

Downregulation of NaPi-IIa and NaPi-IIb Na⁺-coupled Phosphate Transporters by Coexpression of Klotho

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Key Words

Intestine • Kidney • Phosphate transport • Ageing • Life span

Abstract

Klotho, a transmembrane protein, protease and hormone has been shown to exert a profound effect on phosphate metabolism. Klotho overexpression lowers and Klotho deficiency increases the plasma phosphate concentration, effects in part attributed to an inhibitory effect of Klotho on the formation of 1,25-dihydroxycholecalciferol (1,25(OH)₂D₃), the active form of Vitamin D. Beyond that Klotho has been shown to decrease renal tubular phosphate transport more directly. The influence of Klotho on the plasma phosphate concentration contributes to the profound effect of Klotho on ageing and life span. The present study explored whether Klotho influences the major renal tubular (NaPi-IIa) and the major intestinal (NaPi-IIb) phosphate transporters. For functional analysis NaPi-IIa or NaPi-IIb were expressed in *Xenopus* oocytes both, without or with additional coexpression of Klotho and electrogenic phosphate transport was estimated from the phosphate-induced current (Ip). According to RT-PCR Klotho is expressed in the

murine kidney and intestine. Coexpression of Klotho decreased Ip in both NaPi-IIa- and NaPi-IIb-expressing oocytes. Klotho decreased the maximal Ip without appreciably affecting the concentration required for halfmaximal Ip. Treatment of NaPi-IIa- or NaPi-IIb-expressing oocytes with Klotho protein similarly decreased Ip. In conclusion, Klotho down regulates both, renal (NaPi-IIa) and intestinal (NaPi-IIb) phosphate transporters.

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Introduction

Klotho is a transmembrane protein previously shown to be expressed in a variety of tissues including kidney, parathyroid glands and choroid plexus [1, 2]. The extracellular domain of Klotho may be cleaved off and released into the cerebrospinal fluid and blood [3]. Klotho has a profound inhibitory effect on ageing and life span

[4, 5]. As shown in mice, Klotho deficiency is followed by severe growth retardation and accelerated ageing resulting in death within less than 5 months [4]. Conversely, Klotho overexpression is followed by a substantial extension of the life span [4, 5].

Klotho deficiency leads to profound disturbances of mineral metabolism, i.e. hypercalcemia, hyperphosphatemia and vascular calcification [4, 5]. Klotho down regulates phosphate transport in part by inhibiting the 1α -hydroxylase and thus decreasing the formation of $1,25(\text{OH})_2\text{D}_3$ [2, 6, 7], a hormone stimulating intestinal and renal Ca^{2+} and phosphate transport [8, 9]. In the regulation of 1α -hydroxylase, Klotho is partially effective by augmenting the signaling of FGF23 [5]. Klotho deficiency leads to excessive formation of $1,25(\text{OH})_2\text{D}_3$ [2, 6, 7]. The excessive $1,25(\text{OH})_2\text{D}_3$ formation contributes to the increase of plasma Ca^{2+} [10] and phosphate [9] concentration in Klotho-deficient mice [2, 6, 7]. The hyperphosphatemia is considered a decisive determinant of life span [5] and Vitamin D restriction partially reverses the accelerated ageing and early death of Klotho deficient mice [2]. Moreover, Vitamin D deficiency counteracts the growth deficit [2] and premature erythrocyte death [11] of Klotho-deficient mice. Nevertheless, Vitamin D deficiency does not fully reverse the hyperphosphatemia and does not fully normalize the life expectancy of those mice [12]. Thus, the hypothesis was tested, that Klotho has a more direct effect on phosphate transport independent of $1,25$ -dihydroxycholecalciferol ($1,25(\text{OH})_2\text{D}_3$) formation. As shown most recently [13], transgenic Klotho overexpression or intravenous Klotho infusion indeed caused hypophosphatemia and phosphaturia and decreased protein abundance and activity of the major renal transporter NaPi-IIa.

To possibly unravel a direct effect of Klotho on the major renal (NaPi-IIa) and intestinal (NaPi-IIb) phosphate transporters, the carriers were expressed in *Xenopus* oocytes with and without coexpression of Klotho. As phosphate transport is electrogenic [14], the phosphate induced current has been determined by dual electrode voltage clamp and taken as a measure of phosphate transport.

Materials and Methods

RNA extraction and real-time RT-PCR

Total RNA was extracted from mouse tissue in Trizol (Pqlab, Erlangen, Germany) according to the manufacturer's

instructions. After DNase digestion reverse transcription of total RNA was performed using random hexamers (Roche Diagnostics, Penzberg, Germany) and SuperScriptII reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Polymerase chain reaction (PCR) amplification of the respective genes were set up in a total volume of 20 μl using 40 ng of cDNA, 500 nM forward and reverse primer and 2x GoTaq qPCR Master Mix (Promega, Mannheim, Germany) according to the manufacturer's protocol. Cycling conditions were chosen as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 20 sec. For the amplification the following primers were used ($5'$ - $>3'$ orientation): *Klotho*, fw CCC TGT GAC TTT GCT TGG G rev CCC ACA GAT AGA CAT TCG GGT; *Tbp*, fw CAC TCC TGC CAC ACC AGC TT rev TGG TCT TTA GGT CAA GTT TAC AGC C. Specificity of PCR products was confirmed by analysis of a melting curve and in addition with a 2% agarose gel. Real-Time PCR amplifications were performed on a CFX96 Real-Time System (Bio-Rad, Munich, Germany) and all experiments were done in duplicates. Amplification of the housekeeping gene *Tbp* was performed to standardize the amount of sample RNA. Relative quantification of gene expression was performed using the Δct method as described earlier [15].

Expression in *Xenopus laevis* oocytes

Murine full-length Klotho was subcloned from pCR-XL-TOPO vector (Imagines, Berlin, Germany) into pSGEM, a *Xenopus* oocyte expression vector using XhoI - SpeI restriction sites. cRNA encoding wild type NaPi-IIa [16] or NaPi-IIb [17] and encoding Klotho were synthesized as described earlier [18, 19]. Dissection of *Xenopus laevis* ovaries, collection and handling of the oocytes have been described in detail elsewhere [20, 21]. Where not otherwise specified, oocytes were injected with 10 ng of cRNA encoding NaPi-IIa or 15 ng cRNA encoding NaPi-IIb on the first day and 10 ng cRNA encoding Klotho on the second day. For control, the oocytes were injected with the respective volumes of H_2O . Therefore, the number of injections and the injected total volume was equal in all studied oocytes.

Electrophysiology

All experiments were performed at room temperature 3 - 4 days after the second injection of cRNA encoding the respective carrier [22]. Two-electrode voltage-clamp recordings were performed at a holding potential of -50 mV [23]. The data were filtered at 10 Hz, and recorded with a GeneClamp 500 amplifier, a DigiData 1300 A/D-D/A converter and the pClamp 9.0 software package for data acquisition and analysis (Axon Instruments, USA). The control solution (superfusate/ND96) contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 and 5 mM HEPES, pH 7.4. Phosphate was added to the solutions at the indicated concentration. The final solutions were titrated to pH 7.4 using NaOH. The flow rate of the superfusion was 20 ml/min, and a complete exchange of the bath solution was reached within about 10 s. The data of Fig. 2B, Fig. 3 and Fig. 4B, D were normalized by dividing the measured current (in nA) of every single oocyte by the average current of oocytes

expressing the phosphate transporter alone of the respective oocyte batch. The kinetics of Fig. 2C and Fig. 4C were analysed as follows: The arithmetic mean of all measured currents for every phosphate concentration was fitted to a Michaelis-Menten kinetics following the equation:

$$v = v_{\max} \cdot [\text{phosphate}] / (K_m + [\text{phosphate}])$$
 using Microcal Origin 6.0. v_{\max} , K_m and SEM for those parameters were calculated by Origin 6.0. Fig 2C and Fig. 4C display the unfitted raw data as stated in the figure legend.

Detection of Klotho dependent Napi-IIa cell surface expression by chemiluminescence

To determine Napi-IIa cell surface expression by chemiluminescence, defolliculated oocytes were first injected with either 15 ng cRNA encoding Napi-IIa or with water. Injected oocytes were incubated for 48 hours and for further 12 or 24 hours in the presence or absence of 30 ng/ml Klotho protein. Surface expression of Napi-IIa was detected by chemiluminescence. To this end, the oocytes were incubated with primary SLC34A1 (Napi-IIa) rabbit anti-human polyclonal antibody (1:100, Life Span Biosciences, WA, USA) and subsequently with secondary HRP-conjugated goat anti-rabbit IgG (H&L) antibody (1:1000, Cell Signaling Technology, MA, USA). Individual oocytes were placed in 96 well plates with 20 μ l of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) and chemiluminescence of single oocytes was quantified in a luminometer (Walter Wallac 2 plate reader, Perkin Elmer, Juegesheim, Germany) by integrating the signal over a period of 1 s. Results display normalized relative light units.

Statistics

Data are provided as means \pm SEM, n represents the number of oocytes studied. All data were tested for significance using ANOVA or paired or unpaired Student t-test. Only results with $p < 0.05$ were considered statistically significant.

Results

Similar to what has been reported earlier [1, 2], RT-PCR revealed the expression of Klotho in murine kidneys (Fig. 1). More importantly, Klotho transcript was further detectable in murine intestinal tissue pointing to expression of Klotho also in the intestine (Fig. 1). To explore, whether Klotho modifies the activity of the renal phosphate transporter Napi-IIa, cRNA encoding the transporter was injected into *Xenopus* oocytes with and without additional injection of cRNA encoding Klotho, and the phosphate-induced current was determined by dual voltage-clamp experiments. As shown in Fig. 2A, B, phosphate induced a current in Napi-IIa-expressing oocytes, but not in water-injected oocytes. The current was significantly attenuated by coexpression of Klotho.



Fig. 1. Klotho transcript levels in kidney and intestinal tissue from wild type mice. Representative original bands of mRNA encoding Klotho (upper bands) in the kidney (left bands) and intestinal tissue (right bands). The housekeeping gene Tbp (lower bands) served as a calibrator and control.

In order to further characterize the effect of Klotho on Napi-IIa-expressing oocytes, the kinetics of the phosphate-induced current was determined in the absence and presence of coexpressed Klotho (Fig. 2C). As a result, the calculated maximal current v_{\max} was 67.4 ± 2.6 nA ($n = 13 - 17$) in the absence and 49.4 ± 1.9 nA ($n = 13 - 17$) in the presence of Klotho. The maximal current was significantly smaller in *Xenopus* oocytes expressing both, NaPi-IIa and Klotho than in *Xenopus* oocytes expressing NaPi-IIa alone. The calculated phosphate concentration required for a halfmaximal current (K_m) was 96 ± 13 μ M ($n = 13 - 17$) in the absence and 87.0 ± 13 μ M ($n = 13 - 17$) in the presence of Klotho. The K_m was not significantly different between *Xenopus* oocytes expressing NaPi-IIa + Klotho and *Xenopus* oocytes expressing NaPi-IIa alone.

A further series of experiments explored whether the extracellular application of Klotho protein is similarly capable of inhibiting Napi-IIa-mediated currents. As shown in Fig. 3A, the addition of 30 ng/ml Klotho protein to the medium for 24 hours indeed resulted in a significant reduction of the phosphate-induced current. Surface chemiluminescence upon antibody binding was employed to measure Napi-IIa surface abundance. As shown in Fig. 3B, treatment with Klotho protein significantly decreased the Napi-IIa-dependent surface chemiluminescence in a time-dependent manner.

As a next step, we studied whether the intestinal transporter Napi-IIb is also regulated by Klotho. To this end, the phosphate-induced current was determined in Napi-IIb-expressing *Xenopus* oocytes with and

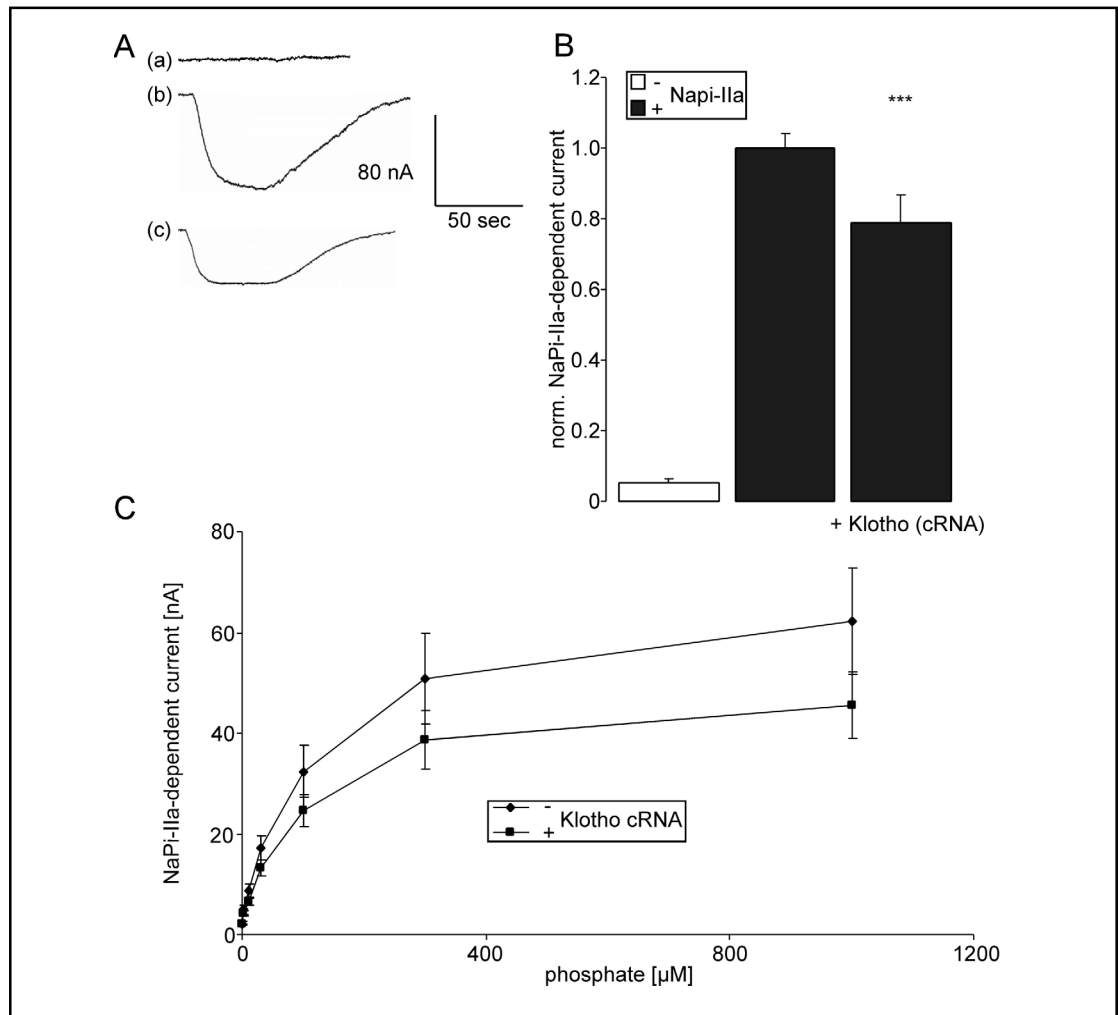


Fig. 2. Coexpression of Klotho downregulates electrogenic phosphate transport in NaPi-IIa- expressing *Xenopus* oocytes. **A.** Original tracings showing phosphate (1 mM) - induced currents (I_{pi}) in *Xenopus* oocytes injected with DEPC-water without (a) or with NaPi-IIa cRNA without (b) or with (c) additional co-expression of Klotho cRNA. Following removal of extracellular phosphate, the current gradually returns to basal levels. **B.** Arithmetic means \pm SEM of the normalized phosphate (1 mM) - induced current (I_p) in *Xenopus* oocytes injected with DEPC-water (left bar, $n = 23$), expressing NaPi-IIa without (middle bar, $n = 72$) or with (right bar, $n = 68$) additional coexpression of Klotho. ***indicates statistically significant difference ($P < 0.001$) from current in *Xenopus* oocytes expressing NaPi-IIa alone. **C.** Arithmetic means \pm SEM of the normalized phosphate induced current (I_p) as a function of phosphate concentration in *Xenopus* oocytes expressing NaPi-IIa without ($n = 13 - 17$) or with ($n = 13 - 17$) additional coexpression of Klotho.

without additional Klotho expression. Similar to the experiments with Napi-IIa-expressing oocytes, Klotho significantly reduced the Napi-IIb-mediated current (Fig. 4A, B). Kinetic analysis (Fig. 4C) revealed that the calculated maximal phosphate-induced current was again significantly reduced from 26.9 ± 0.6 nA ($n = 9 - 13$) in *Xenopus* oocytes expressing NaPi-IIb

alone to 16.9 ± 0.2 nA ($n = 9 - 13$) in *Xenopus* oocytes expressing NaPi-IIb together with Klotho. The calculated phosphate concentration required for a halfmaximal current (K_m) was again not significantly different between *Xenopus* oocytes expressing NaPi-IIb + Klotho (6.0 ± 0.4 μ M, $n = 9 - 13$) and *Xenopus* oocytes expressing NaPi-IIb alone (8.3 ± 0.9 μ M, $n = 9 - 13$).

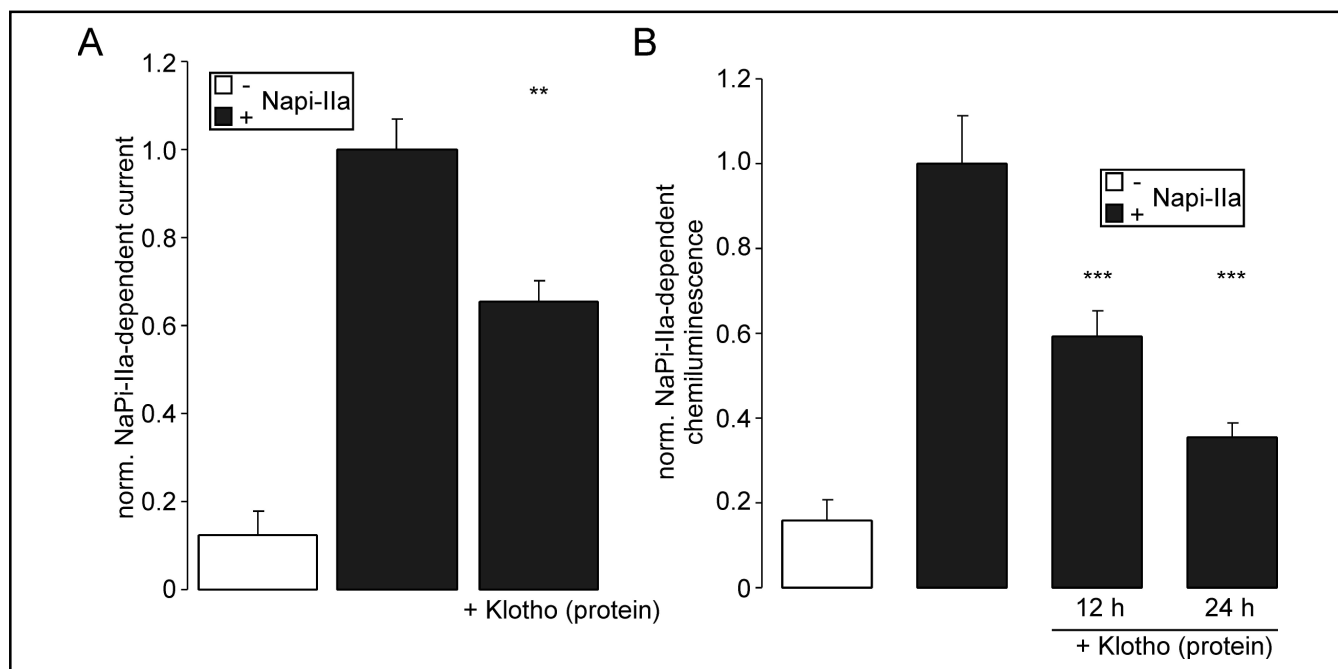


Fig. 3. Treatment with Klotho decreases the electrogenic phosphate transport in NaPi-IIa- expressing *Xenopus* oocytes. A. Arithmetic means \pm SEM of the normalized phosphate (1 mM) - induced current (I_p) in *Xenopus* oocytes injected with DEPC-water (left bar, $n = 7$) or expressing NaPi-IIa and treated for 24 hours without (middle bar, $n = 25$) or with (right bar, $n = 24$) 30 ng/ml Klotho protein. **indicates statistically significant difference ($P < 0.01$) from current in *Xenopus* oocytes expressing NaPi-IIa alone. B. Arithmetic means \pm SEM ($n = 19 - 43$) of the normalized NaPi-IIa-dependent surface chemiluminescence of *Xenopus* oocytes injected with DEPC-water (left bar) or expressing NaPi-IIa and treated for 12 or 24 hours without or with 30 ng/ml Klotho protein. ***indicates statistically significant difference ($P < 0.001$) from chemiluminescence of *Xenopus* oocytes expressing NaPi-IIa alone.

Similar to what was observed in NaPi-IIa expressing *Xenopus* oocytes, extracellular application of Klotho protein (30 ng/ml) in the medium for 24 hours led to a significant reduction of the phosphate-induced current in NaPi-IIb expressing *Xenopus* oocytes (Fig. 4D).

Discussion

The present observations reveal that Klotho down regulates both, the major renal (NaPi-IIa) and intestinal (NaPi-IIb) phosphate carriers. The present experiments were made in the absence of 1α -hydroxylase, $1,25(\text{OH})_2\text{D}_3$ and/or FGF23 and thus reflect a more direct effect of Klotho on the phosphate transporters. The present observations are consistent with a most recent study [13] demonstrating a phosphaturic effect of Klotho in FGF23 null mice, indicating that the effect of Klotho was not restricted to augmentation of FGF receptor signaling [13].

Klotho decreases the maximal transport rate of both carriers, reflecting a decrease of carrier protein abundance in the plasma membrane. As a matter of fact, the effect was mimicked by the Klotho protein, which is known to be effective as protease [5]. Along those lines, the effect of Klotho on NaPi-IIa could be reversed by a beta-glucuronidase inhibitor [13]. Presumably, externally applied Klotho is effective by proteolytic cleavage of extracellular components of the phosphate carriers eventually resulting in decreased abundance of functional carrier protein.

The present paper does, however, not rule out further mechanisms participating in the regulation of the phosphate carriers. In theory, Klotho may be effective by interfering with carrier regulating signaling. Renal tubular NaPi-IIa regulation involves a wide variety of protein kinases including PKA [24, 25], PKC [25-27] PKG [26], MAPK [28], GSK3 [29], mTOR [30] and Akt2/PKB [16]. Moreover, NaPiIIa expression is regulated by GABA(A) receptor-associated protein (GABARAP) [31], epidermal growth

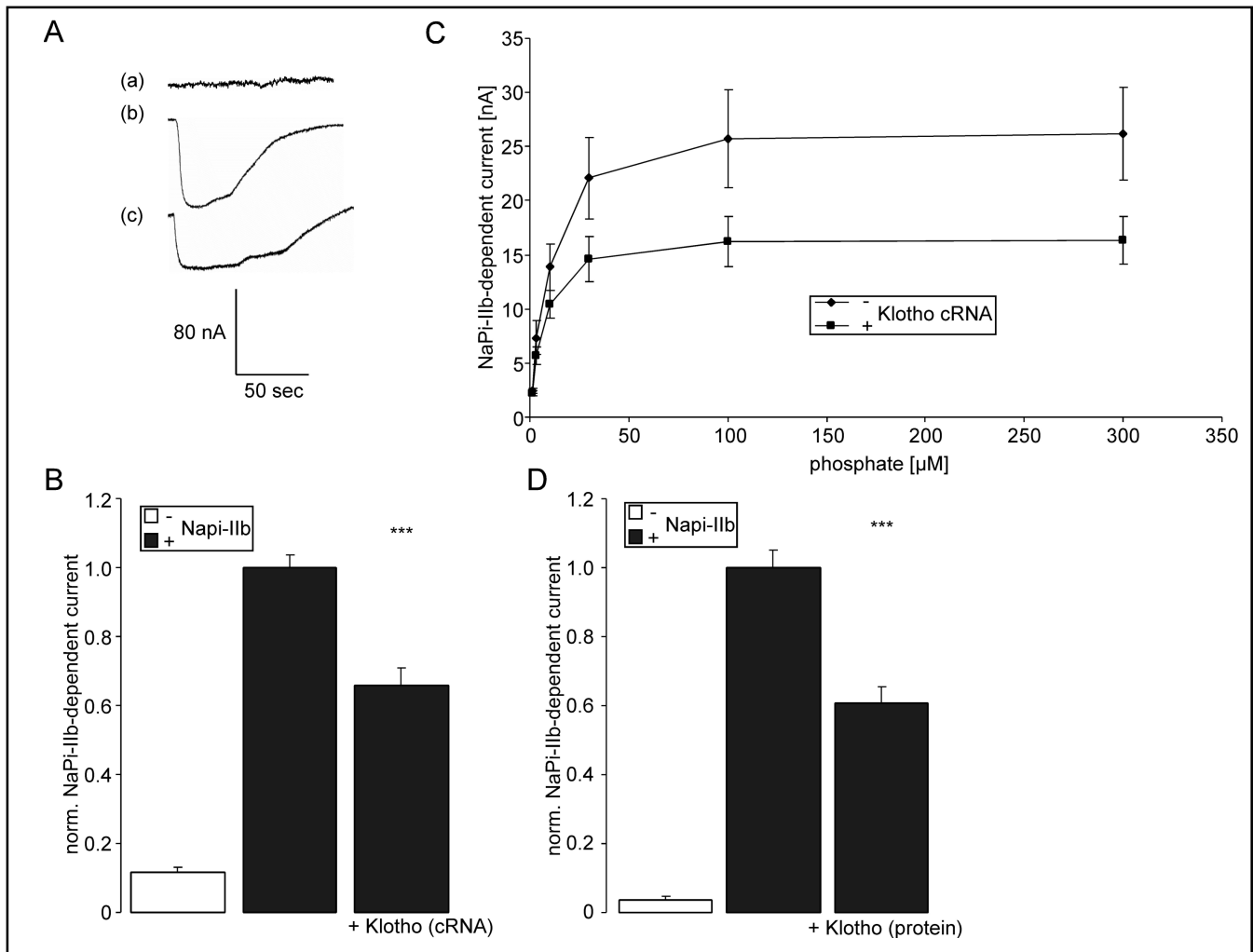


Fig. 4. Coexpression of or treatment with Klotho downregulates electrogenic phosphate transport in NaPi-IIb-expressing *Xenopus* oocytes. **A.** Original tracings showing phosphate (1 mM) - induced currents (I_{pi}) in *Xenopus* oocytes injected with DEPC-water without (a) or with NaPi-IIb cRNA without (b) or with (c) additional co-expression of Klotho cRNA. **B.** Arithmetic means \pm SEM of the normalized phosphate (1 mM) - induced current (I_p) in *Xenopus* oocytes injected with DEPC-water (left bar, $n = 27$), expressing NaPi-IIb without (middle bar, $n = 50$) or with (right bar, $n = 52$) additional co-expression of Klotho. ***indicates statistically significant difference ($P < 0.001$) from current in *Xenopus* oocytes expressing NaPi-IIb alone. **C.** Arithmetic means \pm SEM of the normalized phosphate induced current (I_p) as a function of phosphate concentration in *Xenopus* oocytes expressing NaPi-IIb without ($n = 9 - 13$) or with ($n = 9 - 13$) additional co-expression of Klotho. **D.** Arithmetic means \pm SEM of the normalized phosphate (1 mM) - induced current (I_p) in *Xenopus* oocytes injected with DEPC-water (left bar, $n = 8$) or expressing NaPi-IIb and treated for 24 hours without (middle bar, $n = 19$) or with (right bar, $n = 20$) 30 ng/ml Klotho protein. ***indicates statistically significant difference ($P < 0.001$) from current in *Xenopus* oocytes expressing NaPi-IIb alone.

factor EGF [32], glucocorticoids [33], and dietary phosphate [34, 35]. Regulators of intestinal NaPiIIb include the serum and glucocorticoid inducible kinase SGK1 [36], mTOR [17], ubiquitin ligase Nedd4-2 [36], pH [37], GABARAP [31], EGF [38], estrogen [39] and dietary phosphate [34, 35]. As oocytes were injected with mRNA encoding NaPi-IIa or NaPi-IIb, genomic regulators of the carriers are not likely to modify carrier abundance or activity. Whether Klotho influences the

activity of any of the above kinases, remains to be shown.

In theory, klotho could be effective by altering the driving force for Na^+ coupled phosphate transport. Klotho has, however, most recently been shown to up-regulate endogenous Na^+/K^+ ATPase in *Xenopus* oocytes [40], an effect expected to decrease cytosolic Na^+ concentration. Thus, Klotho enhances rather than decreases the driving force for Na^+ coupled phosphate transport.

In conclusion the present observations disclose an effect of Klotho on renal and intestinal phosphate carriers in the absence of FGF23 and 1,25(OH)₂D₃. The effect contributes to the hyperphosphatemic effect of Klotho deficiency, and thus to the impact of Klotho on ageing.

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