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Contributions of different NaPi cotransporter isoforms to dietary regulation of P transport in the pyloric caeca and intestine of rainbow trout

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Summary

The anatomical proximity and embryological relationship of the pyloric caeca (PC) and small intestine of rainbow trout has led to the frequent assumption, on little evidence, that they have the same enzymes and transporters. In trout, the PC is an important absorptive organ for dietary nutrients, but its role in dietary P absorption has not been reported. We found that apical inorganic phosphate (Pi) transport in PC comprises carrier-mediated and diffusive components. Carrier-mediated uptake was energy- and temperature-dependent, competitively inhibited and Na^+-independent, and greater than the Na^+-dependent intestinal uptake. Pi uptake in PC was pH-sensitive in the presence of Na^+. Despite the active Pi transport system in PC, high postprandial luminal Pi concentrations (~20 mmol l^{-1}) indicate that diffusive uptake represents ~92% of total Pi uptake in PC of fed fish. The nucleotide sequence of a sodium-phosphate cotransporter (NaPi-II) isoform isolated from PC was ~8% different from the intestinal NaPi cotransporter. PC-NaPi mRNA was abundant in PC but rare in the intestine, whereas intestinal NaPi mRNA was abundant in the intestine but scarce in PC. Dietary P restriction reduced serum and bone P concentrations, increased intestine-type, but not PC-type, NaPi mRNA in PC, and increased Pi uptake in intestine but not in PC. Intestine-type NaPi expression may be useful for predicting dietary P deficiency.

Key words: NaPi cotransport, phosphorus, rainbow trout, pyloric caeca, intestine.

Introduction

Many species of fish have a unique digestive/absorptive organ called the pyloric caeca (PC). PC are finger-like diverticula stemming from the first half of the proximal intestine. Rainbow trout *Oncorhynchus mykiss* has about 56 PC, which collectively contribute about 70% of the total gut surface area (Buddington and Diamond, 1987). Embryologically, the PC are only extensions of the proximal intestine (Yasutake and Wales, 1983), hence they are thought to be digestive compartments active in resorption of certain nutrients (Fange and Grove, 1979). Because of the anatomical proximity, histological similarity and embryological relationship of the PC and small intestine, it was assumed that their enzymes and transport systems are the same. As a consequence, there have been very few studies on PC. In the present work, we tested the hypotheses that PC possess the same or similar transporters to those in the intestine, and that dietary regulation of phosphorus (P) transport is also similar in PC and intestine. We also tested the hypotheses that PC play an important role in the absorption of dietary inorganic phosphate (Pi) in rainbow trout, and that changes of NaPi cotransporter expression and function in PC precede clinical P deficiency.

In mammals, the main site of dietary P absorption is the proximal small intestine (Danisi and Murer, 1991). In fish, however, little is known about the site of dietary P absorption. In brook trout, serum ^{32}P concentrations increased rapidly in the first few hours following consumption of a calcium-free synthetic diet containing ^{32}P (Phillips et al., 1961). The rapid increase of ^{32}P in the blood was thought to be due to transmural transport *in vivo* of Pi through the stomach. In carp, Pi transport *in vitro* from mucosa to serosa occurred against a concentration gradient, and this active uptake was greatest in the middle intestine (Nakamura, 1985). Active Pi transport in rainbow trout was also higher in the proximal than distal intestine (Avila et al., 2000). We have previously detected NaPi cotransporter mRNA and protein in trout PC (Sugiura et al., 2003), but no functional study of the role of PC in dietary P absorption and transport has been reported.

Despite the absence of knowledge on the caecal role of dietary P absorption, the absorption of some other nutrients in PC is known. Digestibility *in vivo* of 18 amino acids in trout PC was 60–80% in fish fed a fishmeal-based diet (Dabrowski and Dabrowska, 1981). The contribution of PC to the total uptake capacity (of the entire gut) for glucose, the dipeptide

carnosine and nine amino acids was 68–81%, which corresponded to the contribution of PC to the gut total surface area in trout (~70%; Buddington and Diamond, 1987). These studies suggest that uptake per unit surface area is similar in PC and intestine and that PC plays a dominant role in total amino acid absorption in trout.

Dietary regulation of Pi transport and the NaPi-II transporter mRNA/protein expression in the intestine has been studied in mammals (Hattenhauer et al., 1999; Huber et al., 2000, 2002; Katai et al., 1999) and fish (Coloso et al., 2003), but nothing was known regarding Pi transport, NaPi-II expression or their dietary regulation in PC. Since PC is a primary site of dietary nutrient absorption in trout (Buddington and Diamond, 1987), it is crucial to study its regulatory mechanism.

Rainbow trout is an important aquaculture species in many parts of the world, and fed intensively with pellet feeds. Unfortunately, the absorption of dietary P in contemporary commercial feeds appears to be only about 40–50% (Hardy and Gatlin, 2002), meaning that the majority of dietary P is excreted into the aqueous environment where P is the limiting nutrient for eutrophication. Excessive discharge of P from aquaculture facilities, therefore, causes excessive algal blooms, eventually leading to the destruction of ecosystems. Knowledge of the mechanisms underlying absorption of dietary P may help improve absorption of dietary P by the fish, and alleviate the environmental burden of aquaculture.

We initially measured Pi and H^+ concentrations in the lumen of the gastrointestinal (GI) tract, and determined the kinetics and energy-, Na- and pH-dependence of Pi transport in PC. We then compared partial sequences and sites of expression of NaPi cotransporter in the GI tract, and dietary regulation of P uptake between PC and intestine. Finally, we correlated NaPi cotransporter gene expression and function to traditional indices of dietary P deficiency. We found that PC is the major site of dietary P absorption, and that the absorption is modulated by several factors, including temperature, luminal pH, luminal (dietary) Pi concentration and fish P status. We also found that the nucleotide sequence and dietary regulation of NaPi cotransporter in PC differ from those in the intestine.

Materials and methods

Fish and sampling

Post juvenile, immature rainbow trout, *Oncorhynchus mykiss* Walbaum (mean body mass 150–200 g) were fed to satiation, once daily in the morning. Fish were kept in three large (265 l) temperature-controlled (15°C) recirculating tanks, and were used in all experiments as needed except in the regulation study. In the regulation study, fish (mean body mass 222±11 g, N=40) were initially fed the commercial diet, then at day 0 each fish was force-fed either a low-P diet (LP) containing 0.07% available P, or a high-P diet (HP) containing 0.84% available P, for 20 days at 1.5% of their individual body mass daily. Fish were slightly anesthetized with tricane methane sulfonate (100 mg l^{-1}), and the feed pellets were directly introduced into the stomach of each fish using a polished glass tube and a plunger according to Post et al. (1965). The feeding itself took less than a few seconds per fish. There was no mortality during the experimental period. All fish were treated according to the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Medicine and Dentistry of New Jersey.

Fish were killed 6 h after feeding by severing the spinal cord immediately posterior to the cranium. PC, intestine and blood samples were collected immediately. PC and intestine were gently perfused with an ice-cold Krebs Ringer buffer, kept in the ice-cold buffer, and used for the uptake assay within 1 h, or immediately stored in the RNAlater solution (Ambion, Inc., Austin, TX, USA) for later measurements of NaPi cotransporter mRNA abundance. Blood samples were collected from the caudal peduncle, and the serum was separated within 1 h by centrifugation (12 000 ***g***, 5 min) and stored (–20°C) until analysis. Bone samples (anterior 1/3 of the vertebral column) were collected from whole fish stored at –20°C. Fish were briefly heated to 80–90°C, and skeletal muscle was removed. The vertebral column was washed in warm tapwater, dried, defatted (methanol:chloroform=1:1, v/v), and redried to constant mass.

Diet composition

Commercial trout feed used in the uptake study had the following analytical composition (%, dry basis): crude protein 46.4; fat 21.8; crude fiber 2.6; ash 10.39; Ca 2.45; total P 1.50; available P 0.716. Experimental LP and HP diets used in the regulation study contained the following ingredients (%, dry basis): commercial trout feed (acid-washed) 70; wheat gluten 5; egg albumin 5; wheat flour 10; soybean oil 10. To this mixture were added vitamin and mineral premix 25 g kg^{-1}, $CaCO_3$ 30 g kg^{-1}, $NaH_2PO_4{\cdot}H_2O$ 38 g kg^{-1} (HP diet only), and NaCl 16.4 g kg^{-1} (LP diet only). The dough was cold-extruded, air-dried and crumbled to make pellets. The diets had the following analytical compositions (%, dry basis): total P 0.27 (LP) or 1.30 (HP); available P 0.07 (LP) or 0.84 (HP); total Ca 1.82 (LP) or 1.83 (HP). The available P contents were previously determined in a separate experiment, following a standard method using chromic oxide as a marker (Bolin et al., 1952).

Pi uptake assay

Tissue Pi uptake rates were determined *in vitro* according to Karasov and Diamond (1983) modified by Avila et al. (2000). Briefly, each sleeve of PC (1 cm long) was carefully everted and tied to a stainless steel rod of an appropriate diameter. The sleeve was then pre-incubated for 5 min at 15°C in a Ringer solution gassed with 99%O_2/1%CO_2. The Ringer solution had the following composition (mmol l^{-1}): NaCl 136.6; KCl 4.83; $CaCl_2$ 1.53; Hepes 5. The final pH was adjusted to 7.3–7.4 with HCl or KOH (15°C). To this, NaH_2PO_4 was added as needed, replacing a portion of NaCl on an iso-osmotic basis. The sleeve was incubated at 15°C in the Ringer solution (10 ml) for 5 min with vigorous stirring (1200 r.p.m.) to reduce unstirred layer effects. All the incubation (Ringer) solutions contained

Table 1. *Preincubation and incubation procedure used during the first* experiment on inhibition of Pi uptake*

	Incubation components (mmol l^{-1} Ringer)	
Treatment	Pre-incubation*	Incubation
Normal	5 Pi	tracer Pi
Na– choline	5 Pi, choline chloride	tracer Pi, choline chloride
Na– KCl	5 Pi, KCl	tracer Pi, KCl
Ouabain	5 Pi, 5 ouabain	tracer Pi, 5 ouabain
P++	5 Pi	tracer Pi, 10 Pi
PFA	5 Pi, 5 PFA	tracer Pi, 5 PFA
NaN_3	5 Pi, 10 NaN_3	tracer Pi, 10 NaN_3
Cold	5 Pi	tracer Pi, ice-cold

Pre-incubation conditions were ice-cold (2°C), 30 min; incubation conditions were 15°C, 6 min.

Normal incubation medium was Ringer solution containing Pi as indicated.

*In experiment 2, tissues were pre-incubated at 15°C for 5 min in medium containing no Pi.

Na– choline: iso-osmotic choline chloride replaced all NaCl (see text for details).

Na– KCl: iso-osmotic KCl replaced all NaCl (see text for details).

Tracer Pi, see text.

PFA, phosphonoformic acid; NaN_3, sodium azide.

$H_3{}^{32}PO_4$ (Perkin Elmer Life Sciences, Boston, MA, USA) and 1,2-^{3}H polyethylene glycol (^{3}H-PEG), (Perkin Elmer). The ^{3}H-PEG was added as a non-absorbable inert polymer to correct adhering fluid on the tissue surface. The tissue radioactivity was determined for ^{32}P and ^{3}H using a liquid scintillation counter (LS 7800, Beckman Instruments, Irvine, CA, USA). We chose a 5 min incubation period since, in a preliminary assay, this duration was long enough to measure Pi uptake accurately and uptake was linear within this incubation duration. The initial and final radioactivity (^{32}P and ^{3}H) of the incubation media was the same in all experiments.

The inhibition assay was conducted twice (Table 1). All the incubation media, except that containing excess unlabelled Pi (P++), contained only tracer Pi (^{32}P as H_3PO_4) in order to measure primarily the active component of Pi transport. The tracer ^{32}P concentration was 0.284 nmol l^{-1} (experiment 1) and 0.170 nmol l^{-1} (experiment 2). Two Na^+-free media were prepared in which choline chloride or KCl replaced all NaCl in the Ringer on an iso-osmotic basis. The pH was adjusted to 7.3–7.4 with KOH. Three potential inhibitors of Pi uptake were also tested: (i) an inhibitor of Na^+/K^+ ATPase, ouabain, 5 mmol l^{-1} (Lucu and Flik, 1999), to determine dependence of Pi uptake on Na^+ gradient across the apical membrane; (ii) a known specific inhibitor of mammalian NaPi, phosphonoformic acid (PFA), 5 mmol l^{-1} (Loghman-Adham, 1996), to determine involvement of NaPi in Pi uptake; and (iii) an inhibitor of cytochrome *c* oxidase in the mitochondrial respiratory chain, sodium azide (NaN_3), 10 mmol l^{-1} (Utoguchi et al., 1999), to determine ATP-dependence of Pi uptake. All the inhibitors were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Excess P (P++) was also added to determine whether tracer P uptake by trout PC is carrier-mediated. Trout can survive at freezing temperature, hence the effect of a colder temperature was assessed by determining uptake in an ice-cold (2°C) medium. We then estimated the temperature coefficient Q_{10} from the following equation:

$$Q_{10} = (k_2/k_1)^{10/(T_2-T_1)}, \quad (1)$$

where k_1 and k_2 = rates at temperatures T_1 and T_2, respectively.

Luminal pH and Pi concentration

Luminal contents were collected immediately after killing fish (6 h after feeding with commercial trout feed). Surgical clamps were used to separate the gut sections before dissecting the gut. Luminal contents were immediately centrifuged after collection (12 000 ***g***, 5 min) in microfuge tubes (1.5 ml) to obtain luminal fluid. The pH of the fluid was measured immediately after centrifugation using pH strip paper (colorpHast, EM Science, Gibbstown, NJ, USA) calibrated to standard buffer solutions. The pH of the adherent fluid (fluid remaining on the tissue after luminal fluid was collected) was determined by applying the pH strip paper directly onto the luminal surface of the dissected tissue. The remaining luminal fluid was stored (–20°C) in microfuge tubes. The Pi concentration was determined (Taussky and Shorr, 1953) on the frozen-stored samples after they had been thawed, acidified (5% TCA) and completely solubilized. Fish used for luminal pH and [Pi] measurements were not used for other experiments.

Serum Pi and bone P concentrations

Serum Pi and bone P concentrations of fish fed LP or HP diets were determined to verify the P status of the fish. The serum Pi was determined on the frozen-stored samples (Taussky and Shorr, 1953). Bone samples (defatted and dried) were ashed at 550°C overnight, acid-solubilized (hydrochloric:nitric acid, 1:1), partially neutralized, diluted and analyzed for P (Taussky and Shorr, 1953).

Degenerate RT-PCR and subcloning

Total RNA was extracted from RNAlater-stabilized tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and reverse-transcribed using NaPi-specific degenerate primers designed from consensus sequences of other species (forward: 5′-GCTGGIGAYATCTTCMAGG-3′, reverse: 5′-AAGTGR-CASARIGCAATCTG-3′, corresponding to nt positions 344–362 and 1403–1422, respectively, in the flounder sequence; GenBank accession number U13963). The PCR product of the expected size (1079 bp) was gel-purified, subcloned and sequenced (GenBank accession number AY500241).

RT-PCR–Southern blot

Specific primers were designed from a unique region where the two closely related NaPi cotransporters have different nucleotide base sequences. Thus, the primers amplified either intestine-type NaPi (I-NaPi) or PC-type NaPi (PC-NaPi)

cotransporters, but not both. This specificity was verified by amplifying plasmid clones containing either the I-NaPi or PC-NaPi insert (result not shown). The forward primer was common (5′-GTCTTCTGGATTGCTGGAGGTC-3′, corresponding to nt positions 469–490 in the flounder sequence), and only the reverse primers differed, although they were derived from the same corresponding position (I-NaPi: 5′-GGTCAGGCAGGTT-AGCATAGG-3′, PC-NaPi: 5′-GGTCAGGCAGGTTAGCA-AAAAC-3′, corresponding to nt positions 1011–1031 and 1010–1031, respectively, in the flounder sequence). β-actin primers were as follows: forward: 5′-ACATCAAGGAGA-AGCTGTGCTAC-3′, reverse: 5′-TACGGATGTCCACGTC-ACAC-3′, nt positions 683–705 and 903–922, respectively, in rainbow trout β-actin sequence (GenBank accession number AF157514).

Total RNA (0.5 μg) from PC and the proximal intestine was reverse-transcribed (RT) using oligo-dT_{20} to make cDNA from mRNA, and 1.0 μl of this solution was PCR-amplified using either the I-NaPi-specific primer or the PC-NaPi-specific primer for 14 cycles at 94°C 30 s, 63°C 30 s, 72°C 60 s, per cycle. β-actin served as the control housekeeping transcript, being independent of dietary P intake or NaPi cotransporter distribution, and was amplified for 7 cycles at 94°C 30 s, 58°C 30 s, 72°C 60 s, per cycle. I-NaPi, PC-NaPi and β-actin mRNA abundances were determined from the same RT preparations. Possible genomic DNA contamination was negligible since the number of PCR cycles was small. PCR products were separated on a 1% TAE-agarose gel, and Southern-transferred onto Hybond-NX nylon membranes (Amersham Biosciences, Piscataway, NJ, USA) according to a standard protocol (Brown, 1995). A mixture of pure cDNAs (I-NaPi, PC-NaPi and β-actin, on an equimolar basis) was labeled with ^{32}P-dCTP using Rediprime II DNA labeling system (Amersham). The membranes were pre-hybridized in FPH solution (5× SSC, 5× Denhardt solution, 50% formamide, 1% SDS, and 100 μg yeast tRNA ml^{-1}) for 2 h at 42°C, to which the labeled probes were added and hybridized overnight at 42°C. The membranes were washed twice at 42°C with 2× SSC–0.1% SDS for 30 min each, then once at 50°C and 2–3 times at 60°C with 0.1× SSC–0.1% SDS for 30 min each, and exposed to BioMax MR film (Kodak, Rochester, NY, USA) for 6–12 h at –80°C.

The RT-PCR-Southern blotting procedure was chosen to quantitate I-NaPi and PC-NaPi abundance because northern blots might not clearly distinguish between the two closely related transcripts. Since the number of PCR amplification cycles was very low, there was no risk of reaching the asymptote of the amplification plateau indicating the end of PCR-induced exponential increases in product concentration. To validate this procedure, I-NaPi mRNA abundance was also quantified by northern blot in the proximal intestine where PC-NaPi was almost absent, and the result was almost identical to that of RT-PCR-Southern blot (data not shown).

Statistical methods

Values are means ± S.E.M. (N). The treatment means were compared by a two-tailed t-test (for two treatments) or a one-way ANOVA followed by the Newman–Keuls multiple comparison test (for three or more treatments). The population variances of uptake and concentration data were tested by Bartlett's test, and when the variances were heterogeneous at $P<0.05$, the data were log-transformed before analysis of variance (ANOVA). The kinetic parameters of Pi uptake were determined by nonlinear regression using the Michaelis–Menten equation:

$$\dot{V}_{[S]} = (\dot{V}_{max}[S]) / (K_m+[S]) + K_d[S] , \quad (2)$$

where $\dot{V}_{[S]}$ = uptake rate at concentration [S], [S] = Pi concentration; $\dot{V}_{max}$ = maximum uptake rate, K_m = Michaelis constant and K_d = diffusion coefficient. The kinetic parameters of the saturable component were confirmed using the Eadie–Hofstee plot. Potential correlations among serum Pi concentration, bone P concentration, relative mRNA abundance and uptake rates from the dietary regulation study were determined by subjecting the data to a linear regression analysis, using the significance of the slope as the criterion determining degree of correlation. The significance threshold was set at $P<0.05$. All statistical calculations were performed using Prism 4 (Graphpad Software, Inc., San Diego, CA, USA).

Results

The Pi concentration of the luminal fluid of trout fed commercial diet was high (~40 mmol l^{-1}) in stomach, moderately high in PC (~20 mmol l^{-1}), and very low in all sections of the intestine (<5 mmol l^{-1}) (Fig. 1A). The pH of the adherent and luminal fluids was low in stomach (~3.8), high in PC (7.0–7.5) and even higher in the intestine (8.0–8.5) (Fig. 1B).

Total Pi transport continued to increase as the Pi concentration in the incubation medium increased (Fig. 2A). The increase was linear at Pi concentrations higher than 1 mmol l^{-1}. The slope of the linear component (K_d=0.039±0.002 min^{-1}) was similar to the slope (0.042±0.008) of uptakes determined in the presence of the NaPi-competitive inhibitor PFA; both slopes were assumed to represent passive transport. When the linear component was subtracted from the total Pi transport, the remainder was the saturable component of Pi transport. The saturable component, with a $\dot{V}_{max}$=64.6±7.6 nmol g $tissue^{-1}$ min^{-1} and a K_m=0.474±0.199 mmol l^{-1}, was assumed to represent the carrier-mediated component of transport. These kinetic parameters calculated by nonlinear regression correlated well with those estimated by the Eadie–Hofstee plot (Fig. 2B).

Tracer Pi uptake was not inhibited by replacement of NaCl by choline chloride or KCl in the preincubation and incubation media (Fig. 3A). Preincubation and incubation of the tissue in ouabain also had no effect on Pi uptake. Addition of excess non-labeled Pi (10 mmol l^{-1}) dramatically reduced but did not abolish tracer Pi uptake. Addition of PFA and, to a lesser extent, NaN_3 also significantly reduced Pi uptake. These

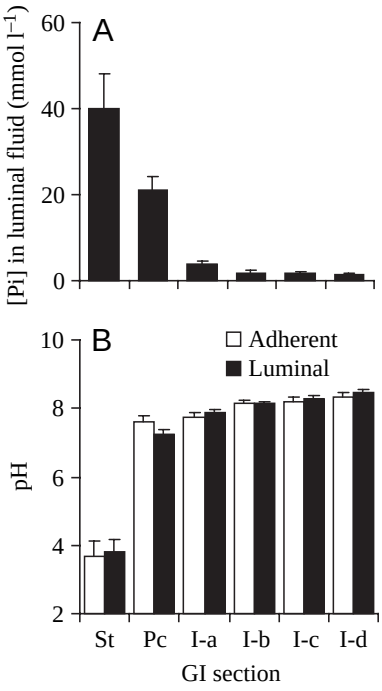

Fig. 1. Pi concentrations of the luminal fluid (A) and pH of the adherent and luminal fluid (B) from various sections of the alimentary canal in rainbow trout consuming commercial feed pellets. GI, gastro-intestinal; St, stomach; Pc, pyloric caeca; I-a, pyloric caeca region of the proximal intestine; I-b, post pyloric caeca region of the proximal intestine; I-c, first 1/2 of the distal intestine; I-d, second 1/2 of the distal intestine; Adherent, adherent fluid; Luminal, luminal fluid. Each column represents the mean ± S.E.M. (*N*=5 fish). Samples were collected 6 h postprandial.

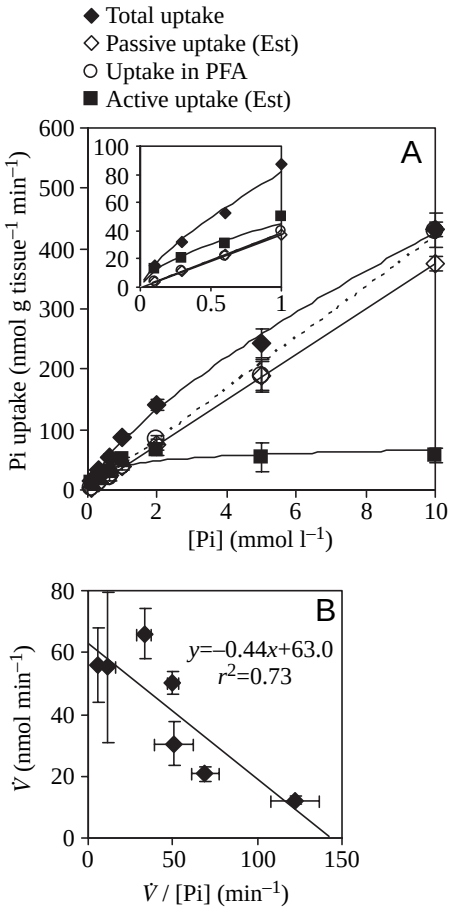

Fig. 2. Total, passive and active Pi uptake in the pyloric caeca of rainbow trout in incubation media containing various [Pi]: (A) Nonlinear plot of total, passive and active components; inset shows enlarged section of first part of curve. (B) Eadie–Hofstee plot of active component. Values are means ± S.E.M. (*N*=6–10 tissues from 3–4 fish). Total Pi uptake was determined in the normal incubation medium, and passive Pi uptake was estimated (Est.) from the slope of normal incubation medium (>1 mmol l^{-1}). The active component of Pi uptake in the pyloric caeca was estimated by difference. Pi uptake in PFA-containing medium (broken line) was slightly higher than the estimated passive uptake at high Pi concentrations (>5 mmol l^{-1}). Using Equation 2, $\dot{V}_{max}$=64.6±7.6 (mean ± S.E.M.) nmol g $tissue^{-1}$ min^{-1}; K_m=0.474±0.199 mmol l^{-1}; K_d=0.039±0.002 min^{-1}. In A, percentages of the active component in total Pi transport were as follows (*N*=6–10): 99.8 (extrapolation) at 0.28 nmol l^{-1} (tracer concentration); 74.5±2.6 at 0.1 mmol l^{-1}; 62.7±3.1 at 0.3 mmol l^{-1}; 51.2±6.2 at 0.6 mmol l^{-1}; 56.5±1.9 at 1 mmol l^{-1}; 45.9±3.0 at 2 mmol l^{-1}; 17.8±8.4 at 5 mmol l^{-1}; 12.5±2.5 at 10 mmol l^{-1}; and 7.7 (extrapolation) at 20 mmol l^{-1}.

observations were confirmed in another experiment with slightly different conditions and using a different batch of fish (Fig. 3B).

Tissues incubated at 2°C had lower uptake rates (54.5%, *P*=0.04) compared with those incubated at 15°C. The Q_{10} of carrier-mediated Pi uptake was estimated to be 1.630±0.144 (*N*=8). Pi uptake in Na^+-containing medium increased markedly between pH 7 and 9 (Fig. 4). Pi uptake in Na^+-free medium was significant but did not change significantly between pH 4 and 9.

The nucleotide sequence of NaPi cotransporter mRNA isolated from trout PC (GenBank accession number AY500241) was different from those of all previously known NaPi cotransporter mRNAs, including that of the trout intestinal NaPi cotransporter mRNA. The PC-NaPi cotransporter mRNA, however, was most closely related to trout I-NaPi mRNA (92% identity in nucleotide base sequence), followed by flounder kidney/intestine NaPi cotransporter mRNA (80%), and carp kidney NaPi-IIb2 transporter mRNA (77%). The identity of PC-NaPi mRNA with trout renal NaPi cotransporter mRNA was low (67%). The unique bases were evenly distributed over the sequenced stretch (~1.08 kb), and were mostly conservative substitutions.

In the stomach, neither I-NaPi nor PC-NaPi mRNA was detected (Fig. 5). In PC, PC-NaPi mRNA was generally more abundant than I-NaPi mRNA. The total abundance of these two isoforms, however, was lower in PC than in the intestine. In the proximal intestine, I-NaPi mRNA was very abundant, whereas only a trace amount of PC-NaPi mRNA was detected. In the distal intestine, I-NaPi mRNA was abundant, whereas PC-NaPi mRNA was not detected.

In both the intestine and PC, total Pi-uptake was independent

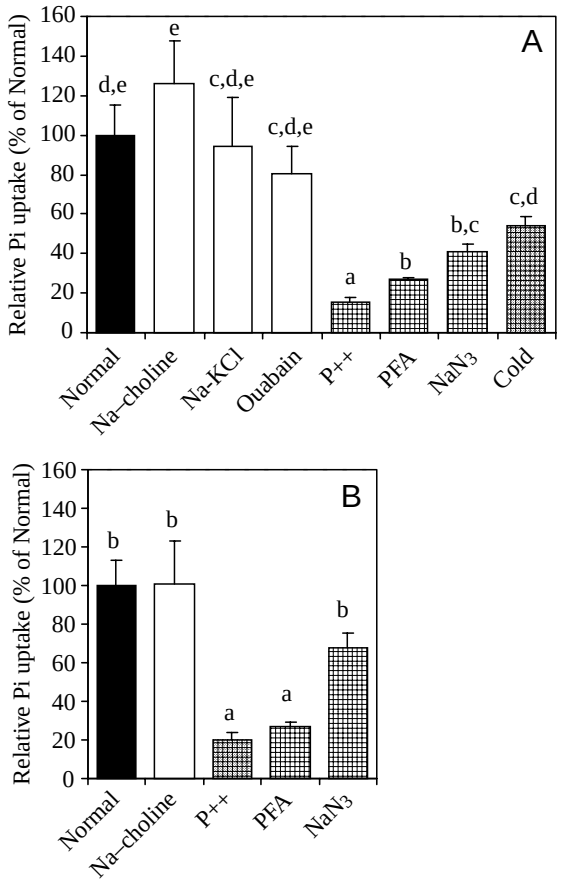

Fig. 3. Effects of various inhibitors and inhibitory conditions on tracer Pi uptake in the pyloric caeca of rainbow trout. (A) Experiment 1; (B) experiment 2. Normal (Ringer); Na-choline (Na– free Ringer, Na replaced by choline); Na– KCl (Na-free Ringer, Na replaced by K); Ouabain (Normal plus ouabain, 5 mmol l^{-1}); P++ (Normal plus 10 mmol l^{-1} non-labeled Pi); PFA (Normal plus phosphonoformic acid, trisodium hexahydrate, 5 mmol l^{-1}); NaN_3 (Normal plus sodium azide, 10 mmol l^{-1}). Cold [tissues incubated as in Normal but ice-cold (<2°C) medium]. All the tissue incubation media (except P++) contained only tracer Pi (^{32}P) as the Pi source. Estimated active Pi uptake at tracer Pi concentration was ~99.8% of the total uptake. Values are means ± S.E.M. (N=6–8 tissues in A; N=5 tissues in B). Columns with uncommon letters are different (P<0.05) by the Newman–Keuls test. All data were log-transformed before ANOVA. See Materials and methods and Table 1 for the assay procedures.

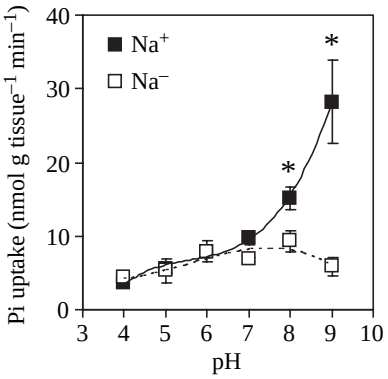

Fig. 4. Effect of pH on the total uptake of Pi in pyloric caeca of rainbow trout. Values are means ± S.E.M. (N=5). *At the same pH, Pi uptake in Na-containing (Na+) medium was significantly greater than that in Na-free (Na–) medium (two-tailed t-test). The incubation medium contained 0.1 mmol l^{-1} Pi, and estimated active Pi uptake was ~75% of the total uptake. In Na-free medium, choline chloride replaced NaCl of the normal medium iso-osmotically. The pH of the medium was adjusted using HCl and KOH. A single buffer system (Tris-citrate) was used for all the incubation media. Pi uptake increased at high pH in Na+ medium, but not in Na-free medium. Significant Pi uptake was seen in Na-free medium at pH 4–9.

of dietary P levels at days 2 and 5 (Fig. 6). By day 20, however, the uptake was markedly higher in LP than HP fish in the intestine (P=0.002) but not in PC (P=0.35). Pi uptake in the intestine increased in LP (day 2 < day 5 < day 20, P=0.0003) and decreased in HP (day 2 > day 5 > day 20, P=0.007) over time, whereas in PC, the uptake increased only modestly in LP (P=0.14) and did not change significantly (P=0.76) in HP. Pi uptake per wet mass of tissue, however, was always higher in PC than in the proximal intestine.

At day 20, serum Pi concentration was much less (P=0.0005) in fish fed LP (2.44±0.26 mmol l^{-1}, N=5) than in fish fed HP (4.86±0.35 mmol l^{-1}, N=5). Bone P levels were also lower (P=0.001) in fish fed LP (10.12±0.12%, N=5) than in fish fed HP (10.75±0.05%, N=5). Serum Pi and bone P concentrations correlated well (P=0.0001–0.0048) to I-NaPi mRNA abundance in both the proximal intestine and PC (Fig. 7A,B), but not to PC-NaPi mRNA abundance in PC (P=0.04 and 0.15, respectively) (Fig. 7A,B). Serum Pi and bone P concentrations also correlated to the Pi uptake rate in the intestine (P=0.001 and 0.007, respectively), but not in PC (P=0.4 and 0.7, respectively) (Fig. 7C,D). In the proximal intestine (P=0.0003), but not in PC (P=0.4), the abundance of I-NaPi mRNA was positively correlated with the rate of Pi uptake. In PC, the abundance of PC-NaPi mRNA was weakly correlated to the rate of Pi uptake (P=0.048).

In the proximal intestine, only trace amounts of PC-NaPi mRNA could be detected in both LP and HP fish. In the proximal intestine, I-NaPi mRNA abundance was higher (P=0.0002) in LP than in HP fish (~1.5-fold) (Fig. 7A,B). In PC, I-NaPi mRNA abundance was markedly higher (P=0.0006) in LP than in HP fish (4–9-fold), whereas that of PC-NaPi was only slightly higher (P=0.01) in LP than in HP fish (~2-fold) (Fig. 7A,B). Total NaPi cotransporter mRNA abundance (relative to beta-actin mRNA) was higher in the intestine than in PC, especially in HP fish (Fig. 7A,B). Pi uptake rate (g^{-1} tissue), however, was higher in PC than in the intestine, especially in HP fish (Fig. 7C,D).

Discussion

In this study we show, for the first time, marked differences in function and regulation of dietary P transport between PC

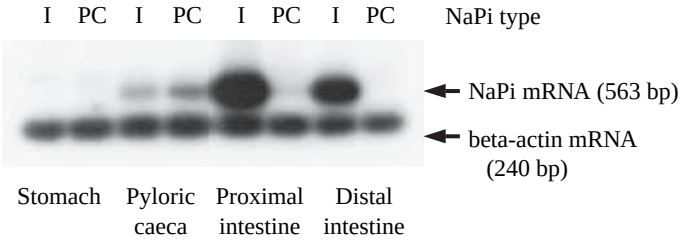

Fig. 5. Tissue distribution of intestine-type NaPi (I) and pyloric caeca-type NaPi (PC) cotransporter mRNA in rainbow trout consuming a normal (sufficient) P diet. Total RNA was reverse-transcribed using oligo-dT_{20} primer, and PCR-amplified for 14 cycles for both intestine-type NaPi (I-NaPi) and pyloric caeca-type NaPi (PC-NaPi), or 7 cycles for beta-actin. The PCR products were Southern-transferred onto a nylon membrane, and probed with ^{32}P-labelled cDNA (a mixture of I-NaPi, PC-NaPi and beta-actin cDNA, equimolar basis). Three fish were examined, and the representative blot is shown above. Specific primers, designed from a unique region, amplified either I-NaPi or PC-NaPi, but not both.

and intestine. The major differences are in sodium dependency and in patterns of dietary regulation.

Kinetics of Pi transport in trout PC

The present study shows that brush border Pi uptake in trout PC, as in trout intestine, is a carrier-mediated active process. Excess 'unlabeled' Pi reduced tracer Pi uptake by ~85% in PC (Fig. 3) and ~95% in the intestine (Avila et al., 2000). PFA reduced both PC and intestinal Pi uptake by ~70%. These findings directly demonstrate the general similarity of these transporters to those of mammalian intestinal and renal carrier-mediated Pi uptake known to be inhibitable by PFA. Passive Pi uptake estimated from the slope was almost identical to that estimated from PFA incubation at low Pi concentrations (<1 mmol l^{-1}) (Fig. 2), suggesting that most of the carrier-mediated Pi transport in PC is PFA-sensitive or NaPi-mediated transport. Slightly higher passive Pi uptake estimated from PFA than that estimated from the slope at higher Pi concentrations (5–10 mmol l^{-1}; Fig. 2) suggests that the inhibition of active Pi transport by PFA was incomplete.

Sodium azide clearly reduced Pi uptake by ~60%, indicating the energy (ATP) dependence of Pi uptake in PC. The Q_{10} of tracer Pi transport was 1.6, indicating, as with all energy-dependent processes, its sensitivity to changes in temperature. None of these inhibitors or ice-cold incubation, however, inhibited Pi uptake completely.

There are modest differences in Pi transport kinetics between intestine, as reported previously (Avila et al., 2000), and PC from the present study. The K_m and V_{max} values are, respectively, 2.5 and 3.4 times higher in the intestine (1.2 mmol l^{-1}, 220 nmol g^{-1} min^{-1}) than in PC (0.474 mmol l^{-1}, 64.6 nmol g^{-1} min^{-1}). These differences may be due to differences in the transporters (see below) or to developmental stages, as the intestinal Pi transport (Avila et al., 2000) was determined using much smaller fish (11 g body mass). The K_m of PC, however, is similar to the values reported for mammalian intestine (Danisi and Murer, 1991).

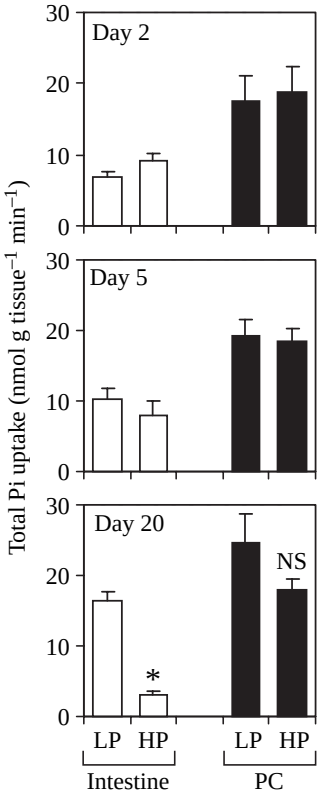

Fig. 6. Effect of dietary P restriction on rate of Pi uptake in the proximal intestine (open bars) and pyloric caeca (PC; filled bars) of rainbow trout. Fish were fed either low-P (LP) or high-P (HP) diet for 20 days. Sleeves of intestine and PC were sampled from the fish at days 2, 5 and 20, and Pi-uptake analyzed *in vitro*. Values are means ± S.E.M. (*N*=5 fish, 2 tissue sleeves per fish assayed). The tissue incubation medium contained 0.1 mmol l^{-1} Pi. At this Pi concentration, the active component represents ~75% of total Pi uptake in PC (see Fig. 3). Between LP and HP fish, Pi-uptake was not different at days 2 and 5 in intestine and PC; however, at day 20, the uptake was markedly different between LP and HP fish in the intestine (**P*=0.002), but not in PC (NS; *P*=0.35). Among sampling days (day 2, 5 and 20) in each treatment, Pi uptake was markedly different in the intestine (ANOVA *P*=0.001 and 0.03, regression *P*=0.0003 and 0.007 in LP and HP fish, respectively), but not in PC (ANOVA *P*=0.34 and 0.95, regression *P*=0.14 and 0.76 in LP and HP fish, respectively).

As shown by RT-PCR-Southern blotting, both PC-NaPi and I-NaPi cotransporters are present in PC, and thus the carrier-mediated active Pi uptake must be considered as the sum of both transporters. Although, in PC, PC-NaPi is generally more abundant than I-NaPi, the contribution of I-NaPi to active Pi uptake can be significant, especially under dietary P restriction where I-NaPi mRNA abundance in PC dramatically increases (Fig. 7A,B).

Na^+-independence

Incubation in Na^+-free medium decreased intestinal Pi

uptake by >90% (Avila et al., 2000) but did not decrease Pi uptake in PC. The Pi uptake in PC was the same whether extracellular Na^+ was substituted by either extracellular K^+ or choline, or whether intracellular Na^+ was increased by ouabain, thereby eradicating the Na^+ gradient required for Pi entry into the cell. Pi uptake increased dramatically at high pH in Na^+-containing, but not in Na^+-free, medium. Hence, the Na^+-dependency is pH dependent.

The molecular basis of the difference in Na^+-dependency must be the difference in amino acid sequence between PC-NaPi and I-NaPi. Putative amino acids responsible for the difference in Na^+-affinity (though not Na^+ dependency) between renal and intestinal isoforms in mammalian NaPi-II (de la Horra et al., 2001), however, differ from those corresponding amino acids in trout PC and intestine. In zebrafish and flounder kidneys (Graham et al., 2003), the corresponding amino acids in the NaPi cotransporter sequence that are thought to confer Na^+-affinity are the same as those in trout. However, both zebrafish and flounder NaPi-IIb are Na^+-dependent. Also, trout intestinal NaPi-II is Na^+-dependent (Avila et al., 2000). Na^+-independent, active (carrier-mediated) Pi transport has been reported in rat and chick intestinal basolateral membrane, and in dog renal basolateral membrane (Danisi and Murer, 1991). Thus, PC-NaPi may be related to the mammalian and avian basolateral Pi transporter.

pH-dependence

When Na^+ is not required to transport Pi in trout PC, what is the alternative cation in the transport system? In ruminant duodenum, where the pH of the digesta is quite acidic, H^+ rather than Na^+ drives brush border Pi uptake (Shirazi-Beechey et al., 1996). In goat jejunum, Pi uptake was stimulated ~60% when pH was decreased from 7.4 to 5.4, but this effect was abolished in the absence of Na^+ (Schroder and Breves, 1996). In frog and rabbit intestine, H^+ stimulated glucose transport in Na^+-free medium (Hoshi et al., 1986). When expressed in *Xenopus laevis* oocytes, the intestinal sodium-glucose cotransporter (SGLT1) is capable of acting both as a low affinity/high capacity transporter and a high affinity/low capacity transporter, depending on the driver cation (Hirayama et al., 1994).

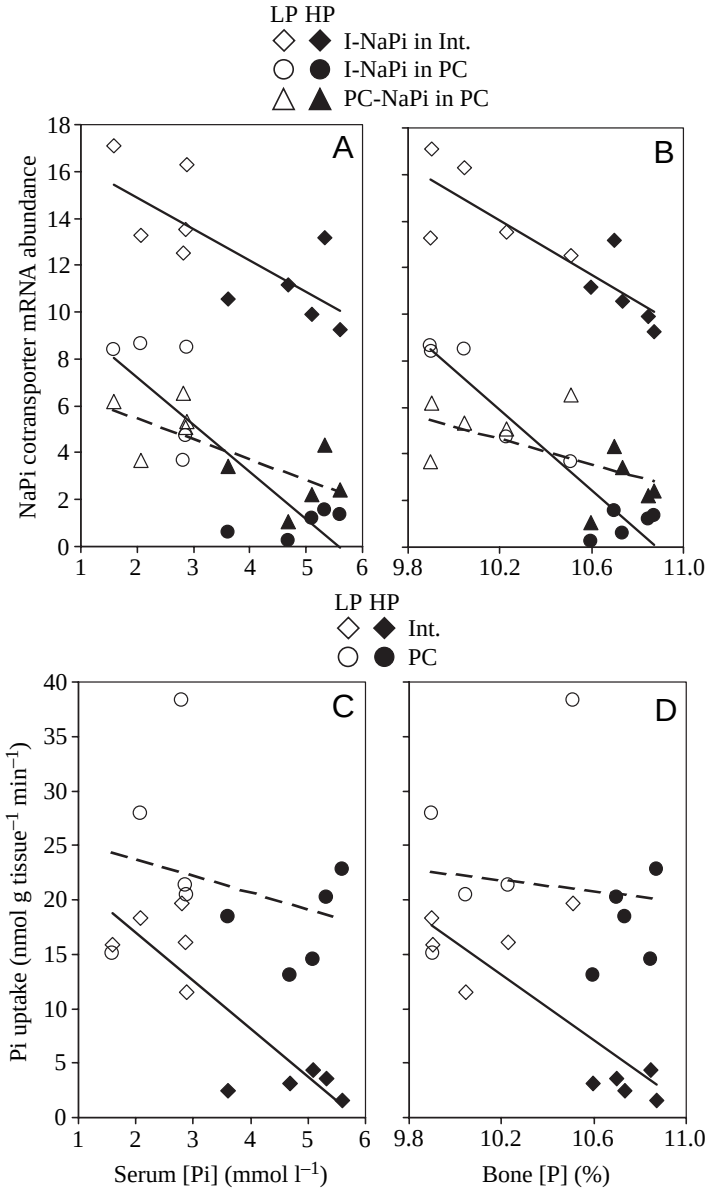

Fig. 7. Effects of dietary P levels on NaPi cotransporter mRNA abundance (A,B) and Pi uptake (C,D) in the proximal intestine (Int.) and pyloric caeca (PC) of rainbow trout. Fish were fed either low-P (LP; open symbols) or high-P (HP; filled symbols) diet for 20 days. Serum Pi and bone P concentrations (*x*-axis) represent the P status of each fish. I-NaPi and PC-NaPi mRNA abundances were determined by RT-PCR-Southern blot (PC-NaPi in the intestine was negligible). The mRNA abundance was normalized by β-actin mRNA abundance and expressed relative to the average of I-NaPi in PC of HP fish (=1.0). Pi uptake was determined *in vitro* in the incubation medium containing 0.1 mmol l^{-1} Pi. At this Pi concentration, the active component represented ~75% of total Pi uptake in PC (Fig. 3). (A) Significant correlations were found between serum Pi concentration and I-NaPi mRNA abundance in the intestine (slope P=0.002) and in PC (slope P=0.002), whereas correlation between serum Pi concentration and PC-NaPi mRNA abundance in PC (broken line) was weak (P=0.04). (B) Significant correlations were also found between bone P concentration and I-NaPi mRNA abundance in the intestine (slope P=0.005) and in PC (slope P<0.0001), whereas correlations between bone P concentration and PC-NaPi mRNA abundance in PC (broken line) were insignificant (P=0.2). In both A and B, PC-NaPi was dominant in PC in HP fish, whereas I-NaPi was dominant in PC in LP fish. I-NaPi abundance in the intestine was higher in both LP and HP fish than the total NaPi cotransporter abundance in PC. (C) Correlation between serum [Pi] and Pi uptake was significant in the intestine (slope P=0.001), but not in PC (broken line; slope P=0.4). (D) Correlation between bone [P] and Pi uptake was significant in the intestine (slope P=0.007), but not in PC (broken line; slope P=0.7). In both C and D, Pi uptake (g^{-1} tissue) was higher in PC than in the intestine, especially in HP fish.

Thus, in the duodenum and proximal jejunum where the pH of chyme is quite acidic and the glucose concentration high, SGLT1 can act as a low affinity/high capacity glucose transporter using the proton gradient. These studies suggest that H^+ can substitute for Na^+ as the driver cation under Na^+-free or Na^+-limiting conditions.

Pi uptake in PC markedly increased at alkaline pH, and this increase was strictly Na^+-dependent. This pH dependency is similar to that of the mammalian renal NaPi cotransporter isoform that is Na^+-dependent and has higher transport rates at alkaline pH (Hilfiker et al., 1998; Murer et al., 2001). It is different from that of the mammalian intestinal NaPi cotransporter isoform, which is also Na^+-dependent but has higher transport rates at neutral to acidic pH (Berner et al., 1976; Borowitz and Ghishan, 1989; Danisi et al., 1984; Lee et al., 1986; Tenenhouse, 1999; Xu et al., 2002). In chick, pig and sheep intestine, however, Pi transport rate is higher at alkaline pH than acidic pH (Danisi and Murer, 1991), which is in agreement with our data in trout PC and also with data from the intestinal and renal NaPi cotransporter isoforms of zebrafish and flounder (Forster et al., 1997; Graham et al., 2003; Kohl et al., 1996; Nalbant et al., 1999). The amino acid motifs that apparently confer the pH dependency in the NaPi-II intestinal and renal transporter isoforms of mammals (de la Horra et al., 2000) are different from the corresponding sequence in trout PC and intestinal NaPi cotransporter and in zebrafish NaPi cotransporter isoform found in both kidney and intestine. Since at pH levels higher than 7.2, divalent ions (HPO_4^{2-}) will be the dominant species over monovalent ions ($H_2PO_4^-$), the preferred ions for the fish NaPi cotransporter isoforms and mammalian renal NaPi cotransporter isoform may be the divalent form, whereas for mammalian intestinal transporter isoform, the preferred species may be the monovalent form. Further study is needed to clarify the pH and Na^+ dependency of Pi transport systems.

One feature of trout PC is that the luminal pH is fairly high, and in the intestine it becomes even higher toward the distal intestine. The differences in luminal pH and Pi concentration between PC and the intestine might have led to the development of two functionally different Pi transporters. It is unclear why the caecal pH of trout is well below the optimal pH for the transporter. Since in PC, both PC-NaPi and I-NaPi are present, it is possible that only I-NaPi is pH sensitive, whereas PC-NaPi is not. Our experimental procedure did not distinguish this.

Differences in patterns of regulation

The PC-NaPi mRNA abundance is only weakly regulated by dietary P. In contrast, I-NaPi mRNA abundance is tightly regulated by dietary P, and the diet-induced difference is inversely but linearly proportional to diet-induced changes in serum Pi and bone P levels. Hence, an abundance of I-NaPi, but not PC-NaPi, indicates the P status (or adequacy of dietary P intake) of fish.

Also, an abundance of I-NaPi and PC-NaPi mRNA in the same fish (using the same RT preparation) were only weakly correlated with one another. These differences in regulation of mRNA abundance lead to differences in regulation of function. In the intestine, where I-NaPi is predominant, saturable Pi uptake is diet-dependent, whereas in the PC, where PC-NaPi is predominant, saturable Pi uptake is mostly independent of diet (Fig. 6).

At the physiological luminal Pi concentration of 20 mmol l^{-1}, when trout are fed P-sufficient diets, the contribution of active, carrier-mediated Pi transport to the total Pi transport in PC is only about 7.7%. However, under conditions of chronic dietary P restriction, there can be a higher V_{max} with lower luminal P concentrations, which increases the significance of active Pi transport in PC. Although this kind of adaptive upregulation was remarkable at a molecular level (i.e. I-NaPi mRNA abundance in PC increased ~eightfold at day 20 in LP fish), the functional difference between LP and HP fish in PC was not pronounced compared with that in the intestine (Fig. 7), suggesting that Pi uptake in PC is mediated mainly by PC-NaPi cotransporter.

Because the diffusive component predominates at high luminal Pi concentrations, the absorption of Pi in PC is poorly regulated, and there might be little functional significance of PC-NaPi cotransporter in fish consuming commercial fish feeds of normal-to-high P content. The predominant passive Pi transport also explains our previous finding of high *in vivo* fractional P absorption even at excess dietary P intakes (Sugiura et al., 2003), and our previous observation of acute tetany (typical sign of hypocalcemia) when trout were fed a low-calcium high-P diet (S. H. Sugiura, unpublished observation).

Role of PC in dietary P absorption

Substituting the prevailing luminal Pi concentration (Fig. 1) and kinetic constants of Pi uptake in PC (Fig. 2) and in intestine (Avila et al., 2000) into Equation 2, one can calculate that the total rate of transport g^{-1} PC is about 850 nmol min^{-1}, whereas transport g^{-1} intestine is 240 nmol min^{-1}. Since mass is directly proportional to mucosal surface area (Ferraris and Diamond, 1989), and PC represents 70% of surface area (Buddington and Diamond, 1987), approximately 89% of total Pi absorption takes place in PC. Clearly, Pi absorption through the PC is more significant than that through the intestine. About 92% of PC uptake is diffusive and cannot be regulated, which suggests that there are limitations to physiological or endocrinological approaches towards enhancing intestinal or caecal Pi uptake in trout, since only carrier-mediated transport can be physiologically regulated.

In the present study, localization of NaPi mRNA within a caecum was not studied. The I-NaPi and PC-NaPi cotransporters may be uniquely distributed within a caecum, and the distribution of I-NaPi, but not PC-NaPi, could be modulated by dietary P intake or P status of fish. The distribution of PC-NaPi among PC in the same fish may also be different, since some PC are located adjacent to the pyloric sphincter of the stomach, which could receive more acidic chyme than the PC that are located almost in the middle part

of the proximal intestine. The large within-treatment variance in the uptake assay might be partly explained by the possibly unique adaptation of each caecum, depending on the anatomical location.

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