NaPi IIb. The higher sodium affinity of goat NaPi IIb can partly explain the higher  $P_i$  absorption capacity in goat jejunum compared with that of monogastric animals.

From these functional and structural studies it can be assumed that  $P_i$  transport in goat jejunum is almost completely  $Na^+$  dependent, but it is still unclear to what extent this  $Na^+$ -dependent transport is mediated by NaPi IIb cotransporters.

Low dietary P significantly increased the  $P_i$  transport capacity in goat and sheep small intestines in both the  $H^+$ - $P_i$  transport system in the duodenum (13) as well as the  $Na^+$ - $P_i$  transport system in the jejunum (15). The upregulation of  $Na^+$ - $P_i$  cotransport in monogastric animals was based on an increase in the specific NaPi IIb protein without increasing amounts of NaPi IIb mRNA, indicating a posttranscriptional process for adaptation to dietary P restriction (3).

It is still unknown how  $P_i$  transport systems in small ruminants adapt to dietary P restriction. For caprine NaPi IIb cotransporter, the molecular biological tools

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are now available to study its expression at the mRNA and at the protein level. Therefore, it can be clarified whether the process of adaptation to dietary P restriction in goats is mediated by NaPi IIB.

It was the aim of the present study to characterize the properties of duodenal and of jejunal P<sub>i</sub> transport mechanisms in goats influenced by dietary P or Ca restriction. In contrast to monogastric animals, intestinal P<sub>i</sub> transport is mediated in goats by two different transport systems. These transporters are distinct in morphological localization, in their type of ion dependency, and in their ability to adapt to dietary P restriction.

## MATERIAL AND METHODS

### *Animals and Feeding*

The protocol of the animal treatment was approved and its execution supervised by the animal welfare officer of the School of Veterinary Medicine, Hannover.

Male White Saanen goats (4–5 mo old) were subdivided into three groups with different feeding regimens with adequate or reduced P or Ca supply. Each group of up to 12 animals was housed separately. Water was available at all times. In the control group, daily P and Ca intake was 1.8 g P and 6.1 g Ca; in the P-restricted group (–P), the intake was 0.8 g P and 5.9 g Ca; and in the Ca-restricted group (–Ca), the intake was 1.9 g P and 2.9 g Ca. Reduction of P and Ca supply was maintained for at least 6–8 wk. Plasma samples were obtained to define P status of the animals. In general, the experimental design was equivalent to previous studies (11, 14).

Animals were killed by stunning using a captive bolt pistol and bled from the carotid arteries. Duodenal and mid-jejunal segments were obtained within 3–5 min after slaughtering. After being rinsed with ice-cold saline (0.9% NaCl wt/vol), the mucosa was stripped off underlying muscle layers, frozen in liquid nitrogen, and stored at –80°C for structural and functional analyses (preparation of tissues for immunohistochemistry, see below). For Ussing chamber experiments, duodenal segments were rinsed with ice-cold saline and kept in a glucose-containing buffer solution at 4°C continuously gassed with O<sub>2</sub>-CO<sub>2</sub> (95:5 vol/vol). The slaughtering and tissue sampling procedure has been described in detail by Schröder and Breves (14).

### *Northern Blot Analyses*

Poly(A)<sup>+</sup>RNA from duodenal and jejunal mucosa was fractionated in 1.0% formamide-agarose gels and transferred by capillary blotting onto nitrocellulose membranes. After baking the membranes in a vacuum oven for 2 h at 80°C, prehybridization/hybridization was performed in a solution containing 5 × standard saline citrate (SSC), 5 × Denhardt's solution, 0.1% SDS, and 40% formamide. For blocking unspecific binding sites of membranes, denatured herring sperm DNA (20 µg/cm<sup>2</sup>) was added. Radioactively labeled NaPi IIB and β-actin-specific probes (50 µCi [<sup>32</sup>P]dCTP for each probe, specific activity 3,000 Ci/mmol, NEN Life Science Products, Boston, MA) were created by using a random prime labeling system (AmershamPharmaciaBiotech, Rediprime II, Buckinghamshire, UK). Hybridization was performed at 42°C overnight. The membranes were washed three times for 10 min with 2 × SSC-0.1% SDS first at room temperature, then with 0.5 × SSC-0.1% SDS at 37°C and, finally, with 0.2 × SSC-0.1% SDS at 42°C. The membranes were analyzed after

exposure to a phosphor imager screen for 2–4 h with a phosphor imager system (BioRad). Relative abundance of specific mRNA was quantified by reference to β-actin as the internal standard using the quantification software Quantity One (BioRad).

### *Western Blot Analyses*

BBMV that had been isolated for uptake studies were fractionated by SDS-PAGE in an 8.3% discontinuous polyacrylamide gel according to Laemmli (9) (for the preparation of BBMV, see below). Probes were not heat denatured before electrophoresis because of the heat instability of NaPi IIB protein. The separated proteins were transferred onto nitrocellulose membranes by tank blotting (blotting time 2 h). After being blocked with 2% fat-free milk solution overnight, membranes were incubated for 2 h with the mouse-specific anti-NaPi type IIB antibody (1:2,000, derived against a synthetic oligopeptide of the NH<sub>2</sub> terminus of the transporter). The antibody was kindly provided by Prof. Dr. J. Biber and Prof. Dr. H. Murer, Institute of Physiology, University of Zürich-Irchel, Zürich, Switzerland. Specificity of the murine antibody in goats was controlled by preincubation of the antibody with the antigenic peptide (data not shown). Immunolocalization of the primary antibody was performed using an anti-rabbit secondary antibody (1:1,500) coupled with horseradish peroxidase. Bands were detected by the enhanced chemiluminescence system (AmershamPharmacia-Biotech) according to the manufacturer's protocol. To quantify relative protein expression levels, β-actin-specific immunodetection was performed on the same blots. Bands were analyzed quantitatively using the Quantity One software.

### *Immunohistochemistry*

Small pieces of jejunal and duodenal gut wall of five goats were sliced and fixed by immersion in 3% paraformaldehyde (wt/vol), 100 mM cacodylate, and 100 mM sucrose (pH 7.4) overnight. Subsequently, tissues were frozen in liquid propane and stored at –80°C. For immunohistochemical analysis, cryosections of ~8-µm thickness were prepared on poly-L-lysine-coated slides. After permeabilization and blocking in PBS (100 mM) containing 0.5% Triton X, 4% goat serum, and 0.1% NaN<sub>3</sub> 1 h at room temperature, the preparations were incubated with anti-NaPi type IIB antibody (1:500 diluted in PBS) overnight in a moisture chamber. Subsequently, the tissues were washed three times for 10 min with PBS at room temperature and were incubated for 2 h with an anti-rabbit secondary antibody labeled with the fluorophore dichlorotriazinyl aminofluorescein (1:200, Jacksons Labs, purchased from Dianova). A second slide was incubated only with labeled secondary antibody to determine the background fluorescence. The preparations were examined with an epifluorescence microscope (IX70, Olympus) using a modified U-MNIBA filter block with the following specifications: beam splitter DM 505 (505 nm), excitation filter BP 470–490 (470–490 nm), barrier filter D 520/20(510–530 nm). Pictures were made with a black and white video camera (model 4910, Cohu, San Diego, CA) connected to a Macintosh Computer and controlled by IPLab Spectrum 3.0 software (Signal Analytics, Vienna, VA). Frame integration and contrast enhancement were employed for image processing. The pictures were colored by means of the IPLab software.

### *Preparation of BBMV and Uptake Measurements*

BBMV were prepared from duodenal and jejunal epithelia with a modified Mg<sup>2+</sup>-EGTA precipitation method, and P<sub>i</sub>

Table 1. Plasma concentrations of  $P_i$  and Ca in control, P-, or Ca-restricted goats

	$P_i$	Ca
Control	2.3 ± 0.3*	2.8 ± 0.02*
-P	1.0 ± 0.1†	3.5 ± 0.1†
-Ca	3.0 ± 1.2*	2.7 ± 0.1*

Values are means ± SE in mM;  $n = 6$  goats/group. Significance level between values within 1 column was at least \* $P < 0.01$  (1-way ANOVA).

uptake into BBMV was quantified using the rapid filtration technique as described by Schröder and Breves (14).  $P_i$  uptake was determined as total  $P_i$  uptake regardless of concentrations of mono- and divalent  $P_i$  anions because there is no experimental evidence for a preferential transport of one of the ionic forms of  $P_i$  (unpublished results). Duodenal vesicular  $P_i$  transport of each feeding group was analyzed in the presence of an inwardly directed gradient of  $K^+$  (extravesicular buffer: 100 mM KCl, 100 mM mannitol, 10 mM HEPES-Tris) at an extravesicular 5.4 with pH 7.4 intravesicularly (intravesicular buffer: 100 mM mannitol, 100 mM KCl, 10 mM HEPES-Tris).  $P_i$  uptake as a function of time was performed with either an inwardly directed  $Na^+$  (extravesicular: 100 mM; intravesicular: 0 mM) or  $K^+$  gradient at both pH 5.4 and 7.4 extravesicularly with pH 7.4 intravesicularly. Jejunal  $P_i$  transport across the apical membrane was analyzed in the presence of an inwardly directed  $Na^+$  gradient (extravesicular: 100 mM; intravesicular: 0 mM) at an extravesicular pH 7.4 and 5.4, respectively, to determine the  $H^+$  sensitivity of the  $Na^+$ -dependent  $P_i$  transport system. Kinetic parameters  $V_{max}$  (nmol·mg protein<sup>-1</sup>·10 s<sup>-1</sup>) and  $K_m$  (mM) for  $Na^+$ - and  $K^+$ -dependent  $P_i$  uptake in each gut segment were calculated from the Michaelis-Menten kinetic of  $P_i$  uptake into the BBMV (14).

#### Measurement of Duodenal Transepithelial $P_i$ Flux Rates

Duodenal transepithelial  $P_i$  flux rates were determined using the Ussing chamber technique as described in detail by Schröder et al. (15). Mucosal  $Na^+$  was present or absent with pH 7.4 or 5.4, and unidirectional  $P_i$  flux rates [mucosal to serosal ( $J_{ms}$ ), serosal to mucosal ( $J_{sm}$ )] were measured in the absence of a transepithelial electrical gradient in all experimental groups allowing to calculate  $P_i$  net flux rates ( $J_{net} = J_{ms} - J_{sm}$ ). Under those conditions, significant positive  $J_{net}$  values have to be interpreted as active  $P_i$  absorption.

#### Determination of Plasma $P_i$ and Ca

Plasma  $P_i$  was determined colorimetrically using the vanadate-molybdate method (8) and plasma Ca by the standard o-cresolphthalein complex method (12)

#### Statistics

Values were given as means ± SE/SD, with  $n =$  number of animals. Significance of differences was tested by one-way ANOVA and as a posttest by Tukey's  $t$ -test assuming a Gaussian distribution of data (software Graphpad prism 2.01, San Diego, CA; www.graphpad.com).  $P$  values < 0.05 were set to be significant. Linear regression (see Fig. 4) was also calculated with Graphpad prism 2.01. All immunodetection and hybridization experiments were performed at least in duplicate. The significance of the effects of group, Ca, pH, and the interaction of  $Na \times pH$  on duodenal transepithelial

$P_i$  flux rates were tested by three-way ANOVA [BMDP-92 software program (2)].

## RESULTS

### $P$ Status of the Animals

Dietary P restriction resulted in hypophosphatemia associated with hypercalcemia, which was used as an indicator of a shortage in  $P_i$  (Table 1). Dietary Ca restriction led to a slight increase in  $P_i$  concentrations at unchanged Ca levels.

### $P_i$ Transport in the Duodenum of Goats

**Structural and functional characteristics of duodenal  $P_i$  transport.** In Northern and Western analyses, neither NaPi IIb transcript nor NaPi IIb protein was found in the duodenum of goats of each feeding group (Fig. 1).

Using the Ussing chamber technique, duodenal transepithelial  $P_i$  flux rates were measured either in the presence or in the absence of mucosal  $Na^+$  at pH 7.4 and 5.4, respectively. At a mucosal pH of 7.4,  $J_{net}$  of  $P_i$  ranged between 11 and 22 nmol·cm<sup>-2</sup>·h<sup>-1</sup> irrespective of mucosal  $Na^+$  concentrations and different P or Ca supply (Table 2). Reducing the mucosal pH to 5.4 increased  $J_{net}$  of  $P_i$  to ~200 nmol·cm<sup>-2</sup>·h<sup>-1</sup> at high mucosal  $Na^+$  concentrations and to ~50 nmol·cm<sup>-2</sup>·h<sup>-1</sup> under  $Na^+$ -free conditions. Enhancement of transepithelial flux rates was due to respective increases  $J_{ms}$  at unchanged  $J_{sm}$ . Na affected  $J_{net}$  of  $P_i$  only at low pH as indicated by significant interactions between Na and pH in the ANOVA. Again, dietary regimens had no significant effects on  $P_i$  flux rates.

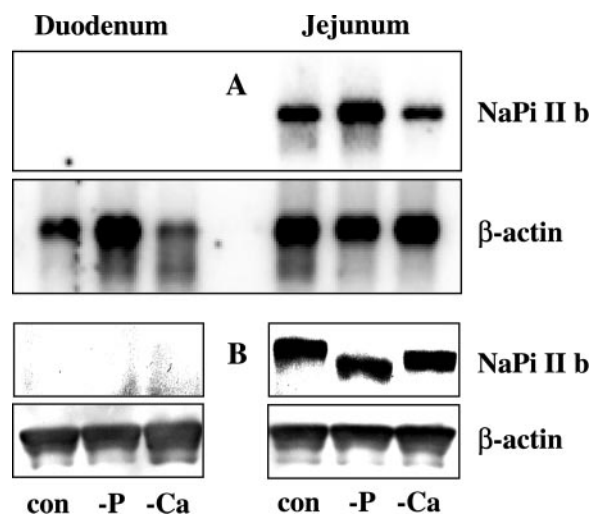


Fig. 1. NaPi IIb gene expression in the small intestines of goats. Northern analysis (A) of jejunal and duodenal poly(A)<sup>+</sup>RNA of 1 animal of each feeding group (con, control animal; -P, P-restricted animal; -Ca, Ca-restricted animal) was performed. Hybridization with a goat-specific NaPi IIb probe revealed strong bands in the jejunum, but not in the duodenum. The lack of NaPi IIb expression in goat duodenum was confirmed by Western analysis (B). NaPi IIb protein was immunodetected with a mouse-specific NaPi IIb antibody only in the jejunum. Integrity of poly(A)<sup>+</sup>RNA and proteins was proven by  $\beta$ -actin detection.

Table 2. Unidirectional and net flux rates of  $P_i$  of duodenal epithelia as affected by mucosal  $Na^+$  and  $H^+$  concentrations. Influence of dietary P or Ca restriction.

Grouping	Na+/-	pH	n	$J_{ms}$ , nmol·cm <sup>-2</sup> ·h <sup>-1</sup>	$J_{sm}$ , nmol·cm <sup>-2</sup> ·h <sup>-1</sup>	$J_{net}$ , nmol·cm <sup>-2</sup> ·h <sup>-1</sup>
Control	+Na	7.4	4	31.6 ± 2.5	14.5 ± 2.1	17.1 ± 3.3
-P	+Na	7.4	4	40.3 ± 8.4	18.4 ± 6.5	21.9 ± 6.3
-Ca	+Na	7.4	4	40.6 ± 8.8	21.2 ± 6.4	19.4 ± 6.0
Control	-Na	7.4	5	30.4 ± 3.2	19.6 ± 3.8	10.8 ± 3.0
-P	-Na	7.4	4	36.9 ± 3.7	18.6 ± 2.2	18.3 ± 5.8
-Ca	-Na	7.4	5	32.1 ± 7.4	18.5 ± 6.5	13.6 ± 9.6
Control	+Na	5.4	4	232.6 ± 45.5	12.0 ± 2.2	220.6 ± 44.5
-P	+Na	5.4	4	200.9 ± 70.7	17.9 ± 7.3	183.0 ± 77.7
-Ca	+Na	5.4	4	215.0 ± 35.0	18.6 ± 4.4	196.4 ± 37.6
Control	-Na	5.4	5	63.4 ± 9.4	15.8 ± 1.1	47.6 ± 9.8
-P	-Na	5.4	4	84.7 ± 17.3	17.3 ± 3.4	67.4 ± 18.5
-Ca	-Na	5.4	5	45.3 ± 7.4	18.4 ± 3.6	29.9 ± 4.4

P Values of 3-Way ANOVA

Source of Variation	$J_{ms}$	$J_{sm}$	$J_{net}$
Group	NS	NS	NS
Na	<0.001	NS	<0.001
pH	<0.001	NS	<0.001
Interaction Na × pH	<0.001	NS	<0.001

Values are means ± SE; n = no. of goats.  $J_{ms}$ , mucosal to serosal flux rate;  $J_{sm}$ , serosal to mucosal flux rate;  $J_{net}$ , net flux rate ( $J_{net} = J_{ms} - J_{sm}$ ). Interactions between Group and Na or pH or between Group, Na, and pH were not significant for all tested parameters.

*Kinetic parameters of proton-dependent  $P_i$  transport in the duodenum.* Time-dependent  $P_i$  uptake in duodenal BBMVs of each feeding group exhibited an overshoot phenomenon if an inwardly directed  $H^+$  gradient was established irrespective of the presence of an inwardly directed  $Na^+$  gradient (Fig. 2). In each animal, extravascular  $Na^+$  slightly stimulated  $H^+$ -dependent  $P_i$  uptake.  $P_i$  uptake in the absence of an inwardly directed  $H^+$  gradient was linear representing the diffusional part of  $P_i$  passover. The kinetic parameters of the duodenal  $H^+$ -dependent  $P_i$  transport are presented in Table 3. Neither in the P- nor in the Ca-restriction

experiment were any significant differences in  $V_{max}$  or  $K_m$  values observed.  $K_m$  values were similar in all groups and ~10-fold higher (0.4 mM) compared with jejunal NaPi IIB transporter (0.04 mM) (see below).

*$P_i$  Transport in Goat Jejunum*

*Immunohistochemical detection of NaPi type IIB in the jejunum of goats.*  $Na^+$ -dependent  $P_i$  transporters of type IIB were found in the apical membranes of the mature jejunal enterocytes of control goats by immunohistochemical analysis (Fig. 3B). In the apical membranes of duodenal enterocytes, no NaPi IIB-specific signal could be detected (Fig. 3A).

*Influence of dietary P or Ca restriction on  $Na^+$ -dependent  $P_i$  transport and NaPi IIB expression.* The influence of the dietary P and Ca supply on the NaPi IIB transcription level was measured for each feeding group by performing quantitative Northern analyses; the ratio of NaPi IIB-mRNA to  $\beta$ -actin for the control group was  $1.26 \pm 0.24$ , for the -P group  $3.41 \pm 2.20$ , and for the -Ca group  $0.78 \pm 0.21$  (mean ± SD, n = 3). The high variance in the -P group was due to a single

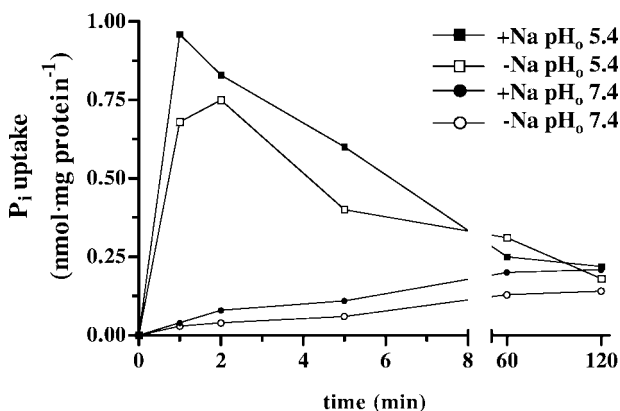


Fig. 2.  $P_i$  uptake in duodenal brush-border membrane vesicles (BBMV) as a function of time. The experiments were performed for 2 animals of each feeding group, resulting in identical overshoot phenomenon. Due to the high interindividual variation, data are given exemplarily for 1 -P goat. +Na/-Na means the presence and the absence of an inwardly directed  $Na^+$  gradient, respectively; pH<sub>0</sub> 5.4/pH<sub>0</sub> 7.4 means the presence and the absence of an inwardly directed  $H^+$  gradient, respectively.  $P_i$  overshoot was only observed in the presence of an inwardly directed  $H^+$  gradient. The slight stimulation of  $H^+$ -dependent  $P_i$  uptake by  $Na^+$  was seen in each animal.

Table 3. Kinetic parameters of  $H^+$ -dependent  $P_i$  transport in goat duodenal BBMV

	$V_{max}$ , nmol·mg protein <sup>-1</sup> ·10 s <sup>-1</sup>	$K_m$ , mM
Control	2.10 ± 1.25	0.45 ± 0.37
-P	1.28 ± 1.02	0.32 ± 0.33
-Ca	0.97 ± 0.45	0.36 ± 0.17

Values are given as means ± SD; n = 4 goats/group.  $H^+$  gradient was generated by setting extravascular pH at 5.4 and intravesicular pH at 7.4 in the absence of an inwardly directed  $Na^+$  gradient. BBMV, brush-border membrane vesicles.

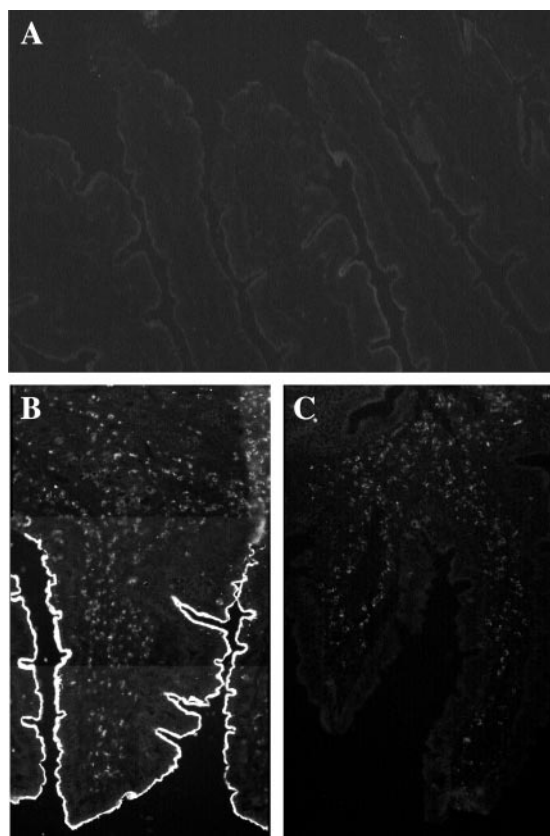


Fig. 3. Immunohistochemical detection of NaPi IIb in the apical membrane of goat midjejunum (B; control animal) but not of goat duodenum (A; control animal). NaPi IIb staining was performed in paraformaldehyde-fixed cryosections with mouse-specific anti-NaPi IIb antibody (1:500). Specific signal was generated using a secondary antibody labeled with DTAF (1:200). Incubation of jejunal tissues with secondary antibody only did not reveal any signal (C).

animal with a transcript level equivalent to that of the control animals. P restriction did not significantly increase the amount of NaPi IIb transcript. Reduced Ca supply did not influence the transcript level.

The relative abundance of specific NaPi IIb protein (ratio NaPi IIb to  $\beta$ -actin) as a function of transport capacity ( $V_{\max}$ ) of  $\text{Na}^+$ -dependent  $\text{P}_i$  transport into jejunal BBMV is shown in Fig. 4 for control and P-restricted goats. Higher  $\text{P}_i$  transport rates into BBMV were associated with increased amounts of specific NaPi IIb protein ( $r^2 = 0.63$ ). P restriction (Fig. 4 and Fig. 5, top left) stimulated this NaPi IIb-mediated  $\text{Na}^+$ - $\text{P}_i$  transport. The transport affinity ( $K_m$ ) of  $\sim 0.038$  mM was not changed by P restriction (Fig. 5, bottom left). In Ca-restricted animals, NaPi IIb protein levels were not affected (data not shown).

**pH sensitivity of jejunal  $\text{Na}^+$ - $\text{P}_i$  transport.** The proton sensitivity of  $\text{Na}^+$ - $\text{P}_i$  transport was studied in jejunal BBMV. Lowering the extravesicular pH from 7.4 to 5.4 at an intravesicular pH of 7.4 stimulated  $V_{\max}$  values significantly in both the control group (7.4/7.4:  $0.132 \pm 0.015$ ; 5.4/7.4:  $0.343 \pm 0.037$  nmol $\cdot$ mg protein $^{-1}\cdot 10$  s $^{-1}$ ) and the -P group (7.4/7.4:  $0.290 \pm 0.049$ ; 5.4/7.4:  $0.544 \pm 0.065$  nmol $\cdot$ mg protein $^{-1}\cdot 10$  s $^{-1}$ ) (Fig. 5, top). The increases in transport capacities

were associated with corresponding increases in  $K_m$ . Thus the affinities for  $\text{P}_i$  were reduced in response to an inwardly directed proton gradient. P restriction did not affect the pH-dependent changes in affinity (Fig. 5, bottom).

## DISCUSSION

$\text{P}_i$  transport was characterized structurally and functionally in goat duodenal and jejunal epithelia and in isolated BBMV from duodenal and jejunal enterocytes. Ion dependency, kinetic parameters, and adaptation to dietary P and Ca restriction were studied.

### *P<sub>i</sub> Transport in the Duodenum of Goats*

For sheep,  $\text{H}^+$ - $\text{P}_i$  transport in the upper small intestines has been described (13). This is in contrast to monogastric animals, in which duodenal  $\text{P}_i$  transport has been identified as a secondary active  $\text{Na}^+$ -coupled process (1).  $\text{Na}^+$ - $\text{P}_i$  cotransporter type IIb gene expression was found in the duodenum of mice (4) but not in goats (Fig. 1). Therefore,  $\text{P}_i$  transport in goat duodenum was studied to permit characterization of trans-epithelial  $\text{P}_i$  transport as affected by  $\text{H}^+$  and  $\text{Na}^+$ . The kinetic parameters of  $\text{H}^+$ - $\text{P}_i$  transport across brush-border membranes and influences of dietary P and Ca restriction were investigated.

**Functional properties of duodenal  $\text{P}_i$  transport system.** The significantly higher  $\text{Na}^+$ -independent  $\text{P}_i$  net transport at pH 5.4 compared with pH 7.4 on the mucosal side indicates an  $\text{H}^+$ -dependent  $\text{P}_i$  transport process in the duodenum of goats (Table 2). In the presence of high luminal  $\text{Na}^+$  concentrations, this transport is effectively stimulated (Table 2). These findings are confirmed by  $\text{P}_i$  uptake studies in duodenal BBMV. In the absence of extravesicular  $\text{Na}^+$ ,  $\text{P}_i$  uptake as a function of time exhibited an overshoot phenomenon only at low pH. This indicates a carrier-mediated  $\text{P}_i$  transport that is dependent of  $\text{H}^+$ . The

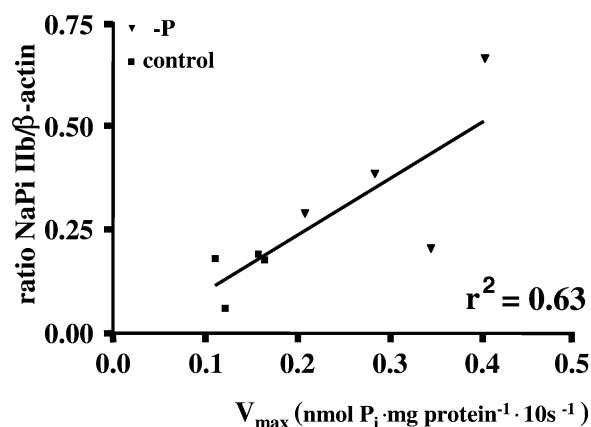


Fig. 4. Relative amounts of NaPi IIb protein in brush-border membrane of goat jejunum (given as ratio of NaPi IIb to  $\beta$ -actin) as a function of  $V_{\max}$  of jejunal  $\text{Na}^+$ -dependent  $\text{P}_i$  transport (given as nmol  $\text{P}_i \cdot \text{mg protein}^{-1} \cdot 10$  s $^{-1}$ ). Higher capacity of  $\text{Na}^+$ - $\text{P}_i$  transport was correlated with an increased abundance of NaPi IIb protein, indicating that the major extent of  $\text{Na}^+$ - $\text{P}_i$  transport was mediated by NaPi IIb.

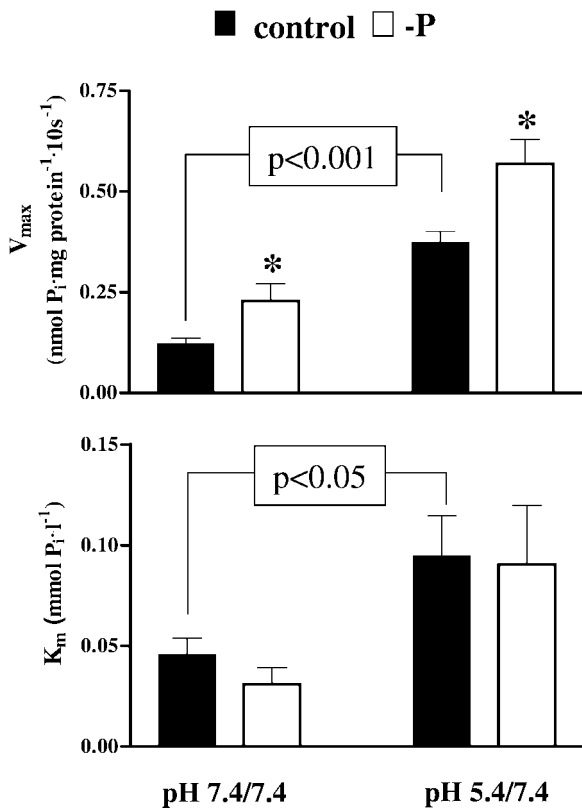


Fig. 5. Influence of dietary P restriction and pH on kinetic parameters of  $Na^+$ -dependent  $P_i$  transport in goat jejunum. Data are given as means  $\pm$  SE,  $n = 8$ /group, significance level for the effect of P restriction was  $P < 0.05$  (\*).  $V_{max}$  values of  $Na^+$ - $P_i$  transport into BBMVs were significantly increased in P restriction ( $*P < 0.05$ ) independent of an inwardly directed  $H^+$  gradient. Reducing the extravesicular pH to 5.4,  $V_{max}$  as well as  $K_m(P_i)$  values increased significantly ( $P < 0.001$  and  $P < 0.05$ , respectively).  $K_m(P_i)$  was not affected by P restriction. Interaction between P restriction and pH was not significant.

presence of extravesicular  $Na^+$  at low pH had a slight stimulating effect on  $P_i$  uptake (Fig. 2). This might have been due to a direct positive interaction of  $Na^+$  ions with the  $P_i$  carrier in analogy to that which has been described for the  $H^+$  ion as “competitive inhibition” interaction with the  $Na^+$  binding site of renal NaPi IIa (10). The affinity of the duodenal  $H^+$ -dependent  $P_i$  transporter is substantially lower than that of  $Na^+$ - $P_i$  cotransporter type IIb. The lack of NaPi IIb expression, the difference in affinity, and the  $H^+$  dependency of  $P_i$  transport in the goat duodenum indicate a novel kind of  $P_i$  transporter for which molecular structure still has to be identified.

**Influence of dietary P or Ca restriction on duodenal  $P_i$  transport.** Dietary P and Ca restriction did not change transepithelial net  $P_i$  flux rates (Table 2) or  $P_i$  transport capacities across brush-border membranes (Table 3) in goat duodenum. The order of magnitude of duodenal  $H^+$ - $P_i$  transport capacity ( $V_{max}$ : 2.1 nmol·mg protein $^{-1}$ ·10 s $^{-1}$ ) as well as transporter affinity ( $K_m$ : 0.4 mM) in goats correspond to those reported for sheep [ $V_{max}$ : 3.6 nmol·mg protein $^{-1}$ ·10 s $^{-1}$ ;  $K_m$ : 0.6 mM (13)]. Unlike in goats, in sheep, dietary P restriction (low-P

diet) resulted in a higher duodenal  $P_i$  transport capacity compared with control (high-P diet) (13). However, because P intake of the sheep was not precisely defined in that study, this discrepancy cannot be explained. A surplus of dietary P could effect a decrease of  $P_i$  transporter expression as observed for jejunal NaPi IIb in goats getting twice as much dietary P as required (unpublished results). This would also result in differences of transport capacity between low- and high-P groups.

#### *P<sub>i</sub> Transport in the Jejunum of Goats*

$Na^+$  dependency and kinetic parameters of jejunal  $P_i$  transport in goats are well defined (14). Structurally, an  $Na^+$ - $P_i$  transporter could be identified that belongs to the NaPi IIb family (5), but it is still not clear whether  $Na^+$ - $P_i$  transport is exclusively mediated by NaPi IIb transporters. There is now strong evidence for the importance of NaPi IIb for  $Na^+$ - $P_i$  transport for the following reasons. First, NaPi IIb is located in the apical membrane of goat enterocytes (Fig. 3). Second, the affinity of goat jejunal  $Na^+$ - $P_i$  transport as determined by studies of  $P_i$  uptake into isolated BBMVs [ $K_m \sim 0.03$  mM; Fig. 5 (14)] is in the same range as that described for the murine NaPi IIb (4). Third, the transport capacity of goat jejunal  $Na^+$ - $P_i$  transport is positively correlated to the amounts of specific NaPi IIb protein in the brush-border membranes (Fig. 4). Therefore, it is concluded that NaPi IIb mediates the major part of  $Na^+$ -dependent  $P_i$  transport in goat jejunum.

**Influence of dietary P and Ca restriction on the NaPi IIb expression.** Dietary P restriction in mice resulted in increased intestinal NaPi IIb protein levels at unchanged NaPi IIb mRNA levels, indicating a posttranscriptional process for adaptation (3). Until recently, adaptation of jejunal  $P_i$  transport to dietary P and Ca restriction in ruminants has been studied at the functional level only. P restriction in goats increased transepithelial net  $P_i$  flux rates (15); it increased transport rates into BBMVs without influencing  $P_i$  affinity of the transport system (Fig. 5). These processes are now being studied at the molecular basis in ruminants. Dietary P restriction did not induce significant increases in specific expression of NaPi IIb at the mRNA level. However, the ability of individual animals to adapt to low dietary P intake cannot be excluded. The expression of specific NaPi IIb protein was stimulated significantly (Fig. 4). Therefore, it is suggested that molecular adaptation to dietary P restriction in ruminants is mediated by an increased specific transporter protein level without increased corresponding gene transcription rate. However, an unaffected amount of NaPi IIb mRNA might also have been the result of an increased transcription rate with a corresponding increase in mRNA turnover rate. Ca restriction enhanced neither mRNA nor protein level, although in earlier experiments, an increase in  $V_{max}$  of  $P_i$  uptake into jejunal BBMVs of Ca-restricted goats was observed (14). Jejunal  $P_i$  transport of Ca-restricted goats may be

upregulated by changing the sodium affinity of the transport system.

*Influence of protons on the function of the Na<sup>+</sup>-dependent P<sub>i</sub> transport.* H<sup>+</sup> strongly stimulated goat jejunal Na<sup>+</sup>-P<sub>i</sub> transport in both control and P-restricted groups (Fig. 5, top), whereas transporter P<sub>i</sub> affinity was significantly lowered by H<sup>+</sup> (Fig. 5, bottom). Na<sup>+</sup> affinity was not examined in this study, but the changes in transport rates observed here could be due to an increase in Na<sup>+</sup> affinity resulting in a higher P<sub>i</sub> transport across apical membranes. From studies on renal P<sub>i</sub> transport, it is already known that H<sup>+</sup> can influence Na<sup>+</sup> binding at the NaPi II transporters. For renal NaPi type IIa, another member of the Na<sup>+</sup>-P<sub>i</sub> cotransporter family that is expressed only in the kidney, a significant but opposite pH sensitivity has been described (6, 10). NaPi IIa activity decreases with increased proton concentrations by the competition of H<sup>+</sup> with Na<sup>+</sup> for interaction with the carrier. Preferential transport of divalent P<sub>i</sub> also contributes to the pH dependency observed here (10). Three charged amino acids in the third extracellular loop (REK) were identified as the molecular determinants for pH sensitivity by site-directed mutagenesis (6). In intestinal murine NaPi IIb, three neutral amino acids (GNT) corresponded to this position, resulting in no or a slight increase in P<sub>i</sub> transport activity at higher luminal proton concentrations (4). Unlike in mice, the stimulating effect was more pronounced in goats (where the pH was lowered to 5.4 as opposed to 6.0 in mice studies); however, no molecular mechanism of a direct H<sup>+</sup>-Na<sup>+</sup> "interaction" on the transporter is as yet known. A preferential transport of monovalent P<sub>i</sub> ions could also contribute to intestinal pH sensitivity. Furthermore, the pH sensitivity could be due to the coexpression of the duodenal H<sup>+</sup>-P<sub>i</sub> transport system and the jejunal NaPi IIb transporter in the goat jejunum. Therefore, P<sub>i</sub> uptake studies into jejunal BBMV were performed in the absence of Na<sup>+</sup> in the extravesicular fluid at pH 7.4 and 5.4, respectively. Under these conditions, P<sub>i</sub> uptake seemed to be linearly dependent on P<sub>i</sub> concentration at both pH levels and was ~10-fold lower than in the presence of Na<sup>+</sup> (unpublished observations), indicating that the H<sup>+</sup>-P<sub>i</sub> transport system is apparently not expressed in goat jejunum.

The physiological relevance of this marked H<sup>+</sup> responsiveness of intestinal P<sub>i</sub> transport in goats could be reflected by the lower pH values in the digesta of the upper small intestines. The comparably low bicarbonate secretions into the upper small intestines of sheep are responsible for the relatively acid pH levels of ~2–4 up to the upper jejunum, whereas in nonruminant animals the pH of digesta rises rapidly to values above 6 at the beginning of the duodenum (7).

The present study has shown clear evidence that at least two different mechanisms are involved in goat

intestinal P<sub>i</sub> absorption. In the duodenum, there is a proton-dependent, sodium-sensitive system that is not influenced by dietary P restriction. In the jejunum, P<sub>i</sub> transport is mediated by a sodium-dependent, proton-sensitive system, mainly by NaPi cotransporter type IIb. This P<sub>i</sub> transport system adapts to dietary P restriction by increasing the transporter capacity due to higher transporter protein expression.

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