GABARAP deficiency modulates the expression of NaPi-IIa in renal brush border membranes

Sonja C. Reining¹, Serge M. Gisler¹,², Daniel Fuster², Orson W. Moe²,³, Gregory A. O'Sullivan⁴, Heinrich Betz⁴, Jürg Biber¹, Heini Murer¹, Nati Hernando¹

1 Institute of Physiology and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Winterthurerstr. 190, 8057 Zurich, Switzerland
2, 3 Departments of Internal Medicine, and the Charles and Jane Pak Center of Mineral Metabolism, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, Texas 75390-8856, USA
4 Department of Neurochemistry, Max-Planck Institute for Brain Research, Deutschordenstraße 46, 60528 Frankfurt am Main, Germany

Corresponding author:
Nati Hernando
hernando@physiol.uzh.ch

Running title: Renal Pᵢ handling in GABARAP⁻/⁻ mice
Abstract

Renal reabsorption of inorganic phosphate (P_i) is mainly mediated by the Na-dependent P_i-cotransporter NaPi-IIa that is expressed in the brush border membrane (BBM) of renal proximal tubules. Regulation and apical expression of NaPi-IIa are known to depend on a network of interacting proteins. Most of the interacting partners identified so far associate with the C-terminal PDZ-binding motif (TRL) of NaPi-IIa. In this paper GABARAP was identified as a novel interacting partner of NaPi-IIa using a membrane yeast-two-hybrid system (MYTH 2.0) to screen a mouse kidney library with the TRL-truncated cotransporter as bait. GABARAP mRNA and protein are present in renal tubules and the interaction of NaPi-IIa and GABARAP was confirmed using GST-pull downs from BBM and co-immunoprecipitations from transfected HEK293 cells. Amino acids 36-68 of GABARAP were identified as the determinant for the described interaction. The in vivo effects of this interaction were studied in a murine model. GABARAP^{-/-} mice have reduced urinary excretion of P_i, higher Na-dependent \(^{32}\)P_i-uptake in BBMV, and increased expression of NaPi-IIa in renal BBM compared to GABARAP\(^{+/+}\) mice. The expression of NHERF1, an important scaffold for the apical expression of NaPi-IIa, is also increased in GABARAP\(^{-/-}\) mice. The absence of GABARAP does not interfere with the regulation of the cotransporter by either parathyroid hormone (PTH) or acute changes of the dietary P_i content.

Keywords: epithelial transport, renal proximal tubules, phosphate homeostasis
Introduction

Membrane transport systems for inorganic phosphate (P\textsubscript{i}) are the key players to maintain whole organism P\textsubscript{i} homeostasis. In the kidney, P\textsubscript{i} excretion is determined by reabsorption of filtered P\textsubscript{i} that is mediated by the Na\textsuperscript{+}-dependent P\textsubscript{i}-cotransporters NaPi-IIa and NaPi-IIc. Both transporters are expressed in the brush border membrane (BBM) of proximal tubules (PT) (1, 11, 40). NaPi-IIa and NaPi-IIc use the lumen-to-cell electrochemical gradient of Na\textsuperscript{+} to accomplish uphill transport of P\textsubscript{i} from the urine back into the cells. However, due to their different Na\textsuperscript{+}:P\textsubscript{i} stoichiometry, the transport mediated by NaPi-IIa is electrogenic whereas NaPi-IIc is electroneutral (15, 40). In the adult kidney the amount of reabsorbed P\textsubscript{i} is mainly determined by the apical expression of NaPi-IIa which is regulated by different factors, among them dietary P\textsubscript{i} intake and parathyroid hormone (PTH) (for review, see (16). Upregulation involves de novo protein synthesis and membrane insertion whereas downregulation occurs by membrane retrieval and lysosomal degradation of NaPi-IIa with little or no recycling back to the membrane (for review, see (16).

Apical expression of NaPi-IIa depends on the presence of its last three amino acids (TRL) as illustrated by the observation that a ΔTRL cotransporter partially accumulates intracellularly (22). These last three amino acids constitute a PSD95/DgIA/ZO-1 (PDZ)-binding motif, which mediates interactions with various PDZ-domain containing proteins including the four members of the Na/H-exchanger regulatory factor (NHERF) family (18). NHERF1 harbors two PDZ-domains and a Merlin-Ezrin-Radizin-Moesin (MERM)-binding domain. The first PDZ-domain of NHERF1 binds to NaPi-IIa whereas the MERM-binding domain interacts with proteins of the MERM family and thus may link NaPi-IIa to the actin cytoskeleton (38). Disruption of the interaction between NaPi-IIa and NHERF1 leads to a reduced apical
Renal Pi handling in GABARAP−/− mice

expression of NaPi-IIa in cells (20). Moreover, basal expression of NaPi-IIa as well as its upregulation in response to low Pi diet is impaired in NHERF1+/− mice (41, 51).

Here, we use a novel screen technique that enables the detection of interaction between transmembrane proteins. This screen led to the identification of the GABA_A-receptor associated protein (GABARAP) as a new interacting partner of NaPi-IIa. In addition to GABA_A-receptors (48), GABARAP interacts with a variety of other receptors (9, 19), as well as with various cytoplasmic proteins important for intracellular trafficking such as tubulin, gephyrin, the N-ethylmaleimide-sensitive factor (NSF) and the clathrin heavy chain (24, 26, 34, 49). This pattern of interactions suggested a role of GABARAP in protein trafficking by modulating either the sorting, the surface expression or the turnover rate of membrane proteins. Supporting this hypothesis is the finding that overexpression of GABARAP in cultured cells increases the cell-surface expression of not only of GABA_A-receptors (27) but also of the Angiotensin II type I receptor (9). However, the synaptic localization of GABA_A-receptors is not affected in GABARAP+/− mice (36). In addition to a role in intracellular trafficking, GABARAP may function as an ubiquitin-like protein. The C-terminus of GABARAP shares strong similarity to ubiquitin (10) and it undergoes a post-translational modification that resembles ubiquitylation. Thus, GABARAP is cleaved at G116, activated by E1- and E2-like enzymes and finally conjugated to other molecules, most likely phospholipids (42, 44). Insertion of lipopeptides into the membrane could influence the membrane expression of proteins.

In the present study, we show that GABARAP is a novel interacting partner of NaPi-IIa. We postulated that GABARAP may regulate the expression of NaPi-IIa in the proximal brush border membrane, similar to its effect on other membrane proteins. To test this hypothesis we compared the expression of the cotransporter in GABARAP+/+ and GABARAP−/− mice. Knock-out of GABARAP leads to increased
expression of NaPi-IIa but not NaPi-IIc in renal BBM. The increased NaPi-IIa abundance in GABARAP−/− mice correlates with a higher Na⁺-dependent Pi transport activity in BBM vesicles (BBMV) and reduced urinary excretion of Pi. The expression of NHERF1 is also increased in GABARAP−/− mice. Finally, we show that the absence of GABARAP does not prevent endocytosis of NaPi-IIa in response to PTH administration neither the acute dietary regulation of the cotransporter.
Experimental procedures

Split-Ubiquitin Yeast-Two-Hybrid Screen
A mouse kidney cDNA library with inserts linked to the N-terminal half of ubiquitin (NubG) in the vector pADSL-Nx was generated using the SMART cDNA construction kit (Clontech). The library was screened using as bait NaPi-IIa lacking the C-terminal PDZ-binding motif (TRL). As recently published (17), the N-terminus of NaPi-IIa was fused to a chimera consisting of the C-terminal half of ubiquitin (Cub) and a transcription factor (TF) derived from the LexA DNA-binding domain and the VP16 activation domain. Upon interaction of bait with prey, split ubiquitin is complemented to induce the proteolytic release of TF and to ultimately allow activation of HIS3 and lacZ reporter genes. In this membrane yeast two-hybrid system, determination of bait-prey interaction was done by colorimetric measurement of β-galactosidase activity after growth selection in the absence of histidine as reported (17).

Animal studies
GABARAP<sup>−/−</sup> mice were derived from a library (Omnibank) of embryonic stem cells in which various genes were inactivated by gene-trap methodology (55). One of the clones was used to generate a heterozygous GABARAP mouse by blastocyst injection (Lexicon Pharmaceuticals, Texas, USA). GABARAP<sup>−/−</sup> mice were generated from transgenic 129SvEvBrd embryonic stem cells, injected into C57Bl/6J albino blastocysts and back crossed for 7 generations with the C57Bl/6J line. Mice were genotyped by PCR amplification of genomic DNA extracted from tail tissue as indicated (36). All experiments were performed with 12 to 14 weeks old male mice.
Mice were injected i.p. with 0.5 µg/g body weight of 1-34 PTH (Sigma) or with 0.9 % NaCl as control. Kidneys were harvested 45 minutes after injection and rapidly frozen until further use.

For metabolic studies, mice were housed separately in metabolic cages with free access to food and water. Urine was collected under mineral oil. Blood was withdrawn from the vena cava of anesthetized mice. Urinary and plasma ion composition was analyzed by Ion Chromatography (Metrohm 820 Professional IC). Creatinine and phosphate concentrations were determined according to the Jaffe (Wako Chemicals) and Fiske Subarov methods (Sigma Diagnostics) respectively. Urinary concentration of cAMP was determined using a cAMP [³H] assay system (Amersham).

For dietary adaptation, mice placed in individual cages were trained for 4 days to eat either a high (1.2 %) or a low (0.1 %) Pi content diet (Kliiba, NAFAG). Food was provided for only two hours daily, while animals had free access to water. On the fifth day, each initial group was further divided into two subgroups that were fed for two hours with either the high or the low Pi diet.

All animal experiments were performed according to Swiss Animal Welfare laws, with approval of the local veterinary authority (Kantonales Veterinäramt Zürich).

**Plasmids**

To obtain GST-fusion constructs, PCR products encoding either full length or amino acids 1-68 and 36-117 of GABARAP were subcloned into the BamHI/Sall site of pGEX-TK-Ras (56). To generate myc-fused GABARAP, the nucleotide sequence encoding the myc epitope (EQKLISEEDL) immediately preceded by a Kozak consensus sequence were first introduced into the pcDNA3.1 plasmid (Invitrogen) by bi-directional PCR. Then, full-length GABARAP was subcloned into the myc-
pcDNA3.1 plasmid using XhoI/KpnI restriction sites. Mouse NaPi-IIa was subcloned into the mammalian expression vector pHA-MEX (Dualsystems Biotech) using XhoI/EcoRI restriction sites.

**Immunoblots and Glutathione S-Transferase Pull-Downs**

Kidney homogenate or renal BBM were prepared as described previously (5). For Immunoblot analysis, kidney homogenate (40 µg) or BBM (15 µg) were separated on 9% SDS/PAGE gels, transferred to PVDF membranes and then incubated with antibodies against NaPi-IIa (11), PDZK1 (18), NHERF-1 (Abcam) and GABARAP (19). After incubation with HRP-linked secondary antibodies (GE Healthcare), immunoreactive signals were detected by chemiluminescence (ECL, Amersham Pharmacia Biotech) using the DIANA III-chemiluminescence detection system (Raytest). The densitometry of all images was analyzed using appropriate software (Advanced Image Data Analyser AIDA, Raytest) to calculate, for each given experiment, the ratio between the protein of interest to β-actin.

GST pull-downs were performed with isolated BBM or HEK293 cell lysates as reported (18). Solubilized BBM/cell lysates were incubated over night at 4 °C with glutathione beads coupled either to GST alone or to GST-fusion proteins. Samples were centrifuged at 13000 g for 1 min. The pelleted beads were washed 5-6 times with TBS containing 0.1% (v/v) Nonidet P-40 and 0.1% (v/v) Tween 20. Proteins were eluted by incubation in loading buffer at 95°C for 5 min. Samples were subsequently analyzed by Immunoblot as described above.

**Cell culture and Co-Immunoprecipitation**

HEK293 cells were plated in 10 cm dishes and cultured in DMEM supplemented with 10 % FCS, 5 mM L-Glutamine, 100 U/ml penicillin and 100 U/ml streptomycin in 5 %
Renal Pi handling in GABARAP−/− mice

CO₂ at 37 °C. Subconfluent cultures were transfected with 6 µg of plasmids encoding myc-GABARAP and/or NaPi-IIa, using Lipofectamine 2000 (Invitrogen) according to the manufacturer`s instructions. Two days after transfection cells were lysed in 1 ml IP-buffer containing 50 mM Tris pH 7.4, 72 mM NaCl, 0.75 % Triton X-100, 0.75 % Na-deoxycholate, 1 mM PMSF, 5 µg/µl leupeptin, 5 µg/µl pepstatin A. After preclearing with 40 µl Protein A/G agarose beads (Calbiochem) anti-myc antibody (Invitrogen) was added in a dilution of 1:250. After incubation overnight at 4ºC on a rotary shaker, 30 µl Protein A/G beads were added to the lysates and further incubated at 4ºC on a rotary shaker for 60 min. Beads were pelleted by short centrifugation steps (microfuge) and washed 4 times with IP-buffer containing detergents and once with IP-buffer without detergents. Proteins were eluted by incubation in loading buffer at 95 ºC for 5 min. Samples were subsequently analyzed by Immunoblot.

**Uptake in isolated BBMV**

The Na⁺-dependent transport rate of ³²P⁻ into renal BBMV was determined in the presence of 0.1 mM potassium phosphate as described previously (4). Similar protocols were used to measure uptake of radioactively labeled L-Glutamine and D-Glucose.

**RNA Isolation and real-time PCR**

Total RNA was extracted from kidneys homogenized in RLT buffer using the RNeasy Mini Kit (Qiagen). Similarly, RNA from dissected nephron segments was extracted using the RNeasy Micro Kit (Qiagen) according to manufacturer`s instructions. Either 300 ng of RNA from total kidney or 80 ng of RNA from nephron segments were used as template for reverse transcription using TaqMan Reverse Transcription Kit
Renal Pi handling in GABARAP−/− mice

Real-Time PCR was performed on the ABI PRISM 7700 Sequence Detection System, using commercial primers and probes for NHERF1 and GABARAP (Taqman Gene Expression Assays) as well as primers and probes for NaPi-IIa and NaPi-IIc (31) and β-ENaC (14). Amplification was carried out using the TaqMan Universal PCR Master Mix (PCR machine and reagents from Applied Biosystems). The expression of the gene of interest was calculated in relation to hypoxanthine guanine phosphoribosyl transferase (HPRT). Relative expression ratios were calculated as $R = 2^{[\text{Ct}(\text{HPRT})-\text{Ct}(\text{test gene})]}$, where Ct represents the cycle number at the threshold 0.2.

Immunostainings

Mouse kidneys were perfusion-fixed through the left ventricle with a fixative solution, and subsequent immunohistochemistry was performed as described previously (12). Cryosections (6 µm) were taken and pretreated for 5 min with either 0.1% SDS (for NaPi-IIa and NaPi-IIc) or 0.5% SDS (for NHERF1/2 and CD98/4F2). After blocking with 2% BSA, 0.02% Na-azide in PBS, sections were incubated overnight at 4°C with antibodies against NaPi-IIa 1:600 (11), NaPi-IIc 1:400 (35), NHERF1 1:300 (52) and CD98/4F2 1:200 (Santa Cruz) in blocking solution. Binding sites of the primary antibodies were detected using Alexa 488-conjugated anti-rabbit antibodies (Invitrogen). F-actin was visualized using Texas-Red-coupled phalloidin (Invitrogen). Sections were studied by epifluorescence with a Polyvar microscope (Reichert Jung), and digital images were acquired with a charged coupled device camera.
Results and Discussion

Using a classical yeast-two hybrid (YTH) screen, we previously identified several proteins that interact with the C-terminal intracellular tail of NaPi-IIa (18). Most of these interactions were PDZ-based and depended on the last 3 residues of NaPi-IIa (TRL). Classical YTH analysis requires soluble baits to screen for protein-protein interactions. In comparison, the advantage of the split-ubiquitin membrane YTH system (MYTH) is that full-length integral membrane proteins can be used as baits (17, 43). We used the MYTH 2.0 system to screen a mouse kidney library targeting the identification of interactions independent on the C-terminal PDZ-binding domain of NaPi-IIa. To this end, we used the TRL-truncated (instead of full-length) cotransporter as bait. Three interacting clones were identified as GABARAP. Two of these clones contained the full length open reading frame (ORF), whereas the third cDNA was a partial fragment that lacked the first 34 nucleotides of the ORF. The GABARAP protein consists of 117 amino acids and shares similarity with the light chain-3 of microtubule-associated proteins 1A and 1B (48).

GABARAP mRNA and protein are expressed in the kidney

GABARAP mRNA was previously shown to be expressed in all tested tissues, including kidney (53). To further address the distribution of GABARAP mRNA within the kidney, Real-Time PCR was performed on dissected murine nephron segments. GABARAP mRNA was found to be uniformly expressed in all tested nephron segments (Fig. 1A). To rule out cross-contaminations with mRNA from different nephron segments, we analyzed the profile of known marker genes differentially expressed along the nephron. As a marker for proximal tubules, we chose the putative interacting partner NaPi-IIa. As shown previously (31), NaPi-IIa mRNA was
highly abundant in proximal convoluted tubules (S1+2) and expressed to a lesser extend in proximal straight segments (S3) whereas expression in more distal segments was hardly detectable (Fig. 1B). To demonstrate the enrichment of distal nephron segments, we determined the expression of β-ENaC mRNA, which has been shown to be present in the distal convoluted tubule, the cortical collecting duct (CCD) and the outer medullary collecting ducts (47). As expected, β-ENaC mRNA was highly abundant in our preparation of CCD and absent in proximal segments (Fig. 1B). Therefore it is unlikely that the uniform GABARAP mRNA distribution along the nephron segments is due to segments cross-contamination. Importantly, GABARAP mRNA expression was detected in proximal convoluted and proximal straight tubules, those segments where NaPi-IIa mRNA is also present.

Endogenous GABARAP protein expression has been reported in rat brain, liver, kidney, spleen, skeletal muscle and heart (45) as well as in various human cell lines (19). Immunoblot using an antibody raised against human GABARAP (19) confirmed its expression in murine renal BBM preparations (Fig. 1C). In addition to the band at the expected molecular weight of 17 kDa (48), we observed a second product of faster mobility which most likely represents a lipidated form of GABARAP (45). None of these bands was detected in BBM preparations of GABARAP<sup>−/−</sup> mice, confirming the specificity of the signal.

Regarding the subcellular localization of GABARAP, a punctate distribution within the cytoplasm has been reported in breast epithelial cells, HeLa cells and neurons (19, 25, 26, 48). Unfortunately, our attempts to identify the localization of GABARAP in the kidney failed, since similar signals were detected in cryosections from GABARAP<sup>+/+</sup> and GABARAP<sup>−/−</sup> kidneys (data not shown). These signals could represent either non-specific binding of the antibody to renal tissue or cross-reaction with the GABARAP-like proteins GABARAPL1 and GABARAPL2. Both paralogs are expressed in kidney,
Renal Pi handling in GABARAP−/− mice

albeit at very low levels (39, 50). Despite this technical caveat, the presence of GABARAP in BBM preparations points to a subcellular proximity of GABARAP and NaPi-IIa, making the interaction between both proteins plausible.

**NaPi-IIa interacts with an internal sequence of GABARAP**

The interaction of GABARAP and NaPi-IIa was verified by pull-downs of NaPi-IIa from BBM using GST-GABARAP and by co-immunoprecipitation from transfected HEK293 cells. In the pull-down assay, a band at the expected molecular weight for NaPi-IIa was detected using GST-GABARAP but not with GST alone (Fig. 1D). The association of GABARAP with the γ-subunit of GABA_A-receptors depends on a region between the amino acids 36-68 of GABARAP (48). In order to find the molecular determinant of GABARAP that mediates the interaction with NaPi-IIa, we expressed two overlapping halves of GABARAP (aa 1-68 and aa 37-117, respectively) fused to GST as described (48). As shown in Fig. 1D, both fusion proteins were capable of pulling down NaPi-IIa from BBM, suggesting that similar to the GABA_A-receptor γ-subunit the interaction of GABARAP with NaPi-IIa involves the region between the amino acids 36-68 of GABARAP. GABARAP 36-117 has been shown to interact also with the transferrin receptor (19). Therefore, these internal residues might represent a common motif for protein-protein interactions.

The specific interaction between NaPi-IIa and GABARAP was further confirmed by co-immunoprecipitation of myc-tagged GABARAP and NaPi-IIa from transfected HEK293 cells (Fig.1E). Cells were transfected either with NaPi-IIa alone or cotransfected with NaPi-IIa and myc-GABARAP. Then, an antibody against the myc-epitope was used to immunoprecipitate myc-GABARAP. A band with the expected molecular weight for NaPi-IIa was seen only in the immunoprecipitation from cotransfected cells in the presence of myc-antibody (Fig.1E top panel) but not in
negative controls lacking either myc-GABARAP or the anti myc-antibody. Taken together, the GST-pull down and the co-immunoprecipitation data suggest that NaPi-IIa and GABARAP may interact with each other in vivo.

In an attempt to identify the molecular determinant for the interaction within NaPi-IIa, we fused several discrete intracellular domains of NaPi-IIa to GST in order to pull down GABARAP from transfected HEK293 cells. However, under similar conditions to the ones used in the pull-down experiments described above, we were unable to detect interaction between both partners (data not shown). The failure of these discrete domains of NaPi-IIa to associate with GABARAP could be due either to the need of more than one single intracellular loop for the association or to the requirement of more physiological conditions. A recent report proposes a consensus sequence for GABARAP-binding sites build around a highly conserved tryptophan residue (46). The C-terminal intracellular tail of NaPi-IIa contains a sequence that would fit to this motif. Therefore, pull downs were performed with lysates from HEK293 cells transfected either with the full length or a truncated cotransporter (Δ571) which lacks the tryptophan-containing peptide. Both the full length and the truncated NaPi-IIa were pulled down by GST-GABARAP (Fig. 1F). Although these data does not rule out its potential implication in vivo, it suggests that the mentioned C-terminal peptide of NaPi-IIa is not required for the in vitro interaction with GABARAP.

Metabolic studies with GABARAP+/+ and GABARAP−/− mice

Generation of GABARAP−/− mice has been described elsewhere (36). These animals show a normal phenotype with regard to the expression of GABA receptor γ2-subunit in synapses, possibly due to a compensatory effect of GABARAPL1 and/or GABARAPL2. In particular GABARAPL2 has also been shown to interact with the GABA receptor and tubulin (33). We found that GABARAP−/− mice were slightly
though significantly heavier than GABARAP\textsuperscript{+/+} despite the fact that both groups consume similar amounts of food (Table 1). In addition, the GABARAP\textsuperscript{+/−} animals drank less water which translated in a reduced urinary output. GABARAP has been recently reported to interact with the angiotensin II type 1 receptor and to regulate its membrane expression (9). It remains to be investigated if this finding is connected with the reduced water intake in GABARAP\textsuperscript{−/−} mice. The reduced urinary excretion tightly correlates with the increased osmolarity and creatinine concentration in the urine from GABARAP\textsuperscript{−/−} animals. Antidiuresis in response to increased plasma osmolarity is mediated by transcriptional and posttranscriptional upregulation of Aquaporin 2 (AQP2) (13), but HPRT-normalized AQP2 mRNA levels were comparable in GABARAP\textsuperscript{+/+} and GABARAP\textsuperscript{−/−} mice (18.29 \pm 2.35 vs. 19.83 \pm 3.56). This suggests that the absence of GABARAP does not induce changes of AQP2 mRNA levels. Interestingly, the urinary excretion of P\textsubscript{i} (as a ratio to creatinine) was significantly reduced in GABARAP\textsuperscript{−/−} as compared to GABARAP\textsuperscript{+/+} mice (Table 1). However, excretion of all other tested ions (Cl\textsuperscript{−}, Na\textsuperscript{+}, K\textsuperscript{+}, Mg\textsuperscript{2+} and Ca\textsuperscript{2+}) was similar in both groups when normalized to urinary creatinine (Table 1). Similar levels of cAMP were detected in the urine of GABARAP\textsuperscript{+/+} and GABARAP\textsuperscript{−/−} mice. This suggests that the basal cAMP status of tubular cells, a major regulator of Pi transport, is not affected by the absence of GABARAP.

Despite the reduced urinary P\textsubscript{i} excretion in GABARAP\textsuperscript{−/−} mice, the concentration of P\textsubscript{i} in serum did not differ between both groups (Table 1). Whether this is related to a higher consumption of P\textsubscript{i} in GABARAP\textsuperscript{−/−} mice, due for instance to their higher body weight, or to some compensatory mechanism(s) such as reduced intestinal absorption or enhanced bone deposition remains unknown. The Ca\textsuperscript{2+} concentration
Renal Pi handling in GABARAP\(^{-/-}\) mice

in blood was also similar in both groups of animals whereas the circulating levels of Cl\(^-\), Na\(^+\) and K\(^+\) were all increased in GABARAP\(^{-/-}\) as compared to GABARAP\(^{+/+}\) mice.

**NaPi-IIa expression is increased in GABARAP\(^{-/-}\) mice**

Urinary excretion of Pi is mostly controlled by the rate of reabsorption in the proximal tubule (16). Based on the severe renal wasting phenotype of NaPi-IIa\(^{-/-}\) mice (1) as well as on RNA hybrid depletion experiments (40) it has been proposed that NaPi-IIa is the major Pi transporter in the adult murine kidney, whereas NaPi-IIc was considered to play only a minor role. However, mutations of NaPi-IIc identified in families with several hypophosphatemic syndromes and in particular the detectable phenotype in the heterozygotes (2, 21, 30) points to either dramatic differences between humans and mice or an incomplete understanding of renal handling of Pi. Therefore, we next analyzed whether the reduced urinary excretion of Pi detected in GABARAP\(^{-/-}\) mice was due to changes on the expression of NaPi-IIa and/or NaPi-IIc. We found that NaPi-IIa abundance was increased approximately two-fold in renal BBM of GABARAP\(^{-/-}\) as compared to GABARAP\(^{+/+}\) mice (Fig. 2A). However, the expression of NaPi-IIc was similar in both groups (Fig. 2B).

Immunohistochemistry was performed to determine whether the loss of GABARAP alters the segmental and/or subcellular distribution of NaPi-IIa and NaPi-IIc. We have shown that NaPi-IIa expression is highest in proximal convoluted tubules (S1, S2) and gradually decreases towards the end of the proximal straight tubule (S3) in superficial and juxtamedullary nephrons (11). In adults, NaPi-IIc expression is found preferentially in S1 segments of proximal convoluted tubules of juxtamedullary nephrons (37, 40). Compared with GABARAP\(^{+/+}\), the segmental distribution and subcellular localization of NaPi-IIa and NaPi-IIc were not altered in kidneys from GABARAP\(^{-/-}\) (Fig.3A and B). The two-fold increase of NaPi-IIa abundance in BBM of
Renal Pi handling in GABARAP\(^{-/-}\) mice

GABARAP\(^{-/-}\) mice revealed by Immunoblots (Fig. 2A) could not be detected by the Immunofluorescence analysis (Fig.3A).

Changes on NaPi-IIa abundance in response to acute stimuli are due to regulated insertion/retrieval from the BBM rather that to changes on mRNA levels (16, 28) whereas chronic adaptation may (28) or may not involve transcriptional regulation (31). Therefore, we quantified the amount of NaPi-IIa mRNA in kidneys from GABARAP\(^{+/+}\) and GABARAP\(^{-/-}\) mice by Real-Time PCR. As shown in Fig. 2C both groups of animals had similar levels of NaPi-IIa mRNA, suggesting that upregulation of the cotransporter in GABARAP\(^{-/-}\) mice is independent of changes on transcription. As reported previously (31), the expression of NaPi-IIc mRNA was very low as compared to NaPi-IIa and its levels were similar in GABARAP\(^{+/+}\) and GABARAP\(^{-/-}\) kidneys (Fig. 2C). Therefore, under normal conditions GABARAP seems to play a suppressive role in the expression/stabilization of NaPi-IIa in renal proximal tubules and its absence cannot be compensated by GABARAP-like paralogs. This negative effect is in contrast to the proposed role of GABARAP in the trafficking and synaptic targeting of GABA\(_A\)-receptors in neurons. Although GABARAP\(^{-/-}\) mice have normal expression of GABA\(_A\)-receptors, GABARAP increases the surface expression as well as clustering of receptors in transfected neurons (8, 27). Both effects seem to require binding of GABARAP to tubulin.

**Na\(^+\)**-dependent transport of Pi is increased in GABARAP\(^{-/-}\) mice

Changes on renal reabsorption of Pi are mostly determined by modifications of the amount of NaPi-IIa which is present in the membrane rather than regulation of the activity of the cotransporter (16). In order to test whether the increased amount of NaPi-IIa in GABARAP\(^{-/-}\) mice leads to changes on Pi reabsorption, we measured Na\(^+\)-dependent uptake of \(^{32}\)Pi in BBMV. As shown in Fig 4, this uptake was significantly
Renal $P_i$ handling in GABARAP$^{-/-}$ mice

higher in BBMV prepared from GABARAP$^{-/-}$ as compared to GABARAP$^{+/+}$ mice. The lack of GABARAP specifically upregulates Na$^+$/P$_i$-cotransport in BBM as Na$^+$-dependent uptake of L-Glutamine and D-Glucose were not altered (Fig. 4). The effect observed in the transport studies (Fig. 4) is smaller than the increase on the expression of NaPi-IIa in GABARAP$^{-/-}$ mice seen in Immunoblots (Fig. 2A). This is likely due to the presence of other Na$^+$-dependent P$_i$ cotransporters, such as NaPi-IIc, which also contribute to the Na$^+$/P$_i$-uptake in renal BBM.

**NHERF1 expression is increased in GABARAP$^{-/-}$ mice**

As indicated above, the upregulation of NaPi-IIa observed in GABARAP$^{-/-}$ mice seems to be a post-transcriptional phenomenon since mRNA levels are similar in wild type and mutant mice. We have previously shown that the stability of NaPi-IIa in the BBM is at least partially controlled by the association of its C-terminal PDZ-binding domain with several PDZ-containing proteins. Among these partners, NHERF1 has been shown to play a major role. Overexpression of the interacting PDZ-domain of NHERF1 in OK cells disturbs the apical expression of NaPi-IIa by competing with the association of the endogenous partners (20). Furthermore, in NHERF1$^{-/-}$ mice NaPi-IIa is mislocalized to intracellular sites and its abundance in BBM is reduced (41). Therefore, we next analyzed whether the increased expression of NaPi-IIa in the BBM of GABARAP$^{-/-}$ mice was associated with changes in the expression of NHERF1. As shown in Fig. 5A, the abundance of NHERF1 was increased in GABARAP$^{-/-}$ as compared to GABARAP$^{+/+}$ mice. Upregulation of NHERF1 is independent on changes at the transcriptional level, since NHERF1 mRNA abundance was not altered in GABARAP$^{-/-}$ mice (Fig. 5B). Thus, the increased NaPi-IIa expression in the BBM of GABARAP$^{-/-}$ mice is paralleled by increased levels of its scaffold partner NHERF1. Attempts to pull down myc-NHERF1 from transfected
HEK293 cells using GST-GABARAP did not give evidence for interaction between both proteins (data not shown). This observation, together with the data from the yeast two hybrid analysis were the ΔTRL-NaPi-IIa was found to interact with GABARAP but not with NHERF1, strongly suggest that the association of NaPi-IIa with GABARAP does not requires the presence of NHERF1. Surprisingly, no change of NHERF1 signal intensity or subcellular localization could be detected in immunofluorescence stainings of kidney cryosections obtained from GABARAP^{+/+} and GABARAP^{-/-} mice (Fig. 3C). Although NHERF1 is not an integral membrane protein, the capacity of its C-terminus to associate with actin-binding proteins from the Ezrin-Radizin-Moesin family may provide a link to the actin cytoskeleton and therefore promote stabilization of membrane proteins (38).

Unlike in NHERF1^{-/-} animals, the expression of NaPi-IIa is not altered in mice lacking PDZK1/NHERF3, a second PDZ-partner of the cotransporter (7, 18). As shown in Fig. 5A, the expression of PDZK1/NHERF3 was similar in GABARAP^{-/-} and GABARAP^{+/+} mice.

**PTH-induced endocytosis of NaPi-IIa is not impaired in GABARAP^{-/-} mice**

PTH is one of the best understood humoral factors that control the apical expression of NaPi-IIa. PTH is a potent phosphaturic hormone and works by inducing endocytosis of NaPi-IIa, at least partially, via clathrin mediated pits (16). GABARAP has been shown to associate with the heavy chain of clathrin (34). To test whether the downregulation of NaPi-IIa in response to PTH is affected by the loss of GABARAP, we injected animals with PTH and quantified the amount of NaPi-IIa remaining in renal BBM. Administration of PTH led to ~50 % reduction of NaPi-IIa in BBM of GABARAP^{+/+} mice (Fig. 6A). PTH also induced a reduction of NaPi-IIa in GABARAP^{-/-} mice. Moreover, despite the higher basal levels in GABARAP^{-/-} animals,
the amount of NaPi-IIa remaining upon PTH administration was similar in both groups of mice (Fig. 6A). In agreement with the Immunoblot data, similar Na⁺-dependent uptakes of $^{32}$P$_i$ were measured in BBMV isolated from GABARAP$^{+/+}$ and GABARAP$^{-/-}$ mice upon PTH treatment, despite the higher basal uptake measured in mutant mice (Fig. 6B). Therefore, the absence of GABARAP does not prevent the acute endocytosis of NaPi-IIa in response to PTH.

**Dietary regulation of NaPi-IIa is not impaired in GABARAP$^{-/-}$ mice**

The content of P$_i$ in the diet is a well known metabolic factor that affects the expression of NaPi-IIa in the apical microvilli. The expression of NaPi-IIa in the BBM increases in response to a low dietary intake of P$_i$, whereas in conditions of high dietary Pi the level of NaPi-IIa decreases (3). Both responses are fast and can be detected few hours (acute adaptation) after Pi ingestion (29). Acute downregulation of NaPi-IIa relies on its endocytic retrieval and lysosomal degradation, similar to the PTH effect (23). Acute upregulation does not require *de novo* synthesis; instead it depends on an intact microtubular cytoskeleton, suggesting the sorting to the BBM of intracellularly stored NaPi-IIa. As expected, the acute switch from low to high P$_i$ diet downregulates NaPi-IIa in the BBM of GABARAP$^{+/+}$ mice (Fig. 7C). This acute response is accompanied by an increased excretion of P$_i$ in the urine (Fig. 7A) and an increased concentration of P$_i$ in serum (Fig. 7B). The acute switch from low to high P$_i$ diet effectively downregulates the amount of NaPi-IIa in the BBM of GABARAP$^{-/-}$ mice (Fig. 7C). Furthermore, the changes on urinary excretion of P$_i$ (Fig. 7A) and circulating levels of P$_i$ (Fig. 7B) are also similar in GABARAP$^{-/-}$ and GABARAP$^{+/+}$ animals. As expected, the acute switch from high to low P$_i$ diet results in the upregulation of NaPi-IIa in the BBM of GABARAP$^{+/+}$ mice (Fig. 8C). In addition this switch results in a decrease in the urinary excretion of P$_i$ (Fig. 8A) and the P$_i$
Renal P\textsubscript{i} handling in GABARAP\textsuperscript{--/--} mice

concentration in serum (Fig. 8B). The acute switch from high to low P\textsubscript{i} diet results in similar changes in GABARAP\textsuperscript{--/+} mice as those described above for GABARAP\textsuperscript{+/+} mice (Fig. 8A-C). Taken together, these findings indicate that the acute dietary regulation of NaPi-IIa does not require the presence of GABARAP.

Taken together, we have identified GABARAP as a new interacting partner of NaPi-IIa and shown that NaPi-IIa interacts with an internal domain of GABARAP. The apical expression of NaPi-IIa is increased in GABARAP\textsuperscript{--/--} mice under normal dietary conditions. This increased NaPi-IIa abundance results in a higher Na\textsuperscript{+}-dependent P\textsubscript{i}-uptake into renal BBMV and a reduced urinary excretion of P\textsubscript{i}. The upregulation of NaPi-IIa in the BBM is independent on changes on mRNA levels. Instead, it is paralleled by an increased expression of NHERF1 (but not PDZK1/NHERF3). The regulation of the cotransporter by either PTH or acute changes of dietary P\textsubscript{i} is not affected by the absence of GABARAP, suggesting that the regulated endo/exocytosis processes are not altered. However, due to the higher expression of NHERF1 in GABARAP\textsuperscript{--/--} mice and its potential to regulate the PTH receptor signaling (6, 32), it remains to be investigated if the intracellular mechanism of the hormonal action is similar in wild type and GABARAP-deficient mice. Our finding that the abundances of both NaPi-IIa and NHERF1 are increased in GABARAP\textsuperscript{--/--} mice suggests that, under normal condition, both proteins are negatively regulated by GABARAP. This is in contrast to the proposed role of GABARAP in promoting membrane expression of receptors (9, 27). However, it is worth to mention that in addition to its role in intracellular trafficking, GABARAP also functions as an ubiquitin-like protein. Evidence comes not only from the crystal-structure of GABARAP (10) but also from the function of its yeast ortholog Atg8. Atg8 is involved in autophagy, an essential process for lysosomal degradation of organelles and proteins in response to
starvation (for review see (45). In this process Atg8 is modified in an ubiquitin-like manner. The E1/E2-like enzymes and the modification of GABARAP in mammalian cells have been identified (44). So far, the only known targets of GABARAP after its modification are phospholipids (42) and the lipid composition of the plasma membrane has been reported to regulate NaPi-IIa (54).

In summary, GABARAP interacts with NaPi-IIa and controls the expression of the cotransporter in the proximal BBM. The absence of GABARAP influences the abundance of NaPi-IIa, and therefore renal reabsorption of Pi, without affecting its acute regulation. Further work will be required to characterize the molecular mechanism responsible for these findings.
Renal Pi handling in GABARAP<sup>−/−</sup> mice

**Acknowledgements**

We thank C.A. Enns, Oregon Health and Science University, and E. Weinman, University of Maryland, for providing us with the antibodies against GABARAP and NHERF1. A. Velic, N. Kampik and C.A. Wagner, University of Zurich, kindly shared dissected nephron segments and primers/probes for RealTime PCR. F. Verrey, University of Zurich, provided primers/probe for the β-ENaC RealTime PCR. This work was supported by Swiss National Science Foundation Grant 44342003 (to HM), the Sixth European Frame Work EuReGene Project Grant 005085 (to HM), the National Institutes of Health DK-48482 and DK-20543 (to OWM), the Simmons Family Foundation (to OWM) and the Fritz-Thyssen-Stiftung (to HB). SC Reining is supported by a PhD student fellowship from the University Research Priority Program „Integrative Human Physiology“ at the University of Zurich. SM Gisler and DG Fuster were supported from Fellowships Grants and Seed Funds from the Pak Center of Mineral Metabolism. Present address GA O'Sullivan: Max-Planck Institute for Molecular Cell Biology & Genetics, Pfotenhauerstraße 108, 01307 Dresden, Germany.
References


Renal Pi handling in GABARAP−/− mice


Renal Pi handling in GABARAP−/− mice


Figure Legends:

Figure 1: Renal expression of GABARAP mRNA and protein. Interaction of GABARAP and NaPi-Ila. Quantification of (A) GABARAP and (B) NaPi-Ila and β-ENaC mRNAs in dissected nephron segments. The abundance of GABARAP, NaPi-Ila and β-ENaC mRNA was measured by Real-Time PCR. Values were normalized to Hypoxanthine-guanine phosphoribosyltransferase (HPRT) (n=2). For NaPi-Ila and β-ENaC, the highest value was set as 1 and used to normalize the other values. (C) Immunoblot analysis to detect the presence of GABARAP in BBM. BBM isolated from GABARAP−/− mice were used to show specificity of the antibody. (D) GST-Pull down analysis to show interaction between GABARAP and NaPi-Ila. Full length and overlapping halves of GABARAP were used to pull down NaPi-Ila from BBM. The top panel shows an Immunoblot probed with an anti-NaPi-Ila antibody. The lower panel shows the Ponceau staining of the PVDF-membrane (as grayscale figure) to demonstrate that similar amounts of GST and its fusion proteins were used in the different pull downs. (E) Co-immunoprecipitation of NaPi-Ila with myc-GABARAP from cotransfected HEK293 cells. Immunoblots of the immunoprecipitated material (top panels) and HEK293 cell lysates (lower panels) were probed with anti-NaPi-Ila (upper blot) or anti-myc (lower blot) antibodies. (F) GST-Pull downs on lysates from HEK293 cells transfected with full length or truncated NaPi-Ila. The top panel shows an Immunoblot of the pulled down material probed with the NaPi-Ila antibody. The middle panel shows the Ponceau staining of the PVDF-membrane (as grayscale figure). The lowest panel shows an Immunoblot on HEK293 cell lysates probed with the NaPi-Ila antibody.
Renal Pi handling in GABARAP−/− mice

Figure 2: Expression of NaPi-IIa and NaPi-Ilc protein and mRNA in GABARAP+/+ and GABARAP−/− mice. Immunoblot on BBM isolated from GABARAP+/+ and GABARAP−/− mice to analyze the expression of (A) NaPi-IIa and (B) NaPi-Ilc. Expression of both transporters was normalized to the abundance of β-actin for the quantification. (C) Real-Time PCR to determine mRNA levels of NaPi-IIa (left) and NaPi-Ilc (right) on mRNA extracted from kidney. NaPi-IIa and NaPi-Ilc mRNA expression was normalized to HPRT (n=6). Data are means ± SEM. (** p ≤ 0.01, unpaired student t-test).

Figure 3: Immunohistochemical analysis of NaPi-IIa, NaPi-Ilc and NHERF1 in GABARAP+/+ and GABARAP−/− mice. Immunostaining of (A) NaPi-IIa, (B) NaPi-Ilc and (C) NHERF1 on kidney cryosections obtained from GABARAP+/+ (left) and GABARAP−/− mice (right). Renal cortical overviews are displayed as grayscale figure. The images of single proximal tubules show (A) NaPi-IIa and (B) NaPi-Ilc, both green, co-stained with β-actin (red) as a marker for BBM. (C) NHERF1 (green) was co-stained with anti-CD98/4F2 (red) as marker for proximal tubules.

Figure 4: Na+−dependent uptake into renal BBMV. Na+−dependent uptake of P_i (n=10), L-Glutamine (n=4) and D-Glucose (n =5) into BBMV isolated from kidneys of GABARAP−/− and GABARAP+/+. Uptakes were analyzed after 1 minute incubation as indicated. Data are means ± SEM. (* p ≤ 0.05, unpaired student t-test)

Figure 5: Renal expression of NHERF1 and PDZK1 in GABARAP−/− and GABARAP+/+ mice. (A) Immunoblot analysis of NHERF1 and PDZK1 on homogenates of kidneys extracted from GABARAP+/+ and GABARAP−/− mice. For quantification, expression was normalized to β-actin. (B) Real-Time PCR to
Renal Pi handling in GABARAP−/− mice
determine NHERF1 mRNA levels normalized to HPRT (n=6). Data are means ± SEM. (** p ≤ 0.01, unpaired student t-test)

Figure 6: PTH-induced endocytosis of NaPi-IIa in GABARAP+/+ and GABARAP−/−
mice. (A) Representative Immunoblot of NaPi-IIa on BBM isolated from GABARAP+/+ and GABARAP−/− mice injected with either saline or PTH. Expression was normalized to β-actin for the quantification. Similar results were obtained in six animals per group. (B) Na+−dependent uptake of Pi into BBMV isolated from kidneys of GABARAP−/− and GABARAP+/+ mice injected with saline (n=10) or PTH (n=6). Uptakes were analyzed after 1 minute incubation as indicated. Data are means ± SEM. (* p ≤ 0.05, ** p ≤ 0.01, unpaired student t-test).

Figure 7: Acute adaptation of GABARAP+/+ and GABARAP−/− mice to a high
dietary Pi content. Mice were adapted to a low Pi diet (↓) for 4 days as indicated in Experimental procedures. On the fifth day they were divided in two subgroups that received either a low Pi diet (n=3) or were switched to a high Pi diet (↑) (n=5). (A) Urinary Pi excretion as ratio to urinary creatinine. (B) Serum Pi concentration. (C) Representative Immunoblot of NaPi-IIa on BBM. Expression was normalized to β-actin for the quantification.

Figure 8: Acute adaptation of GABARAP+/+ and GABARAP−/− mice to a low
dietary Pi content. Mice were adapted to a high Pi diet (↑) for 4 days as indicated in Experimental procedures. On the fifth day they were divided in two subgroups that received either a high Pi diet (n=3) or were switched to a low Pi diet (↓) (n=5). (A) Urinary Pi excretion as ratio to urinary creatinine. (B) Serum Pi concentration. (C)
Renal P\textsubscript{i} handling in GABARAP\textsuperscript{−/−} mice

Representative Immunoblot of NaPi-IIa on BBM. Expression was normalized to β-actin for the quantification.

**Table 1: Metabolic cage studies and urinary analysis of GABARAP\textsuperscript{−/−} and GABARAP\textsuperscript{+/+} mice.** Values are means ± SEM with either n=15 mice per group (for metabolic cage data and urine analysis) or n=10 mice per group (for blood analysis). (*p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.0001, unpaired student t-test).
Figure 2

A

GABARAP^{+/+}  GABARAP^{−/−}

NaPi-IIa

β-actin

B

GABARAP^{+/+}  GABARAP^{−/−}

NaPi-IIc

β-actin

C

2^{ΔΔCT} HPRT - ct NaPi-IIa

2^{ΔΔCT} HPRT - ct NaPi-IIc
Figure 3

A
NaPi-IIa
+/+

B
NaPi-IIc
+/+

C
NHERF1
+/+
Figure 4
Figure 7

A

B

C

NaPi-IIa

β-actin

NaPi-IIa/actin
Figure 8
Table 1: Metabolic cage studies and urinary analysis of GABARAP\(^{+/−}\) and GABARAP\(^{+/+}\) mice.

<table>
<thead>
<tr>
<th></th>
<th>GABARAP(^{+/+})</th>
<th>GABARAP(^{+/−})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Metabolic cages</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>28.48 ± 0.56</td>
<td>30.53 ± 0.34</td>
</tr>
<tr>
<td>Food intake (mg / g body weight)</td>
<td>0.137 ± 0.005</td>
<td>0.148 ± 0.004</td>
</tr>
<tr>
<td>Water intake (ml / g body weight)</td>
<td>0.203 ± 0.010</td>
<td>0.170 ± 0.009</td>
</tr>
<tr>
<td>Stool (g / g body weight)</td>
<td>0.052 ± 0.004</td>
<td>0.052 ± 0.003</td>
</tr>
<tr>
<td>Urine (ml / g body weight)</td>
<td>0.059 ± 0.006</td>
<td>0.041 ± 0.002</td>
</tr>
<tr>
<td><strong>Urine chemistry</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.10 ± 0.04</td>
<td>6.15 ± 0.08</td>
</tr>
<tr>
<td>Osmolality (mosm/kg H(_2)O)</td>
<td>2494 ± 164</td>
<td>3634 ± 124</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>59.28 ± 4.28</td>
<td>94.96 ± 5.93</td>
</tr>
<tr>
<td>cAMP (pmol) / creatinine (mg/dl)</td>
<td>25.14 ± 0.91</td>
<td>23.68 ± 0.68</td>
</tr>
<tr>
<td>Na(^+) (mM) / creatinine (mg/dl)</td>
<td>1.95 ± 0.11</td>
<td>1.80 ± 0.10</td>
</tr>
<tr>
<td>K(^+) (mM) / creatinine (mg/dl)</td>
<td>7.15 ± 0.23</td>
<td>7.02 ± 0.38</td>
</tr>
<tr>
<td>Ca(^{2+}) (mM) / creatinine (mg/dl)</td>
<td>0.037 ± 0.009</td>
<td>0.032 ± 0.003</td>
</tr>
<tr>
<td>Mg(^{2+}) (mM) / creatinine (mg/dl)</td>
<td>0.62 ± 0.02</td>
<td>0.56 ± 0.03</td>
</tr>
<tr>
<td>P(_i) (mM) / creatinine (mg/dl)</td>
<td>1.79 ± 0.07</td>
<td>1.24 ± 0.09</td>
</tr>
<tr>
<td>Cl(^−) (mM) / creatinine (mg/dl)</td>
<td>5.13 ± 0.38</td>
<td>4.32 ± 0.24</td>
</tr>
<tr>
<td><strong>Blood chemistry</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na(^+) (mM)</td>
<td>143.3 ± 0.33</td>
<td>146 ± 0.86</td>
</tr>
<tr>
<td>K(^+) (mM)</td>
<td>3.84 ± 0.09</td>
<td>4.39 ± 0.18</td>
</tr>
<tr>
<td>Ca(^{2+}) (mM)</td>
<td>1.28 ± 0.01</td>
<td>1.27 ± 0.01</td>
</tr>
<tr>
<td>P(_i) (mM)</td>
<td>2.54 ± 0.06</td>
<td>2.66 ± 0.14</td>
</tr>
<tr>
<td>Cl(^−) (mM)</td>
<td>110.5 ± 0.45</td>
<td>112.1 ± 0.64</td>
</tr>
</tbody>
</table>

* p ≤ 0.05
** p ≤ 0.01
*** p ≤ 0.001