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Expression of rat renal sodium/phosphate cotransporter in *Xenopus laevis* oocytes

Hasan A. Al-Mahrouq and Stephen A. Kempson

Department of Physiology and Biophysics, Indiana University School of Medicine, Indianapolis, IN (USA)

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In an attempt to identify the renal Na^+/P_i cotransporter, *Xenopus laevis* oocytes were used to express mRNA isolated from the renal cortex of rat kidney. Na^+-dependent uptake of P_i in oocytes, injected with mRNA, resulted in an increase of 2–4-fold as compared to oocytes injected with water. Both the new expressed and endogenous Na^+-dependent P_i uptake activity were inhibited with 2 mM phosphonoformic acid (PFA). Expression of P_i uptake into oocytes was dose-dependent with the amount of mRNA injected. When mRNA was fractionated on a sucrose gradient, a mRNA fraction of 2.5 kilobases expressed the Na^+/P_i cotransport activity in oocytes. This fraction resulted in a 6-fold stimulation of Na^+-dependent P_i transport when compared to oocytes injected with water. The K_m and V_{max} for Na^+-dependent P_i uptake were 0.18 mM and 118 pmol/oocyte per 30 min, respectively.

Introduction

Phosphate reabsorption in the proximal tubules occurs through an active transcellular mechanism that involves a Na^+-P_i cotransport system at the apical brush border membrane [1]. P_i entry across the brush border membrane (BBM) is driven by an electrochemical gradient for Na^+. In contrast P_i exits the cell via passive Na^+-independent transport through the basolateral membrane. The biochemical characteristics of the renal Na^+-P_i cotransporter protein(s) are largely unknown. Identification of the Na^+-P_i cotransporter protein(s) is hindered by the lack of specific covalent labels. Peerce [2] used a fluorescent probe which appears to label the intestinal Na^+-P_i cotransporter and reported an approximate molecular mass of 145 kDa. *N*-Acetylimidazole which interacts with proline and/or lysine residue, has been used to label the Na^+-P_i cotransporter in established renal epithelial cell line (OK cells). Four different proteins with molecular mass of 31–176 kDa have been reported to be involved in Na^+-P_i cotransport in OK cells [5].

When an azido derivative of NAD was used to photolabel the renal Na^+-P_i cotransport, proteins with approximate molecular mass of 70 and 97 kDa were identified as likely candidates for the Na^+-P_i cotransporter in BBM vesicles prepared from rats [3].

Recent advances in molecular biology have made possible the identification and isolation of clones from cDNA libraries by using expression cloning techniques. Preparative purification of specific mRNA and expression cloning have been used to clone the Na^+-glucose cotransporter from rabbit intestinal BBM [4,11]. Two clones from the human renal Na^+-glucose cotransporter also have been isolated by the use of the clone encoding the human intestinal Na^+-glucose cotransporter [6]. Both cDNA, encoding for the renal and intestinal Na^+-glucose cotransporter, showed >99% identity in their nucleotide sequences [13].

Preparative purification of specific mRNA from rabbit kidney cortex has been used to selectively express the Na^+-P_i cotransporter activity in oocytes. Werner et al. [7] have shown expression of Na^+-dependent P_i transport in *Xenopus laevis* oocytes injected with mRNA isolated from rabbit kidney cortex. They reported a fraction of mRNA, with an average size of 3.4 kilobases, produced a maximum stimulation of Na^+-dependent P_i uptake when injected into oocytes.

Most of the physiological studies, concerning the regulation of the renal Na^+-P_i cotransporter, has been

Correspondence: H.A. Al-Mahrouq, Department of Physiology and Biophysics, Indiana University School of Medicine, 635 Barnhill Drive, Indianapolis, IN 46202-5120, USA.

performed on rat kidneys. Therefore a rat clone is likely be more useful than rabbit for studying regulatory mechanisms of the renal phosphate transport system. In this report, we show injection of oocytes with rat kidney mRNA induced expression of a unique Na^+-dependent P_i transport system which also is inhibited by PFA.

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Materials and Methods

Total RNA and poly(A)$^+$RNA were isolated as described [8,10]. The outer cortex of rat kidney was dissected and homogenized in guanidine thiocyanate buffer followed by extraction with phenol (water saturated)/chloroform/isoamyl alcohol. The aqueous phase was collected by centrifugation at $10000 \times g$ for 20 min at 4°C. The RNA was precipitated by adding isopropanol and incubation at −20°C. The precipitated RNA was resuspended in 1 ml of 75% ethanol, centrifuged for 5 min and the pellet was dried under vacuum. The concentration of RNA was calculated at 260 nm, and the ratio of $A_{260}/_{280}$ was greater then 1.8. Poly(A)$^+$RNA was purified by chromatography on oligo(dT) cellulose and purified in the presence of 0.3 M sodium acetate [8].

Linear sucrose gradient (5–25%, w/w) of the mRNA was performed in the presence of 10 mM Tris buffer (pH 7.4), 0.25% (w/w) sarcosyl, and 5 mM EDTA. The total mRNA was applied to the gradient and centrifuged for 4 h at 20°C at 45000 rpm in a SW 50.1 rotor [9]. The sample was fractionated into 15 fractions. The concentration of RNA was determined by absorbance at 260 nm, and the RNA was stored at −70°C until used. The integrity and the size of poly(A)$^+$RNA was confirmed by formaldehyde/agarose gel electrophoresis and ethidium bromide staining.

Oocytes were prepared from *Xenopus laevis* ovary fragments by treatment with collagenase as described by Hediger et al. [11]. Oocytes were selected and microinjected with 50 nl of water (controls) or 50 nl solution containing RNA. The injected oocytes were maintained at 19°C in Barth's medium (100 mm NaCl, 1.8 mM $CaCl_2$, 2 mM KCl, 1 mM $MgCl_2$, 2.6 mM sodium pyruvate, 10 mg/l gentamicin, 5 mM Hepes, pH 7.5) for 3 days. The medium was changed daily and damaged oocytes were discarded.

The P_i transport by oocytes was assayed at low P_i concentration (0.2 mM), which is in the range of the K_m for the renal Na^+-dependent P_i cotransporter. The uptake medium contained 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes-Tris buffer (pH 7.5), and either 100 mM NaCl or 100 mM choline chloride. After incubation for 30 min at 25°C, the uptake was terminated by washing the oocytes five times with 2-ml aliquots of ice-cold choline-Cl buffer. Each oocyte was dissolved in 0.5 ml of 7% sodium dodecyl sulfate, sonicated briefly and the ^{32}P was counted by liquid scintillation spectrophotometer.

K_m and V_{max} were determined by linear regression of the double-reciprocal plots of the uptake values vs. the P_i concentrations. The Student's t-test was used in comparing groups, and values for $P > 0.05$ were considered not significant.

Results and Discussion

Endogenous P_i transport activity in oocytes was characterized. Uptake in oocytes is a Na^+-dependent process. Na^+-independent P_i uptake was noticed in freshly isolated oocytes and after 3 days of incubation of oocytes in Barth's solution. Fig. 1 shows the P_i uptake by oocytes in the presence (100 mM NaCl) or absence (100 mM choline chloride) of a Na^+ gradient. The amount of Na^+-dependent uptake of P_i in non-injected oocytes ranges from 10–30 pmol/oocyte per h. Injection of oocytes with mRNA extracted from kidney cortex resulted in an enhancement of Na^+-dependent P_i activity. PFA is a competitive inhibitor of Na^+-dependent P_i transport in renal BBM vesicles [12]. We examined the effect of PFA on P_i uptake into control oocytes and oocytes injected with mRNA. Both systems were sensitive to PFA. The concentration of PFA required to inhibit both activities was in the range of 1–2 mM. The time course for P_i transport was linear up to one hour.

Expression of the Na^+-P_i cotransport activity in oocytes was dependent on the injected amount of

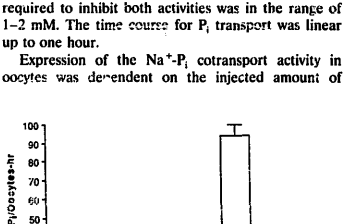

Fig. 1. Expression of rat kidney P_i transport in *Xenopus* oocytes. Oocytes were injected with 50 nl of water or water containing 50 ng mRNA. After 3 days uptake of [^{32}P]P_i (pmol/oocyte per h) was measured. The uptake of 0.2 mM P_i was measured in the presence of 100 mM NaCl (open bars) or 100 mM choline chloride (solid bars). PFA was present at 2 mM (hatched bars). Uptakes are presented as the means obtained with four or five oocytes, and bars indicate S.E.

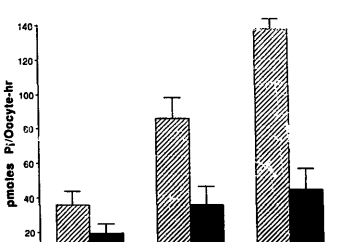

Fig. 2. Effect of different mRNA doses on P_i transport expression in oocytes. Three days after injection of mRNA (0, 20, and 40 ng), P_i transport in four or five oocytes was measured. The uptake of 0.2 mM P_i was measured in the presence of 100 mM NaCl (hatched bars) or 100 mM choline chloride (solid bars). Uptakes are presented as the means obtained with four or five oocytes, and bars indicate S.E.

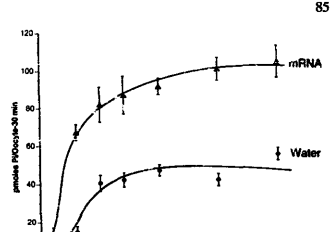

Fig. 3. A representative study of kinetics of Na^+-dependent uptake of P_i in oocytes. Oocytes were injected with 50 nl water containing about 50 ng mRNA (▲) or 50 nl water (●). Oocytes were incubated for 3 days and then assayed for Na^+-dependent uptake of ^{32}P. The plot represents the initial velocity (the uptake of ^{32}P/oocyte per 30 min) vs. the initial substrate concentrations. Data corrected by subtraction of the Na^+-independent component of P_i transport.

mRNA. The expression of the Na^+-dependent transport of P_i, calculated by subtracting the Na^+-independent component from the total P_i uptake in presence of Na^+, increases 2-fold when the injected amount of mRNA was doubled (Fig. 2). Injection of higher amount of mRNA did not result in increasing the expression of the Na^+-dependent P_i transport activity.

Dependence of the initial rate of P_i transport in oocytes as a function of external P_i concentration was measured in the presence of 100 mM NaCl. To determine kinetic constants, oocytes were incubated for 30 min at 22°C in the medium containing various P_i concentrations from 0.05–2.00 mM since Na^+-dependent P_i uptake was linear up to one hour (data not shown). Water and mRNA injected oocytes showed a saturable P_i uptake (Fig. 3). V_{max} and K_m were calculated by linear regression analysis. The V_{max} in oocytes injected with mRNA is 119 ± 2 (mean ± S.E.) pmol/oocyte per 30 min, which is higher ($P < 0.05$, $n = 4$) than that of oocytes injected with water (65 ± 5 pmol/oocyte per 30 min). However, the apparent K_m was lower in oocytes injected with mRNA (0.18 ± 0.04 mM) than that injected with water (0.26 ± 0.07 mM), but this change in the K_m was not statistically significant. Similar results were reported for the expression of Na^+-glucose cotransport system in oocytes after injection of mRNA extracted from rabbit small intestine mucosa; K_m was lowered from 0.39 to 0.10 mM following injection of mRNA [11]. The K_m in oocytes injected with mRNA is in the range of K_m reported for Na^+-P_i cotransport system in BBM vesicles prepared from rat kidney [3].

Total mRNA was fractionated on 5–25% sucrose gradient and the gradient was fractionated into 15 fractions. mRNA was precipitated from each fraction and the same amount of mRNA (about 10 ng) was injected into oocytes and ^{32}P uptake was studied both in the presence and absence of Na^+. As shown in Fig. 4, fractions 6 and 7 produced a higher activity of the expressed Na^+-dependent P_i uptake than the unfractionated mRNA. Specifically, fraction 5 produced a

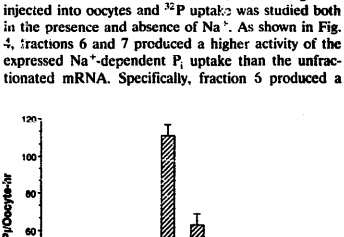

Fig. 4. Uptake of ^{32}P (pmol/oocyte per h) into oocytes injected with mRNA fractions obtained from sucrose gradient centrifugation. Oocytes were injected with 50 nl of water containing about 10 ng of unfractionated or fractionated mRNA. After 3 days the uptake of ^{32}P was measured in the presence of 100 mM NaCl (hatched bars) or 100 mM choline chloride (solid bars) as described above. Uptakes are presented as the means obtained with 4–6 oocytes, and bars indicate S.E.

2.5-fold increase in Na^+-dependent P_i transport compared to total unfractionated mRNA. Oocytes injected with pooled and sized fractionated mRNA (fractions 8–15) give much less expressed P_i transport activity. However, fractions 5 and 7 have a comparable expressed P_i transport activity when compared to unfractionated mRNA. Fraction 6–7 corresponds to a size of 2.5–2.8 kilobases as determined by formaldehyde gel electrophoresis (data not shown). The apparent K_m and V_{max} obtained in these results is similar to that obtained for the expression of Na^+-dependent P_i cotransport system following injection of mRNA extracted from rabbit kidney [7]. The average size (2.5–2.8 kilobases) of mRNA, encoding the Na^+-P_i cotransport system in rat renal BBM, is comparable to that obtained for rabbit BBM (3.4 kilobases). Na^+-independent uptake rate of P_i was increased with incubation time in oocytes injected with mRNA (Figs. 1–3). This phenomena, observed in most of these studies, might be due to the expression of an Na^+-independent P_i transport system or an activator of endogenous Na^+-independent transport system present in oocytes.

Oocytes possess an endogenous Na^+-dependent P_i transport activity which is sensitive to PFA. In spite of the presence of an intrinsic P_i transporter, the oocyte expression system can be used for expression of rat kidney Na^+-dependent P_i transporter by focusing on the stimulation of Na^+-dependent uptake of P_i after injection of mRNA. In conclusion, our results clearly show that the rat renal Na^+-P_i cotransport system can be expressed as a functional protein on the membrane of *Xenopus laevis* oocytes injected with mRNA prepared from rat kidney cortex. This procedure will be useful in cloning the rat renal Na^+-P_i cotransporter. This will open the way to detailed studies on the mode of action of drugs and hormonal control on the rat renal Na^+-P_i cotransport system, and on factors which control the synthesis and incorporation of cotransporters into the membrane.

Acknowledgments

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